M.Sc. Previous Year
Zoology, Paper-V

TECHNIQUES AND TOOLS FOR BIOLOGY AND ENVIRONMENTAL PHYSIOLOGY

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SYLLABI-BOOK MAPPING TABLE

Techniques and Tools for Biology and Environmental Physiology

Syllabi	Mapping in Book
UNIT - 1: Tools and Techniques - I 1. **Principles and Uses of Analytical Instruments:** A. Colorimeter. B. Spectrophotometer. C. Ultracentrifuge. D. Densitometric Scanner. E. Differential Scanning Calorimeter F. ESR and NMR Spectrometers. G. Microtomy. 2. **Microscopy:** Principle of Light Transmission, Electron, Phase - Contrast, Confocal Microscopes. 3. **Microbiological Techniques:** A. Media Preparation and Sterilization. B. Inoculation and Growth Monitoring. C. Use of Fermenters. D. Microbial Assays. 4. **Cell Culture Techniques:** A. Design and Functioning of Tissue Culture Laboratory. B. Cell Proliferation Measurements. C. Cell Viability Testing. D. Culture media Preparation and Cell Harvesting Methods,. 5. **Cryotechniques:** A. Cryopreservation: Cells, Tissues, Organisms. B. Cryotechniques for Microscopy.	**Unit-1:** Tools and Techniques - I **(Pages 3-136)**
UNIT - II Tools and Techniques - II 1. **Separation Techniques in Biology:** A. Molecular Separation by Chromatography, Electrophoresis, Precipitation etc. B. Organelle Separation by Centrifugation. 2. Computer Aided Techniques for Data Presentation Data Analyses, Statistical Techniques. 3. Radioisotope and Mass Isotope Techniques in Biology: A. Sample Preparation for Radioactive Counting. B. Autoradiography. 4. Immunological Techniques based on Antigen - Antibody Interactions. 5. Surgical Techniques: A. Organ Ablations. B. Perfusion Techniques. C. Indwelling Catheters. D. Stereotaxy. E. Parabiosis. F. Biosensors.	**Unit-2:** Tools and Techniques - II **(Pages 137-196)**
UNIT - III Environmental Physiology - I 1. Adaptation: A. Levels of Adaptation. B. Mechanism of Adaptation. C. Significance of Body size. 2. Physiological Adaptations to Different Environments: A. Marine. B. Shores and Estuaries. C. Fresh water. D. Extreme aquatic Environments. E. Terrestrial life. F. Extreme Terrestrial Environments. G. Parasitic habitats.	**Unit-3:** Environmental Physiology - I **(Pages 197-218)**

UNIT - IV Environmental Physiology - II

1. Basic concept of Environmental Stress and Strain, Concept of Elastic and Plastic Strain, Stress Resistance, Stress Avoidance and Stress Tolerance.
2. Adaptation, Acclimation and Acclimatization.
3. Concept of Homeostasis.
4. Endothermy and Physiological Mechanism of Regulation of Body Temperature.
5. Physiological Adaptation to Osmotic and Ionic Stress. Mechanism of Cell Volume Regulation.
6. Osmoregulation in Aqueous and Terrestrial Environments.
7. Physiological response to oxygen deficient stress.
8. Physiological respone to body exercise.
9. Meditation, yoga and their effects.

Unit-4: Environmental Physiology - II
(Pages 219-283)

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INTRODUCTION

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In the study of biology, various new methods and techniques have developed, like microscopy, liquid chromatography, distillation techniques and paper chromatography. Simple Microscopes are most commonly used to study the microorganisms.

Now a day's science is quite advanced and well developed and new methods and techniques have been developed that helped in studying physiology and anatomy of various organisms as well as it helped in a detailed study of the cellular and sub-cellular organelles of these organisms like; chromatography, spectroscopy, electrophoresis, microscopy, distillation techniques, radiography, blotting techniques, etc.

This book, *Techniques and Tools for Biology and Environmental Physiology*, is designed to be a comprehensive and easily accessible book covering the basic concepts of various tools and techniques used in biology and environmental physiology. It will help readers to understand the basics of various kinds of chromatography techniques, spectrometric techniques, microscopy, microbial techniques, cell culture techniques, cryo-techniques, etc., along with the separation techniques, radio isotopic techniques, immunological techniques, and surgical techniques as well. This book also comprehends on the various physiological adaptions of the organisms to different environments and their habits and habitats. Basic concepts of environmental stress, strain, acclimatization, homeostasis, osmoregulation, and other physiological activities like yoga and body exercises and their physiological response have also been explained in detail.

The book is divided into four units that follow the self-instruction mode with each unit beginning with an Introduction to the unit, followed by an outline of the Objectives. The detailed content is then presented in a simple but structured manner interspersed with Check Your Progress Questions to test the student's understanding of the topic. A Summary along with a list of Key Terms and a set of Self-Assessment Questions and Exercises is also provided at the end of each unit for understanding, revision and recapitulation. The topics are logically organized and explained with related mathematical theorems, analysis and formulations to provide a background for logical thinking and analysis with good knowledge of calculus. The examples have been carefully designed so that the students can gradually build up their knowledge and understanding.

UNIT 1 TOOLS AND TECHNIQUES - I

NOTES

Structure

1.0 INTRODUCTION

In the study of biology, various new methods and techniques have developed, like microscopy, liquid, chromatography, distillation techniques and paper chromatography. Analytical instruments provide information on the composition of a sample of matter. They are employed, in some instances, to obtain qualitative information about the presence or absence of one or more components of the sample, whereas in other instances they provide quantitative data. Analytical instruments are a large class of instruments used for analytical applications in chemical, pharmaceutical, clinical, food-processing laboratories and oil refineries. The instruments help in analyzing materials and establishing the composition. Among the most common types of analytical equipment's are spectrophotometer,

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refractometer, calorimeter, conductivity meter, differential scanning calorimeter densitometric scanner, etc.

Simple microscopes are most commonly used to study the microorganisms. Microscopy is the technical field of using microscopes to view objects and areas of objects that cannot be seen with the naked eye. Optical microscopy and electron microscopy involve the diffraction, reflection, or refraction of electromagnetic radiation/electron beams interacting with the specimen, and the collection of the scattered radiation or another signal in order to create an image. Confocal laser scanning microscopy uses a focused laser beam that is scanned across the sample to excite fluorescence in a point-by-point fashion.

Microbiology techniques are methods used for the study of microbes, including bacteria and microscopic fungi and protists. They include methods to survey, culture and stain, identify, engineer and manipulate microbes. There are some technique like cell culture that is the process by which cells are grown under controlled conditions, generally outside their natural environment. After the cells of interest have been isolated from living tissue, they can subsequently be maintained under carefully controlled conditions. Cryo-preservation or cryo-conservation is another process where organelles, cells, tissues, extracellular matrix, organs, or any other biological constructs susceptible to damage caused by unregulated chemical kinetics are preserved by cooling to very low temperatures.

In this unit you will study about principles and uses of analytical instruments like colorimeter, spectrophotometer, ultracentrifuge, densitometric scanner, differential scanning calorimeter, ESR and NMR spectrometers, microscopy, microbiological and cell culture techniques, and cryotechniques.

1.1 OBJECTIVES

After going through this unit you will be able to:

- Understand the principles and uses of analytical instruments
- Comprehend microscopy
- Analyze microbiological techniques
- Comprehend cell culture techniques
- Explain cryotechniques

1.2 PRINCIPLES AND USES OF ANALYTICAL INSTRUMENTS

A scientific instrument is a specific device or tool that is typically used for scientific determinations. Instrumentation is a combined term for measuring those instruments which are characteristically used for indicating, measuring and recording physical quantities, specifically in scientific instrument-making.

Instrumentation refers to very simple devices, such as direct-reading thermometers or exceptionally complex devices, such as multi-sensor components

of industrial control systems. Today, instruments can be found in laboratories, refineries, factories and vehicles, as well as in everyday household use, for example smoke detectors and thermostats.

Analytical instruments are specially designed to provide information about the composition of a given matter. In addition, the analytical instruments also help in obtaining qualitative information about the occurrence or absence of one or more components of the given matter, however, in some cases they provide quantitative data.

Analytical technique is used for determining the chemical or physical properties of a chemical substance, chemical element, or mixture. Following are some standard methods of analysis:

Classical Methods of Analysis: Classical methods of analysis include basic analytical methods that are generally used in laboratories. Gravimetric analysis measures the weight of the sample. Titrimetry is the technique used to determine the concentration of the analyte.

Spectro-Chemical Analysis: Spectrometer helps to determine chemical composition by means of its measure of spectrums. The commonly used spectrometer in analytical chemistry is 'Mass Spectrometry'. In mass spectrometer, very small amount of sample is first ionized and then converted into gaseous ions, where the samples are first separated and then analysed on the basis of their mass-to-charge ratios.

Electroanalytical Analysis: Electroanalytical methods make use of the potential and current of an electrochemical cell. The three main parts of electroanalytical analysis are potentiometry, coulometry and voltammetry. Potentiometry is used to measure the potential of the cell, coulometry is used for measuring the current of the cell, and voltammetry is used for measuring the change in current when the potential of the cell changes.

Chromatography: Chromatography is referred as the specific method for separating the analyte from the given sample for measuring the sample without the interference from other compounds. Different types of chromatography methods are used for separating the analyte and the sample. In the 'Thin Layer Chromatography' method, the analyte mixture moves up and get separated along the coated sheet under volatile mobile phase. In the 'Gas Chromatography' method the gas is used for separating the volatile analytes. When the 'Liquid' is used as mobile phase in 'Chromatography' then a common method is used which is termed as 'High-Performance Liquid Chromatography'.

Spectroscopy

Spectroscopy is used for measuring the interaction of the molecules with the electromagnetic radiation. Spectroscopy method includes several different types, such as Atomic Absorption Spectroscopy, Atomic Emission Spectroscopy, Ultraviolet-Visible (U-V) Spectroscopy, X-Ray Fluorescence Spectroscopy, InfraRed (IR) Spectroscopy, Raman Spectroscopy, Nuclear Magnetic Resonance (NMR) Spectroscopy, Photoemission Spectroscopy, MössBauer Spectroscopy (MBS), Circular Dichroism Spectroscopy, etc.

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The 'Nuclear Spectroscopy' method uses the properties of a nucleus for exploring the properties of the material, specifically the general structures of the materials. Commonly used methods include Nuclear Magnetic Resonance (NMR) Spectroscopy, MössBauer Spectroscopy (MBS), Perturbed Angular Correlation (PAC), etc.

1.2.1 Colorimeter

Colorimetry is the technique used to measure colours. People have been using colour to decorate, to represent images and beliefs, as a status symbol etc. for thousands of years. The knowledge of colour and appearance has made the people more demanding with respect to choice and aesthetics. This has created an increasing demand for more accurate and easier ways to measure colour and appearance. Colorimetry is a valuable way of determining the concentration of a chemical in a coloured solution. It also helps in measuring the intensity of the colour and relates the intensity of the colour to the concentration of the solution.

Principle

Colorimetry uses the basic principles of photometry. It utilizes the basic properties of colour to detect the concentration of a substance in a solution. The only thing important is that it should be coloured: if it is not coloured, various pigments are mixed so that it becomes coloured and can be analyzed with a colorimeter.

Colour is actually the electromagnetic radiation in the wavelength region between approximately 380–780 nm. The colour that you see with your eye is influenced by the spectral distribution of the light source, the reflectance or transmittance properties of the object the sensitivity of the eye and the brain. The human eye is most sensitive at 555nm.

There are three dimensions to colour, which are as follows:

- Hue
- Value
- Intensity

This makes colour multidimensional-any colour appearance can be described in terms of the above three dimensions.

Hue

This dimension, which is the colour you see. It is the contrast between redness, blueness, and greenness.

Value

This dimension refers to the degree of lightness or darkness of the colour. It is often related to a gray scale where white is the lightest value followed by a series of grays to black, the darkest value.

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Chroma

Chroma refers to the colour's level of intensity and richness. It is the intensity of the colour. Chroma refers to the purity or impurity of a hue. The more pure hue a given colour contains, the more intense it is.

Tristimulus Values

The tristimulus values of an object can be calculated by combing the reflectance or transmittance of the object with the spectral power distribution of an illuminant and the colour matching functions of a standard observer. Two objects (that matched earlier) may not look the same as before when the source or observer changes. Metameric coloured objects have the same tristimulus values for the illuminant under which they match but different tristimulus values for illuminants where they do not match.

Principle and Working

When light is passed through a homogeneous medium, a part of the light is reflected, a part is absorbed within the medium and rest is transmitted.

The intensity of the light emerging from the medium decreases exponentially as the concentration and thickness of the absorbing medium increase arithmetically, as per the Beer's-Lambert's law. If the container and the thickness of the medium is kept constant, the intensity of light emerging out of the medium, with respect to the incident light, is directly proportional to the concentration of the medium.

With this principle the concentration of a coloured solution is determined by measurement of relative absorption of light with respect to a solution of known concentration.

Instrumentation

The essential components of a colorimeter are as follows:

- **Light Source:** The light source is often an ordinary low-voltage filament lamp. In modern colorimeters, the filament lamp and filters may be replaced by several light-emitting diodes of different colours. Previously, the colorimeters had incandescent white light as the light source. However, newer colorimeters make use of Light Emitting Diodes (LED) as light source.
- **Aperture:** The aperture limits the incoming light. It is adjustable and helps to pass only a beam of light from the light source. Hence, unwanted or stray light is prevented.
- **Filters:** The filters are usually made of coloured glass. These are generally used for selecting the light of narrow wavelength, that is, for selecting monochromatic light. Filters also absorb light of unwanted wavelength and allow only monochromatic light to pass through them. In order to maximize accuracy, changeable filters are used in the colorimeter to select the wavelength of light which the solute absorbs the most. The usual wavelength range is from 400 to 700 nanometres (nm).

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Table 1.1 *List the Filters Normally Used in a Colorimeter.*

Solution colour	Wavelength range	Filter
Blue	650-700	Red
Blue Green	580-650	Orange
Orange Purple	500-590	Green
Yellow	460-540	Blue
Yellow Green	390-490	Violet

- **Cuvette or Cell:** It is a sample holder that holds the working solution. The monochromatic light from the filter passes through the colored solution in the cuvette. The colored solution in the cuvette absorbs part of light and remaining is allowed to fall on the detector. In a manual colorimeter the cuvettes are inserted and removed by hand. An automated colorimeter is fitted with a flowcell through which solution flows continuously.
- **Detector:** The detectors are photosensitive elements (usually a photoresistor is used) and they convert light energy into electrical signal. This electric signal is directly proportional to the intensity of light falling on the detector. Hence, higher is the electric signal higher is the intensity of light.
- **Display Meter:** The output is displayed by an analogue or digital meter and may be shown as transmittance (a linear scale from 0-100%) or as absorbance (a logarithmic scale from zero to infinity). The useful range of the absorbance scale is from 0-2, but it is desirable to keep within the range 0-1 because, above 1, the results become unreliable due to scattering of light. In addition, the output may be sent to a chart recorder, data logger or computer.

Figure 1.1 shows the various components of a colorimeter.

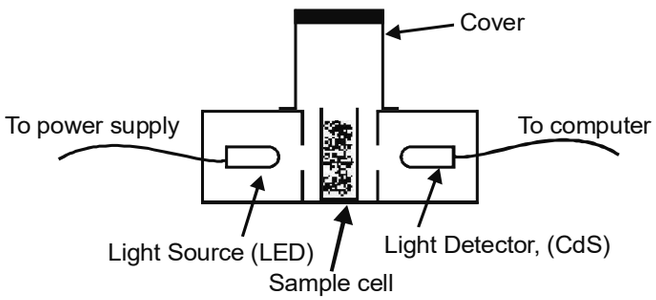

Fig. 1.1 *Colorimeter*

Applications

There are many applications of colorimetry in different fields such as:

- In television and videos, to analyze the colour contrast and brightness adjustments for obtaining an optimized picture display on the screen
- In printing industry
- In electronics, checking the electronic components
- In paper industry, to assess the quality of pulp paper and measuring the quality of printing ink

- In jewellery, to measure the optical properties of precious stones
- In biochemical tests, to assess the water quality of a region
- In agriculture, to determine concentration of plant nutrients
- In dentistry, to assess colour matching of porcelain crown
- In dermatology, to measure the sun protection factor
- In medicine, to assess haemoglobin in blood
- In digital imaging, it measures the luminosity and chromaticity coordinates of different spatial locations

1.2.2 Spectrophotometer

The absorption of light is a well-known concept. The property of absorption of visible light makes the objects look coloured. For example, blue ink appears blue because the light at the red end of the light spectrum is absorbed and the blue light is reflected from the ink to the observer's eye.

Principle

UV-VIS Spectrometry runs on the Beer–Lambert law that is related to absorption of light of that region of the electromagnetic spectrum for which this law was developed.

Beer Lambert Law

The absorbance or optical density and transmission of light through a sample can be calculated by measuring the light intensity entering and exiting the sample.

The following terms are defined:

- Light Intensity entering a sample is "I_0"
- Light Intensity exiting a sample is "I"
- The concentration of analyte in sample is "C"
- The length of the light path in glass sample cuvette is "L".
- "K" is a constant for a particular solution and wave length

The Beer-Lambert Law is given by the following equations:

Light Absorbance (A) = $\log (I_0 / I) = KCL$

Light Transmission (T) = $I/I_0 = 10\text{-}KCL$

The law can be stated in a single equation as:

A=ebc

Where A is absorbance

e is the molar absorbtivity ($L\ mol^{-1}\ cm^{-1}$)

b is the path length of the sample (cm).

c is the concentration of the compound in solution, (mol L-1)

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Instrumentation

All spectrophotometer instruments have the basic components as follows:

1. Source of radiant energy (Light).
2. Wavelength selector: It helps in isolation of a desired wavelength from the source (filter or monochromator).
3. Transparent container (cuvette) for the sample and the blank.
4. Radiation detector (phototube): It converts the radiant energy received to a measurable signal; and a readout device that displays the signal from the detector.

The materials used in the instruments actually depend on the wavelength selected.

Wavelength selection is an important task. If the visible region is used, the analyte may not absorb, but can be transformed chemically to produce a coloured product. The use of coloured filters limits the range of wavelength. This limited range is referred to as band width of a filter.

The methods which employ filter selectors and depend on the production of a coloured compound are the basis of colorimetry.

If the wavelength is selected using prisms or gratings, the technique is referred to as spectrophotometry.

Working

The samples to be tested are used in solution and are placed in a small silica cell. Two types of lamps are used: a hydrogen or deuterium lamp and the tungsten/halogen lamp. The tungsten/halogen lamp is used for the ultraviolet region and a deuterium lamp for the visible region. Hence, both the ultraviolet and the visible regions are covered.

Another cell called as reference cell containing only solvent is used. Light is passed simultaneously through the sample cell (sample cuvette) and reference cell (reference cuvette). The spectrometer compares the light passing through the sample cell with that passing through the reference cell. The transmitted radiation is detected by the spectrometer. Then the absorption spectrum is recorded by scanning the wavelength of the light passing through both these cells.

The two laws governing the working of spectrometer are:

- Beer's law
- Lambert's law

For most spectra the solution obeys Beer's Law.

Beer's Law

It says that the light absorbed is proportional to the number of absorbing molecules, *i.e.,* to the concentration of absorbing molecules. But this law is only valid for dilute solutions.

Lambert's Law

It says that the fraction of radiation absorbed is independent of the intensity of the radiation.

If we combine these two laws, we get what is called as the Beer–Lambert law.

It says $\log_{10} I_0/I = \alpha\, lc$

where;

I_O = the intensity of the incident radiation

I = the intensity of the transmitted radiation

α = the molar absorption coefficient

l = the path length of the absorbing solution (cm)

c = the concentration of the absorbing species in mol dm^{-3}

Two useful pieces of information are obtained by using Beer Lambert law. These are

- the molar absorption coefficient
- the wavelength at which maximum absorption occurs.

These help us in identifying a substance and if these two values are known we can calculate the concentration of a compound in a solution.

Figure 1.2 shows the working of a UV-VIS spectrometry.

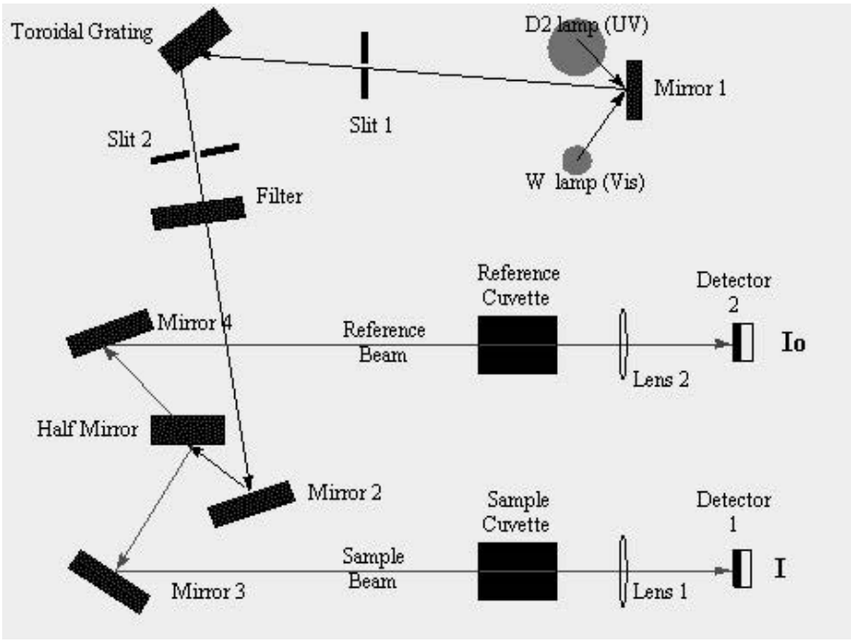

Fig. 1.2 *Working of a UV-VIS Spectrometry*

Applications

Ultraviolet-visible spectrometry is used in the following fields:

- Analysis of pigments, paints, ink and dyeing material etc.
- Colour matching analysis

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- Denture materials
- Study of metal oxidation
- Determination of protein content
- Biochemical analysis of limited sample solution
- Dissolution testing in pharmaceutical industry
- Analysis of sunscreen cream potential in dermatology.

1.2.3 Ultracentrifuge

The 'Ultracentrifuge' is referred as a centrifuge optimized method for spinning a rotor at very high speeds and is efficient to generate acceleration as high as 1 000000 g (approx. 9 800 km/s^2). Two types of ultracentrifuge methods are used, namely the 'Preparative Ultra Centrifuge (PUC)' and the 'Analytical Ultra Centrifuge (AUC)'. Both types of ultracentrifuge instruments are significantly used in molecular biology, biochemistry, and polymer science.

Analytical Ultracentrifugation: This method is used to:

- Determine of the purity and oligomeric state of macromolecules by recording sedimentation velocity data.
- Determine the average molecular mass of solutes in their natural state.
- Study the changes in the molecular mass of supramolecular complexes using the sedimentation velocity or sedimentation equilibrium or both.
- Detect the conformation and conformational changes.

Preparative Ultracentrifugation: This method is used to,

- Evaluate subcellular fractionation.
- Determine the affinity purification of membrane vesicles.
- Separate DNA components.
- Determine the colloid separation.
- Analyse Virus purification.

Ultracentrifuge Instrumentation: Ultracentrifuges are of different types based on the specific structure of rotors appropriate for different experiments. Most of the rotor's design have tubes inside for containing the samples. The 'Swinging Bucket Rotors' contain the tube that hangs on the hinges such that the tubes reorient towards the horizontal when the rotor initially increases its speed or accelerates. The 'Fixed Angle Rotors' are typically made from a single block of material for holding the tubes in the specific holes or spaces that are already bored for rotating at a predetermined angle. The 'Zonal Rotors' are exceptionally designed for containing large volume of sample in a single central cavity instead of tubes. Some zonal rotors can dynamically load and unload samples even though the rotor is spinning at high speed.

Preparative rotors are used in biological investigations specifically for pelleting of fine particulate fractions, such as cellular organelles (mitochondria, microsomes, ribosomes) and viruses. In addition, the gradients of 'Sucrose' are typically used

to separate cellular organelles, gradients of 'Caesium Salts' are used to separate nucleic acids.

1.2.4 Densitometric Scanner

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Densitometric scanner is used in the 'Densitometry' which is the quantitative measurement of optical density in light-sensitive materials, such as photographic paper or photographic film, due to exposure to light. Following are the types of densitometers:

Transmission Densitometers: Transmission densitometers are used to measure transparent materials, to measure transparent surfaces and to measure color transparencies. Film and transparent substrates are some examples of common transparent surface measures.

Reflection Densitometers: Reflection densitometers are specifically used to measure light reflected from a surface.

A densitometer is a device specifically used for measuring the degree of darkness (the optical density) of a photographic or semi-transparent material or of a reflecting surface. The densitometer is essentially a light source aimed at a photoelectric cell since it determines the density of a sample from differences in the readings that is placed between the light source and the photoelectric cell. Modern densitometers have electronic integrated circuitry for improved reading.

Optical density is obtained as a result of the darkness of a built or developed image or picture that is absolutely expressed as the number of dark spots in a given area, but generally it is a relative value expressed in a scale. Density is also termed as absorptiometry, which refers to the measure of light absorption through the medium, because the density is generally measured by means of the decrease in the amount of light which shines through a transparent film. The measuring device is called a absorptiometer or densitometer. The decadic or base-10 logarithm of the reciprocal of the transmittance is termed as the absorbance or density.

The material can produce maximum and minimum density termed as DMax and DMin refer, respectively. The key difference between the DMax and DMin is the difference in the density range. The density range can be associated to the dynamic range, which is referred as the range of light intensity represented by the recorded through the Hurter–Driffield curve.

Bone Density: Bone Density, or Bone Mineral Density (BMD), is defined as the amount of bone mineral in bone tissue. The basic concept states that the bone density is, the 'Mass of Mineral Per Volume of Bone', even though upon imaging it is measured clinically through proxy as per the definition of optical density per square centimetre of bone surface. Bone density measurement is typically used in the clinical medicine as an indirect indicator of osteoporosis and fracture risk. Densitometry measurement is used to measure the bone density, normally done in the radiology or nuclear medicine departments of hospitals or clinics. The bone density measurement is painless and non-invasive and involves low radiation exposure. Measurements are most commonly made over the lumbar spine and over the upper part of the hip. The forearm may be scanned if the hip and lumbar spine are not accessible. The poor bone density can be the reason for higher probability of bone fracture.

Bone density measurements are specifically done by screening the people for risk of osteoporosis and to identify the people who have poor bone density so that their bone strength can be improved after measuring the bone density.

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1.2.5 Differential Scanning Calorimeter

Differential Scanning Calorimetry (DSC) is referred as a thermoanalytical technique in which the difference in the amount of heat required to increase the temperature of a sample and reference is measured as a function of temperature. Both the sample and reference are maintained at approximately the same temperature throughout the experiment. Usually, the temperature program for a DSC analysis is designed in such a way that the sample holder temperature increases linearly as a function of time. The reference sample should have a well-defined heat capacity over the range of temperatures to be scanned.

The Differential Scanning Calorimetry (DSC) technique was developed by E. S. Watson and M. J. O'Neill in 1962 and was commercially introduced at the 1963 Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy. The first adiabatic differential scanning calorimeter that could be used in biochemistry was developed by P. L. Privalov and D. R. Monaselidze in 1964 at Institute of Physics in Tbilisi, Georgia. The term DSC was coined to describe this instrument, which measures energy directly and allows precise measurements of heat capacity.

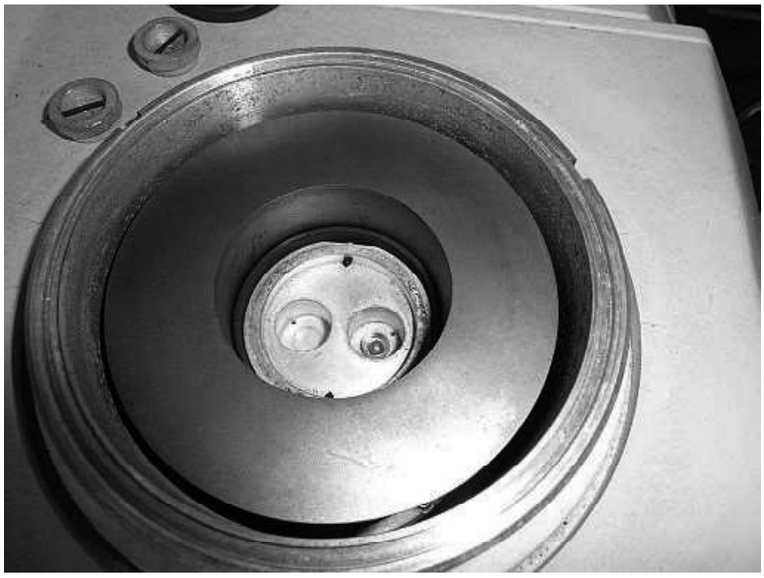

Fig.1.3 *Differential Scanning Calorimeter*

Types of Differential Scanning Calorimetry (DSC)

There are two main types of Differential Scanning Calorimetry (DSC), namely the 'Heat-Flux DSC' which measures the difference in heat flux between the sample and a reference and 'Power Differential DSC' which measures the difference in power supplied to the sample and a reference.

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Heat-Flux DSC: With Heat-Flux DSC, the changes in heat flow are calculated by integrating the DT_{ref-} curve. For Heat-Flux DSC experiment, a sample and a reference crucible are placed on a sample holder with integrated temperature sensors for temperature measurement of the crucibles. This arrangement is performed in a temperature-controlled oven. Contrary to this classic design, the distinctive attribute of MC-DSC is the vertical configuration of planar temperature sensors surrounding a planar heater. This arrangement allows a very compact, lightweight and low heat capacitance structure with the full functionality of a DSC oven.

Power Differential DSC: For Power Differential DSC type of setup, also known as Power Compensating DSC, the sample and reference crucibles are placed in thermally insulated furnaces and not next to each other in the same furnace like in Heat-Flux DSC type experiments. Then the temperature of both chambers is controlled such that the same temperature is always present on both sides. The electrical power is required for obtaining and remaining in this state is then recorded instead of the temperature difference of the two crucibles.

Fast-Scan DSC: The Fast-Scan DSC (FS DSC) was developed in the year 2000s, a novel calorimetric technique that employs micromachined sensors. The key advances of this technique are the ultrahigh scanning rate, which can be as high as 106 K/s, and the ultrahigh sensitivity with a heat capacity resolution typically improve than 1 nJ/K. Nanocalorimetry has attracted much attention in materials science, where it is applied to perform quantitative analysis of rapid phase transitions, particularly on fast cooling.

Detection of Phase Transitions

The basic principle underlying detection of phase transitions technique is that when the sample undergoes a physical transformation, such as phase transitions, more or less heat will be required to flow to it as compared to the reference to maintain both at the same temperature. Whether less or more heat should flow to the sample depends on whether the process is exothermic or endothermic. For example, as a solid sample melts to a liquid, it will require more heat flowing to the sample to increase its temperature at the same rate as the reference. This is due to the absorption of heat by the sample as it undergoes the endothermic phase transition from solid to liquid. Similarly, as the sample undergoes exothermic processes, such as crystallization, less heat is required to raise the sample temperature. By observing the difference in heat flow between the sample and the reference, the differential scanning calorimeters are capable to measure the amount of heat absorbed or released during such transitions. Differential Scanning Calorimeter or DSC may also be used to observe more subtle physical changes, such as glass transitions. It is widely used in industrial settings as a quality control instrument due to its applicability in evaluating sample purity and for studying polymer curing.

Differential Thermal Analysis (DTA)

An alternative technique, which shares much in common with Differential Scanning Calorimeter or DSC is a specific Differential Thermal Analysis (DTA). In Differential Thermal Analysis (DTA) technique it is the heat flow to the sample and reference

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that remains the same rather than the temperature. When the sample and reference are heated identically, phase changes and other thermal processes cause a difference in temperature between the sample and the reference. Both DSC and DTA provide analogous or similar information. DSC measures the energy required for keeping both the reference and the sample at the same temperature whereas DTA measures the difference in temperature between the sample and the reference when the same amount of energy has been introduced into both.

Differential Scanning Calorimeter (DSC) Curves

The result of a Differential Scanning Calorimeter (DSC) experiment is a curve of heat flux versus temperature or versus time. There are two different conventions, the exothermic reactions in the sample shown with a positive or negative peak depending on the kind of technology used in the experiment. This curve is specifically used for calculating enthalpies of transitions. This is done by integrating the peak corresponding to a given transition. The enthalpy of transition can be expressed using the following equation:

$$DH = KA$$

Where, DH is the enthalpy of transition, K is the calorimetric constant, and A is the area under the curve. The calorimetric constant will vary from instrument to instrument and can be determined by analysing a well-characterized sample with known enthalpies of transition. Following illustrations will make the concept clear.

Following Figure 1.4 is a schematic DSC curve of amount of energy input (y) required to maintain each temperature (x), scanned across a range of temperatures for protein denaturation.

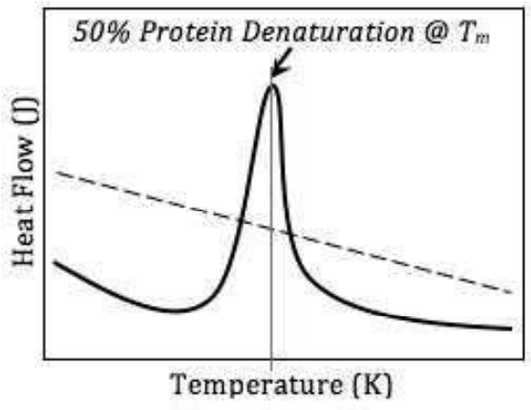

Fig. 1.4 *A Schematic DSC Curve*

Following Figure 1.5 illustrates normalized curves setting the initial heat capacity as the reference. Buffer-buffer baseline is shown as dashed and protein-buffer variance is shown as solid.

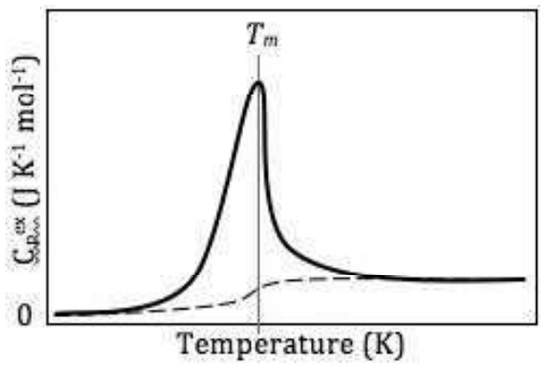

Fig. 1.5 *Normalized Curves Setting the Initial Heat Capacity as the Reference*

Following Figure illustrates the normalized DSC curves using the baseline as the reference (left side), fractions of each conformational state (y) existing at each temperature (right side), for two-state (top), and three-state (bottom) proteins. The minuscule broadening in the peak of the three-state protein's DSC curve, which may or may not appear statistically significant to the naked eye.

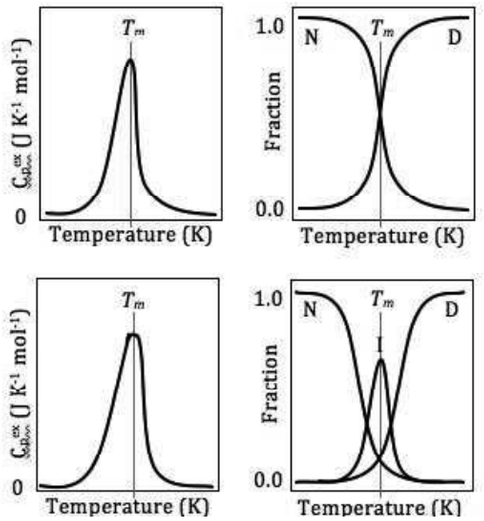

Fig. 1.6 *Normalized DSC Curves Showing Minuscule Broadening in the Peak of the Three-State Protein's DSC Curve*

1.2.6 ESR and NMR Spectrometers

Electron Spin Resonance (ESR)

Electron Spin Resonance (ESR) or Electron Paramagnetic Resonance (EPR) spectroscopy is a method used to study materials having unpaired electrons. The basic concepts of ESR are analogous to those of Nuclear Magnetic Resonance (NMR), but the spins excited are those of the electrons instead of the atomic nuclei. ESR spectroscopy is particularly useful for studying metal complexes and organic radicals. ESR was first observed in Kazan State University by Soviet physicist Yevgeny Zavoisky in 1944 and was developed independently at the same time by Brebis Bleaney at the University of Oxford.

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Electron Spin Resonance (ESR) spectroscopy has been widely applied in the research of biological free radicals for quantitative and qualitative analyses of Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS).

ESR spectroscopy is used in various branches of science, such as biology, chemistry and physics, for the detection and identification of free radicals in the solid, liquid, or gaseous state, and in paramagnetic centers, such as F-centers.

ESR is a sensitive, specific method for studying both radicals formed in chemical reactions and the reactions themselves. For example, when ice (solid H_2O) is decomposed by exposure to high-energy radiation, radicals, such as H, OH, and HO_2 are produced. Such radicals can be identified and studied by EPR. Organic and inorganic radicals can be detected in electrochemical systems and in materials exposed to UV (Ultra-Voilet) light.

ESR spectroscopy is a particularly useful tool to investigate their electronic structures, which is fundamental to understand their reactivity. ESR spectroscopy can be applied only to systems in which the balance between radical decay and radical formation keeps the free radical's concentration above the detection limit of the spectrometer used.

Medical and biological applications of EPR are also significant. Although radicals are very reactive, so they do not normally occur in high concentrations in biology, special reagents have been developed to attach 'Spin Labels', also called 'Spin Probes', to molecules of interest. Specially designed nonreactive radical molecules can attach to specific sites in a biological cell, and ESR spectra then give information on the environment of the spin labels. Spin-labelled fatty acids have been extensively used to study dynamic organisation of lipids in biological membranes, lipid-protein interactions and temperature of transition of gel to liquid crystalline phases. Injection of spin-labelled molecules allows for electron resonance imaging of living organisms.

Nuclear Magnetic Resonance (NMR)

Nuclear Magnetic Resonance (NMR) is a physical phenomenon in which nuclei in a strong constant magnetic field are perturbed by a weak oscillating magnetic field and respond by producing an electromagnetic signal with a frequency characteristic of the magnetic field at the nucleus. This process occurs near resonance, when the oscillation frequency matches the intrinsic frequency of the nuclei, which depends on the strength of the static magnetic field, the chemical environment, and the magnetic properties of the isotope involved; in practical applications with static magnetic fields up to ca. 20 tesla, the frequency is similar to VHF (Very High Frequency) and UHF (Ultra High Frequency) television broadcasts (60–1000 MHz). NMR results from specific magnetic properties of certain atomic nuclei. Nuclear Magnetic Resonance (NMR) spectroscopy is widely used to determine the structure of organic molecules in solution and study molecular physics and crystals as well as non-crystalline materials. NMR is also routinely used in advanced medical imaging techniques, such as in Magnetic Resonance Imaging (MRI).

The most commonly used nuclei are ^{1}H and ^{13}C, even though isotopes of several other elements can be studied by means of high-field NMR spectroscopy.

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To interact with the magnetic field in the spectrometer, the nucleus must have an intrinsic nuclear magnetic moment and angular momentum. This occurs when an isotope has a nonzero nuclear spin, i.e., an odd number of protons and/or neutrons. Nuclides with even numbers of both have a total spin of zero and are therefore NMR-Inactive.

A key feature of NMR is that the resonance frequency of a particular sample substance is usually directly proportional to the strength of the applied magnetic field. Since the resolution of the imaging technique depends on the magnitude of the magnetic field gradient, many efforts are made to develop increased gradient field strength.

The principle of NMR usually involves following three sequential steps:

Step 1: The alignment (polarization) of the magnetic nuclear spins in an applied, constant magnetic field $\mathbf{B_0}$.

Step 2: The perturbation of this alignment of the nuclear spins by a weak oscillating magnetic field, usually referred to as a Radio-Frequency (RF) pulse. The oscillation frequency required for significant perturbation is dependent upon the static magnetic field ($\mathbf{B_0}$) and the nuclei of observation.

Step 3: The detection of the NMR signal during or after the RF pulse, due to the voltage induced in a detection coil by precession of the nuclear spins around $\mathbf{B_0}$. After an RF pulse, precession usually occurs with the nuclei's intrinsic Larmor frequency and does not involve transitions between spin states or energy levels.

The two magnetic fields are usually chosen to be perpendicular to each other as this maximizes the NMR signal strength. The frequencies of the time-signal response by the total Magnetization (M) of the nuclear spins are typically analysed in NMR spectroscopy and Magnetic Resonance Imaging (MRI). Both the methods use applied magnetic fields ($\mathbf{B_0}$) of great strength, often produced by large currents in superconducting coils, in order to achieve dispersion of response frequencies. The information provided by NMR can also be increased using hyperpolarization, and/or using two-dimensional, three-dimensional and higher-dimensional techniques.

The biochemists use NMR to identify proteins and other complex molecules. Besides identification, NMR spectroscopy provides detailed information about the structure, dynamics, reaction state, and chemical environment of molecules. The most common types of NMR are Proton and Carbon-13 NMR spectroscopy, but it is applicable to any kind of sample that contains nuclei possessing spin. NMR phenomena are also utilized in low-field NMR, NMR spectroscopy and MRI in the Earth's magnetic field (referred to as Earth's field NMR), and in several types of magnetometers.

1.2.7 Microtomy

Microtome is made up of two words, micro meaning small and tome meaning cut. A microtome is an instrument that is capable of cutting a specimen in sections of predetermined sizes. Microtomes are important instruments in microscopy preparation. These help in preparation of sections of the sample under study that are to be observed under light or electron microscopes.

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Microtomes use different type of blades to cut the specimen. The blade can be made up of steel, glass, or diamond. The type of blade to be used depends on the sample being sectioned and the thickness of the section required. Steel blades are used to make sections of animal or plant tissues samples for light microscopic histological studies. Glass knives are used to make sections for light microscopic studies and to make very thin sections for electron microscopic examination. Diamond knives are used to make sections of hard materials such as bone, teeth and plant matter for both light microscopic and for electron microscopic examination.

Microtomy is a method for the preparation of thin sections using a microtome. Microtomes are available in various types and operations. Manual type microtomes are operated manually and they are generally used for clinical histopathology. Advanced versions like rotary microtones are also useful for histopathology applications. All microtomes are constructed to be rigid structures and also have facilities to clamp the tissue blade and block firmly. They also have some means of accurate advance of the tissue in relation to the blade that is measurable in micro meters. The sections prepared by a microtome can be as thin as 0.05 and 100 μm. These sections can now be studied under light and electron microscopes.

Historical Background

It was seen by researchers many centuries back that to observe the structure of the specimen under observation it was important to make clean reproducible cuts on the order of 100 μm, through which light can be transmitted and the specimen could be studied. This allowed for the observation of samples using light microscopes in a transmission mode.

In 1770, George Adams, Jr was the first researcher to make an instrument which could make sample sections of that order which could be studied. Later Alexander Cummins further developed this instrument. The instrument prepared was hand operated, and the sample was to be held in a cylinder and sections created from the top of the sample using a hand crank.

In 1835, Andrew Prichard developed a table based model of microtome. This allowed for the vibration to be isolated by affixing the device to the table, separating the operator from the knife.

Some researchers attribute an anatomist Wilhelm His, Sr. for the invention of the microtome.

Whereas other sources attribute the development of microtome to a Czech physiologist Jan Evangelista Purkyne. Several sources describe the Purkyne model as the first microtome which came in practical use.

At the end of the 1800s, the development of very thin and consistently thin samples by microtomy procedure advancements, together with the selective staining of important cell components or molecules allowed the researchers and scientists to visualize the microscope details of the specimens under study.

Today, the majority of microtomes are a knife-block design with a changeable knife, a specimen holder and an advancement mechanism. In most devices the specimens of the sample are made by moving the sample over the knife, where the

advancement mechanism automatically moves forward such that the next section of the sample of the required thickness can be made. The section thickness is controlled by an adjustment mechanism, allowing for precise control. Hence before starting to operate the microtome it is imperative to check whether all adjustment screws are tightly secured.

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Parts of a Microtome

A microtome typically consists of three basic parts:

1. A block holder
2. A knife and its carrier
3. Adjustment screws, ratchet feed wheels and pawl

Block Holder

The block holder holds the tissue to be sectioned in position. The block holder should be adjusted with adjustment screws and there should be no play in the holder.

Knife and its Carrier

The tissue is cut into sections using a knife. The cutting edge of the knife is kept parallel to the sample block and with the help of automated advancement; thin and consistent sections can be prepared.

Adjustment Screws, Ratchet Feed Wheels and Pawl

Pawl, ratchet feed wheels and adjustment screws help in lining up the tissue block in position, so that correct width of specimen sections are cut. There should be no play in these; else irregular sections might be prepared which may not be desirable.

Figure 1.7 shows the parts of microtome.

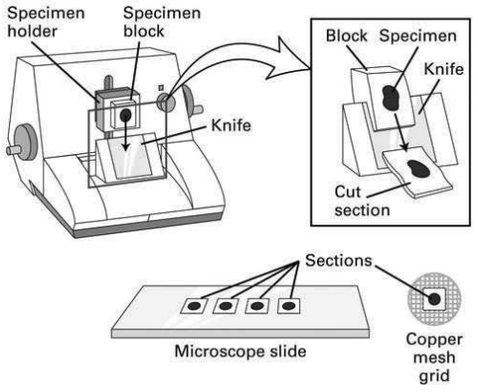

Fig. 1.7 *Parts of a Microtome*

Principle of Microtome

The principle guiding the working of microtome to get consistent same sized thin section is to place the edge of knife parallel to the sample block. Next the spring-balanced teeth or pawl is brought into contact with, and turns a ratchet feed wheel connected to a micrometer screw, which is in turn rotated, moving the tissue block

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to a predetermined distance towards the knife for cutting sections of uniform thickness. All the adjustments of the block and knife holders should be done so that the knife cuts the section at the pre determined distance.

A microtome should have the following features to cut the thin sections successively and consistently:

- All movements in a microtome should be free of vibrations
- The advancement mechanism should be free of static friction
- The incremental advancement of the specimen to be cut should be adjustable to about 0.01μm.
- The specimen should pass the knife edge only once.

Procedure of Using a Rotary Microtome

The procedure of using a rotary microtome involves the following steps:

1. Microtome is always placed in a stable location. It should not be moved every time we need to prepare sections. This is the most important point to remember for all operators.
2. The handle-wheel on the Microtome should be in the locked position before a sample is mounted.
3. Remove the microtome knife holder from its box and ensure that the disposable blade has been removed.
4. Attach a new blade to the holder with the help of a disposable blade dispenser and position the blade. Turn screws to secure the blade into its place.
5. Oil must be removed from the disposable blade of the microtome. These blades are covered with oil to prevent it from rusting during storage.
6. A tissue wet with xylene is used to gently wipe the exposed area of the disposable blade.
7. Fix the disposable blade and handle into position in the microtome. Use the vertical and horizontal tilt controlling adjustment screws as appropriate to adjust the angle and inclination of the specimens correctly with respect to the knife edge.
8. Lock the orientation head in position when the optimum orientation is obtained.
9. Keep the block holder to its rearmost point by turning the Coarse Advance Knob anticlockwise. There is an alarm fitted in most microtome which gives a sound signal when the limit is reached.
10. Adjust the section width that is required. This is usually about 4 – 5 μm.
11. Place a knife into the knife holder, release the clamping lever, located to the right of the clamp plate.
12. Put the knife guard over the cutting edge of the knife blade.
13. The paraffin block is inserted into the tissue clamp. Push the lever forward and place the block into clamp and release.

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14. Manually advance the block forward towards the microtome blade.
15. As the microtome handle is rotated, the chuck will move up and down and advance forward with each revolution.
16. Test if the block is close enough, release the handwheel brake and slowly rotate the handwheel clockwise—the knife should just trim the block.
17. When the assembly is ready, release the microtome handle-wheel and slowly start to cut the block.
18. Slowly turn the handwheel clockwise. Each time the handle of the handwheel moves from the 12 o'clock position to the 1 o'clock position, turn the Coarse Advance Knob approximately one eighth of a turn to advance the specimen toward the knife by approximately 25 μm.
19. The first few cuts will trim the block so you have an even surface and expose the embedded tissue.
20. Once the block has been trimmed it is a good idea to place it back onto the ice for a few minutes then re-attach to the tissue clamp.
21. Continue turning the handwheel and advancing the specimen until clean sections of the specimen are taken.
22. Engage the handwheel brake when trimming is completed and the specimen presents a clean smooth surface to the knife.
23. Start cutting the block and ribbons should begin to form.
24. If your ribbons are not forming adequately, there is some fault in the above said steps.
25. When the ribbons are of the desired quality, move them to the water bath using forceps and/or brushes. The water bath temperature should be set about 10°C less than the melting point of the wax.
26. When you have finished cutting ensure that you lock the microtome handle-wheel into place.
27. Remove the microtome knife or disposable blade handle when finished cutting.
28. If using a disposable blade loosen screws and use the blade dispenser to remove the used blade.
29. Never store blade holders with disposable blades still attached.
30. Never leave the microtome with a knife or disposable blade attached, and ensure that the rotary wheel is in the locked position when not in use.
31. Always place the knife guard over the knife blade when not in use.

Advantages of Using Microtome

There are many advantages of using a microtome such as:

- Sections are of uniform thickness. This cannot be regulated in free hand sections.
- Sections can be obtained at any desired thickness.

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- Sections obtained are not oblique.
- Sections of entire tissue can be obtained.

The above advantages help the observer of the specimen sections to get a clear picture of the cut section. This helps the observer to make correct diagnosis.

Steps in Blocking

The sample is placed in a block to be sectioned by a microtome. The block can be prepared by following the under given steps properly:

- Arrange the L mould on the sheet of glass. Place specimen the bottom of cavity made out of two l mould
- Pour melted paraffin in the cavity over the tissue freeze it for 15-20 minutes
- When moulds are hard, tap them on the table so that they get separated from the paraffin block
- Tissue should be atleast 1-3 mm from each side after trimming of the paraffin mould.

Section Cutting

The steps involved in section cutting are as follows:

1. All parts of microtome are checked and the block holder is mounted on the on microtome.
2. The angle of knife is adjusted (20 degrees) and the microtome is set at the thickness which is required for sections
3. The drive wheel is rotated. As soon as paraffin block makes contact with the razor, sections of paraffin are cut. As each section is cut, the impact of the knife edge on the block slightly meets the same and the subsequent sections adhere to the earlier one.
4. As soon as the ribbon is around an inch length, a brush is inserted below the same with the left hand and the ribbon is held while more sections are being cut.
5. When the ribbon is around 5 inch long the rotation of the wheel is stopped and the ribbon is cut against the middle part of knife with good scalpel leaving around 9 inch long ribbon attached to knife
6. The ribbon is spread on clean white paper. Section cutting is continued till the half of block is cut out.
7. In preparation of staining, paraffin has to be removed.

Microtome Knives

Microtome knives have two parts:

- Heel
- Toe

Heel is the angle formed by the cutting edge and the end of knife nearest the handle.

Figure 1.8 shows the different parts of a microtome knife.

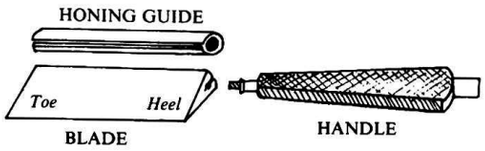

Fig. 1.8 *Parts of Microtome a Knife*

Toe is the angle formed by the cutting edge and the end of knife farthest from the handle.

The knife used in sectioning should have the following characteristics:

- It should be of sufficient hardness so that it does not chip off wen it cuts the hard block.
- It should be made up of homogenous material
- It should be stable under varying temperatures.
- It should not undergo chemical decomposition.

Earlier razor blades were used for sectioning. These days glass knives and diamond are used for sectioning. Whatever the type of microtome knife used, it should be sharp and well adjusted. The microtome knives are classified in many types.

Classification I

- Heiffor type knives
- Large type knives

Generally, Heiffor type knives are used in rocking microtome. The handle of this type is fixed.

Large type knives are used for freezing and base sledge microtomes. The handles of this type are detachable.

Classification II (based on the profile of the knife blade)

- Planar concave; sharp, but are delicate and hence only used with very soft sample.
- Wedge shaped; more stable and used in moderately hard materials, such as, in epoxy or cryogenic sample cutting.
- Chisel shaped; has blunt edge which increases the stability of the knife but also requires significantly more force to achieve the cutting.

Figure 1.9 shows the various microtome knives based on their profiles.

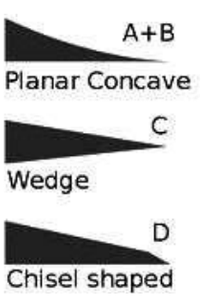

Fig.1.9 *Different types of Microtome Knives*

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Types of Microtomes

Microtomes are mechanical devices for cutting thin uniform slices of tissue sections. The tissue is supported in its embedding medium—usually paraffin wax, but possibly resin or nitrocellulose— and is normally moved, one step at a time between cuts, by an automatic advance towards the knife. In certain designs, the knife is moved towards the tissue block. In both cases, the amount of the advance is operator determined, most commonly in graduated 1um stages. When cutting paraffin-wax embedded tissue, the properties of the wax causes each section to adhere by its edge to the previous one forming a ribbon of sections.

Excluding ultramicrotomes there are five basic types, named according to the mechanism-rocking, rotary, base sledge, and freezing microtomes. Occasionally a microtome may be referred to as a retracting microtome, referring to the withdrawal of the block from the back of the knife on the return stroke. It is essential for the production of resin sections, and usually a feature of rotary microtomes.

The Rocking Microtome

This instrument is one the oldest in design, cheap and simple to use. It is also extremely reliable, requiring the very minimum of maintenance. The knife is fixed and the block of tissue moves through an arc to strike the knife; between strokes the block is moved towards the knife for the required thickness of the sections, by means of a ratchet operated micrometer thread. The name of the microtomes derives from the rocking action of the cross-arm.

A disadvantage is that the size of block that can be cut is limited, although designs have been introduced to overcome this. Moreover, since the block moves through an arc when cutting, the sections are cut in a curved plane. This is more of a theoretical than a practical disadvantage unless the block is subsequently to be cut perfectly flat type of microtome. However, rocking microtomes designed to cut perfectly flat sections, the block moving through an arc at right angles to the knife edge are available.

In view of the lightness of this type of microtomes it is advisable either to fit it into a tray which is screwed to the bench, or to place it on a damp cloth to avoid movement during cutting. The movement of the cutting arm should depend on the type of tissue to be cut; normally a steady forward and backward movement of the handle will give ribbons of good sections, but with difficult tissues there are two alternative movements worth trying:

1. Pulling the handle forward and releasing it from this position, allowing the spring to pull it back sharply, or, if this does not produce a good section.
2. Pulling the handle forward and letting it back very slowly. One of these methods will usually result in a ribbon of good sections.

Figure 1.10 shows a rocking microtome.

Fig. 1.10 *Rocking Microtome*

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Rotary Microtome

This instrument is a common microtome design. The rotary microtomes are so called because a rotary action of the hand-wheel sets in motion the cutting movement. The block-holder is mounted on a steel carriage which moves up and down in grooves, and is advanced by a micrometer screw. The rotary microtome operates with a staged rotary action such that the actual cutting is part of the rotary motion. At the highest point of the rotary motion, the sample holder is advanced by the same thickness as the section that is to be made, allowing for the next section to be made. In a rotary microtome, the knife is typically fixed in a horizontal position. The sections are cut perfectly flat consistently of same thickness.

The flywheel in many microtomes can be operated by hand. This has the advantage that a clean cut can be made, as the relatively large mass of the flywheel prevents the sample from being stopped during the sample cut. The flywheel in newer models is often integrated inside the microtome casing. The typical cut thickness for a rotary microtome is between 1 to 60 μm. For hard materials, such as a sample embedded in a synthetic resin, this design of microtome can allow for good 'Semi-thin' sections with a thickness of as low as 0.5 μm.

Figure 1.11 shows a rotary microtome.

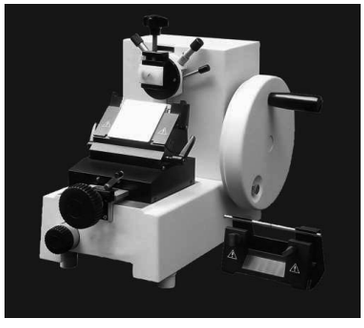

Figure 1.11 *Rotary Microtome*

Advantages of a Rotary Microtome

It has the advantages of being the following:

- Heavier and therefore more stable than the rocking type.

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- Ideal for cutting serial sections.
- Larger blocks of tissue may be cut on this machine, and the cutting angle of the knife is adjustable.
- Less likelihood of vibration, since a heavier and larger knife is used with this type of microtome, when cutting exceptionally hard tissue.
- By using a special holder to set the knife obliquely it may be used for cutting celloidin-embedded sections also.

Sledge Microtome

A sledge microtome is a device where the sample is placed into a fixed holder (shuttle), which then moves backwards and forwards across a knife. Originally designed for cutting sections of very large blocks of tissue (e.g., whole brain), the sledge microtome has become a popular machine for routine use since the Second World War. The block-holder is mounted on a steel carriage which slides backwards and forwards on guides against a fixed horizontal knife. By adjusting the angles between the sample and the microtome knife, the pressure applied to the sample during the cut can be reduced. This microtome is heavy and consequently very stable and not subject to vibration; the knife used is a large one (24 cm in length) and usually wedge-shaped, which again reduces the possibility of vibration and requires less honing. The knife-holding clamps are adjustable and allow the tilt and the angle (slant) of the knife to the block to be easily set. There is an adjustable knife holder in the microtome and hence, this machine may be used for cutting celloidin sections by setting the knife obliquely. A freezing stage is available on this machine.

Typical applications for this design of microtome are of the preparation of large samples, such as those embedded in paraffin for biological preparations. The microtome normally gives a cut thickness between 1to 60 μm.

A limitation of this type of microtome is that it is slower than rocking or rotary microtomes; this is true only when a change from one instrument to another is made, after using the microtome for sometime the sections can be cut quite efficiently and there is no time wastage.

Figure 1.12 shows a sledge microtome.

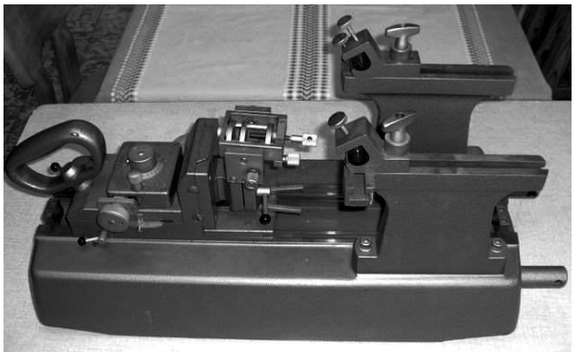

Fig. 1.12 *Sledge Microtome*

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Sliding Microtome

In this type, the knife is moved horizontally against a fixed block which is advanced against it up an inclined place.

The sliding microtome was designed for cutting celloidin-embedded sections, and is probably the best type if sufficient celloidin sections are cut to justify its purchase. It can also be used for paraffin-wax embedded sections.

Figure 1.13 shows a sliding microtome.

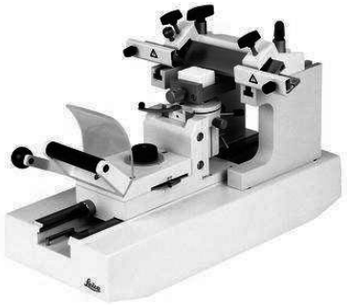

Fig. 1.13 *Sliding Microtome*

Freezing Microtome

For the cutting of frozen samples, many rotary microtomes can be modified to cut in a liquid nitrogen chamber, in a so-called cryomicrotome setup. However, this type will give the best results and is used almost universally.

The machine is clamped to the edge of a bench and is connected to a cylinder of CO_2 by a strengthened flexible metal tube. Some precautions to be taken for the position of the cylinder: liquid CO_2 must reach the valve in the chuck; therefore, ordinary cylinders must be held upside down in a special holder, or blocks placed under them to ensure that the following is maintained:

1. The cylinder valve is the lowest point of cylinder
2. That the cylinder valve is above the level of the chuck of microtome.

A special cylinder fitted with a central tube is also available.

The reduced temperature allows for the hardness of the sample to be increased, such as, by undergoing a glass transition, which allows for the preparation of semi-thin samples. However the sample temperature and the knife temperature must be controlled in order to optimize the resultant sample thickness.

Figure 1.14 shows a freezing microtome.

Fig. 1.14 *Freezing Microtome*

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Ultramicrotome

An ultramicrotome can allow for the preparation of extremely thin sections, with the device functioning in the same manner as a rotational microtome, but with very tight tolerances on the mechanical construction. As a result of the careful mechanical construction, the linear thermal expansion of the mounting is used to provide very fine control of the thickness. These extremely thin cuts are important for use with transmission electron microscope (TEM), and are sometimes also important for light-optical microscopy. The sections of thickness is between 40 and 100 nm for transmission electron microscopy. Thicker sections up to 500 nm thick are also taken for specialized transmission electron microscopy applications or for light microscopy survey sections to select an area for the final thin sections.

Figure 1.15 shows an ultramicrotome.

Fig. 1.15 *Ultramicrotome*

Laser Microtome

The laser microtome is an instrument for contact free slicing i.e. there is no contact of the knife blade with the sample. There is no requirement of prior preparation of the sample and hence saves time and chances of error. This microtome can be used for hard materials, such as bones or teeth and some ceramics. The device operates using a cutting action of an infra-red laser. The thickness of sections achievable is between 10 and 100 μm.

Figure 1.16 Shows a laser microtome.

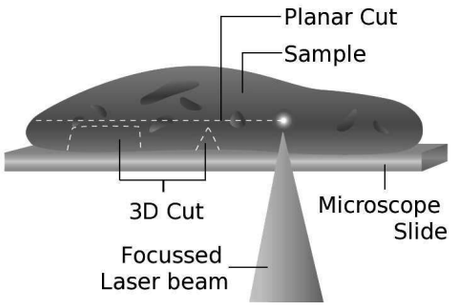

Fig. 1.16 *Laser Microtome*

Table 1.2 summarizes the various types of microtomes generally used for microtomy.

Table 1.2 *Various Types of Microtomes Used for Microtomy*

Rocking Microtome	Rotary Microtome	Base sledge Microtome	Sliding Microtome	Freezing Microtome	Ultra Microtome
This type of microtome is used for cutting sections from small blocks of paraffin embedded tissues and serial sections tissue cutting. Rocking microtome is not suitable for large blocks of hard material. The knife of the mictotome is fixed with edge upward and its cutting stroke is spring operated. The block of the tissue moves horizontally and strikes against the knife edge.	like rocking microtome the knife is fixed with the edge upward. The downward movement of the block holder is not spring operated and is under the control of the operator. The instrument is ideal for cutting of serial sections, large blocks of tissue, paraffin wax embedded material.	the knife of the microtome is fixed, specimen of tissue is mounted in a heavy base which moves to and fro on runners against knife. The instrument is used for cutting large and hard specimens like bone and teeth, and for celloidin embedded tissue.	this is used for mainly celloidin embedded tissue but can also be used for cutting paraffin embedded tissue. The knife is obliquely set and moves horizontally against a fixed block.	it is specially designed for the preparation of frozen sections of fixed or unfixed tissues without preliminary embedding. Liquid carbon dioxide is used for rapid cooling of the specimen and the microtome knife. The knife moves horizontally across the surface of the specimen.	very thin sections (.005 to .1 micron) can be prepared for study under electron microscope. In ultra microtome very small blocks of tissue which is embedded in a hard synthetic resin can be cut. Its knife is specially prepared with plate glass (diamond). Ultra microtome is fitted with a binocular microscope above the object for making proper orientation.

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Various Factors Affecting Microtomy

The factors that affect microtomy have been discussed here.

Paraffin Wax: Various characteristics of paraffin wax required for making blocks to hold the specimen are:

- Knife of the microtome should cut the paraffin wax smoothly. The paraffin wax should be pre treated to adequate temperature.
- There should be continuous shearing seen in the paraffin wax.

Temperature: The temperature desirable for effective cutting of blocks has following characteristic features:

- Temperature of the block should be lower than the surrounding atmospheric temperature. Aerosol freezing compound or ice cube can be used for this.
- The block should be adequately moistened.
- If the atmospheric temperature is very cold, then the surrounding atmosphere needs to be warmed first and then sectioning be done.

Angle of Knife: The smooth cutting of the block can be achieved by a flatter angle of knife. The flat angle of knife means that the rake angle is high. This high

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rake angle is required for cutting the soft tissue samples and a lower rake angle is required for cutting harder tissue samples.

Rake Angle: It is the angle between the leading edge of a knife and a perpendicular to the surface being cut, i.e., in case the block.

Figure 1.17 shows the rake angle being positive when the blade of the microtome is behind the perpendicular drawn.

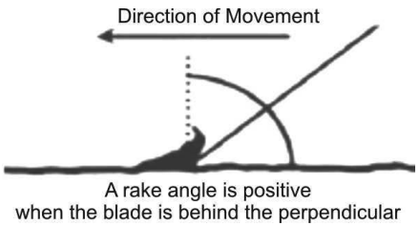

***Fig. 1.17** Positive Rake Angle*

Speed of cutting section: It depends on the hardness or softness of the tissue specimen being sectioned. As a general rule, soft tissue specimen cut well with slow speed and harder tissues cut better with high speed. However, with experience he operator can determine the accurate speed for various tissue samples.

Working

The procedure for cutting the tissue specimen starts with fixing the microtome. The knife is adjusted at a proper angle in the knife holder. Next, adjust the block on the platform and check whether the whole surface of block moves parallel in relation to knife. Next the paraffin block containing specimen is prepared by exposing the specimen in the paraffin block. Set the thickness gauge of the microtome to the required thickness of specimen sections. The block is secured tightly and brought close to the knife edge. Next the cutting is started.

Once the section is cut, it is mounted on the slide. This requires pretreating the section to remove any creases that might have occurred during cutting. This is done by two methods:

- Hot plates
- Water bath method

Hot Plate

The hot plate is set at a temperature of 55-60ºC for 15 minutes, once the section on the slide is kept on the hot plate. This helps in desiccation of the specimen. Another way is to flood the hot plate with distilled water and then the section with slide is put and the section keeps floating in distilled water. The heat conducted through water flattens the section and then the excess water is drained off, the section is now transferred back to hot plate.

Water Bath

The water bath is first filled with distilled water and the water bath is set at a temperature of 10º C below the melting point of paraffin wax. The creases in

section disappears when the section are placed in water and are separated from other sections by touching the junction of the specimens and letting them to spring open. The sections get flattened when kept in warm water. However, flattening the sections can cause cracks in the section; the sections should therefore be prepared as flat as possible.

The air bubbles which are present under the sections in water bath should also be removed. A wax roll is prepared and is gently placed under the section to touch the air bubble. The air bubble adheres to the wax roll and both of them are removed from the water bath.

Mounting of the section is done next by immersing half of the slide in water and touching the edge of the section with the slide. Now the slide is gently removed bringing the flattened section with it. Now the section is oriented on a wet slide and the slide is transferred to the draining rack. Once the excess water has drained off, the sections may then be placed in hot oven set at a temperature of 50ºC for an hour or so to completely dry the section and the slide. The section is now set for staining procedure.

Faults and Remedial Measures for Section Cutting

There are various faults which occur during section cutting; however, there are certain remedial measures also available to reduce the occurrence of the faults.

Some of these faults are as follows:

1. **Cutting of Section Vertically:** It happens because the knife used in the cutting is blunt, dirty or may have small nicks on the cutting edge. calcium salts in the specimen tissue is also one of the reason. Hence always use a sharp and clean knife for cutting sections.
2. **Sections Thickness Varies:** Sometimes the sections are cut alternatively as thick and thin. This happens because the tissue is hard and needs to be softened. It can also occur because the microtome screws were not adjusted properly or the knife used is blunt. Hence always use a sharp knife and soften a hard tissue specimen before cutting.
3. **Curled Sections:** This happens because the knife angle of tilt is high or the knife is blunt. Hence always use a sharp knife at proper angulations for cutting.
4. **Crumbling of Sections while Cutting:** This happens because the knife used is blunt or the paraffin wax in which the tissue specimen is embedded is too soft or crystallized. Hence always use a sharp knife and the wax should be pretreated to bring it to correct temperature.
5. **Creases cannot be Removed:** This happens due to compression of the blocks which results in the breadth of the block is more than the breadth of the sections. The block gets compressed because the wax used may be too soft or the knife is blunt or there is loss of bevel of the knife. Hence the knife edge needs to be honed to restore it. Also wax used should be pretreated to bring it to the correct temperature and consistence before cutting.

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Section Adhesives

The section adhesives are used for simply sticking the section on the slide. For various ester and polyester wax blocks embedded specimens, adhesives are useful. However, for haematoxylin and eosin slides, adhesives are optional. Various adhesives used are albumen, starch, gelatin, araldite etc. amongst these araldite is the most used and successful adhesive.

Albumin

Mix one white of an egg with an equal volume of glycerin. Beat vigorously to obtain a foamy mass. Let stand for a few hours, discard the supernatant foam. Add a crystal of thymol if the adhesive is to be kept for adhesion of other sections. This will increase the shelf life to upto one month. Ideally the adhesive should be prepared every day.

Spread a thin film on a slide and let the albumen dry on it. Use boiled distilled/deionized water, cooled down to room temperature, to stretch the sections on a hotplate or above a flame.

Gelatin

This can be utilized as 0.5% solution in distilled water as albumen-glycerin adhesive.

Araldite

This is one of the most successful adhesive. It is an epoxy resin. It is used in 1 in 10 dilution of the resin in acetone and is coated on the clean slide just before using it. As the section dries, the epoxy resin forms a rigid bond between the tissue and the slide.

Starch

It was once a very popular adhesive but because of its staining reactivity with many dyes, it has lost its popularity.

Sectioning of Specimens Embedded in Celloidin Blocks

The standard method of sectioning the specimens embedded in celloidin blocks is the wet method. 70% alcohol is used to keep the block as well as the knife wet. Various microtomes can be used to cut the sections like sliding microtome, sledge microtome, rotary microtome etc. Amongst these sections are best cut by sliding microtome.

The blocks used for celloridin or paraffin may be represented as shown in Figure 1.18.

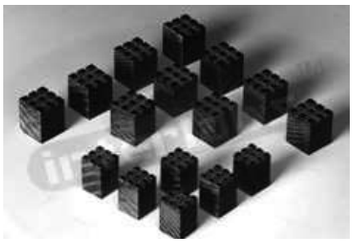

Fig. 1.18 *Blocks Used for Celloidin or Paraffin*

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Technique for Wet Method

The technique for wet method of section cutting is as follows:

- The microtome is fixed and the block is set in a block holder.
- The knife is set obliquely in the knife holder at an angle of 30 to 40°.
- The block is made to touch the knife edge such that the block is parallel to the knife edge.
- The section thickness gauge is set at an approximately 15µm.
- The block is moistened with 70% alcohol.
- The sections can be cut by a jerking section or by first cutting till half of the block and then moving the knife back and then going forward to cut through the whole block.
- It is mandatory to keep the cut sections in 70% alcohol.

Technique for Dry Method

The technique for dry method of section cutting is similar to the wet method except for the moistening of blocks with 70% alcohol. However, the cut sections are stored in 70% alcohol.

Serial Sectioning

Successive sections should be cut since with celloidin, the ribbons cannot be cut.

Fixing

The steps of fixing are as follows:

- The cut sections of specimens which are stored in 70% alcohol are transferred to 90% alcohol for a couple of minutes.
- The section is then taken and orient it on the slide.
- At the same time, let the alcohol drain from the section and slide.\
- The sections are the treated with vapors of ether. The vapors dissolve the thin celloidin attached to the section and will help in adhering the section to the slide.
- The slide is placed with section in 80% alcohol for five minutes. This helps in hardening the celloidin.
- The next step is to clean the slide with water and stain the section.

Technique for Frozen Sections

A freezing microtome is required to make sections of frozen tissue specimens. This technique is useful for detecting the fats and lipids and for rapid penetration of sections for use in diagnosis.

Fixatives Used

The fixation of frozen specimen is done by use of the following fixatives:

- Formal saline

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- Fixatives containing alcohol
- Chrome osmium fixative
- Mercuric chloride fixative
- Gum syrup solution fixative

Formal Saline: Frozen sections require only a few seconds for fixation/ the fixation facilitates easy cutting of the sections. Ten percent pre heated formal saline is used. The frozen sections are kept in 10% pre-heated formal saline for ten minutes at a temperature of 60 ºC. Alternatively, frozen sections can be dipped in boiling formal saline for 5 minutes. This will harden and stabilize the section.

Alcohol Based Fixatives: The alcohol containing fixatives can also be used to fix the frozen sections. However, before sectioning, the specimen should be thoroughly washed with water.

Chrome Osmium Fixative: Another alternative for fixation of frozen sections is using chrome osmium fixative. However, the tissues get hardened and can sometimes make sectioning difficult. The remedy is to wash the specimen in water overnight followed by soaking in gum syrup solution for 2-3 hours before proceeding for sectioning.

Mercuric Chloride Fixative: A fixative containing mercuric chloride also hardens the tissue like chrome osmium fixative and makes sectioning difficult. Hence, the sections should be immersed in iodine alcohol for at least half an hour followed by immersion in nathio sulphate for a period of fifteen minutes. It is washed for several hours in water before being sectioned.

Gum syrup Fixative: Immersing the specimen in gum syrup solution for one minute forms a hard rubbery layer on the specimen making sectioning relatively easier.

Cutting Frozen Sections

The steps involved in cutting frozen sections are as follows:

- The microtome as well as the carbon dioxide cylinder are fixed.
- The gum syrup solution is applied on the stage of microtome and then the tissue specimen is placed.
- The light is set at a proper angle making sure that the upper surface of tissue is just touching the edge of the knife and are parallel to each other.
- Short bursts of carbon dioxide are given onto the specimen.
- Each burst should be of 1-2 seconds and with a pause of 3-4 seconds in between.
- As the gum/water gets frozen, more of it is applied.
- This process is continued till a thickness of 1/8 inch is achieved all around the tissue specimen.
- The firm, rubbery consistency of tissue is checked.
- The set up gauge is set up to the require thickness. Generally, 10-20 µm sections are easily cut.

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Fixing

Fixing may be accomplished either by floating the section on the slide, celloidinization or by using gelatinized or albuminized slide. These methods may be described as follows:

Floating on Slide

The section is floated in water. The slide is immersed from one slide beneath the section. The section gets stuck to the slide by slowly and smoothly removing the slide from water. The excess water is drained off from the slide. The slide is inserted back into the water taking care that only half of the cut section is submerged. This procedure helps in removing any creases that were formed in the cut section. This procedure is repeated for the other half of the cut section. The cut section is now immersed in 70% percent alcohol for a few seconds and then immersed in water. A filter paper that is slightly larger in size to the cut section is placed squarely or oblong on the cut section. The slide is held in water close to the section such that the slide is slightly contacting the section. The slide is now removed gently. It is to be seen that the filter paper remains above the section. The excess water is drained off the slide and the section is kept over a bunsen flame for not more than 1 second. The filter paper becomes white and can be easily separated from the slide.

Celloidinization

In celloidinization, once the section is on the slide and excess water is drained off, a filter paper is kept on the section and it is moistened with alcohol for a few seconds. The alcohol is then blotted off. The slide is now immersed in 1% celloidin for five minutes followed by immersion in 70% alcohol for ten minutes. This helps to harden the celloidin.

Gelatinized Slides

In gelatinized slides, once the section is on the slide and the excess water is drained off, the gelatinized slid is treated with formalin vapors. The gelatine converts to an irreversible gel. This holds the section on the slide. The slide with section is washed after half an hour with water for at least ten minutes. Now the slide is ready to be stained.

Albuminized Slides

In albuminized slides, a mixture of clove and aniline oil in equal parts is poured on the slide. It takes 3 minutes to coagulate the albumin. Xylol is poured on the slide followed by alcohol. The slide is now ready to be stained.

Technique for Cryostat Sections

The introduction of cryostat has led to the demise of the freezing microtome from the laboratories. The potential application of cryostat has made this microtome to be widely adopted by the laboratories. The first cryostat was introduced by Linderstrom-Lang and Mogensen in 1938. The microtome offers the performance, ease-of-use and versatility to make it the ideal instrument in all types of laboratory work. The Cryostat Microtome is used for cutting thin sections of fresh, frozen material. The temperature in a cryostat varies between –10 to –40°C. the microtome is capable of cutting sections in the range of 2-16 m.

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Freezing Tissue for Cryostat Sectioning

All tissue types except muscle tissue can be frozen. Remember the following points:

1. The tissue to be sectioned is snap-frozen by quenching in liquid nitrogen.
2. Quenching is accomplished by putting a small amount of OCT compound onto a cork disc. Next the tissue is placed on it. It is then dropped upside down into the liquid nitrogen.
3. The frozen sample is attached to a cryostat chuck.
4. The sample is then put into the cryostat for 15-20 minutes to get warm.
5. If the sample is not pre-treated with liquid nitrogen quenching, it may be frozen using CO_2 apparatus.
6. After fixing the sample, it is washed in running water for several hours to remove the fixative.

Cutting of the Sections: The steps of cutting sections with a cryostat are as follows:

- The knife used should be sharp and brought to the working temperature of the cryostat. An antiroll plate is adjusted which ensures that the cut sections are flat and do not roll up on the knife edge when it is cut.
- The top of anti roll plate should be projecting about 0.5 mm above the cutting edge of the knife and at the same time parallel to it.
- As the section is cut, the anti roll plate is swung back and the section is picked up.

