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TOTAL	**8.5/10**

LABORATORY REPORT

GROUP NUMBER: w3

EXPERIMENT NUMBER: 5

UV-IS Spectrophotometry: Visible Spectrum of Hemoglobin

DATE: February 25, 2000.

ROLE ASSIGNMENTS

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ABSTRACT

The absorption spectra of bovine oxy- and deoxy-hemoglobin and their molar extinction coefficients were determined at a wavelength of 410 nm using the Spectronic Genesys 5 Spectrometer. This was done by making a working solution of a 1:75 dilution of bovine blood to a phosphate buffered saline solution that yielded absorption of 1.013. A series of dilutions was made from the original 100% working solution by 20% intervals and the final was a 10% dilution of the original. A regression plot of concentration versus absorption data revealed a slope of $3.16E^5 \pm$ (YOU SHOULD IDENTIFY THIS AS THE UNCERTAINTY $0.07E^5$) L/mol-cm, which is the molar extinction coefficient ($\Delta A/\Delta C$) for the wavelength used. The uncertainty of $0.07E^5$ L/mol-cm was determined by looking at the upper and lower region of the 95% confidence interval, which was between $3.09E^5$ and $3.23E^5$ L/mol-cm. Determination of the absorbance of 1:75 dilution of bovine oxy- and deoxy- hemoglobin across different wavelengths show that their maximum absorbance occur at wavelengths of 411 nm and 405 nm, respectively. In addition, two minor absorption peaks at higher wavelengths are present in the bovine oxy-hemoglobin, at wavelengths of 540 nm and 576 nm. No such peaks were present in the deoxy-

hemoglobin. The difference arose from the conformational change that occurs when oxygenated bovine hemoglobin becomes deoxygenated. No literature value was found for bovine hemoglobin. HOW DOES IT COMPARE TO HUMAN HEMOGLOBIN?

RESULTS

Results for the absorbance spectra of bovine oxy- and deoxy-hemoglobin and molar extinction coefficients using the Spectronic Genesys 5 Spectrophotometer at a wavelength of 410nm are listed and explained below.

Table 1: Tabulation of Absorbance Values, Protein Concentrations, and Molar Extinction Coefficients

Protein	Concentrations (M)	Absorbance	Notes	Molar Extinction Coefficient	Uncertainty (%)	Uncertainty (Absolute Value)
100% Deoxy	3.17E-06	0.705	*cuvette had a fingerprint	2.22E+05	3.8	± 0.08 E+05
100% Oxy	3.17E-06	1.013		3.2E+05	3.8	± 0.1 E+05
80% Oxy	2.54E-06	0.795		3.1E+05	6.3	± 0.2 E+05
60% Oxy	1.90E-06	0.6		3.2E+05	4.5	± 0.1 E+05
40% Oxy	1.27E-06	0.41		3.2E+05	4.5	± 0.1 E+05
20% Oxy	6.35E-07	0.212		3.3E+05	6.3	± 0.2 E+05
10% Oxy	3.17E-07	0.099		3.1E+05	10.7	± 0.3 E+05

The 100% concentration denotes a dilution of 1:75 of the bovine blood. Subsequent dilutions are made from the 1:75 working solution as seen in Table 1. Absorbances were tabulated from the spectrophotometer. By using the gravimetric data of the stock solution, the concentration values for each protein concentration was determined.[1] Molar extinction coefficients for each protein concentration were determined by dividing absorbance values by their corresponding concentrations and the length of the cuvette (1 cm).

The uncertainty was determined by converting the absolute uncertainty on the mass scale (± 0.1 g) to a percentage. This was done by taking 0.1 g and dividing it by the amount of solution that was added each time. The uncertainty as given above is the practical uncertainty, derived by taking the square root of the sum of the uncertainties squared. The only other source of error, which was the error arising from the spectrophotometer, was insignificant compared to the error derived from using the mass scale.

The graph for the above data follows as Figure 1.

[1] Bioengineering Course Web Page (http://www.seas.upenn.edu/~be210).

