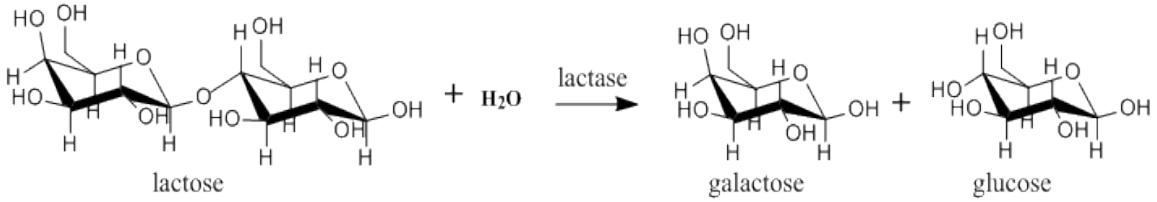
Experiment 17

Kinetic Study of the Enzyme Lactase

GOAL:
In this experiment we will study the kinetics of the enzyme lactase and calculate its Michaelis constant.

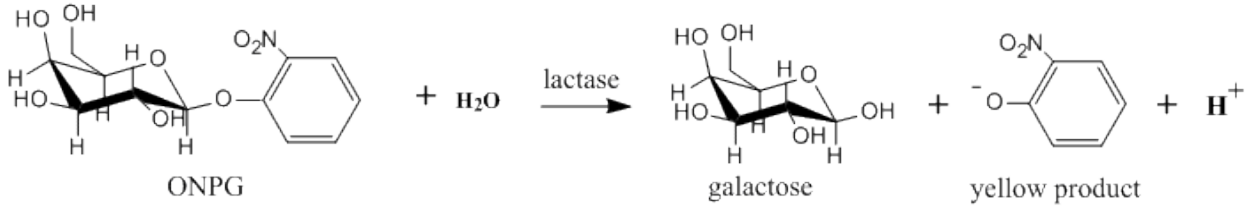
INTRODUCTION:
Lactose, the disaccharide found in milk, requires the enzyme lactase for proper digestion. Unfortunately, many people consume more lactose than their bodies can properly digest. They don't necessarily lack the enzyme, but may have it present in smaller amounts so that the consumption of a significant amount of milk overwhelms the enzyme that is present. Lactose molecules not properly hydrolyzed by the lactase enzyme travel to the large intestine where they ferment, produce CO_2 gas, and cause bloating and diarrhea in these lactose-intolerant individuals. Because milk products are such an important source of calcium and other nutrients, artificial sources of lactase have become popular. Among the most widely used is Lactaid®, which we will study in this experiment.

Enzymes are biological catalysts that speed up otherwise slow reactions. Lactase catalyzes the hydrolysis of lactose(usually called the substrate) to glucose and galactose, as seen in Eqn 1.



Eqn 1

To keep our kinetic study as simple as possible, we will use the synthetic substrate, o-nitrophenyl-β-D-galactopyranoside (ONPG), in place of lactose. The hydrolysis of the ONPG substrate produces o-nitrophenolate, which is yellow (see Eqn 2). As a result, we can monitor the reaction by measuring the increasing absorbance of this product with time.



Eqn 2

Because the enzyme is not consumed in these reactions, one enzyme molecule is able to catalyze the reaction of many substrate molecules. Since the enzyme isn't consumed, we might expect to always have kinetics that depend only on the substrate and give a simple first-order rate law where rate = k $[S]^1$, but things aren't quite that simple. Each substrate molecule spends a finite amount of time at the enzyme's active site. When the substrate

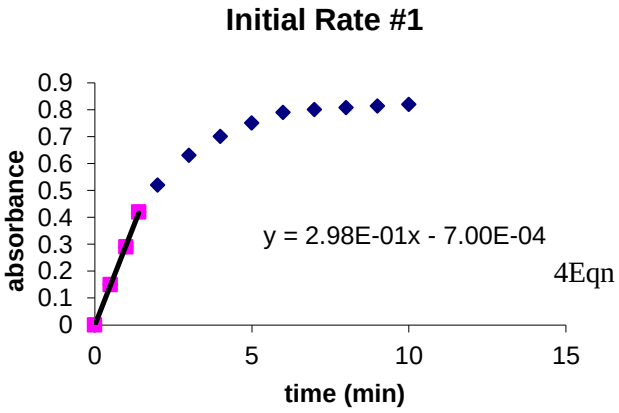
concentration becomes large enough, all the enzyme active sites are occupied and we say that the enzyme is saturated. When this happens, further increases in [S] can't increase the rate of reaction because those substrate molecules can't reach the enzyme active sites. In the case of lactose digestion, the unhydrolyzed lactose molecules pass lower in the digestive tract and cause the unpleasant symptoms.