Handling of Section: The sections are attached directly to warm slide. This is accomplished by slightly touching the section against the slide. The slide is then air dried for at least 5 minutes and then treated with a fixative.

Fixative Used: The fixative generally used for cryostat sections acetic alcohol. Once the section is fixed it is ready to be stained.

Applications of Microtomes

The most common applications of microtomes include:

- **Traditional histology technique:** Tissues are hardened by replacing water with paraffin. The tissue is then cut in the microtome at thicknesses varying from 2 to 50 µm thick. From there the tissue can be mounted on a microscope slide, stained with appropriate aqueous dyes after prior removal of the paraffin, and examined using a light microscope. This provides a complete clear picture of the cut section.
- **Cryosectioning:** This technique is much faster than traditional histology and is used in conjunction with medical procedures to achieve a rapid intraoperative diagnosis. Cryosections can also be used in immunohisto-chemistry as freezing tissue stops degradation of tissue faster than using a fixative and does not alter or mask its chemical composition as much. The frozen sections are also used for:

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- o Diagnostic and research enzyme histochemistry.
- o Diagnostic and research non enzyme histochemistry
- o Immunohistochemistry methods
- o Immunofluorescent methods
- o Silver methods, mainly in the field of neuropathology.

- **Electron microscopy:** After embedding tissues in epoxy resin, a microtome equipped with a glass or gem grade diamond knife is used to cut very thin sections (typically 60 to 100 nanometers). Sections are stained with an aqueous solution of an appropriate heavy metal salt and examined with a transmission electron microscope. This instrument is often called an ultramicrotome. The ultramicrotome is also used with its glass knife or an industrial grade diamond knife to cut survey sections prior to thin sectioning. These survey sections are generally 0.5 to 1 micrometer thick and are mounted on a glass slide and stained to locate areas of interest under a light microscope prior to thin sectioning for the TEM. Thin sectioning for the TEM is often done with a gem quality diamond knife. Ultramicrotomes are also used for SEM and TEM studies polymeric systems with metal containing clusters. An ultramicrotome can also be used with a scanning probe microscope for the serial section tomography of the wide range of biological and polymer materials.
- **Botanical microtomy:** Hard materials like wood, bone and leather require a sledge microtome. These microtomes have heavier blades and cannot cut as thin as a regular microtome. However a clear picture of the sections of plants and trees wood etc. Can be achieved.
- Spectroscopy, especially FTIR or infra-red spectroscopy, where thin polymer sections are needed in order that the infra-red beam will penetrate the sample under examination. It is normal to cut samples to between 20 and 100 micrometres in thickness. For more detailed analysis of much smaller areas in a thin section, FTIR microscopy can be used for sample inspection. Infra red spectra of the cut sections can be studied as they give a very close picture of the specimen.

Check Your Progress

1. What is a scientific instrument?
2. Which technique is used to determine the concentration of the analyte?
3. What are three dimensions to colour?
4. On which law UV-VIS spectrometry runs?
5. Define ultracentrifuge.
6. State Beer's law.
7. Define densitometer.
8. What is Differential Scanning Calorimetry (DSC)?
9. What do you understand by Nuclear Magnetic Resonance (NMR)?

1.3 MICROSCOPY

NOTES

Microbiology is the study of microorganisms. Microorganisms are all single-cellular microscopic organisms and include the virus as well, which are microscopic but not cellular. Microbial cells differ from the cells of plants and animals in that, microorganisms are independent entities that carry out their life processes independently of other cells. Because of the nature of discipline microbiology, the microscope is of crucial importance and demand attention. Thus, it is important to understand how the microscope works and the way in which specimens are prepared for examination. Microscopes are used for a variety of purposes. They are used for medical purposes, in the diagnosis of illnesses. They are used for biology research, scientific research, medical research, and in environmental scientific research.

Definition: The word microscope is derived from the Latin word *'micro'*, which means small, and the Greek word '*skopos'*, to look at. So, microscopic means invisible to the eye unless aided by a microscope. A microscope is an instrument used to see objects that are too small to be seen by the naked eye. Microscopes are specialized optical instruments designed to produce magnified visual or photographic (including digital) images of the objects or specimens that are too small to be seen with the naked eye. Microscopy is the science of investigating small objects and structures using such an instrument. In general terms, microscopy is the technology of making very small things visible to the human eye.

Principle: Modern compound microscopes operate using a dual stage magnifying design that incorporates a primary imaging lens, the objective, coupled to a secondary visualizing lens system known as the eyepiece or ocular mounted at the opposite ends of a body tube. The objective is responsible for primary image formation at varying magnifications, while the eyepiece is used to observe the image created by the objective.

History: The study of the minute organisms was started after the invention of the microscope. Robert Hooke (1635–1703), an English mathematician and natural historian, was also an excellent microscopist. Robert Hooke (in 1665) devoted his first famous book *Micrographia* to explain the microscopic observations. The first person to see bacteria was the Dutch draper and amateur microscope builder Antoni van Leeuwenhoek (1632–1723). Antony van Leeuwenhoek invented a single lens microscope in the 1660s that could magnify a sample 200 times. He constructed simple microscopes capable of magnifying objects 100 to 300 times. As compare to van Leeuwenhoek, Robert Hooke built compound microscopes which have multiple lenses. However, these early compound microscopes were of poor quality and could not be used to see bacteria. It was about in 1830 that a significantly better microscope was developed by Joseph Jackson Lister (the father of Joseph Lister). In 1625, the name 'microscope' was chosen by Giovanni Faber to reference the compound

microscope created by Galileo Galilei's. Zacharias Jansen introduced microscope currently used in most laboratories.

1.3.1 Microscopy: Principle of Light Transmission

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Microscopy is the technique which uses microscopes to view objects and areas of objects that cannot be seen with the naked eye. i.e., objects that are not within the resolution range of the normal eye. There are three branches of microscopy, the optical microscopy, the electron microscopy, and the scanning probe microscopy, along with the emerging field of X-ray microscopy.

Optical microscopy and electron microscopy involve the diffraction, reflection, or refraction of electromagnetic radiation/electron beams interacting with the specimen, and the collection of the scattered radiation or another signal in order to create an image. Scanning probe microscopy involves the interaction of a scanning probe with the surface of the object under study. X-ray microscopy is three-dimensional and non-destructive technique. A 3D X-ray microscope uses the technique of Computed Tomography (microCT), rotating the sample 360 degrees and reconstructing the images. Computed Tomography (CT) is typically carried out with a flat panel display. A 3D X-ray microscope employs a range of objectives, for example from 4X to 40X, and can also include a flat panel.

Optical microscopes are not capable of showing things that are smaller than light waves, because of the diffraction limit. Following microscopes are used to see smaller things:

- Atomic Force Microscope (AFM)
- Scanning Electron Microscope (SEM)
- Scanning Ion-Conductance Microscopy (SICM)
- Scanning Tunnelling Microscope (STM)
- Transmission Electron Microscopy (TEM)
- Ultra-Violet (UV) Microscope
- X-Ray Microscope

Transmitted Light Microscopy

Transmitted light microscopy is one of the techniques of the light microscopy. It's associated for any type of microscopy where the light passes from the source to the opposite side of the lens. This method it's used to distinguish the morphological characteristics and optics proprieties of the observed material.

Principle

The light rays are organized and conducted through the instrument so the light can be polarized and redirected.

The light from the condenser fills the plane of the objective and a ray of light will be project to lighten the field, basically the condenser is able to control the angle of the illumination which permits the right balance of resolution and contrast in the microscope.

The light passes through the sample and it will go to the objective where the image will be magnified. The second step it's going to the oculars when the

enlargement can be observed. This type of microscopy permits the capture of high-quality images but the most important factor it's the illumination because permits the capture of images bright enough to be useful.

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Techniques Involving a Transmitted Light Path

Bright-Field: This method of microscopy is most commonly used. It helps to observe tissues because it makes the object appear against a bright background this is caused by the absorption of part of the transmitted light in dense areas. The inventor was Köhler hence it is called Köhler illumination.

Dark-Field: This method is used for biological samples, such as bacteria and microorganisms. In this technique the entrance of the light is bigger which permit the diffraction of the lights rays and it is illuminated obliquely. As a result, the field around the specimen is generally dark because of which the bright parts can be clearly observed.

Phase Contrast: The light that passes through the sample is retarded so the sample is illuminated by the rays of light. This technique is important in biology because it reveals many cellular structures. Sometimes these light rays are associated to the 'Normal' rays and occurs interference, that could be constructive and destructive, leading to the production of light and dark characteristic in the image.

Polarisation: Normally polarised light is used to analyse birefringent structures. The birefringent structures are structures with two different indices of refraction. From the interaction of the birefringent samples with the polarized light the image contrast is obtained. The function of polarised light microscopy is to measure the quantity of retardation and provides molecular information about birefringent specimen.

Differential Interference Contrast Optics: This method is used to expand the contrast in transparent/non-coloured specimens so that the invisible features can be observed to obtain information about the optic density of it. With this technique black and white images can be achieved on a grey background. The main difference between this type of method and the phase contrast is bright diffraction aureole.

Facts About the Microscope

- The earliest microscopes were known as the '**Flea glasses**' because they were used to study small insects.
- A father-son duo, Zacharias and Han Jansen, created the first compound microscope in the 1590s.
- Antonie van Leeuwenhoek created powerful lenses that could see teeming microbes (bacteria) in a drop of water.
- Leeuwenhoek was also unwilling to part with any of the 419 microscopes he made. It was only near the time of his death that his daughter, on his direction, sent 100 of them to the Royal Society.
- Robert Hooke discovered cells by studying the honeycomb structure of a cork under a microscope.
- Marcello Malpighi, known as the father of microscope anatomy, found taste buds and red blood cells.

- German engineer Carl Zeiss revolutionized the quality of lenses in the 19th century.

Depending upon the lens used, the microscopes basically are of two types:

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Simple Microscope: When there is only single lens fitted inside the microscope. The very first microscopes had only one lens, and were referred to as simple microscopes. The simplest form of light microscope consists of a single glass lens mounted in a metal frame is called as a magnifying glass.

Compound Microscope: It consists of two or more than two lens system. A compound microscope invented in the 1590s and has at least two lenses, including one at the eye called the eye piece, and one at the end closest to the sample called the objective.

The **monocular** compound light microscopes are equipped with one eyepiece as compare to **binocular** microscope which has two eye pieces (Refer Figure 1.19). Binocular microscopes are generally used in pathological labs. They provide with better field view than the monocular microscope. The resolution power of the binocular microscope is also better than the monocular microscope. There are two basic types of compound light microscope on the basis of stand (that holds all of the components firmly in position); **upright** and an **inverted microscope**. In upright microscope, the light source is at the bottom and situated below the condenser lens. The objectives are above the specimen stage as in the most commonly used microscopes for viewing specimens. The inverted microscope is configured so that the light source and the condenser lens are above the mechanical stage, i.e., specimen, and the objective lenses are beneath it. It allows additional room for manipulating the specimen directly on the stage. Inverted microscope is mainly used for the microinjection of macromolecules into tissue culture cells, for in-vitro fertilization of eggs or for viewing developing embryos over time.

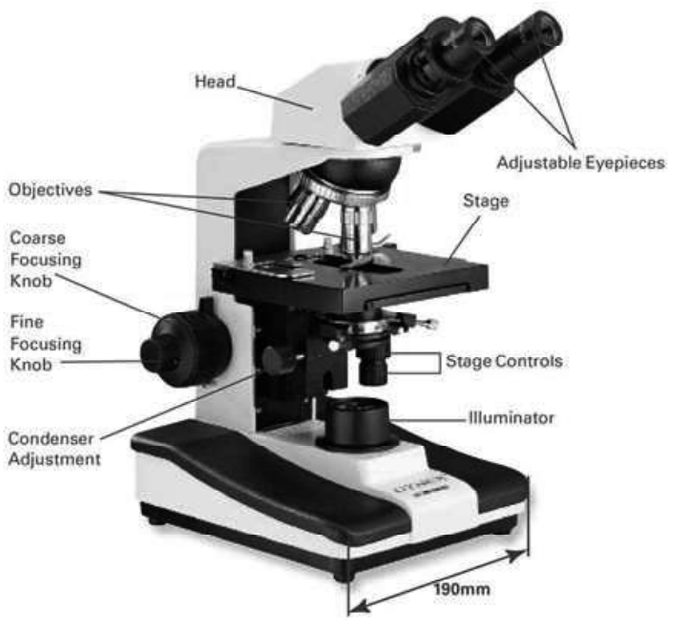

Fig. 1.19 *Binocular Microscope*

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Basic Configuration of Microscope: Microscope comprises of two main parts, the **supporting stand** (non-optical components) and the **optical system**. The supporting stand includes various parts which have role in providing mechanical strength to microscope. All microscopes have a basic frame structure, which includes the **arm** and **base.** All the parts are attached to these basic frameworks (Refer Figure 1.20).

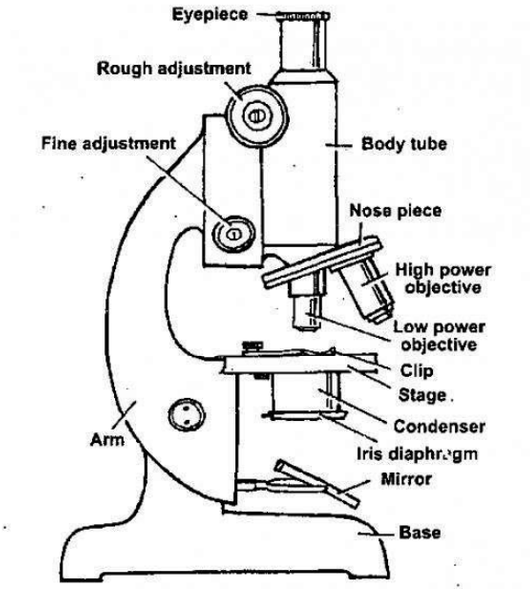

Fig. 1.20 *Diagram Showing Different Parts of Monocular Light Microscope*

- **Stage:** The horizontal platform that supports the microscopic slide on which the object to be examined is placed. It is usually equipped with mechanical devices that hold the glass slide firmly and on which the object is mounted so that it can be moved from place to place by setscrews.
- **Base:** It is U or horseshoe-shaped metallic structure that supports the whole microscope. It provides firm support and stability to the microscope.
- **Arm (Limb):** It is a curved metallic handle that connects with the base by inclination joint. It supports stage and body tube. It is also used for carrying the microscope can be set in a comfortable position.
- **Inclination Joint:** It is used for tilting the microscope if required for observation in sitting position.
- **Stage**: It is a metallic platform with a central hole fitted to the lower part of the arm. It acts as a platform which holds the microscopic slide in position. Microscopic slides held on the stage by either simple side clips or by a mechanical stage clip.
- **Body Tube**: It is meant for holding ocular and objective lenses at its two ends. The end holding ocular lens is called head while the end containing objective lens is called nose piece. The body tube has an internal pathway (mirrors and prisms) for the passage of light rays which form the enlarged image or microscopic objects.

- **Adjustment Screws**: There is two pair of screws for moving the body tube in relation to stage, larger for coarse adjustment and smaller for fine adjustment. In fine adjustment the body tube or stages moves for extremely short distances. The coarse adjustment is meant for setting objective lens at a proper distance from the object so as to form image of the same at the ocular end. Fine adjustment is required to obtain sharp image.

Optical System: Optical system is comprises of objective and ocular lenses (eyepieces) for imaging.

- **Diaphragm:** It is fitted just below the condenser for regulating the amount of light falling on the object. Opening and closing of iris diaphragm controls the light reaching the object. Diaphragm is of two types, disc and iris.
- **Condenser:** It is attached below the diaphragm. Condenser can be moved up and down to focus light on the object. Lowering the condenser diminishes illumination whereas raising the condenser increases the illumination. A correctly positioned condenser lens produces illumination that is uniformly bright and free from glare across the viewing area of the specimen.
- **Reflector (Mirror):** It is attached just above the base. Both its surface bear mirrors, plane on one side and concave on other side. Plane side is used in strong light and concave side in weak light. Reflector directs the light on the object through the condenser and diaphragm system.
- **Objective Lenses:** The objective lens consists of several lenses to magnify an object and project a larger image. They are fitted over the nose piece. According to the difference of the focal distance, objective lenses are of three types – low power (commonly 10X or 5X), high power (commonly 45X) and oil immersion (commonly 100X). Most objectives below 40X are air (dry) objectives, and those above 40X are immersion (oil, glycerol or water). It forms real inverted image of the object inside the body tube.
- **Ocular Lens or Eyepiece:** It is lens through which image of the microscopic object is observed. The image magnified by the objective lens is further magnified by the ocular lens for observation. Depending upon magnification, the eye piece is of four types-5X, 10X, 15X, and 20 X. Advanced microscopes have two eye pieces so that both the eyes can be used for observation. Microscope head having device for using two eye pieces is called binocular head. It contains a number of internal mirrors and prisms for the passage of light.

Magnification and Resolution

Microorganisms and their structural components are measured in very small units, such as micrometers (μm) and nanometers (nm). A micrometer is equal to 0.000001 m (10^{-6}m) and a nanometer (nm) is equal to 0.000000001 m (10^{-9} m). The **magnification** is the process of enlarging the image of an object or

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specimen. The **magnifying power** of a microscope is an expression of the number of times the object being examined appears to be enlarged. It is usually expressed in the form X, for example 10X for an image magnified 10-fold. The total magnification of a compound light microscope is the product of the magnification of its objective and ocular lenses. The total magnification of a specimen can be calculated by multiplying the objective lens magnification (power) by the ocular lens magnification (power). For example, if a 45X objectives is used in combination with a 10X eyepiece, the total magnification of the specimen will be 450X. A 500 nanometer long object was the smallest sample seen through a light microscope.

Total Magnification = Objective Magnification x Eyepiece Magnification

On the other way, the **resolution** (also called **resolving power**) of a microscope is a measure of the smallest detail of the object that can be observed. It is the ability of the lenses to distinguish two points a specified distance apart. Resolution is expressed in linear units, usually micrometres (μm). For example, if we say that a microscope has a resolving power of 0.4 nm, then it can only distinguish two points as separate objects if they are at least 0.4 nm apart. Light microscopes use glass lenses to bend and focus light rays and produces enlarged image of small objects. The resolution of a light microscope is determined by the numerical aperture of its lens system (a measure of light-gathering ability) and by the wavelength of the light it employs. The minimum distance (*d*) between two objects that separates them as individual entities is given by the Abbe equation. As *d* becomes smaller, the resolution increases, and finer detail can be discerned in a specimen. A general principle of microscopy is that the shorter the wavelength of light used in the instrument, the greater the resolution. The white light used in a compound light microscope has a relatively long wavelength and cannot resolve structures smaller than about 0.2 μm. As compare to visible light, ultraviolet light and high voltage electron beams are two sources of illumination with shorter wavelengths and used in the electron microscope. The resolution and the light-collecting capability of the microscope are determined by The Numerical Aperture (NA) of the objective lens. **Numerical Aperture** can be defined as function of the diameter of the objective lens in relation to its focal length. The NA is a measure of the ability of a lens to collect light from the specimen (Refer Figure 1.3). It is an important factor when considering the performance of the objective lens. Lenses with a low NA collect less light than those with a high NA. As the NA increases, the resolution and brightness of the lens improves. The working distance of an objective is the distance between the front surface of the objective lens and the surface of the cover glass or the specimen when it is in sharp focus. Objectives with large numerical apertures and great resolving power have short working distances.

$$\text{Minimum distance } (d) = \frac{\lambda}{2 \times NA} \quad \text{or} \quad \frac{0.5\,\lambda}{\eta \sin\theta}$$

Where, λ = Wavelength of light used which is usually set at 550 nm

Numerical Aperture (NA) = $\eta \sin \theta$

Here, η represents the refractive index of the medium through which light passes before entering the objective lens. And sin θ is the trigonometric sign of one half the angles formed by light rays coming from the condenser and passing through the specimen. Large NA objectives sometimes require the use of immersion oils between the object under inspection and the front of the objective. This is because the highest NA that can be achieved within air is an NA of 1 (corresponding to 90° angle of light). To get a larger angle and increase the amount of light entering the objective, it is necessary to use immersion oil (Refractive Index = 1.25) to change the refractive index between the object and the objective.

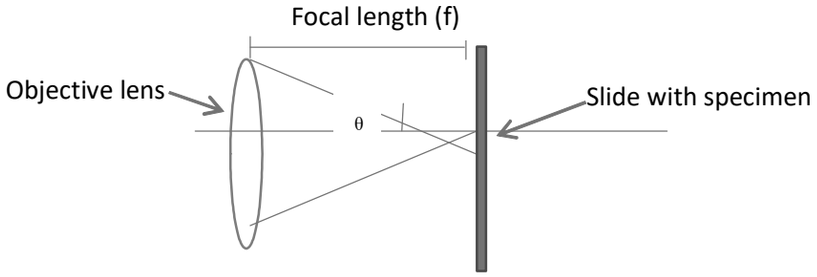

Fig. 1.21 *Diagramamatic Representation of Numerical Aperture*

The maximum theoretical resolving power of a microscope with an oil immersion objective (numerical aperture of 1.25) and blue-green light is approximately

$$\text{Resolving Power} = \frac{0.5\lambda}{\eta \sin\theta} = \frac{(0.5)(530nm)}{1.25} = 212\text{nm or } \mathbf{0.2\mu m}$$

(Here, $\theta = 90^{O}$; $\sin 90^{O} = 1$)

Hence, a bright-field light microscope can distinguish between two dots around 0.2 μm apart (the same size as a very small bacterium).

Working of Compound Microscope

A specimen AB to be magnified is placed in front of the objective lens just beyond its principal focus (F_O). The objective lens form an enlarged real image (A'B') within the microscope, and the eyepiece lens further magnifies this primary image into highly magnified virtual (A"B") image (Refer Figure 1.22). A'B' acts as an object for the eye piece lens, whose position is adjusted so that A'B' lies between optical centre C2 and the focus (F_e) of eye piece. The virtual image appears to lie just beyond the stage about 25 cm away. The lenses of the light microscope have a fixed focal length and are focused by moving them nearer to or further from the specimen with the help of coarse and fine adjustments.

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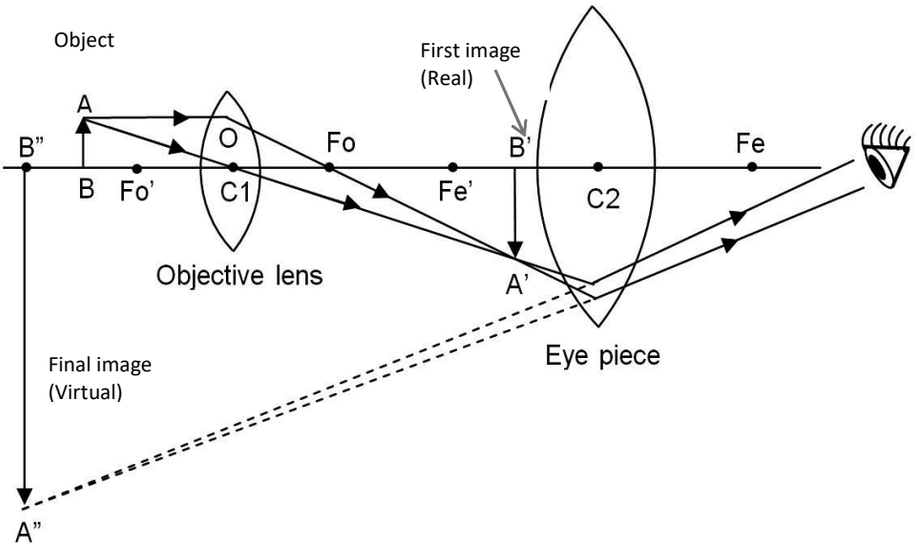

Fig. 1.22 *A Ray Diagram of Compound Microscope Showing Image Formation*

The **real image** implies the representation of an actual object, produced when the light rays arising from a single source converge at a particular (real) point. On the contrary, the **virtual image** can be understood as the image produced due to the apparent divergence of rays of light from a definite point.

Types of Microscope

Depending upon the source of illumination, the microscope can be classified as light microscope and electron microscope. In general, light microscopes are used to examine cells at relatively low magnifications, and electron microscopes are used to look at cells and cell structures at very high magnification.

Light Microscopy refers to the use of any kind of microscope that uses visible light to make specimens observable. In **Electron Microscope**, the image is formed on a fluorescent screen by electron beams instead of light rays and focused by magnets instead of lenses.

Over the years, several kinds of light microscopes have been developed, each adapted for making certain kinds of observations. Presently, there are mainly four types of compound light microscopes as described here: the bright-field, dark-field, phase-contrast, and fluorescence microscopes (Refer Table 1.3).

Table 1.3 *Advantages and Appearance of Specimens in commonly used Microscopes*

Sr. No.	Types of Microscopes	Appearance of Specimen	Advantages
1.	Bright Field Microscopy	Specimen generally stained and appear color of stain	Observing stained specimens and counting microbes
2.	Dark-field Microscopy	Specimen generally unstained and appears bright in an dark background	It allows living microbes to be viewed. Observation of unstained cells is possible.
3.	Phase-Contrast Microscopy	Structures varying degrees of darkness	Allows observation of motility, phagocytosis and intracellular structures
4.	Fluorescence Microscopy	Specimen color depends upon fluorescent dye used to stain it	Helpful in detecting specific infectious agents in tissue. Detecting immunological reactions.
5.	Electron Microscopy	Viewed on fluorescent screen	Allows viewing of very small objects like viruses and ultrastructure of cells

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Bright-Field Microscope: A microscope that allows light rays to pass directly through to the eye without being deflected by an intervening opaque plate in the condenser is called a bright-field microscope. The ordinary microscope is called a bright-field microscope because it forms a dark image against a brighter background. All bright-field microscopes have certain things in common, yet they differ somewhat in mechanical operation. The compound bright-field microscope is commonly used in laboratory courses in biology and microbiology.

Dark-Field Microscope: In dark field microscopy, the condenser is designed to form a hollow cone of light as compare to a full cone of light in bright-field microscopy (Refer Figure 1.23). To view a specimen, an opaque disc (dark-field stop) is placed underneath the condenser lens system. The condenser then produces a hollow cone of light, so that only light entering the objective lens comes from the specimen on the slide. The light travels only around the objective lens, but does not enter the cone shaped area. This hollow cone of light is focused on the specimen in such a way that unreflected and unrefracted rays do not enter the objective. Instead of coming up through the specimen, the light is reflected by particles on the slide. Only light that has been reflected or refracted by the specimen forms an image. The sample appears bright against a dark background when placed on the stage.

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Phase-Contrast Microscope: Due to the little difference in contrast between the cells and water (unstained living cells absorb practically no light), un-pigmented living cells are not clearly visible in the bright-field microscope. Phase-contrast microscopy is based on the principle that cells differ in refractive index (a factor by which light is slowed as it passes through a material) from their surroundings. Light passing through a cell thus differs in phase from light passing through the surrounding liquid. This difference in phase is amplified by a device in the objective lens of the phase-contrast microscope called the phase ring, resulting in a dark image on a light background. The condenser of a phase-contrast microscope has an annular stop, an opaque disk with a thin transparent ring, which produces a hollow cone of light. Phase-contrast microscopy is especially useful because it permits detailed examination of the internal structures in living microorganisms. It is very useful to observe living cells in their natural state without previous fixation or labeling.

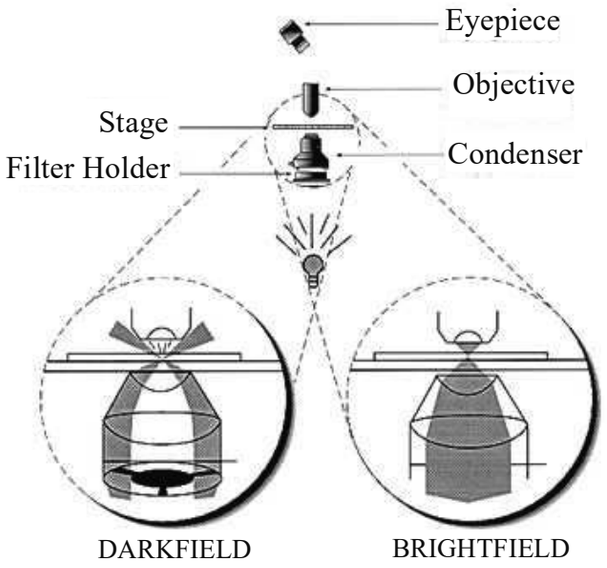

Fig. 1.23 *Basic Difference between Dark-Field and Bright-Field Microscopy: (A) Hollow Cone of Light Formed by Opaque Disc in Dark-field Microscopy; (B) Full Cone of Light in Bright-Field Microscope*

Fluorescence Microscopy

Fluorescence microscopy takes advantage of fluorescence, the ability of substances to absorb short wavelengths of light (ultraviolet) and give off light at a longer wavelength (visible). So, we can observe specimens that give off light of one color when subjected to light of another color. It illuminates a fluorochrome-labelled specimen and forms an image from it fluorescence. A fluorescence microscope is same as a conventional light microscope with some added features to enhance its capabilities. As compare to the conventional microscope which uses visible light (400-700 nm) to illuminate a sample, a fluorescence microscope uses a much higher intensity light source (ultraviolet light) which excites a fluorescent material

present in the specimen. This fluorescent specimen in turn emits a lower energy light of a longer wavelength that produces the magnified image. The light given off by the fluorescent material is of orange, yellow, or green color depending upon the type of fluorescent material.

Some specimens exhibit **autofluorescence** (contain naturally fluorescent materials) when they are irradiated with ultraviolet radiation, for example *Pseudomonas*. But, in most of the cases, the samples, such as *Mycobacterium tuberculosis* and *Treponema pallidum* are first labeled with a fluorescent substance (DiAmidino-2-Phenylindole (DAPI), rhodamine, auramine and fluorescein) known as a fluorophore and then illuminated through the lens with the higher energy source. The illumination source is usually a high-pressure mercury or xenon vapour lamp, and more recently lasers and LED sources, which emit from the UV into the red wavelengths. The illumination light is absorbed by the fluorophores that attached to the sample and causes them to emit a longer lower energy wavelength light. There are usually four main filters: a heat barrier, an excite filter, a dichromatic mirror (often called a dichroic) and a barrier filter (Table 1.4).

Table 1.4 *Different Parts of Optical System Used in Fluorescence Microscopy and their Functions*

Sr. No.	Important Parts	Functions
1.	Mercury Vapor Arc Lamp	A mercury vapor/xenon arc lamp produces an intense beam of ultraviolet, violet, or blue light.
2.	Heat Filter	Microscope uses a direct light source for illumination.
3.	Excite Filter	It allows only short wavelength light to pass forward. (About 400nm).
4.	Dichromic Mirror	A dichroic mirror allows light of a certain wavelength to pass through, while light of other wavelengths is reflected.
5.	Barrier Filter	It removes any remaining exciter wavelengths (up to about 500 nm) without absorbing longer wavelengths of fluorescing objects and prevents viewer's eyes.
6.	Dark Field Condenser	It provides a black background against which the fluorescent objects glow.

A mercury vapor arc lamp or other source produces an intense beam, and heat transfer is limited by special infrared filters that are called **heat filters**. Then, the light passes through an **exciter filter** that transmits only the desired wavelength. An excite filter allows a light of specific wavelength to pass through it that excites the fluorescent molecules and fluorophore in the specimen. For example, the commonly used fluorophore fluorescein is optimally excited at a wavelength of 488 nm, and emits maximally at 518 nm. The 488nm light is then directed to the specimen *via* the **dichromatic mirror**. Any fluorescein label in the specimen is excited by the 488 nm light, and the resulting 518 nm light that returns from the specimen passes through the **barrier filter** to the detector. The barrier filters only

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allow light of 518nm to pass through to the detector, and ensure that only the signal emitted from the fluorochrome of interest reaches it. This fluorescent light can be separated from the surrounding radiation with filters designed for that specific wavelength allowing the viewer to see only that which is fluorescing. A dark field condenser provides a black background against which the fluorescent objects glow. The filters and the dichroic mirror are often plugged in together in a filter cube. The fluorescence emitted from the specimen is often too low to be detected by the human eyes. But a sensitive digital camera, for example CCD or PMT easily detects such signals (Refer Figure 1.24).

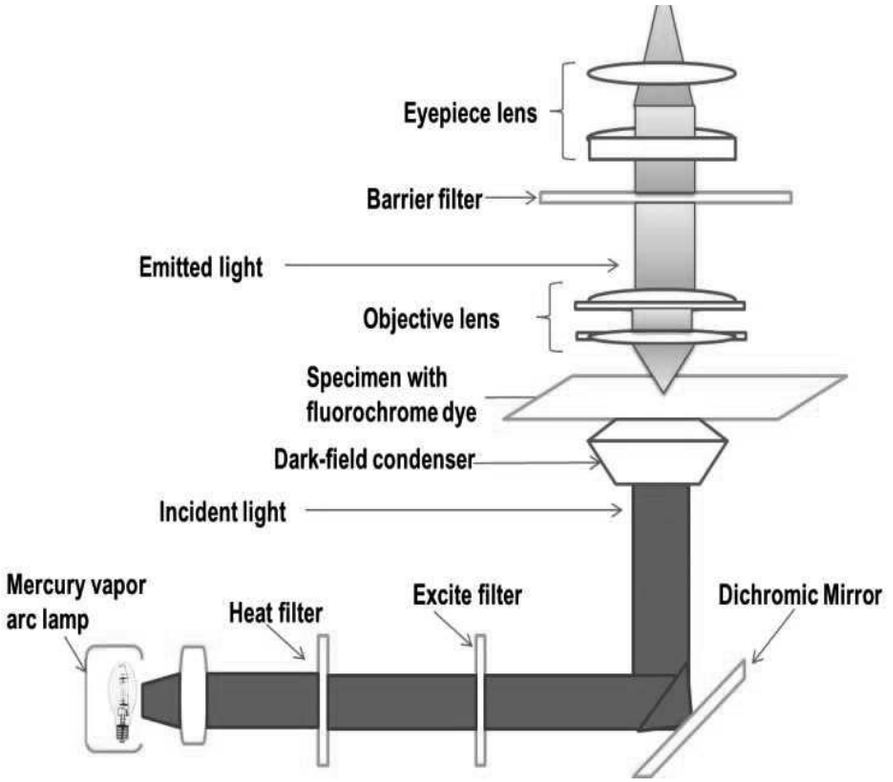

Fig.1.24 *Mechanism of Working for Fluorescence Microscope*

Applications: British scientist Sir George G. Stokes, first described fluorescence in 1852 and was responsible for coining the term when he observed that the mineral fluorspar emitted red light when it was illuminated by ultraviolet excitation. However, it was not until the 1930s that the use of fluorochromes was initiated in biological investigations to stain tissue components, bacteria and other pathogens. Several of these stains were highly specific and stimulated the development of the fluorescence microscope. Fluorescence microscopy is useful tool to visualize and enumerating cells/ microbes in various natural habitats, such as; soil, water, food, or a clinical specimen. It also helps in rapid detection and identification of an unknown microbe. **Fluorescent antibody staining** is widely used in diagnostic procedures to determine whether an *antigen*, i.e., a foreign substance, such

as a microbe is present in clinical samples. Immunofluorescence microscopy is used to map the spatial distribution of macromolecules in cells and tissues. A related technique, **Fluorescence In-Situ Hybridisation** (FISH), employs the specificity of fluorescently labelled DNA or RNA sequences (nucleic acid probes). This technique has been used for the rapid screening of chromosomal and nuclear abnormalities in inherited diseases.

Confocal Microscope: Confocal microscopy, most frequently confocal laser scanning microscopy or laser confocal scanning microscopy, is an optical imaging technique for increasing optical resolution and contrast of a micrograph by means of using a spatial pinhole to block out-of-focus light in image formation. A confocal microscope uses a laser to excite a thin layer of the sample and collects only the emitted light coming from the target layer, producing a sharp image without interference from fluorescent molecules in the surrounding layers.

Conventional light microscope uses a mixed wavelength light source and illuminates a large area of the specimen. Even if not in focus, images of bacteria from all levels within the field will be visible. As a result the image can be murky, fuzzy, and crowded. The solution to this problem is the **Confocal Scanning Laser Microscope (CSLM)** or confocal microscope. A focused laser beam strikes a point in the specimen which is fluorescently stained. Light from the illuminated spot is focused by an objective lens onto a plane above the objective. The laser is scanned over a plane in the specimen (beam scanning) or the stage is moved (stage scanning) and a detector measures the illumination from each point to produce an image of the optical section

Differential Interference Contrast Microscopy

Differential Interference Contrast (DIC) microscopy is a modified form of light microscopy. In this microscopy, a polarizer is used in the condenser to produce polarized light (light in a single plane). This polarized light passes through a special prism that generates two distinct beams. These beams traverse the specimen and enter the objective lens where they are again recombined to form one beam. Because the two beams pass through different substances with slightly different refractive indices, the combined beams are not totally in phase but instead create an interference effect. This effect visibly enhances subtle differences in cell structure. Thus, by DIC microscopy, cellular structures, such as the nucleus of eukaryotic cells or endospores, vacuoles, and granules of bacterial cells, appear more three-dimensional.

1.3.2 Electron Microscopy

The light microscope could not resolve objects separated by less than 0.2 μm and the view was limited to observations at the level of whole cells and their arrangements. The advent of the electron microscope (Refer Table 1.5) allowed

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small subcellular structures and viruses to be visualized and studied. It has transformed the whole microbiology and added immeasurably to our knowledge and concepts. Electron microscopes use a focused beam of electrons instead of a beam of light (Like light, free electrons travel in waves). When a high voltage of between 40,000 and 1,00,000 volts (the accelerating voltage) is passed between the cathode and the anode, a tungsten filament emits electrons. Whereas, the condenser of a light microscope consists of one or a few glass lenses, the condenser of the electron microscope consists of several large, circular electromagnets. Electromagnets function as lenses in the electron microscope, and the whole system operates in a vacuum. All the components like the specimen, the filament, the electromagnets, and the zinc sulfide screen are all mounted within a sealed compartment that is connected to a vacuum pump. The resolving power of the electron microscope is far greater than that of the other microscopes described above. The reason behind for better resolution of the electron microscopes is due to the shorter wavelength of electrons; the wavelengths of electrons are about 100,000 times smaller than the wavelengths of visible light. The wavelength of radiations used with the electron microscope is typically about 0.005 nm (0.05 Å). But electron microscopes are much more expensive than light microscopes. They also take up much more space and require additional rooms for preparation of specimens and for processing of photographs. The image of the electron microscope is viewed after its magnetic projection onto a zinc sulfide screen. The molecules of the screen are excited by the impinging electrons and emit visible light during their return to the ground state.

Table 1.5 *Some of the Basic Differences Between Light and Electron Microscopy*

S.No.	Features	Light Microscopy	Electron Microscopy
1.	Magnification	Maximum magnification can be 1000-1500 times in light microscope.	Electron microscopes are capable of magnifying to a maximum of approximately 200000 times.
2.	Source of Illumination	Electric Lamp or Light Emitting Diode	Electron microscopes use a focused beam of electrons a source of illumination.
3.	Lens	Glass lenses are used in the light microscope.	Electromagnets function as lenses in the electron microscope.
4.	Image	The last lens of the light microscope is the ocular, through which the image may be viewed with the eye.	The image of the electron microscope is viewed after its magnetic projection onto a zinc sulfide screen.
5.	Resolution	Light microscope has maximum resolution up to 0.2 µm.	Electron microscope has resolution about 0.2 nm.
6.	Condenser	It consists of glass lenses.	It consists of several large, circular electromagnets.
7.	Focusing	Lenses are focused by moving them nearer to or further from the specimen.	In the electron microscope, focusing is accomplished by manipulating the amount of current flowing through series of electromagnetic lenses.
8.	Observation	Both living and dead specimens are viewed with a light microscope.	Only dead ones are viewed with an electron microscope.

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There are two types of electron microscopes: The **Transmission Electron Microscope (TEM)** and the **Scanning Electron Microscope (SEM)**. The first electron microscope was the transmission electronic microscope, invented in 1931 by Ernst Ruska and was in use in many laboratories by the early 1940s. The scanning electron microscope was invented in 1935 by Max Knoll.

Transmission Electron Microscope

Transmission Electron Microscopy (TEM) is a microscopy technique in which a beam of electrons is transmitted through a specimen to form an image. The specimen is most often an ultrathin section less than 100 nm thick or a suspension on a grid. An image is formed from the interaction of the electrons with the sample as the beam is transmitted through the specimen. The image is then magnified and focused onto an imaging device, such as a fluorescent screen, a layer of photographic film, or a sensor, such as a scintillator attached to a Charge Coupled Device (CCD).

Transmission electron microscopes are capable of imaging at a significantly higher resolution than light microscopes, owing to the smaller de Broglie wavelength of electrons. This enables the instrument to capture fine detail, even as small as a single column of atoms, which is thousands of times smaller than a resolvable object seen in a light microscope. Transmission Electron Microscopy (TEM) is a major analytical method specifically used in the physical, chemical and biological sciences.

With the development of TEM, the associated technique of Scanning Transmission Electron Microscopy (STEM) was re-investigated. TEM instruments have multiple operating modes including conventional imaging, Scanning TEM (STEM) imaging, diffraction, spectroscopy, and combinations of these. The TEM mechanism or technique gives images on the contrast mechanism and also defines the settings of lenses, apertures, and detectors.

The key factors when considering electron detection include Detective Quantum Efficiency (DQE), Point Spread Function (PSF), Modulation Transfer Function (MTF), pixel size and array size, noise, data readout speed, and radiation hardness.

Imaging systems in a TEM consist of a phosphor screen, which may be made of fine (10–100 ìm) particulate zinc sulfide, for direct observation by the operator, and, optionally an image recording system, such as photographic film, doped YAG (Yttrium Aluminium Garnet) screen coupled CCDs (Charge Coupled Device) or another digital detector. Typically these devices can be removed or inserted into the beam path by the operator as required. While photograph film can record high resolution information, it is not simple to automate and the results cannot be viewed in real time.

The resolution of Transmission Electron Microscopes (Refer Figure 1.25), about 0.2 nm, permits fine structural details to be distinguished, and their powerful magnification would make a 1 µm long bacterial cell look like a soccer

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ball. Most of the organelles in eukaryotic cells and the double-layered structure of the plasma membrane were first observed with electron microscopes. Even individual protein and nucleic acid molecules can be visualized in the transmission electron microscope due to its high magnification and powerful resolution. It has a practically resolution roughly 1000 times much better than the commonly used light compound microscope. In Transmission Electron Microscopy (TEM), specimens are cut into very thin sections and placed under a high vacuum. A beam of electrons is focused on a small area of the specimen by an electromagnetic condenser lens that direct the beam of electrons in a straight line to illuminate the specimen (Refer Figure 1.26).

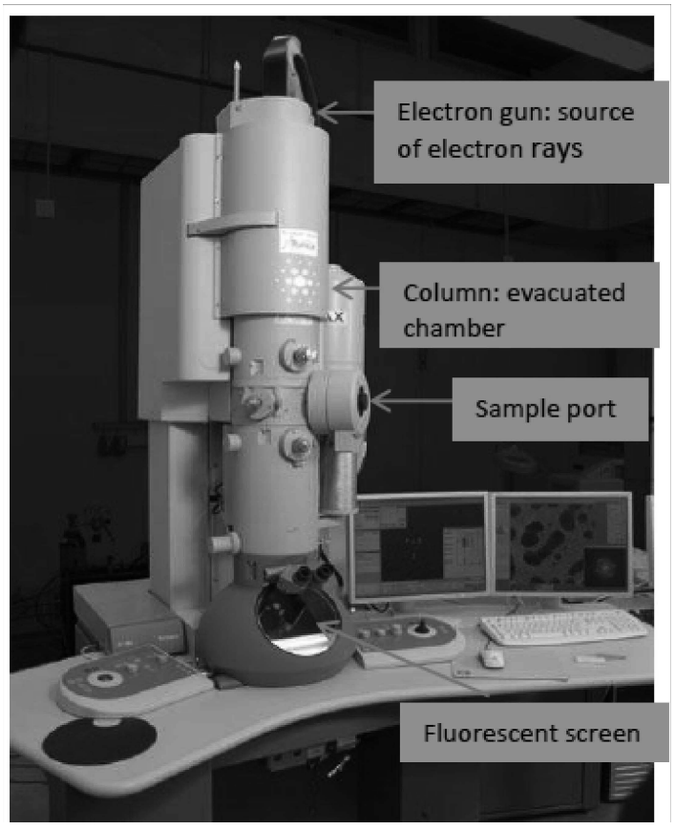

Fig. 1.25 *Electron Microscope, Instrument Encompasses both Transmission and Scanning Electron Microscope Functions*

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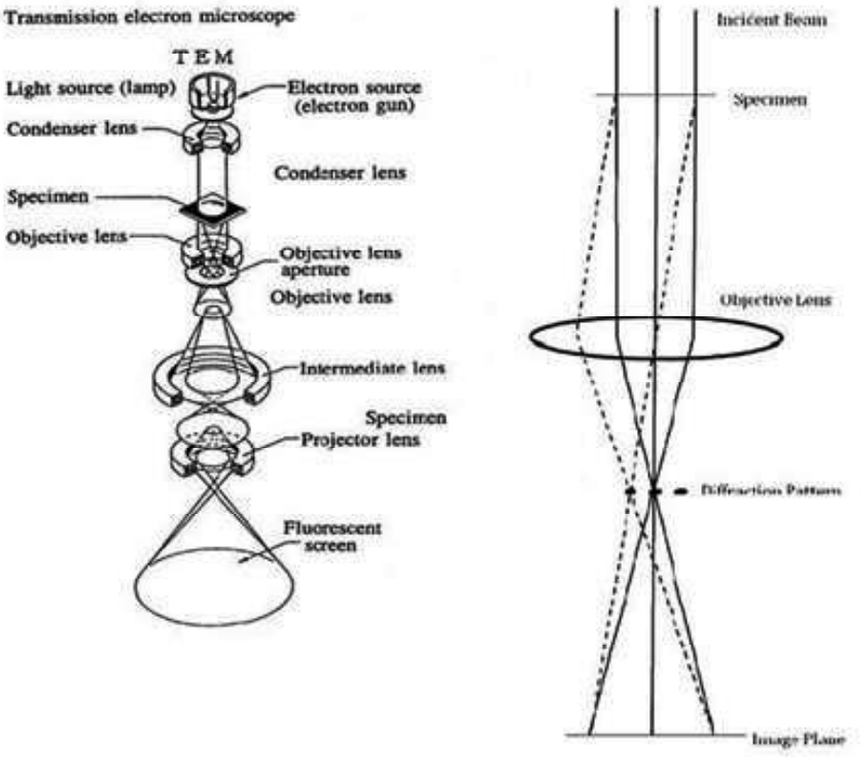

Fig. 1.26 *Path of Electron Rays in Transmission Electron Microscope*

The microscope produces its image by transmitting electrons through the specimen. It gives a better view of the internal structure of microbes than do other types of microscopes. The final image, called a Transmission Electron Micrograph (TEM), appears as many light and dark areas, depending on the number of electrons absorbed by different areas of the specimen (Refer Figure 1.27).

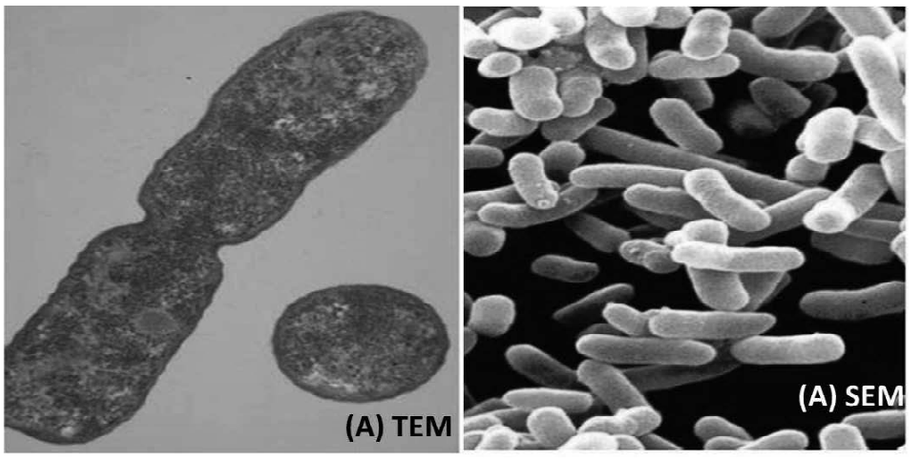

Fig. 1.27 *Electron Micrographs of E.coli Produced by; (A) Transmission Electron Microscope and; (B) Scanning Electron Microscope*

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Section Cutting: Because electrons cannot easily penetrate thick preparations, the special techniques of thin sectioning are needed to prepare specimens before observing them. Such thin slices are cut down by providing support to specimen of some kind (the necessary support is provided by plastic). A single bacterial cell, for instance, is cut into many very thin (20–60 nm) slices, which are then examined individually by TEM. To prepare specimens to view in transmission electron microscopy, a specimen may be embedded in a block of plastic that provides necessary support to specimen. Since electrons are quite easily absorbed and scattered by solid matter, only extremely thin slices of a microbial specimen can be viewed in the TEM. After fixation with chemicals like glutaraldehyde or osmium tetroxide to stabilize cell structure, the specimen is dehydrated with organic solvents, for example acetone or ethanol. The specimen is soaked in unpolymerized, liquid epoxy plastic up to it is completely permeated, and then the plastic is hardened to form a solid block. A special instrument called as ultramicrotome, is used to cut thin sections from this block with the help of a glass or diamond knife.

Staining: These sections are placed on thin wire grids for viewing so that a beam of electrons will pass directly through the section. To obtain sufficient contrast, the preparations are treated with stains, such as osmic acid, or permanganate, uranium, lanthanum, or lead salts. Because these substances are composed of atoms of high atomic weight, they scatter electrons well and thus improve contrast. A special staining technique is used to view the specimen in transmission electron microscopy which is called as **shadow casting/shadowing**. In this procedure, a specimen is coated with thin film of heavy metal, such as platinum or gold at an angle of about 45° from horizontal, so that metals strikes the microbe from only one side. The area coated with metal scatters electrons and appears light, whereas the uncoated side on the opposite side of the specimen leaves a clear area behind it as a shadow. This gives a three-dimensional effect to the specimen and provides a general idea of the size and shape of the specimen.

Freeze-Fracturing: It is also possible to view the interior of a cell with a TEM by a technique called **freeze-fracturing**. In this technique, the cell is frozen and then fractured with a knife. The cleaving of a specimen reveals the surfaces of structures inside the cell. **Freeze-etching** involves the evaporation of water from the frozen and fractured specimen. Cells are rapidly frozen in liquid nitrogen and then exposed to -100°C in a vacuum chamber. Next a knife that is precooled with liquid nitrogen (-196°C) fractures the frozen cells, which are very brittle and break along lines of greatest weakness. Usually it breaks down the middle of internal membranes. The specimen is left in the high vacuum for a minute or more so that some of the ice can sublimate away and uncover more structural detail.

Scanning Electron Microscope (SEM)

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The scanning probe microscope was created in the 1980s, by Gerd Binnig and Heinrich Rohrer. In the last decade, the Scanning Electron Microscope (SEM) has become an increasingly important tool of the cell biologist. A scanning electron microscope is a type of electron microscope that generates images of a sample by scanning its surface with a focused beam of electrons. The electrons interact with atoms present in the sample and further produce various signals that can be used to obtain information about the surface topography and composition of the sample. The electrons are generated at the top of the column by the electron source and are emitted when their thermal energy overcomes the work function of the source material. They are then accelerated and attracted by the positively-charged anode Like all the components of an electron microscope, the electron source is sealed inside a special chamber in order to preserve vacuum and it also allows the user to acquire a high-resolution image. In the absence of vacuum, other atoms and molecules can be present in the column. Their interaction with electrons causes the electron beam to deflect and reduces the image quality.

In SEM, a focused beam of electrons is scanned across the specimen surface in an evacuated chamber and the back scattered and secondary electrons produced are processed to provide a topographical image of the specimen surface. The electron beam also generates characteristic X-rays of the specimen. With the use of Energy-Dispersive Spectroscopy (EDS), it is possible to obtain an elemental map of the specimen surface layer. In traditional SEM, the specimen must be coated with a conducting layer to overcome charging of the surface by the electron beam. However, recent developments mean that a lower-energy electron beam (approximately 1-3keV) can be used which can eliminate the problem of surface charging and hence the necessity for coating the specimen surface. The **condenser** lens is the first lens that electrons meet as they travel towards the sample. Condenser lens converges the beam before the electron beam cone opens again and is converged once more by the **objective** lens before hitting the sample. The condenser lens defines the size of the electron beam, while the main role of the objective lens is to focus the beam onto the sample.

The interaction of electrons with a specimen results in the generation of many different types of electrons, photons or irradiations. In the case of SEM, the two types of electrons used for imaging are the Back Scattered Electrons (BSE) and the Secondary Electrons (SE). Backscattered electrons belong to the primary electron beam and are reflected back after elastic interactions between the beam and the sample. On the other hand, secondary electrons originate from the atoms of the sample: they are a result of inelastic interactions between the electron beam and the sample. BSE come from deeper regions of the sample, while SE originates from surface regions. Therefore, BSE and SE carry different types of information. BSE

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images show high sensitivity to differences in atomic number: the higher the atomic number, the brighter the material appears in the image.

Five basic steps involved in Scanning Electron Microscopy are:

- A stream of high voltage electrons (usually 5-100 KeV) is formed by the electron source (usually a heated tungsten or field emission filament) and accelerated in a vacuum toward the specimen using a positive electrical potential (Anode).
- This stream is confined and focused using metal apertures and magnetic lenses into a thin, focused, monochromatic beam.
- This beam is focused onto the sample using electromagnetic lens.
- Interactions occur inside the irradiated sample, affecting the electron beam.
- These interactions and effects are detected and transformed into an image (Refer Figure 1.28).

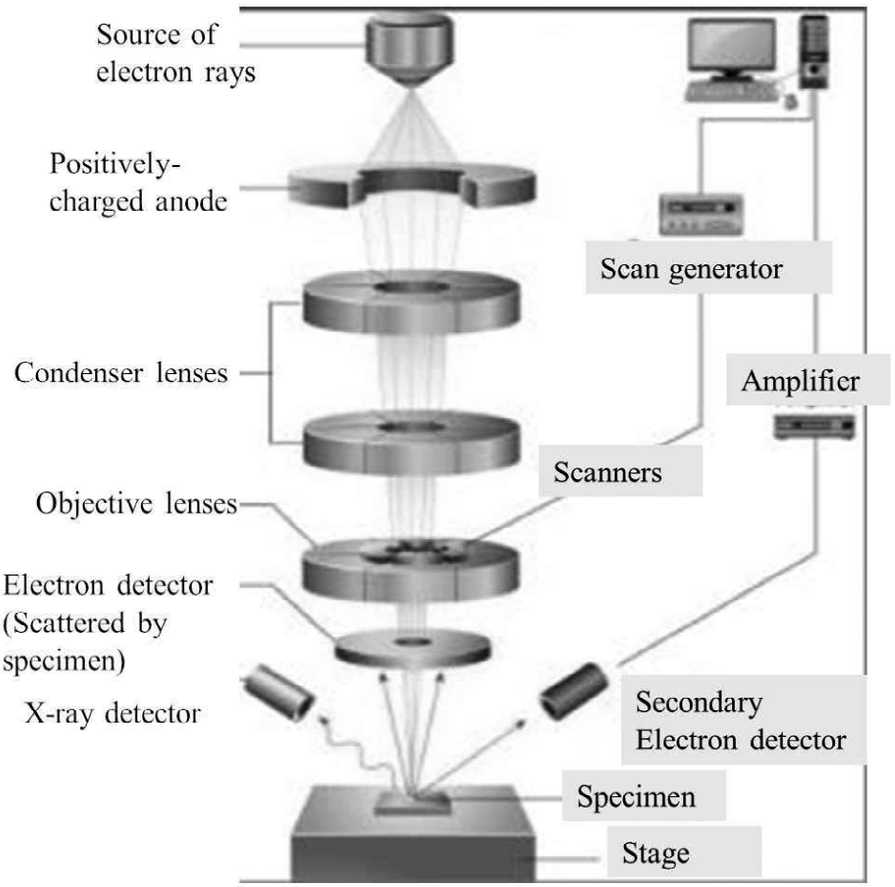

Fig. 1.28 *Diagram Showing Working of Scanning Electron Microscopy*

Although, in both types of electron microscopes, we uses electron beams to produce magnified image of the sample. But there are some basic differences occurred between them. These differences include light source, purpose, way of

sample preparation, magnification, resolution, processing of samples and image formation (Refer Table 1.6). We can easily understand the difference by observing the images formed in TEM and SEM.

Table 1.6 *Differences between Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM)*

Properties	**Transmission Electron Microscopy (TEM)**	**Scanning Electron Microscopy (SEM)**
Light Source	In TEM, electrons are used as source of light. TEM is based on transmitted electrons and operates on the same basic principles as the light microscope.	SEM is based on electrons emitted from the surface of a specimen (scattered electrons).
Purpose	TEM is used to view many characteristics of the sample, such as internal composition, morphology, crystallization, etc.	SEM provides detailed surface images of the cell. SEM focuses on the sample's surface and its composition, so SEM shows only the morphology of samples.
Sample Preparation	The sample in TEM needs to be cut into thin sections (70-90 nm) because electrons cannot penetrate deep into samples.	Sample is coated with a thin layer of a heavy metal, such as gold or palladium.
Magnification	The magnifying power of TEM is up to 2 million times.	The magnifying power of SEM is up to 50,000X.
Resolution	TEM has much higher resolution than SEM. It can resolve objects as close as 1 nm.	SEM can resolve objects as close as 20 nm.
Processing of Sample (s)	With TEM only small amount of sample can be analyzed at a time.	SEM allows for large amount of sample to be analyzed at a time.
Image Formation	Transmitted electrons hit a fluorescent screen giving rise to a 'shadow image' of the specimen with its different parts displayed in varied darkness according to their density. The image can be studied directly by the operator or photographed with a camera.	Secondary or backscattered electrons arising from interaction of electron beam and metal coated specimen are collected and the resulting image is displayed on a computer screen.

1.3.3 Phase - Contrast Microscope

The technique of phase contrast microscope was invented by Dutch physicist Frits Zernike in the 1934. He received the Nobel Prize in physics in 1953 for this contribution. Phase contrast microscopy is a contrast-enhancing optical technique that can be utilized to produce high-contrast images of transparent specimens such as

- Living cells (usually in culture)
- Microorganisms

- Thin tissue slices
- Sub cellular particles (including nuclei and other organelles)

Principle

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The phase contrast microscope produces high-contrast images by converting the slight differences in refractive index and thickness into changes of amplitude.

The differences in light absorption are often negligible between living cells and their surrounding nutrient medium, as well as between the various intracellular components and plasma membranes, rendering them barely visible when observed by brightfield illumination. The human eye can perceive changes in light amplitude (intensity) and changes in wavelength but not to changes in phase, where one wave is retarded in relation to other. Unstained biological specimens, such as living cells, are essentially transparent to our eyes, but they interact with light in a fairly uniform way, by slowing the passage of a light beam by approximately a quarter of a wavelength. This slowing of a light beam relative to another light beam that had passed though the surrounding medium occurs since the specimen under study alters the phase of the beams. Intensity (amplitude) is additive and light rays that are ½ out of phase are perceived as darkness. Zernicke took advantage of **minute refractive index differences** within cellular components and between unstained cells and their surrounding aqueous medium to separate the light beams interacting with the specimen from those that do not encounter the specimen and was able to generate changes in amplitude within the living cells, so that they can be seen easily.

When an annulus, a transparent ring, is placed in the substage condenser, an image of the annulus is formed in the back of focal plane of the objective. Another complementary ring is placed inside the objective lens. Nearly all of the light that passed through the sample but missed the specimen then was through the objective lens through this complimentary ring. Unstained living cells have slight non-homogenicities and when these are placed in the object plane, a halo of light both inside and outside the annular image is produced. This halo formed comprises of light rays that are diffracted by the object and are a quarter out of phase in respect to the direct light rays.

It follows that if the glass plate holding the ring is designed in such a manner that all light missing the ring would encounter an additional ¼ of retardation relative to the beams of light that had not interacted with the specimen, then the phase difference between the direct and the diffracted rays would be1/2 and interference would take place in the final image plane, creating some parts of the unstained specimen being darker and other parts of the unstained specimen brighter relative to the background.

Zernicke developed this glass plate or phase plate, also called as “z” plate, which brought this about. The phase plate consists of an optically plane glass disc out of which is cut a channel to coincide with the image of light annulus. The depth of the channel should be exact to retard the diffracted rays which travel through the channel.

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Although, there will be interference now, the great difference in amplitude between the two sets of rays will prevent the maximum contrast from being obtained. However, to surmount this factor a light absorbing material is placed in the area of channel thus reducing the amplitude of direct light rays without affecting the diffracted light rays. This helps in achieving the maximum contrast.

The theory of phase contrast microscopy is true only when monochromatic light is used as an illuminant. If white light is used instead, it would split into its constituent colours when diffracted and contrast is decreased. In practise, white light is used; however, better contrast can be achieved using a green filter in conjunction with a compound high intensity lamp.

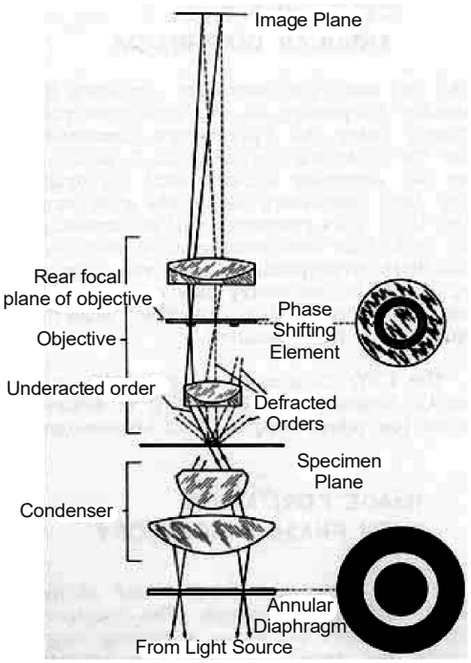

Fig. 1.29 *Schematic Representation of the Working of a Phase Contrast Microscope*

Parts of Phase Contrast Microscope

The phase contrast microscope consists of

1. Lamp
2. Annulus
3. Objective
4. Auxillary Telescope

Lamp

The source of illumination should be strong like a high intensity compound lamp with a mercury green filter.

Annulus

Each objective lens requires a different sized annulus. They are inserted separately in each objective lens. They can be adjusted by the centring screws.

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Objective Lenses

The phase plate is pre fitted in the objective lens.

Auxillary Telescope

It is used for examining the back focal plane of the objective and ensuring that the objective phase plate and the condenser annulus are aligned properly.

It is used in place of an eyepiece.

Figure 1.30 illustrates the parts of a phase contrast microscope:

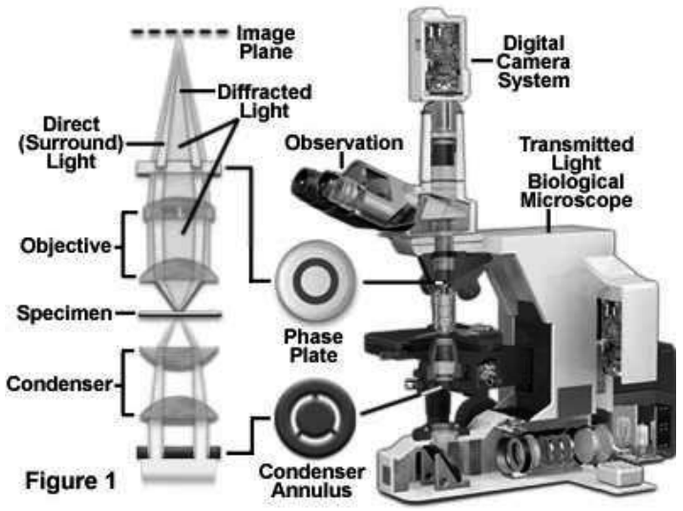

Fig. 1.30 *Phase Contrast Microscope*

Operation of Phase Contrast Microscope

- The microscope is set in a usual manner making certain that there is no annulus in the substage.
- Set up the correct brightfield illumination.
- Focus on the object.
- Rotate the condenser turret cylinder until the number on the condenser turret matches the number engraved on the objective lens.
- Remove the eyepiece and replace it with auxillary telescope without disturbing the focus.
- This lens enables you to see the rear focal plane of the objective lens, the plane where the ring resides. [A bright circle of light will be observed (the condenser annulus) and a dark ring (present within the objective). The dark ring is stationary, but the bright annulus is not.]
- Align the annulus with the ring so that the two are superimposed.
- Adjustment screws present on the back side of the condenser aid in the alignment.
- Place the eyepiece back into the microscope.
- A phase contrast image can now be observed.

Advantages of phase contrast microscopy

The major advantages of phase contrast microscopy are:

- Living cells can be inspected in their natural state without being killed, fixed, and stained. As a result, the dynamics of continuing biological processes can be observed and recorded in high contrast with sharp clarity of microscopic specimen detail.
- The performance of modern phase contrast microscopes is so refined that it enables specimens containing very small internal structures, or even just a few protein molecules, to be detected when the technology is coupled to electronic enhancement and post-acquisition image processing.

The development of phase contrast microscopy by Zernicke is an excellent example of how research can give way to pioneering developments. During the Second World War, the Zeiss Optical Works in Jena, Germany, was the first manufacturer to incorporate practical phase contrast optics into their microscopes.

Mathematical Relations Helpful in Phase Contrast Microscopy

The mathematical relationship between the various light waves generated in phase contrast microscopy can be described basically as:

$P = S+D$

S is the surround wave, i.e., undeviated or zeroth order planar wavefront

D is the Diffracted wave

P is the resultant particle wave after interference of S wave and D wave

If the amplitudes of the particle and surround waves are significantly different in the intermediate image plane, then the specimen acquires a considerable amount of contrast and is easily visualized in the microscope eyepieces. Otherwise, the specimen remains transparent and appears as it would under ordinary brightfield conditions (in the absence of phase contrast or other contrast-enhancing techniques).

The difference in location of an emergent wavefront between the specimen and surrounding medium is termed the phase shift (δ) and is defined in radians as:

$$\delta = 2\pi\Delta/\lambda$$

In the equation above, the term Δ is referred to as the optical path difference, which is similar to the optical path length:

Optical Path Difference (OPD) = $\Delta = (n_2 - n_1) \times t$

where

n(2) is the refractive index of the specimen and

n(1) is the refractive index of the surrounding medium.

The optical path difference results from the product of two terms: the thickness of the specimen, and its difference in refractive index with the surrounding medium.

When the refractive index of the specimen equals that of the surrounding medium, the optical path difference is zero regardless of whether the specimen thickness is large or small.

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The phase shift of the diffracted wave relative to the surround wave is presented as Φ, where:

$$\Phi = \pm 90° + \varphi/2$$

In the equation,

φ is the relative phase shift (a function of the optical path difference) between the surround (S) and particle (P) wave vectors.

For specimens displaying a negligible optical path difference (in effect, no phase shift), the latter term of the equation equals zero and Φ becomes ± 90 degrees.

Applications

Phase contrast is an excellent method for enhancing the contrast of thin, transparent specimens without loss of resolution, and has proven to be a valuable tool in the study of dynamic events in living cells. The technique of phase contrast is widely applied in biological and medical research, especially throughout the fields of cytology and histology.

As such, the methodology is utilized to examine

- Living cells and tissues; and
- Microorganisms which are transparent under brightfield illumination.

The uses and applications are as follows:

- Phase contrast microscopy facilitates internal cellular components, like the cell membrane, nuclei, mitochondria, chromosomes, and Golgi apparatus of various cells and tissues to be examined.
- Also, phase contrast microscopy is widely employed to study the growth and dynamics of a wide variety of living cells. It is also helpful in tissue culture investigations.
- Other areas in the biological field that gain advantage from phase contrast observation are hematology, virology, bacteriology, parasitology, etc.
- Industrial and chemical applications for phase contrast include mineralogy, crystallography, and polymer morphology research.
- It is used for examining unstained paraffin, resin and frozen sections.
- Incident light phase contrast microscopy, although largely replaced by differential interference contrast techniques, is useful for assessment of surfaces, including crystal dislocations, defects as well as for lithography.

In the early twentieth century another vista in the microscopy world began to unfold, the world's tiniest objects as seen by electron microscope.

Electron microscope is a specialized field of science employing electron microscope as a tool. The electron microscope uses a particle beam of highly energetic electrons to illuminate the specimen and create an image of it. It uses electrons that have wavelengths about 1 lakh times shorter than visible light (photons), and can achieve magnifications of up to 10 lakh times. The electron

microscope has helped us understand the intricate details of cells and tissues and their organization.

The Transmission Electron Microscope (TEM) was the first type of electron microscope to be developed. It differed from the standard light microscope because a beam of electrons is used instead of light. It was developed by Max Knoll and Ernst Ruska in Germany in 1931.

The first Scanning Electron Microscope (SEM) was developed in 1942.

Figure 1.31 shows the difference in working of light microscope, transmission electron microscope and scanning electron microscope.

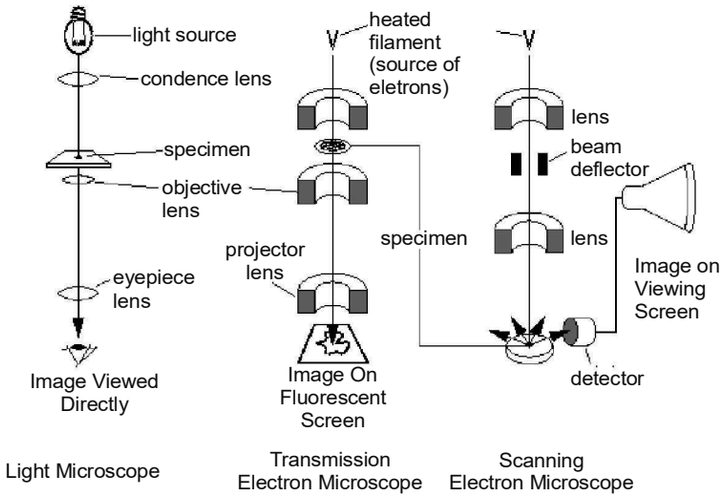

Figure 1.31 *Difference in Light Microscope, Transmission Electron Microscope and Scanning Electron Microscope*

The electron microscope reveals information about the sample including external morphology (texture), chemical composition and crystalline structure.

1.3.4 Confocal Microscopes

Confocal microscopy has following benefits in comparison to conventional widefield optical microscopy.

- Elimination or reduction of background information away from the focal plane, which causes image degradation
- Ability to control depth of field
- Capability to collect serial optical sections from thick samples

The fundamental of confocal approach is the usage of spatial filtering techniques for elimination of the out-of-focus light or glare in samples whose thickness exceeds the immediate plane of focus. In recent years, the popularity of confocal microscopy has increased, because of the relative ease with which exceptionally high-quality images can be obtained from samples that are prepared for conventional fluorescence microscopy, and the increase in the number of

applications in cell biology, which depends on imaging both fixed and living cells and tissues. Confocal technology has been proved to be the most important advances ever achieved in optical microscopy.

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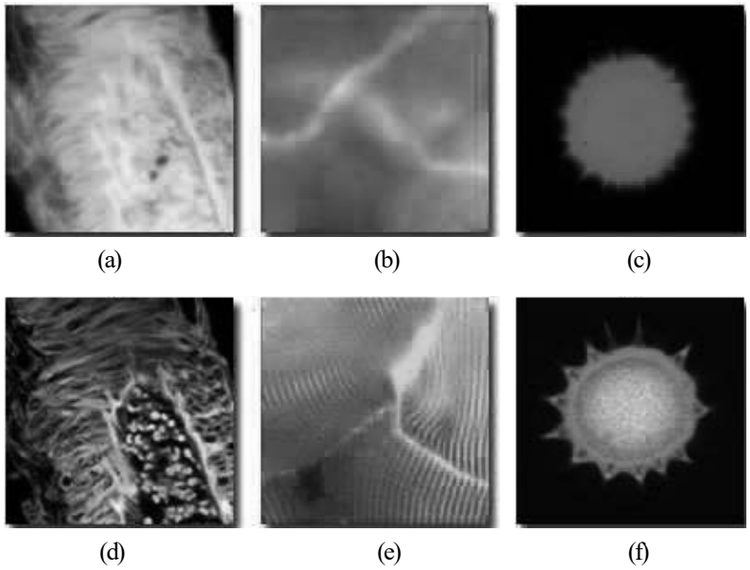

Fig. 1.33 *Confocal and Widefield Fluorescence Microscopy*

In a conventional widefield optical epi-fluorescence microscope, secondary fluorescence released by the sample is occurred through the excited volume and obscures resolution of features that are in the objective focal plane. The problem is compounded by thicker samples, which are greater than 2 micrometers, usually exhibit high degree of fluorescence emission in which most of the fine detail is lost. Confocal microscopy gives only a marginal improvement in both axial (z; along the optical axis) and lateral (x and y; in the specimen plane) optical resolution. However, it is able to exclude secondary fluorescence in areas which are removed from the focal plane from resulting images. The resolution can be enhanced with confocal microscopy over conventional widefield techniques, but it is still less than that of the transmission electron microscope. In this aspect, confocal microscopy is considered as a bridge between these two classical approaches.

In Figure 1.33, there are image series that compare certain view fields in traditional widefield and laser scanning confocal fluorescence microscopy. In widefield fluorescence, as seen in Figure 1.33 a, thick section of fluorescently stained human medulla exhibits a large amount of glare from fluorescent structures above and below the focal plane. When imaged with a laser scanning confocal microscope as shown in Figure 1.33 d, the medulla thick section exposes a significant degree of structural detail. Similarly, widefield fluorescence imaging of whole rabbit muscle fibers stained with fluorescein produce blurred images displayed in Figure 1.33 b lacks detail, while the same sample field in confocal microscopy (Figure 1.33 e) reveals a highly striated topography. In Figure 1.33 c, autofluorescence in a sunflower pollen grain emits an indistinct outline of the basic external morphology, but produces no indication of the internal structure. Comparatively, a thin optical section of the same grain as displayed in Figure 1.33 f,

acquired with confocal techniques shows a dramatic difference between the particle core and the surrounding envelope.

Historical Perspective

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In the mid-1950, Marvin Minsky developed the concept of confocal microscopy during his studies in Harvard University. Later, in 1957, Minsky got patent for it. Minsky's reason behind the invention was to image biological events and neural network of brain tissue in the living system. Minsky's invention was not noticed because of the lack of intense light sources which was necessary for imaging and horsepower of the computer required for handling large data. In 1960, Mojmir Petran and M. David Egger fabricated a multiple-beam confocal microscope that used a spinning (Nipkow) disk for examination of ganglion cells and unstained brain sections. In 1973, Egger developed the first mechanically scanned confocal laser microscope and issued the first recognizable images of cells. In Late 1970s and 80s, interest in confocal microscopy increases, because of advances in computer and laser technology, and introduction of new algorithms for digital manipulation of images.

After the expiration of Minsky's patent, many investigators translated practical laser scanning confocal microscope designs into working instruments. In 1979, Dutch physicist G. Fred Brakenhoff developed a scanning confocal microscope and Colin Sheppard also contributed a technique with a theory of image formation simultaneously. In late 1980s, Brad Amos, John White and Tony Wilson developed the utility of confocal imaging in the examination of fluorescent biological specimens. In 1987, instruments for commercial use were demonstrated. In 1990s, optics and electronics afforded high-throughput fiber optics, better thin film dielectric coatings, stable and powerful lasers, detectors with reduced noise characteristics and high-efficiency scanning mirror units. Along with this, fluorochromes started to synthesized. In 1990s, with the advancement of enhanced displays, computer processing speeds and large-volume storage technology, science was ready for a virtual explosion in many applications that could be directed with laser scanning confocal microscopy.

Today's confocal microscopes is considered as complete integrated electronic systems. In these systems, the optical microscope plays a crucial role in a configuration which, consists a computer, electronic detectors, and several laser systems that can be combined with beam scanning assembly and wavelength selection devices. In many instances, the entire confocal microscope is collectively called as a digital or video imaging system which is capable of creating electronic images. Presently, these microscopes are used for routine investigations on molecules, cells, and living tissues.

Principles of Confocal Microscopy

Figure 1.34 explains the confocal principle in epi-fluorescence laser scanning microscopy diagrammatically. Coherent light is emitted by the laser system (excitation source) passes through a pinhole aperture that is situated in a conjugate plane (confocal) with a scanning point on the sample and a second pinhole aperture positioned in front of the detector, which is a photomultiplier tube. Dichromatic

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mirror reflects the laser and scanned across the sample in a defined focal plane, secondary fluorescence emitted from points on the sample, which is in the same focal plane pass back through the dichromatic mirror. Then they are focused as a confocal point at the detector pinhole aperture.

