

ENZYME REACTIONS–Peroxidase

I – BACKGROUND

Introduction:

In your discussion section, you have decided, or will decide, upon a particular tissue of a particular plant to examine. You probably chose either to compare it to turnip root (for which we already have information; turnip serves as a control), or to compare it to a different tissue from the same plant. In either case, you need to know how to make a comparison experimentally, and how to determine whether the comparison is valid. Only if you have a valid comparison can you use your data to learn something about the world.

Before beginning, it is important to think about what enzymes do, and what enzyme reactions are like. This will help us design the experiment properly, so that it can address the questions we want to answer. To do so, let's consider what an enzyme does in the cell: it binds to a "substrate" molecule and catalyzes a reaction, thereby converting the substrate into "product." Sometimes, there are two or more substrates, or two or more products—it depends on the reaction. In the test tube, we try to re-create the reaction from purified components. The conditions of the reaction are not identical to the conditions inside the cell, but we can often identify conditions that work.

To make our comparison of the enzymes from our two species or two tissues, we need to know how to measure the reaction. Typically, this means that we need a way to measure the appearance of product, or the disappearance of substrate. Recall that you monitored the disappearance of blue-colored DCIP as a way to follow the Hill reaction of photosynthesis. In the peroxidase reactions we will study here, we will monitor the appearance of blue-colored product (using the spectrophotometer). We also need to know how much enzyme we have in a given amount of material, and how much substrate to use for the reaction to proceed effectively. Most importantly, we need to know how to tell if the reaction is proceeding normally.

A normal chemical reaction proceeds at some rate (called velocity by enzymologists)—X molecules of product produced per minute. The actual rate can be affected both by the amount of enzyme present in the test tube, and by the amount of substrate. Think of it this way: inside the tube, there will be enzyme molecules and substrate molecules floating around in solution bumping into one another. They have to bump into each other in the right orientation for the enzyme to bind the substrate and catalyze the reaction. With this image of the reaction, it is easy to see why the rate depends on the concentrations of both enzyme and substrate. The more of each that there is, the more frequently they will encounter one another in the correct orientation for the reaction to occur.

We can also see that at low concentrations of substrate, the reaction rate will slow down as the reaction proceeds, because the enzyme continuously decreases the concentration of substrate by converting it into product. If this happens, it is not very easy to determine what the actual rate of reaction is, since it keeps changing. We would have to try to determine the rate at the very instant the reaction starts, before it has a chance to slow down significantly. At very high concentrations of substrate—what we might call a "vast excess" of substrate—the reaction rate

Enzymes

will not slow down nearly as quickly, since it will take longer for the same small amount of enzyme to act upon the larger amount of substrate. Under these conditions, the reaction rate should be constant for a longer period of time.

From this, we can see that there is a general principle of enzyme reactions that can guide our experimental design: the rate is slow and variable at low substrate concentrations, but faster and more constant at high substrate concentrations. Therefore, to make a valid comparison of our two enzyme preparations, we will need to be sure we use substrate concentrations that are high enough to avoid technical difficulties. To accommodate this in the laboratory, our experiment must be broken into three parts, that will take place over the course of the next three weeks. In the first part, we will learn about the assay for peroxidase enzymatic activity and how enzymatic reactions can be studied. In the second part, we will determine the substrate dependence of the enzymes, so that we can learn the best way to assay the enzymes accurately. In the third experiment, we will compare the relative stabilities of the two enzymes to heat—using the assay conditions that we determined in the second experiment to be the best for each enzyme.

Linearity

The preceding discussion indicates that if we have enough of an excess of substrate molecules relative to enzyme molecules, the rate should remain constant for a reasonable period of time. That is, it should be linear—if we make a graph with time on the X axis, and amount of product produced on the Y axis, we should find that the data points fall upon a straight line for the first several minutes of the reaction. If the reaction is linear, then we have a good criterion for whether we have set up the experiment properly: the data points should fall upon a straight line when we graph “product produced” vs “time of reaction.” What if the data points do *not* fall on a straight line? What if they jump up and down? If this happens, we cannot blame the enzyme reaction; rather, it must be an error in experimental technique. For example, if we do not mix the tube after adding all the components, we will obtain data that do not reflect the reaction conditions accurately.

How do you tell whether your data *do* reflect the conditions accurately, or that your “straight line” is a valid interpretation of the data? The first clue, of course, is that the data should be reproducible. If you are concerned about your reactions, repeat them. Secondly, you can use a statistical test to determine the validity of the straight line: you can (and should) use a linear regression and calculate the value for “ r^2 .” You can do this with Cricket Graph, or manually. If you do not already know why this is important, *read Appendix A* at the end of this section. It describes an analogous situation that should help make the points clear.

Enzyme Kinetics

The subject of enzyme kinetics is examined in detail in upper-level biochemistry courses. But it’s really not difficult, and we need to know a little about it to be able to make a valid comparison of our two enzymes. As we said above, we need to be sure we have enough substrate to do the reactions properly...how do we know how much is “enough”?

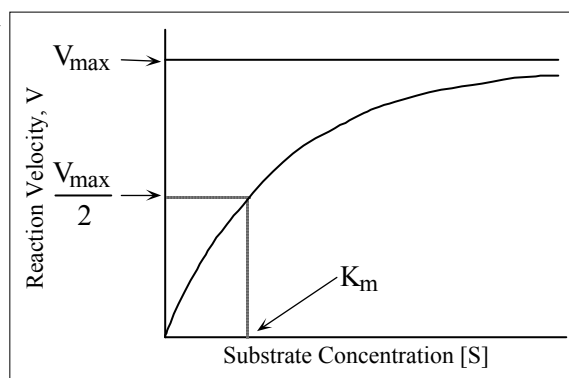
Every enzyme has a certain ability to bind to its substrate. Some kinds of enzymes bind their substrates very tightly, and can work at very low substrate concentrations, while others bind

rather weakly, and require much higher substrate concentrations to be able to catalyze their reactions effectively. This “affinity” is an inherent property of the enzyme, and plays an important role in designing experiments that seek to compare enzymes from different species, or different tissues. If we try to use a substrate concentration that is below the inherent affinity of the enzyme, we will find that the reaction doesn’t work very well. We must use substrate concentrations that are high enough that the enzyme can function.

The purpose of the first week’s experiments is to determine how much substrate is enough. In the process, we will determine the affinity of the enzyme for its substrate. It is interesting to see how this works.

At low substrate concentrations, the enzyme reaction is slow. After releasing the product(s), any particular enzyme molecule floats free in the reaction tube, unable to react until it encounters another substrate molecule. At higher concentrations, the time between encounters decreases, so the rate of the reaction increases. At very high concentrations, the time between encounters could conceivably become less than the time it takes for the enzyme to catalyze the reaction and release the products. This results in an interesting phenomenon: the rate of the reaction increases with increasing substrate concentration, but only up to a point. There is a maximum rate (or maximum velocity, V_{\max}) that cannot be exceeded, and that is determined by the time it takes for the enzyme to catalyze the reaction and release the products. Therefore, if we perform a series of enzyme reactions using different substrate concentrations, then determine the rate of reaction for each, and then make a graph of the reaction rates as a function of substrate concentration, we would see a curve something like that shown here.

The reaction rate (velocity) would increase rapidly at first, then level off. The theoretical maximum velocity is called the V_{\max} . Obviously, it is possible to define a reaction rate that is exactly half of V_{\max} . The substrate concentration at which this occurs turns out to be constant for any particular enzyme (regardless of how much enzyme is used in the reaction), and is called the K_m (for the “Michaelis Constant”, named for the person who first recognized its importance). The K_m is related to the actual affinity of the enzyme



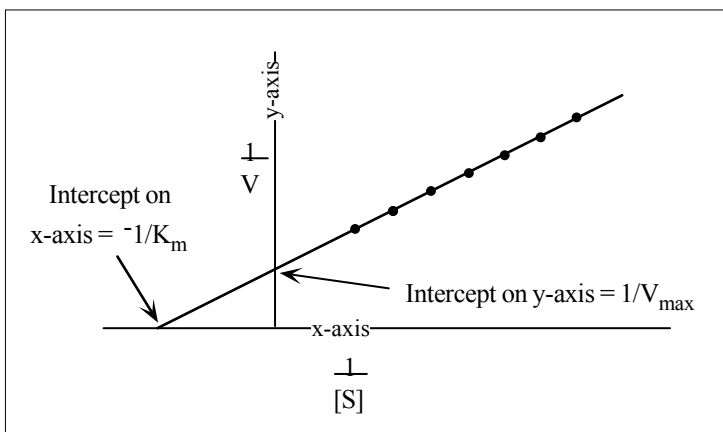
for the substrate. A higher affinity for the substrate is reflected in a smaller K_m . Conversely, if one enzyme has a lower substrate affinity than another, it will have a higher K_m .

In our experiments, we are seeking to identify a substrate concentration that is high enough to give us reliable data in our comparisons. According to the graph shown here, this should be a concentration on the level part of the graph, not on the steep part. Then, slight changes in reaction conditions will not influence our data significantly. In the jargon of enzyme kinetics, we want to perform our experiments with a substrate concentration that “is well above the K_m .” Because it is so easy to determine the K_m , and because it is necessary to do so before we can carry out the later portion of the experiments, we will devote the first week to this set of experiments: the substrate concentration curves.

The Double-Reciprocal Plot: how to determine K_m and V_{\max} more easily

Enzymes

As noted above, a graph of reaction rate (or “velocity,” V) as a function of substrate concentration should give a smooth curve that levels off as it approaches the maximum rate (V_{\max}). But, it will never actually reach the V_{\max} . Thus, this kind of graph allows only an estimate of the V_{\max} , and therefore only an estimate of K_m . It is possible to determine the V_{\max} accurately by plotting the data in a different way: as the



reciprocal of reaction rate ($1/V$) vs the reciprocal of substrate concentration ($1/[S]$). From this curve (which is linear instead of hyperbolic), the two kinetic constants are determined from the X and Y intercepts. This is fairly straightforward for the V_{\max} —as the concentration of substrate increases, the rate of reaction increases. Thus, when $[S]$ is infinite, and the V_{\max} is finally achieved, $1/[S]$ is zero. Thus, the Y-intercept is $1/V_{\max}$. The K_m can be determined from the X-intercept. As indicated here, the X-intercept is $-1/K_m$.

