**Absorption Spectroscopy**

The amount of light transmitted through a solution or suspension may be diminished by the absorption of light by colored compounds and/or the scattering of light rays by particulate matter. An instrument that measures the amount of light passing through a sample is called a spectrophotometer. Absorption spectroscopy has many uses; it can be used to study pigmented molecules, to monitor the density of bacteria in a culture, and to follow the progress of an enzymatic reaction. The main requirement is that light be absorbed or scattered by some substance in the sample under investigation.

In this exercise you will examine some of the principles and applications of visible absorption spectroscopy. The purpose of this lab is to learn as much as you can about these techniques and the analysis of the data they generate. In particular, you will determine the absorption spectrum of a molecule, construct a calibration curve that can be used to quantify the concentration of that molecule, and use spectroscopy to monitor the changes in the molecule's concentration over time. (The molecule we will study today is the dye methylene blue, which appears blue when oxidized and colorless when reduced.) Later in the semester, you will be expected to use these techniques to investigate cellular and biochemical processes with little or no instruction provided.

Please note that, in this lab, *it is particularly important to carefully label information and data tables in your lab notebook.* The TAs will be looking for specific information, and you won't get credit if they can't easily locate the information. This has been a huge problem in the past, and I don't want to to lose points because of it.)

PRE-LAB ASSIGNMENT

Read the rest of this handout and page 766 in your textbook. Then answer the questions below in your lab notebook.

1. In the world of absorption spectroscopy, what is a "blank" and why is it necessary?

2. The Lambert-Beer law can be used to calculate the concentration of a substance based upon its absorbance. Why, then, are you going to the trouble of constructing a standard curve that relates concentration to absorbance? (Aside from the fact that your tyrannical professor is making you do it, that is.)

3. If you perform four two-fold serial dilutions (as noted in Part 2 below), what will be the concentration of the final (most dilute) sample relative to that of your starting sample? Show your work.

4. In Part 3 of the lab, do you think that that the absorbance of methylene blue will change *linearly* or *nonlinearly* over the time course of your experiment? Briefly explain your reasoning.

GENERAL NOTES ABOUT THE EQUIPMENT

In this class we will use two types of spectrophotometers: the smaller, single-beam Spectronic 20 Genesys Spectrophotometer (which replaces the Turner spectrophotometer you may have used in other classes) and the dual-beam Shimadzu Recording Spectrophotometer. This lab will introduce you to both instruments.

Samples for spectroscopy are placed in transparent holders called cuvettes. Correct use and care of cuvettes is essential for producing reliable data.

Tips for identifying and using cuvettes

There are two different styles of cuvettes used in this department, round and square. I will describe both styles because it is important for you to recognize cuvettes and treat them appropriately. The round cuvettes (for the Turner and Spectronic), unfortunately, resemble test tubes. The way to distinguish between cuvettes and test tubes is to look for a label near the opening of the tube: test tubes will sport a "Pyrex" or "Kimax" brand label or no label at all, whereas a cuvette will have some other mark (a logo in blue, red or white, or a conspicuous colored dot). If in doubt, please ask for assistance.

The square cuvettes (for the Shimadzu and Spectronic) are elongated cubes. We will use plastic square cuvettes for most of our work because they are less expensive and less breakable. If you encounter glass cuvettes, you should treat them with great care; depending on the type of glass, a single cuvette may cost up to $100. While the flat bottoms of the square cuvettes enable them to sit on bench tops, it's a wise idea to use the plastic holders whenever possible -- there's nothing more heartbreaking than having your last bit of sample spill across the lab bench.

Anything that hinders light passage through the cuvette will produce abnormally high absorbance readings. For example, scratches on the cuvette are a major problem. To avoid scratches, cuvettes should *always* be hand-washed (the jostling that occurs in the glassware tubs is damaging) using a cotton swab dipped in a soap solution. After the cuvette is scrubbed inside and out, the soap should be removed by rinsing with tap water and then distilled water.