Enzyme kinetics are described by the Michaelis-Menten mechanism, which yields the expression

$$v = \frac{V_{max}\,[S]}{K_M + [S]} \qquad \text{Eqn 3}$$

where v is the initial rate (or velocity), V_{MAX} is the maximum reaction velocity, [S] is the initial substrate concentration, and K_M is the Michaelis constant. K_M is a measure of how easily the enzyme can be saturated by the substrate at a given temperature and pH. The larger the value of K_M, the greater the substrate concentration the enzyme can handle before it becomes saturated.

The simplest way to find the value of K_M is to measure reaction rates at several substrate concentrations. The initial rate at each [S] is determined by plotting absorbance vs. time. While the entire set of data gives a curve, we can draw a straight line through the first few data points. The slope of this straight line is the initial reaction rate, v, for this [S]. In Figure 1, the data points for times 0-1.5 min. appear to form a straight line. The equation for the linear trendline from just these points is shown on the graph. The slope of the trendline gives us the reaction's initial rate, v, of 2.98 x 10^{-1}/min.



Equation 3 can be rearranged to a more useful form

$$\frac{1}{v} = \frac{K_M}{V_{MAX}}\left(\frac{1}{[S]}\right) + \frac{1}{V_{MAX}} \qquad \text{Eqn 4}$$

which matches the $y = mx + b$ form of a straight line. Thus if we plot $1/v$ vs. $1/[S]$, the slope of the resulting line equals K_M/V_{MAX} while the y-intercept equals $1/V_{max}$. This graph is called a Lineweaver-Burk plot.

4Eqn

PRELAB: Remember to complete your prelab quiz in Inquire and to prepare your notebook entry.

HAZARDS:
None of the compounds used in this experiment are particularly hazardous, but as always they should be treated with respect. All wastes should be placed in the designated waste container. Look up the MSDS (http://hazard.com/msds/index.php, or Trexler 464) for o-nitrophenyl-β-D-galactopyranoside. If looking this up on line, you don't want to type that entire name, just do a partial word search for galactopyranoside, and then choose the correct entry. Record the following information in your Hazards section of your pre-lab entries:

- correct handling and storage
- types of personal protective equipment you should wear (Exposure Control section)
- toxicological and environmental information

Given what you found under toxicological and environmental information, should we feel free to allow free exposure to this chemical for people and the environment? Explain.

LABORATORY OBSERVATIONS AND DATA:
For this experiment you will work in pairs to gather the data and make observations in lab. You should each, however, make your own notebook entries. You will each write up your own lab report.

You will be measuring the absorbance of each of four solutions at 420 nm every 15 seconds for the first minute and then every 30 seconds for 5 minutes. You need a data table with columns labeled 1-4 for the four solutions, and rows labeled with times, starting at 0 minutes and continuing to 5 minutes. It should look something like the one at right. Make sure you leave enough room to go all the way to 5 minutes. Note that absorbance at time 0 is equal to 0 in all cases.

Time (min)	# 1	# 2	# 3	# 4
0	0	0	0	0
0.25				
0.5				
0.75				
1.0				
1.5				
2.0				

PROCEDURE:

Part 1: Preparation of the diluted substrate solutions

1. Take a small beaker to the reagent bench and get 25 mL of the 1.5×10^{-2}M substrate stock solution. Use a marker to label your beaker, #1.

2. Obtain three 10-mL volumetric flasks and a 10-mL graduated measuring pipet and bulb. Label the flasks #2 - #4.

3. Rinse the pipet with a small amount of the substrate solution. Pipet 8.0 mL into flask #2, 5.0 mL into flask #3, and 2.0 mL into flask #4.

4. Dilute each to the mark with distilled water, seal with parafilm, and invert to mix. Place the containers numbered 1-4 in a dish of slushy ice to store until use.

