

Using the Spectrophotometer

OVERVIEW

Color provides us with both beauty and useful information. Color is a source of our pleasure in a sunset, in the autumn leaves, or in a beautiful bouquet of flowers. Color can also be an indicator of when vegetables or fruits are ripe, when our coffee is strong enough, or when a storm is coming.

In this laboratory, you will explore how a **spectrophotometer** uses the colors of the light spectrum to determine the concentration of light-absorbing molecules in a solution.

The visible light spectrum, like X-rays, radio waves, and infrared waves, is part of the spectrum of electromagnetic radiation. Types of electromagnetic radiation differ in both wavelength and energy level, but all types travel through space in waves. The height of a wave at its crest is called its **amplitude**; the intensity or the brightness of visible light is proportional to its amplitude. The distance from the crest of one wave to the crest of the next wave is called the **wavelength** (λ); in the visible spectrum, the color of the light we see depends on its wavelength. Wavelength is measured in units called nanometers (1×10^{-9} m). Wavelengths of 400 to 700 nm comprise the “visible light spectrum”—the part of the electromagnetic spectrum that can excite photoreceptors within the human eye.

STUDENT PREPARATION

Prepare for this laboratory by reading Exercise A, Part I.

✓ EXERCISE A How the Spectrophotometer Works

✓ PART I Principles of Spectrophotometry

Molecules either absorb or transmit energy in the form of electromagnetic radiation. White light (normal daylight) is made up of all the wavelengths of electromagnetic radiation in the visible spectrum. How objects or chemical substances absorb and transmit the light that strikes them determines their color.

What we see as the color of an object, or a solution, is determined by what wavelengths of light are “left over” to be transmitted or reflected by the object after certain wavelengths are absorbed by its constituent molecules. For example, the pigment chlorophyll, present in the leaves of plants, absorbs a high percentage of the wavelengths of light in the red and violet to blue ranges (Figure 4A-1). Green light, not absorbed by chlorophyll molecules, is reflected from the surface of the leaf—thus most plants appear to be green. A solution of chlorophyll extracted from a leaf would also be green.

The spectrophotometer can be used to measure the amount of light absorbed or transmitted by molecules in a solution. The spectrophotometer operates on the following principle. When a specific

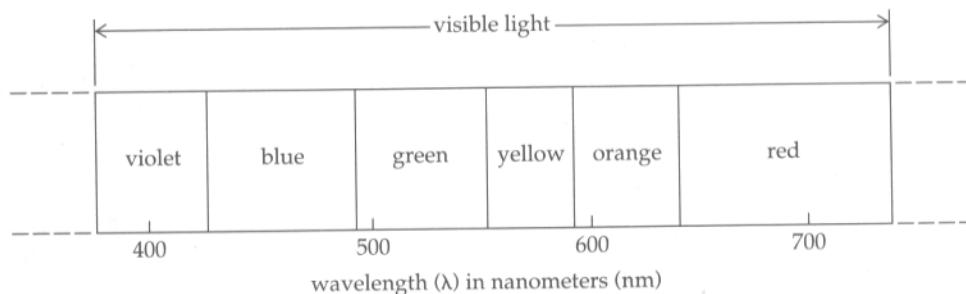


Figure 4A-1 Electromagnetic wavelengths in the visible light spectrum. For a diagram illustrating the full range of electromagnetic radiation, see Figure 12A-2, page 12-5.

wavelength of light is transmitted through a solution, the radiant light energy absorbed, **absorbance** (A), is directly proportional to (1) the absorptivity of the solution—the ability of the solute molecules to absorb light of that wavelength; (2) the concentration of the solute; and (3) the length of the path of light (usually 1 cm) from its source, through the solution, to the point where the percentage of light energy transmitted or absorbed is measured by a phototube.

