Biyani's Think Tank

Concept based notes

INSTRUMENTATION AND BIOTECHNIQUES

[B.Sc. Biotech Sem II]

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Preface

I am glad to present this book, especially designed to serve the needs of the students. The book has been written keeping in mind the general weakness in understanding the fundamental concepts of the topics. The book is self-explanatory and adopts the ―Teach Yourself‖ style. It is based on question-answer pattern. The language of book is quite easy and understandable based on scientific approach.

Any further improvement in the contents of the book by making corrections, omission and inclusion is keen to be achieved based on suggestions from the readers for which the author shall be obliged.

I acknowledge special thanks to Mr. Rajeev Biyani, Chairman & Dr. Sanjay Biyani, Director (Acad.) Biyani Group of Colleges, who are the backbones and main concept provider and also have been constant source of motivation throughout this Endeavour. They played an active role in coordinating the various stages of this Endeavour and spearheaded the publishing work.

I look forward to receiving valuable suggestions from professors of various educational institutions, other faculty members and students for improvement of the quality of the book. The reader may feel free to send in their comments and suggestions to the under mentioned address.

Author

Detailed Syllabus

BTH- 52T-255 INSTRUMENTATION AND BIOTECHNIQUES

UNIT-I

Buffers- Preparation and principle of pH meter

Microscopy – Principle and application of Dissecting and compound Microscope, phase contrast, Fluorescence and Electron microscopy (SEM and TEM)

Spectroscopy: basic principle, instrumentation, application, UV visible spectrophotometer

IR & Raman spectroscopy – Basic principle, theory and qualitative interpretation of I.R. spectra, quantitative methods.

UNIT-II

Fluorescence spectroscopy- Principle, Instrument Design, Methods & Applications

Centrifugation & Ultracentrifugation-Basic principles, Forces involved, RCF Centrifugation, techniques-principal, types and applications.

Chromatography-Basic Concepts of Adsorption & Partition Chromatography; TLC, Paper, GC, GLC, HPLC, Ion exchange.

UNIT-III

Electrophoresis: Principle, Electrophoretic mobility (EPM) estimation, factors affecting EPM, Instrument design & set-up, Methodology & Applications of Free & Zone (Paper, Cellulose acetate, Agarose & Starch gel, Pulse-field, PAGE, SDS-PAGE, Capillary) Applications isoelectric focusing, 2D electrophoresis

UNIT-IV

General biophysical methods- Measurement of pH, Radioactive labelling and counting, Autoradiography, X ray crystallography- X Ray diffraction, Bragg equation, Reciprocal lattice, Miller indices and unit cell, concept of different crystal structure, determination of crystal structure (Concept of rotating crystal method, powder method)

Unit I

Buffers, Microscopy, Spectroscopy

Q-1 What is a buffer?
Ans A buffer is a solution that resists changes in pH when small amounts of acid or base are added.

Q-2. What is the principle of a pH meter?
Ans A pH meter measures the hydrogen ion concentration using a glass electrode and reference electrode to determine pH.

Q-3. Name two applications of a compound microscope.
Ans Used in biology for observing cell structures and in microbiology for identifying microorganisms.

Q-4. What is the function of SEM in microscopy?
Ans Scanning Electron Microscope (SEM) provides detailed 3D surface images of a sample.

Q-5. What is the basic principle of UV-visible spectroscopy?
Ans It is based on the absorption of ultraviolet or visible light by electrons in molecules, causing electronic transitions.

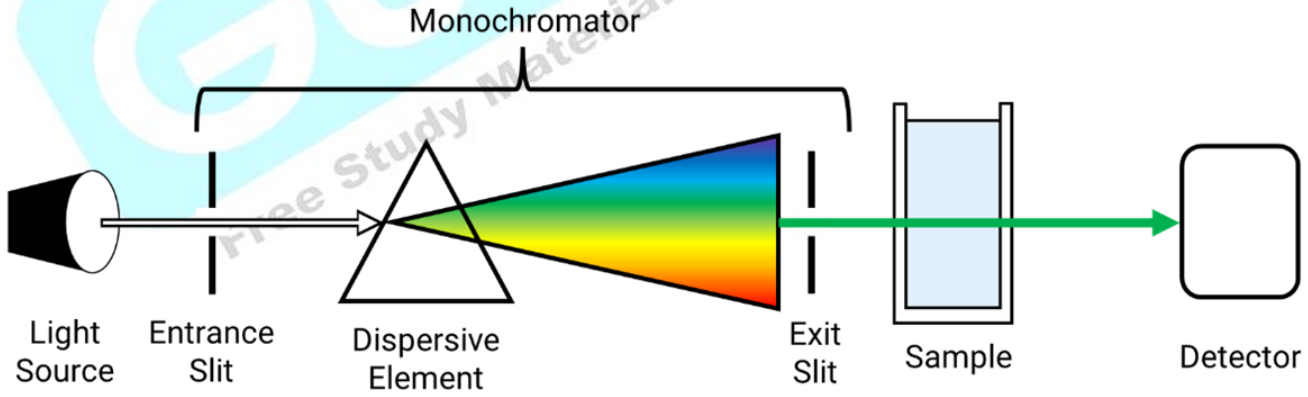

Fig 1. U.V. Vis spectrophotometer working principle.

Q-6. How are buffers prepared in the laboratory?
Ans Buffers are prepared by mixing a weak acid with its conjugate base or a weak base with its conjugate acid. For example, an acetic acid and sodium acetate buffer is made by mixing acetic acid with sodium acetate in a desired ratio to achieve a specific pH. The exact pH can be calculated using the Henderson-Hasselbalch equation. The pH is adjusted using small amounts of strong acid or base.

Q-7. Describe the principle and use of a compound microscope.
Ans A compound microscope uses multiple lenses (objective and ocular) to magnify small specimens. Light passes through the sample and then through objective lenses that provide primary magnification. The eyepiece further magnifies this image. This microscope is crucial in biological studies for observing cells, tissues, and microorganisms.

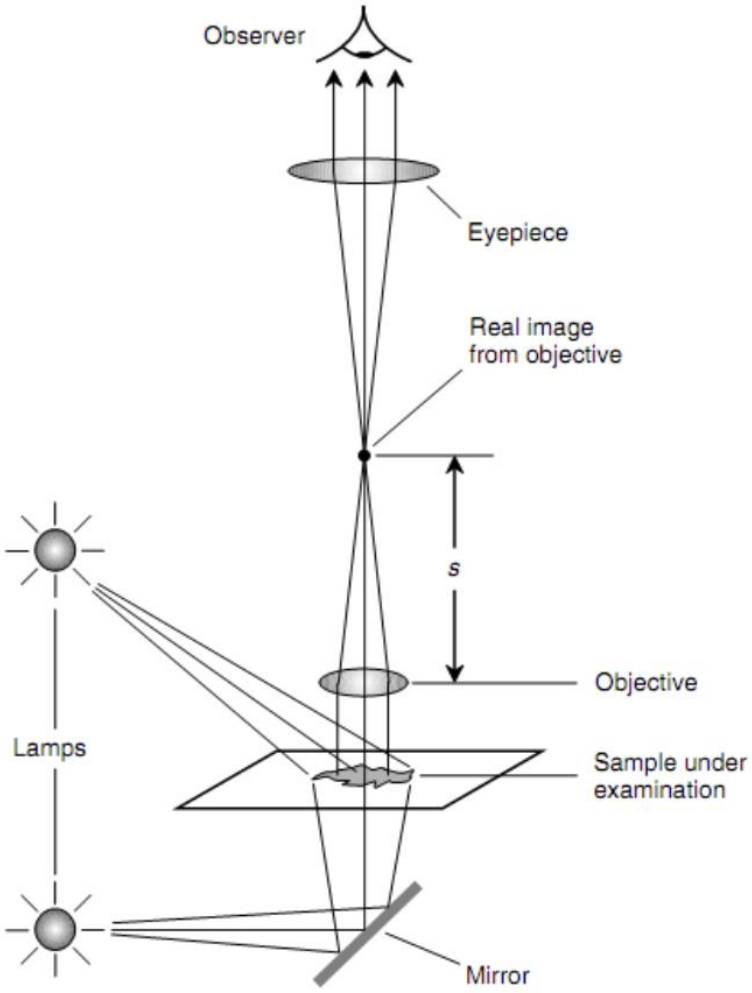

Fig 2. llumination and focusing in a compound optical microscope.

Q-8. What is the principle behind fluorescence microscopy?
Ans Fluorescence microscopy is based on the emission of light by a substance that has absorbed light. When fluorochromes (fluorescent dyes) are excited by specific wavelengths, they emit light at a different (longer) wavelength, which is used to visualize specific components of cells or tissues.

Q-9. Explain the role of UV-Visible spectroscopy in quantitative analysis.
Ans In UV-Vis spectroscopy, the amount of light absorbed at a specific wavelength correlates with the concentration of the compound in solution. This is described by Beer-Lambert's law, which allows quantitative determination of sample concentration.

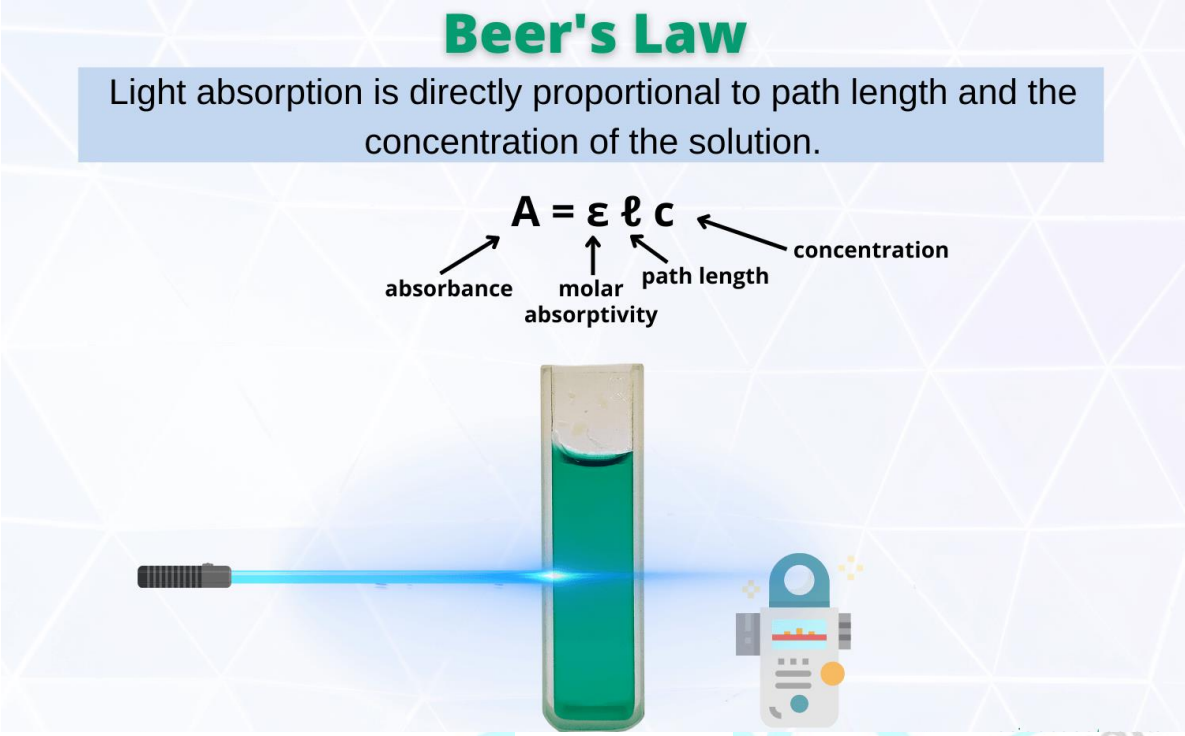

Fig 3. Beer Lambert's law.

Q-10. What is Raman spectroscopy and where is it used?
Ans Raman spectroscopy is based on inelastic scattering of monochromatic light, usually from a laser. It provides molecular fingerprints of substances and is widely used in material science, chemistry, and pharmaceutical analysis.

Q-11. Discuss in detail the principle, instrumentation, and applications of electron microscopy (SEM and TEM).

Ans Electron microscopy is a powerful imaging technique that uses a beam of electrons to visualize specimens at very high resolution. Unlike light microscopes that use visible light, electron microscopes utilize electrons with much shorter wavelengths, allowing for magnifications up to two million times and resolving structures at the nanometer and even atomic levels. The two primary types of electron microscopes are the Scanning Electron Microscope (SEM) and the Transmission Electron Microscope (TEM). Both have distinct principles, instrumentation, and applications, making them indispensable tools in modern scientific research.

Principle

Electron microscopy operates on the principle that electrons have wave-like properties. The wavelength of electrons is much smaller than that of visible light, enabling a significantly higher resolution. The resolution of an optical microscope is limited to around 200 nm due to the diffraction limit of light. In contrast, electron microscopes can achieve resolution below 1 nm.

- **SEM**: SEM scans a focused beam of high-energy electrons across the surface of a specimen. These electrons interact with atoms in the sample, producing various signals such as secondary electrons, backscattered electrons, and characteristic X-rays. These signals are used to construct detailed 3D images of the sample's surface.

- **TEM**: TEM operates by transmitting a beam of electrons through an ultra-thin specimen. As electrons pass through different parts of the sample, they are scattered differently, creating an image based on the internal structure of the specimen. It provides highly detailed 2D images at atomic-scale resolution.

Instrumentation

Although both SEM and TEM share some similarities in electron generation, they differ significantly in design and components:

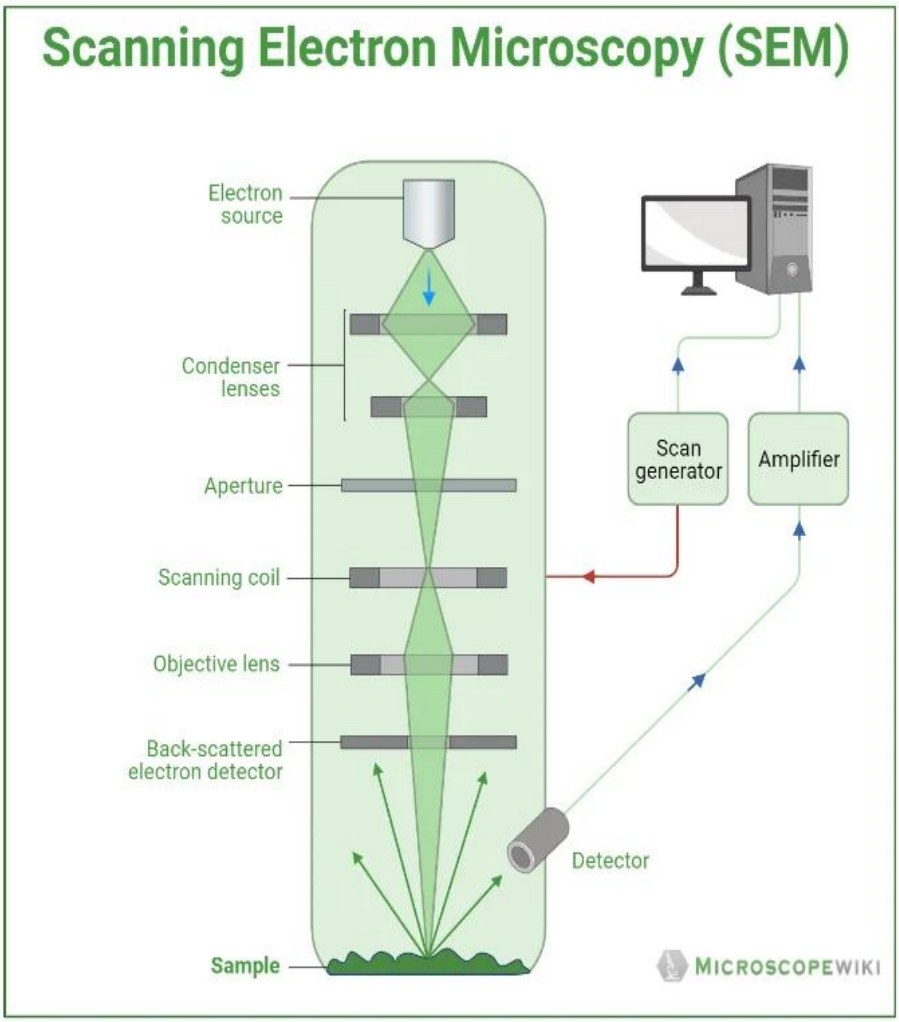

Fig 4. Scanning Electron Microscope

Scanning Electron Microscope (SEM)
Key components:

- **Electron gun**: Produces a beam of electrons, typically via thermionic or field emission.
- **Condenser and objective lenses**: Focus the electron beam into a fine probe.

- **Scan coils**: Deflect the beam in a raster pattern over the sample surface.
- **Sample chamber**: Holds the specimen under vacuum.
- **Detectors**:
    - *Secondary electron detector* (for surface topology)
    - *Backscattered electron detector* (for compositional contrast)
    - *X-ray detector* (for elemental analysis via EDS)

Image formation: The detectors collect emitted signals and convert them into an electronic image displayed on a monitor.

Transmission Electron Microscope (TEM)
Key components:

- **Electron gun**: Emits high-energy electrons (usually 60-300 kV).
- **Condenser lenses**: Condense the beam before it hits the sample.
- **Specimen stage**: Requires ultra-thin sections (usually <100 nm thick).
- **Objective lens**: Magnifies the image formed by transmitted electrons.
- **Projector lenses**: Further magnify the image for viewing.
- **Viewing screen or digital camera**: Captures high-resolution 2D images.

Sample preparation: TEM requires thin sectioning (using ultramicrotomes), staining with heavy metals (like uranyl acetate), and careful mounting on grids.

Applications of SEM and TEM

Both SEM and TEM are utilized in diverse fields such as materials science, nanotechnology, biology, medicine, and engineering. Below are detailed applications of each:

SEM

- **Material Science**: Analyzing surface morphology, fracture surfaces, and coatings.
- **Biology**: Examining the surface structure of cells, tissues, and microorganisms.
- **Forensics**: Identifying gunshot residue, tool marks, and other microscopic evidence.
- **Electronics**: Investigating semiconductor devices and failure analysis.
- **Geology**: Studying mineral textures, compositions, and microstructures.

