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S.Y.B.Sc. SEM-4

Subject: Biochemistry

Paper-401: BIOPHYSICAL & BIOCHEMICAL TECHNIQUES

Unit -1

SPECTROSCOPIC TECHNIQUES

Unit -1 SPECTROSCOPIC TECHNIQUES

- Spectrophotometry and colorimetry are analytical methods of measuring the amount of light absorbed by a substance in solution. They are commonly used techniques for quantitatively determining substances encountered in biochemistry. All substances in solution absorb light of one wavelength and transmit light of other wavelength.
- Absorbance is a characteristic of a substance just like melting point, boiling point, density and solubility. But because it can be related to the amount of the substance in solution, absorbance can be used to quantitatively determine the amount of substance in solution.

Electromagnetic radiation and spectra:

- Light or electromagnetic radiation is composed of photons moving in a wave that oscillates along the path of motion. The wavelength of light is defined as the distance between adjacent peaks in the wave and can be further by the equation:
- **λ= c /υ** where λ is wavelength of light, c is speed of light and **υ** is frequency.
- Photos of different wavelengths have different energies that are given by the following equation:
- **E=hc/λ = hυ** where h is plank's constant.
- Thus, shorter the wavelength, greater would be the energy of the electromagnetic radiation.
- Electromagnetic radiation can be divided into various region according to the wavelength.

Region	Wavelength
Gamma rays	0.01 nm – 0.1 nm
X rays	0.1 nm – 10 nm
Ultraviolet (UV) rays	200 nm – 400 nm
Visible rays	400 nm – 700 nm
Infra-red (IR) rays	700 nm – 500 um
Microwaves (Radar)	1.0 cm – 10 cm
Radio waves	1.0 m – 5.0 m

- In the visible region, light of different wavelengths has different colors: violent and blue in the lower wavelength region and orange and red in the higher wavelength region. When a substance in solution in solution appears blue, it means that substance that appears red is absorbing blue light and transmitting red light. A graph of wavelength v/s absorbance is called absorption spectra.

- Molecules possess both kinetic energy and the energy associated with their bonding electrons. The absorption of light does not directly affect the kinetic energy, but it can affect the energy of the bonding electrons. The energy of bonding electrons can be further divided into three types: electronic, vibration and rotational. Absorption of electromagnetic radiation involves a change in one or more of these bonding energies.

- Each of electronic, vibration and rotational energy components can have only certain definite values of energy levels. A molecule whose electronic, vibrational and rotational energies are all at their lowest value is said to be in the ground state. When a molecule

is irradiated by photons, the photons are absorbed and the molecule is raised to higher energy level.

- Spectra arise when molecules absorb photons of specific energy. Transition between different electronic levels give rise to spectra in UV or visible regions: transitions between vibrational levels within the same electronic state give rise to spectra in near infrared region (700 nm – 200 um) and transitions between rotational levels belonging to the same vibrational state give rise to spectra in the far infrared region (> 200um).
- The molecules once arrived at, an excited electronic, vibrational or rotational state does not continue forever because the energy originally gained from photon absorption is lost by collisions with other molecules such as solvent molecules. Eventually, the energy is transformed into kinetic or thermal energy and the molecule is returned to the ground state with liberation of heat.
- In general, the absorption of visible and UV light by organic compounds can occur when there is some un-saturation in the molecules. A specific grouping is of atoms having un-saturation and absorbing light is called chromophore or chromophoric groups. The common chromophoric groups include: carbon-carbon double and triple bonds and the carbonyl, amido, azo, nitroso, nitro, imidazole, indole, purine and pyrimidine groups.

Quantitative aspects of light absorption:

Two laws govern the absorption of monochromatic light by any absorbing material:

- **1. Beer's Law:** The amount of light absorbed by a substance in a solution is proportional to the number of absorbing molecules i.e. the concentration of the absorbing solution.
- **2. Lambert's Law:** The amount of light absorbed by a substance in a solution is proportional to the thickness of the absorbing material i.e. the path length and is independent of the intensity of the incident light.

