ENZYME ACTIVITY

Pre-lab Preparatory Information

Know this material prior to attending your lab. You may be quizzed on this material.

Objectives: by the end of the lab, you will be able to…

…use a Spectronic 20 to quantify enzyme activity under different conditions.

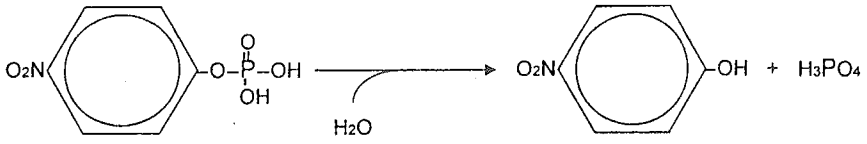
…determine and explain the effect of enzyme and substrate concentration upon the reaction rate.

...identify some of the factors that affect the rate of an enzyme-catalyzed reaction and explain how each influences this rate

…describe and explain chemical reaction in that the studied enzyme catalyzes.

1. Enzyme-catalyzed reactions: The importance of enzymes in biological systems cannot be overstated. A review of the enzyme section of Chapter 7 in your textbook (p 162), paying particular attention to factors that influence the rate of enzyme-catalyzed reactions, will help you interpret your results. Based on previous research, the enzyme you will study in this laboratory is hypothesized to be a type of phosphatase, a group of enzymes that catalyze the removal of phosphate groups from other molecules. Recall that MOST enzyme names end with **–ase** to denote their enzymatic activity. The specific enzyme you will research, alkaline phosphatase, as its name implies, it is hypothesized to be a phosphatase capable of converting p-Nitro-Phenyl Phosphate (**pNPP**) to p-Nitrophenol (**pNP**) and inorganic phosphate (Pi). The hypothesized reaction is:

SUBSTRATE:	ENZYME:	PRODUCTS:	
pNPP	alkaline phosphatase	pNP	+ P_i



Like most biochemical reactions, this reaction is reversible (not shown).

While pNPP is colorless at alkaline pH, pNP is bright yellow.

What tools are you familiar with that could quantify the production of pNP and enable the testing the hypothesis that alkaline phosphatase is a phosphatase?

test tube,
vortex
pH 30

In-Laboratory Exercises:

Work in groups of 4.

Exercise I. Semi-Quantitative Estimates of Enzyme Activity

a. Recall that a hypothesis we are considering is that alkaline phosphatase is a phosphatase capable of removing a phosphate group from pNPP. As part of your assay, some samples will also contain what is thought to be a phosphatase inhibitor. Using your hypothesis, predict and justify what you think will happen in regard to pNP production in these samples:

1. no enzyme:
2. no substrate:
3. complete (both enzyme and substrate):
4. complete plus sodium molybdate:
5. complete plus inorganic phosphate:
6. complete plus phenyl phosphonate:

b. Procedure:

1. Label 6 clean Spec 20 tubes from 1 to 6. Place water in another tube for the BLANK.

2. Set the wavelength of your Spec 20 to **415 nm**. (Why? Would 540 nm, which you used in the glucose assay in Lab 3 work just as well? For help, consult Fig 9-1 in your textbook or Table 1 from Lab 3. What color is 540 nm light? What color is 410 nm light?) Set the dark and light controls (Lab 3, steps 3 to 5, p 5).

3. Pipette the amounts of buffer (pH 8.0) and substrate (pNPP) stated in Data Sheet 4.1 (page 5).

4. Add 1 mL of sodium molybdate into tube 4, of sodium phosphate to tube 5, and of phenyl phosphonate to tube 6.

5. To start the reaction (zero time = o'clock), add 1.0 mL of enzyme to tubes 2 through 6. NOTE: Do NOT add enzyme to tube 1!

6. Cover the openings of the tubes tightly with parafilm. Press your thumb over the covered opening of each tube and mix the contents by inverting the tube several times.

7. Place tube 3 (buffer + enzyme + substrate) in the Spec 20. Watch the needle slowly move as the amount of yellow product increases. When the absorbance has reached between 0.3 and 0.4 – probably in 5-10 minutes – record this absorbance and the elapsed time. **Then quickly read all other tubes in order, starting with tube 6 and ending with tube 1, re-reading tube 3 as you go.**

