Supplemental Material

CBE—Life Sciences Education

Whitworth *et al.*

Supplementary File for "Interactive computer simulations as pedagogical tools in biology labs", by Whitworth, K., Rakes, C. Leupen, S., and Bustos, M.M.

Laboratory description and protocols

Enzymes are responsible for many of the chemical reactions that take place in all living organisms. Many of these reactions are slow, such as metabolic processes, yet these reactions are essential to sustain life. An enzyme is a catalytic protein that allows reactions to occur at much higher rates. The amount of energy needed to begin the bond-breaking process in a reaction is called the activation energy. Enzymes speed up the reaction rate by lowering this energy barrier, and thus allowing reaction to proceed much earlier when a lower energy level is reached. In today's lab exercise, you will study conditions that alter the efficiency of an enzyme.

Most enzymes are proteins, and it is the protein's structure that determines how an enzyme will function. Proteins are composed of amino acids connected to one another by peptide bonds. There are 20 major amino acids, each with different chemical properties. Thus, the series of amino acids that make up a typical protein can be almost infinitely variable. The amino acid sequence of a protein is termed its **primary structure**.

However, a sequence of amino acids is not linear. The side chains of the amino acids interact with each other and with water to form various weak non-covalent bonds. As a result of these interactions, the protein spontaneously folds to form a three dimensional shape. Scientists are still uncovering the rules that allow prediction of this shape based upon the primary structure. This folding forms the **secondary** and **tertiary structure** of a protein.

Before coming to leadoff lecture, review protein structure. You learned about this topic in both BIOL 141 and BIOL 302. For quizzes and tests, I expect you to know the structures that all amino

acids share: alpha carbon, amino group, carboxyl group, side groups, the structure of peptide bonds and how they form, what primary, secondary, tertiary and quaternary structures are, and what kinds of interactions/ bonds cause those structures.

Proteins that are enzymes have a three dimensional structure that allows them to bind other molecules. The simplest analogy for this interaction is a **lock and a key**. Each lock has a specific internal shape such that only one key can function in it; each key fits one lock only. Just as every lock is specific to one key, every enzyme is specific for one substrate.

The "key" is called the substrate molecule; the "lock" is called the active site of the enzyme. Once substrate binds at the active site, a series of electrochemical reactions occur where charges are rearranged and covalent bonds are made or broken. The ultimate product of these unstable transition states is no longer able to bind and is thus released from the active site of the enzyme. The enzyme is unchanged and is then free to interact with the next substrate molecule it encounters. A "typical" enzyme converts substrate to product at a maximum rate of about 1000 per second, although rates of up to a million per second are possible.

The energy needed to convert substrate to product is called the activation energy. An enzyme lowers this activation energy, in part by bringing the reactive groups of the substrate and enzyme into close proximity.

The environment in which the reaction takes place can also affect its rate. For example, a change in pH may change the rate of a reaction. Thinking about this on a molecular level, how could pH affect the rate of an enzyme-catalyzed reaction?

In this lab, you will study the kinetic properties of one enzyme, as well as the factors that affect its activity. The enzyme is **acid phosphatase**. Acid phosphatases are a family of enzymes that are widespread in nature and are found in both animal and plant species. The name of the enzyme is quite revealing about what this enzyme does as well as under what conditions it is functional. This enzyme is a phosphatase, meaning that its substrate contains a phosphate group that is

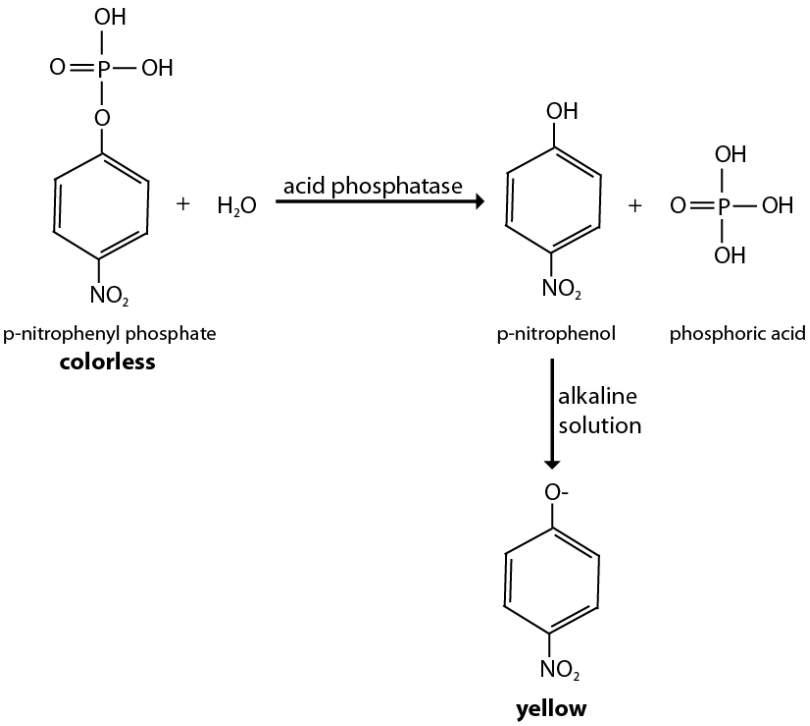
removed to form a product free of this phosphate, and this enzyme is functional under acidic conditions.

One of the most important characteristics of an enzyme is its activity - this is a measure of how quickly it works and is defined as follows:

Enzyme activity or rate = substrate converted (moles) / time (s)

In order to measure the rate of an enzyme, the enzyme is incubated with its substrate(s) under appropriate conditions and then you can measure the rate of product formation. The progress of products produced over time is the **reaction rate** (you can also measure loss of substrate over time). The rate of reaction is proportional to the concentration of enzyme.

A good way to assay this enzyme is to use a substrate which will be converted by the enzyme from a colorless substrate to a colored product. In this experiment, acid phophatase will convert p-nitrophenyl phosphate and water into **p-nitrophenol** and phosphoric acid. All components of this system are colorless except p-nitrophenol, which is yellow. Therefore, measurement of the amount of colored product formed is a direct measurement of the amount of substrate hydrolyzed by the enzyme.



The structures of the substrate and products.

WEEK 1 EXPERIMENTS

The effect of temperature on enzymatic and non-enzymatic hydrolysis of substrate to product.