Peroxidase

Peroxidase can be extracted from any of a number of plants, but we will discuss here the enzyme extracted from turnips, which happen to have a high concentration of peroxidase. All peroxidases are remarkably stable and easy to assay. This makes peroxidase a good enzyme to use for comparisons among species.

Information: These virtues have been recognized commercially. Many ELISA (enzyme-linked immunosorbent assay) diagnostic tests use a closely related peroxidase (from horseradish) and the same color-producing agent (TMB). Because the enzyme is so stable, the diagnostic kit can be stored by hospitals and clinics for relatively long periods of time. So, in some cases, human survival may be linked to a plant enzyme.

Your AI will prepare a very crude enzyme extract by homogenizing a small amount of turnip root in water (using a Waring blender) and then filtering the homogenate (see Appendix C for details). This extract will contain all of the soluble proteins from the turnip root. However, we need not be concerned about the thousands of other kinds of proteins present because only peroxidase will catalyze the reaction that you will measure.

In this case, the peroxidase reaction is:

$$\text{H}_2\text{O}_2 + \text{ZH}_2 \xrightarrow{\text{peroxidase}} 2 \text{H}_2\text{O} + \text{Z}$$

That is, the enzyme converts hydrogen peroxide to water, obtaining the two hydrogen atoms it needs for this from a “donor” molecule—called ZH_2 in this example. Thus, at the same time that hydrogen peroxide is being reduced, ZH_2 is oxidized to Z . In this example, the letter “Z” is not the symbol for a specific chemical; rather, it indicates that the enzyme can use several different molecules as the source of the hydrogen atoms. For our experiments, we will use a chemical that does not occur in plants, but that changes color—a fact that makes the reactions very easy to monitor. The chemical we will use in the lab is called TMB (which stands for 3,3',5,5'-tetramethylbenzidine). In its reduced form (i.e., as ZH_2), TMB is colorless. Oxidized TMB (Z) is blue. You can therefore measure the progress of the reaction by monitoring the appearance of

blue color in the tube. This is done by placing the tube in a spectrophotometer, and measuring the amount of red light (655 nm) absorbed by it. The more the reaction proceeds the more product, Z, is produced. In this reaction, Z is oxidized TMB. The more oxidized TMB present, the more blue the solution in the tube becomes, and the more it absorbs at 655 nm. **In other words, the rate of blue color development is a direct measure of the rate of the peroxidase reaction.**

Information: 1)TMB is 3,3',5,5'-tetramethylbenzidine, a chemical that was developed originally as a very sensitive agent to detect blood stains (hemoglobin exhibits peroxidase-like activity). TMB is colorless, but the oxidized form is blue with a maximum absorption at 655 nm. At that wavelength, the molar absorptivity index = 5400; (that is, a 1.0 M solution in a 1-cm light path would have a theoretical absorbance of 5400).

2)TMB (unlike many other benzidine-containing compounds) does not have any carcinogenic activity.

3)You may note that the enzyme reaction will continue, even after you stop making measurements. As a result, the concentration of oxidized TMB becomes high enough to cause it to precipitate from the solution.

Question: How do you know that 655 nm is the best wavelength for the experiment you will be doing? How could you check?

The enzyme assay is easy and fast. Essentially, all you do is take a test tube, and add water, buffer (to maintain a constant pH), TMB, a small amount of crude enzyme, and some H_2O_2 ; then you watch the tube turn blue. To collect meaningful data, you put the tube into a spectrophotometer that can quantitate the amount of red light (655 nm) absorbed by the solution, and record the absorbance every few seconds for 2 minutes (at 15 second intervals). You then calculate the velocity of the process—which, as described above, should be linear.

Comparing Amounts of Peroxidase Between Samples

In addition to properties of the enzyme (e.g., K_m) that can differ between different sources of peroxidase, it is also possible that the amount of enzyme may differ. That is, some tissues may produce a large amount of peroxidase, while others produce a small amount. These differences are often important for the biology of the plant.

However, when we prepare our extracts, we adjust for these differences. If our tissue has very little activity, we grind a large amount of it in water; if the tissue has a great deal of activity, we grind a small amount. When your AI prepares your extracts, s/he will take care to use an amount of tissue that gives about the same amount of activity in the standard assay. This makes it easy to do the assays, but makes it difficult to determine from the extract alone how much peroxidase was in the original tissue. For this, it is necessary to keep track of how much tissue was used to prepare the extract.

What is commonly done is to define a “unit” of enzyme activity as that amount of enzyme which produces a specific amount of product in a specific amount of time under standardized conditions. For peroxidase in L113, we can define our “unit” as the amount of enzyme it takes to increase the absorbance of TMB by 0.4 in 2 minutes (= 0.2 in 1 min) under the conditions of the standard assay. By this definition, every extract should have about 1 unit of activity per 50 μ l of extract (the quantity we use in the reactions). There may be slight variations between extracts, but for the purposes of this discussion, we will assume that your AI prepares an extract that really is very close to 1 unit per 50 μ l.

Because the extracts will all be nearly the same concentration in terms of units/volume, the main variable that will be helpful for comparing amounts of peroxidase between samples will be the amount of tissue that your AI ground in water (your AI will use 300ml). Ask your AI how much tissue s/he used to make the extract, so you can learn how your different samples compare. In your minireport, this should be expressed as grams of tissue per 100 ml of water (g/100ml).

Note: these comparisons can be quite striking. For turnip roots, we may need about 1g of tissue, while for horseradish (the source of commercially important peroxidase), we may need only 0.05g, and for cactus stems, we may need as much as 20g.

II – LABORATORY EXPERIMENT

Experiment 1. The Standard Assay and Controls

Before working with any enzyme, it is essential to make sure that the solutions are working correctly. It is also a good idea to run through the reaction yourself to be sure you can do it efficiently. Here, we will ask two questions:

- i. Does the reaction depend on peroxidase?
- ii. Does the reaction depend on TMB?

How would you go about asking these two questions? As a general rule with enzyme assays (or any experiment, for that matter), you should *change only one thing at a time*. That is, you can ask whether the reaction depends on peroxidase by setting up a reaction mix that is exactly the same as always, but from which only the plant extract has been omitted. You then collect data from this tube just like any other, and calculate the reaction rate just like any other. To ask whether the reaction depends on TMB, you set up another tube that is exactly the same as always, but lacks only the TMB. Again, you treat it like any other tube.

What do you do about the fact that leaving out TMB or peroxidase results in a decrease in the final volume of the reaction? Do you need to increase the amount of water added to the tube to make up for it? Do Experiment A and Experiment B from the first week give you any clues? You *must* keep the final volume the same, or you will change the concentration of the other components of the reaction!

To do these kinds of “variations” on the reaction conditions, you need to have a “standard” set of reaction conditions—what we will call a “standard assay.” The protocol for a standard assay, as determined for turnip root peroxidase, is presented on page D-8. This is the protocol you will follow for this week’s reactions as well as for next week’s reactions. Study it carefully before you begin.

For this week:

First, turn to Enzyme Data Sheet #1 (page D-11). This is a simplified chart on which you can write the components of each reaction you will do, and on which you can record the data. This keeps the information on each reaction in a single place where you can refer to it easily. You should fill in the relevant portions of the data sheet **before you come to lab**. Then, as you do the experiment, you can refer to the protocol sheet for the sequence of manipulations that are common to all reactions, and to the data sheet for the details of each separate reaction.

Fill in columns 1-4 on the top part of Enzyme Data Sheet #1. Columns 1 and 2 are for identical reactions, the “standard assay,” so the quantities of reagents will be exactly the same as those on the protocol sheet for the standard assay. Why two tubes? As with the dilution experiment earlier, it is important to verify that your technique is good—and demonstrating that you can perform the reaction reproducibly is a way to do so. These two reactions should give nearly identical data.

Column 3 is a test of the requirement for peroxidase, and asks question **i** posed above. Fill in the appropriate information for this reaction on Enzyme Data Sheet #1. Remember, if you omit enzyme, you must increase the water accordingly so that the final volume remains the same.

Enzymes

Column 4 is a test of the requirement for TMB, and asks question **ii** posed above. Fill in the appropriate information; again, keep the final volume the same.

When you come to the lab, perform these 4 reactions, setting up one tube at a time. Record the data in the lower part of the table on Enzyme Data Sheet #1, and make a graph of the data.

Data interpretation

Although it is possible that the water used in the reactions is contaminated with chemicals that act directly on TMB (this *has* happened), it is most likely that the reactions do, indeed, depend on peroxidase and TMB. We expect that tubes 3 and 4 will show no reactions.

For the tubes 1 and 2, however, it is important to complete the data analysis. An individual reaction, of course, shows you how fast the enzyme produces its product for the particular conditions inside that tube. To compare different tubes, you need to compare the *rates* of the reactions. Therefore, you need to calculate the rate of each reaction. The “rate” is most easily expressed as the “change in absorbance at 655 nm” (or ΔA_{655}) per minute. You could estimate this by dividing the A_{655} of several of your data points by the number of minutes that had elapsed since adding the H_2O_2 to that tube. (i.e., divide your 1-minute reading by 1 and your two-minute reading by 2, then take the average). A more accurate way is to use all the data points for each assay to calculate the best-fit line by linear regression, and determine the slope of the line (which will be the $\Delta A_{655}/\text{min.}$).

The easiest way for you to do the linear regression is to use the computer. A reminder on how to use the computer: open Cricket Graph, enter your time points and your A_{655} readings for each tube, and then graph the data. With the graph selected, first choose “curve fit” under the “Options” menu; then choose “Linear” under Methods and “r squared” under Coefficient of Display. The line is the best-fit curve, and the equation gives the linear regression values, including r^2 . You can plot both curves on one set of axes, and fit curves to each of them. (See your L113 Computer Manual for more directions.)