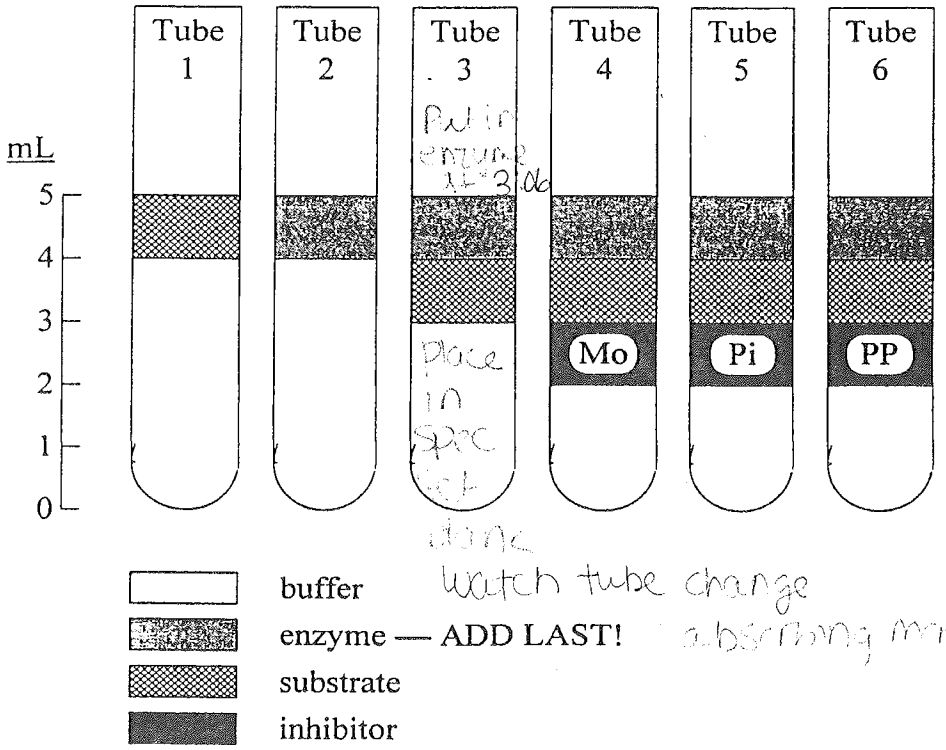
   Record your data in Data Sheet 4.1, page 5, and calculate the rates as $\Delta A/10$ minute.

Name _____; Section _____; Date _____ TA initials ____

Data Sheet 4.1: General features of enzyme-catalyzed reactions.

Tube No.	Volume (mL): Buffer	pNPP	Treatment	PRODUCT FORMED A after 8 min	RATE (A/10 min)
1	4.0	1.0	no enzyme	.058	.058 − 0 / 8 − 0 × 10 = .0725
2	4.0	0.0	no substrate	.059	.059 / 8 × 10 = .0737
3	3.0	1.0	complete	.322	.322 / 8 × 10 = .4025
4	2.0	1.0	plus sodium molybdate	.344	.344 / 8 × 10 = .43
5	2.0	1.0	plus inorganic phosphate	.036	.036 / 8 × 10 = .045
6	2.0	1.0	plus phenyl phosphonate	.208	.208 / 8 × 10 = .26

The reaction was started by adding 1.0 mL of enzyme to each tube EXCEPT tube no.1.



9. Now **analyze** your results by answering the following questions.

   a. Tube 1 had no enzyme: Was there any change in absorbance in this tube? What might a color change indicate?

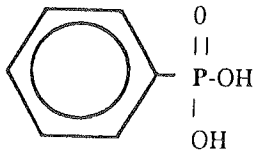
   b. Tube 2 had no substrate: Did the absorbance change in this tube? What might this indicate?

   c. What components were in tube 3? Thinking about the rest of the experiment, which tubes were the controls?

   d. Tube 4 contained the complete mixture plus sodium molybdate, a salt that contains the heavy metal molybdenum. What did you predict would be the effect of this compound? Was your prediction consistent with your observation? Since alkaline phosphatase requires zinc as a cofactor, what might be the mechanism for the inhibition by the sodium molybdate? (The zinc is bound to the phosphatase when the enzyme is isolated.)

   e. In tube 5 you added inorganic phosphate (as sodium phosphate) to the complete mixture: What did you predict would be the effect of this compound? Was your prediction consistent with your observation? What does this tell you about the reaction?

   f. Tube 6 had a compound that can bind to the enzyme but has no bonds that can be hydrolyzed. What did you predict would be the effect of this compound? Was your prediction consistent with your observation? The structural formula for phenyl phosphonate is given below. Draw the structural formula for p-nitro-phenyl phosphate beside it. How do the two molecules differ? (Ignore the nitro-portion.)



What effect did this compound have upon the rate of breakdown of pNPP? Why?

