Principles and Applications of Spectrophotometric and Spectrofluorometic Techniques

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Optical Techniques

The *electromagnetic spectrum* is the distribution of electromagnetic radiation according to energy. Ultra-violet and visible regions of the electromagnetic spectrum and their associated techniques are probably the most widely used both for routine analytical work and research into biological problems. UV-visible spectrophotometry and spectrofluorometry are two optical techniques, which operate on the principle of absorption and emission of a part of the radiation (*viz.* UV and visible range) of electromagnetic spectrum respectively.

Optical transitions

Absorption and emission of light by atoms or molecules between two energy states, ground state, Em and excited state, En, take place to give rise to absorption or emission spectra respectively.

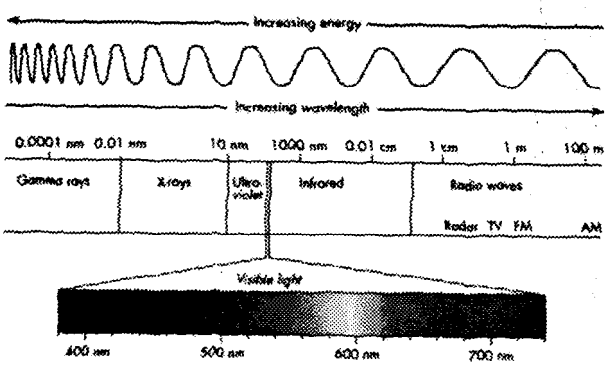
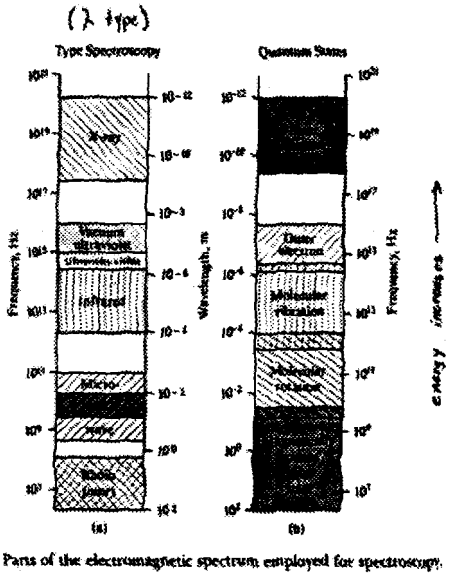


Fig 1 Schematic diagram of electromagnetic spectrum



Parts of the electromagnetic spectrum employed for spectroscopy.

Fig 2 Schematic of different parts of the electromagnetic spectrum employed for varied types of spectroscopy

Types of transitions (refer to fig. 3)

Stimulated Absorption: A transition form state m to n can be stimulated by incoming radiation and in the process the excited atoms or molecules absorb radiation.

Spontaneous Emission: An excited atom or molecule can give up its energy upon spontaneous emission of a photon. Direction and phase of the emitted photons are

random and emission is independent on the incoming radiation density. (Principle involved in spectrofluorometry)

Stimulated Emission: A transition from n to m can be stimulated by incoming radiation resulting in the emission of light, which is exactly in phase with the incoming light. In a laser (light amplification by stimulated emission of radiation) this process dominates over all others and as a result all photons emitted from the source are emitted in phase. This results in exceptional brightness, and directionality of the emitted light. Such light is said to be coherent as opposed to classical light, which is incoherent. (Principle involved in LASER)

Stimulated emission is also involved in the principle of Inductively Coupled Plasma Atomic Emission Spectroscopy ICP-AES. When energy is imparted to a compound through high temperature, the elements in the sample are converted to excited free atoms and ions. As they return to ground state, they emit radiations at characteristic wavelengths.

UV-Visible Molecular Absorption Spectrometry

UV-Visible Molecular Absorption Spectrometry is one of the most common analytical techniques in the analytical laboratory. It involves absorption of ultraviolet or visible, radiation by organic molecules, metals and metal-organic complexes.

Most absorption measurements are conducted by dissolving the analyte in a solvent. The attenuation of light as it passes through a sample is measured and is governed by Beer-Lambert's law.

The Beer-Lambert law is a combination of two laws, each dealing separately with the absorption of light, related to the concentration of the absorber and the path length or thickness of the layer. When the absorbing substance is partially transparent it will transmit a portion of the incident radiation. The ratio of the intensities of transmitted and incident light gives the transmittance, T, expressed as

$$T = I / I_o$$

where I_o is the intensity of incident radiation, and I is the intensity of transmitted radiation.

