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Programme: M.Sc., Biochemistry

Course Title: Analytical Biochemistry

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Unit V

Fluorimetry

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Fluorimetry

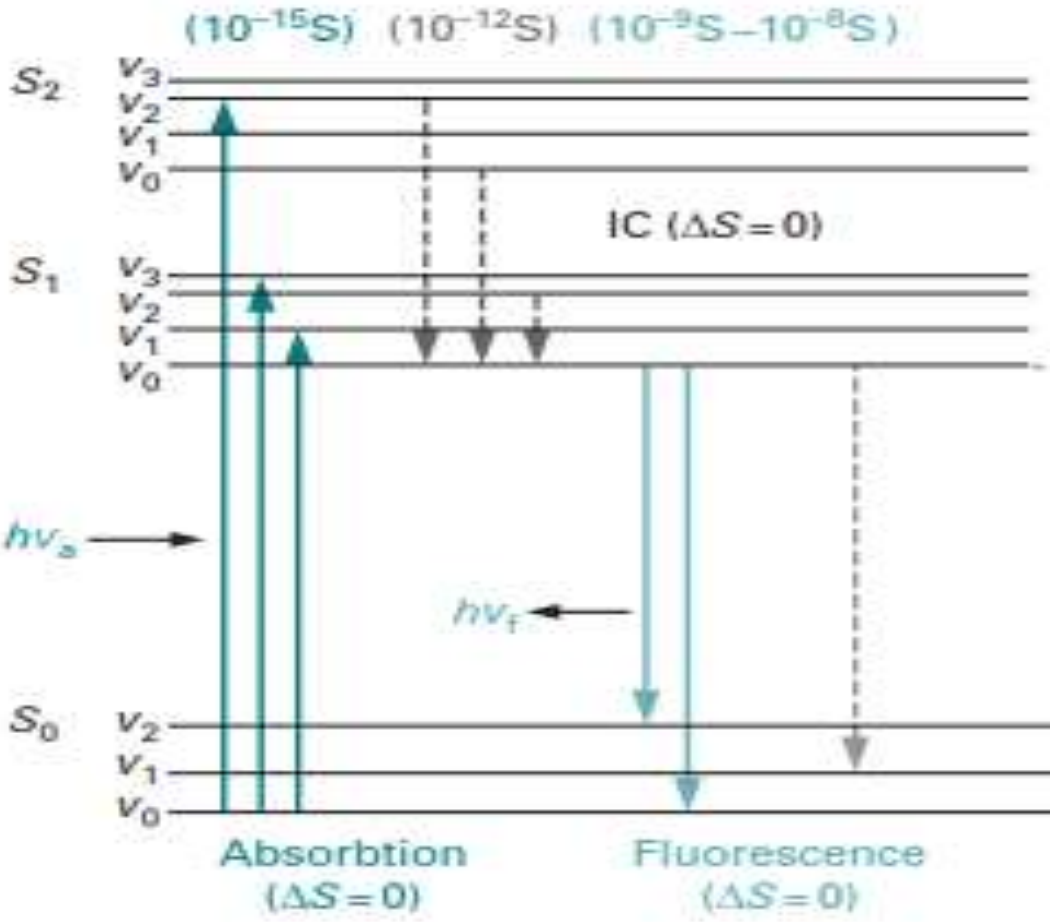
- **Principle:** Fluorescence is the phenomenon where by a molecule, after absorbing radiation emits radiation of longer wavelength. At room temperature, most organic molecules are in the ground state. When a beam of light is passed, absorption of photons elevates an electron in the molecules to a higher energy state in less than 10^{-15} sec. After absorption, energy is lost very rapidly by collision degradation from high energy state to the lowest ground state in less than 10^{-8} sec. The energy emitted by these molecules in regaining the ground state within 10^{-8} sec gives rise to a fluorescent peak. The intensity of fluorescence varies with the concentration of the solute.

