

Biophysical and Biochemical Techniques

Spectroscopic Techniques

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Introduction to Spectroscopy

Molecular spectroscopy is the study of the interaction of electromagnetic radiation with matter. Different regions of the electromagnetic spectrum provide different kinds of information as a result of such interactions. Electromagnetic radiation has two wave motions, magnetic (M) and electrical (E), which are at right angle to each other. Electromagnetic waves are generated by oscillating electric or magnetic dipoles and are propagated through a vacuum at a velocity of light (c). The electromagnetic spectrum covers a wide range of wavelengths and photon energies which is seen in the figure below:

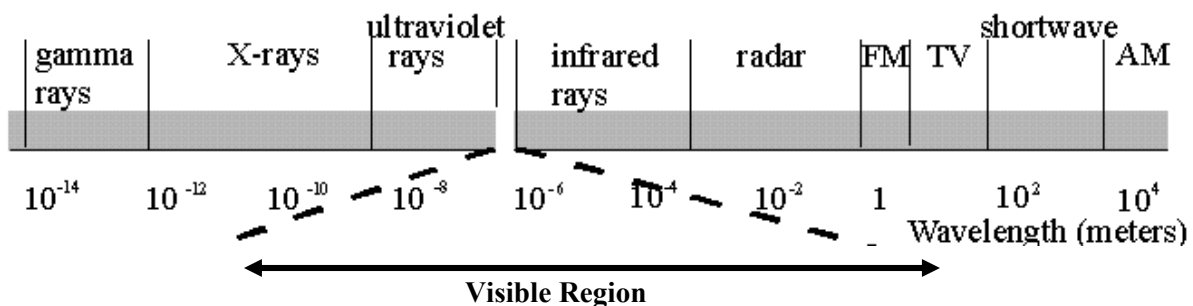


Fig. 1: Different regions of electromagnetic radiation

The energy (E) of the electromagnetic wave is given by:-

$$E = hc / \lambda = h\nu \dots(1)$$

where h is Planck's constant, c is the velocity of light, λ is the wavelength and ν is the frequency. When electromagnetic radiation encounters molecules, it can be either scattered (i.e. its direction of propagation changes) or absorbed (i.e. its energy is transferred to molecule). The relative probability of the occurrence of each process is the property of a particular molecule. If the electromagnetic energy of light is absorbed, the molecule is said to be excited or in an excited state. An excited molecule can possess any one discrete amount (quantum) of energy called 'energy levels' of the molecule. The major energy levels are determined by the possible spatial distributions of the electrons called electronic energy levels, on these are superimposed vibrational levels (ν_1 , ν_2 , etc) that indicate the various modes of vibration of molecules which have further smaller energy levels called rotational levels (r_1 , r_2 , r_3 etc) (Fig 2).

Ultraviolet-Visible Spectroscopy

The UV-VIS range of the EMR consists of radiations with a wavelength range of 200 to 800 nm. Absorption of this relatively high-energy light causes "electronic excitation". This region shows absorption only if conjugated pi-electron systems are present in the molecule. When ultraviolet /visible light is passed through a sample in solution, some light energy may be absorbed by the molecules. Molecules (part of molecule) capable of absorbing light are called "Chromophore". Light energy is used to promote electrons from the ground state to various excited states. Each chemical structure absorbs different frequencies of light since each has a characteristic electronic structure. The ground and excited electronic levels differ from each other by smaller energy increments, ΔE .

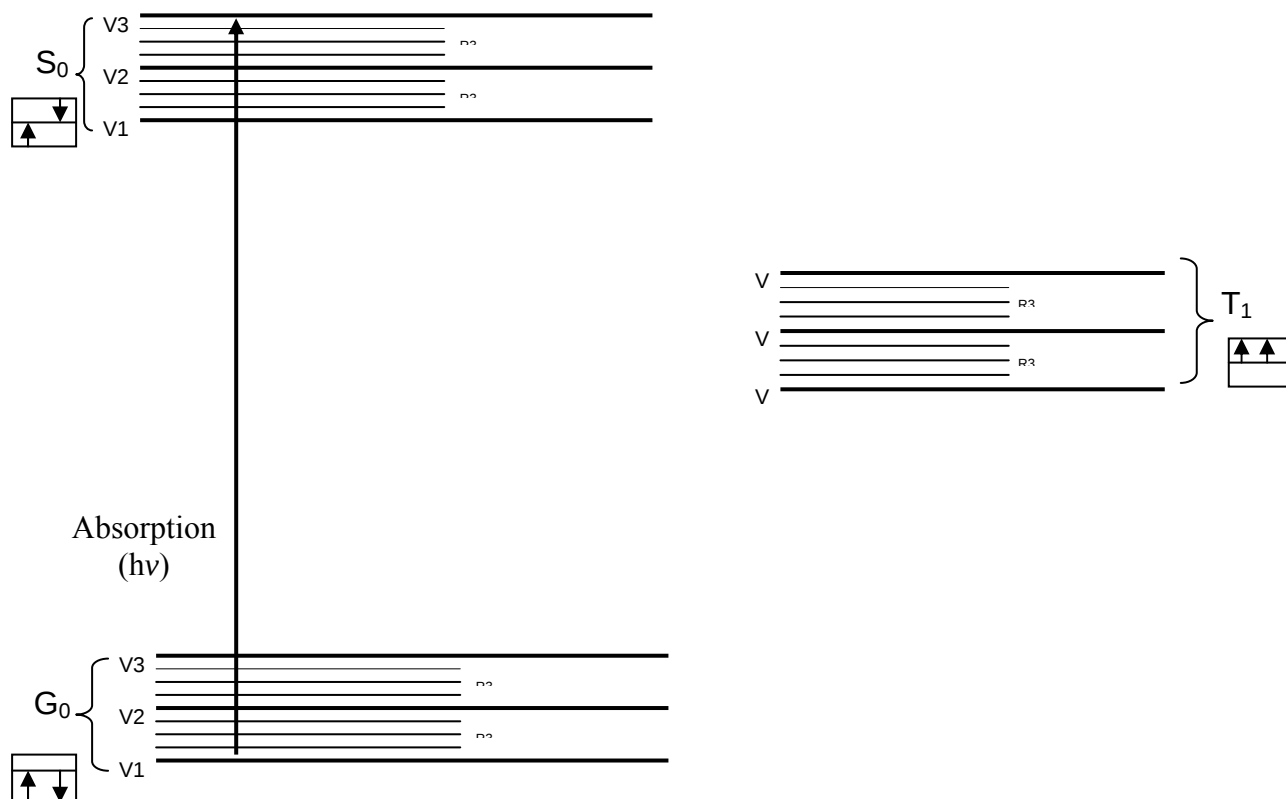


Fig. 2: Energy levels of a molecule showing various rotational, vibrational and electronic states

Excited molecule can return to the ground state by various ways which will be discussed under “fluorescence”. The absorption of UV or visible radiation by a molecule corresponds to the excitation of outer electrons and are two types of electronic transitions: i) transitions involving π , σ , and n electrons, and ii) transitions involving charge-transfer electrons. The absorption spectroscopy of organic compounds is based on transitions of n or π electrons to the π^* excited state. This is because the absorption peaks for these transitions fall in an UV-VIS region of the spectrum (200 - 700 nm).

These transitions have unsaturated groups in the molecules to provide the π electrons. The solvent in which the absorbing species is dissolved also has an effect on the spectrum of the species. Peaks resulting from n , π , π^* transitions are shifted to shorter wavelengths (*blue shift*) with increasing solvent polarity. This arises from increased solvation of the lone pair, which lowers the energy of the n orbital. Often (but *not* always), the reverse (i.e. *red shift*) is seen for n , π , π^* transitions. This is caused by attractive polarization forces between the solvent and the absorber, which lowers the energy levels of both the excited and unexcited states. This effect is greater for the excited state, and so the energy difference between the excited and unexcited states is slightly reduced, resulting in a small red shift. This effect also influences n , π , π^* transitions but is overshadowed by the blue shift resulting from solvation of lone pairs.

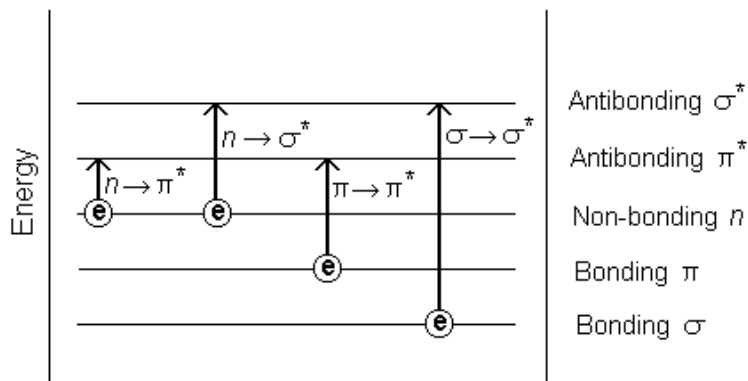


Fig. 3: Possible electronic transitions of π , σ , and n electrons

Beer - Lambert Law

This is also known as 1st law of photochemistry. If an incident light with intensity I_0 falls on a molecule, it may absorb (A) a part of it and rest of it is transmitted. The intensity of transmitted light (T) is given by

$$T = I / I_0 \dots\dots(2)$$

So, if all the light passes through a solution without any absorption, then absorbance is zero, and percent transmittance is 100. If all the light is completely absorbed, then percent transmittance is zero, and absorption is infinite. In other words the intensity of light falls off exponentially as it passes through the absorbing sample. Lambert's law assumes that the medium (solution) is divided in a number of thin layers each with a thickness of dl and absorption is proportional to the dl . Therefore, the absorption of light is proportional to the pathlength of the absorbing medium which corresponds to the amount of absorber in that thin layer, dl . However, Beer's law states that the amount of radiation absorbed by the chromophore is directly proportional to its concentration, $[C]$. The two laws put together give

$$\text{Absorbance (A)} \propto dl \times [C] \dots\dots(3)$$

If intensity of the incident light is I_0 , one can derive

$$\log (I_0/I) = \epsilon \times [C] \times l \quad \text{or} \quad A = \epsilon \times [C] \times l \dots\dots(4)$$

In equation 4), I_0 is intensity of incident light, I is intensity of transmitted light, $[C]$ is molar concentration and l is the pathlength (cm), ϵ is extinction coefficient and A is absorption and has no units. If a molecule absorbs a part of the radiation, I of the incident radiation, I_0 then T , the transmittance and absorbance are related by $A = \log (1/T)$.