Background information

Temperature affects the rate of an enzyme catalyzed reaction. An increase in temperature increases the thermal activity of substrate molecules, making a collision with an enzyme more likely. It also may increase the energy of the substrate molecule itself. If enough heat is applied, substrate may be converted to product even in the absence of enzyme, as the heat supplied overcomes the energy of activation for the reaction. In this exercise, you will determine the effect of temperature on the enzyme catalyzed reaction. Using the same set of tubes, you can also determine the effect of temperature on the non-enzymatic reaction.

Most biochemical reactions proceed very slowly at room temperature, because the reacting molecules do not collide with each other with sufficient energy to form an "activated complex." It is only when such an "activated complex" is formed that the biochemical reaction can go to completion. By raising the temperature, a large proportion of the molecules will achieve this minimal energy, and the rate of the reaction will increase accordingly. However, biological systems are not able to function at high temperatures, usually because proteins begin to unfold at higher temperatures. This is called a **denatured protein**. If an enzyme active site is improperly folded at higher temperature, then the reaction will be unable to proceed with the help of this enzyme.

Enzyme-catalyzed reactions are able to proceed very rapidly at room temperature because the enzyme combines with the substrate to form an "enzyme-substrate complex," thereby lowering the energy barrier of activation. Thus the **activation energy** for a given biochemical reaction may be defined as the amount of energy that must be imparted to the reacting molecules in order to permit them to react. Different kinds of reactions require different amounts of activation energy. The activation energy for a given reaction can be calculated from measurements of rates of reaction at different temperatures, using the **Arrhenius equation**:

$$\log (v_2/v_1) = (Ea/4.6)\ (1/T_1 - 1/T_2)$$

The temperature (T) in this calculation is expressed as degrees Kelvin, which is equal to 273 + °C. Ea is the activation energy expressed calories/mole.

If heat activates the molecules taking part in a biochemical reaction, it should follow that the more heat supplied, the faster the reaction will occur. Indeed, it has been found that for most reactions for every temperature increase of 10°C, the rate of the reaction approximately doubles. A **temperature coefficient**, usually designated by the letter Q, expresses how many times a reaction is speeded up by any stated increase of temperatures. The temperature coefficient (Q_{10})

for a 10° rise in temperature can readily be calculated for a given reaction by using the **van't Hoff equation**:

$$\log Q_{10} = (10/[T_2 - T_1]) (\log [v_2/v_1])$$

Procedure

1. In this experiment, you will vary the temperature of incubation. We have several baths around the room, set at temperatures of approximately 0, 23, 37, 50, 70 and 95 degrees C.

2. Obtain 24 tubes, four for each temperature. At each temperature, you will have an experimental, a control, an enzyme source tube, and a water source tube. Label all your tubes with masking tape and a waterproof marker. *Write your name or initials on the label too.*

3. Into each experimental tube and each control tube, put 1.0 ml of buffer (pH 4.8) and 1.0 ml of substrate (0.004 M).

4. Into each of the six 'enzyme' tubes, put 2 mls of enzyme. Into each of the six 'water' tubes, put about 2 mls of distilled water.

5. Choose one bath. Place all four tubes for that temperature in the bath for at least two minutes so the tube contents come to temperature. Add 1.0 ml of enzyme (from the enzyme tube in the same bath) to the experimental tube. Mix and record the time. Add 1.0 ml of water (from the water tube in the same bath) to the control tube. Mix and record the time.

6. Repeat step #5 for all of the remaining temperature baths.

7. <u>Exactly</u> 15 minutes after adding enzyme, stop the reaction with 3 mls of 0.1N NaOH (one shot of the repipettor). Do the same to the control. Right away, take those four tubes from the bath and put them on your desk to equilibrate to room temperature. Repeat with the remaining temperature baths, but in every case the reaction MUST be stopped EXACTLY 15 minutes after addition of enzyme!

8. To measure the effect of temperature on the enzymatic reaction, read each of the six experimental tubes (one per temperature) using its same-temperature control tube as a blank. Record absorbances in your lab notebook.

9. Now, measure the effect of temperature on the non-enzymatic reaction. To so, use the ice bath control tube as a blank, and read the other five control tubes against it. Record the absorbances in your lab notebook.

> Important note: Absorbance values larger than 0.80 are not accurate on our spectrophotometers. If you have any such tubes (*you will!*), the contents must be diluted (using 0.1 N NaOH as diluent, not water!) until the absorbance is less than 0.80. And if you dilute one tube, its corresponding control tube must be diluted in the same way. Keep track of how much diluent you add. Rather than guessing on how much to dilute a tube, consult with your TA first.

Finally, have your TA approve your data before you dump your tubes.

The effect of pH on acid phosphatase activity.

In addition to the importance of temperature, changing the concentration of hydrogen ions in the environment can affect the shape of the enzyme. That's because the 3-D shape of the enzyme is held together, in part, by **hydrogen bonds**, which are affected by pH. In this exercise, you will see how a change in pH affects the ability of the enzyme to carry out the reaction.

Procedure

1. Place 10 Bellco tubes in a rack. Label 1-10 with waterproof ink on a tape label. Label an 11th tube 'enzyme' and a 12th tube 'water'. Include your name or initials on the label.

2. In tubes 1-10, place 1.0 mls of 0.004M substrate.

3. In tubes 1 and 2 place 1.0 mls of buffer pH 3.0. In tubes 3 and 4 place 1.0 mls of buffer pH 4.0. Continue in this fashion for pH buffers 5.0, 6.0 and 7.0.

4. Fill the 'enzyme' tube with 7 mls of enzyme. Fill the 'water' tube with 7 mls of distilled water.

5. Place all 12 tubes in the 37 degree water bath for about 2 minutes to bring them to temperature.

6. Add 1.0 mls from your 'enzyme' tube to tube 1. Note the exact time. Add 1.0 mls of 'enzyme' to tube 3. Repeat for tubes 5, 7 and 9, noting the exact time of enzyme addition.

7. Now add 1.0 mls from your 'water' tube to tube 2, then to tube 4, 6, 8, and 10. So at this point, all the odd numbered tubes received enzyme, while even numbered tubes received water.

8. Exactly 15 minutes after adding the enzyme to tube 1, add 3.0 mls of 0.1 N NaOH (one shot from the repipettor) to tube 1. Do the same for the remaining 9 tubes, being certain that the time between addition of enzyme (or water) and addition of NaOH is exactly 15 minutes.

9. Take the tubes out of the water bath, place them on your desk, and give them 10 minutes or so to reach room temperature.

