Introduction:

Metabolism requires enzymes. Why? Consider that a molecule of glucose can break down to carbon dioxide and water on its own, but the process might take decades. That same conversion takes just seconds inside your cells. Enzymes make the difference. An enzyme makes a reaction run much faster than it would on its own. The enzyme is unchanged by participating in the reaction, so it can work again and again.

Each kind of enzyme interacts only with specific reactants, or substrates, and alters them in a specific way. Such specificity occurs because an enzyme’s polypeptide (or nucleotide) chains fold to form a pocket called an active site, where substrates bind and a reaction proceeds. An active site is complementary in shape, size, polarity, and charge to the enzyme’s substrate. That fit is the reason why each enzyme acts in a specific way on a specific substrate (Figure 1).

A. An active site binds substrates that are complementary in shape, size, polarity, and charge.

B. Binding at an active site squeezes substrates together, influences their charge, or causes some change that lowers activation energy, and the reaction proceeds.

C. The product leaves the active site after the reaction is finished. The enzyme is unchanged, so it can work again.

Figure 1: How the active site of an enzyme works
An enzyme speeds a reaction by reducing activation energy, so it lowers the barrier that prevents the reaction from proceeding. When we talk about activation energy, we are really talking about the energy required to bring reactant bonds to the breaking point. An active site can bring reactants to this state by (for example) holding them in a certain position, squeezing them, or redistributing their charge.

**Factors That Influence Enzyme Activity:**

Environmental factors such as pH, temperature, salt, and pressure influence an enzyme's shape, and so influence its function. Each enzyme works best in a particular range of conditions that reflect the environment in which it evolved.

![Enzymes, temperature, and pH](image)

**A.** The pH-dependent activity of two digestive enzymes. Pepsin acts in the stomach, where the normal pH is 2. Trypsin acts in the small intestine, where the pH is normally around 7.5.

**B.** Temperature-dependent activity of an enzyme from two bacteria: *E. coli*, which inhabits the human gut (normally 37°C); and *Thermus aquaticus*, which lives in hot springs around 70°C.

Consider pepsin, a digestive enzyme that works best at low pH (Figure 2A). Pepsin begins the process of protein digestion in the very acidic environment of the stomach (pH 2). During digestion, the stomach's contents pass into the small intestine, where the pH rises to about 7.5. Pepsin denatures (unfolds) above pH 5.5, so this enzyme becomes inactive in the small intestine. Here, protein digestion continues with the assistance of trypsin, an enzyme that functions well at the higher pH.

Adding heat boosts energy, which is why the jiggling motion of atoms and molecules increases with temperature. The greater the energy of reactants, the closer they are to reaching
activation energy. Thus, the rate of an enzymatic reaction typically increases with temperature—but only up to a point. An enzyme denatures above a characteristic temperature. Then, the reaction rate falls sharply as the shape of the enzyme changes and it stops working (Figure 2B). Body temperatures above 42°C (107.6°F) adversely affect the function of many of your enzymes, which is why such severe fevers are dangerous.

The activity of many enzymes is also influenced by the amount of salt in the surrounding fluid. Too little salt, and polar parts of the enzyme attract one another so strongly that the molecule’s shape changes. Too much salt interferes with the hydrogen bonds that hold the enzyme in its characteristic shape, so the enzyme denatures.

Regulatory molecules (or ions) enhance or inhibit the activity of many enzymes. Some of these substances exert their effects by binding directly to an active site; others bind elsewhere on the enzyme. In the latter case, binding of the regulatory molecule alters the overall shape of the enzyme (Figure 3).

Figure 3: Regulatory molecule binding to enzymes. Some types of regulatory molecules (red) bind to an enzyme in a place other than the active site. This binding changes the shape of the enzyme in a way that enhances or inhibits its function.

Most enzymes (and many other proteins) can function properly only with assistance from metal ions or small organic molecules. These helpers are called cofactors. Many dietary vitamins and minerals are essential because they are cofactors or become converted into cofactors. Coenzymes, which are organic cofactors, carry chemical groups, atoms, or electrons from one reaction to another, and often into or out of organelles. In some reactions, coenzymes stay tightly bound to the enzyme. In others, they participate as separate molecules.
Unlike enzymes, many coenzymes are modified by taking part in a reaction. They are regenerated in separate reactions.

**The Experiment:**

There are two ways to measure enzyme activity: (1) determine the rate of disappearance of the substrate, and (2) determine the rate of appearance of the product.

This exercise will investigate the result of diphenol oxidase activity. In the presence of oxygen, diphenol oxidase catalyzes the removal of electrons and hydrogens from catechol, a phenolic compound found in plant cells. Catechol is converted to benzoquinone, a pigment product. The hydrogens combine with oxygen, forming water (Figure 4). The pigment products are responsible for the darkening of fruits and vegetables, such as apples and potatoes, after exposure to air.

![Figure 4: The oxidation of Catechol. In the presence of diphenol oxidase, catechol is converted to benzoquinone. Hydrogens removed from catechol combine with oxygen to form water](image)

In this initial experiment you will use an extract of potato tuber to learn to study the activity of diphenol oxidase and to establish the appearance of the products when the reaction takes place. The enzymatic reaction of catechol oxidase in potatoes was first reported by Richter (1934), and was further studied and optimized over the years, most notably by Dawson and Magee (1955) and Harel et al. (1966).

The general methodology of this assay to be used in a teaching laboratory was first published by Archer and Palmer (1975). The final adjustments to the assay we will use in this lab came a few years later (Volk et al., 1978; Marumo and Waite, 1986).

