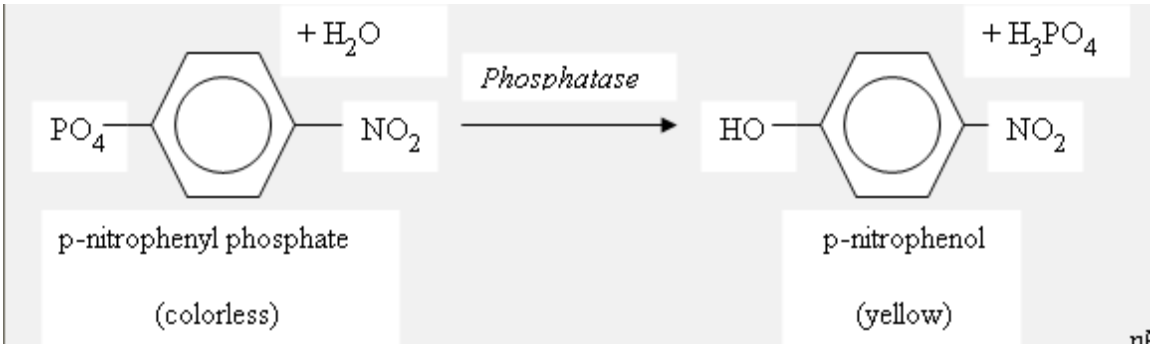
Week 2 – Effect of Enzyme & Substrate Concentration on Enzyme Activity

Week 2 Learning Goals:

- To understand the role of enzymes in catalyzing chemical reactions
- To understand the effect of enzyme and substrate concentration on reaction rate
- To learn how to perform enzyme assays
- To learn how to make serial dilutions and calculate concentrations after dilution

Background

Within the crude protein extract that we obtained from wheat germ last week, is an enzyme called acid phosphatase. Enzymes are proteins that catalyze or accelerate chemical reactions beyond their spontaneous rate. Their activity is often so great that it is possible to measure their activity in very dilute solutions. A reliable assay is the key to successful enzyme studies. Wheat germ acid phosphatase catalyzes the hydrolysis of phosphate from many different phosphorylated molecules such as nucleotides and phosphorylated intermediates of glycolysis. For the experiments here, we will use p-nitrophenyl phosphate as a **substrate**. This synthetic substrate is used because the hydrolysis product, p-nitrophenol, dissociates at alkaline pH to yield the brightly colored nitrophenolate anion. This form absorbs strongly at 405 nm and appears yellow to the eye.



Note that the p-nitrophenol product does not turn yellow until it is brought to an alkaline pH, by adding NaOH, in the procedure below.

When an enzyme catalyzes a chemical reaction, the substrate first diffuses into and binds to the active site of the enzyme. Binding is mediated by non-covalent interactions such as vanderwaals contacts, hydrogen bonds and ionic bonds. The position of amino acid residues within the active site is critical for these interactions and as a result, conditions that alter protein structure will inactivate enzymes. After the reaction occurs, the products are released and new substrate may then bind.

To maintain a constant temperature and pH during the experiment, reaction tubes will be kept in a 37^{o}C water bath and a buffer will be added to prevent changes in pH. One goal is to measure the rate of the reaction under different conditions. To do this, we must measure the amount of product formed after different lengths of incubation. Reactions will be initiated by adding the enzyme to pre-warmed “reaction tubes” containing substrate and buffer and then terminated at specific times by transferring **aliquots** (portions) to “stop tubes” containing 0.1M NaOH. The high pH in the stop tube will prevent any further product formation in the aliquot that was removed, while the remaining enzyme in the reaction tube will continue making product, so that an aliquot can be removed at a later time. After completing each reaction, the absorbance of the stop tubes will be measured at 405 nm and recorded for more detailed analysis during week 3 of the experiment. In these studies, we will vary the concentration of enzyme, source of enzyme, and concentration of substrate.

C. Effect of Enzyme Concentration on Production of NP

All assays MUST BE performed at 37^{O}C. Allow tubes with buffer, substrate (and inhibitor if necessary) to equilibrate at 37^{O}C for 3-4 minutes before adding stock enzyme.
Here we will test 4 enzyme preparations: 0.1x P, 0.01x P, 0.1x E and 0.01x E.