SEM can be used to observe the surface features of pollen grains, revealing detailed texture and patterns

that are species-specific.

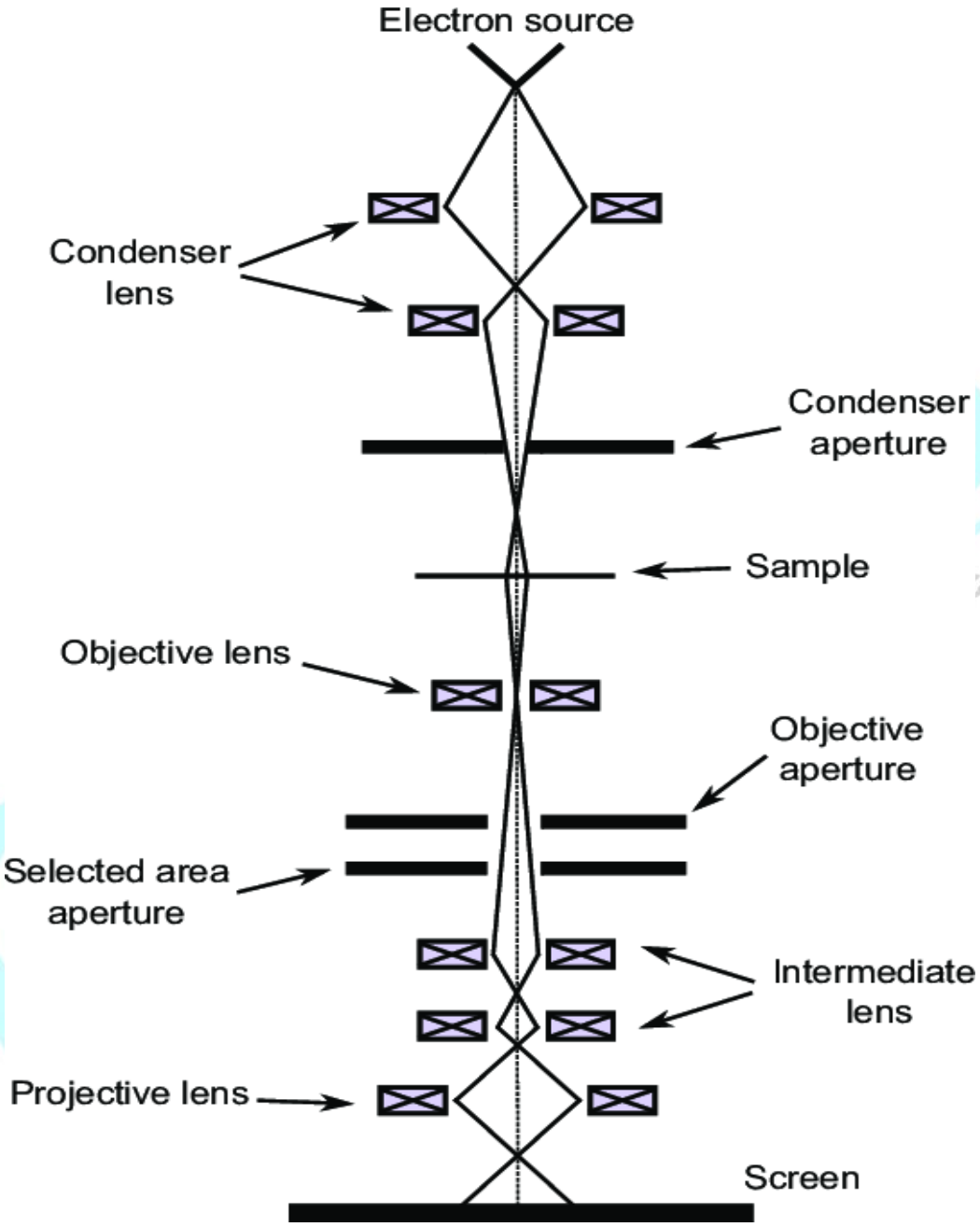

Fig 5. Transmission Electron Microscope

TEM

- **Cell Biology**: Visualizing organelles, viruses, and protein complexes at molecular resolution.
- **Virology**: Identifying and characterizing virus morphology and interactions with host cells.
- **Crystallography**: Determining crystal structure using electron diffraction techniques.
- **Nanotechnology**: Measuring and characterizing nanoparticles and nanomaterials.

- **Metallurgy**: Investigating dislocations, grain boundaries, and internal defects in metals.

TEM has been pivotal in determining the double-helix structure of DNA and visualizing ribosomes during protein synthesis.

Advantages and Limitations

Aspect	SEM	TEM
Resolution	~1–10 nm	<1 nm
Sample	Bulk or surface samples	Ultrathin sections only
Image Type	3D surface image	2D internal structure
Sample Prep	Relatively easy	Complex and time-consuming
Cost	Less expensive than TEM	More expensive and delicate

Despite their immense capabilities, both SEM and TEM require vacuum environments, complex sample preparation, and trained personnel. Specimens may also be damaged by the electron beam, especially in biological samples.

Q-12. Explain the types and role of buffer solutions along with the working of a pH meter in laboratory and biological settings.

Ans Buffer solutions and pH meters are essential tools in both laboratory research and biological systems. Buffer solutions maintain a constant pH despite the addition of acids or bases, while pH meters provide accurate and real-time measurements of hydrogen ion concentration. These tools are crucial for biochemical reactions, pharmaceutical formulations, medical diagnostics, and environmental monitoring.

Buffer Solutions

A buffer is a solution that resists changes in pH upon the addition of small amounts of acidic or basic substances. Buffers are composed of a weak acid and its conjugate base, or a weak base and its conjugate acid. They function according to Le Chatelier's Principle, which allows the equilibrium to shift and neutralize added hydrogen (H^+) or hydroxide (OH^-) ions.

Henderson-Hasselbalch Equation : The pH of a buffer solution can be calculated using this equation:

pH=pKa+log⁡([A−]\[HA])

Where:

- pKa is the dissociation constant of the weak acid,

- [A−] is the concentration of the conjugate base,
- [HA] is the concentration of the weak acid.

Types of Buffer Solutions

A. Acidic Buffers

- Consist of a weak acid and its salt with a strong base.
- Maintain a pH below 7.
- Example: Acetic acid (CH_3COOH) and sodium acetate (CH_3COONa).

B. Basic Buffers

- Comprise a weak base and its salt with a strong acid.
- Maintain a pH above 7.
- Example: Ammonia (NH_3) and ammonium chloride (NH_4Cl).

C. Biological Buffers

- Designed to be biocompatible, often used in physiological systems.
- Examples:
    - Phosphate buffer (used in DNA extraction),
    - Tris buffer (used in electrophoresis),
    - HEPES (used in cell culture).

Role and Applications of Buffer Solutions

A. In Laboratories

- Enzyme reactions: Most enzymes function within narrow pH ranges. Buffers ensure that pH remains optimal.
- Titration and analytical chemistry: Buffers stabilize pH for accurate measurements.
- Sample preparation: Ensures biomolecules remain in native forms.

B. In Biological Systems

- Blood buffering system: Maintains pH near 7.4 using carbonic acid (H_2CO_3) and bicarbonate (HCO_3^-).
- Cellular processes: Buffers like phosphate ions help maintain intracellular pH.
- Digestive system: Buffers in saliva and stomach regulate pH to aid digestion and enzyme activity.

pH Meter

A pH meter is an electronic device used to measure the hydrogen-ion activity (H^+) in a solution, which is expressed as pH. It is widely used in laboratories, industry, agriculture, environmental monitoring, and medicine.

Principle

The pH meter works on the principle of electrochemical potential. It measures the voltage

(electromotive force or EMF) generated between two electrodes (a reference and a glass electrode) immersed in a solution. This voltage is directly related to the hydrogen ion concentration, hence the pH.

Mathematically, the relationship is given by the **Nernst equation**:

$E = E^\circ - 2.303\frac{RT}{nF} \cdot \log[H^+]$

Since: $pH = -\log[H^+]$

The output voltage is directly related to the pH of the solution.

Components of a pH Meter

1. Glass Electrode (Measuring Electrode):
    - Sensitive to hydrogen ions.
    - Made of special pH-sensitive glass that allows exchange of H^+ ions with the solution.
    - Contains an internal buffer solution (usually pH 7).
2. Reference Electrode:
    - Provides a constant reference voltage.
    - Commonly a silver/silver chloride (Ag/AgCl) or calomel (Hg/Hg_2Cl_2) electrode.
    - Filled with potassium chloride (KCl) solution.
3. Temperature Sensor (optional but important):
    - Since pH readings are temperature-dependent, a thermistor or RTD is often used to compensate.
4. High-Impedance Voltmeter (pH meter circuitry):
    - Converts the tiny voltage difference (millivolts) between the electrodes into a readable pH value.
    - Often includes a microprocessor, display, and calibration controls.
5. Display Unit:
    - Shows the pH value, sometimes temperature as well.
6. Calibration System:
    - Uses standard buffer solutions (e.g., pH 4, 7, 10) to ensure accuracy

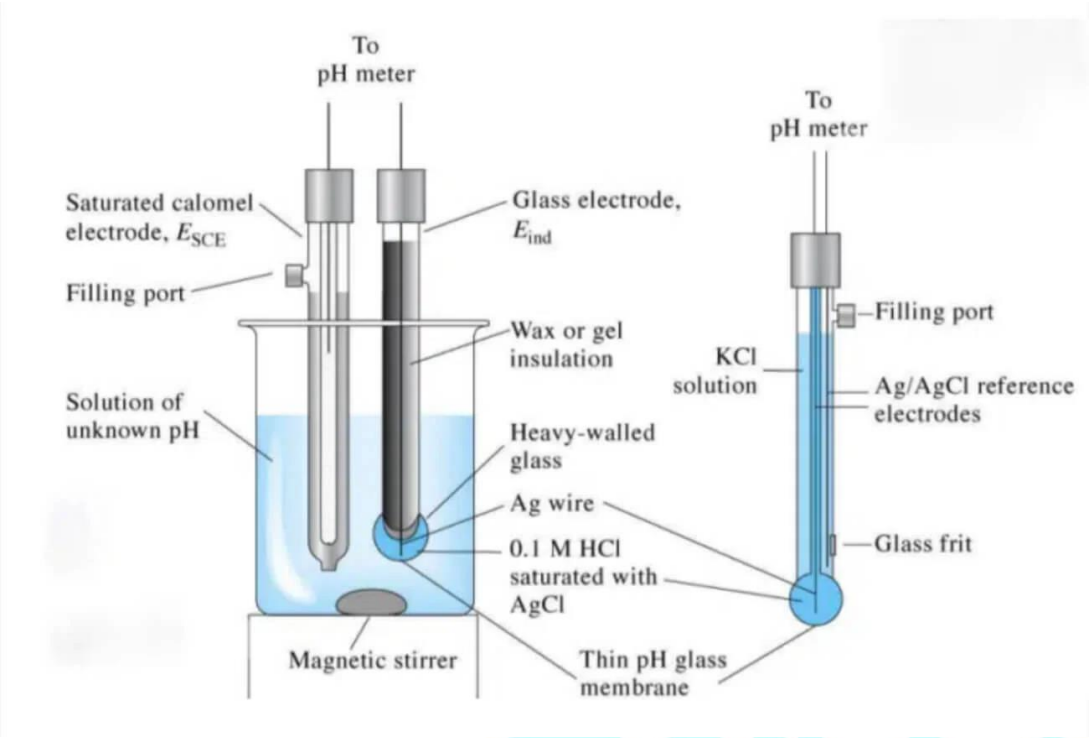

Fig 6. pH meter components and principle

Working of a pH Meter (Step-by-Step)

1. Immersion:
   - The glass and reference electrodes are immersed in the solution to be tested.

2. Ion Exchange:
   - H^+ ions from the solution interact with the outer layer of the glass membrane.
   - This creates a potential difference due to ion exchange.

3. Voltage Generation:
   - The difference in H^+ concentration between the internal solution (inside the glass bulb) and the sample solution creates an electrochemical potential.

4. Reference Voltage:
   - The reference electrode provides a stable voltage for comparison.

5. Signal Measurement:
   - The voltmeter measures the potential difference between the two electrodes.

6. Conversion to pH:
   - The device calculates the pH based on the voltage, using the Nernst equation.
   - Most modern meters auto-compensate for temperature.

7. Display:
   - The pH value is shown digitally or analogically on the display.

Applications of pH Meters

In Laboratories

- Essential in biochemistry, pharmaceuticals, and analytical chemistry.
- Used to monitor the pH of enzyme assays, cell cultures, and reactions.
- Helps formulate buffer solutions, shampoos, cosmetics, and cleaning products**.**

In Medical and Biological Fields

- pH of blood and urine is routinely measured to diagnose conditions like acidosis or alkalosis.
- Ensures correct pH in IV fluids, eye drops, and wound care products.

In Industrial and Environmental Applications

- Monitors pH in water treatment, food processing, soil testing, and chemical manufacturing.
- Crucial for aquaculture, ensuring healthy environments for fish and aquatic plants.

Precautions:

- Calibration is crucial. pH meters need to be calibrated regularly using standard buffer solutions.
- Cleaning the electrodes between uses is essential to maintain accuracy.
- Temperature compensation improves precision, especially in scientific and industrial applications.
- Storage of electrodes (usually in KCl solution) keeps them hydrated and functional.

Q-13. Elaborate on the theory, instrumentation, and applications of IR and Raman spectroscopy in biological and chemical analysis.

Ans Infrared (IR) Spectroscopy

Principle:

- Based on the absorption of infrared radiation by molecules, causing them to undergo vibrational transitions.
- Only vibrations that change the dipole moment of a molecule are IR active.

Types of Vibrations:

- Stretching (symmetric, asymmetric)
- Bending (scissoring, rocking, wagging, twisting)

IR Region: Divided into

  - Near IR (14,000–4,000 cm^{-1})
  - Mid IR (4,000–400 cm^{-1}) – most commonly used
  - Far IR (400–10 cm^{-1})

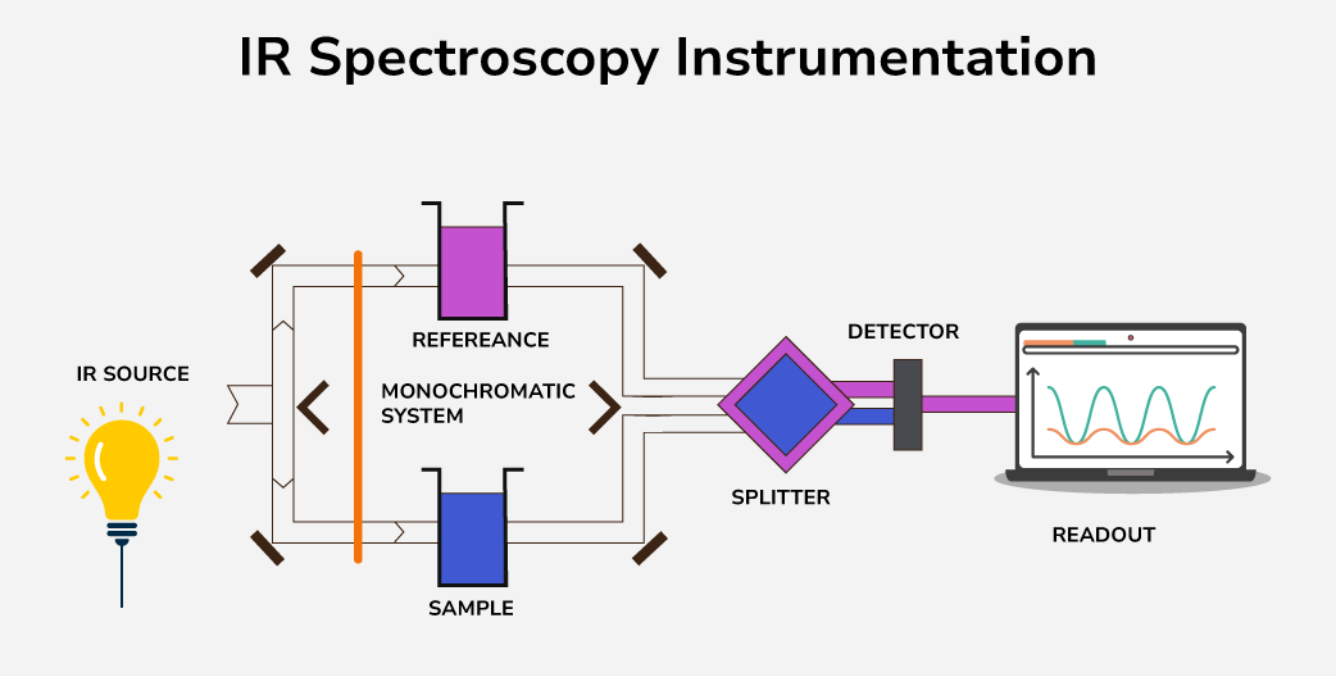

Fig 7. IR Spectroscopy

Instrumentation

Main Components:

1. IR source (Globar or Nernst glower)
2. Sample holder (solid, liquid, or gas cells; ATR crystals)
3. Monochromator or Interferometer (in FTIR systems)
4. Detector (DTGS, MCT – for fast or sensitive detection)
5. Computer/Data system (for spectra processing)

Techniques:

- FTIR (Fourier Transform IR) is widely used for higher sensitivity and resolution.
- ATR (Attenuated Total Reflectance) simplifies solid/liquid sample analysis.

Working of IR Spectroscopy

IR spectroscopy identifies and studies chemical substances based on how they absorb infrared light and undergo **molecular vibrations**. The core idea is that different **functional groups** absorb characteristic frequencies of IR light.

Step-by-Step Working Process

1. IR Radiation Source

- An IR light source (like a **Globar** or **Nernst glower**) emits a continuous spectrum of infrared radiation across a wide range of frequencies (usually **4000–400 cm^{-1}**, the mid-IR region).

2. Sample Interaction

- The IR beam is directed at the **sample**, which can be in **solid, liquid, or gas** form.
- Sample preparation varies:
    - **Solids**: Pressed in KBr pellets or analyzed with ATR.
    - **Liquids**: Sandwiched between IR-transparent plates (e.g., NaCl).
    - **Gases**: Placed in gas cells with long pathlength.

