Experiment 1
(Lab period 1)

Spectrophotometry: Absorption spectra and the use of light absorption to measure concentration

Spectrophotometry is a procedure that is frequently utilized in biological laboratories. Probably the most common application in biology of this technique is in the measurement of the concentration of a compound in solution. In this lab you will be introduced to the concepts of spectrophotometry as well as how it is used to measure the concentration of compunds in solution. Over the rest of the course you will apply spectrophotometry on several occasions.

Spectrophotometry is the measurement of the interaction of light with matter. Many kinds of biological substances absorb visible light (400 to 700 nm) selectively and, as a result, appear colored. Substances that do absorb visible light are called pigments. Other substances do not absorb light of visible wavelengths and appear colorless. We can study colorless compounds in two different ways. One is that they can often react with other substances to form colored derivatives that we can see and measure (as you will see later on in this course). Another is that colorless compounds usually absorb light in the region of the spectrum that is not visible to the naked eye. Absorption of this light can be measured, even if we cannot observe it unaided.

Using a spectrophotometer, which measures the absorption by a solution of light of specific wavelengths (visible or not), allows us to determine concentration as discussed below. A second application of spectrophotomerty is the determination of the absorption spectrum of a compound. (Both of these can be applied to colorless as well as colored solutions since a spectrophotometer can measure absorbance of light that we cannot see.) These two applications are discussed separately below.

Absorption spectra and color

Regardless of whether a solution is colorless or colored, the wavelength(s) absorbed are distinctive. A solution of a particular compound, such as hemoglobin, always absorbs light of specific wavelengths and reflects light of other wavelengths. Furthermore, absorption of light is not absolute. A compound will characteristically absorb a certain proportion, anywhere from 0% to 100%, of light of a specific wavelength. For every compound, if you measure the proportion of light absorbed, for any wavelength, you will always get the same answer. This is the basic idea of the absorption spectrum of a specific compound, which is the proportion of light absorbed for each wavelength of the spectrum.

The wavelength(s) absorbed by a substance in the visible part of the spectrum is complementary to the color that we perceive. The "color" is a function of human perception, but absorption of specific wavelengths of light is a function of molecular interaction with light. If a substance absorbs blue and red light, but not green light, we will see it as green since that is the only light that reaches us from that substance. Therefore, when you look at the absorption spectrum of a green solution, it should show low absorbance of wavelengths in the green part of the spectrum but high absorption of light in the red and blue parts of the visible spectrum.

While the "color" of a substance depends on the person observing it, absorption of specific wavelengths depends on the molecular structure of the substance. This allows qualitative

analysis (i.e., identification) of some substances by determination of the absorption spectrum. In this lab you will determine the absorption spectrum of a red pigment, hemoglobin.

Measuring concentration

The basis of this application of spectrophotometry is that the proportion of light that is absorbed by a solution of a particular compound is a function of the concentration of that compound. This allows for a quantitative analysis of concentration of a substance from the Beer-Lambert relationship (below). A spectrophotometer will direct light of a specific wavelength on your solution. This light is the incident light. The light that passes through the solution is the transmitted light. The absorbance (A) of the solution is the log of the ratio of these two measures:

A (Absorbance, or Optical Density) = $\log_{10}$ (Intensity of incident light / Intensity of transmitted light)

The spectrophotometer will calculate and display the absorbance. Once we know the absorbance, concentration of the solution follows from the **Beer-Lambert equation**:

$$A = E * C * L$$

in which:

- **E** (Molar Absorption) = absorbance of a l M solution of the substance measured through a l-cm light path. This is a constant for the substance at a given wavelength.
- **C** = concentration, in moles/liter.
- **L** = length of the light path through the solution, in cm. For the spectrophotometer we will be using, L is equal to 1 cm.

Therefore, *since L equals 1*, **C = A / E.**

In order to apply the Beer-Lambert equation you must know the Molar Absorption Value (E) for the substance (compound) and wavelength you are using. This is defined as the Absorbance of a 1 M solution so it can be measured easily by the obvious - reading Absorbance of a 1 M solution. However, for substances with strong absorbance, the Absorbance of a l M solution is too great to read in an analytical instrument. In this case a standard curve is produced by measuring Absorbance of a number of dilute solutions, each of known concentration. *When Absorbance is plotted against concentration the slope of the line is the relationship between concentration and Absorbance*. This is the E value (E=A/C, if L=1) so the slope of the standard curve gives you E. Once you have measured E, you can find the concentration of any solution (of that compound) by measuring Absorbance at the *same* wavelength used to calculate E.