Enzyme Data Sheet #1 has space available for you to write the linear regression values (the Y-intercept, the slope, and the square of the correlation coefficient, r^2). The slope is the best determination of the rate of the reaction, $\Delta A_{655}/\text{minute}$.

How similar are the rates of reaction that you calculate for tubes 1 and 2?

Protocol for the Standard Assay

Read this first (and the comments on the following page) before doing it! If you think about the protocol, and about how to do it in the lab, your work will go more smoothly.

a. Prepare the initial mixture in the tube:

To a 13 x 100 mm test tube, **add:**

2.77 ml distilled water (room temperature)

30 μ l 1.0 M Citrate Buffer, pH 6.0

50 μ l 0.5% TMB Working Solution

- Mix completely by covering the tube with parafilm, and inverting it several times. Then add:

50 μ l crude peroxidase preparation

the peroxidase is prepared according to the procedure shown in Appendix C

- Mix completely by covering the tube with parafilm, and inverting it several times.

b. Mark the tube (so that you can keep the same orientation in the spectrophotometer), make sure the spectrophotometer is set at 655 nm, and place the tube in the spectrophotometer.

- Using this tube as a “blank,” “zero” the spectrophotometer.

c. Start the reaction:

Add 100 μ l H₂O₂ Working Solution

Immediately cover the tube with parafilm, invert the tube several times to mix it, and *start timing the reaction*.

Place the tube in the spectrophotometer in the correct orientation.

d. Record data:

Take readings at 15 sec intervals for 2 min for a total of eight readings. Your first reading will be at 15 seconds (you will not have a data point for “zero seconds”).

e. Plot the data:

Plot your data as a graph of A₆₅₅ vs time.

- Your curves should be linear. If they are not, consult your AI.
- You actually did not collect data at “zero seconds.” That was the time you put the H₂O₂ into the reaction. Therefore, although it seems likely that your curves should pass through the origin (0, 0) on your graph, you may not assume that this is true. Do not put a datapoint at 0, 0.

Technical Tips:

1. Be sure everything is ready before you start:

- a. Be sure you have filled out the relevant Enzyme Data Sheet, so that you can pay attention to *doing* the experiment, rather than figuring it out.
- b. Be sure that the spectrophotometer is turned on and is set for 655 nm.
- c. Be sure you have all the tubes you need. Get an appropriate number of clean, dry 13 x 100 mm tubes from the front of the lab.
- d. With a wax pencil, label each tube with an appropriate number or letter. Mark the top so you can *always* place the tube in the spectrophotometer in the same orientation to eliminate variation due to scratches or aberrations in the glass.
- e. Practice moving your wrist! **Second only to pipetting error, failure to mix the reagents is the most common source of error in this experiment.**

2. As you do the experiment:

- a. It is most efficient to add the water to all of your tubes, then add the buffer to all of them. But, don't add the TMB or enzyme or substrate until you are ready to collect data from that particular tube. TMB is sensitive to light, and may be damaged if you put it in the tube too far ahead of time.
- b. Make additions to a tube in the order the reagents are listed on the data sheet. (Be sure to note the difference between ml and μl : $1000\ \mu\text{l} = 1\ \text{ml}$ and $50\ \mu\text{l} = 0.050\ \text{ml}$.)
- c. As you make *each* addition to a tube, check it off on the data sheet.
- d. When you finish a series, plot the data for that tube. Your data must give a reasonably straight line in order to use linear regression to calculate velocity.
- e. Record your absorbance readings directly on the data sheet provided. Note that time (on the sheet, and in all your calculations) is expressed in terms of minutes to 2 decimal places (1 min 15 sec = 1.25 min).
- f. At some point, you may make a mistake (like forgetting to mix the tube), or you may obtain a non-linear reaction for reasons you cannot immediately determine. If this happens, set up another tube. The assays are easy to run, and there should be time.
The more accurate your data, the more straightforward your interpretation and lab writeup will be!

Unless your section is really small, you will be working in groups of 2, 3, or 4 students. It is extremely convenient to share this work with one or more co-workers. Identify one person in the group to do the pipetting, one to check off the reagents on the list as they are added to the tubes, and one to verify that the numbers are, in fact, correct. When

you collect the data, identify one person to watch the time carefully, another to read the spectrophotometer accurately, and a third to write the data on the data sheet. Identify one person to enter the data into the computer as you acquire it, in order to draw the graphs as soon as the data are available. It is a good idea to rotate these responsibilities as you proceed through the experiment.

Enzyme Data Sheet #1

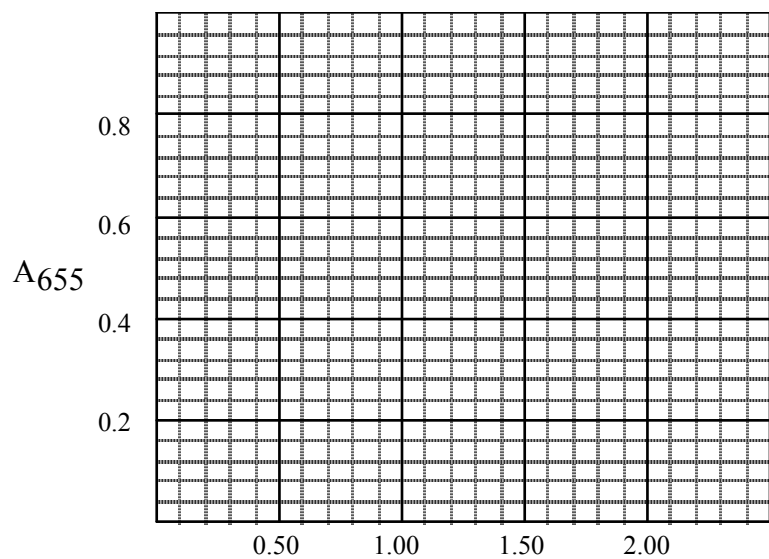
Name: _____ Lab Section: _____ Date: _____
 Experiment: _____ Tissue: _____ g/300ml used: _____

	<u>TUBE</u>			
	<u>1</u> Standard assay	<u>2</u>	<u>3</u> no-enzyme	<u>4</u> no-TMB
Distilled water (ml)	_____	_____	_____	_____
1.0M Citrate pH 6.0 (μl)	_____	_____	_____	_____
0.5% TMB (μl)	_____	_____	_____	_____
crude enzyme (μl)	_____	_____	_____	_____
H ₂ O ₂ (μl)	_____	_____	_____	_____

Time (minutes)	<u>ABSORBANCE</u>			
	0.50	_____	_____	_____
0.75	_____	_____	_____	_____
1.00	_____	_____	_____	_____
1.25	_____	_____	_____	_____
1.50	_____	_____	_____	_____
1.75	_____	_____	_____	_____
2.00	_____	_____	_____	_____

Information from linear regression (curve fit, $y = Ax + B$)

y intercept (B)	_____	_____	_____	_____
slope (A)	_____	_____	_____	_____
rate of reaction, V (=A)	_____	_____	_____	_____
r ²	_____	_____	_____	_____



Enzymes

The Effect of Substrate Concentration: a Simulated Enzyme Reaction

Next week, you will do a series of reactions using different substrate concentrations. These are important experiments, but it is often difficult to relate the data to the actual chemical reaction. The following is a simulation* that can help make this connection clear.

In the lab, there are a series of bags containing segments of yellow and blue sides of Ziploc bags—the ones that are designed to turn green when they are sealed, to give a visual indication of whether the bag is really closed. In the bags of Ziploc bits, the number of pieces of each color varies from bag to bag thereby changing the concentration of the less common color bit. (See the outside of each bag for the numbers of each and for concentration information.)

The “enzyme reaction” consists of one person reaching into the bag and removing pieces of Ziploc, then pressing them together if they are of complementary colors, while another person records the number of sealed pieces as a function of time.

This “reaction” simulates the substrate concentration experiments you will do next week. In these experiments, the concentration of TMB is constant and high in every tube (analogous to the constant Ziploc pieces), while the concentration of H_2O_2 varies. In both the experiment and the simulation, the rate of reaction (product vs time) shows a dependence on the concentration of the substrate that varies in concentration.

Do the simulation as follows:

Each lab group should take a bag of Ziploc pieces and carry out the reaction. One person needs to be the enzyme, another the timekeeper, and a third the recorder. When the timekeeper says to start, the “enzyme” reaches into the bag with both hands and, without looking, withdraws two Ziploc pieces. If the two are complementary, the enzyme zips them together and places the product on the lab bench. Don't get too involved in the zipping part; just press them together as well as you can without taking too much time. Also, *don't rush to be the first ones done!* Then the enzyme returns to the bag for two more pieces. If the two fragments are not complementary, the enzyme tosses them back into the bag, and proceeds to withdraw two more pieces. Every 30 sec, the timekeeper calls out the time, and the recorder records the time along with the total number of zipped seals, i.e. products, that now lie on the lab bench. (At each time point, you need to record the number of zipped seals produced since the reaction began and not the number produced since the last time point. This is what we will be doing in lab when we take an absorbance reading of the reaction test tube to measure the amount of blue TMB-product.) The reaction continues in this manner for 3 - 5 min.

After completing the “reaction,” each group should graph their results (using Cricket Graph or the Ziploc Data Sheet) and calculate the rate of reaction in zipped seals per min. This will be the slope of the line determined by plotting the number of zipped seals vs. the elapsed time in min.

Once each group has determined the reaction rate for the substrate concentration they tested, you will pool your results, and as a section work together to determine the V_{max} and K_m for human Ziploc seal-zipping enzyme.