3. Molecular Absorption and Vibrations

- When the IR radiation hits the sample:
    - Specific frequencies are **absorbed** by the molecules.
    - Absorption causes **vibrational excitation** (stretching or bending) in the chemical bonds.
- Only bonds with a **change in dipole moment** during vibration absorb IR (i.e., IR active vibrations).

4. Transmission Through Sample

- After passing through the sample, some IR light is **absorbed**, and the rest is **transmitted**.
- The amount of absorption depends on:
    - Type of bond
    - Bond strength
    - Molecular environment

5. Dispersion or Interference (in FTIR)

In Dispersive IR:

- A **monochromator** (like a prism or grating) separates different wavelengths.
- The IR beam is scanned across the IR range one frequency at a time.

In FTIR (Fourier Transform IR):

- Uses an **interferometer** (usually Michelson type) to modulate all IR frequencies simultaneously.
- An **interferogram** is created and then mathematically converted to a spectrum using **Fourier Transform**.

6. Detection

- The transmitted IR beam is measured by a **detector** (commonly:
    - **DTGS** (Deuterated triglycine sulfate)
    - **MCT** (Mercury cadmium telluride) – more sensitive

- The detector records **intensity** of the IR beam across different wavelengths.

7. Spectrum Generation

- The instrument software plots a graph of:
    - % Transmittance (or Absorbance) vs. Wavenumber (cm^{-1})

- The resulting spectrum shows **peaks** where IR radiation was absorbed.

8. Interpretation

- Each peak corresponds to a specific **vibrational mode** of a bond or functional group.
- Analysts use **correlation charts** to identify the types of chemical bonds (e.g., C=O stretch ~ 1700 cm^{-1}, O-H stretch ~ 3200–3600 cm^{-1}).

IR Term	Meaning
Wavenumber (cm^{-1})	Frequency scale used in IR, inversely proportional to wavelength
Transmittance	Percentage of IR light that passes through the sample
Absorbance	Logarithmic measure of IR absorbed (used in quantitative analysis)
Fingerprint Region	1500–400 cm^{-1}; unique to each compound

Raman Spectroscopy:

Principle:

- Based on inelastic scattering of monochromatic light (usually from a laser) by molecules.
- When light hits a molecule, most scatter elastically (Rayleigh), but a small fraction scatters inelastically (Raman effect), with a shift in energy corresponding to vibrational modes.
- Only vibrations that change the polarizability of the molecule are Raman active.

Stokes and Anti-Stokes Lines:

- Stokes lines: energy lost by photon (more intense)
- Anti-Stokes lines: energy gained (less intense)

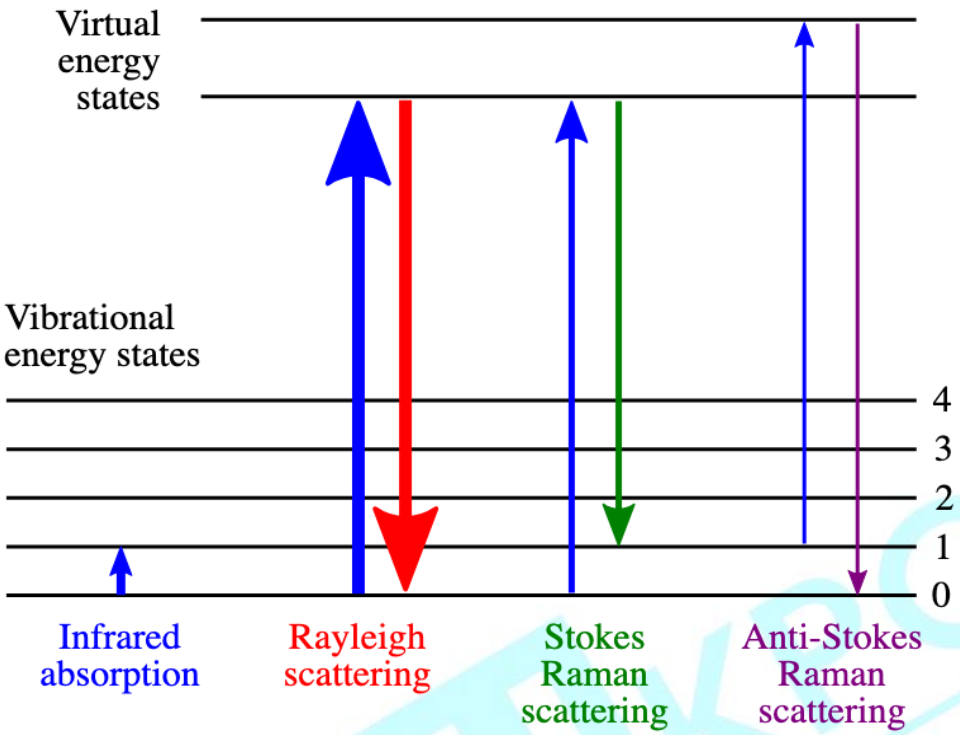

Fig 8. Different energy level in Raman spectra

Raman Spectrometer:

Main Components:

1. Laser source (commonly 532 nm, 633 nm, 785 nm)
2. Sample illumination system (focuses laser on sample)
3. Optical filters (block Rayleigh scattering)
4. Monochromator/Spectrograph
5. Detector (CCD or photomultiplier tube)
6. Computer system (for spectrum acquisition and analysis)

Working of Raman Spectroscopy

Raman spectroscopy is based on the inelastic scattering of monochromatic light (usually from a laser) when it interacts with molecular vibrations. Unlike IR, Raman measures changes in polarizability rather than dipole moment.

Step-by-Step Working Process

1. Laser Light Source

- A high-intensity monochromatic laser (commonly 532 nm, 633 nm, or 785 nm) is directed at the sample.
- This laser serves as the excitation source and initiates the Raman scattering.

2. Interaction with Molecules

- As the laser light hits the sample, photons interact with the molecules:
    - Most photons are scattered elastically (no energy change) – this is Rayleigh scattering.
    - A small fraction of photons (~1 in 10^7) are scattered inelastically – this is the Raman effect.

3. Raman Scattering

- In Raman scattering, the energy of the scattered photon is shifted due to energy being transferred to or from molecular vibrations.

Two Types of Raman Scattering:

- Stokes Scattering: The molecule gains energy, and the scattered photon loses energy (most common).
- Anti-Stokes Scattering: The molecule loses energy, and the scattered photon gains energy (less intense).

The energy difference between the incident and scattered light corresponds to the vibrational energy levels of the molecule.

4. Light Collection and Filtering

- The scattered light is collected at an angle (often 90° or backscattered geometry).
- Optical filters (such as notch or edge filters) are used to remove the intense Rayleigh line and allow only the Raman-shifted light to pass.

5. Wavelength Dispersion

- The filtered light enters a monochromator or spectrograph, which disperses the light based on wavelength.
- This separates the Raman-shifted wavelengths from the unshifted ones.

6. Detection

- A CCD (Charge-Coupled Device) or photomultiplier tube (PMT) detects the Raman-scattered light.
- These detectors are highly sensitive to low-light signals, crucial since Raman scattering is weak.

7. Spectrum Generation

- The spectrometer generates a Raman spectrum, a plot of Intensity vs. Raman shift (in cm^{-1}).
- The Raman shift is calculated as the difference between the incident laser frequency and the scattered light frequency.

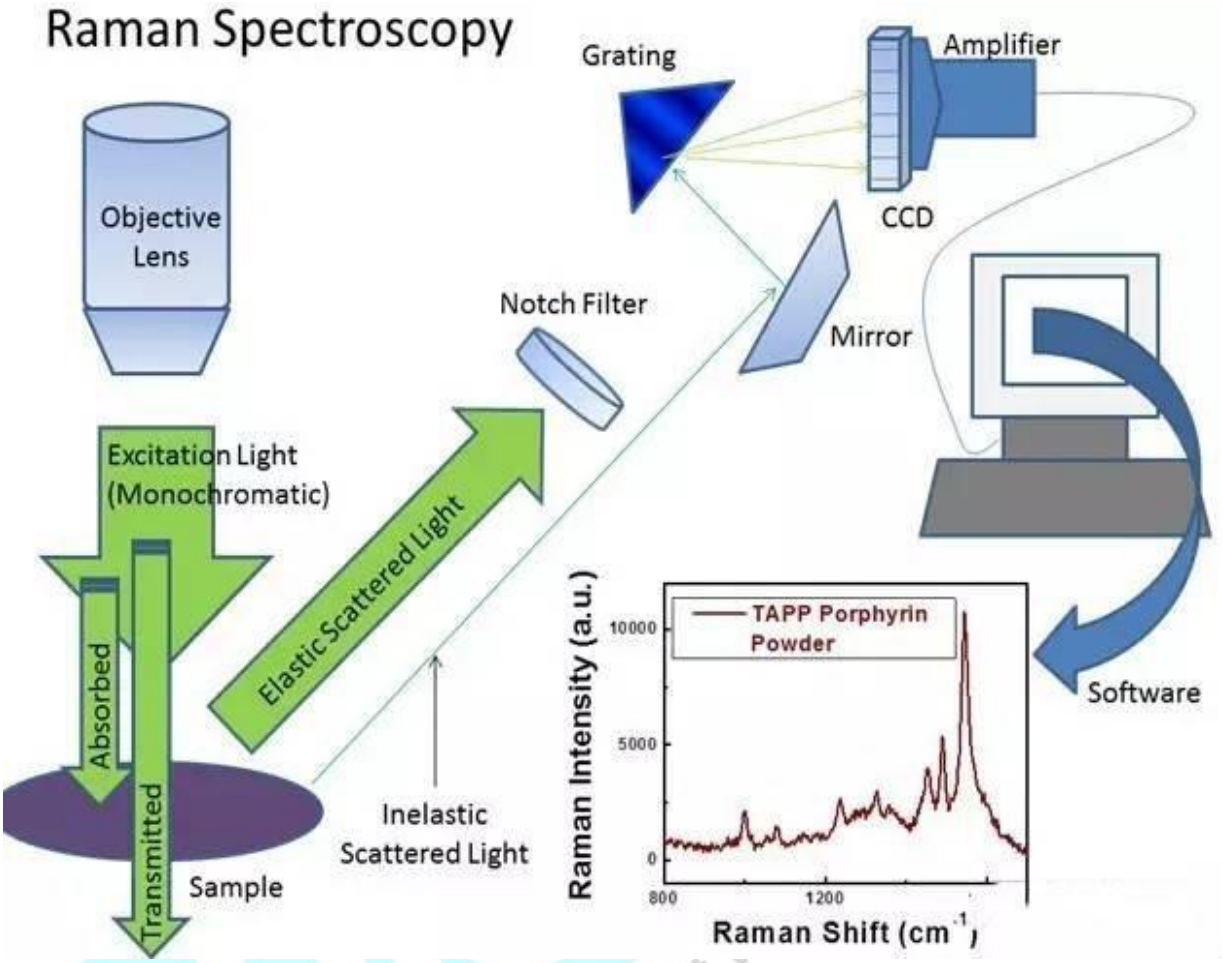

Fig 9. Instrumentation of Raman Spectroscopy

APPLICATIONS in BIOLOGICAL & CHEMICAL ANALYSIS

In Chemical Analysis:

IR:

- Functional group identification (e.g., –OH, –NH_2, –COOH, –C=O)
- Qualitative and quantitative analysis of organic compounds

- Polymer characterization
- Identification of isomers based on fingerprint region

Raman:

- Complementary to IR (detects non-polar bonds better)
- Analysis of symmetrical molecules (e.g., O_2, N_2)
- Crystallinity, polymorphism studies
- Petrochemical and material sciences

In Biological Analysis:

IR:

- Protein secondary structure analysis via amide I & II bands
- Lipid, nucleic acid, and carbohydrate profiling
- Disease diagnosis through serum/tissue IR spectra
- Monitoring drug–biomolecule interactions

Raman:

- Live cell imaging (label-free, non-invasive)
- Protein and DNA conformation studies
- Cancer diagnosis and biomarker detection
- Single-cell biochemical fingerprinting
- Drug delivery monitoring at cellular level

Feature	IR Spectroscopy	Raman Spectroscopy
Principle	Absorption of IR light	Inelastic scattering of light
Sample prep	Requires more (especially solids)	Minimal (can be done in water)
Water interference	Strongly absorbs IR	Weak in Raman (ideal for biological)
Best for	Polar bonds, functional groups	Non-polar bonds, molecular symmetry
Common sample states	Solid, liquid, gas	Solid, liquid, aqueous

Unit II

Buffers, Microscopy, Spectroscopy

Q-1 What is the basic principle of fluorescence spectroscopy?
Ans Fluorescence spectroscopy is based on the emission of light by a substance that has absorbed light or other electromagnetic radiation. It typically involves excitation of molecules by UV or visible light, which then emit light of a longer wavelength as they return to the ground state.

Q-2 What does RCF stand for in centrifugation?
Ans RCF stands for *Relative Centrifugal Force*. It is the measure of the force exerted by a centrifuge on a sample, expressed in terms of gravity (×g), and depends on the speed of rotation (rpm) and the radius of the rotor.

Q-3 Name one application of paper chromatography.
Ans Paper chromatography is commonly used for separating and identifying amino acids, inks, and dyes in a mixture due to differences in their solubility and interaction with the stationary phase.

Q-4 What type of molecules are separated using ion exchange chromatography?
Ans Ion exchange chromatography is used to separate proteins, peptides, and other charged biomolecules based on their net charge.

Q-5 What is ultracentrifugation used for?
Ans Ultracentrifugation is used to separate particles such as viruses, ribosomes, and cellular organelles based on their size, shape, and density under very high centrifugal forces.

Q-6 Describe the instrumentation of a fluorescence spectrophotometer.
Ans A fluorescence spectrophotometer consists of a light source (usually a xenon or mercury lamp), an excitation monochromator to select the excitation wavelength, a sample holder, an emission monochromator to isolate emitted light, and a detector such as a photomultiplier tube. The system measures the intensity of emitted light as a function of wavelength, providing data on the fluorescent properties of the sample.

Q-7 Explain the principle and application of TLC (Thin Layer Chromatography).
Ans TLC operates on the principle of adsorption chromatography. A thin layer of adsorbent material (usually silica gel) is spread on a glass or plastic plate. The sample is spotted on the plate and placed in a solvent chamber. As the solvent ascends by capillary action, components in the sample travel at different rates, allowing separation. It's used for analyzing plant pigments, pharmaceutical compounds, and checking reaction progress.

Q-8 What is the difference between adsorption and partition chromatography?
Ans Adsorption chromatography separates components based on their affinity to the stationary solid

phase. In contrast, partition chromatography involves a stationary liquid phase and separation based on solubility differences between the mobile and stationary phases.

Q-9 Define sedimentation and its role in centrifugation.
Ans Sedimentation is the process by which particles settle to the bottom of a liquid under the influence of gravity or centrifugal force. In centrifugation, sedimentation is accelerated using high-speed rotation, allowing rapid and efficient separation of components based on size and density.

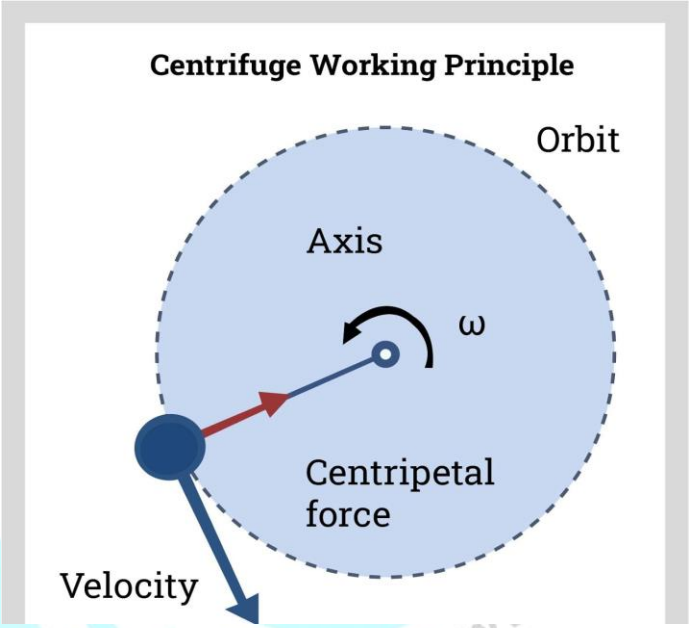

Fig 10. Centrifugation forces

Q-10 What are the forces involved in centrifugation?
Ans Centrifugation involves centrifugal force, gravitational force, and frictional force. The centrifugal force is responsible for pushing particles outward, while gravitational force acts downwards, and frictional force resists movement through the medium, influencing sedimentation rate.

Q-11 What is chromatography? Elaborate different types of chromatography techniques, their principles, and comparative advantages.

Ans Chromatography is a powerful and versatile analytical technique used to separate, identify, and purify components of a mixture. The process is based on the differential distribution of the components between two phases: a stationary phase and a mobile phase. As the mobile phase moves through the stationary phase, different substances interact differently with each phase, leading to separation.

Chromatography has become indispensable in biochemical analysis, pharmaceuticals, environmental testing, forensic science, and food safety.

Basic Principles of Chromatography

Chromatography works on the principle of differential partitioning between the mobile and stationary

phases:

- Adsorption chromatography relies on the differential adsorption of solutes on the surface of the solid stationary phase.
- Partition chromatography is based on the differential solubility of components in two liquid phases (one stationary, one mobile).
- The speed at which different substances move depends on their affinity to either phase, leading to separation.

Types of Chromatography Techniques

1. Paper Chromatography

Principle: Partition chromatography.

Stationary Phase: Water trapped in cellulose fibers of paper.

Mobile Phase: A solvent or solvent mixture.

Application: Separation of amino acids, sugars, and pigments.

Advantages: Simple, inexpensive, requires minimal equipment.

Limitations: Low resolution and not suitable for non-polar substances.