Equation 4 represents Beer-Lambert law. It is important to note that Beer-Lambert law is applicable only for dilute solutions and therefore $[C]$ of the chromophore must be very low. The Beer - Lambert law is obeyed only if I_0 is monochromatic.

The extinction coefficient is a measure of the absorbing power of the compound and is clearly dependent at the wavelength of light. The extinction coefficient is expressed as ϵ_m if concentration is given as moles per liter and $\epsilon \%$ if the concentration is given as gm/100 ml. The absorption spectrum represents variation in ϵ as wavelength is changed. Spectra that contain

several absorption bands are characterized by the absorption maximum, λ_{max} , and the corresponding value of ϵ .

A typical single beam absorption spectrophotometer consists of a light source, (hydrogen or deuterium lamp which gives light UV range and a tungsten lamp or Xe arc lamp which gives radiation in the VIS range), a monochromator (consisting of an entrance slit, prism or grating and an exit slit) to give radiation of a single wavelength, a sample compartment, and a photomultiplier which converts the light signal into electric signal and finally an amplifier to amplify the signal (Fig 4a). The final signal is transferred to a recorder to get the data in the form of a spectrum. The quantitative data can also be obtained in the form of a table. The sample is taken in glass cuvette to study in the visible range and quartz cuvette is used both for the UV as well as VIS range.

The single beam spectrophotometer has some inherent problems associated with it. The light passed through the solvent (usually buffer) is assumed to have exactly the same intensity as that passed in solution containing the sample; however, in practice it is not true. Secondly, the measurement method is cumbersome involving two steps, first to run the buffer, and then remove the buffer to take solution in the same cuvette. More importantly, major advantage of the double beam spectrophotometer is that the voltage fluctuations that affects the flux of radiant energy falling on the photocell is cancelled out in double (cell) beam instrument, one containing reagent blank and the other sample. Therefore, a Split - beam Spectrophotometer was developed which simultaneously divides the light into two beams, one going to solvent compartment and the other to the sample compartment (Fig 4b). A matched pair of cuvettes is used to record the spectrum.

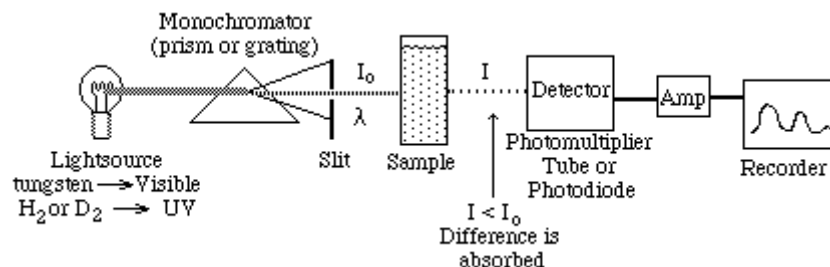


Fig. 4 a: Single Beam Spectrophotometer

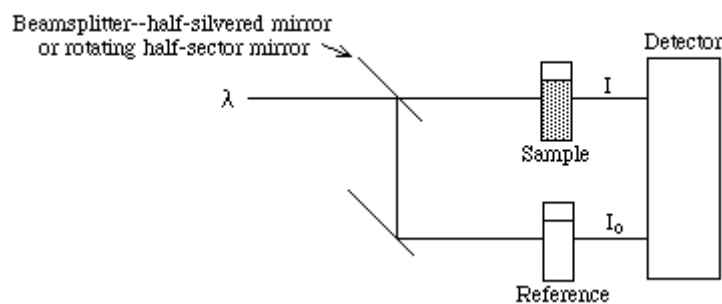


Fig. 4b: Double - Beam Spectrophotometer

Absorption Spectroscopy of Proteins and Nucleic Acids

(a) Proteins

Protein absorption mainly comes from two different regions, one is the backbone peptide bond and the other includes some of the aromatic side chains of tryptophan, tyrosine etc. The n to π^* transition is typically observed at 210-220 nm (ϵ max, 100) while main transition π to π^* occur at ≈ 190 nm (ϵ max, 7000). Side chains of a number of other amino acids like His, Arg, Glu, Gln, Asn and Asp have transition around 210 nm. However, these are not usually observed in proteins because they are swamped by the absorption from the more numerous and intense absorbing amide backbone groups which absorb at around 230nm. However, the absorption between 240nm and 290 nm consists of at least three (unresolved) electronic transitions. The side chain of the disulfide group of cysteine also shows a weak absorption band with a λ max of 250 nm (ϵ max = 300).

The most useful UV range for protein is at wavelengths greater than 230 nm where maximum absorption comes from the aromatic side chains of phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp).

Fig 5 shows the absorption spectrum of aromatic amino acid Phe, Tyr, Trp with λ max at 257, 274, and 280nm respectively. The absorbance of phenylalanine in this range is very low (at 257nm which arises from a π to π^* spin forbidden transition). Usually tyrosines and tryptophans together present in a protein have most intense absorbance than phenylalanine. UV absorption of a typical protein, BSA is also shown in Fig 5 the absorbance is used for determining protein concentration.

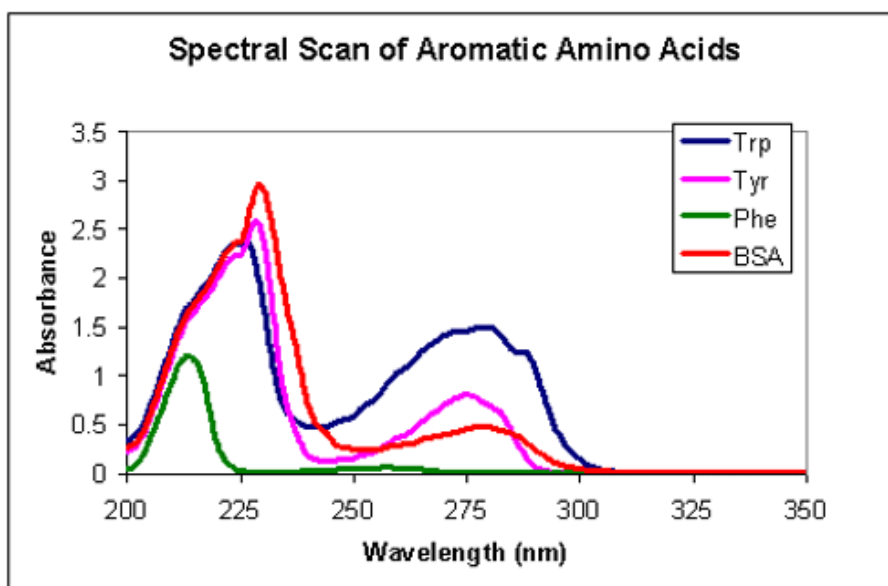


Fig. 5: Absorbance spectrum of amino acids tryptophan, tyrosine and Phenylalanine and a representative protein, BSA

Additional moieties like prosthetic groups, cofactors etc present in proteins also contribute to the absorption spectrum. Molecules with a large number of unsaturated groups that are all conjugated resulting in high degree of delocalization often show spectrum in visible region. Some examples for the non-protein compounds are porphyrin ring system of heme (550nm) in Cytochrome C, FMN (443nm) in flavodoxin and 4-pyridoxal phosphate (415nm) in threonine deaminase.

(b) Nucleic acids

The absorption spectrum of nucleic acids mainly is due to the purine and pyrimidine bases. These bases undergo $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ transitions. The UV spectrum of purine and pyrimidine bases occur between 200 and 300 nm for example, adenine, guanine, cytosine and thymine show λ_{max} at 260.5, 275, 267 and 264.5nm, respectively as shown in Fig 6a. The nucleic acids however show absorption peak around 260nm depending on the composition of bases. Native double helical DNA shows lower absorption as compared to the denatured single stranded DNA (Fig 6b). This is because in native DNA, due to base pairing ($A = T$, $G \equiv C$) the bases are not completely exposed to UV light, this phenomenon is called “hypochromicity”

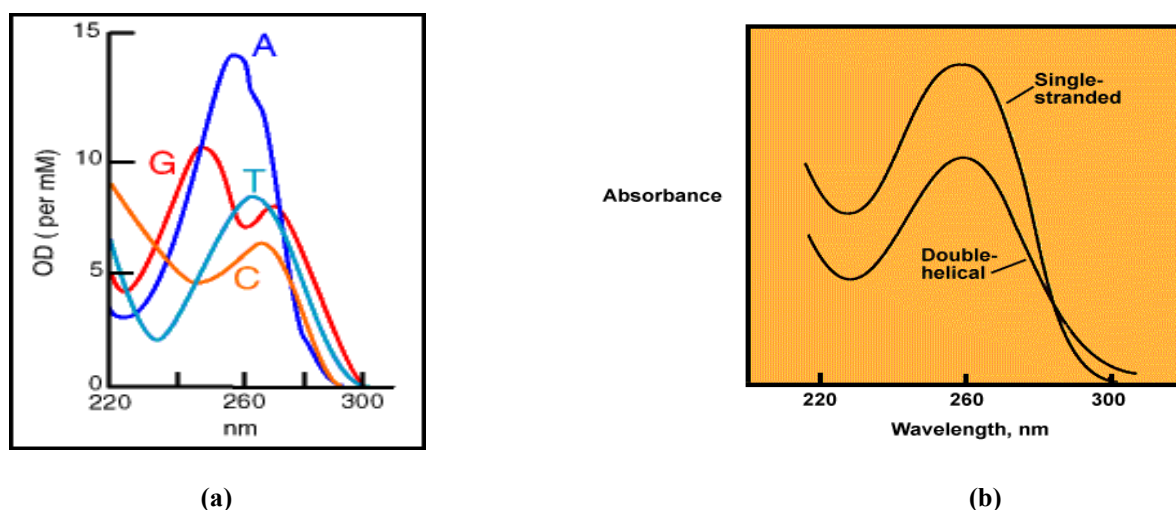


Fig. 6: Absorbance spectrum of (a) different DNA bases and (b) single and double stranded DNA Application to proteins and nucleic acids

UV-VIS absorption spectroscopy has found applications in analyzing chemical and structural aspects of various molecules.

