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Subject: Biochemistry

Production of Courseware
e-Content for Post Graduate Courses
Paper : P-12 Biochemical Techniques
Module : UV -Visible Spectroscopy



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| Description of Module | |
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| Subject Name | Biochemistry |
| Paper Name | 12 Biochemical Techniques |
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1. Objectives

- 1.1 To understand laws involved in light absorption.
- 1.2 To explain instrumentation designs available in UV-visible spectroscopy.
- 1.3 What are the applications of UV-visible spectroscopy?

2.0 Electromagnetic radiation (EMR):

Electromagnetic radiation or waves is composed of an electric vector and magnetic vector which oscillates in phase and 90 degrees (right angles) to each other and both the vectors are also right angles to the direction of propagation of wave. EMR radiates out from a source in all the directions and includes radiowaves, microwaves, infrared, visible light (400-700 nm), ultraviolet (200-400 nm), X-rays and Gamma rays depending on the source. Electromagnetic spectrum consists of radiations of different wavelength, each possessing different energy, frequency and intensity. High energy radiations are characterized by short wavelength and high frequency. Low energy radiations are characterized by long wavelength and low frequencies.

The interaction of electromagnetic radiation with a particular matter involves not only the properties of the radiation but also involves the structural characteristics of the matter. The electrons in a molecule are distributed at different energy levels, but mainly present in the lowest energy state (E_1) until promoted to the excited state (E_2). The difference in the energy, $\Delta E = E_2 - E_1$, in terms of quantum is required by the electron to reach the higher energy state (excited state). If this energy is derived from the EMR, it gives rise to the **absorption spectrum** (range of wavelengths which are absorbed by the electrons of the molecule to reach the excited state). Additionally, the source of radiation is also due to the energy transitions in the matter itself. This occurs when the electrons falls back from higher energy excited state to lower energy ground state. The range of wavelength released gives rise to **emission spectrum**. Therefore, absorption and emission spectrum is characteristic of the wavelength of EMR and the material involved.

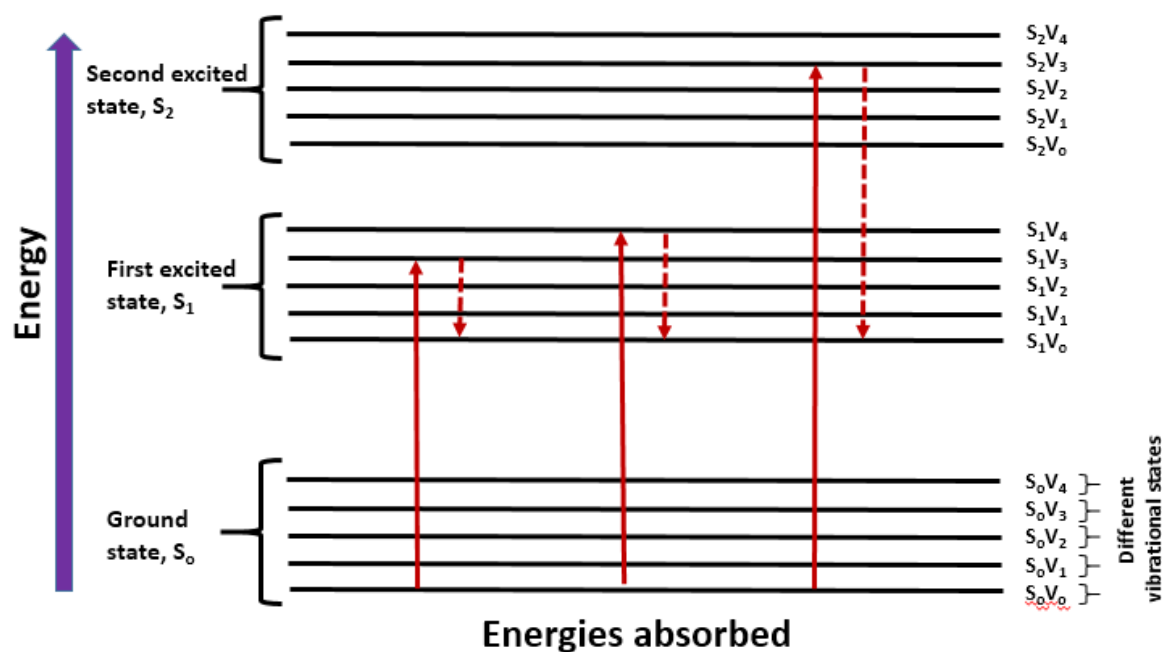
During the absorption and emission which involves single electron transition, one quantum of the energy is absorbed or emitted respectively and is equivalent to ΔE .