1. **Enzyme Stock:** Prepare 0.1xP by adding 0.4 mL of 1xP to 3.6 mL dH_2O. Prepare 0.01xP by adding 40 μL of 1xP to 3.96 mL dH_2O.

2. **Reaction tubes:** Prepare 4 tubes containing both 1.5 ml citrate buffer, pH 4.8, and 1.5 ml 2.5 mM p-nitrophenyl phosphate (NPP). – Label them 0.1x P, 0.01x P, 0.1x E and 0.01x E. Place in 37^{o}C water bath.

3. **Stop tubes:** Set up 4 tubes with 3 mL 0.1 M NaOH and 16 tubes with 2 mL 0.1 M NaOH.

4. Add 0.75 ml of 0.01x P enzyme stock to its reaction tube, mix and IMMEDIATELY transfer 0.75 ml to stop tube containing 3.0 ml 0.1 M NaOH (t = 0 point). Return reaction tube to 37^{o}C

5. Repeat step 4 for each of the other 3 extracts at 1 minute intervals.

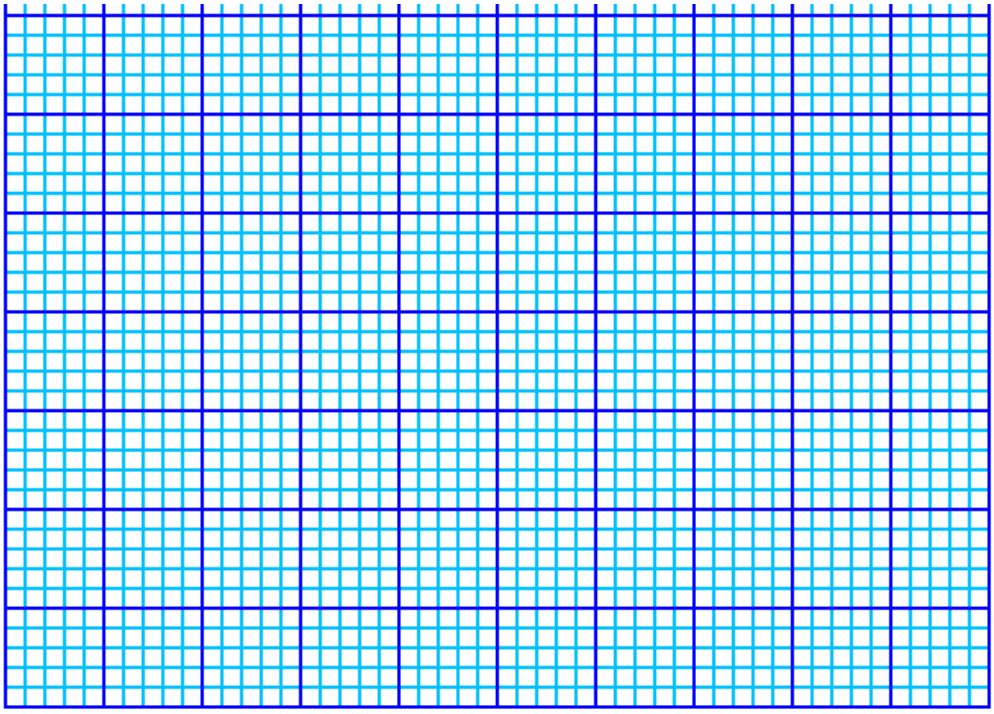
6. Transfer 0.5 ml samples from reaction tubes to 2.0 ml 0.1 M NaOH (stop solution) at exactly 5, 10, 15, 20 minutes.

7. Read A_{405} for all 5 samples, using dH_2O to set zero on spectrophotometer. Record the data in the table on the next page.

Time	0.01X P	0.1X P	0.01X E	0.1X E
0 min				
5 min				
10 min				
15 min				
20 min				

Table 2. Effect of Enzyme Concentration

8. Graph Absorbance vs. time for each of the samples.
   - Which variables go on which axes? _____
   - What is the independent variable in this experiment?_____
   - What is the dependent variable in this experiment?_____
   - Are some of the reactions linear over the 20 minute interval tested? _____



D. Effect of Substrate Concentration

1. Choose the purified enzyme preparation from part C that yielded the highest activity while still being linear over the entire 20 min. period.

2. **Reaction tubes**: Prepare a series of 5 tubes containing 25 μL, 50 μl, 100 μl, 200 μl and 500 μl of 25 mM NPP. Add 1.0 ml of citrate buffer and sufficient dH_2O to make final volume of 2.0 ml.

