1 Introduction

Biochemistry is the chemistry that occurs in biological systems. It is elegantly simple, yet extraordinarily complex. In order to understand this branch of science, one must 1) be skilled in many of the basic techniques of chemistry and 2) be familiar with the theories and principles of both biological and physical disciplines. As such, a good biochemist must accept and use the theories and techniques from all allied areas and disciplines.

An aim of this course is to develop your understanding of biochemical and molecular biological experimentation. Your course instructor is a biochemistry enthusiast who has certain expectations of you. First, when you walk into the laboratory, you must be familiar with the general background material concerning each experiment. As such, you must read and study the experiment before you get to class. Reading and studying the experiment before coming to class not only help you do well on the quizzes and examinations, but will enable you to efficiently perform the experiments, and analyze and interpret the experimental data/results as well. Additionally, you will be required to perform and interpret many calculations during the course of an experiment. Sometimes, the calculations from one part of the experiment will be used in another. Other times, the calculations are required for data interpretation. Consequently, it is very important that you understand what you are doing at each step and the rationale for doing it.

As is the case in all of your chemistry laboratory courses, you must keep a laboratory notebook containing all of your data, calculations, graphs, tables, results, and observations. Your notebook does not have to be so neatly written that it takes you in an inordinate amount of time to write down the required information or to draw a table. However, it must be written in such a way that anyone, and that includes a middle-aged professor with failing eyesight and frequent “senior moments”, can quickly understand what has been done and the results that were obtained.

You will also be required to write and submit typed laboratory reports. Communication is very important in science. As such, a successful scientist must be able to let the scientific community know what s/he has achieved in both oral and written form. The laboratory report will give you practice in honing your written communication skills. Consequently, most of the elements required for your laboratory notebook must also be included in the type-written report that you will submit to your instructor. Additionally, writing the report gives you the opportunity to enhance your analytical and critical-thinking skills because you will have to interpret your experimental results, relate them to the theory that you learn in the lecture, and draw conclusions from them. Your instructor will discuss the specific requirements for your notebook and laboratory report with you on the first day of class.

This manual contains a compilation of experiments that introduce students to some basic biochemical techniques and concepts. The experiments have been developed, tested, modified, and tested again! As such, there is a high rate of success for all of them.

However, there will be times when your results do not appear to be particularly useful or when the data are ambiguous. When these things occur, you must be able to give a plausible explanation of your results. It is never sufficient, nor is it acceptable, to simply state "the experiment didn't work!" If you have done the background reading prior to coming to class and paid attention to the pre-lab lecture given by your instructor, the task of explaining your results will be easier than if you come to class unprepared. At this level in your education, it is expected that you are able to, *and will*, **think** about your results and adequately interpret them. Remember R.I.F. (Reading Is Fundamental)? Well, at this point you must also remember T.I.F. (***T***hinking ***I***s ***F***undamental)!

This compilation has been assembled over thirty some odd years by efforts of two really terrific classical biochemists, Dr. Leonard Price (1968 – 2002) and Dr. Donald Robinson (1975* – 2001), with more recent contributions made by Dr. Tuajuanda Jordan (1994 -) , Dr. Nitsa Rosenzweig (1998* -2004), and Dr. David Wolfgang (2002 -). This compilation evolved into the first laboratory manual assembled by Dr. Jordan in 1995 and it has continued to evolve into this tenth edition, published in Fall 2005. It is my humble and unsolicited opinion that this is the best one yet. It is my hope that, by the end of the semester, you will understand some applications of the biochemical theory to which you are introduced in lecture. Additionally, it is equally important that you will have enjoyed your experience of being a budding / blossoming biochemist and, perhaps, aspire to become one!*

Have a safe and wonderful semester!

dr. tj, July 2003

**These start dates are only approximations since, at the time of this writing, there is no one around to correct me! The only one that is an absolute certainty is my own. What you should get out of it is that there were two people who got the biochemistry laboratory course up and running a long time ago and three other people who have made more recent contributions.*