1. Chemical analysis

(a) Concentration measurement

If we know the ϵ of the molecule and path length of the cuvette is also known, then we calculate concentration of molecule directly by measuring O.D. and using equation (4). This is routinely done in biochemistry and molecular biology for determining the concentration of proteins, DNA, RNA and small ligands like drugs etc.

(b) Enzyme Assay

Enzyme assays are also done using UV-VIS absorption spectroscopy by utilizing the fact that the ligand before and after the enzyme treatment has different absorption properties. For example, beta-lactamase cleaves the nitrocefin which is measured by change in absorbance (486 nm) of nitrocefin. Beta-glactosidase cleaves o-nitrophenyl galactoside (absorb at 420 nm) to form o-nitrobenzene. Similarly, NAD⁺ dependent dehydrogenase assay is done by measuring absorbance of formed NADH at 340 nm.

(c) DNA Purity

During nucleic acid extraction, the purity of nucleic acid is checked by measuring the ratio of absorbance at 260 and 280 nm (i.e. A_{260}/A_{280}). Absorbance ratio of 1.8 is said to be free from any protein contamination.

2. Structural analysis**(a) Helix–Coil Transition in DNA**

Double helical DNA when heated denatures leading to two single strands, this is studied by following O.D at 260nm. This denaturation causes increase in O.D at 260nm which is called “hyperchromicity” which is exploited to see the helix-coil transitions. The temperature at which 50% DNA is melted is termed as melting temperature (T_m).

(b) Estimation of pK of amino acids in Proteins

pK values for proton dissociation from ionizable amino side chains can be calculated because dissociation causes specific spectral changes. Tyrosine which absorbs at pH 7.0 shows absorption maximum of 280nm while at pH greater than 10, the absorption maximum shifts to 295nm.

(c) Conformational changes of Proteins and enzymes

Conformational changes of proteins brought by the change in pH, temperature or presence of denaturants like urea, guanidinium hydrochloride can be very conveniently studied using UV/VIS absorption spectroscopy. Also, binding of substrate to the active site of an enzyme produces spectral changes in chromophores in or near the active site by affecting the polarity of the region or the accessibility to solvent. For example the addition of various substrates to enzyme lysozyme produce a shift in λ_{max} for tryptophan to longer a wavelength. The magnitude of the change is expected from transfer of one tryptophan from polar to a non polar environment. This suggests that tryptophan is present in binding site of the enzyme.

Fluorescence Spectroscopy

Fluorescence spectroscopy is used to measure the emitted light by compounds called ‘fluorophores’. Light emission can also reveal some properties of biological molecules which may not be observed by UV-Vis absorption spectroscopy. Fluorescence occurs in certain molecules having delocalized π -electrons in their valence shells, there are generally poly-aromatic or heterocyclic hydrocarbons. The process of fluorescence is illustrated by the simple electronic-state diagram, Jablonski diagram, shown in Figure 7.

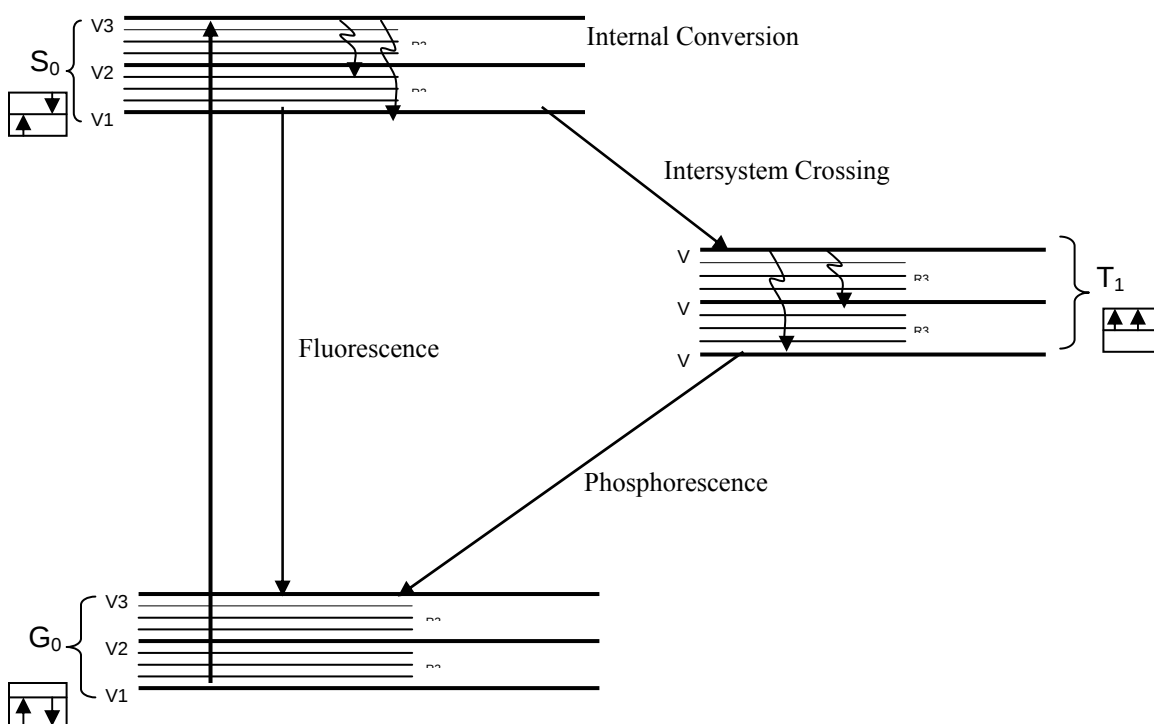


Fig. 7: Jablonski diagram showing excitation of ground state (G_0) singlet electrons to their next excited state (S_0) singlet state and coming back to their ground state (G_0) by radiative emission i.e. fluorescence. The excited electrons undergo ‘inter-system crossing’ from S_0 to T_1 and return from T_1 to S_0 by emitting phosphorescence

When a photon of energy $h\nu_{\text{ex}}$ is supplied by an external source such as an incandescent lamp or a laser and absorbed by the fluorophore, it excites the π -delocalized ground singlet state (G_0) electrons to next excited electronic singlet state (S_0). The molecule stays in the excited state for a finite time typically 1–10 nanoseconds and excited electrons emit fluorescence by coming to G_0 . Not all the molecules initially excited by absorption return to the ground state (G_0) by giving out energy in the form of emission. The processes such as collision, quenching, fluorescence resonance energy transfer (FRET), etc simultaneously depopulate S_0 and these competitive radiation-less ways by which electron loses its energy actually determine whether a chromophore can be a fluorophore. Due to energy dissipation by collision, energy transfer, etc during the excited-state lifetime, the energy of the emitted photon is very often lower than that it has absorbed ($h\nu_{\text{ex}}$). Therefore, the fluorescence energy is always lower than that of absorbed. In other words, if the molecule absorbs at $h\nu_{\text{ex}}$, its fluorescence will always be at higher wavelength (i.e. $\lambda_{\text{em}} > \lambda_{\text{ex}}$). The difference in energy or wavelength represented by $(h\nu_{\text{ex}} - h\nu_{\text{em}})$ is called the Stokes shift (Fig 8). The Stokes shift is fundamental to the sensitivity of fluorescence techniques because it allows emission photons to be detected against a low background, isolated from excitation photons.

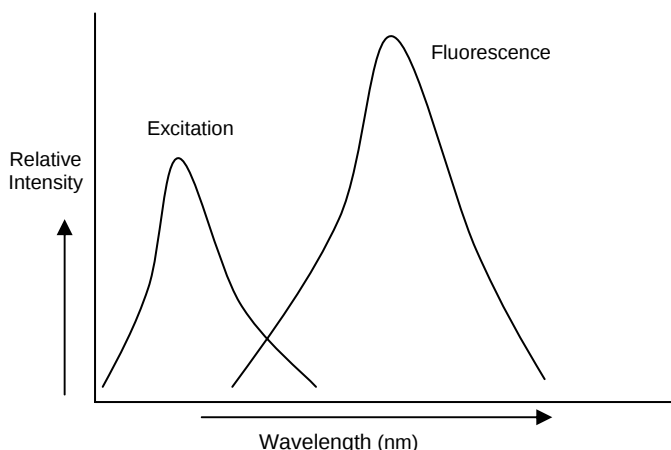


Fig. 8: The excitation and emission spectra of a fluorophore. The difference in energy of the two a wavelength maxima is called as ‘Stokes shift’

Phosphorescence

When electromagnetic radiation of appropriate energy is given to some heavy atoms (for e.g. molecular oxygen), possessing even numbers of electrons in their singlet ground state (G_0), their excited electrons change their spin by intersystem crossing and go to triplet state (T_1). When these electrons from T_1 come back to their native ground state (G_0), they emit energy as radiation which is called phosphorescence (Figure 7). Phosphorescence occurs in rigid media at low temperature and its life time is generally about 10^{-4} sec, much higher than fluorescence. Therefore, phosphorescence is also known as “delayed-fluorescence”. Though some of the energy is spent in changing the orientation of the excited electrons back to singlet state and in vibrational collisions, heat, etc, so the emitted phosphorescence also has lesser energy than the absorbed rays ($h\nu_{em} < h\nu_{ex}$).

Factors affecting fluorescence

Several factors like chemical structure, environment, solvent, temperature, presence of impurities, etc. influence the fluorescence of a compound.

a) Structural factors

Molecules/compounds that delocalize the π -electrons like $-\text{NH}_2$, $-\text{OH}$, $-\text{F}$, $-\text{OCH}_3$ etc. increase fluorescence because they tend to increase the transition probability between lowest excitation singlet and ground state. Electron withdrawing groups like $-\text{Cl}$, $-\text{Br}$, $-\text{I}$, $-\text{NO}_2$, etc. decrease or quench the fluorescence. Molecular rigidity decreases the possibility of non-radiative transitions by decreasing vibrations. This in turn decreases the intersystem crossing to triplet state and collision heat loss. Those molecules that are most planar, rigid and sterically uncrowded are usually most fluorescent because they capture energy. For example, fluorescein and eosin are strongly fluorescent while similar compound phenolphthalein is non fluorescent (Fig. 9).