A100% value of T represents a totally transparent substance, with no radiation being absorbed, whereas a zero value of T represents a totally opaque substance, which, in effect, represents complete absorption. For intermediate values we can define the absorbance (A) or extinction (E), which is given by the logarithm (base 10) of the reciprocal of the transmittance:

$$A = E = \log (1/T) = \log (I_o/I)$$

Absorbance is a logarithm it is by definition unit less and has a range of values from 0 ($\equiv$100% T) to $\propto$($\equiv$ 0% T).

Thus Beer-Lambert law, which, as described above, states that the absorbance is proportional to both the concentration of absorber and thickness of the layer, as

$$A = \varepsilon_\lambda cl \text{ or } a_e cl$$

where ε_λ is the molar absorbance coefficient (or molar extinction coefficient) for the absorber at wavelength λ, (*L/mol* cm)

a = absorptivity (L/g cm)

c is the concentration of absorbing solution, (g/L) and l is the path length through the solution (or thickness) (cm).

Beer's law is a fundamental law governing (molecular and atomic) spectroscopy and there is a linear relationship between absorbance and concentration (but not absorbance and transmission)

Limitations to Beer's law:

- At high concentrations charge distribution effects occur causing electrostatic interactions between absorbing species
- Analyte dissociates/associates or reacts with solvent
- Most light sources are polychromatic not monochromatic.
- Stray light comes from reflected radiation in the monochromator reaching the exit slit.

Advantages of absorption measurements are that they are insensitive to quenching, the strength of the absorption is directly related to the absolute concentration of the analyte and that they are relatively simple to perform. The measurements are self-calibrating: *Disadvantage*: Signal appears as a small dip on a very large positive background signal. This makes the technique less sensitive than fluorescence based methods and also lowers their dynamic range.

Instrumentation

Wavelength selection

Wavelength selection is of crucial importance. In the visible region where the analyte may not absorb, but can be readily modified chemically to produce a coloured product, coloured filters are used which absorb all but a certain limited range of wavelengths. This limited range is known as the bandwidth of the filter.

In a colorimeter, the bandwidth of the wavelengths is determined by the filter. A filter that appears red to the human eye transmits red light and absorbs almost everything else. This kind of filter would be used to examine blue solutions as they would absorb red light. In general the filter should be of a colour complementary to that of the solution under test. The arrangement in such an instrument can be very simple, consisting merely of a light source (lamp), filter, cuvette and photosensitive detector to collect the transmitted light. Another detector is required to measure the incident light, or a single detector is used to measure incident and transmitted light alternately. This latter design is both cheaper and analytically better as it eliminates variation between detectors.

The spectrophotometer is more sophisticated instrument. Here the bandwidth is selected by the monochromator, which selects a single wavelength of monochromatic radiation, The optical systems used are either prisms, which split the multi wavelength source radiation into its component parts by refraction, or gratings, which achieve the same thing by diffraction. The resolution of wavelengths is greater from gratings than from prism but is very expensive. With the advent of photo-reproduction in the semiconductor industry, gratings of high quality can be reproduced in large numbers and hence are now relatively cheap. In the ultraviolet region it is necessary to use prisms, gratings, reflectors and cuvettes made of silica. Above 350 nm wavelength, borosilicate glass may be used but also there are now some plastic materials (e.g. disposable cuvettes) available that are

transparent over virtually the whole of the visible region and into the near ultraviolet.

The detector in the photometer is generally a photocell in which a sensitive surface receives photons and a current is generated that is proportional to the intensity of the light beam reaching the surface. In instruments for measuring ultraviolet/visible light, two lamps are usually required: one, a tungsten filament lamp, produces wavelengths in the visible regions; the second, a hydrogen or deuterium lamp, is suitable for the ultraviolet. There is a switchover point, and the 'switch' is a mechanical means of directing the appropriate beam along the optical axis, using mirrors or lenses. The optical arrangement in a single-beam instrument is such that the blank and then the sample must be moved into the beam, adjustments made and readings taken.

In the double-beam device the beam is split into two parts, one passing through the blank, or reference, at the same time as the other part passes through the sample. This approach prevents any problems of variation in light intensity. The resultant measured absorbance is the difference between the two transmitted beams of light recorded by the matched detectors.