A. Laboratory Safety

Although biochemists study the chemistry that occurs in living organisms, and we know that the compounds at the *level* that they occur in your *healthy body* are not particularly detrimental, the experiments in this laboratory manual employ some potentially hazardous reagents. Biochemists oftentimes find themselves working with strong acids and bases, corrosive materials, mutagens, carcinogens, flammable compounds, sharp objects, and electricity. Consequently, you must always practice laboratory safety when you are in this class. To protect your eyes, goggles must be worn at all times. The best goggles for this course should both be resistant to chemicals and provide ultraviolet protection. At the very least, they must be chemically resistant. Laboratory coats are not required but are recommended. Latex or vinyl gloves are required for some manipulations and are optional for others. Your laboratory instructor will always supply them when they are needed or desired.

The laboratory is equipped with a safety shower, eye wash solution, first aid kit, emergency exit, glass disposal boxes, and a fire extinguisher. Broken glass, razor blades, Pasteur and serological pipets must be disposed of in the glass disposal boxes regardless of whether they are glass, plastic, or sharp metal. Take the time to familiarize yourself with the location of each of these items. Your laboratory instructor will instruct you on the proper use and disposal of all hazardous reagents. If you do become injured or have any questions about your potential health risk, do not hesitate to consult with your instructor.

B. Equipment to Measure/Dispense Liquids

Supply/ Equipment	*Use*		*Common sizes*	*Notes*
	Measure/ Dispense	Mix		
Micropipette	M/D		0.5 – 10 microliters 1 – 100 2 – 200 100 - 1000	Generally used to accurately measure & dispense volumes of ≤ 1 milliliters. Requires disposable tip to accurately measure and dispense volume.
Maxipipette	M/D		1 – 10 milliliters	Generally used to accurately measure & dispense volumes between 1 – 10 milliliters. Requires disposable tip to accurately measure and dispense volume.
Serological pipet	M/D		0.01 – 1 milliliter 0.1 - 5 0.1 - 10 0.2 - 25	Requires a Pipet-Aid® or rubber bulb to accurately measure and dispense volume.
Graduated cylinder	M/D		10 milliliters 50 100 250 500 1000	Not as accurate as pipet. Very good for measuring large volumes. Graduations are usually $^{1}/_{10}$ total volume
Volumetric flask	M	✔	10 milliliter 50 100 250 500 1000	Most accurate flasks. Excellent for preparing large volumes (> 10 milliliters). Disadvantage is that can only be used for preparing a solution of a single volume. Should ONLY be used to mix solutions containing liquid components.
Erlenmeyer flask	M/D	✔	50 milliliters 125 250 500	Primarily used for mixing solutions containing both solid and liquid components and those containing just liquids. Can be used to estimate volumes, not as accurate as any of the above.
Beaker		✔	50 milliliters 100 250 400 600	Should ONLY be used for MIXING.

C. Equipment used in CHEM 4130L

Item	*Class*	*Use*
Low Speed Centrifuge	Centrifuge	Separation of macromolecules or organelles based on centrifugal force. Max. speed ~6,000 r.p.m.; max. tube volume = 50mL; max. number of tubes = 6. Temperature range: 0^o – 37^oC.
Microcentrifuge	"	As above. Max. speed ~13,000 rpm.; max. tube volume = 2mL; max. number of tubes = 24. Temperature range: 0^o – 37^oC. Also referred to as "microfuge".
Plate reader	Spectrophotometer	Measurement of transmitted or absorbed light in the visible region of the spectrum
BioDocIt	Camera	Photo documentation
Horizontal Gel System	Electrophoresis	Macromolecular separation through a support medium in an electric field; separation based on charge &/or size
pH meter	pH meter	Measurement of the negative log of the $[H^+]$ concentration in an aqueous solution