A general guideline for filling the cuvettes is to add enough solution so that the cuvette is 2/3 full. After filling the cuvette, hold the cuvette near the rim and wipe fingerprints off the lower portion of the cuvette. Insert the cuvette into the spectrophotometer. It is important that the round cuvettes be inserted into the machine with a consistent orientation, i.e., the logo or marking should always face one direction. The Shimadzu cuvettes are marked with an arrow near the top; the side with the arrow should be placed in the light path.

PART 1: DETERMINING AN ABSORPTION SPECTRUM

All colored compounds absorb certain wavelengths of visible light and transmit other wavelengths. This pattern of light absorption over a range of wavelengths is called an absorption spectrum. Each molecule has a characteristic absorption spectrum that depends upon its structure.

1. Obtain two square cuvettes that have been cleaned inside and out. One will serve as your *blank* (or *reference*) and should be filled with distilled water (or whatever solvent is used for the colored compound). Use this cuvette to adjust zero absorbance (100% transmittance). (See the instructions below on how to do this.) Start with a wavelength of 400 nm. It is important to use matched cuvettes; to check this, place at least 2.0 ml of water (or whatever liquid is in the reference cuvette) in the other cuvette (called the *sample* cuvette) and read the absorbance. Is it identical to that of the first cuvette (within 0.003 absorbance units)? If not, get two that read the same. (If you're having problems, check with me.)

The blank must be used to adjust the spectrophotometer every time the wavelength is changed. If you are working with a single wavelength, the blank should be placed in the spectrophotometer periodically to check for drift.

2. Recording all calculations, prepare a dilution of the methylene blue stock solution. The methylene blue stock concentration will be specified as a weight/volume %. (Percentage solutions can be made on a weight/volume or volume/volume basis. In a weight/volume solution, the weight refers to the number of grams of solute in 100 ml of solution. Solutions in which the solute is a liquid [such as ethanol] are indicated as vol/vol, where the percentage refers to the number of ml of the liquid in 100 ml of final solution [such as 70% ethanol]. Water is assumed to be the solvent unless otherwise specified.) A recommended dilution will be provided in lab. (Hint: consider the total volume you will need. Will this solution be used for another part of this lab?)

3. Determine the absorbance at 10 nm intervals from 400 nm to 700 nm. *Remember to zero the spectrophotometer with the blank each time you change the wavelength.* At what wavelength is the absorbance at its maximum? This is referred to as the λmax. There may be more than one λmax for a compound. The absorbances over the full range of wavelengths constitutes the absorption spectrum.

4. Referring to the instructions for the Shimadzu recording spectrophotometer, use the "spectrum" mode of the Shimadzu to prepare an absorption spectrum for methylene blue. Use the data processing features to determine the position of absorption peaks. Also try the expansion/compression feature to become familiar with its features. Print a copy of the spectrum to be turned in (consult the Shimadzu instructions). Note: any time you record a spectrum, each group member should print and keep a copy.

PART 2: CONSTRUCTING A CALIBRATION CURVE

Once the λmax of a substance is known, this wavelength can be used to monitor it and, under the right circumstances, to provide a quantitative determination of its concentration. In order for absorption spectroscopy to be useful for quantitative studies, it is necessary to relate the absorbance of a compound to the amount of compound present. The Lambert-Beer law provides the mathematical basis of this relationship:

A = ε \* l \* c

where A is the absorbance or optical density (no units), l is the path length of the cuvette (in cm),

c is the concentration (in M, mM, or μM), and ε is the extinction coefficient (what are the units?). The molar (or millimolar or micromolar) extinction coefficient is a constant that relates the absorbance of a particular substance to its concentration.

According the Lambert-Beer law, an increase in the concentration of a compound leads to a linear increase in the absorbance of the solution. (Note that the Lambert-Beer equation is a form of the general equation of a line, y = mx + b.) In practice, an increase in concentration produces a linear increase in absorbance only over a certain range because spectrophotometers are less sensitive to absorbance changes above a reading of 1.0 absorbance unit (something to keep in mind if you are doing quantitative work).

If the extinction coefficient is known, the Lambert-Beer law can be used to calculate the concentration of the solution. However, the molar extinction coefficient is not always known (or not readily available), so it is a common practice to experimentally produce a *calibration curve* (also called a *standard curve*), a graph that relates concentration to absorbance.