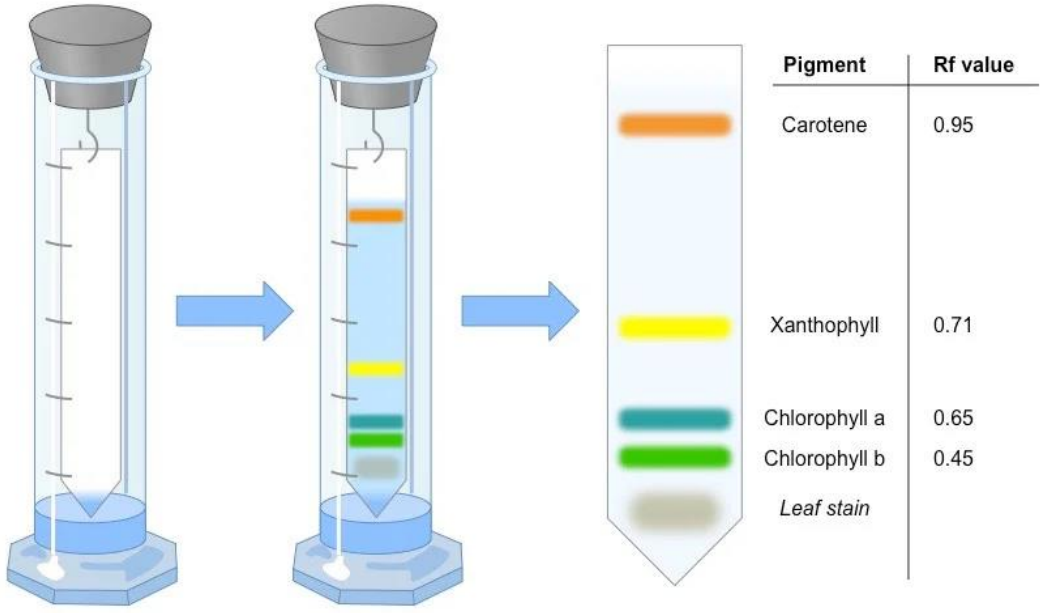

Fig 11. Paper Chromatography

2. **Thin Layer Chromatography (TLC)**

Principle: Adsorption chromatography.

Stationary Phase: Thin layer of silica gel or alumina on glass/plastic.

Mobile Phase: A suitable solvent or solvent mixture.

Application: Analyzing pharmaceuticals, lipids, alkaloids.

Advantages: Quick results, high sensitivity, better resolution than paper chromatography.

Limitations: Semi-quantitative, manual observation.

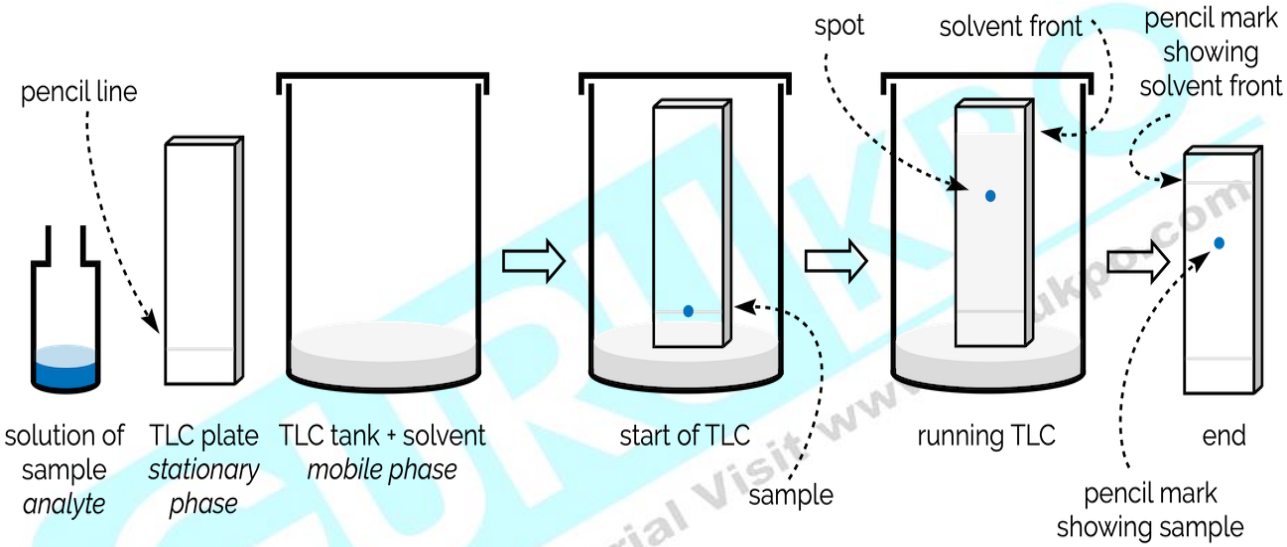

Fig 12. Thin Layer Chromatography

3. Gas Chromatography (GC) / Gas-Liquid Chromatography (GLC)

Principle: Partition chromatography.

Stationary Phase: Liquid coated on an inert solid support in a column.

Mobile Phase: An inert carrier gas (e.g., helium, nitrogen).

Application: Volatile organic compounds, forensic analysis, environmental toxins.

Advantages: High resolution, fast analysis, precise quantification.

Limitations: Only volatile and thermally stable compounds can be analyzed.

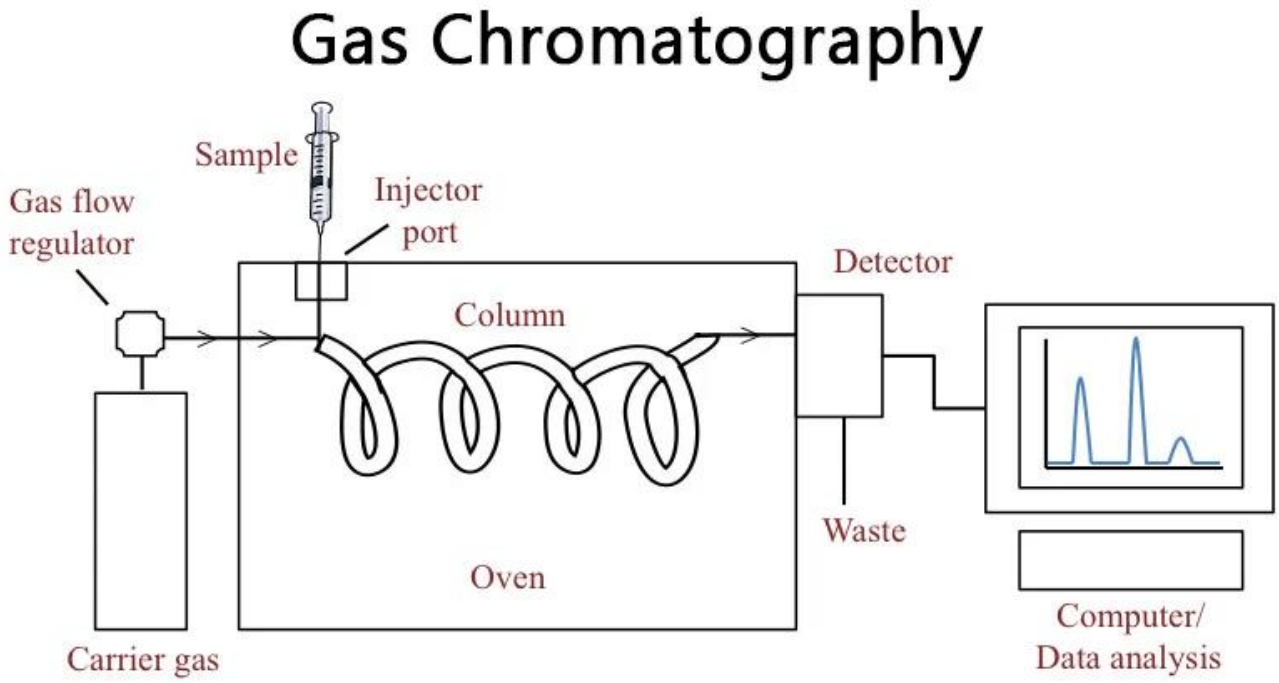

Fig 13. Gas Chromatography

4. High Performance Liquid Chromatography (HPLC)

Principle: Partition or adsorption chromatography (depending on column type).

Stationary Phase: Column packed with silica or polymer-based particles.

Mobile Phase: Liquid solvent pumped at high pressure.

Application: Drug analysis, clinical diagnostics, food industry.

Advantages: High accuracy, automation, applicable to a wide range of molecules.

Limitations: Expensive equipment, requires skilled operation.

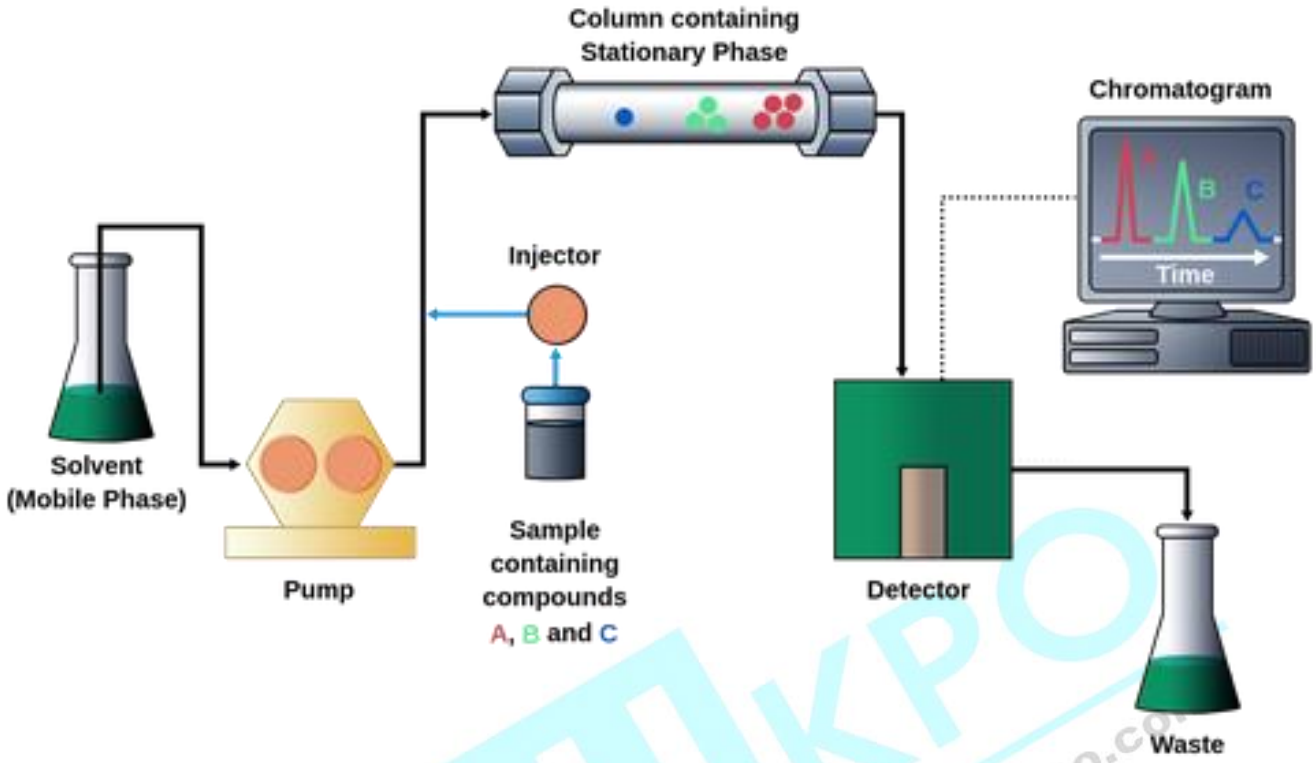

Fig 14. High Performance Liquid Chromatography

5. Ion Exchange Chromatography

Principle: Separation based on charge interactions.

Stationary Phase: Resins with charged functional groups.

Mobile Phase: Buffered solution.

Application: Protein purification, desalting, amino acid analysis.

Advantages: High selectivity for charged molecules, effective in protein purification.

Limitations: Affected by pH and ionic strength of buffer.

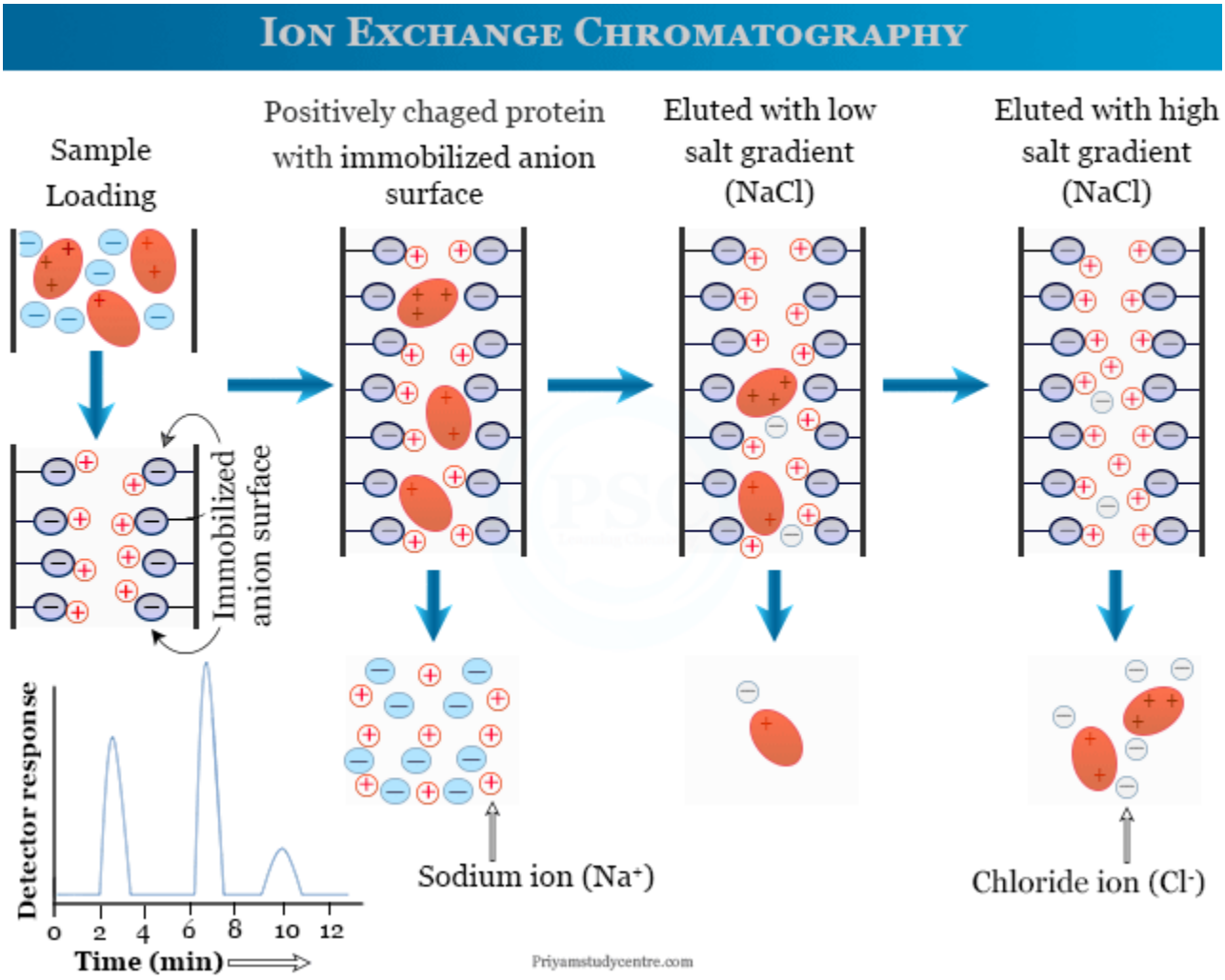

Fig 15. Ion Exchange Chromatography

Comparative Advantages

Technique	Best For	Advantages	Limitations
Paper Chromatography	Simple compounds (e.g., amino acids)	Cost-effective, simple setup	Low sensitivity and resolution
TLC	Small-scale analysis	Faster than paper, more accurate	Not suitable for large-scale separation
GC/GLC	Volatile organics	High precision and sensitivity	Limited to volatile, thermally stable samples
HPLC	Broad range of compounds	High resolution, automation possible	Cost and technical expertise needed
Ion Exchange Chromatography	Charged biomolecules	Very selective, useful for proteins	Sensitive to changes in pH and salt conditions

Q-12 Describe in detail the principle, instrumentation, types, and applications of fluorescence spectroscopy.

Ans Fluorescence spectroscopy is a highly sensitive analytical technique used to detect and quantify fluorescent molecules in various samples.

Principle

Fluorescence spectroscopy is based on the phenomenon of fluorescence, where certain molecules (called fluorophores) absorb light at a particular wavelength (excitation) and then emit light at a longer wavelength (emission). This process involves:

- Excitation: A molecule absorbs energy from incident photons, promoting electrons from the ground state (S_0) to an excited singlet state (S_1 or S_2).
- Relaxation: The excited electron loses part of its energy non-radiatively (e.g., through vibrational relaxation).
- Emission: The electron returns to the ground state by emitting a photon with less energy (longer wavelength) than the absorbed light.

This emission is measured to provide qualitative and quantitative information about the fluorophores.

Instrumentation

A typical fluorescence spectrophotometer consists of:

a. Light Source

Common sources include xenon arc lamps, mercury vapor lamps, or lasers.
They provide high-intensity, continuous or monochromatic light for excitation.

b. Excitation Monochromator

Selects a specific wavelength from the light source to excite the sample.
This ensures the excitation light matches the absorbance peak of the fluorophore.

c. Sample Holder

Holds the cuvette or sample.
Usually made of quartz (transparent to UV and visible light).

d. Emission Monochromator

Isolates the emission wavelength for detection.
Ensures that only the fluorescence signal is measured, avoiding scattered excitation light.