Spectrophotometers that employ ultraviolet or visible light are the types most often used to study biological structures and reactions. The investigator selects a wavelength of light that will be maximally absorbed by a particular solute in solution. (If visible light is used and the molecule of interest does not absorb light, it is often possible to set up a chemical reaction that will yield a colored product.) After passing through the solution, the light energy received at the phototube is expressed as the ratio of transmitted light I_T (the light that passes through the sample) to incident light I_0 (the intensity of light at the source before it enters the sample). The light received at the phototube is measured as percent transmittance (T), or as the log of its inverse, absorbance (A):

$$\%T \text{ (percent transmittance)} = \frac{I_T}{I_0} \times 100$$

$$A \text{ (absorbance)} = \log \frac{I_0}{I_T}$$

By measuring the absorbance (or transmittance) it is possible to determine the concentration of the absorber (molecule) in solution. Concentration can be calculated directly if the molar absorptivity of the molecule (the amount of light at a specific wavelength absorbed by a specified concentration of solute in moles per liter) is known. Usually, however, molar absorptivity is not known and absorbance readings indicate only relative concentrations—a higher absorbance (A) resulting from a higher concentration. In such cases, the concentration can be found by locating the absorbance reading of the unknown concentration on a graph of the absorbances of known concentrations (standard curve).



PART 2 Using the Spectrophotometer

The Bausch & Lomb Spectronic 20 Colorimeter (Figure 4A-2) is an extremely versatile instrument that is useful for the spectrophotometric, or colorimetric, determinations of solutions.

Within the optical system of the spectrophotometer, rotation of a prism (diffraction grating) allows the investigator to select specific wavelengths of light in a range from 375 to 625 nm. Light of a selected wavelength is passed through the sample and is picked up by a measuring phototube, where the light energy is converted to a reading on the meter of the spectrophotometer (Figure 4A-3).

Most spectrophotometers have two scales—one is a linear scale (the *transmittance scale*) given as percent transmittance, the other is a logarithmic scale with the same gradations as the percent transmittance scale (the *absorbance scale*) (Figure 4A-2). (Since transmittance is related to absorbance as the log of the inverse, values of 0.0 absorbance occur at 100% transmittance and values of infinite absorbance

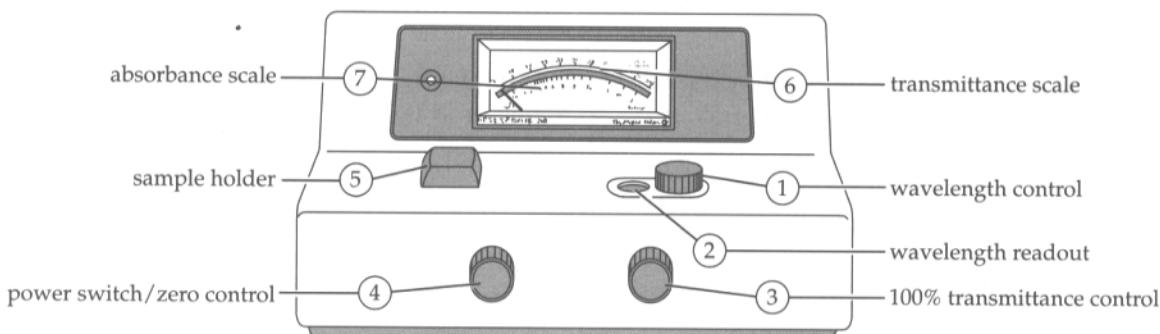


Figure 4A-2 Features of the Bausch & Lomb Spectronic 20 Colorimeter. The transmittance and absorbance scales run in opposite directions. Why?

