

## **Section B and C**

### **Volume-29**

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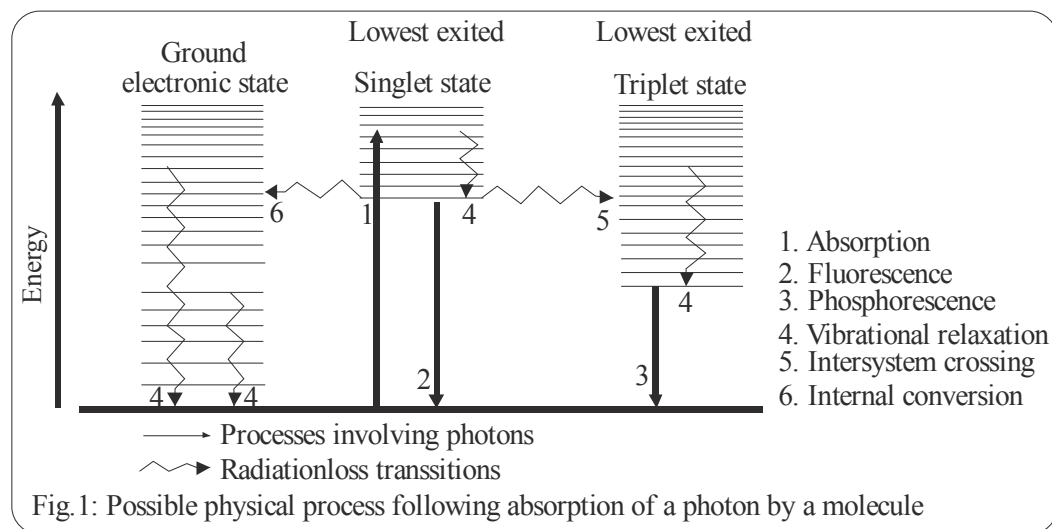
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## 13- METHODS IN BIOLOGY

### C. BIOPHYSICAL METHODS

#### 1. Ultraviolet and Visible Absorption Spectroscopy

Matter can capture electromagnetic radiation and convert the energy of a photon into internal energy. This process is called absorption. We describe the energy change of the absorber as a transition or an excitation from a lower energy level to a higher energy level. Since the energy levels of matter are quantized, only light of energy that can cause transitions from one level to another will be absorbed. An absorption spectrum is the absorption of light as a function of wavelength. The spectrum of an atom or molecule depends on its energy-level structure, making absorption spectra useful for identifying compounds.



#### Instrumentation

The UV-VIS spectral range is approximately 190 to 900 nm, as defined by the working range of typical commercial UV-VIS spectrophotometers. The short-wavelength limit for simple UV-VIS spectrometers is the absorption of ultraviolet wavelengths less than 180 nm by atmospheric gases. The light source is usually a deuterium discharge lamp for UV measurements and a tungsten-halogen lamp for visible and NIR measurements. The instruments automatically swap lamps when scanning between the UV and visible regions. The wavelengths of these continuous light sources are typically dispersed by a holographic grating in a single or double monochromator or spectrograph. The spectral band-pass is then determined by the

monochromator slit width or by the array-element width in array-detector spectrometers. Spectrometer designs and optical components are optimized to reject stray light, which is one of the limiting factors in quantitative absorbance measurements. Most commercial UV-VIS absorption spectrometers use one of three overall optical designs: a fixed or scanning spectrometer with a single light beam and sample holder, a scanning spectrometer with dual light beams and dual sample holders for simultaneous measurement of the reference and test samples, or a non-scanning spectrometer with an array detector for simultaneous measurement of multiple wavelengths. In single-beam and dual-beam spectrometers, the light from a lamp is dispersed before reaching the sample cell. In an array-detector instrument, all wavelengths pass through the sample and the dispersing element is between the sample and the array detector.

### **Beer-Lambert Law**

Measuring the concentration of an absorbing species in a sample is accomplished by applying the Beer-Lambert law. Transmittance is the ratio of the intensities of the transmitted light ( $I$ ) to the incident light ( $I_0$ ). According to the Beer's law, transmittance ( $T$ ) is inversely proportional to the concentration ( $c$ ) of the solute and the Lambert's law says that the transmittance is inversely proportional to the path length ( $l$ ). Combining these two, we get

$$I = I_0 10^{-\epsilon \lambda c l}$$

$$\log (I / I_0) = -\epsilon \lambda c l$$

$$\log (I_0 / I) = \epsilon \lambda c l$$

where  $\epsilon \lambda$  a wavelength-dependent molar extinction coefficient,  $l$  is the path length (cm) and  $c$  is the analyte concentration (M).  $\epsilon \lambda$  has units of  $M^{-1} \text{ cm}^{-1}$ . Modern absorption instruments can usually display the data as either % transmittance ( $100 \times I_0/I$ ) or Absorbance ( $\log(I_0/I)$ ). When  $d = 1 \text{ cm}$ ,  $A$  is commonly called optical density (OD). The linear relationship between concentration and absorbance is both simple and straightforward, which is why we prefer to express the Beer-Lambert law using absorbance as a measure of the absorption rather than %T. Measuring the amount of light that a sample absorbs and applying Beer's law can determine an unknown concentration of an analyte. If the extinction coefficient is not known, the unknown concentration can be determined using a working curve of absorbance versus concentration derived from standards.

The linearity of the Beer-Lambert law is limited by chemical and instrumental factors. Some of the factors resulting in the deviation of linearity include:

- (1) Deviations in absorptivity coefficients at high concentrations due to interactions between molecules in close proximity
- (2) Scattering of light due to particulates in the sample,
- (3) Fluorescence or phosphorescence of the sample,
- (4) Changes in refractive index at high analyte concentration,
- (5) Shifts in chemical equilibria as a function of concentration,
- (6) Non-monochromatic radiation, deviations can be minimized by using a relatively flat part of the absorption spectrum such as the maximum of an absorption band and
- (7) Stray light.

### **Probing the Structure by Spectroscopy**

Proteins contain several chromophores that absorb light in the ultra-violet region. The most important chromophores in proteins are the peptide bonds, aromatic rings of Phe, Tyr and Trp and in nucleic acids, purine and pyrimidine bases. UV absorbance is a useful probe of structure and structural changes. This is due to the fact that chromophores display shifted spectra upon increasing or decreasing polarity of their environment, with changes in wavelength of maximum absorbance ( $\lambda_{max}$ ) and possible molar extinction coefficient.

Some of the empirical rules for the interpretation of absorption spectra of biological molecules: the  $\lambda_{max}$  and  $\epsilon$  of Tyr, Trp, Phe and His increase if they are exposed to less polar environment (Be sure your less polar solvent does not denature the proteins); the  $\lambda_{max}$  and  $\epsilon$  of titratable groups (OH of Phe, imidazole of His, SH of Cys) increase if they are ionized; stacking of nucleic acid bases decreases the  $\epsilon$ .

### **Quantitation of Tryptophan and Tyrosine**

It is often very difficult to accurately determine the number of Trp and Tyr residues in a protein. Conventional amino acid analysis frequently underestimates the contents of these two important amino acids. A useful and simple approach is the use of 4<sup>th</sup> derivative Spectroscopy of unfolded proteins. In this approach the fourth derivative of the UV-spectrum is calculated .....