

SPECTROPHOTOMETRIC DETERMINATION OF pK_a AND MOLAR ABSORPTIVITY

This experiment has two theory objectives and one practical objective:

1. To determine the molar absorptivity of a dye and estimate an unknown concentration of the dye in solution.
2. To estimate the pK_a of an indicator dye from spectrophotometric data.
3. (Omit from report) To provide experience in operating single- and double-beam spectrophotometers.

We will explore how solute concentration and pH of the solvent affect absorption of monochromatic light by an indicator dye (bromophenol blue). This compound is a weak acid that happens to have a complex structure. When ionized (negatively charged, since it has lost a proton), it absorbs all wavelengths but blue. When protonated, the dye appears yellow. At pH values in the vicinity of the pK_a , some dye molecules will be ionized and some will not, giving the solution a more or less green color.

The section which immediately follows deals with pK_a determination. Later there is a description of basic colorimetry and determination of molar absorptivity. These sections can be done in either order. Staggering them will ease the crunch on equipment.

EXPERIMENTAL SECTION

A. DETERMINATION OF pK_a

There will be available for you a series of citrate buffers of known pH and a solution of bromophenol blue of known concentration. Use these to prepare the following set of solutions in dry test tubes. **Pipet accurately!** Volumetric errors here will greatly affect your results. *Record the pH values and concentrations written on the stock bottles, which may not be exactly those below.*

Set up 8 clean, dry 13 x 100mm test tubes. To each tube add 0.1 mL (=100 μ L) of 0.001 M bromophenol blue stock solution plus 4.9 mL of 0.1 M citrate buffer of appropriate pH (indicated below):

Sample number	1	2	3	4	5	6	7	8
pH	3.0	3.4	3.8	4.2	4.6	5.0	5.4	>8

In these samples, what is varied and what is held constant?

Thoroughly mix each sample by inversion several times (use a piece of Parafilm). Use the Hitachi double-beam spectrophotometer in Murdock 104 or an Ocean Optics instrument to record a spectrum for each. Detailed instructions on how to operate the instruments will be made available. Set the scan range for 700-370 nm and record a baseline. Using the same cuvet, record sample spectra, starting with the bluest one. Position the cursor at the wavelength of maximum absorption near 590 nm. Record that wavelength. Record the absorbance at this wavelength of each sample. When each scan is finished, pour the sample back into its original test tube, rinse out the cuvet, and then shake it out well to minimize carryover and dilution. After filling with the next sample, wipe the optical faces clean if necessary before you insert it into the instrument. Insert the cuvet the same way each time (don't rotate it 180°). Each spectrum will automatically be recorded under a different name.

Overlay all 8 spectra and print the overlay on a single sheet of paper. Label each line clearly with the corresponding sample number.

When you return to the lab, measure the pH of each sample with a standardized electrode. You'll need to pour each sample into a small vial and assure that the porous plug of the reference electrode is below the liquid surface. Rinse the vial between readings. Make a table of $A_{(\lambda, \max)}$ vs. **measured** sample pH.

Compute a value for the pK_a of bromophenol blue (BPB) using the absorbance vs. pH data. Since the anion absorbs strongly near 590 nm but the protonated form does not, high absorbance indicates a high ratio of anionic form to uncharged form. A low absorbance naturally indicates a low ratio. Use the Henderson-Hasselbalch equation and the relationship



to develop a linear equation which allows you to graphically determine pK_a . (*Hints:* Recall that $A = \epsilon c l$, so A is proportional to c . In sample 8, which is alkaline, all the dye is in the form BPB^- . Its A_{590} value is proportional to the total quantity of dye present (T). T is the same for all samples. For samples 1 - 7, what experimental value is proportional to $[\text{BPB}^-]$? How can you calculate a value that is proportional to $[\text{HBPB}]$?)

The plot (which is a single line) and the pK_a value you determine should be presented in your results section. In an Appendix, show how you derived the equation on which your plot is based. The *Handbook of Biochemistry* gives the pK_a of bromophenol blue as 4.1*. Compare your result, and comment on any discrepancy. *Tempted to report a % error? Note that % error has no meaning for logarithmic values.*

*ed. H. Sober, CRC Press, Cleveland, OH, 1973, p. J-225.

(The following material is pertinent to Part B of this handout)

OPERATION of Turner and Spectronic 20 Single-Beam Colorimeters

See illustration on next page. Instructions for Spectronic 20 instruments differ slightly – check with instructor. Remember the procedure – you will need it every time you use a single-beam instrument.