Part 2: Enzyme assay

In this part of the experiment, you mix the enzyme with the substrate and then monitor the reaction with a Spec-20. Since you have just prepared four different concentrations of the substrate, you will do the assay four times, once with each concentration of substrate. You should recall that when we use the Spec-20, we need a "blank" solution that includes everything in the main solution except for the active ingredient. So, for each of the four measurements, you make

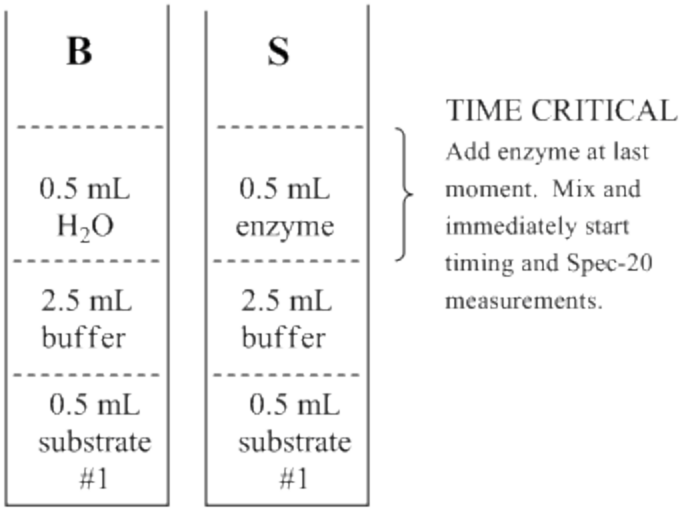
a. A sample solution containing substrate, buffer, and enzyme, and
b. A blank solution containing substrate, buffer, and distilled water (in place of the enzyme)

For the each substrate concentration, you will

Add substrate solution to both Blank and Sample cuvettes
Add buffer solution to both Blank and Sample cuvettes
Add water to just the Blank
Mix, and use to zero the Spec-20
Add enzyme to just the Sample cuvette at the last possible moment
Quickly mix, and start measuring Absorbance values on the Spec-20

You then repeat the process for the other three substrate concentrations.

As illustrated by the figure below. Preparing the blank is NOT time critical. You can prepare it, mix it, and let it sit around for awhile. However, timing on the sample cuvette is CRITICAL. As soon as the enzyme goes into the tube, you mix, and immediately starting timing and measuring absorbance.



1. Obtain two Spec-20 cuvettes. Use a marker to label them near the top: one S for sample, and the other B for blank.

2. Get 40 mL of pH 8 potassium phosphate buffer solution in a beaker.

3. Get one 5-mL graduated measuring pipet and two 1-mL graduated measuring pipets. You will also need a small beaker of distilled water.

4. Use a 1-mL measuring pipet to add 0.5 mL of substrate solution #1 into **both** the blank and sample cuvettes. Save this pipet for future measurements of substrate.

5. Now use the 5-mL measuring pipet to add 2.5mL of buffer into **both** the blank and sample cuvettes.

6. Use the second 1-mL graduated pipet to add 0.5 mL distilled water **to the blank only**. Save this pipet for measuring water only.

7. Top the blank cuvette with Parafilm and mix by inversion.

8. Take both cuvettes to the Spec-20, which should be set at 420 nm.

9. Use your blank cuvette to zero the instrument.

10. You will find the Lactaid® enzyme solution and an automatic pipet next to the Spec-20. Add 0.5 mL of this enzyme solution to your **sample cuvette**. **Immediately** top it with parafilm, invert to mix, and start timing.

11. As quickly as possible, insert the sample cuvette into the Spec-20. Record the absorbance every 15 seconds for the first minute and then at 30 second intervals for 5 minutes.

12. Dispose of your solutions in the waste container, then rinse your cuvettes with distilled water and allow the excess water to drain from the tubes.

13. Repeat the procedure of Steps 8-16 with each substrate solution 2-4, each time preparing both a blank and sample solution. Rinse your pipet with the new substrate solution before measuring it into the Spec-20 cuvettes. Use the same Spec-20 each time.

RESULTS AND DISCUSSION:

Go to the Inquire site for your lab section or the online lab manual and find the Excel template for this experiment. When you open it in Excel, you will find color-coded tables needed to complete your report. While the format of the tables has been set, you must enter you own data (in the pink blocks), and use Excel formulas to calculate values (in the yellow blocks). Sometimes you must enter data that is read from a graph or other sources (in the blue blocks).