$$\Delta E = hv$$

where,

h = Planck constant (6.63×10^{-34} Js)

ν = frequency of the electromagnetic radiation (number of oscillations made by the wave per second)



Energy levels and transitions of electrons

2.0 Principle Ultraviolet-visible (UV/Visible) spectroscopy-

It is based on the principle of absorbance and works in the visible and UV region of the electromagnetic spectrum. This region of the electromagnetic spectrum when absorbed by the molecule it affects the electronic transition of the molecule and therefore, transition occurs from ground state to the excited state. The law which governs light absorption is referred as Lambert-Beer Law. The law is combination of two different laws and these are described here.

2.1 Lambert's law:

When a ray of monochromatic light of initial intensity (I_0) passes through a solution in a transparent vessel, the intensity of the transmitted light (I) is inversely proportional to

the path length (l) of the absorbing medium. **OR** When a ray of monochromatic light passes through an absorbing medium, its intensity decreases exponentially as the length of the absorbing medium increases. **OR** The absorbance of a material sample is directly proportional to its thickness (path length).

$$I = I_0 e^{-k_1 l} \dots\dots (1)$$

In 1852, August Beer discovered another relation

2.2 Beer's law:

When a ray of monochromatic light of initial intensity (I_0) passes through a solution in a transparent vessel, the intensity of the transmitted light (I) is inversely proportional to the concentration absorbing solution. **OR** When a ray of monochromatic light passes through an absorbing solution, its intensity decreases exponentially as the concentration of the absorbing solution increases. **OR** The absorbance of a material sample is directly proportional to concentration.

$$I = I_0 e^{-k_2 c} \dots\dots (2)$$

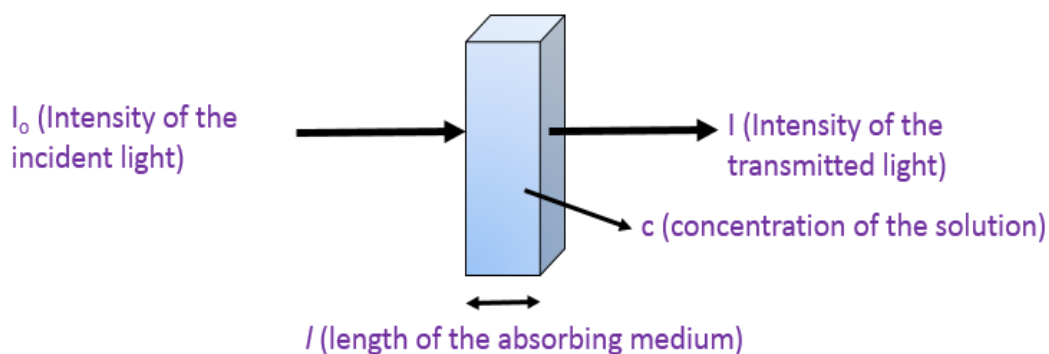
2.3 Lambert-Beer Law

When a ray of monochromatic light of initial intensity I_0 passes through a solution in a transparent vessel, some of the light is absorbed so that the intensity of the transmitted light I is less than I_0 and is inversely proportional to the path length (l) of absorbing medium and concentration (c) of absorbing solution.

