Date: _____ Name and Period: _____

AP Biology Lab 2
ENZYME CATALYSIS

OVERVIEW

In this lab you will:

1. observe the conversion of hydrogen peroxide (H_2O_2) to water and oxygen gas by the enzyme catalase, and
2. measure the amount of oxygen generated and calculate the rate of the enzyme-catalyzed reaction.

OBJECTIVES

Before doing this lab you should understand:

- the general functions and activities of enzymes;
- the relationship between the structure and function of enzymes;
- the concept of initial reaction rates of enzymes;
- how the concept of free energy relates to enzyme activity;
- that changes in temperature, pH, enzyme concentration, and substrate concentration can affect the initial reaction rates of enzyme-catalyzed reactions; and
- catalyst, catalysis, and catalase.

After doing this lab you should be able to:

- measure the effects of changes in temperature, pH, enzyme concentration, and substrate concentration on reaction rates of enzyme-catalyzed reaction in a controlled experiment; and
- explain how environmental factors affect the rate of enzyme-catalyzed reactions.

INTRODUCTION

In general, **enzymes** are proteins produced by living cells; the act as catalysts in biochemical reactions. A **catalyst** affects the rate of a chemical reaction. One consequence of enzyme activity is that cells can carry out complex chemical activities at relatively low temperatures.

In an enzyme-catalyzed reaction, the substance to be acted upon, the **substrate (S)**, binds reversibly to the active site of the **enzyme (E)**. One result of this temporary union is a reduction in the energy required to activate the reaction of the substrate molecule so that the **products (P)** of the reaction are formed. In summary:

E + S -> ES -> E + P

Note that the enzyme is not changed in the reaction and can be recycled to break down additional substrate molecules. Each enzyme is specific for a particular reaction because its amino acid sequence is unique and causes it to have a unique three-dimensional structure. The **active site** is the portion of the enzyme that interacts with the substrate, so that any substance that blocks or changes the shape of the active site affects the activity of the enzyme. A description of several ways enzyme action may be affected follows:

1. **Salt Concentration.** If the salt concentration is close to zero, the charged amino acid side chains of the enzyme molecules will attract each other. The enzyme will denature and form an inactive precipitate. If, on the other hand, the salt concentration is very high, normal interaction of charged groups will be blocked, new interactions will occur, and again the enzyme will precipitate. An intermediate salt concentration, such as that of human blood (0.9%) or cytoplasm, is the optimum for many enzymes.

2. **pH.** pH is a logarithmic scale that measures the acidity, or H^+ concentration, in a solution. The scale runs from 0 to 14 with 0 being highest in acidity and 14 lowest. When the pH is in the range of 0-7, a solution is said to be acidic; if the pH is around 7, the solution is neutral; and if the pH is in the range of 7-14, the solution is basic. Amino acid side chains contain groups, such as –COOH and $-NH_2$, that readily gain or lose H^+ ions. As the pH is lowered an enzyme will tend to gain H+ ions, and eventually enough side chains will be affected so that the enzyme's shape is disrupted. Likewise, as the pH is raised, the enzyme will lose H^+ ions and eventually lose its active shape. Many enzymes perform optimally in the neutral pH range and are denatured at either an extremely high or low pH. Some enzymes, such as pepsin, which acts in the human stomach where the pH is very low, have a low pH optimum.

3. **Temperature.** Generally, chemical reactions speed up as the temperature is raised. As the temperature increases, more of the reacting molecules have enough kinetic energy to undergo the reaction. Since enzymes are catalysts for chemical reactions, enzyme reactions also tend to go faster with increasing temperature. However, if the temperature of an enzyme-catalyzed reaction is raised still further, a **temperature optimum** is reached; above this value the kinetic energy of the enzyme and water molecules is so great that the conformation of the enzyme molecules is disrupted. The positive effect of speeding up the reaction is now more than offset by the negative effect of changing the conformation of more and more enzyme molecules. Many proteins are denatured by temperatures around 40-50^oC, but some are still active at 70-80^oC, and a few even withstand boiling.