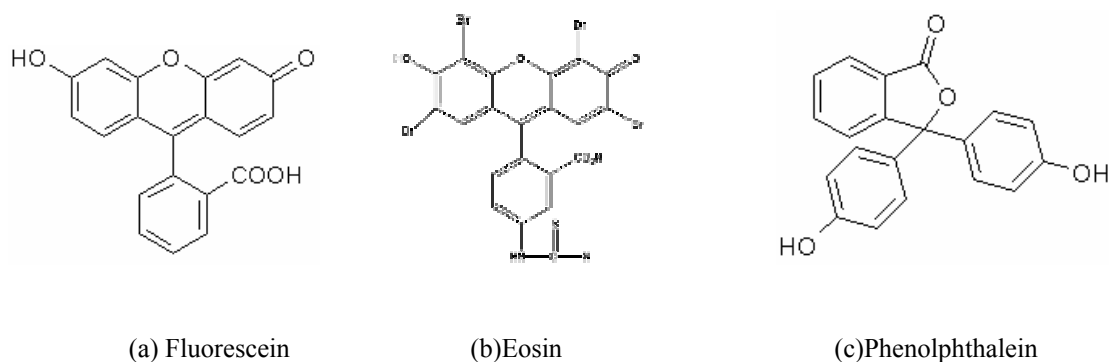


Fig. 9: Rigidity and planar conformation influence the fluorescence efficiency (a) Fluorescein and (b) Eosin exhibit fluorescence while (c) Phenolphthalein is non-fluorescent.

b) Surroundings

Solvents- fluorescence of some fluorophores is quenched strongly by aqueous environment while in non-polar or rigid environments, it is enhanced. In fact, excited molecules will tend to interact with polar solvents so as to align the dipoles. This alignment decreases the energy of excited state and causes the emission spectrum to shift towards red (longer wavelength). Beside the accessibility of fluorophore, it is very important to note that a fluorophore free in aqueous solution is quite susceptible to quenching, but when incorporated into a macromolecular structure, it may be shielded from solvent and quenching can be avoided and therefore its becomes more fluorescent. pH also affects the status of a chromophore in solution and this pH effect can be explained by comparing resonance forms of cations and anions. For example, at pH 7.0 both phenol and anisole give fluorescence but at pH 12 phenol is converted into non-fluorescent anion and fails to fluoresce.

c) Quenching

It is an intermolecular non-radiative process in which the fluorescence emission of a fluorophore is lost. Internal quenching is due to some internal structural features of the excited molecule involving structural rearrangements. While, external quenching arises either from interaction of the excited molecule with another molecule present in the sample or absorption of exciting or emitted light by another chromophore in the sample. External quenching may be due to contaminants present in preparations or deliberately introduced into experiment. Examples- acrylamide, ascorbic acid, iodide etc are very good external quenchers. External quenching involves two main possible mechanisms. First is dynamic quenching which involves collision between two molecules with fluorophores losing energy as kinetic energy. This depends on collisions between the excited fluorophore and the quencher and result in a decrease in the excited lifetime, T . It is a diffusion controlled process which increase with temperature. Secondly, static quenching involves the more stable complex between fluorophore and quencher. The complex is also called as Dark complex. This process does not affect excitation lifetime and occurs at higher temperatures.

Quantum yield

To quantitate the fluorescence, quantum yield is used and it is defined as the fraction of excited singlet electrons that become de-excited due to the fluorescence. The fluorescence quantum yield (Φ_F) is given by

$$\Phi_F = \text{photons emitted} / \text{photons absorbed.}$$

Under a given set of conditions, Φ_F usually has a fixed value for a particular fluorophore, with a maximum value of one. But, experimentally it is difficult to measure Φ_F accurately; we often use relative measurements of fluorescence in practice.

Fluorescence Spectroscopy - Instrumentation

The basic components of a fluorescence spectrophotometer are shown schematically in figure 10.

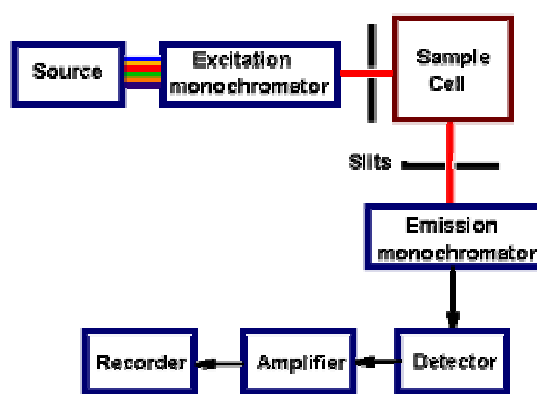


Fig. 10: Basic components of a typical fluorescence spectrophotometer

In spectrofluorometer, an incident beam of radiation of a given wavelength is passed through a sample cuvette containing the fluorophore. Emitted radiation is detected by the photoemission tube. The construction of fluorometer is almost same as spectrophotometer but the main differences are that emitted radiation is detected at 90° to the direction of the incident light beam and that a second monochromator is required to select for the different wavelength of the emitted light. Since, fluorescence is emitted in all directions from the fluorophore, this design excludes inadvertent detection of the incident beam.

Background fluorescence- interference in fluorescence signal detection

Fluorescence detection sensitivity is severely compromised by background signals, which may originate from endogenous sample constituents (autofluorescence) or from unbound or nonspecifically bound probes (reagent background). Detection of autofluorescence can be minimized either by selecting filters that reduce the transmission or by selecting probes that absorb and emit at longer wavelengths. Although, narrowing the fluorescence detection bandwidth increases the resolution, it also compromises the overall fluorescence intensity detected. Signal distortion caused by autofluorescence of cells, tissues and biological fluids is most readily minimized by using probes that can be excited at >500 nm. Furthermore, at longer wavelengths, light scattering by dense media such as tissues is much reduced, resulting in greater penetration of the excitation light.

Applications

Protein fluorescence spectroscopy

Intrinsic fluorescence in a protein mainly comes from tryptophan (Trp) and tyrosine (Tyr). The contribution from phenylalanine (phe) is practically negligible (Table 1).

	Absorption Wavelength (nm)	Fluorescence Wavelength (nm)	Quantum Yield	Life Time (ns)
Tryptophan	280	348	0.20	2.6
Tyrosine	274	303	0.14	3.6
Phenylalanine	257	282	0.04	6.4

Table 1- Excitation wavelength and respective fluorescent emission wavelength and quantum yield of aromatic amino acids present in proteins

Fluorescence of tyrosine in proteins is frequently quenched as a result of proton transfer in the excited state. Therefore, if a protein contains several tyrosines and a single tryptophan residue, the observed fluorescence emission of the protein is only that of tryptophan (335-350nm). However, if there are no tryptophans in a protein, the fluorescence is from tyrosine. These aromatic residues have an average occurrence of only 3.5%, 1.1% and 3.5% in proteins, respectively.

The extrinsic fluorescence can be due to fluorophores other than proteins, like cofactors such as FMN, FAD, NAD and porphyrins or some external agents attached to the protein which usually exhibit weak fluorescence. In many cases, a fluorophore can be introduced into the molecule to be studied either by chemical coupling or by simple binding (as in the use of reporter groups in absorption spectroscopy). But the fluorophore should be tightly bound to protein without changing the features of the protein and sensitive to the environment change. Examples are ANS (1-Anilino-8-naphthalene sulphonate), dansyl chloride, fluorescein, etc.

Nucleic acids

Though nucleic acids, DNA/RNA, highly absorb at 260 nm but they do not emit fluorescence. The purine and pyrimidine bases present in nucleic acids absorb radiation but dissipate energy by various radiation-less transitions like giving to the surrounding molecules. Therefore, to study the DNA/RNA structures, extrinsic fluorophores should be attached. Different fluorescent ligands are routinely used in nucleic acid structural and binding studies. These molecules either bind to grooves (Hoechst 33258 for DNA and SYBR GREEN II for RNA) or intercalate between base pairs of the helical form of nucleic acids (ethidium bromide, acridine orange). It is well known that ethidium bromide is also conventionally used to visualize DNA by staining in several molecular biological experiments (Table 2).

Fluorescent probes	Excitation λ_{max} (nm)	Emission λ_{max} (nm)	Quantum yield
Dansyl chloride	330	510	0.1
ANS	374	454	0.98
Ethidium bromide	515	600	1.0
Proflavin	445	516	0.02

Table 2: Excitation and emission wavelengths and quantum yield of some fluorescent probes

DNA/RNA quantification is done by using different fluorescent probes like Hoechst 33258 dye and Ethidium Bromide (for DNA), SYBR GREEN II (for RNA). These dyes show a linear relationship of their fluorescence with subsequent increase in nucleic acid concentration i.e. the fluorescence intensity of the nucleic acid-dye complex is directly proportional to the concentration of the nucleic acid present in the sample. This method of nucleic acid quantification is very popular because it is rapid, accurate, and very less expensive.

Other applications of Fluorescence Spectroscopy

Structural studies of DNA/RNA/Proteins are done by using extrinsic fluorophores to them. The use of emitted light is captured and visualized in fluorescence microscope for live cells observation. In immunoassays, attachment of fluorophores to either primary or secondary antibodies has proved very sensitive technique which is called ELISA. Enzyme assays and assessment of S-S bonds and SH groups are also included in long list of applications of this technique. Fluorescence is more sensitive to fluorophore environment than UV-VIS absorption due to the increased time the molecule stays in the excited state.

Chemiluminescence

Chemiluminescence is the generation of electromagnetic radiation as light by the release of energy from a chemical reaction. Light-emitting reactions arising from a living organism, such as the firefly or jellyfish, are commonly termed bioluminescent reactions. Chemiluminescent and bioluminescent reactions usually involve the cleavage or fragmentation of the O-O bond in an organic peroxide compound. Peroxides, especially cyclic peroxides, are prevalent in light emitting reactions because the relatively weak peroxide bond is easily cleaved and the resulting molecular reorganization liberates a large amount of energy. Molecules such as luciferin and luminol show this property and are oxidized by enzymes luciferase and peroxidase respectively in firefly and emit light. These reactions are highly efficient and are incorporated into sensitive enzyme assays.