Figure 1: Absorbance versus Concentration at 410nm for Oxy- and Deoxy- hemoglobin

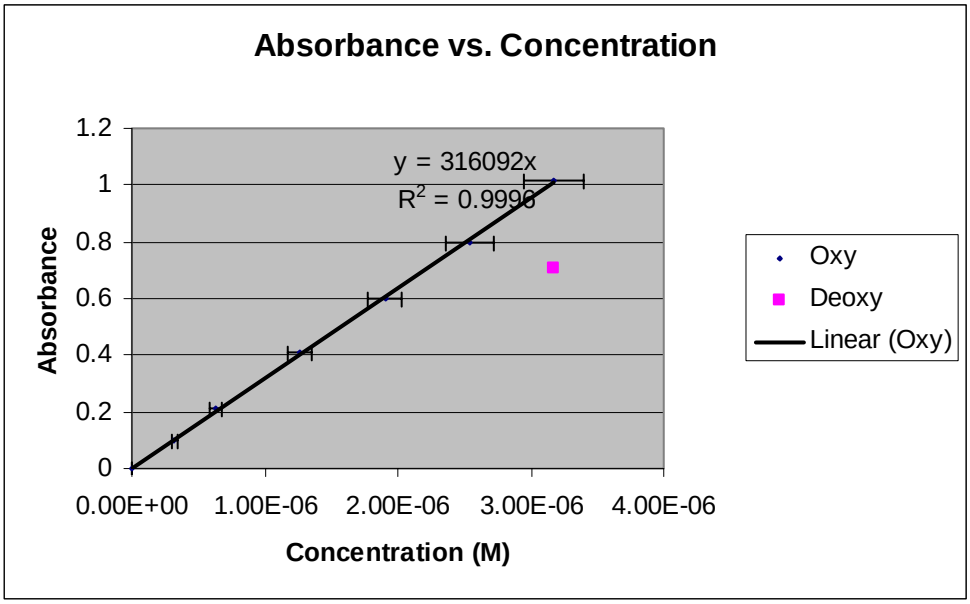


Figure 1 displays the varying absorbance of oxy-hemoglobin as a function of concentration at 410 nm. YOU SHOULD EXPLAIN WHAT THE ERROR BARS REPRESENT. There is also a single point correlating to the absorption of 100% deoxy-hemoglobin at 410 nm. As can be seen on the figure, this is a linear relationship correlating the amount of absorbance and concentration of the oxy-hemoglobin. The slope of this curve is 3.16E5 L/mol-cm and is the value of the molar extinction coefficient of oxy-hemoglobin at this wavelength. The regression analysis of this curve follows as Table 2.

Table 2: Regression Analysis for the Absorbance versus Concentration Graph

SUMMARY OUTPUT

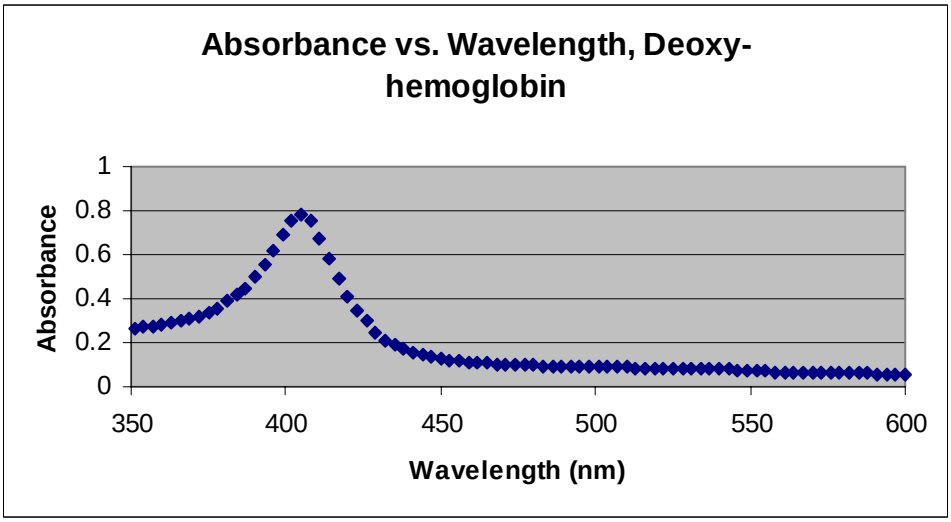
Regression Statistics	
Multiple R	0.999815003
R Square	0.99963004
Adjusted R Square	0.999556049
Standard Error	0.007894872
Observations	7

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	0.002843003	0.004848333	0.586387815	0.583087444	-0.009620013	0.01530602
X Variable 1	316092.4982`		116.232486	8.93968E-10	309101.851	323083.1454

Regression analysis for the absorption spectra of the bovine oxy- and deoxy-hemoglobin are shown in Table 2. As seen through the data (YOU MEAN THE REGRESSION STATISTICS) shown above the y intercept (0.00284) is not significantly different from zero, it lies between the upper (0.0153) and lower (-0.00962) 95% confidence interval for