4. **Activations and Inhibitors.** Many molecules other than the substrate may interact with an enzyme. If such a molecule increases the rate of the reaction it is an **activator**, and if it decreases the reaction it is an **inhibitor**. These molecules can regulate how fast the enzyme acts. Any substance that tends to unfold the enzyme, such as an organic solvent or detergent, will act as an inhibitor. Some inhibitors act by reducing the –S-S- bridges that stabilize the enzyme's structure. Many inhibitors act by reacting with side chains in or near the active site to change its shape or block it. Many well-known poisons, such as potassium cyanide and curare, are enzyme inhibitors that interfere with the active site of critical enzymes.

The enzyme used in this lab, catalase, has four polypeptide chains, each composed of more than 500 amino acids. This enzyme is ubiquitous in aerobic organisms. One function of catalase within cells is to prevent the accumulation of toxic levels of hydrogen peroxide formed as a byproduct of metabolic processes. Catalase might also take part in some of the many oxidation reactions that occur in all cells.

The primary reaction catalyzed by catalase is the decomposition of H_2O_2 to form water and oxygen:

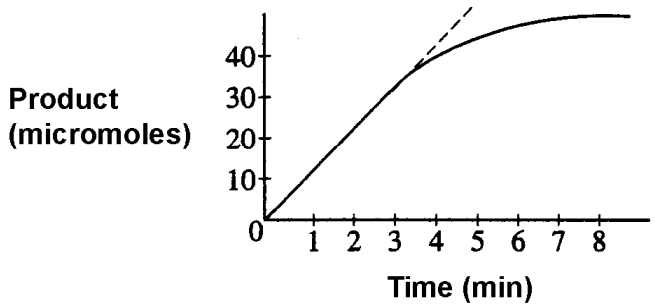
$$2\ H_2O_2 \rightarrow\ 2H_2O_2 + H_2O_2\,(\text{gas})$$

In the absence of catalase, this reaction occurs spontaneously but very slowly. Catalase speeds up the reaction considerably. In this experiment, a rate for this reaction will be determined.

Much can be learned about enzymes by studying the kinetics (particularly the changes in rate) of enzyme-catalyzed reactions. For example, it is possible to measure the amount of product formed, or the amount of substrate used, from the moment the reactants are brought together until the reaction has stopped.

If the amount of product formed is measured at regular intervals and this quantity is plotted on a graph, a curve like the one in Figure 2.1 is obtained.

Figure 2.1



Study the solid line on the graph of this reaction. At time 0 there is no product. After 20 seconds, 5 micromoles (μmoles) have been formed; after 1 minute, 10 μmoles; after 2 minutes, 20 μmoles. The rate of this reaction could be given at 10 μmoles of product per minute for this initial period. Note, however, that by the third and fourth minutes, only about 5 additional μmoles of product have been formed. During the first three minutes, the rate is constant. From the third minute through the eighth minute, the rate is changing; it is slowing down. For each successive minute after the first three minutes, the amount of product formed in that interval is less than in the preceding minute. From the seventh minute onward, the reaction rate is very slow.

In the comparison of the kinetics of pf one reaction with another, a common reference point is needed. For example, suppose you wanted to compare the effectiveness of catalase obtained from potato with that of catalase obtained from liver. It is best to compare the reactions when the rates are constant. In the first few minutes of an enzymatic reaction such as this, the number of substrate molecules is usually so large compared with the number of enzyme molecules that changing the substrate concentration dies not (for a short period at least) affect the number of successful collisions between substrate and enzyme. During this early period, the enzyme is acting on substrate molecules at a nearly constant rate. The slope of the graph line during this early period is called the **initial rate** of the reaction. The initial rate of any enzyme-catalyzed reaction is determined by the characteristics of the enzyme molecule. It is always the same for any enzyme and its substrate at a given temperature and pH. This also assumes that the substrate is present in excess.

The rate of the reaction is the slope of the linear portion of the curve. To determine a rate, pick any two points on the straight-line portion of the curve. Divide the difference in the amount of product formed between these two points by the difference in time between them. The result will be the rate of the reaction, which if properly calculated, can be expressed as μmoles product/sec. The rate, then, is:

$$\frac{\mu moles_2 - \mu moles_1}{t_2 - t_1}$$

or from the graph,

$$\frac{\Delta y}{\Delta x}$$

In the illustration of Figure 2.1, the rate between two and three minutes is calculated:

$$\frac{30 - 20}{180 - 120} = \frac{10}{60} = 0.17 \text{ μmoles/sec}$$

The rate of the chemical reaction may be studied in a number of ways, including the following:

1. measuring the rate of disappearance of substrate (in this example H_2O_2);

2. measuring the rate of appearance of product (in this case, O_2, which is given off as a gas);
3. measuring the heat released or absorbed in the reaction.