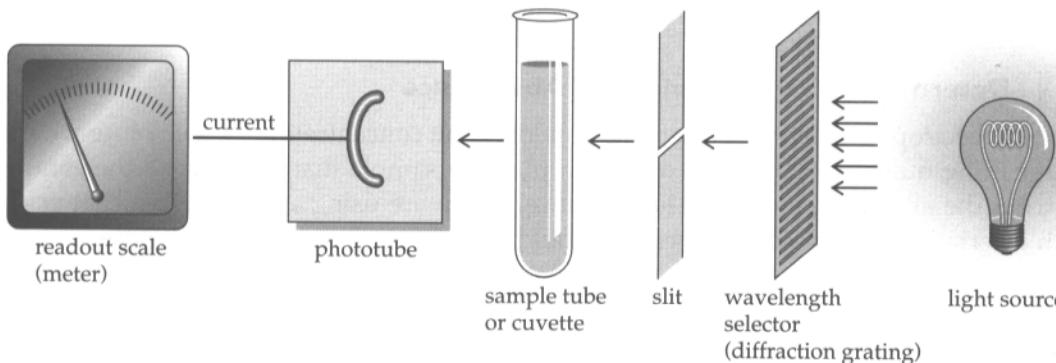


Figure 4A-3 Operation of the spectrophotometer. Light of 340 to 750 nm is emitted from a light source and passes through a diffraction grating (wavelength selector) that generates light of a particular wavelength. Light intensity is adjusted as the light passes through a slit before reaching the sample. Some light is absorbed by the sample, and the rest is transmitted and detected by a phototube on the other side of the sample. The current generated by the phototube is proportional to the intensity of light hitting it and is registered as transmittance (or its inverse, absorbance) on the readout scale (meter) of the spectrophotometer.

occur at 0% transmittance.) Examine the readout meter on the spectrophotometer you will be using. Note that the scales run in opposite directions.

Objectives

- Describe the relationship between absorbance and transmittance.
- Describe how absorbance and transmittance of light are related to the color of an object.
- Explain how the optical system of a spectrophotometer works.
- List the steps in the proper use of the spectrophotometer.

Procedure

1. Familiarize yourself with the parts of the spectrophotometer shown in Figure 4A-2.
2. Work in pairs. Cut a strip of white paper to just fit the diameter and length of a Spectronic 20 tube or cuvette. Slide the paper into the tube and insert the tube into the sample holder of the spectrophotometer.

3. Leave the sample holder open and place a cylinder of black paper around the opening.
4. Set the wavelength control to 620 nm and adjust the position of the tube containing the white paper until you see the maximum amount of red light on the right side of the paper.
5. Have your partner turn the wavelength dial in both directions and record the range of each wavelength at which you see a particular color. Range of wavelengths:

Red _____

Yellow _____

Green _____

Blue _____

Violet _____

6. Trade positions and let your partner check your observations by repeating steps 3–5. Compare your results with the wavelengths given in Figure 4A-1.



PART 3 Determining Transmittance and Absorbance

To assure that spectrophotometer readings indicate only the concentration of the solute we wish to measure, a reading must first be obtained using a **blank**, a sample that contains all the components of the solution except the absorbing molecule. For instance, if you are using a reagent that changes color when mixed with a certain solute molecule, a blank should contain *all* the components of the test solution, including the colorimetric reagent, *except* the substance (solute) to be measured.

Example A 1-ml sample of substance X is mixed with 5 ml of water and 1 ml of colorimetric reagent to give a volume of 7 ml in a sample tube. A blank is prepared by mixing 5 ml of water with an additional 1 ml of water (as a substitute for substance X) and 1 ml of colorimetric reagent. The volume in the blank tube is 7 ml. Note that the volume of the blank should always be the same as the volume of the sample.

With the blank inserted into the spectrophotometer, the instrument is adjusted to 100% transmittance (zero absorbance). This step is similar to taring a balance: the transmittance of light through the blank will be less than 100% because of substances (including the colorimetric reagent) present in the blank.

However, the instrument can be adjusted to accept this reading as 100% transmittance, so that when the blank tube is replaced by the sample tube to measure absorbance of the sample, the only thing absorbing light will be the sample molecule of interest (the solute that reacts with the colorimetric reagent).

Procedure

1. Prepare a sample tube. Place 10 drops of albumin solution into a spectrophotometer tube and add 1 ml of distilled water. Add 5 ml of Coomassie brilliant blue (a colorimetric reagent used to identify protein) and allow the tube to stand until a blue color develops.
2. Prepare a blank: place 10 drops of water into a spectrophotometer tube and add 1 ml of distilled water and 5 ml of Coomassie blue.
3. Turn the power switch on, and allow a 5-minute warm-up period. The on/off switch is operated by the zero control knob on the left.
4. Use the wavelength control knob to adjust the spectrophotometer to any wavelength between 550 and 600 nm. The selected wavelength is indicated on the wavelength readout in the window next to the knob.
5. When using the Spectronic 20, the meter must be adjusted to read across its full scale—0% transmittance to 100% transmittance. With *no* sample tube in the machine, use the zero (left-hand) control knob to set the scale to 0% transmittance (infinite absorbance). (With no sample tube, the light path is automatically blocked, and no light reaches the phototube; thus, 0% transmittance and infinite absorbance are simulated.) Be sure the cover on the sample holder is closed when you perform this step.