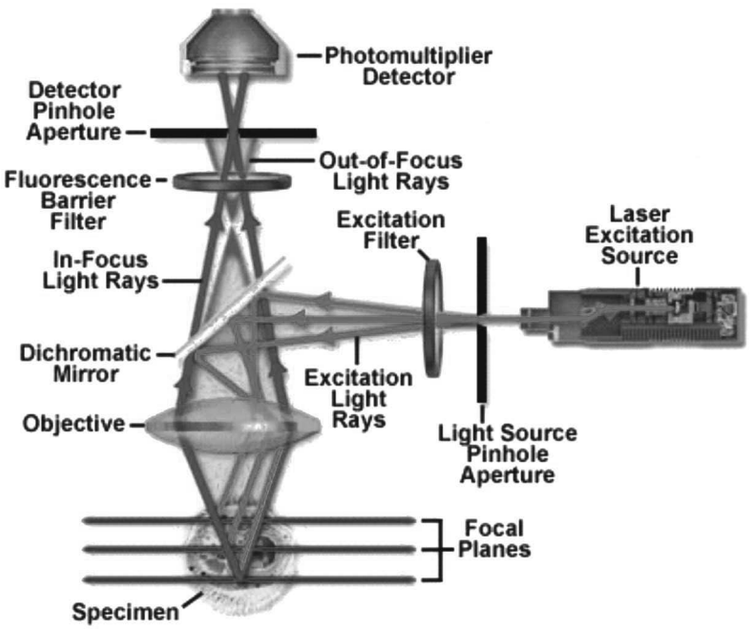

Fig. 1.34 *Laser Scanning Confocal Microscope Optical Configuration*

The substantial amount of fluorescence emission, which occurs at points above and below the objective focal plane is not confocal with the pinhole. It is termed as Out-of-Focus Light Rays. It forms extended Airy disks in the aperture plane. As only a small fraction of the out-of-focus fluorescence emission is transported through the pinhole aperture, most of this extraneous light is not detected by the photomultiplier and does not result to the resulting image. In a wide field epi-fluorescence microscope, the dichromatic mirror, excitation filter, and barrier filter perform similar functions to identical components. When the objective is refocused in a confocal microscope, it shifts the excitation and emission points on a sample to a new plane that becomes confocal with the pinhole apertures of the light source and detector.

In traditional widefield epi-fluorescence microscopy, the entire sample is subjected to intense illumination from an incoherent mercury or xenon arc-discharge lamp. The resulting image of secondary fluorescence emission is viewed directly in the eyepieces or projected onto the surface of an electronic array detector or traditional film plane. Contrary to the simple concept, the mechanism of image formation in a confocal microscope is fundamentally different. The confocal fluorescence microscope consists of multiple laser excitation sources, a scan head with optical and electronic components, electronic detectors (usually photomultipliers), and a computer for acquisition, processing, analysis, and image display.

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The scan head is at the heart of the confocal system. It is responsible for rasterizing the excitation scans, and collection of the photon signals from the sample that are required to assemble the final image. A typical scan head contains inputs from the external laser sources, a galvanometer-based raster scanning mirror system, fluorescence filter sets and dichromatic mirrors, variable pinhole apertures for generating the confocal image, and photomultiplier tube detectors that are tuned for different fluorescence wavelengths. For a typical commercial unit, the general arrangement of scan head components is presented in Figure 1.35.

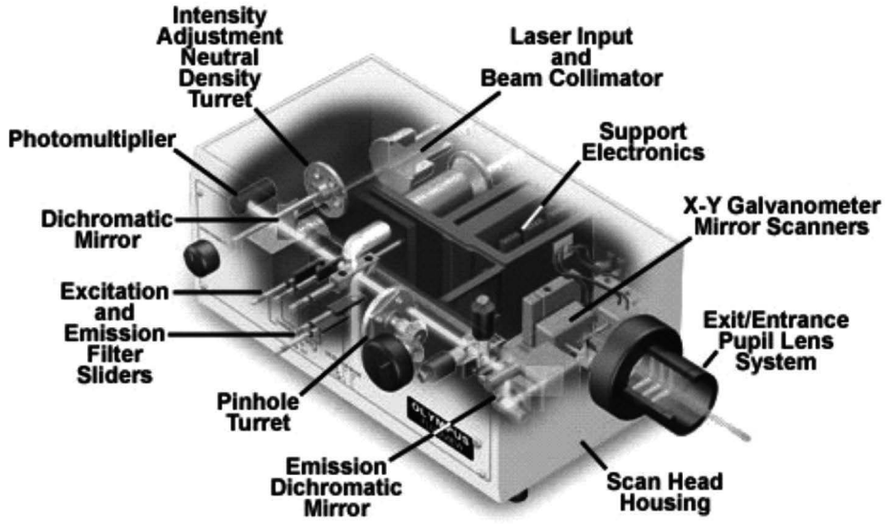

Fig. 1.35 *Confocal Microscopy Scanning Unit*

In epi-illumination scanning confocal microscopy, the laser light source and photomultiplier detectors are both separated from the sample by the objective that functions as an objective combination and well-corrected condenser. Internal fluorescence filter components such as the excitation and barrier filters and the dichromatic mirrors and neutral density filters are contained within the scanning unit as shown in Figure 1.35. Interference and neutral density filters are housed in rotating turrets or sliders, which are inserted into the light path by the operator. The excitation laser beam is coupled to the scan unit with a coupler of fiber optic. It is followed by a beam expander, which enables the thin laser beam wrist to completely fill the objective rear aperture. Expanded laser light is passes through the microscope objective that forms an intense diffraction-limited spot. It is scanned by the coupled galvanometer mirrors in a raster pattern across the sample plane (point scanning).

Pinhole aperture is the essential component of the scanning unit. It acts as a spatial filter at the conjugate image plane positioned directly in front of the photomultiplier. Several apertures of varying diameter are usually contained on a rotating turret, which enables the operator to adjust pinhole size and optical section thickness. Secondary fluorescence that are collected by the objective is descanned by the same galvanometer mirrors that form the raster pattern. Then

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it passes through a barrier filter before reaching the pinhole aperture. The aperture serves to exclude fluorescence signals from out-of-focus features positioned above and below the focal plane that are instead projected onto the aperture as Airy disks. These disk have a diameter much larger than those image formations. These oversized disks are distributed over a relatively large area so that only a small fraction of light originating in planes away from the focal point can pass through the aperture. The pinhole aperture also serves for elimination of the stray light that passes through the optical system. Coupling of aperture-limited point scanning to a pinhole spatial filter at the conjugate image plane is an important feature of the confocal microscope.

Traditional widefield epi-fluorescence microscope objectives focus a wide cone of illumination over a large volume of the sample that is uniformly and simultaneously illuminated as demonstrated in Figure 1.36. A majority of the fluorescence emission directed back towards the microscope is gathered by the objective, which is depend upon the numerical aperture and projected into the eyepieces or detector. The result is a significant amount of signal due to emitted background light and auto fluorescence originating from areas above and below the focal plane. This helps in reduction of resolution and image contrast.

Fig. 1.36 *Widefiled Versus Confocal Point Scanning of Specimens*

In Figure 1.36, it is shown that the laser illumination source in confocal microscopy is first expanded to fill the objective rear aperture, and then focused by the lens system to a small spot at the focal plane. The size of the illumination point depends upon the objective numerical aperture, ranges from 0.25 to 0.8 micrometers approximately in diameter and 0.5 to 1.5 micrometers deep at the brightest intensity. Confocal spot size is determined by the microscope design, objective characteristics, scanning unit settings, wavelength of incident laser light, and the sample. Figure 1.36 is a demonstration of the comparison between the typical illumination cones of a widefield (Refer Figure 1.36) and point scanning confocal (Refer Figure 1.36) microscope at the same numerical aperture. The entire depth of the sample over a wide area is illuminated by the widefield microscope. The sample is scanned with a finely focused spot of illumination, which is centered in the focal plane in the confocal microscope.

In laser scanning confocal microscopy, extended sample image is generated by scanning the focused beam across a defined area in a raster pattern, which is controlled by two high-speed oscillating mirrors driven by galvanometer motors. One mirror moves the beam from left to right along the x lateral axis, and the other

translates the beam in the y direction. After every single scan along the x axis, the beam is rapidly transported back to the starting point and shifted along the y axis to start a new scan in a process termed as flyback. During the flyback operation, image information is not collected, and the area of interest on the sample in a single focal plane is excited by laser illumination from the scanning unit.

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Laser Scanning Confocal Microscope Configuration

Significantly, fluorescent probes, which are employed to add contrast to biological samples and other associated technologies with optical microscopy techniques have been improved. The explosive growth and confocal approach development is a direct result of a renaissance in optical microscopy that are largely fueled by modern optical and electronics technology advances. Among these are stable multi-wavelength laser systems that deliver better coverage of the ultraviolet, visible, and near-infrared spectral regions, sensitive low-noise wide band detectors, improved interference filters (including dichromatic mirrors, barrier, and excitation filters), and powerful computers. Now, the powerful computers are available with relatively low-cost memory arrays, high-resolution video displays, image analysis software packages, and high quality digital image printers. In Figure 1.37, the flow of information is presented diagrammatically through a modern confocal microscope.

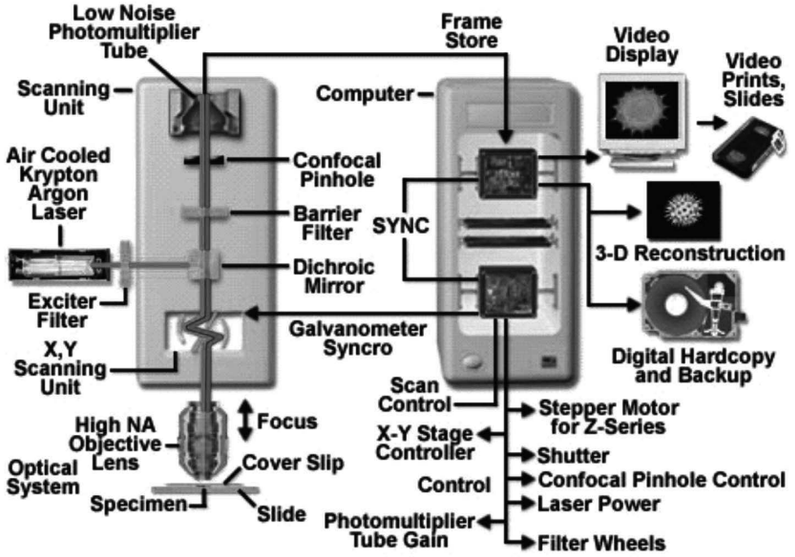

Fig. 1.37 *Confocal Microscopy Information Flow Schematic Diagram*

Many of these technologies are developed independently for a variety of specifically-targeted applications. They have been gradually incorporated into mainstream commercial confocal microscopy systems. In modern microscope systems, classification of designs is based on the technology utilized to scan samples. Scanning can be performed either by translating the stage in the x, y, and z directions while the laser illumination spot is held in a fixed position, or the beam itself can be raster-scanned across the space. As, three-dimensional translation

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of the stage is cumbersome and prone to vibration, modern instruments employ some type of beam-scanning mechanism.

In modern confocal microscopes, two fundamentally different techniques for beam scanning are developed, single-beam and multiple-beam scanning.

1. **Single-Beam Scanning** - It is one of the popular methods employed in a majority of the commercial laser scanning microscopes. It uses a pair of computer-controlled galvanometer mirrors to scan the sample in a raster pattern at a rate of one frame per second approximately. Faster scanning rates (to near video speed) can be achieved using oscillating mirrors or acousto-optic devices.
2. **Multiple-Beam Scanning –** In multiple-beam scanning, confocal microscopes are equipped with a spinning Nipkow disk that contains an array of pinholes and micro lenses. These instruments use arc-discharge lamps for illumination instead of lasers to decrease sample damage and improves the detection of low fluorescence levels during real time image collection. It also has the ability to readily capture images with an array detector, such as a charge-coupled device (CCD) camera system.

Laser scanning confocal microscope designs are positioned around a conventional upright or inverted research-level optical microscope. However, instead of the mercury arc-discharge or standard tungsten-halogen lamp, one or more laser systems are used as a light source to excite fluorophores in the sample. Image information is collected point by point with a specialized detector such as an avalanche photo-diode or photo-multiplier tube, and then digitized for processing by the host computer that also controls the scanning mirrors and/or other devices to facilitate the collection and exhibition of images. After a series of images (usually serial optical sections) has been developed and stored on digital media, analysis can be conducted by using various image processing software packages, which are available on the host or a secondary computer.

Advantages and Disadvantages of Confocal Microscopy

Advantages of Confocal Microscopy

Laser scanning confocal microscopy has the ability to produce thin (0.5 to 1.5 micrometer) optical sections consecutively through fluorescent specimens. It has a thickness ranging up to 50 micrometers or more. By using a stepper motor, the image series cam be collected by coordinating incremental changes in the microscope fine focus mechanism with sequential image acquisition at every step. Image information is constrained to a well-defined plane, instead of signals complications that arises from remote locations in the sample. Because of the reduction in background fluorescence and improved signal-to-noise, definition and contrast are radically improved over widefield techniques. Additionally, optical sectioning eliminates artifacts which, occur during physical sectioning and fluorescent staining of tissue samples for traditional forms of microscopy. The non-invasive confocal optical sectioning technique enables the analysis of both living and fixed specimens under various conditions with enhanced clarity.

With confocal microscopy software packages, optical sections can be collected and demonstrated in transverse planes. They are not restricted to the perpendicular lateral (x-y) plane. Vertical sections that are parallel to the microscope optical axis in the x-z and y-z planes, can be created by confocal software programs. Hence, the sample appears to be sectioned in a plane, which is perpendicular to the lateral axis. Practically, vertical sections are achieved by combination of a series of x-y scans and the z axis with the software and projects a view of fluorescence intensity.

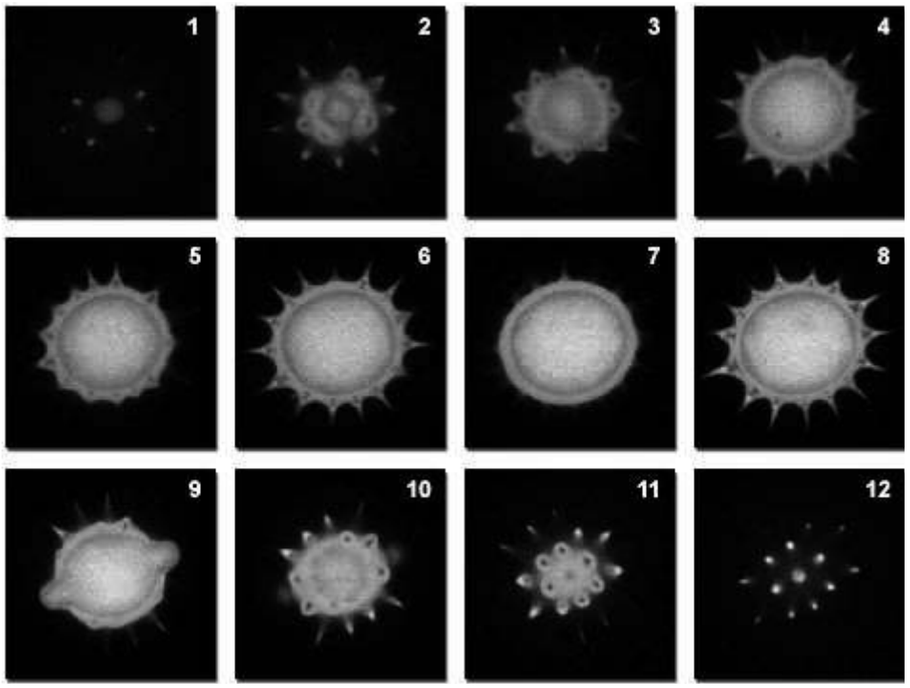

Fig. 1.38 *Pollen Grain Serial Optical Sections by Confocal Microscopy*

In Figure 1.38, auto fluorescence emission wavelengths are illustrated from a stack of optical sections, which is termed a z-series through a sunflower pollen grain that reveals internal variation.

Optical sections are gathered in 0.5-micrometer steps, which is perpendicular to the z-axis, i.e., microscope optical axis) that uses a green helium/ neon (543 nanometers; red fluorescence) laser system and dual argon-ion (488 nanometers; green fluorescence).

In many instances, a composite or projection view obtained from a series of optical sections provides important information about a three-dimensional specimen than a multi-dimensional view. For example, a fluorescently labeled neuron that have numerous thin, extended processes in a tissue section is difficult to image using widefield techniques because of out-of-focus blur. Confocal thin sections of the same neuron reveals portion of several extensions, but frequently appear as fragmented streaks and dots and also lack continuity. Composite views that are created by flattening a series of optical sections from the neuron, reveals all of the extended process in sharp focus with well-defined continuity. Functional and

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structural analysis of other cell and tissue sections also benefits from composite views. It can be opposed to, or combined with, three-dimensional volume rendering techniques.

Because of the advancement in confocal microscopy, multi-dimensional views of living cells and tissues are possible. It includes image information in the x, y, and z dimensions as a function of time. It can also be presented in multiple colors by the use of two or more fluorophores. After volume processing of each image stacks, the resulting data can be demonstrated as three-dimensional multicolor video sequences in real time. Unlike, conventional widefield microscopy, all fluorochromes in multiply labeled sample appear in register by the use of confocal microscope. Temporal data are collected either from time-lapse experiments conducted over extended time-periods or through real time image acquisition in smaller frames for short time period. The potential for use of multi-dimensional confocal microscopy as a powerful tool in cellular biology is continuously growing, as new laser systems are developed to limit cell damage. It also speeds the computer processing and improves storage capacity.

Digitization of the sequential analog image data are collected by the confocal microscope photo-multiplier or any other similar detector helps in facilitating computer image processing algorithms. It is done by transforming the continuous voltage stream into discrete digital increments, which correspond to variations in light intensity. Along with the benefits and speed, which grows from processing digital data, images can be prepared for print output or publication. In cautiously controlled experiments, quantitative measurements of spatial fluorescence intensity are obtained from the digital data either by statically or as a function of time.

Disadvantages of Confocal Microscopy

The limitation of number of excitation wavelengths that are available with common lasers also known as laser lines, that occur over very narrow bands is the main disadvantage of confocal microscopy. They are also quite expensive to produce in the ultraviolet region. Compare to it, conventional widefield microscopes use xenon or mercury based arc-discharge lamps that provides a full range of excitation wavelengths in the visible, ultraviolet and near-infrared spectral regions.

Another disadvantage is the destructive nature of high-intensity laser irradiation to living cells and tissues. This issue is recently addressed by multiphoton and Nipkow disk confocal imaging.

The high cost of purchasing and operating multi-user confocal microscope systems that can range up to an order of magnitude higher than comparable widefield microscopes can limits their implementation in smaller laboratories. This problem can be solved by cost-shared microscope systems that have service in one or more departments in a central facility. Recently, the introduction of personal confocal systems has competitively lowered the price of low-end confocal microscopes and increased the number of individual users.

Check Your Progress

10. Write the names of various branches of microscopy.
11. Who created the first compound microscope?
12. Who invent the technique of phase contrast microscope
13. What are the benefits of confocal microscopy in comparison to conventional widefield optical microscopy?

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1.4 MICROBIOLOGICAL TECHNIQUES

A microbiological culture or microbial culture is a method of multiplying microbial organisms by letting them reproduce in predetermined culture medium under controlled laboratory conditions. Microbial cultures are foundational and basic diagnostic methods used as a research tool in molecular biology.

Microbial cultures are used to determine the type of organism, its abundance in the sample being tested, or both. It is one of the primary diagnostic methods of microbiology and used as a tool to determine the cause of infectious disease by letting the agent multiply in a predetermined medium. For example, a throat culture is taken by scraping the lining of tissue in the back of the throat and blotting the sample into a medium to be able to screen for harmful microorganisms, such as *Streptococcus pyogenes*, the causative agent of strep throat. Furthermore, the term culture is more generally used informally to refer to 'Selectively Growing' a specific kind of microorganism in the lab.

It is often essential to isolate a pure culture of microorganisms. A pure or axenic culture is a population of cells or multicellular organisms growing in the absence of other species or types. A pure culture may originate from a single cell or single organism, in which case the cells are genetic clones of one another. For the purpose of gelling the microbial culture, the medium of agarose gel (agar) is used. Agar is a gelatinous substance derived from seaweed. A cheap substitute for agar is guar gum, which can be used for the isolation and maintenance of thermophiles.

1.4.1 Media Preparation and Sterilization

Culture Media: It is a medium (liquid or solid) that contains nutrients to grow bacteria in vitro. Because sometimes we cannot identify with microscopically examination directly, and sometimes we do culture for antibiotic sensitivity testing. The medium is sterilized before usage in the lab. Culture media contains nutrients and physical growth parameters necessary for microbial growth. All microorganisms cannot grow in a single culture medium and in fact many can't grow in any known culture medium. Organisms that cannot grow in artificial culture medium are known as obligate parasites. *Mycobacterium leprae, Chlamydias,* and *Treponema pallidum* are obligate parasites. Bacterial culture media can be distinguished on the basis of composition, consistency and purpose.

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Classification of culture media used in Microbiology laboratory on the basis of consistency

- **Solid Medium:** Solid medium contains agar at a concentration of 1.5-2.0% or some other, mostly inert solidifying agent. Solid medium has physical structure and allows bacteria to grow in physically informative or useful ways, for example as colonies or in streaks. Solid medium is useful for isolating bacteria or for determining the colony characteristics of the isolate.
- **Semisolid Media:** They are prepared with agar at concentrations of 0.5% or less. They have soft custard like consistency and are useful for the cultivation of microaerophilic bacteria or for determination of bacterial motility.
- **Liquid (Broth) Medium:** These media contains specific amounts of nutrients but do not have trace of gelling agents such as gelatin or agar. Broth medium serves various purposes such as propagation of large number of organisms, fermentation studies, and various other tests, for example sugar fermentation tests, MR-VR broth.

Classification of culture media based on the basis of composition, is as follows:

- Synthetic or chemically defined medium: A chemically defined medium is one prepared from purified ingredients and therefore whose exact composition is known.
- Non-synthetic or chemically undefined medium.

Non-synthetic medium contains at least one component that is neither purified nor completely characterized nor even completely consistent from batch to batch. Often these are partially digested proteins from various organism sources. Nutrient broth, for example, is derived from cultures of yeasts. Synthetic medium may be simple or complex depending up on the supplement incorporated in it. A simple non-synthetic medium is capable of meeting the nutrient requirements of organisms requiring relatively few growth factors whereas complex non-synthetic medium support the growth of more fastidious microorganisms.

Classification of Bacterial Culture Media based on the basis of Purpose/ Functional Use/ Application

Many special purpose media are needed to facilitate recognition, enumeration, and isolation of certain types of bacteria. To meet these needs, numerous media are available.

- **General Purpose Media/ Basic Media:** Basal media are basically simple media that supports most non-fastidious bacteria. Peptone water, nutrient broth and nutrient agar are considered as basal medium. These media are generally used for the primary isolation of microorganisms.
- **Enriched Medium (Added Growth Factors):** Addition of extra nutrients in the form of blood, serum, egg yolk, etc. to basal medium makes them enriched media. Enriched media are used to grow nutritionally exacting (fastidious) bacteria. Blood agar, chocolate agar, Loeffler's serum slope,

etc. are few of the enriched media. Blood agar is prepared by adding 5-10% (by volume) blood to a blood agar base. Chocolate agar is also known as heated blood agar or lysed blood agar.

- **Selective and Enrichment Media** are designed to inhibit unwanted commensal or contaminating bacteria and help to recover pathogen from a mixture of bacteria. While selective media are agar based, enrichment media are liquid in consistency. Both these media serve the same purpose. Any agar media can be made selective by addition of certain inhibitory agents that do not affect the pathogen of interest. Various approaches to make a medium selective include addition of antibiotics, dyes, chemicals, alteration of pH or a combination of these.
  - o **Selective Medium** is designed to suppress the growth of some microorganisms while allowing the growth of others. Selective medium are agar based (solid) medium so that individual colonies may be isolated. Examples of selective media include:
  - Thayer Martin Agar used to recover *Neisseria gonorrhoeae* contains antibiotics; vancomycin, colistin and nystatin.
  - Mannitol Salt Agar and Salt Milk Agar used to recover *S.aureus* contains 10% NaCl.
  - Potassium tellurite medium used to recover *C.diphtheriae* contains 0.04% potassium tellurite.
  - MacConkey's Agar used for Enterobacteriaceae members contains bile salt that inhibits most gram positive bacteria.
  - Pseudosel Agar (Cetrimide Agar) used to recover *P. aeruginosa* contains cetrimide (antiseptic agent).
  - Crystal Violet Blood Agar used to recover *S. pyogenes* contains 0.0002% crystal violet.
  - Lowenstein Jensen Medium used to recover *M.tuberculosis* is made selective by incorporating malachite green.
  - Wilson and Blair's Agar for recovering *S.typhi* is rendered selective by the addition of dye brilliant green.
  - Selective media such as TCBS Agar used for isolating *V.cholerae* from fecal specimens have elevated pH (8.5-8.6), which inhibits most other bacteria.
  - o **Enrichment Culture Medium:** Enrichment medium is used to increase the relative concentration of certain microorganisms in the culture prior to plating on solid selective medium. Unlike selective media, enrichment culture is typically used as broth medium. Enrichment media are liquid media that also serves to inhibit commensals in the clinical specimen. Selenite F broth, tetrathionate broth and Alkaline Peptone Water (APW) are used to recover pathogens from fecal specimens.
- **Differential/ Indicator Medium:** Certain media are designed in such a way that different bacteria can be recognized on the basis of their colony

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colour. Various approaches include incorporation of dyes, metabolic substrates, etc. so that those bacteria that utilize them appear as differently colored colonies. Such media are called differential media or indicator media. Differential media allow the growth of more than one microorganism of interest but with morphologically distinguishable colonies. Examples of differential media include:

- Mannitol salts agar (mannitol fermentation = yellow).
- Blood agar (various kinds of hemolysis, i.e., α, β and γ hemolysis).
- MacConkey agar (lactose fermenters, pink colonies whereas non-lactose fermenter produces pale or colorless colonies.
- TCBS (*Vibrio cholerae* produces yellow colonies due to fermentation of sucrose).

- **Transport Media:** Clinical specimens must be transported to the laboratory immediately after collection to prevent overgrowth of contaminating organisms or commensals. This can be achieved by using transport media. Such media prevent drying (desiccation) of specimen, maintain the pathogen to commensal ratio and inhibit overgrowth of unwanted bacteria. Some of these media (Stuart's and Amie's) are semi-solid in consistency. Addition of charcoal serves to neutralize inhibitory factors. Examples of transport media include:
  - Cary Blair transport medium and Venkatraman Ramakrishnan (VR) medium are used to transport feces from suspected cholera patients.
  - Sach's buffered glycerol saline is used to transport feces from patients suspected to be suffering from bacillary dysentery.
  - Pike's medium is used to transport streptococci from throat specimens.
- **Anaerobic Media:** Anaerobic bacteria need special media for growth because they need low oxygen content, reduced oxidation –reduction potential and extra nutrients. Media for anaerobes may have to be supplemented with nutrients like hemin and vitamin K. Such media may also have to be reduced by physical or chemical means. Boiling the medium serves to expel any dissolved oxygen. Addition of 1% glucose, 0.1% thioglycollate, 0.1% ascorbic acid, 0.05% cysteine or red hot iron filings can render a medium reduced. Before use the medium must be boiled in water bath to expel any dissolved oxygen and then sealed with sterile liquid paraffin. Examples of Anaerobic media include:

  Robertson Cooked Meat (RCM) medium that is commonly used to grow *Clostridium* spp. contains a 2.5 cm column of bullock heart meat and 15 ml of nutrient broth. Thioglycollate broth contains sodium thioglycollate, glucose, cystine, yeast extract and casein hydrolysate. Methylene blue or resazurin is an oxidation-reduction potential indicator that is incorporated in the medium. Under reduced condition, methylene blue is colorless.
- **Assay Media:** These media are used for the assay of vitamins, amino acids and antibiotics. For example, antibiotic assay media are used for

determining antibiotic potency by the microbiological assay technique and for enumeration and maintenance of Bacteria.

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Sterilization: The sterilization is an absolute term which denotes the complete killing or removal of microorganisms of all kinds, while the disinfection is a relative term indicating a mere removal of burden of pathogenic microorganisms. Sterilization is the process of freeing of an article from all living organisms, including viruses, bacteria and their spores, fungi and their spores, both pathogens and non-pathogens. Sterility is an absolute state. An article can never be 'relatively sterile'. Sterilization of culture media, containers and instruments is essential in bacteriological work for the isolation and maintenance of pure culture. In nursing practice, surgery and medicines, the sterilization of the instruments, drugs and other supplies is important for the prevention of the disease and it is also required for medical and surgical instruments and materials used in procedures that involve the pen-etration into the blood, tissues and other normally sterile parts of the body, for example surgical operation, intravenous transfusion, hypodermic injections and diagnostic aspirations. Disinfection is a method of freeing an article or instrument from some or all living pathogens which may produce the infection during the use of the instruments contaminated with the pathogens. This term is relative since the effectiveness of disinfection depends upon the proportion of the pathogenic microorganisms, killed or removed. In circumstances where the sterility is not necessary or sterilizing procedures are unwanted and impracticable, the disinfection should be adopted, for example bedpans, baths, wash basins, furniture, eating utensils, bed clothes which may spread the infection in the hospitals, cannot be sterilized but they can be easily disinfected. Similarly, it is not practicable to apply sterilizing procedures to the skin. The non-spore forming bacteria present in the skin mostly infect the surgical wound, hence it is of great necessity to disinfect the operative site as a valuable preoperative precaution to kill the vegetative bacteria.

Methods of Sterilization: Exposure to harmful microorganisms can be prevented by utilizing the correct sterilization methods. Though they are invisible to the naked eye, organisms capable of causing infection are everywhere. Sterile bandages, instruments, and equipment are necessary for preventing infection in animals receiving veterinary care.

- **Steam**: The use of steam under pressure is most commonly used by veterinary hospitals to sterilize items. The three factors that dictate the success of steam sterilization are temperature, pressure and exposure time. Increasing pressure of steam in a closed container causes the temperature of the steam to rise. When microbes are exposed to the correct temperature and pressure for the right amount of time, they are destroyed and the items they were on become sterile. The device used for steam sterilization is called an autoclave. The minimum time, temperature, and pressure required to sterilize items is 10 minutes at 275 ºF or 15 minutes at 250 ºF and 15 pounds per square inch of pressure.
- **Chemical (Gas)**: Some items will be destroyed when exposed to the temperatures and pressures required for steam sterilization. These items

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include plastics, power cables and endoscopes. Ethylene oxide is a gas that can be used to sterilize these types of items. Exposure to the gas at under appropriate conditions results in sterility. Ethylene oxide is flammable, carcinogenic, can cause numerous health problems and is environmentally harmful.

- **Plasma:** A safer method of sterilizing heat-sensitive items is plasma sterilization. This method uses reactive ions, electrons and neutrons to sterilize items in about 45 minutes at temperatures as low as 122 ºF.
- **Ionizing Radiation**: Most prepackaged sterile items like surgical gloves and suture packets have been sterilized with ionizing radiation. Exposure of these items to a radioactive source, such as cobalt 60, destroys microorganisms. This process is expensive and limited to commercial use. In practice, sterilization by radiation is achieved by the use of high speed cathode rays (electrons), X-rays and short X-rays (gamma rays) from an apparatus (linear accelerator) or gamma rays from an isotope source (cobalt 60).This method is very costly for hospital use; but it is used commer-cially only for the sterilization of prepacked disposable plastic syringes, transfusion sets and catheters which cannot withstand the heat. It is known as cold sterilization.
- **Cold Chemical:** Cold chemical sterilization is a common and inexpensive method of sterilizing items that cannot be exposed to steam sterilization. The most common chemical used is glutaraldehyde. A 2 % glutaraldehyde solution is noncorrosive to metal and delicate equipment like endoscopes. Immersion times in the solution vary depending on the item. Items should be thoroughly rinsed with sterile water prior to being used on a patient.

1.4.2 Inoculation and Growth Monitoring

Inoculation is a set of methods of artificially inducing immunity against various infectious diseases. The terms inoculation, vaccination, and immunization are often used synonymously, but there are some important differences among them.

The word 'Inoculation' comes from the Latin word '*inoculare*' which has the meaning 'To Graft'. In middle English, inoculate meant 'To insert a bud in a plant'. The bud of a plant resembles an eye (Latin: *oculus*) and therefore inoculate was used to describe grafting or implanting in horticulture. This term for grafting or implanting was then applied to the process of 'Inoculating' a person against a disease, initially regarding smallpox, then following 1799, the term was widened for inoculating via vaccination against many different diseases.

In microbiology, monitoring the growth of any microorganism in culture is important for studying and optimizing the growth kinetics, the biomass and the metabolite production. In this work, we show that laser speckle imaging is a reliable technique that can be used to perform real-time monitoring of bacteria growth kinetic in liquid culture media. Speckle parameters, specifically speckle grain size and the spatial contrast of the speckle images, and standard analytical parameters (optical density, pH and colony forming units) were measured during the culture of

different strains of Bacillus thuringiensis. Our results show that both speckle grain size and spatial contrast decrease with bacterial growth. Furthermore, speckle parameters are sensitive to the fermentation conditions. Statistical analysis revealed a relatively high correlation between speckle and analytical parameters.

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Growth of Microorganisms

The growth of microorganisms is a highly complex and coordinated process, ultimately expressed by increase in cell number or cell mass. The process of growth depends on the availability of requisite nutrients and their transport into the cells, and the environmental factors, such as aeration, O_2 supply, temperature and pH.

Doubling time refers to the time period required for doubling the weight of the biomass while generation time represents the period for doubling the cell numbers. Doubling times normally increase with increasing cell size and complicity as given below.

Bacteria 0.30 – 1 Hour

Yeasts 1 – 2 Hours

Animal Cells 25 -48 Hours

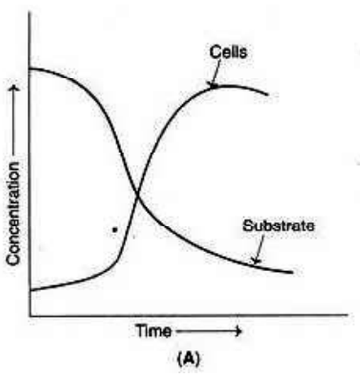
Plant Cells 20 -70 Hours

Basically, when all other conditions are kept ideal, growth of the microorganisms is dependent on the substrate (nutrient) supply. The microorganisms can be grown in batch, fed-batch, semi-continuous or continuous culture systems in a bioreactor.

A diagrammatic representation of microbial cell growth in relation to substrate is depicted in Figure (A, B, C) given below. In batch fermentation, the growth medium containing the substrates is inoculated with microorganisms, and the fermentation proceeds without the addition of fresh growth medium.

In fed-batch fermentation, substrates are added at short time intervals during fermentation. In batch and fed-batch fermentation, the growth of the cells is quite comparable. And in both cases, growth medium is not removed until the end of fermentation process.

Figure given below illustrates the diagrammatic representation of microbial cell growth in relation to substrate (A) Batch Fermentation, (B) Fed-Batch Fermentation and (C) Continuous Fermentation.



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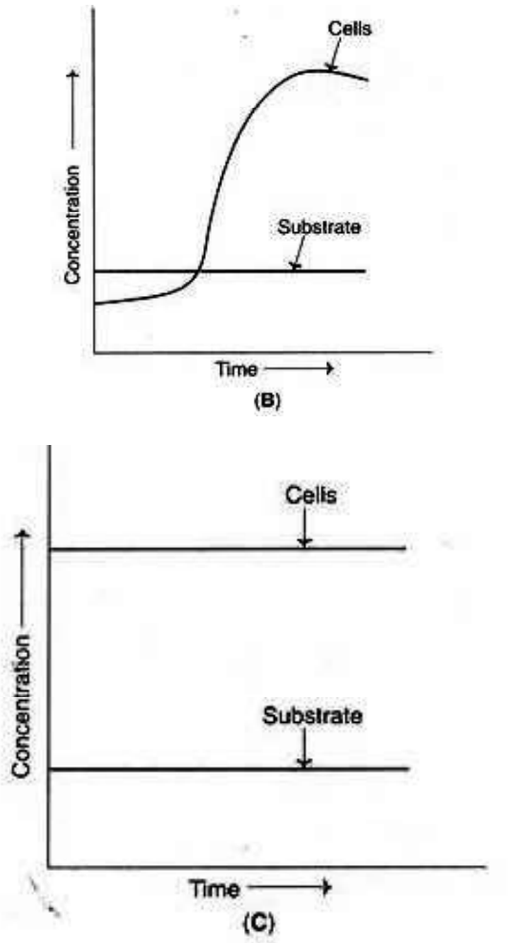

Fig 1.39 *Diagrammatic Representation of Microbial Cell Growth in Relation to Substrate (A) Batch Fermentation, (B) Fed-Batch Fermentation and (C) Continuous Fermentation*

In case of continuous fermentation, as the fermentation proceeds, fresh growth medium is added continuously. Simultaneously, an equal volume of spent medium containing suspended microorganisms is removed. This enables the cells to grow optimally and continuously (Figure C).

Batch Culture or Batch Fermentation

A batch fermentation is regarded as a closed system. The sterile nutrient culture medium in the bioreactor is inoculated with microorganisms. The incubation is carried out under optimal physiological conditions (pH, temperature, O_2 supply, agitation, etc.). It may be necessary to add acid or alkali to maintain pH, and anti-foam agents to minimise foam. Under optimal conditions for growth, the following six typical phases of growth are observed in batch fermentation as shown below in the given Figure 1.40.

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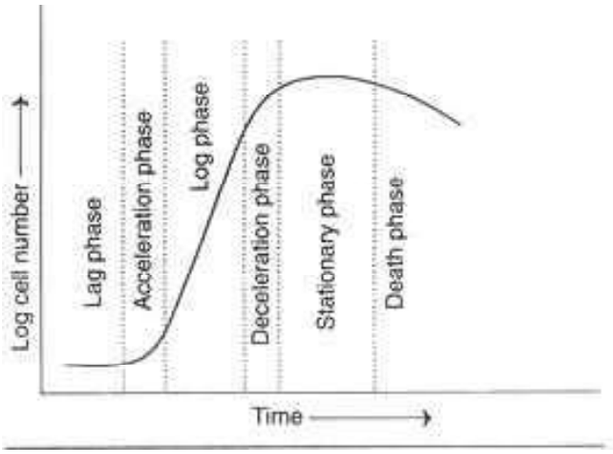

Fig 1.40 *Pattern of Microbial Cell Growth in Batch Culture or Batch Fermentation*

The above Figure illustrates the pattern of microbial cell growth in batch culture or batch fermentation. It includes the following six phases:

1. Lag Phase
2. Acceleration Phase
3. Logarithmic (Log) Phase (Exponential Phase)
4. Deceleration Phase
5. Stationary Phase
6. Death Phase

1. **Lag Phase:** The initial brief period of culturing after inoculation is referred to as lag phase. During the lag phase, the microorganisms adapt to the new environment—available nutrients, pH, etc. There is no increase in the cell number, although the cellular weight may slightly increase.

   The length of the lag phase is variable and is mostly determined by the new set of physiological conditions, and the phase at which the microorganisms were existing when inoculated. For example, the lag phase may not occur if the culture inoculated is at exponential phase, i.e., log phase and growth may start immediately.

2. **Acceleration Phase:** The acceleration phase is a brief transient period during which cells start growing slowly. In fact, acceleration phase connects the lag phase and log phase.

3. **Log Phase:** The most active growth of microorganisms and multiplication occur during log phase. The cells undergo several doublings and the cell mass increases. When the number of cells or biomass is plotted against time on a semi logarithmic graph, a straight line is obtained, hence the term log phase.

   Growth rate of microbes in log phase is independent of substrate (nutrient supply) concentration as long as excess substrate is present, and there are no growth inhibitors in the medium. In general, the specific growth rate of microorganisms

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for simpler substrates is greater than for long chain molecules. This is explained on the basis of extra energy needed to split long chain substrates.

Two log phases are observed when a complex nutrient medium with two substrates is used in fermentation, and this phenomenon is referred to as **diauxy**. This happens since one of the substrates is preferentially metabolised first which represses the breakdown of second substrate. After the first substrate is completely degraded second lag phase occurs, during which period, the enzymes for the breakdown of second substrate the synthesized. Now a second log phase occurs.

4. **Deceleration Phase:** As the growth rate of microorganisms during log phase decreases, they enter the deceleration phase. This phase is usually very short-lived and may or may not be observable.
5. **Stationary Phase:** As the substrate in the growth medium gets depleted, and the metabolic end products that are formed inhibit the growth, the cells enter the stationary phase. The microbial growth may either slow down or completely stop. The biomass may remain almost constant during stationary phase. This phase, however, is frequently associated with dramatic changes in the metabolism of the cells which may produce compounds (secondary metabolites) of biotechnological importance, for example production of antibiotics.
6. **Death Phase:** The death phase is associated with cessation of metabolic activity and depletion of energy reserves. The cells die at an exponential rate so that a straight line may be obtained when the number of surviving cells are plotted against time on a semi logarithmic plot. In the commercial and industrial fermentations, the growth of the microorganisms is halted at the end of the log phase or just before the death phase begins, and the cells are harvested.

Fed-Batch Culture or Fed-Batch Fermentation

Fed-batch fermentation is an enhancement of batch fermentation wherein the substrate is added in increments at different times throughout the course of fermentation. In batch culture method, substrate is added only at the beginning of the fermentation. Periodical substrate addition prolongs log and stationary phases which results in an increased biomass. Consequently, production of metabolites, such as antibiotics during stationary phase is very much increased.

As it is difficult to directly measure substrate concentration in fed-batch fermentation, other indicators that correlate with substrate consumption are used. The formation of organic acids, production of CO_2 and changes in pH may be measured, and accordingly substrate addition carried out. In general, fed-batch fermentation requires more careful monitoring than batch fermentation, and is therefore not a preferred method by industrial biotechnologists.

The Growth Curve

Microorganisms grown in closed culture (also known as a batch culture), in which no nutrients are added and most waste is not removed, follow a reproducible growth pattern referred to as the growth curve. An example of a batch culture in

nature is a pond in which a small number of cells grow in a closed environment. The culture density is defined as the number of cells per unit volume. In a closed environment, the culture density is also a measure of the number of cells in the population. Infections of the body do not always follow the growth curve, but correlations can exist depending upon the site and type of infection. When the number of live cells is plotted against time, distinct phases can be observed in the curve (Figure given below).

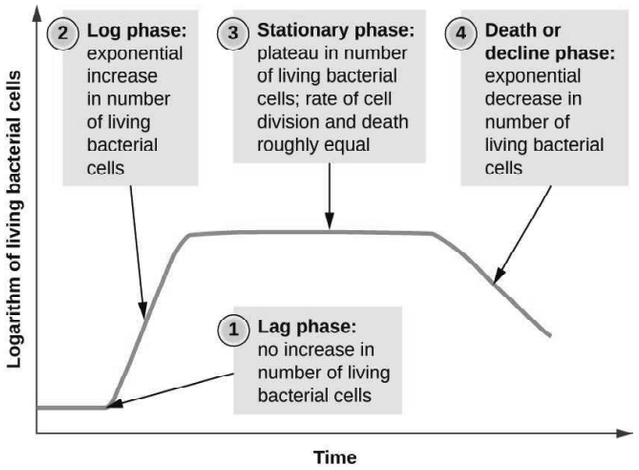

Fig. 1.41 *The Growth Curve of a Bacterial Culture*

Figure given above shows the growth curve of a bacterial culture which is represented by the logarithm of the number of live cells plotted as a function of time. The graph can be divided into four phases according to the slope, each of which matches events in the cell. The four phases are lag, log, stationary, and death.

1.4.3 Use of Fermenters

Fermenters are designed to contain an internal environment for the optima metabolization and efficient reproduction of microorganisms. In biology, the fermenters or stirred-tank bioreactors are process vessels used to cultivate heterotrophic microalgae. Fundamentally, eukaryotic microalgae grow in a bioreactor under lower mixing intensity, and prokaryotic microalgae grow in a fermenter.

De Becze and Liebmann (1944) used the first large scale (above 20 litre capacity) fermenter for the production of yeast. But it was during the First World War, a British scientist named Chain Weizmann (1914-1918) developed a fermenter for the production of acetone.

When the significance of aseptic conditions was recognized, then the steps were taken to design and construct piping, joints and valves in which sterile conditions could be achieved and manufactured when required. The fermenter consisted of a large cylindrical tank with air introduced at the base via network of perforated pipes.

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Later, with the modifications in technology, the mechanical impellers were used to increase the rate of mixing and to break up and disperse the air bubbles. This process led to the compressed air requirements. Baffles on the walls of the vessels prevented a vortex forming in the liquid. In the year 1934, Strauch and Schmidt patented a system in which the aeration tubes were introduced with water and steam for cleaning and sterilization.

Types of Fermenters

Fluidized Bed Bioreactor: It is more popular in chemical industry rather new to biochemical industries. These are mostly used in conjunction with immobilized cells or enzyme system and are operated continuously.

Loop or Air Lift Bioreactor: In the conventional bioreactor, oxygen is supplied by vigorous agitation of the bioreactor content. The heat is generated which is a problem in conventional type. In air lift fermenter, cooling becomes simpler due to the position of inner or outer loop.

Membrane Bioreactor: These consist of a semipermeable membrane made up of cellulose acetate or other polymeric materials. The primary purpose of the membrane is to retain the cells within the bioreactor, thus increasing their density, while at the same time allowing metabolic products to pass through the membrane.

Pulsed Column Bioreactor: The essential component of a pulsed column bioreactor is a column bioreactor generator connected to the bottom of the column. A pulsed column bioreactor can be utilized as an aerobic bioreactor, enzyme bioreactor or as a separation unit since its original successful application was in the extraction of uranium.

Bubble Column Bioreactor: Multistage bubble column bioreactor are suitable in the equivalent batch process. It is necessary to vary the environmental conditions over the course of the reaction. In this bioreactor, it is possible to provide different environmental conditions in various stages. The system may not be suitable for fungal fermentation due to high oxygen demanding system.

Photo Bioreactor: For the growth and production of photosynthetic organisms, a light source is retired. In photo-bioreactor, there is an important 'Reactant', the photons which must be absorbed in order to react and produce products. Therefore, the design of the light source is critical in the performance of this type of bioreactor.

One of the most interesting photochemical reactors is the annular reactor. In this source of radiation is a cylinder with an annular section, which encloses the lamp completely. The nutrient passing from the product is removed from the top. This is used for Spirulina (SCP) and other algal protein production.

Packed Tower Bioreactor: It consists of cylindrical column packed with inert material like wood shavings, twigs cake, polyethylene or sand. Initially, both medium and cells are fed into the top of the packed bed. Once the cells adhered to the support and were growing well as a thin film, fresh medium is added at the top of the packed bed and the fermented medium removed from the bottom of the column.

This is used for vinegar production, sewage effluent treatment and enzymatic conversion of penicillin to 6-amino penicillanic acid. The design of fermenter involves the cooperation between experts in microbiology, biochemistry, mechanical engineering and economics.

Applications

Microbial biosensors have several uses in clinical analysis, general health care monitoring, veterinary and agricultural applications, industrial product processing and monitoring besides control of environmental pollution. In addition to low cost and small size, these are easy to use sensitive and selective in nature.

A large number of bacteria and eukaryotic cell culture are used in manufacturing new products. The monitoring of these items is essential by using microbial biosensors. This allows in minimizing the cost of production. Biosensors have immense importance to military and defense in detection of chemical and biological species used in weapons.

1.4.4 Microbial Assays

Microbial assays or microbiological assays can be considered as a type of bioassays specifically designed to analyse the compounds or substances that impact the microorganisms. They estimate the concentration and efficiency of antibiotics.

As per Britannica, "*Microbiological assay is applicable only to the B vitamins. The rate of growth of a species of microorganism that requires vitamin is measured in growth media that contain various known quantities of a foodstuff preparation containing unknown amounts of the vitamin. The response (measured as rate of growth) to the unknown amounts of vitamin is compared with that obtained from a known quantity of the pure vitamin*".

Microbiological Assay of Antibiotics

The inhibition of growth under standardized conditions are utilized for demonstrating the therapeutic efficacy of antibiotics.

Any subtle change in the antibiotic molecule which may not be detected by chemical methods will be revealed by a change in the antimicrobial activity and hence microbiological assays are very useful for resolving doubts regarding possible change in potency of antibiotics and their preparations.

Estimation and Properties

The potency of an antibiotic is estimated by comparing the inhibition of growth of sensitive microorganisms produced by known concentrations of the antibiotic to be examined and a reference substance.

The reference substances used in the assays are substances whose activity has been precisely determined with reference to the corresponding international standard or international reference preparation.

The assay is typically designed to examine the validity of the mathematical model on which the potency equation is based.

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If a parallel line model is selected, then the 2 log dose-response lines of the preparation is examined and the reference preparation is referred to be parallel for which it is linear on the range of doses used in the calculation.

These conditions must be validated or verified by means of validity tests for a given probability, usually P=0.05.

The mathematical models, such as the slope ratio model, may be used provided that proof of validity is determined.

Microorganisms have been extensively used in the implementation of bioassays to:

- Determine the concentration of certain compounds, such as amino acids, vitamins and some antibiotics in complex chemical mixtures or in body fluids.
- Diagnose certain diseases.
- Test chemicals for potential mutagenicity or carcinogenicity.
- Monitor the use of immobilized enzymes.
- Determine the pharmacokinetics of drugs in animal and human.

The microbial assays are also typically used in sterility testing of antibiotics. Microbiological assays are used to determine the potency and quality control. In antimicrobial chemotherapy, the microbial assays are used to monitor, manage and control the chemotherapeutic agents.

An assay is an investigative (analytic) procedure in laboratory medicine, mining, pharmacology, environmental biology and molecular biology for qualitatively assessing or quantitatively measuring the presence, amount, or functional activity of a target entity. The analyte can be a drug, biochemical substance, chemical element or compound, or cell in an organism or organic sample. The measured entity is often called the analyte, the measurand, or the target of the assay. An assay usually aims to measure an analyte's intensive property and express it in the relevant measurement unit, for example molarity, density, functional activity in enzyme international units, degree of effect in comparison to a standard, etc.

Principle

The microbiological assay is based upon a comparison of the inhibition of growth of microorganisms by measured concentration of the antibiotics to be examined with that produced by known concentrations of a standard preparation of the antibiotic having a known activity.

Following two general methods are normally used:

1. The Cylinder-Plate or Cup-Plate Method.

2. The Turbidimetric or Tube Assay Method

Preparation of Media

The 'Media' required for the preparation of test organisms are made from the ingredients. Minor modifications of the individual ingredients may be made or reconstituted dehydrated media may be used provided that the resulting media

have equal or better growth promoting properties and give a similar standard curve response.

Dissolve the ingredients in sufficient water to produce 1000 ml and add sufficient 1M Sodium Hydroxide or 1M Hydrochloride Acid, as required so that after sterilization the pH is between 6.5 to 7.5.

Preparation of Buffer Solutions

Buffer solutions are prepared by dissolving the quantities of Dipotassium Hydrogen Phosphate and Potassium Dihydrogen Phosphate in sufficient water to produce 1000 ml after adjustive the pH with 8 M Phosphoric Acid or 10M Potassium Hydroxide as shown below.

Table 1.7

Buffer No.	Dipotassium Hydrogen Phospahate, K_2HPO_4 (g)	Potassium Dihydrogen Phosphate, KH_2PO_4 (g)	pH adjusted after sterilisation to :
1	2.0	8.0	6.0 ± 0.1
2	16.73	0.532	8.0 ± 0.1
3	-	13.61	4.5 ± 0.1
4	20.0	80.00	6.0 ± 0.1
5	35.0	-	10.5 ± 0.1*
6	13.6	4.0	7.0 ± 0.2

Check Your Progress

14. What is a microbial culture?
15. Define inoculation.
16. Write the difference between the doubling time and generation time.
17. What are fermenters?
18. What do you understand by microbial assays?

1.5 CELL CULTURE TECHNIQUES

Cell culture is the process by which cells are grown under controlled conditions, generally outside their natural environment. After the cells of interest have been isolated from living tissue, they can subsequently be maintained under carefully controlled conditions. These conditions vary for each cell type, but generally consist of a suitable vessel with a substrate or medium that supplies the essential nutrients (amino acids, carbohydrates, vitamins, minerals), growth factors, hormones, and gases (CO_2, O_2), and regulates the physio-chemical environment (pH buffer, osmotic pressure, temperature). Most cells require a surface or an artificial substrate

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(adherent or monolayer culture) whereas others can be grown free floating in culture medium (suspension culture). The lifespan of most cells is genetically determined, but some cell culturing cells have been 'Transformed' into immortal cells which will reproduce indefinitely if the optimal conditions are provided.

Actually, the term 'Cell Culture' refers to the culturing of cells derived from multicellular eukaryotes, especially animal cells, in contrast with other types of culture that also grow cells, such as plant tissue culture, fungal culture, and microbiological culture (of microbes). The historical development and methods of cell culture are closely interrelated to those of tissue culture and organ culture. Viral culture is also related, with cells as hosts for the viruses.

The laboratory technique of maintaining live cell lines, i.e., a population of cells descended from a single cell and containing the same genetic makeup, separated from their original tissue source became more robust in the middle 20th century.

Much of the study of microbiology depends on the ability to grow and maintain microorganisms in the laboratory, and this is possible only if suitable culture media are available. In the natural habitats, microorganisms usually grow in complex mixed populations with many species. This presents problem for microbiologists because a single type of microorganism cannot be studied in a mixed culture, one needs a pure culture, a population of cells arising from a single cell, to characterize individual species. However, to isolate microorganisms from the natural environment, it is necessary to perform serial dilution to reduce or thin out the population of microorganisms in the sample sufficiently.

Serial Dilution: Ten test tubes are filled with 9ml of water and then sterilized, (they are labeled -1 to -10). With a sterile pipette, 1ml of the original sample is taken and transferred into the first test tube (-1), vortexed, and 1ml taken and transferred into the next test tube (-2). The process is repeated till the last test tube (-10). Depending on the type of plating to be carried out, a known volume is taken from any of the dilutions with a sterile pipette, and dropped on the agar in the petri dishes, spreading is done with a sterile glass rod. The petri dishes are allowed to dry, and the incubated. Isolated cells grow into colonies and can be used to establish pure cultures (Refer Figure 1.42).

As certain disadvantages are encountered in this method like heat sensitive microorganisms might not grow and also bacteria when they grow within the nutrient medium might not be useful for diagnostic purposes. In order to avoid these problems, spread plate method is mostly used. A 0.1ml of the diluted sample is added to the surface of the nutrient medium and spread uniformly with the help of a glass spreader and after incubation, the colonies can be counted and the concentration of the bacterial cells in the original sample is calculated.

Number of bacteria/ml = Number of colonies on plate x Reciprocal of dilution of sample.

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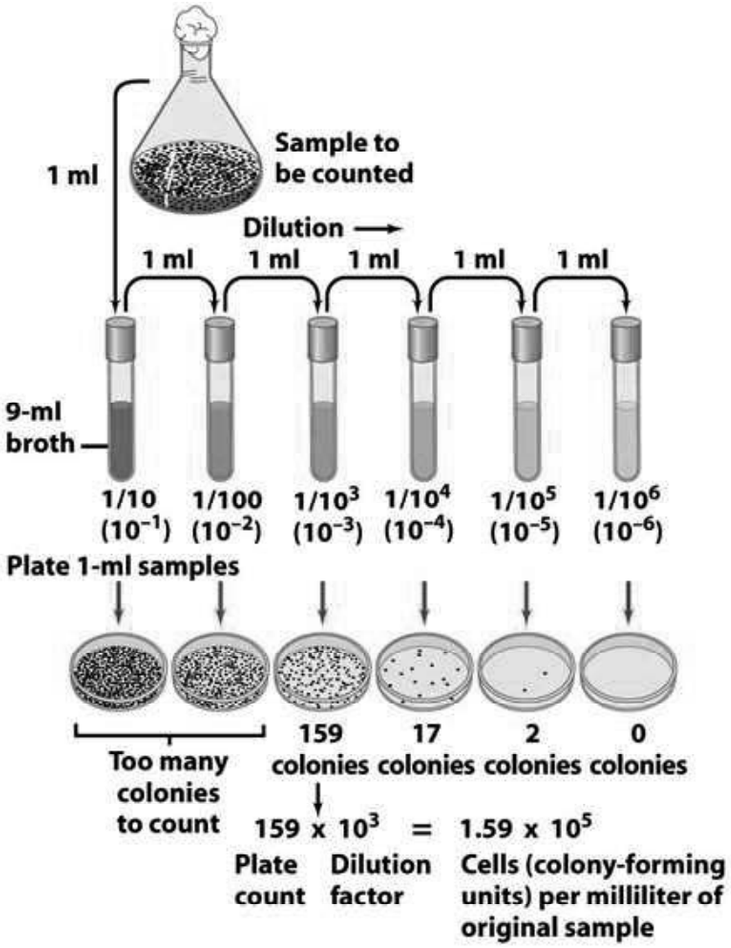

Fig. 1.42 *Process of Serial Dilution Method*

The Spread Plate: A small volume of dilute microbial mixture containing around 30 to 300 cells is transferred to the center of an agar plate and spread evenly over the surface with sterile bent-glass rod. The dispersed cells develop into isolated colonies. Because the number of colonies should equal the number of viable organisms in the sample, spread plate can be used to count the microbial population (Refer Figure 1.43).

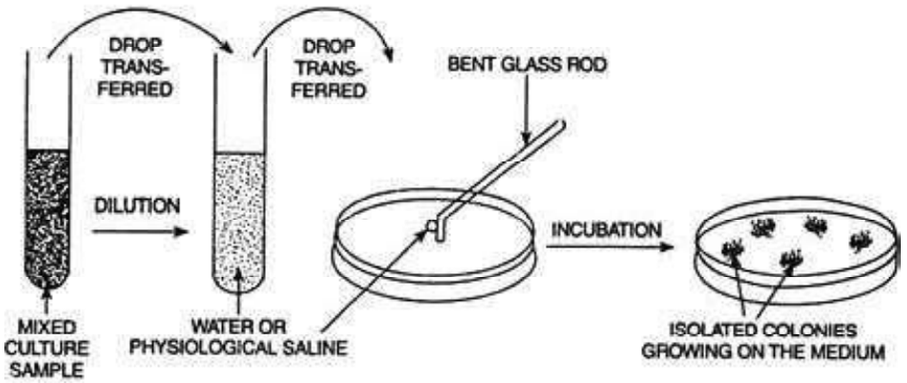

Fig. 1.43 *The Process of Spread Plate Method*

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The Streak Plate: The microbial mixture is transferred to the edge of an agar plate with an inoculating loop or swab and then streaked out over the surface in one of several patterns. After the first sector is streaked, the inoculating loop is sterilized and an inoculum for the second sector is obtained from the first sector. A similar process is followed for streaking the third sector, except that the inoculum is from the second sector. Thus, this is essentially a dilution process. Eventually, very few cells will be on the loop, and single cells will drop from it as it is rubbed along the agar surface. These develop into separate colonies. In both spread-plate and streak plate techniques, successful isolation depends on spatial separation of single cells (Figure 1.44).

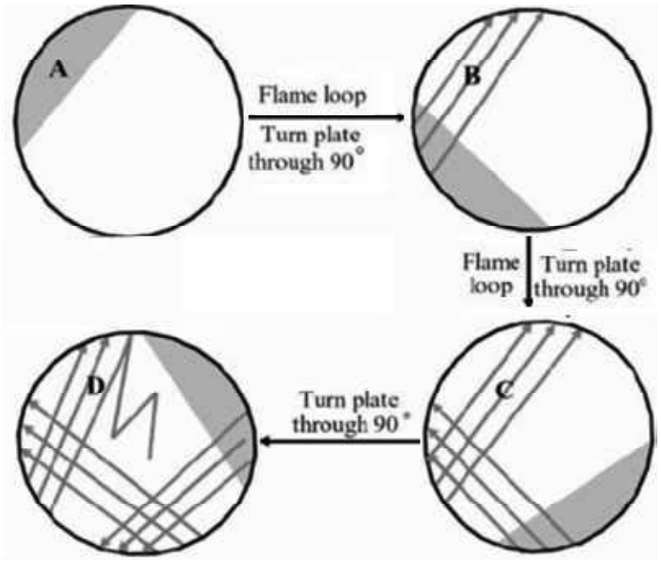

Fig. 1.44 *The Process of Streak Plate Method*

The Pour Plate: The original sample is diluted several times to reduce the microbial population sufficiently to obtain separate colonies when plating. Then small volumes of several diluted samples are mixed with liquid agar that has been cooled to about 45°C, and the mixtures are poured immediately into sterile culture dishes. Most bacteria and fungi are not killed by a brief exposure to the warm agar after the agar has hardened; each cell is fixed in place and forms an individual colony. Like the spread plate, the pour plate can be used to determine the number of cells in a population. Plates containing between 30 and 300 colonies are counted. The total number of colonies equals the number of viable microorganism in the sample that are capable of growing in the medium as colonies growing on the surface also can be used to inoculate fresh medium and prepare pure cultures (Refer Figure 1.45).

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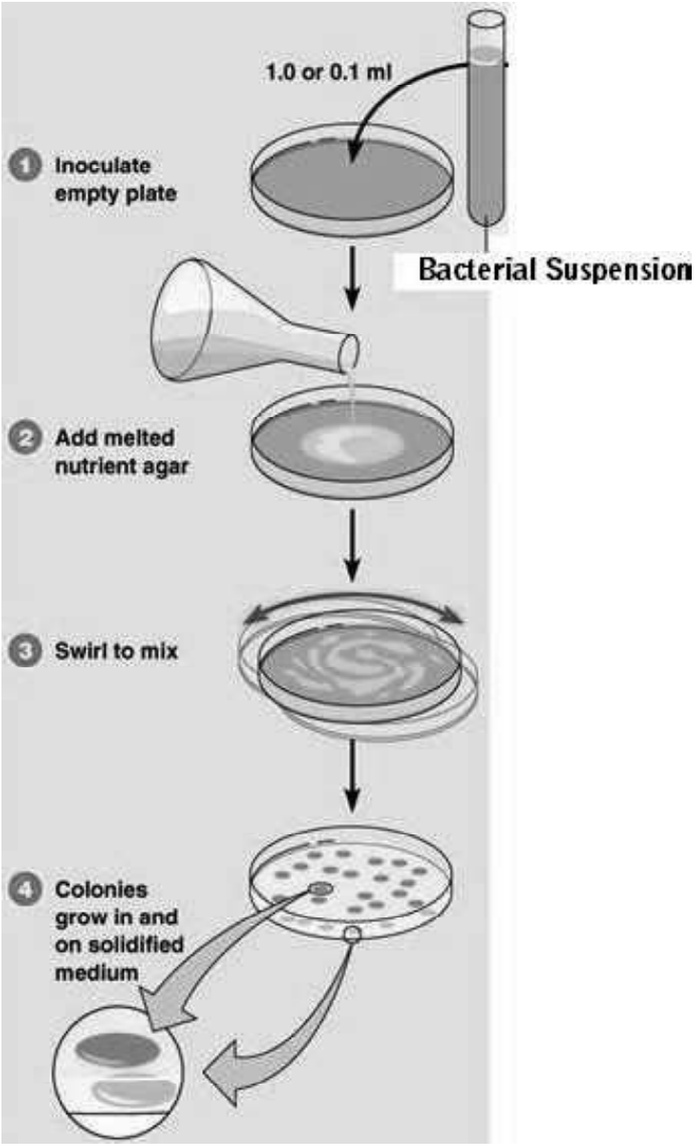

Fig. 1.45 *Process of Pour Plate Method*

1.5.1 Design and Functioning of Tissue Culture Laboratory

Tissue culture is the growth of tissues or cells in an artificial medium separate from the parent organism. This technique is also called micropropagation. This is typically facilitated via use of a liquid, semi-solid, or solid growth medium, such as broth or agar. Tissue culture commonly refers to the culture of animal cells and tissues, with the more specific term plant tissue culture being used for plants. The term 'Tissue Culture' was coined by American pathologist Montrose Thomas Burrows.

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General principles of tissue culture laboratory planning include the following.

Designated working areas are the significant prerequisites to providing a smooth running cell culture laboratory. By isolating each activity, contamination risks to both the operator and materials are reduced to a minimum.

Following are the key activities, each of which ideally should have their own dedicated work area:

- Preparation of sterile equipment and media.
- Function and culture of newly established or incoming cells.
- Culture of cells which have received in-house testing for microbial contamination.
- Banking of cells requiring special conditions to prevent contamination, for example cells used in the production of diagnostic and therapeutic agents.
- Cryopreservation and cell storage.

Laboratories must be planned with the natural flow of activity, while at the same time minimizing the possibility of contamination of cells and of workers by extraneous organisms, such as bacteria, fungi, mycoplasmas and viruses. It is important that waste and contaminated material should not be allowed to accumulate, as accidents are potentially more hazardous with larger volumes of waste which will be more awkward for laboratory staff to handle. Appropriate operation is more easily achieved by providing separate working areas designated for specific functions.

Setting Up a Tissue Culture Lab

Any laboratory, in which tissue culture techniques are performed, regardless of the specific purpose, must contain the following basic facilities:

- A general washing area
- A media preparation, sterilization, and storage area
- An aseptic transfer area
- Environmentally controlled incubators or culture rooms
- An observation/data collection area

Washing Area: The washing area should contain large sinks, some lead-lined to resist acids and alkalis, draining boards, and racks, and have access to demineralized water, distilled water, and double distilled water. Space for drying ovens or racks, automated dishwashers, acid baths, pipette washers and driers, and storage cabinets should also be available in the washing area.

Media Preparation Area: The media preparation area should have ample storage space for the chemicals, culture vessels and closures, and glassware required for media preparation and dispensing. Bench space for hot plates/stirrers, pH meters, balances, water baths, and media dispensing equipment should be available. Other necessary equipment may include air and vacuum sources, distilled and double-distilled water, Bunsen burners with a gas source, refrigerators and freezers for storing stock solutions and chemicals, a microwave or a convection

oven, and an autoclave or domestic pressure cooker for sterilizing media, glassware, and instruments. In preparing culture media, analytical grade chemicals should be used, and good weighing habits practiced. To ensure accuracy, and exact step-by-step routine should be developed for media preparation and a complete checklist required of all media preparers even for the simplest media.

The water used in preparing media must be of the utmost purity and highest quality. Tap water is unsuitable because it may contain cations (ammonium, calcium, iron, magnesium, sodium, etc.), anions (bicarbonates, chlorides, fluorides, phosphates, etc.), microorganisms (algae, fungi, bacterial), gases (oxygen, carbon dioxide, nitrogen), and particulate matter (silt, oils, organic matter, etc.).

Transfer Area: Under very clean and dry conditions, tissue culture techniques can be successfully performed on an open laboratory bench. However, it is advisable that a laminar flow hood or sterile transfer room be utilized for making transfers. Within the transfer area there should be a source of electricity, gas, compressed air, and vacuum. The most desirable arrangement is a small dust-free room equipped with an overhead ultraviolet light and a positive pressure ventilation unit.

The ventilation should be equipped with a High-Efficiency Particulate Air (HEPA) filter. A 0.3- μm HEPA filter of 99.97-99.99% efficiency works well. All surfaces in the room should be designed and constructed in such a manner that dust and microorganisms should not get accumulated and the surfaces can be thoroughly cleaned and disinfected.

Culture Room: All types of tissue cultures should be incubated under conditions of well-controlled temperature, humidity, air circulation, and light quality and duration. These environmental factors may influence the growth and differentiation process directly during culture or indirectly by affecting their response in subsequent generations. Protoplast cultures, low-density cell suspension cultures, and anther cultures are particularly sensitive to environmental cultural condition.

1.5.2 Cell Proliferation Measurements

Cell proliferation is the process by which a cell grows and divides to produce two daughter cells. Cell proliferation leads to an exponential increase in cell number and is therefore a rapid mechanism of tissue growth. Cell proliferation requires both cell growth and cell division to occur at the same time, such that the average size of cells remains constant in the population. Cell division can occur without cell growth, producing many progressively smaller cells (as in cleavage of the zygote), while cell growth can occur without cell division to produce a single larger cell (as in growth of neurons). Thus, cell proliferation is not synonymous with either cell growth or cell division, despite the fact that these terms are sometimes used interchangeably.

Stem cells undergo cell proliferation to produce proliferating 'Transit Amplifying' daughter cells that later differentiate to construct tissues during normal development and tissue growth, during tissue regeneration after damage, or in cancer.

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The total number of cells in a population is determined by the rate of cell proliferation minus the rate of cell death.

Total Number of Cells in a Population = Rate of Cell Proliferation - Rate of Cell Death

Cell size depends on both cell growth and cell division, with a disproportionate increase in the rate of cell growth leading to production of larger cells and a disproportionate increase in the rate of cell division leading to production of many smaller cells. Cell proliferation typically involves balanced cell growth and cell division rates that maintain a roughly constant cell size in the exponentially proliferating population of cells. Cell proliferation occurs by combining cell growth with regular "G1-S-M-G2" cell cycles to produce many diploid cell progeny.

In single-celled organisms, cell proliferation is largely responsive to the availability of nutrients in the environment or laboratory growth medium.

In multicellular organisms, the process of cell proliferation is tightly controlled by gene regulatory networks encoded in the genome and executed mainly by transcription factors including those regulated by signal transduction pathways elicited by growth factors during cell–cell communication in development. In addition, intake of nutrients in animals can induce circulating hormones of the Insulin/IGF-1 family, which are also considered growth factors, and that function to promote cell proliferation in cells throughout the body that are capable of doing so.

Uncontrolled cell proliferation, leading to an increased proliferation rate, or a failure of cells to arrest their proliferation at the normal time, is a cause of cancer. Following Figure illustrates the cell division, growth, and proliferation.

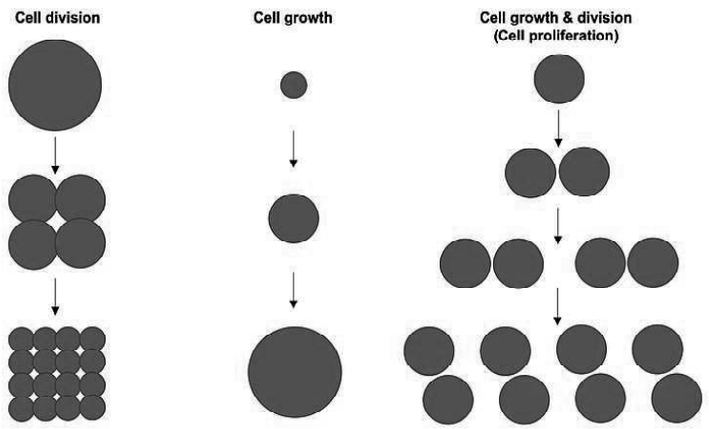

Fig. 1.46 *Cell Division, Growth and Proliferation*

Methods for Measuring Cell Proliferation

Cell proliferation is an increase in the number of cells resulting from the normal, healthy process by which cells grow and divide. By the way, cell proliferation can be a good indicator of general cell health. Cells that are subject to a variety of disease states may exhibit different rates of proliferation than normal cells. Therefore, measuring rates of cell proliferation between different cell populations may provide insight into the relative health of those cells.

There are several methods available to measure cell proliferation rates. One method is to measure the overall metabolic activity inside a cell. Several dyes are available that can permeabilize a cell and react with certain enzymes and other factors and form a coloured end-product which can be easily detected. One such dye, MTT, is a well-published molecule that produces a distinct purple colour when added to proliferating cells. Another dye, WST-1, provides a similar colour change in proliferating cells, but has the advantage of a single-step method that eliminates the detergent solubilization step required for MTT.

Fluorescence dyes are also available to measure rates of cell proliferation. These dyes have the advantage of providing greater sensitivity than colorimetric dyes, which can be a benefit when measuring smaller differences in cell proliferation rates between different cell populations.

Besides overall metabolic activity, cell proliferation may be measured by examining one or more specific markers within a cell. A well-published example is the BrdU incorporation assay. In this assay, cells are treated with BrdU, a thymidine analog that is incorporated into the DNA during cell proliferation. The BrdU can be detected with a special antibody, and high rates of BrdU incorporation correlate with high cell proliferation rates. Another such marker is Proliferating Cell Nuclear Antigen (PCNA), which promotes DNA replication in actively proliferating cells.

In addition to measuring relative rates of proliferation between different cells, cell proliferation assays can be useful for determining the effects of compounds that may have the potential for increasing or decreasing cell proliferation rates.

1.5.3 Cell Viability Testing

Cell viability is a measure of the proportion of live, healthy cells within a population. Cell viability assays are used to determine the overall health of cells, optimize culture or experimental conditions, and to measure cell survival following treatment with compounds, such as during a drug screen.

Typically, cell viability assays provide a readout of cell health through measurement of metabolic activity, ATP content, or cell proliferation. Cell viability can also be assessed using cell toxicity assays that provide a readout on markers of cell death, such as a loss of membrane integrity.

Cell viability assays are used to determine the number of healthy cells in a sample. Aside from proliferation assays described above, other cell viability assays report on the overall health of a population, without distinguishing dividing cells from non-dividing cells.

Testing Cell Viability

The most common readout of cell viability is with vital dyes, such as Propidium Iodide, however cell viability assays also typically measure the metabolic activity or ATP content of healthy cells.

Metabolic assays, such as the MTT and XTT assays quantify cell health by measuring reduction of a colorimetric substrate by mitochondrial enzymes. The MTT assay quantifies the relative quantity of viable cells using this approach. Cultures are incubated with the yellow Tetrazolium Dye MTT (3-(4,5-

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Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) which, in healthy cells, is converted by mitochondrial enzymes into an insoluble purple Formazan Product. After solubilization by detergents or isopropanol, the number of viable cells can be determined by measuring absorbance at 450 nm in a microplate reader. The XTT cell viability assay is an alternative to the MTT assay which yields a formazan product that is soluble in aqueous solutions, and thus does not require an additional solubilization step.

A viability assay is an assay that is created to determine the ability of organs, cells or tissues to maintain or recover a state of survival. Viability can be distinguished from the all-or-nothing states of life and death by the use of a quantifiable index that ranges between the integers of 0 and 1 or, if more easily understood, the range of 0% and 100%. Viability can be observed through the physical properties of cells, tissues, and organs. Some of these include mechanical activity, motility, such as with spermatozoa and granulocytes, the contraction of muscle tissue or cells, mitotic activity in cellular functions, and more. Viability assays provide a more precise basis for measurement of an organism's level of vitality.

Viability assays can lead to more findings than the difference of living versus non-living. These techniques can be used to assess the success of cell culture techniques, cryopreservation techniques, the toxicity of substances, or the effectiveness of substances in mitigating effects of toxic substances.

1.5.4 Culture Media Preparation

Essentially, **cell culture** involves the distribution of cells in an artificial environment (in-vitro) which is composed of the necessary nutrients, ideal temperature, gases, pH and humidity to allow the cells to grow and proliferate.

- **In-vivo**: When the study involves living biological entities within the organism.
- **In-vitro**: When the study is conducted using biological entities (cells, tissue, etc.) that has been isolated from their natural biological environment, for example tissue or cells isolated from the liver or kidney.

Whereas pieces of tissue can be put in the appropriate culture to produce cells that can then be used for culture (explant culture), cells from tissues (soft tissue) can be obtained through enzymatic reactions. Here, such enzymes as trypsin is used to break down the tissue and release the desired cells. When cells have been obtained directly from the organism/animal tissue (or even plant tissue) through enzymatic or mechanical techniques, such cells are referred to as primary cells. However, cells that continue to proliferate indefinitely (after the first subculture) under special conditions are referred to as cell lines. These particular cells tend to have been passaged for a long period of time, which causes them to acquire homogenous (similar) genotypic and phenotypical traits (Refer Figure 1.47).

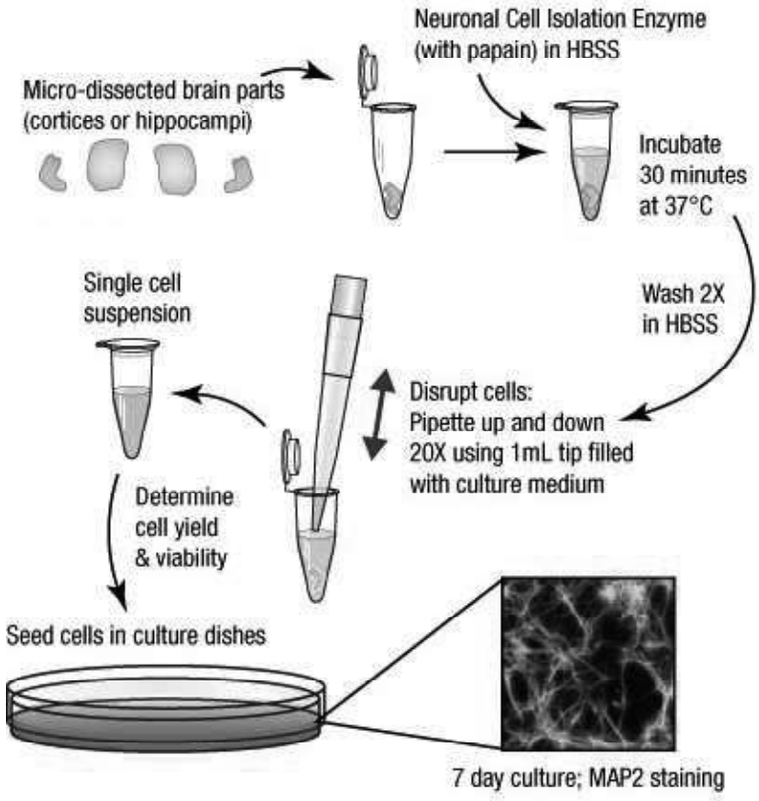

Fig. 1.47 *Process of Cell Culturing and Staining*

Morphology

Based on their appearance, cells in culture can be categorized in to three main groups:

- **Fibroblastic:** This includes cells that tend to be bipolar/multipolar with elongated shapes. These cells are attached to the substrate as they grow.
- **Epithelial:** Epithelial like cells attain a polygonal shape with regular dimensions. Although they tend to grow in discrete patches, these cells also grow attached to the substrate.
- **Lymphoblast:** These cells are usually spherical in shape and do not attach to the surface of the substrate. As a result, they are grown in suspension.