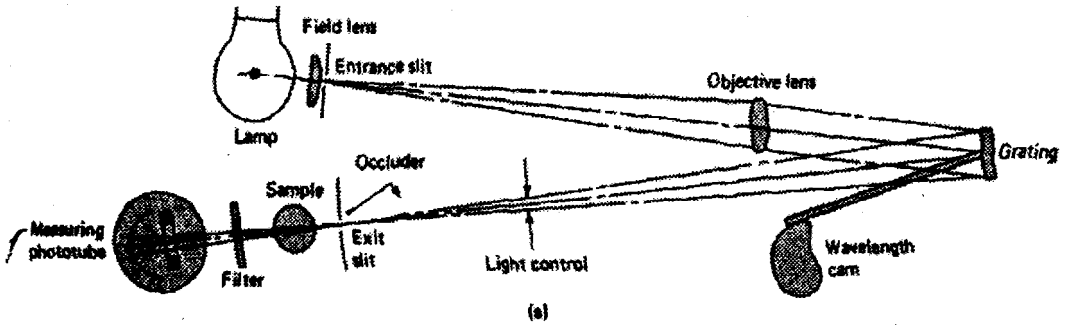
One of the advantages of the spectrophotometer is the facility to scan the wavelength range over both ultraviolet and visible and obtain absorption spectra. These are plots of absorbance versus wavelength.

Cuvettes should be optically matched for accurate work, the optical faces parallel and the path lengths identical, one containing a blank in which all the materials are mixed except the sample under test, an equivalent volume of solvent being added to this mixture, and the other containing the coloured material to be measured.

Multi-beam instruments allow the simultaneous recording of absorbance changes at two or more predetermined wavelengths.

It is necessary to standardize or zero the instrument using the blank, change cuvettes and read the absorbance. It is apt to work from the most dilute to the most concentrated because even if the cuvette is rinsed between each measurement the possibility of carryover should be minimized.

In both colorimetry and spectrophotometry, the usual procedure is to prepare a set of standards and produce a concentration versus absorbance calibration curve. Absorbance of unknowns is then



(a)

measured and the concentration interpolated from the linear region of the plot. Interpolation is critical because:

i) One should never extrapolate beyond the region for which any instrument has been calibrated

ii) In colorimetry, a phenomenon known as the Job effect occurs. If measurements are made beyond the colour reagent limit, the linearity of the Beer-Lambert calibration ceases and forms a plateau, which indicates that there is insufficient reagent to produce any more color. To extrapolate beyond the linear portion of the curve, therefore, would introduce enormous errors.

It is important to note that when plotting calibration curves, despite the fact that the Beer-Lambert relationship implies that there is zero absorbance at zero concentration, and that the instrument is physically zeroed, it is wrong to force the drawn line through zero. This would be to give greater credence to this point than any other and assume an unjustified level of precision. The best straight line should be drawn through the points, either by eye or by regression methods.

Applications

- Qualitative analysis can be performed in the ultraviolet/visible regions to identify certain classes of compound both in the pure state and in biological mixtures.
- Quantitative analysis may be performed by making use of the fact that certain chromophores, for example the aromatic amino acids in proteins and the heterocyclic bases in nucleic acids, absorb at specific wavelengths.
- The amounts of substances with overlapping spectra, such as chlorophylls a and b in diethylether may be estimated if their extinction coefficients are known at two different wavelengths.
- Used to follow enzyme kinetic by studying the extent of interaction between an enzyme and its substrate. The binding of a substrate to a heam group containing a ferric ion in the high spin state perturbs the spectrum by displacing the ligand water from the sixth position of the ferric ion, causing it to change to the low spin state. The process may be followed spectrophotometrically.
- Structural studies of biological macromolecules such as proteins and nucleic acids can be performed. In proteins, the spectrum of a chromophore depends on the polarity of the microenvironment. A change in the polarity changes the spectrum of a particular amino acid chromophore without changing the conformation of the protein. This phenomenon is known as solvent perturbation and to be accessible to the solvent, the amino acid residue must be on the surface of the protein.
- Aromatic amino acids are powerful chromophores in the ultraviolet region. Processes such as denaturation of a polypeptide chain by pH, temperature and ionic strength can be monitored as more of these residues become exposed to the incident radiation.
- If the amino acid residue tyrosine is involved, it is possible to study protein-

protein binding, protein-metal or protein-small molecule interactions. The range may be extended by the use of reporter group techniques in which an artificial chromophore is attached to the appropriate region of the protein.