D. Significant Figures

Mathematically, the measurement of a physical quantity, regardless of the precision of that measurement, is still unreliable. As such, a certain amount of uncertainty is associated with it. For instance, if the mass of NaCl sample is found to be 0.123g and 0.129g on successive measurements, the answer is unreliable in the third decimal place. Consequently, the measurement is said to contain three significant figures (i.e., three of the digits are obtained with a certain degree of reliability. The average mass of the NaCl sample is 0.126g, so the answer, rounded off to the correct number of significant digits is 0.130g. The point of this illustration is that the results of a calculation cannot be more reliable than the least reliable number used. This means that one must drop digits that are unreliable. "Rounding off" is governed by three rules.

1. If the digit to be dropped is less than five, leave the last significant digit unchanged.

2. If the digit to be dropped is greater than five, increase the last significant digit by one.

3. If the digit to be dropped is five followed by a zero, the last remaining significant digit is left even.

Example 1: Indicate the number of significant digits in the following numbers – 9870.0, 21.2, 6.103, 0.0785, and 0.52980
(Answer: 5, 3, 4, 3, and 5)

Example 2: Multiply 7.9 x 300. Give the answer to the correct number of significant digits.
(Answer: 2400)

E. Metric Prefixes and Conversions

1. Common prefixes

Prefix	*Accepted symbol*	*Definition*
milli-	m	10^{-3}
micro-	µ	10^{-6}
nano-	n	10^{-9}
pico-	p	10^{-12}
femto-	f	10^{-15}
atto-	a	10^{-18}

2. Relationships

1 mole = 10^3 mmol, 10^6 µmol, 10^9 nmol, 10^{12} pmol, 10^{15} fmol, and 10^{18} amol

Example 3: How many milliliters (mL) are equivalent to 1 microliter (µL)?
(Answer: $1\mu L(L/10^6\mu L)(10^3 mL/L) = 1 \times 10^{-3} mL$)

Example 4: How many picomoles (pmol) are equivalent to 10 millimoles (mmol)?
(Answer: $(10 mmols)(mol/10^3 mmols)(10^{12} pmols/mol) = 10^{10}$ pmols

F. Solution Preparation

1. "Percent Solution" Terminology

Designation	*Meaning of Abbreviation*	*Definition*
X% (v:v)	volume:volume	mL Solute / 100 mL Solution

Y% (w:v)	weight:volume	g Solute / 100 mL Solution
Z% (w:w)	weight:weight	g Solute / 100 g Solution

2. Concentration Based on Volume (v:v or w:v)

Concentrations based on the amount of dissolved solute per unit volume are the most widely used in biochemistry labs. The most common convention is based on molarity. Weight to volume percent is often used for routine laboratory solutions where exact concentrations are not too important.

Example 5: How should you prepare 1L of a 10% (v:v) ethanol solution?
(Answer: 10% (v:v) = 10mL solute/100mL solution; 100mL ethanol / L solution. To 100mL ethanol add water to 1L)

Example 6: How many grams of NaOH are required to prepare 100mL of a 25% (w:v) solution?
(Answer: 25% (w:v) = 25g solute / 100 mL solution. Dissolve 25g NaOH pellets in ~50mL of water; then bring volume to 100mL with more water)

Example 7: How should you prepare 500mL of 2M NaCl?
(Answer: Molecular weight of NaCl is 58.88 g/mol. (0.5L)(2mol / L)(58.88g / mol) = 58.88g. Dissolve the NaCl crystals in ~250mL of water then add water until the volume of the solution is 0.5L)

Example 8: How should you prepare a 250mL 0.5M glucose solution from a 5M glucose stock solution?
(Answer: $M_1V_1 = M_2V_2$; $V_1 = M_2V_2 / M_1$ = (250mL)(0.5M)/5M = 25mL of 5M stock + water to bring the volume to 250)

3. Concentration Based on Weight (w:w)

This calculation is not as accurate as molarity but problems of this nature would be solved in the same manner as Example 5 above with the substitution of g solute per 100g solution. Note, however, that 100g of solvent is equal to 100mL of solvent *only when* the solvent is water.