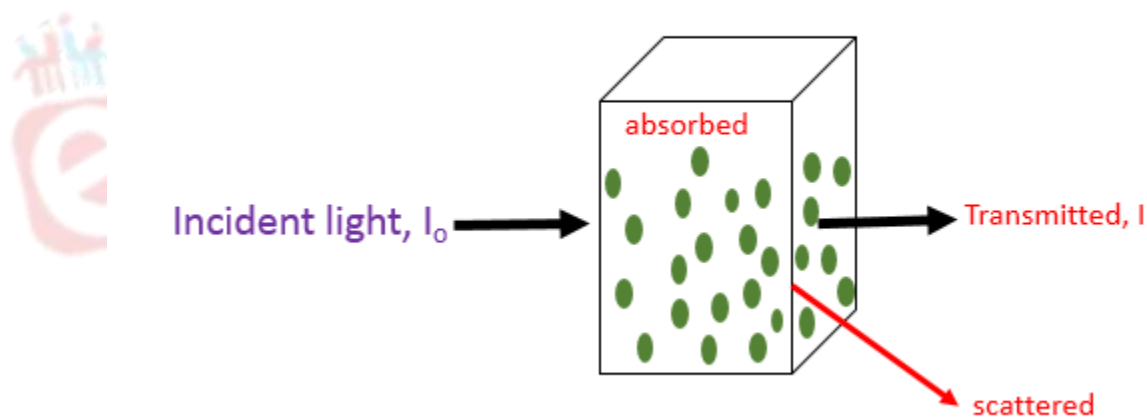
The relationship between I and I_0 depends on the path length of the absorbing medium, l and the concentration of the absorbing solution, c . These factors are related in the Beer and Lambert Law.

On combining (1) and (2)

$$I = I_0 e^{-k_3 c l} \dots\dots (3)$$



Intensity of the transmitted light (I) is always less than the intensity of the incident light (I_0). Decrease in the intensity of transmitted light is mainly because of the absorption of light by the analyte present in the solution. There is some loss of light intensity from scattering by particles present in the solution or by reflection at the interfaces.



Transmittance is the ratio of intensity of the transmitted light (I) and intensity of the incident light (I_0).

$$\text{Transmittance (T)} = I/I_0$$

100% T represents a solution which does not absorb anything and 100% photons reaches the detector.

60% T represents a solution which absorbs 40% of the photons and 60% of the photons reaches the detector.

0% T represents a solution which does not transmits but absorbs all the photons (occurs in case of opaque solutions)

Absorbance (A) is the reciprocal of transmittance = $(1/T) = I_0/I$.

A = 0, represents no photons are absorbed by the solution (no analyte is present).

A = 1, represents 90% of the photons are absorbed by the analyte present in the solution and only 10% of the photons reaches the detector.

A = 2, represents 99% of the photons are absorbed by the analyte present in the solution and only 1% of the photons reaches the detector.

$$I = I_0 e^{-k_1 l} \text{ (Lambert Law) (1)}$$

$$I = I_0 e^{-k_2 c} \text{ (Beer Law) (2)}$$

$$I = I_0 e^{-k_3 c l} \text{ (Lambert-Beer Law) (3)}$$

$$I / I_0 = e^{-k_3 c l}$$

$$I_0 / I = 1 / e^{-k_3 c l}$$

$$\log (I_0 / I) = \log (1 / e^{-k_3 c l})$$

$$A = (k_3 c l) / 2.303$$

$$A = \epsilon_{\lambda} c l$$

Absorbance is unitless

where,

A = absorbance of the solution

ϵ_{λ} = extinction coefficient (ϵ_{λ} varies with the wavelength of the light)

l = pathlength of the cuvette through which the light travels

c = concentration of the solution

Molar Extinction (Absorbance) Coefficient/Molar absorptivity, $\epsilon_\lambda = A$, when concentration of the solution is 1 mol/L and pathlength of the cuvette is 1cm. Unit of molar extinction coefficient is **L mol⁻¹ cm⁻¹ or M⁻¹ cm⁻¹**. In SI, unit of ϵ_λ is **mol⁻¹ m²**.

3.0 INSTRUMENTATION OF UV-VISIBLE SPECTROPHOTOMETRY

Spectrophotometer covers the range of wavelengths in the both UV and Visible region. Materials used in the spectrophotometer depends upon the wavelength used.

3.1 Light Source:

Tungsten filament lamps are used in the visible region and hydrogen and deuterium lamps are used in the UV range. In UV-Visible spectrophotometer both the lamps are switched on while working, and it depends on the user which lamp to be used. For this, a mechanical switch is available for directing the light.