One Word Question

Sr. No	Question	Answer
1	Colorimeter and spectrophotometer are work on which Principle?	Light Adsorption
2	Each and every light energy contain specific_____?	Wavelength
3	What is the wavelength of UV Rays?	200 nm – 400 nm
4	What is the wavelength of Visible Rays?	400 nm – 700 nm
5	What is the wavelength of Infrared Rays?	700 nm – 500 um
6	Which law apply on Spectrophotometer and colorimeter?	Beer's & Lambert's Law

Beer – Lambert's Law:

$A=\Sigma CI$

Where, A = Absorbance, Σ = Extinction coefficient, C = Concentration of substances, I = Path length

Limitations of Beer – Lambert's law:

- 1. These laws are applicable for monochromatic light and not applicable for polychromatic light.
- 2. These laws are applicable only at lower solute concentrations but not applicable at very high solute concentrations. At lower concentrations, with increase in concentration there is proportionate increase in absorbance while at very high solute

concentrations the increase in the absorbance is not proportionate to the concentration.

♣ 3. They are applicable only for clear solutions and not applicable for turbid solutions. As the high turbidity of the solutions gives false higher absorbance readings leading to over estimation of solute concentration in the solutions.

♣

A quantity **A** (absorbance) or **E** (extinction) or **O.D.** (cptical density) is defined as

A or $E = \log I_0/I$

Where, I_0 = intensity of incident light

I = intensity of transmitted light.

Comparison of percent transmittance and absorbance scales:

$A = 2 - \log T$

Where, T = % transmittance.

Therefore, when T = 100 then A = 0

And when T = 0 then A = ∞.

- For most of the practical purposes the colorimeter readings are taken in the form of absorbance or optical density and not as percent transmittance because; the relationship between concentration and optical density is linear while the relationship between concentration and percent transmittance is exponential.

- The determination of concentration of the solute form the unknown solution is easier with the graph of concentration v/s O.D. while it is more complicated with the graph of concentration v/s percent transmittance.

Definition of Extinction coefficients:

- The units of the extinction coefficient depend on the units of concentration and vice a versa. Frequently Σ is expressed as the molar extinction coefficient (Σ_M) and C is then expressed as moles per liter. The molar extinction coefficient may be defined as "The absorbance given by 1M solution when the path length is 1 cm". Σ can also be expressed as millimolar (Σ_{mM}) or micromolar (Σ_{uM}) extinction coefficient and the concentration is then expressed as millimolar or micromolar respectively. The units for Σ_M are moles per liter per centimeter.
- When molecular weight of a substance is not known, as frequently occurs in the biochemical measurements of macromolecules such as proteins and nucleic acids, the extinction coefficient may be based on weight/volume concentration such as 1% (w/v) solutions (10mg/ml solutions) and would be designated as $\Sigma_{1\%}$.

Instrumentation:

The main types of instruments used for measuring either absorbance or % transmittance are colorimeter and spectrophotometer. The basic components of these instruments include a light source, a monochromator or colored filter (wavelength selector) to give a selected wavelength, variable slit, a sample holder (cuvette), a photo detector and a meter.

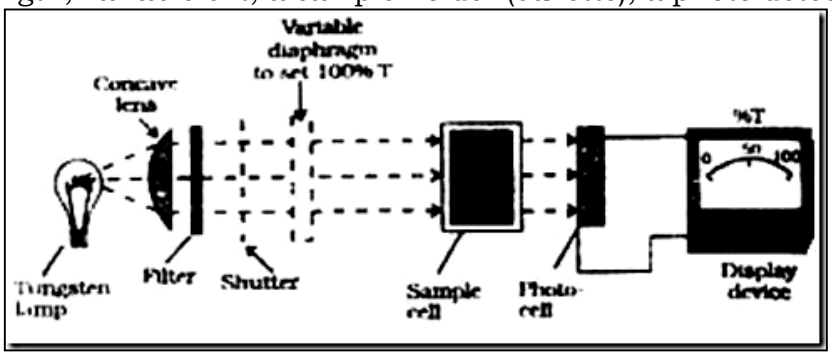


Figure: Schematic diagram of the parts of single beam colorimeter and spectrophotometer

Radiation (light source):

a) Visible radiation

Tungsten filament lamp is the most commonly used source of visible radiation. It is inexpensive and emits continuous radiation in the region between 350 and 2500 nm. Carbon arc, which provides more intense visible radiation, is used in a small number of commercially available instruments.

b) UV radiation

Hydrogen or deuterium lamps are most commonly used sources of ultraviolet light. Both the systems consist of a pair of electrodes enclosed in a glass tube provided with a quartz window. The glass tube is filled with hydrogen or deuterium gas at low pressure. When a stabilized high voltage is applied they emit radiation, which is continuous in the region roughly between 180 to 350 nm. Xenon lamp may also be used for UV radiation, but the radiation produced is not as stable as hydrogen lamp.