G. Dimensional Analysis:

Many problems in biochemistry require a conversion of one unit to another. **Dimensional analysis** (unit cancellation) should be used during the conversion to ensure accuracy during the manipulations. For example, an *E. coli* cell is 2000nm long. What is its length in meters?

$$2{,}000\ \cancel{\text{nm}} \times \frac{10^{-9}\ \text{m}}{\cancel{\text{nm}}} = 2 \times 10^{-6}\ \text{m}$$

Another example: The hydrogen ion concentration in a neutral solution is 1 x 10^{-7}M. What is its concentration in μM?

$$1 \times 10^{-7}\ \cancel{\text{M}} \times \frac{10^{6}\ \mu\text{M}}{\cancel{\text{M}}} = 0.1\ \mu\text{M}$$

Dimensional analysis should be used during problem solving because it provides a quick check on the accuracy of a solution (i.e., does the solution of the problem produce the expected units?) and may give some insight into possible alternate approaches. Dimensional analysis is especially critical when carrying out multi-step operations on a calculator, which, typically, does not carry units.

H. Statistical Analysis

This is not intended to be a class in statistics, but we do expect you to know some of the basics.

In Biochemistry as in any science, making a single measurement is not sufficient. Imagine if an alien came to our planet and abducted Shaquille O'Neal. The alien race might conclude that earthlings are all 7' 1" or 216 cm tall. If they abducted Shaq and Dr. Wolfgang they might conclude that the average height is 6' 5" tall, closer to the truth but still far from the truth. If we include Dr. Carroll we are now down to an average of about 6' 3". Generally speaking the more measurements one takes the closer one gets to the truth.

For some of our experiments we will be doing triplicates. So we need to understand some basic statistics.
Mean: this is the average, add the value from each data point and divide by the number of data points (n). Mean denoted as $x = 1/n \Sigma xi$
If Shaq is 216 cm, Dr. Carroll is 183 cm, and Dr. Wolfgang is 178 cm what is their mean height?
$x = 1/3$ (216 cm + 183 cm + 178 cm) =192.33 cm
Remember significant figures, the individual heights have only three significant figures so the answer should only have three significant figures 192 cm.
A group of three people who are each 192 cm tall also has a mean of 192 cm.

Standard deviation (SD) tells us how closely the measurements match.
$SD = \{(1 / n\text{-}1)\ \Sigma\ (xi - x)^2\}^{1/2}$
$SD = (1 / 3\text{-}1)^{1/2}\ \{(216\text{-}192)^2 + (183\text{-}192)^2 + (178\text{-}192)^2\}^{1/2}$
$SD = (1/2)^{1/2}\ \{24^2 + (-9)^2 + (-14)^2\}^{1/2}$
$SD = (0.707)\ (853)^{1/2}$
SD = 20.6

So we would report the value as 192 ± 20.6 cm
Sometimes the error is expressed as "Standard Error of the Mean" (SEM, or SE)
$SEM = SD / n^{1/2}$
SEM = 20.6 / 1.73
SEM = 11.9

This analysis is important for two reasons:

Calculating when a measured value is an "outlying value"
Calculating a statistically significant difference between two groups

The procedure for determining if a point can be discarded is as follows:

1. Calculate the mean and SD for the data WITHOUT the suspected outlying value

2. Find the absolute value of the difference between the suspected outlying value and the mean. Then divide by the calculated SD
3. For triplicate samples, if this number is > 25.5 the suspected outlying value can be disregarded.

For example: Triplicate values; 0.302, 0.322, and 0.524

Mean = 0.312

SD = 0.01

(0.524 - .312) / 0.01 = 21.2

Although this point (0.524) looks very different from the other two, it **can't** be thrown out.

Try this example:

Our triplicate values are 0.304, 0.320, and 0.524

Now can we discard the 0.524 data point?