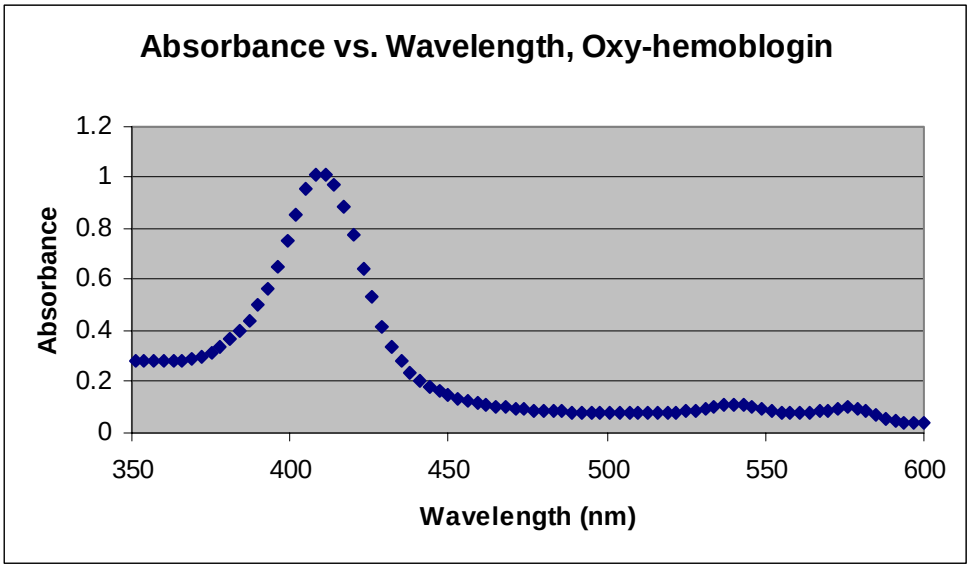
the linear region. Data shows that zero lies within the confidence interval and therefore the experimental data is significant.

Figure 2: Absorption Spectra of Deoxy-hemoglobin (1:75 dilution of bovine blood)



An absorption spectrum of deoxy-hemoglobin was done using Survey Scan mode on the spectrophotometer. As seen on Figure 2, the maximum absorbance of 0.783 occurs at a wavelength of 405 nm. There is no other maximum absorbance at any other wavelengths.

Figure 3. Absorption Spectra of Oxyhemoglobin (1:75 dilution of bovine blood)



An absorption spectrum of oxy-hemoglobin was done using Survey Scan mode on the spectrophotometer. As seen on Figure 3, the maximum absorbance of 1.013 occurs at a wavelength of 411 nm. There are two peaks at two higher wavelengths. These are: a

maximum absorbance value of 0.108 at wavelength of 540 nm, and a maximum absorbance value of 0.102 at wavelength of 576 nm. Discussion of these two peaks follows in the analysis.

ANALYSIS

In the lab conducted, UV-IS Spectrophotometry, the visible spectrum of bovine hemoglobin was determined by using the Spectronic Genesys 5 Spectrophotometer at a 410nm wavelength. Figure 1 shows absorbance versus concentration at 410nm for oxyhemoglobin. The regression analysis (Table 2) for oxy-hemoglobin revealed a linear relationship between the concentration and absorption (nm) (R^2 =0.996). The y intercept on the graph in Figure 1 is 0.00284 and is not significantly different from zero, because zero lies between the upper (0.0153) and lower (-0.00962) 95% confidence interval for y intercept of the linear region.

The experimental value determined for the molar extinction coefficient of bovine oxy-hemoglobin at 410 nm is 3.16E5 ± 0.07E5 L / mol-cm and is the slope of the absorbance vs. concentration graph (Figure 1). The 95 % confidence limits for the molar extinction coefficient were determined by regression analysis to be from 3.09E5 L / mol-cm to 3.23E5 L-mol/ cm. This means that the molar extinction coefficients for 100%, 80%, 60%, 40%, and 10% bovine oxy-hemoglobin solutions ($3.19E^5$, $3.13E^5$, $3.15E^5$, $3.23E^5$, $3.12E^5$ respectively) were not significantly different than the extinction coefficient ($3.16E^5$ L / mol-cm) calculated from the slope of the regression plot, because they were all within the 95% confidence range. The extinction coefficient for 20% bovine oxy-hemoglobin solution ($3.34E^5$), though, lay outside of the 95% confidence range of the data and thus was significantly different.

Another way to analyze the data is by looking at the individual uncertainties. Qualitatively, it is clear that the lower concentration solutions had higher uncertainties because the method of dilution (mass balance) was accurate only to 0.1g, and at the lower concentrations, accuracy to the hundredths decimal was needed for accurate dilution. This resulted in a very large uncertainty for the concentrations in the experiment. For example, the 20% bovine oxy-hemoglobin solution had an uncertainty of ± 6.3% for the molar extinction coefficient, giving it an allowable range for values from $3.1E^5$ to $3.6E^5$. Since the experimental value obtained ($3.16E^5$ ± 0.0 $7E^5$ L/ mol-cm) falls within the uncertainty, it is acceptable given the uncertainty of the experiment. A similar analysis reveals that all experimental values fall within the uncertainty range. However, the uncertainty range is far too large. One way to decrease the amount of uncertainty in this experiment would have been to use a more accurate balance, or create more volume of diluted sample so that the group would not be as limited by the significant digits of the scale.