Wavelength Selector:

- All the sources discussed so far emit radiation over wide range of wavelengths. However, the laws of absorption apply only to the monochromatic radiation. Thus absorption of narrow bandwidth will tend to show greater adherence to Beer's law. Moreover, narrow bandwidth radiation will allow the resolution of absorption bands, which are quite close to each other.

- Therefore, a narrow bandwidth will go a long way in increasing the sensitivity of absorbance measurement. Narrow bandwidths are made possible by using wavelength selectors.

- Wavelength selectors are of two types: Filters and Monochromators.

Filters:

- A light filter is a device that allows light of the required wavelength to pass but absorbs light of other wavelengths wholly or partially. Filters resolves polychromatic light into a relatively wide bandwidth of about 40 nm and are used as wavelength selectors only in colorimeters.
- Filters used generally are of complementary colors i.e. a blue colored solution absorbs the red color maximally therefore for blue colored solution a red colored filter is used. For absorbance measurement of a red colored solution a blue colored filter is used because red colored solution absorbs maximally the blue colored light.
- The filters are of two types; **absorption filters** work by selective absorption of unwanted wavelengths. An absorption filter is generally made from colored tinted glass plates. Gelatin filters are made of a layer of gelatin, colored with organic dyes and sealed between glass plates.
- Another type of filters is known as **interference filters**. When a ray of light is focused on the interference filters, a part of light is reflected back whereas the remaining light is transmitted. Part of the incident light is reflected repeatedly by the metal layer but at each reflection some is transmitted outwards. The several outgoing rays undergo constructive interference for those wavelengths, which the interference filters, are made for. The interference filters isolates monochromatic light with relatively narrow bandwidths compared to the absorption filters.

One Word Question

Sr. No	Question	Answer
1	Beer's Law note applicable for?	Transparent & Turbid Solution
2	The determination of concentration of the solute form the unknown solution	OD vs. Wavelength graph
3	The molar extinction coefficient may be defined as "The absorbance given by _____when the path length _____.	1Molar, 1 Cm
4	Visible radiation given by _____light Source.	Tungsten lamp
5	UV radiation given by _____light Source.	Hydrogen lamp
6	Name of Wavelength selectors	Filters and Monochromators.
7	How many types of filter?	Adsorption filter Interference filter

Monochromators:

- The monochromators are preferred wavelength selectors in spectrophotometers because of the several advantages they offer compared to the filters. As the name suggests, a monochromator resolves polychromatic light into its individual wavelengths and isolates these wavelengths into very narrow bands.
-
- This increases the sensitivity of detection by closely obeying the Beer's law. Secondly with the help of monochromators it is possible to resolve the two compounds in a mixture which are having the absorption maxima quite close to each other. Both the compounds in the same solution can be estimated quantitatively without separation.
  The essential components of monochromators are:

♣ An entrance slit
♣ A collimating device such as lens or mirror
♣ A wavelength resolving device like prism or grating
♣ A focusing lens or mirror
♣ An exit slit

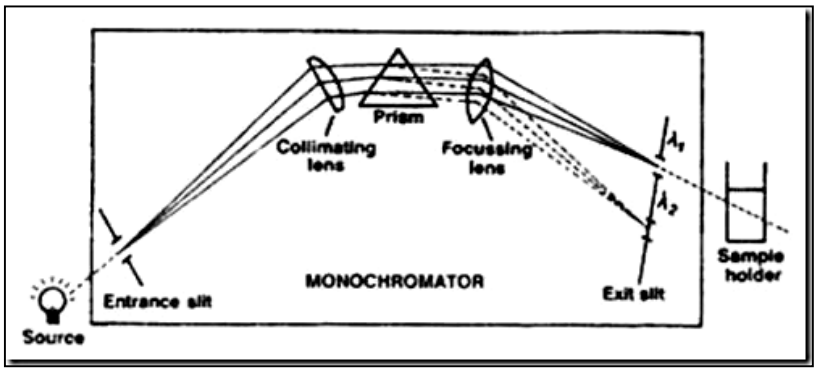


Fig: Prism monochromator

- A prism splits the incoming light into its components by refraction. Refraction occurs because radiation of different wavelengths travels along different paths in medium of higher density.

- In order to maintain the principle of velocity conservation, light of shorter wavelength (higher speed) must travel a longer distance (i.e. blue sky effect). At a grating, the splitting of wavelengths is achieved by diffraction. Diffraction is a reflection phenomenon that occurs at a grid surface, in this case a series of engraved fine lines. The distance between the lines has to be of the same order of magnitude as the wavelength of the diffracted radiation.

