ENZYME KINETICS: EXPERIMENTS

Laboratory Supplies

Construction of Standard Graph for PNP - All Groups	
PNP stock solution, 2 x 10^{-4} M	5 ml/group
Distilled water, 50 ml	1/group
Small test tubes	7/group
Pipets, 5 ml	2/group
Pipets, 1 ml	8/group
Small test tube rack	1/group
Na_2CO_3, 1 M	6 ml/group
Determination pH Optimum-Group 1	
pH 2, pH 4, pH 5, pH 6, pH7, pH 8 and pH 10 buffers	each 2 ml/tt
NPP, 2 x 10^{-4} M	8 ml
Distilled water	10 ml
Na_2CO_3, 1 M	6 ml
Test tubes	5
Pipets, 1 ml	4
Pipets, 5 ml	1
Undiluted enzyme solution	1 ml
Effect of Enzyme Concentration-Group 2	
pH 5 buffer in a test tube	15 ml
NPP, 2 x 10^{-4} M	15 ml
Distilled water	15 ml
Na_2CO_3, 1 M	15 ml
Test tubes	13
Pipets, 1 ml	8
Undiluted enzyme solution	5 ml
Time Course (Kinetic Assay)-Group 3	
pH 5 buffer in a test tube	15 ml
NPP, 2 x 10^{-4} M	15 ml
Undiluted enzyme solution	1 ml
Na_2CO_3, 1 M	10 ml
Distilled water	25 ml
Test tubes	9
Pipets, 1 ml	9
Pipets, 5 ml	9
Pipets, 10 ml	3
Beaker, 100 ml	1
Effect of Substrate Concentration-Group 4	
pH 5 buffer in a test tube	10 ml
NPP, 2 x 10^{-4} M	10 ml
Distilled water	15 ml
Test tubes	8
Pipets, 1 ml, 5 ml	4 of each
Undiluted enzyme solution	1 ml
Na_2CO_3, 1 M	10 ml

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Procedures

The class will be divided into 4 groups and each group will do a section of the experiment. However, every group should prepare a standard graph on its own and also, everyone should obtain all the data from the other experiments, interpret and plot the data, and discuss all of the work in the lab report, if one is assigned. To draw your graphs, divide each page of graph paper into 4 sections and use one section per experiment.

In all of the following protocols, add the indicated components in the order listed, being especially careful to add the enzyme last to all reaction mixtures. Incubate all reaction mixtures at 37°C for 30 min unless otherwise indicated. Then add 1.0 ml Na_2CO_3 to each tube to stop the reaction and develop color in the product. The last tube in each protocol is the blank for that experiment. (Remember: the blank is used to zero the spectrophotometer. The λ_{max} for PNP is 420 nm.)

Construction of Standard Graph for PNP - All Groups

1. Obtain 7 small test tubes and label them 1 through 7. Add 2 ml of water to tubes 2 through 7.

2. The stock solution of PNP has a molarity of 2×10^{-4} M. Add 2 ml of this stock to tubes 1 and 2. Mix tube 2 thoroughly by a vortexer and transfer 2 ml of it to tube 3. Mix tube 3 well. Note that the concentrations of PNP in tubes 1, 2 and 3 are 2×10^{-4}, 1×10^{-4} and 5.0×10^{-5} M, respectively. (See Fig. 8).

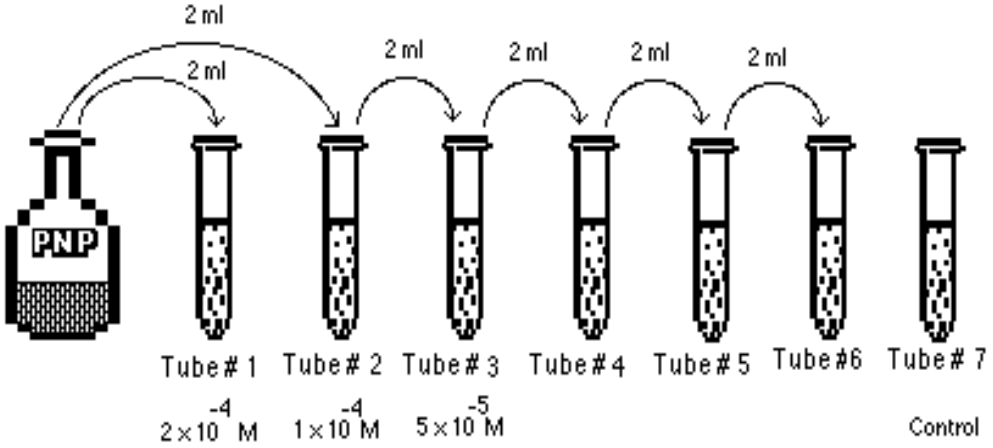

Figure 8. A flow diagram of preparation of dilution tubes for use in standard graph construction.