To construct a calibration curve, a series of dilutions (of known concentration) are prepared and absorbance readings are obtained for these solutions. The calibration curve is constructed by graphing the absorbance readings for the series of concentrations.

1. Using the dilution prepared for Part 1, prepare four two-fold serial dilutions. The term "two-fold" indicates that each sample should be 1/2 as concentrated as the previous sample, and the term "serial" means that each sample should be made from the previous sample. Plan your dilutions so that you are adding the same volume of solvent at each step; this repeatable pattern is most efficient and less subject to error than using different volumes at each step. It is most convenient to make all dilutions first and then take the readings. Be sure you have a sufficient volume of each dilution; in making serial dilutions, you always need to start with a greater volume than you'll eventually need (how much greater?).

2. To take absorbance readings of the 5 samples (original concentration plus the 4 dilutions) on the Spectronic spectrophotometer, select the wavelength, zero the instrument using the blank, and read the samples (what wavelength will you choose and why?). You will need to rinse and drain the sample cuvette well before reading the unknown sample. You can use one sample cuvette to read all samples; as long as you work from dilute to concentrated samples, it is sufficient to drain the cuvette by tapping it upside down on a Kimwipe.

3. Take the methylene blue solution of unknown concentration. Record your strategy for determining its concentration, and then take any readings needed to carry out your plan.

4. Now use the photometric mode of the Shimadzu to determine the absorbances of your 5 samples and the unknown solution. (Consult the Shimadzu instructions.)

A calibration curve is prepared by plotting absorbance relative to concentration. (Which is the independent variable?) From the calibration curve, or its equation, one can determine the concentration of an unknown solution.

PART 3: MONITORING CHANGING ABSORBANCE

If an enzyme uses a colored substrate or produces a colored product, the progress of the reaction can be monitored by following the appearance or disappearance of the colored compound. In many cases, biochemists are able to use indicator dyes, which aren't the primary substrate or product, but react with the product to produce a color change.

Glucose, in an alkaline solution, can reduce methylene blue, converting the dye to its colorless form. Though this does not involve an enzyme catalyst, it will serve as a convenient reaction with which to practice this technique. Several glucose-KOH solutions will be available; your instructor will specify which one to use.

1. Place 3 ml of the recommended glucose-KOH solution in a square cuvette. Select the wavelength you will use for this assay (what did you choose and why?), and adjust the machine with the blank (what is your reference solution for this portion of the lab?). When you are ready to begin the assay, add enough of the concentrated methylene blue stock solution to give you the same final concentration as used for Part 1, cap with parafilm and quickly invert twice to mix. Place the cuvette in the spectrophotometer as soon as possible. Record readings every 10 seconds until the absorbance reaches zero.

2. Use the "time scan" mode of the Shimadzu to monitor samples with changing absorbance. You will need a fresh sample of glucose-KOH. Add the dye just before taking a reading, cover the cuvette with parafilm and invert quickly a couple times to mix.

3. Use the data processing feature to "trim" the graph to cover the time frame over which the reaction occurred. You will need a copy of the graph that appears on the Shimadzu screen and also a printout of numerical absorbance values at 5-second intervals. Consult your instructions and follow them explicitly; a wrong move can erase the data, and then the reaction would need to be repeated.

A rate curve is generated by plotting the change in the amount of the measured compound (in moles or micromoles, not in concentration). The initial reaction rate can then be determined by the slope of the best-fit line or tangent line (depending on whether the reaction is linear or curved).

CLEANUP

Hand-wash cuvettes using a cotton swab. (Cuvettes never go into the glassware tub.)  
Rinse well with tap water. Do a final rinse with distilled water. Drain upside down in a drying rack.

Remove any labels from the glass pipets and test tubes. Rinse them and put them in the glassware tub.

HOW TO USE THE SPECTRONIC 20 GENESYS SPECTROPHOTOMETER

1. Check to see that the sample compartment is empty by raising the hinged cover on the right side of the spectrophotometer.

