Introduction to the Spectrophotometer:

Wavelength, Absorbance, and Concentration

In Methylene Blue

Kevin Donnelly

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TA: Alex Trachtenberg

Lab partners: Tamara Jette and Wayne Thornton

INTRODUCTION

The spectrophotometer is an essential tool for biologists and chemists in analyzing chemical and biological samples. Gaining familiarity with its operating protocols and understanding what its outputs mean are very important in the development of lab technique for students of cell biology. This experiment will help laboratory students gain experience in using the spectrophotometer.

This instrument takes advantage of the regular light absorption and scattering patterns of chemical structures (Lab Manual, p.19). Specifically, it detects compounds absorbing light at selected wavelengths and produces a number corresponding to its absorption (Alberts, 2004). Each compound absorbs and scatters this light more than others at specific wavelengths. Years of research have yielded information on a myriad of compounds, permitting one to use spectrophotometry as a way of identifying unknown compounds and determining the concentration of a substance.

Specifically, the spectrophotometer measures quantitatively the amount of light passing through a compound in solution as a fraction of the light emitted by the machine ("Spectrophotometry"). A monochromator is used to produce light in very small ranges of wavelength. A photodetector detects how much of the light emitted by the machine actually transmits through the solution. A specific wavelength is input, and a display indicating percent transmittance (and its inverse, absorption or optical density) communicates to the user the absorption of a compound at this wavelength.

This experiment includes two pieces related to the spectrophotometer. First, a determination of the wavelength at which a compound (in this case, methylene blue) absorbs light best is made. Second, using this wavelength, solutions of varied concentrations are measured for their absorptions; this measurement relies on the idea that a greater number of molecules in a given volume will absorb more light than one that has fewer (Lab Manual, p. 23).

Two hypotheses may be made prior to performing this experiment. First, the absorption spectrum obtained from methylene blue should peak at an intermediate range approximately equal to 668 nm ("Optical Absorption of Methylene Blue"). In addition, the absorptions of serial dilutions of methylene

blue should yield a linear relationship, as the absorbance of a substance should be proportional to its concentration.

METHODS

This experiment consisted of three parts. First, an absorption spectra was created using the Spectronic-20 spectrophotometer. Using information gleaned from the absorption spectra and serial dilutions of the substance subsequently measured for absorption, a standard curve of absorption against concentration was created. Finally, a sample of unknown concentration was tested for its absorption level and, using the standard curve, its concentration was determined.

To begin the experiment, the spec-20 spectrophotometer was turned on to allow time for it warm up. This was done before all else, and left on for approximately 15 minutes prior to any use of the machine. To prepare for the measurement of the absorbance spectrum, two test tubes were prepared. A test tube containing 5 ml of the stock solution of methylene blue, stated to be a 3.5×10^{-5} M concentration, was prepared with the label "stock." Also, a test tube containing 5 ml water was made, labeled "blank." Separate pipettes were used for each test tube filling, to protect from contamination of the blank or dilution of the stock. With the machine properly warmed and ready for experimentation, the wavelength was set to 400 nm. Next, the machine was set to zero. With no tube in the machine, the zero control was turned to adjust the needle to a 0% transmittance/infinite optical density (OD). The blank tube was placed in the sample chamber, the cover to the chamber was closed, and the transmittance/absorbance control knob was adjusted so the needle read 100% transmittance/0 OD. The blank tube was then removed.

With the machine warmed and properly zeroed, data for the absorbance spectrum was collected. With the wavelength set to 400 nm, the stock solution tube was placed in the sample chamber, and its OD was recorded. The tube was removed, the wavelength control set to 425 nm, and the machine was zeroed again. Though the dial for 0% transmittance was not observed to be adjusted when the machine was empty and the wavelength adjusted, it was necessary to place the blank tube in the sample chamber and the dial adjusted to reestablish a reading of 100% transmittance. The blank tube was replaced by the stock tube, and a second OD reading was recorded. This same set of steps (adjust the wavelength control

by 25 nm, zero the machine, measure the OD of the stock solution at the new wavelength) was repeated 21 times, until readings for the same stock solution across a wide spectrum of wavelengths was recorded. The only extra adjustment needed during this process occurred at the 600 nm reading, when the machine's filter lever needed be flipped to its second position; this was forgotten at first, and resulted in initial readings at 600nm, 625nm, and 650nm inconsistent with the hypothetical trend. The final readings recorded, however, reflect the proper adjustment of the lever as required by the spec-20. The results of this part of the experiment were recorded in a table, and then plotted using Microsoft Excel. A peak wavelength was recorded for use in the next step in the experiment.