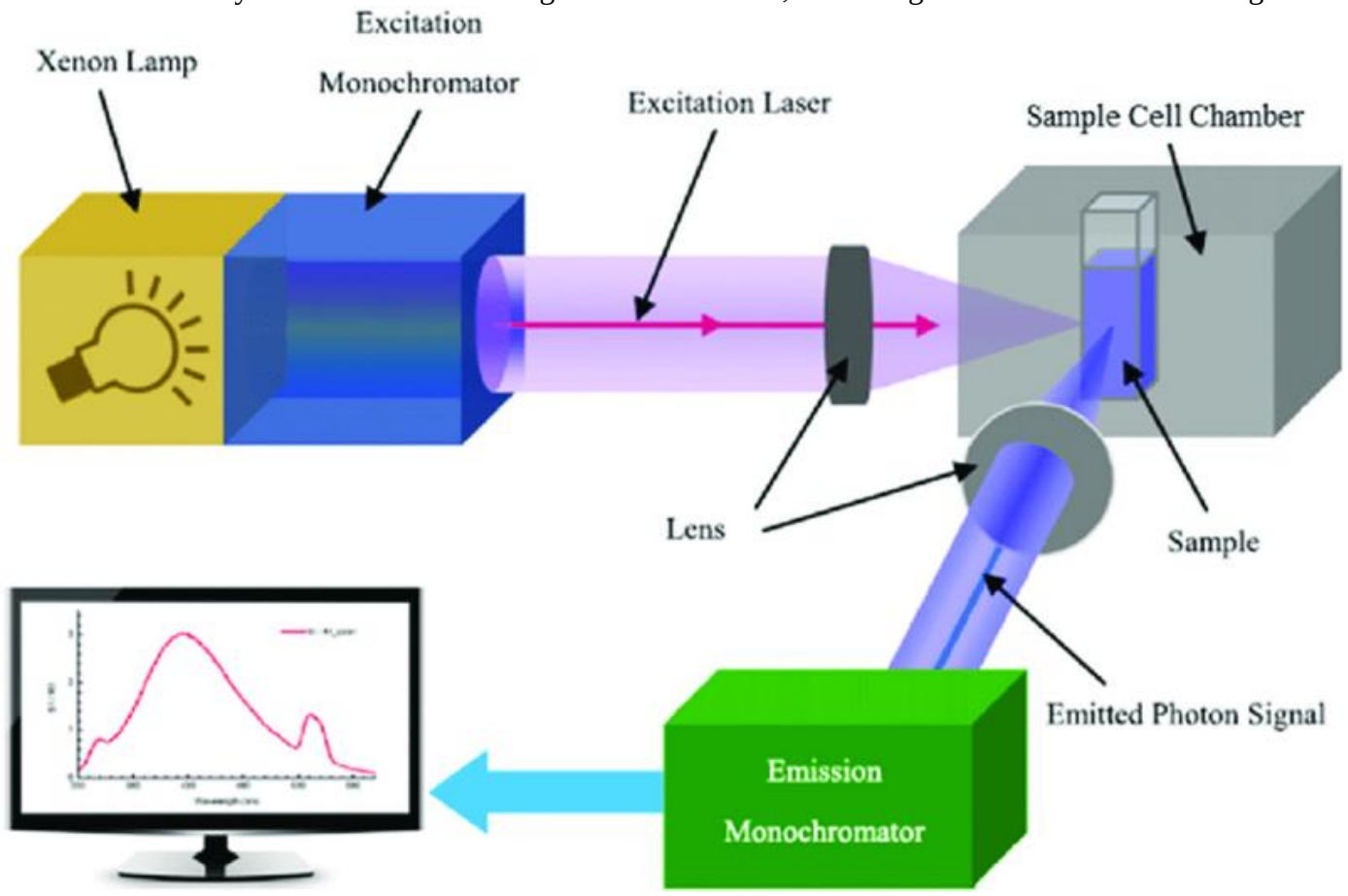

Fig 16. Fluorescence spectrophotometer

e. Detector

Photomultiplier tube (PMT) is commonly used for high sensitivity.
Converts the emitted light into an electrical signal.

f. Computer Interface

Controls the instrument and processes the output data.
Provides excitation and emission spectra, intensity measurements, etc.

Types

a. Steady-State Fluorescence Spectroscopy

Measures the average fluorescence intensity over time.
Used for routine qualitative and quantitative analysis.

b. Time-Resolved Fluorescence Spectroscopy

Measures the fluorescence decay over time after a brief excitation pulse.
Provides information on fluorescence lifetime, helpful for studying dynamic processes.

c. Synchronous Fluorescence Spectroscopy

Simultaneously scans excitation and emission wavelengths with a fixed wavelength difference.
Improves spectral resolution and selectivity.

d. Fluorescence Anisotropy (or Polarization)

Measures the polarization of emitted light.
Useful for studying molecular size, shape, and interactions.

e. Fluorescence Lifetime Imaging Microscopy (FLIM)

Combines spatial and lifetime data to image biological samples.
Often used in cell imaging and biophysics.

Applications

a. Biological and Medical Sciences

Detection of biomolecules: Proteins, nucleic acids, vitamins, etc.
Immunofluorescence: Tagging antibodies for detection of specific antigens.
DNA sequencing and microarrays.
Fluorescent markers in cell imaging.

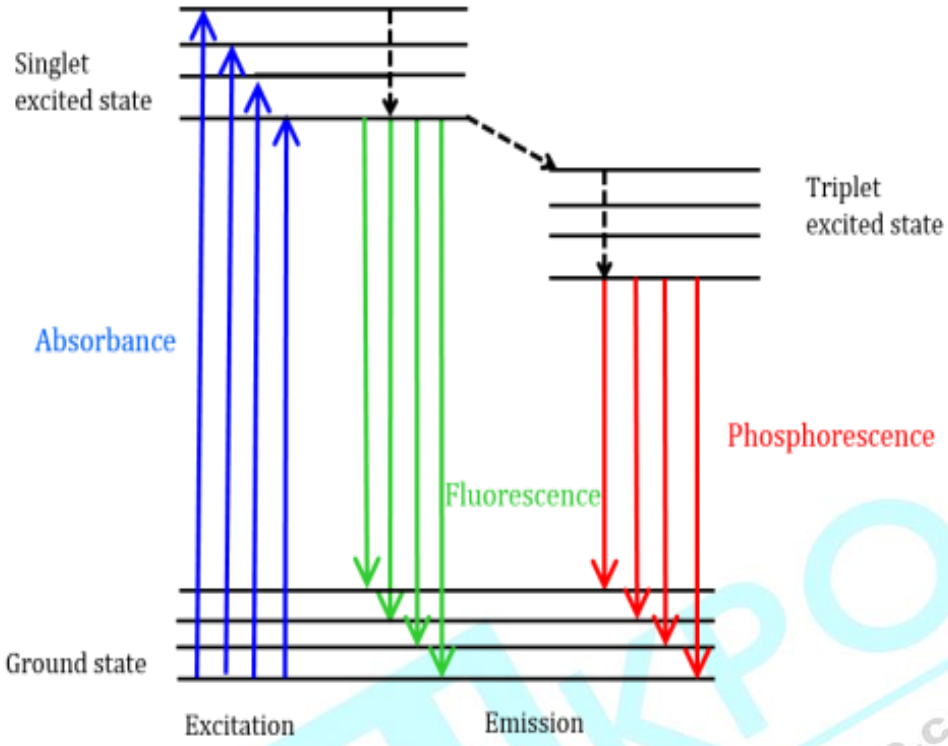

Fig 17. Different energy levels during Fluorescence, Absorbance and Phosphorescence

b. Chemical and Environmental Analysis

Detection of trace metals (e.g., via chelation with fluorescent ligands).
Monitoring pollutants in water, air, and soil.
Study of chemical kinetics and reaction mechanisms.

c. Pharmaceutical Industry

Drug discovery: Screening compounds with fluorescence-labeled targets.
Pharmacokinetics: Tracking drug distribution using fluorescent tracers.
Quality control: Identifying and quantifying active ingredients.

d. Material Science

Characterization of polymers and nanomaterials.
Quantum dots and fluorescent dyes used in optoelectronics and sensors.

Q-13 Centrifugation and Ultracentrifugation: Explore the physics, methods, types, instrumentation, and research significance.

Ans Centrifugation and ultracentrifugation are essential techniques in modern laboratories used to separate particles from a solution based on their size, shape, and density. These methods apply centrifugal force to accelerate sedimentation of particles, enabling isolation of cellular components, macromolecules, or nanoparticles**.**

Principle

Centrifugation is based on the principle of sedimentation, where centrifugal force is used to push particles outward in a spinning system. The Relative Centrifugal Force (RCF) is calculated using the formula:

$$RCF = 1.118 \times 10^{-5} \times r \times (RPM)^2$$

Where:
RCF is in ×g (times gravity),
r is the radius in centimeters from the center of the rotor,
RPM is the speed in revolutions per minute.

Larger and denser particles sediment faster than smaller or lighter ones.

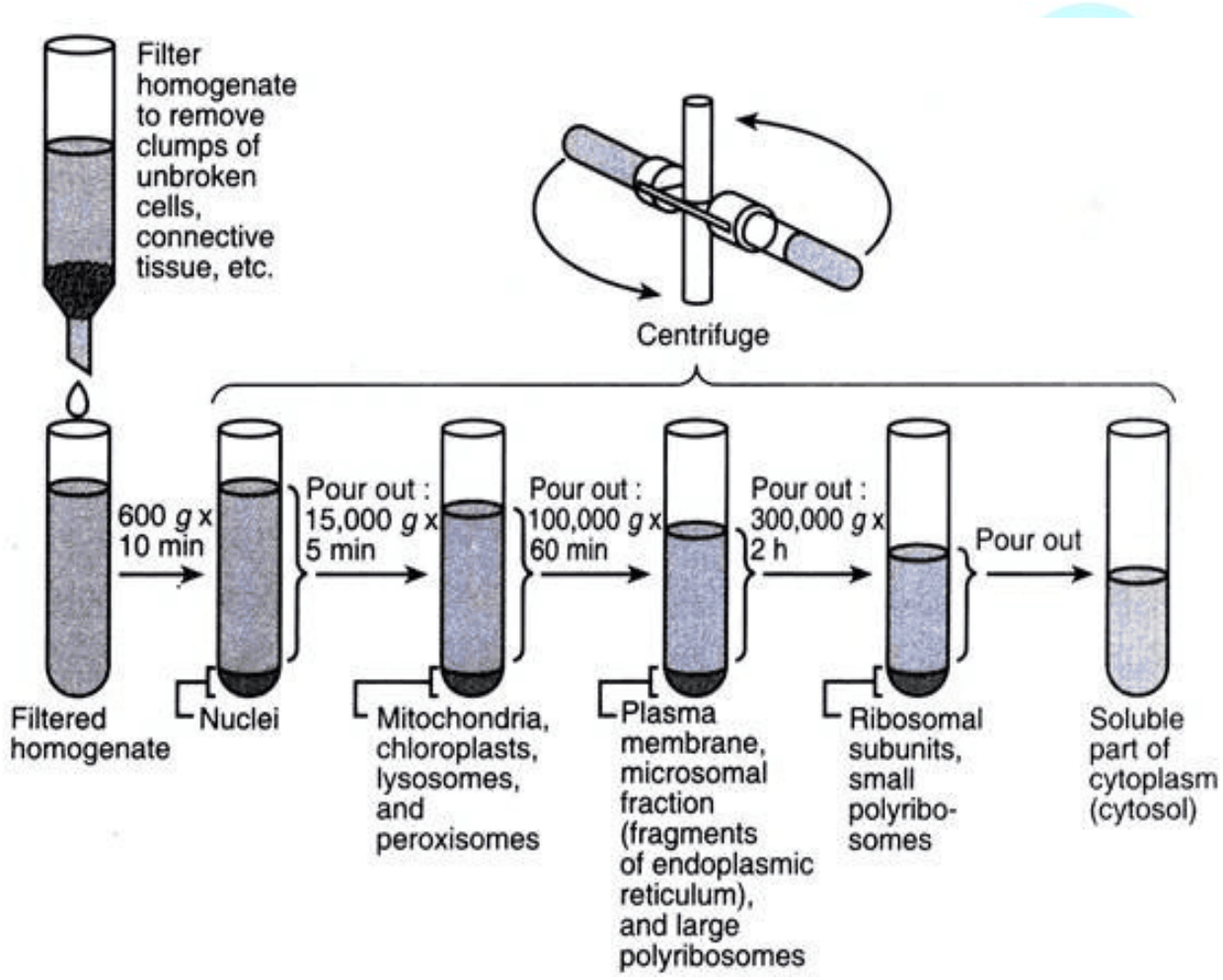

Fig 18. Centrifugation process

Types

1. Differential Centrifugation
    - Separates components by size and mass through sequential spinning at increasing speeds.
    - Used for separating nuclei, mitochondria, lysosomes, etc.
2. Density Gradient Centrifugation
    - Uses a medium (like sucrose or cesium chloride) that forms a density gradient.
    - Two subtypes:
        - Rate-zonal centrifugation: Particles separated based on their sedimentation rates.
        - Isopycnic centrifugation: Particles migrate to a zone matching their density.
3. Ultracentrifugation
    - High-speed centrifugation (up to 1,000,000 ×g).
    - Allows separation of very small particles such as ribosomes, viruses, and DNA.

Instrumentation

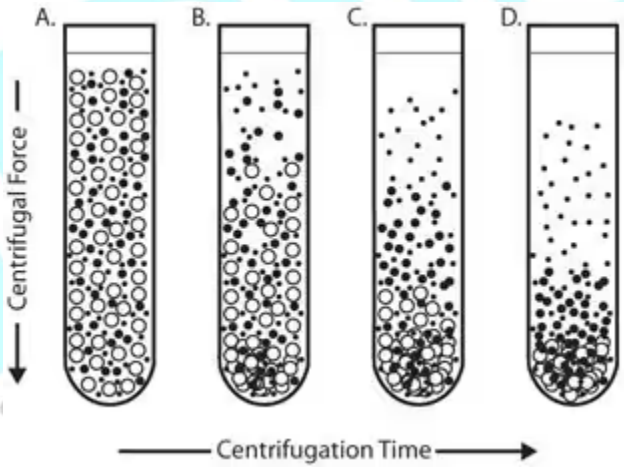

Fig 19. Centrifugation

Basic Centrifuge Components:

Rotor: Holds tubes and spins them; can be fixed-angle or swing-bucket.

Motor: Provides the rotational force.

Chamber: Houses the rotor and maintains temperature.

Control panel: Sets speed, time, and temperature

Ultracentrifuge Specifics:

High-speed rotors: Made from strong, lightweight materials like titanium or carbon fiber.

Vacuum system: Reduces friction and heating.

Refrigeration unit: Maintains constant temperature for sample stability.

Analytical ultracentrifuge: Equipped with optical systems to monitor sedimentation in real time.

Applications and Research Significance

1. Cell Biology
    - Separation of cell organelles (e.g., nuclei, mitochondria, chloroplasts).
    - Preparation of subcellular fractions for biochemical analysis.
2. Molecular Biology
    - Isolation and purification of DNA, RNA, and proteins.
    - Used in plasmid preparation and virus purification.
3. Structural Biology
    - Ultracentrifugation helps determine molecular weights, shapes, and conformational changes of macromolecules.
    - Analytical ultracentrifugation is critical for studying protein-protein and protein-DNA interactions.
4. Nanotechnology
    - Size-based separation and characterization of nanoparticles.
    - Ensures uniformity in nanomaterial synthesis.
5. Pharmaceuticals
    - Drug delivery studies, liposome separation, and formulation development.

Advantages and Limitations

Advantages:

- Highly efficient separation method.
- Scalable for both analytical and preparative purposes.
- Preserves biological activity of sensitive components.

Limitations:

- Equipment is expensive and requires maintenance.
- High-speed centrifugation may cause heat damage.
- Sample overload can lead to poor resolution.

Q-14 Explain in detail the principle, instrumentation, working applications of HPLC.

Ans High Performance Liquid Chromatography (HPLC) is an advanced analytical technique used to separate, identify, and quantify components in a mixture. It is highly efficient, accurate, and widely used in pharmaceuticals, environmental analysis, food safety, and biochemical research.

Principle

HPLC is based on the principle of **partition chromatography** or **adsorption chromatography**, depending on the stationary phase used. The sample mixture is dissolved in a solvent (the mobile phase) and passed through a column packed with a stationary phase under high pressure.

- Components of the mixture interact differently with the stationary and mobile phases.
- Those with greater affinity for the stationary phase move slower, while those with more affinity for the mobile phase move faster.
- This differential migration causes the components to separate as they exit the column at different times, known as retention times.

Instrumentation

An HPLC system consists of several critical components:

1. Solvent Reservoirs
Contain the mobile phase, which may be a single solvent or a mixture.
Common solvents include water, methanol, acetonitrile, etc.

2. Pump
Delivers the mobile phase through the system at high pressures (typically 1,000 to 5,000 psi).
Ensures constant flow and helps in achieving reproducible results.

3. Injector
Introduces the sample into the mobile phase stream.
Manual or automatic injectors are used, often via a loop system.

4. Column
The heart of the HPLC system, packed with stationary phase materials like silica or polymer-based particles.
Columns vary in length, diameter, and particle size depending on the application.

5. Detector
Detects the separated components as they elute from the column.

Common detectors:

UV/Vis Detector: Measures absorbance.
Refractive Index Detector: For compounds with low UV absorbance.

Fluorescence Detector: For highly sensitive applications.

6. Data System
Connected to the detector, records signals, and produces a chromatogram.
Used for data analysis, peak integration, and quantification.

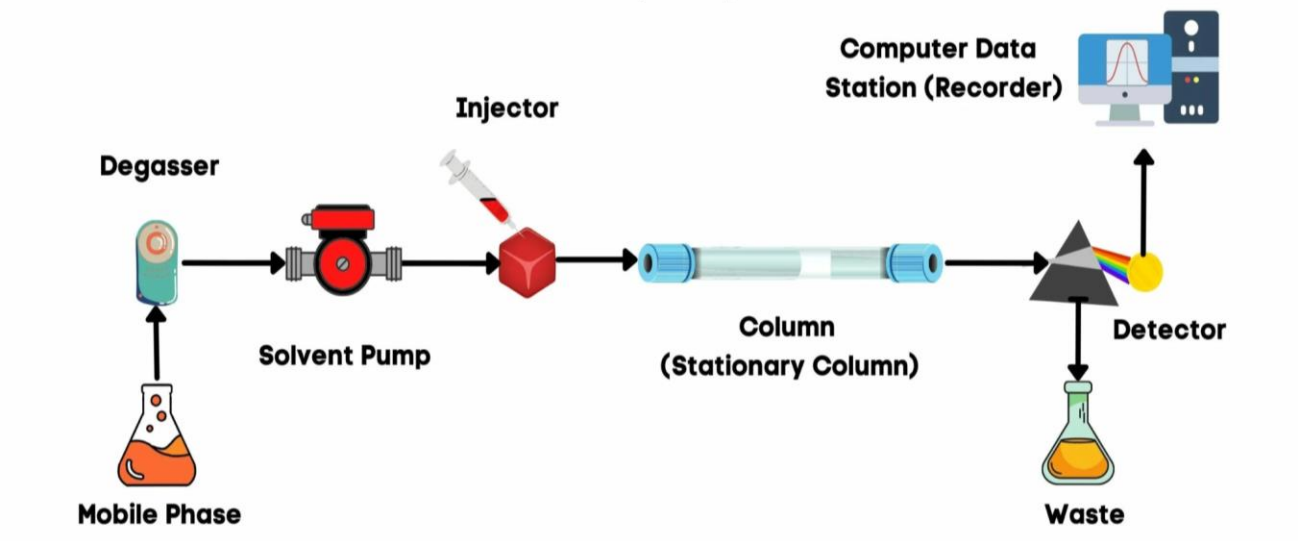

Fig 20. HPLC working principle

Working of HPLC

1. **Sample Preparation**: The sample is dissolved in a suitable solvent.
2. **Injection**: The sample is injected into the flow of the mobile phase.
3. **Separation**: As the mobile phase moves through the column, different components interact differently with the stationary phase, causing separation.