Significance of Cell Culture

Cell culture is an important technique in both cellular and molecular biology given that it provides the best platform for studying the normal physiology and biochemistry of cells. A cell is the basic structural, functional and biological unit of all living things. In order to understand an organism or given tissues, it is important to understand how its cells work. Through cell culture, this becomes possible especially due to the fact the primary cells resemble the parental cells from the organism/ tissue. Therefore, whatever is learnt about the cells in-vitro is representative of

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what is happening to the organism/tissue. This makes cell culture significantly important for vaccine development, screening (drugs, etc.) and diagnosis of given diseases/conditions. Given that different types of cells require different environments for proliferation, there are different types of media used for culture such as serum-free media and serum containing media among others. Once the right requirements have been provided, the cells will increase in numbers and may form colonies, which can then be easily seen and identified. However, all this requires that the purpose of the procedure be understood.

Having a good understanding of what the procedure is meant to achieve, it becomes easier to prepare the culture with the right components. For instance, by understanding what the procedure is aimed for, the researcher will know whether to prepare a selective media (which allow for specific cells to grow) or differential media (allowing for different types of cells to grow).

Primary Cell Culture

Cell culture is a process where cells (animal or plant cells) are removed from the organism and introduced in to an artificial environment with favorable conditions for growth. This allows for researchers to study and learn more about the cells. There are three major types of cell culture, which include:

- Primary Cell Culture
- Secondary Cell Culture
- Cell Line

There are Two Types of Primary Cells

- **Adherent Cells**: Also referred to as anchorage dependent cells, these are the type of cells that require attachment for growth. Adherent cells are immobile, and obtained from such organs as kidney.
- **Suspension Cells**: These are the type of cells that do not require attachment in order to grow. They are therefore also referred to as anchorage independent cells, and include such cells as lymphocytes found in the blood system.

In primary cell culture, cells obtained from such parental tissues (living tissues) as the liver and kidney, are introduced into suitable media for growth. Once the cells have been obtained, they can either be cultured as explants culture, suspension or monolayer. In primary cell culture, the cells must have been obtained from the parental/living tissue. That is, they are not from another culture process. Before the cells are cultured, they are first subjected to enzymatic treatment for dissociation. However, has to be for a minimal amount of time to avoid damaging or killing the cells. Once single cells are obtained, they are then appropriately cultured in media to allow them to grow (divide) are reach the desired numbers. Initially, the culture

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tends to be heterogeneous in that it is composed of different types of cells obtained from the tissue. Although this can be maintained through the in vitro process (in a culture in a suitable media) this would only be for a limited period of time. However, through the transformation process, the primary cells may be used for a long period of time, changing the culture over time. These cells are refers to as continuous cell lines. However, primary cells are typically preferred over continuous cell lines because of the fact that they are more similar (physiologically) to in vivo cells (cells from the living tissue). In addition, continuous cell lines may undergo certain changes (phenotypic and genotypic changes) which would result in discrepancies during analysis. As such, they cannot be used to determine what is happening to the in-vivo cells. It is for this reason that primary cells are preferred. Given that the primary cells significantly resemble the cells obtained from living tissue, they are important for research purposes in that they can be used to study their functions, metabolic regulations, cell physiology, development, defects and conditions affecting the tissue of interest. In addition, they are used for such purposes as vaccine production, genetic engineering drug screening as well as toxicity testing and prenatal diagnosis among others.

Cell Culture Media

In cell culture techniques, cells (or tissues) are removed from a plant or an animal and introduced into a new, artificial environment that can support their proliferation (survival and growth). Some of the requirements of such an environment for the proliferation of the cells include; a substrate (source of nutrition), ideal temperature range (controlled), growth medium, and ideal pH among others. Although there are different types of culture media (for different types of cells) they are typically composed of glucose, amino acids, vitamins, inorganic salts and attachment factors, etc. There are two major types of culture media. These include:

- **Natural Media**: Natural culture media is composed of biological fluids that are naturally occurring. Although, this type of media can be used for a range of cells, its biggest disadvantage is that it may lack the exact components required by given cells, which can greatly affect reproducibility.
- **Artificial Media**: Also referred to as synthetic media, artificial media refers to the type of media that is produced by adding such nutrients as vitamins, gases (oxygen and carbon dioxide) and protein among others. These organic and inorganic nutrients are added so as to meet the specific needs of given cells, and thus provide the ideal environment for their growth. As such, they can be used for a number of purposes including:
    - o Providing immediate survival of the cells
    - o Allowing for prolonged survival of the cells
    - o Allowing for indefinite growth of the cells
    - o Providing for specialized functions

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On the other hand, culture may be categorized as:

- **Selective Media**: This is a special type of media that only allows for certain cells to grow. For instance, blood agar (used to isolate Streptococcus and Moraxella species) can be turned in to a selective media by adding antibiotics.
- **Differential Media**: This type of media allows for different types of cells/ microorganisms to grow depending on their metabolism. As mentioned above, different types of synthetic media are prepared in a manner that will provide the ideal proliferation environment for given cells. For this reason, synthetic media can be divided in to four major categories. These include:
  - **Serum Containing Media**: In these types of media, serum (fetal bovine serum) is used as a carrier for nutrients and growth factors among others that tend to be water insoluble.
  - **Serum-Free Media**: These types of media is typically produced for the purposes of supporting single cell type of culture. As such, it provides specified nutrients and other factors required by the cell type. In this media, serum is absent because it present some disadvantages and can result in misinterpretation of immunological results.
  - **Chemically Defined Media**: Like the name suggests, this type of media is composed of contamination- free pure organic and inorganic ingredients. Constituents of this type of media are typically produced through genetic engineering in bacteria/yeast.
  - **Protein-Free Media**: Protein- free media are typically lacking of any type of protein. It is largely used to promote superior growth of the cells as well as protein expression in addition to facilitating for the purification of any expressed product.

Some of the major components of cell culture media include:

- Nutrients provided for peptides and amino-acids, which are the building blocks of proteins.
- Carbohydrates for energy.
- Essential minerals such as calcium, magnesium, phosphates and iron among others buffering agents such as acetates to stabilize the culture media.
- Vitamins.
- pH change indicators such as phenol red.

Cell culture media are used for the proliferation of cells, which can then be identified and studied. As such, it can be used for various purposes including for education, diagnosis and treatment of a disease among others.

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Cell Suspension

In culture methods, cell suspension refers to a type of culture where cells are suspended in a liquid medium. To obtain single cells, a friable callus (small tissue that falls apart easily) is put in agitated liquid medium (agitation allows for gaseous exchange unlike solid medium), breaking it up. This allows for single cells to be released, which are then transferred to another fresh medium. Cell suspension cultures have a big advantage over the stationary ones given that it allows for the cells to be uniformly bathed. Moreover, given that the medium tends to be agitated, it allows for aeration of the medium, providing gases to the cells. Given that the medium is a suspension, it also becomes easy to manipulate the contents of the culture. Like any other culture, suspension cell culture has to be under controlled conditions, proving the cells with an ideal environment to proliferate. Once they reach about 80 percent confluence, it is time to subculture in order to ensure continued proper growth. Eighty percent confluence refers to the state where 80 percent of the culture surface is covered with the growing cells. In some cases, the cells in suspension may adhere on to the plastic surface of the culture flask or even form clumps. In such cases, a pipette can be used to pick these cells and expel them on to the surface of the flask and therefore away from the plastic surface. This helps obtain single cells given that they are no adhere on to the plastic surface.

Counting Cells

Counting the number of cells in a suspension is a process that involves the use of a stain. For instance, when trypan blue is used, it penetrates the cell membrane of the dead cells, but not the living cells. The cells are then gently expelled into a haemocytometer (contains the counting chamber) under the cover slip and observed under a microscope. Cells are then counted within a given number of squares for calculations.

Significance

This method is largely preferred due to the fact that it allows for cells to be suspended in a solution rather than being held in a solid media. Here, therefore, it becomes easier to manipulate the contents thereby preventing them from forming clusters. With cell suspensions, it is also easier to observe single cells under the microscope. In this case, it becomes possible to not only study the structure of the cells, but also get to observe how well they have differentiated; viewing dead and living cells under the microscope.

Cell Culture Protocol

Cell culture protocols are meant to ensure that culture procedures are carried out to the required standards. This is not only meant to prevent the contamination of the cells, but to also ensure that the researchers themselves are protected from any form of contamination. Moreover, the nature of the work is expected to conform

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to the appropriate ethical guidelines. Therefore, before anything else, it is essential to ensure that the entire procedure conforms with both medical-ethical and animal experiment guidelines. This is because going against such legislation and guidelines can result in heavy penalties and even shutting down of the laboratory.

Before any work starts, carry out the following procedure:

- Ensure that the working are is sanitized (using 70 percent ethanol).
- Always use a new pair of gloves. If a pair of gloves has to be used for another cell culture procedure, they should be sanitized using 70 percent ethanol and allowed to air dry.
- Any equipment that had been taken out of the cabinet should also be sanitized to prevent any contamination.
- Such equipment as pipette, glass jars and plastics to be used for the procedure should be autoclaved.

Although, there are a wide range of culture media for cells, it is important to keep in mind that cell cultures, and particularly primary cell cultures are easily prone to contamination in addition to the risk of containing undetected viruses. For this reason, all material should be handled as potentially infectious in order to avoid any infections. In addition, for safety purposes, work on cell culture should be carried out in the appropriate laminar flow hood, where air is directed away from the researcher.

Protocols for Cell Culture Preparation

Always check the information on the container to ensure that the medium is appropriate for the cell to be cultured, Once prepared, the cell culture should be maintained under the recommended temperature range, Monitor the culture every 30- 48 hours and check for confluency (when cells completely cover the surface of the culture), However, this is largely dependent on the type of cells. Once the procedure is completed and the cells have been analyzed, the culture should be appropriately discarded. Here, it is important to take a lot of caution given that by this time, cells have already proliferated and increased in numbers. Moreover, there are high chances that the specimen has been contaminated, which increase the risks of causing infections to the researcher if not handled appropriately.

Disposal

- For such items as scalpels, glass slides and cover slips among others, decontamination may involve autoclaving at 121 degrees Celsius and 15 psi (pressure) for at least 30 minutes. However, if they are to be discarded, it is important that they are put into a plastic bag and into the appropriate container to be incinerated later.
- With liquid waste, chemical disinfection is one of the best methods through which the waste product can be deactivated. Such chemicals as bleach can be used for this purpose before pouring the liquid down the sink drain.

- For solid waste products, they are collected into a plastic bag for incineration. On the other hand, they can first be autoclaved before being incinerated.

Conclusion

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In general, cell culture, whether it involves using a suspension or a stationary media, involves the growth of cells in an artificial environment with favorable conditions. Whereas enzymatic action can be used to obtain cells for culture, it is the mechanical disaggregation method that is most preferred given that it provides a simpler and less traumatic way of obtaining cells. This method simply involves the slicing of a tissue into smaller pieces from which the spill out cells is then collected. On the other hand, primary explants technique can be used to obtain the cells. However, this method is mostly useful for the disaggregation of smaller quantities of tissue. While any cells can be used in cultures to observe their behavior, embryonic tissues are preferred (for primary cells) as compared to adult cells because they provide cells that are more viable and can rapidly proliferate. Here, it is also important to ensure that that the cells are of higher quantity given that their survival rate tends to be lower in comparison to the sub-cultures. In order to enhance the success of cultures, it is also important that ensure that damage to the cells is minimized both during cell collection and processing. This is in addition to using the appropriate medium for the cells in question.

1.5.5 Cell Harvesting Methods

Cells harvested from human blood and human tissues are being used to research basic biology, mechanisms of disease and to develop and test therapeutics. Harvesting cells from an organism, starts with a biopsy which depending on the tissue type can be performed by needle aspiration, scalpel, punch biopsy or laser. In the case of cell therapy, the harvested cells are the treatment, but they may first be cultured and then harvested from cell culture before being administered to patient.

Cell harvesting is a step in the production of therapeutics like insulin that are grown in bacteria or antibodies produced in mammalian cell culture. In addition to the biomedical field, cell harvesting is a key step in bioprocessing of products like meat and leather made through cellular agriculture. To harvest cells from culture, cells, if they are adherent, are detached from the tissue culture surface and then they are separated from the culture medium by centrifugation or filters.

Harvesting from Cell Culture

Harvesting cells from culture is routinely done in research labs to refresh culture medium or split cell cultures into more culture dishes or flasks. Also, cells are harvested before analysis or extraction of cellular materials.

Centrifugation: For cells that are grown in suspension, centrifugation can be the first step in cell harvesting. There needs to be enough centrifugal force to cause the dense solid cells to collect at the bottom of the centrifugation tube, but not so much as to destroy cell membranes. Disk stack centrifuges, originally developed for the dairy industry to separate fat from milk without shearing the fat

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particles, have been applied to cell culture cell harvesting because it is gentle on mammalian cells, which are particularly shear sensitive.

Depth Filtration: Depth filters are made of a porous filtration medium that traps particles or cells throughout the medium rather than particles remaining on the surface only. Depth filters can replace centrifugation as a method of capturing cells or can be used as a second step after centrifugation. Depth filters can be small single use devices that are connected to a pump system or they can be industrial-scale depth filtration systems. The filters are flushed with buffers to remove loose particles and flushed to recover the bound cells.

Cell Detachment: Before centrifugation or filtration, cells that adhere to surfaces must first be detached. Manual cell detachment by scraping is quick but may compromise cell viability and may not be feasible on an industrial scale. Trypsin, a protease enzyme commonly used in the lab for this purpose, works by cleaving the amino acids lysine and arginine in proteins that interact with the support surface. Trypsin is purified from pig pancreas but there are animal origin-free cell dissociation agents. Trysinization is often combined with shaking, combining mechanical and chemical methods. In microfluidic systems, where cells are cultured on internal walls of channels, a strong fluid flow passing through results in detachment, but this method causes significant damage to cells. Non-enzymatic treatments like EDTA or citric saline are gentle detachment treatments. Thermoresponsive cell culture supports are coated in the polymer poly(N-isopropylacrylamide) (pNIPAAm) and cells detach upon temperature change. Similarly, pH-responsive polymers like chitosan, electro-responsive substrates and photo-responsive substrates can be used to release cells off supports.

Check Your Progress

19. What is the need of cell culture in laboratory?
20. Define cell culture.
21. What do you understand by cell proliferation?
22. Define cell viability.
23. Why is cell culture considered as an important technique in both cellular and molecular biology?

1.6 CRYOTECHNIQUES

Cryotechniques are a group of related procedures for stabilizing, or 'fixing' specimens for microscopic observation. Samples are rapidly frozen to maintain cellular structure and composition as it exists under physiological conditions, such as electrolyte concentration and protein antigenicity. Alternatively, samples may be lightly fixed and cryoprotected first before freezing. These techniques preserve the native structures of tissues without the artifacts associated with chemical fixation. Some cryotechniques facilitate investigations of membranes and membrane proteins.

Examples of cryotechniques include:

- **Cryo-Ultramicrotomy:** Ultra-thin frozen sections cut for the TEM. It is generally regarded as the most sensitive TEM immuno prep technique.
- **Freeze Fracture:** Samples are fixed, frozen, fractured and a replica of the fracture surface is imaged in the TEM. Usually performed on cells or lipid suspensions. It is one of the only ways to demonstrate the presence of tight junctions.
- **Frozen Hydrated TEM:** A thin layer of unfixed aqueous sample is rapidly frozen to form a sheet of vitreous ice with the sample suspended in it. This technique is often used to image liposome suspensions and record images of viruses for 3-D reconstruction.
- **Cryosubstitution:** This technique avoids the denaturing effects of room temperature dehydration and resin infiltration. Samples are frozen and transferred to the Cryosubstitution unit and placed in, typically, acetone at -90°C. At this low temperature the water ice is replaced by the acetone in a sublimation type process thereby minimizing adverse effects on antigenicity. The sample is then infiltrated with special low viscosity resins, such as the Lowicryl series and cured by UV light.

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1.6.1 Cryopreservation Cells, Tissues, Organisms

Cryopreservation or cryoconservation is a process where organelles, cells, tissues, extracellular matrix, organs, or any other biological constructs susceptible to damage caused by unregulated chemical kinetics are preserved by cooling to very low temperatures (typically –80 °C using solid carbon dioxide or –196 °C using liquid nitrogen). The preservation of cells, tissue and organs in liquid nitrogen is called **cryobiology**. At low enough temperatures, any enzymatic or chemical activity which might cause damage to the biological material is effectively stopped. Cryopreservation methods seek to reach low temperatures without causing additional damage caused by the formation of ice crystals during freezing. Traditional cryopreservation has relied on coating the material to be frozen with a class of molecules termed cryoprotectants. New methods are constantly being investigated due to the inherent toxicity of many cryoprotectants. By default it should be considered that cryopreservation alters or compromises the structure and function of cells unless it is proven otherwise for a particular cell population. Cryoconservation of animal genetic resources is the process in which animal genetic material is collected and stored with the intention of conservation of the breed.

Discovery of Cryopreservation

Ernest John Christopher Polge, a English biologist in 1949 –was the first person to solve the mystery of how to preserve living cells and tissues at very low temperatures. He accidentally discovered the cryoprotective properties of glycerol on fowl sperm.

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Objective of Cryopreservation

The primary purpose of cryopreservation is to preserve biological specimens. Individual cells and biological tissues may be cryopreserved in a living state of suspended cellular metabolism at the temperature of liquid nitrogen (-196°C). This practice is crucial for biomedical research, clinical medicine, zoology, botany, and biotechnology. When frozen and kept properly, specimens may remain in a state of suspended cellular metabolism indefinitely and can be thawed as needed.

Basic Aspect of Cryopreservation

The purpose of cryopreservation is to store cells indefinitely by halting the cell's metabolism with ultralow temperatures. The freeze-thaw process is stressful to all cells and tissues. Therefore, effective techniques were developed to prevent cell death and damage. One common cryopreservation technique involves changing cell maintenance media to culture media containing a cryopreservation agent, such as Dimethyl Sulfoxide (DMSO). Cells are then cooled at a rate of -1°C/min (for mammalian cells) by transferring to -80°C in a specialized cooling container. After cells have cooled to -80°C, they are transferred to ultralow temperature storage of below -135°C. The most common ultralow temperature storage is liquid nitrogen in liquid or vapor form. After freezing, cells are stored as original seed and working stock assets. Ultralow storage temperatures work by maintaining cells below the glass transition temperature (Tg) of pure water. This suspends all molecular processes and prevents free radical generation that negatively effects cryopreserved cultures.

Cells survive by cooling the cultures to a 'glassy' state slowly, so that less ice forms within the cell. Too much intracellular ice can cause mechanical damage to the cell when thawing. In addition, cells must not freeze too slowly because cells will shrink and dehydrate. Physical stress of shrinkage and dehydration results in high solute concentrations that increase toxicity. Damage is also prompted by pH shifts and protein degradation that compromises the cell membrane or metabolic pathways. Technologies for the cryopreservation of cells and tissues are constantly improving. The best cryopreservation results are obtained through research-based approaches that are adjusted for the specific purpose of cryopreservation. There are six applications of cryopreservation that include preservation of cells or organs, cryosurgery, biochemistry or molecular biology, food sciences, ecology or plant physiology, and medical applications (transfusion, bone marrow and cells transplantation, artificial insemination, and in vitro fertilization). This guide will cover the basics of cellular cryopreservation for research and clinical use.

Cryoinjury

Cryopreservation can cause damage to cells mainly during the freezing stage, which include: solution effects, extracellular ice formation, dehydration and intracellular ice formation. These effects are discussed briefly as follows:

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- **Solution Effects:** As ice crystals grow in freezing water, solutes are excluded, causing them to become concentrated in the remaining liquid water. High concentrations of some solutes can be very damaging.
- **Extracellular Ice Formation:** When tissues are cooled slowly, water migrates out of cells and ice forms in the extracellular space. Too much extracellular ice can cause mechanical damage to the cell membrane due to crushing.
- **Dehydration:** Migration of water, causing extracellular ice formation, can also cause cellular dehydration. The associated stresses on the cell can cause damage directly.
- **Intracellular Ice Formation:** While some organisms and tissues can tolerate some extracellular ice, any appreciable intracellular ice is almost always fatal to cells.

The biological effects of cooling are dominated by the freezing of water, which results in the concentration of the solutes that are dissolved in the remaining liquid phase. Rival theories of freezing injury have envisaged either that ice crystals pierce or tease apart the cells, destroying them by direct mechanical action, or that damage is from secondary effects via changes in the composition of the liquid phase. Many of these adverse effects can be reduced by application of **cryoprotectants**. Once the preserved material has become frozen, it is relatively safe from further damage.

Cryoprotectants, simply by increasing the total concentration of all solutes in the system, reduce the amount of ice formed at any given temperature; but to be biologically acceptable they must be able to penetrate into the cells and have low toxicity. Many compounds have such properties, including glycerol, dimethyl sulfoxide, ethanediol, and propanediol. In fact, both damaging mechanisms are important, their relative contributions depending on cell type, cooling rate, and warming rate.

Cryoprotectants

Cryoprotectants are an essential ingredient for cell freezing; protecting the cell on its journey from room temperature to -196 °C. The general properties required for cryoprotectants are that they have low molecular weight, are nontoxic and cheap. They also need to provide high levels of cell viability post thaw, and have no other influence on the cells, i.e., they must not affect the cells in a way that causes them to behave differently after exposure to the cryoprotectants.

Classes of Cryoprotectants

These are divided into two main classes:

- **Intracellular Agents**, like DMSO, Glycerol, PEG, which penetrate inside the cell preventing the formation of ice crystals that could result in membrane rupture.

- **Extracellular Agents** that do not penetrate in cell membrane and act to improve the osmotic imbalance that occurs during freezing, for example sucrose, trehalose, and dextrose.

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Cryoprotective Agents (CPAs): The CPA, which is usually a fluid, reduces the freezing injury from the cryopreservation process. CPAs should be biologically acceptable, be able to penetrate the cells, and have low toxicity. Various CPAs have been developed and used to reduce the amount of ice formed at any given temperature, depending on the cell type, cooling rate, and warming rate. In order to achieve the best survival rate of cells and tissues, the sample volume, cooling rate, warming rate, and CPA concentrations should be optimized depending on the different cell types and context of tissues. It should be mentioned that the macroscopic physical dimension of the tissue is a major point to be defined in a cryopreservation protocol because of heat and mass transfer limitations in these bulk systems. CPAs can be divided into two categories:

- Cell membrane-permeating cryoprotectants, such as Dimethyl Sulfoxide (DMSO), glycerol, and 1,2-propanediol
- Non-membrane permeating cryoprotectants, such as 2-methyl-2, 4-pentanediol and polymers, such as polyvinyl pyrrolidone, hydroxyethyl starch, and various sugars.

Unlike synthetic chemicals, biomaterials, such as alginates, polyvinyl alcohol, and chitosan can be used to impede ice crystal growth, along with traditional small molecules. The direct inhibition of ice crystal formation and application of antioxidants and other compounds have been used to attempt to reduce cell death from processes, such as apoptosis during the freezing and thawing cycle.

Some of the examples of cryoprotectants (Refer Figure 1.48) are as follows:

- **Glycerol:** Glycerol has good kosmotropic properties; it forms hydrogen bonds with water molecules. This condition makes difficult to form ice crystals by mixture (70% glycerol and 30% water), unless and until the temperature is very low, such as –37.8°C. Compare to other cryoprotectant glycerol is less toxic in high concentration. Glycerol is less toxic compared to DMSO although it may cause other problems, such as osmotic lysis of eukaryotic cell culture.
- **Dimethyl Sulfoxide (DMSO):** DMSO is an organosulfur compound with the formula $(CH_3)_2SO$. It is also a polar aprotic solvent which can dissolve polar and nonpolar compounds and can be easily miscible with wide range of organic solvents and with water. It has garlic like taste. DMSO has low cost and minor cytotoxicity, which makes it more prominent candidate for cryopreservation. DMSO has typical property as it freezes within 18.5°C. This means, below room temperature DMSO transformed into solids, and this property makes it most suitable for

cryoprotectant. At any particular temperature, DMSO reduces the electrolytic concentration in the residual chilled contents in and around of a biological cell, during cryopreservation. However rising of altered demarcated cells due to DNA methylation and histone alteration is a drawback of DMSO based cryopreservation. DMSO is an excellent cryoprotectant but it may sometimes trigger differentiation of certain cell lines, for example, stem cells and thus, alternatives, such as glycerol may be chosen for specific applications.

- **Polymers*:*** The entrapment of CPAs within a capsule during cell resuspension in an encapsulating material is another strategy for the modulation of cell location. Among encapsulating materials, synthetic non penetrating polymers can provide cryoprotection of cells within the scaffold, there by bypassing the limitations of diffusion in higher-dimensional cryopreservation. Vinyl-derived polymers, such as polyethylene glycol, polyvinyl alcohol and hydroxyethyl starch have a capacity to decrease the size of formed ice crystals.
- **Others*:*** Some proteins (Sericin) and sugar molecules (Trehalose) have also been reported as cryoprotectants. Sericin is a water-soluble sticky protein (~30 kDa) isolated from the silkworm cocoon and has been developed as a fetal bovine serum- or DMSO-replacing CPA for human adipose tissue-derived stem or progenitor cells, or hepatocytes. Small antifreeze proteins derived from marine teleosts or fishes have also attracted attention as CPAs.

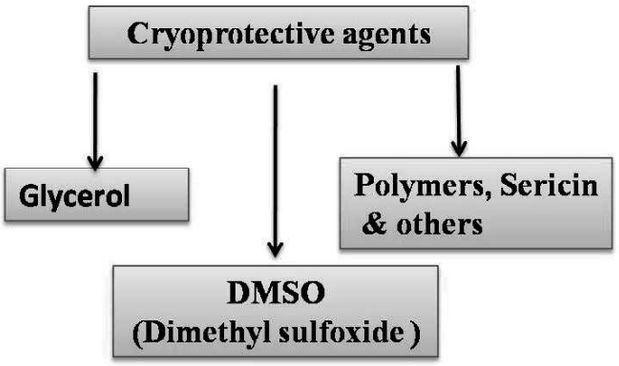

Fig. 1.48 *Various Available Cryoprotective Agents*

A newly developed cell banker series allows for rapid cell cryopreservation at and has been shown to achieve better survival rates following freezing and thawing. The cell banker series of cryopreservation media contain 10% DMSO, glucose, a prescribed high polymer, and pH adjustors. Serum-containing Cell Bankers can be used for the cryopreservation of almost all mammalian cells. Indeed, conventional cryopreservation media include fetal bovine serum, which contains a mixture of growth factors, cytokines, and undefined substances, such as bovine

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exosomes, rendering its use forbidden in the establishment of a standardized cryopreservation protocol for clinical use in humans. In this aspect, the non serum-type Cell Banker 2 is optimal for the cryopreservation of cells in serum-free culture conditions. Cell Banker3 (or Stem cell Banker) is composed of 10% DMSO and other inorganic compounds and satisfies the criterion of a chemically defined known ingredient that is xeno-free and is thus suitable for the preservation of somatic stem cells and induced pluripotent stem cells.

Cryoprotective agent development has improved cell viability post-thaw as well as stem cell pluripotency. Increased knowledge of the physiochemical processes involved during cell cryopreservation has resulted in reproducible protocols. Freezing techniques exist for tissues, single cells, embryos and microorganisms. Challenges with cryopreservation include post-thaw cell growth, viability, maintenance of pluripotency, changes in gene expression, and the ability to differentiate post-thaw. By overcoming the challenges of cell freezing, researchers and clinicians will continue to bring valuable treatments to patients.

Mechanism of Cryopreservation: The technique of freeze preservation is based on the transfer of water present in the cells from a liquid to a solid state. Due to the presence of salts and organic molecules in the cells, the cell water requires much more lower temperature to freeze (even up to -68°C) compared to the freezing point of pure water (around 0°C). When stored at low temperature, the metabolic processes and biological deteriorations in the cells/tissues almost come to a standstill.

Methods of Cryopreservation

There are three main methods of freeze samples at ultra-low temperature, i.e., with liquid nitrogen:

- Slow Freezing
- Vitrification
- Ultra-Rapid Freezing

Slow Freezing: It involves step-wise programmed decrease in temperature. The procedure is lengthy and requires the use of expensive instrumentation. The process does not exclude ice crystal formation.

Vitrification: It refers to any process resulting in 'glass formation', the transformation from a liquid to a solid in the absence of crystallization. It involves the use of a medium that has a very high solute concentration to begin with. Thus ice cannot form. The vitrified state and the associated physico-chemical condition obtained using vitrification methods are to some extent similar to those obtained by slow cooling, but the way of reaching those point is quite different. It is rapid cooling of a sample in the presence of a cryopreservation that increases viscosity and depresses the freezing temperature inside of the cell. It is a simple, inexpensive and rapid process of more newly developed technology. It increase the embryos and oocyte survival rate. Unfortunately common cryoprotectants are toxic and the immersion of solution directly in liquid nitrogen can be cause of contamination of embryos and oocytes with bacterium, mushroom and virus.

Ultra-Rapid Freezing: It is a midway technique between slow freezing and vitrification. It is quicker than slow-freezing technique, does not involve the use of programmable machines and requires lower concentrations of Cryoprotectant Agents (CPA) than those used in vitrification.

Technique of Cryopreservation

It includes various steps as:

- Development of sterile tissue cultures
- Addition of cryoprotectants and pretreatment
- Freezing
- Storage
- Thawing
- Re-culture
- Measurement of survival/viability
- Regeneration

Applications of Cryopreservation

The applications of cryopreservation can be categorized into the following areas:

- Cryopreservation of Cells or Organs
- Cryosurgery
- Biochemistry and Molecular Biology
- Food Sciences
- Ecology and Plant Physiology

Many medical applications, such as blood transfusion, bone marrow transplantation, artificial insemination, and In-Vitro Fertilization (IVF). Some suggested advantages of cryopreservation include the possible banking of cells for human leukocyte antigen typing for organ transplantation; the allowance of sufficient time for transport of cells and tissues among different medical centers; and the provision of research sources for identifying unknown transmissible diseases or pathogens.

Furthermore, the long-term storage of stem cells is still the initial step toward tissue engineering, which holds promise for regeneration of the soft tissue esthetic function and for the treatment of known diseases that have currently no therapy option. Potent applications of cryopreservation in different fields of sciences are as follows:

- **Animal Husbandry:** Cryopreservation of bull semen has been used to propagate the rare and endangered species using assisted reproduction techniques. Every year, more than 25 millions cows are artificially inseminated with frozen-thawed bull semen and many bovine calves have been produced using the transfer of cryopreserved embryos into cow.

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- **Fishery Science:** In fish aquaculture the successful cryopreservation of gametes and embryos could offer new commercial possibilities, allowing the unlimited production of fry and potentially healthier and better conditioned fish as required. Cryopreservation of aquatic sperm is relatively common in the breeding and management of fish species. However, cryopreservation of embryos and oocytes of aquatic species have not been successful, except for eastern oyster eggs (*Crassotrea virginica*).

Advantage of fish semen is not only useful management tool, it offers several benefits, such as stock protection from being totally eliminated due to sudden outbreak of disease, natural disaster, over exploitation, etc. Fish germplasm also plays a significant role in human genomic studies because its relatively small size of the genome makes it easier for sequencing and ideal models for studying the human disease. More than 200 species of fresh water and marine fish have been cryopreserved and have been adequated for the purpose of cryobanking.

- **Medical Science:** Low temperature has been used in medicine and to prevent food spoilage. It is also used in fertility treatment the transport of human organs and the long- term storage of biological specimens, either for future or simply as a record of biodiversity.
- **Cryopreservation of Sperm:** Germ cell depletion caused by chemical or physical toxicity, disease, or genetic predisposition can occur at any age. Fertility preservation is of great importance to guarantee the quality of life of patients facing chemo- and radiotherapy. Human sperm cryopreservation is widely used to store donor and partner spermatozoa before assisted reproduction treatments to preserve spermatozoa before therapy for malignant diseases, vasectomy or surgical in fertility treatments and to ensure the recovery of a small number of spermatozoa in several male factor infertility. Sperm and semen can be used almost indefinitely after proper cryopreservation. Procedure of preservation of sperm cells is commonly called sperm-banking. For human sperm the longest successful storage is 21 years.
- **Cryopreservation of Testicular Tissue:** Cryopreservation of immature testicular tissue is a developing method to avail reproduction to young boys who need to have gonado toxic therapy. There are new trials for cryopreserving testicular tissues in the form of cell suspensions, tubular pieces, and entire gonads, but this technique is still premature. Overall, cryopreservation can be used as a first-line means of preserving fertility for men undergoing vasectomy or treatments that may compromise their fertility, such as chemotherapy, radiotherapy, or surgery.
- **Embryo Cryopreservation:** Embryo cryopreservation is used most often to store good-quality excess embryos resulting from an IVF treatment cycle. Embryos can be stored for a patient who elects to have

her eggs fertilized with donar sperms. Pregnancies have been reported from embryos stored for 16 years18. The first case of embryo cryopreservation for fertility preservation took place in 1996, with the application of a natural IVF cycle prior to chemotherapy in a woman diagnosed with breast cancer. Results from a retrospective study of 11,768 cryopreserved human embryos that underwent at least one thaw cycle from 1986 to 2007 showed that there was no significant impact of the duration of storage on clinical pregnancy, miscarriage, implantation, or live birth rate, whether from IVF or oocyte donation cycles.

- **Cryopreservation of Oocyte:** Cryopreservation of mature oocytes is a proven technique for preserving the reproductive capacity. This is a technology in which a woman's eggs are extracted, frozen or stored. Egg freezing benefits two groups of women:
  - o Those women who are diagnosed with a medical condition whereby the necessary treatments for cure may render them sterile or unable to produce viable eggs.
  - o Those who are delaying their childbearing for personal reasons.

Eggs frozen at the age of 35 are more usable than fresh oocytes produced at age 43 years of age. Since oocytes are highly prone to chilling injury, cryopreservation of immature oocytes and ovarian tissue is a promising approach, with reports of live births, but the need for investigational improvement remains.

- **Cryopreservation of Ovarian Tissue:** Ovarian tissue cryopreservation is considered to be an experimental technique for fertility preservation. This procedure is an option for patients who require immediate gonadotoxic treatment of aggressive malignancies when there is insufficient time to allow the woman to undergo ovulation induction, oocyte retrieval and cryopreservation oocytes and/or embryos. Ovarian tissue cryopreservation is the only option available for fertility preservation in young girls who are prepubertal or in woman who have hormone-sensitive malignancies or whose reproductive potential is threatened by future of cryopreservation.
- **Cryopreservation of Stem Cell:** Adult stem cells are capable of differentiating into multiple types of specific cells and can be obtained from various locations other than bone marrow, including fat tissue, the periosteum, amniotic fluid, and umbilical cord blood. Stem cells can be subdivided into embryonic stem cells, mesenchymal stromal cells, and hematopoietic stem cells, all of which are considered as goldmines for potential application in regenerative medicine.

An important application of cryopreservation is in the freezing and storage of hematopoietic stem cell, which are found in the bone marrow rescue, hematopoietic stem cells are collected from a patient's bone marrow prior to treatment with high-dose chemotherapy. Following treatment, the patient's cryopreserved cells are thawed and infuse back into the body. This procedure is necessary, since high dose chemotherapy is extremely toxic to the bone marrow.

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Clearly, the fields of tissue engineering, gene therapy, regenerative medicine, and cell transplantation are largely dependent on the ability to preserve, store, and transport these stem cells without modification of their genetic and/or cellular contents.

- **Cryopreservation of Hepatocytes:** Primarily isolated hepatocytes have found important applications in science and medicine over the past 40 years in a wide range of areas, including physiological studies, investigations on liver metabolism, organ preservation and drug detoxification, and experimental and clinical transplantation. In addition, there is currently increasing interest in the applications of liver progenitor cells across a range of scientific areas, including both regenerative medicine and biotechnology, which raises a need for cryobanking.
- **Preservation of Micro-Biology Cultures:** Bacteria and fungi can be preserved or refrigerated for short term storage where cell division and metabolism is not completely arrested. It is not an optimal option for long term storage or to preserve cultures genetically or phenotypically because cell divisions can lead to mutations.
- **Conserve Plant Biodiversity:** The conservation of plant biodiversity is an important issue concerning the human population worldwide. Conservation of plant biodiversity can be performed in-situ and ex-situ. These two methods are complementary and are not exclusive. They offer different alternatives for conservation, but selection of the appropriate strategy should be based on a number of criteria, including the biological nature of the species and the feasibility of applying the chosen methods. Various biotechnological methods have been used to conserve endangered, rare crop ornamental, medicinal and forest species for different time period.

For long-term conservation cryopreservation is the most effective tool, as it maintain the living cells, tissues, organs at ultralow temperature(usually that of liquid nitrogen,-196ºC). At liquid nitrogen temperature, all metabolic activity and cell divisions are stopped and cells will not undergo genetic changes during storage. Cryopreservation is the only technique that ensures the safe and cost-efficient long term conservation of various categories of plants, including non-orthodox seed species, vegetatively propagated plants, rare and endangered species and biotechnology products.

Cryopreservation of Plants

Germplasm broadly refers to the hereditary material (total content of genes) transmitted to the offspring through germ cells. Germplasm provides the raw material for the breeder to develop various crops. Thus, conservation of germplasm assumes significance in all breeding programmes. The main objective of germplasm conservation (or storage) is to preserve the genetic diversity of a particular plant or genetic stock for its use at any time in future.

A global body namely International Board of Plant Genetic Resources (IBPGR) has been established for germplasm conservation. Its main objective is to provide necessary support for collection, conservation and utilization of plant genetic resources throughout the world.

Preservation of Germplasm

Enormous increase in the population is continuously imposing pressure on the forest and the land resources. This results in depletion of population of medicinal and aromatic plant species. Even some of the plant species are at the verge of vanishing from the forest. The list of endangered species is growing day by day. The conventional methods of germplasm preservation are prone to possible catastrophic losses because of:

- Attack by Pathogen and Pests
- Climatic Disorders
- Natural Disasters
- Political And Economic Causes
- Loss Of Seed Viability Under Storage

Methods for Germplasm Conservation

The conservation of germplasm can be done by two methods:

- **In-Situ Preservation:** Preservation of the germplasm in their natural environment by establishing biospheres, national parks, etc.
- **Ex-Situ Preservation:** In the form of seeds or by in-vitro cultures (plant cells, tissues or organs).

Seeds are the most common and convenient materials to conserve plant germplasm. This is because many plants are propagated through seeds, and seeds occupy relatively small space. Further, seeds can be easily transported to various places. But preservation of the plant germplasm in the form of seeds has the following disadvantages:

- Discrete clones cannot be maintained in the form of seeds.
- Some plants do not produce fertile seeds.
- Loss of seed viability.
- Seed destruction by pests.
- Poor germination rate.
- It is useful for seed propagating plants and is not applicable to vegetatively propagated crops, like potato, ginger, etc.

In-vitro methods employing shoots, meristems and embryos are ideally suited for the conservation of germplasm of vegetatively propagated plants. The plants with recalcitrant seeds and genetically engineered materials can also be preserved by this in vitro approach.

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In-vitro preservation by tissue culture has several advantages over seed preservation:

- Large amount of materials can be stored in a small area.
- The material could serve as an excellent form of nucleus stock to propagate large number of plants rapidly, when required.
- Under special storage conditions the plants do not require frequent splitting and pruning.
- Being free from known viruses and pathogens, the clonal plant material could be sent from country to country, thus, minimizing the obstructions imposed by quarantine systems on the movement of live plants across national boundaries.
- Protection from natural hazards.
- The plants are not exposed to the threat of changing government policies and urban development.

Disadvantages of in-vitro system used for conservation of plant material are as follows:

- It is a costly process.
- In cultures, plants can be maintained by serial subcultures at frequent intervals for virtually unlimited periods.

However, the storage of germplasm by serial subcultures risks the loss of plant material by microbial contamination due to human error and also, is uneconomical. Moreover, in long-term callus and suspension cultures, the regeneration potential, biosynthetic properties and genetic make-up of the cells suffer. The basic requirement of a plant tissue culture method is the preservation of genetic resources, therefore, is to reduce the frequency of subcultures to a bare minimum.

Approaches for the In-Vitro Conservation of Germplasm

There are mainly three approaches for in-vitro preservation of germplasm as:

- Cryopreservation (freeze-preservation).
- Cold storage.
- Low-pressure and low-oxygen storage.

These methods (Refer Figure 1.49) are used for short term as well as long term storage of germplasm. The most effective method of preservation of plant material is cryopreservation.

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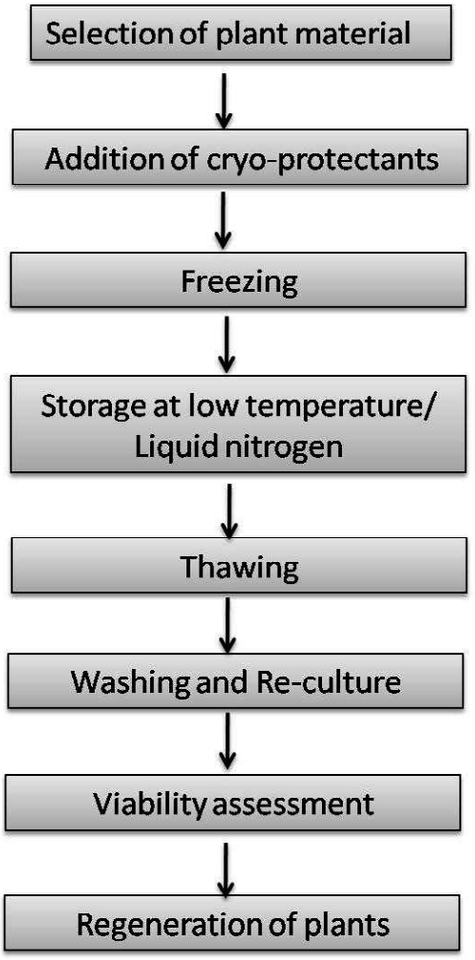

Fig. 1.49 *General Method of Cryopreservation of Plant Material*

Cryopreservation: Cryopreservation means preservation in the frozen state. The principle involved in cryopreservation is to bring the plant cell and tissue cultures to zero metabolism or non-dividing state by mean of storage of germplasm at a very low temperatures,

- Over Solid CO_2 (-79°C)
- Deep Freezers (-80°C)
- In Vapor Phase Nitrogen (-150°C)
- In Liquid Nitrogen (-196°C)

Among these, the most commonly used non-lethal storage of biological material at ultra-low temperature is by employing liquid nitrogen. At the temperature of liquid nitrogen (-196°C), almost all the metabolic activities of cells are ceased and the sample can then be preserved in such state for extended periods.

Steps Involved in Cryopreservation Technique

The technique of cryopreservation involves the following steps:

- Selection of plant material
- Pre-culture

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- Cryoprotective treatment
- Freezing and storage
- Thawing
- Re-culture
- Measurement of survival/viability
- Plant regeneration

Selection of Plant Material: The morphological and physiological conditions of the plant material, prior to freezing, considerably influence its ability to survive freezing at -196°C. Generally, small, richly cytoplasmic and meristematic cells survive better than the larger, highly vacuolated cells. Therefore, cell suspensions should be frequently subcultured and frozen in the late lag phase or exponential phase when the majority of the cells are in the preferred condition. While preservation of cell lines remains useful with respect to in-vitro production of secondary metabolites, cultured cells are not the ideal system for germplasm storage. Instead, organized structures, such as shoot apices, embryos or young plantlets are preferred. The reasons to shift from cell cultures to organized cultures are as follows:

- The genetic instability of cells in long term callus and cell suspension cultures is a very common phenomenon and there is no effective measure to control it so far. Moreover, most of the callus cultures are initiated from non-meristematic cells of the plant body which might exhibit polysomaty. Hence, the cultured cells may exhibit genetic heterogeneity from the very beginning. In contrast, plants raised from shoot apices have generally proved to be true- to-type.
- Cultured cells of several important plants do not exhibit totipotency. Moreover, in few cases these cells initially form organs/ embryos and whole plants but this potentiality is often lost after some time in culture. Besides, shoot apices possess a high regeneration ability which is retained in prolonged cultures. Shoot apices are mostly preferred to develop a virus free plants and also for the rapid clonal multiplication.
- Haploidy, which is highly unstable in callus and suspension cultures, can be maintained through shoot tip culture and axillary-bud proliferation.
- The cells of shoot-tip and young embryos are small and meristematic. They appear to be better suited than larger cells to survive Liquid Nitrogen (LN) freezing and thawing.

Pre-Culture: In several cases, a brief culture of shoot apices for at least 48h at 4°C before freezing has proved beneficial for consistently high frequency of survival of shoot apices after freezing in liquid nitrogen. The other treatments include the application of additives that known to enhance plant stress tolerance, for example ABA, proline, osmoticum (sucrose, mannitol), Dimethyl Sulfoxide (DMSO, 1-5%). Sugars acts as osmotically effective agents, although they do not penetrate inside the cells. Dehydration of cells/tissues occurs in the presence of sugars during

the preculture, which prevents lethal ice crystal formation during freezing. Proline may act by reducing the level of latent injury to the cells or it may actively participate in recovery metabolism.

Cryoprotective Treatments: There are two potential sources of cell damage during cryopreservation.

- Formation of large ice crystals, inside the cells, leading to rupture of organelle and the cell itself.
- Intracellular concentration of solutes increases to toxic levels before or during freezing as a result of dehydration.

Addition of cryoprotectants controls the appearance of ice crystals in cells and protects these cells from the toxic solution effect.

Cryoprotectants are categorized as:

- Penetrating, which exert their protective colligative action.
- Non-penetrating, which affect through osmotic dehydration.

A large number of heterogeneous groups of compounds have been shown to possess cryoprotective properties with different efficiencies, for example glycerol, DMSO, etc. Cryoprotectants depress both the freezing and super-cooling point of water, i.e., the temperature at which the homogeneous nucleation of ice occurs, thus, retarding the growth of ice crystal formation in cells and protect cells from toxic effect. The cryoprotectants used in cryopreservation are:

- **Alcohols:** Ethylene glycol, glycerol, propylene glycol, sorbitol, mannitol.
- **Sulphur Containing Compounds:** Amino acids, Dimethyl Sulphoxide (DMSO), sugar (glucose, saccharose).
- **Polymers:** Hydroxyethyl amidon, polyethylene glycol, polyvinyl pyrrolidine.

Mechanism of Action of Cryoprotectants

The protective mechanisms are as follows:

- **Vitrification:** At a sufficiently low temperature, highly concentrated aqueous solutions of cryoprotective agents become so viscous that they solidify into an amorphous 'glassy' state, without ice crystal formation (crystallization) at practical cooling rates, this phenomenon is called vitrification. The significance of vitrification in cryopreservation of biological materials is that the cells applied with highly concentrated solution of osmotically active compounds, are protected from internal damage from ice crystal formation during freezing. This pretreatment also causes dehydration of cells. The commonly used cryoprotectants are employed for vitrification like DMSO.
- **Cryoprotective Dehydration:** If cells are sufficiently dehydrated they may be able to withstand immersion in liquid nitrogen without further application

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of traditional cryoprotectant mixtures. Dehydration can be achieved by growing the cultures in the presence of high concentration of osmotically active compounds (sugars) and /or air desiccations in laminar-air-flow cabinet or over silica gel. Dehydration reduces the amount of water available for the ice formation.

- **Encapsulation and Dehydration:** Encapsulation and Dehydration involves the encapsulation of tissues in calcium alginate beads which are pre-grown in liquid culture media containing high concentrations of sucrose. The beads are transferred to sterile airflow in a laminar cabinet and desiccated further. After these treatments, the cells are able to withstand exposure to liquid nitrogen without application of chemical cryoprotectants.

Freezing and Storage: The type of crystal water within stored cells is very important for survival of the tissue. Different tissues have different sensitivities for cooling rates. In general, there are three different types of freezing procedures:

- **Rapid Freezing:** The plant material is placed in vials, liquid nitrogen is poured directly in the vial and dipping the vial into an open flask filled with liquid nitrogen. In this procedure, cooling between -10°C and -70°C occurred at the rate of >1000°C/min. The quicker the freezing is done, smaller the intracellular ice crystals are formed. In combination with desiccation or vitrification pre-treatments, ultra-rapid cooling is proved to be the most attractive method for cryopreservation of plant materials. This method has been successfully used for the cryopreservation of shoot-tips, somatic embryos and embryonal axes from zygotic embryos of a number of plant species. The survival rate of cryopreserved tissues by this method is high and when the desiccation pretreatment is applied even the cryoprotectants are not required.
- **Slow Freezing:** The tissue is slowly frozen at a slow cooling rate of 0.5-4°C/min from 0 to -100°C, and then transferred to liquid nitrogen. Survival of cells frozen at slow freezing rates may involve some beneficial effects of dehydration, which minimizes the amount of water that freezes intracellularly. Slow cooling permits the flow of water from the cells to the outside, thereby promoting extracellular ice formation instead of intracellular freezing. It is generally agreed that upon extracellular freezing the cytoplasm will be effectively concentrated and plant cells will survive better when adequately dehydrated. This has been successfully employed for cryopreservation of meristems of few plants and has proved especially successful with cells from suspension cultures.
- **Stepwise Freezing:** Firstly, the material is cooled gradually (ca 1°C/min) or step-wise (5°C/min) to an optimum intermediate temperature (-30°C to -50°C) for about 30 min, and then rapidly cooled by dipping into liquid nitrogen. The method is highly favorable for freeze preservation of shoot apices and buds. It is equally successful to cells from suspension cultures.

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The initial slow freezing reduces the amount of intracellular freezable water by dehydrating the cells. Early in the freezing process ice is formed first outside the cells, and the unfrozen protoplasm of cells loses water due to the vapor pressure deficit between the supercooled protoplasm and the external ice. This initial cooling, thus, acts as another pre-treatment for dehydration of the cells.

Storage: Maintaining the frozen material at the correct temperature is as important as proper freezing itself. Temperatures above -130°C may allow ice-crystal growth inside the cells and, as a result reduce their viability. Long-term storage of the material frozen at -196°C, therefore, requires a liquid nitrogen refrigerator.

Generally, the frozen cells or tissues are immediately kept for storage at temperature ranging from -70°C to -196°C. The storage is ideally done in liquid nitrogen refrigerator at -150°C in the vapor phase or -196°C in the liquid phase. The temperature should be sufficiently low for long term storage of cells to arrest all metabolic activities and to prevent biochemical injury.

Thawing: Rapid thawing of the material frozen at -196°C is achieved by plunging it into water at 37 to 40°C which gives thawing rate of 500-750°C/min. After about 90s, the material is transferred to an ice bath and maintained there until recultured or its viability is tested. The transfer is necessary because the cells might get damage if it is left long in the water bath 37-45°C. Rapid thawing protects the cells from the damaging effects of ice crystal formation, which may occur during slow warming.

Re-Culturing: The material after thawing should be washed several times to remove the cryoprotectant which may otherwise be toxic to the cells. A gradual dilution of the cryoprotectant is desirable in-order to avoid any deplasmolysis injury to the cells. The plant material frozen at -196°C may need some special requirements for better survival when re-cultured. Shoot-tips from frozen seedlings of tomato directly developed into plantlets only if the medium was supplemented with GA_3. In its absence, apices callused, followed by the differentiation of adventitious shoots.

Measurement of Survival/Viability: The viability/survival of the frozen cells can be measured at any stage of cryopreservation or after thawing or reculture. The techniques employed to determine viability of cryopreserved cells are the same as used for cell cultures .Staining techniques using Triphenyl Tetrazolium Chloride (TTC), Evan's blue and Fluorescein Diacetate (FDA) are commonly used. The best indicator to measure the viability of cryopreserved cells is their entry into cell division and regrowth in culture. This can be evaluated by the following expression:

$$\frac{\textbf{No. of cells or organs growing}}{\textbf{No. of cells or organs thawing}} \times \mathbf{100}$$

Plant Regeneration: The ultimate purpose of cryopreservation of germplasm is to regenerate the desired plant. For appropriate plant growth and regeneration, the cryopreserved cells/tissues have to be carefully nursed, and grown.

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Precautions/Limitations for Successful Cryopreservation

- Good technical and theoretical knowledge of living plant cells and as well as cryopreservation technique are essential.
- Formation ice crystals inside the cells should be prevented as they cause injury to the organelles and the cell.
- High intracellular concentration of solutes may also damage cells.
- Sometimes, certain solutes from the cell may leak out during freezing.
- Cryoprotectants also affect the viability of cells.
- The physiological status of the plant material is also important.

1.6.2 Cryotechniques for Microscopy

Cryogenic Electron Microscopy (cryo-EM) is an Electron Microscopy (EM) technique applied on samples cooled to cryogenic temperatures and embedded in an environment of vitreous water. An aqueous sample solution is applied to a grid-mesh and plunge-frozen in liquid ethane or a mixture of liquid ethane and propane.

Transmission Electron Cryomicroscopy

Cryogenic Transmission Electron Microscopy (cryo-TEM) is a transmission electron microscopy technique that is used in structural biology and materials science.

Cryogenic Electron Tomography (Cryo-ET), a specialized application where samples are imaged as they are tilted.

Electron Crystallography method is used to determine the arrangement of atoms in solids using a TEM or Transmission Electron Microscopy.

MicroED, method to determine the structure of proteins, peptides, organic molecules, and inorganic compounds using electron diffraction from 3D crystals.

Single particle analysis cryo-EM, an averaging method to determine protein structure from monodisperse samples.

Check Your Progress

24. What are cryotechniques?
25. Name the first person who solve the mystery of preserving living cells and tissues at very low temperatures.
26. Define Cryoprotective Agents (CPAs).
27. Write the various steps included in cryopreservation.
28. Define cryogenic electron microscopy.

1.7 ANSWERS TO 'CHECK YOUR PROGRESS'

1. A scientific instrument is a specific device or tool that is typically used for scientific determinations.

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2. Titrimetry is the technique used to determine the concentration of the analyte.
3. Hue, Value and Intensity are three dimensions to colour.
4. UV-VIS Spectrometry runs on the Beer-Lambert law that is related to absorption of light of that region of the electromagnetic spectrum for which this law was developed.
5. The 'Ultracentrifuge' is referred as a centrifuge optimized method for spinning a rotor at very high speeds and is efficient to generate acceleration as high as 1,000,000 g (approx. 9,800 km/s^2).
6. Beer's law says that the light absorbed is proportional to the number of absorbing molecules, i.e., to the concentration of absorbing molecules. But this law is only valid for dilute solutions.
7. A densitometer is a device specifically used for measuring the degree of darkness (the optical density) of a photographic or semi-transparent material or of a reflecting surface.
8. Differential Scanning Calorimetry (DSC) is referred as a thermoanalytical technique in which the difference in the amount of heat required to increase the temperature of a sample and reference is measured as a function of temperature.
9. Nuclear Magnetic Resonance (NMR) is a physical phenomenon in which nuclei in a strong constant magnetic field are perturbed by a weak oscillating magnetic field and respond by producing an electromagnetic signal with a frequency characteristic of the magnetic field at the nucleus.
10. There are three branches of microscopy, the optical microscopy, the electron microscopy, and the scanning probe microscopy, along with the emerging field of X-ray microscopy.
11. A father-son duo, Zacharias and Han Jansen, created the first compound microscope in the 1590s.
12. The technique of phase contrast microscope was invented by Dutch physicist Frits Zernike in the 1934. He received the Nobel Prize in physics in 1953 for this contribution.
13. Confocal microscopy has following benefits in comparison to conventional widefield optical microscopy.
    - Elimination or reduction of background information away from the focal plane, which causes image degradation,
    - Ability to control depth of field.
    - Capability to collect serial optical sections from thick samples
14. A microbiological culture or microbial culture is a method of multiplying microbial organisms by letting them reproduce in predetermined culture medium under controlled laboratory conditions.
15. Inoculation is a set of methods of artificially inducing immunity against various infectious diseases.

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16. Doubling time refers to the time period required for doubling the weight of the biomass while generation time represents the period for doubling the cell numbers.
17. In biology, the fermenters or stirred-tank bioreactors are process vessels used to cultivate heterotrophic microalgae.
18. Microbial assays or microbiological assays can be considered as a type of bioassays specifically designed to analyse the compounds or substances that impact the microorganisms. They estimate the concentration and efficiency of antibiotics.
19. In the natural habitats, microorganisms usually grow in complex mixed populations with many species. This is a problem for microbiologists because a single type of microorganism cannot be studied in a mixed culture, one needs a pure culture, a population of cells arising from a single cell, to characterize individual species. So cell culture in laboratory become necessary for studying microorganisms.
20. Cell culture is the process by which cells are grown under controlled conditions, generally outside their natural environment.
21. Cell proliferation is the process by which a cell grows and divides to produce two daughter cells. Cell proliferation leads to an exponential increase in cell number and is therefore a rapid mechanism of tissue growth.
22. Cell viability is a measure of the proportion of live, healthy cells within a population.
23. Cell culture is an important technique in both cellular and molecular biology as it provides the best platform for studying the normal physiology and biochemistry of cells.
24. Cryotechniques are a group of related procedures for stabilizing, or 'fixing' specimens for microscopic observation.
25. Ernest John Christopher Polge, an English biologist in 1949 -was the first person to solve the mystery of how to preserve living cells and tissues at very low temperatures.
26. Cryoprotective Agent (CPA) is usually a fluid, reduces the freezing injury from the cryopreservation process. CPAs should be biologically acceptable, be able to penetrate the cells, and have low toxicity.
27. It includes various steps as:
    - Development of sterile tissue cultures
    - Addition of cryoprotectants and pretreatment
    - Freezing
    - Storage
    - Thawing
    - Re-culture

- Measurement of survival/viability
- Regeneration

28. Cryogenic Electron Microscopy (cryo-EM) is an Electron Microscopy (EM) technique applied on samples cooled to cryogenic temperatures and embedded in an environment of vitreous water

NOTES

1.8 SUMMARY

- A scientific instrument is a specific device or tool that is typically used for scientific determinations.
- Analytical instruments are specially designed to provide information about the composition of a given matter.
- Spectrometer helps to determine chemical composition by means of its measure of spectrums.
- The tristimulus values of an object can be calculated by combing the reflectance or transmittance of the object with the spectral power distribution of an illuminant and the colour matching functions of a standard observer.
- When light is passed through a homogeneous medium, a part of the light is reflected, a part is absorbed within the medium and rest is transmitted.
- The property of absorption of visible light makes the objects look coloured. For example, blue ink appears blue because the light at the red end of the light spectrum is absorbed and the blue light is reflected from the ink to the observer's eye.
- If the wavelength is selected using prisms or gratings, the technique is referred to as spectrophotometry
- The 'Ultracentrifuge' is referred as a centrifuge optimized method for spinning a rotor at very high speeds and is efficient to generate acceleration as high as 1000000 g (approx. 9800 km/s^2).
- Densitometric scanner is used in the 'Densitometry' which is the quantitative measurement of optical density in light-sensitive materials, such as photographic paper or photographic film, due to exposure to light.
- The material can produce maximum and minimum density termed as DMax and DMin refer, respectively.
- Bone density measurements are specifically done by screening the people for risk of osteoporosis and to identify the people who have poor bone density so that their bone strength can be improved after measuring the bone density.
- The Differential Scanning Calorimetry (DSC) technique was developed by E. S. Watson and M. J. O'Neill in 1962.

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- The Fast-Scan DSC (FS DSC) was developed in the year 2000s, a novel calorimetric technique that employs micro-machined sensors.
- In Differential Thermal Analysis (DTA) technique it is the heat flow to the sample and reference that remains the same rather than the temperature.
- ESR spectroscopy is used in various branches of science, such as biology, chemistry and physics, for the detection and identification of free radicals in the solid, liquid, or gaseous state, and in paramagnetic centers, such as F-centers.
- ESR spectroscopy is a particularly useful tool to investigate their electronic structures, which is fundamental to understand their reactivity.
- The biochemists use NMR to identify proteins and other complex molecules. Besides identification, NMR spectroscopy provides detailed information about the structure, dynamics, reaction state, and chemical environment of molecules.
- Microtomes are important instruments in microscopy preparation.
- Microtomes use different type of blades to cut the specimen. The blade can be made up of steel, glass, or diamond.
- In 1770, George Adams, Jr was the first researcher to make an instrument which could make sample sections of that order which could be studied.
- The principle guiding the working of microtome to get consistent same sized thin section is to place the edge of knife parallel to the sample block.
- The rotary microtomes are so called because a rotary action of the hand-wheel sets in motion the cutting movement.
- The section adhesives are used for simply sticking the section on the slide. For various ester and polyester wax blocks embedded specimens, adhesives are useful.
- In celloidinization, once the section is on the slide and excess water is drained off, a filter paper is kept on the section and it is moistened with alcohol for a few seconds.
- In gelatinized slides, once the section is on the slide and the excess water is drained off, the gelatinized slid is treated with formalin vapors.
- In albuminized slides, a mixture of clove and aniline oil in equal parts is poured on the slide. It takes 3 minutes to coagulate the albumin.
- The word microscope is derived from the Latin word *'micro'*, which means small, and the Greek word '*skopos'*, to look at. So, microscopic means invisible to the eye unless aided by a microscope.
- Microscopy is the technique which uses microscopes to view objects and areas of objects that cannot be seen with the naked eye.

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- Optical microscopy and electron microscopy involve the diffraction, reflection, or refraction of electromagnetic radiation/electron beams interacting with the specimen, and the collection of the scattered radiation or another signal in order to create an image.
- Optical microscopes are not capable of showing things that are smaller than light waves, because of the diffraction limit.
- The monocular compound light microscopes are equipped with one eyepiece as compare to binocular microscope which has two eye pieces.
- Transmitted light microscopy is one of the techniques of the light microscopy.
- The real image implies the representation of an actual object, produced when the light rays arising from a single source converge at a particular (real) point.
- Fluorescence microscopy takes advantage of fluorescence, the ability of substances to absorb short wavelengths of light (ultraviolet) and give off light at a longer wavelength (visible).
- British scientist Sir George G. Stokes, first described fluorescence in 1852 and was responsible for coining the term when he observed that the mineral fluorspar emitted red light when it was illuminated by ultraviolet excitation.
- The light microscope could not resolve objects separated by less than 0.2 μm and the view was limited to observations at the level of whole cells and their arrangements.
- A special staining technique is used to view the specimen in transmission electron microscopy which is called as shadow casting or shadowing.
- The scanning probe microscope was created in the 1980s, by Gerd Binnig and Heinrich Rohrer.
- The technique of phase contrast microscope was invented by Dutch physicist Frits Zernike in the 1934.
- If the amplitudes of the particle and surround waves are significantly different in the intermediate image plane, then the specimen acquires a considerable amount of contrast and is easily visualized in the microscope eyepieces.
- The electrical system consists of a high tension unit, lens current supply unit as well as a voltage and current stabilizer unit.
- In traditional widefield epi-fluorescence microscopy, the entire sample is subjected to intense illumination from an incoherent mercury or xenon arc-discharge lamp.
- Microbial cultures are used to determine the type of organism, its abundance in the sample being tested, or both.
- Microorganisms grown in closed culture (also known as a batch culture), in which no nutrients are added and most waste is not removed, follow a reproducible growth pattern referred to as the growth curve.

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- Microbial biosensors have several uses in clinical analysis, general health care monitoring, veterinary and agricultural applications, industrial product processing and monitoring besides control of environmental pollution.
- Microbial assays or microbiological assays can be considered as a type of bioassays specifically designed to analyse the compounds or substances that impact the microorganisms.
- Buffer solutions are prepared by dissolving the quantities of Dipotassium Hydrogen Phosphate and Potassium Dihydrogen Phosphate in sufficient water.
- Cell culture is the process by which cells are grown under controlled conditions.
- Tissue culture is the growth of tissues or cells in an artificial medium separate from the parent organism. This technique is also called micropropagation.
- The media preparation area should have ample storage space for the chemicals, culture vessels and closures, and glassware required for media preparation and dispensing.
- Essentially, cell culture involves the distribution of cells in an artificial environment (in-vitro) which is composed of the necessary nutrients, ideal temperature, gases, pH and humidity to allow the cells to grow and proliferate.
- The most common readout of cell viability is with vital dyes, such as Propidium Iodide, however cell viability assays also typically measure the metabolic activity or ATP content of healthy cells.
- In culture methods, cell suspension refers to a type of culture where cells are suspended in a liquid medium.
- Cell culture protocols are meant to ensure that culture procedures are carried out to the required standards.
- The primary purpose of cryopreservation is to preserve biological specimens.
- The biological effects of cooling are dominated by the freezing of water, which results in the concentration of the solutes that are dissolved in the remaining liquid phase.
- An important application of cryopreservation is in the freezing and storage of hematopoietic stem cell, which are found in the bone marrow rescue, hematopoietic stem cells are collected from a patient's bone marrow prior to treatment with high-dose chemotherapy.

1.9 KEY TERMS

- **Colorimetry**: Colorimetry is the scientific technique of measuring colours.
- **Chroma**: Chroma refers to the colour's level of intensity and richness. It is the intensity of the colour.

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- **Detector:** The detectors are photosensitive elements (usually a photo-resistor) and they convert light energy into electrical signal.
- **Beer's Law:** It says that the light absorbed is proportional to the number of absorbing molecules, i.e., to the concentration of absorbing molecules.
- **Lambert's Law:** It says that the fraction of radiation absorbed is independent of the intensity of the radiation**.**
- **Transmission Densitometers:** Transmission densitometers are used to measure transparent materials, to measure transparent surfaces and to measure color transparencies.
- **Reflection Densitometers:** Reflection densitometers are specifically used to measure light reflected from a surface.
- **Bone Density:** Bone Density, or Bone Mineral Density (BMD), is defined as the amount of bone mineral in bone tissue.
- **Differential Scanning Calorimetry (DSC)**: Differential Scanning Calorimetry (DSC) is referred as a thermoanalytical technique in which the difference in the amount of heat required to increase the temperature of a sample and reference is measured as a function of temperature.
- **Electron Spin Resonance**: Electron Spin Resonance or Electron Paramagnetic Resonance (EPR) spectroscopy is a method used to study materials having unpaired electrons.
- **Nuclear Magnetic Resonance** (NMR): Nuclear Magnetic Resonance is a physical phenomenon in which nuclei in a strong constant magnetic field are perturbed by a weak oscillating magnetic field and respond by producing an electromagnetic signal with a frequency characteristic of the magnetic field at the nucleus.
- **Microtomy:** Microtomy is a method for the preparation of thin sections using a microtome.
- **Sledge Microtome**: A sledge microtome is a device where the sample is placed into a fixed holder (shuttle), which then moves backwards and forwards across a knife.
- **Ultramicrotome:** An ultramicrotome can allow for the preparation of extremely thin sections, with the device functioning in the same manner as a rotational microtome, but with very tight tolerances on the mechanical construction
- **Laser Microtome:** The laser microtome is an instrument for contact free slicing, i.e., there is no contact of the knife blade with the sample.
- **Microscopy**: Microscopy is the technique which uses microscopes to view objects and areas of objects that cannot be seen with the naked eye.
- **Light Microscopy:** Light Microscopy refers to the use of any kind of microscope that uses visible light to make specimens observable.
- **Fluorescence:** Fluorescence is the ability of substances to absorb short wavelengths of light (ultraviolet) and give off light at a longer wavelength (visible).

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- **Microbiological Culture:** A microbiological culture or microbial culture is a method of multiplying microbial organisms by letting them reproduce in predetermined culture medium under controlled laboratory conditions.
- **Culture Media**: It is a medium (liquid or solid) that contains nutrients to grow bacteria in vitro.
- **Fermenters:** The fermenters or stirred-tank bioreactors are process vessels used to cultivate heterotrophic microalgae.
- **Tissue Culture:** Tissue culture is the growth of tissues or cells in an artificial medium separate from the parent organism. This technique is also called micropropagation.
- **Cell Proliferation**: Cell proliferation is the process by which a cell grows and divides to produce two daughter cells.
- **Cell Viability:** Cell viability is a measure of the proportion of live, healthy cells within a population.
- **Cryotechniques**: Cryotechniques are a group of related procedures for stabilizing, or 'fixing' specimens for microscopic observation.
- **Cryopreservation:** Cryopreservation or cryoconservation is a process where organelles, cells, tissues, extracellular matrix, organs, or any other biological constructs susceptible to damage caused by unregulated chemical kinetics are preserved by cooling to very low temperatures.
- **Cryoprotective Agents (CPAs):** The CPA, which is usually a fluid, reduces the freezing injury from the cryopreservation process.
- **Cryogenic Electron Microscopy:** Cryogenic Electron Microscopy (cryo-EM) is an Electron Microscopy (EM) technique applied on samples cooled to cryogenic temperatures and embedded in an environment of vitreous water.

1.10 SELF-ASSESSMENT QUESTIONS AND EXERCISES

Short-Answer Questions

1. What is the basic principle of colorimeter?
2. State the Beer-Lambert law.
3. Which two ultracentrifuge methods are used in various fields of science?
4. What are the types of densitometers?
5. What do DMax and DMin refer to? What is the key difference between the DMax and DMin?
6. What are various factors affecting microtomy?
7. State the techniques involving a transmitted light path.
8. What is the basic difference between dark-field and bright-field microscopy?

9. Write the major advantages of phase contrast microscopy.
10. How the image production occurs in transmission electron microscope?
11. What are the advantage and disadvantages of confocal microscopy?
12. Write classification of bacterial culture media on the basis of purpose, functional use, and application.
13. Why the monitoring the growth of any microorganism in culture is important in cell culture?
14. State about the types of fermenters.
15. Which basic facilities are needed for setting up a tissue culture lab?
16. How the total number of cells in a population is determined?
17. What is the significance of cell culture?
18. What is the primary purpose of cryopreservation?
19. What are cryoprotectants? Why they are an essential ingredient for cryopreservation?

Long-Answer Questions

1. Describe the essential parts of a colorimeter with the help of diagram. Also write its applications.
2. Explain Differential Scanning Calorimetry (DSC) and its types in detail.
3. Explain microtome, its principle, advantage and types of microtomes in detail.
4. Analyze the appearance of specimens in commonly used microscopes.
5. Draw a ray diagram of compound microscope showing image formation and explain its working.
6. Describe the mathematical relationship between the various light waves generated in phase contrast microscopy.
7. Explain the working principle of confocal microscopy in detail.
8. Elaborate on various methods of sterilization.
9. Illustrate the various phases in the pattern of microbial cell growth in batch culture or batch fermentation.
10. What are cell culture protocols? Why they are needed?
11. Briefly explain cell harvesting methods.
12. Elaborate on the various methods for cryopreservation.

1.11 FURTHER READING

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UNIT 2 TOOLS AND TECHNIQUES - II

NOTES

Structure

2.0 INTRODUCTION

A separation process is a method that converts a mixture or solution of chemical substances into two or more distinct product mixtures. At least one product mixture of the separation is enriched in one or more of the source mixture's constituents. Cells are complex little structures comprised of wildly different components that each serve specific functions. One of the most basic ways of separating cell components is to do so by mass. The size and weight of the organelles or other large particles can be sorted using a centrifuge, a device that spins cells at high speeds to generate substantial g-forces, or gravity due to centrifugal forces. Some centrifuges also implement heat or vacuum as a secondary means of separation Chromatography is a widely used analytical technique because of its resolving power, being able to separate sample components from one another prior to measurement. Chromatography is conducted with computer-controlled instrumentation for high precision and unattended operation. Chromatography is an important biophysical technique that enables the separation, identification, and purification of the components of a mixture for qualitative and quantitative analysis. Chromatography techniques are based on the stationary bed, including column,

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thin layer, and paper chromatography. Column chromatography is one of the most common methods of protein purification.

The surgical techniques is a popular subject. It is a subject in which we analyze the anatomy of the living and pulsating organism. This is regarding the handling and suturing the tissues, as well as the control of the bleeding. Surgical procedures are commonly categorized by urgency, type of procedure, body system involved, the degree of invasiveness, and special instrumentation.

In this unit you will study about separation techniques in biology, such as chromatography, electrophoresis, precipitation, etc., computer aided techniques for data presentation, data analyses, statistical techniques, radioisotope and mass isotope techniques in biology, immunological techniques based on antigen antibody interactions, surgical techniques, such as organ ablations and perfusion techniques.

2.1 OBJECTIVES

After going through this unit you will be able to:

- Explain separation techniques in biology
- Understand molecular separation by chromatography electrophoresis, precipitation, etc.
- Comprehend organelle separation by centrifugation.
- Analyse computer aided techniques for data presentation data analyses, statistical techniques
- Explain radioisotope and mass isotope techniques in biology
- Elaborate on autoradiography
- Explain immunological techniques based on antigen - antibody interactions
- Understand various surgical techniques, such as organ ablations, perfusion techniques, indwelling catheters, stereotaxy, parabiosis and biosensors

2.2 SEPARATION TECHNIQUES IN BIOLOGY

Various separation techniques are routinely used in chemical and life science laboratories for separation of desired molecule from mixture of molecules. The molecules are separated on the basis of different properties including structure, function, charge, affinity and molecular size. The molecules can be separated, purified and analyzed using suitable separation technique (Refer Figure 2.1). Various types of chromatographic techniques and electrophoresis techniques are currently included in routine experimentation.

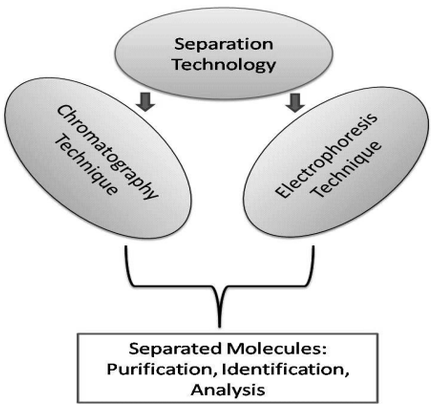

Fig. 2.1 *Separation Technology and its Main Components: Chromatography and Electrophoresis Techniques*

2.2.1 Chromatography: Concept, Types and Applications

Principle of Chromatography: Currently, chromatography technique has application in every branch of physical and biological. Chromatography is a method of separation based on the different interactions of various compounds with two phases, mobile phase and stationary phase. According to Keith Wilson, the basis of all forms of chromatography is the distribution or partition coefficient (K_d) which describes the way in which a component distributes between two immiscible phases. The two phases can be considered as phase A and phase B. the distribution coefficient of molecule 1 can be describe as:

K_d = Concentration in phase A/ Concentration in phase B.

The phase A is generally stationary phase B is mobile phase.

The chromatography technique involves use of following three basic components:

Mobile Phase: This phase is composed of liquid or gaseous component. Generally, a suitable solvent is used that moves or travels down through stationary phase.

Stationary phase: The phase is composed of solid phase. The stationary phase uses specific molecules on the supporting medium that interact with the analyte to be separated.

Analyte (Separated molecules): The analyte or molecule to be separated from mixture.

Types of Chromatography and Applications: Chromatography is a method for separating the components of a mixture by differential adsorption between a stationary phase and a mobile (moving) phase. Various chromatography methods have been developed by researchers for different applications. These include (Refer Figure 2.2):

- Paper Chromatography
- Column Chromatography

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- Adsorption Chromatography
- Partition Chromatography
- Affinity Chromatography
- Ion-Exchange Chromatography
- Gel-Permeation Chromatography
- Gas Chromatography
- Hydrophobic Interaction Chromatography
- Thin-Layer Chromatography
- High-Pressure Liquid Chromatography (HPLC)

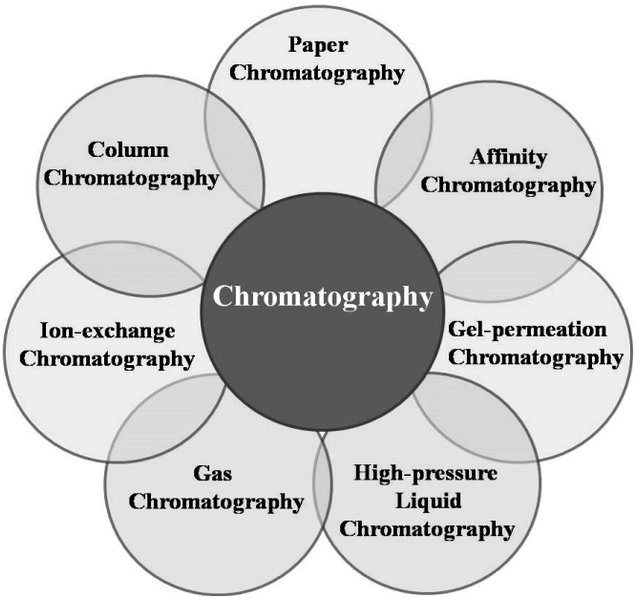

Fig. 2.2 *Major Forms of Chromatography Technique*

Paper Chromatography: Paper chromatography is a technique used to separate mixture of chemical substances into its individual compounds. Cellulose based chromatography paper strip or sheet is used as adsorbent stationary phase in this technique. The mixture of chemical compound moves along with the mobile phase through stationery phase. This technique is simple, economic and gives reproducible results. It is frequently used for identifying and separating small molecules, sugars, amino acids, lipids, nucleic acids and other molecules of interest. Paper chromatography is particularly useful for separation of amino acids.