The denaturation of the helical structure of DNA in solution may be investigated when the double-stranded DNA is heated through its transition temperature. The extinction at 260 nm increases (hyperchromic shift) on denaturation and decreases again (hypochromic shift) on renaturation, which occurs on cooling. Effects on the secondary structure of DNA by pH and ionic strength may be studied in a similar way.

In certain situations an action spectrum may be shown as a plot of physiological (non-extinction) parameter against wavelength. In many complex biological systems such a spectrum often corresponds to the absorption spectrum of a single key compound. An example is the plotting of the rate of oxygen evolution by green plant tissue against the wavelength of light used to irradiate the system. This results in a graph similar to the spectrum of the chlorophylls.

Spectrofluorometry

Fluorescence is an emission phenomenon. The energy transition from a higher to lower state within the molecule as a result of absorption of electromagnetic radiation is measured by the detection of emitted radiation. The wavelength(s) of absorbed radiation are at lower values (higher energy) than the emitted (fluoresced) wavelength. The difference between these two wavelengths is known as the Stokes shift and in general the best results are obtained from compounds involving large shifts. It is possible for a compound to absorb (be excited) in the ultraviolet region and emit or fluoresce in the visible.

Fluorescence measurements are the most sensitive of all concentration measurements; in some situations it is possible to detect individual molecules. This is because fluorescence emission appears as a positive signal on zero background. The technique of spectrofluorometry is most accurate at very low concentrations, whereas absorption spectrophotometry is least accurate at these concentrations. The technique also allows great spectral selectivity because, due to the Stokes shift, two monochromators may be used, one for the exciting wavelength and the other for the emitted fluorescence.

Susceptibility to pH, temperature, solvent polarity and the inability to predict whether a particular compound will fluoresce, are disadvantages but the major one is the phenomenon of quenching. This occurs because energy that might have been emitted as fluorescence is lost to other molecules by collisional interaction. Quenching collisions make quantification difficult, and the method is not inherently self-calibrating. Quantification is most often done by comparisons with known concentration standard which are often difficult and expensive to prepare.

Theory behind fluorescence

Most electrons occupy the ground state at room temperature. Elevation to a higher energy level is achieved by absorption of electromagnetic energy (photons) in less

than 10^{-15}s. Electrons in this state return to the ground state in less than 10^{-8}s, the emitted energy being manifested as fluorescence. Aromatic compounds with delocalised π-electrons sometimes fluoresce. Fluorescence spectra give information about events that occur in less than 10^{-8}s.

The ratio, Q = *quanta fluoresced* quanta absorbed gives Q as the quantum efficiency and is usually independent of the exciting wavelength. At low concentrations, the intensity of fluorescence (I_f) is related to the intensity of the incidence radiation (I_o) by

$$I_f = 2.3\, I_o\, \varepsilon_\lambda\, cdQ,$$

i.e. I_f á c

where c is the concentration of the fluorescing solution (molar), d is the light path in fluorescing solution (cm), and ε_λ is the molar extinction coefficient for the absorbing material at wavelength λ ($dm^3 mol^{-1} cm^{-1}$).

The above equation has important consequences:

Q is linear for concentrations spanning many orders of magnitude in contrast to absorption spectroscopy. The operating range in absorption spectroscopy is therefore limited to 0.2 to 2.0 whereas the limit of detection of fluorescence spectroscopy is the 2 to 3 order of magnitude higher than absorption.

Instrumentation

A typical spectrofluorometer set-up is depicted in fig. 4. The major components are a light source, an excitation monochromator, the sample chamber, the emission monochromator and the detector. Note that the design shown allows both excitation and emission scans to be performed.

Light Source

An ideal light source would emit a flat spectrum over all frequencies; a Xe arc lamp is usually used. Its light is produced by inducing an electric discharge in a high pressure Xe gas that ionises the Xe atoms. The free electrons thus produced subsequently recombine with the Xe ions to produce neutral atoms during the process of which photons are emitted. Other possible sources used are quartz-tungsten-halogen (QTH) and high pressure Hg lamps. In recent years light emitting diodes are available which exhibit an incredible spectral brightness and these are set to replace all other sources in the near future.