General Procedure

In this experiment the disappearance of the substrate, H_2O_2 , is measured as follows (see Figure 2.2):

1. A purified catalase extract is mixed with substrate (H_2O_2) in a beaker. The enzyme catalyzes the conversion of H_2O_2 to H_2O and O_2 (gas).

2. Before all the H_2O_2 is converted to H_2O and O_2, the reaction is stopped by adding sulfuric acid (H_2SO_4). The H_2SO_4 lowers the pH, denatures the enzyme, and thereby stops the enzyme's catalytic activity.

3. After the reaction is stopped, the amount of substrate (H_2O_2) remaining in the beaker is measured. To assay (measure) this quantity, potassium permanganate is used. Potassium permanganate ($KMnO_4$) in the presence of H_2O_2 and H_2SO_4 reacts as follows.

$$\mathbf{5H_2O_2 + 2KMnO_4 + 3H_2SO_4 \rightarrow K_2SO_4 + 2MnSO_4 + 8H_2O + 5O_2}$$

Note that H_2O_2 is a reactant for this reaction. Once all the H_2O_2 has reacted, any more $KMnO_4$ added will be in excess and will not be decomposed. The addition of excess $KMnO_4$ causes the solution to have a permanent pink or brown color. Therefore, the amount of H_2O_2 remaining is determined by adding $KMnO_4$ until the whole mixture stays a faint pink or brown, permanently. Add no more $KMnO_4$ after this point. The amount of $KMnO_4$ added is a proportional measure of the amount of H_2O_2 remaining (2 molecules $KMnO_4$ of reacts with 5 molecules H_2O_2 of as shown in the equation).

Figure 2.2: The General Procedure

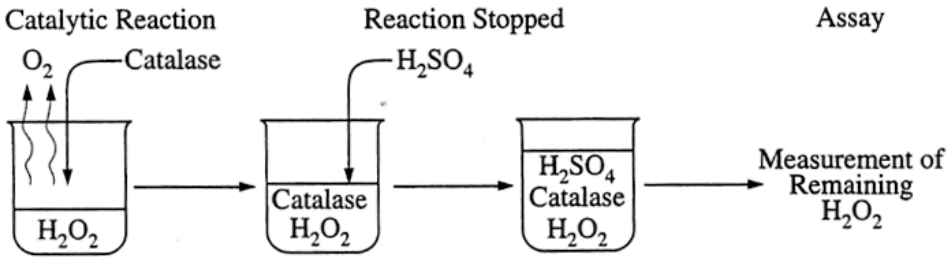
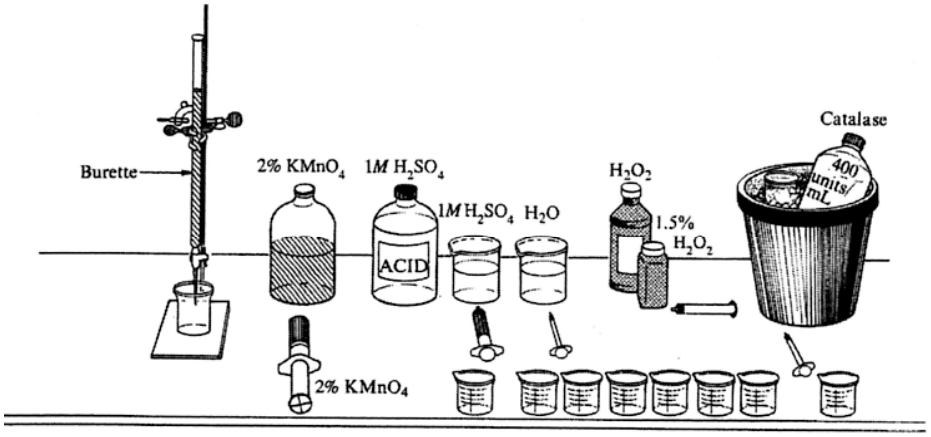


Figure 2.3: The Apparatus and Materials



EXERCISE 2A: Test of Catalase Activity

Procedure

1. To observe the reaction to be studied, transfer 10 mL of 1.5% (0.44M) H_2O_2 into a 50mL glass beaker and add 1 mL of the freshly made catalase solution. The bubbles coming from the reaction mixture are O_2, which results from the breakdown of by catalase. Be sure to keep the freshly made H_2O_2 by catalase solution on ice at all times.

a. What is the enzyme in this reaction?_____

b. What is the substrate in this reaction?

