AS-2239

M.Sc. (First Semester) Examination, 2013

BIOTECHNOLOGY

Paper: Third [LBTM-103]

Bioinstrumentation

Answer keys/Model answer

Section 'A' **10 X 2 = 20**

Objective type question

1. Choose the correct alternative

(i) c. Dichroic mirror

(ii) a. $G = w^2r$

(iii) b. Specific absorption coefficient

(IV) c. Hookes law

(V) a. Flow rate

(VI) c. Distribution coefficient

(VII) d. Sudan black

(VIII) c. Ammonium per sulphate

(IX) c. $t_{1/2} = 0.693/\lambda$

(X) c. 5000 years

Section 'B'

2. Descriptive type questions:

Ans 1. A. Phase contrast microscope:

Most microorganisms and their organelles are colorless and often difficult to see by ordinary bright-field or dark-field microscopy. Phase-contrast microscopy permits the observation of otherwise indistinct, living, unstained bacteria and their associated structures (such as endospores).

Certain transparent, colorless living microorganisms and their internal organelles are often impossible to see by ordinary bright-field or dark-field microscopy because they do not absorb, reflect, refract, or diffract sufficient light to contrast with the surrounding environment or the rest of the microorganism. Microorganisms and their organelles are only visible when they absorb, reflect, refract, or diffract more light than their environment. The **phase-contrast microscope**

permits the observation of otherwise invisible living, unstained microorganisms. In the phase-contrast microscope, the condenser has an annular diaphragm, which produces a hollow cone of light; the objective has a glass disk (the phase plate) with a thin film of transparent material deposited on it, which accentuates phase changes produced in the specimen. This phase change is observed in the specimen as a difference in light intensity**.** Phase plates may either retard (positive phase plate) the diffracted light relative to the undiffracted light, producing dark-phase-contrast microscopy**,** or advance (negative phase plate) the undiffracted light relative to the directed light, producing bright-phase contrast microscopy.

Application of phase contrast microscope:

1. Identification of unstained bacterial e.g. *Bacillus, Clostridium*
2. Visualization of endospore

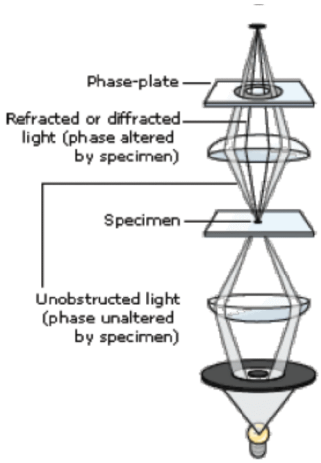


Fig. working of phase contrast microscope

B. **Fluorescent microscope**

It works on the basis of specific fluorescence shown by stained biomolecules and cellular components. The sample is kept in a slide stained with suitable fluorescent dyes. The light of excitation wavelength is incident on the sample through dichroic mirror assembly. The sample fluoresces and the fluorescent wavelength is passed through the specific filter in dichroic mirror assembly.

Fluorescence microscopy is commonly used in the clinical laboratory for the rapid detection and identification of bacterial antigens in tissue smears, sections, and fluids, as well as the rapid identification of many disease-causing microorganisms. For example, a sputum specimen can be quickly screened for *M. tuberculosis* by staining it with a fluorescent dye that binds specifically to *M. tuberculosis*. Only the stained bacterium of interest will be visible when the specimen is viewed under the fluorescence microscope.

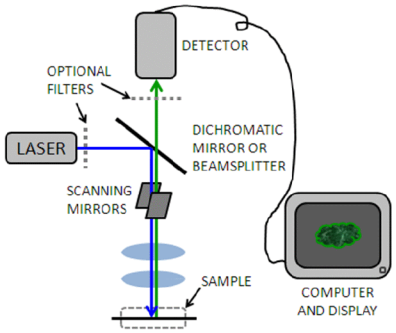


Fig. Working of fluorescence microscope

Ans. 2.

The atomic nuclei with odd mass numbers has the property spin, this means they rotate around a given axis. The nuclei with even numbers may or may not have this property. A spin angular momentum vector characterizes the spin. The nucleus with a spin is in other words a charged and spinning particle, which in essence is an electric current in a closed circuit, well known to produce a magnetic field. The magnetic field developed by the rotating nucleus is described by a nuclear magnetic moment vector, μ, which is proportional to the spin angular moment vector.