10. Read the absorbance at 400 nm of the odd numbered tubes in the spec, using the corresponding even numbered tubes as blanks. That is, tube 2 is the blank for tube 1, tube 4 is the blank for tube 3, etc. Record the five absorbances in your lab notebook. Ask a TA if you need help using the spec. Have a TA check over your data before you clean up.

Preparing a standard curve

For this lab, you will need to create a standard curve. This standard curve will reveal the relationship between varying amounts of the enzyme's product, p-nitrophenol, and their absorbances as measured in a spectrophotometer. The standard curve you are about to make will be similar in concept to the one you made in the protein determination lab.

1. You will be given a stock solution of p-nitrophenol, 10 micromoles/ml.

2. You will need 8 tubes: 7 different amounts of p-nitrophenol and one blank with no p-nitrophenol. Label each tube with tape. Begin by adding to each tube 1.0 ml buffer (pH 4.8), 1.0 ml enzyme, 1.0 ml distilled water and 3.0 ml NaOH. Use the Pipetman pipeting device to place 2.5 microliters of p-nitrophenol in tube 1, 5ul in tube 2, 7.5ul in tube 3, 10ul in tube 4, 12.5ul in tube 5, 15ul in tube 6 and 17.5ul in tube 7. The blank (tube 8) should have no p-nitrophenol. The final volume of all tubes should be 6.0 mls; check visually make sure that the liquid level is equal in all tubes (note that although each tube has a different volume of p-nitrophenol, this volume is so tiny that it does not *significantly* change the overall volume of the tube).

3. Set the spec for 400 nm. Measure the absorbance of each tube in the spec, first setting the absorbance of the blank to zero. Record your data in your notebook. Ask a TA for help if you need a review on how to use the spec. Do not clean up yet; save your tubes in case there is a problem with your data.

4. Knowing the concentration of the stock p-nitrophenol solution and the volume of it used in each tube, calculate the *amount* of p-nitrophenol in each tube (in moles). Record the data in your notebook.

5. Graph your data points on a scatter plot in Excel to generate the standard curve, with amount of p-nitrophenol on the X axis and absorbance on the Y axis. Insert a best-fit straight line (ie, a trendline) through your data points, and include the equation for this line on the graph. Label each axis and include units on each axis, if warranted. Write an informative, scientific title for the graph. Have your TA check your data and graph. This graph should be printed out and taped into your notebook for full credit on the notebook check.

Dealing with the data

Part A: The effect of temperature on the rate of enzymatic and non-enzymatic hydrolysis of substrate to product.

You measured absorbance in a spec. How do you get from absorbance to rate?

The answer is to use the standard curve you generated in Part C. The standard curve shows the linear relationship between amount of p-nitrophenol product (in micromoles) and the absorbance of that p-nitrophenol. So use the equation for the trendline on your standard curve to find the amount of p-nitrophenol formed in each the tubes. Record the amount in your notebook.

Rate is amount of p-nitrophenol formed per unit time. Divide the amount of p-nitrophenol formed by the reaction time (15 minutes) to get the rate in micromoles per minute. Record the rate in your notebook. For each of your six 'enzymatic' absorbances and each of your five 'non-enzymatic' absorbances, calculate the amount of product formed. Divide by the 15 minute reaction time to get the rate.

Construct one Excel graph of rate of reaction as a function of temperature. The graph will have

two sets of data: enzymatic and non-enzymatic. Chose a graph that includes a smooth line through your clearly indicated data points. Make sure the graph has an informative, scientifically correct title, axis labels with units, and a clearly labeled key to distinguish between the two data sets represented on this graph. This graph should be printed out and taped into your notebook for full credit on the notebook check.

Calculate two Ea's, one from the enzymatic temperature data and one from the non-enzymatic temperature data. Do not use data points where the rate is zero or almost zero. Use data points on the part of the curve where the enzyme is not being denatured.

Calculate two Q10's one from the enzymatic temperature data and one from the non-enzymatic temperature data. Do not use data points where the rate is zero or almost zero. Use data points on the part of the curve where the enzyme is not being denatured.

Part B: The effect of pH on *rate* of acid phosphatase activity.

Calculate the rate of the reaction at the various pH conditions.

Construct one Excel graph of rate of reaction as a function of pH. Chose a graph that includes a smooth line through your clearly indicated data points. Make sure the graph has an informative, scientifically correct title, axis labels with units, and a clearly labeled key to distinguish between the two data sets represented on this graph. This graph should be printed out and taped into your notebook for full credit on the notebook check.

Part D: Write a brief explanation to these questions in your lab notebook:

1) What temperature is most efficient for the enzymatic reaction? How about for the non-enzymatic reaction?

2) At which pH did acid phosphatase work best? Was this expected?

3) Describe the importance of adding enzyme to the standard curve. Do you expect that the enzyme contributed to the coloration in the tubes? Why or why not?

4) Describe the importance of adding NaOH at the end of the reactions. In particular, what effects does it have on the enzyme as well as the product?

Homework for week #1 of the enzyme lab

1. Finish any calculations, graphs, or questions that you did not finish in lab, for parts A through D. While you take the quiz, we will do a notebook check, making sure the three graphs are done properly, the Ea's and Q10's are present, and the questions in Part D are correct.

2. Study for a 10-point quiz at the start of next week's lab. Expect a number of questions from the lab lecture, as well as questions on any aspect of the lab you just did.