To determine the standard curve of absorbance versus concentration, several steps were necessary. First, serial dilutions of the stock solution were made. Using the pipettes from step 1, 5 ml of stock solution was placed in one vial without any water. A second vial containing 4.5 ml and 0.5 ml of stock solution and water, respectively, was made. This process was continued for a total of 9 vials (each totaling 5 ml, with remaining ratios of stock:water::4.0:1.0, 3.5:1.5, 3.0:2.0, 2.5:2.5, 2.0:3.0, 1.5:3.5, 1.0:4.0). The wavelength was held constant through this experiment. The wavelength used was the peak wavelength from the previous portion of this experiment: 625nm.[1] The machine was zeroed for this wavelength using the steps outlined earlier. Each dilution tube was placed individually in the sample chamber and the resulting OD was recorded. Alongside this data, the molarity of each sample was calculated and their corresponding g/L concentrations were, as well. This data was plotted using Microsoft Excel in an X-Y scatter plot. A best fit line (standard curve) was calculated and plotted using Excel, and the equation for this line included.

A tube of the stock solution with unknown concentration labeled "B" was obtained from the instructor for evaluation. This tube was placed in the sample chamber of the spec-20 without adjustment from the previous step in the experiment; its OD was recorded at a wavelength of 625 nm and its OD was

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[1] 625 nm is not the actual peak wavelength recorded for the stock solution used. Two higher results were excluded because there was instruction to exclude wavelengths with correlated OD values greater than 0.8. See Table 1 in the results section for these values.

recorded. Having established the standard curve for this wavelength, the sample of unknown

concentration was plotted on the best fit line using OD. Its correlating concentration was then found.

RESULTS

The first experiment yielded wavelength and absorbance (OD) readings as shown in Table 1.

These results were plotted in Figure 1. Figure 1 shows the absorbance increasing as the wavelength is

increased until approximately 650 nm is reached; from this point, the absorbance pattern decreases

rapidly. Therefore the peak wavelength for Methylene Blue is approximately 650 nm.

Table 1

Wavelength (nm)	Absorbance (OD)	Wavelength (nm)	Absorbance (OD)
400	0.018	675	0.800
425	0.010	700	0.165
450	0.025	725	0.035
475	0.038	750	0.010
500	0.055	775	0.008
525	0.068	800	0.008
550	0.180	825	0.009
575	0.300	850	0.005
600	0.560	875	0.001
625	0.725	900	0.005
650	0.975		

**Figure 1:**
Absorbance vs. Wavelength
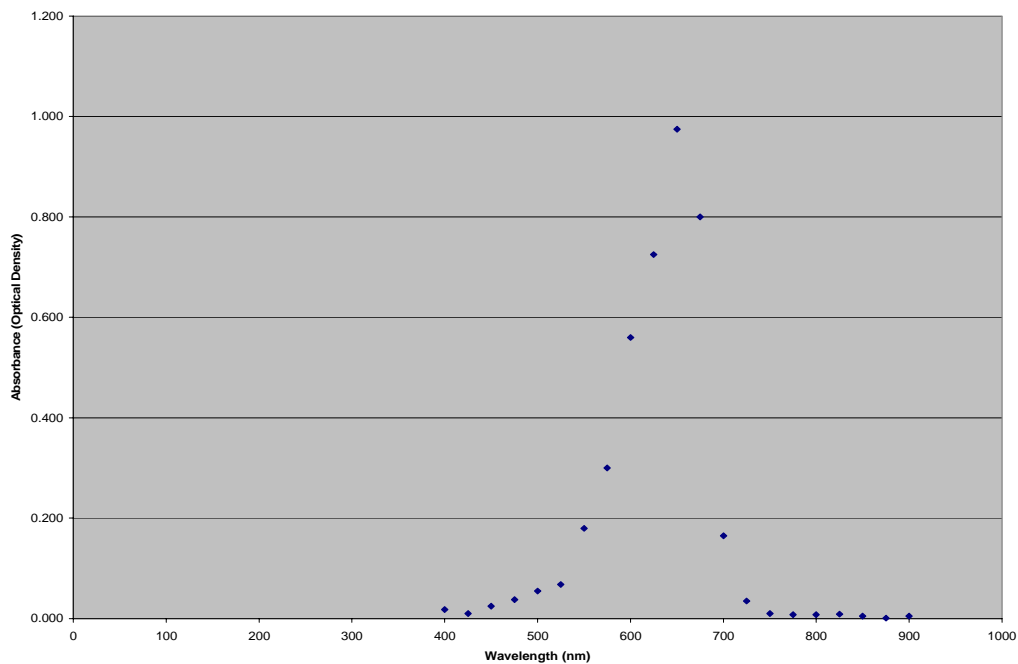
Using the peak wavelength (below 0.8 OD), Table 2 and its corresponding Figure 2 were created. The equation for the best line is: $Y = 20476X + 0.0211$. Also, the value $R2 = 0.987$ for this line was determined by Excel. Finally, the value for the unknown concentration B shown in Table 2 was calculated based on the equation for the best fit line. This value was measured on the graph using a ruler to be approximately 1.00×10^{-6} M; using the formula it was determined to be 9.76×10^{-6} M. In addition, concentration in g/L were calculated, and included in Table 2. The unknown concentration B in these terms was found to be 3.11×10^{-6} g/L.