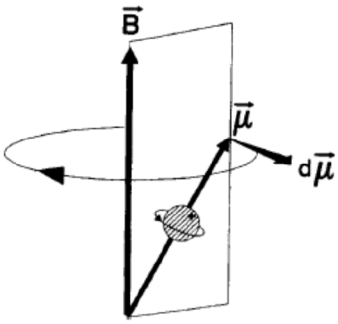


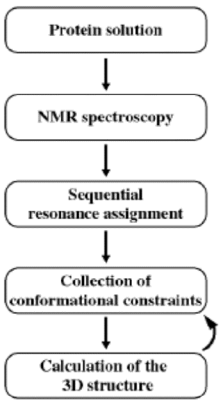
Fig. Spinning nucleus with a charge processing in a magnetic field

The basis of all NMR experiments is a quantum mechanical property of the nucleus: the spin. The nuclei of interest in NMR of biological macromolecules carry a spin of which allows only two different spin states, often referred to as spin up and spin down. Associated with the spin is a magnetic moment which for a spin can be interpreted as a magnetic dipole. These very small atomic dipoles can only orient parallel or antiparallel to an external magnetic field. The two possible orientations correspond to slightly different energies and spins are allowed to jump from one orientation to the other, absorbing or emitting the energy difference in the form of electromagnetic radiation. Its is remarkable that the difference between the number of spins in the two states is extremely small even if the spins are in a very strong magnetic field. For example, in one million hydrogen (^{1}H) nuclei, the numbers of spins orienting parallel or anti-parallel differ only by about 60 at room temperature and in the highest magnetic field strengths available for NMR. Only the very small difference between the number of parallel and anti-parallel spins contributes to the NMR signal.

Structure characterization using NMR:

Structure determination by NMR is an established technique and is routinely used to determine three-dimensional structures of biological macromolecules in solution with molecular weights up to 30 kDa.

Schematic diagram for structure determination of protein using NMR:



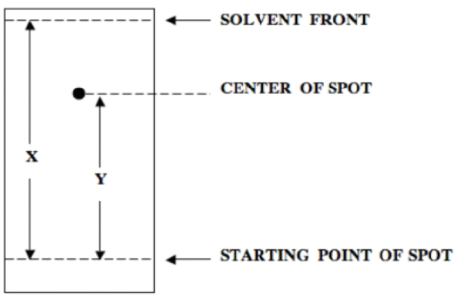
Answer 3.

Thin layer chromatography, or TLC, is a method for analyzing mixtures by separating the compounds in the mixture. TLC can be used to help determine the number of components in a

mixture, the identity of compounds, and the purity of a compound. By observing the appearance of a product or the disappearance of a reactant, it can also be used to monitor the progress of a reaction. TLC is a sensitive technique - microgram (0.000001 g) quantities can be analyzed by TLC and it takes little time for an analysis (about 5-10 minutes).

TLC consists of three steps - spotting, development, and visualization.

1. The sample to be analyzed is dissolved in a volatile (easily evaporated) solvent to produce a very dilute (about 1%) solution. Spotting consists of using a micro pipet to transfer a small amount of this dilute solution to one end of a TLC plate, in a thin layer of powdered silica gel that has been coated onto a plastic sheet. The spotting solvent quickly evaporates and leaves behind a small spot of the material.
2. Development consists of placing the bottom of the TLC plate into a shallow pool of a development solvent, which then travels up the plate by capillary action. As the solvent travels up the plate, it moves over the original spot. A competition is set up between the silica gel plate and the development solvent for the spotted material. The very polar silica gel tries to hold the spot in its original place and the solvent tries to move the spot along with it as it travels up the plate. The outcome depends upon a balance among three polarities that of the plate, the development solvent and the spot material.
3. Different components in the original spot, having different polarities, will move different distances from the original spot location and show up as separate spots. When the solvent has traveled almost to the top of the plate, the plate is removed, the solvent front marked with a pencil, and the solvent allowed to evaporate.



Ans. 4

PAGE (Polyacrylamide Gel Electrophoresis), is an analytical method used to separate components of a protein mixture based on their size. The technique is based upon the principle that a charged molecule will migrate in an electric field towards an electrode with opposite sign. The general electrophoresis techniques cannot be used to determine the molecular weight of biological molecules because the mobility of a substance in the gel depends on both charge and size. To overcome this, the biological samples needs to be treated so that they acquire uniform charge, then the electrophoretic mobility depends primarily on size. For this different protein molecules with different shapes and sizes, needs to be denatured (done with the aid of SDS) so that the proteins lost their secondary, tertiary or quaternary structure .The proteins being covered by SDS are negatively charged and when loaded onto a gel and placed in an electric field, it will migrate towards the anode (positively charged electrode) are separated by a molecular sieving effect based on size. After the visualization by a staining (protein-specific) technique, the size of a protein can be calculated by comparing its migration distance with that of a known molecular weight ladder (marker).