6. Insert your blank (be sure it is clean and dry on the outside) into the sample holder, and turn the right-hand control knob to set the meter scale to 100% transmittance, zero absorbance. This adjustment regulates the amount of light reaching the phototube in the absence of the absorber. *Whenever the wavelength is changed, the 100% transmittance adjustment must be reset.* Also, when operating at a fixed wavelength for an extended period of time, periodically check the 100% and 0% transmittance readouts and adjust if necessary.
 7. If you are beginning an experiment, repeat steps 5 and 6 to make sure the machine is stable.
 8. Insert the sample tube into the chamber; read absorbance directly on the absorbance scale (lower scale). The reading on the absorbance scale is proportional to the concentration of your sample substrate. *Note:* The absorbance scale reads from *right to left*, opposite to the direction of the transmittance scale. Record your data: wavelength _____ nm, absorbance _____, transmittance _____.
- Do not discard your sample and blank tubes!***
9. The steps that follow provide a brief checklist for using the spectrophotometer during this laboratory and in later lab work.

QUICK CHECKLIST FOR USING THE SPECTROPHOTOMETER

1. Turn the power on and allow a 5-minute warm-up period before taking sample readings.
2. Select the wavelength.
3. Check that the sample holder is empty and the cover is closed.
4. Use the zero control knob to set the meter to 0% transmittance.
5. Wipe off fingerprints from the reference blank, insert it into the sample holder, and set transmittance to 100%.
6. Wipe off fingerprints from the unknown sample tube, insert it into the sample holder, and read the meter display in percent transmittance or absorbance.

For best results when using the spectrophotometer, always remember the following:

- All solutions *must* be free of bubbles.
- All sample holders *must* be at least one-half full.
- For best performance with test tube holders, be sure that the index mark on the tube or cuvette aligns with the mark on the adapter (if tubes and cuvettes are marked).
- All sample tubes *must* be clean and free of scratches. Use lens paper to remove all fingerprints from the sample tubes and cuvettes.
- During extended operation at a fixed wavelength, make occasional checks for meter drift: use the blank to check for 100% transmittance.

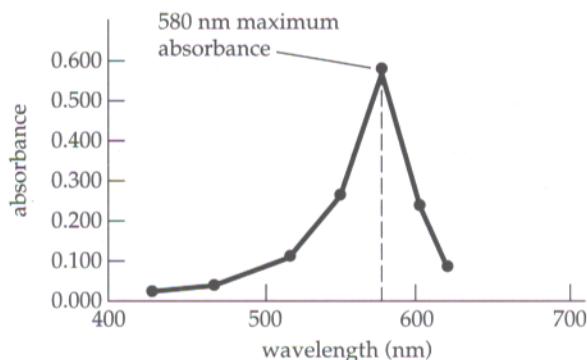


EXERCISE B

Determining the Maximum Absorption Wavelength

Molecules in solution (solute) will absorb light maximally within a narrow range of wavelengths. When deciding upon the wavelength to use in measuring concentration, an “absorbance spectrum” is generated in which the absorbance of a particular solute, or “absorber,” is measured at a continuous selection of wavelengths. A curve such as that shown for bromphenol blue (Figure 4B-1) is generated, and *maximum absorbance* can be determined. The most accurate measurements of absorbance are obtained by selecting a wavelength of light that is maximally absorbed by the solute of interest.