Circular Dichroism

Optical activity is the ability of a chiral molecule to rotate the plane of polarized light. A plane polarized light wave can be resolved into right- and left- circularly polarized components. If

these two circularly polarized components are absorbed to different extents at any wave-length, then it turns out that the sample will also have a different index of refraction (n) for the two components at virtually all wavelengths. This means that one will propagate more rapidly than the other through the medium. The result is a phase shift between the two components, proportional to the refractive index difference, $n_L - n_R$. In other words, the two circularly-polarized components travel at different speeds, and are absorbed in differing degrees by the substance. Thus, the light passing through the substance is elliptically polarized (Figure 11) and the substance is said to have “circular dichroism (CD)”. This effect is called as ‘circular birefringence’. When the two components are combined, the phase shift results in a permanent rotation of the long axis of the elliptically polarized light. Thus circular birefringence is equivalent to optical rotation.

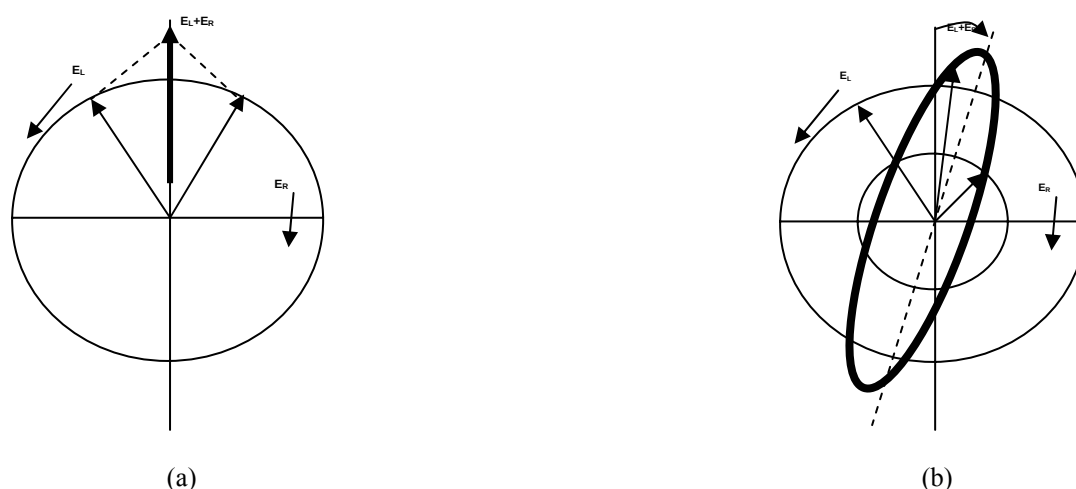


Fig. 11: (a) Circularly polarized light (b) Origin of birefringence

Optically active samples have distinct molar extinction coefficients for left (ϵ_L) and right (ϵ_R) circularly polarized light. The magnitude of circular dichroism is usually expressed in terms of molecular ellipticity $[\theta]$, which is determined according to the following relationship $[\theta] = 4500/\pi (\epsilon_L - \epsilon_R)$, where, ϵ_L and ϵ_R are the molecular extinction coefficients for the right and left circularly-polarized beams of light. The difference between ϵ_L and ϵ_R may be expressed as $\Delta\epsilon$. If $\Delta\epsilon$ or ellipticity is plotted against wavelength (λ), a CD spectrum may be obtained.

CD spectra are measured in a special type of spectrophotometer called a CD spectropolarimeter of which an outline design is shown in figure 12.

The light source (normally a xenon lamp) covers the UV- VIS spectral range, 180 - 800 nm. Since CD depends on differential absorption, which is selectively exposing sample to left and right circularly polarized light is necessary. This is achieved by passing a beam of plane polarized light through a photoelastic modulator which is normally quartz piezoelectric crystal subjected to an oscillating electric field. Differential absorption of left and right circularly polarized light is detected at photomultiplier and converted into ellipticity, θ which has units of milidegrees.

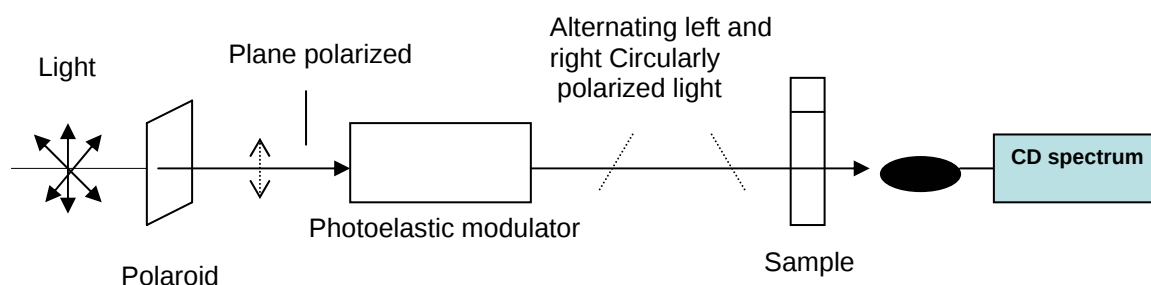


Fig. 12: CD spectropolarimeter. A photoelastic modulator selects at any one time for either left or right circularly polarized components of plane polarized light. Selective absorption is detected at the photomultiplier and gives a CD Spectrum for the sample

Determination of Protein Secondary Structure

Secondary structure can be determined by CD spectroscopy in the "far-uv" spectral region (190-250 nm). At these wavelengths the chromophore is the peptide bond, and the signal arises when it is located in a regular, folded environment. Alpha-helix, beta-sheet, and random coil structures each give rise to a characteristic shape and magnitude of CD spectrum. (Fig. 13).

Typically, α -helix gives positive band at 190nm with –ve bands at 222nm and 208nm, β - sheet gives +ve band at 195nm with -ve bands at 217 and 180nm and random coli gives +ve band at 195nm and –ve band at 195nm. Like all spectroscopic techniques, the CD signal reflects an average of the entire molecular population. Thus, while CD can determine that a protein contains about 50% alpha-helix, it cannot determine which specific residues are involved in the alpha-helical portion. The CD spectra are used as thumb rule similar to IR spectroscopy.

Protein Tertiary Structure from Circular Dichroism

The CD spectrum of a protein in the "near-uv" spectral region (250-350 nm) can be sensitive to certain aspects of tertiary structure. At these wavelengths the chromophores are the aromatic amino acids and disulfide bonds, and the CD signals they produce are sensitive to the overall tertiary structure of the protein. Signals in the region from 250-270 nm are attributable to phenylalanine residues, signals from 270-290 nm are attributable to tyrosine, and those from 280-300 nm are attributable to tryptophan. Disulfide bonds give rise to broad weak signals throughout the near-uv spectrum. If a protein retains secondary structure but no defined three-dimensional structure (*e.g.* an incorrectly folded or "molten-globule" structure), the signals in the near-uv region will be nearly zero. On the other hand, the presence of significant near-uv signals is a good indication that the protein is folded into a well-defined structure. The near-uv CD spectrum can be sensitive to small changes in tertiary structure due to protein-protein interactions and/or changes in solvent conditions.

Determination of nucleic acid conformation

For nucleic acids not only the base composition, but in fact some actual sequence information must be taken into account to explain CD spectra. From CD spectrum we can deduce the conformation of various forms of DNA; like A-form gives +ve band at 165nm with –ve 140nm

band, B-form gives +ve band at 172nm with -ve band at 140nm while Z-form gives reverse pattern of B-form. One other useful application of CD is in studying the binding of small molecules to protein and nucleic acids. An optically active small molecule may show a change in CD upon binding to a macromolecule, either because of electronic interactions with its binding site or because it may undergo a conformational change when it binds. The bands of nucleic acids and protein overlap but in the longer wavelength region of DNA, there is no contribution. Hence, conformational differences between free nucleic acid and the nucleic acid in the complex can be monitored.

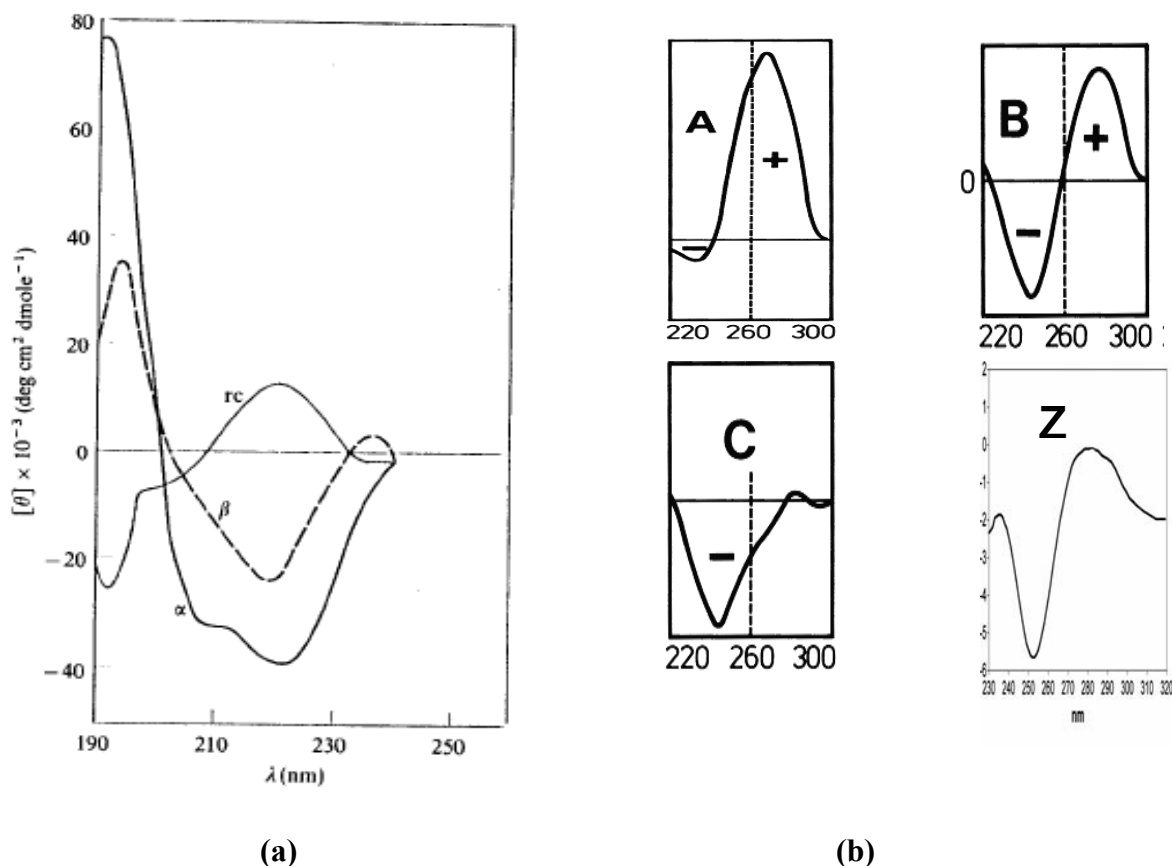


Fig. 13: Characteristic CD spectra of (a) α -helix, β -sheet and random coil (rc) conformation in a protein. (b) Spectra of A-DNA, B-DNA, C-DNA and Z-form DNA