WEEK2 EXPERIMENTS

Enzyme kinetics

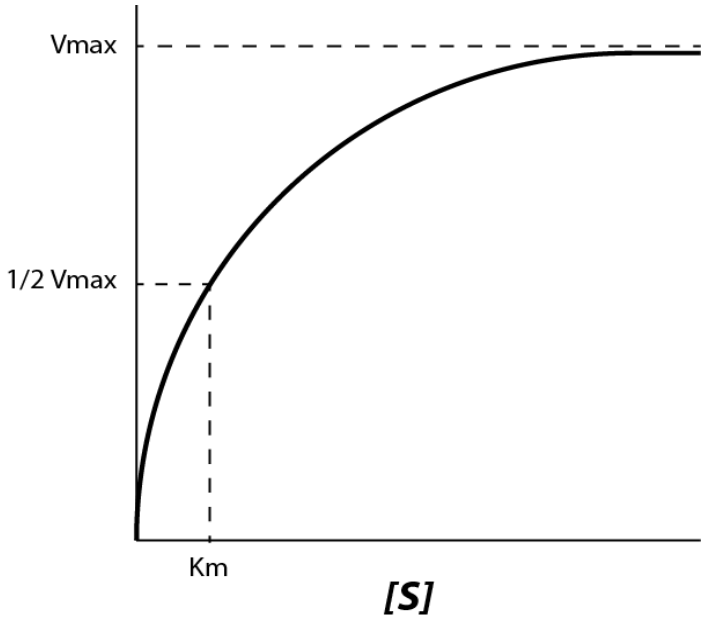
Enzyme catalyzed reactions show a distinctive feature not found in other chemical reactions: **saturation**. That is, every enzyme has a characteristic concentration of substrate at which the rate of the reaction is maximal; addition of more substrate will not increase the rate. On a molecular level, this can be visualized as follows: as soon as a product molecule is released, another substrate molecule binds to the active site. There is no "dead time" in which the active site is unoccupied, because the substrate concentration is so high that the enzyme encounters substrate virtually instantaneously. The rate at saturation is termed **Vmax** (or, the maximum velocity or rate of the enzyme). The substrate concentration at one half Vmax is termed **Km**. Vmax and Km are the kinetic parameters that define an enzyme; every enzyme has its own characteristic Km and Vmax, thus virtually every investigation involving an enzyme reports the Km and Vmax found by the authors. In today's laboratory exercise, you will determine the Km and Vmax for the common cellular enzyme, acid phosphatase.

To find Km and Vmax, set up an experiment where you vary substrate concentration, **[S]**, and measure rate, **v** (v for velocity). The resulting data form a curve (shown below), as you will see when you graph your data.

Two biochemists (Michaelis and Menten) derived an equation to describe this curve:

$$v = \frac{V_{max}[S]}{K_m + [S]}$$

where [S] = a given concentration of substrate, v = the rate of reaction at that substrate concentration, Vmax = the maximum rate of the reaction, and Km = the concentration of substrate it takes to reach one half the maximum rate.



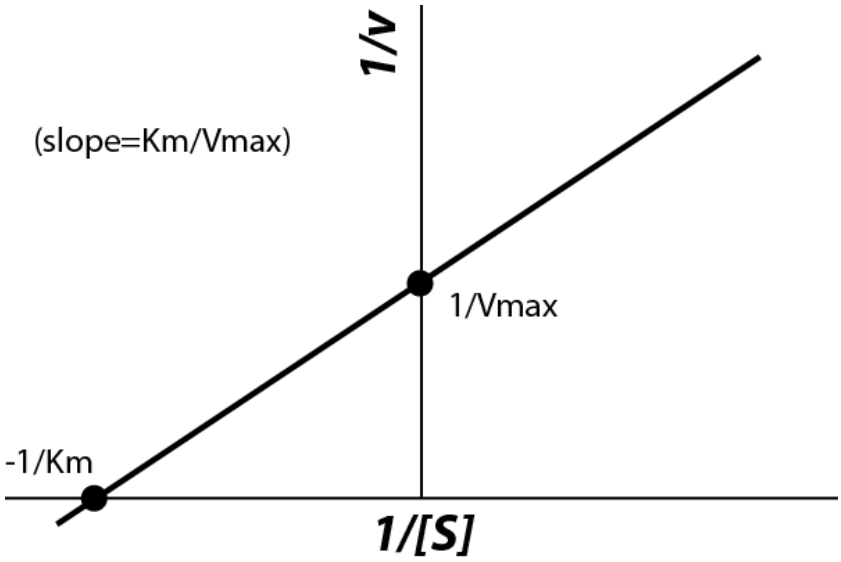
This graph, called a **Michaelis - Menten plot**, takes on a logarithmic shape as shown on the right. Also shown on the above graph is how Vmax, and then Km, can be *estimated*.

Note that since the curve approaches Vmax in a logarithmic fashion, there is some level of uncertainty about where exactly to draw the line that yields Vmax. (Of course, unless you repeat the experiment many, many times, the data points will have some scatter in them due to experimental error, and you will again be uncertain about where to draw the line the yields Vmax.) Also, you won't know in advance if you have chosen a substrate concentration that really reaches saturation; in this instance it will be impossible to estimate Vmax off the Michaelis - Menten plot. And if your estimate of Vmax is inaccurate, your estimate for Km will be also.

To over come these limitations and to simplify the determination of Km, two later biochemists, Lineweaver and Burk, took the reciprocal of the Michaelis-Menten equation and obtained:

$$\frac{1}{v} = \frac{Km}{Vmax} \cdot \frac{1}{(S)} + \frac{1}{Vmax}$$

The advantage of this equation is evident if one plots 1/v against 1/S; a straight line is obtained, whose slope is equal to Km/Vmax. Where the line intersects the Y axis gives 1/Vmax. Where the line intersects the negative X axis gives negative 1/Km. Since Vmax and Km can be determined mathematically from the intercepts, its value is not a "guesstimate" as it was in the Michaelis-Menten plot, and is thus more accurate. In practice, the Lineweaver - Burk plot is always favored over the Michaelis - Menten plot.



A **Lineweaver - Burk plot** is shown below, as is how to calculate the kinetic parameters Vmax and Km.

Valuable information regarding the structure of the active site of the enzyme, the mechanism of the reaction, and how the enzyme works in a cell, has been obtained through the use of inhibitors. Many drugs are actually inhibitors of enzymes that work inside of our cells. The most intuitively obvious kind of inhibitor is the **competitive inhibitor**. Competitive inhibitors compete with substrate for the active site on the enzyme. Because the shape of the competitive inhibitor is similar to the substrate, it binds at the active site. No catalysis occurs, however, and the inhibitor is released. The presence of a competitive inhibitor thus slows the rate of product formation relative to the rate in its absence. The only exception to this is at Vmax. Theoretically, for a given concentration of competitive inhibitor, it is possible to add such a high concentration of substrate that the chances of an active site encountering the competitive inhibitor are essentially zero. Thus, in the presence of a competitive inhibitor, Vmax is unchanged, but Km (the concentration of substrate needed to reach one half Vmax) is always larger. The competitive inhibitor we are using is phosphate ion.

Another kind of inhibitor is the **non-competitive inhibitor**. Such an inhibitor binds equally well to the enzyme and the enzyme-substrate complex, usually not in the active site, so it does not compete with substrate. However, once an enzyme is bound by the non-competitive inhibitor, it can no longer catalyze the reaction. In the presence of an non-competitive inhibitor, the Vmax is reduced, while the Km remains the same. The non-competitive inhibitor we will use in this experiment is fluoride ion. Finally, there is such a thing as an *uncompetitive inhibitor*, but we will not study that in this class.