Exercise II. Quantitative Determination of Enzyme Activity

1. Responsibility for All Data: Before you leave the lab, be certain that you have the rates from other members of the class who performed different experiments so that you can complete BOTH Fig. 4.1 and 4.2. **YOU ARE RESPONSIBLE FOR ALL PARTS OF EXERCISE II AND III WHETHER YOU PERFORMED THEM OR NOT! Your instructor will tell your group which assay(s) to perform.**

2. Hypothesis generation: based on your knowledge of enzymes from lecture and the textbook, summarize what modern science hypothesizes about enzyme action and product generation.

3. Predictions: Using your hypothesis above, state some predictions about the effect of enzyme concentration and substrate concentration on rate of an enzyme-catalyzed reaction before you conduct any assay.

The following assays will help test these predictions.

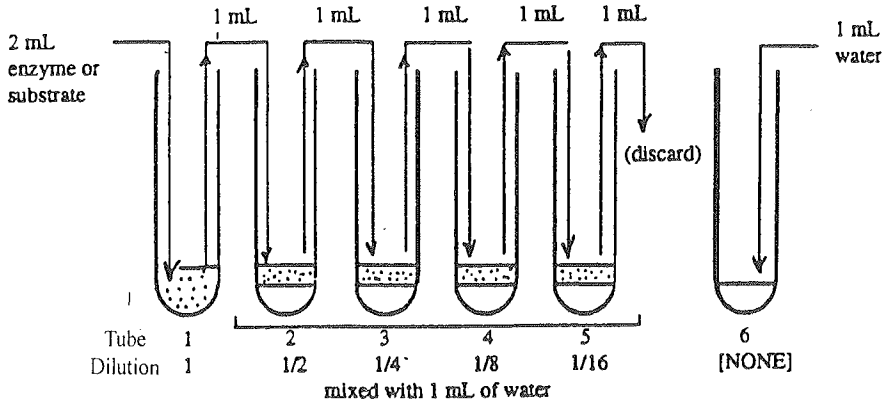
4. Controls: the enzyme assays in this exercise should have two controls, one with no enzyme and one with no substrate.

Assay 1. Effect of Enzyme Concentration

1. Label six clean Spec 20 tubes from 1 through 6. Pipette 5 mL of water into a seventh tube labeled "B" (BLANK).

2. Use the enzyme to make the following dilutions. Mix each dilution thoroughly before making the next dilution. Use the figure below for guidance.

Tube no.	Contents	Dilution
1	2.0 mL of undiluted enzyme	1.0
2	1.0 mL from tube 1 + 1.0 mL of water	0.5
3	1.0 mL from tube 2 + 1.0 mL of water	0.25
4	1.0 mL from tube 3 + 1.0 mL of water	0.12
5	1.0 mL from tube 4 + 1.0 mL of water	0.06
6	1.0 mL of water	none



After preparing Tube 5, remove and discard 1.0 mL of the mixture from tube 5 so that all tubes contain 1.0 mL.

3. Add 3.0 mL of buffer, pH 8, to each Spec tube (except the BLANK). Mix thoroughly.

4. To start the reaction, add 1.0 mL of substrate (pNPP) to each Spec tube (except the BLANK). Mix thoroughly.

IMMEDIATELY determine the absorbance (A) at **410 nm** for each sample using distilled water as a blank (tube B). Record your numbers on the data sheet (Data Sheet 4.2, page 14). (zero time = o'clock)

5. At 5-minute intervals, redetermine the absorbances for each tube, recording your data in Data Sheet 4.2, page 14. Continue until tube 1 has an absorbance of 0.3-0.5.
6. Graph the absorbance at each time for tube 1 (undiluted enzyme). The absorbances should be on the vertical axis; the times, horizontal. Plot the absorbance at each time for tube 1 (undiluted), 3 (diluted 1/4), and 6 (no enzyme) on the graph below the data sheet. Select a time to use to calculate the rates for all of the samples. Place the rates at the bottom of table.
7. Now transfer the rates to the top of Data Sheet 4.3, page 15, *noting that the tubes are arranged in the reverse order*. Plot the rate (vertical axis) against the enzyme dilution (x-axis). Draw a line or curve to best fit your data.

What is the effect of enzyme concentration upon the rate of an enzyme-catalyzed reaction? Did increased enzyme always cause a proportional increase in rate? Or was there a point after which additional enzyme did NOT increase the rate?

How can you explain your results in terms of what you know about the interaction of enzymes and their substrates?

Assay II. Effect of Substrate Concentration

1. Label six clean Spec 20 tubes from 1 through 6. Pipette 5 mL of water into a seventh tube labeled B (BLANK).
2. Make the following substrate (pNPP) dilutions. *Use the diagram in Assay 1 instructions, page 8* for guidance.