In the experiments where we do triplicates we expect you to do the statistical analysis, and throw away any outlying values.

The other use for statistics is looking for statistically significant differences between groups. **This is called a t-test**

Consider the following data:

Sample 1	Sample 2
1458	1561
1480	1794
1603	1722
1531	1846
1518 ± 32 (SEM)	1731 ± 62 (SEM)

Are these two groups statistically different?

$t = (\text{mean 1- mean 2}) / \{SEM_1^2 + SEM_2^2\}^{1/2}$

This t-test has 6 degrees of freedom $(n_1 - 1)$ plus $(n_2 - 1)$

For a p value* of 0.05 and 6 degrees of freedom the "cutoff" is 2.4469

If t > 2.4469 then the two samples are different.

The p value of 0.05 means that if all the points were from the same group there is only a 5% chance that we would see a t score > 2.4469. If the t score is above 2.4469 we can say with 95% certainty that these two groups are different (95% confidence level).

A p value of 0.05 is often used in biochemical and medical research.

For our example the t score is 3.053 therefore these two groups are statistically different.

I would not expect you to do the t test without giving you the equation for the t score and the "cutoff" number. I would expect you to be able to determine the mean, the SEM and the degrees of freedom.

Questions

1. How many grams of NaCl in 400 mL of a 15 % (w/v) solution?

2. How many mL of acetone in 250 mL of a 30 % (v/v) solution?

3. What is the maximum volume of a 12 % NaOH solution you can make if you have 65 grams of NaOH and 2 liters of water?

4. If you have 10 mL of 1 M HCl and you add it to 85 mL of H_2O (remember always add acid to water), what is the new concentration of HCl?

5. If you have 25 mL of 0.3 M sodium acetate buffer and you add enough water to make a total volume of 1.5 L, what is the new concentration of sodium acetate?

6. You have a stock solution of 4 mg/mL protein. You wish to make 2 mL of 0.3 mg/mL protein, what volume of stock solution do you need, and what volume of water do you need?

7. Manny Ramirez is the left fielder for the Boston Red Sox; he has played 10 full seasons (1995 – 2004) and hit 31, 33, 26, 45, 44, 38, 41, 33, 37, and 43 home runs. What is his mean home run total and the standard deviation? (Many calculators will calculate mean and standard deviation, if you use them make sure you understand what information they are giving you)

8. You measure the height of three people: 216 cm, 183 cm, and 178 cm. Is the 216 cm data point considered an outlying value?

9. A small study was conducted using drug X. Drug X was designed to reduce cholesterol levels. Ten people, matched for age, gender, general health and cholesterol level were divided into two groups. Group A was given drug X and placed on an exercise regimen. Group B was given a placebo and placed on the same exercise regimen. After one month their serum cholesterol levels were measured. Group A subjects had the following decreases in serum cholesterol (units mg/dL): 43, 38, 27, 33, and 39. Group B subjects had the following decreases in serum cholesterol (units mg/dL): 24, 27, 28, 19, and 37.

Degrees of freedom	P = 0.10	P = 0.05	P = 0.01
4	2.1318	2.7764	4.6041
6	1.9432	2.4469	3.7074
8	1.8595	2.3060	3.3554
10	1.8125	2.2281	3.1693
12	1.7823	2.1788	3.0545

Consider the above chart, and recall that $t = (\text{mean 1- mean 2}) / \{SEM_1^2 + SEM_2^2\}^{1/2}$
What is the t score?

Does drug X have a statistically significant effect on serum cholesterol to a P value of 0.05 (95% confidence level)?

Does drug X have a statistically significant effect on serum cholesterol to a P value of 0.10 (90% confidence level)?