NMR, ESR and PET

(a) Nuclear Magnetic Resonance Spectroscopy

Nuclear Magnetic Resonance (NMR) spectroscopy uses the magnetic properties of the nucleus of an atom and this technique is routinely used by chemists to determine chemical structure using simple one-dimensional techniques. Two-dimensional techniques are used to determine the structure of more complicated molecules. NMR is replacing X-ray crystallography for the determination of protein structure. Later, Magnetic Resonance Imaging (MRI) which gives clinically relevant information was developed and now it has become a very common non-invasive technique to detect cancer etc.

All nuclei carry a charge. In some nuclei, this charge “spins” on the nuclear axis. The angular momentum of the spinning charge can be described in terms of spin numbers I ; these number have values of 0, $1/2$, 1, $3/2$, and so forth ($I=0$ denotes no spin). If a nucleus has a finite spin, then it has a magnetic moment. The intrinsic magnitude of the generated dipole is expressed in terms of nuclear magnetic moment, μ . Each proton and neutron has its own spin, and I is a resultant of these spins. If the sum of protons and neutrons is even, I is zero or integral (0, 1, 2,....). But if the sum is odd, I is half-integral ($1/2, 3/2, 5/2, \dots$); if both protons and neutrons are even numbered, I is zero. Both ^{12}C and ^{16}O fall in the last category and give no NMR signal. If the transition between these energy levels is $h\nu$ (h is Planck’s constant; ν is the frequency of electromagnetic radiation) in a magnetic field of given strength H_0 , the fundamental NMR equation correlating electromagnetic frequency with magnetic field is, $\nu = \gamma H_0 / 2\pi$. A frequency of 60 MHz is needed at a magnetic field H_0 of 14,092 gauss for the proton. The constant γ is called magnetogyric ratio and is a fundamental nuclear constant; it is the proportionality constant between the magnetic moment μ and the spin number I , $\gamma = 2\pi\mu / hI$. In the absence of an external magnetic field, the nuclear magnets (protons) are randomly oriented in space. When they are subjected to an external field H_0 , they align themselves either parallel or antiparallel to the field.. The spins do not align perfectly along H_0 ; this gives to a permanent torque on μ_N . nucleus also has the property of angular momentum because of its spin. The H_0 precessional angular velocity, ω_0 is equal to the product of the magnetogyric ratio and the strength of the applied magnetic field H_0 , and is given by $\omega_0 = \gamma H_0$. (See Figure 14). This ω_0 is also known as resonant or the Larmor frequency.

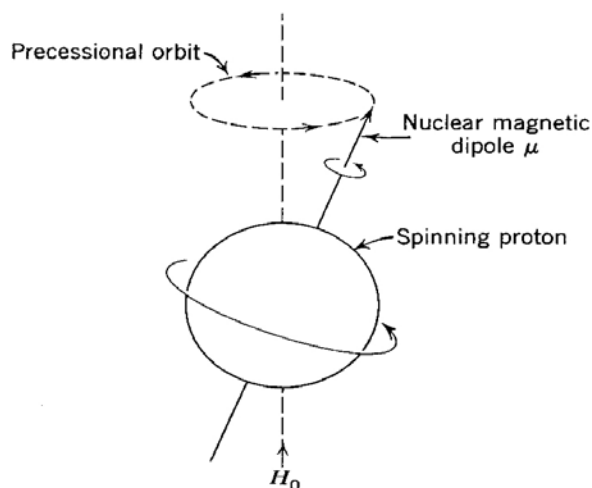


Fig. 14: Proton precession in an external magnetic field H_0

The protons and neutrons of the nucleus have a magnetic field associated with their nuclear spin and charge distribution. Under appropriate conditions, a sample can absorb electromagnetic radiation in the radio-frequency region at frequencies governed by the characteristics of the sample. A plot of the frequencies of the absorption peaks versus peak intensities constitutes an NMR spectrum. Once the MR spectrum is obtained, it can be analyzed in terms of the following parameter: (i) chemical shift, (ii) relaxation (T_1 , T_2), (iii) signal intensity, (iv) Spin-spin

coupling. Nuclei are surrounded by electrons, which shield them from the applied magnetic field, B_0 . The B_0 induces currents in these electron clouds that reduce the effective field experienced at the nucleus because they tend to oppose B_0 . The induced fields are directly proportional to B_0 . Thus we can write $B_{\text{eff}} = B_0 (1 - \sigma)$, where σ is a shielding constant that depends on the nature of the electrons around the nucleus. Thus, different nuclei within a sample experience different fields, depending on their immediate chemical environment.

Rather than use a scale involving magnetic fields, it is more convenient to use a frequency scale and to normalize this scale by using a signal from a reference compound. The scale now used in NMR is called the chemical shift scale. The intrinsic shifts of different groups and of different nuclei can vary widely shifts for ^1H resonances one measured with respect to a reference compound of the type $\text{R Si (CH}_3)_3$. For example, aromatic NH given 10-15PPM, peptide NH at 10-5 PPM, aliphatic CH_2 7-0 PPM and methyl CH_3 0-4 PPM.

High resolution NMR spectra exhibit multiplet structure that arises from weak interactions between magnetic nuclei. These interactions are communicated between the nuclei by the electrons in a chemical bond. The size of the interaction is defined by the spin-spin coupling constant (J) which is expressed in hertz. Consider, for example, a hypothetical molecule consisting of two bonded nuclei. Each nucleus can be oriented in two ways. These two orientations cause slightly different electron distributions, which result in small chemical shifts. These shifts cause each of the two nuclear resonances to split in two, making a pair of doublets. The spectrum of a nucleus coupled to two equivalent nuclei is a triplet with intensities in the ratio 1: 2: 1 because the following orientations are possible: $\uparrow \uparrow, \downarrow \uparrow, \uparrow \downarrow, \downarrow \downarrow$, two of which are equivalent. Similarly, three equivalent nuclei give rise to a quartet with intensity ratios 1:2:2:1, and so on with coupling constant J . If $\delta/J \gg 1$, then simple pair of doublets is observed.

For example in ethanol $\text{CH}_3 - \text{CH}_2 - \text{OH}$, the resonance from the CH_3 group has a relative area of three units, and because it is adjacent to two hydrogens on the CH_2 group, it is a triplet. The CH_2 resonance has a relative area of two units and is a quartet because it is next to the CH_3 group. The coupling between the OH hydrogen and the CH_2 group is removed because of rapid chemical exchange of this hydrogen between ethanol molecules.

(b) Electron Spin Resonance

Electron spin resonance (ESR) or **Electron Paramagnetic Resonance (EPR)** spectroscopy is a spectroscopic technique which detects species that have unpaired electrons, generally it must be a free radical. Because most stable molecules have a closed-shell configuration without a suitable unpaired spin, the technique is not so widely used as compared to NMR. In ESR, the basic concept is similar to that of NMR, but instead of the spins of the nuclei, "electron spins" are excited. Because of the difference in mass between nuclei and electrons, weaker magnetic fields and higher frequencies are used as compared to NMR. For electrons in a magnetic field of 0.3 tesla, spin resonance occurs at around 10 GHz.

An electron has a magnetic moment and when placed in an external magnetic field of strength B_0 , this magnetic moment can align itself parallel or antiparallel to the external field. The former is a lower energy state than the latter which is known as the Zeeman effect, and the energy separation between the two is given by $\Delta E = g_e \mu_B B_0$, where g_e is the gyromagnetic ratio of the

electron, the ratio of its magnetic dipole moment to its angular momentum, and μ_B is the Bohr magneton. To jump to a higher energy level, the electron can absorb electromagnetic radiation of the correct energy (ΔE). The paramagnetic centre is placed in a magnetic field and the electron is made to resonate between the two states. A free electron on its own has a g_e which is the electronic g factor a value of 2.0. This means that for radiation at the commonly used frequency of 9.5 GHz gives rise to X-band spectra, resonance occurs at a magnetic field of about 0.34 tesla (3400 gauss).

EPR signals can be generated by measuring resonance energy absorption at different electromagnetic radiation frequencies (ν) in a constant external magnetic field. This can be achieved by scanning a range of different frequency radiation at constant magnetic field. Conversely, measurements can be provided by changing the magnetic field B and using a constant frequency radiation due to technical reasons, the second method is more common. Therefore an EPR spectrum is normally plotted with the magnetic field along the x-axis, and peaks are seen at the fields which cause resonance. Figure 15 is a diagram of the main components of the ESR instrument.

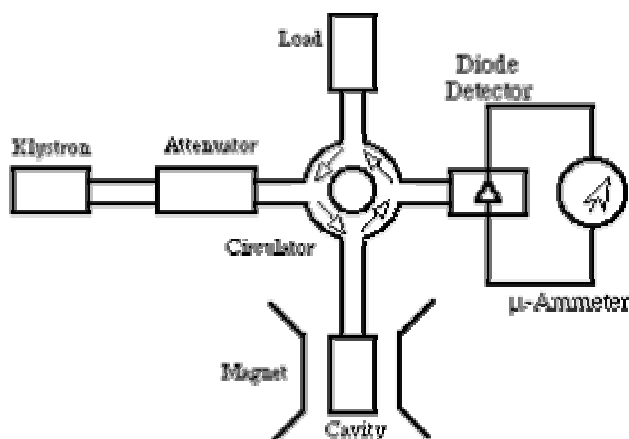


Fig. 15: Block diagram of an ESR spectrometer

The field strengths generated by the electromagnets are of the order of 50 to 500 millitesla, and variations of less than 1 in 10^6 are required for highest accuracy.