Figure 4B-1 Absorption spectrum for bromphenol blue.



|||| Objectives |||||

- Determine the maximum absorbance wavelength for a light-absorbing substance in solution.
- Relate the shape of an absorbance curve to the absorption of light at different wavelengths.

|||| Procedure |||||

1. Set the wavelength on your spectrophotometer to 540 nm.
2. Use the zero control knob to set the spectrophotometer to 0% transmittance.
3. Adjust the spectrophotometer to 100% transmittance using the same blank as you used in Exercise A.
4. Remove the blank tube and place the albumin sample tube (used in Exercise A, Part 3) into the chamber. Record the absorbance and transmittance readings at 540 nm in Table 4B-1.
5. Readjust the wavelength to 560 nm. Repeat steps 2–4.
 - a. *Why do you need to use the blank to adjust the spectrophotometer at each wavelength used for a reading?* _____
6. Continue to increase the wavelength until you reach 640 nm, repeating steps 2–4 at each wavelength and recording the data in Table 4B-1.
7. Verify the relationship between transmittance and absorbance by calculating absorbance from the transmittance data ($T = \%T/100$). Show your calculations in the last column of Table 4B-1.

Table 4B-1 Absorbance and Transmittance at Various Wavelengths

Wavelength (nm)	Absorbance	% Transmittance	Calculations $A = \log 1/T$ (or $-\log T$)
540			
560			
580			
600			
620			
640			

8. Graph the absorbance data on graph paper.
- At what range of wavelengths is absorbance at a maximum for Coomassie blue? _____*
 - Within this range of wavelengths, determine the maximum wavelength for absorption, $A_{\max} = \underline{\hspace{2cm}}$*
 - Do you think the wavelength at which absorbance is maximum would change if the concentration of albumin in the sample tube were doubled? _____ Why or why not?*

EXTENDING YOUR INVESTIGATION: ABSORBANCE AND TRANSMITTANCE

Your instructor will provide you with a tube of solution that appears red/red-orange. Consider the way in which light waves are absorbed or transmitted. What wavelengths of light would you expect to be absorbed by this solution? _____

Formulate a hypothesis that predicts what the absorption spectrum would look like for this solution.

HYPOTHESIS:

NULL HYPOTHESIS:

What do you predict will be the outcome of your experiment?

What is the **independent variable**?

What is the **dependent variable**?

Follow steps 1–8 to test your hypothesis, using the appropriate range of wavelengths for your sample. Collect absorbance data in the table below.

Wavelength	Absorbance
420	
470	
520	
570	
620	
670	

Graph the absorbance data on graph paper. From your results, describe the absorbance properties of the red solution. _____

Do your results support your hypothesis? Your null hypothesis?

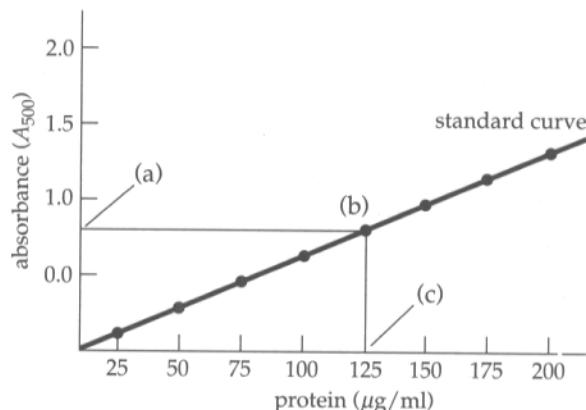
Was your prediction correct?

**EXERCISE C****Exploring the Relationship between Absorbance and Concentration**

Coomassie brilliant blue forms a colored complex with proteins. In Exercise B, you determined the wavelength at which this complex maximally absorbs. Now, using this wavelength, you can determine the concentration of protein in a solution by measuring the intensity of color developed when Coomassie blue is mixed with the solution.

Color intensity can be accurately measured by determining the amount of light absorbed by the solution. Absorbance is a function of concentration and, by comparing the absorbance of a solution containing an unknown amount of protein to a **standard curve**, a graph of absorbances plotted from a series of samples of known concentrations of the same material, the concentration of protein in an “unknown” can be determined (Figure 4C-1).

Figure 4C-1 Absorbance measurements of known concentrations of a protein molecule are used to generate the standard curve (in this case the “curve” is linear). An absorbance reading (a) is obtained for a solution containing an unknown amount of protein. By drawing a straight line from (a) to the curve at (b) and dropping a line from (b) to (c), the concentration of the protein can be determined. A_{500} (Y-axis) indicates absorbance at 500 nm.