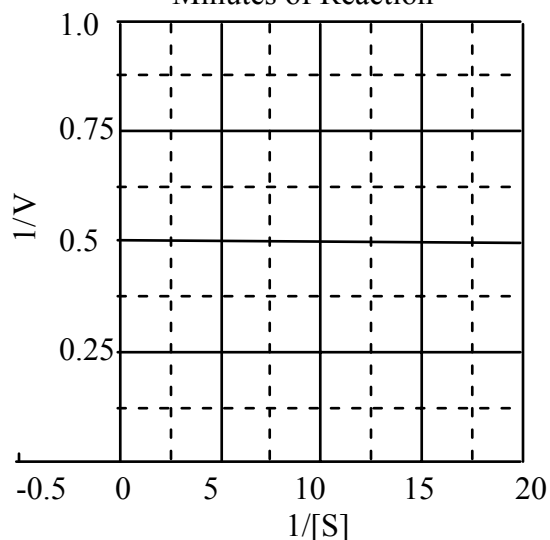
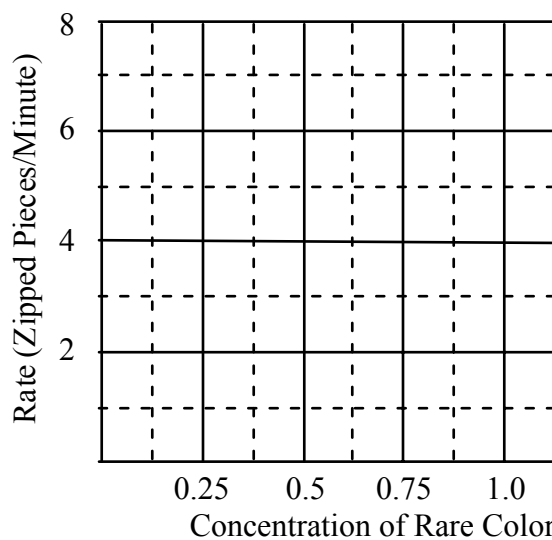
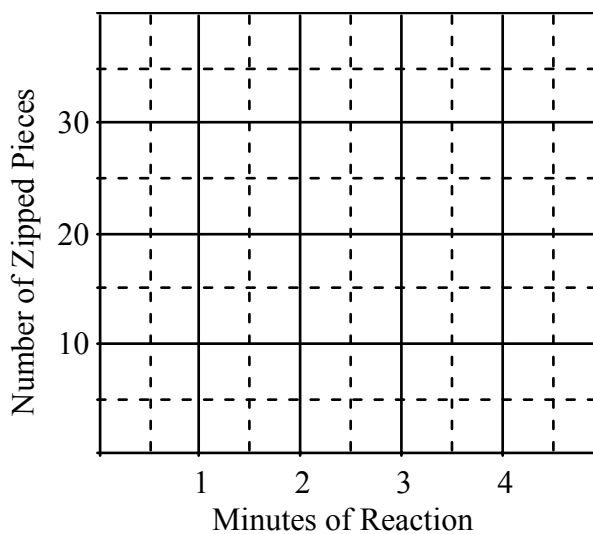
*Simulations are very important in gaining understanding. Difficult biological problems are often examined by computer-aided simulations, which help the researcher see how the variables interact. For us, as students, it is *extremely* helpful to perform simulations (or “demos”) of the concepts we are learning. In L211, you will do a simulation of DNA replication using a pipe-cleaner demo. For meiosis, you can use a pop-bead demo (see section F). If there is *anything* you don’t see clearly, grab some handy objects (pencils, hunks of clay, pipe cleaners, etc) and simulate it with a demo. It will really help your understanding.

Enzymes

Ziploc Simulation Datasheet

Ratio of Colors	Concentration of Rare Color	Number of Zipped Pieces at Time...							
		0.5 min.	1 min.	1.5 min.	2 min.	2.5 min.	3 min.	3.5 min.	4 min.
1:1	0.5								
1:2	0.33								
1:4	0.2								
1:8	0.11								
1:16	0.06								
1:32	0.03								

You should make the graphs using the computer. Here are some axes so you can make copies here, too:



Enzymes

Experiment 2: How Does Changing the Substrate Concentration Affect the Reaction Rate?

To determine the temperature-dependence of your enzyme, you need to know how much substrate to use in the reactions—the concentration must be high enough to work well. It is therefore necessary to do a series of reactions that use different amounts of H_2O_2 and that allow us to determine the K_m . This is essential so that we can use substrate concentrations that are well above the K_m for our experiments next week.

A second reason to do this experiment is to obtain the information that it can yield about the enzyme itself. Every enzyme binds to its substrate with some inherent affinity reflected in the K_m —some bind tightly, some loosely. Enzymes that bind tightly are capable of functioning effectively when there is very little substrate present, while those that bind their substrates poorly require much higher concentrations of substrate to work effectively. Not only is this inherently interesting from the molecular/biochemical viewpoint, but, as we will see later in the semester, this kind of information can be of significant value in understanding how individuals in a population can be affected by their environments.

To prepare yourself for this experiment, fill out columns 1-8 of Enzyme Data Sheet # 2 (p. D-19). Fill out the top portion of these columns, designing reaction tubes that contain different amounts of H_2O_2 (but that are otherwise identical to the Standard Assay). There is nothing magical about what concentrations you use, nor is there a “scientific requirement” to use some concentrations and not others. The critical thing is to have a range of concentrations, some of which are fairly low, and some of which are fairly high. You could, for example, use 14.3 μl , 42 μl , 61.8 μl , 103 μl , 185 μl , 213 μl , and such. However, it is easier to do the calculations and plot the data if you stick with round numbers like 0 μl , 20 μl , 40 μl , 60 μl , 100 μl , 150 μl , 200 μl and 250 μl . This range will give you concentrations of H_2O_2 that are both higher and lower than that of the Standard Assay. [Remember, of course, to keep the final volume constant.]

Do this before you come to lab!

When you come to lab, follow the procedure that is outlined for the standard assay, but use the volumes of reagents that you have calculated on Enzyme Data Sheet #2. Collect the data directly onto Enzyme Data Sheet #2, and plot the data.

Data Interpretation

For the next experiments, it is important to use a substrate concentration that is well above the K_m of the enzyme. This will ensure accurate determination of enzyme activity. The data from these enzyme reactions can be used to determine the K_m .

The first step is to graph your data. Use Cricket Graph to determine the best-fit curve, and determine the rate of reaction just as you did last week for your standard assay. A reminder of what to do: open Cricket Graph, enter your time points and your A_{655} readings for each H_2O_2 concentration, and graph the data. With the graph selected, first choose "curve fit" under the "Options" menu; then choose "Linear" under Methods and "r squared" under Coefficient of Display. The line is the best-fit curve, and the equation gives the linear regression values, including r^2 .

Enzyme Data Sheet #2 has space available for you to write the linear regression values (the Y-intercept, the slope, and the square of the correlation coefficient, r^2). The slope is the best determination of the rate of the reaction, $\Delta A_{655}/\text{minute}$.

Enzyme Data Sheet #2, and the graphs you have drawn, provide you with the raw data. To answer the question we have asked ("how does changing the substrate concentration affect the reaction rate?"), it is necessary to present the data in a form that reveals the relationship between substrate concentration and reaction rate. That is, we need to make a graph of reaction rate as a function of H_2O_2 concentration. To do so, turn to **Enzyme Data Sheet #3** (p. D-21).

For each of your reactions, convert the "amount of substrate used" (the number of μl of H_2O_2 you put into each reaction) into the H_2O_2 concentration. This is easy to do: $100\mu\text{l}$ of the H_2O_2 stock solution gives $1.00\ \mu\text{M}$ in the $3.00\ \text{ml}$ reaction. Use this relationship to calculate the concentration, in μM , in the other tubes.

For reaction rate, use the slopes of the lines you determined on Enzyme Data Sheet #2, as $\Delta A_{655}/\text{minute}$.

The first graph should resemble the idealized graph on page D-3. However, unlike the graph on page D-3, it probably does not level off. This makes it difficult to determine V_{max} . Therefore, convert the data into the double-reciprocal plot to determine V_{max} and K_m more accurately. You will need to use the equation for the best fit line that Cricket Graph will give you, and a bit of algebra, to calculate the value of x when $y=0$. This is the x -intercept, and for this line, is equal to $-1/K_m$. Use this relationship to calculate the K_m from the x -intercept of your graph, and record your answers at the bottom of Enzyme Data Sheet #3. Your section will need to review these results to determine how much H_2O_2 to use in your reactions next week, so carry out these calculations before your next discussion session.

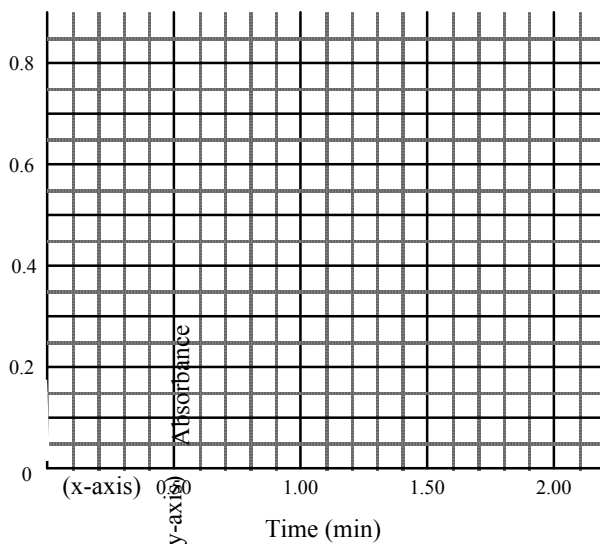
Enzyme Data Sheet #2

NAME: _____ LAB SEC. _____ DATE: _____ PAGE ___ of ___
 EXPERIMENT: _____ TISSUE: _____ g/300ml used in extract: _____

	TUBE NUMBER							
	1	2	3	4	5	6	7	8
dist. water (ml)	_____	_____	_____	_____	_____	_____	_____	_____
1.0 M citrate, pH 6.0 (μl)	_____	_____	_____	_____	_____	_____	_____	_____
0.5% TMB (μl)	_____	_____	_____	_____	_____	_____	_____	_____
crude enzyme (μl)	_____	_____	_____	_____	_____	_____	_____	_____
H ₂ O ₂ (μl)	_____	_____	_____	_____	_____	_____	_____	_____