- By varying the distance between the lines, different wavelengths are selected. This is achieved by rotating the grating perpendicular to the optical axis. The resolution achieved by gratings is much higher than the one available by prisms. Nowadays instruments almost exclusively contain gratings as monochromators as they can be reproducibly made in high quality by photo reproduction.

Sample containers (holders):

- Borosilicate glass and normal plastics absorb UV light; such cuvettes can only be used for applications in the visible range of the spectrum (up to 350 nm). For UV measurements, quartz cuvettes need to be used. However, disposable plastic cuvettes have been developed that allow for measurements over the entire range of the UV/Vis spectrum.
- Standard path length is usually 1 cm. however cuvette of path length of 1 mm to 10 cm are available.

Detection devices:

Most modern instruments rely on photoelectric transducers, detection devices that convert photons into an electrical signal. Photoelectric transducers have a surface that can absorb radiant energy. The absorbed energy either causes the emission of electrons, resulting in a photocurrent or moves electrons into the conduction band of a solid semiconductor, resulting in an increase in conductivity. There are several common forms of these detectors including barrier layer cells, photomultiplier tubes, and semiconductor detectors.

Phototubes or Photo Emissive Tubes

The components of a phototube include:

- ♣ i) An evacuated glass envelope (with a quartz window),
- ♣ ii) A semi-cylindrical cathode whose inner surface is coated with alkali or alkaline earth oxide, and
- ♣ iii) A centrally located metal wire anode.

A potential difference of approximately 90 volts is applied across the electrodes. The quartz window allows the passage of radiation, which strikes the photo emissive surface of the cathode. The energy of the photon is transferred to the loosely bound electrons of the cathode surface. The electrons become excited and finally leave the surface and travel towards the anode causing current to flow in the circuit. If the electron collection is 100% efficient, the phototube current should be proportional to the light intensity. A schematic diagram of a phototube and the associated circuitry is shown in Fig.

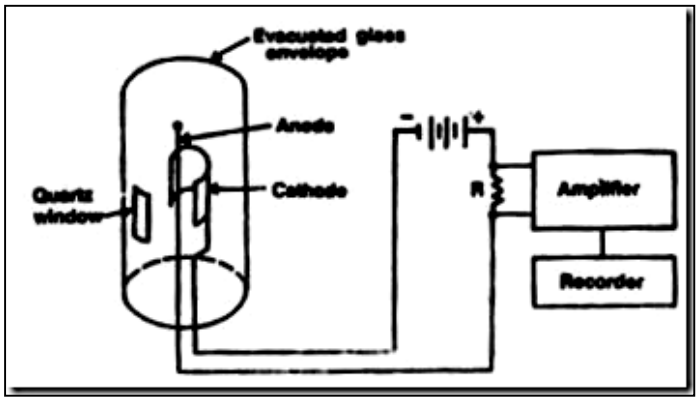


Fig. Photo Emissive Tube

Phototube currents are quite small and require amplification. This is usually accomplished by placing a high resistance (R in the Figure) in the phototube circuit.

Photo multiplier Tube

These detectors are designed to amplify the initial photo electric effect and are suitable for use at very low light intensities. A photomultiplier consists of:

- i) An evacuated glass tube into which is sealed the cathode and the anode
- ii) Additional intervening electrodes known as dynodes.

- The arrangement is shown in Fig. The external circuitry is arranged so that a high voltage (1000 volts) exists between anode and the cathode. As the radiation strikes lie photocathode, electrons are liberated the applied potential difference accelerates the electrons towards the first dynode.

- Each successive dynode is at a higher electric potential and thus acts, as an amplification stage for the original photon. The applied voltage causes sufficient electron acceleration to knock out other electrons from each dynode surface. The liberated electrons are dragged onto the next dynode where electrons are released and this process goes on as cascade till the last dynode.

