

## GENERAL BIOLOGY LABORATORY II



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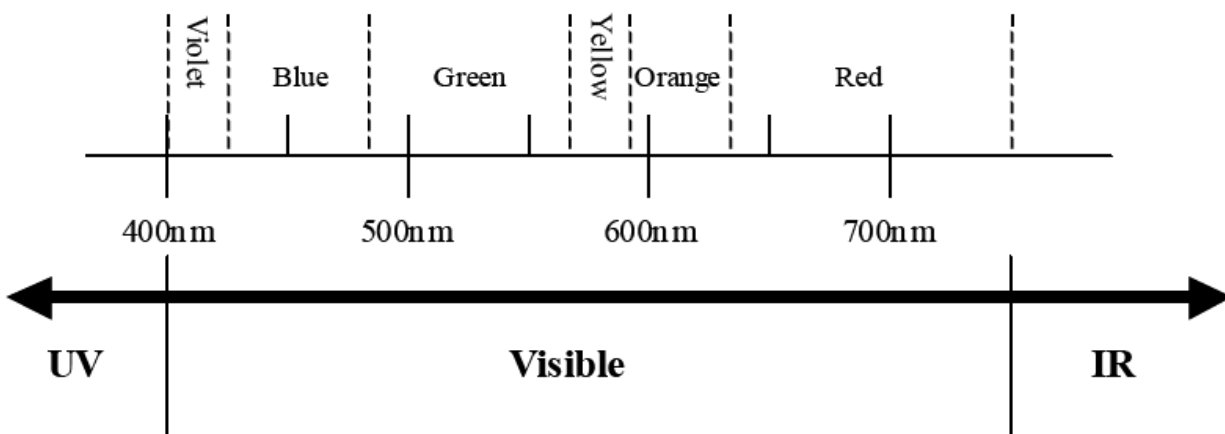
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Week 10

### **Spectrophotometry: Dance of light with molecules**

In spectrophotometry, we use light at different wavelengths to measure the amount of a sample (e.g., proteins, nucleic acids, small organic molecules, etc). In other words, spectrophotometry is the quantitative measurement of reflection or transmission properties of a substance as a function of wavelength. Spectrophotometry is one of the most useful methods of quantitative analysis in various fields such as biology, chemistry, physics and clinical and industrial applications. In previous lab (i.e., *Lowry Assay of proteins*) you found the concentration of a protein sample in a solution utilizing a standard curve. Here, as you remember we have treated protein solution with certain reagents (e.g., copper sulfate, Folin reagent) to stain them and then looked to their absorbance characteristic under appropriate visible light spectrum (around 600 nm).



However, at certain wavelengths (e.g., in ultraviolet range) the concentration of many biological molecules in solution can be measured without any extensive manipulation. For example, nucleic acids characteristically absorb light maximally at 260 nm, while proteins do so at 280 nm, and NADH at 340 nm. To relate the concentration to the absorbance at UV range is the simplest and most direct assay method for proteins, nucleic acids and other characteristic compounds such as NADH.

Amino acids with aromatic side chains (i.e., tyrosine, tryptophan and phenylalanine) exhibit strong UV-light absorption at 280 nm. Although, peptide bonds absorb light strongly at 190 nm, 280 nm is the characteristic standard wavelength to measure protein concentration in a solution.

- Without interfering substances, 1  $A_{280}$  for a pure protein solution is considered to have about 1 mg protein/ml solution. That is;

$$1 A_{280} \cong 1 \text{ mg/ml}$$

For example, if the absorbance of our protein solution at 280 is 0.24, the protein concentration is about 0.24 mg protein/ml.

If we have a partially purified protein solution in which nucleic acids are also present;

$$1.55 (A_{280}) - 0.76 (A_{260}) = \text{mg/ml protein}$$

Let say the absorbance of protein sample at 280 is 0.48 and at 260 is 0.09. The protein concentration in this mixture,

$$1.55 (0.48) - 0.76 (0.09) = 0.68 \text{ mg/ml protein}$$

Once we know the protein concentration of unknown by such a rough method, now we can determine its concentration in a more specific and sensitive assay such as the Lowry assay.

- Similarly,  $1 A_{260}$  for a DNA solution is considered to have about 50 microgram ( $\mu\text{g}$ ) DNA/ml solution and  $1 A_{260}$  for a RNA solution is considered to have about 40 microgram ( $\mu\text{g}$ ) RNA/ml solution. That is;

$$1 A_{260} \cong 50 \mu\text{g/ml DNA}$$

$$1 A_{260} \cong 40 \mu\text{g/ml RNA}$$

For example, if the absorbance of a DNA solution at 280 is 0.64, DNA concentration is about 32 mg DNA /ml.