Two categories of buffer systems are available for SDS PAGE: continuous and discontinuous. Continuous systems use the same buffer in both the gel and tank. While continuous gels are easy to prepare and give adequate resolution for some applications, bands tend to be broader and resolution consequently poorer in these gels. Discontinuous buffer systems employ different buffers for tank and gel, and often two different buffers within the gel, with a third buffer in the tank. Discontinuous systems concentrate, or 'stack' the protein samples into a very narrow zone prior to separation, which results in improved band sharpness and resolution.

In the classic SDS PAGE system developed by Laemmli, the gel is divided into an upper 'stacking' gel of low percentage (i.e. large pore size) and low pH (6.8) and a resolving gel with a pH of 8.8 with much smaller pores. Both gels contain only Cl^- as the mobile anion. The tank buffer has glycine as its anion, at a pH of 8.8. When electrophoresis begins, glycine enters the stacking gel, where equilibrium favors the zwitterionic form with zero net charge. The glycine front moves slowly through the stacking gel, lagging behind the strongly charged, smaller Cl^- ions. As these two current carrying species separate, a region of low conductivity, with a consequent high voltage

drop, is created between them. This zone (a Kohlrausch discontinuity) 'sweeps' the proteins rapidly through the large pores of the stacking gel, collecting the sample and depositing it at the top of the resolving gel in a focused narrow band. When the Kohlrasch discontinuity enters the resolving gel, the increase in pH ionizes the glycine so that it runs faster, dissipating the discontinuity. This allows the proteins to unstack and separate through the small pore resolving gel.

The term "**discontinuous"** refers to the fact that there are actually two layers in the gel matrix. The lower layer (separating, or resolving, gel) is responsible for actually separating polypeptides by size. The upper layer (stacking gel) includes the sample wells. It is designed to sweep up proteins in a sample between two moving boundaries so that they are compressed (stacked) into micrometer thin layers when they reach the separating gel.

PAGE is working upon the principle in which, the charged molecule will migrate towards the opposite charged electrode through highly cross linked matrix. Separation occurs due to different rates of migration occurs by the magnitude of charge and frictional resistance related to the size. The gel used is divided into an upper "stacking" gel of low percentage (with large pore size) and low pH (6.8), where the protein bands get squeezed down as a thin layer migrating toward the anode and a resolving gel (pH 8.8) with smaller pores. Cl^- is the only mobile anion present in both gels. When electrophoresis begins, glycine present in the electrophoresis buffer, enters the stacking gel, where the equilibrium favors zwitterionic form with zero net charge. The glycine front moves through the stacking gel slowly, lagging behind the strongly charged, Cl- ions. Since these two current carrying species separate, a region of low conductivity, with high voltage drop, is formed between them. This zone sweeps the proteins through the large pores of the stacking gel, and depositing it at the top of the resolving gel as a narrow band.

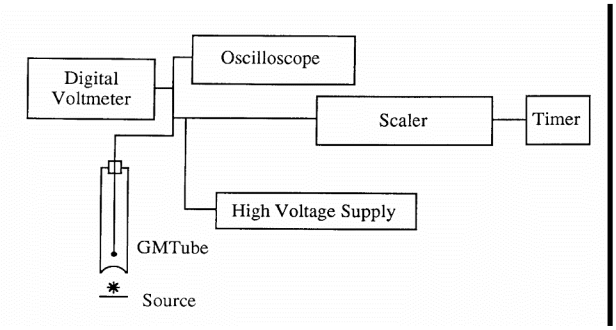
Stacking occurs by the differential migration of ionic species, which carry the electric current through the gel. When an electrical current is applied to the gel, the negatively charged molecules start migrating to the positively charged electrode. Cl- ions, having the highest charge/mass ratio move faster, being depleted and concentrated at anode end. SDS coated proteins has a higher charge/mass ratio than glycine so it moves fast, but slower than Cl-. When protein encounters resolving gel it slows the migration because of increased frictional resistance, allowing the protein to stack in the gel.

When glycine reaches resolving gel it becomes negatively charged and migrates much faster than protein due to higher charge/mass ratio. Now proteins are the main carrier of current and separate according to their molecular mass by the sieving effect of pores in gel.

Ans 5.

The Geiger counter is a gas-ionization device. The basic design consists of a metal tube, often with a glass or mica window at one end. At the center of the tube runs a wire with a strong positive charge. The tube is sealed and filled at low pressure with an inert gas such as argon, helium or neon with some gases added. In the absence of radiation, the detector does not conduct charge. Whenever ionizing radiation passes through, however, an electric signal is generated because the metal casing of the Geiger-Muller tube acts as a cathode, and the central wire is the anode. The anode transfers the pulses of current through a resistor, where they are converted to pulses of voltage. The voltage pulses are then recorded by a counting device. Finally, an oscilloscope, LED screen, or other display conveys the particle count to the user.