Given the above information about competitive and noncompetitive inhibitors, you should be

able to sketch in what the curve would look like for each inhibitor on the M-M plat and what the line would look like for each inhibitor on the L-B plot.

Procedure

1. Work in pairs. *Each* person should prepare 10 numbered tubes as shown in the chart below. One person should use regular citrate buffer pH 4.8; the partner should use citrate buffer pH 4.8 plus competitive inhibitor. (Other than the presence or absence of competitive inhibitor, you and your partner's experiments will be identical.) When finished, each of the 10 tubes should have 1 ml of buffer and 1 ml of substrate. Label all tubes. Be sure to include your name or initials.

	Tubes 1 and 2	Tubes 3 and 4	Tubes 5 and 6	Tubes 7 and 8	Tubes 9 and 10
Buffer (+/- Inihibitor)	1ml	1ml	1ml	1ml	1ml
0.0005 moles/L substrate	1ml				
0.001 moles/L substrate		1ml			
0.002 moles/L substrate			1ml		
0.004 moles/L substrate				1ml	
0.006 moles/L substrate					1ml

2. Into an eleventh tube put about 6 mls of enzyme. Label. This will be your source of enzyme for this experiment. Into a twelfth tube put about 6 mls of distilled water. Incubate all tubes at 37°C for a few minutes to equilibrate temperature.

3. To the even numbered tubes add 1.0 ml of distilled water.

4. To the odd numbered tubes add 1.0 ml enzyme and note the exact time of addition. Since time is absolutely critical in this experiment and must be held constant at exactly 15 minutes, it may be beneficial to stagger the time of enzyme addition by one minute per tube.

5. After exactly 15 minutes of incubation at 37°C, add 3.0 mls of 0.1 N NaOH to each tube. Remove the tubes from the water bath and allow them to cool to room temperature.

6. Use the even-numbered tubes as blanks for the corresponding odd-numbered tubes. Read the absorbances and record in your lab notebook. Using the standard curve you made last week, calculate the amount of p-nitrophenol formed. Record. To get the rate of p-nitrophenol formed, divide this amount by the time of the reaction. Record. Have your TA approve your data before you clean up.

7. Now repeat steps 1-6, but use the <u>noncompetitive inhibitor</u> (in buffer) instead. Yes, you have to include the tubes without inhibitor as well, even though you have such a set of tubes from

the experiment you set up an hour ago. Why?

Dealing with the data

1. In Excel, plot the competitive inhibitor and control reaction rates as a function of substrate concentration. This is your Michaelis Menton plot. Chose a graph that includes a smooth line through your clearly indicated data points. Make sure the graph has an informative, scientifically correct title, axis labels with units, and a clearly labeled key to distinguish between the two data sets represented on this graph. This graph should be printed out and taped into your notebook for full credit on the notebook check. Next, create a Michaelis-Menton plot for the noncompetitive inhibitor data.

2. Build two Lineweaver-Burk plots using the Excel scatter plot: one of the competitive data and another of the noncompetitive data (include the control data on each). Insert best-fit straight lines (ie, a trendlines) through your data points, and include each equation for this line on the graph. Make sure that each equation is clearly associated with its associated data set. Label each axis and include units where appropriate. Write an informative, scientific title for the graph. This graph should be printed out and taped into your notebook for full credit on the notebook check.

3. From **each** of the four graphs, calculate the Km and Vmax for the inhibited and uninhibited cases. Thus, you will have 8 Km's and 8 Vmax 's. For the Michaelis-Menten plot, estimation of Vmax is difficult but necessary so that you can understand the strengths and weaknesses of each method of graphing. For the Lineweaver-Burk, you should use the equation to solve for the x and y intercepts.

Finally, assemble all this kinetic data into a logically designed table, complete with an informative title and units.

Homework for week #2 of the enzyme lab

Whatever you don't finish among items 1-3 above in class must be completed as homework. If you can't figure out how to do this assignment, it is your responsibility to see your TA or the instructor to find out before your next lab period. This homework will be checked and graded (as a notebook check) at the start of the next lab period, while you take your quiz.

There will be a quiz next week at the start of lab on this material. It will be heavily mathematical, and you should feel comfortable with the two plots used in today's lab as well as how to determine Km and Vmax.

WEEK3 ACTIVITIES

Data analysis and interpretation

This lab period will be spent analyzing and interpreting the data from the enzyme lab. Please use this time to ensure that you understand everything covered over the last two weeks.

If you have one, bring your laptop to lab.

1. Hand in your lab notebook. While you take the quiz, your TA will do a notebook check, making sure you've done the homework assignments. Your grade (out of 10) will be recorded in the notebook so you know what grade you got.

2. Take the quiz.

3. After the quiz, your notebook will be returned. As a class, led by your instructor or TA, you will generate an outline on the board of the results of the entire enzyme lab. This outline will form the basis for an Abstract. You will write this Abstract during lab time, as a group with your lab partner(s). Consult the handout on "How to write an abstract". Show this rough draft of your Abstract to your TA, who will make comments on your draft.

4. On the teaching desk at the front of the room, write your Km and Vmax values on the paper there. Make sure you report your values in the requested units! Only report kinetic parameters from one person of the pair, whichever is more accurate. Once collected, the instructor will place it in an Excel spreadsheet that will be posted on Blackboard. See the homework section for further instructions.

5. Calculate the specific activity of acid phosphatase from your data.

Background Information:

Specific activity is a measurement of enzyme purity. As an enzyme is extracted from a tissue and then purified by a number of biochemical procedures, it becomes increasingly more pure. Thus, during the purification process, less and less enzyme is needed to produce the same rate of substrate conversion. Specific activity is defined as the number of micromoles of substrate converted to product, per minute, per mg enzyme protein, at 25 degrees Centigrade under optimal conditions. Specific activity of the enzyme used in this exercise, acid phosphatase, can be calculated if you know the Vmax (in micromoles per minute), and how many mg enzyme protein is used in each tube. The number of mg protein used in each tube can be obtained by a Bradford assay on the enzyme solution. You did this in the third lab of the semester, and can obtain this value from your notebook

Homework for week #3 of the enzyme lab

1. Finish writing your abstract, if you did not finish it in class. Rewrite for clarity, and edit. Type it (double spaced), and save it. If your partner helped you, make sure both names are on it. It's OK if your partner's abstract is identical to yours. Print out two hard copies. Tape one hard copy in your lab notebook. Hand in the second hard copy at the start of lab next week; it will be graded as a notebook check.