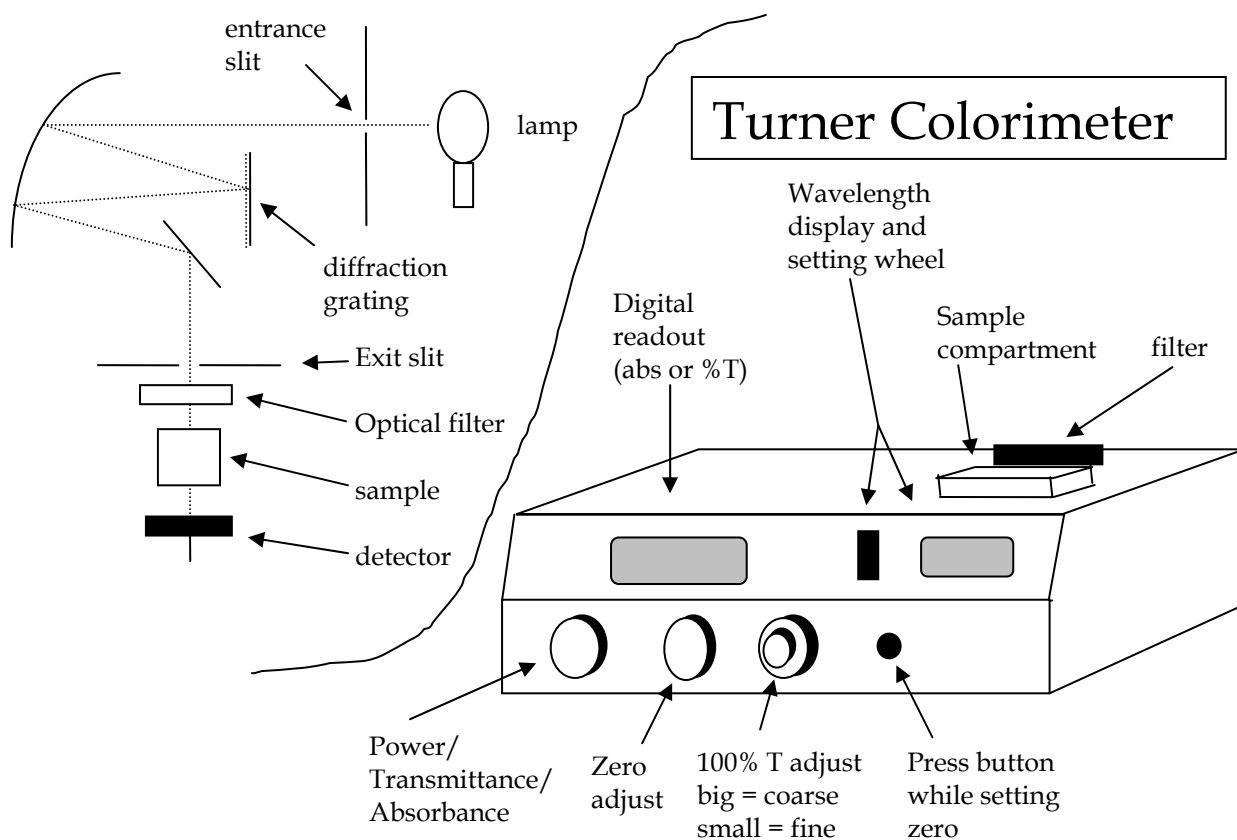
1. SELECT WAVELENGTH using the thumb wheel on the front or adjustment on upper right side of instrument. The digits in the window read in nanometers (nm). Also INSERT THE CORRECT FILTER (range is stamped on metal carrier) in the slot immediately to the rear of the sample compartment.
2. TURN ON the instrument with the lid on the sample compartment closed. On digital display instruments the knob should be set to "transmittance". Adjust the 100% T knob so the digital display reads below 100 or the meter pointer is not "pinned" off scale. Allow about 3-5 minutes warm up time.
3. HOLD IN THE *ZERO SET* button and TURN *ZERO ADJUST* knob so that the display or meter reads 0% T. This adjustment sets the instrument for complete darkness.
4. Rinse a cuvet and fill with "blank" solution (frequently water). Gently wipe the optically clear faces of cuvet with a Kimwipe. Open the sample compartment lid and raise the cuvet holder wire at the left of the holder. Place the cuvet onto holder - optically clear faces should face front and rear - and lower it into sample compartment using the wire. Be sure the cuvet has reached the bottom of its well (it sometimes sticks part way down). Close the cover.
5. With the *100% T ADJUST* knob, adjust to read 100% T. This adjustment sets the lamp intensity. Do not change the *ZERO ADJUST* knob. Wait a few seconds to be sure the reading is stable.