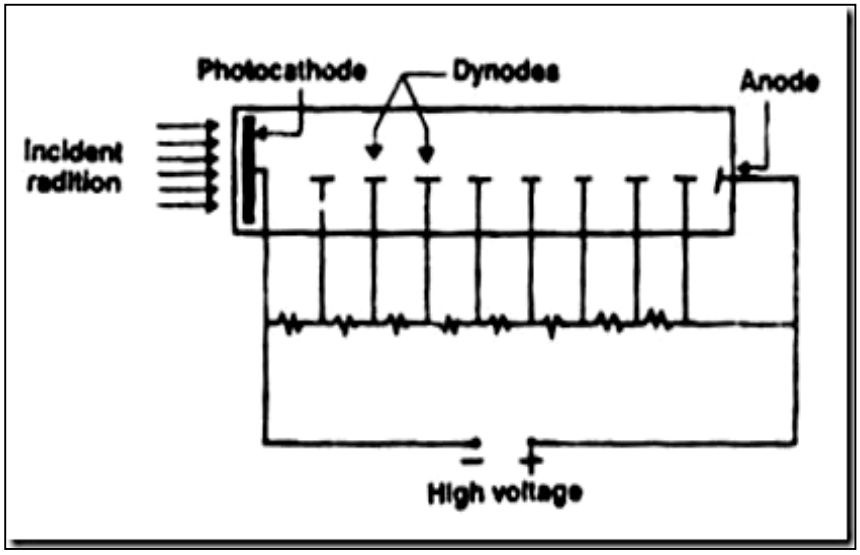
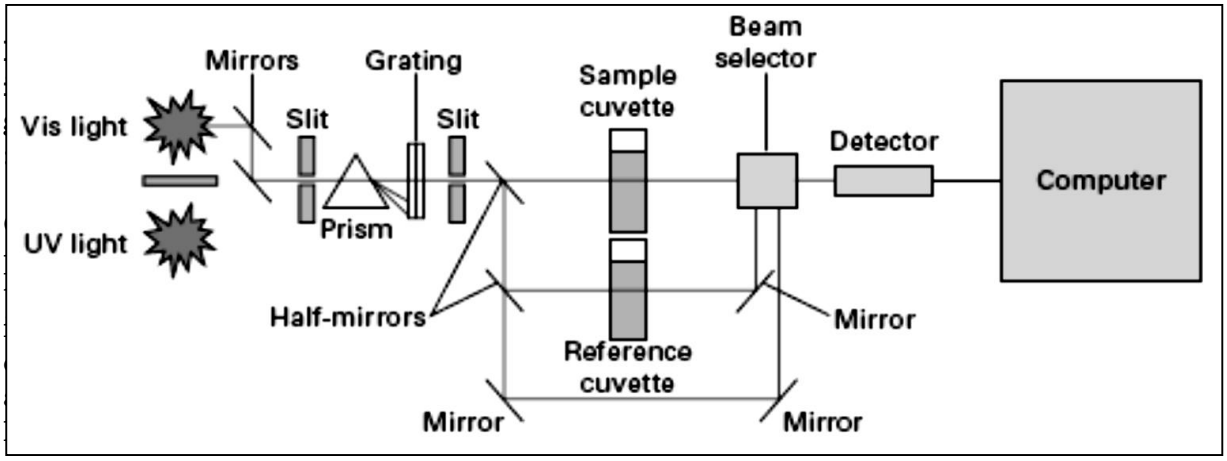


Fig: 7.9: A Photo Multiplier tube

By the time the electrons arrive at the collecting anode, the initial photoelectric current is amplified by a factor of approximately 10^6. In practice, photomultiplier tubes are used only for low light intensities. At higher light intensities, due to their great amplification power, photomultipliers exhibit great instability. In spite of this tendency to be unstable, photomultipliers are the detectors of choice in all modem spectrophotometers.

Double Beam Spectrophotometer:



Double beam spectrophotometer are arrangements in a dual-beam spectrophotometer. Either a prism or a grating constitutes the monochromatic of the instrument. Optical paths are shown as green lines.

Double beam spectrophotometers use a beam chopper to separate the reference beam from the sample beam. These beams pass through sample and reference solutions and fall on two detectors separately. The final read is in absorbance or transmittance, obtained after electronic manipulation of the above two detectors.

Advantages of double beam spectrophotometers

- ♣ speed of operation
- ♣ automatic compensation for variation in lamp output
- ♣ solvent absorption at various 8
- ♣ changes in detector sensitivity
- ♣ spectra scan

Disadvantages of double beam spectrophotometers

- ♣ cost is higher

Spectra scan

Spectra scans have two coordinated: the X axis is normally wavelength, and the Y axis is either absorbance or transmittance. The resulting graph is called a spectra scan. A spectra scan is similar to a fingerprint of a compound.