Save this Excel file, naming it: Your Name Exp 17. Use the file to do your calculations and record your results. When you finish your report, email the file to your instructor. Be sure that your name is in the file name!

Enter your data in the pink sections of the "Absorbances and Initial Rates" page of the spreadsheet.

Find initial rates

For each of your four Spec-20 enzyme assay solutions, plot absorbance vs. time on the "Absorbances and Initial Rates" page of the spreadsheet. Your graphs should be similar to Figure 1. Now notice that the first several data points, including (0,0), form a straight line. (See Figure 1) Look at each graph and decide how many points fit this straight line region. (The linear region may be 3-6 data points. You may wish to enlarge your graph temporarily to get a better look to determine which points starting from zero form a straight line.)

You need to add a trendline that includes just these first few linear points. Click on your graph so that you get the Chart Tool tabs across the top of the screen. Choose the **Chart Tool Design** tab. Choose the **Select Data** button. Click on **Add** in the Legend Entries (Series) area. Name the series "linear." Now you need to specify the data points for this linear series. In the middle of the small window you should see a place for *x*-values and a small arrow on the far right end of this box. Click the arrow and then highlight the cells in your spreadsheet that should be included in the linear region. Remember this will be just the first couple of data points. Press **Enter**. Now follow the same procedure to specify the *y*-values. Click **OK** and return to looking at your graph. Be sure that the point (0,0) is included in the new linear series. Now following the usual procedure, add a linear trendline for the linear series: Switch to the Layout tab, Choose Trendline, Choose More Trendline Options, Choose the linear series, and display the equation on the graph. (Your graph should look a lot like Figure 1 on Page 2.) The slope of this trendline is the initial rate, *v*, for this solution.

Calculate substrate concentrations

Number	Substrate Solution	[S] in Spec-20 tube	v	1/[S]	1/v
1	1.5×10^{-2}				
2					
3					
4					

Now go to the Lineweaver-Burk plot page of the spreadsheet, where you will find a table like the one shown. Recall that you did two sets of dilutions. In Part 1, you diluted Substrate #1 to make Substrates #2, #3, and #4. Substrate solution #1 had a concentration of 1.5×10^{-2} M. The other solutions were prepared by diluting this one. Use Excel formulas to calculate the concentrations of substrate solutions in volumetric flasks 2-4 that appear down the left hand column. Recall that $M_1V_1 = M_2V_2$

In Part 2, you did a second level of dilution when each of the substrate solutions was mixed with buffer and enzyme to make the final solution that you put into the Spec-20. You need to calculate the [S] that was actually present in the Spec-20 tubes. Use the Substrate solution concentrations you just calculated above and recorded in the left hand column to calculate [S] in the final Spec-20 enzyme assay solutions. This new concentration goes into the column labeled "[S] in Spec-20 tube." In each case, 0.5 mL of the appropriate substrate solution was diluted to 3.5 mL of enzyme assay solution. Enter an Excel formula to do this calculation for solution #1, and then copy this formula down the rest of the column.

Lineweaver-Burk plot

The next column in your table is labeled *v*, for initial rate. Recall that each *v* value is the slope of a graph you did on the "Absorbances and Initial Rates" page of the spreadsheet. Record these values in the blue column of the table. Use Excel formulas to calculate 1/[S] and 1/*v* values. Plot 1/*v* (on the *y*-axis) vs. 1/[S] (on the *x*-axis). Add a linear trendline and put the equation for that line on your graph.

As noted in Eqn 4 in the Introduction, your y-intercept equals $1/V_{MAX}$. Calculate V_{MAX} and then use it and the slope, which equals K_M/V_{MAX}, to calculate K_M. Record values of V_{MAX} and K_M in the blue fields of the spreadsheet.

Save your Excel file (with your name in the file name). Email it to your instructor.
Print both pages of the spreadsheet and include these in your report, which will include

- a cover sheet
- print copies of the spreadsheet pages
- word processed answers to the questions below

QUESTIONS:

1. Enzymes like lactase are catalysts, and like all catalysts speed up reactions. How do they do that? Your answer should include some reference to energy profiles such as Figure 13.17 of your textbook.

2. Did you find that initial rate increased or decreased with higher substrate concentrations? Cite examples from your data. Will this trend continue indefinitely? [Read the Introduction!] Discuss what happens when substrate concentrations become large, and the meanings of V_{MAX} and K_M.