_____

c. What is the product in this reaction? _____

d. How could you show that the gas evolved is H_2O_2?

_____

2. To demonstrate the effect of boiling on enzymatic activity, transfer 5 ml of purified catalase extract to a test tube and place it in a boiling water bath for five minutes. Transfer 10 mL of 1.5% H_2O_2 into a 50 mL of the cooled, boiled catalase solution. How does the reaction compare to the one using the unboiled catalase.? Explain the reason for this difference.

3. To demonstrate the presence of catalase in living tissue, cut 1 cm^3 of potato or liver, macerate it and transfer it to a 50 mL glass beaker containing 10 mL of 1.5%. H_2O_2. What do you observe? What do you think would happen if the potato or liver was boiled before being added to the H_2O_2?

EXERCISE 2B: The Base Line Assay

To determine the amount of H_2O_2 initially present in a 1.5% solution, one needs to perform all the steps of the procedure without adding catalase (enzyme) to the reaction mixture. This amount is known as the baseline and is an index of the initial concentration H_2O_2 of in solution. In any series of experiments, a base line should be established first.

Procedure for Establishing a Base Line

1. Put 10 mL of 1.5% H_2O_2 into a clean glass beaker.

2. Add 1 ml of H_2O (instead of enzyme solution).

3. Add 10 mL of H_2SO_4 (1.0M) **Use extreme caution in handling reagents.** Your teacher will instruct you about the proper safety procedures for handling hazardous materials.

4. Mix well.

5. Remove a 5 mL sample. Place this 5 mL sample into another beaker and assay for the amount H_2O_2 of as follows. Place a beaker containing the sample over a piece of white paper. Use a burette, a syringe or a 5 mL pipette to add $KMnO_4$, a drop at a time, to the solution until a persistent pink or brown color is obtained.

Remember to gently swirl the solution after adding each drop. Check to be sure that you understand the calibrations on the burette or syringe (See Figure 2.4). Record your reading in the box below.

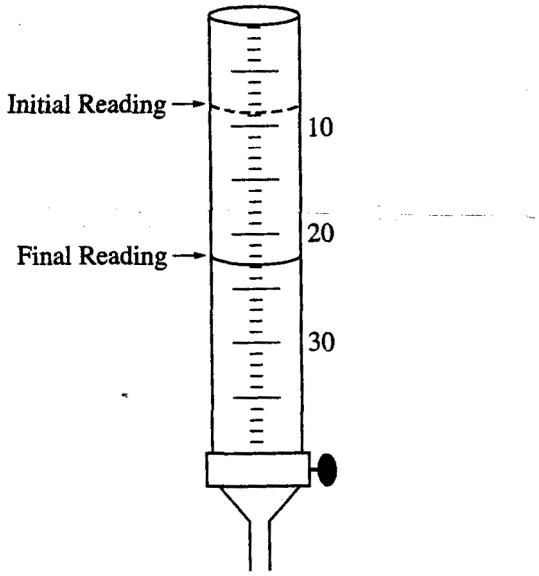
Base line calculation

Final reading of burette _____ mL

Initial reading of burette _____ mL

Base line (Final-Initial) _____mL $KMnO_4$

Figure 2.4: Proper Reading of a Burette



The base line assay value should be nearly the same for all groups. Compare your results to another team's before proceeding.

Remember the amount of $KMnO_4$ used is proportions to the amount of H_2O_2 that was in solution.

Note: Handle with $KMnO_4$ care. Avoid contact with skin and eyes.

EXERCISE 2C: The Uncatalyzed H_2O_2 Rate of Decomposition

To determine the rate of spontaneous conversion of H_2O_2 to H_2O and O_2 in an uncatalyzed reaction, put a small quantity of 1.5% H_2O_2 (about 15 ml) in a beaker. Store it uncovered at room temperature for approximately 24 hours. Repeat Steps 2-5 from Exercise 2B to determine the proportional amount of H_2O_2 remaining (for ease of calculation assume the 1 mL of $KMnO_4$ used in the titration represents the presence of 1 mL of H_2O_2 in the solution). Record your readings in the box below.