In paper chromatography, the mixture of chemical compounds is applied or spotted near one end of paper (Refer Figure 2.3). The sheet is placed vertically in a solvent, which rises through the paper by capillary action. The chemical components move along with the solvent. The chromatogram is developed by flow of solvent. The components move at different rates due to differential absorption, difference in solubility and are separated due to different partition

coefficient. The paper is dried and the different components form a line of spots along the paper. Colorless substances are detected by using ultraviolet radiation or by spraying with a substance that reacts to give a colored spot, for example ninhydrin used for detection of amino acids. The ratio of the distance moved by the compound to the distance moved by solvent is measured that is known as Retention Factor (R_f). The components can be identified by the distance they move in a given time. The R_f values are affected by several factors including temperature, concentration, impurities in solvent system, homogeneity of paper, quantity of sample and others.

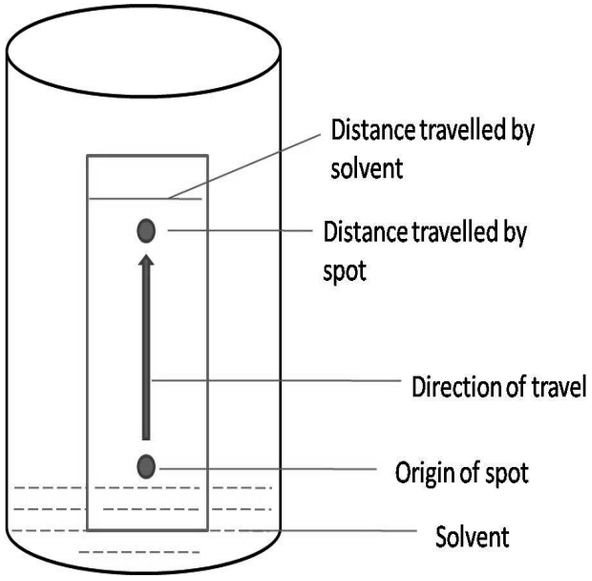

Fig. 2.3 *Basic Diagram for Paper Chromatography*

Column Chromatography: The column chromatography involves use of glass or metal column for packaging of stationary phase. The basic set up of column chromatography is relatively simple (Refer Figure 2.4**)**. It has simple instrumentation with minimal requirements.

The suitable stationary phase or solid phase (matrix) is packed in column. The particles of stationary phase should be uniform in size and shape. Impurities may affect the separation of analytes. Therefore, stationary phase particles or matrix molecules should be free from impurities.

The matrix particles should also be chemically inert and should not react with acids, bases and other solvents used in to the procedure. It should be easily available, inexpensive and colorless. Further, stationary phase should allow free flow of mobile phase and also suitable for separation of wide variety of compounds.

The mixture of components or analytes is applied from the top end of column. The mobile phase in column chromatography is commonly known as eluent. The mobile phase may be liquid or gas in nature. The eluent is passed through the column. The eluent flows through the column and components (analytes) are

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separated on the basis of their distribution coefficient. The analytes are collected separately in form of eluate. Basic components of column chromatography include stationary phase, column and mobile phase. In addition to the above components, an injector system to add the samples is also required for faster performance. A mechanized fraction collector can be used for collecting the separated analytes or components. This technique is common in various chemical and life science laboratories. It is frequently used for separation of chemical compounds, proteins, alkaloids, phytochemicals, vitamins and other molecules of interest. High Performance Liquid Chromatography (HPLC) and Gas Chromatography (GC) are two common forms of column chromatography.

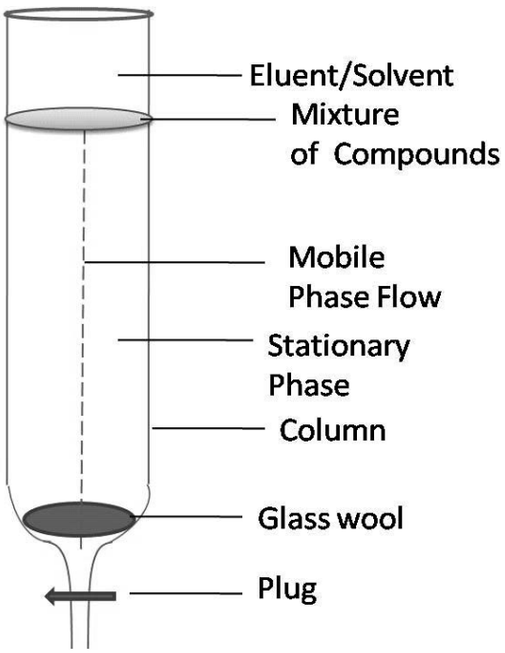

Fig. 2.4 *Basic Diagrammatic Presentation of Column Chromatography*

Adsorption Chromatography: It is considered as classic form of chromatography. The principle of adsorption chromatography is that certain solid materials can adsorb or hold the various molecules at their surface. These solid materials are known as adsorbents. In adsorption chromatography, separation is based on different adsorption affinities of the sample components for the surface of solid phase or matrix. Adsorption chromatography is based on the interaction between the solute molecules and active sites on the stationary phase. This chromatography involves binding of components based on the weak and non-ionic van der Waals forces and hydrogen bonding based interactions. The stationary phase may be based on inorganic adsorbents (silica gel, aluminium hydroxide, aluminium oxide, magnesium oxide, hydroxyapatite and others) and organic porous polymer based resins. This technique is frequently used for purification of enzymes, antibiotics, steroids, amino acids, proteins and other components. The mobile phase may be liquid or gaseous. Hydrophobic interaction chromatography,

hydroxylapatite chromatography and affinity chromatography are the examples of adsorption chromatography.

Partition Chromatography: This form of chromatography is also based on distribution coefficient of the analyte. The analytes distribute themselves into two phases, liquid stationary and mobile phase. Partition chromatography method is comparatively simple, low operating cost and has broad specificity. It is subdivided into liquid-liquid chromatography and bonded-phase liquid chromatography. Cellulose, starch or silica matrix can be used in partition chromatography.

Affinity Chromatography: This technique is widely used for protein and enzyme purification. It is also used for purification of hormones, antibodies and nucleic acids. Affinity chromatography involves biological interaction among the molecules. The affinity chromatography separates the proteins due to its reversible binding with specific ligand attached to chromatography matrix. The protein to be separated should be able to bind the ligand coupled with chromatography matrix. In other words, the protein to be separated should have affinity for the immobilized ligand. The combination of different types of interactions, including hydrophobic interactions, electrostatic interactions, van der Waals forces, or hydrogen bonding results in affinity interactions among the molecules. High fold of purity is obtained in affinity chromatography due to selective and specific affinity interaction among the specific molecules. The chromatography matrix with immobilized ligand is packed in a column. The commonly used matrix is generally based on agarose, dextrans, polyacrylamide, cellulose, silica and Poly Methyl Methacrylate (PMMA). Agarose is frequently used material. The nature of ligand depends on molecule to be purified. Some examples of ligand include avidin, lectin, calmodulin, 5'-AMP, etc. Suitable spacer arms may be used to couple the ligand with base matrix. The spacer arm links the ligand with base matrix. The spacer arm is generally linear molecules with different chain length, are used to bridge ligands and matrix. The mixture containing molecule to be purified is applied to the column. The molecule to be purified binds to ligand immobilized on the matrix. Other molecules are removed in form of wash. The binded molecules are released with the help of eluent. The purified and concentrated molecules are collected and analyzed.

Ion-Exchange Chromatography: The Ion-Exchange Chromatography (IEX) is used for purification of proteins. Other biological molecules including peptides and nucleic acids can also be purified by this technique. The principle of ion exchange chromatography is the separation of molecules on the basis of reversible electrostatic interaction between opposite charges. The separation of proteins depends on isoelectric point. Ion exchange can be defined as the reversible exchange of ions between a liquid phase and solid medium. The selectivity of the ion exchange media depends on the nature of functional group coupled to the matrix. The oppositely charged stationary phase is known as ion exchanger. Depending on the ionic exchange, two types of ion exchange chromatography have been developed: cation exchange chromatography and anion exchange

chromatography. The important parameters affecting the results of ion exchange chromatography include choice of exchanger, elution pH and elution (stepwise, continuous, and gradient).

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Cation Exchange Chromatography: The functional group coupled to matrix has negative charge. Therefore, it shows affinity towards the positively charged molecules. The commonly used matrices include agarose, cellulose, dextran, polystyrene, etc. Generally, the cation exchange matrix contains carboxymethyl, sulphonic acid, sulphomethyl, phosphonic acid or carboxylic acid as functional group. Carboxymethyl cellulose is a commonly used cation exchange matrix.

Anion Exchange Chromatography: The functional group coupled to matrix has positive charge and therefore, shows affinity towards the negatively charged molecules. The commonly used matrices include agarose, cellulose, dextran, polystyrene, etc. Generally, the anion exchange matrix contains secondary amine, quaternary amine, etc. as functional group. Diethylaminoethyl (DEAE) cellulose is an example of commonly used matrix.

Gel-Permeation Chromatography: The gel permeation chromatography is also known as gel filtration and size exclusion chromatography. This technique separates the molecules on the basis of their molecular sizes. In gel permeation chromatography, the stationary phase gel or matrix has small pores. The molecules small in size diffuses rapidly into the gel as compared to larger molecules. The small size molecule penetrates in the pores of gel to greater degree. Therefore, they take longer period of time to elute from the column. The larger molecules are not able to permeate through the small pores of gel. Therefore, these larger molecules move faster and eluted first from the column. The larger molecules travel through the space between the porous particles of the gel. Various gel materials are available commercially. Cross linked dextrans based gels (Sephadex and Sephacryl) and cross linked agarose (Sepharose) with various pore sizes are used routinely for the purification. This technique is useful in protein molecular weight determination, purification and removal of salts (Refer Figure 2.5).

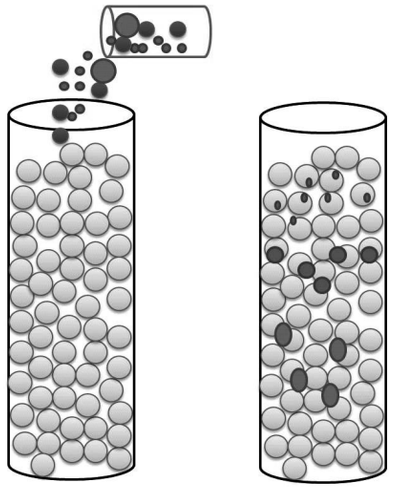

Fig. 2.5 *Basic Diagrammatic Presentation of Gel Permeation or Gel Filtration Technology*

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Gas Chromatography (GC): Gas Chromatography is a gas-liquid chromatography. It is a commonly used technique for identification, purification, quantification and analysis of compounds in a sample. GC is used in various quality control processes. Gas chromatography is a simple, multifaceted and highly sensitive technique. Molecules in very small amount can be separated with this technique. Gas chromatography is also used to monitor industrial processes. A large variety of samples can be analyzed as long as the compounds are sufficiently thermally stable and reasonably volatile. In gas chromatography, stationary phase consists of a packed column. The column contains an inert solid support coated with liquid stationary phase. Its mobile phase or carrier phase consists of gases like helium, argon, or nitrogen. Theses gases are known as carrier gases. The mobile phase (inert gas) is passed through the column under high pressure. Liquid samples are vaporized before injection into the carrier stream. The vaporized sample enters the gas stream which transports the sample into a separation column. The various components are separated inside this column. The attached detector measures the quantity of the components that exit the column. The concentration of sample can be determined by using a standard sample with known concentration. The peak retention time (appearance time) and area of standard sample are used for calculation of test sample concentration.

Hydrophobic Interaction Chromatography: Hydrophobic Interaction Chromatography (HIC) separates the molecules depending on their hydrophobicity. This technique is particularly useful for separation and purification of proteins in their native states. This method is based on the property of protein molecules to interact with hydrophobic adsorbents. This interaction is dependent on the salt concentration of the solution. The media of HIC consisted of base matrix and ligand. The base matrix functions as a solid support on which the hydrophobic ligand is immobilized. Cross-linked agarose is commonly used base matrix for HIC. Hydrophobic ligands are attached to the surface of base matrix by covalent bonds. Widely used ligands for HIC are linear chain alkanes and phenyl. The polysaccharide-based Butyl-Sepharose, Octyl-Sepharose and Phenyl-Sepharose materials derived from polymers has been found suitable for chromatographic separation of proteins. Sample molecules containing hydrophobic and hydrophilic regions are applied to an HIC column in a high-salt buffer. The salt in the buffer (commonly ammonium sulfate) reduces the solvation of sample solutes and results in exposure of hydrophobic regions. The hydrophobic regions are adsorbed by the hydrophobic areas on the solid media. The more hydrophobic the molecule, the less salt is needed to promote binding. The decrease in salt concentration results in elution of samples from the column. Mild organic modifiers or detergents can be used in elution buffer to assist the sample elution.

Thin Layer Chromatography (TLC): It is a solid-liquid adsorption chromatography. Thin layer chromatography is used to separate and analyze complex biological or non-biological samples into their constituents. It is also used for testing the purity of a sample. In thin layer chromatography, the stationary

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phase consisted of solid adsorbent substance coated on glass plate. The solid adsorbent material can be based on silica or alumina. In general laboratory experimentation, silica coated on glass plate is used for performing TLC. The sample is applied in form of spot and allowed to run in presence of suitable mobile phase (solvent). The mobile phase (solvent) runs from bottom towards top by diffusion. The sample travels along with the mobile phase and get distributed on the stationary phase and solvent. The different molecules are separated on the stationary phase due to different mobility. The movement depends on nature of the substance, functional groups on substance, polarity of the substance, stationary phase, solvent system, functional groups on stationary phase and other factors. If the components in sample are colorless, then suitable chemical substance or fluorescence can be used for development of color of the separated molecules. The developed color can be checked by visible light or UV light. The position of each molecule in the mixture can be measured by calculating the ratio between the distances travelled by the molecule and the solvent. The ratio is denoted by R_f.

High Performance Liquid Chromatography (HPLC): High Performance Liquid Chromatography (HPLC) is an improved or advanced form of column chromatography. It can be used in various forms depending on the stationary phase. It may be normal phase HPLC, reverse phase HPLC, ion exchange HPLC or size exclusion HPLC. It is useful in separation, purification, analysis of structural, analysis of functional aspects and quantification. This technique is fast and efficient. HPLC can be used effectively for purification and analysis of amino acids, lipids, carbohydrates, nucleic acids, proteins, steroids, and other biologically active molecules. The results can be obtained in very short time. HPLC is used in a variety of industrial and scientific applications including pharmaceutical, environmental, forensics, and chemicals. The general instrumentation for HPLC includes pump, injector, column, detector, data acquisition and display (Refer Figure 2.6).

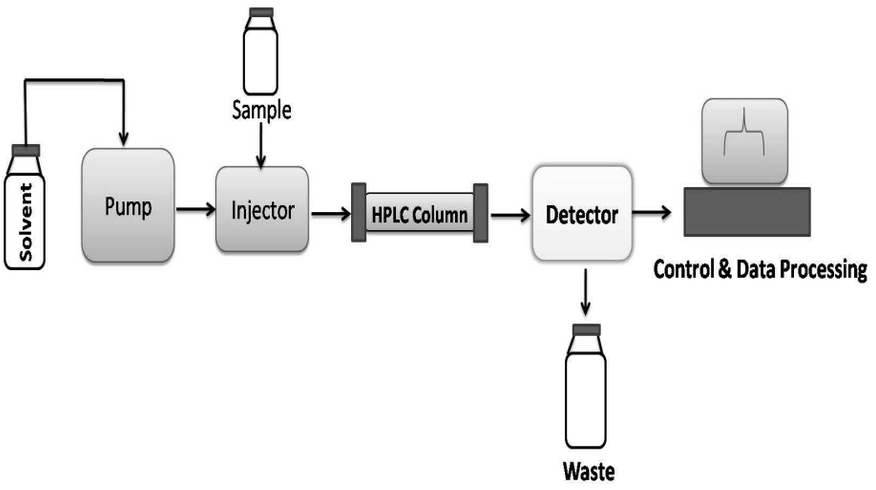

Fig. 2.6 *General Instrumentation or Setup for HPLC*

The stationary phase should be resistant to mechanical and chemical damage. High pressure is applied in HPLC to derive the solvent through packed column. In HPLC, sample mixture or analyte in a solvent (known as the mobile phase) is pumped at high pressure through a column having suitable chromatographic packing material (stationary phase). The sample is carried by a moving carrier gas stream of helium or nitrogen. Through HPLC, the trace concentrations of compounds in a sample can be separated and identified. The HPLC operate with basic principle similar to other chromatographic techniques. Separation of a sample into its constituent parts depends on the difference in the relative affinities of different molecules for the mobile phase and the stationary phase used in the separation.

2.2.2 Electrophoresis: Concept, Types and Applications

Gel electrophoresis is the commonly used technique in molecular biology. It is routinely used for separation and molecular size/weight determination of charged biological molecules (DNA, RNA, nucleotides, peptides and proteins). It can be defined as the migration of charged particles or molecules under effect of an applied electric field. Various biologically important molecules including amino acids, peptides, nucleotides, nucleic acids and proteins can be separated under the influence of electric field.

The basic equipment of electrophoresis consists of electrophoresis unit and power supply. In electrophoresis, two electrodes of opposite charge (anode and cathode) generally made of an inert metal (platinum) are immersed and connected by appropriate electrophoresis buffer. The electrophoresis buffer is also known as conducting medium (electrolyte). The buffer remains in two chambers which are not fully isolated from each other. An electric power supply is used to generate Electric Potential (E) between the two electrodes. The charged particles migrate between the two interconnected chambers. These particles are driven by the electric potential between the two electrodes. Negatively charged ions (anions) move towards the positively charged anode, while positively charged ions (cations) move towards the positively charged cathode. The ionic particles separate due to differences in their Velocity (v), which is the product of the particle's Mobility (m) and the Field Strength (E). The Mobility (m) of an ionic particle depends on particle charge, size, shape and temperature during the electrophoretic separation and is constant under defined electrophoretic conditions. Basic electrophoretic conditions cover the electrical parameters (current, voltage and power) and various factors like ionic strength, pH value, viscosity, pore size of the gel and others.

A suitable polymer based gel (media) is used for separation of biological molecules in laboratory. The gel acts as a molecular sieve. It affects the movement of molecules depending on their size. Generally, small molecules travels faster while large molecules move slowly. The concentration of gel has a profound effect on its pore size and therefore, affects the movement or migration of the molecules. All gels are characterized by an average pore size. When ions with sizes in the range of the pore size are migrated through the gel by electrophoresis, the gel exerts a pronounced size-dependent dragging force on them. The concentration

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and pore size of the gel determines the size range of molecules to be separated by the electrophoresis. The gel or the gel media should be hydrophilic, stable in buffer system, free of ionic charge (neutral), easy to stain, transparent and should not affected by the applied electric charge.

Two materials particularly useful in gel electrophoresis are agarose and polyacrylamide. Depending on gel matrix electrophoresis can be categorized in two major forms: (a) Agarose gel electrophoresis and; (b) Polyacrylamide gel electrophoresis (Refer Figure 2.7). These are commonly used material for preparation of gels for electrophoresis. Agarose is generally used for nucleic acids while polyacrylamide is used for proteins.

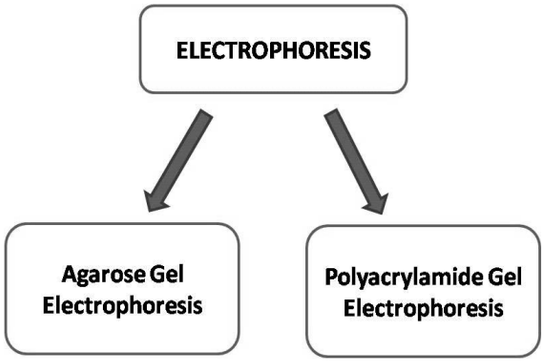

Fig. 2.7 *Two Major Forms of Electrophoresis*

Agarose: Agarose is a linear galactan hydrocolloid isolated from agar or agar-bearing marine algae. Agarose is natural colloid extracted from seaweed. It is a linear polysaccharide made up of the repeating unit agarobiose. Gels prepared from agarose have a substantially larger pore size than polyacrylamide gels. Agarose gels can be prepared faster than polyacrylamide, but the resolution is low. Structurally, it is a linear polymer consisting of alternating D-galactose and 3, 6-anhydro-L-galactose units. As a gelling agent, agarose is used to separate nucleic acids electrophoretically because its gels have larger pore sizes than polyacrylamide gels at low concentrations. The consistency of the agarose gel is more solid as compared to acrylamide gel. Agarose is also used in various immunological assays.

Agarose Gel Electrophoresis: In agarose gel electrophoresis, horizontal electrophoresis unit is used. Agarose is a polysaccharide derived from seaweed. It is isolated from the seaweed genera *Gelidium* and *Gracilaria*. Structurally, it consists of repeated agarobiose (L- and D-galactose) subunits. During gel formation, agarose polymers associate non-covalently. The pore sizes determine a gel's molecular sieving properties. Agarose gel electrophoresis is routinely used for large DNA/RNA molecules. The pore size of agarose gels is larger as compared to acrylamide gels. The pore size of the agarose gel can be controlled by adjusting the concentration of the agarose solution. For gel preparation, the suspension of agarose (in buffer) is heated until a clear solution state is obtained. After proper heating, it is allowed to cool down to room temperature to reach the gel state. The DNA or RNA sample is loaded in to wells (depressions of uniform size in gel) of gel. The gel should remain flooded with the running buffer. The electric current of

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fix strength is applied for a period of time. Generally, 50-100 volts is applied for wide range of size of DNA. The DNA/RNA possesses negative charge due to phosphate backbone. Therefore, when placed in an electric field, DNA/RNA fragments migrate towards the positively charged electrode (anode). Due to uniform mass/charge ratio, DNA molecules are separated on the basis of size within an agarose gel. The distance traveled by the molecule is inversely proportional to the log of its molecular weight. Smaller molecules travel faster while larger molecules travel slowly in gel of specific concentration. The standard apparatus and gel position in agarose gel electrophoresis has been shown in Figures 2.8 and 2.9.

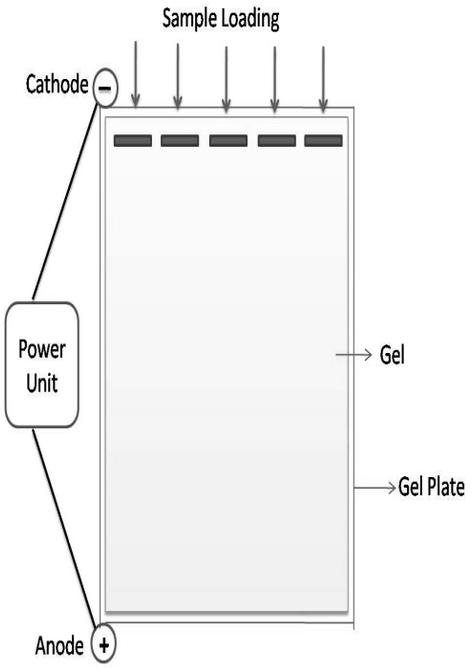

Fig. 2.8 *A Simple Schematic Presentation for the Agarose Gel Electrophoresis*

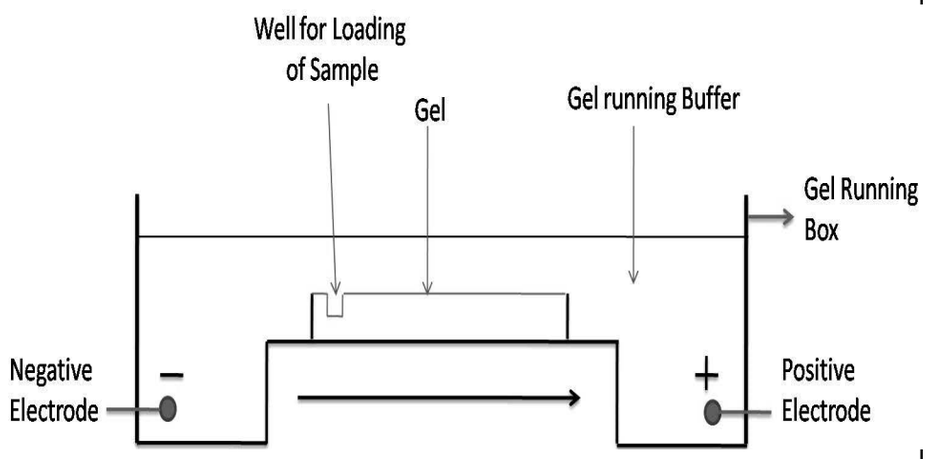

Fig. 2.9 *Side View of Agarose Gel Electrophoresis Set Up*

Various factors affect the migration of DNA molecule through the gel. These include concentration of agarose, size of DNA molecule, DNA conformation,

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voltage, agarose, ethidium bromide and electrophoresis buffer. After electrophoresis, the nucleic acids (DNA/RNA) can be visualized under UV light by staining the gel with dye ethidium bromide. The ethidium bromide can be added in buffer during gel preparation. Alternately the gel can be stained after electrophoresis (Refer Figure 2.10).

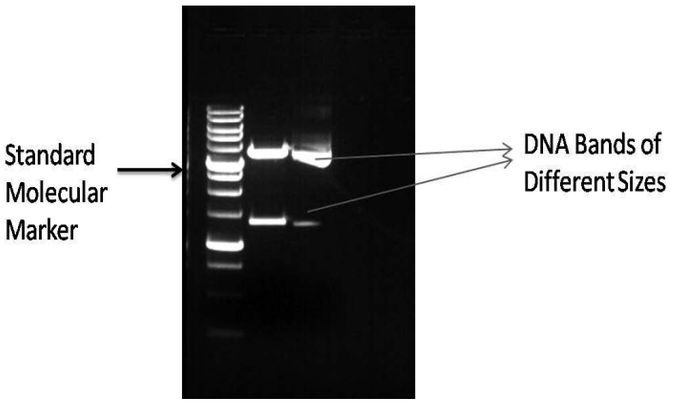

Fig. 2.10 *Real Agarose Gel after Electrophoresis Showing DNA Bands Of Different Sizes*

The above Figure 2.10 shows the real agarose gel after electrophoresis showing DNA bands of different sizes. The size can be determined by running a standard molecular marker having different known sizes of DNA. The DNA molecular marker is also known as DNA ladder.

Some basic points about agarose gel electrophoresis are as follows:

- Most commonly, 0.5-2.5% agarose (w/v) is used for regular electrophoresis experiments.
- Most common gel running buffer are TAE (Tris-acetate, EDTA) and TBE (Tris-borate, EDTA).
- Gel loading dye is used to load the DNA/RNA sample exactly in well.
- Gel loading dye also helps in tracking the sample during electrophoresis. The gel loading dye travels along with the sample. The distance travelled by sample is estimated by the movement of loading dye in gel during electrophoresis.
- Gel loading dye is typically made of bromophenol blue, xylene cyanol and glycerol.
- Commonly ethidium bromide at concentration of 0.5 mg/ml is added to gel before its solidification.
- Gel can be stained after electrophoresis for 15-30 minutes with ethidium bromide (added in running buffer).
- During electrophoresis the surface of gel should be covered by running buffer.

- Ethidium bromide is harmful. It is potent mutagen. Care should be taken while preparing the gel. Wear gloves during handling of gel.
- Discard the gel according to specified guidelines.

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The molecular size (bp) of DNA can be approximated by agarose gel electrophoresis. The DNA sample is run along with the standard molecular size marker of DNA and the band position is compared with the standard DNA marker.

General Procedure for Agarose Gel Electrophoresis

- Take gel casting plate and seal its sides with transparent adhesive tape.
- Prepare the 1X TAE buffer by diluting the 5X TAE buffer with double distilled water.
- Take appropriate volume of 1X TAE buffer in a flask or beaker and add specific amount of agarose (amount depends on the gel percentage).
- Boil the buffer to dissolve the agarose in TAE buffer. A clear solution will be obtained.
- Allow to cool the agarose solution up to 55-60 °C. *Ethidium bromide can be added at this stage.
- Adjust the comb in the electrophoresis unit. The comb should not touch the base of casting tray.
- Pour the agarose solution in gel tray slowly to avoid the bubble entrapment.
- Allow the agarose to solidify. Do not disturb the set up until agarose solidified.
- Remove the tape carefully.
- Remove the comb carefully and check the well formation. Wells should be uniform, undamaged and firm.
- Transfer the gel plate in buffer tank of electrophoresis unit.
- Pour the 1X TAE buffer slowly into tank in manner that buffer stands 0.5-0.8 cm above the gel surface.
- Connect the electrodes of power supply and electrophoresis unit with appropriate connecting wires.
- Load the sample DNA and marker DNA mixed with the tracking dye with the help of micropipette.
- Power on the system and set at 50-100 V.
- Allow running the loaded sample and marker till the blue tracking dye approached the other end of gel.
- Switch off the power supply.
- Remove the electrode wires from the electrophoresis unit.
- Remove the gel form buffer tank slowly and carefully.

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- Take ethidium bromide solution in a staining tray and satin the gel for about 20 minutes. Alternately, ethidium bromide can be added in agarose suspension while preparing or casting the gel.
- De-stain the gel with water for about 15 minutes.
- Place the gel on UV transilluminator and switch on the UV to visualize the DNA bands.
- Molecular weight can be assessed by comparing with the bands of standard molecular weight marker.

Precautions for Agarose Gel Electrophoresis

- Chemicals should be better quality.
- Water for solution and buffer preparation should be double distilled and of good quality.
- Use precise and accurate measurement while preparing the buffers, solutions and gel.
- Use micropipettes for precise volumes.
- Ethidium bromide is potent carcinogen. Use and handle carefully.
- UV rays are harmful. Proper safety measures should be followed before observing DNA under UV rays. Protect the eyes from UV rays by using good quality glasses.
- Wear gloves while performing experiment.
- Carefully adjust the comb for well preparation.
- Carefully remove the adhesive tape and comb from the gel.
- Carefully transfer the gel from electrophoresis unit to staining tray.
- Be careful, while connecting the wires with electrodes and electrophoresis unit.
- Do not touch the power unit, electrophoresis unit, electrodes and wires when running the gel.
- Carefully monitor the movement of tracking dye through gel. It should not run out of gel.

Polyacrylamide Gel Electrophoresis: Polyacrylamide gels can be used for the separation and analysis of proteins and small nucleic acid molecules. The pores size in polyacrylamide gels is much smaller than agarose gels. The polyacrylamide gel is formed by the radical polymerisation of acrylamide monomers. This process of polymerization alone would lead to very long polymer chains instead of a three-dimensional gel. The three-dimensional network is formed by the incorporation of N, N'-methylenebisacrylamide into the polymerizing chains. The polyacrylamide gel is held together by covalent bonds. The pore size of polyacrylamide gels can be adjusted by the concentration of

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the acrylamide monomer and the ratio of the crosslinking agent, N, N'-methylenebisacrylamide. The separation or resolution capacity of polyacrylamide gels is very high. The size range that can be separated depends on the pore size of gel. Higher amount of acrylamide (generally 4-20%) is used as compared to bisacrylamide (generally 1-3%). Gel buffers of specific pH are used for gel formation. Ammonium persulfate and Tetramethylethylenediamine (TEMED) are used as catalyst and initiator for gel formation. Acrylamide concentration and pH are important factors. Various forms of polyacrylamide gel electrophoresis have been developed depending on the different molecular properties of protein. The major forms of polyacrylamide gel electrophoresis are native PAGE, SDS-PAGE and 2D PAGE (Refer Figure 2.11).

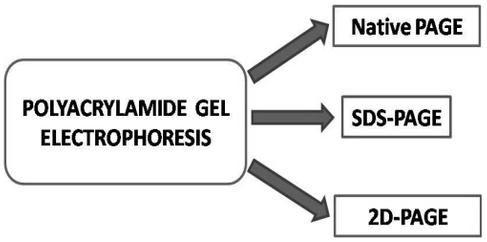

Fig. 2.11 *Major Forms of Polyacrylamide Gel Electrophoresis*

Native PAGE: Native PAGE is non-denaturing PAGE. SDS is not used in native PAGE for protein electrophoresis. Proteins are separated on the basis of charge to mass ratio. The amino acid sequence of protein, isoelectric point and pH of buffer affects the mobility of proteins. Proteins with identical molecular weight can be separated with native PAGE. In native PAGE, the proteins retain their native confirmation state. The proteins remain functional. Therefore, enzymes and proteins can be detected with their specific assay or test. Moreover, proteins/ enzymes can be resolved and recovered in functional form. Under specific conditions, enzymes can be selectively detected within the gel through specific staining reaction. After electrophoresis, the gel is soaked in a substrate containing solution. The substrate diffuses into the gel and interacts specifically with the enzyme. This interaction results in development of specific colour. Native PAGE can also be used to detect complex formation between proteins.

SDS-PAGE: SDS-PAGE is a very useful tool to separate protein molecules by size (Refer Figures 2.12 – 2.14). SDS is a detergent that denatures secondary and non-disulfide-linked tertiary structures. SDS treatment results in development of net negative charge on surface of proteins. All the treated proteins have almost same strength of negative charge. Therefore, proteins migrate in gel on the basis of molecular weights. Mobility through the running gel can be affected by the state of the protein and their form (monomer or multi unit).

The SDS-PAGE system is a discontinuous gel with an upper stacking gel and lower resolving gel that have different polyacrylamide concentrations and different pH values. The upper stacking gel has a lower percentage of

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polyacrylamide. Due to low percentage of polyacrylamide, proteins move faster through the gel and got 'stacked' into a tight band just before entering into the higher percentage polyacrylamide resolving gel for separation. The percentages of polyacrylamide can be optimized for the size range of molecules present in the sample. Gradient gels can be prepared allowing a greater range of separation in a single gel if both large and small proteins need to be resolved simultaneously. Small proteins will move through the resolving gel more quickly than large proteins. The SDS-PAGE can be used for separation, purification and molecular weight determination of proteins. The standard molecular weight marker can be run along with the test sample to determine the molecular weight of test sample. The purity and homogeneity of the purified proteins ca be determined by SDS-PAGE. These days, improved mini-gel systems are available which allow simultaneous running of 2-4 gels in the same system. Commonly mini-gels are used for fast and improved results. The protein bands in SDS-PAGE can be visualized easily by using suitable dye.

Coomassie brilliant blue staining is used commonly for visualization of protein bands after electrophoresis. The mobility and distance traveled by the proteins during electrophoresis can be tracked by using bromophenol blue. The protein sample is mixed and treated with sample buffer of specific composition. A standard and optimized protocol for SDS-PAGE using mini gels system has been given below. The method can be further optimized.

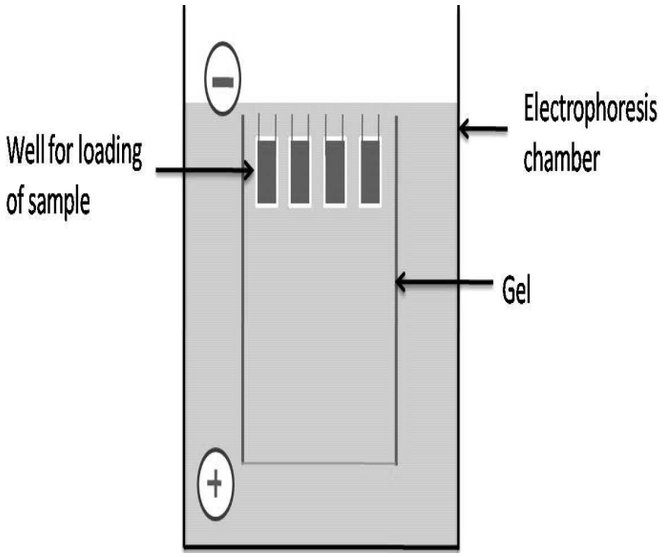

Fig. 2.12 *General Set Up for SDS-PAGE*

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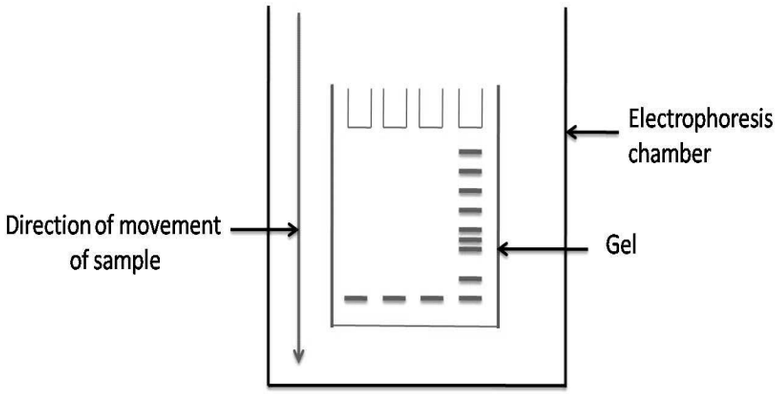

Fig. 2.13 *General Presentation of SDS-PAGE Electrophoresis*

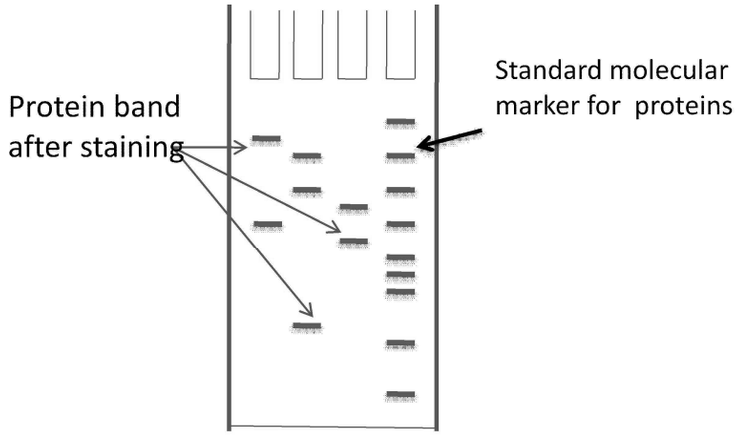

Fig. 2.14 *General Presentation of SDS-PAGE Gel after Staining*

General method for molecular mass determination of proteins by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE):

The purity and homogeneity of the proteins can be determined by the basic Laemmli SDS-PAGE procedure (Refer Figure 2.15). Gels can be run on electrophoretic systems at a constant voltage (50-100 V). The mini-gel protein electrophoresis system is available at various companies. Following materials and methods can be used for protein electrophoresis. This method has been used for electrophoresis of a specific protein.

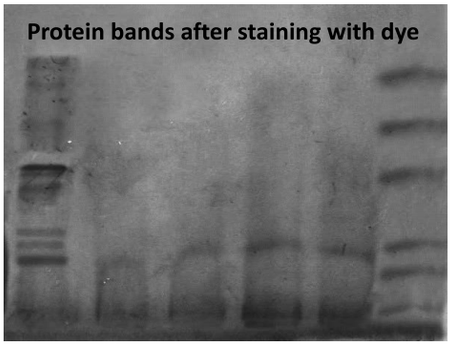

Fig. 2.15 *General Picture of Stained SDS-PAGE showing various Protein Bands*

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Reagents

- Separating (4x) gel buffer: Dissolve Tris HCl (18.3 g) in 100 ml of deionized water and adjust pH to 8.8 with 1 N HCl.
- Stacking (4x) gel buffer: Dissolve Tris HCl (6.055 g) in 100 ml of deionised water and adjust pH to 6.8 with 1 N HCl.
- Acrylamide-bisacrylamide (30%): Dissolve acrylamide (29.2 g) and bisacrylamide (0.8 g) in 100 ml of deionised water.

Sample Buffer: Prepare the sample buffer by mixing the following components:

Tris HCl buffer (pH 6.8)	:	0.4 ml
SDS (10%)	:	2.5 ml
2-Mercaptoethanol	:	0.4 ml
Glycerol	:	2.0 ml
Bromophenol blue	:	0.002g
Deionised water	:	4.7 ml

Running Buffer: Prepare the running buffer by mixing the following components:

Tris HCl	:	6.05 g
Glycine	:	28.8 g
SDS	:	2.0 g
Deionised water	:	2.0 l
pH	:	8.3

Separating Gel (12.5%): Prepare the solution for separating gel as follow.

Deionised water	:	6.4 ml
Acrylamide-bisacrylamide	:	8.3 ml
4x Separating gel buffer	:	5 ml
SDS (10%)	:	0.2 ml
Ammonium persulphate (2%)	:	0.1 ml
TEMED	:	6.7 µl

Stacking Gel (4%): Prepare the solution for stacking gel as under.

Deionised water	:	6.1 ml
Acrylamide-bisacrylamide	:	1.3 ml
4x Stacking gel buffer	:	2.5 ml
SDS (10%)	:	0.1 ml
Ammonium persulphate (2%)	:	50 µl
TEMED	:	10 µl

Standard Procedure: Prepare the loading sample by mixing the protein sample with sample buffer in equal ratios and heat in a boiling water bath for 2-3 min. Run the gels at 100 V and stain with coomassie brilliant blue stain.

Coomassie Brilliant Blue Staining: This method of staining is fast and detects as little as 0.1 µg of protein in a single band (Bollag *et al.*, 1996).

Solutions

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Prepare the following solutions:

- Fixing solution: Add 25 ml of methanol and 10 ml of acetic acid to 65 ml of distilled water.
- Coomassie brilliant blue gel stain: Dissolve 0.12 g of coomassie brilliant blue R-250 in 100 ml of acetic acid (10%, v/v).
- Destaining solution: Add 10 ml of acetic acid and 10 ml of methanol to 80 ml of distilled water.

Procedure: Transfer the washed gel to fixing solution and keep on rocker shaker for 40 min to fix the protein bands. Transfer it to staining solution and again put on rocker shaker with gentle shaking for 2 h. Transfer the gel to destaining solution for 2 h. Wash the gel with deionised water and preserve the gel in acetic acid solution (10%, v/v).

2D-PAGE: Two-Dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE) is a technique used for detection and analysis of proteins. It is a primary technique in proteomics work. In this technique, the complex mixture of proteins can be separated and analyzed on the basis of two different properties of proteins. In first step (first dimension), proteins are separated on the basis of pI value while in second step (second dimension) proteins are separated on the basis of molecular weight (Refer Figure 2.16). Thousands of proteins can be resolved by 2D-PAGE gel electrophoresis.

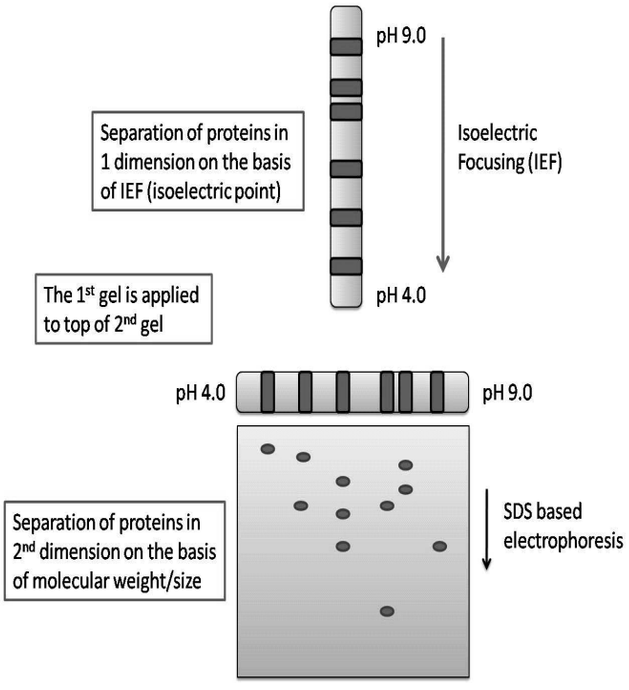

Fig. 2.16 *General Presentation of 2D-PAGE*

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The 2-D electrophoresis begins with 1-D electrophoresis followed by separation of the protein molecules by a second property in a 90 degrees direction from the first. In 1-D electrophoresis, all the proteins will position along a lane but remains separated from each other depending on the isoelectric point. The separation of proteins by isoelectric point is known as Isoelectric Focusing (IEF). A gradient of pH is applied to the gel and an electric potential is applied across the gel, making one end more positive than the other.

The proteins applied in the first dimension will move along the gel and will position at their isoelectric point (the point at which the net neutral charge on the protein). After completion of the first dimension electrophoresis, the complexes are destroyed by applying the denaturing SDS-PAGE in the second dimension, where the proteins are separated by their mass. Proteins are treated with Sodium Dodecyl Sulfate (SDS) along with other reagents. The proteins are denatured by SDS and binds with a number of SDS molecules. SDS is negatively charged molecule. The binding of SDS molecules to proteins results in negative charge coating on their surface. After treatment with SDS, proteins have same mass-to-charge ratio. In the second dimension, an electric potential is again applied, but at a 90 degree angle from the first field. The proteins will be attracted to the more positive side of the gel proportionally to their mass-to-charge ratio. Due to SDS treatment all proteins have same mass-to-charge ration. Therefore, gel acts like a molecular sieve and proteins are separated on the basis of their molecular weight. The larger proteins move slower as compared to smaller proteins. The different proteins are separated on gel depending on their molecular weight. The separated proteins can be detected by various methods, but the most commonly used methods are silver staining and coomassie staining. In 2D electrophoresis, some molecules other than proteins can also be separated.

General Workflow for Protein Sample Preparation

The general work flow for protein sample preparation is given in form of Figure 2.17. It may include disruption of cells for release of protein, removal of debris, removal of contaminants and salts, quantification of protein, concentrating the protein sample and electrophoresis.

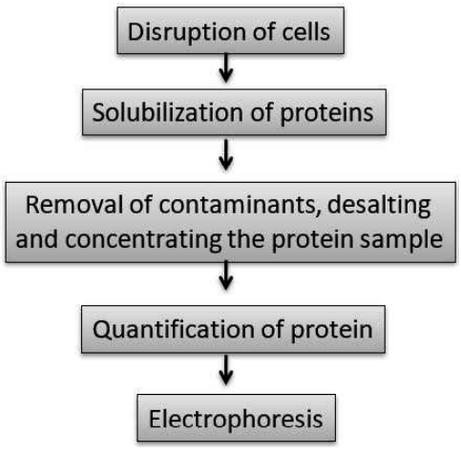

Fig. 2.17 *General Workflow for Protein Sample Preparation*

General Workflow for Protein Electrophoresis

The general work flow for protein electrophoresis is given below (Refer Figure 2.18). It includes selection of suitable electrophoresis method, sample preparation, preparation of buffers and gel, electrophoresis and analysis.

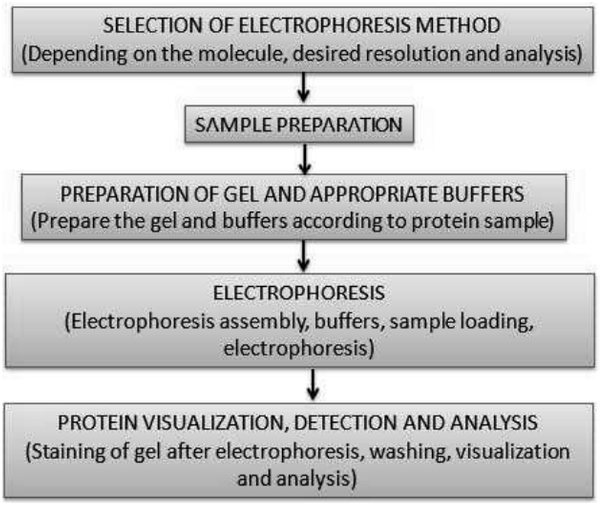

Fig. 2.18 *The General Workflow for Protein Electrophoresis*

Basic Difference between the Agarose Gel and Poly-Acrylamide Gel

The difference between agarose and polyacrylamide depends primarily on the size range and desired resolution of separation of nucleic acid samples, although gel casting and sample recovery methods may be considered. The basic differences between agarose and polyacrylamide are given in Table 2.1. Agarose forms matrices with pore sizes ideal for separating nucleic acid molecules in the range of 0.1-25 kb. Polyacrylamide forms smaller pore sizes which resolve nucleic acid molecules smaller than 1 kb. In some cases, single-base resolution between fragments of <100 bp may be obtained with polyacrylamide gels.

Table 2.1 *Basic Differences between Agarose and Polyacrylamide Gel*

Property	Agarose gel	Poly-acrylamide gel
Source of polymer	Obtained from sea weed	Crosslinked acrylamide and bis-acrylamide
Gel casting approach	Melt and solidify	Initiate chemical reactions
Nucleic acid recovery	Melt and extract	Dissolve and diffuse, or electroelute
Resolving power	5–10 nucleotides	Single nucleotides
DNA separation range	50–50,000 bp	5–3,000 bp
Gel cast dimension	Gel casted horizontally	Gel casted vertically
Toxic nature	Non-toxic	Potent toxic
Staining aspect	Staining can be done before or after electrophoresis	Staining can be done only after electrophoresis
Molecule separation	Commonly used for DNA separation	Commonly used for DNA or protein separation

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2.2.3 Precipitation

A method which is used for the separation of a mixture on the basis of the solubility of its components is known as precipitation. In other words, it is the process that transforms a dissolved substance into an insoluble solid from a supersaturated solution. The solid formed is called the precipitate. The formation of a precipitate takes place by a chemical reaction. There are various factors that affect the solubility of a compound. These factors are the ionic strength of the solution, its pH and temperature. Any change in these factors can cause a compound to become an insoluble solid that gets separated from a solution.

In a solution, the separation of a mixture consisting of metal ions can be done by precipitation with anions. When a metal ion or a group of metal ions form insoluble salts with a particular anion, the method of precipitation can be used to separate them from others**.** The anions can be separated by precipitating them with appropriate metal ions. For instance, a reaction between a barium chloride solution and sulphuric acid leads to the formation of a white precipitate of barium sulfate.

Some of the common methods used to separate the precipitate from the rest of the mixture are filtration, centrifugation, sublimation, evaporation, etc.

2.2.4 Organelle Separation by Centrifugation

Biological centrifugation is a process that uses centrifugal force to separate and purify mixtures of biological particles in a liquid medium. The smaller the particles, the higher the g-forces required for the separation. It is a key technique for isolating and analysing cells, subcellular fractions, supramolecular complexes and, with higher g-force instruments or 'ultra'-centrifuges (up to 60,000 revolutions per minute corresponding to ~ 2,00,000×g) isolated macromolecules such as proteins or nucleic acids. Such high-speed devices require a vacuum to avoid overheating of samples. The development of the first analytical ultracentrifuge with a specially designed optical system for monitoring and recording the sedimentation process by Svedberg in the late 1920s and the technical refinement of the preparative centrifugation technique by Claude and colleagues in the 1940s positioned centrifugation technology at the centre of biological and biomedical research for many decades. Today, centrifugation techniques represent a critical tool for modern biochemistry and are employed in almost all invasive subcellular studies. While analytical ultracentrifugation is mainly concerned with the study of purified macromolecules or isolated supramolecular assemblies, preparative centrifugation methodology is devoted to the actual separation of tissues, cells, subcellular structures, membrane vesicles and other particles of biochemical interest.

Most undergraduate students will be exposed to preparative centrifugation protocols during practical classes and might also experience a demonstration of analytical centrifugation techniques. This unit is accordingly divided into a short introduction into the theoretical background of sedimentation, an overview of

practical aspects of using centrifuges in the biochemical laboratory, an outline of preparative centrifugation and a description of the usefulness of ultracentrifugation techniques in the biochemical characterization of macromolecules. To aid in the understanding of the basic principles of centrifugation, the general designs of various rotors and separation processes are diagrammatically represented. Often, the learning process of undergraduate students is hampered by the lack of a proper linkage between theoretical knowledge and practical applications. Analytical ultracentrifugation – which unlike other analytical separation techniques does not require a separation medium, i.e., it is 'matrix-free' – has become a preferred or 'gold standard' technique for establishing the purity or homogeneity and state of aggregation of macromolecular or nanoparticle solutions, and to illustrate this point, we show how the purity of preparations of monoclonal antibodies can be routinely analysed with the modern ultracentrifuge, and how the inclusion of a density gradient, when appropriate, can enhance the resolution of the method even further.

Basic Principles of Sedimentation

From everyday experience, the effect of sedimentation due to the influence of the Earth's gravitational field ($G = g = 9.81\ m\ s^{-2}$) versus the increased rate of sedimentation in a centrifugal field ($G > 9.81\ m\ s^{-2}$) is apparent. To give a simple but illustrative example, crude sand particles added to a bucket of water travel slowly to the bottom of the bucket by gravitation, but sediment much faster when the bucket is swung around in a circle. Similarly, biological structures exhibit a drastic increase in sedimentation when they undergo acceleration in a centrifugal field. The relative centrifugal field is usually expressed as a multiple of the acceleration due to gravity. Below is a short description of equations used in practical centrifugation classes.

When designing a centrifugation protocol, it is important to keep in mind that:

- The more dense a biological structure is, the faster it sediments in a centrifugal field.
- The more massive a biological particle is, the faster it moves in a centrifugal field.
- The denser the biological buffer system is, the slower the particle will move in a centrifugal field.
- The greater the frictional coefficient is, the slower a particle will move.
- The greater the centrifugal force is, the faster the particle sediments.
- The sedimentation rate of a given particle will be zero when the density of the particle and the surrounding medium are equal.

Biological particles moving through a viscous medium experience a frictional drag, whereby the frictional force acts in the opposite direction to sedimentation and equals the velocity of the particle multiplied by the frictional coefficient. The frictional

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coefficient depends on the size and shape of the biological particle. At the same time, the particles also encounter a frictional drag that is proportional to their velocity. The frictional force of a particle moving through a viscous fluid is the product of its velocity and its frictional coefficient, and acts in the opposite direction to sedimentation. When the conditions for the centrifugal separation of a biological particle are described, a detailed listing of rotor speed and radial dimensions of centrifugation has to be provided. Essentially, the rate of sedimentation, v, is dependent upon the applied centrifugal field G (measured in cm s –2). G is determined by the radial distance, r, of the particle from the axis of rotation (in cm) and the square of the angular velocity, ω, of the rotor (in radians per second):

$$G = \omega^2 \times r \quad \text{.....(Eq. 2.1)}$$

The average angular velocity of a rigid body that rotates around a fixed axis is defined as the ratio of the angular displacement in a given time interval. One radian, usually abbreviated as 1 rad, represents the angle subtended at the centre of a circle by an arc with length equal to the radius of the circle. Since 360° equals 2π radians (or rad), one revolution of the rotor can be expressed as 2π rad. Accordingly, the angular velocity of the rotor, given in rad s^{-1}. Note that rad is treated as a scalar and is related to the rotor speed in revolutions per minute (rpm = 1 min^{-1}) by

$$\omega = 2\pi \text{ rad} \times \text{rpm} \quad \text{..... (Eq. 2.2)}$$

and therefore the centrifugal field can be expressed as:

$$G = 4\pi^2 \text{ rad}^2 \times \text{rpm}^2 \times r \quad \text{..... .... (Eq. 2.3)}$$

Where, the variable rpm is the rotor speed (measured in revolutions per minute, i.e., the non-italicised 'rpm' denotes the unit) and r is the radial distance from the centre of rotation. Note that 60 revolutions per minute is the same speed as one revolution per second, i.e. rpm = 60 min^{-1} = 1s^{-1}.

Types, Care and Safety Aspects of Centrifuges

Types of Centrifuges: Centrifugation techniques take a central position in modern biochemical, cellular and molecular biological studies. Depending on the particular application, centrifuges differ in their overall design and size. However, a common feature in all centrifuges is the central motor that spins a rotor containing the samples to be separated. Particles of biochemical interest are usually suspended in a liquid buffer system contained in specific tubes or separation chambers that are located in specialized rotors. The biological medium is chosen for the specific centrifugal application and may differ considerably between preparative and analytical approaches. As outlined below, the optimum pH value, salt concentration, stabilizing cofactors and protective ingredients such as protease inhibitors have to be carefully evaluated in order to preserve biological function. The most obvious differences between centrifuges are:

- The maximum speed at which biological specimens are subjected to increased sedimentation.
- The presence or absence of a vacuum.

- The potential for refrigeration or general manipulation of the temperature during a centrifugation run.
- The maximum volume of samples and capacity for individual centrifugation tubes.

Many different types of centrifuges are commercially available including:

- Large-capacity low-speed preparative centrifuges.
- Preparative high-speed ultracentrifuges.
- Refrigerated preparative centrifuges/ultracentrifuges.
- Analytical ultracentrifuges.
- Large-scale clinical centrifuges.
- Small-scale laboratory microfuges.

Ultracentrifugation has decisively advanced the detailed biochemical analysis of subcellular structures and isolated biomolecules. Preparative ultracentrifugation can be operated at relative centrifugal fields of up to 9 00,000×g. In order to minimize excessive rotor temperatures generated by frictional resistance between the spinning rotor and air, the rotor chamber is sealed, evacuated and refrigerated. Depending on the type, age and condition of a particular ultracentrifuge, cooling to the required running temperature and the generation of a stable vacuum might take a considerable amount of time. To avoid delays during biochemical procedures involving ultracentrifugation, the cooling and evacuation system of older centrifuge models should be switched on at least an hour prior to the centrifugation run. In contrast, modern ultracentrifuges can be started even without a fully established vacuum and will proceed in the evacuation of the rotor chamber during the initial acceleration process. For safety reasons, heavy armour plating encapsulates the ultracentrifuge to prevent injury to the user in case of uncontrolled rotor movements or dangerous vibrations. A centrifugation run cannot be initiated without proper closing of the chamber system. To prevent unfavorable fluctuations in chamber temperature, excessive vibrations or operation of rotors above their maximum rated speed, newer models of ultracentrifuges contain sophisticated temperature regulation systems, flexible drive shafts and an over speed control device. Although slight rotor imbalances can be absorbed by modern ultracentrifuges, a more severe misbalance of tubes will cause the centrifuge to switch off automatically. This is especially true for swinging-bucket rotors. The many safety features incorporated into modern ultracentrifuges make them a robust piece of equipment that tolerates a certain degree of misuse by an inexperienced operator. In contrast to preparative ultracentrifuges, analytical ultracentrifuges contain a solid rotor that incorporates one counterbalancing cell and typically either three or seven analytical cells. A specialized optical system enables the sedimenting material to be observed throughout the duration of a centrifuge run. Using either an absorption optical system or a Rayleigh interference optical system, or a combination of both, concentration distributions of macromolecules in solution can be recorded at any time during ultracentrifugation. From these records, information about the purity/ heterogeneity, sedimentation coefficient distribution, average molar mass and molar mass distributions, and ligands interaction information can be obtained.

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Types of Rotor

To illustrate the difference in design of fixed-angle rotors, vertical tube rotors and swinging-bucket rotors. Companies usually name rotors according to their design type, the maximum allowable speed and sometimes the material composition. Depending on the use in a simple low-speed centrifuge, a high-speed centrifuge or an ultracentrifuge, different centrifugal forces are encountered by a spinning rotor. Accordingly, different types of rotors are made from different materials. Low-speed rotors are usually made of steel or brass, while high-speed rotors consist of aluminium, titanium or fibre-reinforced composites. The exterior of specific rotors might be finished with protective paints. For example, rotors for ultracentrifugation made out of titanium alloy are covered with a polyurethane layer. Aluminium rotors are protected from corrosion by a tough, electrochemically formed layer of aluminium oxide. In order to avoid damaging these protective layers, care should be taken during rotor handling.

Fixed-angle rotors are an ideal tool for pelleting during the differential separation of biological particles where sedimentation rates differ significantly, for example when separating nuclei, mitochondria and microsomes. In addition, isopycnic (matching density) banding, where the density of the substance matches that of the gradient at that radial position, may also be routinely performed with fixed-angle rotors. For isopycnic separation, centrifugation is continued until the biological particles of interest have reached their isopycnic position in a gradient. This means that the particle has reached a position where the sedimentation rate is zero because the density of the biological particle and the surrounding medium are equal. Centrifugation tubes are held at a fixed angle of between 14° and 40° to the vertical in this class of rotor (Refer Figure 2.19). Particles move radially outwards and since the centrifugal field is exerted at an angle, they only have to travel a short distance until they reach their isopycnic position in a gradient using an isodensity technique or before colliding with the outer wall of the centrifuge tube using a differential centrifugation method.

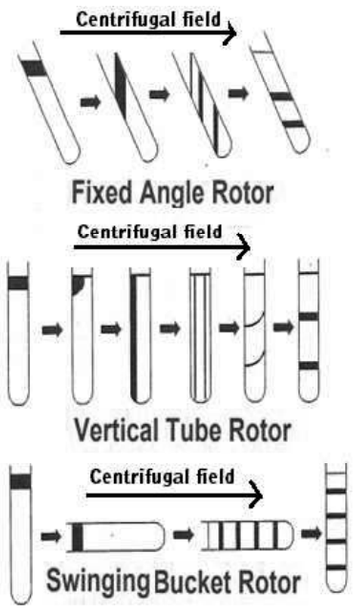

Fig. 2.19 *Diagrammatic Presentation of Difference in Design of Rotors*

Vertical rotors may be divided into true vertical rotors and near-vertical rotors. Sealed centrifuge tubes are held parallel to the axis of rotation in vertical rotors and are restrained in the rotor cavities by screws, special washers and plugs. Since samples are not separated down the length of the centrifuge tube but, across the diameter of the tube. Isopycnic separation time is significantly shorter as compared to swinging-bucket rotors. In contrast to fixed-angle rotors, near-vertical rotors exhibit a reduced tube angle of 7° to 10° and also employ quick-seal tubes. The reduced angle results in much shorter run times as compared to fixed-angle rotors. Near-vertical rotors are useful for gradient centrifugation of biological elements that do not properly participate in conventional gradients. Hinge pins or a crossbar is used to attach rotor buckets in **swinging-bucket rotors**. They are loaded in a vertical position and during the initial acceleration phase, the rotor buckets swing out horizontally and then position themselves at the rotor body for support.

Analytical ultracentrifugation is most often employed for:

- The determination of the purity (including the presence of aggregates) and oligomeric state of macromolecules, from recording the distribution of sedimentation coefficients from sedimentation velocity.
- The determination of the average molecular mass, or distribution of molecular mass of solutes in their native state, from sedimentation equilibrium.
- The examination of changes in the molecular mass of supramolecular complexes, using either sedimentation velocity or sedimentation equilibrium (or both).
- The detection of conformation and conformational changes using sedimentation velocity.
- Ligand-binding studies.

Since the mass of one molecule is extremely small, when researchers refer to the 'molecular mass' of a molecule, they really mean the molar mass M which describes the mass of 1 mol (= 6.023×10^{23} molecules) of macromolecules, in units of g mol^{-1}, or, equivalently, the relative molecular mass M_r, which is the mass of a macromolecule per one twelfth of the mass of a carbon-12 atom. Molar mass and relative molecular mass are numerically the same, but being a relative measure, M_r has no units. In addition, manufacturers of analytical ultracentrifuges offer a large range of excellent brochures on the theoretical background of this method and its specific applications available.

Check Your Progress

1. Define chromatography.
2. What do you understand by paper chromatography?
3. Which chromatography is considered as classic form of chromatography?
4. Which chromatography used to separate and analyze complex biological or non-biological samples into their constituents?
5. Define electrophoresis.
6. Which two materials are particularly useful in gel electrophoresis?
7. Define biological centrifugation.

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2.3 COMPUTER AIDED TECHNIQUES FOR DATA PRESENTATION, DATA ANALYSES, STATISTICAL TECHNIQUES

Computer applications are now routinely used in various life science related disciplines. Initially, use of computers was very limited due to cost and software/ programs availability. Now, small sized, cheaper and program based computer systems are available. Computers are now integral part of life science program, education and research. Computers are mainly used for following functions:

- Data Logging and Storage
- Data Analysis
- Software Assisted Control of Processes
- Robotics
- Application Specific Software Programs
- Coding
- Data Linking
- Graphical Analysis
- Annotation
- Data Search and Storage
- Mapping of Data

Some of the programs for daily usage include:

- **Microsoft Office:** Software programs used for typing, editing, retrieving, storage, managing word documents and other functions.
- **Microsoft Excel:** Software programs used for data recording, numerical value analysis, graphical presentation of data, basic statistical, computation of data and other functions.
- **Microsoft Publisher:** For data presentation in form of sheets, posters and other formats.

Computer Assisted Statistical Analysis

Statistics is a branch of science that deals with the collection, organization, and analysis of data and drawing of inferences from the samples to the whole population. For analysis, a proper design of the study, an appropriate selection of the study sample and choice of a suitable statistical test is required. Statistical methods involved in carrying out a study include planning, designing, collecting data, analyzing, drawing meaningful interpretation and reporting of the research findings. The statistical analysis gives meaning to the meaningless numbers, thereby breathing life into a lifeless data. The results and inferences are precise only if proper statistical tests are used. Different tests are used for statistical analysis of data. Some of the generally used tests are:

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- **Student's *t*-Test:** The *t* test (also called Student's T Test) compares two averages (means) and tells if they are different from each other. The t test also tells significance of the differences.
- **Analysis Of Variance (ANOVA):** Analysis Of Variance (ANOVA) is a collection of statistical models and their associated estimation procedures (such as the variation among and between groups) used to analyze the differences among group means in a sample.
- **Repeated Measures Analysis of Variance:** Repeated measures ANOVA is an adaptation of ANOVA for cases where measures are repeated on the same statistical units.
- **Sign Test:** The sign test is a statistical method to test for consistent differences between pairs of observations, such as the weight of subjects before and after treatment.
- **Wilcoxon's Signed Rank Sum Test:** The Wilcoxon signed rank sum test is used is used to test the null hypothesis that the median of a distribution is equal to some value. It can be used a) in place of a one-sample t-test b) in place of a paired t-test or c) for ordered categorical data where a numerical scale is inappropriate but where it is possible to rank the observations.
- **Kolmogorov-Smirnov Test:** The Kolmogorov–Smirnov test is a non-parametric goodness-of-fit test and is used to determine whether two distributions differ, or whether an underlying probability distribution differs from a hypothesized distribution. It is used when we have two samples coming from two populations that can be different.
- **Kruskal-Wallis Test:** The Kruskal-Wallis H test, sometimes also called the 'one-way ANOVA on ranks is a rank-based nonparametric test that can be used to determine if there are statistically significant differences between two or more groups of an independent variable on a continuous or ordinal dependent variable.
- **Jonckheere Test:** The Jonckheere–Terpstra test is a variation that can be used when the treatments are ordered.
- **Friedman Test:** The Friedman test is an extension of the sign test for matched pairs and is used when the data arise from more than two related samples.

Various computer based statistical software systems are available that can be used in different analysis objectives. The commonly used software systems are:

- **Statistical Package for the Social Sciences (SPSS):** The Statistical Package for the Social Sciences (SPSS) is a software package used in statistical analysis of data.
- **Statistical Analysis System (SAS):** Statistical Analysis System (SAS) is an integrated system of software products provided by SAS Institute Inc., which enables programmers to perform: Information retrieval and data management, report writing and graphics, statistical analysis, econometrics

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and data mining, business planning, project management, applications development, data mining, data alter, data manage and retrieve data from a variety of sources and perform statistical analysis on it. Its ability to help business develop models and do predictive analytics has made SAS as an irreplaceable business management tool.

- **R Project:** R is a free software environment for statistical computing and graphics. It compiles and runs on a wide variety of UNIX platforms, Windows and MacOS.
- **Minitab Statistical Software (Minitab):** Minitab is a statistics package developed at the Pennsylvania State University by a group of researchers.
- **Software for Statistics and Data Science (Stata):** It is software for statistics and data science, to obtain and manipulate data, explore, visualize, model, make inferences. Collect results into reproducible reports.
- **Microsoft Excel:** To manage charts, data analyses, calculations, graphical presentation of data, statistical measures and more. To create, view and edit the spreadsheets.

Statistical Tool 'RSM' for Optimized Production of Industrial Microbial Products and 'ANN' as Mathematical/ Statistical Modeling

Currently, Response Surface Methodology (RSM) and Artificial Neural Network (ANN) are used routinely as computer based statistical software and artificial intelligence, respectively for optimized product formation in applied biology including industrial microbiology, biotechnology and food technology. Initially researchers used one-variable-at-a-time approach for various optimization studies in biotechnology and microbiology. Even, these days also sometimes this approach is used. It is particularly useful in fermentation media optimization in biotechnology to produce industrial enzymes, amino acids, pigments, antibiotics and other important metabolites. This traditional approach of one-variable-at-a-time method for media optimization requires several sequential experiments to find the optimal conditions. The optimization of media constituents or biological transformation is generally expensive, labourious and time consuming and can be used only if there is no interaction between the variables. Moreover, the complexity of microbial processes and variable responses to different nutrients adds to the unreliability of the traditional approach. In last decades, researchers used Response Surface Methodology (RSM) to optimize media. RSM is a statistical tool used to elucidate the interaction of independent variables and relationship among responses. Response Surface Methodology (RSM) emerged as most common method used for media optimization. Central Composite Rotatable Design (CCRD), Plackett-Burman and Box-Behnken were used widely. In RSM, a number of factors and their interactions regulating the common response can be studied. RSM has been used both at small and large scale for the optimization of production of various bio-molecules, but many research groups have reported used of Artificial Neural Networks (ANN) as the mathematical or statistical modeling tool in a wide range

of biotechnology applications particularly optimization of bioprocesses, media optimization for various biomolecules production and to increase enzyme yield from different source microorganisms. ANN consists of dense interconnected computing units that are simple models for complex neurons in biological systems. ANN is biologically motivated and mimics human brain. Commonly nonlinear activation functions, such as sigmoid and step functions are used. ANNs are trained by experience, when applied a new input to the network it can generalize from past experiences and produce a new result. The simple structure of ANN normally consists of an input layer, a hidden layer and an output layer. By applying algorithms that mimic the processes of real neurons, the network can learn to solve many types of problems. It provides a mathematical alternative to the quadratic polynomial for representing data derived from statistically designed experiments. ANN is also able to handle a large amount of data to approximate functions to any desired degree of accuracy, thus make it attractive as empirical model. ANN has been used in different filed of science including physics, chemistry and management also. ANN has also been used in predicting the secondary structures of globular proteins.

Check Your Progress

8. What is the use of statistical analysis?
9. Define sign test.
10. What is Analysis Of Variance (ANOVA)?
11. What do you understand by Statistical Analysis System (SAS)?

2.4 RADIOISOTOPE AND MASS ISOTOPE TECHNIQUES IN BIOLOGY

Isotopes are two or more types of atoms that have the same atomic number (number of protons in their nuclei) and position in the periodic table (and hence belong to the same chemical element), and that differ in nucleon numbers (mass numbers) due to different numbers of neutrons in their nuclei. While all isotopes of a given element have almost the same chemical properties, they have different atomic masses and physical properties.

The number of protons within the atom's nucleus is called atomic number and is equal to the number of electrons in the neutral (non-ionized) atom. Each atomic number identifies a specific element, but not the isotope; an atom of a given element may have a wide range in its number of neutrons. The number of nucleons (both protons and neutrons) in the nucleus is the atom's mass number, and each isotope of a given element has a different mass number.

For example, carbon-12, carbon-13, and carbon-14 are three isotopes of the element carbon with mass numbers 12, 13, and 14, respectively. The atomic number of carbon is 6, which means that every carbon atom has 6 protons so that the neutron numbers of these isotopes are 6, 7, and 8, respectively.

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Some isotopes/nuclides are radioactive, and are therefore referred to as radioisotopes or radionuclides, whereas others have never been observed to decay radioactively and are referred to as stable isotopes or stable nuclides. For example, Carbon-14 is a radioactive form of carbon, whereas, Carbon-12 and Carbon-13 are stable isotopes. There are about 339 naturally occurring nuclides on Earth, of which 286 are primordial nuclides, meaning that they have existed since the Solar System's formation.

Isotope-Ratio Mass Spectrometry (IRMS) is a specialization of mass spectrometry, in which mass spectrometric methods are used to measure the relative abundance of isotopes in a given sample. This technique has two different applications in the Earth and environmental sciences. The analysis of 'Stable Isotopes' is normally concerned with measuring isotopic variations arising from mass-dependent isotopic fractionation in natural systems. On the other hand, radiogenic isotope analysis involves measuring the abundances of decay-products of natural radioactivity and is used in most long-lived radiometric dating methods.

2.4.1 Sample Preparation for Radioactive Counting

In order not to lose radioactivity on the walls of the containers when preparing the sources, a preliminary stage will allow saturation of the sites. For this purpose, the material to be used is rinsed with a carrier solution containing the stable isotope of the radionuclide or at least a chemically close element with the same oxidation and complexing state.

The radioactive samples are prepared from concentrated solutions whose activity can be accurately measured. Weighing being more precise than volume measurements, all dilutions and deposits of active substance are carried out by gravimetry.

The sources (excluding large volumes for γ spectrometry) are prepared by differential weighing using a pycnometer (plastic vial with stretched end), on a high-precision balance (microbalance), which makes it possible to avoid uncertainties in linearity and accuracy of the balance. Radioactivity sources are generally calibrated by mass activity.

For liquid scintillation measurements, the radioactive solution is solubilized in a scintillating liquid in a glass or plastic bottle while for ionization chamber measurements, the radioactive solution is sealed in a vial.

Sealing a Vial

For measurements by means of α spectrometry, electro-deposited sources are prepared.

For γ spectrometry, several types of sources can be measured, liquid sources of various geometries (1 to 3000 mL) or point sources by deposition on mylar® film of a few milligrams of radioactive solution which will be dried before being sandwiched with terphane®. The $4\pi\gamma$ well-type measurement sources are prepared by depositing a few milligrams of radioactive solution on mylar® film. The deposit is then dried and sandwiched with terphane®.

For 4π proportional counter measurements, very thin film deposits are necessary. This technique consists in making a thin film that will be metallized (laying a gold conductive layer). A coating of a layer of latex is then electro-pulverized. The metal thickness can be adapted according to the emission energies of the radionuclides studied, while latex microspheres optimize the drop spreading on the support. The radioactive solution is then deposited on the support and dried.

The deposition of radioactive material in liquid form can be dried at room temperature by conventional heating, optimized heating (primary vacuum with nitrogen blowing) or freeze-drying.

The laboratory also has shielded production lines, allowing the handling of highly active elements and/or short half-life elements, for example radiopharmaceuticals, reducing radiation exposure.

2.4.2 Autoradiography

An autoradiograph is an image on an x-ray film or nuclear emulsion produced by the pattern of decay emissions (e.g., beta particles or gamma rays) from a distribution of a radioactive substance. Autoradiography is an imaging technique that uses radioactive sources contained within the exposed sample. In vitro autoradiography methods involve the isolation of cellular components such as, DNA, RNA, proteins or lipids, followed by labelling with suitable radioisotopes. In in vivo autoradiography, radioisotopes are coupled with radioactive tracers and administered orally or via injection, and the distribution of radiation is evaluated in thin tissue or whole-body cryosections. In vivo autoradiography using laboratory animals is widely used in metabolic studies, disease monitoring and new drug development experiments.

X-Ray Diffraction (XRD) relies on the dual wave/particle nature of X-rays to obtain information about the structure of crystalline materials. X-ray crystallography is a technique for determining the three-dimensional structure of molecules, including complex biological macromolecules, such as proteins and nucleic acids. A primary use of the technique is the identification and characterization of compounds based on their diffraction pattern. X-ray diffraction techniques are superior in elucidating the three-dimensional atomic structure of crystalline solids. The properties and functions of materials largely depend on the crystal structures. X-ray diffraction techniques have, therefore, been widely used as an indispensable means in materials research, development and production.

Principle

X-ray crystallography is based on the principle that the crystalline atoms cause a beam of X-rays to diffract into many specific directions (Refer Figure 2.20). The electrons surrounding the molecule diffract as the X-rays hit them. **Diffraction** is the slight bending of light as it passes around the edge of an object. By measuring the angles and intensities of these diffracted beams, a crystallographer can produce a 3-dimensional picture of the density of electrons within the crystal. From this electron density image, the mean positions of the atoms in the crystal can be

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determined, as well as their chemical bonds, their disorder, and various other information. Data is collected by diffracting X-rays from a single crystal, which has an ordered, regularly repeating arrangement of atoms. Based on the diffraction pattern obtained from X-ray scattering off, the periodic assembly of molecules or atoms in the crystal can be reconstructed.

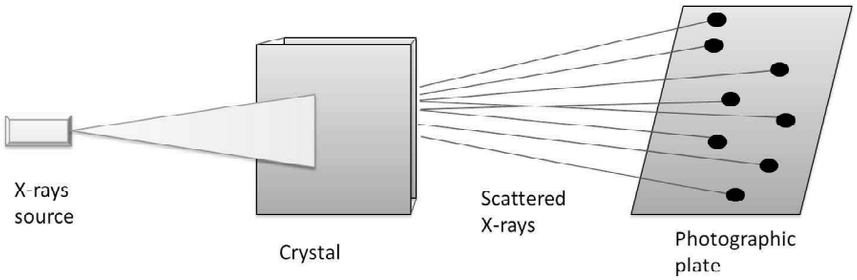
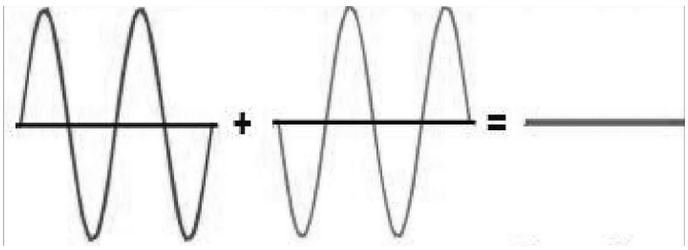


Fig. 2.20 *Principle of X-Ray Crystallography*

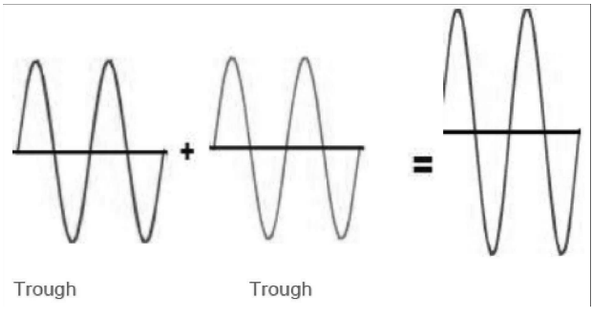
Bragg's Law

The dominant effect that occurs when an incident beam of monochromatic X-rays interacts with a target material is scattering of those X-rays from atoms within the target material. In materials with regular structure, i.e., crystalline, the scattered X-rays undergo constructive and destructive interference. This is the process of diffraction. Destructive interference occurs when there is a collision of photons in such a way that their intensities cancel out. Constructive interference occurs when there is a collision of protons in a way that their intensities combine. The constructive interference from a diffracting crystal is observed as a pattern of points on the detector.