The bovine oxy- and deoxy-hemoglobin Survey Scans (Figure 2 and 3) highlighted the differences between oxygen being and not being present in bovine hemoglobin. The spectral data analysis at 410 nm concludes that the bovine deoxy-hemoglobin sample has

a molar extinction coefficient of 2.22E+05, whereas the extinction coefficient for bovine oxy-hemoglobin was 3.16E+05. Because of conformational changes in the hemoglobin protein that result when oxygen is added (the addition of the oxygen molecules make the structure more dense – YOU MEAN IT WEIGHS MORE? THE FOLDING OF THE PROTEIN CHANGES AND THIS ALTERS ITS OPTICAL PROPERTIES) the molar extinction coefficient was higher for bovine oxy-hemoglobin than for deoxy-hemoglobin. Also while the absorption of the bovine oxy-hemoglobin is 1.013, with an absorbance peak wavelength of 411nm, the absorption of bovine deoxy-hemoglobin was considerably lower – 0.783, and it occurred at a wavelength of 405 nm. The structural change in the bovine hemoglobin molecule when it is oxygenated creates a 6nm difference in the maximum absorbance wavelength between bovine oxy- and deoxy-hemoglobin, as well as a 0.230 difference in absorption values. Also, two minor absorption peaks at higher wavelengths are present in the bovine oxy-hemoglobin. These miniature peaks of A = 0.104 and A = 0.102 occur at wavelengths 540nm and 576nm respectively. The molar extinction for these peaks was $3.29E^4$ and $3.22E^4$ respectively. No peaks were present in the deoxy-hemoglobin.

As can be witnessed in the absorbance of 100% ($3.17E^{-5}$ M concentration) oxyhemoglobin graph (Figure 3), there are two minor absorbance peaks at higher wavelengths. These are: a maximum absorbance value of 0.108 at wavelength of 540 nm, and a maximum absorbance value of 0.102 at wavelength of 576 nm. The molar extinction coefficients and their respective uncertainties for these two wavelengths are $3.41E^4 \pm 0.04\ E^4$ and $3.22\ E^4 \pm 0.04\ E^4$. Because the focus of this experiment was on the major peak at 410 nm, the scale makes it very hard to determine the molar extinction coefficients of the minor absorbance peaks without a large amount of uncertainty. If an experiment wanted to look more closely at the peaks, it would be necessary that the concentration of the working solution be increased to give a bigger absorbance. This is because the uncertainty on the spectrophotometer is given as an absolute scale: the spectrophotometer measures only up to the thousandth digit, and thus the digits beyond that are uncertain. To target the absorbance value at 1.5, the concentration of the working solution at 540 nm wavelength should be $4.40E^{-5}$ M (or 13.89 times the current working solution) and that at 576 nm should be $4.66E^{-5}$ M (or 14.70 times the current working solution). Increasing the concentration of the working solution to $4.5E^{-5}$ M would allow for accurate determination of both minor absorbance peaks.

In conclusion, bovine hemoglobin was diluted serially and its absorbance on the spectrophotometer was measured. The molar extinction coefficient for bovine hemoglobin was determined at 410 nm to be $3.16E^5 \pm 0.07\ E^5$, with the uncertainty value obtained from 95% confidence interval. Absorption spectra were determined on 1:75 dilution oxy-hemoglobin and deoxy-hemoglobin. Absorption spectrum of oxy-hemoglobin reveals a major peak of absorbance and two minor ones, at 411nm, 540 and 576 nm respectively. Absorption spectrum of deoxy-hemoglobin reveals a single absorbance peak of 405 nm. ARE THESE RESULTS SIMILAR TO MOLAR EXTINCTION VALUES FOR HUMAN BLOOD?

The experimental and educational objectives were both met. Group members measured absorbency levels of a working solution of bovine hemoglobin at different dilutions as a function of concentration, determined molar extinction coefficients, and quantitatively analyzed both the data obtained in the lab and possible techniques for minimizing future error in the apparatus.

REFERENCES

Litt, Mitchell. UV-Visible Spectrophotometry:Visible Spectrum of Hemoglobin. *Bioengineering Laboratory Manual*. pp. 1-7, 2000

Bioengineering Course Web Page (http://www.seas.upenn.edu/~be210).