	ABSORBANCE							
time (min) 0.25	_____	_____	_____	_____	_____	_____	_____	_____
0.50	_____	_____	_____	_____	_____	_____	_____	_____
0.75	_____	_____	_____	_____	_____	_____	_____	_____
1.00	_____	_____	_____	_____	_____	_____	_____	_____
1.25	_____	_____	_____	_____	_____	_____	_____	_____
1.50	_____	_____	_____	_____	_____	_____	_____	_____
1.75	_____	_____	_____	_____	_____	_____	_____	_____
2.00	_____	_____	_____	_____	_____	_____	_____	_____

	LINEAR REGRESSION VALUES (y = Ax + B)							
(Y-intercept) B	_____	_____	_____	_____	_____	_____	_____	_____
(Slope = V) A	_____	_____	_____	_____	_____	_____	_____	_____
r ²	_____	_____	_____	_____	_____	_____	_____	_____



Enzyme Data Sheet #3

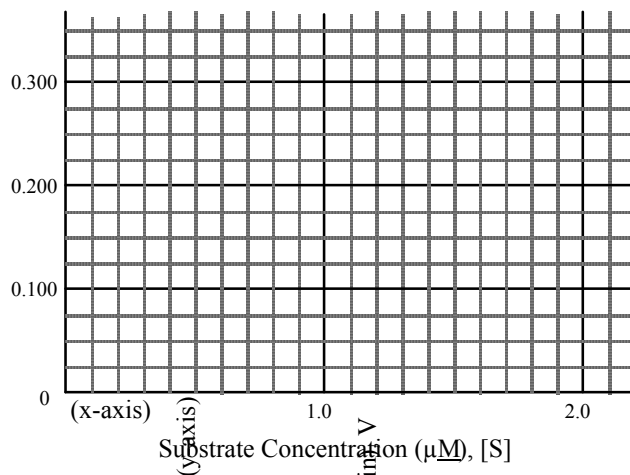
NAME: _____ LAB SEC. _____ DATE: _____ PAGE ___ of ___

EXPERIMENT: _____ TISSUE: _____ g/300ml used in extract: _____

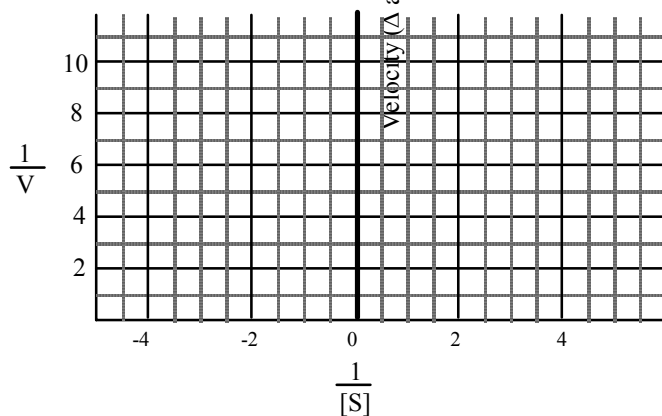
(Used) Substrate Amt. (μ l)	(Calculated) Substrate Conc. (μ M)	Velocity (2 Abs/min)
_____	_____	_____
_____	_____	_____
100	1.00	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____

Estimated Vmax = _____ 2 Abs/min

Estimated Km = _____ μ M



$\frac{1}{[S]}$	$\frac{1}{V}$
_____	_____
_____	_____
_____	_____
_____	_____
_____	_____
_____	_____



Linear Regression Values:

Y-intercept = $\frac{1}{V_{max}}$ = B = _____ Vmax = _____ 2 abs/min

Slope = A = _____

r^2 = _____

Calculation of Line Equation: $y = Ax + B$ (therefore, $x = -\frac{B}{A}$ when $y = 0$)

Value of x when $y = 0$ (i.e., $-\frac{1}{K_m}$) = _____ Km = _____ μ M

Experiment 3. How stable is peroxidase to thermal denaturation?

Enzymes are destroyed by high temperature. The three-dimensional structure of any protein is disrupted by thermal energy, resulting in a high probability of folding incorrectly. Typically, proteins from organisms adapted to life at high temperatures are more resistant to thermal “denaturation” than are enzymes from organisms adapted to life at low temperature. Yet, within an organism, some enzymes are more stable than others, so thermal stability does not necessarily parallel the growth temperatures of organisms. To determine how your peroxidase is affected by high temperature, you can simply incubate a preparation of the enzyme at high temperature, and at various times remove a small sample to assay for how much activity the enzyme still has. The following is the most efficient way to do this:

1. Prepare six Reaction tubes by adding the correct amount of **distilled water** and **Citrate Buffer** to each. Label each tube. Record what you do on **Enzyme Data Sheet #4** (p. D-25).
2. With **tube # 1**, add TMB, enzyme, and H_2O_2 , and perform a standard assay of peroxidase, recording data at 15-second intervals. The volume of enzyme extract and H_2O_2 that you will use will depend on what you learned last week. Check with your AI. Record the data on **Enzyme Data Sheet 4**. This tube will be your “0 minute” sample since the enzyme in this tube was never exposed to the $70^\circ C$ water bath.
3. To a clean, empty test tube, add 2.0 ml of the enzyme extract that you are studying. Cover the tube with a marble or small, glass beaker to minimize evaporation. Place the tube in a $70^\circ C$ water bath, and record the time at which you do this. *Check the temperature of the water bath with a thermometer, and record the actual temperature that you use.*

At various times, remove a sample of enzyme from the tube in the water bath (the same volume of enzyme as you used for tube 1), and add it to one of the prepared reaction tubes to which you have added TMB shortly before this. Record the time (to determine the length of time the enzyme was in the water bath), mix the tube, and use it to zero the spectrophotometer. Then add the H_2O_2 , mix again, put the tube in the spectrophotometer and follow the reaction. Each assay is identical to the standard assay, except that the enzyme was incubated at $70^\circ C$ beforehand.

While any set of times would work, it might be reasonable to use the times suggested in the table below. With most peroxidases, which are quite stable, these times are likely to show good activity at the shortest time, and very little, if any activity at the longest times. However, it is possible that the enzyme you have chosen will be quite heat-sensitive, and require shorter timepoints. Or, it may be quite stable, and require longer timepoints. If it is completely dead after only 10 minutes, set up a new tube, and use shorter time periods.

Tube #2 -- 2 min		Tube #1 -- ___ min
Tube #3 -- 6 min		Tube #2 -- ___ min
Tube #4 -- 10 min	<u>OR</u>	Tube #3 -- ___ min
Tube #5 -- 15 min		Tube #4 -- ___ min
Tube #6 -- 20 min		Tube #5 -- ___ min

Summary: To each of the reaction tubes you should do the following:

- a) About 1 min before the timepoint, add TMB to the correct reaction tube and mix.
- b) At the appropriate timepoint, remove the *correct* amount of enzyme from the heated tube, and add it to the reaction tube. Mix the tube!
- c) Zero the spectrophotometer with this tube.
- d) Add the correct amount of H₂O₂ to the tube (mix well).
- e) **Read A₆₅₅ at 15 second intervals** as you would for the standard assay, recording data on Enzyme Data Sheet #4.
- f) Determine the rate of reaction for each assay, and plot the data as reaction rate vs time at 70°C (i. e., the time you pre-incubated the extract at 70°C)

Enzyme Data Sheet #4

NAME: _____ LAB SEC. _____ DATE: _____ PAGE ___ of ___

THERMAL INACTIVATION TEMPERATURE USED: XX°C = _____

TISSUE: _____
g/300ml: _____

TUBE NUMBER

	1	2	3	4	5	6	7	8
dist. water (ml)	_____	_____	_____	_____	_____	_____	_____	_____
1.0 M citrate, pH 6.0 (μl)	_____	_____	_____	_____	_____	_____	_____	_____
0.5% TMB (μl)	_____	_____	_____	_____	_____	_____	_____	_____
crude enzyme (μl)	_____	_____	_____	_____	_____	_____	_____	_____
H ₂ O ₂ (μl)	_____	_____	_____	_____	_____	_____	_____	_____
Time tube was placed at XX°C	_____	_____	_____	_____	_____	_____	_____	_____
Time sample was removed for assay	_____	_____	_____	_____	_____	_____	_____	_____
Time (min) at XX°C.	_____	_____	_____	_____	_____	_____	_____	_____

ABSORBANCE

time (min) 0.25	_____	_____	_____	_____	_____	_____	_____	_____
0.50	_____	_____	_____	_____	_____	_____	_____	_____
0.75	_____	_____	_____	_____	_____	_____	_____	_____
1.00	_____	_____	_____	_____	_____	_____	_____	_____
1.25	_____	_____	_____	_____	_____	_____	_____	_____
1.50	_____	_____	_____	_____	_____	_____	_____	_____
1.75	_____	_____	_____	_____	_____	_____	_____	_____
2.00	_____	_____	_____	_____	_____	_____	_____	_____

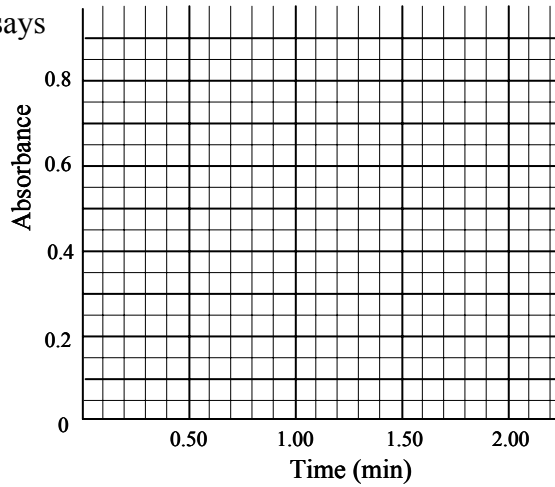
LINEAR REGRESSION VALUES

(Y-intercept) B	_____	_____	_____	_____	_____	_____	_____	_____
(Slope = V) A	_____	_____	_____	_____	_____	_____	_____	_____
r ²	_____	_____	_____	_____	_____	_____	_____	_____