Tube no.	Contents	Initial Concentrations	Final Concentrations
1	2.0 mL of 2 mM pNPP	2.0 mM	0.4 mM
2	1.0 mL from tube 1 + 1.0 mL of water	1.0 mM	0.2 mM
3	1.0 mL from tube 2 + 1.0 mL of water	0.5 mM	0.1 mM
4	1.0 mL from tube 3 + 1.0 mL of water	0.25 mM	0.05 mM
5	1.0 mL from tube 4 + 1.0 mL of water	0.125 mM	0.025 mM
6	1.0 mL of water	0 mM	0 mM

After preparing Tube 5, remove and discard 1.0 mL of the mixture from tube 5 so that all tubes contain 1.0 mL.

3. Add 3.0 mL of buffer, pH 8, to each Spec tube (except the BLANK).

4. To start the reaction, add 1.0 mL of enzyme to each Spec 20 tube. Mix thoroughly.

IMMEDIATELY determine the absorbance (A) at **410 nm** for each sample using distilled water as a blank (tube B). Record your numbers in the data sheet (Data Sheet 4.4, page 16). (zero= o'clock)

5. At 5 minute intervals, re-determine the absorbance for each tube, recording your data in Data Sheet 4.4, page 16. Continue until tube 1 has an absorbance of 0.3 to 0.5.

6. Plot the absorbance at each time for tube 1 (undiluted), 3 (diluted 1/4), and 6 (no enzyme) on the graph below the data sheet. Select a time to use to calculate the rates for all of the samples. Record the rates in the middle of Data Sheet 4.4, page 16.

7. Now transfer the rates to the top of Data Sheet 4.5, page 17, noting that the tubes are arranged in the reverse order. Plot the rate (vertical axis) against the substrate concentration (x-axis). Draw a line or curve to best fit your data.

Note that there is a slight complication. The final concentrations in each case were 20% (1/5) of the initial concentrations, prior to dilution by enzyme and buffer. (Think about it.)

Share your RATES with other class members who did not perform this section.

What is the effect of substrate concentration upon the rate of an enzyme-catalyzed reaction? Was the enzyme ever "saturated" - working as fast as possible so that additional substrate did not increase the rate?

How can you explain your results in terms of what you know about the interaction of enzymes and their substrates?

Exercise III. Other Factors That Affect Enzyme Activity

1. Responsibility for All Data: Before you leave the lab, be certain that you have the rates from other members of the class who performed different experiments so that you can complete BOTH Fig. 4.1 and 4.2. **YOU ARE RESPONSIBLE FOR ALL PARTS OF EXERCISE II AND III WHETHER YOU PERFORMED THEM OR NOT! Your instructor will tell your group which assay(s) to perform.**

IV. Preparing a Report: To produce a good research report reviewing the results of the hypothesis testing done in today's exercises, **at a minimum**, one should be able to…

1. …use your data from Part II (including a graph) to show how to calculate the rate of a reaction and show how this calculation was used to demonstrate the effect of enzyme or substrate concentration on the rate of a reaction (Data Sheets 4.3 and 4.5) more enzyme has higher rate enzyme & subst concen both effect rate
2. …report if the rate of the reaction increased as you added more enzyme and if this could continue to occur even at the highest enzyme concentrations. What would this result indicate about enzyme-catalyzed reactions? reaching saturation where if you add enzyme wont make reaction go faster after sat. Same for Subs
3. …report if the rate of the reaction increased as you added more substrate and if this could continue to occur continue to increase if you keep adding more and more substrate. What would this result about an enzyme-catalyzed reaction?
4. …report if differing pH's altered the reaction rate and if there was the optimum pH for the reaction? In addition, one should be able to review the bonds that hold a protein in its three-dimensional shape (tertiary structure) and discussion how pH levels for affect these bonds and the activity of the enzyme? wont have enzyme function found ideal pH, ideal range enviro too acedic denature protein
5. …report if increasing the temperature (up to 37° C) changed the reaction rate and discuss what might happen to an enzyme at the highest temperatures and what that suggests about the enzyme. ↑temp ↓temp, may get little activity
6. …report if the substrate broke down spontaneously (without the enzyme) at room temperature or at 100° C and what that suggests about the nature of a protein catalyst? 2 w/o enzyme @ 100° form product, see reactn high temp provided reaction for producing product we can't deal w/ it as humans

COMING ATTRACTIONS:

In this lab you used a spectrophotometer to measure the rate of enzyme activity. Next week you will use this instrument to measure the rate of photosynthesis (determine an ACTION SPECTRUM) as well as to quantify the amount of light absorbed at different wavelengths (determine an ABSORPTION SPECTRUM).