2. Class data for Km and Vmax, with and without competitive inhibitor, and with and without non-competitive inhibitor, will be posted on Blackboard. Download the appropriate Excel spreadsheet, and perform four t tests using these data:

a. compare the Km's without inhibitor to the Km's with competitive inhibitor

b. compare the Km's without inhibitor to the Km's with non-competitive inhibitor

c. compare the Vmax's without inhibitor to the Vmax's with competitive inhibitor

b. compare the Vmax's without inhibitor to the Vmax's with non-competitive inhibitor

In your lab notebook, report the four p values you obtain. For each, state whether you rejected or failed to reject the null hypothesis. Also tell what that means with regard to the Km's and Vmax's and with regard to the expected results. This will be a notebook check at the start of lab next week.

3. Prepare for next week's quiz. You will have to calculate a specific activity.

Michaelis-Menten kinetics model

The most general representation of a Michaelis-Menten reaction in the presence of a reversible inhibitor involves seven chemical species $\boldsymbol{E}$(enzyme), $\boldsymbol{S}$ (substrate), $\boldsymbol{C}$ (enzyme:substrate complex), $\boldsymbol{I}$ (inhibitor), $\boldsymbol{EI}$ (enzyme:inhibitor or binary complex), $\boldsymbol{CI}$ (enzyme:substrate:inhibitor or ternary complex), and $\boldsymbol{P}$ (product), and seven elementary or component reactions. The table below summarizes this chemical reaction system, including values of elementary rate constants that were adjusted to fit the behavior exhibited by the wheat germ acid phosphatase used in the labs.

Reaction	Equation	Rate constant
1	$E + S \rightarrow C$	k_f = 1000000
2	$C \rightarrow E + S$	k_b = 242.5
3	$C \rightarrow E + P$	k_{cat} = 1.02e-05
4	$E + I \rightarrow EI$	k_{if} = 10000
5	$EI \rightarrow E + I$	k_{ib} = 190
6	$C + I \rightarrow CI$	k_{iif} = 80000
7	$CI \rightarrow C + I$	k_{iib} = 100

These seven reactions lead to a system of seven mass-action kinetics differential equations that constitute the mathematical model underlying these simulations.

$$\frac{dS}{dt} = -k_f * E * S + k_b * C$$

$$\frac{dE}{dt} = -k_f * E * S - k_{if} * E * I + (k_b + k_{cat}) * C + k_{ib} * EI$$

$$\frac{dC}{dt} = -(k_b + k_{cat}) * C - k_{iif} * C * I + k_f * E * S + k_{iib} * CI$$

$$\frac{dP}{dt} = k_{cat} * C$$

$$\frac{dI}{dt} = -k_{if} * E * I - k_{iif} * C * I + k_{ib} * EI + k_{iib} * CI$$

$$\frac{dEI}{dt} = -k_{ib} * EI + k_{if} * E * I$$

$$\frac{dCI}{dt} = -k_{iib} * CI + k_{iif} * C * I$$

Modeling the effect of pH on the activity of wheat germ acid phosphatase

The effect of pH on the activity of wheat germ acid phosphatase was recapitulated using an equilibrium model. The model rests on three acid-base reactions (below). In reaction 1 a molecule of enzyme $\boldsymbol{E}$ turns into an inactive state $\boldsymbol{EH^+}$ after binding a proton. In reaction 2 the enzyme turns into a different inactive form $\boldsymbol{EOH^-}$ after binding a hydroxyl ion (or equivalently losing a proton).

Reaction	Equation	Equilbrium constants
1	$E + H^+ \rightarrow EH^+$	K_H = 2.0e-4
2	$E + OH^- \rightarrow EOH^-$	K_{OH}= 7.0e-9
3	$H_2O \rightarrow H^+ + OH^-$	K_W = 1.0e-14

The equilibrium concentration of $\boldsymbol{E}$, as a function of pH, is given by the formula

$$\boldsymbol{E} = \frac{\boldsymbol{E_t}}{\left(\mathbf{1} + \frac{\boldsymbol{H^+}}{\boldsymbol{K_H}} + \frac{\boldsymbol{OH^-}}{\boldsymbol{K_{OH}}}\right)}$$

where E_t is total enzyme concentration, while the equilbrium concentrations of and are

$$H^+ = 10^{-pH}$$

$$OH^- = \frac{K_W}{H^+}$$

Modeling the effect of temperature on the activity of wheat germ acid phophatase

The effect of temperature on the rate of reaction is two-fold. An increase in the turn-over rate of an enzymatic reaction can be expected from the Arrehnius equation, $k_{cat} = k_{cat0}\, e^{-\frac{Ea}{RT}}$, which predicts an exponential increase in k_{cat} as a function of the temperature T. This effect is balanced by an opposite change on the stability of the enzyme:substrate complex. The two concepts are combined into the heuristic formula

$$k_{cat} = k_{cat0}\ \frac{e^{\epsilon(T-T_{act})}}{(1+e^{\epsilon(T-T_{act})})}$$

that has the form of a logistic law centered at the characteristic activation temperature T_{act}. A similar argument can be made for the effect of temperature on the degradation (i.e. heat inactivation) of the enzyme that reduces the total concentration of active enzyme E_t, which ia captured by a second heuristic formula

$$E_t = \frac{E_0}{\left(1+e^{\omega(T-T_{deg})}\right)}$$

containing the parameters E_0, the initial enzyme concentration, and T_{deg}, a characteristic degradation temperature. Both heuristic formulas include the same ramping parameter ϵ, which determines how quickly the enzyme activity changes with temperature.