Uncatalyzed H_2O_2 decomposition

Final reading of burette _____ mL

Initial reading of burette _____ mL

Amount of $KMnO_4$ titrant _____mL

Amount of spontaneously decomposed (mL baseline – mL $KMnO_4$) _____ mL

What percent of the spontaneously decomposes in 24 hours? [(mL baseline – mL 24 hours)/ mL baseline] X 100 _____%

EXERCISE 2D: The Enzyme-Catalyzed H_2O_2 Rate of Decomposition

In this experiment you will determine the rate at which 1.5% H_2O_2solution decomposes when catalyzed by purified catalase extract. To do this, you should determine how much H_2O_2 has been consumed after 10, 30, 60, 90, 120, 180 and 360 seconds.

If a day or so has passed since you did Exercise 2B, you must reestablish the base line by determining the amount of present in your 1.5% H_2O_2 solution. Repeat the assay procedure (Steps 1-5) and record your results in the box below. The base line assay should be approximately the same value for all groups. Check with another team before proceeding.

Base line calculation

Final reading of burette _____ mL

Initial reading of burette _____ mL

Base line (Final-Initial) _____mL $KMnO_4$

Procedure for a Time-Course Determination

To determine the course of an enzymatic reaction, you will need to measure how much substrate is disappearing over times. You will measure the amount of substrate decomposed after 10, 30, 60, 90, 120, 180 and 360 seconds. To use lab time more efficiently, set up all of these at the same time and do them together. Stop each reaction at the proper time.

1. **10 seconds**
   - **a.** Put 10 mL of 1.5 % H_2O_2 in a clean 50 ml glass beaker.
   - **b.** Add 1 mL of catalase extract.
   - **c.** Swirl gently for 10 seconds.
   - **d.** At 10 seconds, add 10 mL of H_2SO_4 (1.0 M).

2. **30, 60, 90, 120, 180 and 360 seconds**

   Each time, repeat steps 1 a-d as described above, except for allowing the reaction to proceed for 30, 60, 90, 120, 180 and 360 seconds, respectively, while swirling gently.

Note: Each time, remove a *5 mL* sample and assay for the amount of H_2O_2 in the sample. Use a burette to add $KMnO_4$, one drop at a time, to the solution until a persistent pink or brown color is obtained. Should the end point be overshot, remove another 5 mL sample and repeat the titration. *Do not discard any solutions until the entire lab is completed.* Record your results in Table 2.1 and Graph 2.1.

Table 2.1

$KMnO_4$ (ml)	**Time (seconds)**						
	10	**30**	**60**	**90**	**120**	**180**	**360**
a) Base line*							
b) Final reading							
c) Initial reading							
d) Amount of $KMnO_4$ Consumed (B minus C)							
e) Amount of H_2O_2 Used (A minus D)							