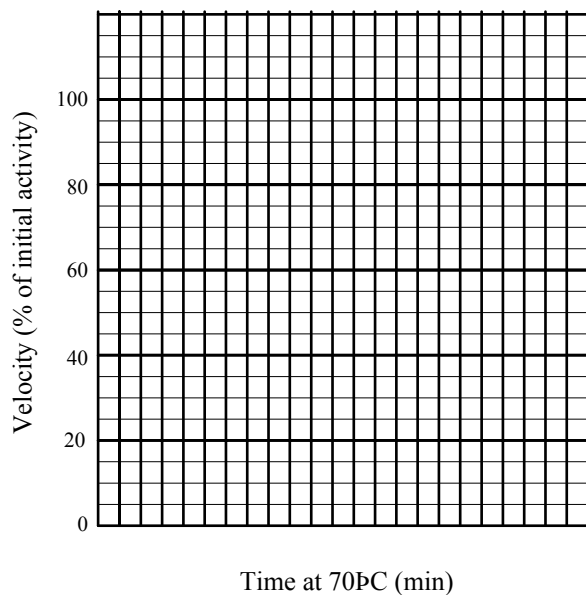
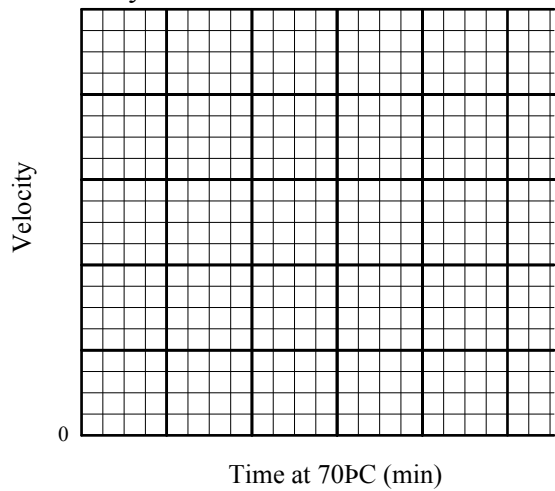
Enzyme Data Sheet #5

NAME: _____ LAB SEC. _____ DATE: _____ PAGE ___ of _____

Graph of Enzyme Assays



Graphs of Enzyme Activity as a Function of Time at 70°C



To compare two enzymes from different sources, it is easiest to graph the "% of initial activity"—divide each velocity measurement by the "zero time" measurement, and express the result as a percent.

Experiment 4. What is the optimum temperature for the peroxidase reaction?

Many enzymes have a temperature optimum that is similar to the optimum growth temperature for the organism. Does peroxidase? If it does, it may be a result of adaptation by natural selection. But, what if it does not? This experiment will give you the data from which to argue for, or against, the idea that peroxidase has undergone direct selection for the optimum reaction temperature.

This experimental approach would be simplified if you and the spectrophotometer could be placed in a room with the appropriate temperature; however, ice-bath temperatures and 55°C would both be uncomfortable—to say the least! So, what you will do is to perform the reaction at the temperature of interest for exactly two minutes, then, very quickly, move the tube to the spectrophotometer to determine the absorbance at 655nm for a single 2-minute time point.

This method has a technical difficulty, of course: it takes time to move the tube from the water bath to the spectrophotometer. Therefore, the spectrophotometer should be as close to the water bath as possible, and you should work quickly. To neutralize as much as possible the effects of variation due to this constraint, collect the data from all groups in your section, and determine the average reaction rate for each temperature.

1. Prepare six reaction tubes, each containing the correct volumes of **distilled water** and **Citrate Buffer**, as you did last week. Use an H₂O₂ concentration that is sufficiently high to be reliable (probably, 100µl of the H₂O₂ working solution will be appropriate—but the enzyme you chose to examine may have different properties and require a higher concentration). Fill out the upper part of **Enzyme Data Sheet #6** (p. D- 31) before you come to lab; when you do the experiment, record your data on Enzyme Data Sheet #6.
2. Working with one tube at a time, do the following:
 - a. Label the tube with the temperature being used
 - b. **Add the TMB**, mix the tube, and put the tube in the appropriate water bath. *Check the temperature of the water with a thermometer, and record this temperature on the data sheet* ; allow 10 minutes for the contents of the tube to come to equilibrium at the desired temperature.
 - c. When the contents of the tube reach the correct temperature:
 - 1) **add the enzyme**, mix well; quickly read A₆₅₅—this gives you a 0.00 min reading. You do not need to "zero" the machine as long as the zero knob remains untouched throughout the experiment.
 - 2) **add the H₂O₂**, mix well (and quickly), **immediately** return to the correct water bath.
 - 3) allow the reaction mixture to remain in the water bath as long as possible, but you *must* have the tube in the spectrophotometer and ready to read A₆₅₅ at *exactly* the 2:00 min time point

Be sure to wipe off the tube with lab tissue before you put it in the spectrophotometer!

- 4) calculate the velocity of the reaction by determining the change in the absorbance ($\Delta A_{655} = A_{655} \text{ at } 2 \text{ min} - A_{655} \text{ at } 0 \text{ min}$) and dividing this by 2 min to give you $\Delta A_{655} / \text{min}$ which is the velocity.

Enzymes

Enzyme Data Sheet #6

NAME: _____ LAB SEC. _____ DATE: _____

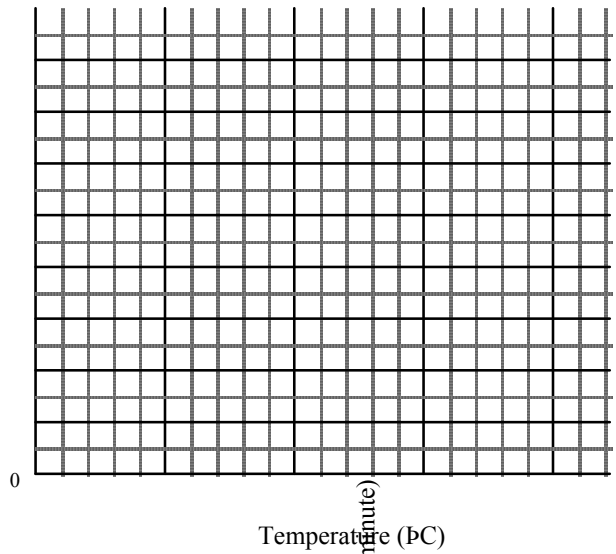
EXPERIMENT: _____ TISSUE: _____ g/300ml used in extract: _____

TUBE NUMBER

	1	2	3	4	5	6	7	8
dist. water (ml)	_____	_____	_____	_____	_____	_____	_____	_____
1.0 M citrate, pH 6.0 (μl)	_____	_____	_____	_____	_____	_____	_____	_____
0.5% TMB (μl)	_____	_____	_____	_____	_____	_____	_____	_____
crude enzyme (μl)	_____	_____	_____	_____	_____	_____	_____	_____
H ₂ O ₂ (μl)	_____	_____	_____	_____	_____	_____	_____	_____
Temp (°C)	_____	_____	_____	_____	_____	_____	_____	_____

ABSORBANCE

0.00 minutes	_____	_____	_____	_____	_____	_____	_____	_____
2.00 minutes	_____	_____	_____	_____	_____	_____	_____	_____
² A ₆₅₅	_____	_____	_____	_____	_____	_____	_____	_____
² A ₆₅₅ /minute	_____	_____	_____	_____	_____	_____	_____	_____



III – POST LABORATORY DISCUSSION

Once again, first consider each experiment individually, before looking at all of the results together.

Before the post-laboratory discussion, you need to review all of your data, plot all of the graphs called for in the Lab Manual, and calculate values such as the K_m .

Be sure that you know what your section chose as its question to be addressed or hypothesis to be tested in these experiments. If you are unsure about this, get it clarified during the post-laboratory discussion. This will make writing the minireport much easier.

It might also be helpful to do some background research on the plants that were the sources of the peroxidases you studied. You might want to check in the library or on the Web for information on where the plants grow, what their growth habit is like, and what the temperature range typically is during their growing season.

Experiment 1. The standard assay and controls (Enzyme Data Sheet #1)

- a) Did your reaction depend on the presence of peroxidase enzyme?
- b) Did your reaction depend on TMB?
- c) These two experiments are called “controls.” What do they control for?

Think about the reaction that peroxidase catalyzes, and about the role of the enzyme, of H_2O_2 , and of TMB. Make sure you know what’s going on inside the tube! Then, think about things that could interfere with what you are trying to measure, like contaminants in the water or in the extract that could react chemically with the TMB in the absence of the enzyme, or that

could cause something *other than* TMB to turn blue. Do these controls suggest that you are

Experiment 2. How does changing the substrate concentration affect the reaction rate? (Enzyme Data Sheets #2 and #3)

Simple enzymes

For simplicity, we will assume that the enzyme you analyzed was “well behaved” and did not show unusual behavior. [see below if your data were more complex.] For a simple enzyme, the reaction displays an increasing reaction rate as the concentration of the substrate increases, but there seems to be an upper limit. The rate stops increasing at very high concentrations of the substrate. You should be able to explain why this happens.

c) For you to know what to do next, you need to understand the idea of K_m . Do you? Your data should give you some help. If an enzyme doesn't work very well except when given a very high concentration of substrate, it has (by definition) a high K_m .

d) How similar were the enzymes you studied? Were their K_m 's similar or different?

e) From these results, how much H_2O_2 did you conclude was enough for the rest of the experiments on your enzymes? Why?

Remember that this is an important experiment, for which the *only* “right answer” comes from the actual data.

Complex enzymes

Some enzyme preparations show complex behavior. The simplest is “substrate inhibition,” in which the reaction rate *decreases* when high concentrations of substrate are used. In this case, you can still determine K_m from the double-reciprocal plot, using the data from the low concentrations of substrate. Other preparations appear to contain a mixture of two or more enzymes, each with its own complex behavior. For these, you cannot determine the K_m .