NOTE: Be sure you insert a cuvet with the same face toward the front each time. You can mark cuvetts if you wish. Because of optical differences, it is most accurate to read both the blank and the sample in the same cuvet. *On a digital instrument, once you have set Transmittance to 100% on the blank, remove the cuvet and note the new, higher transmittance reading. If the value is less than 150.0, record it. If the instrument drifts, you can restore it to the proper calibration without the cuvet in the path by adjusting the "100% T" knob to restore this value. Check with instructor for a variation on this if you are using a "meter" instrument.*

On digital display instruments, once the display is steady at 100.0, change the left knob to "absorbance". The display should read "0.000". Remove the "blank" solution.

6. Put the unknown sample solution into a sample cuvet and read its absorbance. Make sure that the optically clear faces of the cuvet are clean, that they face front and rear, that the cuvet is all the way down, and that the lid on the sample compartment is closed during the reading.

7. REPEAT STEP 6 for all solutions to be measured.



BASIC SPECTROPHOTOMETRY

Look first at the left side of the picture above. Light (all wavelengths) from the lamp passes through the entrance slit and then is diffracted by a diffraction grating. In our single-beam spectrophotometers, the "monochromatic" light produced by this process has a bandwidth of about 10 nanometers (nm). This band of selected wavelengths is passed through the sample, which may absorb part of the energy, and then on to a photodetector. Colorimeters have a single beam, which means that one must read the blank and the sample successively. One adjusts the instrument with a blank solution (which usually contains all solutes except the colored or light-absorbing one) and then reads the sample solution. Any absorbance (transmittance of less than 100%) is caused by colored solute. (A double beam instrument does the comparison with the blank at the same time that the sample is read.)

The "blank" calibration (i.e., 100% T) of the instrument does not change (assuming that the instrument is reasonably stable) provided the wavelength selector is not changed. If you want to measure the absorbance of a series of samples at a single wavelength, it is usually sufficient to read the blank at the beginning and end of the series. However, if the wavelength is changed, it is necessary to "rezero" the instrument (re-set the 100% T reading) using the blank. This is because lamp output, optics throughput and detector efficiency all vary with wavelength.

B. DETERMINATION OF MOLAR ABSORPTIVITY

We could use the data from part A to calculate the molar absorptivity of the chromophore, but this would not be scientifically sound since we cannot tell if the dye is obeying the Beer-Lambert Law (which says that $A \propto C$) at the concentration used. Accuracy demands construction of a standard curve. This is one of the most frequently needed operations in biochemistry.

Label two small test tubes. In one put about 1 mL of 10^{-3} M dye (copy its actual concentration from the stock bottle label – *be sure you write the correct number of significant figures!*), and in the second put 1 mL of your assigned unknown. **Record the identity (letter) of your unknown.**

First, you need to dilute the stock solution of dye to about 5×10^{-5} M, since the provided 10^{-3} M stock is too opaque. There are two ways to do this. Choose the way you think will work best for you.

(a) The old-fashioned way using glass pipets: Work out a set of serial dilutions of the standard dye. A good strategy is to pick a dilution factor in the range of 2 - 10 for each step (it can be smaller than 2 if necessary). That way, the volume of the amount you pipet each time is a significant fraction of the total volume after dilution. This minimizes volumetric errors. Perform these serial dilutions accurately (this includes thorough mixing at each step!) and keep clear records of what you do. Consider the hazard of carryover (or dilution) from liquid on the outside or the inside of your pipet(s) and other glassware you use.

(b) Use micropipettors: Work out the necessary volume of concentrated stock solution to transfer *via* micropipet and the needed final volume. It is recommended that you make replicate dilutions to verify the repeatability of your technique. Note that use of this technique forces you to rely on the instrument's ability to deliver a very small volume accurately and repeatably.

Once you have your diluted stock solution of dye, compute its concentration to the proper number of significant figures and prepare the following set of 6 further dilutions of it in dry test tubes (shaking out is OK – don't wipe inside with a Kimwipe (lint!)). Pipet carefully and mix each thoroughly. After the series is complete, make a carefully prepared dilution of the unknown **whose color is fairly dark but no darker than that of your 5×10^{-5} M dye stock solution**. Put 1.0 mL of this in tube 7.