2. Turn on the spectrophotometer and allow 30 minutes to warm up before using.

3. Press A/T/C to select absorbance mode for your experiment. The absorbance mode is indicated by the capital "A," which appears on the far right side of the display.

4. Press nm △ or nm ▽ to select the wavelength.

5. Insert your blank into the cell holder positioning tube so that the light passes through the clear walls (light path indicated by arrow). Close the sample door.

6. Press 0 ABS / 100% T. The instrument will automatically set the blank to 0 absorbance or 100% transmission.

7. Remove your blank and insert your sample into the cell holder, again aligning the clear part of the tube with the arrow. The sample measurement appears on the LCD display.

8. Be sure to use a blank to re-zero the spectrophotometer between experimental readings.

HOW TO USE THE SHIMADZU UV/VIS RECORDING SPECTROPHOTOMETER

## General instructions

1. Familiarize yourself with the parts of the instrument using the photographic guide.

2. Turn on the power switch. The spectrophotometer takes about 3 minutes to initialize itself and make baseline corrections. When it is finished, a menu will appear on the screen.

3. Are your cuvettes matched? If so, proceed to step 4. If you haven’t already selected matched cuvettes, insert 2 cuvettes with the "blank" solution and press AUTO ZERO. Remove the forward cuvette, pour out the reference solution (tap the cuvette on a Kimwipe to dislodge larger drops of remaining liquid) and fill it with a sample. (If you use a 3rd cuvette for the sample, the auto zeroing will have been in vain.) The other cuvette is a reference cuvette. Be careful not to spill anything into the sample chamber.

4. Select the desired Mode number. Then press ENTER.

5. Next set your parameters. More specific instructions will be given for each specific mode (see below). After each parameter change you will be asked "Parameter Change?" After the final change, reply NO to this question.

6. Once the parameters are set and the sample and reference solutions are in place, press the START/STOP button to begin scanning.

7. Wait for the scanning to end. The results will be displayed on the screen.

8. Many of the modes offer data processing features. These will be described below.

9. When finished with the machine, be sure to remove cuvettes, wipe up anything that has spilled on or in the machine, and turn it off.

### Spectrum mode

This mode sequentially scans a series of wavelengths and is useful for the determining the pattern of absorbance (absorption spectrum) of a substance.

1. Parameter changes:

- wavelength range to be scanned

- absorbance or transmittance?

2. Run spectrum.

3. You now have a choice of Data Processing. If you choose not to, then press NO. You may now obtain a hard (printed) copy of your spectrum by pressing COPY. If you wish to perform Data Processing, press YES. Procedures to choose from are displayed at the top of the screen. Press the desired number, then ENTER.

(1) SAVE: This allows you to store a spectrum. You won't need to do this.

(2) EXPANSION/COMPRESSION: This allows you to expand or compress your spectrum field. Press 2, then ENTER, then set your new parameters. The cursor will flash in front of the number. If you wish to change it, type in the new number then ENTER. If you wish it to remain unchanged, just press ENTER.

(3) PEAKS AND VALLEYS: This will mark each peak and valley with a "|" and automatically print out the data.

(4) DERIVATIVE: This will give 1st through 4th order derivatives of the spectrum. You won't need to do this.

(5) PRINT: This will print out absorbance values at regular wavelength intervals. This allows you to choose an interval from 1-99 nm and print out the values of the spectrum at each of the interval points. Press 5, then ENTER and then press the interval you wish (1-99) then ENTER again. The data will automatically print out.

(6) PLOT: Allows you to plot an X-Y curve of the spectrum. This requires an additional instrument and won't be done.

4. Replying NO to the data processing question will generate a spectrum with designated places to label the graph. You can obtain a printed copy of this screen by pressing COPY. (You can generate a hard copy of the screen display at any time by pressing the COPY button.)

## Photometric mode

In the photometric mode, the Shimadzu takes readings at one or more fixed wavelengths (up to 5 different wavelengths). Much of the work you do will involve only a single wavelength; however, other applications require the use of two or more wavelengths. For example, DNA quantification is carried out by taking readings at 260 and 280 nm. The ratio of these two numbers enables the investigator to assess the degree of protein contamination (which has an absorbance maximum at 280 nm) of the DNA (which has an absorbance maximum at 260 nm).