ESR is used for the identification and quantification of radicals molecules with unpaired electrons to identify reaction pathways in chemistry as well as in biology and medicine for tagging biological spin probes. Since radicals are very reactive, they do not normally occur in high concentrations in biological environments. However, it is possible with the help of specially designed nonreactive radical molecules that attach to specific sites in a biological cell.

(c) Positron Emission Tomography

A positron can be thought of as a positive electron. Most radioactive isotopes decay by release of a gamma ray and electrons, some decay by the release of a positron. Positron emission tomography (PET) imaging of a particular class of radioactive isotopes is used for medical

purpose. PET technology was stimulated by development of reconstruction algorithms associated with X-ray, CT, MRI . PET is now a tool for medical diagnosis, for dynamic studies of human metabolism and for studies of brain activation.

Beta particles fast electrons or positrons produced in the weak interaction decay of neutrons or protons in neutron- or proton-rich nuclei. Firstly, in a neutron-rich nucleus a neutron can transform into a proton via the process, $n \rightarrow p + \tilde{e} + \tilde{\nu}_e$, by emitting an electron and an antineutrino. The daughter nucleus now contains one extra proton so that its atomic number Z has increased by one unit. This can be written as $(Z,A) \rightarrow (Z+1, A) + \tilde{e} + \tilde{\nu}_e$. This is how a free neutron decays with a half-life of 10.25 minutes.

Secondly, in proton-rich nuclei a positron and neutrino are emitted in, $p \rightarrow n + e^+ + \nu_e$ and the corresponding decay is written as $(Z,A) \rightarrow (Z-1, A) + e^+ + \nu_e$. The daughter nucleus now contains one proton less and therefore the atomic number has decreased by one unit. A third process called “electron capture”, in this process an atomic electron that is "close" to the nucleus is captured by the nucleus $p + e^- \rightarrow n + \nu_e$

A basic characteristic of the β -decay process is the continuous energy spectrum of the β - particles. This is because the available energy in the decay is shared between the β - particle and the neutrino or antineutrino. The positron emitted in β^+ - decay combine with electrons and annihilate resulting in emission of gamma rays which are detected in the PET detector system.

PET imaging begins with the injection of a metabolically active tracer-a biological molecule that carries with it a positron-emitting isotope (for example, ^{11}C , ^{13}N , ^{15}O , or ^{18}F). Immediately, the isotope accumulates in an area of the body for which the molecule has an affinity. As an example, glucose labeled with ^{11}C (half-life, 20 min), or a glucose analog labeled with ^{18}F (half-life, 1.8 hr), accumulates in the brain, where glucose is used as the primary source of energy. The radioactive nuclei then decay by positron emission. The emitted positron collides with a free electron usually within less than 1 mm from the point of emission. The interaction of the two subatomic particles results in a conversion of matter to energy in the form of two gamma rays. These high-energy gamma rays emerge from the collision point in opposite directions, and are detected by an array of detectors which surround the patient. When the two photons are recorded simultaneously by a pair of detectors, the collision that gave rise to them must have occurred somewhere along the line connecting the detectors. After 500,000 or more annihilation events are detected, the distribution of the positron emitting tracer is calculated by tomographic reconstruction procedures. PET then reconstructs a two-dimensional image. Three dimensional reconstructions can also be done using 2D projections from multiple angles.

PET has a manyfold sensitivity over other techniques used to study regional metabolism and neuroreceptor activity in the brain and other body tissues. Since the nanomolar range is the concentration range of most receptor proteins in the body, positron emission tomography is ideal for this type of imaging. The major clinical applications of PET have been in cancer detection of the brain, breast, heart, lung and colorectal tumors. Another application is the evaluation of coronary artery disease by imaging the metabolism of heart muscle. Thus, with the combination of 8 full scans, a whole body image of the distribution of ^{18}F -deoxyglucose (or other common

radioisotopes) can be acquired in 40 minutes; as is commonly done for evaluation of breast cancer.

Mass Spectrometry

Mass spectrometry (MS) is a powerful analytical technique that is used to identify unknown compounds, to quantify known compounds, and to elucidate the structure and chemical properties of molecules. Detection of compounds can be accomplished with very minute quantities as little as 10^{-12} g, 10^{-15} moles for a compound with a mass of 1kDa. In principle, in MS the sample molecules are converted into ions in the gas phase and separated according to their mass: charge (m/z) ratio. It measures the molecular weight of molecules based upon the motion of a charged particle in an electric and magnetic field. Basically, a mass spectrometric analysis can be envisioned to be made up of the following steps: a) sample introduction b) ionization and c) ion separation and detection. The following are the ionization types, commonly used in mass spectrometry.

Electron Impact and Chemical Ionization

Volatile substances can be ionized by electron (impact) ionization in a process involving the interaction of the gaseous sample with an electron beam generated by a heated filament in the ion source Figure 16.

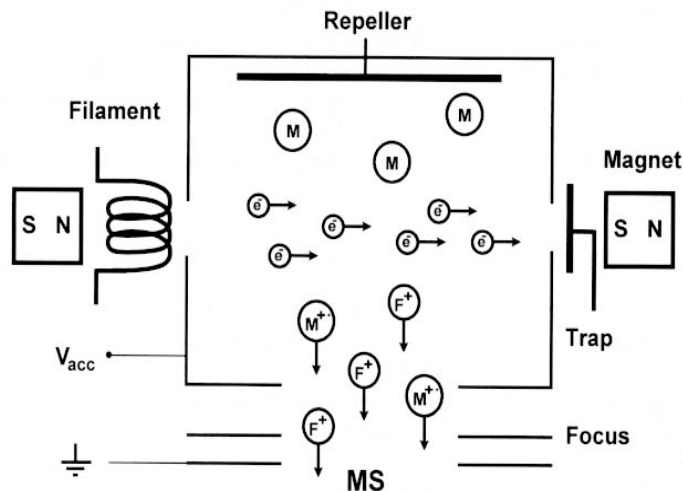
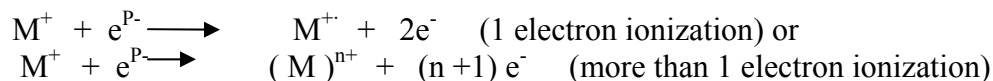


Fig. 16: Schematic representation of an electron ionization ion source. M represents neutral molecules; e^- , electrons; M^+ , the molecular ion; F^+ , fragment ions; V_{acc} , accelerating voltage; and MS, the mass spectrometer analyzer

A magnetic field keeps the electron beam focused across the ion source and onto a trap. Upon impact with a 70 eV electron, the gaseous molecule may lose one of its electrons to become a positively charged radical ion (M^+ represents the molecular ion).



It carries an unpaired electron and can occupy various excited electronic and vibrational states. If these excited states contain enough energy, bonds will break and fragment ions and neutral particles will be formed. With electron energy of 70 eV, enough energy is transferred to most molecules to cause extensive fragmentation. All ions are subsequently accelerated out of the ion source by an electric field produced by the potential difference applied to the ion source and a grounded electrode. Depending on the lifetime of the excited state, fragmentation will either take place in the ion source giving rise to stable fragment ions, or on the way to the detector, producing metastable ions. The mass spectrum obtained from recording all of these ions contains signals of varying mass to charge ratio, m/z and intensities, depending on the numbers of ions that reach the detector.

Electrospray ionization (ESI)

ES ionization has a tremendous impact over the last few years on the use of mass spectrometry in biological research. It was the first method to extend the useful mass range of instruments to well over 50,000 Da. The sample is usually dissolved in a mixture of water and organic solvent, commonly methanol, isopropanol or acetonitrile. It can be directly infused, or injected into a continuous-flow of this mixture, or be contained in the effluent of an HPLC column or CE capillary.

Matrix-Assisted Laser Desorption (MALDI)

MALDI was introduced independently in 1988 by Hillenkamp and Tanaka as a method of transferring large, labile molecules into the gas phase as intact ions. Briefly, the technique involves mixing the analyte of interest with a large molar excess of a matrix compound, usually a weak organic acid. This mixture is placed on a vacuum probe and is irradiated with a laser beam (usually 337 nm). The laser causes the desorption and ionization of the matrix and analyte and then ions are accelerated to mass spectrometer for analysis. A wide range of matrices have been used for MALDI. The three widely used matrices for peptides and proteins are α -cyano-4 hydroxy cinnamic acid, 2,5 dihydroxybenzoic acid (2,5 DHB) and sinapinic acid

Ion Separation

After the gas phase ions have been produced, they are accelerated from the ion source and guided into the analyzer, the region of ion separation. The ions are separated according to mass: charge ratio. Time-of-Flight (TOF) analyzer is one of the simplest and widely used mass analyzer. TOF measures the m/z ratio of an ion by determining the 'time required for it to traverse the length of a flight tube' and is based on the principle that ions of different m/z values have the same energy, but different velocities, after acceleration out of the ion source. The time of flight of an ion is proportional to the square root of mass/charge ratio when given a constant accelerating voltage. Time of flight is given by, $T = k (m/z)^{1/2}$

The combinations of time-of-flight mass spectrometry with MALDI and ESI have produced effective tools in the laboratory of biochemists, due to their relatively low cost, high sensitivity, speed and ease of operation.