- The intensity of fluorescence is given by the following calculation.
- $I_f = I_o\ 2.3\varepsilon\ cdQ$ ie $I_f \alpha\ c$
- I_f – Intensity of fluorescence
- I_o – Incident radiation
- Q – Quantum efficiency
- ε – Molar extinction coefficient
- c – molar concentration of absorbing solution
- d – light path in the absorbing material in centimeters.

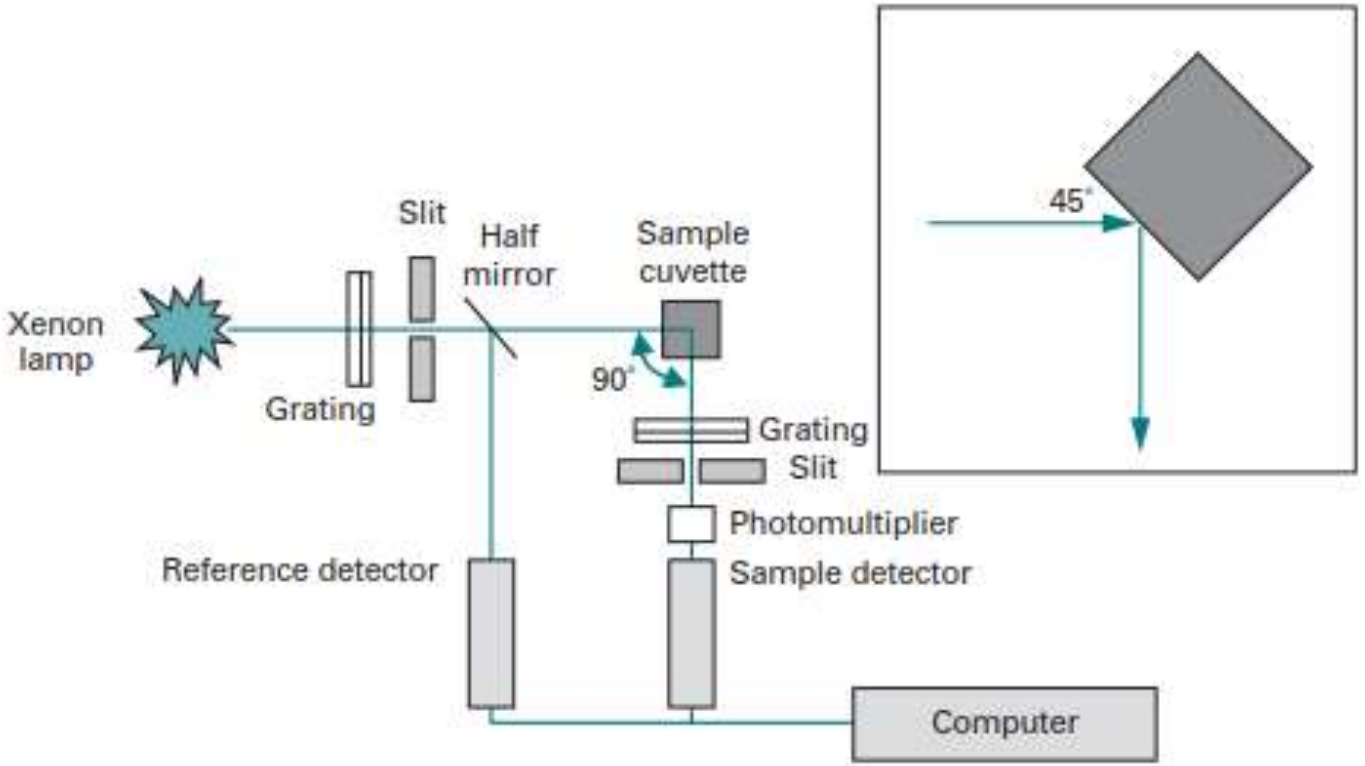
Stokes shift

- Stokes shift: Fluorescence is a phenomenon whereby a molecule, after absorbing radiation, emits radiation of a longer wavelength. This increase in wavelength is known as the Stokes shift. Example: if a compound absorb radiation in the UV region and emit visible light.

Fluorescence



Instrumentation



Schematics of a spectrofluorimeter with 'T' geometry (90°). Optical paths are shown as green lines. Inset: Geometry of front-face illumination.