Table 2

Tube	ml of stock	ml of water	Concentration (mol/L)	Absorbance (OD)	grams/L
1	5.0	0.0	3.50E-05	0.725	1.12E-02
2	4.5	2.5	3.15E-05	0.680	1.01E-02
3	4.0	1.0	2.80E-05	0.580	8.95E-03
4	3.5	1.5	2.45E-05	0.530	7.84E-03
5	3.0	2.0	2.10E-05	0.450	6.72E-03
6	2.5	2.5	1.75E-05	0.370	5.60E-03
7	2.0	3.0	1.40E-05	0.360	4.48E-03
8	1.5	3.5	1.05E-05	0.220	3.36E-03
9	1.0	4.0	7.00E-06	0.145	2.40E-04
Unknown (B):			**9.76E-06**	0.220	**3.11E-03**

Figure 2:
Absorbance vs. Concentration
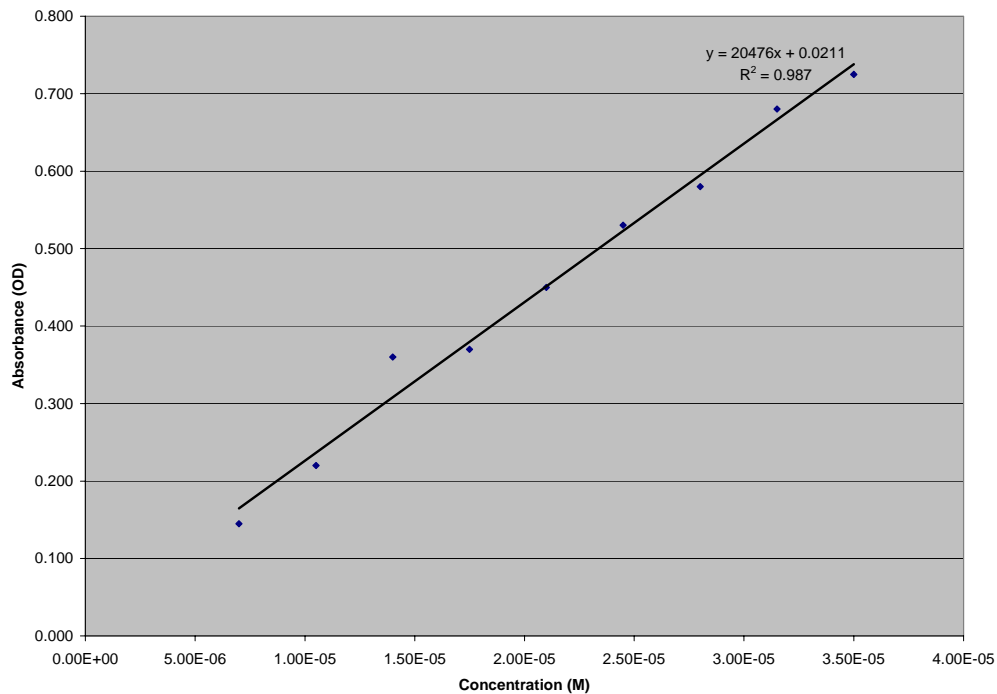
DISCUSSION

        Two hypotheses were evaluated in the course of this experiment. The first hypothesis, that methylene blue would absorb light at 668 nm, was not able to be falsified. The peak wavelength occurred at 650 nm. The next highest occurred at 675 nm, and the third at 625 nm. Given that the OD measured at 675 nm was slightly higher than that at 625 nm, it is likely that the peak wavelength actually exists somewhere between the measures evaluated at 650 nm and 675 nm. The second hypothesis, that a linear relationship between absorbance and concentration would be found, was also not falsified based on the experiment. The R^2 value for the best fit line was 0.987; in statistics, the R^2 value can be anywhere from 0 to 1, with 0 being least likelihood of correlation, and 1 indicating a perfect correlation between events. As the R^2 value was very close to 1, a high degree of correlation was found.

        Given the results, little error appears likely in the experimental procedures. However, any doubts regarding the results may be traced to a few elements of the experiment that lend themselves to possible error. The need to zero the machine between each of the readings in obtaining the absorption spectrum, and resulting peak wavelength, leaves room for error between each reset procedure. Though the student performing the zeroing was supported in his procedure by two lab partners, the precision with which a person can accurately adjust the needle on the spectrophotometer to zero is limited. Another place where error is likely is in the serial dilution of methylene blue. Though there is a presumption that the pipetting was done accurately, similar room for human error in measurement is possible as in the repeated zeroing of the instrument. Finally, reading the OD outputs became less precise the higher the OD reads; the machine measures more precisely smaller increments, with larger increments closer together on the dial.

        A lack of precision in all three of the stated procedures above is possible. However, error appears to have been minimized in the experiment, assuming the hypotheses made were not misguided. One way of determining the accuracy of these results would be to repeat this experiment several times, finding a mean between the data obtained.

REFERENCES

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