3.2 Monochromator:

Monochromatic light refers to the light of single color or single wavelength or narrow band of wavelengths. Wavelength is selected by the monochromator (prisms and gratings). Monochromator is an optical instrument which selects the light of single or narrow range wavelengths (bandwidth) from wide range of wavelengths emitted from light source. Prism separates the beam of light into its components by the phenomenon of refraction or dispersion whereas gratings do this by the phenomenon of diffraction. The resolution is higher from gratings than from prisms. It is also to be noted that prisms have higher dispersion in UV range, therefore are best suited to work in UV region. Monochromators are also equipped with collimators which converts the diverging light to the collimated light (parallel rays of light).

3.3 Slits:

Optical slit width affects the bandwidth. Light from a source is emitted in all directions. Slit allows only a portion of the light to reach the monochromator. Selected light is again allowed to pass through the slit. The reproducibility of the absorbance values increases

as the slit width decreases. With the decrease in slit width, sensitivity also decreases as less radiation passes to the detector.

3.4 Cuvettes:

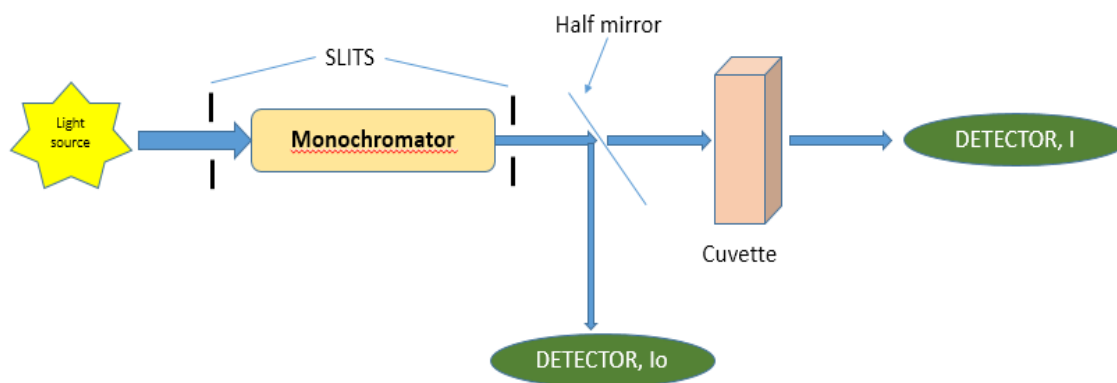
Samples for the detection should be placed in a transparent cuvette, mostly rectangular in shape and commonly have internal width, 1 cm. Small test tubes are also used in some instruments. Cuvettes are made of silica for the detection in the visible range. Borosilicate glass is used in visible region as well as in near UV region. Fused silica and quartz cuvette are required for the UV range but also covers the visible region.

3.5 Chromophores:

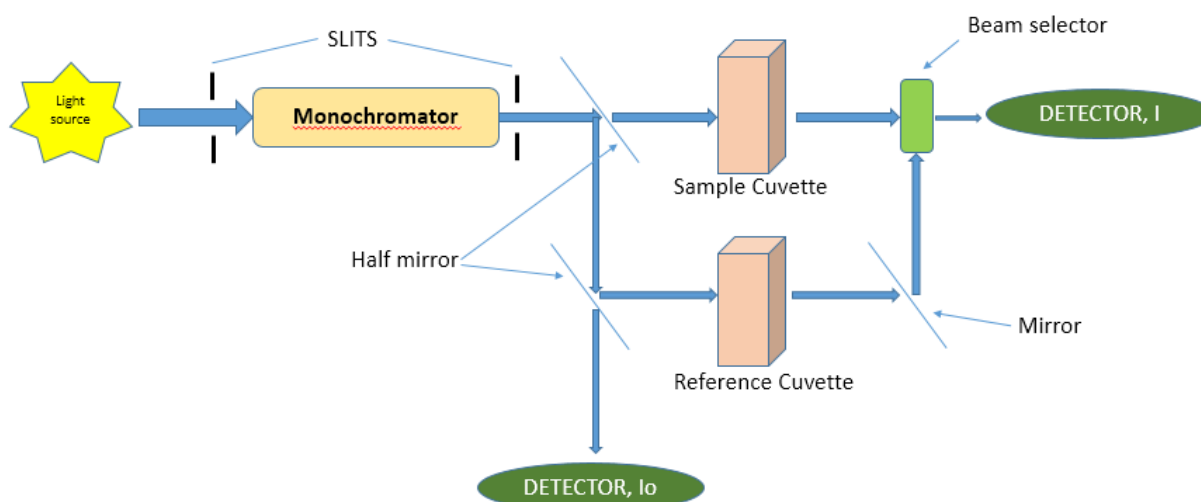
It is the part/moiety of molecule which is responsible for imparting absorbance. This phenomenon occurs when a molecule absorbs certain wavelength in the visible range and promoted to the excited state. Biological molecules also cause conformation change when exposed to light. Examples include molecules or compounds that have unpaired electrons or unsaturated bonds and can absorb the radiation such as alkenes, ketones, aldehydes, phenyl and other aromatic species. Organic compounds absorb in the UV or visible region. For the determination of organic compounds in the UV range, organic solvents are not suitable as these solvents absorb in the UV region. Therefore, ethanol is best suited as a solvent for organic soluble compounds and water for water soluble compounds.