1. Parameter changes:

In order to select the wavelength(s) to be read, you first need to deal with parameter #5. Parameter #5 specifies the number of different wavelengths to be read for each sample.

Always begin parameter changes in the photometric mode by selecting parameter #5 (even if it already shows the number of wavelengths you plan to use). Once you have indicated the number of wavelengths to be measured, the cursor will jump to the wavelength designation at parameter #1. If you try to change parameter #1 directly, it changes the "Go to l = \_\_\_\_\_\_ nm" but will not change the l designation to the right (which happens to be the critical wavelength).

Choose absorbance or transmittance.

2. Set the reference and sample cuvettes. Take a reading by pressing the START/STOP button.

3. You can print out the data from the screen by pressing the COPY button.

No data processing options are given.

## Time scan mode

This mode takes a series of sequential readings at a fixed wavelength, which allows you to follow changes in optical density over time.

1. Parameter changes:

- select the wavelength

- specify absorbance or transmittance

- time (set at the maximum of 600 seconds; if the reaction doesn't take the full time, you can always press the STOP button to interrupt)

2. Set the reference cuvette. Start the reaction and set the sample cuvette as quickly as possible. Press the START/STOP button as soon as the door to the sample chamber is closed.

3. Data processing. The following functions may be of use to you in this course:

EXPANSION/COMPRESSION: This allows you to expand or compress the axes on the plot. The operation of this feature is similar to that found in the Spectrum Mode.

PEAKS: This will mark each peak with a "|" and automatically print out the data. Since all of our reactions will increase or decrease in absorbance rather than alter the wavelength at which absorbance occurs, this feature is not useful for our applications.

PRINT: This will print out at regular time intervals in seconds. This allows you to choose an interval from 1-600 sec and print out the absorbance/transmittance values at each of the interval points. This is a very useful function.

4. Once no further data processing is required, replying with a NO to the data processing question will generate a graph with designated places to label the graph. You can obtain a printed copy of this screen by pressing COPY.

POST-LAB ASSIGNMENT

Please turn in all Shimadzu printouts, all graphs requested below, and answers to the questions below. You may type or hand-write your answers. (You do not need to write the answers in your lab notebook.)

## Absorption spectrum

1. Using data from the Spectronic spectrophotometer, plot A vs. λ to prepare an absorption spectrum. Turn in both Spectronic and Shimadzu absorption spectra for methylene blue. (Prepare these figures as for a formal report, remembering to include appropriate figure legends.)

## Calibration curve

2. Prepare a calibration curve (half page or larger, formal report style) of the Spectronic data relating methylene blue absorbance to its concentration (in micromoles/liter, or μM). (Remember to think about which variable should be plotted on which axis.) When all absorbance readings are taken at one wavelength, the wavelength is often specified on the axis as "Aλ", such as A260. Be sure to show all calculations used to determine the concentrations. (Label calculations and show units.)

3. Determine the concentration (in micromoles/liter) of the unknown solution using data from the Spectronic spectrophotometer. (You should include enough information for your TA to follow your work; label your calculations and show units.)

4. From the information you have on hand, can you determine the extinction coefficient for methylene blue? Describe your procedure and calculations.

## Time scan

5. Plot your Spectronic results (half page or larger, formal report style) so that your graph shows the change in oxidized methylene blue (in micromoles, *not* in micromoles/liter) over time. (Are you able to plot your results directly? If not, document the intermediate steps you need to take.) Draw the best-fit line or smooth curve for the data points.

6. Determine the rate of change for this reaction. If the reaction progress is best shown by a straight line, the reaction rate will be determined by finding the slope of the line. If a curve fits the data points best, the rate can be determined by drawing a tangent line to the curve and finding the slope of this tangent line. The point at which you draw the tangent line is a decision you will need to consider carefully; if you were working with a series of curves, you would need to be consistent in drawing tangent lines to the other curve(s) within one data set. Describe your strategy for the rate determination and show all calculations.

7. Describe how you would use the Shimadzu data to determine rates. (You don't actually need to do the calculations for this step.)