Applications

Mass spectrometry has found several applications in chemistry viz) molecular mass, structural analysis, identification of unknown compound, presence of impurities and distinction between cis- and trans- isomers. In biology, it is extensively used for proteins, peptides, the following are the various uses of mass spectrometry.

a) Proteins

i) Molecular mass

The mass spectrometry is unique among the analytical methods in providing the information on the molecular mass accurately. Much of the work with proteins falls into two categories; analysis of intact proteins and analysis of peptides derived from chemically or enzymatically cleaved proteins. The analytical aspects of concern for a given technique are the mass range, resolution, sensitivity, susceptibility to salts, precision, speed, and most of all, mass measurement accuracy.

ii) Sequence analysis

For sequence analysis, two general approaches have been used. The first involves MS/MS methodology to perform the ionization, peptide ion backbone cleavage, and final identification of products. And the second utilizes specific enzymatic or chemical reactions to form truncated peptides and subsequent use of mass spectrometry to analyze the reaction products.

iii) Post-Translational Modifications

A number of chemical changes to a protein can occur after synthesis on the ribosome, including partial proteolytic hydrolysis, glycosylation, acylation, phosphorylation, cross-linking through disulfide bridges, etc. What these have in common is that they change the mass of the original molecule. Further, using the proper procedures, the position of these modifications within the polypeptide chain can be identified.

iv) Location of Disulfide Linkages

The determination of the numbers and positions of disulfide links in proteins, both intra- and intermolecular can be accomplished quite effectively by mass spectrometry. The number of Cysteine residues in a protein can be quickly determined by alkylation of the protein, for example, with vinylpyridine. Each pyridylethyl group adds 105 Da for each modified Cysteine residue. The molecular mass analysis, before and after alkylation using ESI or MALDI- MS, thus provides the total number of cysteine residues modified.

b) Nucleic Acids

The use of mass spectrometry for the analysis of nucleic acids has come in two general areas, the first involving nucleosides, nucleotides and small oligonucleotides, and the second polynucleotides like DNA, RNA. By far, most work has come from the small molecule area, due primarily to the highly charged, polar nature of these molecules. Generally nucleosides, nucleotides and small oligonucleotides do not desorb efficiently as compared to peptides and are considerably less sensitive with current methods of ionization processes. Only recently has ESI and MALDI been applied with some success to polynucleotide analysis.

Infrared Spectroscopy

As shown in the Fig 1, the infrared radiation (IR) is that part of the electromagnetic spectrum between the visible and microwave regions. The ordinary infrared region extends from $2.5\text{--}15\ \mu$ ($4000\text{--}667\ \text{cm}^{-1}$); the region from $0.8\text{--}2.5\ \mu$ ($12500\text{--}4000\ \text{cm}^{-1}$) is called the near- infrared and the region from $15\text{--}200\ \mu$ ($657\text{--}50\ \text{cm}^{-1}$) is called the far- infrared. Infrared radiation is absorbed by organic molecules and energy is converted into molecular rotation and vibration. The study of the absorptions of radiation that results from transitions among the vibrational energy levels leads to further detailed insight into the nature of molecules. IR radiation induces transitions among the vibrational energy levels only when the vibration of a molecule leads to an oscillating dipole moment and a vibrational spectrum of that molecule can be generated. Also during transition, change in vibrational quantum number can be only ± 1 i.e., $\Delta v = \pm 1$. There are two kinds of fundamental vibrations for molecules- stretching and bending. Stretching in which the distance between two atoms increases or decreases, but the atoms remain in the same bond axis. However, in bending, the position of the atoms changes relative to the original bond axis.

A polyatomic non-linear molecule having n atoms has a total of $3n$ (due to X,Y,Z directions) degrees of freedom. Three of these are overall translational degrees of freedom and 3 (or 2 if the molecule is linear) are rotational degrees of freedom, there will be $3n-6$ ($3n-5$ for a linear molecule) vibrational degrees of freedom. Therefore, for each of these $3n-6$ energy-level patterns, different $v=0$ to $v=1$ transitions modes of vibration are possible. If the vibrations corresponding to all these patterns are associated with oscillating dipole moments, there will be $3n-6$ (or $3n-5$) observed absorption bands. Molecules like H_2 , N_2 , O_2 , etc. do not give infrared spectrum whereas molecules like HCl , H_2O , NO_2 , etc. do give infrared spectrum because $3n-6$ is zero. Water molecule, H_2O with 3 atoms, gives IR absorption at 1595 , 3652 , $3756\ \text{cm}^{-1}$. So a polyatomic molecule with n number of atoms, $3n-6$ of vibrations is very large and there will be several vibrational transitions giving rise to a very complicated spectral pattern.

Infrared spectrum of NO_2 (with 3 atoms) gives three peaks at 750 , 1323 and $1616\ \text{cm}^{-1}$. This proves that NO_2 is a bent molecule and not a linear molecule because a linear molecule should give 4 peaks according to formula $3n-5$ but only two peaks should be expected as two of the vibrations are not associated with oscillating dipole moment.

The characteristic frequencies of some of the important groups are: Primary alcohol ($\text{CH}_2\text{O-H}$) at 3630 , secondary alcohol (CH-OH) at $3620\ \text{cm}^{-1}$, Dialkyl amines ($\text{N(R}_2\text{)-H}$) at $3400\ \text{cm}^{-1}$, hydrocarbon (CH_3) at $2900\ \text{cm}^{-1}$, aliphatic aldehyde (HC=O) at $1730\ \text{cm}^{-1}$, aliphatic amines (C-NH_2) at $1220\text{--}1020\ \text{cm}^{-1}$, alkyl nitro compounds (NO_2) at $920\text{--}830\ \text{cm}^{-1}$, primary and secondary nitro at $1565\text{--}1545\ \text{cm}^{-1}$, aromatic nitro at $1550\text{--}1510\ \text{cm}^{-1}$. Please note that it is the transmittance and not absorption on Y axis which gives sharp minima in the spectra at the corresponding functional groups.

In general, hydrogen bonding to an X-H molecule results in a decrease and broadening of the absorption band due to X-H stretching vibration. In dilute solution, in a non-polar solvent like CCl_4 (or in the gas phase), where association between molecules is minimal, ethanol, for example, shows an O-H stretching (V O-H) band at $3640\ \text{cm}^{-1}$. The bonding of hydrogen to the second oxygen weakens the O-H bond, lowers the energy and hence the frequency of vibration. Infrared technique has been used in the detection of end groups and chain-branching as well as

study of crystallinity in polymers. Use of polarized infrared radiation has been made in the study of certain properties of polymers.

Therefore, IR spectroscopy is a very useful tool to analyze the compound's structure, identify some important functional groups, assess its purity etc.. Analysis of a spectrum of an unknown organic compound IR spectra are also analyzed by comparing observed spectra with spectra of known compounds. The IR spectra are also known as “finger print” of the molecule. match with your compound. From IR spectra one can deduct the shape or symmetry of a molecule, presence of hydrogen bonding, branching in polymers etc.

Atomic Absorption Spectroscopy and Flame Photometry

All the above mentioned techniques, UV-VIS spectrophotometry, C.D, fuorescence etc. are based on the absorption of molecules and ions in solution. However, the atomic absorption spectroscopy (AAS) and flame photometry (FP) deal with absorption of “atoms”. As the concentration of atoms can not be measured directly in solution, they are volatilized either electro thermally or in a flame. Elements particularly metals which have an important role in biological systems, eg iron (present in hemoglobin), magnesium (in chlorophyll) and various other metals present in several toxins, can be analyzed by these methods.

Here the ground state atom absorbs light energy of a specific wavelength and enters into the excited state and emits light when returning back to the ground state. The wavelength at which absorption or emission from the atom occurs is associated with transitions where the minimal energy change is possible. For example, the sodium ion species are first decomposed and then reduced to give atoms, $\text{Na}^+ + \text{e}^- \rightarrow \text{Na}$.

The electronic transitions occuring in an atom are limited by the availability of empty orbital or levels. These limitations mean that emission and absorption lines are absolutely characteristic of the particular element. Atoms in the vapour state give line spectra and not band spectra like in electronic transitions). This is because no covalent bonds are involved in excitation and hence no vibrational sub-levels to cause broadening. The use of special light sources and careful selection of wavelengths allow the specific determination of individual elements.

Atomic absorption spectroscopy (AAS)

This technique for analyzing the metals involves the atomization of elements followed by assay of the element in vapour state. The concentrations of various metal ions can be directly corelated by the absorbed light through the Beer Imbert's law. A discharge lamp emits radiation in a narrow bandwidth, a wavelength specific for the element being assayed is chosen. The sample from the test solution sent to a nebulizer which creates a fine aerosol. The sample is then mixed with fuel and oxidant thoroughly and introduced into the flame.

Ions or atoms in a sample must undergo desolvation and vaporization in a high-temperature source such as a flame or graphite furnace. Monochromator removes scattered light of other wavelengths from the flame and isolate the absorption line from background light which can otherwise cause interferences. Finally a photomultiplier is used as detector to convert into electric signal. (see Fig 17) .Various metals like sodium, potassium, lithium, calcium can be measured respectively at 589.0, 776.5, 670.7 and 442.7 nm . Pharmacological and physiological sample can be measured directly while some metals require prior extraction from biological

source eg copper ,lead ,iron, mercury. Element determination in soil and plant extracts eg metal detection in macromolecules, organelles, cells and tissues is commonly performed by AAS. The disadvantage of these narrow-band light sources is that only one element is measurable at a time.

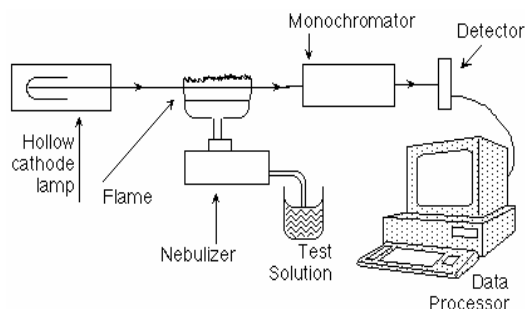


Fig. 17: Schematic diagram of Atomic Absorption Spectrometer

Flame photometry

This is a simple, relatively inexpensive, high sample throughput atomic emission spectroscopy method used for clinical, biological, and environmental analysis. This is similar to AAS but the emission is measured instead of absorption. The sensitivity of these two types of spectroscopy varies widely for example, calcium can be detected upto 0.1 ppm by AAS while upto 0.005 ppm by flame photometry similarly potassium can be detected till 0.001 ppm and sodium can be detected till 0.0001ppm with the help of flame photometry while AAS can detect them only upto 0.03 ppm level.

Suggested Readings

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