Excitation Monochromator

This is used to spectrally narrow the excitation light to correspond to the molecular resonance of the sample under investigation. The most vital part of this instrument is the diffraction grating. It consists of a series of very narrow parallel grooves, which scatter incoming light into all directions.

Sample Chamber

The excitation light is focused on the sample cuvette, and the corresponding fluorescence is imaged at right angles to the entrance slit of the emission monochromator. Some light is split off and passed through a reference cell, the fluorescence of which is imaged onto a filtered detector, photomultiplier tube, (PMT).

Photomultiplier Tube

PMT's are extremely sensitive light detectors, capable of detecting individual

photons under optimal circumstances (Fig. 5). PMT operation is based on the photoelectric effect: Photons kick electrons from a photocathode material. These get accelerated through a series of dynodes. Each electron impact onto the dynodes produces further electrons and this cascade process results in greatly amplified signals. Different cathode materials exist to maximize the sensitivity for the wavelength region of interest. For UV sensitivity the tube material must be made of UV transmitting quartz glass. PMT's operate typically in the region from 180 nm to about 1 ¼ m.

Demerit of a PMT is dark current which is the output *generated without light* impinging on PMT detector. It is caused by *thermal* electron excitation, cosmic radiation, etc. It can be reduced by cooling of photomultiplier tube. Attention has to be paid to this aspect if signals are collected over a long period of time.

Emission Monochromator

This is used to adjust the detection spectral bandwidth to the signal line width to improve the signal to noise ratio.

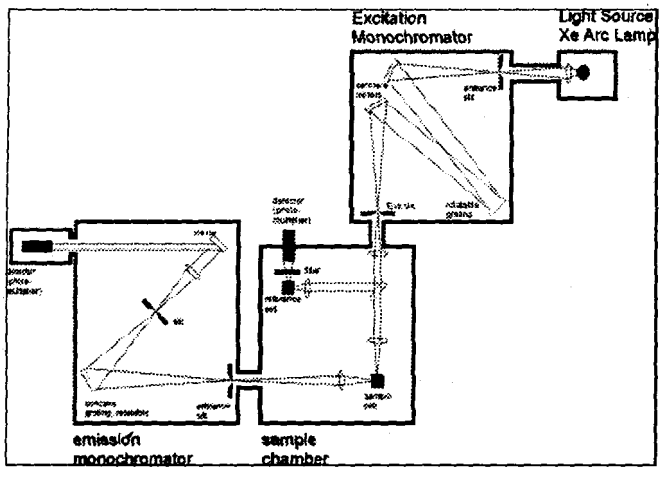


Fig 4: Schematic diagram of a spectrofluorometer

Sample preparation

Concentration: If the concentration is too high, signals are reduced by light absorption and fluorescence trapping.

Impurities: Contaminated solvents or species, which fluoresce near the analyte under investigation, can produce experimental artifacts.

Particulate scatter: Produces noise from scattered excitation light, which can be very intense

Applications

Applications of the technique are *many* and varied, despite the fact that relatively few compounds exhibit the phenomenon.

Fluorescence measurements from biochemical molecules

Intrinsic Fluorescence These occur naturally in the sample under investigation. For example proteins fluoresce when excited in the UV because of the aromatic amino acid residues (tyrosine, tryptophan, phenylalanine) (Fig 6). Tryptophan's fluorescence spectrum shifts to shorter wavelength and the intensity of the fluorescence increases as the polarity of the solvent surrounding the tryptophan residue decreases. Tryptophan residues, which are buried in the hydrophobic core of proteins, can have spectra, which are shifted by 10 to 20 nm, compared to residues on the surface of the protein. Thus fluorescence from Try is often used as a reporter to monitor protein denaturing. Other

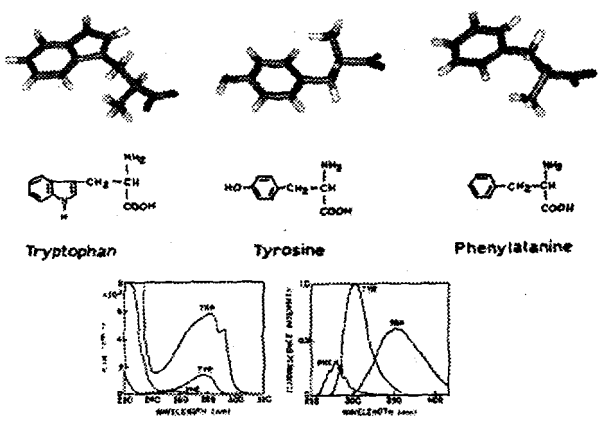


Fig 6: Structure and spectral properties of aromatic amino acid residues.

naturally occurring fluorophores are NADH, flavin, derivatives of pyridoxal, chlorophyll, and many others.