In this lab, you will examine both of these aspects of spectrophotometry. First, you will determine the absorption spectrum of hemoglobin by measuring the absorbance of light of different wavelengths. Second, you will construct a standard curve for protein serum and

calculate E for this compound. Then, using this value you will determine the concentration of various dilutions of a serum solution and then calculate the concentration of the serum solution.

The analytical instrument you will use is a spectrophotometer. As mentioned above, a spectrophotometer measures the intensity of light transmitted through a solution. It consists of two principal parts: a spectrometer and a photometer. Using a white light source and a monochromator (a prism), the spectrometer of the instrument is designed to provide discrete wavelengths of light at a known intensity. The photometer consists of a photoelectric tube sensitive to the wavelengths of light provided by the spectrometer and a galvanometer to quantitate the intensity of the light. The sample to be measured is inserted between the spectrometer and the photometer. By comparing the intensity emitted by the spectrometer to the intensity measured by the photometer, absorbance by the solution can be calculated by the machine.

Procedure

A - ABSORPTION SPECTRUM OF HEMOGLOBIN

Almost all of the oxygen carried in the blood is combined with hemoglobin within the red blood cells. Hemoglobin is composed of a protein (globin) to which the red, iron-containing heme molecule is attached. The absorption spectrum of hemoglobin in the visible range is characteristic of many heme-containing proteins.

l. Prepare and label two cuvettes:

Cuvette l containing 4 ml distilled water

Cuvette 2 containing 4 ml blood diluted in distilled water

Cuvette l is the reference cuvette, or blank; cuvette 2 is the sample cuvette. The reference cuvette is used to 'Zero' the spectrophotometer. This means that you adjust the Absorbance reading to zero while the reference is in the spectrophotometer. As a result, when you put your sample into the spectorphotometer, the Absorbance measures only absorption by the compound in solution, not absorption by the solvent (see Appendix 1 on operating the Spectrophotometer).

2. Read the Absorbance of the sample of diluted blood every l0 nm from 400 to 700 nm. Wherever Absorbance rises significantly, read at 5 nm intervals. Record your data in your notebook.

3. Place a strip of white paper in a cuvette and place the cuvette in the sample holder. Leaving the cover open, rotate the wavelength control slowly from 650 nm to 400 nm. Record the wavelengths at which the color of the incident light clearly changes color; you should see a spectrum from dark red light to pale violet.

B - BIURET COLORIMETRIC ASSAY OF SERUM PROTEIN

Proteins react with copper ions in alkaline solution to form a violet-colored complex that absorbs light at 550 nm. This reaction is the basis of the biuret assay for protein. Biuret reagent is a solution of $CuSO_4$ in NaOH. When you add biuret reagent to a protein solution the reaction produces a solution of the protein-biuret complex in a concentration that is the same as the original concentration of protein. Therefore, by measuring the concentration of the complex, using A_{550} (Absorbance at 550 nm), you are also measuring the concentration of protein.

The assay consists of two parts that will be conducted simultaneously. The first part consists of establishing a standard curve by using several known quantities of a pure protein. In this experiment bovine serum albumin (BSA) will be used as the protein to be reacted with the biuret reagent. Since all proteins react with the biuret agent in the same manner, this standard curve will give us an E value that can be applied to any protein solution reacted with biuret.

The second part consists of reacting measured quantities of serum (protein) with the biuret reagent and measuring the Absorbance of each reaction mixture.

l. Prepare and label eleven test tubes as shown in the table. Use one l-ml pipet to dispense the NaCl solution to all tubes, then a second l-ml pipet to dispense the BSA solution, and a third l-ml pipet to dispense the serum.

Standard curve

Tube	Volume of 0.9% NaCl (ml)	Volume of 10mg/ml BSA (ml)
1(Blank)	1.0	0
2	0.9	0.1
3	0.8	0.2
4	0.6	0.4
5	0.4	0.6
6	0.2	0.8
7	0	1.0

Serum measurement

Tube	Volume of 0.9% NaCl (ml)	Volume of serum sample
1a	0.9	0.1
2a	0.8	0.2
3a	0.7	0.3
4a	0.6	0.4

2. When the eleven tubes have been prepared, mix the contents of each tube well by closing the tube with Parafilm and inverting the tube. Then, using the pipet pump (**do not mouth- pipet biuret reagent**) add 4 ml of biuret reagent to each tube and mix well again.