4. **Detection**: Each component is detected and recorded as a peak on a chromatogram.
5. **Analysis**: The retention time and peak area are used to identify and quantify the components.

Types of HPLC

Applications of HPLC

1. Pharmaceutical Industry

Drug purity testing.
Stability studies.
Quantitative analysis of active pharmaceutical ingredients (APIs).

2. Clinical and Biomedical Research

Detection of biomarkers.
Hormone level analysis.
Therapeutic drug monitoring.

3. Environmental Analysis

Detection of pollutants, pesticides, and herbicides in soil and water.

4. Food and Beverage Industry

Analysis of additives, preservatives, vitamins, and contaminants.

5. Forensic Science

Identification of drugs and toxic substances in biological samples.

Advantages of HPLC

High resolution and sensitivity.
Fast and reproducible results.
Applicable to both small and large molecules.
Automation and integration with other systems is possible.

Limitations

High equipment cost.
Requires skilled operation.
Some compounds may require derivatization for detection.

Unit III
Electrophoresis

Q-1. What is the basic principle of electrophoresis?
Ans Electrophoresis is a technique that separates charged particles in an electric field. Molecules move toward the electrode with opposite charge; their rate of movement depends on size, charge, and the medium used.

Q-2. Define Electrophoretic Mobility (EPM).
Ans EPM is the velocity of a particle per unit electric field strength. It reflects how fast a particle moves under the influence of an electric field and is affected by the particle's charge, size, and the medium.

Q-3. Name two factors affecting electrophoretic mobility.
Ans Two key factors are:

- The net charge of the molecule
- The viscosity and pore size of the supporting medium.

Q-4. What is SDS-PAGE used for?
Ans SDS-PAGE is used to separate proteins based on their molecular weight by denaturing them and giving them uniform negative charges using SDS detergent.

Q-5. What is isoelectric focusing?
Ans Isoelectric focusing separates proteins based on their isoelectric point (pI), the pH at which a protein has no net charge and therefore does not move in an electric field.

Q-6. Describe the instrument design and basic setup of an electrophoresis unit.
Ans An electrophoresis setup includes a power supply, buffer reservoirs, electrodes, and a supporting medium (like agarose or polyacrylamide gel). The power supply generates a controlled voltage/current. Electrodes are placed at each end of the gel chamber to establish an electric field. The gel acts as the medium through which molecules migrate. The sample is loaded into wells formed in the gel. When the current is applied, charged molecules migrate toward the opposite electrode. The rate of migration depends on the molecule’s charge, size, and shape, and the gel matrix's pore size. The setup may also include a cooling system to dissipate heat, and visualization systems (such as UV transilluminators for nucleic acids stained with ethidium bromide).

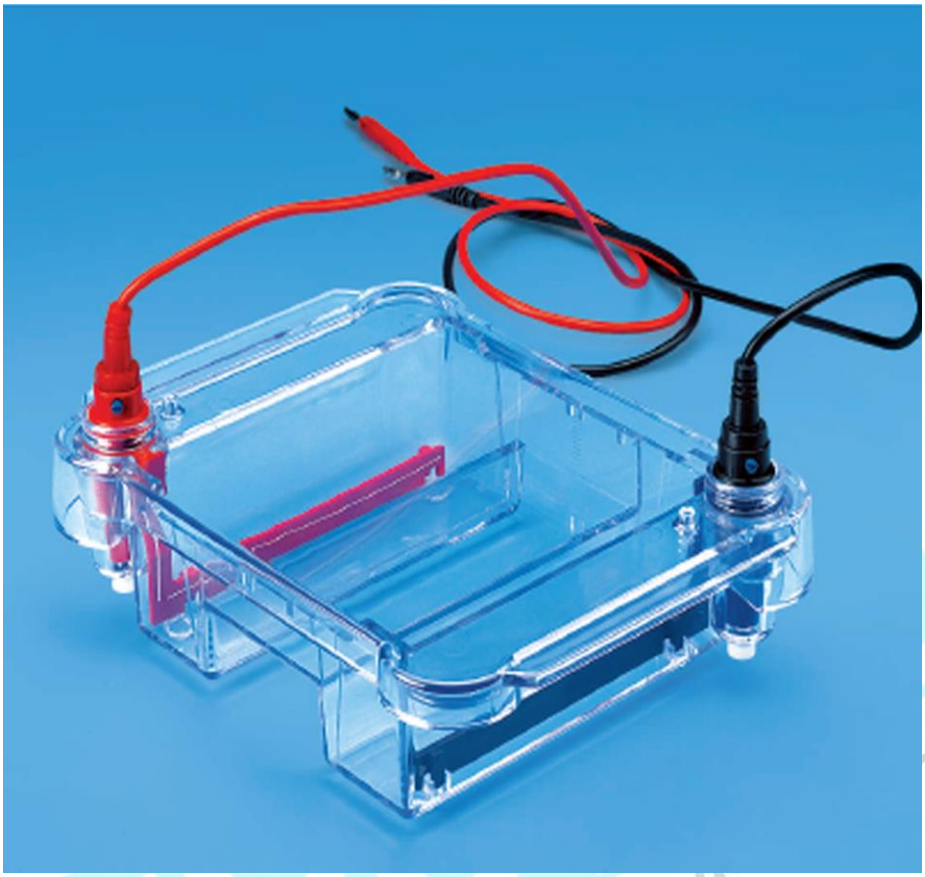

Fig 21. Electrophoresis unit

Q-7. What are the differences between free electrophoresis and zone electrophoresis?
Ans Free electrophoresis occurs in a solution without a supporting medium, often in a capillary tube, where particles move freely under an electric field. It offers high-resolution separation but has limitations like convection disturbances. Zone electrophoresis uses a supporting medium (e.g., paper, gel) which stabilizes the system, minimizing diffusion and allowing better separation. It separates molecules into zones or bands based on their electrophoretic mobility. Zone methods are widely used due to better reproducibility and clearer results.

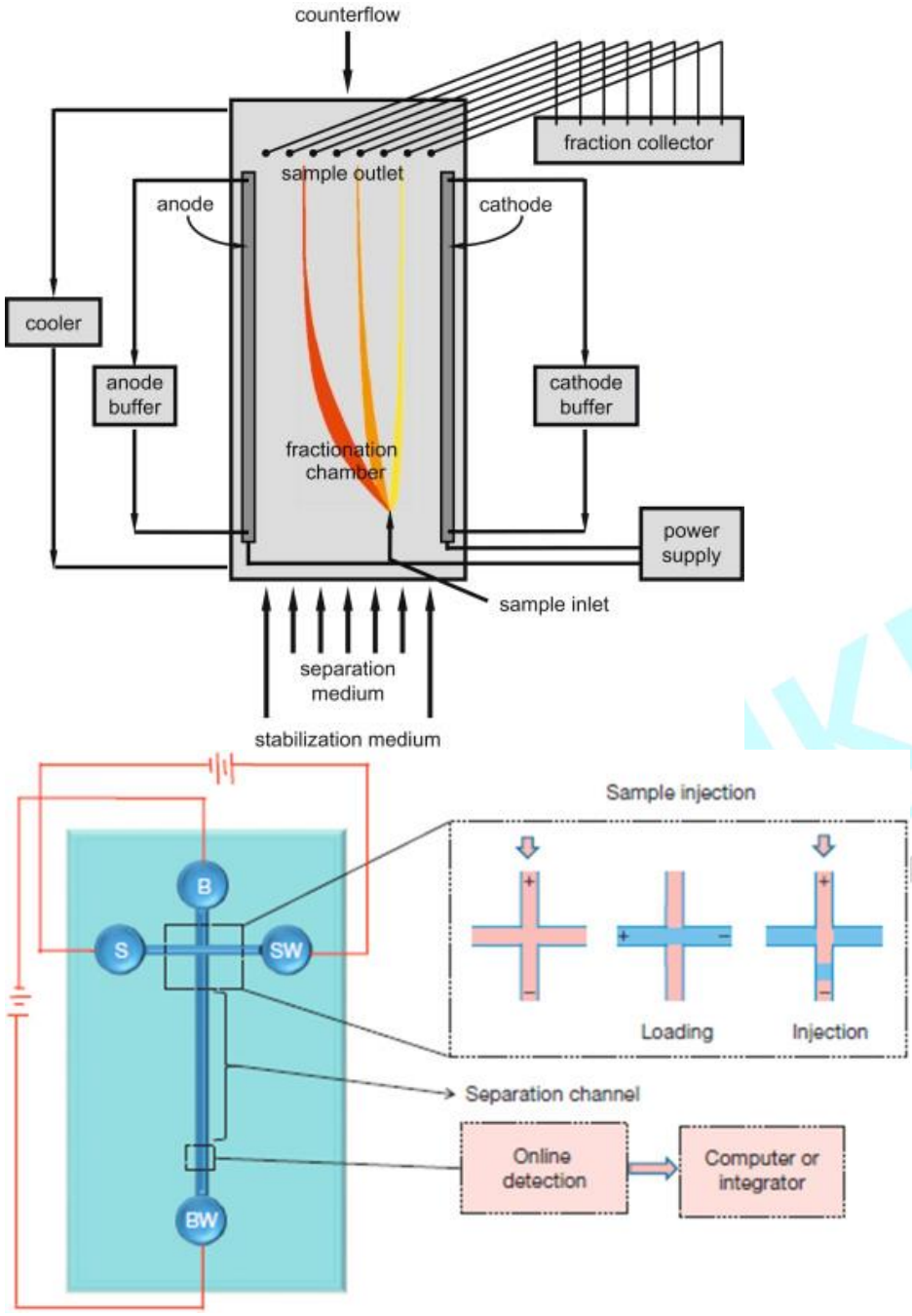

Fig 22. A) Free Electrophoresis and B) Zone Electrophoresis

Q-8. Explain the methodology and significance of 2D electrophoresis.
Ans 2D electrophoresis combines isoelectric focusing (IEF) and SDS-PAGE. First, proteins are separated by IEF based on their isoelectric point (pI). Then the strip is laid onto an SDS-PAGE gel for separation by molecular weight. This technique provides high-resolution separation, ideal for complex protein mixtures in proteomics studies. It helps identify post-translational modifications and is widely used in disease biomarker discovery and comparative protein analysis.

Q-9 Elaborate on the principle, procedure, and applications of pulse-field gel electrophoresis (PFGE) in detail.

Ans Pulse-Field Gel Electrophoresis (PFGE)

Principle:

Pulse-Field Gel Electrophoresis (PFGE) is a modification of traditional gel electrophoresis that allows the separation of very large DNA molecules, typically ranging from 10 kilobases (kb) to over 10 megabases (Mb).

In regular agarose gel electrophoresis, large DNA molecules migrate poorly or together in a single band because they can't properly orient themselves in the gel matrix. PFGE overcomes this limitation by alternating the direction of the electric field, which forces large DNA fragments to repeatedly reorient themselves before they can continue moving forward.

This reorientation process is size-dependent: larger DNA molecules take more time to reorient, and hence migrate more slowly. Thus, PFGE enables high-resolution separation of large DNA fragments, based on size.

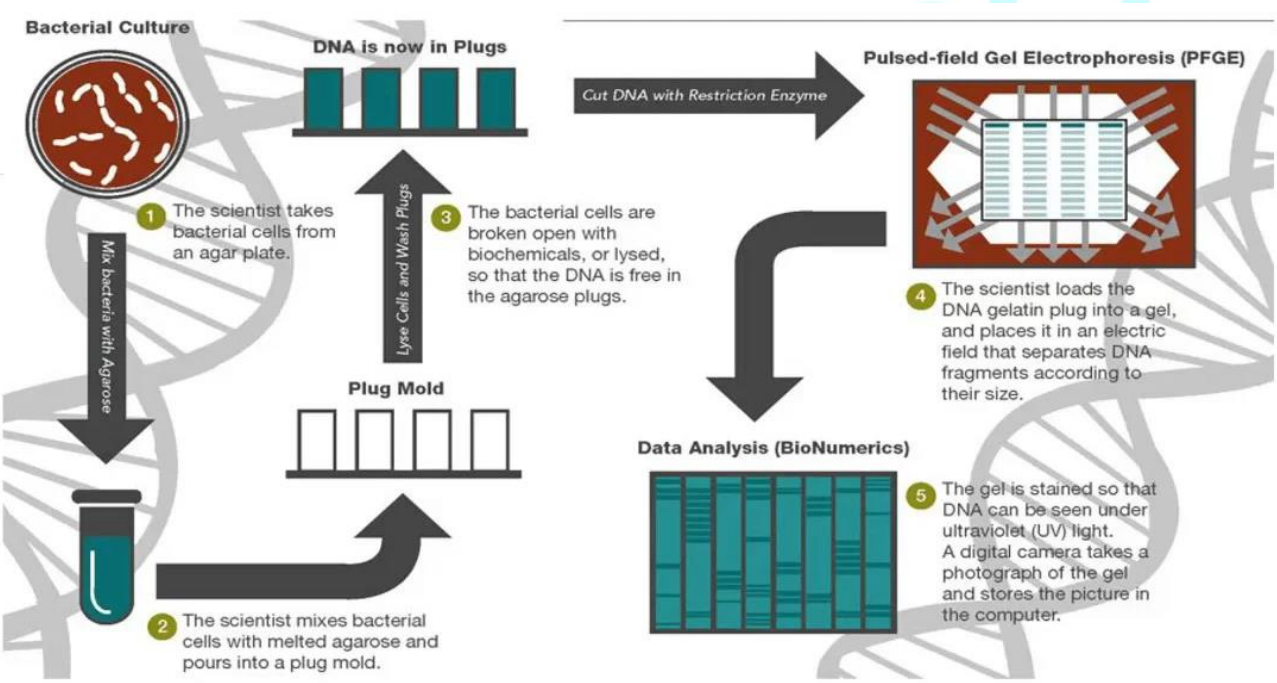

Fig 23. Pulse-Field Gel Electrophoresis (PFGE)

Procedure:

1. Sample Preparation

- Cells (e.g., bacteria) are embedded in agarose plugs to protect DNA from shearing during extraction.
- Inside the plugs, cells are lysed and treated with enzymes (e.g., proteinase K) to digest proteins and release intact chromosomal DNA.

2. Restriction Digestion

- The DNA in the plugs is digested with a **rare-cutting restriction enzyme** (e.g., *XbaI*, *NotI*, *SmaI*) that creates a small number of very large DNA fragments.

3. Gel Casting and Loading

- A **low-concentration agarose gel** (to allow large DNA to migrate) is cast.
- The DNA-containing plugs are placed in wells and sealed with molten agarose.

4. Electrophoresis with Pulse Field

- The gel is run in a **special PFGE system** that uses **alternating electric fields**—typically from three directions at defined angles (e.g., 120°).
- The **field is pulsed** at intervals (called **switch times**), and this switching causes DNA fragments to change direction.

5. Staining and Visualization

- After electrophoresis, the gel is stained with **ethidium bromide or another DNA-binding dye**, and the DNA bands are visualized under **UV light**.

Key Technical Parameters:

Switch time: Interval at which the direction of the field changes. Lower switch times resolve smaller fragments; longer switch times resolve larger ones.

Run time: Can be 18 to 48 hours, depending on fragment size.

Voltage gradient: Usually 6 V/cm.

Temperature: Maintained around 14°C to prevent overheating.

Applications of PFGE:

1. Epidemiological Typing of Microorganisms

PFGE is the gold standard for bacterial strain typing, especially in outbreak investigations (e.g., *Listeria*, *Salmonella*, *E. coli*).

It helps determine whether strains from different patients or food sources are genetically identical.

2. Genomic Mapping

PFGE helps create physical maps of genomes by generating large DNA fragments that can be ordered and analyzed.

3. Detection of Chromosomal Rearrangements

Used in genetic studies to identify large-scale DNA deletions, duplications, or rearrangements that aren't detectable with standard methods.

4. Molecular Characterization of Bacteria

Helpful in characterizing bacteria based on restriction fragment length polymorphism (RFLP) patterns.

5. Comparative Genomics

Useful in comparing genomic diversity between strains or species.

Advantages

High-resolution separation of large DNA fragments.
Allows comparison of whole genomic patterns.
Useful in forensic and public health investigations.

Limitations:

Time-consuming (often >24 hours).
Requires specialized equipment.
Interpretation can be subjective without standardized protocols.

Feature	**PFGE**
DNA size range	10 kb to >10 Mb
Matrix	Low-melting agarose gel
Electric field	Alternating (pulsed)
Run time	18–48 hours
Resolution	Very high for large DNA
Key use	Strain typing, large DNA separation

Q-10. Compare SDS-PAGE and native PAGE in terms of principle, methodology, and applications.

Ans SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis)

Principle:

SDS-PAGE is a type of polyacrylamide gel electrophoresis used to separate proteins based on molecular weight. It utilizes Sodium Dodecyl Sulfate (SDS), an anionic detergent, to:

- Denature proteins (i.e., unfold them by disrupting non-covalent bonds).
- Impart a uniform negative charge to all proteins, regardless of their native charge.