Extrinsic Fluorophores Some molecules such as DNA and lipids are devoid of any fluorescence. The detection of such compounds may be achieved by coupling a fluorescent probe (or fluor), extrinsic fluorophores. The fluor should be tightly bound at a specific site, its fluorescence should be sensitive to environmental changes and it should not have adverse effects on the system being studied. The use of such probes is valuable in both qualitative and quantitative analysis.

- Amino acids and peptides separated by chromatography or electrophoresis can be identified by coupling to their primary amino groups either dansyl chloride or o-phthalaldehyde. The latter conjugates fluoresce intensely blue and the total oligopeptide fingerprint may be determined at only 10^{-5}g of protein.
- Acridine orange is an extrinsic fluor that can be used to determine the strandedness of polynucleotides conjugates of single and double-stranded polynucleotides, which fluoresce red and green, respectively.
- Modern DNA sequencers are based on selective labeling with extrinsic fluorophores. Individual nucleic acids (AGCT) are labeled with fluorophores of 4 different colors. The type of nucleic acid passing through the sequencer can thus be identified from the fluorescence spectrum of its extrinsic fluorophore.
- Quantitative determination of materials present in concentrations too low for absorption spectrophotometry can be made. Assays of vitamin B_1 in foodstuffs, NADH, hormones, drugs, pesticides, carcinogens, chlorophyll, cholesterol, porphyrins and some material ions can be done.
- Ca^{2+} can be measured in the cytoplasm by the chelating agent Quin-2, which preferentially binds the metal. The fluorescence increases about five-fold on binding. More sensitive probes for this analysis are Fura-2 and Indo-1. Quin-1 is also used as a fluorescent probe to monitor intracellular pH changes in the range 5 to 9. Over this range, there is a 30-fold increase in fluorescence.

Enzyme assays and kinetic analysis

- Group specific hydrolytic enzymes hydrolyze ether or ester derivatives of the flour 4-methylumbelliferone

to produce the anion of the flour, which fluoresces at 450 nm. Its rate of appearance and kinetics may be monitored by fluorescence measurement.

- It is possible to detect as low as one molecule of β-galactosidase when it acts on fluorescein bis (β-D-galactopyranoside) as substrate. Hence actual numbers of molecules in a single bacterial cell may be determined.
- Spectrofluorometry can be applied in metabolic studies where NAD forms are involved as cofactors because NADH and NADPH fluoresce, whereas the oxidized equivalents do not.

Membrane structure

- The fluorescent properties of a molecule are affected by its mobility and polarity of the environment. Various probes have charged and hydrophobic regions (2-anilino-6-naphthalene sulphonate (ANS) and N-methyl-2-anilino-6-naphthalene sulphonate (MNS)) and hence are able to orient themselves across lipid/aqueous interfaces. These probes may be used to study membrane structure and gain information about the properties of such interfaces.
- Incorporation of phospholipids containing 12-(9-anthroanoyl)-stearic acid and 2-(9-anthroanoyl)-palmitic acid into membranes yields information about membrane structure, the effects of temperature and certain biological phenomena.
- Changes in mitochondrial membranes during energy transduction have also been monitored using an ANS probe.

Fluorescence bleaching recovery

If a fluor is exposed to a pulse of high intensity radiation it may be irreversibly bleached. Fluorescently labeled phospholipids incorporated into a biological membrane may be subjected to this treatment and then the motion of such entities (in the membrane) can be studied by monitoring (with low intensity radiation) the re-emergence of fluorescence as the bleached and unbleached molecules interdiffuse. Applications include study of

- the lateral motion of extrinsically labeled rhodopsin in the photoreceptor membrane,
- polymerization of proteins such as actin
- diffusion of fluorescently labeled proteins microinjected into cells.