3. **Stop tubes**: Prepare 15 tubes with 2.0 mL 0.1 M NaOH

4. In the next step, 0.5 mL of enzyme will be added to the reaction tube.

   What will be the total volume of the reaction mixture? _____

   Using the $V_1C_1 = V_2C_2$ equation, calculate the substrate concentration ([S]) for each reaction and enter your results in the table below.

5. Add 0.5 ml of stock enzyme to each reaction tube at 1 minute intervals. Immediately after adding enzyme to each tube transfer 0.5 ml to a tube of 2.0 ml 0.1 M NaOH. These are t = 0 samples.

6. At exactly 7.5 min and 15 min after starting each reaction, transfer 0.5 ml to appropriately labeled tubes of 2.0 ml 0.1 M NaOH (t = 7.5 and 15 min samples).

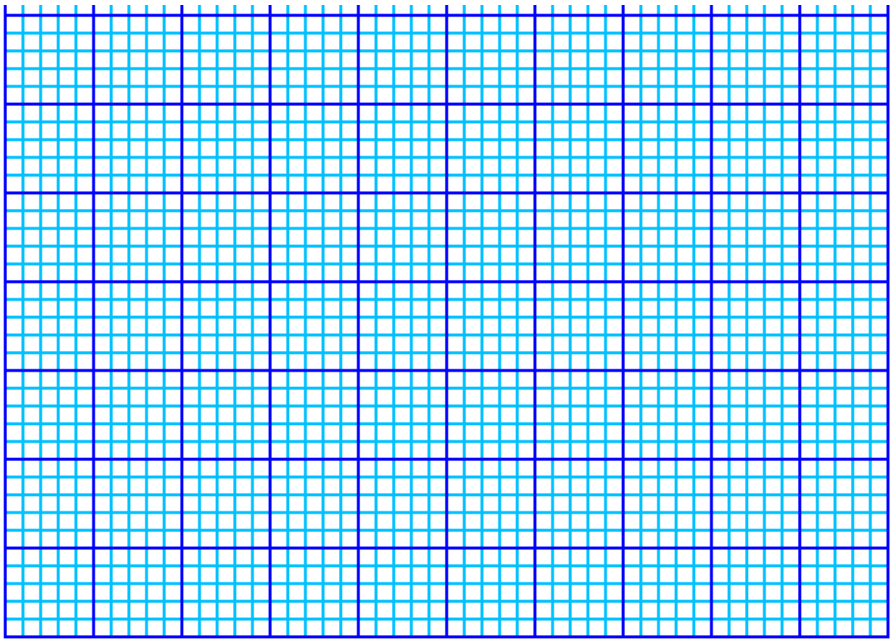
7. Read A_{405} for all 15 tubes of NaOH-quenched acid phosphatase reactions. Zero spectrophotometers with dH_2O. Calculate reaction rates by subtracting the 0 time reading from the 15 minute reading (change in absorbance over 15 min) and multiplying by 4 to convert to change in absorbance per hour (ΔAU/hr).

Table 3. Absorbance Data Using Different Substrate Concentrations

Amount of Substrate	25 μL	50 μL	100 μL	200 μL	500 μL
[S] (mM) (in rxn tube)					
0 min					
7.5 min					
15 min					
Reaction rate (ΔAU/hr)					