As a result, when an electric field is applied, the proteins migrate only based on size—smaller proteins move faster through the gel matrix, and larger ones move slower.

Methodology:

1. **Sample Preparation**: Proteins are treated with:
    - SDS, to denature them and provide uniform negative charge.
    - Reducing agents (e.g., β-mercaptoethanol or DTT) to break disulfide bonds and ensure full denaturation.

2. **Gel Preparation**: Polyacrylamide gel is used, often composed of,
    - Stacking gel: Lower acrylamide concentration to concentrate the protein samples into sharp bands.
    - Resolving gel: Higher acrylamide concentration to separate proteins based on size.

3. **Electrophoresis**

The sample is loaded into wells.

An electric field is applied (proteins move from cathode to anode).

Migration depends solely on molecular weight.

4. **Staining and Visualization:** Proteins are visualized using
    - Coomassie Brilliant Blue
    - Silver staining
    - Western blotting (for protein identification)

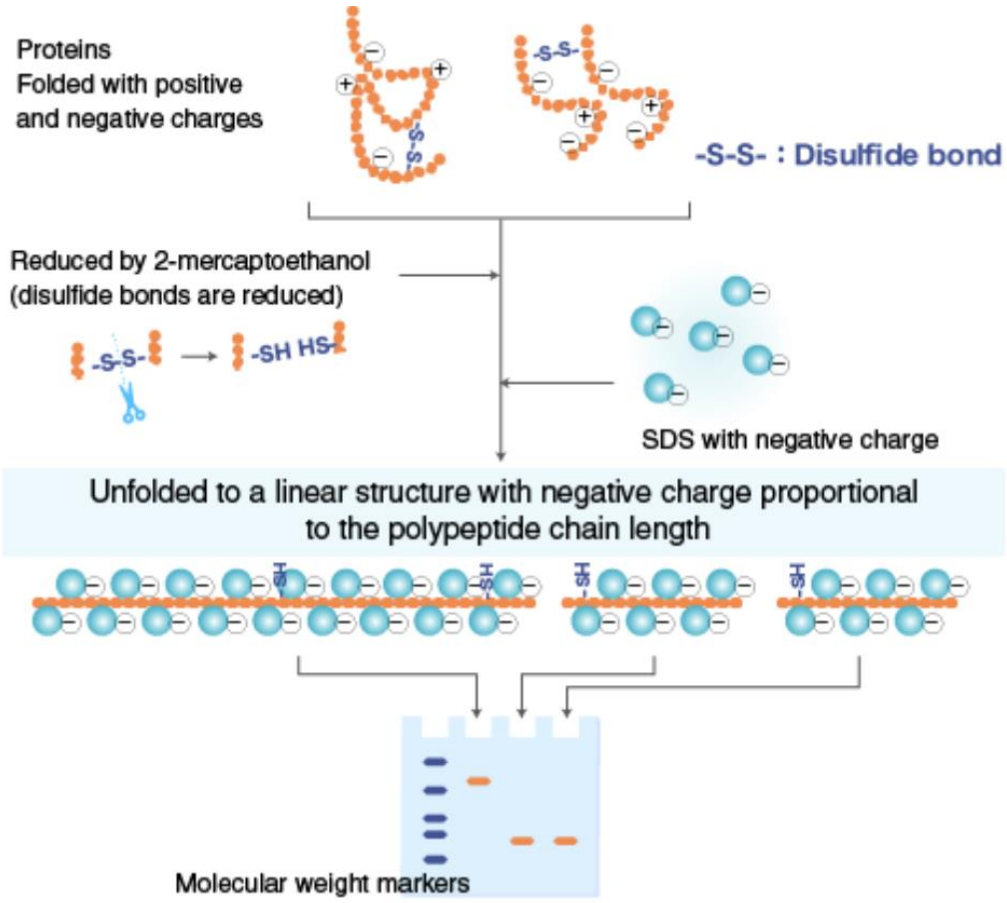

Fig 24. SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Applications of SDS-PAGE:

- Determining molecular weight of proteins
- Checking protein purity
- Monitoring protein expression in biotechnology
- Verifying results after protein purification
- Western blotting precursor for protein identification
- Comparing protein expression across different conditions or organisms

Native PAGE (Non-denaturing PAGE)

Principle:

Native PAGE separates proteins based on their native charge, size, and shape without denaturing them. Unlike SDS-PAGE, it does not use SDS or reducing agents, so proteins retain:

- Their natural tertiary or quaternary structure
- Their biological activity
- Their intrinsic charge differences

As proteins maintain their native structure and net charge, the migration is influenced by a combination of charge-to-mass ratio, size, and shape.

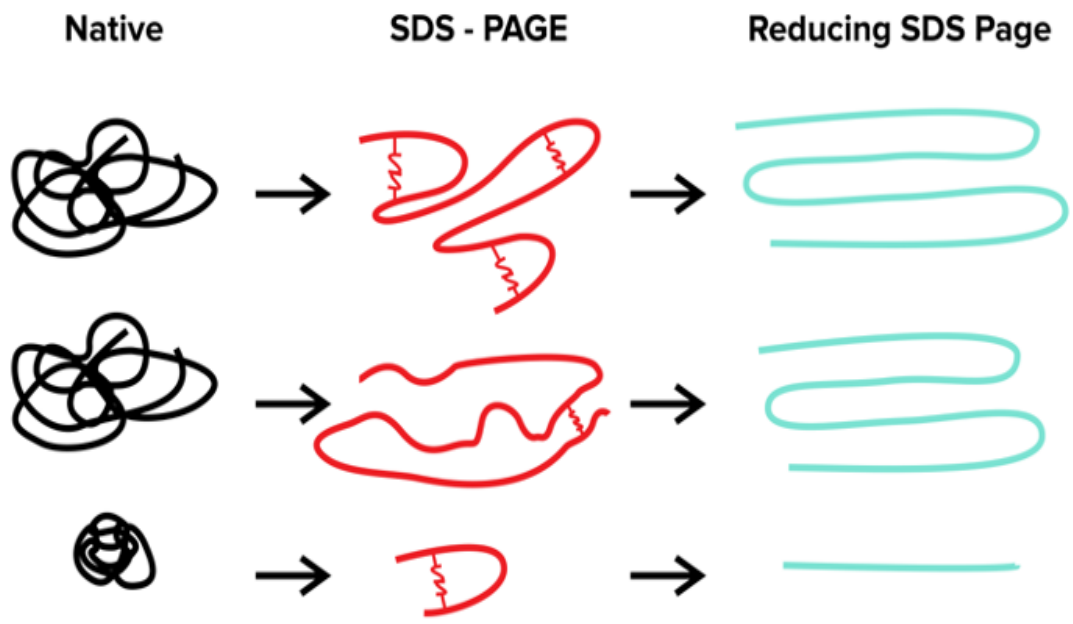

Fig 25. Conversion of Native DNA into its reduced form

Methodology:

1. Sample Preparation

- No SDS or reducing agents.
- Proteins remain in their functional, native conformation.

2. Gel Preparation

- Standard polyacrylamide gel, but without SDS.
- Buffer systems (e.g., Tris-glycine) maintain pH to preserve protein activity.

3. Electrophoresis

- Proteins migrate based on net charge at the buffer pH and size/shape.
- Movement is from cathode to anode if the protein is negatively charged at that pH.

4. Staining and Visualization

- Same as SDS-PAGE (e.g., Coomassie or silver staining).
- Can also be followed by enzyme staining to detect active proteins.

Applications of Native PAGE:

- Studying enzyme activity (since structure is preserved)
- Analyzing protein-protein interactions (e.g., complexes, oligomers)
- Separating isoforms of a protein that differ in charge
- Electrophoretic mobility shift assays (EMSA) to study DNA-protein binding
- Comparing conformational states of a protein

Feature	SDS-PAGE	Native PAGE
Denaturation	Yes (SDS + reducing agents)	No
Protein Structure	Denatured	Native (functional)
Charge Uniformity	Uniform negative charge (due to SDS)	Varies with native charge
Separation Based On	Molecular weight only	Charge, size, and shape
Protein Activity	Lost	Preserved
Use for Complexes	No	Yes (can detect complexes)
Typical Use	Molecular weight estimation, purity check	Functional analysis, activity, complexes

Unit IV
General biophysical methods

Q-1. What is the pH scale, and what does it measure?
Ans The pH scale measures the hydrogen ion concentration in a solution. It ranges from 0 (very acidic) to 14 (very basic), with 7 being neutral. A lower pH indicates higher acidity, and a higher pH indicates greater alkalinity.

Q-2. What is autoradiography used for?
Ans Autoradiography is used to detect radioactive molecules in a sample. It involves placing the sample against a photographic film to visualize the distribution of radioactive substances, often used in molecular biology and tissue studies.

Q-3. What is the Bragg equation?
Ans The Bragg equation, nλ=2dsin⁡θn\lambda = 2d\sin\thetanλ=2dsinθ, relates the wavelength of incident X-rays and the angle at which they are diffracted by the crystal lattice planes. It's fundamental in determining crystal structures through X-ray diffraction.

Q-4. Define a unit cell in crystallography.
Ans A unit cell is the smallest repeating unit of a crystal lattice that retains the overall structure and symmetry of the entire crystal. It is defined by its edge lengths and angles between edges.

Q-5. What are Miller indices used for?
Ans Miller indices are a notation system in crystallography to denote the orientation of planes and directions in a crystal lattice. They are expressed as a set of three integers (h, k, l).

Q-6. Explain the principle and applications of radioactive labeling and counting.
Ans Radioactive labeling involves attaching radioactive isotopes (like 14C^{14}C14C, 32P^{32}P32P, or 3H^{3}H3H) to molecules such as DNA, RNA, or proteins. This process allows researchers to track the movement or transformation of these molecules. Detection is done using scintillation counters or Geiger-Müller tubes.

Applications include:

- Studying metabolic pathways
- Tracking gene expression
- Measuring enzyme activity
- Studying protein-protein interactions

Q-7. Describe the concept of reciprocal lattice in crystallography.
Ans The reciprocal lattice is a mathematical construct used in crystallography to simplify the interpretation of diffraction patterns. Each point in the reciprocal space corresponds to a set of crystal planes in real space. The concept is crucial for understanding Bragg's law and determining interplanar spacing and orientation in crystals. Reciprocal lattices help convert observed diffraction data into real lattice structures.

Q-8. Discuss the rotating crystal method for crystal structure determination.
Ans In the rotating crystal method, a single crystal is mounted on a goniometer and rotated about a fixed axis. Monochromatic X-rays are directed at the crystal, and the resulting diffraction pattern is recorded. This method helps in determining unit cell dimensions and symmetry by analyzing angles and intensities of diffracted beams.

Q-9. What is the powder method in X-ray crystallography?
Ans The powder method is used for studying polycrystalline or powdered samples. The sample is exposed to monochromatic X-rays, and the diffracted rays form concentric rings on a photographic film or detector. By measuring these angles and using Bragg's law, the crystal structure and lattice parameters can be determined. This method is widely used in materials science and mineralogy.

Q-10. Describe the principle, working, and applications of X-ray diffraction in determining crystal structures.
Ans X-ray Diffraction (XRD)

Principle

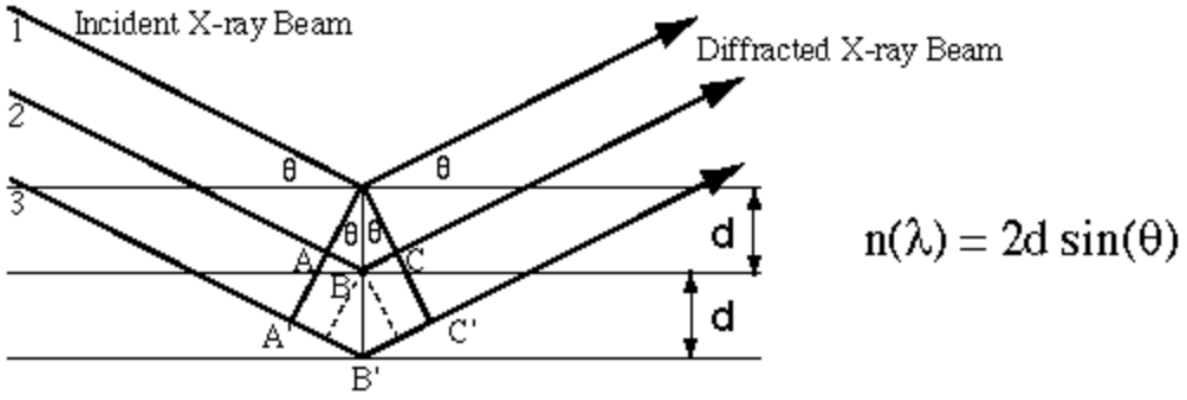

Fig 26. X-Ray Diffraction Bragg's law

X-ray diffraction (XRD) is a non-destructive technique used to analyze the atomic and molecular structure of crystals. The fundamental principle is based on the constructive interference of monochromatic X-rays and a crystalline sample. When X-rays interact with the periodic arrangement of atoms in a crystal, they are scattered in specific directions. This scattering leads to the formation of a diffraction pattern, which is characteristic of the internal arrangement of atoms within the crystal.

The behavior of the diffracted X-rays is described by Bragg's Law:

nλ=2dsinθn\lambda = 2d\sin\theta

Where:

- nn = Order of reflection (usually 1)
- λ\lambda = Wavelength of the X-ray
- dd = Distance between atomic layers (interplanar spacing)
- θ\theta = Angle of incidence/diffraction

This equation explains how diffraction peaks arise only when the conditions of constructive interference are met.

Working

1. Sample Preparation:
    - The material to be analyzed must be crystalline (can be a single crystal or powdered form).
    - In powder XRD, the sample consists of many tiny crystals in random orientations.
2. X-ray Generation:
    - X-rays are produced by bombarding a metal target (e.g., copper) with high-energy electrons in an X-ray tube.
    - The emitted X-rays are collimated and filtered to produce a monochromatic beam.

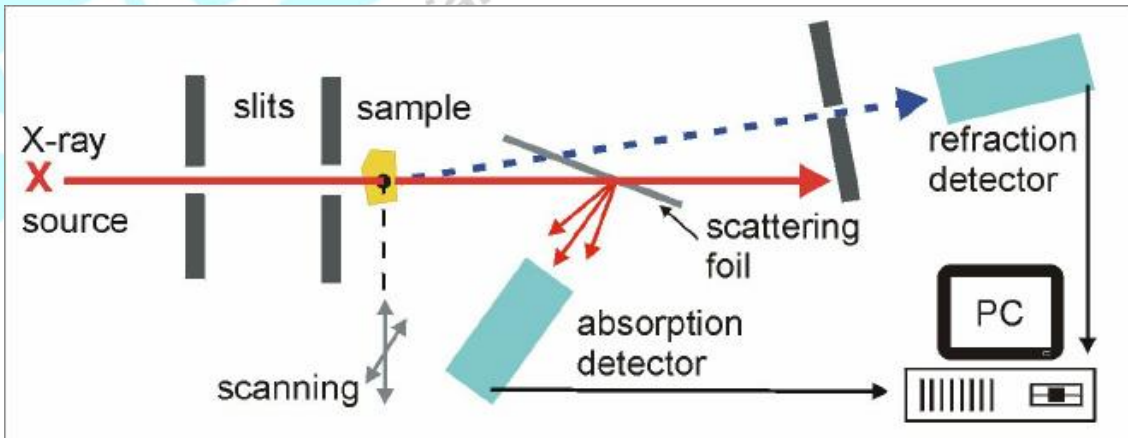

Fig 27. X-Ray Diffraction Instrumentation

3. Diffraction Process:
    - X-rays are directed onto the sample.

- As the X-rays strike the crystal lattice, they are diffracted by the planes of atoms.
- The diffracted rays interfere constructively at specific angles, producing a diffraction pattern.

4. Detection and Analysis:
    - Detectors (e.g., photographic film, CCD detectors, or goniometers) record the intensity and angles of diffracted beams.
    - The resulting pattern consists of peaks, where each peak corresponds to a set of atomic planes.
    - The peak positions and intensities are used to determine the lattice spacing, symmetry, and atomic positions.

Applications

1. Identification of Crystalline Phases:
    - Each crystalline substance has a unique XRD pattern.
    - Comparing the sample's diffraction pattern to standard databases (e.g., JCPDS) allows identification.
2. Determination of Unit Cell Dimensions:
    - The geometry and size of the unit cell can be calculated by analyzing the position of diffraction peaks.
3. Crystal Structure Determination:
    - For complex structures (like proteins), single-crystal XRD is used to determine the exact position of atoms in 3D space.
4. Quality Control in Industries:
    - Used in pharmaceuticals to verify polymorphic forms.
    - In metallurgy and ceramics, to ensure phase purity and monitor phase changes.
5. Residual Stress and Texture Analysis:
    - XRD can measure internal stress in materials and preferred orientation of grains (texture).
6. Thin Film and Surface Studies:

- XRD is used to assess thickness, crystallinity, and orientation of thin films used in electronics and coatings.

Q-11.Explain in detail the concept and significance of Miller indices in crystallography.

Ans Introduction

In crystallography, Miller indices are a set of three integers (h, k, l) that denote the orientation of a crystal plane in a three-dimensional lattice. Named after the British mineralogist William Hallowes Miller, these indices provide a standard way of describing the geometry of planes and directions within a crystal structure.

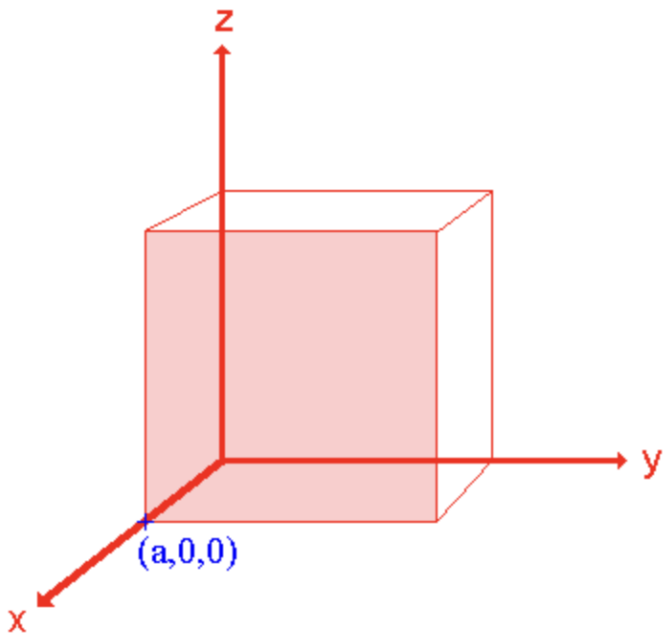

Fig 28. Miller Indices

Concept of Miller Indices

1. Miller indices are the reciprocals of the intercepts that a crystal plane makes with the crystallographic axes, which are then cleared of fractions and reduced to the smallest set of integers.