Reagent ↓	Tube #						
	1	2	3	4	5	6	7
5×10^{-5} M bromophenol blue (mL)	0.1	0.2	0.4	0.6	0.8	1.0	---
0.1 M Na citrate (mL)	4.9	4.8	4.6	4.4	4.2	4.0	4.0
Diluted Unknown (tube 7 should be no darker than tube 6)	---	---	---	---	---	---	1.0
Final conc. of dye in tube?→	___	___	___	___	___	___	___

(0.1 M Na citrate is used rather than a buffer since it is mildly alkaline and so converts all of the dye into its blue (charged) form.)

Set a single beam colorimeter to 590 nm and zero it on a blank consisting of 5.0 mL of 0.1 M Na citrate in the cuvet you plan to use for all samples. Between samples, shake out the cuvet and rinse it with about one mL of the next sample to minimize carryover from the previous contents. Begin with the most dilute sample.

- Compute the final concentrations of dye in tubes 1-7 (*with the correct number of significant figures*). Plot the measured A_{590} (dependent variable, thus ordinate) against the concentration of the dye (independent variable, thus abscissa). Remember that the blank reading (which should be zero, because the solute concentration is zero) is always part of your data. The lower concentrations should fall along a straight line; the highest ones may deviate. The absorbance of the dye follows the Beer-Lambert Law where the data define a straight line. Calculate the molar absorptivity (ϵ) from the slope of this line (what are its units?). It represents the efficiency with which the chromophore (BPB⁻) absorbs photons of the chosen wavelength.

The value of ϵ is a physical constant which depends on the physical structure of the chromophore (affected, in this case, by the pH of the solvent) **and** on the wavelength of light used. Thus it is very important to specify both the wavelength and the chemical conditions under which the measurement was made.

- Use your value of ϵ to calculate the original concentration (**before dilution**) of dye in the unknown stock bottle. **In your report, identify by letter which unknown you took!** Caution: how many dilutions did you do on your unknown? Did you take into account the last one (in Table above)?

LAB REPORT

An *assay* is a procedure used to measure the amount of something you are interested in. Many students do not know how to describe assay protocols in a professional manner. Typical errors include reporting the number of tubes used and actual volumes of each reagent used in each tube, or presenting a table with this information. None of this is normally necessary, and you should avoid doing it. In this experiment, the protocol for generating the standard curve should be identified by a clear subheading that states the purpose served, and should resemble the following, in which the *italicized* letters stand for numbers that you provide:

"Standard curve for bromophenol blue: The provided *X M* stock solution of bromophenol blue was diluted *y* fold with water to create a working solution (*Z M*). Various proportions of this working solution and sodium citrate (0.1 M) were mixed, and the A_{590} values of the resulting solutions were measured."

This short, undetailed protocol is preferred because the concentrations and related A_{590} values are on the Figure that you show. The actual numbers needed for computation of concentrations are in your notebook, from which they can be retrieved should that become necessary. Note that contents of samples are identified first by chemical name. Following this, in parentheses, you should state first the concentration, and then, if necessary, the pH. An example of this format is shown in the sentence below for a more complicated assay (not related to the present experiment):

"Each assay contained pyruvate (0.03 – 0.2 mM), NADH (0.13 mM), oxamate (0.3 mM), and sufficient phosphate buffer (0.1 M, pH 7) to bring the volume to 2.9 mL." Note that final concentrations of reagents and the final volume of the assay mixture are reported, not the stock concentrations and individual volumes used. (The buffer is an exception, since its final concentration isn't very important.)

Please remember this strategy when you write protocols for assays that you describe in future experiments. Overly detailed protocols will be returned for revision.

QUESTIONS to answer in an appendix

1. Assuming that you followed the protocol as described in the experimental section, would a scratch on the sample cuvet affect a reading on a double-beam instrument? Why? On a single beam instrument? Why?
2. Suppose your sample has an absorbance greater than 1.5 (this means that less than 5% of the original light is getting to the detector). You only have a simple colorimeter which does not offer high precision for strongly absorbing samples. What is the best way to get reliable data?
3. a. On a single-beam instrument, why is it essential to re-zero every time the wavelength is changed? Give two instrument-related reasons.
b. Why is this not a problem on double-beam instruments?
4. You are given a bottle that contains a pure solid for which you must determine the ϵ value at a particular wavelength. You know its molecular weight, and it is sufficiently soluble in the chosen solvent. You have a spectrophotometer of excellent quality. In spite of all this, the ϵ value will have some degree of error. In order to gauge the reliability of ϵ , you need to know the precision of two different measurements that must be used to compute ϵ . What are these measurements?
(They are not related to the spectrophotometer. Think about **what physical operations** you have to do in the lab to prepare the necessary solutions of the solid.)