**|||| Objectives |||||**

- Describe the relationship between absorbance and the concentration of a light-absorbing substance in solution.
- Use a standard curve to determine the concentration of a light-absorbing substance in solution.

|||| Procedure |||||

On your laboratory table you will find a tube marked BSA. It contains 240 $\mu\text{g}/\text{ml}$ of bovine serum albumin (BSA), a more highly purified form of albumin. To develop a standard curve for bovine serum albumin, you and your laboratory partner should prepare at least five dilutions (see Appendix III or Laboratory 2, Exercise D).

1. Prepare dilutions as follows. Add 0.5 ml of distilled water to each of five test tubes and label the tubes 1 through 5.
2. To the first tube, add 0.5 ml of BSA stock solution and *mix well*. This will give a protein concentration of 120 $\mu\text{g}/\text{ml}$.
3. Take 0.5 ml of solution from test tube 1 and add it to test tube 2. Mix well.
4. Take 0.5 ml from test tube 2 and add it to test tube 3. Mix well. Repeat this procedure until you have added protein solution to each test tube.

5. Discard 0.5 ml from the last test tube. *a. Why is this necessary?* _____
6. To prepare a blank, add 0.5 ml of distilled water to another test tube and label it "blank." *b. Why is a blank necessary?* _____
- c. What type of serial dilution have you performed—1:2, 1:5, 1:10, and so on, or some other?* _____
7. Tube C on your laboratory bench contains an unknown amount of BSA. Add 0.5 ml of this solution to a test tube, label it "unknown," and set it in the test tube rack with the standards and the blank.
- You should now have a series of five test tubes containing protein concentrations of 120, 60, 30, 15, and 7.5 $\mu\text{g}/\text{ml}$; a blank containing only distilled water; and a tube containing an unknown concentration of protein.
8. Add 5 ml of Coomassie blue to each of tubes 1 through 5 and to the blank. Wait at least 3 minutes, but no longer than an hour, then read absorbances at 595 nm. Follow the Spectronic 20 procedure steps outlined in Exercise A, Part 3, or refer to the quick checklist. Record your data in Table 4C-1.

Table 4C-1 Data for Determining Concentration from Absorbance

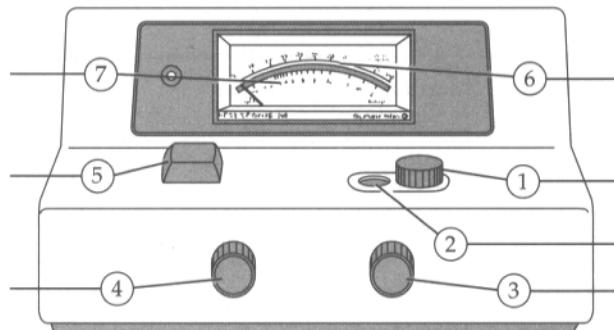
	Protein Concentration ($\mu\text{g}/\text{ml}$)	Absorbance (595 nm)
Tube 1	120	
Tube 2	60	
Tube 3	30	
Tube 4	15	
Tube 5	7.5	
Unknown		

9. Obtain a sheet of graph paper. Label the abscissa (X-axis) "Concentration" and the ordinate (Y-axis) "Absorbance." Make a graph as large as possible on the paper; plot the absorbance data for the solutions of known concentration. This is your standard curve. (Keep in mind that a "standard curve" can be used as a standard only when known and unknown concentrations have been prepared according to the same procedure. For example, for this standard curve to be useful, knowns and unknowns must be prepared by using 0.5 ml of the sample and 5 ml of reagent.)
10. Add 5 ml of Coomassie blue to your unknown. Wait 5 minutes or the same amount of time as in step 8, and read its absorbance at 595 nm using the Spectronic 20. Record absorbance in Table 4C-1. From the standard curve you have prepared, determine the concentration of your unknown:

_____ $\mu\text{g}/\text{ml}$ BSA (Record this value in Table 4C-1.)

Laboratory Review Questions and Problems

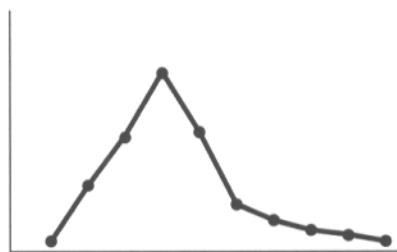
1. You have a solution that appears green. What color light is being transmitted? What color(s) of light are most strongly absorbed?
2. How is absorbance related to transmittance of light through a solution?
3. List the steps in using a spectrophotometer. What is meant by "zeroing" the spectrophotometer?
4. You want to use the Spectronic 20 to find the wavelength of light you should use for determining the concentration of protein in an unknown solution. Using the following diagram, indicate the number of the dial or scale that you would use to accomplish each of the steps listed below.