3.6 Single-beam and double beam spectrophotometer

A spectrophotometer may be single beam or double beam. In **single-beam spectrophotometer**, beam of light passes through the cuvette present in the sample cell. Therefore, sample cell is first used to zero the instrument by using blank or reference solution in the cuvette and later by placing the sample cuvette in the same place and the absorbance is recorded. This is to minus the blank absorbance readings from the sample absorbance readings. In double-beam spectrophotometer, incident light is split into two beams by half mirror, one beam passes through the reference cuvette and the other beam passes through the sample cuvette. The transmitted light from both the cuvettes then reaches the detectors.



SINGLE-BEAM SPECTROPHOTOMETER



DOUBLE-BEAM SPECTROPHOTOMETER

4.0 Applications Of UV-Visible Spectroscopy

4.1 Qualitative analysis:

The technique is used for the identification of molecules/ compounds in its pure state as well as in biological mixtures. Examples include proteins, nucleic acids, chlorophylls,

cytochromes. This technique also gives the structural details of proteins and the intermediates arising in a biological system. A plot of absorbance or %T against wavelength can be obtained however getting a plot of absorbance against wavelength is a common practice. It is also used in the analysis of transition metal ions as they absorb in the visible range because of which the d electrons are excited to the higher energy state and gives color to the metal ion solutions.

4.2 Quantitative analysis:

With the help of standard curve, quantity of unknown molecule in a sample can be determined. Many colorimetric assays are used in the visible range for the easy detection of the molecule. A molecule which is to be detected may be colored or may be chemically modified to produce colored product.

4.3 Purity of macromolecules:

Proteins and nucleic acids are also measured in UV range. Proteins are measured at 280nm and nucleic acids are measured at 260 nm. For pure DNA, $A_{260/280}$ is ~1.8. If the ratio is more than 1.8, it indicates the RNA contamination and if the ratio is less than 1.8, it indicates the protein contamination. For pure RNA, $A_{260/280}$ is ~2. For pure protein, $A_{280/260}$ is ~2.

4.4 Determination of concentration of analyte from extinction coefficient:

If the value of the molar extinction coefficient (ϵ) is known, it is possible to calculate concentrations directly from absorbance readings at specific wavelength. For pure proteins of known coefficient, the concentration (mg/ml) can be calculated by dividing the absorbance at 280 nm by the extinction coefficient. The concentration (mg/ml) of the proteins with possible nucleic acid contamination can be calculated by the following formula $(1.55 \times A_{280}) - (0.76 \times A_{260})$

Although proteins and nucleic acids can be measured in UV range, there is an additional requirement of corrections for interfering substances.

| At a wavelength of 260 nm | EXTINCTION COEFFICIENT ($\mu\text{g/ml}$) ⁻¹ cm ⁻¹ | ABSORBANCE CORRESPONDS TO CONCENTRATION |
|---------------------------|---|---|
| double-stranded DNA | 0.020 ($\mu\text{g/ml}$) ⁻¹ cm ⁻¹ | 50 $\mu\text{g/ml}$ |
| single-stranded DNA | 0.027 ($\mu\text{g/ml}$) ⁻¹ cm ⁻¹ | 37 $\mu\text{g/ml}$ |
| single-stranded RNA | 0.025 ($\mu\text{g/ml}$) ⁻¹ cm ⁻¹ | 40 $\mu\text{g/ml}$ |
| Protein | Varies and depends on the ϵ of a particular protein ($c=A/\epsilon l$) | |