Applications

- Applications: Fluorimetry applications are extended by chemical modifications such as oxidation, reduction, hydrolysis, coupling and self condensation. For example, Morphine is measured by oxidizing it to pseudomorphine which fluoresces. B – adrenergic blocking agents may be detected fluorimetrically on a thin layer chromatography plate by coupling them to dansyl chloride. Tetracycline when bound to calcium fluoresces, used to detect gastric ulcers and intestinal carcinomas.

- Qualitative analysis: The determination and comparison of both excitation and fluorescence spectra of a compound may help to identify it. The effect of pH and solvent composition on the fluorescence of a compound, and the polarization of its fluorescence may provide information about its structure.

- Quantitative analysis: Substances at very low concentrations can also be identified and measured by fluorimetry.
- 1. Vitamin B1 in food stuffs assayed.
- 2. NADH in intact mitochondria and microorganisms under different metabolic conditions.
- 3. Hormones such as cortisol and oestradiol, drugs such as lysergic acid and barbiturates in blood can be assayed.
- 4. Organophosphorous pesticides in soil and animal tissues.
- 5. Carcinogen in tobacco smoke.
- 6. Assay of chlorophyll, cholesterol, porphyrins and some metal ions.

- Extrinsic fluorescence: Detection of non fluorescent compound by coupling with fluorescent probe is known as extrinsic fluorescence.
- Nucleic acid by acridine orange. Amino acid and proteins by dansyl chloride or O – phthalaldehyde. Ca^{++} in cytoplasm is measured by chelating agent Quin -2 which binds the metal. Fluorescence increases 5 fold. Quin I is a chelating agent used as a fluorescent probe to monitor intracellular pH changes in the range of 5 – 9.

- Enzyme assays and kinetic analysis: Group specific hydrolases may be readily assayed by measuring the rate of appearance of fluorescence at 450nm of the anion of 4 – methyl umbelliferone when the enzyme acts upon on ether or ester derivative of 4 – methyl umbelliferone.

- NAD+ and NADP+ linked reactions: Since NADH and NADPH fluoresce, numerous metabolic reactions that are coupled to the oxidation of NADH or NADPH, or the reduction of NAD+ or NADP+ are studied by fluorimetry. The kinetics of oxidation and reduction of the endogenous material in intact organelles or cells may be measured, eg mitochondrial NADH.

- Studies on protein structure: Since some proteins contain fluorescent chromophores (eg, tyrosine and FAD), they fluoresce. The binding of substances such as inhibitors, coenzymes and allosteric effectors to them can be measured by the changes in their fluorescence spectra. This further may give information about the conformation and polymerization of the proteins.

- Studies on membrane structure: The fluorescent properties of a molecule vary with its mobility and polarity of its environment, monitoring such properties yields information about the environment of a fluorescent molecule or probe.Various fluorescent probes are designed to study the membrane structure.

- Anilinonaphthalene – 8 – sulphonate (ANS), N-methyl-2-anilino-6-naphthalene sulphonate (MNS) contain both charged and hydrophobic areas and locate at the water – lipid interface. By incorporating phospholipids containing 12 – (9-anthroanoyl) – stearic acid and 2-(9-anthroanoyl) – palmitic acid into membranes, information about the region 0.5 and 1.5nm respectively from the phosphate headgroups of the lipid bilayer may be obtained. Such experiments provides the basic structure of biological membranes, effect of temperature on membrane structure and structural changes occur in mitochondrial membranes during energy transduction.

- Microspectrofluorimetry: Combination of spectrofluorimeter with a microscope allows the subcellular location of fluorescent compounds. For eg. nucleic acids may be detected in subcellular structures within living cells using acridine orange. It is used to detect malignant cells which tend to contain more nucleic acid than normal cells.

References

- Principles and Techniques of Biochemistry and Molecular Biology 7th edition.Eds Keith Wilson and John Walker, 2010.
- Introductory Practical Biochemistry. Eds Sawhney SK and Randhir Singh, 2000.