Dr. Mark Wallert, Bemidji State University, handed this lab onto me some years ago for its continued use at MSUM (Wallert, personal communication). So, with all the history to this assay, it might make you feel like you are standing on the shoulders of giants (Sir Isaac Newton, 1675). I just hope it doesn’t leave you cold (R.E.M., King of Birds, 1987). Anyway, on with the lab class.........

4
The assay:
1. Turn on the Spec 20 Genesys. It needs to run at least fifteen minutes prior to use. Turn the instrument on by flipping the toggle switch on the lower left of the back of the instrument.

2. Using Table 1, prepare the 6 tubes used for this experiment. Label them C (control), B (the blank), 1, 2, 3,4.

3. Note that all tubes should contain the same total amount of solution.

4. Prepare the solutions in test tubes as directed for tubes 1, 2, 3, 4, but omitting the potato extract until you are ready to take readings.

The blank will contain 8 ml of distilled water and 2 ml of potato extract only.

DO NOT add catechol to the blank.

Table 1. Content of Control, Blank and Experimental Tubes.

<table>
<thead>
<tr>
<th>Tube #</th>
<th>Distilled H₂O</th>
<th>Catechol</th>
<th>Potato Extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>8 ml</td>
<td>0</td>
<td>2 ml</td>
</tr>
<tr>
<td>Control</td>
<td>7 ml</td>
<td>3 ml</td>
<td>0 ml</td>
</tr>
<tr>
<td>1</td>
<td>7 ml</td>
<td>1.0 ml</td>
<td>2 ml</td>
</tr>
<tr>
<td>2</td>
<td>6 ml</td>
<td>2.0 ml</td>
<td>2 ml</td>
</tr>
<tr>
<td>3</td>
<td>5 ml</td>
<td>3.0 ml</td>
<td>2 ml</td>
</tr>
<tr>
<td>4</td>
<td>5 ml</td>
<td>5.0 ml</td>
<td>2 ml</td>
</tr>
</tbody>
</table>

5. Record the absorbance for each tube in Table 2 at time zero and at 5-minute intervals for 20 minutes.

Table 2A. Absorbance Values over Time – Distilled Water

<table>
<thead>
<tr>
<th>tube/time</th>
<th>0 min</th>
<th>5 min</th>
<th>10 min</th>
<th>15 min</th>
<th>20 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2B. Absorbance Values over Time – Repeated Experiment

<table>
<thead>
<tr>
<th>tube/time</th>
<th>0 min</th>
<th>5 min</th>
<th>10 min</th>
<th>15 min</th>
<th>20 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

6. To record the absorbance of the solutions follow these instructions:

   A. Press the mode selection key until A appears in the LCD display window to select absorbance readout.

   B. Press the wavelength selection keys until it reaches 540 nm appears in the LCD display.

   C. Raise the sample compartment door and place the cuvette with the blank into the sample holder.

      Note: Be sure to position your cuvette so that the clear walls are in the light path.

   D. Close the sample compartment door.

   E. Press the automatic blank key to set the absorbance to zero.

   F. Remove your blank and insert a sample cuvette.

   G. The machine is now ready to read the absorbance of your sample.

   H. Continue to take readings at 5 minute intervals for 20 minutes.

      Note: Tubes should be thoroughly mixed and wiped off prior to each reading.

   I. Immediately repeat with cuvettes 1, 2, 3 and 4.

7. Repeat the entire experiment a second time.
8. Calculate the initial rate of the chemical reaction in each of the tubes for the first two runs.
The initial rate is calculated over the region of the reaction where product formation is occurring in a linear fashion.
Calculate initial rate as follows:
a. Plot Absorbance versus time for each test tube.
b. Determine the time over which product formation is linear. This time will be 5, 10, or 15 minutes.

c. Determine the change in absorbance which occurs over that time.
d. Initial Rate = Change in Absorbance = \( \frac{\Delta \text{Abs}}{\text{time}} \)

Plot Initial Rate (velocity, \([V_i]\)) versus catechol concentration (ml). This is a Michaelis-Menten plot for diphenol oxidase.

\[ V_i = \text{initial velocity (moles/time)} \]
\[ [S] = \text{substrate concentration (molar)} \]
\[ V_{\text{max}} = \text{maximum velocity} \]
\[ K_m = \text{substrate concentration when } V_i \text{ is one-half } V_{\text{max}} \]

(Michaelis-Menten constant)

Figure 5: An example plot of absorbance versus time under any given experimental conditions.

Figure 6: Example of a Michaelis-Menten plot, including how to determine the \( V_{\text{max}} \) and the \( K_m \)
The substrate concentration that produces a $V_i$ that is one-half of $V_{\text{max}}$ is designated the Michaelis-Menten constant, $K_m$ (named after the scientists who developed the study of enzyme kinetics). $K_m$ is (roughly) an inverse measure of the affinity or strength of binding between the enzyme and its substrate. The lower the $K_m$, the greater the affinity (so the lower the concentration of substrate needed to achieve a given rate).

**For week Two:**

As a lab group you will be given a variable to investigate its potential effect on this enzyme assay. As a group, you will plan your experiment accordingly and show me your plan for the following week. You will carry out your planned experiment at least in duplicate and begin analysis of your data and experimental system.

Lastly, begin planning your PowerPoint presentation for week three.

**For week Three:**

After 30 minutes of final polishing – 10 min PowerPoint presentation from each group. If you do not work on this with the rest of your group or if you are not present during week three – **you cannot earn any points for this assignment.**

**References:**