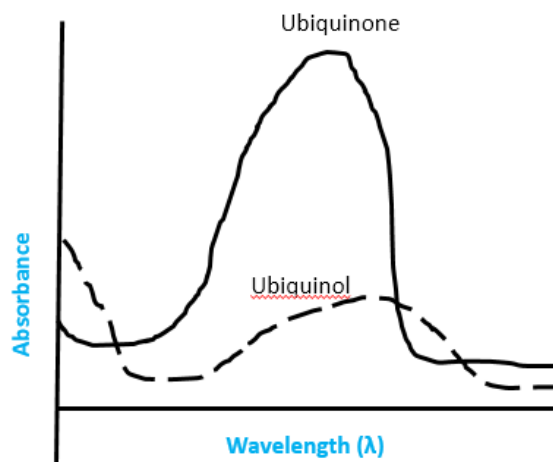
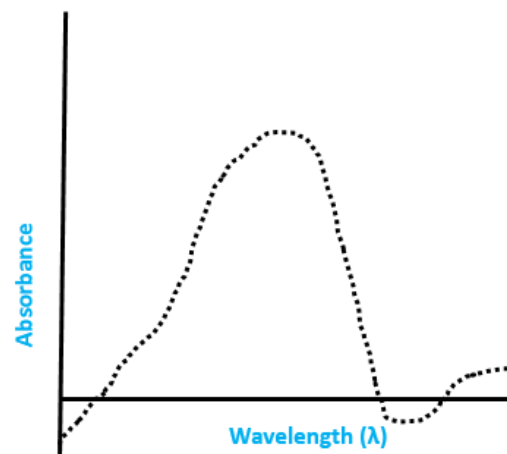
4.5 Difference spectra:

A difference spectrum is the difference of two absorption spectra. There are two methods of obtaining the difference spectra:

- By subtraction of one absolute spectrum from another (reduced - oxidized)
- By placing one test compound in the reference cell (containing the compound in reduced state) and the other in the test cuvette (containing the same compound in oxidized state).

Difference spectra may have negative values. The absorption maxima and minima are different from the individual spectra of reduced and oxidized compound. Isobestic points are also wavelengths where absorbance under two different conditions (eg. reduced and oxidized state of analyte) are same. Presence of interfering substance can be checked at isobestic points.

An example: Ubiquinone (oxidized) and ubiquinol (reduced). Difference spectra is obtained by subtracting the absorbance values of ubiquinone from ubiquinol.


 Absorption spectrum of ubiquinol and ubiquinone

 Difference spectrum of Ubiquinol and ubiquinone

The main advantage of difference spectra is that it enables the detection of small changes in absorbance occurring in a biological system with a high background absorbance. Examples of such system are the mitochondria and chloroplast where it is possible to detect the changes in the oxidation states of its respiratory chains components.

4.6 Binding spectra:

Binding spectra is also known as substrate binding spectra. As the name suggests, this is used to study the level of interaction between an enzyme and its specific substrate.

Binding of a substrate or drug to enzymes can be detected by the changes in the absorbance or changes in the λ_{max} . Increase in absorbance is known as hyperchromic shift and decrease in absorbance is known as hypochromic shift. An example of hyperchromic shift is noted when DNA is subjected to heat or placed under alkaline conditions, it causes disruption of the hydrogen bonds of each DNA duplex to yield single-stranded structures and thereby enhances UV light absorption. With the renaturation, the absorbance will decrease and causes hypochromic shift.

Binding of substrate also cause shifts in the λ_{max} . Increase in λ_{max} is known as bathochromic shift. Conjugation of double bonds lowers the energy required for the

electronic transition and therefore chromophore absorbs at longer wavelength. Red color in the visible spectrum has a longer wavelength than most other colors and thus this effect is also commonly called a red shift. Decrease in λ_{max} is hypsochromic shift (blue shift). Decrease in conjugation increases the demand for higher energy wavelength to be absorbed by the chromophore and this causes decrease in λ_{max} .