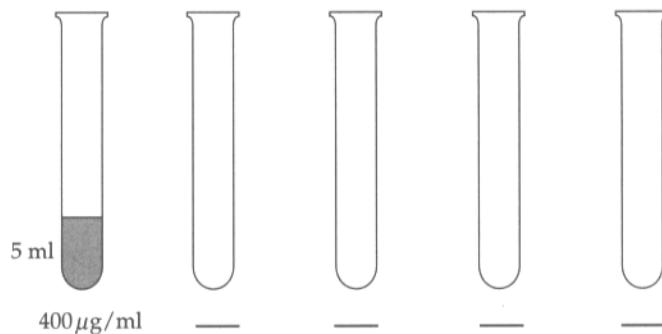
- _____ Insert protein sample.
- _____ Adjust to 100% transmittance and 0 absorbance using a blank.
- _____ Change wavelength.
- _____ Adjust to maximum absorbance and 0% transmittance.
- _____ Read wavelength.
- _____ Read absorbance of sample.
- _____ Read transmittance of sample.

5. What is the purpose of a "blank" or reference tube?

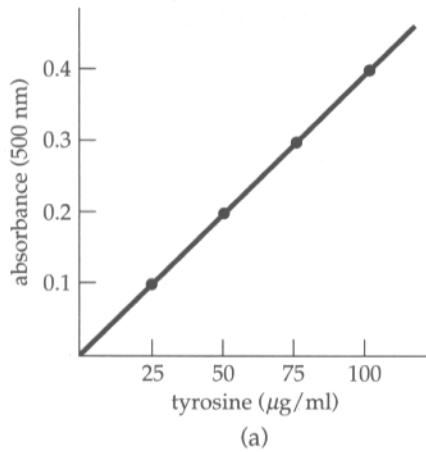
6. You are making a standard to be used to measure protein concentration. You have added 2 ml of protein, 3 ml of water, and 4 ml of Coomassie blue. How do you make a blank for this experiment?
7. You generate an absorbance spectrum for a red solution as shown below. Label the axes of this graph (include units where necessary). What wavelength (or color) of light would you expect to show the maximum reading? _____



8. a. You are conducting an experiment in which an extract of cytoplasm is mixed with a red dye. When the mitochondria in the cytoplasmic extract are functioning, the red dye fades. You wish to measure the rate of the reaction that causes the decolorization. What wavelength of light might you use to make your measurements if you wanted to measure the decrease in absorbance by the red solution? _____
- b. What would you use as a reference blank?
- c. What would you use as a control for this experiment?
- d. How might you determine the rate (decrease in concentration/time) of the reaction? (*Hint:* How would you measure concentration?)
9. In order to determine the concentration in an unknown solution, you need to prepare a standard curve. You are given a tube containing 5 ml of a 400- $\mu\text{g}/\text{ml}$ solution of protein. You are told to make *four* dilutions and record final concentrations for each of the dilutions. Use the tubes below to indicate how you would make these dilutions, and record the final concentration in each tube.



10. You have developed a standard curve for several dilutions of tyrosine, as shown below (a). Tyrosine can be oxidized by an enzyme, tyrosine oxidase, found in liver. Tyrosine reacts with nitrosonaphthol. You begin with 100 μg of tyrosine. This is mixed with tyrosine oxidase and absorbance readings are obtained (b). Use the graph to complete the table.



Time (minutes)	Absorbance (500 nm)	Tyrosine ($\mu\text{g}/\text{ml}$)
0	0.40	
0.5	0.35	
2	0.2	
5	0.1	

- a. How much tyrosine has been oxidized during a 5-minute period? _____
- b. At what rate does the tyrosine disappear during the first 2 minutes of the reaction?
_____ c. During the last 3 minutes? _____