In a GM counter, the avalanche produced near the anode wire spreads along its entire length. That is why the output signal is independent of the magnitude of the triggering event. After the ionizing event creates electrons and positive ions, the electrons are collected very quickly while it takes a longer time for the ions to move slowly. When the discharge is quenched, the space charge sheath surrounding the anode moves towards the cathode. After it reaches sufficient distance from the anode, the voltage is sufficient to re-establish the threshold for Geiger action. If another ionizing event occurs immediately after this, a second pulse is recorded. In the circuit, the collector electrode is maintained at high voltage above the ground but this is blocked by a capacitor from the counter input. The cathode is maintained at ground potential for safe operation.



Ans 6.

- There are distinct ways that molecules vibrate, sometimes referred to as vibrational modes. Molecules stretch and bend, and do so symmetrically and asymmetrically. For bending, there are four variations – rocking, scissoring, wagging and twisting. The first two bending modes are called "in-plane", and the second two are "out of plane". All of these vibrations happen at specific frequencies. A frequency is a measure of how often, or how frequently, the molecule vibrates in a given period of time. Frequencies are measured in units of wavenumber, or in number of vibrations over a distance of centimeters. The specific frequencies of a molecule are very much like a fingerprint: every molecule vibrates at very distinctive frequencies.
- FT-IR stands for Fourier Transform InfraRed, the preferred method of infrared spectroscopy. In infrared spectroscopy, IR radiation is passed through a sample. Some of the infrared radiation is absorbed by the sample and some of it is passed through (transmitted). The resulting spectrum represents the molecular absorption and transmission, creating a molecular fingerprint of the sample.
- An infrared spectrum represents a fingerprint of a sample with absorption peaks which correspond to the frequencies of vibrations between the bonds of the atoms making up the material. Because each different material is a unique combination of atoms, no two compounds produce the exact same infrared spectrum. Therefore, infrared spectroscopy can result in a positive identification (qualitative analysis) of every different kind of material. In addition, the size of the peaks in the spectrum is a direct indication of the amountof material present. With modern software algorithms, infrared is an excellent tool for quantitative analysis.

Application of FTIR:

• Food analysis: additives, preservatives, colorants
• Environmental analysis: water, atmospheric particles, gases
• Forensic science: paints, textiles, cosmetics,
• Pharmaceuticals.
• Multilayer compounds: polymers, paintings, films

Ans 7.

X-Ray Diffraction (XRD):

X-ray diffraction (XRD) is an analytical technique looking at X-ray scattering from crystalline materials. Each material produces a unique X-ray "fingerprint" of X-ray intensity versus scattering angle that is characteristic of it's crystalline atomic structure. Qualitative analysis is possible by comparing the XRD pattern of an unknown material to a library of known patterns. The process of reflection is described here in terms of incident and reflected (or diffracted) rays, each making an angle θ with a fixed crystal plane knows as Bragg's law.

Bragg's law : $2d \sin \theta = n \lambda$

In x-ray diffraction work we normally distinguish between single crystal and polycrystalline or powder applications. The single crystal sample is a perfect crystal with a cross section of about 0.3 mm. The single crystal diffractometer and associated computer package is used mainly to elucidate the molecular structure of novel compounds, either natural products or man made molecules. Powder diffraction is mainly used for "finger print identification" of various solid materials, e.g. asbestos, quartz. In powder or polycrystalline diffraction it is important to have a sample with a smooth plane surface. If possible, we normally grind the sample down to particles of about 0.002 mm to 0.005 mm cross section. The ideal sample is homogeneous and the crystallites are randomly distributed. The sample is pressed into a sample holder so that we have a smooth flat surface. Only crystallites having reflecting planes parallel to the specimen surface will contribute to the reflected intensities

Scanning Electron Microscope (SEM):

The scanning electron microscope (SEM) uses electrons to form an image. A beam of electrons is produced at the top of the microscope (electron gun) and follows a vertical path through the column of the microscope, it makes its way through electromagnetic lenses which focus and direct the beam down towards the sample. The beam passes through pairs of scanning coils or pairs of deflector plates in the electron column, typically in the final lens, which deflect the beam in the x and y axes so that it scans over a rectangular area of the sample surface.

The scanning electron microscope has many advantages over traditional microscopes. The SEM has a large depth of field, which allows more of a specimen to be in focus at one time. The SEM also has much higher resolution, so closely spaced specimens can be magnified at much higher levels. Because the SEM uses electromagnets rather than lenses, the researcher has much more control in the degree of magnification. All of these advantages, as well as the actual strikingly clear images, make the scanning electron microscope one of the most useful instruments in research today.

The basic steps involved in SEM sample preparation include surface cleaning, stabilizing the sample with a fixative, rinsing, dehydrating, drying, mounting the specimen on a metal holder, and coating the sample with a layer of a material that is electrically conductive.