Here, again, this is an extremely important experiment, for which the *only* “right answer” comes from the actual data. For the next set of experiments, you must use the substrate concentration that gives the “best” reaction, regardless of the actual K_m .

Experiment 3. How stable is peroxidase to thermal denaturation? (Enzyme Data Sheets #4 and #5)

a) Do you understand the theory? At the molecular level, what happens to an enzyme when it is denatured? Why does this usually inactivate the enzyme, that is, make it unable to catalyze a chemical reaction? In the lab, we used heat to denature our peroxidases. Why does heat denature enzymes/proteins?

b) What do your data show? Did your enzyme show a steady decrease in activity as it sat in the hot water bath, or was the response more complex? Many enzyme denaturation curves give straight lines when graphed with logarithmic scales on both axes, and a “power” curve-fit. Try this with your data. Be prepared to offer possible explanations for what you observed.

d) How did the sensitivity to thermal denaturation of your section's two enzymes compare? Was the result consistent with your expectations? Be prepared to speculate on what might be the reasons for any unexpected results.

Reasoning from the data rather than from “what you're supposed to get:” your data tell you how stable these enzymes are, regardless of what your predictions may have been. One might be more stable than the other, or maybe they were the same. However it came out, use your data to derive your answers.

Experiment 4. What is the optimum temperature for the peroxidase reaction? (Enzyme Data Sheet #6)

First, deal with the “technical” aspects of the work:

a) As you may recall, the Lab Manual suggested that your section pool all of its data from this experiment to try to minimize variations in the results caused by running the reactions at one temperature and then measuring the absorbance at room temperature. Be prepared to share your data ($\Delta A_{655}/\text{min}$ for each temperature) with your section, and to record others’ data.

b) Were there unexpected results for this experiment—such as two peaks on the graph of activity *vs* temperature? If so, estimate the accuracy/reproducibility of each measurement, and consider drawing a best-fit curve without using the mathematical functions of Cricket Graph (that is, by eye). Some points may be above or below the line, due to experimental variation.

Next, think about what the data tell you.

c) What was the optimum temperature for the reaction for each enzyme?

d) What is the optimum growth temperature for the plant you examined (at least, to the best of your knowledge or according to your best ability to predict).

Reasoning from the data rather than from “what you’re supposed to get:” your data might not match the growth temperatures of the plants. Why not? Can you explain the difference by “technical error” in the way you did the reactions (such as using the 30° water bath when you thought you were using the 50° water bath), or is the result actually valid? The result is probably valid, since you are using data from many replications done by each group in your section. You have no choice but to derive your conclusions based on your data, regardless of your preconceived notions.

For these experiments, there are two main comparisons you must do: (1) compare the thermal stability and temperature optimum of each peroxidase to the growth conditions of the plants, and (2) compare peroxidase X to peroxidase Y, to determine whether the two enzymes show the kinds of differences you predicted.

If you expected to see that the enzymes show “perfect adaptation” to the environment of the plant, ask whether your data support this hypothesis. Does the plant grow at the temperature at which peroxidase shows its optimum activity? If your data do not match your predictions, think about all the processes that can occur evolutionarily. There is an evolutionary history to your plants and their peroxidases. What different scenarios can you propose that might have led to the situation your data reveal?

Minireport

The experiments you have done the last few weeks are actually part of a single research project, and should be reported together. The substrate concentration curves were required to determine how best to do the next set of experiments. How best can you develop a lab report on these experiments?

The main question we asked about peroxidase was whether enzymes show thermal adaptation. Your experimental approach was to perform a comparison among samples, which also gives you information on the characteristics of the enzymes (e.g. K_m) and concentration in the starting tissue (amount of enzyme activity per gram of tissue). For the sake of example here, let’s consider the question, “How similar is peroxidase from X compared to peroxidase from Y?”

What experimental approach did you use to address this question? You performed a series of enzyme assays under different conditions (substrate concentration, temperature, etc.) for each enzyme, then compared the findings. [This may seem trivial, but it is necessary to mention this in a scientific report. Other approaches are possible, but you did not use them. You did not determine the amino acid sequence of the proteins, or solve their 3-dimensional structures. You focussed on the kinetic parameters of the enzymes.]

Did you develop an hypothesis before starting the assays? Perhaps you did—you may have hypothesized that the temperature optimum of enzymes approximates the growth temperature of the organisms that produce them. You may then have predicted that each species' peroxidase should exhibit a temperature optimum approximately the same as the optimum growth temperature. Alternatively, you may not have stated an hypothesis beforehand; it is not required in all scientific approaches.

For the Purpose section of the minireport, it is sufficient to state these things in a simplified format: your question and/or hypothesis and the approach used to address or test it.

The Methods section: For a paper in the biochemical tradition, you would say “experiments were performed as described (Bonner, 2001)” and cite the Lab Manual in the references section. You would also need to state how much of each tissue was ground to make the extract, and mention any other things that are unique to your plant tissues.

For a paper in the organismal tradition, you would explain how you approached the problem, identify the experiments that you did, and describe the relevant variables and other aspects of the experiment that are critical to understanding the nature of the work. You would tell your reader what variable you varied in each experiment (for example, time, or the temperature), what characteristic you measured (for example, absorbance, or the rate of reaction), and why you did this (i.e., what this measurement has to do with the thing you want to study, such as the progress of a biochemical reaction or the activity of an enzyme). You would list the actual temperatures that you used in Experiments 3 and 4.

Your AI may prefer one or the other format, and recommend that you use it.

The Results section: For a paper in the biochemical tradition, you need to introduce each experiment, and describe enough of the logic that the reader can follow what is important. (Some, but not all, of what would have been in Methods for a paper in the organismal tradition). Then, you would describe the results. For a paper in the organismal tradition, you would simply identify each experiment before describing the results. In either case, you have the same 4 experiments, regardless of how much introductory text you might use.

For each experiment (controls, effect of substrate concentration, test of thermal denaturation, and determination of the temperature optimum), you use a separate paragraph or section. For each experiment, direct the reader to the figures that are relevant to it—here, the graphs you prepared from your data. (Since you have used Cricket Graph, print out clean copies for the lab report. The key is to have clean, easy-to-read figures that communicate your results to the reader.) Then point out the important features of the data: for example, that the activity of peroxidase was highest at temperature XX, and lower at temperatures above or below this. This may be obvious from the figures, but it is essential to point it out in words also, because the figure and the text are different modes of communication. Once you have led the reader through the data, stop. Interpretation is for later.

In the Interpretation section, look at the results from each experiment separately, and explain to the reader what the data tell you about each enzyme. Explain how the data force you to suggest that the enzymes differ in some respects, or are similar in some respects (regardless of what you may have predicted!).

Enzymes

In the Conclusions section, try to consider the four experiments as a whole. What do your results indicate about the enzymes you studied? How would you answer the question “How similar is the peroxidase from X compared to the peroxidase from Y?” Did you accomplish your goals or answer your question or support or refute your hypothesis? If you made predictions, based on an hypothesis, but they were not borne out by the data, what changes might you need to make to your hypothesis? For example, if peroxidase does not show the characteristics you expected for adaptation to the thermal environment, offer alternate suggestions for its evolutionary history. You could ramble forever if you tried, but for a minireport, you should identify the most important, and most valid, conclusions. Practice being concise. It might be hard at first, but it is a very valuable skill to learn. If you need help, ask your AI and visit the professor during office hours.

APPENDIX A

A Preliminary Exercise on the Value of Linear Regression

Introduction:

The purpose of this brief exercise is to examine:

- (1) how velocity (or rate) calculations are made for a process that is assumed to have a uniform (or constant) velocity;
- (2) how to address the question of whether the calculations are reasonable and useful.
- (3) the use of linear regression for various data sets.

The reason for considering velocity calculations now is to help you prepare for the lab exercise that deals with "Enzyme Reactions" (and by that we simply mean studies involving changes in the velocity of an enzyme reaction). For that lab, you need to be quite familiar with velocity calculations.

In this exercise, you will be working with situations that probably seem more obvious intuitively than enzyme reactions, but take the examples seriously -- They have been selected carefully to illustrate a number of important points. Each of the three examples involves someone you might know, an L113 student headed home for the upcoming vacation and he or she must get to the Indianapolis airport in 60 minutes. Before we consider any concepts, let's look at the first example.

Example #1:

1. Osgood McDirk has borrowed his roommate's car and, to his dismay, the accelerator "sticks" on the way out of Bloomington. Fortunately, he was already up to 55 miles/hr (on Walnut!) and all of the stop lights are just right for a non-stop trip. Osgood runs out of gas 5 miles this side of I-465 and hitches a ride on to the airport with another L113 student, Rip Unser, who happens to come by a few minutes later. The roommate's car is towed to Indianapolis two days later.
2. If you had observed the progress of the car, you might have collected the following data from a few check-points along the way:

<u>Lapsed Time (min)</u>	<u>Distance Traveled (miles)</u>
11	10
20	18
27	25
44	40
2950	55 (remember, this is for the car)

3. Notice that the data are collected in pairs: a time and the corresponding distance measurement. In this case, you have 5 pairs of numbers (if you count the last pair).
4. Plot these data using Cricket Graph, or on the graph on page D-41, with time (min) along the X-axis and distance (miles) along the Y-axis.

Enzymes

5. As you can see, until the car ran out of gas, the velocity was constant. That is, it took 11 min to cover every 10-mile segment of the trip (again, except the last 15 miles -- which we will ignore for a while). As a result, the data pairs plot as a straight line and that line is a visual demonstration that the velocity was constant over the first 44 min of the trip.
6. For a process with a constant rate (as evidenced by the straight line), the velocity (or rate) is the slope of the line (provided that you use time on the X-axis) -- where the **slope** is the amount of change in y (here distance in miles) per unit change in x (here time in min). That is, the slope will have the units: miles/min.

If you measure some increment in time and then divide that into the corresponding change in distance, you would come out with about 0.9 miles/min. (Clearly, you must stick with the linear part of the curve for this to be true). In this instance, the correct line was easy to draw and the slope could be measured rather accurately.