3. Allow the protein to react with the reagent for 30 min.

4. Read the Absorbance of each mixture at 550 nm. (It is usually not necessary to check the blank before each measurement, but the instrument may drift, so it is wise to check the blank occasionally and readjust it to read 0 % Absorbance with the light control knob if necessary.)

Calculations

A - ABSORPTION SPECTRUM OF HEMOGLOBIN

C1. On a page of graph paper, plot the Absorbance readings (on the Y-axis) as a function of wavelength (on the X-axis). Above the plotted spectrum, indicate the color of each wavelength region as observed in step 3 of the procedure.

B - BIURET COLORIMETRIC ASSAY OF SERUM PROTEIN

C1. On a page of graph paper, plot the Absorbance readings of the BSA samples (on the Y-axis) as a function of BSA (i.e. protein) concentration in each reaction mixture (on the X-axis). Note that each is a separate dilution of a 10mg/ml solution. Therefore, the concentration used in the plot is for the *dilution*. This is calculated by multiplying the original concentration and the dilution factor. The dilution factor is the volume of the original solution that you added divided by the final volume. For example, for tube 3 the dilution factor is 0.2ml/1.0ml = 0.2. The concentration, therefore, is 10mg/ml*0.2 = 2mg/ml. Using any two points on the straight line, calculate the slope of the standard curve. This is E from the Beer-Lambert equation, the relationship between concentration and absorbance.

C2. Determine the concentration of protein in the serum sample dilutions using the E value you have calculated and the Beer-Lambert equation and record the results in your notebook (do not forget the units).

C3. Each sample is a different dilution of one undiluted serum sample and you want to calculate the concentration of that original solution. To do this, first take the concentration of each sample you measured (C2) and divide by the dilution factor of that sample. This will give you several estimates of the concentration of the serum solution so they should be roughly the same. Next, take the average from all of your measures to find the overall estimate of the concentration.

Lab Report

There is a lab report due for this experiment. Following the outline for lab reports discussed in the first recitation, present the results you obtained for the spectrum and for the concentration of the serum sample.

APPENDIX 1
USE OF THE SPECTRONIC 20 SPECTROPHOTOMETER

A - Anatomy and Functions

This type of spectrophotometer consists of five external parts with which you should become familiar in order to use the instrument.

l. The on/off knob (at the left on the front of the instrument); it is also used as the "dark control."
2. The "light control" knob (at the right on the front of the instrument); this knob is rotated to set the reading (see 5) to Absorbance = zero (l00 % transmittance) when a reference solution is in the light path.
3. The cuvette holder (at the left on the horizontal table of the instrument); a cuvette is a glass tube of known diameter that will be used to insert samples into the instrument; there is a cover on the cuvette holder that MUST be closed during all readings and adjustments of the instrument, to prevent entry of light from the room into the photometer.
4. The wavelength control knob and its scale (at the right on the horizontal table of the instrument); rotating the knob rotates the prism of the spectrometer so that light of the wavelength indicated on the scale passes through the cuvette.
5. The meter (on the upper face of the instrument); the upper scale displays %Transmittance, and the lower scale displays Absorbance; the light control knob (see 2) controls the movement of the needle across the meter when a cuvette is in place.

B - Operation

l. Turn on the Spectronic 20 and allow l5 min. for the instrument to warm up.
2. Set the dark control so that the needle reads 0 % transmittance.
3. Set the wavelength with the wavelength control knob.
4. Prepare a cuvette containing a liquid that contains all the components of the sample solution except the substance whose absorbance is to be measured. This is the reference cuvette or blank. Place the reference cuvette in the sample holder (close the cover) and use the light control to adjust the needle to read 100 % transmittance.
5. Replace the reference cuvette with a sample cuvette containing the sample solution (close the cover). Allow the needle to come to rest and read the Absorbance. (Note that the Absorbance scale reads from right to left.)
6. To read another sample at the same wavelength, replace the first sample cuvette with the next one.
7. To read the same sample at another wavelength, reset the wavelength, insert the reference cuvette, adjust to l00 % transmittance with the light control knob, then insert the sample and read Absorbance. The instrument must be reset with the reference cuvette for each wavelength.