Parameter	
***k*cat optimum**	k_{cat0} =1.0e-5
Ramping constant	$\epsilon = 0.1260$
Degradation constant	$\omega = 0.1260$

Activation Tm	T_{act} = 46.25
Degradation Tm	T_{deg}= 46.25

Psychometric survey

	Strongly Agree	Agree	Neither Agree/Disagree	Disagree	Strongly Disagree
1. Laboratory experiments are something that I enjoy doing.	a	b	c	d	e
2. The computer simulation allowed me to spend more time considering the predicted outcome of an enzymatic reaction than the laboratory experiment.	a	b	c	d	e
3. I enjoyed working with the computer simulation more than completing the laboratory experiment.	a	b	c	d	e
4. Working with computers is challenging for me.	a	b	c	d	e
5. The laboratory experiment allowed me to do deeper analysis of the parameters that determine how enzymatic reactions work than the simulation did.	a	b	c	d	e
6. Computer simulations make science more interesting.	a	b	c	d	e
7. The laboratory experiment provided better training on how enzymes work than the computer simulation.	a	b	c	d	e
8. The laboratory experiment was more fun than the computer simulation.	a	b	c	d	e
9. Computer simulations are useful in helping to solve the problems of everyday life.	a	b	c	d	e
10. Quantitative reasoning is easy for me.	a	b	c	d	e
11. I performed deeper analysis of the parameters that determine how enzymatic reactions work with the computer simulation than in the laboratory experiment.	a	b	c	d	e

<u>Optional Questions:</u>

12. What is your Gender?
    a. Female
    b. Male

13. Do you consider English your primary language?
    a. Yes
    b. No

Responses to the psychometric survey. The total number of responses obtained for each response category are shown together with the corresponding percentage response rates (in parenthesis).

	Q1	***Q2***	***Q3***	***Q4***	***Q5***	***Q6***	***Q7***	***Q8***	***Q9***	***Q10***	***Q11***
Strongly agree	**148 (39.9)**	**86 (28)**	**48 (15.6)**	**8 (2.2)**	**63 (20.6)**	**41 (11.2)**	**49 (16.1)**	**77 (25.2)**	**80 (21.7)**	**74 (20.2)**	**53 (17.6)**
Agree	**147 (39.6)**	**111 (36.2)**	**50 (16.2)**	**19 (5.1)**	**102 (33.3)**	**114 (31.3)**	**121 (39.7)**	**102 (33.3)**	**170 (46.2)**	**147 (40.1)**	**90 (29.9)**
Neither agree\disagree	**45 (12.1)**	**79 (25.7)**	**92 (29.9)**	**37 (10.0)**	**86 (28.1)**	**139 (37.9)**	**98 (32.1)**	**84 (27.5)**	**92 (25.0)**	**116 (31.6)**	**96 (31.9)**
Disagree	**23 (6.2)**	**18 (5.9)**	**88 (28.6)**	**129 (34.8)**	**41 (13.4)**	**53 (14.4)**	**30 (9.8)**	**31 (10.1)**	**16 (4.3)**	**27 (7.4)**	**49 (16.3)**
Strongly disagree	**8 (2.2)**	**13 (4.2)**	**30 (9.7)**	**178 (48)**	**14 (4.6)**	**20 (5.4)**	**7 (2.3)**	**12 (3.9)**	**10 (2.7)**	**3 (0.8)**	**13 (4.3)**

Supplementary figure legends

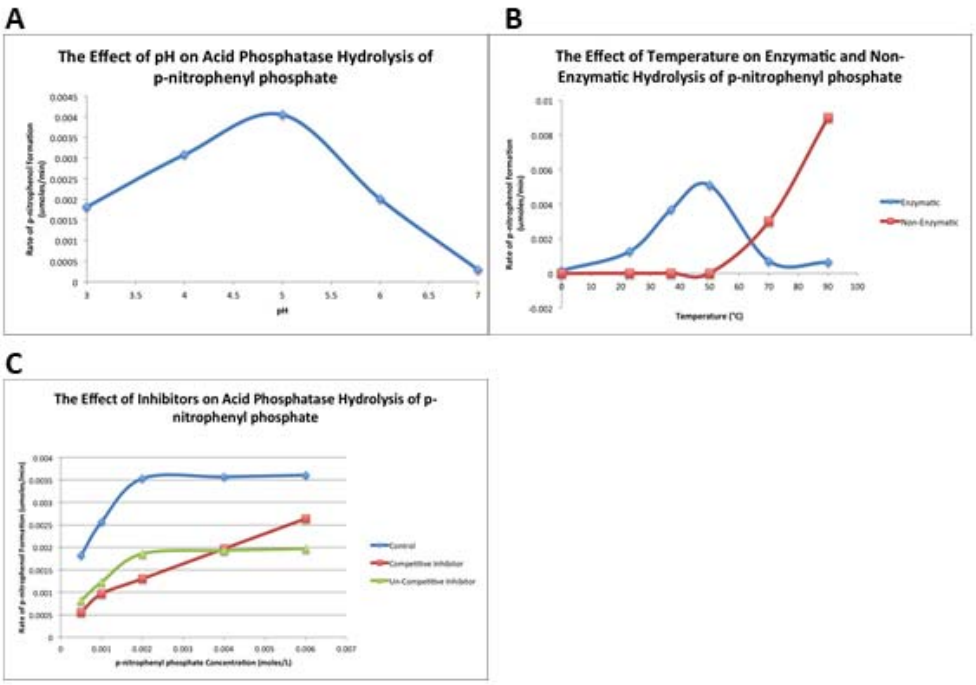


Figure S1. Experiments of the enzyme kinetics laboratory exercise. Acid phosphatase activity (µmol p-nitrophenol min^{-1}) was measured by the standard laboratory protocol (Supplementary file). Experiments shown in panels A and B are carried out in week 1, while the experiment shown in panel C corresponds to week 2. **A)** Effect of pH; **B)** Effect of temperature; **C)** Effect of competitive (phosphate ion) and non-competitive (fluoride ion) inhibitors on the enzyme's V_{max} and K_M.