Destructive Interference



Constructive Interference



The diffraction of X-rays by crystals is described by Bragg's Law. English physicists Sir W.H. Bragg and his son Sir W.L. Bragg developed a relationship in 1913 to explain why the cleavage faces of crystals appear to reflect X-ray beams at certain angles of incidence (theta, θ). The Braggs were awarded the Nobel Prize in physics in 1915 for their work in determining crystal structures beginning with NaCl, ZnS and diamond. The variable (d) is the distance between atomic layers in a crystal, and the variable lambda (λ) is the wavelength of the incident X-ray beam; n is an integer (Refer Figure 2.21). When this equation is satisfied, X-rays scattered by the atoms in the plane of a periodic structure are in phase and diffraction occurs in the direction defined by the angle θ. In the simplest instance, an X-ray diffraction experiment consists of a set of diffracted intensities and the angles at which they are observed. This diffraction pattern can be thought of as a chemical fingerprint, and chemical identification can be performed by comparing this diffraction pattern to a database of known patterns. This observation is an example of X-ray wave interference.

$$n\lambda = 2d\sin\theta \quad \text{.....Bragg's Law}$$

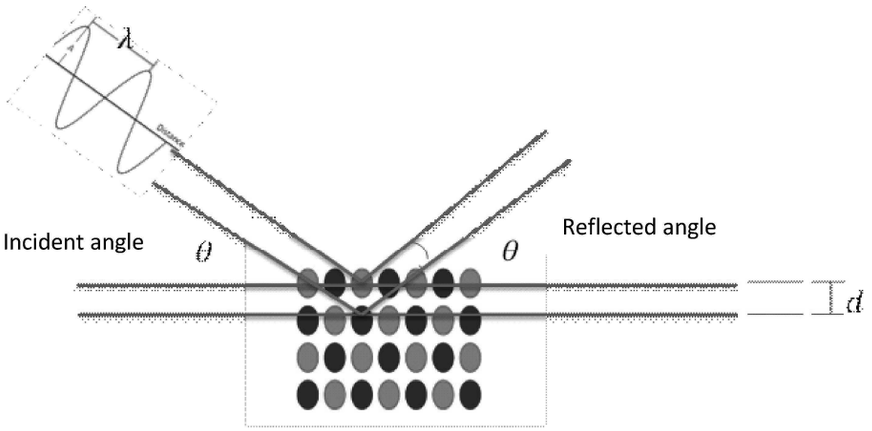


Fig. 2.21 *Diagram showing Diffraction of the Incident Rays by Atoms in the Crystal*

Method of Working

- The specimen crystal is mounted on a goniometer to keep it in place during the process and gradually rotated while being bombarded with X-rays.
- X-ray beams are shot through a crystal of the atom.
- The crystal causes the beam of X-Ray to diffract in a predictable pattern based on their crystal lattice structure.
- The result is a diffraction pattern generally. The two-dimensional images taken at different rotations are converted into a three dimensional structure. The diffraction pattern is first converted to an electron density map. This is accomplished using computers and a mathematical technique called **Fourier Transforms**. Using the electron density map as a guide,

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the crystallographers place every atom into a computer model, kind of like solving a puzzle.

Applications: Using X-ray crystal data, Watson and Crick were able to determine the double helix structure of DNA in 1953. The method also revealed the structure and function of many biological molecules, including vitamins, drugs, proteins and nucleic acids. X-Ray Diffraction (XRD) is a powerful nondestructive technique for characterizing crystalline materials. It provides information on structures, phases, preferred crystal orientations (texture), and other structural parameters, such as average grain size, strain and crystal defects.

Precautions should be taken in the handling of microscope:

- Always carry the microscope by holding it from the arm and one hand below the base.
- Focusing should be done by moving the objective lens away from the glass slide, avoid focusing downwards.
- Set the mirror and light on the specimen while using low power objective to give adequate illumination.
- Firstly focus the lens on the specimen by adjusts the coarse adjustment knob until it nearly touches the upper surface of the mounted specimen. Then focus by moving the fine adjustment knob.
- While observing the unstained objects, the Iris diaphragm should be barely open to achieve good contrast. Iris diaphragm is fully open with higher magnification and while viewing stained objects.
- Always clean the lenses before and after use with lens paper. Do not touch the lenses with figures. Leave the objectives with lowest power in working position after use.
- Keep the microscope covered when not in use.
- Place a drop of immersion oil on the illuminated area of slide and then shift to 100X objective lens.

Check Your Progress

12. Define an isotope.
13. What do you understand by Isotope-Ratio Mass Spectrometry (IRMS)?
14. What is an autoradiograph?

2.5 IMMUNOLOGICAL TECHNIQUES BASED ON ANTIGEN - ANTIBODY INTERACTIONS

Immunochemical techniques are based on a reaction of antigen with antibody, or more exactly, on a reaction of an antigenic determinants with the binding site of the antibody. The antibodies used are produced by various ways.

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Monoclonal antibodies are products of a single clone of plasma cells derived from B-lymphocytes, prepared in the laboratory by hybridoma technology, based on cellular fusion of tumour (myeloma) cells with splenic lymphocytes of immunised mice. Monoclonal antibodies are directed against single epitope; and are all identical copies of immunoglobulin molecule with the same primary structure and specificity of antigen binding site. They typically display excellent specificity, but poor ability to precipitate antigen.

Polyclonal antibodies (conventional antibodies) are prepared by immunisation of animals (rabbits, goats, sheep) with the antigen. Blood serum of the immunised animal that contains antibodies against the antigen used, is called an **antiserum**. If one antigen, for example one protein, is used for immunisation, monospecific antibodies (antiserum) result. However, as every epitope stimulates different clone of B cells, and complex antigens bear several epitopes, the antiserum contains mixture of monoclonal antibodies, differing in their affinity and specificity towards particular epitopes on the antigen used for immunisation. Immunisation of an animal with mixture of antigens results in production of **poly specific antibodies**, containing immunoglobulins against many antigens, for example antiserum against human serum proteins used in **immune-electrophoresis**.

Antigen-antibody interaction, or antigen-antibody reaction, is a specific chemical interaction between antibodies produced by B cells of the white blood cells and antigens during immune reaction. The antigens and antibodies combine by a process called **agglutination**. It is the fundamental reaction in the body by which the body is protected from complex foreign molecules, such as pathogens and their chemical toxins. In the blood, the antigens are specifically and with high affinity bound by antibodies to form an antigen-antibody complex. The immune complex is then transported to cellular systems where it can be destroyed or deactivated.

The first correct description of the antigen-antibody reaction was given by Richard J. Goldberg at the University of Wisconsin in 1952. It came to be known as "**Goldberg's Theory**" of antigen-antibody reaction.

Antigens are macromolecules of natural or synthetic origin; chemically they consist of various polymers – proteins, polypeptides, polysaccharides or nucleoproteins. Antigens display two essential properties: first, they are able to evoke a specific immune response, either cellular or humoral type; and second, they specifically interact with products of this immune response, i.e., antibodies or immunocompetent cells. A complete antigen – immunogen – consists of a macromolecule that bears antigenic determinants (epitopes) on its surface.

Antibodies are produced by plasma cells that result from differentiation of B lymphocytes following stimulation with antigen. Antibodies are heterogeneous group of animal glycoproteins with electrophoretic mobility $\beta - \gamma$ and are also called Immunoglobulins (Ig).

There are several types of antibodies and antigens, and each antibody is capable of binding only to a specific antigen. The specificity of the binding is due to specific chemical constitution of each antibody. The antigenic determinant or epitope is recognized by the paratope of the antibody, situated at the variable region of the polypeptide chain. The variable region in turn has hyper-variable regions which

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are unique amino acid sequences in each antibody. Antigens are bound to antibodies through weak and noncovalent interactions, such as electrostatic interactions, hydrogen bonds, Van der Waals forces, and hydrophobic interactions.

The principles of specificity and cross-reactivity of the antigen-antibody interaction are useful in clinical laboratory for diagnostic purposes. One basic application is determination of ABO blood group. It is also used as a molecular technique for infection with different pathogens, such as HIV, microbes, and Helminth parasites.

Molecular Basis

Immunity developed as an individual is exposed to antigens is called adaptive or acquired immunity, in contrast to immunity developed at birth, which is innate immunity. Acquired immunity depends upon the interaction between antigens and a group of proteins called antibodies produced by B cells of the blood. There are many antibodies and each is specific for a particular type of antigen. Thus immune response in acquired immunity is due to the precise binding of antigens to antibody. Only very small area of the antigens and antibody molecules actually interact through complementary binding sites, called epitopes in antigens and paratopes in antibody.

Antibody Structure

In an antibody, the Fab (Fragment, antigen-binding) region is formed from the amino-terminal end of both the light and heavy chains of the immunoglobulin polypeptide. This region, called the variable (V) domain, is composed of amino acid sequences that define each type of antibody and their binding affinity to an antigen. The combined sequence of Variable Light (V_L) chain and Variable Heavy (V_H) chain creates three hypervariable regions (HV1, HV2, and HV3). In V_L these are roughly from residues 28 to 35, from 49 to 59, and from 92 to 103, respectively. HV3 is the most variable part. Thus, these regions may be part of a paratope, the part of an antibody that recognizes and binds to an antigen. The rest of the V region between the hypervariable regions are called framework regions. Each V domain has four framework domains, namely FR1, FR2, FR3, and FR4.

Chemical Basis of Antigen-Antibody Interaction

Antibodies bind antigens through weak chemical interactions, and bonding is essentially non-covalent. Electrostatic interactions, hydrogen bonds, Van der Waals forces, and hydrophobic interactions are all known to be involved depending on the interaction sites. Non-covalent bonds between antibody and antigen can also be mediated by interfacial water molecules. Such indirect bonds can contribute to the phenomenon of cross-reactivity, i.e., the recognition of different but related antigens by a single antibody.

Affinity of the Interaction

Antigen and antibody interact through a high affinity binding much like lock and key. A dynamic equilibrium exists for the binding. For example, the reaction is a reversible one, and can be expressed as:

$$[\mathrm{Ab}] + [\mathrm{Ag}] \rightleftharpoons [\mathrm{AbAg}]$$

Where [Ab] is the antibody concentration and [Ag] is the antigen concentration, either in free ([Ab], [Ag]) or bound ([AbAg]) state.

The equilibrium association constant can therefore be represented as:

$$K_a = \frac{k_{on}}{k_{off}} = \frac{[AbAg]}{[Ab][Ag]}$$

Where *K* is the equilibrium constant.

Reciprocally the dissociation constant will be:

$$K_d = \frac{k_{off}}{k_{on}} = \frac{[Ab][Ag]}{[AbAg]}$$

However, these equations are applicable only to a single epitope binding, i.e., one antigen on one antibody. Since the antibody necessarily has two paratopes, and in many circumstances complex binding occurs, the multiple binding equilibrium can be summed up as:

$$K_a = \frac{k_{on}}{k_{off}} = \frac{[AbAg]}{[Ab][Ag]} = \frac{r}{c(n-r)}$$

Where, at equilibrium, *c* is the concentration of free ligand, *r* represents the ratio of the concentration of bound ligand to total antibody concentration and *n* is the maximum number of binding sites per antibody molecule (the antibody valence).

The overall strength of the binding of an antibody to an antigen is termed its avidity for that antigen. Since antibodies are bivalent or polyvalent, this is the sum of the strengths of individual antibody-antigen interactions. The strength of an individual interaction between a single binding site on an antibody and its target epitope is termed the affinity of that interaction.

Avidity and affinity can be judged by the dissociation constant for the interactions they describe. The lower the dissociation constant, the higher the avidity or affinity, and the stronger the interaction.

2.6 SURGICAL TECHNIQUES

Surgery is a medical specialty that uses operative manual and instrumental techniques on a person to investigate or treat a pathological condition, such as, a disease or injury, to help improve bodily function, appearance, or to repair unwanted ruptured areas. The act of performing surgery may be called a surgical procedure, operation, or simply 'Surgery'.

Definition: Surgery is an invasive technique with the fundamental principle of physical intervention on organs/organ systems/tissues for diagnostic or therapeutic reasons.

As a general rule, a procedure is considered surgical when it involves cutting of a person's tissues or closure of a previously sustained wound.

2.6.1 Organ Ablations

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Organ ablation is a procedure of surgical removal of a body part, organ, or tissue. For example removal of a cancerous growth by an oncologist, amputation of limb and pulling of teeth by the dentist are known as ablation. Here we will discuss the removal of ovaries and adrenal glands to prevent the cancerous growth and other disorders.

Ovariectomy: It is a surgical procedure where the ovaries (either one or both) are removed and can be done with or without a hysterectomy. It is also known as Oophorectomy. Ovaries are almond-shaped organs present on each side of the uterus in pelvis. Ovaries contain eggs and produce hormones that control menstrual cycle. Oophorectomy is divided into following types:

- **Unilateral Oophorectomy:** Removal of one ovary, usually done when a woman still wants to become pregnant.
- **Bilateral Oophorectomy:** Removal of both ovaries to prevent disorders or spread of cancer cells.
- **Prophylactic Oophorectomy:** This procedure is done to reduce the risk of future diseases. It is also called preventative oophorectomy.
- **Salpingo-Oophorectomy:** Removal of the fallopian tube along with the ovary, often to treat cancers or other disorders. It does not involve removal of the uterus which is known as Hysterectomy. Salpingo-oophorectomy is further of three types:
  - **Unilateral Salpingo-Oophorectomy:** Removal of one ovary and fallopian tube.
  - **Bilateral Salpingo-Oophorectomy:** Removal of both ovaries and fallopian tubes.
  - **Risk-Reducing Salpingo-Oophorectomy:** Removal of ovaries and fallopian tubes to prevent ovarian cancer in those women who are at particularly high risk.

Need of Oophorectomy: This procedure is used to treat various risk factors, such as:

- Ovarian cancer or breast cancer
- Endometriosis
- Benign tumors or cysts
- Tubo-ovarian abscess (a pus-filled pocket involving a fallopian tube and an ovary)
- Ovarian torsion (twisting of the ovary), a pelvic infection
- Pelvic Inflammatory Disease (PID)
- Ectopic pregnancy

Different Ways to Perform Oophorectomy

An oophorectomy can be performed two ways:

- **Laparotomy:** In this surgical approach, the surgeon makes one long incision in your lower abdomen to access your ovaries. The surgeon separates each ovary from the blood supply and tissue that surrounds it and removes the ovary.
- **Minimally Invasive Laparoscopic Surgery:** In this surgical approach, the surgeon makes three or four very small incisions in your abdomen. The surgeon inserts a tube with a tiny camera through one incision and special surgical tools through the others. The camera transmits video to a monitor in the operating room that the surgeon uses to guide the surgical tools.

Each ovary is separated from the blood supply and surrounding tissue and placed in a pouch. The pouch is pulled out of your abdomen through one of the small incisions. Laparoscopic oophorectomy may also be robotically assisted in certain cases. During robotic surgery, the surgeon watches a 3-D monitor and uses hand controls that allow finer movement of the surgical tools.

Preparation before Oophorectomy

- To drink a solution to clear your intestines the day before surgery.
- To stop eating the day before your surgery and limit liquids.
- To stop taking certain medications.
- Undergo blood test, urine test and imaging tests (ultrasound and Computerized Tomography (CT)), to help surgeons plan for the procedure.

Technique of Oophorectomy

An oophorectomy is performed using either open abdominal surgery or laparoscopic surgery. Both operations should take no more than a few hours to complete but may require staying one or several nights in the hospital. These techniques are discussed below:

- **Open Abdominal Surgery:** In an open abdominal surgery, a surgeon will make an incision in the abdomen and then carefully separate the abdominal muscles. Blood vessels will be temporarily tied off to prevent bleeding. The surgeon will remove the ovary or ovaries and then seal up the incision.
- **Laparoscopic Surgery:** During laparoscopic surgery, a thin, cord-like instrument is inserted into a small cut near the navel. A tiny camera allows the surgeon to see and remove the ovary or ovaries. The process may leave less noticeable scars and have a shorter recovery time than open abdominal surgery.

Risks Associated with Oophorectomy

It includes the following risks:

- Bleeding
- Infection
- Fever

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- Nausea and vomiting
- Difficulty in urination
- Damage to nearby organs
- Chronic abdominal pain
- Shortness of breath or chest pain
- Mood swings
- Depression
- Nerve damage
- Hernia due to weakened abdominal muscles
- Rupture of a tumor, spreading potentially cancerous cells
- Inability to get pregnant, if both ovaries are removed
- Ovarian remnant syndrome (retention of ovary cells that continue to cause signs and symptoms, such as pelvic pain, in premenopausal women)

Risks of Premature Menopause

Ovaries produced estrogen and progesterone hormones in the body. Removal of both ovaries stops the production of these hormones in the body which causes premature menopause in females. This will further leads to the following complications:

- Menopause signs and symptoms, such as hot flashes and vaginal dryness
- Depression or anxiety
- Heart disease
- Memory problems
- Decreased sex drive
- Osteoporosis
- Premature death

Adrenalectomy

The **adrenal glands** are two small organs, one located above each kidney. They are triangular in shape and about the size of a thumb. Each gland is comprised of two parts, an outer layer called the adrenal cortex, and the central part called the adrenal medulla. The adrenal glands are known as endocrine glands because they produce hormones. These adrenal-produced hormones include cortisol, aldosterone, the adrenaline hormones – epinephrine and norepinephrine – and a small fraction of the body's sex hormones (estrogen and androgens). These hormones regulate metabolism, immune system, blood pressure, blood sugar and other essential functions. Although individuals have two adrenal glands, only one normal gland is needed to provide adequate hormonal function. Where both glands must be removed, patients must take steroid supplements of cortisone and hydrocortisone.

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Disease or Major Problem with Adrenal Glands

Diseases of the adrenal gland are relatively rare. The most common problem of the adrenal gland is excess hormone production by a tumor located within the adrenal gland. Adrenal tumors associated with excess hormone production include pheochromocytomas, aldosterone-producing tumors, and cortisol-producing tumors. These tumors have the following characteristics:

- **Pheochromocytomas:** It produce excess hormones that can cause very high blood pressure and periodic spells characterized by severe headaches, excessive sweating, anxiety, palpitations, and rapid heart rate that may last from a few seconds to several minutes.
- **Aldosterone:** Excess of this hormone produces tumors that cause high blood pressure and low serum (blood) potassium levels. It may also result in variety of symptoms, such as weakness, fatigue and frequent urination.
- **Cortisol:** Cortisol producing tumor cause a syndrome termed as Cushing's syndrome. This is characterized by obesity (especially of the face and trunk), high blood sugar, high blood pressure, menstrual irregularities, fragile skin, and prominent stretch marks.
- **Adrenal Mass:** An incidentally found mass in the adrenal may be any of the above types of tumors, or may produce no hormones at all. Most incidentally found adrenal masses do not make excess hormones, cause no symptoms, are benign, and do not need to be removed. Surgical removal of incidentally discovered adrenal tumors is indicated only if:
  - o The tumor is found to make excess hormones.
  - o Is large in size (more than 4-5 centimeters or 2 inches in diameter).
  - o There is a suspicion that the tumor could be malignant.
- **Adrenal Cortical Cancer:** Adrenal gland cancers are rare tumors that are usually very large at the time of diagnosis. Removal of these tumors is usually done by open adrenal surgery.

  Adrenalectomy: Adrenalectomy is the surgical removal of one or both adrenal glands. This procedure is performed to remove tumors of the adrenal glands including:
  - Benign tumors and cysts.
  - Malignant primary tumors.
  - Metastatic tumors that have spread from other organs of the body.

Adrenalectomy is usually advised for patients with tumors of the adrenal glands. Most adrenal tumors are noncancerous (benign). Surgery may be required to remove an adrenal gland if the tumor is producing excess hormones or is large in size (> 2 inches or 4 to 5 centimeters). Surgery may also be required if the tumor is cancerous (malignant) or suspected to be cancerous (adrenalectomy).

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An adrenalectomy is traditionally performed laparoscopically as a minimally invasive procedure through the abdomen or as an open procedure where a laparoscopic approach not feasible.

Technique of Adrenalectomy: An adrenalectomy can be performed to remove cancer that has spread (metastasized) from another location, such as the kidney or lung. Adrenalectomy can be performed through an open incision (laparotomy) or laparoscopic technique or by using cryoablation. The technique used by the surgeon for removal of adrenal gland is influenced by several factors:

- **Size of Tumor:** Larger tumors are more likely to be cancer. This may require a larger incision for a more thorough dissection and removal of the cancer, including other organs it may be attached to.
- **Type of Tumor** (benign versus cancer): When cancer of the adrenal gland is found, the technique of choice for removing the cancer is open adrenalectomy.
- Relationship of tumor to surrounding organs
- **History of Prior Abdominal Surgery:** In this scenario, laparoscopic adrenalectomy may still be possible. Significant scar tissue from another surgery may limit the success of the laparoscopic technique and open adrenalectomy may be needed.
- Experience of the surgeon: Surgeons without training in laparoscopic surgery should not perform laparoscopic adrenalectomy.

Commonly used techniques for adrenalectomy are as follows:

- Open Adrenalectomy
- Laparoscopic Adrenalectomy
- Cryoablation

These are as follows:

Open Adrenalectomy: The surgeon may operate from any of four directions, depending on the exact problem and the patient's body type. In the anterior approach, the surgeon cuts into the abdominal wall. Usually the incision will be horizontal, just under the rib cage. If the surgeon intends to operate on only one of the adrenal glands, the incision will run under just the right or the left side of the rib cage. Sometimes a vertical incision in the middle of the abdomen provides a better approach, especially if both adrenal glands are involved.

In the posterior approach, the surgeon cuts into the back, just beneath the rib cage. If both glands are to be removed, an incision is made on each side of the body. This approach is the most direct route to the adrenal glands, but it does not provide quite as clear a view of the surrounding structures as the anterior approach. In the flank approach, the surgeon cuts into the patient's side. This is particularly useful in massively obese patients. If both glands need to be removed, the surgeon must remove one gland, repair the surgical wound, turn the patient onto the other side, and repeat the entire process.

The last approach involves an incision into the chest cavity, either with or without part of the incision into the abdominal cavity. It is used when the surgeon anticipates a very large tumor, or if the surgeon needs to examine or remove nearby structures as well.

Specific Conditions for Open Adrenalectomy

There are different factors which may increase the possibility of choosing this procedure, such as:

- Obesity.
- A history of prior abdominal surgery causing dense scar tissue.
- Inability to visualize the adrenal gland clearly.
- Bleeding problems during the operation.
- Certain tumor characteristics, such as large size or invasion into adjacent structures.

Laparoscopic Adrenalectomy: Laparoscopic adrenalectomy technique does not require the surgeon to open the body cavity. Instead, four small incisions (about 0.5 in [1.27 cm] diameter each) are made into a patient's flank, just under the rib cage. A laparoscope enabling the surgeon to visualize the inside of the abdominal cavity on a television monitor is placed through one of the incisions. The other incisions are for tubes that carry miniaturized versions of surgical tools. These tools are designed to be operated by manipulations that the surgeon makes outside the body.

An alternative approach to laparoscopic surgery is a Posterior Retroperitoneoscopic Adrenalectomy (PRA), in which surgeons make small incisions in your back. Sometimes surgeons use the robotic da Vinci Surgical System to perform laparoscopic adrenalectomies.

Procedure of laparoscopic method of adrenalectomy

It involves the following steps:

Step 1: The surgery is performed under a complete general anesthesia, so that the patient is asleep during the procedure.

Step 2: A cannula (a narrow tube-like instrument) is placed into the abdominal cavity in the upper abdomen or gives the surgeon a magnified view of the patient's internal organs on a television screen.

Step 3: Other cannulas are inserted which allow your surgeon to delicately separate the adrenal gland from its attachments. Once the adrenal gland has been dissected free, it is placed in a small bag and is then removed through one of the incisions. It is almost always necessary to remove the entire adrenal gland in order to safely remove the tumor.

Step 4: After the surgeon removes the adrenal gland, the small incisions are closed.

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Advantages of Laparoscopic Removal of Adrenal Glands

Laparoscopic surgery has many benefits, including smaller scars, less pain and a shorter recovery period than traditional open surgery. The common advantages of this technique are as follows:

- Less postoperative pain
- Reduced risk of herniation or wound separation
- Quicker return to normal activity
- Shorter hospital stay
- Improved cosmetic result

Cryoablation: The procedure of cryoablation uses CT imaging to guide the insertion of a probe that freezes and destroys adrenal tumors. Interventional radiologists may use cryoablation as a treatment option for small tumors that have spread to the adrenal gland (metastasis), particularly when surgery carries a high risk.

Risk Associated with Adrenalectomy

The risks of adrenalectomy include major hormone imbalances, caused by the underlying disease, the surgery, or both. These can include problems with healing, blood pressure fluctuations, and other metabolic problems. Other risks are typical of many operations. These include:

- Bleeding
- Damage to adjacent organs (spleen, pancreas (/knowledge/Pancreas.html))
- Loss of bowel function
- Blood clots in the lungs
- Lung problems
- Surgical infections
- Pain
- Scarring

2.6.2 Perfusion Techniques

Perfusion is the passage of fluid through the circulatory system or lymphatic system to an organ or a tissue, usually referring to the delivery of blood to a capillary bed in tissue. Perfusion is measured as the rate at which blood is delivered to tissue, or volume of blood per unit time (blood flow) per unit tissue mass. The SI unit is $m^3/(s \cdot kg)$, although for human organs perfusion is typically reported in ml/min/g. The word is derived from the French verb 'Perfuser' meaning to 'Pour Over or Through'. All animal tissues require an adequate blood supply for health and life. Poor perfusion (malperfusion), that is, ischemia, causes health problems, as seen in cardiovascular disease, including coronary artery disease, cerebrovascular disease, peripheral artery disease, and many other conditions.

Tests verifying that adequate perfusion exists are a part of a patient's assessment process that are performed by medical or emergency personnel. The

most common methods include evaluating a body's skin color, temperature, condition (dry/soft/firm/swollen/sunken/etc.), and capillary refill.

During major surgery, especially cardiothoracic surgery, perfusion must be maintained and managed by the health professionals involved, rather than left to the body's homeostasis alone. As the lead surgeons are often too busy to handle all hemodynamic control by themselves, specialists called perfusionists manage this aspect. There are more than one hundred thousand perfusion procedures annually.

Malperfusion

'**Malperfusion**' refers to any type of incorrect perfusion though it usually refers to '**Hypoperfusion**'. The meaning of the terms '**Overperfusion**' and '**Underperfusion**' is relative to the average level of perfusion that exists across all the tissues in an individual body. Perfusion levels also differ from person to person depending on metabolic demand.

Following are some examples of 'Perfusion'.

1. Heart tissues are considered overperfused because they normally are receiving more blood than the rest of tissues in the organism; they need this blood because they are constantly working.
2. In the case of skin cells, extra blood flow in them is used for thermoregulation of a body. In addition to delivering oxygen, blood flow helps to dissipate heat in a physical body by redirecting warm blood closer to its surface where it can help to cool a body through sweating and thermal dissipation.
3. Many types of tumors, and especially certain types, have been described as 'Hot and Bloody' because of their overperfusion relative to the body overall.

Overperfusion and underperfusion should not be confused with hypoperfusion and hyperperfusion, which relate to the perfusion level relative to a tissue's current need to meet its metabolic needs. For example, hypoperfusion can be caused when an artery or arteriole that supplies blood to a volume of tissue becomes blocked by an embolus, causing either no blood or at least not enough blood to reach the tissue. Hyperperfusion can be caused by inflammation, producing hyperemia of a body part. Malperfusion, also called poor perfusion, is any type of incorrect perfusion. There is no official or formal dividing line between hypoperfusion and ischemia; sometimes the latter term refers to zero perfusion, but often it refers to any hypoperfusion that is bad enough to cause necrosis.

2.6.3 Indwelling Catheters

In medicine, a catheter is a thin tube made from medical grade materials serving a broad range of functions. Catheters are medical devices that can be inserted in the body to treat diseases or perform a surgical procedure. By modifying the material or adjusting the way catheters are manufactured, it is possible to tailor catheters for cardiovascular, urological, gastrointestinal, neurovascular, and ophthalmic applications. The process of inserting a catheter is 'Catheterization'.

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Basically, a catheter is a thin, flexible tube, 'Soft' Catheter, though catheters are available in varying levels of stiffness depending on the application. A catheter left inside the body, either temporarily or permanently, may be referred to as an '**Indwelling Catheter'**, for example, a peripherally inserted central catheter. A permanently inserted catheter may be referred to as a 'Permcath' originally a trademark.

Catheters can be inserted into a body cavity, duct, or vessel, brain, skin or adipose tissue. Functionally, they allow drainage, administration of fluids or gases, access by surgical instruments, and also perform a wide variety of other tasks depending on the type of catheter. Special types of catheters, also called probes, are used in preclinical or clinical research for sampling of lipophilic and hydrophilic compounds, protein-bound and unbound drugs, neurotransmitters, peptides and proteins, antibodies, nanoparticles and nanocarriers, enzymes and vesicles.

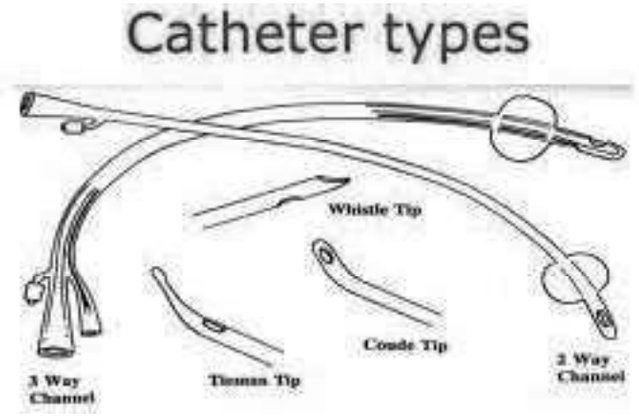

Fig 2.22 *Catheter Types*

Materials used in Catheter

A range of polymers are used for the construction of catheters, including silicone rubber, nylon, polyurethane, Poly Ethylene Terephthalate (PET), latex, and thermoplastic elastomers. Silicone is one of the most common implantable choice because it is inert and unreactive to body fluids and a range of medical fluids with which it might come into contact. On the other hand, the polymer is weak mechanically, and number of serious fractures have occurred in catheters. For example, silicone is used in Foley catheters where fractures have been reported, often requiring surgery to remove the tip left in the bladder.

Polyimides are used to manufacture vascular catheters for insertion into small vessels in the neck, head and brain.

Some catheters have a thin hydrophilic surface coating. When immersed in water, this coating swells to a smooth, slippery film making the catheter safer and more comfortable to insert. Since this wire is too slippery to handle, torque devices are used to control the direction and spin the wire. It is useful in subintimal angioplasty. However, care should be taken as it can easily cause dissection of vascular wall. Some catheters are packed in a sterile saline solution.

2.6.4 Stereotaxy

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Stereotaxy, 'Stereo' meaning 'Solidity', and 'Tactile' meaning 'Touch', refers to any technique that involves the recording and reproduction of three-dimensional haptic information or creating an illusion of depth to the sense of touch within an otherwise flat surface. Unlike the current trend in haptic technology to provide haptic perception of simulated, virtual objects within an augmented reality, i.e., within a most realistic setting when the stereohapty is applied as a field of study, then it is known as stereohaptics or stereotactics. Stereotaxy aims to provide an illusion of three-dimensional depth to the sense of touch by the human body. This resembles how stereoscopy, its visual counterpart, is meant to provide a visual illusion of depth to otherwise-flat images (such as, 3-D films), a process known as stereopsis.

Stereotactic surgery is a minimally invasive form of surgical intervention that makes use of a three-dimensional coordinate system to locate small targets inside the body and to perform on them some action, such as ablation, biopsy, lesion, injection, stimulation, implantation, radiosurgery (SRS), etc.

In theory, any organ system inside the body can be subjected to stereotactic surgery. However, there are difficulties in setting up a consistent frame of reference, such as bone landmarks, which have a constant spatial relation to soft tissues, limited to brain surgery. Plain X-ray images, radiographic mammography, Computed Tomography (CT), and Magnetic Resonance Imaging (MRI) are specifically used in the procedure.

Procedure

Stereotactic surgery works on the basis of following three main components:

1. A stereotactic planning system, including atlas, multimodality image matching tools, coordinates calculator, etc.
2. A stereotactic device or apparatus.
3. A stereotactic localization and placement procedure.

Modern stereotactic planning systems are computer based. The stereotactic atlas is a series of cross sections of anatomical structure, for example, a human brain, depicted in reference to a two-coordinate frame. Thus, each brain structure can be easily assigned a range of three coordinate numbers, which will be used for positioning the stereotactic device. In most atlases, the three dimensions are: Latero-Lateral (X), Dorso-Ventral (Y) and Rostro-Caudal (Z).

The stereotactic apparatus uses a set of three coordinates (X, Y and Z) in an orthogonal frame of reference, Cartesian Coordinates or alternatively cylindrical coordinates system with three coordinates - Angle, Depth and Antero-Posterior or Axial location. The mechanical medical devices have head-holding clamps and bars which puts the head in a fixed position in reference to the coordinate system, referred as zero or origin. In small laboratory animals, these are usually bone landmarks which are known to bear a constant spatial relation to soft tissue. For example, brain atlases often use the external auditory meatus, the inferior orbital ridges, the median point of the maxilla between the incisive teeth or the bregma, the confluence of sutures of frontal and parietal bones, as such landmarks.

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Guide bars in the X, Y and Z directions or alternatively in the polar coordinate holder, fitted with high precision vernier scales allow the neurosurgeon to position the point of a probe, an electrode, a cannula, etc., inside the brain, at the calculated coordinates for the desired structure, through a small trephined hole in the skull.

Currently, number of manufacturers produce stereotactic devices fitted for neurosurgery in humans, for both brain and spine procedures, as well as for animal experimentation.

Stereotactic Radiosurgery

Stereotactic radiosurgery utilizes externally generated ionizing radiation to inactivate or eradicate defined targets in the head or spine without the need to make an incision. This concept requires steep dose gradients to reduce injury to adjacent normal tissue while maintaining treatment efficacy in the target. As a consequence of this definition, the overall treatment accuracy should match the treatment planning margins of 1-2 millimeter or better.

To use this paradigm optimally and treat patients with the highest possible accuracy and precision, all errors, from image acquisition over treatment planning to mechanical aspects of the delivery of treatment and intra-fraction motion concerns, must be systematically optimized. To assure quality of patient care the procedure involves a multidisciplinary team consisting of a radiation oncologist, medical physicist, and radiation therapist. Dedicated, commercially available stereotactic radiosurgery programs are provided by the irrespective Gamma Knife, Cyber Knife, and Novalis Radiosurgery devices.

2.6.5 Parabiosis

Parabiosis means 'Living Beside'. This laboratory technique is used to study physiology. It combines two living organisms which are joined together surgically to develop a single, shared physiological system. Parabiosis is used in the study of areas, such as obesity, biological aging, stem cell research, tissue regeneration, diabetes, organ transplantation, tumour biology, and endocrinology. It can also describe a communal ecology of separate species of ants in colonies.

Parabiotic Experiments

Parabiosis combines two living organisms which are joined together surgically and develop single, shared physiological systems. Researchers can prove that the feedback system in one animal is circulated and affects the second animal via blood and plasma exchange.

Parabiotic experiments were pioneered by Paul Bert in the mid-1800s. He postulated that surgically connected animals could share a circulatory system.

The term is also applicable to spontaneously occurring conditions, such as in conjoined twins.

2.6.6 Biosensors

A **biosensor** is an analytical device, used for the detection of a chemical substance, that combines a biological component with a physicochemical detector. The sensitive

biological element, for example tissues, microorganisms, organelles, cell receptors, enzymes, antibodies, nucleic acids, etc., is a biologically derived material or biomimetic component that interacts with or recognizes the analyte under study.

The biologically sensitive elements can also be created by biological engineering. The **transducer** or the detector element, which transforms one signal into another one, works in a physicochemical way, namely optical, piezoelectric, electrochemical, electrochemiluminescence, etc., resulting from the interaction of the analyte with the biological element, to easily measure and quantify.

The **biosensor reader device** connects with the associated electronics or signal processors that are primarily responsible for the display of the results in a user-friendly way. This sometimes accounts for the most expensive part of the sensor device however it is possible to generate a user-friendly display that includes transducer and sensitive element, holographic sensor.

Biosensors are classified by means of their biotransducer type. The most common types of biotransducers used in biosensors include the following:

- Electrochemical Biosensors
- Optical Biosensors
- Electronic Biosensors
- Piezoelectric Biosensors
- Gravimetric Biosensors
- Pyroelectric Biosensors
- Magnetic Biosensors

Biosensor System

A **biosensor** typically consists of a bio-receptor (enzyme/antibody/cell/nucleic acid/aptamer), transducer component (semi-conducting material/nanomaterial), and electronic system which includes a signal amplifier, processor and display. Transducers and electronics can be combined, for example, in CMOS-based (Complementary Metal-Oxide Semiconductor) microsensor systems. The recognition component, often called a **bioreceptor**, uses biomolecules from organisms or receptors modelled after biological systems to interact with the analyte of interest. This interaction is measured by the **biotransducer** which outputs a measurable signal proportional of the target analyte in the sample.

Bioreceptors

In a biosensor, the **bioreceptor** is typically designed to interact with the specific analyte of interest to produce an effect which is measurable by the transducer. High selectivity for the analyte among a matrix of other chemical or biological components is a key requirement of the bioreceptor. The biosensors can be classified according to common types of bioreceptor interactions involving the antibody/antigen, enzymes/ligands, nucleic acids/DNA, cellular structures/cells, or biomimetic materials.

NOTES

Check Your Progress

15. Immunochemical techniques are based on which reactions?
16. How are the monoclonal antibodies produced?
17. What do you understand by ovariectomy (state)?
18. State about organ ablation.
19. What is the name given to the surgical procedure where the ovaries (either one or both) are removed.
20. What are the benefits of laparoscopic surgery?
21. Define perfusion.
22. What is a catheter?
23. State about biosensor reader device.

2.7 ANSWERS TO 'CHECK YOUR PROGRESS'

1. Chromatography is a method of separation based on the different interactions of various compounds with two phases, mobile phase and stationary phase.
2. Paper chromatography is a technique used to separate mixture of chemical substances into its individual compounds.
3. Adsorption Chromatography is considered as classic form of chromatography.
4. Thin layer chromatography is used to separate and analyze complex biological or non-biological samples into their constituents.
5. Electrophoresis can be defined as the migration of charged particles or molecules under effect of an applied electric field.
6. Two materials particularly useful in gel electrophoresis are agarose and polyacrylamide.
7. Biological centrifugation is a process that uses centrifugal force to separate and purify mixtures of biological particles in a liquid medium.
8. The statistical analysis gives meaning to the meaningless numbers, thereby breathing life into a lifeless data. The results and inferences are precise only if proper statistical tests are used.
9. The sign test is a statistical method to test for consistent differences between pairs of observations, such as the weight of subjects before and after treatment.
10. Analysis Of Variance (ANOVA) is a collection of statistical models and their associated estimation procedures (such as the variation among and between groups) used to analyze the differences among group means in a sample.

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11. Statistical Analysis System (SAS) is an integrated system of software products provided by SAS Institute Inc., which enables programmers to perform: Information retrieval and data management, report writing and graphics, statistical analysis, econometrics and data mining, business planning, project management, applications development, data mining, data alter, data manage and retrieve data from a variety of sources and perform statistical analysis on it.
12. Isotopes are two or more types of atoms that have the same atomic number (number of protons in their nuclei) and position in the periodic table (and hence belong to the same chemical element), and that differ in nucleon numbers (mass numbers) due to different numbers of neutrons in their nuclei.
13. Isotope-Ratio Mass Spectrometry (IRMS) is a specialization of mass spectrometry, in which mass spectrometric methods are used to measure the relative abundance of isotopes in a given sample.
14. An autoradiograph is an image on an x-ray film or nuclear emulsion produced by the pattern of decay emissions (e.g., beta particles or gamma rays) from a distribution of a radioactive substance.
15. Immunochemical techniques are based on a reaction of antigen with antibody, or more exactly, on a reaction of an antigenic determinants with the binding site of the antibody.
16. Monoclonal antibodies are products of a single clone of plasma cells derived from B-lymphocytes, prepared in the laboratory by hybridoma technology, based on cellular fusion of tumour (myeloma) cells with splenic lymphocytes of immunised mice.
17. Ovariectomy is a surgical procedure where the ovaries (either one or both) are removed.
18. Organ ablation is a procedure to remove a body part, organ, or tissue surgically. This is a general term that applies to the surgical removal of any part of the body.
19. Surgery is an invasive technique with the fundamental principle of physical intervention on organs/organ systems/tissues for diagnostic or therapeutic reasons.
20. Laparoscopic surgery has many benefits, including smaller scars, less pain and a shorter recovery period than traditional open surgery.
21. Perfusion is the passage of fluid through the circulatory system or lymphatic system to an organ or a tissue, usually referring to the delivery of blood to a capillary bed in tissue.
22. A catheter is a thin tube made from medical grade materials serving a broad range of functions.
23. The biosensor reader device connects with the associated electronics or signal processors that are primarily responsible for the display of the results in a user-friendly way.

NOTES

2.8 SUMMARY

- Various separation techniques are routinely used in chemical and life science laboratories for separation of desired molecule from mixture of molecules.
- Chromatography is a method of separation based on the different interactions of various compounds with two phases, mobile phase and stationary phase.
- The principle of adsorption chromatography is that certain solid materials can adsorb or hold the various molecules at their surface.
- The Ion-Exchange Chromatography (IEX) is used for purification of proteins. Other biological molecules including peptides and nucleic acids can also be purified by this technique.
- Thin layer chromatography is used to separate and analyze complex biological or non-biological samples into their constituents.
- Gel electrophoresis is the commonly used technique in molecular biology. It is routinely used for separation and molecular size/weight determination of charged biological molecules (DNA, RNA, nucleotides, peptides and proteins).
- The basic equipment of electrophoresis consists of electrophoresis unit and power supply.
- Two materials particularly useful in gel electrophoresis are agarose and polyacrylamide.
- Polyacrylamide gels can be used for the separation and analysis of proteins and small nucleic acid molecules.
- The SDS-PAGE system is a discontinuous gel with an upper stacking gel and lower resolving gel that have different polyacrylamide concentrations and different pH values.
- Various computer based statistical software systems are available that can be used in different analysis objectives.
- Minitab is a statistics package developed at the Pennsylvania State University by a group of researchers.
- Statistical Tool 'RSM' for Optimized Production of Industrial Microbial Products and 'ANN' as Mathematical/ Statistical Modeling
- Isotopes are two or more types of atoms that have the same atomic number (number of protons in their nuclei) and position in the periodic table (and hence belong to the same chemical element), and that differ in nucleon numbers (mass numbers) due to different numbers of neutrons in their nuclei.
- The number of protons within the atom's nucleus is called atomic number and is equal to the number of electrons in the neutral (non-ionized) atom.
- Carbon-14 is a radioactive form of carbon, whereas Carbon-12 and Carbon-13 are stable isotopes.

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- The radioactive samples are prepared from concentrated solutions whose activity can be accurately measured.
- An autoradiograph is an image on an x-ray film or nuclear emulsion produced by the pattern of decay emissions (e.g., beta particles or gamma rays) from a distribution of a radioactive substance.
- Immunochemical techniques are based on a reaction of antigen with antibody, or more exactly, on a reaction of an antigenic determinants with the binding site of the antibody.
- Polyclonal antibodies (conventional antibodies) are prepared by immunization of animals (rabbits, goats, and sheep) with the antigen.
- The antigens and antibodies combine by a process called agglutination.
- Immunity developed as an individual is exposed to antigens is called adaptive or acquired immunity, in contrast to immunity developed at birth, which is innate immunity.
- Antigen and antibody interact through a high affinity binding much like lock and key.
- Heart tissues are considered overperfused because they normally are receiving more blood than the rest of tissues in the organism; they need this blood because they are constantly working.
- Many types of tumors, and especially certain types, have been described as 'Hot and Bloody' because of their overperfusion relative to the body overall.
- Catheters can be inserted into a body cavity, duct, or vessel, brain, skin or adipose tissue.
- A range of polymers are used for the construction of catheters, including silicone rubber, nylon, polyurethane, Poly Ethylene Terephthalate (PET), latex, and thermoplastic elastomers.
- Stereotactic radiosurgery utilizes externally generated ionizing radiation to inactivate or eradicate defined targets in the head or spine without the need to make an incision.
- Parabiosis means 'Living Beside'. This laboratory technique is used to study physiology.
- A biosensor is an analytical device, used for the detection of a chemical substance, that combines a biological component with a physicochemical detector
- The biosensor reader device connects with the associated electronics or signal processors that are primarily responsible for the display of the results in a user-friendly way
- In a biosensor, the **bioreceptor** is typically designed to interact with the specific analyte of interest to produce an effect which is measurable by the transducer.

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2.9 KEY TERMS

- **Chromatography:** Chromatography is a method of separation based on the different interactions of various compounds with two phases, mobile phase and stationary phase.
- **Paper Chromatography:** Paper chromatography is a technique used to separate mixture of chemical substances into its individual compounds.
- **Column Chromatography:** The column chromatography involves use of glass or metal column for packaging of stationary phase.
- **Gel-Permeation Chromatography:** The gel permeation chromatography is also known as gel filtration and size exclusion chromatography. This technique separates the molecules on the basis of their molecular sizes.
- **Gas Chromatography (Gc):** Gas Chromatography is a gas-liquid chromatography. It is a commonly used technique for identification, purification, quantification and analysis of compounds in a sample.
- **Hydrophobic Interaction Chromatography (HIC):** Hydrophobic Interaction Chromatography (HIC) separates the molecules depending on their hydrophobicity.
- **Agarose:** Agarose is a linear galactan hydrocolloid isolated from agar or agar-bearing marine algae.
- **Biological Centrifugation:** Biological centrifugation is a process that uses centrifugal force to separate and purify mixtures of biological particles in a liquid medium.
- **Microsoft Office:** Software programs used for typing, editing, retrieving, storage, managing word documents and other functions.
- **Microsoft Excel:** Software programs used for data recording, numerical value analysis, graphical presentation of data, basic statistical, computation of data and other functions.
- **Microsoft Publisher:** For data presentation in form of sheets, posters and other formats.
- **Statistics**: Statistics is a branch of science that deals with the collection, organization, and analysis of data and drawing of inferences from the samples to the whole population
- **Sign Test:** The sign test is a statistical method to test for consistent differences between pairs of observations, such as the weight of subjects before and after treatment.
- **Isotope-Ratio Mass Spectrometry (IRMS)**: Isotope-Ratio Mass Spectrometry (IRMS) is a specialization of mass spectrometry, in which mass spectrometric methods are used to measure the relative abundance of isotopes in a given sample.

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- **Autoradiograph:** An autoradiograph is an image on an x-ray film or nuclear emulsion produced by the pattern of decay emissions (e.g., beta particles or gamma rays) from a distribution of a radioactive substance.
- **Organ Ablation:** Organ ablation is a procedure to remove a body part, organ, or tissue surgically. This is a general term that applies to the surgical removal of any part of the body.
- **Ovariectomy:** It is a surgical procedure where the ovaries (either one or both) are removed and can be done with or without a hysterectomy.
- **Adrenalectomy:** Adrenalectomy is the surgical removal of one or both adrenal glands.
- **Surgery:** Surgery is an invasive technique with the fundamental principle of physical intervention on organs/organ systems/tissues for diagnostic or therapeutic reasons.
- **Perfusion:** Perfusion is the passage of fluid through the circulatory system or lymphatic system to an organ or a tissue, usually referring to the delivery of blood to a capillary bed in tissue.
- **Catheters:** Catheters are medical devices that can be inserted in the body to treat diseases or perform a surgical procedure.
- **Indwelling Catheter:** A catheter left inside the body, either temporarily or permanently, may be referred to as an indwelling catheter, for example, a peripherally inserted central catheter.
- **Stereotactic Surgery:** Stereotactic surgery is a minimally invasive form of surgical intervention that makes use of a three-dimensional coordinate system to locate small targets inside the body and to perform on them some action, such as ablation, biopsy, lesion, injection, stimulation, implantation, radiosurgery (SRS), etc.
- **Biosensors:** A biosensor is an analytical device, used for the detection of a chemical substance, that combines a biological component with a physicochemical detector

2.10 SELF-ASSESSMENT QUESTIONS AND EXERCISES

Short-Answer Questions

1. Which are the three basic components involved in the chromatography technique?
2. What are the types of chromatography?
3. Write the principle of chromatography.
4. Write the general procedure for agarose gel electrophoresis.

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5. What are the components of sample buffer?
6. State about the types of centrifuges.
7. Name some commonly used computer based statistical software systems.
8. Write the process of preparing samples for radioactive counting.
9. What is the "Goldberg's Theory" of antigen-antibody reaction?
10. What are the types of oophorectomy?
11. Why stereotactic surgery is considered as a very useful surgical intervention.
12. What is parabiosis?

Long-Answer Questions

1. Describe the Ion-Exchange Chromatography (IEX) in detail.
2. Explain the concept, types and applications of electrophoresis in detail.
3. Analyze the general work flow for protein electrophoresis.
4. Describe the care and safety aspects of centrifuges.
5. Explain various tests used in computer assisted statistical analysis.
6. Elaborate on the chemical basis of antigen-antibody interaction and affinity of this interaction.
7. Explain the procedure of stereotactic surgery.
8. Write an explanatory note on biosensors.

2.11 FURTHER READING

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UNIT 3 ENVIRONMENTAL PHYSIOLOGY - I

NOTES

Structure

3.0 INTRODUCTION

An amazing fact of life on Earth is ?that organisms live nearly everywhere on its surface despite extreme variation in environmental conditions, including temperature, pressure, and availability of oxygen and water. Environmental physiology a branch of physiology that studies the dependence of human and animal functions on conditions of life and activity in different physico-geographical zones. Ecophysiology or environmental physiology is a biological discipline that studies the response of an organism's physiology to environmental conditions.

Physiological adaptation is any alteration in the structure or function of an organism or any of its parts that results from natural selection and by which the organism becomes better fitted to survive and multiply in its environment. It may be a metabolic or physiologic adjustment within the cell, or tissues, of an organism in response to an environmental stimulus resulting in the improved ability of that organism to cope with its changing environment. It may also be the response of the organism to a specific external stimulus in order to maintain homeostasis. An adaptation is a genetically controlled structural, behavioral or physiological feature that enhances the survival of an organism in particular environmental conditions. Adaptation is the process of evolution where an animal or plant becomes better suited to its habitat. There are many examples of physiological processes and special functions that animals and plants use to thrive.

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In this unit you will study about adaptations, levels of adaptation, mechanism of adaptation significance of body size, physiological adaptations to different environments, such as, marine, shores and estuaries, fresh water, extreme aquatic environments, terrestrial life, extreme terrestrial environments and parasitic habitats.

3.1 OBJECTIVES

After going through this unit you will be able to:

- Understand adaptation
- Comprehend levels of adaptation.
- Explain the mechanism of adaptation
- Discuss the significance of body size
- Elaborate on physiological adaptations to different environments like marine shores, fresh and water extreme aquatic environments
- Analyze physiological adaptations to terrestrial life, extreme terrestrial environments and parasitic habitats.

3.2 ADAPTATION

Adaptation is related to biological fitness, which governs the rate of evolution as measured by change in gene frequencies. Often, two or more species co-adapt and co-evolve as they develop adaptations that interlock with those of the other species, such as with flowering plants and pollinating insects. Features evolved for one purpose may be co-opted for a different one, as when the insulating feathers of dinosaurs were co-opted for bird flight.

Definition: "*Adaptation is the physical or behavioural characteristic of an organism that helps an organism to survive better in the surrounding environment*."

Living things are adapted to the habitat in which they live because of the special features they have that help them to survive in that environment. The development of these special features is because of the result of the evolution process that happens due to gene mutation. These mutations help in survival and reproduction and pass on from one generation to the other.

According to Charles Darwin, "*Only those species which have a high 'Biological Fitness', i.e., the ability to adapt to changes in one's environment, will be selected by nature to pass on their genes to the next generation. So, adaptations are basically the results of natural selection*".

As per the 'Encyclopaedia Britannica', "***Adaptation, in biology, the process by which a species becomes fitted to its environment; it is the result of natural selection's acting upon heritable variation over several generations. Organisms are adapted to their environments in a great variety of ways: in their structure, physiology, and genetics, in their***

locomotion or dispersal, in their means of defense and attack, in their reproduction and development, and in other respects".

The following definitions of adaptation are given by the evolutionary biologist Theodosius Dobzhansky:

1. Adaptation is the evolutionary process whereby an organism becomes better able to live in its habitat or habitats.
2. Adaptedness is the state of being adapted, the degree to which an organism is able to live and reproduce in a given set of habitats.
3. An adaptive trait is an aspect of the developmental pattern of the organism which enables or enhances the probability of that organism surviving and reproducing.

In biology, adaptation has following three related consequences.

1. It is the dynamic evolutionary process that positions or fits organisms to their environment. Basically, in a physiological sense, an animal or plant can adapt by adjusting to its immediate environment, for example by changing its temperature or metabolism with an increase in altitude.
2. This specific state is reached by the population during that process. Characteristically, the word 'Adaptation' refers either to the process of becoming adapted or to the characteristics or features of organisms that stimulate reproductive success relative to other feasible or possible characteristics or features. In this state, the process of adaptation is determined by genetic variations among individuals that become adapted to the environment. The most historic and classic example is displayed by the melanistic (dark) phenotype of the peppered moth, *Biston betularia*, whose numbers increased greatly in Britain due to the Industrial Revolution. The adaptation process happens through an eventual or ultimate change in the gene frequency corresponding to advantages that are typically conferred by means of a specific characteristic.
3. It is a phenotypic trait or adaptive trait, with a functional role in each individual organism, that is maintained and has evolved through natural selection. Examples include the long necks of giraffes which is an adaptation to reach the tops of trees for their food, the streamlined bodies of aquatic fish and mammals, the light bones of flying birds and mammals, and the long dagger like canine teeth of carnivores.

Historically, adaptation has been described from the time of the ancient Greek philosophers, namely Empedocles and Aristotle. Charles Darwin proposed instead that it was explained by natural selection.

Adaptation is a major topic in the philosophy of biology, as it concerns function and purpose (teleology).

3.2.1 Levels of Adaptation

The US psychologist Harry Helson (1898–1977) proposed the theory for levels of adaptation in an article in the American Journal of Psychology in 1947. The

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theory proposed that, "*The adaptation level is determined for a class of stimuli by members of the class already sampled or attended to, by stimuli having a background or contextual influence, and by recollections of past judgements of similar stimuli, the adaptation level being the logarithm of the mean of the relevant stimuli, weighted according to their effectiveness in terms of nearness, recency, salience, and so on*".

According to the theory, subjective judgements are essentially relative to the prevalent and established norm or adaptation level. The phenomenon is referred as a type of context effect and is also called AL or Adaptation Level theory.

3.2.2 Types of Adaptations

Basically, there are three types of adaptations, namely structural adaptations, behavioural adaptations, and physiological adaptations.

1. Structural Adaptations

An organism's environment shapes its appearance through structural adaptations. The 'Structural Adaptations' typically involve the physical features of an organism that help them to survive in the environment as well as the different types of terrestrial habitat. The physical changes are associated with the changes in the physical environment. For example, Desert foxes have large ears for heat radiation and Arctic foxes have small ears to retain body heat. Seals have flippers to navigate water and Raccoons have separate, flexible digits to manipulate food. Camouflage is the protective colouration that helps an organism to blend in its environment. This protects them from predators increasing their chances of survival.

2. Physiological Adaptations

In biology, the 'Physiological Adaptation' is a metabolic or physiologic adjustment within the cell or tissues of an organism in response to an environmental stimulus resulting in the improved ability of that organism to cope with its changing environment. It may also be the response of the organism to a specific external stimulus in order to maintain homeostasis. Based on body chemistry and metabolism, physiological adaptations generally not observed from the outside. Characteristically, the physiological adaptations involve physical changes in the species, i.e., this type of adaptation can be either due to changes in the environment or due to the behaviour of other species. For example, consider a fish living in water, suddenly the water becomes more acidic, in such condition the fish must shift its body chemistry to adapt itself to the more acidic environment.

3. Behavioural Adaptations

Adaptations that influence how an organism acts are termed as behavioural adaptations it is the change that influences the behaviour of an organism. This could be caused due to the changes in the surrounding environment or due to the actions of other species. For example, the Rabbit freezes if it feels that it has been seen by a predator, Bears hibernate to escape cold, Birds and Whales migrate to warmer winter climates, Desert animals are active at night during hot summer weather, and many more. Changes in reproductive strategy, feeding habits,

migration, hibernation, communication methods are a few relevant examples of behavioural adaptations.

3.2.3 Mechanism of Adaptation

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In evolutionary theory, adaptation is the biological mechanism by which organisms adjust to new environments or to changes in their current environment. Charles Darwin and Alfred Russel Wallace developed the theory of natural selection. Wallace believed that the evolution of organisms was connected in some way with adaptation of organisms to changing environmental conditions. In developing the theory of evolution by natural selection, Wallace and Darwin both went beyond simple adaptation by explaining how organisms adapt and evolve. The idea of natural selection is that traits that can be passed down allow organisms to adapt to the environment better than other organisms of the same species. This enables better survival and reproduction compared with other members of the species, leading to evolution.

Organisms can adapt to an environment in different ways. They can adapt biologically, meaning they alter body functions. An example of biological adaptation can be seen in the bodies of people living at high altitudes, such as Tibet. Tibetans thrive at altitudes where oxygen levels are up to 40 percent lower than at sea level. Breathing air that thin would cause most people to get sick, but Tibetans' bodies have evolved changes in their body chemistry. Most people can survive at high altitudes for a short time because their bodies raise their levels of haemoglobin, a protein that transports oxygen in the blood. However, continuously high levels of haemoglobin are dangerous, so increased haemoglobin levels are not a good solution to high altitude survival in the long term. Tibetans seemed to have evolved genetic mutations that allow them to use oxygen far more efficiently without the need for extra haemoglobin.

Scientists who studied adaptation prior to the development of evolutionary theory included Georges Louis Leclerc Comte de Buffon. He was a French mathematician who believed that organisms changed over time by adapting to the environments of their geographical locations. Another French thinker, Jean Baptiste Lamarck, proposed that animals could adapt, pass on their adaptations to their offspring, and therefore evolve. The example he gave stated the ancestors of giraffes might have adapted to a shortage of food from short trees by stretching their necks to reach higher branches. In Lamarck's thinking, the offspring of a giraffe that stretched its neck would then inherit a slightly longer neck. Lamarck theory stated that behaviours acquired in a giraffe's lifetime would affect its offspring. However, it was Darwin's concept of natural selection, wherein favourable traits like a long neck in giraffes survived not because of acquired skills, but because only giraffes that had long enough necks to feed themselves survived long enough to reproduce. Natural selection, then, provides a more compelling mechanism for adaptation and evolution than Lamarck's theories.

3.2.4 Significance of Body Size

Body size is a key indicator of an animal's ability to survive and reproduce, so records of size variation in response to past temperature changes can help in concluding how the biota may respond to present and future climate change.

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The palaeontologists study body size in very different contexts. Prof Twitchett focuses on marine organisms and looks at reductions in size, while Prof Lister studies fluctuations in the size of 'Ice Age Mammals' and 'Island Dwarfing'.

Shrink and Swim: In the immediate aftermath of extinction events, the surviving animals tend to become temporarily a lot smaller.

Prof Twitchett studies this 'Lilliput Effect' in marine animals that survived the three major extinction events of the Mesozoic Era (252 to 66 million years ago). He investigates why the phenomenon seems ubiquitous across geological time and faunal groups. He looked at size reduction in the surviving taxa, so it's not simply about large species being more vulnerable to extinction. Following the decrease in size, he observed an increase and stated that - the 'Lilliput Effect' is a temporary thing.

"It is basically about environmental stress, as these post-extinction times are also peak greenhouse times."

The proportion of gases in the atmosphere that absorb infrared radiation rises in greenhouse periods, causing an overall increase in global temperature. For marine organisms, that equals stress, and due to stress the body size can become smaller.

Stressed Shellfish: Greenhouse conditions are common after major extinction events, and marine survivors find themselves living in waters that are warmer and therefore less able to hold oxygen. Less oxygen means organisms must reduce the share of their cellular energy that goes towards growth in order to maintain other essential processes, such as keeping a hard shell around their body.

Scientists concluded after research that soft-bodied creatures, such as polychete worms and burrowing invertebrates might have been protected from the Lilliput Effect, since they had no need to expend energy maintaining a hard shell.

But Prof Twitchett's research shows that they too experienced a size reduction, indicating that severe environmental stresses affect the entire spectrum of marine fauna.

Temperature itself is another strain on marine organisms.

As temperatures rise, the animal's metabolic rate increases. This may cause the animal to mature faster and to a smaller size.

Other potential triggers for size reduction in marine organisms include water salinity, high sedimentation rates and low food availability.

Body size is a key indicator of an animal's fertility, lifespan, and ability to survive a food shortage or drought. This means that any environmentally driven change in body size may significantly affect the broader ecosystem, as well as the species' own success.

Recent studies have confirmed that the body size of many animals is predicted to decrease in the near future in response to a warming climate and increasingly variable levels of rainfall.

Scientists like Prof Twitchett and Prof Lister are quantifying biotic responses, bringing the hazards of environmental change further into the global consciousness.

Check Your Progress

1. Define adaptation.
2. What do you understand by the term physiological adaptation?
3. What are behavioural adaptations?
4. Name the scientist who studied adaptation prior to the development of evolutionary.

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3.3 PHYSIOLOGICAL ADAPTATIONS TO DIFFERENT ENVIRONMENTS

In this section we will study about the various physiological adaptations of the organisms in different environments.

3.3.1 Marine

Marine life, sea life, or ocean life includes the plants, animals, and other organisms that live in the salt water of the sea or ocean, or the brackish water of coastal estuaries. At a fundamental level, marine life affects the nature of the planet. Marine organisms, mostly microorganisms, produce oxygen and sequester carbon. Marine life in part shape and protect shorelines, and some marine organisms even help create new land, for example coral building reefs. Most life forms evolved initially in marine habitats. By volume, oceans provide about 90% of the living space on the planet. The earliest vertebrates appeared in the form of fish, which live exclusively in water. Some of these evolved into amphibians, which spend portions of their lives in water and portions on land. Other fish evolved into land mammals and subsequently returned to the ocean as seals, dolphins, or whales. Plant forms, such as kelp and other algae grow in the water and are the basis for some underwater ecosystems. Plankton forms the general foundation of the ocean food chain, particularly phytoplankton which are key primary producers.

Marine invertebrates exhibit a wide range of modifications to survive in poorly oxygenated waters, including breathing tubes as in mollusc siphons. Fish have gills instead of lungs, although some species of fish, such as the lungfish, have both. Marine mammals, for example dolphins, whales, otters, and seals need to surface periodically to breathe air.

Marine species range in size from the microscopic like phytoplankton, which can be as small as 0.02 micrometres, to huge cetaceans like the blue whale – the largest known animal, reaching 33 m (108 ft) in length. Marine microorganisms, including protists and bacteria and their associated viruses, have been variously estimated as constituting about 70% or about 90% of the total marine biomass. Marine life is studied scientifically in both marine biology and in biological oceanography. The term marine comes from the Latin mare, meaning "Sea" or "Ocean".

Fig.3.2 *Pelagia noctiluca*

Most organisms do not tolerate large variations in salinity so they are called 'Stenohaline' and only a few can survive when it changes 'Euryhaline', as can happen in coastal lagoons. Very few are the species able to switch from fresh to salt water or vice versa during their life. Salmon and Eel are the most famous examples.

The evolutionary history of prey-predator relations has led to a wide variety of morphological and chromatic adaptations. The aim of these adaptations is to increase the chances of survival of the species in the environment. Many types of mimicry have been described, among them defensive, aggressive and reproductive mimicry.

3.3.2 Shores and Estuaries

An estuary is a partially enclosed coastal body of brackish water with one or more rivers or streams flowing into it, and with a free connection to the open sea. Estuaries form a transition zone between river environments and maritime environments and are an example of an ecotone. Estuaries are subject both to marine influences such as tides, waves, and the influx of saline water and to fluvial influences such as, flows of freshwater and sediment. The mixing of seawater and freshwater provides high levels of nutrients both in the water column and in sediment, making estuaries among the most productive natural habitats in the world.

Most existing estuaries formed during the Holocene epoch with the flooding of river-eroded or glacially scoured valleys when the sea level began to rise about 10,000–12,000 years ago. Estuaries are typically classified according to their geomorphological features or to water-circulation patterns. They can have many different names, such as bays, harbors, lagoons, inlets, or sounds, although some of these water bodies do not strictly meet the above definition of an estuary and could be fully saline.

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Many estuaries suffer degeneration from a variety of factors including soil erosion, deforestation, overgrazing, overfishing and the filling of wetlands. Eutrophication may lead to excessive nutrients from sewage and animal wastes; pollutants including heavy metals, polychlorinated biphenyls, radionuclides and hydrocarbons from sewage inputs; and diking or damming for flood control or water diversion.

Mangrove trees and blue crabs are some of the estuarine species that have adapted to unique environmental conditions. In almost all estuaries the salinity of the water changes constantly over the tidal cycle. To survive in these conditions, plants and animals living in estuaries must be able to respond quickly to drastic changes in salinity.

Plants and animals that can tolerate only slight changes in salinity are called 'Stenohaline'. These organisms usually live in either freshwater or saltwater environments. Most stenohaline organisms cannot tolerate the rapid changes in salinity that occur during each tidal cycle in an estuary.

Mangrove Trees

Mangrove trees have become specialized to survive in the extreme conditions of estuaries. Two key adaptations they have are the ability to survive in waterlogged and anoxic (no oxygen) soil, and the ability to tolerate brackish waters.

Some mangroves remove salt from brackish estuarine waters through ultra-filtration in their roots. Other species have special glands on their leaves that actively secrete salt, a process that leaves visible salt crystals on the upper surface of the leaves.

All mangrove species have laterally spreading roots with attached vertical anchor roots. These roots are very shallow. Because the soil in shallow areas of mangal forests is typically flooded during high tides, many species of mangrove trees have aerial roots, called pneumatophores, that take up oxygen from the air for the roots. Some species also have prop roots or stilt roots extending from the trunk or other roots that help them withstand the destructive action of tides, waves, and storm surges.

Many mangrove trees also have a unique method of reproduction. Instead of forming seeds that fall to the soil below and begin growing, mangrove seeds begin growing while still attached to the parent plant. These seedlings, called propagules, even grow roots. After a period of growth, these seedlings drop to the water below and float upright until they reach water that is shallow enough for their roots to take hold in the mud.

Mangrove forests, or mangals, grow at tropical and subtropical latitudes near the equator where the sea surface temperatures never fall below 16°C. Mangals line about two-thirds of the coastlines in tropical areas of the world.

There are about 80 species of mangrove trees, all of which grow in hypoxic (oxygen poor) soils where slow-moving waters allow fine sediments to accumulate. Many mangrove forests can be recognized by their dense tangle of prop roots that make the trees appear to be standing on stilts above the water. This tangle of roots helps to slow the movement of tidal waters, causing even more sediments to settle

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out of the water and build up the muddy bottom. Mangrove forests stabilize the coastline, reducing erosion from storm surges, currents, waves and tides.

Just like the high and low areas of salt marshes where specific types of grasses are found, mangals have distinct zones characterized by the species of mangrove tree that grows there. Where a species of mangrove tree exists depends on its tolerance for tidal flooding, soil salinity, and the availability of nutrients. Three dominant species of mangrove tree are found in Florida. The Red Mangrove (***Rhizophora mangle***) colonizes the seaward side of the mangal, so it receives the greatest amount of tidal flooding. Further inland and at a slightly higher elevation, Black Mangroves (***Avicennia germinanas***) grow. The zone in which Black Mangrove trees are found is only shallowly flooded during high tides. White Mangrove (***Laguncularia racemosa***) and Buttonwood Trees (***Conocarpus erectus***), a non-mangrove species, face inland and dominate the highest parts of the mangal. The zone where white mangrove and buttonwood trees grow is almost never flooded by tidal waters.

A unique mix of marine and terrestrial species lives in mangrove ecosystems. The still, sheltered waters among the mangrove roots provide protective breeding, feeding, and nursery areas for Snapper, Tarpon, Oysters, Crabs, Shrimp and other species important to commercial and recreational fisheries. Herons, Brown Pelicans, and Spoonbills all make their nests in the upper branches of mangrove trees.

Blue Crabs

Blue crabs live in estuaries along the United States' Atlantic and Gulf coasts. They are mobile predators whose salinity requirements change at different stages in their lives.

Adult male crabs live in the low-salinity waters upstream, while adult female crabs live in the higher-salinity waters near the mouth of the estuary. During the crabs' mating season (May to October), the high-salinity preference of the female overlaps with the lower-salinity preference of the male. After mating, female crabs migrate offshore, sometimes up to 200 km, to high-salinity waters to incubate their eggs. The females release their larvae, called zoeae, during spring high tides. The zoeae, resembling tiny shrimp, develop in the coastal waters. Zoeae require water with a salinity over 30 ppt (parts per thousand) for optimal development, which is only found in the ocean. Winds and coastal currents keep the larvae near the ocean shore, until they return to the estuary as young crabs, called megalops.

When the megalops return to the estuary, they swim up and down in the water in response to light and tides. This is called vertical migration. The young crabs use night-time flood tides to move upriver into the shallow parts of the estuary. Eventually, the young crabs take up life on the bottom of the estuary, seeking out shallow-water habitats like seagrass beds and submerged aquatic vegetation, where they feed and gain protection from predators.

Plants and animals that can tolerate a wide range of salinities are called 'Euryhaline'. These are the plants and animals most often found in the brackish waters of estuaries. There are far fewer euryhaline than stenohaline organisms because it requires a lot of energy to adapt to constantly changing salinities. Organisms that can do this are rare and special. Some organisms have evolved

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special physical structures to cope with changing salinity. The Smooth Cordgrass (***Spartina alterniflora***) found in salt marshes, for example, has special filters on its roots to remove salts from the water it absorbs. This plant also expels excess salt through its leaves.

Unlike plants, which typically live their whole lives rooted to one spot, many animals that live in estuaries must change their behaviour according to the surrounding waters' salinity in order to survive. Oysters and Blue Crabs are good examples of animals that do this.

3.3.3 Fresh Water

Freshwater biomes like rivers and lakes have water with a salt concentration of less than 1%. Additionally, most water bodies have movement. Hence, this means that the plants and animals adapt themselves accordingly.

Many plants and animals have adapted to the freshwater biome and could not survive in water having a higher salt concentration. As this ecosystem covers a vast portion of the world, the animal life found can vary considerably.

Fish are able to obtain oxygen through their gills. Fish such as trout have adapted to living in rivers and streams where the water is cooler, clearer and has a higher oxygen level. At the mouth of these water sources, the sediments create a murkier environment with lower oxygen levels and fish, such as Catfish and Carp have adapted to exist in these areas.

There are three zones in lakes and ponds:

The **littoral zone**, the topmost and warmest is home to Snails, Clams, Insects, Crustaceans, Fishes and Amphibians and the eggs and larvae of Dragonflies and Midges. These resources provide food for Turtles, Snakes and Ducks.

The **limnetic zone** is close to the surface and consequently receives a good deal of light. This zone contains a variety of freshwater fish.

The **profundal zone** is very dense and cold, with little light penetrating this region. Only heterotrophs, animals that eat dead organisms, are found in this region

Mammals, such as Badgers, Otters, Mink live near water and are capable of swimming to catch their main food source, fish.

Amphibians and reptiles, such as Toads, Frogs, Alligators, Crocodiles, Salamanders and newts start life underwater as eggs and tadpoles, and then move to ground as adults.

Insects, such as Skaters, Water Beetles, Mosquitoes and Dragonflies can skim over the surface of ponds, playing a critical role in the food supply for other animals. Some spiders can actually take a bubble of air with them underwater.

Many species of Ducks, Geese and Swans also call the freshwater biome their home, feeding on a number of different items including fish, while wading birds such as herons and egrets wander through the mud shallows searching for insects.

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Manatees have adapted to survive in warm water and migrate south. Some have found the warm water near power plants, and consequently do not have to migrate.

Beavers shape their environment more than most other animal species on Earth, utilizing their ever-growing teeth to cut down trees and plants to create dams to create their dens. Their actions are not always appreciated by nearby humans, but they are vital to the ecology, causing a build-up of water which in turn creates a new wetland. Beavers also help to purify water because the sediments and any toxins are trapped behind the dam.

Check Your Progress

5. Give examples of some species able to switch from fresh to salt water or vice versa during their life.
6. What is an estuary?
7. Which factors are responsible for degeneration of many estuaries?
8. What are the two key adaptations mangrove trees have to survive in estuary?
9. What is the habitat of blue carbs?

3.4 EXTREME AQUATIC ENVIRONMENTS

An extreme environment is a habitat that is considered very hard to survive in due to its considerably extreme conditions, such as temperature, accessibility to different energy sources or under high pressure. For an area to be considered an extreme environment, it must contain certain conditions and aspects that are considered very hard for other life forms to survive. Pressure conditions may be extremely high or low; high or low content of oxygen or carbon dioxide in the atmosphere; high levels of radiation, acidity, or alkalinity; absence of water; water containing a high concentration of salt or sugar; the presence of sulphur, petroleum, and other toxic substances.

Examples of extreme environments include the geographical poles, very arid deserts, volcanoes, deep ocean trenches, upper atmosphere, outer space, and the environments of every planet in the Solar System except the Earth. Any organisms living in these conditions are often very well adapted to their living circumstances, which is usually a result of long-term evolution. Physiologists have long known that organisms living in extreme environments are especially likely to exhibit clear examples of evolutionary adaptation because of the presumably intense past natural selection they have experienced.

The distribution of extreme environments on Earth has varied through geological time. Humans generally do not inhabit extreme environments. There are organisms referred to as extremophiles that do live in such conditions and are so well-adapted that they readily grow and multiply. Extreme environments are usually hard to survive in.

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3.4.1 Types of Extreme Environments

Among extreme environments are places that are alkaline, acidic, or unusually hot or cold or salty, or without water or oxygen. There are also places altered by humans, such as mine tailings or oil impacted habitats.

Alkaline: Broadly conceived as natural habitats above pH 9 whether persistently, or with regular frequency or for protracted periods of time.

Acidic: Broadly conceived as natural habitats below pH 5 whether persistently, or with regular frequency or for protracted periods of time.

Extremely Cold: Broadly conceived habitats periodically or consistently below -17 °C either persistently or with regular frequency or for protracted periods of time. Includes montane sites, polar sites, and deep ocean habitats.

Extremely Hot: Broadly conceived habitats periodically or consistently in excess of 40 °C either persistently or with regular frequency or for protracted periods of time. Includes sites with geothermal influences, such as Yellowstone and comparable locations worldwide or deep-sea vents.

Hypersaline: Environments with salt concentrations greater than that of seawater, i.e., >3.5%. Includes salt lakes.

Under Pressure: Broadly conceived as habitats under extreme hydrostatic pressure, i.e., aquatic habitats deeper than 2000 meters and enclosed habitats under pressure. Includes habitats in oceans and deep lakes.

Radiation: Broadly conceived as habitats exposed to abnormally high radiation or of radiation outside the normal range of light. It includes habitats exposed to high UV and IR radiation.

Without Water: Broadly conceived as habitats without free water whether persistently, or with regular frequency or for protracted periods of time. It includes hot and cold desert environments, and some endolithic habitats

Without Oxygen: Broadly conceived as habitats without free oxygen – whether persistently, or with regular frequency, or for protracted periods of time. Includes habitats in deeper sediments.

Altered by Humans: Anthropogenically impacted habitats. Includes mine tailings, oil impacted habitats, and pollution by heavy metals or organic compounds.

Without Light: Deep Ocean environments and habitats, such as caves.

Void of Food: Areas on earth that lack an abundance of food, such as the vast ocean, desert and high country.

Extreme Pressure: Deep Ocean Areas.

3.5 TERRESTRIAL LIFE

Terrestrial adaptations are exhibited by the plants and animals living in land habitats. As there are varied types of land habitats, the adaptations shown by organisms also are of diverse kinds.

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All animals that live on land must overcome certain common problems. Terrestrial animals must have the following:

1. A moist membrane that allows for an adequate gas exchange between the atmosphere and the organism.
2. A means of support and locomotion suitable for land travel.
3. Methods to conserve internal water.
4. A means of reproduction and early embryonic development in which large amounts of water are not required.
5. Methods to survive the rapid and extreme climatic changes that characterize many terrestrial habitats.