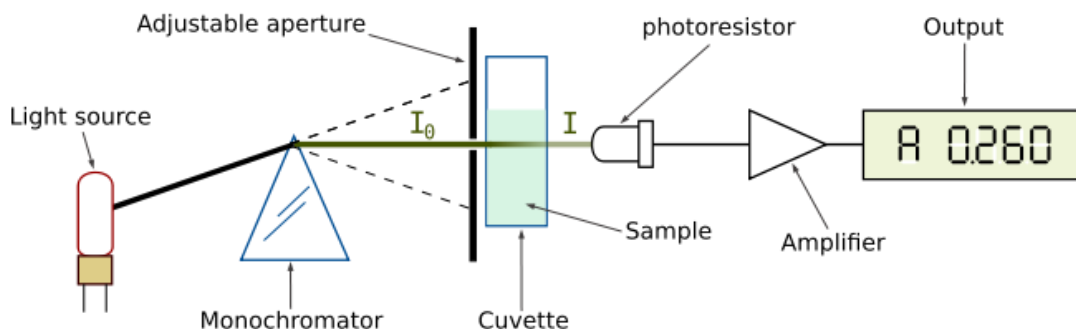
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Objectives of this laboratory are (1) to make you become familiar with operating the spectrophotometer, an instrument that operates by passing a beam of light through a sample and measuring the intensity of light reaching a detector, (2) how to use the spectrophotometer in determining the absorption spectrum of a compound in solution and in constructing a standard curve, (3) to use the absorption spectrum to calculate the molar extinction coefficient of compound (e.g., NADH) at its absorbance maximum, and (4) to use the Beer-Lambert Law to calculate the concentration of compound in solution from the standard curve.

the Beer-Lambert Law states that absorbance ( $A$ ) of a compound, its concentration ( $c$ , generally Molar) and its characteristic absorption coefficient (sometimes called extinction coefficient,  $\epsilon$ ) is related:

$A = \epsilon cl$  where  $l$  is the the light path which is 1 cm.

## Week 10: Spectrophotometry



To measure the concentration of an unknown sample, first the intensity of light ( $I_0$ ) passing through a blank is measured. The intensity is the number of photons per second. The blank is a solution that is identical to the sample solution except that the blank does not contain the solute that absorbs light. Second, the intensity of light ( $I$ ) passing through the sample solution is measured. Third, the experimental data is used to calculate two quantities: the transmittance ( $T$ ) and the absorbance ( $A$ ) which are related as shown below:

$$T = I/I_0$$
$$A = -\log T$$
$$\log(I_0/I) = \epsilon cl$$
$$A = \epsilon cl$$

The transmittance is simply the fraction of light in the original beam that passes through the sample and reaches the detector. The remainder of the light,  $1 - T$ , is the fraction of the light absorbed by the sample.

In most applications, the amount of light absorbed is related to the concentration of the light absorbing molecule and the absorbance rather than the transmittance is used to find out the concentration. If no light is absorbed, the absorbance is zero (100% transmittance). Each unit in absorbance corresponds with an order of magnitude in the fraction of light transmitted. For  $A = 1$ , 10% of the light is transmitted ( $T = 0.10$ ) and 90% is absorbed by the sample. For  $A = 2$ , 1% of the light is transmitted and 99% is absorbed. For  $A = 3$ , 0.1% of the light is transmitted and 99.9% is absorbed.

### State in your report

1. What are the similarities and differences between direct spectrophotometric readings at UV range and spectrophotometric readings at visible range?
2. Why did we use the 280 nm wavelength before we start the Lowry method?
3. Calculate the concentrations of your unknowns from the standard curve you have constructed, taking into account your dilutions of original sample.

### Problems to solve

1. If  $\epsilon l = 0.347$  liter/mole, what is the concentration if;
  - a. the absorbance is 0.362?
  - b. the % transmittance is 63.2?

2. If the transmittance is 50.8% in a 1 cm cuvette, what is the absorbance in a 3 cm cuvette?
3. A 1 mg/ml protein solution transmits 60% of light in a 1 cm cuvette in 280 nm. What is the transmission of the same protein when its concentration is 0.5 mg/ml, 2 mg/ml, 4 mg/ml?
4. NADH absorbs UV strongly at 340 nm. This coenzyme has specific extinction coefficient ( $\epsilon$ ) of 6220. What is the concentration of a NADH solution with  $A_{340}$  0.32.
5. If a compound at 2 mg/ml has 0.2 absorbance and 4 mg/ml has 0.4 absorbance at 280 in a 1 cm cuvette, find out the % transmission of these solutions.
6. Find the molar absorption coefficient ( $\epsilon_M$ ) of 0.5 M solution whose absorbance in a tube of path length 2 cm is 0.42.
7. A solution contains 2mg/ml of a light absorbing compound in a cuvette with 1 cm light path and transmits 75% of incident ( $I_o$ ) light at a certain wavelength. Find the transmission of the solution containing (a) 1 mg/ml, (b) 4 mg/ml, and (c) if the molecular weight of compound is 250 (that is 250 g/mol) calculate the molar absorption coefficient ( $\epsilon_M$ ) of this compound [tip: if concentration is expressed in molar,  $\epsilon$  is molar extinction coefficient (i.e.,  $\epsilon_M$ ). If concentration is expressed in g/l,  $\epsilon$  is specific extinction coefficient (i.e.,  $\epsilon_S$ ) and  $\epsilon_M = \epsilon_S \cdot \text{MWt}$ ].
8. A  $10^{-5}$  M ATP solution has a transmission of 0.54 (54%) at 260 nm in a 1 cm cuvette. (a) Calculate absorbance of this solution and (b) calculate the absorbance and transmittance of this solution in 2 cm cuvette.