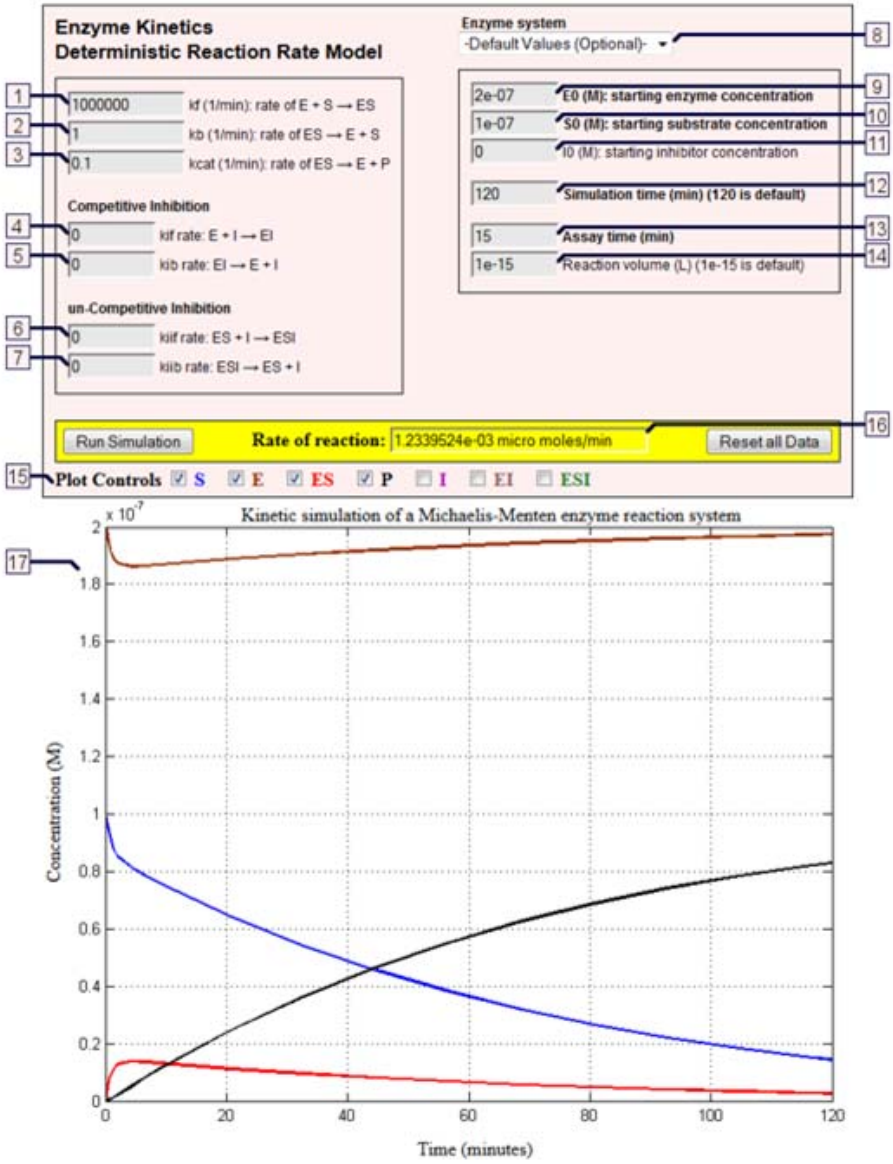

Enzyme Kinetics
Deterministic Reaction Rate Model
Enzyme system
-Default Values (Optional)-
1
1000000
kf (1/min): rate of E + S → ES
2
1
kb (1/min): rate of ES → E + S
3
0.1
kcat (1/min): rate of ES → E + P
Competitive Inhibition
4
0
kif rate: E + I → EI
5
0
kib rate: EI → E + I
un-Competitive Inhibition
6
0
kiif rate: ES + I → ESI
7
0
kiib rate: ESI → ES + I
8
2e-07
E0 (M): starting enzyme concentration
9
1e-07
S0 (M): starting substrate concentration
10
0
I0 (M): starting inhibitor concentration
11
120
Simulation time (min) (120 is default)
12
15
Assay time (min)
13
1e-15
Reaction volume (L) (1e-15 is default)
14
Run Simulation
Rate of reaction:
1.2339524e-03 micro moles/min
Reset all Data
16
15
Plot Controls
S
E
ES
P
I
EI
ESI
17
x 10^-7
Kinetic simulation of a Michaelis-Menten enzyme reaction system
Concentration (M)
Time (minutes)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
0
20
40
60
80
100
120

Figure S2. Screen shot of the GUI for a generic enzyme without inhibitor.

Elementary rate constants that determine the enzyme's V_{max} and K_M: [1,2] kf and kb, forward binding and dissociation of substrate to enzyme, E + S → ES and ES → E + S ; [3] kcat, product formation and release of intact enzyme. ES → E + P.

Elementary rate constants for competitive inhibition: [4,5] kif and kib, forward binding and dissociation of inhibitor to enzyme, E + I → EI and EI → E + I.

Elementary rate constants for uncompetitive inhibition: [6, 7] kiif and kiib, forward binding and dissociation of inhibitor to enzyme-substrate complex, ES + I → ESI; and ESI → E + I.

Enzyme System selection: [8] System selection: Optional. Select from a menu of pre-loaded inputs for standard enzyme systems.

Starting Concentrations: [9, 10, 11] E0, [Molar] Enzyme *Note: The maximum rate of reaction V_{max} is directly proportional to E0*; S0, [Molar] Substrate. *Note: Rate of reaction saturates at V_{max} for large S0 values*; I0: [Molar] Inhibitor. *Note: A reversible inhibitor may alter the kinetics of product formation. Two basic types of reversible inhibition exist, competitive and uncompetitive. A single compound may exhibit both types simultaneously, giving rise to mixed and non-competitive inhibition.*

Simulation time: [12] Simulated reaction time [minutes]. *Note: Times longer than necessary to reach saturation are not recommended.*

Assay time: [13] This represents the time at which the reaction is stopped prior to measuring the amount of product made [minutes] *Note: Typically 10 to 15 minutes.*

Reaction volume: [14] [liters] *Note: reaction volume and starting concentrations E0, S0 and I0*

determine the total number of molecules. There is no lower or upper limit on the total number of molecules for this model.

Inhibition

Plot Controls: [15] *Note: Check a box to display corresponding chemical species on the plot pane.*

Rate of reaction: [16] The average rate of reaction for the period 0 to assay time [µmol min^{-1}] that approximates v_0 the instantaneous rate of reaction at time *t*=0.

Concentration vs Time plots: [17] Concentration [Molar] versus time for each chemical species.

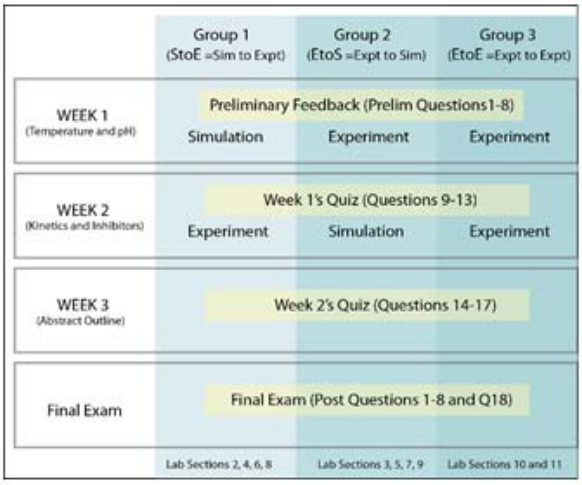


Figure S3. Summary of the assessment schedule used in weeks 1-3