When the first terrestrial animals evolved, there were many unfilled niches; therefore, much adaptive radiation occurred, resulting in a large number of different animal species. Of all the many phyla of animals in the ocean, only a few made the transition from the ocean to the extremely variable environments found on the land. The Annelids (Earthworms and Leeches) and the Mollusks (Land Snails) have terrestrial species but are confined to moist habitats. Many of the Arthropods (Centipedes, Millipedes, Scorpions, Spiders, Mites, Ticks, and Insects) and Vertebrates (Reptiles, Birds, and Mammals) adapted to a wide variety of drier terrestrial habitats.

Terrestrial Arthropods

There are five kinds of terrestrial arthropods: Crustaceans, Millipedes, Centipedes, Arachnids (Mites, Ticks, Spiders, Scorpions), and Insects. The few terrestrial crustaceans are generally confined to moist environments. The first terrestrial animals were Millipedes, which are known from the fossil record from over 400 million years ago. Flightless insects also are early terrestrial organisms. The exoskeleton of marine arthropods was important in allowing some of their descendents to adapt to land. It provides the support needed in the less buoyant air and serves as a surface for muscle attachment that permits rapid movement. The exoskeleton of most terrestrial arthropods has a waterproof, waxy coating that reduces water loss.

Terrestrial Vertebrates

The first vertebrates on land were probably the ancestors of present-day Amphibians (Frogs, Toads, and Salamanders). The endoskeleton of vertebrates is an important prerequisite for life on land. It provides the support in the air and provides the places for muscle attachment necessary to movement. However, appendages are needed to move about. Certain bony fishes have lobe-fins, which can serve as primitive legs. It is likely that the amphibians evolved from a fish with modified fins. The first amphibians made the transition to land about 360 million years ago during the Devonian period. This was 50 million years after plants and arthropods had become established on land. Thus, when the first vertebrates developed the ability to live on land, shelter and food for herbivorous as well as carnivorous animals were available. But vertebrates faced the same problems that the insects, spiders and other invertebrates faced in their transition to life on land.

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3.5.1 Extreme Terresterial Environments

Many different habitats can be considered extreme environments, such as the polar ice caps and the driest spots in deserts. Many different places on the Earth demand that species become highly specialized if they are to survive. In particular, microscopic organisms that can't be seen with the naked eye often thrive in surprising places.

Polar Regions: Owing to the dangerously low temperatures, the number of species that can survive in these remote areas is very slim. Over years of evolution and adaptation to this extremely cold environment, both microscopic and larger species have survived and thrived no matter what conditions they have faced. By changing their eating patterns and due to their dense pelt or their body fat, only a few species have been capable of adapting to such harsh conditions and have learned how to thrive in these cold environments.

Deserts: A desert is known for its extreme temperatures and extremely dry climate. The type of species that reside in this area have adapted to these harsh conditions over years and years. Species that are able to store water and have learned how to protect themselves from the Sun's harsh rays are the only ones that are capable of surviving in these extreme environments.

3.6 PARASITIC HABITATS

In ecology, the term habitat summarises the array of resources, physical and biotic factors that are present in an area, such as to support the survival and reproduction of a particular species. A species habitat can be seen as the physical manifestation of its ecological niche. Thus "habitat" is a species-specific term, fundamentally different from concepts such as environment or vegetation assemblages, for which the term "habitat-type" is more appropriate.

The physical factors may include, for example soil, moisture, range of temperature, and light intensity. Biotic factors will include the availability of food and the presence or absence of predators. Every organism has certain habitat needs for the conditions in which it will thrive, but some are tolerant of wide variations while others are very specific in their requirements. A species habitat is not necessarily a geographical area, it can be the interior of a stem, a rotten log, a rock or a clump of moss; a parasitic organism has as its habitat the body of its host, part of the host's body (such as, the digestive tract), or a single cell within the host's body.

Geographic habitat types include polar, temperate, subtropical and tropical regions. The terrestrial vegetation type may be forest, steppe, grassland, semi-arid or desert. Fresh-water habitats include marshes, streams, rivers, lakes, and ponds; marine habitats include salt marshes, the coast, the intertidal zone, estuaries, reefs, bays, the open sea, the sea bed, deep water and submarine vents.

Habitats may change over time. Causes of change may include a violent event, such as the eruption of a volcano, an earthquake, a tsunami, a wildfire or a change in oceanic currents; or change may occur more gradually over millennia with alterations in the climate, as ice sheets and glaciers advance and retreat, and

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as different weather patterns bring changes of precipitation and solar radiation. Other changes come as a direct result of human activities, such as deforestation, the plowing of ancient grasslands, the diversion and damming of rivers, the draining of marshland and the dredging of the seabed. The introduction of alien species can have a devastating effect on native wildlife - through increased predation, through competition for resources or through the introduction of pests and diseases to which the indigenous species have no immunity.

Parasitism is a close relationship between species, where one organism, the parasite, lives on or inside another organism, the host, causing it some harm, and is adapted structurally to this way of life. The entomologist E. O. Wilson has characterised parasites as. "Predators that eat prey in units of less than one". Parasites include single-celled protozoans, such as the agents of malaria, sleeping sickness, and amoebic dysentery; animals, such as hookworms, lice, mosquitoes, and vampire bats; fungi, such as honey fungus and the agents of ringworm; and plants, such as mistletoe, dodder, and the broomrapes. There are six major parasitic strategies of exploitation of animal hosts, namely parasitic castration, directly transmitted parasitism (by contact), trophically transmitted parasitism (by being eaten), vector-transmitted parasitism, parasitoidism, and micropredation.

Etymology: First used in English in 1539, the word 'Parasite' comes from the Medieval French 'Parasite', from the Latin 'Parasitus', the 'Latinisation' of the Greek 'Parasitos', "One who eats at the table of another" and that from 'Para', 'Beside, By' + 'Sitos'. The related term parasitism appears in English from 1611.

Like predation, parasitism is a type of consumer-resource interaction, but unlike predators, parasites, with the exception of parasitoids, are typically much smaller than their hosts, do not kill them, and often live in or on their hosts for an extended period. Parasites of animals are highly specialised, and reproduce at a faster rate than their hosts. Classic examples include interactions between vertebrate hosts and tapeworms, flukes, the malaria-causing plasmodium species, and fleas.

Parasites reduce host fitness by general or specialised pathology, from parasitic castration to modification of host behaviour. Parasites increase their own fitness by exploiting hosts for resources necessary for their survival, in particular by feeding on them and by using intermediate (secondary) hosts to assist in their transmission from one definitive (primary) host to another. Although parasitism is often unambiguous, it is part of a spectrum of interactions between species, grading via parasitoidism into predation, through evolution into mutualism, and in some fungi, shading into being saprophytic.

People have known about parasites, such as roundworms and tapeworms since ancient Egypt, Greece, and Rome. In Early Modern times, Antonie van Leeuwenhoek observed Giardia lamblia in his microscope in 1681, while Francesco Redi described internal and external parasites including sheep liver fluke and ticks. Modern parasitology developed in the 19th century. In human culture, parasitism has negative connotations.

Parasitism is a kind of symbiosis, a close and persistent long-term biological interaction between a parasite and its host. Unlike saprotrophs, parasites feed on living hosts, though some parasitic fungi, for instance, may continue to feed on

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hosts they have killed. Unlike commensalism and mutualism, the parasitic relationship harms the host, either feeding on it or, as in the case of intestinal parasites, consuming some of its food. Because parasites interact with other species, they can readily act as vectors of pathogens, causing disease. Predation is by definition not a symbiosis, as the interaction is brief, but the entomologist E. O. Wilson has characterised parasites as, "Predators that eat prey in units of less than one".

Within that scope are many possible strategies. Taxonomists classify parasites in a variety of overlapping schemes, based on their interactions with their hosts and on their life cycles, which are sometimes very complex. An obligate parasite depends completely on the host to complete its life cycle, while a facultative parasite does not. Parasite life-cycles involving only one host are called 'Direct'; those with a definitive host (where the parasite reproduces sexually) and at least one intermediate host are called 'Indirect'. An endoparasite lives inside the host's body; an ectoparasite lives outside, on the host's surface.

Mesoparasites, for example enter an opening in the host's body and remain partly embedded there. Some parasites can be generalists, feeding on a wide range of hosts, but many parasites, and the majority of protozoans and helminths that parasitise animals, are specialists and extremely host-specific. An early basic, functional division of parasites distinguished microparasites and macroparasites. These each had a mathematical model assigned in order to analyse the population movements of the host–parasite groupings. The microorganisms and viruses that can reproduce and complete their life cycle within the host are known as microparasites. Macroparasites are the multicellular organisms that reproduce and complete their life cycle outside of the host or on the host's body.

Parasitism has focussed on terrestrial animal parasites of animals, such as helminths. Those in other environments and with other hosts often have analogous strategies. For example, the snubnosed eel is probably a facultative endoparasite (i.e., it is semiparasitic) that opportunistically burrows into and eats sick and dying fish. Plant-eating insects, such as scale insects, aphids, and caterpillars closely resemble ectoparasites, attacking much larger plants; they serve as vectors of bacteria, fungi and viruses which cause plant diseases. As female scale insects cannot move, they are obligate parasites, permanently attached to their hosts.

The sensory inputs that a parasite employs to identify and approach a potential host are known as 'Host Cues'. Such cues can include, for example, vibration, exhaled carbon dioxide, skin odours, visual and heat signatures, and moisture. Parasitic plants can use, for example, light, host physiochemistry, and volatiles to recognize potential hosts.

Major Parasitic Strategies

There are six major parasitic strategies, namely Parasitic Castration; Directly Transmitted Parasitism; Trophically-Transmitted Parasitism; Vector-Transmitted Parasitism; Parasitoidism; and Micropredation. These apply to parasites whose hosts are plants as well as animals. These strategies represent adaptive peaks; intermediate strategies are possible, but organisms in many different groups have consistently converged on these six, which are evolutionarily stable.

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Parasitism is a major aspect of evolutionary ecology; for example, almost all free-living animals are host to at least one species of parasite. Vertebrates, the best-studied group, are hosts to between 75,000 and 300,000 species of helminths and an uncounted number of parasitic microorganisms. On average, a mammal species hosts four species of nematode, two of trematodes, and two of cestodes. Humans have 342 species of helminth parasites, and 70 species of protozoan parasites. Some three-quarters of the links in food webs include a parasite, important in regulating host numbers. Perhaps 40 percent of described species are parasitic.

Check Your Progress

10. What is an extreme environment?
11. Define extremophiles.
12. What is parasitism?
13. Why is parasitism considered as a major aspect of evolutionary ecology?

3.7 ANSWERS TO 'CHECK YOUR PROGRESS'

1. Adaptation is the physical or behavioral characteristic of an organism that helps an organism to survive better in the surrounding environment.
2. The 'Physiological Adaptation' is a metabolic or physiologic adjustment within the cell or tissues of an organism in response to an environmental stimulus resulting in the improved ability of that organism to cope with its changing environment.
3. Adaptations that influence how an organism acts are termed as behavioural adaptations it is the change that influences the behaviour of an organism.
4. Scientists who studied adaptation prior to the development of evolutionary theory included Georges Louis Leclerc Comte de Buffon. He was a French mathematician who believed that organisms changed over time by adapting to the environments of their geographical locations.
5. Some of the species are able to switch from fresh to salt water or vice versa during their life. Salmon and Eel are the most famous examples.
6. An estuary is a partially enclosed coastal body of brackish water with one or more rivers or streams flowing into it, and with a free connection to the open sea.
7. Many estuaries suffer degeneration from a variety of factors including soil erosion, deforestation, overgrazing, overfishing and the filling of wetlands.
8. Two key adaptations mangrove trees have are the ability to survive in waterlogged and anoxic (no oxygen) soil, and the ability to tolerate brackish waters.
9. Blue crabs live in estuaries along the United States' Atlantic and Gulf coasts. They are mobile predators whose salinity requirements change at different stages in their lives.

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10. An extreme environment is a habitat that is considered very hard to survive in due to its considerably extreme conditions, such as temperature, accessibility to different energy sources or under high pressure.
11. There are organisms referred to as extremophiles that do live in such conditions and are so well-adapted that they readily grow and multiply.
12. Parasitism is a close relationship between species, where one organism, the parasite, lives on or inside another organism, the host, causing it some harm, and is adapted structurally to this way of life.
13. Parasitism is considered as a major aspect of evolutionary ecology, as almost all free-living animals are host to at least one species of parasite.

3.8 SUMMARY

- Adaptation is related to biological fitness, which governs the rate of evolution as measured by change in gene frequencies.
- An adaptive trait is an aspect of the developmental pattern of the organism which enables or enhances the probability of that organism surviving and reproducing.
- Historically, adaptation has been described from the time of the ancient Greek philosophers, namely Empedocles and Aristotle.
- Basically, there are three types of adaptations, namely structural adaptations, behavioral adaptations, and physiological adaptations.
- Adaptations that influence how an organism acts are termed as behavioural adaptations.
- In evolutionary theory, adaptation is the biological mechanism by which organisms adjust to new environments or to changes in their current environment.
- Body size is a key indicator of an animal's ability to survive and reproduce.
- Greenhouse conditions are common after major extinction events, and marine survivors find themselves living in waters that are warmer and therefore less able to hold oxygen.
- As temperatures rise, the animal's metabolic rate increases.
- Marine life, sea life, or ocean life is the plants, animals, and other organisms that live in the salt water of the sea or ocean, or the brackish water of coastal estuaries.
- Many are the marine organisms that carry out their lives in the absence of light.
- The evolutionary history of prey-predator relations has led to a wide variety of morphological and chromatic adaptations.
- An estuary is a partially enclosed coastal body of brackish water with one or more rivers or streams flowing into it, and with a free connection to the open sea.

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- Mangrove trees and blue crabs are some of the estuarine species that have adapted to unique environmental conditions.
- Some mangroves remove salt from brackish estuarine waters through ultra-filtration in their roots.
- A unique mix of marine and terrestrial species lives in mangrove ecosystems.
- Blue crabs live in estuaries along the United States' Atlantic and Gulf coasts.
- Plants and animals that can tolerate a wide range of salinities are called 'Euryhaline'.
- Freshwater biomes like rivers and lakes have water with a salt concentration of less than 1%.
- An extreme environment is a habitat that is considered very hard to survive in due to its considerably extreme conditions, such as temperature, accessibility to different energy sources or under high pressure.
- Humans generally do not inhabit extreme environments.
- A desert is known for its extreme temperatures and extremely dry climate.
- The first vertebrates on land were probably the ancestors of present-day Amphibians (Frogs, Toads, and Salamanders).
- Parasitism is a close relationship between species, where one organism, the parasite, lives on or inside another organism, the host, causing it some harm, and is adapted structurally to this way of life.
- Unlike saprotrophs, parasites feed on living hosts, though some parasitic fungi, for instance, may continue to feed on hosts they have killed.
- The sensory inputs that a parasite employs to identify and approach a potential host are known as 'Host Cues'.

3.9 KEY TERMS

- **Adaptation:** Adaptation is the evolutionary process whereby an organism becomes better able to live in its habitat or habitats.
- **Adaptive trait:** An adaptive trait is an aspect of the developmental pattern of the organism which enables or enhances the probability of that organism surviving and reproducing
- **Estuary:** An estuary is a partially enclosed coastal body of brackish water with one or more rivers or streams flowing into it, and with a free connection to the open sea.
- **Stenohaline:** Most organisms do not tolerate large variations in salinity so they are called 'Stenohaline'.
- **Euryhaline:** Plants and animals that can tolerate a wide range of salinities are called 'Euryhaline'.
- Extremophiles: Organisms which can survive in extreme environmental conditions are called extremophiles.

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3.10 SELF-ASSESSMENT QUESTIONS AND EXERCISES

Short-Answer Questions

1. In biology, what are the consequences of adaptation?
2. What is the significance of body size?
3. How organisms adapt to an environment?
4. Why estuaries are among the most productive natural habitat of the world?
5. Which organisms are called stenohaline organisms?
6. Name the different zones in lakes or ponds.
7. What are the type of extreme environments?
8. What are the necessary requirements for living organisms to survive in terrestrial habitat?
9. What are the six major parasitic strategies of exploitation of animal hosts?
10. Define host cues.

Long-Answer Questions

1. Describe the types of adaptation in detail.
2. Explain the mechanism of adaptation in detail.
3. Analyze the factors responsible for size reduction in animals.
4. Discuss the adaptations in mangrove trees.
5. Write an explanatory note on extreme aquatic environment.

3.11 FURTHER READING

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UNIT 4 ENVIRONMENTAL PHYSIOLOGY - II

NOTES

Structure

4.0 INTRODUCTION

In terms of environmental physiology, strain is the functional property of an organism to resist and survive under environmental stress, i.e., physiological adaptation or compensatory response to the external stressor. Stressors are factors that challenge the homeostasis of organism resulting in stress, Environmental stress refers to a negative subjective psychological response to an environmental stimulus. It is important to note that an environmental stimulus that is stressful for one person in

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a particular situation may not be stressful for another or for the same person in a different situation. Elastic strain is a strain which can be recovered during unloading or in other words when the applied stress is within Elastic limit the resultant strain is called Elastic strain, plastic strain is a strain which cannot be recovered after removal of the deforming force.

Stress, either physiological, biological, or psychological is an organism's response to a stressor such as an environmental condition. Stress is the body's method of reacting to a condition such as a threat, challenge or physical and psychological barrier.

Climate is the most important ecological factor determining the growth, development, and productivity of domestic animals. Adaptation refers to a shift in genotype and phenotype over generations in response to prolonged environmental pressure. Acclimation or acclimatization is the process where by an organism adjust in a smooth way to absorb the stress or shock condition that occur in the environment. Adaptation are the changes in an organism over a long period of time, whereas acclimatization are changes over a short period of time.

Organisms in aquatic and terrestrial environments must maintain the right concentration of solutes and amount of water in their body fluids. Osmoregulation is the active regulation of the osmotic pressure of an organism's body fluids. Organisms that maintain an internal osmolarity different from the medium in which they are immersed have been termed osmoregulators. They tightly regulate their body osmolarity, maintaining constant internal conditions.

In this Unit you will study about basic concept of environmental stress and strain, adaptation, acclimation and acclimatization, concept of homeostasis, physiological adaptation to osmotic and ionic stress, mechanism of cell volume regulation, physiological response to oxygen deficient stress, response to body exercise, meditation, yoga and their effects

4.1 OBJECTIVES

After going through this unit you will be able to:

- Understand the basic concept of environmental stress and strain,
- Explain adaptation, acclimation and acclimatization
- Discuss the concept of homeostasis
- Explain endothermy and physiological mechanism of regulation of body temperature
- Elaborate on physiological adaptation to osmotic and ionic stress
- Analyze the mechanism of cell volume regulation
- Define osmoregulation in aqueous and terrestrial environments
- Explain physiological response to oxygen deficient stress
- Analyze physiological response to body exercise
- Explain meditation, yoga and their effects

4.2 BASIC CONCEPT OF ENVIRONMENTAL STRESS

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In biology, 'Stress' is referred as an organism's response to a stressor, such as an environmental condition. Stress can be defined as the method in which the organism's body react to a condition, such as a threat, physical and psychological barrier. Stimuli modifies an organism's environment and are responded by various systems in the body of the organism. In humans and most mammals, the autonomic nervous system and Hypothalamic-Pituitary-Adrenal (HPA) axis are the two key systems that respond to stress.

The SympathoAdrenal Medullary (SAM) axis may activate the fight or flight response through the Sympathetic Nervous System (SNS), which contributes energy to more relevant bodily systems to acute adaptation to stress, while the Parasympathetic Nervous System (PNS) returns the body to homeostasis. The second major physiological stress-response center, the HPA axis, regulates the release of cortisol, which influences many bodily functions, such as metabolic, psychological and immunological functions. The SAM and HPA axes are regulated by several brain regions, including the limbic system, prefrontal cortex, amygdala, hypothalamus, and stria terminalis.

Because of these mechanisms, stress can alter memory functions, immune function, metabolism and susceptibility to diseases. Disease risk is particularly pertinent to mental illnesses, whereby chronic or severe stress remains a common risk factor for several mental illnesses. One system suggests there are five types of stress described as 'Acute Time-Limited Stressors', 'Brief Naturalistic Stressors', 'Stressful Event Sequences', 'Chronic Stressors', and 'Distant Stressors'. An acute time-limited stressor requires a short-term challenge, while a concise natural stressor requires an event that is normal but though difficult. A stressful event sequence is a stressor that occurs, and then continues to yield stress into the immediate future. A chronic stressor involves exposure to a long-term stressor, and a distant stressor is a stressor that is not immediate.

4.2.1 Biological Environment and Stress

Stress can have several profound and theoretical effects on the human biological systems. Biology principally attempts to describe key concepts of stress using a stimulus-response paradigm, generally equivalent to how a psychobiological sensory system function. The Central Nervous System or CNS, which includes brain and spinal cord, plays a crucial and significant role in the stress-related mechanisms in a body. The CNS functions closely with the body's endocrine system in order to regulate these mechanisms. The sympathetic nervous system is primarily active during a stress response in order to regulate the physiological functions of the body using techniques that should make an organism more adaptive to its environment.

Stress can be either severe, acute stress or chronic low-grade stress and may induce or stimulate abnormalities in three principal regulatory systems in the body, namely Serotonin Systems, Catecholamine Systems and the Hypothalamic-

Pituitary-Adrenocortical Axis. Aggressive behaviour can also be associated with abnormalities in these three systems.

4.2.2 Biological Requirement for Equilibrium

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Homeostasis is an essential concept of stress. In biology, most biochemical processes endeavour to maintain equilibrium (homeostasis), a constant state that exists more than as an ideal and less as a feasible and possible condition. Environmental factors, internal or external stimuli, continually disrupt homeostasis; basically, an organism's present condition is considered as a state of constant flux moving about a homeostatic point such that the organism has optimal condition for living. Factors causing an organism's condition to diverge too far from homeostasis can be experienced as stress. A life threatening condition, such as a most important physical trauma or prolonged starvation can greatly disrupt homeostasis. Alternatively, an organism's attempt to restore conditions back to or near homeostasis, often consumes energy and natural resources, can also be interpreted as stress.

The ambiguity in defining this phenomenon was first recognized by Hans Selye (1907–1982) in 1926. In 1951 a commentator roughly summarized Selye's view of stress as, "Something that in addition to being itself was also the cause of itself, and the result of itself".

First to use the term in a biological context, Selye continued to define stress as, "The non-specific response of the body to any demand placed upon it". Neuroscientists, Bruce McEwen and Jaap Koolhaas defined stress based on years of empirical research as, "Should be restricted to conditions where an environmental demand exceeds the natural regulatory capacity of an organism". Certainly, in 1995 Toates defined stress as, "A chronic state that arises only when defense mechanisms are either being chronically stretched or are actually failing", while according to Ursin (1988) stress results from an inconsistency between expected events as "Set Value" and perceived events as "Actual Value" that cannot be resolved satisfactorily, which also puts stress into the broader context of cognitive-consistency theory.

4.2.3 Biology of Stress

In the brain, the endocrine interactions are significant and essential for the translation of stress into physiological and psychological changes. The Autonomic Nervous System (ANS) plays an important role in translating stress into a response. The ANS responds reflexively to both physical stressors, for example baroreceptor and to higher level inputs from the brain.

Baroreceptors are sensors that are located in the carotid sinus, at the bifurcation of external and internal carotids, and in the aortic arch. They sense the blood pressure and relay the information to the brain, so that a proper blood pressure can be maintained. Baroreceptors are specific type of mechanoreceptor sensory neuron that are excited by a stretch of the blood vessel. Thus, increases in the pressure of blood vessel triggers increased action potential generation rates and provides information to the central nervous system. This sensory information

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is used primarily in autonomic reflexes that in turn influence the heart cardiac output and vascular smooth muscle to influence vascular resistance.

The ANS is comprised of the Parasympathetic Nervous System (PNS) and Sympathetic Nervous System (SNS), both the two branches are active with opposing activities. The ANS directly innervates tissue through the postganglionic nerves, which is controlled by preganglionic neurons originating in the intermediolateral cell column. The ANS receives inputs from the medulla, hypothalamus, limbic system, prefrontal cortex, midbrain and monoamine nuclei.

The activity of the sympathetic nervous system describes the 'Fight or Flight' response. The fight or flight response to stress involves mydriasis, increased heart rate and force contraction, vasoconstriction, bronchodilation, glycogenolysis, gluconeogenesis, lipolysis, sweating, decreased motility of the digestive system, secretion of the epinephrine and cortisol from the adrenal medulla, and relaxation of the bladder wall. The parasympathetic nervous response, 'Rest and Digest', is essential to maintain homeostasis, and involves miosis, bronchoconstriction, increased activity of the digestive system, and contraction of the bladder walls. ANS related mechanisms contribute to increased risk of cardiovascular disease after key stressful events.

The HPA axis is considered as a neuroendocrine system that mediates a stress response. Neurons in the hypothalamus, particularly the paraventricular nucleus, release vasopressin and corticotropin releasing hormone, which travel through the hypophysial portal vessel where they travel to and bind to the corticotropin-releasing hormone receptor on the anterior pituitary gland. Multiple CRH (Corticotropin Releasing Hormone) peptides have been identified and receptors have been identified on multiple areas of the brain, including the amygdala. CRH is the main regulatory molecule of the release of ACTH (AdrenoCorticoTropic Hormone).

The secretion of ACTH into systemic circulation allows it to bind to and activate Melanocortin receptor, where it stimulates the release of steroid hormones. Steroid hormones bind to glucocorticoid receptors in the brain, providing negative feedback by reducing ACTH release.

The immune system can be strongly influenced by stress. The sympathetic nervous system innervates various immunological structures, such as bone marrow and the spleen, allowing for it to regulate immune function. The adrenergic substances released by the sympathetic nervous system can also bind to and influence various immunological cells to provide a connection between the systems. The HPA axis ultimately results in the release of cortisol, which generally has immunosuppressive effects. Even though, the effect of stress on the immune system is uncertain, and various models have been defined for both the evidently 'Immunodeficiency' linked diseases and diseases involving hyper activation of the immune system.

4.2.4 Environmental Stress

Environmental stress refers to physical, chemical, and biological constraints on the productivity of species and on the development of ecosystems. The ecological responses result when the intensity of exposure to environmental stressors increases or decreases.

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Stressors can be natural environmental factors, or they may result from the activities of humans. Some environmental stressors exert a relatively local influence, while others are regional or global in their scope. Stressors are challenges to the integrity of ecosystems and to the quality of the environment.

Environmental stressors can be defined into the following significant categories:

1. Physical Stress

Physical stress describes the extreme exposures to kinetic energy, i.e., form of ecological disturbance, for examples volcanic eruptions, windstorms, and explosions.

2. Wildfire

Wildfire is referred as a powerful disturbance through which a large amount of the biomass of an ecosystem is combusted, and the major dominant species may be destroyed.

3. Pollution

Pollution happens when chemicals are present in significant concentrations that affect organisms and cause ecological changes. Pollution is the presence of contaminants into the natural environment that cause adverse change. Pollution can take the form of any substance (solid, liquid, or gas) or energy (such as, radioactivity, heat, sound, or light). Pollutants, the components of pollution, can be either foreign substances/energies or naturally occurring contaminants. Even though environmental pollution can be caused by natural events, the term pollution generally indicates that the contaminants have an anthropogenic source, i.e., a source created by human activities. Pollution is often classed as point source or nonpoint source pollution. Major forms of pollution include air pollution, light pollution, litter, noise pollution, plastic pollution, soil contamination, radioactive contamination, thermal pollution, visual pollution, and water pollution.

Following are the main forms of pollution with the relevant contaminants:

Air Pollution: The release of chemicals and particulates into the atmosphere. Common gaseous pollutants include carbon monoxide, sulfur dioxide, ChloroFluoroCarbons (CFCs) and nitrogen oxides produced by industry and motor vehicles. Photochemical ozone and smog are created as nitrogen oxides and hydrocarbons react to sunlight. Particulate matter, or fine dust is characterized by their micrometre size PM10 to PM2.5.

Electromagnetic Pollution: This is referred as the accumulation of electromagnetic radiation in their non-ionizing form, like radio waves, etc., that people are constantly exposed at, especially in large cities.

Light Pollution: It includes light trespass, over-illumination and astronomical interference.

Noise Pollution: It encompasses roadway noise, aircraft noise, industrial noise as well as high-intensity sonar.

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Plastic Pollution: It involves the accumulation of plastic products and microplastics in the environment that adversely affects wildlife, wildlife habitat, or humans.

Soil Contamination: It occurs when chemicals are released by spill or underground leakage. Among the most significant soil contaminants are hydrocarbons, heavy metals, MTBE, herbicides, pesticides and chlorinated hydrocarbons.

Radioactive Contamination: It results from 20th century activities in atomic physics, such as nuclear power generation and nuclear weapons research, manufacture and deployment.

Thermal Pollution: It refers to the temperature change in natural water bodies caused by human influence, such as use of water as coolant in a power plant.

Water Pollution: It refers to the discharge of industrial wastewater from commercial and industrial waste (intentionally or through spills) into surface waters; discharges of untreated sewage, and chemical contaminants, such as chlorine, from treated sewage; release of waste and contaminants into surface runoff flowing to surface waters (including urban runoff and agricultural runoff, which may contain chemical fertilizers and pesticides); groundwater pollution from waste disposal and leaching into the ground, including from pit latrines and septic tanks; eutrophication and littering.

4. Thermal Stress

Thermal stress happens when releases of heat influence ecosystems, such as in the vicinity of natural hot-water vents on the ocean floor, and near industrial discharges of heated water.

5. Radiation Stress

Radiation stress is associated with excessive loads of ionizing energy. This can occur on mountain tops where there are intense exposures to ultraviolet radiation, and in places where there are exposures to radioactive materials.

6. Climatic Stress

Climatic stress is associated with excessive or insufficient regimes of temperature, moisture, solar radiation, and combinations of these. Tundra and deserts are examples of climatically stressed ecosystems, while tropical rainforests occur under a relatively benign climatic regime.

7. Biological Stress

Biological stresses are associated with the diverse interactions that occur among organisms of the same or different species. Biological stresses can result from competition, herbivory, predation, parasitism, and disease. The harvesting and management of species and ecosystems by humans is a type of biological stress. The introduction of invasive, non-native species may be regarded as a type of biological pollution.

8. Biotic Stress

Biotic stress is stress that occurs as a result of damage done to an organism by other living organisms, such as bacteria, viruses, fungi, parasites, beneficial and

harmful insects, weeds, and cultivated or native plants. It is different from abiotic stress, which is the negative impact of non-living factors on the organisms, such as temperature, sunlight, wind, salinity, flooding and drought. The types of biotic stresses imposed on an organism depend the climate where it lives as well as the species' ability to resist particular stresses. Biotic stress remains a broadly defined term and those who study it face many challenges, such as the greater difficulty in controlling biotic stresses in an experimental context compared to abiotic stress.

The damage caused by these various living and nonliving agents can appear very similar. Even with close observation, accurate diagnosis can be difficult. For example, browning of leaves on an oak tree caused by drought stress may appear similar to leaf browning caused by oak wilt, a serious vascular disease caused by a fungus, or the browning caused by anthracnose, a fairly minor leaf disease.

4.2.5 Stress Resistance

Species and ecosystems have some capacity to tolerate changes in the intensity of environmental stressors. This is known as **resistance**, but there are limits which represent thresholds of tolerance. When these thresholds are exceeded by further increases in the intensity of environmental stress, substantial ecological changes are initiated.

Ability of the organisms to survive under adverse environmental condition is termed as **stress resistance**. The stress resistance of biological organisms is of two main types.

Elastic Resistance: Ability of the organism to prevent reversible or elastic strain (physical or chemical change) when exposed to a specific stress.

Plastic Resistance: Plastic resistance is the ability of an organism to prevent irreversible or plastic strain. The term resistance to environmental stress has been used for plastic resistance.

An organism can show a dynamic physiological response to environmental changes at more extreme range beyond the 'Region of Tolerance' which is defined as the 'Region of Resistance', for example the Polar bears can live at extremely cold arctic regions tolerating the ultimate cold condition.

4.2.6 Stress Avoidance and Stress Tolerance

When organisms are exposed to an extreme environmental stress, one of three possible outcomes takes place, namely the organisms may die, the organisms avoid the environmental stress and survive, or the organisms tolerates the environmental stress and survive. The rare organisms use to survive extreme environmental stresses including freezing, desiccation, intense heat, irradiation, and low-oxygen conditions (hypoxia). The molecular mechanisms involved in environmental stress tolerance defines that these tolerances can be regulated and the organisms can survive in the unavoidable environmental conditions.

Tolerance: Every organism has the capability to compensate for environmental variation as its genetic ability provided that it should not go beyond the specified range of tolerance, for example under the standard conditions, a catfish can survive

at temperatures ranging from 1p C to 35p C. This temperature is referred as the range of tolerance for the catfish, above or below this standard temperature the catfish may die after a resistance time.

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Check Your Progress

1. Define stress.
2. What do you understand by baroreceptors?
3. Define environmental stress.
4. What is electromagnetic pollution?
5. What is resistance?

4.3 BASIC CONCEPT OF STRAIN

In biology, a strain is a genetic variant, a subtype or a culture within a biological species. Strains are often seen as inherently artificial concepts, characterized by a specific intent for genetic isolation. This is most easily observed in microbiology where strains are derived from a single cell colony and are typically quarantined by the physical constraints of a petri dish. Strains are also commonly referred to within virology, botany, and with rodents used in experimental studies.

A strain is a genetic variant or subtype of a microorganism, such as a virus, bacterium or fungus. For example, a 'Flu Strain' is a certain biological form of the influenza or 'Flu' virus. These flu strains are characterized by their differing isoforms of surface proteins. New viral strains can be created due to mutation or swapping of genetic components when two or more viruses infect the same cell in nature. These phenomena are known respectively as antigenic drift and antigenic shift. Microbial strains can also be differentiated by their genetic makeup using metagenomic methods to maximize resolution within species. This has become a valuable tool to analyse the microbiome. Scientists have modified strains of viruses in order to study their behaviour, as in the case of the H5N1 influenza virus.

In terms of environmental physiology, strain is the functional property of an organism to resist and survive under environmental stress, i.e., physiological adaptation or compensatory response to the external stressor.

Stressor

A **stressor** is a chemical or biological agent, environmental condition, external stimulus or an event seen as causing stress to an organism.

Stressors can cause physical, chemical and mental responses internally. Physical stressors produce mechanical stresses on skin, bones, ligaments, tendons, muscles and nerves that cause tissue deformation and (in extreme cases) tissue failure. Chemical stresses also produce biomechanical responses associated with metabolism and tissue repair. Physical stressors may produce pain and impair work performance. Chronic pain and impairment requiring medical attention may result from extreme physical stressors or if there is not sufficient recovery time between successive exposures.

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Stressors may also affect mental function and performance. One possible mechanism involves stimulation of the Hypothalamus, CRF (Corticotropin Release Factor) → Pituitary Gland releases ACTH (AdrenoCorticoTropic Hormone) → Adrenal Cortex secretes various Stress Hormones (e.g., Cortisol) → Stress Hormones travel in the Blood Stream to relevant Organs, e.g., Glands, Heart, Intestines → flight-or-fight response. Between this flows there is an alternate path that can be taken after the stressor is transferred to the hypothalamus, which leads to the sympathetic nervous system; after which the adrenal medulla secretes epinephrine.

Elastic Strain

The elastic strain can be completely reversible, i.e., when the stress is reduced or reversed then the organism becomes normal.

Plastic Strain

In case of the plastic strain, the strain may be partially reversible or partially irreversible. Therefore, the plastic strain is also termed as permanent strain.

4.4 ADAPTATION, ACCLIMATION AND ACCLIMATIZATION

Adaptation is a generic phrase encompassing all changes undertaken by an organism to reduce the negative effects of unfamiliar and undesirable external environment. This includes both acclimatization and acclimation.

Adaptation describes a spectrum of changes that follow acute and repeated exposure incorporating genotypic (changes within the genome) and phenotypic (within an organism's physiology) adaptations. It may also include behavioural responses including reduced activity levels.

Acclimatization describes adaptive physiological or behavioural changes within an organism in response to their natural climate or environment.

Acclimation differs from acclimatization in that rather than adaptive characteristics being augmented in a natural climate or environment, the stimuli for adaptation is artificially induced, typically within an enclosed chamber within which ambient conditions (temperature/oxygen content) are altered.

4.4.1 Adaptation

The changes in the organism by which it suits best in the environment and leave behind offspring's are known as adaptation. Had there been no adaptation, evolution would have been impossible.

The capability of an organism to change itself with the changing world determines its survival. Greater the degree of adaptation, higher is the ability to exploit the environment resulting in the production of greater number of offspring's and consequent higher chances of survival of the species. Adaptation to environment is one of the basic characteristics of the living organisms. Living organisms are

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plastic and possess the inherent properties to respond to a particular environment. Adaptation to environmental dynamics is a biological process eternally operating in nature.

It is a facet of evolution and involve structural diversities amongst living organisms that are heritable. Organisms exhibit numerous structural and functional adaptations that help them to survive as species and to overcome the tremendous competition in nature.

Living organisms show the following two basic properties:

(i) Adaptability

(ii) Adaptation

The term adaptability is applied to the power of orientation of the organisms to new environmental condition. All organisms possess the power of adaptability to a limited extent to various environmental changes. Mammals are adaptable to various climatic conditions.

Adaptability and adaptation are two quite separate biological processes. Adaptation is defined as the permanent moulding in organisms to such an extent that they can live in a particular environment suitably. It is a characteristic of living forms which develop over a length of time, when certain morphological and physiological modifications are initiated that enable them to survive within the jurisdiction of a particular environmental condition.

All animals harmoniously live in different ecological conditions. Every region has a peculiar and characteristic physical condition, which indirectly gives rise to varied forms. The island fauna represents the typical examples. The evolutionary history of the animals also gives big number of instances of adaptive changes.

Fishes are the primary aquatic vertebrates which show all the basic adaptations to their primal aquatic home. From fishes the amphibia, the first tetrapod evolved. Having appeared on land they had to modify to live in a completely different environment. The amphibians show adaptive duality. They show modifications for aquatic medium as well as for terrestrial living.

The reproductive system in amphibia is not adjusted for terrestrial life, they had to come back to the watery home for the purpose of reproduction. The reptiles are the true land adapted forms in the phylogenetic history of vertebrates.

The reptiles hold the pivotal position from which both birds and mammals evolved and underwent parallel evolution. These living organisms have shown all the possible ways of living and furnish extensive adaptive radiations.

Adaptive Convergence and Divergence: As a result of living in a similar environment, organisms of quite distant and unrelated groups show close structural and functional convergence. The reverse is also true in nature where organisms originating from the same stock exhibit adaptive divergence as a response to living in completely different environment. All secondary aquatic vertebrates show adaptive convergence. Animals belonging to different groups living under similar life conditions show similar adaptation. This is known as convergence or parallelism. Animals belonging to same stock migrate to different geographical areas to meet their need

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for food and safety and are subjected to different life conditions. This leads to varied and diverse adaptations. This is called divergence.

Structural and Functional Adaptations: The adaptations exhibited by organisms to a particular environment are both structural as well as functional in nature. In a particular environment, the structural and functional adaptations are almost inseparable. The typical case of functional adaptation is the modifications of the gastrointestinal tract in vertebrate body which is due to adaptation to different types of food. But both these modifications work harmoniously in an organism to suit a particular environment.

Adaptation by Organisms in Relation to Environments: The different adaptations exhibited by the organisms depend upon their surroundings. The environment of an organism is not only the physical environment, but also includes the biogeochemical (biological, geological and chemical) and biotic environments. Of all these, light, temperature and water are the three main factors.

Light causes modifications of the eyes in vertebrates, temperature plays a very important role and the water seems to be a very important physical factor, especially from the ecological point of view. Some animals are adapted completely within watery medium, and others are adapted to land. Structural adaptations for living in these environments are quite obvious and contrasting.

Besides these important factors, such as light, temperature and water, other chemical and nutritional factors also play great role in adaptations. These impelling factors have caused the adaptive radiations amongst animals. Most of the vertebrates except fishes and some secondarily aquatic forms are adapted to terrestrial life. The terrestrial forms also exhibit divergent adaptive lines.

4.4.2 Acclimation and Acclimatization

Acclimatization or acclimatisation, also called acclimation or acclimatation, is the process in which an individual organism adjusts to a change in its environment, such as a change in altitude, temperature, humidity, photoperiod, or pH, allowing it to maintain fitness across a range of environmental conditions. Acclimatization occurs in a short period of time (hours to weeks), and within the organism's lifetime compared to adaptation, which is evolution, taking place over many generations. This may be a discrete occurrence, for example when mountaineers acclimate to high altitude over hours or days, or may instead represent part of a periodic cycle, such as a mammal shedding heavy winter fur in favour of a lighter summer coat. Organisms can adjust their morphological, behavioural, physical, and/or biochemical traits in response to changes in their environment.

Biochemical

In order to maintain performance across a range of environmental conditions, there are several strategies organisms use to acclimate. In response to changes in temperature, organisms can change the biochemistry of cell membranes making them more fluid in cold temperatures and less fluid in warm temperatures by increasing the number of membrane proteins. In response to certain stressors, some organisms express the heat shock proteins that act as molecular chaperones and reduce denaturation by guiding the folding and refolding of proteins. It has

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been shown that organisms which are acclimated to high or low temperatures display relatively high resting levels of heat shock proteins so that when they are exposed to even more extreme temperatures the proteins are readily available. Expression of heat shock proteins and regulation of membrane fluidity are the two significant biochemical methods organisms use to acclimate to novel environments.

Morphological

Organisms are capable to change several characteristics relating to their morphology in order to maintain performance in novel environments. For example, birds often increase their organ size to increase their metabolism. This can take the form of an increase in the mass of nutritional organs or heat-producing organs, like the pectorals with the latter being more consistent across species.

The degree to which organisms are capable to acclimate is defined by their phenotypic plasticity or the ability of an organism to change certain traits. Recent research in the study of acclimation capacity has focused more on the evolution of phenotypic plasticity rather than acclimation responses. Scientists believe that when they understand more about how organisms evolved the capacity to acclimate, they will better understand acclimation.

Animals

Animals acclimatize in many ways. Sheep grow very thick wool in cold, damp climates. Fish are able to adjust only gradually to changes in water temperature and quality. Tropical fish sold at pet stores are often kept in acclimatization bags until this process is complete. Lowe and Vance (1995) were able to show that lizards acclimated to warm temperatures could maintain a higher running speed at warmer temperatures than lizards that were not acclimated to warm conditions. Fruit flies that develop at relatively cooler or warmer temperatures have increased cold or heat tolerance as adults, respectively.

Humans

In humans, the salt content of sweat and urine decreases as people acclimatize to hot conditions. Plasma volume, heart rate, and capillary activation are also affected. Acclimatization to high altitude continues for months or even years after initial ascent, and ultimately enables humans to survive in an environment that, without acclimatization, would kill them. Humans who migrate permanently to a higher altitude naturally acclimatize to their new environment by developing an increase in the number of red blood cells to increase the oxygen carrying capacity of the blood, in order to compensate for lower levels of oxygen intake.

Check Your Progress

6. Define strain.
7. What is a stressor?
8. What do you understand by the term 'adaptation'?
9. Why do animals belong to the same stock migrate to different geographical areas?

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4.5 CONCEPT OF HOMEOSTASIS

The term homeostasis refers to the maintenance of stable condition in living organisms through involuntary mechanism which works against the system trying to disturb the balance. In other words homeostasis means the ability of organisms to maintain uniformity in the internal conditions without being effected by the external changes.

The most complex organism is human beings and the maintenance of internal stability requires regular supervision and adjustment of several things such as:

- Carbon dioxide
- Oxygen
- Temperature
- Fluid level
- Nutrients
- Hormones
- Organic and inorganic substances

Though the changes occur externally, the internal composition of these substances remain constant.

When this mechanism occurs in smaller organisms such as molecules it is known as *feedback inhibition* and it works to regulate the quantity of chemical product produced by an enzyme system.

The chronological arrangement of enzymes that converts the metabolite into the end product needed by the organisms is known as the enzyme system.

The production of the enzymes is regulated by the inhibitory effect that the end product enzyme has on the first one when the end product is used up by the body, the effect that it had on the first enzyme reduces and the need to develop a new end product is acknowledged by the enzyme system and the vacant place of the used enzyme is again filled up by a new end product. This inhibitory effect also regulates the overproduction of the end product. Thus the amount of enzymes remains constant.

Homeostatic systems exhibit several properties which are as follows:

- Stability
- Whole association, internal, structural, and functional, contributes to the maintenance of balance
- Unpredictable

Mechanisms of Homeostasis

There are two main types of feedback to which the system reacts:

- *Negative feedback* is a reaction in which the system responds in such a way as to reverse the direction of change to maintain homeostasis

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- *Positive feedback* is a reaction in which the response intensifies the change in the variable and has a destabilizing effect, so does not result in homeostasis.

The homeostasis that is present in cells is known as *contact inhibition*. In such an event the cell division is regulated at the time when their number increases to the extent that they come in contact with each other. It is assumed that this happens when a *chemical messenger cell* passes on the information to stop the division of cell. This phenomenon is reversed in case of simulated cancer cells which keep on dividing even after they touch each other.

The endocrine system also functions on this basic concept. The homeostasis is illustrated in the endocrine glands by the hormone production process. The hormone production is regulated by the actions that take place in the system. Such an example is seen in the pancreas which secretes insulin as and when the level of blood glucose rises above normal. This helps in removing the glucose from the blood and converting it into glycogen and fats that is stored in the body tissues to be used when required such as during heavy exercises or running.

The process of feeling hungry and thirsty in living beings is also the result of homeostasis.

This basic concept also applies to the fact of all living creatures to survive in controlled population. The quantity of prey required by the predator is survived . When the population of the prey decreases due to predation, the predator population also shrinks and the prey population again gets time to be enlarged. Thus the ecological balance is maintained.

Homeostasis is maintained by several consistent mechanisms in the organism to attain a state of symmetry. The internal stability is acquired by simultaneous adjustment with the external environment. The phenomenon occurs when human beings are exposed to higher temperature than the normal body temperature of 98.6º F. The sweating of the human body is the homeostasis process to cool down the heated body and maintain equilibrium.

Homeostasis is present in regulating the temperature and blood pressure of human beings nearly constant though the activity level of the being or the environment changes.

During accidental cut or bruise, the automatic clotting of blood is also example of homeostasis. This homeostatic interaction between the platelet and blood clotting protein to produce haemostatic plug helps to prevent haemorrhage that might turn into critical incurable condition.

Although there are many haemostatic mechanisms that control the systematic and steady performance of the organisms, still some disorders related to haemorrhagic or prothrombotic diathes are evident that results in critical conditions.

The blood pressure is regulated by haemostatic mechanisms that maintain the blood pressure levels. Stretch-sensitive cells which are present in the arteries in the neck act as sensors. These arteries transport blood from heart to brain. When the blood pressure rises, the sensors sends alarm signal to the brain. The nerve that supplies blood to the heart called vagus releases acetylcholine which slows the heart rate and eventually blood pressure decreases.

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Similar haemostatic mechanisms regulate the amount of blood that flows in the body. When there is any change in the pressure in the capillaries the plasma travel between and the intercellular fluid the capillaries. Sensors are located at the base of the brain. When the volume of blood decreases the sensors secretes some substance that originates contraction of muscles which encloses the blood vessels leading to the capillaries. This contraction reduces the blood flow to the capillaries and the blood volume is thereby restored to normal.

The temperature of the body of mammals is synchronized through sensors located in the hypothal¬amus of the brain. There are many things that effect the body temperature such as:

- Nonspecific muscle activity such as shivering
- Sweating
- Panting
- Vasodilation which is opening of new blood vessels in the skin
- Piloerection which means thickening of fur that decreases heat loss
- Curling up

Overview of Haemostasis

Haemostasis is a dynamic process whereby blood coagulation is initiated and terminated in a rapid and tightly regulated fashion. According to this postulate the stopping of blood flow from an injured blood vessel happens due to the guard mechanism of the human body to fight for survival.

The platelets stick to the macromolecules in subendothelial tissues at the spot of injury and then comprehensively form haemostatic plug to stop bleeding. To further strengthen the haemostatic plug, the platelets stimulate the creation of plasma coagulation feature to create fibrin clot at the injury spot.
As and when the wound gets healed the collected platelet and fibrin clot breaks down and is detached.

The regulation of haemostasis is controlled by three fundamental components namely:

1. Vascular wall
2. Platelets
3. Coagulation cascade

As advocated by Kumar, Abbas, & Faousto in 2005 there is a set of regulated procedures that helps in the occurence of normal haemostasis. This helps in solving two purposes which are as follows:

- To keep blood in clot-free state that is easy to flow
- To stimulate a speedy and enclosed haemostatic plug at the spot of vascular injury

Coagulation of blood results after the thrombin enzyme is created and combines with proteolyzes soluble plasma fibrinogen to form insoluble fibrin polymer.

Hoffbrand, Catovsky, & Tuddenham stated that the mechanism which regulates the formation of collective platelet and fibrin clots are vital to maintain the flowing capacity of blood.

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Historical Background

The clotting of blood at the site of injury was well known by Aristotle, Hippocrates, Celsius, and Galen. This inclination of internal and external bleeding to occur was further illustrated by observation that blood congealed or firms up on cooling and the wound heals up and clots if left in open air. They thought that blood coagulability was not due to concept of haemostasis[2].

In 1720, French surgeon Jean-Louis Petit observed that clots formed in the blood vessels after amputation and that was a result of haemostasis. In 1828, Swiss physician Friedrich Hopff observed that hypocoagulability is the reason behind familial bleeding tendency in males. This tendency is now identified as haemophilia which is an X chromosome linked disorder in males. This paved the way to the belief that coagulability was necessary to prevent bleeding. In 1860, German pathologist Rudolf Virchow explained blood clots and their urge to embolize.The purpose of platelets and various components of the coagulation procedure was recognized leading to standard theory of coagulation by Paul Morawitz in 1905. According to Paul's postulate prothrombin transforms into thrombin in the presence of calcium and thromboplastin. Then thrombin transforms fibrinogen to fibrin. The fact that blood clotting elements were presents in fluid blood and does not clot until a wet surface is available for clotting.

The homeostatic mechanisms involve the neural and endocrine systems of mammals. Such preparations saturate systems from genes to biological communities and they are utilized both by the simplest and the intricate organisms.

Homeostasis in plants occurs when the amount of leaf water is maintained by the opening and closing of the stomata.

The concept of homeostasis is also appropriate to biological communities.

The term 'homeostasis' is also used in cybernetics to allocate any autoregulatory mechanism.

Biological Homeostasis

Homeostasis is one of the primary features of living things. It is the regulation of the internal environment within limits that can be tolerated.

The internal environment of a living organism's body has body fluids in multicellular animals. Blood Plasma, tissue fluid and intracellular fluid comprise the body fluid. The maintenance of a constant state in these fluids is necessary for living things as its lacking causes harm to the genetic material.

With respect to any parameter, an organism can be a conformer or a regulator. Regulators attempt at maintaining the parameter at a steady level, irrespective of what is occuring in its environment. Conformers permit the environment to decide the parameter. For example, endothermic animals keep up a steady body temperature, whereas ectothermic animals show broad differences in body temperature.

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This does not mean that conformers may not have behavioral adaptations that permit them to apply certain control over the parameter in question. For example, reptiles generally sit on sun-heated rocks in the morning to increase their body temperatures.

A merit of homeostatic regulation is that it permits more effective functioning of the organism. For example, ectotherms have the tendency to become sluggish at reduced temperatures, while endotherms are usually active. On the other hand, regulation needs energy. One reason snakes can eat only once a week is due to much less energy utilization by them for maintaining homeostasis.

Homeostasis In The Human Body

All kinds of factors affect the suitability of the human body fluids for sustaining life; characteristics such as temperature, salinity and acidity, and the concentrations of nutrients such as glucose, various ions, oxygen, and wastes, such as carbon dioxide and urea are included in this. As these properties affect the chemical reactions that are responsible for keeping the bodies alive, there are built-in physiological mechanisms for maintaining them at the desired levels.

In any case, it needs to be noted that homeostasis is not the reason for these continuous unconscious adjustments. Homeostasis must be considered as a normal characterization of several usual procedures in concert, not their proximal cause per se. Moreover, there are several biological phenomena which are not in compliance with this model, like anabolism.

4.5.1 Theories of Blood Coagulation

Coagulation is clotting of blood. This process is extremely complicated and complex and is a prime component of haemostasis which is the termination of stopping of loss of blood from a damaged vessel, in which the wall of the blood vessel that is damaged gets covered by a clot that contains fibrin and platelet to mend the vessel and cause the bleeding to cease. Coagulation disorders can cause haemorrhage (excessive bleeding) or thrombosis (obstructive clotting).

Coagulation works with two components namely protein (coagulation factor) and cellular (platelet). Coagulation starts as soon as the lining of a blood vessel (endothelium) is damages by an injury.

When blood gets protein exposure, changes are initiated in the blood platelets and the plasma protein fibrinogen, which is a factor in clotting. Primary haemostasis occurs, which means that the platelets instantly create a plug where the vascular injury is. At the same time, Secondary haemostasis also takes place. The proteins that are present in the blood plasma are referred to as clotting factors/coagulation factors or, react in a intricate cascade to make fibrin strands that provide strength to the platelet plug. Later, as the injury heals the break down and removal of the fibrin clot and platelet aggregate takes place.

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Haemostasis is a dynamic process in which blood coagulation starts and stops in a quick and synchronized manner. It keeps the blood clot free and in fluid state and it produces a swift and contained haemostatic plug at the vascular injury site.

Blood coagulation forms a part of the vital mechanism of host defense.

The three components that regulate haemostasis are as follows:

1. Coagulation cascade
2. Platelets
3. Vascular wall

Blood coagulates when thrombin enzyme is created which proteolyzes soluble plasma fibrinogen, to produce a clot which is insoluble fibrin polymer. Also important for blood are those systems that restrain platelet aggregates from being formed as also fibrin clots at the sites of vascular injury as they keep blood fluid.

The Coagulation/Waterfall Cascade

Roman numerals are used to represent coagulation factors in plasma. The numbers are attached to the factors in the order in which they were discovered. Significantly, the numeric system that was adopted assigned the number to the factor according to the sequence of discovery and not to the point of interaction in the cascade.

The factors are as shown in Table 4.1.

Table 4.1 *Coagulation Factors in Plasma*

Factor	I	(Fibrinogen)
Factor	II	(Prothrombin)
Factor	III	(Thromboplastin)
Factor	IV	(Calcium)
Factor	V	(Ac-Globulin, proaccelerin, labile factor)
Factor	VII	(Proconvertin, Spca)
Factor	VIII	(Antihaemophilic Factor)
Factor	Ix	(Plasma thromboplastin componeint-PTC)
Factor	X	(Stuart-Prower Factor)
Factor	XI	(Plasma thromboplastiin acntecedent-PTA)
Factor	XII	(Hageman Factor

There was no clarity on what the interaction was between factors to prothrombin to thrombin to form a fibrin clot. The year 2003 provided this knowledge to the world and opened new understanding.

In the Waterfall/Cascade model, there are two distinct initiations: intrinsic (contact) and extrinsic pathways (Figure 4.1). These become one at the level of Factor Xa (common pathway).

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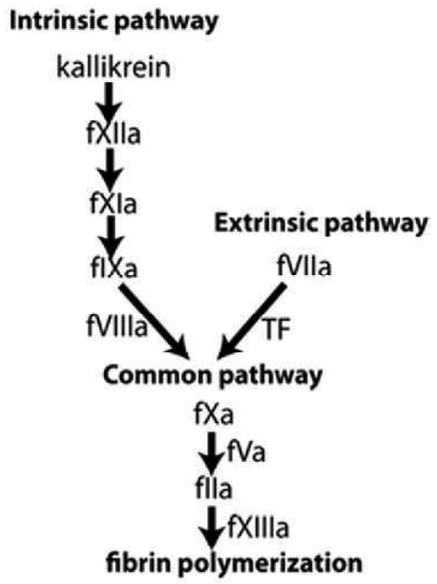

Fig. 4.1 *Fibrin Polymerization*

Intrinsic Pathway

The intrinsic pathway comprises a cascade of protease reactions that are triggered by factors present in the blood. When it touches a surface that has negative charge, like an activated platelet, a plasma protein named FXII(Hageman factor) changes to FXIIa (the 'a' implies an activated version). The molecule HMWK (high molecular weight kininogen) which is a product of platelets that might be connected to the platelet membrane, enable FXII to be attached to the charged surface by playing the role of a cofactor. This process is slow. After some FXIIa accumulates, this protease changes prekallikrein to kallikrein, and HMWK haves as an anchor. The fresh kallikrein speeds up the process of FXII to FXIIa conversion. Besides increasing its own production by creating kallikrein, FXIIa proteolytically cleaves FIX to FIXa, also a protease.

FIXa along with two downstream products of the cascade, thrombin and FXa, cleave proteolytically FVIII, to make FVIIIa that is a cofactor in the succeeding reaction. Lastly, FVIIIa and FIXa along with Ca2+ and phospholipids with negative charge make a trimolecular complex termed tenase. Then, tenase changes FX to FXa, another protease.

A series of parallel interactions, binds FXa to the cofactor FVa. This generates a complex with enzymatic activity known as prothombinase, which changes the proenzyme prothrombin to thrombin, its enzyme form. Thrombins action on fibrinogen creates the fibrin monomer whose swift polymerization makes fibrin clot. Laboratories analyse blood clotting, by using the activated partial thromboplastin time (PTT), to evaluate the intrinsic pathway of blood coagulation.

Extrinsic Pathway

Even the extrinsic pathway comprises enzymes and protein cofactors. Initiation of this pathway is done by the creation of a complex between TF on cell surfaces

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and FVIIa located outside the vascular system. Constitutively, nonvascular cells express the integral membrane protein TF, a plasma protein FVII receptor. In an injury, when endothelium FVII comes in contact with TF, the TF nonproteolytically converts FVII to FVIIa.

The mechanism of the initial conversion of the zymogen FVII to FVIIa is still debated but is most likely due to autocatalytic activation and not a TF effect. This binding of FVIIa to TF forms an enzyme complex that activates FX to FXa. The FVIIa/TF complex, similar in function to the tenase complex, converts FX to its active form (FXa), which binds to the cofactor FV and is bound on membrane surfaces in the presence of calcium ions to generate the prothrombinase complex.

Prothrombin is converted to thrombin by the prothrombinase complex. Thrombin changes fibrinogen to fibrin to cause the fibrin clot. In laboratory analysis of blood clotting, its extrinsic pathway is evaluated by using prothombin time (PT). Irrespective of FXa arises from intrinsic or extrinsic pathway, the cascade moves on to the common pathway.

Common Pathway

When FX is activated within the intrinsic pathway/extrinsic pathway/both pathways, it is the start of the common pathway. The common pathway's first protease is the FXa. In the presence of FV, Ca2 and phospholipids, FXa turns prothrombin to its active form of thrombin. The purpose of thrombin is to catalyze the soluble plasma protein fibrinogen's proteolysis to create soluble fibrin monomers. Polymerizatn of Fibrin monomers takes place form a blood cells trapping gel of fibrin polymers. Thrombin activates FXIII, which get changed to FXIIIa and mediates the covalent cross-linking of the fibrin polymers to form stable fibrin which is a mesh termed of lower solubility than fibrin polymers.

Thrombin can catalyze:

- formation of the cofactors FVIIIa and FVa
- formation of new thrombin from prothrombin

The canalization leads to efficient augmentation of coagulation. Since the factors FII, FV and FX contained in the common pathway, PTT and the PT can monitor these factors.

In recent years, it has been observed that the cascade/waterfall hypothesis is not a complete reflection of the events of haemostasis in vivo.

Faults in the hypothesis have come up. Some being:

- There is no apparent reason for the lack of clinical bleeding tendency in deficiencies of prekallikrein, FXII, kininogen with high molecular weight. It is a fact that if n on eof these factors has deficiencies, there is a distinct prolong surface-activated coagulation assays for haemostasis in vitro.
- There is no explanation why FIX or FVIII deficiency leads to clinically severe bleeding, even though the extrinsic pathway should have bypassed the requirement for FIX and FVIII.

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Cell-based Model of Coagulation

When blood is exposed to cells that express TF on their surface, it becomes essential and also enough to begin blood coagulation in vivo. Though deficiency of FXII deficiency does not cause bleeding problems, its absence protects against pathological thrombosis.

The cell based model defines that haemostasis needs the creation of a fibrin plug and an impermeable platelet at the site of vessel injury and the procoagulant substances that this process activates must stay localized to the site of injury. Blood coagulation process starts when there is exposure of TF expressing cells to flowing blood. Tissue factor is not expressed on resting endothelium, it is expressed constitutively on cells like smooth muscle cells.

It does not remain in contact with blood, but tissue factor is there in the membranes of cells hat surround the vascular bed The disruption of the endothelium/ activation of endothelial cells/ monocytes exposes it to the circulating blood. Evidence shows the presence of FT in blood on cellular microparticles. The membrane fragments derive from varied cell types like platelets, endothelium and white blood cells that might be vital to pathological haemostasis rather than normal clotting.

It is a good idea to look at every biochemical, cellular and physical process contributing to haemostasis not as pathways but as a series of process phases/ stages. The complex processes of maintaining vascular integrity is aptly demonstrated by the three phases (Figure 4.2):

1. Initiation
2. Propagation
3. Termination

Extremely fragile vessels can lead to excessive bleeding. Then again it could be caused by platelet dysfunction or deficiency, derangement of coagulation or even a combination of all these. You can see the coagulation pathways' physiologic significance in the huge number of genetic deficiencies that end up in bleeding disorders. Activities of platelets and coagulation factors are closely related. A deficiency in all factors except FXII causes bleeding disorder

Common hereditary bleeding disorders are as follows:

- Haemophilia A which is caused by FVIII deficiency
- Haemophilia B which is caused by FIX deficiency
- Von Willebrand disease (VWD)

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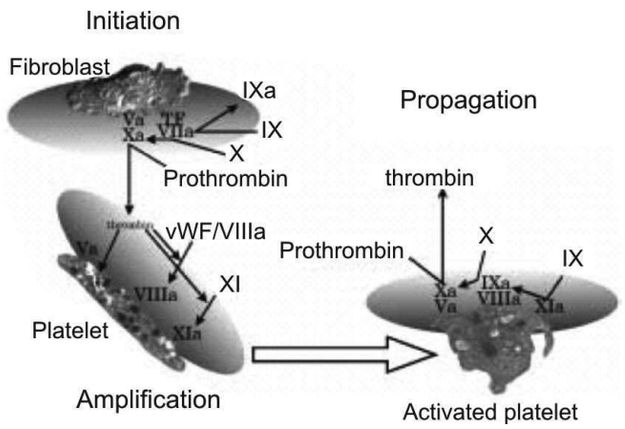

Fig. 4.2 *Three Phases of Coagulation*

Haemophilia A and haemophilia B erupt due to a functional deficiency of a plasma protein inherited in an X-linked manner. FVIII and FIX are needed for normal thrombin generation, and if either protein is not present, the ability to create fibrin and thrombin is damaged. Table 4.2 shows the different phases in coagulation.

Table 4.2 *Phases of Coagulation Proposed by the Current Cell-Based Theory of Coagulation – Process Stages in Haemostasis*

Initiation	Amplification	Propagation	Termination
Vascular endothelium and circulating blood cells are perturbed; interaction of plasma-derived FVIIa with tissue factor	Thrombin activates platelets, cofactors FVa and FVIII on the platelet surface, and FXI on the platelet surface	Results in the production of a significant level of thrombin activity, generation of a stable plug at the site of injury, and cessation of blood loss	Clotting process in limited to avoid thrombotic occlusion in surrounding normal areas of the vasculature

Let us look at each phase in some detail.

Initiation Phase

This initiation phase is contained in the TF expressing cells, generally located outside of the vasculature. Small amounts of FX and FIX are activated by the FVIIa/TF complex. FXa links with its cofactor FVa, to form a prothrombinase complex on the TF-bearing cell's surface. FVa needed for prothrombinase assembly can be produced by FXa activated by FV or by noncoagulation proteases.

Within the extravascular space, low-level activity of the TF pathway happens continuously. Tissue let coagulation proteins seep through, to be found in the lymph sort of in proportion to their molecular size. FVII is bound to extravascular TF even if there is no injury, and extravascular FIX and FX can get activated even while just passing through tissues. The amplification phase is entered when damage to the vasculature lets platelets and FVIII spill out into the extravascular tissues and to stick to TF-bearing cells.

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Amplification Phase

There are many vital functions performed by the tiny amount of thrombin generated on the TF bearing cell. A valuable one being activating platelets, binding sites for activated clotting factors and exposing receptors. Due to this activation, the platelets give out part activated forms of FV onto their surfaces. The thrombin, which during the initiation phase, activates of the cofactors FV and FVIII on the activated platelet surface. During this process, the FVIII/VWF complex is dissociated, permitting VWF to mediate additional platelet adhesion and aggregation at the site of injury. In addition, small amounts of thrombin activate FXI and FXIa on the platelet surface during this phase.

Propagation Phase

The propagation phase of clot formation occurs on the surface of activated platelets. In this process of the cofactors FV and FVIII on the activated platelet surface. This process occur in these steps:

- First step: In this phase, FIXa, activated during the initiation phase, binds to FVIIIa on the platelet surface.
- Second step: In this step additional FIXa is supplied by platelet-bound FXIa.
- Third step: In this step, FXa must be provided directly on the platelet surface by the FIXa/FVIIIa complex as FXa cannot move effectively from the TF-bearing cell to the activated platelet.
- Fourth step: In this step, the FXa rapidly associates with FVa bound to the platelet during the amplification phase. Finally, the completion of this platelet prothrombinase assembly leads to a burst of thrombin generation of sufficient magnitude to clot fibrinogen.

Termination Phase

In this phase, the clotting process is limited to avoid thrombotic occlusion in surrounding normal areas of the vasculature. This phase gets initiated after a fibrin platelet clot is formed over an area of injury. Three types of natural anticoagulants regulate clotting form. These types are as follows:

1. Antithrombin (ATIII) inhibits the activity of thrombin and other serine proteases, such as FIXa, FXa, FXIa, and FXIIa. Binding to heparin-like molecules on endothelial cells activates antithrombin.
2. Proteins C and S deactivate the procoagulant cofactors FVa and FVIIIa. Protein C is a vitamin K–dependent plasma glycoprotein. Protein C inactivates FVa and FVIIIa. Protein S functions as a cofactor to Protein C. Protein S enhances Protein C activity against FVa and FVIIIa).
3. TF pathway inhibitor (TFPI) is a protein secreted by endothelium. It deactivates FXa and to TF/FVIIa complexes and limits coagulation rapidly. Protein C is activated by thrombin that is bound to the transmembrane protein thrombomodulin (TM) on the surface of intact endothelial cells. In human plasma, about 30% of protein S circulates as free protein. The

remaining part is bound to the complement regulatory protein C4b-binding protein.

Platelet – Protein Interactions

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When the vascular wall gets damaged blood gets exposure to subendothelial tissue. In response to vascular damage the primary haemostasis gets triggered. Then the initial sealing of the damaged blood vessel is done by tissue components, plasma proteins, and receptors on platelets through coordinated interactions. This initiates the process of haemostasis. The primary platelet plug formed is temporary and is responsible for the activation of the blood coagulation system.

When the vascular wall gets damaged, platelets undergo a series of events such as adhesion, aggregation, release of granule content, and morphological changes that lead to the formation of the platelet plug. In this process, platelets form an occlusive plug or 'clot'. The process of adhesion and aggregation is mediated by VWF and fibrinogen. Primary platelet adhesion is dependent on the interaction between platelets and VWF.

VWF is a large multimeric plasma protein composed of multiple disulphidelinked monomers. The VWF undergoes proteolytic processing in plasma. This processing is mediated by a metalloprotease termed ADAMTS 13. ADAMTS 13 is a disintegrinlike and metalloprotease (reprolysin type) with thrombospondin type 1 motif.

The multimers of larger larger size are more efficient in platelet adhesion than the smaller ones. The VWF mediates platelet adhesion by serving as a bridge between the tissue and the platelets. The VWF binds both to collagen that is exposed at sites of vascular injury and to the platelet membrane glycoprotein Ib-V-IX (GPIb-VIX).

If blood coagulation doesn't occur properly then excessive bleeding may occur. Other reasons for excessive bleeding are as follows:

- An increased fragility of vessels
- Platelet deficiency or dysfunction
- Derangement of coagulation
- A combination of these three above mentioned reasons

The physiologic significance of the coagulation pathways is evident given the number of genetic deficiencies that result in bleeding disorders. The activities of platelets and coagulation factors are closely related. With the exception of FXII deficiency, which does not cause bleeding, a deficiency in every other clotting factor has been reported to cause a bleeding disorder. Three common hereditary bleeding disorders are as follows:

1. Haemophilia A (FVIII deficiency)
2. Haemophilia B (FIX deficiency)
3. Von Willebrand disease (VWD)

Functional deficiency of a plasma protein, inherited in an X-linked manner, causes both haemophilia A and haemophilia B. Physiologically, the tissue factor

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pathway of FX activation requires FVIII and FIX for normal thrombin generation. Absence of either protein severely impairs the ability to generate thrombin and fibrin. Because of the primary clotting factor deficiency in haemophilia A or haemophilia B, clot formation is delayed and is not robust. Thus, patients with haemophilia do not bleed more rapidly. As there is delayed and abnormal clot formation.

Both haemophilia A and haemophilia B are inherited as X-linked recessive traits because both the FVIII and FIX genes are located near the telomere of the long arm of the X chromosome. Numerous mutations within the FVIII gene have been identified that result in haemophilia A. The most common genetic alteration is a gene inversion. In families in which the index patient has a known molecular abnormality, genetic screening and carrier detection are highly accurate.

Unlike the complexity of the FVIII gene, the FIX gene is considerably smaller, and its defects have been studied more extensively. More than 60% of the FIX gene defects are due to missense point mutations. An identifiable defect in the gene can be found in nearly all patients

The von Willebrand factor is indispensable for the efficient initiation of platelet adherence to collagenrich matrices under high shear. There are 2 other important platelet collagen receptors: GPVI and the integrin, alpha-2-beta-. These receptors are engaged after the binding of VWF to GPIb and rapidly amplify the collagen-dependent response.

Function of VWF include the following:

- To stabilize the procoagulant cofactor FVIII in the circulation
- To serve as FVIII's carrier protein in plasma.
- To protect FVIII from premature protein C–mediated proteoloytic degradation

Any change in plasma VWF level usually leads to an associated change in FVIII plasma concentration. Thus, VWF performs two major roles in haemostasis that include the following:

- It mediates the adhesion of platelets to sites of vascular injury, making it essential for platelet plug formation.
- It functions as a carrier protein that stabilizes coagulation factor FVIII.

von Willebrand disease is notable for the considerable heterogeneity of its molecular basis. The population distribution of VWF levels is broad and does not exhibit a simple genetic basis. Although molecular studies have been successful in defining the genetic defects associated with VWD types 2 and 3, VWD type 1, the most common form of VWD, remains a challenge.

Since 1989 when the structure of the gene for human VWF was identified, numerous studies have attempted to identify potential VWF defect(s) that may lead to an earlier and more accurate diagnosis of VWD type 1. Due to the complexity of the gene structure, this has proven a difficult task. Fortunately, 2 recent studies completed in Europe and Canada have shed some light on this

topic. The studies have confirmed that the genetic basis of VWD type 1 is highly variable and that there are genes in addition to the VWF gene that can result in low plasma VWF levels. However, this work requires validation in independent studies, and additional work needs to be conducted on this. It is expected that these advances in both our understanding of haemostasis and knowledge of the organization of the human genome will pave the way to novel insights into the genetics of bleeding disorders. Ultimately these works will lead to more precise diagnostic tools and therapeutic interventions.

Check Your Progress

10. What do you understand by the term homeostasis?
11. What are the properties of homeostatic systems?
12. How is homeostasis helpful in body functioning?
13. What is the function of regulators?
14. Define coagulation.

4.6 ENDOTHERMY AND PHYSIOLOGICAL MECHANISM OF REGULATION OF BODY TEMPERATURE

An endotherm is an organism that maintains its body at a metabolically favourable temperature, mainly by the use of heat released by its internal bodily functions instead of relying almost purely on ambient heat. Such internally generated heat is mainly an incidental product of the animal's routine metabolism, but under conditions of excessive cold or low activity an endotherm might apply special mechanisms adapted specifically to heat production. Examples include special function muscular exertion, such as shivering, and uncoupled oxidative metabolism, such as within brown adipose tissue. Only birds and mammals are extant universally endothermic groups of animals. Certain lamnid sharks, tuna and billfishes are also endothermic.

Characteristically, endotherms are characterized as 'Warm Blooded'. The opposite of endothermy is ectothermy, although in general, there is no absolute or clear separation between the nature of endotherms and ectotherms.

4.6.1 Mechanisms of Thermoregulation

The animals can be divided into endotherms and ectotherms based on their temperature regulation. Endotherms, such as birds and mammals, use metabolic heat to maintain a stable internal temperature, often one different from the environment.

Ectotherms, like lizards and snakes, do not use metabolic heat to maintain their body temperature but take on the temperature of the environment.

Both endotherms and ectotherms have adaptations that help them maintain a healthy body temperature. These adaptations can be behavioural, anatomical,

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or physiological. Some adaptations increase heat production in endotherms when it is cold. Others, in both endotherms and ectotherms, increase or decrease exchange of heat with the environment. For example, Polar Bears and Penguins maintain a high body temperature in their chilly residences at the poles, while Kangaroo Rats, Iguanas, and Rattlesnakes thrive in Death Valley, where summertime highs are over 100°F (36°C).

Thermoregulation

Thermoregulation is the ability of an organism to maintain a core body temperature, which is 37° C (98°F) within an optimal physiological range. The hypothalamus, a specific portion of a brain plays significant role in regulating the body temperature which acts as a thermostat. Thermoregulation is also termed as the heat regulation. For example, human beings living in a climate of varying temperature are capable to maintain constant body temperature. In both animals and birds, the balance in heat gain and loss is supported by the hair, feathers, and fat skin layers.

Ectothermic Animals: The ectothermic animals are generally termed as cold-blooded animals and mostly gain the heat from external sources. The ectothermic animals produce very less amount of heat for keeping their body warm and has a low metabolic rate. Examples of ectothermic animals include amphibians, fish, lizards, other reptiles, etc.

Endothermic Animals: The endothermic animals are generally termed as warm-blooded animals. The endothermic animals mainly develop most of the heat from their body's metabolisms and produce an essential amount of heat to keep their body warm. The endothermic animals have very high metabolic rate. Examples of endothermic animals include all mammals and birds.

4.6.2 Physiological Mechanism of Regulation of Body Temperature

Thermoregulation is the ability of an organism to keep its body temperature within certain boundaries, even when the surrounding temperature is very different. A thermoconforming organism, by contrast, simply adopts the surrounding temperature as its own body temperature, thus avoiding the need for internal thermoregulation. The internal thermoregulation process is one aspect of homeostasis, a state of dynamic stability in an organism's internal conditions, maintained far from thermal equilibrium with its environment, the study of such processes in zoology has been called **physiological ecology**. If the body is unable to maintain a normal temperature and it increases significantly above normal, a condition known as **hyperthermia** occurs. Humans may also experience lethal hyperthermia when the wet bulb temperature is sustained above 35 °C (95 °F) for six hours. The opposite condition, when body temperature decreases below normal levels, is known as **hypothermia**. It results when the homeostatic control mechanisms of heat within the body malfunction, causing the body to lose heat faster than producing it. Normal body temperature is around 37 °C (99 °F), and hypothermia sets in when the core body temperature gets lower than 35 °C (95 °F). Hypothermia is usually treated by methods that attempt to raise the body

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temperature back to a normal range, usually caused by prolonged exposure to cold temperatures.

With the introduction of thermometers, the exact data on the temperature of animals were obtained. It was then found that local differences were present, since heat production and heat loss vary considerably in different parts of the body, although the circulation of the blood tends to bring about a mean temperature of the internal parts. Therefore, it is important to identify the parts of the body that most closely reflect the temperature of the internal organs. Also, for such results to be comparable, the measurements must be conducted under comparable conditions. The rectum has traditionally been considered to reflect most accurately the temperature of internal parts.

Some animals undergo one of various forms of dormancy where the thermoregulation process temporarily allows the body temperature to drop, thereby conserving energy. Examples include hibernating bears and torpor in bats.

Animals other than humans regulate and maintain their body temperature with physiological adjustments and behaviour. Desert lizards are ectotherms and so unable to metabolically control their temperature but can do this by altering their location. They may do this, in the morning only by raising their head from its burrow and then exposing their entire body. By basking in the sun, the lizard absorbs solar heat. It may also absorb heat by conduction from heated rocks that have stored radiant solar energy. To lower their temperature, lizards exhibit varied behaviours. Sand seas or ergs, produce up to 57.7 °C (135.9 °F), and the sand lizard will hold its feet up in the air to cool down, try to find cooler objects with which to contact, find shade or return to their burrow. They also go to their burrows to avoid cooling when the sun goes down or the temperature falls. Aquatic animals can also regulate their temperature behaviourally by changing their position in the thermal gradient.

Some animals living in cold environments maintain their body temperature by preventing heat loss. Their fur grows more densely to increase the amount of insulation. Some animals are regionally heterothermic and are capable to allow their less insulated extremities to cool to temperatures much lower than their core temperature—nearly to 0 °C (32 °F). This minimizes heat loss through less insulated body parts, like the legs, feet (or hooves), and nose.