NOTE: THERE ARE MANY DIFFERENT WAYS TO ASK PERCENT SOLUTION QUESTIONS AND DILUTION QUESTIONS. DO NOT MEMORIZE A WAY TO SOLVE THESE QUESTIONS, UNDERSTAND THE DEFINTION OF PERCENT SOLUTION AND UNDERSTAND WHY M1V1 = M2V2 WORKS AND YOU CAN APPLY YOUR KNOWLEDGE TO THE CREATIVE WAYS YOUR INSTRUCTOR WILL QUESTION YOU.

I. Linear Regression Analysis Using Microsoft® Excel 2000

The most essential thing to do before doing linear regression is ask yourself "Do I expect this data to fit a linear function?" Absorbance as a function of concentration fits the equation A = εlc, is this linear? Reaction rate or velocity as a function of substrate concentration fits the equation v = V_{max} [S] / (K_M + [S]), is this linear?

1. Open Excel.
2. Create a worksheet. In cell, A1 type the name of the independent variable. This variable will be plotted on the x-axis.
3. In cell B1, type the name of the dependent variable.
4. Input your experimental data in the appropriate column. Remember, significant digits are important. If necessary, data should be formatted so that all values contain the appropriate number of significant digits. To do this
   a. Highlight the appropriate column
   b. Click on FORMAT
   c. Click on CELL
   d. Click on NUMBER. Choose the number of decimal places appropriate for your data. Click on OK.
   e. Repeat 4a – 4d as necessary.
5. Highlight all data (except that for your unknown)
6. Click on the icon that corresponds to the Chart Wizard
7. Under CHART TYPE, choose XY (Scatter)

a. Click on NEXT at the bottom of the window. Your chart will appear on the screen

b. Click on NEXT. A window will appear having your curve and a variety of tabs.

   1. Click on the TITLES tab

      a. Type in the name of your graph

      b. Type in the name of value for x-axis with the units (Example: [Riboflavin] (x 10^{-5} M))

      c. Type in the name of value for y-axis with the units (Example: A_{450})

   2. Click on the GRIDLINES tab

      a. For VALUE (X) AXIS, check MAJOR GRIDLINES

      b. For VALUE (Y) AXIS, check MAJOR GRIDLINES

   3. Click on the LEGEND tab and **un**check SHOW LEGEND

8. Click on NEXT. Indicate you want to show chart AS NEW SHEET. This option puts the full-size graph on a separate sheet.

9. Click on FINISH.

10. Your graph will be shown as a full sheet.

    a. The background, however, will be gray in color and this color should be removed

       1. Click on the plot anywhere within the gray background and the window FORMAT PLOT AREA will appear

       2. For the AREA click on NONE

       3. Click OK

    b. Format the y-axis by clicking anywhere on the graph's y-axis. The FORMAT AXIS window will appear with multiple tabs.

       1. Click on PATTERNS and make certain the following are checked: LINES, *Automatic*; MAJOR TICK MARK TYPE, *Outside*; MINOR TICK MARK TYPE, *Inside*; TICK MARK LABELS, *Next to axis*.

       2. Click OK

    c. Format the x-axis by clicking anywhere on the graph's y-axis. The FORMAT AXIS window will appear with multiple tabs. Repeat 10.b.1-2.

11. To draw the best straight line through the origin, go to the menu bar at the top of the graph

    a. Click on CHART

    b. Click on ADD TRENDLINE

1. Click on the OPTIONS tab
2. Click on the SET INTERCEPT = 0
3. Click on DISPLAY EQUATION ON CHART. This option helps you calculate your unknown's value using the regression analysis
4. Click on DISPLAY R-SQUARED VALUE ON CHART. This option gives you an indication of how straight your line is and, by extension, how good your data are. A perfect correlation has an R^2 of 1.000. Usually, $R^2 \leq 0.995$ do not generate very accurate data.
5. Click on OK

12. Calculate the concentration (or other independent variable) using the equation on the graph.
    a. Rearrange this equation to solve for x.
    b. Plug in the value for your unknown's absorbance (or other dependent variable) to calculate its concentration (or the independent variable)