Energy transfer studies

Energy may be transferred, from a donor to an acceptor fluor, provided there is overlap between the donor fluorescence spectrum and the acceptor absorption spectrum. The transfer efficiency is related to spatial separation. This efficiency can be measured as quenching of the donor fluorescence by acceptor. Intrinsic or extrinsic fluors or fluorescent analogues of substrates, inhibitors, cofactors or phospholipids may be employed in energy transfer experiments to deduce distances within protein molecules. Accuracy is limited to about ±0.5 nm and determinations include

- localization of metals in

metalloproteins,

- measurement of conformational changes in enzymes during substrate binding
- the distances between various pairs of proteins in the ribosome
- the three-dimensional structure of transfer RNAs.

Microspectrofluorimetry

Here a microscope is combined with a spectrofluorometer equipped with fibre optics.

- Enables the examination of single bacterial cells binding fluorescent antibodies and also the fluorescent intensity of subcellular structures.
- The extra amount of nucleic acid that tends to be present in malignant cells will take up more of the fluorecent probe acridine orange than do normal cells. This observation may be used to detect malignant cells in biopsy tissue.

The fluorescence-activated cell sorter

This system makes use of the light emitted by cells carrying a fluorescently labeled antibody to trigger their physical separation from unlabelled cells as they flow through a fine capillary.

Fluorescence immunoassay

One of the most established ways to obtain very specific labels is to label highly specific antibodies with fluorophores. Purified versions of the molecules to be tagged are injected into vertebrates which cause an immune response in the animal. As a result antibodies are produced in the animals which are completely specific to the analyte molecule. The antibodies are extracted and purified from the blood serum and labeled with suitable fluorophores. To see whether an unknown sample contains traces of the analyte molecules, the labeled antibodies are mixed with it and rinsed off. Only the antibodies which attach to their "targets" will show up in fluorescence. The strength of the signal is directly proportional to analyte concentration. This is the principle of immunoassay.

Inductively Coupled Plasma Atomic Emission Spectrometry (ICP- AES)

Atomic Emission Spectrometry (AES) is based on the principle that during reversion to the ground state an excited atom or ion releases absorbed energy as light (photons) of characteristic wavelengths, the positions and intensities of which can be measured. The energy transfer for electrons when they fall back to ground state is unique to each element as it depends upon the electronic configuration of the orbitals. The energy transfer is inversely proportional to the wavelength of electromagnetic radiation,

$$E = hc/\lambda$$

(Where h is Planck's constant, c the velocity of light and λ is wavelength), and hence the wavelength of light emitted is also unique.

The wavelengths used in AES ranges from the upper part of the vacuum ultraviolet (160 nm) to the limit of visible light (800 nm). As borosilicate glass absorbs light below 310 nm and oxygen in air absorbs light below 200 nm, optical lenses and prisms are generally fabricated from quartz glass and optical paths are evacuated or filled by a non-absorbing gas such as Argon. Plasma can be defined as luminous volume of partially ionized gas.

In Inductively Coupled Plasma (ICP) the plasma is generated from radio frequency magnetic fields induced by a copper coil which is wound around the top of a quartz torch. High frequency current flows in the coil generating a rapidly varying magnetic field within it. If the charged particles flow through the field, cutting the magnetic lines of force, ohmic heating results. It is the heat resulting from this interaction that generates the ICP flame. The inductive heating effect maintains the ICP flame at a temperature of 6000 K and upto 10000 K at its hottest point. Samples are injected into the centre of the plasma toroid and are heated rapidly to around 8000 K by conduction, convection and radiation effects. The sample molecules undergo instantaneous desolvation, vaporization, dissociation, ionization and excitation. ICP-AES is less susceptible to interferences. This is because of the high temperature at which no chemical bond survives causing complete atomisation of the analyte solution entering the ICP. The extreme temperature generates strong emission lines for most of the elements of the periodic table. This method is especially suited to many of the refractory elements, rare earths etc. High temperatures ensure complete dissociation and recombination and hence the interferences are negligible. Since each atom emits a photon of characteristic wavelength, we can not only identify the species from the photon energy (qualitative analysis) but also determine the amounts of each element (quantitative analysis) from intensity of the light emitted. It is possible to achieve a linear calibration range extending over five orders of magnitude of analyte concentrations.

Applications

Inductively Coupled Plasma Atomic Emission Spectroscopy is a versatile method for estimation of elements. It finds use in many fields including inorganic chemistry, bio-inorganic chemistry, pharmaceutical industries, biological sciences, geology, oceanography, food industries, polymer industries, pesticide industries, environmental studies and pollution monitoring of water and air and catalyst industries.