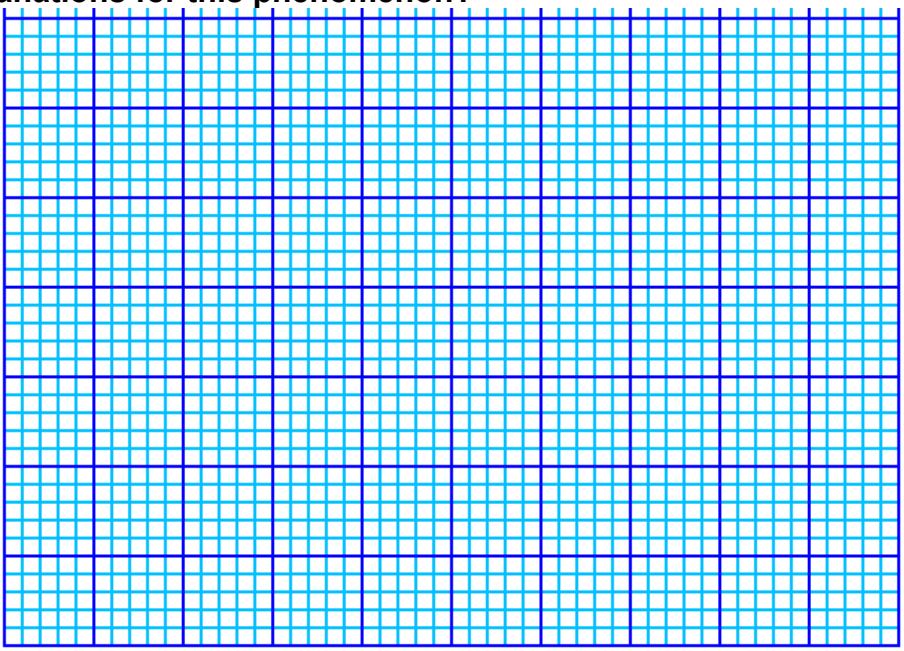
8. Check linearity of product formation over 15 min by graphing absorbance data for each concentration of substrate tested. What is a rate?

How can this data be used to calculate a rate?



9. Create a graph of reaction rate. vs. concentration of substrate.

How would you describe the curve obtained? What are some possible explanations for this phenomenon?



10. Think about the reaction sequence shown below that occurs within a single reaction tube over time, where S is the substrate, P is the product, E is the free enzyme, and S is the enzyme substrate complex. Complete the table and graph below, **estimating** the concentration of each component over time.

E + S ⟷ ES ⟷ E + P

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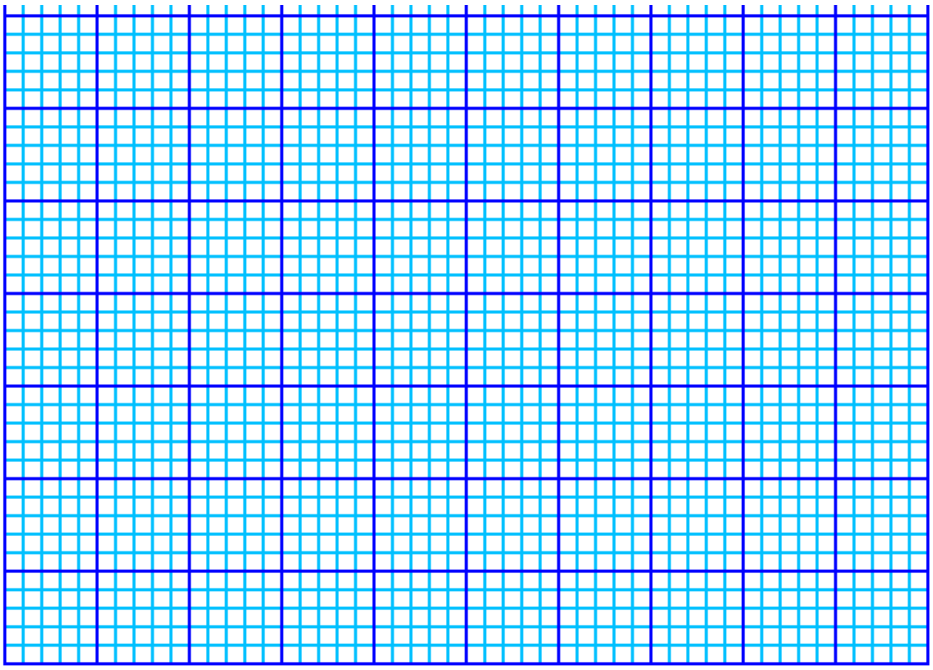
Name →				
Time	**[S]**	**[P]**	**[E]**	**[ES]**
0 min				
1 min				
5 min				
10 min				
15 min				



Summary Questions:

1. Write the full reaction studied in this experiment, and label the enzyme and substrate.

2. Define a unit of enzyme.

3. How did you detect the enzyme's activity on its substrate (explain the assay that you performed)?

4. What was the relationship between the enzyme concentration and the reaction rate? Explain

5. Describe the relationship between amount of enzyme activity and concentration of protein.

6. What was the relationship between the substrate concentration and the reaction rate? Explain.

7. What happened to the reaction rate at high substrate concentrations? Why?