2. Steps to Determine Miller Indices:

    - Step 1: Identify the intercepts of the plane with the x, y, and z axes in terms of lattice constants (a, b, c).

    - Step 2: Take the reciprocal of each intercept.

- Step 3: Clear fractions to get the smallest set of whole numbers.
- Step 4: Enclose the result in parentheses as (hkl).

3. Example:
    - A plane cuts the axes at x=1a,y=2b,z=3cx = 1a, y = 2b, z = 3c
    - Reciprocals: 11,12,13\frac{1}{1}, \frac{1}{2}, \frac{1}{3}
    - Clear fractions: Multiply by 6 → (6, 3, 2)
    - Miller indices = (6 3 2)
4. Special Cases:
    - If a plane is parallel to an axis, the intercept is at infinity, so its reciprocal is zero.
    - Negative intercepts are indicated with a bar over the number, e.g., (1¯10)(\bar{1} 1 0)

Visualization and Representation

Miller indices define a specific plane in a unit cell. For instance:

- (100) represents a plane that cuts the x-axis at 1a and is parallel to the y and z axes.
- (111) cuts all three axes at equal lengths.

Multiple equivalent planes form what is known as a family of planes, denoted with curly braces, e.g., {100}, {111}.

Significance of Miller Indices

1. Describing Crystal Planes and Directions: Miller indices help in identifying and communicating specific planes and directions in a crystal, which is essential for understanding material properties.
2. Interpreting X-ray Diffraction Patterns: Each diffraction peak corresponds to a particular set of

lattice planes (hkl). The Miller indices help assign these peaks to planes, enabling determination of crystal structure.

3. Understanding Slip Systems in Metals: Deformation in metals occurs along specific planes and directions. For example, in FCC crystals, slip usually occurs along the {111} planes in the <110> directions.

4. Surface Science and Thin Films: The properties of surfaces and thin films depend on which crystal plane is exposed. For instance, the catalytic activity or electronic behavior of a material can vary with its surface orientation.

5. Crystal Growth and Etching: Different planes grow or etch at different rates. Controlling the orientation via Miller indices is crucial in semiconductor manufacturing and nanofabrication.

6. Symmetry and Classification: Crystals are classified based on symmetry and lattice systems (cubic, tetragonal, hexagonal, etc.), and Miller indices are used to describe the symmetry of planes within these systems.

Glossary

1. **Buffer** – A solution that resists changes in pH upon the addition of small amounts of acid or base.
2. **pH Meter** – An instrument used to measure the hydrogen ion concentration (pH) in a solution.
3. **Dissecting Microscope** – A low-magnification microscope used for viewing surface details of specimens.
4. **Compound Microscope** – A high-magnification microscope using two lenses for observing thin, transparent samples.
5. **Phase Contrast Microscopy** – Enhances contrast in transparent specimens without staining by converting phase shifts in light.
6. **Fluorescence Microscopy** – Uses high-intensity light to excite fluorophores in the sample, which emit light for visualization.
7. **Electron Microscopy** – Uses beams of electrons for imaging; includes TEM and SEM for high-resolution observation.
8. **Transmission Electron Microscope (TEM)** – Provides detailed internal structure of cells by passing electrons through thin specimens.
9. **Scanning Electron Microscope (SEM)** – Provides 3D surface images by scanning the surface with electrons.
10. **Spectroscopy** – Study of the interaction between matter and electromagnetic radiation.
11. **UV-Vis Spectrophotometry** – Measures absorbance of ultraviolet or visible light by a substance to determine its concentration.
12. **Infrared (IR) Spectroscopy** – Identifies chemical bonds based on molecular vibrations and IR light absorption.
13. **Raman Spectroscopy** – Detects vibrational modes of molecules via inelastic scattering of light.
14. **Fluorescence Spectroscopy** – Measures emitted light from fluorophores to study molecular environments and interactions.
15. **Centrifugation** – Separation of particles from a solution based on density by spinning them at high speed.
16. **Ultracentrifugation** – High-speed centrifugation used to separate small molecules like proteins and nucleic acids.
17. **Relative Centrifugal Force (RCF)** – The force exerted on particles during centrifugation, dependent on

speed and radius.

18. **Chromatography** – A method to separate components of a mixture using stationary and mobile phases.
19. **Adsorption Chromatography** – Separation based on different levels of adherence of substances to the stationary phase.
20. **Partition Chromatography** – Separation based on solubility differences between two liquid phases.
21. **Thin Layer Chromatography (TLC)** – Uses a thin layer of adsorbent for separation and identification of compounds.
22. **Paper Chromatography** – Uses paper as the stationary phase for separation of pigments or small molecules.
23. **Gas Chromatography (GC)** – Separates volatile compounds using a gas as the mobile phase.
24. **Gas-Liquid Chromatography (GLC)** – GC where the stationary phase is a liquid coated onto a solid support.
25. **High-Performance Liquid Chromatography (HPLC)** – High-pressure liquid chromatography used for separating complex mixtures.
26. **Ion Exchange Chromatography** – Separates ions and polar molecules based on charge.
27. **Electrophoresis** – Movement of charged particles in an electric field, used to separate DNA, RNA, or proteins.
28. **Electrophoretic Mobility (EPM)** – Speed of a particle under an electric field, indicating charge-to-size ratio.
29. **Agarose Gel Electrophoresis** – Separates nucleic acids using agarose matrix and electric current.
30. **SDS-PAGE** – Denaturing electrophoresis method for separating proteins based on molecular weight.
31. **PAGE (Polyacrylamide Gel Electrophoresis)** – Electrophoresis in polyacrylamide gel for separating proteins or nucleic acids.
32. **Capillary Electrophoresis** – Uses thin capillaries for high-resolution separation of biomolecules.
33. **Isoelectric Focusing** – Separates proteins based on isoelectric points in a pH gradient.
34. **2D Electrophoresis** – Combines isoelectric focusing and SDS-PAGE for detailed protein analysis.
35. **pH Measurement** – Determining the acidity or alkalinity of a solution using indicators or a pH meter.
36. **Radioactive Labelling** – Using radioactive isotopes to trace molecules in biological studies.
37. **Counting (Radioactivity)** – Measurement of radioactive decay using instruments like scintillation counters.

38. **Autoradiography** – Imaging technique to detect radioactive molecules on a medium like film.
39. **X-ray Crystallography** – Technique to determine atomic structure using X-ray diffraction patterns from crystals.
40. **X-ray Diffraction** – Scattering of X-rays by atoms in a crystal to reveal structure.
41. **Bragg's Equation** – Describes the angles at which X-rays are diffracted by crystal planes.
42. **Reciprocal Lattice** – A mathematical construct used in crystallography to interpret diffraction patterns.
43. **Miller Indices** – Notation system for identifying crystal planes.
44. **Unit Cell** – The smallest repeating unit of a crystal lattice.
45. **Crystal Structure** – Arrangement of atoms in a crystalline solid.
46. **Rotating Crystal Method** – X-ray crystallography technique where a crystal is rotated to obtain diffraction data.
47. **Powder Method** – X-ray method using powdered samples to study polycrystalline materials.
48. **Fluorophore** – A fluorescent chemical compound used in microscopy and spectroscopy.
49. **Monochromator** – An optical device that transmits a specific wavelength from a light source.
50. **Detector (Spectroscopy)** – Device that measures light intensity in spectrophotometry or other optical techniques.

Multiple Choice Questions

1. Which of the following combinations would best prepare an acidic buffer solution?
A) HCl and NaCl
B) CH_3COOH and CH_3COONa
C) NH_4OH and NH_4Cl
D) NaOH and NaCl
Answer: B

2. What is the role of a buffer in a biochemical reaction?
A) Speed up the reaction
B) Change pH as needed
C) Resist changes in pH
D) Precipitate proteins
Answer: C

3. The Henderson-Hasselbalch equation is used to calculate:
A) Reaction rates
B) Ionic strength
C) pH of buffer solutions
D) Molarity
Answer: C

4. What component of the pH meter is sensitive to hydrogen ions?
A) Mercury electrode
B) Glass electrode
C) Salt bridge
D) Platinum wire
Answer: B

5. pH meters measure the potential difference between:
A) Two salt bridges
B) A glass electrode and a reference electrode
C) Acid and base solutions
D) Two magnetic probes
Answer: B

6. Which type of microscope provides a 3D image of the specimen's surface?
A) TEM
B) Compound microscope
C) SEM
D) Fluorescence microscope
Answer: C

7. The dissecting microscope is best used for:
A) Examining cell nuclei
B) Observing ultrathin sections
C) Viewing insects and plant parts
D) Visualizing organelles
Answer: C

8. Phase contrast microscopy enhances contrast in:
A) Fluorescent samples
B) Transparent, unstained samples
C) Radioactive materials
D) Thick opaque samples
Answer: B

9. Fluorescence microscopy relies on:
A) Magnetic fields
B) Reflected light
C) Absorption and emission of light
D) Surface tension
Answer: C

10. Which electron microscope has higher resolution for internal structures?
A) SEM
B) Fluorescence microscope
C) Dissecting microscope
D) TEM
Answer: D

11. The principle of UV-visible spectroscopy is based on:
A) Absorption of IR radiation
B) Vibration of atomic nuclei
C) Electronic transitions of molecules
D) Fluorescence emission
Answer: C

12. Which of the following is NOT a component of a UV-Vis spectrophotometer?
A) Monochromator
B) Light source
C) Electron gun
D) Detector
Answer: C

13. Beer-Lambert law is used to relate absorbance with:
A) Fluorescence intensity
B) Concentration and path length

C) Wavelength and temperature
D) Refractive index
Answer: B

14. In IR spectroscopy, absorption bands correspond to:
A) Electron transitions
B) Changes in vibrational energy
C) Gamma emissions
D) Changes in nuclear spin
Answer: B

15. Which functional group typically shows a strong peak around 1700 cm^{-1} in IR spectra?
A) Alcohol
B) Amine
C) Carbonyl (C=O)
D) Alkene
Answer: C

16. Raman spectroscopy differs from IR spectroscopy because it relies on:
A) Absorption of visible light
B) Inelastic scattering of light
C) Fluorescence emission
D) Radioactive decay
Answer: B

17. In TEM, contrast in images arises due to:
A) Light reflection
B) Electron absorption by thick regions
C) Phase shift
D) Surface charging
Answer: B

18. Which of the following parameters does NOT directly affect pH meter accuracy?
A) Temperature
B) Calibration
C) Light intensity
D) Electrode condition
Answer: C

19. A basic buffer solution can be prepared using:
A) HCl and NaCl
B) H_2SO_4 and Na_2SO_4
C) NH_4OH and NH_4Cl
D) NaOH and NaCl
Answer: C

20. What does the Raman shift in Raman spectroscopy indicate?
A) Wavelength of incident laser
B) Atomic mass

C) Energy difference between incident and scattered light
D) Fluorescence of a compound
Answer: C

21. What is the principle behind fluorescence spectroscopy?
A) Scattering of light
B) Absorption of infrared light
C) Emission of light from excited molecules
D) Conversion of sound to light
Answer: C

22. Which component in a fluorescence spectrophotometer isolates specific wavelengths?
A) Filter paper
B) Sample cuvette
C) Monochromator
D) Detector
Answer: C

23. Fluorescence occurs when a molecule absorbs light and emits it at:
A) The same wavelength
B) A shorter wavelength
C) A longer wavelength
D) Zero wavelength
Answer: C

24. Which of the following is used as a light source in fluorescence spectroscopy?
A) Tungsten lamp
B) UV or Xenon arc lamp
C) LED display
D) Infrared laser
Answer: B

25. A major application of fluorescence spectroscopy is:
A) Determining viscosity
B) Identifying crystal lattices
C) Studying protein-ligand interactions
D) Measuring sound waves
Answer: C

26. Centrifugation separates components based on:
A) pH
B) Size and density
C) Absorbance
D) Boiling point
Answer: B

27. Relative Centrifugal Force (RCF) depends on:
A) Light intensity and wavelength
B) Temperature and viscosity
C) Rotor radius and angular velocity
D) Time and pressure
Answer: C

28. Which of the following is NOT a type of centrifugation?
A) Differential
B) Density gradient
C) Reverse flow
D) Isopycnic
Answer: C

29. Ultracentrifuges are used to:
A) Weigh molecules
B) Generate visible spectra
C) Separate small molecules like proteins and nucleic acids
D) Measure pH
Answer: C

30. Which of the following forces is involved in centrifugation?
A) Electrostatic
B) Gravitational
C) Centrifugal
D) Magnetic
Answer: C

31. Chromatography separates molecules based on:
A) Density
B) Electrical conductivity
C) Differential migration through a stationary phase
D)Acoustic velocity
Answer: C

32. Which chromatography technique uses paper as the stationary phase?
A) TLC
B) Paper chromatography
C) GC
D) HPLC
Answer: B

33. In thin layer chromatography (TLC), the stationary phase is typically made of:
A. Paper pulp
B. Silica gel or alumina
C. Plastic
D. Ion resin
Answer: B

34. Gas chromatography (GC) is best suited for:
A. Ions in solution
B. Non-volatile solids
C. Volatile compounds
D. DNA fragments
Answer: C

35. In ion exchange chromatography, separation is based on:
A. Molecular weight
B. Charge of the molecules
C. Boiling point
D. Optical density
Answer: B

36. What is the basic principle of electrophoresis?
A. Separation based on boiling point
B. Movement of charged particles in an electric field
C. Absorption of UV light
D. Emission of light after excitation
Answer: B

37. Electrophoretic mobility (EPM) is defined as:
A. The mass of a molecule per unit charge
B. The net charge of a molecule
C. The velocity per unit electric field
D. The resistance offered by a molecule
Answer: C

38. Which of the following factors does NOT affect electrophoretic mobility?
A. pH of the buffer
B. Size of the molecule
C. Molecular shape
D. Room temperature only
Answer: D

39. The buffer used in electrophoresis serves to:
A. Fix the molecules in place
B. Maintain pH and conduct current
C. React with proteins
D. Make the gel solid
Answer: B

40. In SDS-PAGE, proteins are separated based on their:
A. Charge
B. Molecular weight

C. Solubility
D. Shape
Answer: B

41. Which of the following is a matrix used in zone electrophoresis?
A. Cellulose acetate
B. Agarose
C. Starch gel
D. All of the above
Answer: D

42. PAGE stands for:
A. Polyacrylamide Gel Electrophoresis
B. Protein Analytical Gel Electrophoresis
C. Partial Agarose Gel Electrophoresis
D. Positive Agarose Gel Electrophoresis
Answer: A

43. In isoelectric focusing, proteins are separated based on their:
A. Net charge
B. Isoelectric point (pI)
C. Size
D. Hydrophobicity
Answer: B

44. Capillary electrophoresis offers which advantage over traditional gel electrophoresis?
A. Less expensive
B. Slower results
C. Higher resolution and faster analysis
D. No separation of molecules
Answer: C

45. Which gel type is commonly used for nucleic acid electrophoresis?
A. Polyacrylamide
B. Starch gel
C. Agarose
D. Cellulose acetate
Answer: C

46. Pulse-field gel electrophoresis is especially useful for:
A. Small peptides
B. Large DNA fragments
C. Ions and salts
D. Sugars and lipids
Answer: B

47. 2D electrophoresis separates proteins based on:
A. Charge only
B. Molecular weight only

C. Isoelectric point and molecular weight
D. Shape and solubility
Answer: C

48. The function of SDS in SDS-PAGE is to:
A. Preserve protein shape
B. Stain the proteins
C. Denature proteins and give them uniform negative charge
D. Lower the pH
Answer: C

49. Which electrophoresis method does NOT use a gel matrix?
A. Free electrophoresis
B. Agarose electrophoresis
C. PAGE
D. SDS-PAGE
Answer: A

50. What is the main purpose of using a tracking dye in electrophoresis?
A. To color the proteins
B. To reduce voltage
C. To monitor the progress of electrophoresis
D. To increase current
Answer: C

51. What does a pH meter actually measure?
A. Oxygen concentration
B. Hydrogen ion activity
C. Water purity
D. Electrical resistance
Answer: B

52. Which electrode is commonly used in pH measurement?
A. Copper electrode
B. Platinum electrode
C. Glass electrode
D. Mercury electrode
Answer: C

53. Radioactive labeling is used to:
A. Identify optical density
B. Measure boiling point
C. Trace molecules in biochemical reactions
D. Increase solubility
Answer: C

54. Which of the following is used to detect radioactive emissions?
A. Thermometer
B. UV lamp

C. Scintillation counter
D. Spectrophotometer
Answer: C

55. Autoradiography is used to:
A. Study pH changes in solutions
B. Determine solubility
C. Visualize radioactively labeled molecules on film
D. Measure viscosity
Answer: C

56. What is the basic principle of X-ray crystallography?
A. Emission of UV light
B. Absorption of gamma rays
C. Diffraction of X-rays by crystals
D. Scattering of visible light
Answer: C

57. The Bragg equation is used to determine:
A. Protein structure
B. DNA sequence
C. Angle of X-ray diffraction
D. pH of a crystal
Answer: C

58. What does the term 'unit cell' refer to in crystallography?
A. A biological cell under a microscope
B. The smallest repeating unit in a crystal lattice
C. A cell used for radioactive storage
D. A water molecule
Answer: B

59. Miller indices are used to:
A. Measure pH
B. Describe crystal planes
C. Estimate radioactivity
D. Classify protein types
Answer: B

60. In the rotating crystal method of X-ray crystallography, the crystal is:
A. Dissolved in buffer
B. Bombarded with sound waves
C. Rotated to expose different lattice planes to X-rays
D. Kept stationary throughout the process
Answer: C

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Notes