3. Record the base line value, obtained in Exercise 2D, in all of the boxes on line A in Table 2.1.

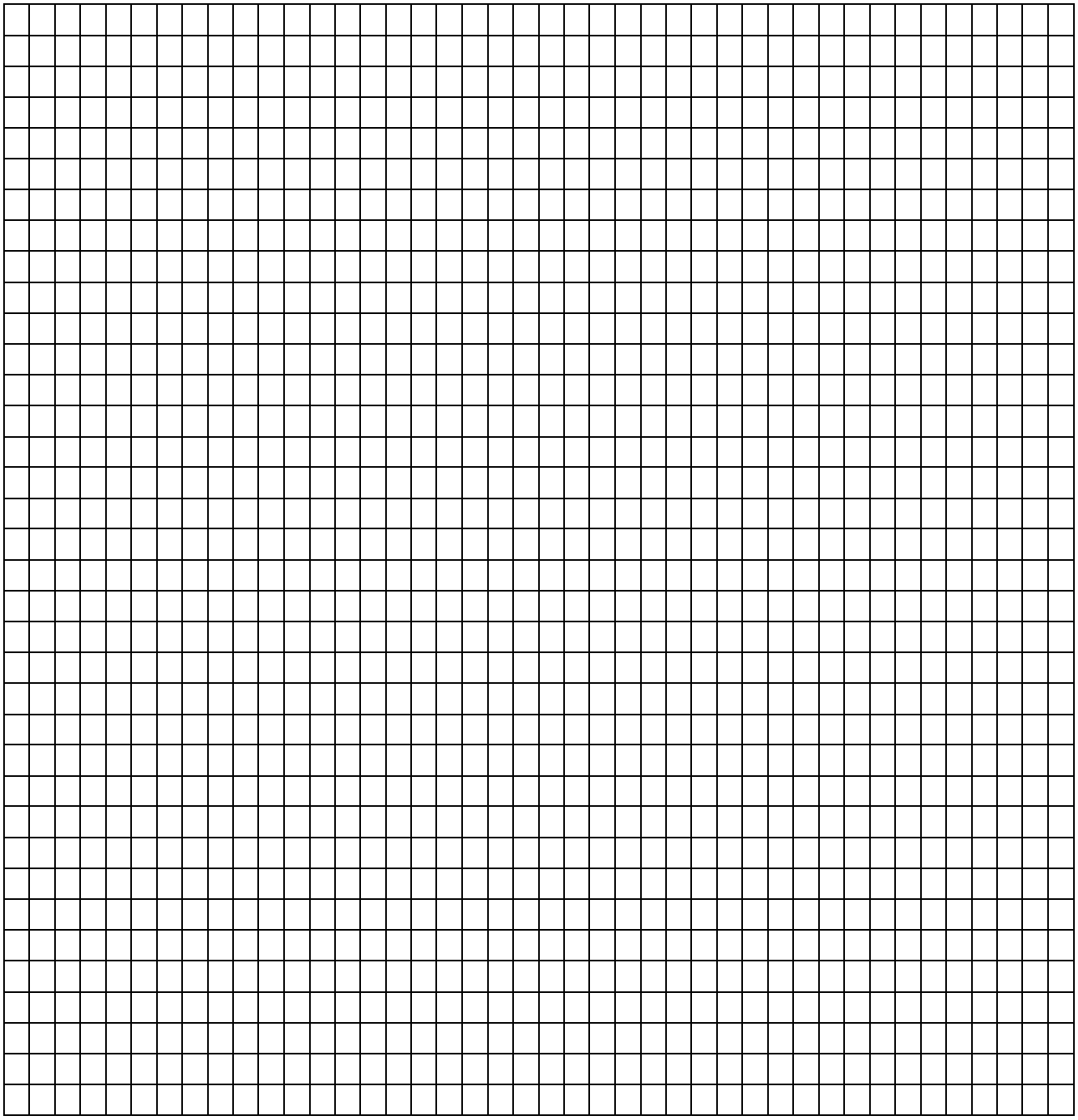
- Remember that the base line tells how much H_2O_2 is in the initial 5 mL sample. The difference between the initial and final readings tells how much H_2O_2 is left after the enzyme-catalyzed reaction. The shorter the time, the more H_2O_2 remains and therefore, the more $KMnO_4$ is necessary to titrate to the endpoint. If syringes are used, $KMnO_4$ consumed may be calculate as c – b.

4. Graph the data for enzyme-catalyzed H_2O_2 decomposition.
   For this graph you will need to determine the following:

   a. The *independent* variable: _____
      Use this value to label the horizontal (x) axis.

   b. The *dependent* variable: _____
      Use this value to label the vertical (y) axis.

Graph 2.1 Title: _____



Analysis of Results

1. From the formula described earlier recall that rate = $\frac{\Delta y}{\Delta x}$

Determine the initial rate of the reaction and the rates between each of the time points. Record the rates in the table below.

	Time Intervals (seconds						
	Initial 0 to 10	10 to 30	30 to 60	60 to 90	90 to 120	120 to 180	180 to 360
Rates*							

* Reaction rate (mL H_2O_2 /sec)

2. When is the rate the highest? Explain why?

3. When is the rate the lowest? For what reasons is the rate low?

4. Explain the inhibiting effect of sulfuric acid on the function of catalase Relate this to enzyme structure and chemistry?

5. Predict the effect that lowering the temperature would have on the rate on enzyme activity. Explain your prediction.

6. Design a controlled experiment to test the effect of varying pH, temperature or enzyme concentration.