Different species of Sonoran Desert Drosophila exploits different species of cacti based on the thermotolerance differences between species and hosts. For example, ***Drosophila mettleri*** is found in cacti namely the Saguaro and Senita; these two cacti remain cool by storing water.

Some flies, such as ***Lucilia sericata***, lay their eggs. The resulting group of larvae, depending on its size, is able to thermoregulate and keep itself at the optimum temperature for development.

4.6.3 Hibernation, Estivation and Daily Torpor

To cope with limited food resources and low temperatures, some mammals hibernate during cold periods. To remain in stasis for long periods, these animals

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build up brown fat reserves and slow all body functions. True hibernators (e.g., groundhogs) keep their body temperatures low throughout hibernation whereas the core temperature of false hibernators (e.g., bears) varies; occasionally the animal may emerge from its den for short periods. Some bats are true hibernators and rely upon a rapid, non-shivering thermogenesis of their brown fat deposit to bring them out of hibernation.

Estivation is similar to hibernation, however, it usually occurs in hot periods to allow animals to avoid high temperatures and desiccation. Both terrestrial and aquatic invertebrate and vertebrates enter into estivation. Examples include lady beetles (***Coccinellidae***), North American desert tortoises, crocodiles, salamanders, cane toads, and the water-holding frog.

Daily torpor occurs in small endotherms like bats and hummingbirds, which temporarily reduces their high metabolic rates to conserve energy.

Homeothermy vs. Poikilothermy

A poikilotherm is an organism whose internal temperature varies considerably. It is the opposite of a homeotherm, an organism which maintains thermal homeostasis. Poikilotherm's internal temperature usually varies with the ambient environmental temperature, and many terrestrial ectotherms are poikilothermic. Poikilothermic animals include many species of fish, amphibians, and reptiles, as well as birds and mammals that lower their metabolism and body temperature as part of hibernation or torpor. Some ectotherms can also be homeotherms. For example, some species of tropical fish inhabit coral reefs that have such stable ambient temperatures that their internal temperature remains constant.

Means of Heat Transfer

Heat can be exchanged between an animal and its environment through four mechanisms, namely radiation, evaporation, convection, and conduction. Radiation is the emission of electromagnetic heat waves. Heat radiates from the sun and from dry skin the same manner. When a mammal sweats, evaporation removes heat from a surface with a liquid. Convection currents of air remove heat from the surface of dry skin as the air passes over it. Heat can be conducted from one surface to another during direct contact with the surfaces, such as an animal resting on a warm rock.

4.6.4 Thermoregulation Mechanism

The hypothalamus is referred as a small portion of a human brain, which is primarily involved in secretion or release of all hormones from their respective glands and controlling several body functions. The mechanisms of thermoregulation are also controlled and regulated by the hypothalamus.

Whenever there is any variation in the internal body temperature, them the sensors in the Central Nervous System (CNS) sends the message to the hypothalamus and in response, the hypothalamus sends signals to various cells, muscles, and other systems in our body.

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To warm up the body, the mechanisms of thermoregulation include the following:

Vasoconstriction: As the blood vessels under the skin receive signals, they become narrower to decrease the blood flow and retain heat to warm the inner body.

Thermogenesis: This process is mainly seen in all warm-blooded animals. The body's organs produce heat in a variety of ways to keep the body warm.

Hormonal Thermogenesis: In this mechanism, the thyroid gland regulates to release hormones in order to increase the body's metabolism, which produces a more amount of heat to maintain a stable internal body temperature.

To cool down the body, the mechanisms of thermoregulation include the following:

Sweating: The sweat glands of the body receive signals to release sweat and it cools the skin as it evaporates. This helps by lowering the internal temperature.

Vasodilatation: In this process, the blood vessels present beneath the skin expand and increases the blood flow, which cools by releasing the body's heat through heat radiation.

Significance of Thermoregulation

The mechanism of thermoregulation are typically designed to regulate the body to homeostasis or a state of equilibrium. This process helps in controlling the loss or gain of heat and maintaining of an optimum temperature range by an organism. In humans, the average healthy body temperature falls within a 37°C to 37.8°C. However, if the body temperature falls from 37°C to 35°C or lower, then a person may suffer from a medical emergency of hypothermia. This condition may lead to cardiac arrest, brain damage, and even death. The factors affecting the hypothermia or lower in the internal body temperature include metabolic conditions, such as an under-functioning thyroid gland, usage of alcohols and other drugs.

4.7 PHYSIOLOGICAL ADAPTATION TO OSMOTIC AND IONIC STRESS

Salinity or osmolality represents an abiotic factor that varies depending on the environment. It has contributed to shaping the evolution of cells and animals for a long time and continues to do so by producing selection pressure on animal phenotypes and physiotypes. Therefore, all cells and all animals have mechanisms to cope with **osmotic stress**. Such mechanisms are based on:

1. Sensing and quantifying changes in environmental/extracellular osmolality.
2. Signal transduction of information about osmolality changes from osmosensors.
3. Compensatory and counteracting effector mechanisms.

In animals, osmoregulatory and osmoprotective effector mechanisms define the osmosensing and osmotic stress signalling. The pathways within the animal osmosensory signal are based on transduction network. Some elements of these

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networks are highly evolutionarily conserved not only in animals, but also in unicellular eukaryotes (yeast), for example mitogen-activated protein kinase cascades. Other elements, in particular molecular osmosensors, have diverged in animal cells from those of other organisms. For example, two-component histidine kinases, a family of proteins that includes potent osmosensors in prokaryotes and yeast, are lacking from animal genomes.

Animal cells utilize an array of osmosensors, each having a different operating range. For example, some osmosensors, such as calcium sensing receptors and transient receptor potential ion channels, are capable of responding to subtle and gradual osmolality changes whereas others, such as cell volume sensors and macromolecular damage sensors, are activated only during severe and acute osmolality changes. Animal cells effectively integrate input from multiple molecular osmosensors with different sensitivity ranges to accurately quantify osmolality and osmotic stress. Such integration is achieved by utilization of a complex signalling network, which generates and amplifies output signals to specifically activate effector mechanisms that are appropriate for the severity of osmotic stress. Appropriate effector mechanisms include, for example, organic osmolyte synthesis for tolerable osmotic stress and induction of apoptosis if tolerance limits are exceeded. In animals, the osmosensory signal transduction network is very extensive and very tightly linked to the signalling mechanisms controlling many other critical physiological functions, such as immunity, apoptotic volume decrease, cell proliferation, cell differentiation, and development.

4.7.1 Osmotic Stress

Osmotic shock or osmotic stress is physiologic dysfunction caused by a sudden change in the solute concentration around a cell, which causes a rapid change in the movement of water across its cell membrane. Under conditions of high concentrations of either salts, substrates or any solute in the supernatant, water is drawn out of the cells through osmosis. This also inhibits the transport of substrates and cofactors into the cell thus shocking the cell. Alternatively, at low concentrations of solutes, water enters the cell in large amounts, causing it to swell and either burst or undergo apoptosis.

All organisms have mechanisms to respond to osmotic shock, with sensors and signal transduction networks providing information to the cell about the osmolarity of its surroundings; these signals activate responses to deal with extreme conditions. Cells that have a cell wall tend to be more resistant to osmotic shock because their cell wall enables them to maintain their shape. Although single-celled organisms are more vulnerable to osmotic shock, since they are directly exposed to their environment, cells in large animals, such as mammals still suffer these stresses under some conditions. Current research also suggests that osmotic stress in cells and tissues may significantly contribute to many human diseases.

In eukaryotes, calcium acts as one of the primary regulators of osmotic stress. Intracellular calcium levels rise during hypo-osmotic and hyper-osmotic stresses.

Calcium plays a significant role in the recovery and tolerance for both hyper and hypo-osmotic stress situations. Under hyper-osmotic stress conditions, increased levels of intracellular calcium are exhibited.

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4.7.2 Ionic Stress

High salinity stress causes an ion homeostasis imbalance inside the cell. The cells produce an ionic stress signal. The salinity stress signal is then perceived by a receptor or salt sensor present at the plasma membrane of the cell, which is generally regulated by the coordinated action of various Salt Overly Sensitive (SOS) pathways and ion pumps which ultimately results in the efflux of excess ions.

The ionic strength of a solution is a measure of the concentration of ions in that solution. Ionic compounds, when dissolved in water, dissociate into ions. The total electrolyte concentration in solution will affect important properties, such as the dissociation constant or the solubility of different salts. One of the main characteristics of a solution with dissolved ions is the ionic strength. Ionic strength can be molar (mol/L solution) or molal (mol/kg solvent) and to avoid confusion the units should be stated explicitly. The concept of ionic strength was first introduced by Lewis and Randall in 1921 while describing the activity coefficients of strong electrolytes.

4.7.3 Mechanism of Cell Volume Regulation

It is essential for the survival of cells to maintain their volume within certain limits. The ability to control cell volume is important for cell function. Cell volume perturbation elicits a wide array of signalling, leading to protective (e.g., cytoskeletal rearrangement) and adaptive (e.g., altered expression of osmolyte transporters and heat shock proteins) measures and, in most cases, activation of volume regulatory osmolyte transport. The cell volume is regulated by the process of Regulatory Volume Decrease (RVD), which involves the activation of KCl cotransport and of channels mediating K^+, Cl^-, and taurine efflux. On the contrary, cell volume is regulated by the process of Regulatory Volume Increase (RVI), which is mediated primarily by Na^+/H^+ exchange, Na-K-2Cl cotransport, and Na^+ channels. The molecular identity of these transport pathways and their regulation can be specified by membrane deformation, ionic strength, Ca^{2+}, protein kinases and phosphatases, etc.

The animal cell membrane is, with a few exceptions, highly permeable to water. Cell water content and cell volume are thus determined by the cellular content of osmotic active compounds and by the extracellular tonicity. The osmotic water permeability of animal cells in orders of magnitude is greater than the permeability towards Na^+, K^+, and Cl^-.

4.7.4 Osmosis

Osmosis is a special type of diffusion of water molecules across a semi-permeable membrane, from a solution of high water potential to a region of low water potential. Water moves in or out of a cell until till equilibrium is reached on both sides of the plasma membrane (Refer Figure 4.3). The solution which has the higher solute

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concentration is called **hypertonic**, the solution with the lower solute concentration is **hypotonic** and solution with equal concentrations of solute and solvent is called **isotonic**. If a cell is placed in a hypertonic solution, the solution has more solute and less water concentration than the cell cytosol and hence water moves out of the cell leading to shrinkage of cell. When the cells are in a hypotonic solution, i.e., less solute and more water concentration, water will move across their membrane leading to swelling of cells.

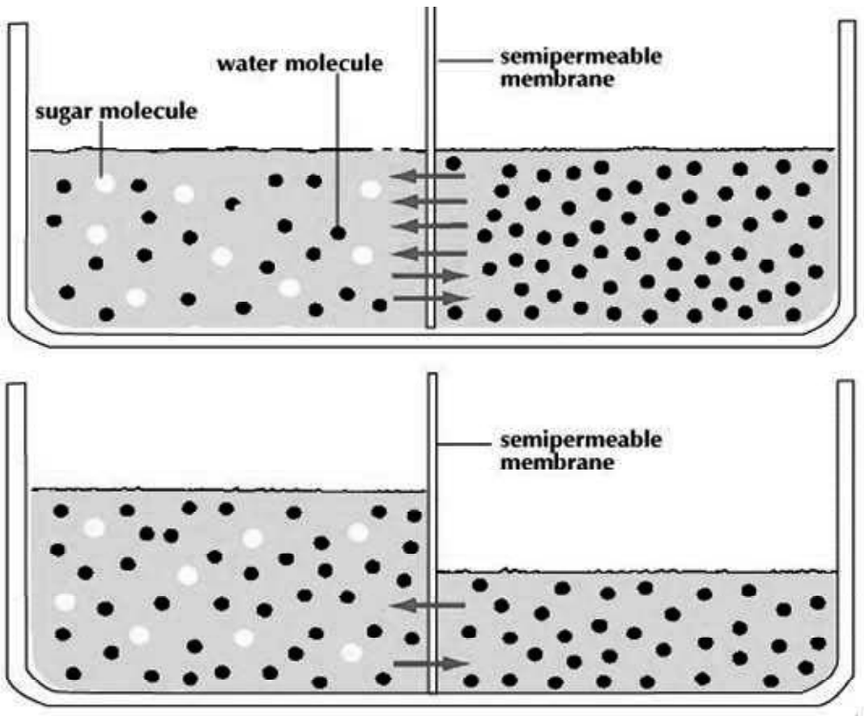

Fig. 4.3 *Osmosis*

So, the cell which does not have a rigid cell wall will swell and burst when placed in a hypotonic solution and when placed in a hypertonic solution, a cell without a cell wall will lose water to the environment, shrivel, and probably die.

Table 4.3 illustrates the difference between the different types of solutions.

Table 4.3 *Difference between the Different Types of Solutions*

Properties	Hypotonic Solution	Isotonic Solution	Hypertonic Solution
Solvent Concentration	Have a low solvent concentration	Have equal solute concentration	Have higher concentration of solvent
Effect on Cell	As solvent flows inside the side, it causes cells to swell	Show no effect on cells	As solvent moves out of the cell, it causes cell to shrink
Function	They are not helpful in food preservation	They are not helpful in food preservation	They are helpful in food preservation

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Osmotic Pressure

It is the minimum pressure which is exerted by cell membrane to prevent the inward flow of its pure solvent across semipermeable membrane. Cell wall helps to maintain the cell's water balance.

The action of osmosis may be very dangerous to organisms, especially ones which lack cell walls. For example, when a saltwater fish is placed in fresh water, its cells will take on excess water, lyse, and the fish will die. Hence, there needs some mechanism which helps them to sustain in hypotonic or hypertonic environment. A contractile vacuole is a type of vacuole that removes excess water from a cell.

Active Transport

Active transport occurs when energy like ATP is needed to move a substance to move across a plasma membrane against the concentration gradient. This is a like moving a ball uphill which requires energy. Like passive transport, active transport may also involve transport Proteins. Cells spend a huge amount of energy to transport the ions/molecules against the concentration gradient and keep the physiology of cells in moving state. Transport of Na^+ and K^+ against the concentration gradient to maintain the internal Sodium and Potassium ions levels is one of the best example of active transport. It is carried out by the means of transport Proteins which are found in the membrane

Active transport mechanisms are divided into two categories. Primary active transport which directly uses a source of chemical energy (ATP) to move molecules across a membrane against their gradient. Secondary active transport (cotransport) is the type of transport which uses an electrochemical gradient as an energy source to move molecules against concentration gradient.

Primary Active Transport

As stated above, this transport uses chemical energy (ATP) to move molecules across a membrane against their gradient. To move these molecules against their concentration gradient, a carrier protein is needed which works both in passive and active transport.

Example: Na^+/K^+-ATPase (Sodium pump).

Sodium-Potassium Pump: It is well known that cells have higher intracellular concentrations of Potassium ions relative to Sodium as compared to their surroundings. The Na^+/K^+-ATPase (Sodium pump) is a protein complex which helps in active transport of Na^+ and K^+ ions across the plasma membrane in the animal cells. It helps in transmembrane transport of three Na^+ outward and two K^+ inward utilizing energy derived from ATP hydrolysis. At rest, the cell consumes 20–30% of ATP production to actively transport Na^+ out of and K^+ into the cell. The electrochemical gradients so generated are essential for maintaining the resting potential of the cells, electrical excitability of nerves and muscles, secondary uptake and exclusion of ions, nutrients and neurotransmitters. ATP transfers a phosphate group directly to a carrier protein which allows the carrier protein to change its

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shape and hence facilitating the movement of molecule/ion from one side of the cell to the other side.

A Sodium-Potassium pump, exchanges Sodium ions for Potassium ions across the plasma membrane of animal cells as stated below (Refer Figure 4.4):

- At the beginning, the pump is open to the inside of the cell and has a high affinity for Sodium ions, and three sodium ions bind to pump.
- Binding of sodium ions leads to the hydrolysis of ATP leading to release of ADP and a Phosphate group. One phosphate group gets attached to the pump and is said to be phosphorylated.
- With this phosphorylation, a conformational change is induced in the pump and it opens towards the extracellular space to release three Na^+ outside the cell.
- In its outward-facing form, the pump has a high affinity for Potassium ions and hence binds to two of them, and Dephosphorylation of the pump takes place.
- With the dephosphorylation of pump, pump reverts to original form and opens towards the interior of the cell.
- As the pump opens towards the interior of the cell, pump loses its affinity with Potassium ions, so the two potassium ions will be released into the cytoplasm giving the possibility to initiate a new reaction cycle.

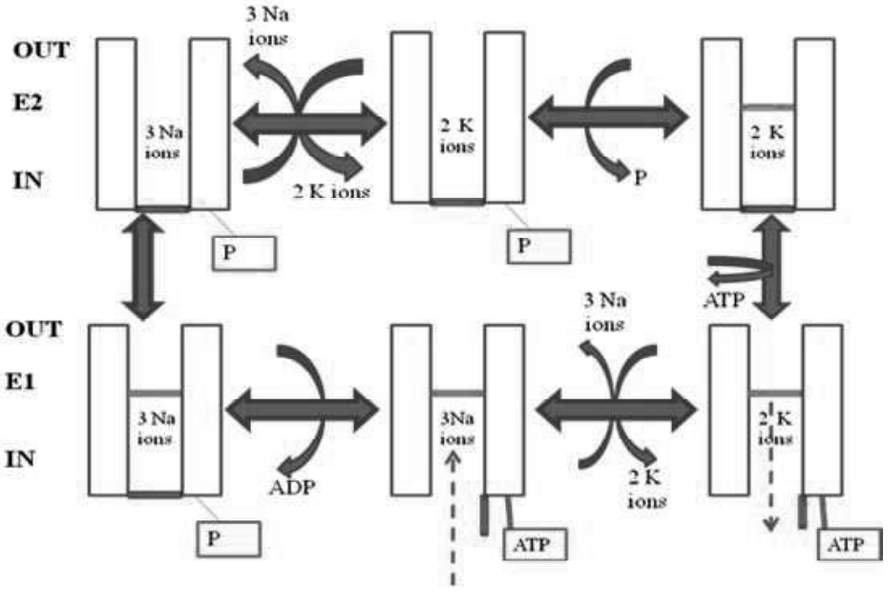

Fig. 4.4 *Working of Sodium-Potassium Pump*

Sodium-Potassium pump involves the protein between two forms: an inward-facing form with high affinity for Sodium (and low affinity for Potassium) and an outward-facing form with high affinity for Potassium (and low affinity for Sodium).

The protein shuffles between these two forms by the addition or removal of a phosphate group.

Check Your Progress

15. Define endotherm.
16. What is thermoregulation?
17. What is the difference between hypothermia and hyperthermia?
18. What do you understand by the term osmotic stress?

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4.8 OSMOREGULATION IN AQUEOUS AND TERRESTRIAL ENVIRONMENTS

Osmoregulation is the active regulation of the osmotic pressure of an organism's body fluids, detected by osmoreceptors, to maintain the homeostasis of the organism's water content; that is, it maintains the fluid balance and the concentration of electrolytes (salts in solution which in this case is represented by body fluid) to keep the body fluids from becoming too diluted or concentrated. Osmotic pressure is a measure of the tendency of water to move into one solution from another by osmosis. The higher the osmotic pressure of a solution, the more water tends to move into it. Pressure must be exerted on the hypertonic side of a selectively permeable membrane to prevent diffusion of water by osmosis from the side containing pure water.

Although there may be hourly and daily variations in osmotic balance, an animal is generally in an osmotic steady state over the long term. Organisms in **aquatic** and **terrestrial environments** must maintain the right concentration of solutes and amount of water in their body fluids; this involves excretion (getting rid of metabolic nitrogen wastes and other substances, such as hormones that would be toxic if allowed to accumulate in the blood) through organs, such as the skin and the kidneys.

Characteristically, the 'Osmoregulation' is the process of maintaining salt and water balance (osmotic balance) across membranes within the body. The fluids inside and surrounding cells are composed of water, electrolytes, and nonelectrolytes. An electrolyte is a compound that dissociates into ions when dissolved in water. A nonelectrolyte, in contrast, does not dissociate into ions in water. The body's fluids include blood plasma, the fluid that exists within cells, and the interstitial fluid that exists in the spaces between cells and tissues of the body. The membranes of the body, both the membranes around cells and the membranes made of cells lining body cavities, are semipermeable membranes. Semipermeable membranes are permeable to certain types of solutes and to water, but typically cell membranes are impermeable to solutes.

The body does not exist in isolation. There is a constant input of water and electrolytes into the system. Excess water, electrolytes, and wastes are transported to the kidneys and excreted, helping to maintain osmotic balance. Insufficient fluid

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intake results in fluid conservation by the kidneys. Biological systems constantly interact and exchange water and nutrients with the environment by way of consumption of food and water and through excretion in the form of sweat, urine, and feces. Without a mechanism to regulate osmotic pressure, or when a disease damages this mechanism, there is a tendency to accumulate toxic waste and water, which can have serious consequences.

Mammalian systems have evolved to regulate not only the overall osmotic pressure across membranes, but also specific concentrations of important electrolytes in the three major fluid compartments, such as blood plasma, interstitial fluid, and intracellular fluid. Since osmotic pressure is regulated by the movement of water across membranes, the volume of the fluid compartments can also change temporarily. Since blood plasma is one of the fluid components, osmotic pressures have a direct bearing on blood pressure.

Regulators and Conformers

Two major types of osmoregulation are osmoconformers and osmoregulators. Osmoconformers match their body osmolarity to their environment actively or passively. Most marine invertebrates are osmoconformers, although their ionic composition may be different from that of seawater. In a strictly osmoregulating animal, the amounts of internal salt and water are held relatively constant in the face of environmental changes. It requires that intake and outflow of water and salts be equal over an extended period of time.

Organisms that maintain an internal osmolarity different from the medium in which they are immersed have been termed osmoregulators. They tightly regulate their body osmolarity, maintaining constant internal conditions. They are more common in the animal kingdom. Osmoregulators actively control salt concentrations despite the salt concentrations in the environment. An example is freshwater fish. The gills actively uptake salt from the environment by the use of mitochondria-rich cells. Water will diffuse into the fish, so it excretes a very hypotonic (dilute) urine to expel all the excess water. A marine fish has an internal osmotic concentration lower than that of the surrounding seawater, so it tends to lose water and gain salt. It actively excretes salt out from the gills. Most fish are stenohaline, which means they are restricted to either salt or fresh water and cannot survive in water with a different salt concentration than they are adapted to. However, some fish show an ability to effectively osmoregulate across a broad range of salinities; fish with this ability are known as euryhaline species, e.g., flounder. Flounder have been observed to inhabit two disparate environments, marine and fresh water, and it is inherent to adapt to both by bringing in behavioural and physiological modifications. Following Figure illustrates the movement of water and ions in freshwater fish.

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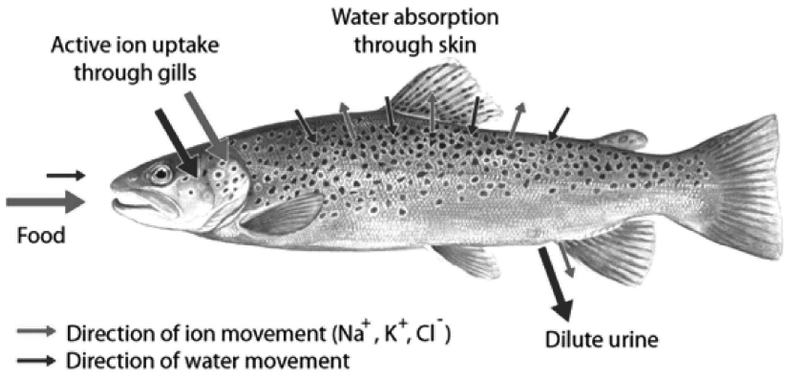

Fig. 4.5 *Movement of Water and Ions in Freshwater Fish*

Following Figure 4.6 illustrates the movement of water and ions in saltwater fish.

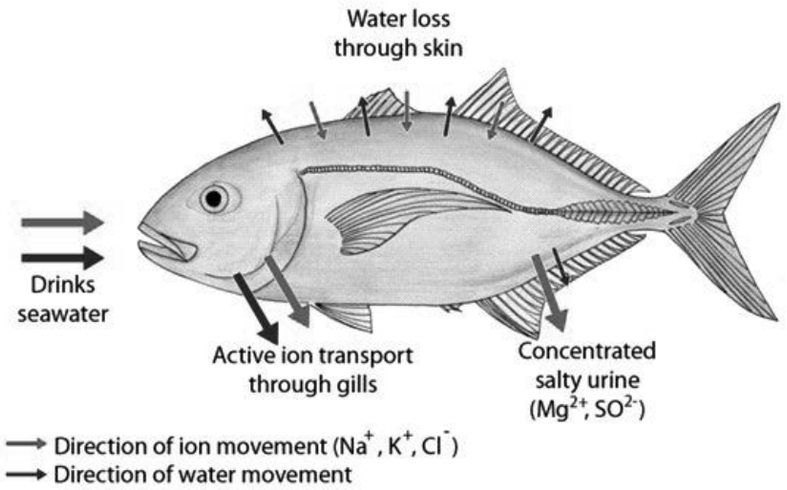

Fig. 4.6 *Movement of Water and Ions in Saltwater Fish*

Some marine fish, like sharks, have adopted a different, efficient mechanism to conserve water, i.e., osmoregulation. They retain urea in their blood in relatively higher concentration. Urea damages living tissues so, to cope with this problem, some fish retain trimethylamine oxide. This provides a better solution to urea's toxicity. Sharks, having slightly higher solute concentration (i.e., above 1000 mOsm which is sea solute concentration).

Humans

Kidneys play a very large role in human osmoregulation by regulating the amount of water reabsorbed from glomerular filtrate in kidney tubules, which is controlled by hormones, such as AntiDiuretic Hormone (ADH), Aldosterone, and Angiotensin II, for example, a decrease in water potential is detected by osmoreceptors in the hypothalamus, which stimulates ADH release from the pituitary gland to increase the permeability of the walls of the collecting ducts in the kidneys. Therefore, a large proportion of water is reabsorbed from fluid in the kidneys to prevent too much water from being excreted.

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The human excretory system functions to remove waste from the body through the skin as sweat, the lungs in the form of exhaled carbon dioxide, and through the urinary system in the form of urine. All three of these systems participate in osmoregulation and waste removal.

Marine Mammals

Drinking is not common behaviour in pinnipeds and cetaceans. Water balance is maintained in marine mammals is by metabolic and dietary water, while accidental ingestion and dietary salt may help maintain homeostasis of electrolytes. The kidneys of pinnipeds and cetaceans are lobed in structure, unlike those of non-bears among terrestrial mammals, but this specific adaptation does not confer any greater concentrating ability. Unlike most other aquatic mammals, manatees frequently drink fresh water and sea otters frequently drink saltwater.

4.9 PHYSIOLOGICAL RESPONSE TO OXYGEN DEFICIENT STRESS

Hypoxia is a condition in which the body or a region of the body is **deprived of adequate oxygen supply at the tissue level**. Hypoxia may be classified as either generalized, affecting the whole body, or local, affecting a region of the body. Although hypoxia is often a pathological condition, variations in arterial oxygen concentrations can be part of the normal physiology, for example, during hypoventilation training or strenuous physical exercise.

Hypoxia differs from hypoxemia and anoxemia in that hypoxia refers to a state in which oxygen supply is insufficient, whereas hypoxemia and anoxemia refer specifically to states that have low or zero arterial oxygen supply. Hypoxia in which there is complete deprivation of oxygen supply is referred to as anoxia.

Generalized hypoxia occurs in healthy people when they ascend to high altitude, where it causes altitude sickness leading to potentially fatal complications, such as High Altitude Pulmonary Edema (HAPE) and High Altitude Cerebral Edema (HACE). Hypoxia also occurs in healthy individuals when breathing mixtures of gases with a low oxygen content, e.g., while diving underwater especially when using closed-circuit rebreather systems that control the amount of oxygen in the supplied air.

In acute or silent hypoxia, a person's oxygen level in blood cells and tissue can drop without any initial warning, even though the individual's chest X-ray shows diffuse pneumonia with an oxygen level below normal.

In aquatic systems, hypoxia stress is often defined as Dissolved Oxygen (DO) levels below 2 mg/L. DO deficiency can affect aquatic animals in adverse ways, including a reduction in growth and reproduction, and can even be lethal in sensitive organisms. The sea cucumber ***Apostichopus japonicus*** is an important species, to cope with hypoxia stress, a series of molecular, physiological and morphological changes are expected to occur in ***Apostichopus japonicus*** for survival.

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The regulation of DO can be done by pumping nitrogen into water and then oxygen gradually decreased to the level needed. The traditional method is to use plastic film covering the water bucket after aeration in order to avoid vapour exchange. Occasionally, measurements are needed and then either nitrogen or oxygen is pumped into the experimental aquatic environment artificially to regulate DO level.

Hypoxia in Fish

Fish are exposed to large oxygen fluctuations in their aquatic environment since the inherent properties of water can result in marked spatial and temporal differences in the concentration of oxygen. Fish respond to hypoxia with varied behavioural, physiological, and cellular responses in order to maintain homeostasis and organism function in an oxygen-depleted environment. The biggest challenge fish face when exposed to low oxygen conditions is maintaining metabolic energy balance, as 95% of the oxygen consumed by fish is used for ATP production releasing the chemical energy of O_2 through the mitochondrial electron transport chain. Therefore, hypoxia survival requires a coordinated response to secure more oxygen from the depleted environment and counteract the metabolic consequences of decreased ATP production at the mitochondria.

4.10 PHYSIOLOGICAL RESPONSE TO BODY EXERCISE

The physiological response to exercise is dependent on the intensity, duration and frequency of the exercise as well as the environmental conditions. During physical exercise, requirements for oxygen and substrate in skeletal muscle are increased, as are the removal of metabolites and carbon dioxide. Chemical, mechanical and thermal stimuli affect alterations in metabolic, cardiovascular and ventilatory function in order to meet these increased demands.

Compared to our resting state, exercise poses a substantial increase in demand for the body. At rest, our nervous system maintains a parasympathetic tone, which affects the respiratory rate, cardiac output, and various metabolic processes. Exercise stimulates the sympathetic nervous system and will induce an integrated response from the body. This response works to maintain an appropriate level of homeostasis for the increased demand in physical, metabolic, respiratory, and cardiovascular efforts.

Understanding the effect of exercise involves studying specific changes in muscular, cardiovascular, and neurohumoral systems that lead to changes in functional capacity and strength due to endurance training or strength training. The effect of training on the body has been defined as the reaction to the adaptive responses of the body arising from exercise or as “an elevation of metabolism produced by exercise”.

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Check Your Progress

19. Define osmotic pressure?
20. What are two major types of osmoregulation?
21. What is hypoxia?
22. How the regulation of dissolve oxygen can be done in water.

4.11 BASIC PRINCIPLES OF YOGA

Before starting yoga, knowing its basic principles will help to open the door of the heart and mind. Saint Patanjali was the compiler of the Yoga Sutras. Yoga Sutras are the compilation of the axioms of yoga practice. According to Patanjali, the yogic path involves two stages to calm the inner thoughts:

- **The First Stage:** These are the methods of developing positive qualities into one's own self. If such qualities are not built up, especially during meditation, then the inner peace will be disturbed by the chaos of the outside world.
- **The Second Stage:** This is path of meditation. This is the stage to practice meditation to transcend the self and attain enlightenment.

These stages are achieved by practicing the ten principles of yoga. Here are those:

1. **Ahinsa (Non-violence):** According to this principle, one must not kill another being. One has to be meek and peaceful.
2. **Satya (Truthfulness):** This is one of the most vital basic principles of yoga. A yoga practitioner's life is based on truth. To be precise, one should be honest with oneself and with others. The yoga principle says, one can only tell a lie in a well-justified situation. For example, if you are telling a lie to save someone's life, then it is justified. Once you practice telling truth always, you will radiate truth. Liars always give negative vibes.
3. **Ateya (Righteousness):** The next principle of yoga is walking on the path of righteousness. One should not cheat and steal anything, ever. Life should be illuminated with the power of honesty. A true yogi/yogini will not look for unwanted advantage. Rather, he/she should always go for fairness in life.
4. **Brahmacharya (Wisdom):** This means one should always live with a spiritual focus. Instead of money, one should be devoted towards inner happiness and look for the inner peace that will bring the true happiness in life.
5. **Aparigraha (Simplicity):** When it comes to external extravaganza, one should be simple and moderate. Once a yogi attains that simplicity, they will feel an inner peace, which will cleanse their body from the inside. Such practices will bring the light of knowledge one day.
6. **Ishvara-Pranidhana (Worship of the Spiritual Goal):** According to this principle, one should never leave the spiritual path. One should remind oneself about the spiritual goal to attain in life, and owning this spiritual life means dedication towards the almighty. One can worship an idol, follow

the guidance of the spiritual persons or chant a mantra. Whatever one does, one should do it with a clear mind.

7. **Shaucha (Sacrifice the Ego):** This principle means self-cleansing. Ego is the evil of the mind. Attaining yoga can purify the soul just by killing the ego. Through true sacrifice, one can attain that the enlightenment.
8. **Tapas (Self-discipline):** This means leading a disciplined life. One must have a clear goal in life and follow it by setting a clear path to achieve it.
9. **Svadhaya (Reading):** One can chant a mantra or go for mediation. Regular reading encourages the mind to stay on the spiritual path, cleanses the soul and makes it possible to establish a contact between practitioner and the enlightened masters.
10. **Santosha (Contentment):** Finally, this principle suggests that one must learn to be happy with what one has.

These are the ten basic principles of yoga that helps in finding the true self by delving deep into the soul while also assisting in connecting with the Almighty.

Proper Relaxation in Yoga

In yoga, proper relaxation is known as 'Savasana'. Life is full of upheavals. Stress and tension are our regular companion. Therefore, proper mental and physical relaxation is needed. Once you relax your five senses (seeing, tasting, hearing, touching and smelling), the senses are cleansed. To attain good health, inner peace and vitality, it is quite important to follow these proper ways of relaxations:

1. Physical Relaxation

The underlying meaning of this is not wasting energy unnecessarily. With yoga asanas and proper relaxation method, one can relax each part of the body. People, who practice asanas regularly, feel energetic and well rested even after getting little sleep.

2. Mental Relaxation

With a tired mind, it is impossible to focus into anything. If the mind is full of sensory impressions, it will get tired easily. If you are anxious about anything, that destroys your energy faster than physical exhaustion. Whenever you feel you are mentally tired, you should breathe slowly. Learning the breathing techniques can help you to deal with your tension and anxiety.

3. Spiritual Relaxation

One can only attain complete mental and physical relaxation just by finding the connection of the soul with the higher power. Spiritual relaxation will help in learning to feel the happiness within yourself and grab the positivity around you. By practicing yoga postures and meditation, it is possible to relax the body, mind and soul.

Proper Exercise

To stay fit, yoga is effective. If you are a beginner, you need to know that the 'Asanas' are the form of yoga exercise. Along with that, one needs to know the breathing techniques. There are different types of yoga poses, which should be practiced every day to be fit. Following are the best yoga poses, which are the proper exercise for the entire body.

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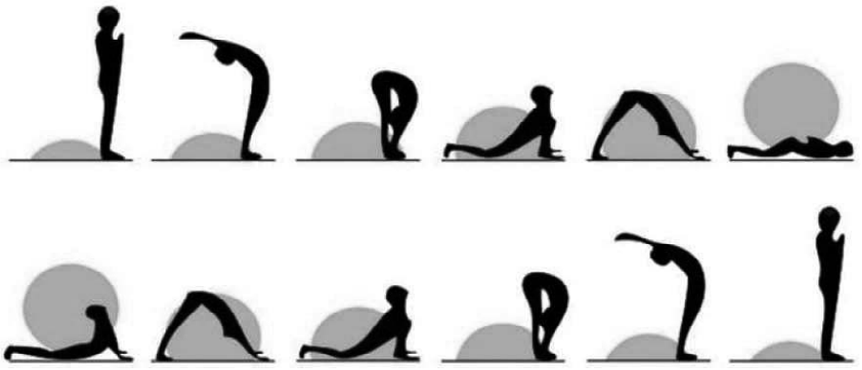
1. Malasana (Garland Pose)

This is a kind of squat. If someone is suffering from back pain and bulging belly, this pose is very effective. This pose opens the hips, releases stress from the lower back, and strengthens the spine. Practicing the pose every day can cure the pain in the back and lower back portion.

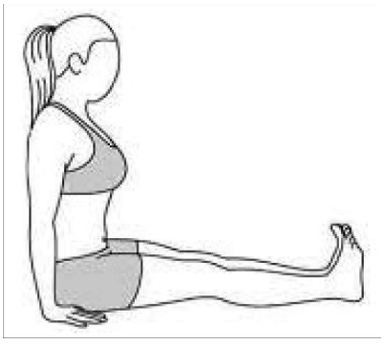
2. Surya Namaskar (Sun Salutation)

This is one of the most essential yoga poses where the entire body is engaged into the position. With seven postures involved in the exercise, each of the limbs gets movement and is quite effective in burning fat with the stretching involved. Surya Namaskar works on the seven major chakras and fills up the body with lots of energy. Besides, it calms the body and mind from inside.



3. Chaturanga Dandasana (Four-limbed Staff Pose)

This asana also includes the entire body and tones up the core muscle. This asana should be practiced under the guidance of an experienced practitioner. It is important to be slow with this pose at the beginner stage to avoid injury.



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4. Utkatasana (Chair Pose)

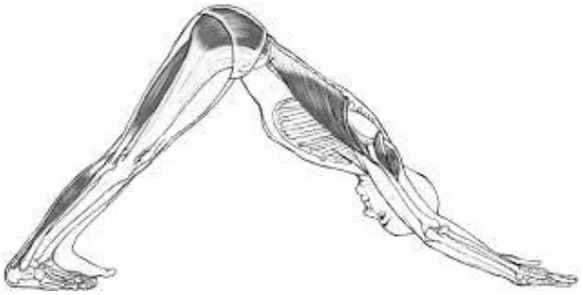
If someone is feeling lethargic and lacking energy, this asana can instantly build heat in the body and boost up the energy level for the next go. This asana is effective for making the core muscles strong and it gives strength to the legs.

5. Utthita Trikonasana (Extended Triangle Pose)

This is a classic yoga pose for the practitioners. Beginners should not try to touch the ground with the hands, but concentrate to make the pose right just by placing the palm on the shin below the knee. Such a pose can put pressure on the lower back and strengthen it. It also helps to expand the body and mind.

6. Adho Mukha Svanasana (Downward Facing Dog)

If there is not much time in the morning for exercise, then doing this can make one feel active and energetic. As the entire body is engaged in this asana, it will give many benefits. It strengthens the core muscle, reduces fat from belly, waistline, and hips and gives a toned body.



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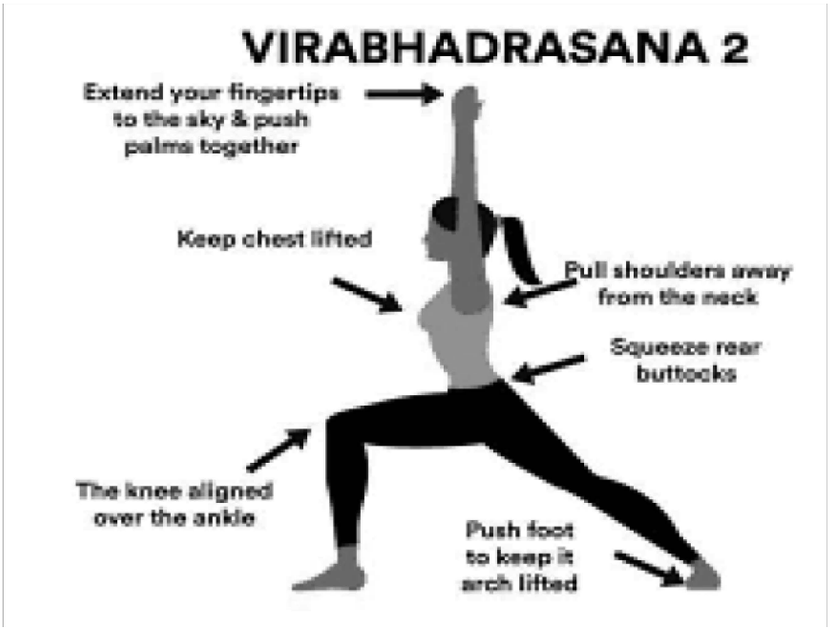
7. Virabhadrasana (Warrior Pose)

One needs to just give 30 seconds to this pose and will feel the difference instantly. This yoga asana activates the core, legs, and strengthens them. Practicing this asana can boost up the solar plexus chakra, Manipura in the body and fills it with willpower, confidence, and determination.

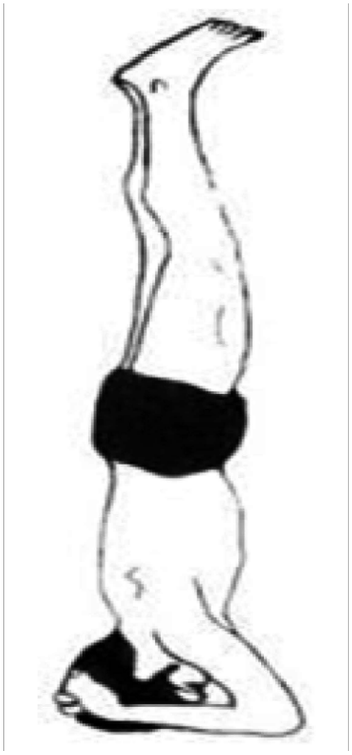


8. Ardha Matsyendrasana (Half Lord of Fish Pose)

This is another classic yoga pose for practicing every day. As it is a twisting posture, it works on the spine and helps to get rid of love handles. Other than that, it stimulates the inner organ of the body, detoxifies and promotes healthy digestion.

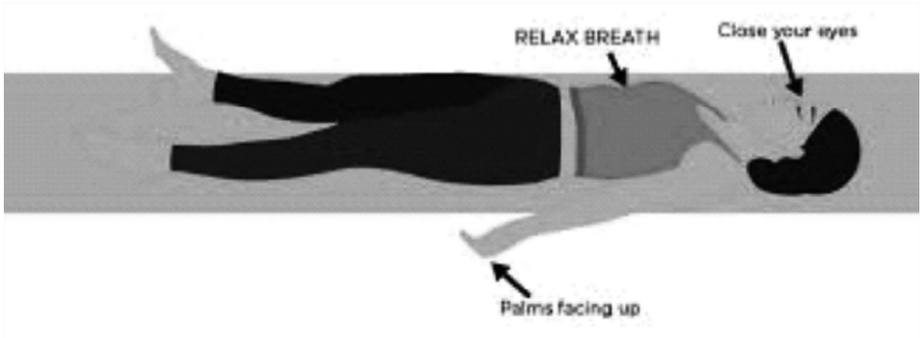
9. Sheersasana (Headstand)

Initially, it may seem like a difficult exercise. It is important to practice balancing well before doing it properly. Inversions are actually interesting to do. This asana strengthens the core muscle, legs, and arms.



10. Savasana

Finally, one should practice this ultimate asana after doing every asana. For this asana, one needs to concentrate on the natural breathing technique and think about positive things in life. This relaxation method is very important to gain back the energy.



These were the top 10 yoga asanas that one should practice every day to expand the mind, body, and soul. It is always better to do the asanas under the guidance of some experts who can assist in pursuing the correct pose and know the actual breathing technique.

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Proper Breathing

Yoga is quite different from mere physical exercises. The yoga poses or asanas are the media to bringing one's body, mind, and soul into a single alignment so that one can feel his true self and are connected to the almighty. Meditation is one of the vital elements of the yogic practice. Besides physical exercises, breathing exercises are also very important here. Breath is one of the pivotal parts of yogic feats. It regulates the mind, helps to stay focused and concentrated and controls the emotions. Learning the breathing control helps to make one feel calm, composed and energetic. Following the below given yogic breathing techniques can bring lots of physical and mental benefits:

1. **Abdominal or Diaphragmatic Breathing:** In this type of breathing technique, one should breathe deep in the abdomen. Focus on the expansion of stomach during inhalation and squeeze the stomach during exhalation.
2. **Clavicular Breathing:** In this type of breathing, one should breathe into the lungs until one feels a pressure due to the expansion of the upper portion of the lungs. During this breathing, the collarbone and the shoulder also move up during inhalation, while the exhalation, should be slow starting from the chest and then through the neck.
3. **Thoracic Breathing:** Lungs get upwards and downwards during this breathing exercise. It goes upward while inhaling and goes down after exhalation.
4. **Yogic Breathing:** This is the combination of all types of breathing techniques. Here, the yoga practitioner should breathe deep and slow by filling up the abdominal areas and then exhale it from stomach, chest, shoulder, and neck, respectively.

Breathing Asanas

One can practice the breathing technique in the perfect way by doing the breathing asanas:

1. **Kapalbhaati**: This is kind of sneezing. According to experts, the practitioner should exhale from the lungs. This is also known as 'Bhasrika'. It is effective in producing heat inside the body and cleanses the frontal brain area (or the forehead).
2. **Anulom-Bilom:** The yogic gurus call it 'Chandra Vedi Pranayama'. The practitioner breathes in and out with alternate nostrils. Practicing this regularly can keep the body calm and cool, by activating the right side of the brain.
3. **Sheetali Pranayam:** This is kind of breathing is done by keeping the tongue rolled out. If someone is suffering from high blood pressure, one should try this one.

Breathing Technique While Doing Asanas

While doing asanas, keeping the perfect rhythm of breathing is very important. Here are the main criteria of that:

- Inhale when in the center position

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- Exhale while bending sideways
- Also, exhale while bending forward
- If bending backward, inhale
- Experts say people with heart issues or high blood pressure should never try breath retention.

Proper Diet

From ancient time, yoga is closely inter-connected with foods, rather than diet. Practicing yoga asanas will not bring any result if the proper diet is not followed. It can even have a reverse effect on the human body. Here are the best foods one should include in the diet while practicing yoga:

1. **Dark Leafy Greens:** Usually, they are one of the most nutritious foods in a three-course-meal every day. These leafy greens must be added in more amounts. It is advisable to have spinach, lettuce, cabbage, and other leafy vegetables in maximum quantity, as they are the powerhouse of nutrition.
2. **Quinoa:** During yoga asana, one needs the energy to stretch body and need a powerful mind to concentrate more on the postures. Quinoa is rich in nine essential amino acids and iron that boost up blood circulation. There is also vitamin B2 that increases energy production in the cells and magnesium can control blood sugar level. This is quite surprising that one can find the mention of quinoa in the ancient manuscripts on yoga and the diets of yogis.
3. **Berries:** Colourful fruits can increase energy level, which is very important for yoga practitioners. As berries are full of anti-oxidants, they have the potential to fight against killer diseases, like cancer. While exercising, one should avoid hunger pangs. If it disturbs a lot, a handful of berries can do well to the satiate and control the hunger pangs.
4. **Porridge:** It is beneficial to include fibre-oriented items in the regular diet. Porridge is a high source of fibre and it is low on the glycemic index scale. According to yogi gurus, porridge is the most important food item in the yogic diet and it works the best while having with cardamom or cinnamon powder.
5. **Lentils:** While you do asanas, your energy level drains out. To keep up the level intact, one should have protein in his/her diet. Having lentil soup can provide enough protein that can keep one energized. A bowl of lentil soup warmed up with spices can make you ready for the next round of exercises.

These are only a few foods one can have regularly while practicing yoga. One must keep in mind to add more fresh fruits and limit the consumption of caffeine and alcohol.

Benefits of Yoga in Life

Yoga is not a mere exercise. It is the way to connect one's body with the soul and mind and finally leads one to the devotion towards the ultimate. It is not a religion. It is a practice depeciting when mind, body, and soul feel contented with the amalgamation to the One. It is a way to eliminate all the negative thoughts and

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vibes around human life and seek ultimate happiness. From ancient times, the yoga gurus have opined the practice yoga to attain the following benefits in life:

1. Direct Your Mind towards the Right

The mind gets diverted. However, with yoga practice, one can increase the method of directing the mind toward the path of love, affection, truth, and contentment.

2. Eliminate Negative Thoughts

Life is difficult. However, once you find out the key to happiness, it becomes easier to live life truly. Fight against your negative thoughts and purify your thoughts. It is important that when life feels submerged in negative thoughts, like depression, failure, darkness, doubts, weakness, etc., there is an effort to redirect the mind towards positive things, like courage, strength, happiness, and satisfaction.

3. Improve Your Thinking

The main principle of Yoga is to improve the thinking. According to yoga gurus, the thoughts of human play an important role in making him what they truly are. Therefore, one needs to fill one's mind with purity of thoughts to be a good person. In addition, to gain all these, meditation can help you. It is the path that connects one to the universe and to nature. Once a person mediates with complete concentration, one can find the happiness within oneself. One can know himself the best through meditation only. Meditation can help to get the real reflection of oneself in the mirror of their mind.

Obesity and other diseases have become a major problem among children. Once children reach their teenage, they become prone to pressures from various areas like education, career, and job prospects. This results in developing stress and doubting their own capabilities. This has made schools and educational institutions realize the need to introduce yoga as a part of the physical education and sports curriculum.

Yoga contributes more than a routine workout, or a sports related exercise. Students can work on their weakness and develop their strengths through yoga. Before playing any sport, warm-up with yoga-based stretches can also help in performing better. For better concentration and for sustaining energy while playing sports, yoga comes to aid. Introduction of yoga as a part of physical education will help achieve better sports, mental and physical skills in students and adults both. Let us look at the benefits of yoga in the context of physical benefits, physiological benefits, biochemical benefits, psychological benefits and other benefits.

Physical Benefits of Yoga

Yoga has many physical benefits and it widely became popular owing to the fact that it aids in weight loss and practicing it every day with proper nourishment will make the weight loss irreversible. Weight gain is the most talked about subject. Yoga cures obesity, aids in digestion and helps improve metabolism. Once metabolism gets better, a person can lead an active and a healthy life. It gives strength to do any given activity more efficiently. As mentioned earlier, it helps athletes perform better in sports. It improves flexibility to a great extent.

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It helps in improving eyesight, hearing and height in children as well. For these particular postures also known as asana are taken into account. For example, Tadasana is an excellent practice for increasing height. Children who start practicing yoga at a very young age can get rid of all the issues related to their eyesight, hearing, and height, among others. Yoga is important to prevent and fight any heart disease, muscular ailments, or any other issues which may be present. It also helps in faster recovery from aches, diseases, and injuries.

Physiological Benefits of Yoga

Yoga helps improve blood pressure, heart rate due to the postures called asana and breathing exercises. Oxygen reach becomes better and that helps improve blood circulation and lowering cholesterol. Better blood flow results in healthier skin, hair and nails too. It helps in reversing signs of ageing as well.

Yoga is considered as the best exercise to prevent spine and other ortho-muscular degradation. It is regarded as the best-known treatment for arthritis. Again, the sun salutations—Surya Namaskar—are excellent for arthritis and spinal strengthening. It greatly improves flexibility. Breathing techniques like Kapalabhati pranayama helps in both diabetes and heart disease.

Biochemical Benefits of Yoga

Yoga functions at the cellular level. By this, we mean that it helps control hormonal changes. It helps the increase of lymphocytes hence, boosting the immune system. Irregular menstrual cycle, can be greatly improved when we control female hormone through yoga. Often particular yoga exercises are incorporated during pregnancy to have a less stressful and safe childbirth. Hemoglobin increase acting at the biochemical level can help in curing many diseases such as anemia.

Yoga helps decreasing cholesterol and sodium levels that help fight stress and heart diseases. Diabetics benefit the most as it lowers blood sugar and improves insulin sensitivity. For example, the asana Supta Matsyendrasana (Recline twist or Reclining Lord of the Fish pose)works on abdominal organs and is one of the most important posture for diabetic patients. Dhanurasana (the bow posture) also regulates the functioning of pancreas and it is good for strengthening spinal and abdominal muscles. It is important to notice that a yoga pose will have many benefits helping all body functions.

Polycystic Ovaries Syndrome (PCOS) is now one of the most common problems in woman and even young girls are now facing this problem. PCOS causes infertility, weight gain, skin problems as acne, hair fall and excessive masculine hair as well. Yoga helps to balance hormones and keeps the ovaries healthy. Bhadrasana (Butterfly pose) helps work against PCOD (polycystic ovaries disease) and menstrual discomfort too. Other famous asanas for PCOD include Kapalbhaati, Chakki Chalanasana (Mill Churning Pose), Bharadvajasana (Seated Twist), etc. Pranayama as Nadisodhan Pranayama, which help reduce stress, is also very important as PCOD is mainly caused by stress.

Psychological Benefits of Yoga

Having mind free of doubt and knowing what is right and concentration is very crucial during the formative years. Yoga helps to nurture mind and improve

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concentration in sports and as well as studies. People who practice yoga and meditation tend to have better concentration, memory, coordination, balance and make better decisions. It helps in relaxing the mind, cures insomnia, and in turn reduces stress and anxiety. Yoga acts as an anti-depressant and helps people cope with problems.

Many schools instead of giving detention are making students practice yoga and the results have been setting up examples for other schools. Some excellent yoga poses help reduce stress. Some of them are Garudasana (Eagle pose), Uttanasana (Standing forward bend), Balasana (Child pose), Vajrayana, Supta Baddha Konasana (Reclining Bound angle pose), etc.

Other Benefits of Yoga

Yoga helps in boosting emotional health. It helps to become more aware of what you do, eat and drink. It helps build a timetable and helps to adopt a healthier lifestyle. It has been used as a tool to help people to get rid of bad habits like drugs, alcohol, smoking, etc.

Yoga helps a person to be spiritually aware and helps build better relationships. Not only spiritual health it helps in curing infertility issues and builds better sex drive too.

Yoga can be done individually, as couples or in groups; it helps in improving social circle as well. Studying on yoga can increase awareness on what is good and bad for the body as well as making the mind help make better decisions.

4.12 MEDITATION

The result of the growing social and cultural rush-taking place around us is that we are getting our mind stressed. We rely upon a good state of mind to be happy, content, and emotionally stable as individuals and at the same time to be kind, thoughtful, and considerate in our relationship with others. Soundness of mind contributes towards us being focused, creative, and spontaneous and to perform at our very best in everything that we do. However, what happens actually is that we use our mind to process many difficult thoughts and confusing emotions and we really do not know how to deal with that. This makes us miss the other things that could have been more important to us.

Different people deal with stress in different ways. Some of them busy themselves with heavy work, grateful for the distraction and others turn to their friends and family for support. However, we often seem to undermine the idea of dealing with stress by living in the present moment, to which meditation paves the right path and is technically proven. While meditating, it is important that our good intentions extend beyond the simple act of closing our eyes. Training the mind is much more difficult than it seems. It is not just limited to stopping thoughts or eliminating feelings. Meditation gifts a person with a greater appreciation and understanding for the present moment. It does not mean not getting lost in thoughts, not being distracted, not with overwhelmed with different emotions, but instead learning how to be in the here and now and to be mindful with healthy thought process. Meditation simply familiarizes ourselves with the present moment.

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Methods of Meditation

Meditation offers time for heightened and relaxation awareness in a stressful world where our senses are dulled. Research tells that meditation has the potential for more than just temporary stress relief.

Concentration meditation involves focusing on a single point. This could entail following the repeating a single word or mantra, breath, listening to a repetitive gong, staring at a candle flame, or counting beads on a mala. The challenging part in meditation is to stay focus. A beginner can meditate for only a few minutes and then can practice for longer durations. Meditation inspires the practitioner to observe wandering thoughts and to keep the focus on them.

Concept of Meditation

Meditation is the practice of balancing and achieving, the physical, mental and emotional aspects. Meditation is used to decrease the symptoms associated with stress, depression, and anxiety. The 'deep rest' that is succeeded through the practice of meditation can eliminate stress and uncertainty. This allows the individual to make better decisions through clearer and more focused thinking.

The concepts of meditation are similar to Yoga as both have same objective. In both these disciplines, self-healing depends on the practitioner to focus on maintaining a balance between physical and mental state.

Meditation, as explained, is the collective name of a process, which helps us to be more and more aware of our actions, thoughts, feelings and emotions. It has no rigid rules. You are not required to sit cross-legged in a particular pose for mediation. You can meditate in the most mundane circumstances. In fact, the best meditation is possible when your body is in the most comfortable state. The requirement of yoga in meditation is only for the purpose of instilling a habit of regularity in you.

Specific Techniques

Meditation is a process of training your mind for peace and calm state by increasing concentration and focus and relaxing the thoughts and body through various techniques. There are few widely used techniques discussed below.

There are many ways to approach relaxation techniques. Our body releases few chemicals for 'flight or fight', during stress. These stressful situations in day-to-day life are lifesaving in an emergency. However, if this stress constantly increases it takes over the emotional and physical health.

Muscle Relaxation

Dr. Edmund Jacobson invented the 'Jacobson's relaxation technique' in the 1920s to help people suffering from anxiety. According to Jacobson's relaxation technique, relaxing the muscles is directly proportional to relaxing the mind. This method involves tightening one of the muscle groups, while taking a deep breath and relaxing the remaining muscles of the body, which results in a release of tension. This is a therapy, which includes tightening the group of muscles while relaxing the other in an order of sequence. Dr. Jacobson says that, by focusing on a specific group of muscles, that is by tensing and relaxing them, we can be aware of our own body

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and physical sensations. This method is also known as progressive muscle relaxation (PMR) therapy.

This PMR is flowed by simple ten steps:

1. In a quiet place which is free from distractions. Recline in a chair or lie on the floor. Rest your hands.
2. Start taking few slow breaths.
3. Forehead – Squeeze the forehead muscles. Be sure to tense only the forehead muscles and the remaining body have to be relaxed. Slowly release the tension in your forehead. Continue to breathe slowly and evenly.
4. Jaw – Tense the jaw muscles and release them slowly. Feel the relaxation without forgetting to breathe evenly and slowly.
5. Neck and Shoulders – Increase tension in the muscles of neck and shoulders while raising the shoulders towards ears followed by releasing the tension created in the neck and shoulders.
6. Arms and Hands – While drawing both hands into fists, pull the fists closer to the chest. Squeeze them as tight as possible. Slowly start releasing the muscles tensed previously while noticing the relaxation in the created in the arms and hands region.
7. Buttocks – Slowly increase the tension in the muscles of buttocks followed by releasing the tension. Continue to breathe evenly and slowly.
8. Legs – Increase the tension in the quadriceps and calves. Melt the tension and start feeling relaxed.
9. Feet – Slowly, create tension in feet and toes and slowly release the tension.
10. Continue to breathe evenly and slowly throughout the process while creating or increasing the tense and relaxing the muscles.

This technique is widely used in insomnia patients. Researches studies provide evidence that Jacobson's relaxation technique helps in patients to reduce epilepsy and the frequency of their seizures.

Deep muscle relaxation is a technique for beginners to tense your muscle and then consciously relax them. When the muscles are tensed, there is decreased blood flow, nerve communication and even nutrient assimilation. This is will help the body to open the pores and allow the fresh blood flow through the muscle and nourishing it. This allows the energy to heal every part of the body.

Logic Relaxation

Meditation involves, a state known as 'logic relaxation'. Many consider there is an increase of the activation of the prefrontal lobe. This region is associated with logic and is not supposed to be activated during the 'logic relaxation'. It was termed by Craven in 1989.

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According to Cardoso et al. 2004 and Cardoso et al., 2007, logic relaxation is one of the most difficult concepts to understand. To obtain logic relaxation there are three major things to follow:

1. Not "to intend" to analyse the possible psychophysical effects
2. Not "to intend" to judge the possible effects
3. Not "to intend" to create any type of expectation regarding the process

Through these three strategies, it helps the individual to become involved in the thoughts which appear during meditation.

Logic relaxation is unclear and paradox in the beginning. In simple terms meditation is an alternative process of 'anchor' (self-focusing skill) and logic relaxation. Meditation gradually increases the 'focused in the anchor'. Despite the person's attention to maintain 'logic relaxation', will involve in the stream of thoughts and return to the anchor.

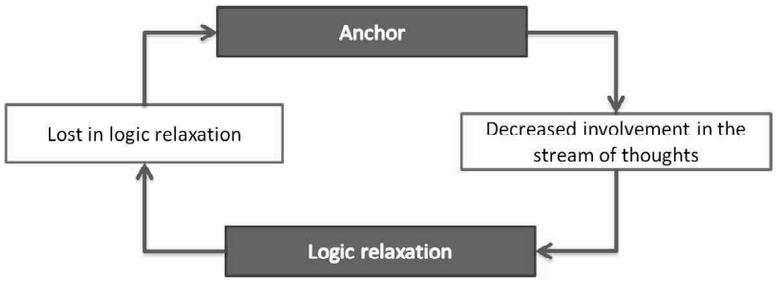

Fig. 4.7 *Method of Logic Relaxation*

In this schematical representation, the higher activity in prefrontal lobe, caused by the use of the 'self-focus skill', which is an exercise to increase the focus of the attention, important in the initial stage of meditating process. The model has explained better in seven steps:

1. Definition of the specific technique
2. Application of the 'anchor exercise'
3. 'Logic relaxation'
4. Loss of 'logic relaxation'
5. Higher effort in the 'anchor exercise'
6. Repeated cycles, every time in more distant
7. Psychophysical relaxation

Self-Induced State

Instead of getting more in touch with reality we humans tend to take a step away from reality. Meditation causes us to take a step away from the model of reality that we have built in our minds, by adding thought after thought through the years, until the only world we can see is the one we built (or others built in you, often enough). On the contrary, the state of meditation results in heightened awareness of reality rather than diminishing it. Many of the techniques of meditation use a mechanism that narrows down and blocks out extraneous stimulus of the senses,

but that is not the state of meditation itself, but is only the technique used to arrive at a state of meditation.

Benefits of Meditation

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Meditation can improve mental and emotional health. It can be done anywhere, without special equipment. Meditation support groups and courses are widely available. There are many styles, each with different benefits and strengths.

Few benefits of meditation are as follows:

1. Stress reduction is one of the most common benefits people say about meditation.
2. Fewer stress results in less anxiety or even anxiety-related mental health issues like phobias, social anxiety and obsessive-compulsive behaviors.
3. Some forms of meditation improve depression and create a more positive outlook on life.
4. Self-inquiry and related styles of meditation can help to "know ourselves."
5. Improvements in attention and clarity of thinking may help keep your mind young.
6. Meditation develops willpower and mental discipline and can help you avoid triggers for unwanted impulses.
7. Meditation helps in improving sleep and reduces insomnia.
8. Meditation can diminish the perception of pain in the brain and helps in treating chronic pain when used as a supplement to medical care or physical therapy.
9. Meditation can also improve physical health by reducing strain on the heart and decreases blood pressure. It also decreases cardiovascular diseases related to hypertension.
10. Due to peace of mind, people feels happier and confident.

There are more than 100 physiological, psychological and spiritual benefits.

Check Your Progress

23. Which two stages yogic path involves to calm the inner thoughts?
24. How one can attain complete mental and physical relaxation?
25. Why is breathing important in yoga?
26. What is meditation?
27. How are yoga and meditation similar?
28. Define concentration meditation.

4.13 ANSWERS TO 'CHECK YOUR PROGRESS'

1. Stress can be defined as the method in which the organism's body react to a condition, such as a threat, physical and psychological barrier.

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2. Baroreceptors are sensors that are located in the carotid sinus, at the bifurcation of external and internal carotids, and in the aortic arch.
3. Environmental stress refers to physical, chemical, and biological constraints on the productivity of species and on the development of ecosystems.
4. Electromagnetic pollution is referred to as the accumulation of electromagnetic radiation in their non-ionizing form, like radio waves, etc., that people are constantly exposed at, especially in large cities.
5. Species and ecosystems have some capacity to tolerate changes in the intensity of environmental stressors. This is known as resistance, but there are limits which represent thresholds of tolerance.
6. In terms of environmental physiology, strain is the functional property of an organism to resist and survive under environmental stress, i.e., physiological adaptation or compensatory response to the external stressor.
7. A stressor is a chemical or biological agent, environmental condition, external stimulus or an event seen as causing stress to an organism.
8. The changes in the organism by which it suits best in the environment and leave behind offspring's are known as adaptation.
9. Animals belonging to same stock migrate to different geographical areas to meet their need for food and safety and are subjected to different life conditions. This leads to varied and diverse adaptations. This is called divergence.
10. Homeostasis means the ability of organisms to maintain uniformity in the internal conditions without being effected by the external changes.
11. Homeostatic systems exhibit several properties which are as follows:
    - Stability
    - Whole association, internal, structural, and functional, contributes to the maintenance of balance
    - Unpredictable
12. Homeostasis helps in solving two purposes which are as follows:
    - To keep blood in clot-free state that is easy to flow
    - To stimulate a speedy and enclosed haemostatic plug at the spot of vascular injury.
13. Regulators try to maintain the parameter at a constant level, regardless of what is happening in its environment.
14. Coagulation is clotting of blood.
15. An endotherm is an organism that maintains its body at a metabolically favourable temperature, mainly by the use of heat released by its internal bodily functions instead of relying almost purely on ambient heat.
16. Thermoregulation is the ability of an organism to maintain a core body temperature, which is 37° C (98°F) within an optimal physiological range.

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17. If the body is unable to maintain a normal temperature and it increases significantly above normal, a condition known as hyperthermia occurs. The opposite condition, when body temperature decreases below normal levels, is known as hypothermia.
18. Osmotic shock or osmotic stress is physiologic dysfunction caused by a sudden change in the solute concentration around a cell, which causes a rapid change in the movement of water across its cell membrane.
19. Osmotic pressure is the minimum pressure which is exerted by cell membrane to prevent the inward flow of its pure solvent across semipermeable membrane. Cell wall helps to maintain the cell's water balance.
20. Two major types of osmoregulation are osmoconformers and osmoregulators.
21. Hypoxia is a condition in which the body or a region of the body is deprived of adequate oxygen supply at the tissue level.
22. The regulation of DO can be done by pumping nitrogen into water and then oxygen gradually decreased to the level needed.
23. Yogic path involves two stages to calm the inner thoughts:
    - The First Stage: These are the methods of developing positive qualities into one's own self. If such qualities are not built up, especially during meditation, then the inner peace will be disturbed by the chaos of the outside world.
    - The Second Stage: This is path of meditation. This is the stage to practice meditation to transcend the self and attain enlightenment.
24. One can only attain complete mental and physical relaxation just by finding the connection of the soul with the higher power. Spiritual relaxation will help in learning to feel the happiness within yourself and grab the positivity around you.
25. Breath is one of the pivotal parts of yogic feats. It regulates the mind, helps to stay focused and concentrated and controls the emotions. Learning the breathing control helps to make one feel calm, composed and energetic.
26. Meditation is the practice of balancing and achieving, the physical, mental and emotional aspects. Meditation is used to decrease the symptoms associated with stress, depression, and anxiety.
27. The concepts of meditation are similar to Yoga as both have same objective. In both disciplines, self-healing depends on the practitioner to focus on maintaining a balance between physical and mental state.
28. Concentration meditation involves focusing on a single point. This could entail following the repeating a single word or mantra, breath, listening to a repetitive gong, staring at a candle flame, or counting beads on a mala.

4.14 SUMMARY

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- In biology, 'Stress' is referred as an organism's response to a stressor, such as an environmental condition.
- The Central Nervous System or CNS, which includes brain and spinal cord, plays a crucial and significant role in the stress-related mechanisms in a body.
- The ambiguity in defining this phenomenon was first recognized by Hans Selye (1907–1982) in 1926.
- In the brain, the endocrine interactions are significant and essential for the translation of stress into physiological and psychological changes.
- The ANS is comprised of the Parasympathetic Nervous System (PNS) and Sympathetic Nervous System (SNS), both the two branches are active with opposing activities.
- The activity of the sympathetic nervous system describes the 'Fight or Flight' response.
- The HPA axis is considered as a neuroendocrine system that mediates a stress response.
- Environmental stress refers to physical, chemical, and biological constraints on the productivity of species and on the development of ecosystems.
- Thermal stress happens when releases of heat influence ecosystems, such as in the vicinity of natural hot-water vents on the ocean floor, and near industrial discharges of heated water.
- Biotic stress is stress that occurs as a result of damage done to an organism by other living organisms, such as bacteria, viruses, fungi, parasites, beneficial and harmful insects, weeds, and cultivated or native plants.
- When organisms are exposed to an extreme environmental stress, one of three possible outcomes takes place, namely the organisms may die, the organisms avoid the environmental stress and survive, or the organisms tolerates the environmental stress and survive.
- In terms of environmental physiology, strain is the functional property of an organism to resist and survive under environmental stress.
- Stressors can cause physical, chemical and mental responses internally. Physical stressors produce mechanical stresses on skin, bones, ligaments, tendons, muscles and nerves that cause tissue deformation and (in extreme cases) tissue failure.
- Adaptation is a generic phrase encompassing all changes undertaken by an organism to reduce the negative effects of unfamiliar and undesirable external environment.
- The changes in the organism by which it suits best in the environment and leave behind offspring's are known as adaptation.

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- The capability of an organism to change itself with the changing world determines its survival.
- In order to maintain performance across a range of environmental conditions, there are several strategies organisms use to acclimate.
- The term homeostasis refers to the maintenance of stable condition in living organisms through involuntary mechanism which works against the system trying to disturb the balance.
- The process of feeling hungry and thirsty in living beings is also the result of homeostasis.
- Homeostasis is present in regulating the temperature and blood pressure of human beings nearly constant though the activity level of the being or the environment changes.
- Coagulation works with two components namely protein (coagulation factor) and cellular (platelet). Coagulation starts as soon as the lining of a blood vessel (endothelium) is damages by an injury.
- An endotherm is an organism that maintains its body at a metabolically favorable temperature, mainly by the use of heat released by its internal bodily functions instead of relying almost purely on ambient heat.
- The ectothermic animals are generally termed as cold-blooded animals and mostly gain the heat from external sources.
- The endothermic animals are generally termed as warm-blooded animals.
- Normal body temperature is around 37 °C (99 °F), and hypothermia sets in when the core body temperature gets lower than 35 °C (95 °F).
- Animals other than humans regulate and maintain their body temperature with physiological adjustments and behaviour.
- To cope with limited food resources and low temperatures, some mammals hibernate during cold periods.
- Daily torpor occurs in small endotherms like bats and hummingbirds, which temporarily reduces their high metabolic rates to conserve energy.
- Heat can be exchanged between an animal and its environment through four mechanisms, namely radiation, evaporation, convection, and conduction.
- Salinity or osmolality represents an abiotic factor that varies depending on the environment.
- All organisms have mechanisms to respond to osmotic shock, with sensors and signal transduction networks providing information to the cell about the osmolarity of its surroundings; these signals activate responses to deal with extreme conditions.
- The ionic strength of a solution is a measure of the concentration of ions in that solution.

- Osmoregulation is the process of maintaining salt and water balance (osmotic balance) across membranes within the body.
- Two major types of osmoregulation are osmoconformers and osmoregulators.
- Hypoxia differs from hypoxemia and anoxemia in that hypoxia refers to a state in which oxygen supply is insufficient, whereas hypoxemia and anoxemia refer specifically to states that have low or zero arterial oxygen supply.
- The physiological response to exercise is dependent on the intensity, duration and frequency of the exercise as well as the environmental conditions.
- In yoga, proper relaxation is known as 'Savasana'. Life is full of upheavals. Stress and tension are our regular companion.
- Yoga is quite different from mere physical exercises. The yoga poses or asanas are the media to bringing one's body, mind, and soul into a single alignment so that one can feel his true self and are connected to the almighty.
- Meditation is one of the vital elements of the yogic practice.
- Yoga is not a mere exercise. It is the way to connect one's body with the soul and mind and finally leads one to the devotion towards the ultimate.
- Yoga and physical education both have the same objective of keeping the body healthy. Schools, colleges, and military forces, among other institutions, have adopted physical education also known as physical training.
- Yoga contributes more than a routine workout or a sports related exercise would do. Students can work on their weakness and develop their strengths.
- Yoga functions at the cellular level. By this, it means it helps control hormonal changes. It helps increase lymphocytes hence boosting the immune system. Yoga helps to nurture mind and improve concentration in sports and as well as studies.
- People who practice yoga and meditation tend to have better concentration, memory, coordination, balance and make better decisions.
- Meditation is an art or skill of developing a focus on a particular object or on an activity while achieving an emotionally calm state.
- Meditation is a way of training the mind, similarly training the body for healthy fitness. Meditation has the power to create peace of mind and unconditional happiness.
- Deep muscle relation is a technique for beginners to tense your muscle and then consciously relax them.
- Meditation involves, a state known as "logic relaxation". Many consider there is an increase of the activation of the prefrontal lobe. This region is associated with logic and is not supposed to be activated during the "logic relaxation".

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- Meditation can improve mental and emotional health. It can be done anywhere, without special equipment. Meditation support groups and courses are widely available. There are many styles, each with different benefits and strengths.

4.15 KEY TERMS

- **Stress:** 'Stress' is referred as an organism's response to a stressor, such as an environmental condition.
- **Wildfire**: Wildfire is referred as a powerful disturbance through which a large amount of the biomass of an ecosystem is combusted, and the major dominant species may be destroyed.
- **Plastic Pollution:** It involves the accumulation of plastic products and microplastics in the environment that adversely affects wildlife, wildlife habitat, or humans.
- **Biotic Stress:** Biotic stress is stress that occurs as a result of damage done to an organism by other living organisms, such as bacteria, viruses, fungi, parasites, beneficial and harmful insects, weeds, and cultivated or native plants.
- **Elastic Resistance:** Ability of the organism to prevent reversible or elastic strain (physical or chemical change) when exposed to a specific stress.
- **Plastic Resistance:** Plastic resistance is the ability of an organism to prevent irreversible or plastic strain. The term resistance to environmental stress has been used for plastic resistance.
- **Stressor**: A **stressor** is a chemical or biological agent, environmental condition, external stimulus or an event seen as causing stress to an organism.
- **Adaptation:** Adaptation is a generic phrase encompassing all changes undertaken by an organism to reduce the negative effects of unfamiliar and undesirable external environment.
- **Acclimatization**: Acclimatization is the process in which an individual organism adjusts to a change in its environment, such as a change in altitude, temperature, humidity, photoperiod, or pH.
- **Homeostasis**: The term homeostasis refers to the maintenance of stable condition in living organisms through involuntary mechanism which works against the system trying to disturb the balance.
- **Enzyme system**: The chronological arrangement of enzymes that converts the metabolite into the end product needed by the organisms is known as the enzyme system.
- **Endotherm:** An endotherm is an organism that maintains its body at a metabolically favourable temperature, mainly by the use of heat released by its internal bodily functions instead of relying almost purely on ambient heat.

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- **Thermoregulation:** Thermoregulation is the ability of an organism to maintain a core body temperature, which is 37° C (98°F) within an optimal physiological range.
- **Ectothermic Animals:** The ectothermic animals are generally termed as cold-blooded animals and mostly gain the heat from external sources.
- **Endothermic Animals:** The endothermic animals are generally termed as warm-blooded animals.
- **Poikilotherm**: A poikilotherm is an organism whose internal temperature varies considerably.
- **Osmosis**: Osmosis is a special type of diffusion of water molecules across a semi-permeable membrane, from a solution of high water potential to a region of low water potential.
- **Osmotic Pressure**: It is the minimum pressure which is exerted by cell membrane to prevent the inward flow of its pure solvent across semipermeable membrane. Cell wall helps to maintain the cell's water balance.
- **Hypoxia:** Hypoxia is a condition in which the body or a region of the body is deprived of adequate oxygen supply at the tissue level.
- **Yoga**: It comes from the Sanskrit word 'Yog' which means union; the union between God and humans
- Yoga Sutras: It refers to the compilation of the axioms of yoga practice
- **Savasana**: In yoga, proper relaxation is known as 'Savasana'
- **Asanas**: It refers to the forms of yoga exercise
- **Insomnia**: It refers to Habitual sleeplessness or the inability to sleep.
- **Metabolism:** It refers to the chemical processes that occur within a living organism in order to maintain life.
- **Meditation**: It is an art or skill of developing a focus on a particular object or on an activity while achieving an emotionally calm state.
- **Deep muscle relaxation**: It is a technique for beginners to tense your muscle and then consciously relax them.

4.16 SELF-ASSESSMENT QUESTIONS AND EXERCISES

Short-Answer Questions

1. Write about various categories of environmental stressors.
2. What is the difference between biotic and abiotic stress?
3. What are the two types of stress resistance in biological organisms?
4. What is the difference between elastic stain and plastic strain?

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5. Define convergence or parallelism.
6. What are the two phases involved in homeostasis mechanism?
7. Write the Mechanisms of thermoregulation.
8. What is the difference between homeothermy and poikilothermy?
9. Why the ability to control cell volume is important for cell function? Write its mechanism.
10. Define osmosis.
11. What are regulators and conformers?
12. What is the effect of exercise on body?
13. Write the benefits of yoga for a good lifestyle.
14. What are the biochemical benefits of yoga for humans?
15. Write the concept of meditation.
16. Write the steps of Progressive Muscle Relaxation (PMR) therapy.

Long-Answer Questions

1. Explain the biology of stress in detail.
2. Analyze the adaptations in the organisms with relation to environments. Explain with examples.
3. Explain the concept of homeostasis
4. Describe the mechanism of homeostasis in detail.
5. Elaborate on the physiological mechanism of regulation of body temperature.
6. Write a comparative note on hibernation, estivation and daily torpor.
7. Explain Sodium-Potassium pump in detail with diagram.
8. Analyze osmoregulation in aqueous and terrestrial environments.
9. Describe the basic principles of yoga in detail.
10. Elaborate on the specific techniques involved in meditation.

4.17 FURTHER READING

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