One Word Question

Sr. No	Question	Answer
1	What is the function of Monochromator	Polychromatic light to monochromatic light
2	How many types of Monochromator	Prism & Greetings
3	UV Spectrophotometer contain _____Cuvette?	Quartz
4	Visible Spectrophotometry contain _____Cuvette?	Glass
5	How Many types of photon energy detection device	3 types
6	Which one id best photon energy detection device	Photo multiplayer tube
7	Double beam spectrophotometer contain which type of Monochromator	Greetings
8	Advantage of Double beam spectrophotometer	speed of operation

Applications of Colorimeter and Spectrophotometers:

UV-Visible spectrophotometry has diverse applications in qualitative and quantitative analysis in biochemistry. Only a few important applications are summarized below:

- ♣ **Qualitative analysis:** Visible and ultraviolet spectra may be used to identify classes of compounds in both pure state and in biological preparations. This is based on the fact that absorption spectra are specific for a class of compounds.
- ♣ **Quantitative analysis:** Some compounds have specific max and have higher extinction coefficient can be estimated quantitatively by absorbance measurements. Many substances do not possess significant extinction coefficient in the visible g\region will react quantitatively with some other reagents to give a colour product. This property is used to estimate such substances. Colorimetry is widely used in biochemistry to assay a wide range of biologically important substances like: inorganic phosphorus, calcium, amino acids, proteins, carbohydrates, nucleic acids, lipids, cholesterol, sterols and steroid hormones, vitamins, drugs etc.
- ♣ **Denaturation of double stranded DNA:**

DNA absorbs at 260 nm. This absorbance increases with rise in temperature, as the double stranded DNA becomes single stranded. This is known as hyper chromicity. The reverse occurs on the renaturation of DNA upon cooling. This can give information about the melting temperature Tm of DNA and also give idea about A=T, G≡C content of DNA.

- **Solvent perturbation of nucleic acids:**
  Spectral characteristics of nucleotides are different in a solvent which is 50% D20 as compared to a solvent 100% H2O. This change in spectral characteristics does not occur in nucleotides that are base paired. This difference allows us to determine how many nucleotides in nucleic acid are base paired. Consequently this assay is useful in determining structure of t-RNAs.
  Protein folding or protein-protein or protein-nucleic acid interaction can be studied by using UV- spectroscopy.
- **Enzyme assay and kinetic studies:**
  The quantitative assay of enzyme activity is carried out when substrate or product is colored or absorbs light in UV range because the rate of appearance or disappearance of light absorbing product or substrate can be followed spectrophotometrically.
  e.g. **Lactate + NAD^+ ↔ Pyruvate + NADH + H^+**
  NADH strongly absorbs at 340 nm while NAD does not.
- **Molecular Weight determination:**
  The molecular weights of amines, picrates, sugars and many aldehyde and ketone compounds can be determined by using the absorbance data with accuracy of ±2%.
- **Study of Cis-Trans isomerism:**
  Tran isomer is usually more elongated than its cis counterpart and has higher absorption maxima. Absorption spectroscopy can be thus utilized to study the cis-trans isomerism.
- **Testing or Purity of Compounds:**
  It can be done based on their specific extinction coefficients.
- **Control of Purity:**
  Impurities in a compound can be detected easily by spectrophometric studies. E.g. carbon disulfide impurity in the carbon tetrachloride can be detected easily by measuring absorbance at 318 nm where only the carbon disulfide absorbs. Similarly benzene impurities in commercial absolute alcohol can be detected by measuring absorbance at 280 nm where alcohol (λmax = 210 nm) does not absorbs.
- **Difference Spectroscopy:**
  Difference spectroscopy provides a sensitive method for detecting small changes in the environment of chromophore. It may also be used to demonstrate ionization of the chromophore leading to identification and quantitation of various components in a mixture. Difference spectroscopy involves comparison of absorption spectra of two samples, which differ slightly in their physical states. Difference spectroscopy can be used for quantitative estimation of contents of different cytochromes in mitochondria; analysis of toxic drugs in toxicological laboratory e.g. barbiturates and studies on conformation of globular proteins
- **Turbidometry and nephlometry:**
  Measurement of the intensity of the transmitted light will give an idea about number of particles in the suspension. Using this turbid metric technique, one can get approximate number of particles in suspension. This technique is routinely used to

measure the number of bacteria in a given suspension. The wavelength used for this purpose is 600 nm.

One word question

Sr. No	Question	Answer
1	UV-Visible spectrophotometry has diverse applications on	Qualitative & Quantitative
2	Denaturation of double stranded DNA absorbed at _____nm?	260 nm
3	Molecular Weight determination carried out on the basis of	Adsorption Spectra
4	Absolute alcohol can be detected by measuring absorbance at	280 nm
5	This technique is routinely used to measure the number of bacteria in a given suspension at _____nm?	600 nm