7. However, this is not always so -- points don't always fall exactly on a straight line and then it is hard to select the "correct" line. Also, it is hard to judge just how well the points actually fit a straight line even though you have determined the one that is presumably "correct".
8. Fortunately, there is a procedure to do these tasks -- a statistical method known as **linear regression**. It is based on the fact that sets of data pairs that plot as a straight line can always be expressed in the form:

$$y = Ax + B$$

where x is the member of the data pair on the X-axis (minutes) and y is the other member of the data pair (miles) which is plotted on the Y-axis.

The constant A is the slope (which tells how much y changes per unit change in x); in our case A will come out in terms of miles/min and is the velocity (V).

The constant B is the "Y-intercept" and tells what the value of y is when x is 0. In this case, since no miles were traveled until the timing began, B should be 0 (or something very close).

Also, the linear regression calculations provide a measure of how well the data points actually fit the best straight line -- by calculating the value for r^2 , where r = the correlation coefficient. For sets of data pairs that match a straight line perfectly $r^2 = 1$; and for sets of data pairs that have no relationship to a straight line, $r^2 = 0$. For now, let's say that any number above 0.95 is OK, numbers above 0.98 are good and a 1.00 is almost too good to be true.

9. For the first four data pairs of Osgood's trip, determine the values for A, B, and r^2 using Cricket Graph on the Macintosh for linear regression.
10. For the linear portion of Osgood's trip, the calculated values are:
Y-intercept (the "B" value) = _____
V = velocity (the "A" value) = _____ miles/min
 r^2 = (a measure of validity) = _____

Write the equation for the line (in the form $y = Ax + B$, but fill in your own values for A and B):

How does the calculated velocity compare with the one that you estimated from the line that you drew earlier? _____

11. Do the same calculation again, including the last (5th) set of data points:

$$\begin{aligned} \text{Y-intercept} &= \underline{\hspace{2cm}} \\ \text{Velocity (V)} &= \underline{\hspace{2cm}} \text{ miles/min} \\ r^2 &= \underline{\hspace{2cm}} \end{aligned}$$

Clearly, the five pairs of data points do not fit a straight line--even the best one that can be calculated.

Velocity calculations by linear regression are valid only for the linear portion of a process (when a single velocity is involved).

12. You might also note that it is not really valid in most cases to calculate velocity from only the first and last data pairs. You really need several pairs to establish that the relationship is linear and that a single velocity is involved.

Example #2:

- After passing the Oliver Winery, Rip Unser decided to "burn the carbon out" of his engine. The Indiana State trooper who usually parks about ten miles beyond there took a dim view of this approach to automotive maintenance, so Rip was delayed for several minutes on this side of Martinsville. However, he managed to get to the Indy airport in time and he did pick up Osgood along the way.
- If you had observed the progress of Rip's car, you might have collected the following data:

<u>Lapsed Time (min)</u>	<u>Distance Traveled (miles)</u>
10	10
15	18
34	25
47	40
60	55

What was Rip's velocity?

- Be sure to plot the actual points, then draw in the line that best fits those points (as determined by linear regression).

Is it reasonable to plot those points as a straight line?

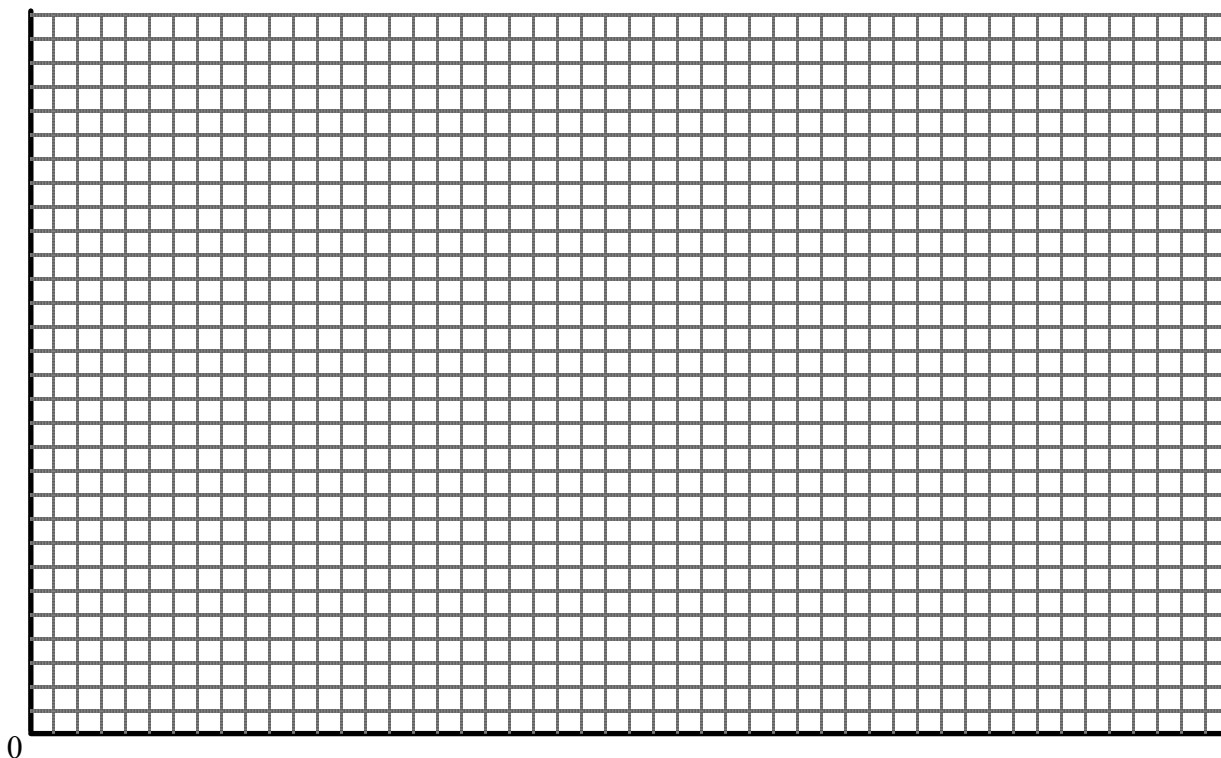
Example #3:

1. Following her usual practice, Holly Golightly (a typical L113 student) rented a helicopter for the trip to the Indy airport. Since she was early, she decided to stop at her favorite cafeteria in Martinsville for bowl of soup.
2. If you had followed the progress of Holly's helicopter, you might have collected the following data:

<u>Lapsed Time (min)</u>	<u>Distance Traveled (miles)</u>
3	10 (not bad!)
6	18
8	25
54	40
60	55

3. What was Holly's velocity?
4. Be sure to plot the actual points, then draw in the line that best fits the data (as determined by linear regression).
It is reasonable to plot these points as a straight line?

Here is a set of graph axes if you want to make a plot of these data before turning to the computer:



APPENDIX B -- SOLUTIONS**1. H₂O₂ Working Solution** (3×10^{-5} M H₂O₂; 20 ml)

To 20 ml cold distilled water add 60 μ l of 30% hydrogen peroxide.

Store at 4°C. Prepare fresh daily.

The 30% Hydrogen Peroxide stock should be reasonably fresh (purchased within the last few months).

2. 0.5% TMB Working Solution (about 0.016 M; 200 ml)

To 50.3 ml cold dist. water, **add**:

16.7 ml acetic acid, chill
then **add**:

1.0 g of 3,3',5,5'-Tetramethylbenzidine Dihydrochloride (Sigma T 8768)
mix thoroughly and then **add**:

133.0 ml cold, dist. water. Mix

Store at 4°C in a foil covered container. This stock will keep for several weeks (good until it turns blue/green).

3. 1.0 M Citrate Buffer, pH 6.0 (1.0 M Na Citrate; 500 ml)

To 400 ml cold dist. water, **add** (with stirring, in a Pyrex dish filled with ice):

105.1g Citric acid monohydrate (H₃C₆H₅O₇·H₂O), FW= 210.14

61.0g NaOH pellets

Continue stirring.

After the temperature drops below 30°C, adjust to pH 6.0 with a few drops of 10 N NaOH.

Adjust the volume to 500 ml with dist. water.

Filter sterilize, using a 500-ml disposable Nalgene device (pore size = 0.2 μ).

Store in the bottom section of the Nalgene unit and cover with the enclosed sterile cap.
Keep at 4°C.

With reasonable care and infrequent opening, this solution should remain uncontaminated for weeks (or even months). The amount suggested is enough for 8,300 assays (about 40 lab sections). 25 ml/lab would be more than 2x that needed. **NOTE**: Considerable heat is generated when that amount of NaOH dissolves in the volume of solution used -- enough to boil the solution if the starting point is room temperature. Be sure to start with cold water and stir mixture in an ice bath while the NaOH is dissolving.

APPENDIX C-- Preparation of the Crude Enzyme Extract

1. To a chilled, clean Waring blender container **add**:
300 ml ice-cold distilled water
1.5 g peeled, inner portion of the turnip root (add as thin slices)
2. Blend at full speed for 3 min (very few small pieces should be seen)
3. Filter through miracloth (using a chilled funnel) into a 500-ml Erlenmeyer flask on ice.
4. Store the extract at 4°C. (It is stable for days.)

Test Activity

The reaction is most convenient to assay if the concentration of enzyme is such that it gives about $\Delta 0.4A_{655} / 2$ minutes. This is fast enough to be efficient, but slow enough that we can work with it. Adjust the concentration as follows:

Perform a standard assay on the extract. If the reaction gives $> \Delta 0.4 A_{655} / 2$ minutes, dilute accordingly. If the reaction gives $< \Delta 0.2 A_{655} / 2$ minutes, prepare a new extract using an appropriately greater amount of fresh weight of material.

NOTE: The enzyme extracts can be prepared from other plant and animal tissue in the same way. The amount of tissue needed may vary, however; one might need a fraction of a gram (horseradish) or several grams (iceberg lettuce).