3. Continue the dilution process for tubes 4 through 6 in the same manner as above. The idea is to halve the concentration of the PNP in each successive tube. Discard 2 ml from tube 6. Do not add any PNP to tube 7 since it is your "control" tube.

4. Add 1 ml of 1 M Na_2CO_3 to all tubes and mix well. Note that no incubation at 37°C is necessary because PNP is the product.

5. Zero the spectrophotometer using tube 7 as blank. Read both the absorbance (A_{420} nm) and transmittance ($\%T_{420}$ nm) of all tubes. This is the only time that you will record

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%T; it is done to illustrate the mathematical relationship between transmittance and absorbance (A = O.D.).

6. Complete the following table and draw graphs of (a) mM PNP (x-axis) vs. A (y-axis), (b) %T (y-axis) vs. mM PNP (x-axis) and (c) % T (x-axis) vs. A (y-axis). Try the third graph on semi-log paper. The A vs. mM PNP is your standard graph.

Tube number	1	2	3	4	5	6	7
ml PNP added	2.0	2.0	0.0	0.0	0.0	0.0	0.0
ml water	0.0	2.0	2.0	2.0	2.0	2.0	2.0
ml 1M Na_2CO_3	1.0	1.0	1.0	1.0	1.0	1.0	1.0
mM PNP present	____	____	____	____	____	____	____
A_{420} nm	____	____	____	____	____	____	____
$\%T_{420}$	____	____	____	____	____	____	____

Note: For simplicity, calculate the mM PNP present without regard to the amount of Na_2CO_3 added.

Determination of pH Optimum-Group 1

1. Prepare a 1/2 dilution of the enzyme; i.e., mix 0.5 ml of the enzyme and 0.5 ml of water.

2. Prepare 7 small test tubes and add the proper amounts of buffer, NPP and water as shown in the following table. Add the enzyme solution last. Mix well.

   Note: First line of table in bold numbers shows pHs of buffers and not their amounts.

3. Incubate the tubes 30 min at 37°C and then add 1.0 ml of 1 M Na_2CO_3 to all tubes and mix well.

4. Measure the absorbance value of each tube using tube 7 as your blank. Plot mM PNP produced/30 min (y-axis) vs. pH (x-axis). What is the pH optimum of your enzyme?

Tube number	1	2	3	4	5	6	7
1.0 ml of buffer of **pH**	**2.0**	**4.0**	**5.0**	**6.0**	**8.0**	**10.0**	**7.0**
ml NPP stock	1.0	1.0	1.0	1.0	1.0	1.0	1.0
ml water	1.9	1.9	1.9	1.9	1.9	1.9	2.0
ml 1/2 enzyme	0.1	0.1	0.1	0.1	0.1	0.1	0.0
Incubate at 37°C for 30 min							
ml 1M Na_2CO_3	1.0	1.0	1.0	1.0	1.0	1.0	1.0
A_{420} nm	____	____	____	____	____	____	____
mM PNP produced	____	____	____	____	____	____	____

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Effect of Enzyme Concentration-Group 2

1. Prepare 1.0 ml each of 1/2, 1/3, and 1/5 dilutions of the enzyme.

2. Label 10 small test tubes and add buffer, NPP and water as shown in the following table. Add the proper amount of the enzyme solutions and incubate the tubes at 37°C for 30 min.

Tube number	1	2	3	4	5	6	7	8	9	10
ml pH 5 buffer	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
ml NPP stock	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
ml water	1.9	1.9	1.9	1.9	1.8	1.6	1.4	1.2	1.0	2.0
ml 1/5 enzyme	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
ml 1/3 enzyme	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
ml 1/2 enzyme	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
ml undil. enzyme	0.0	0.0	0.0	0.1	0.2	0.4	0.6	0.8	1.0	0.0
Incubate at 37°C for 30 min										
ml 1M Na_2CO_3	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
A_{420} nm	____	____	____	____	____	____	____	____	____	____
mM PNP	____	____	____	____	____	____	____	____	____	____

3. After incubation, add 1.0 ml of 1 M Na_2CO_3 to all tubes.

4. Record the absorbance values of each tube. Use tube 10 as the blank. Plot mM of PNP produced/30 min (y-axis) vs. ml of undiluted enzyme (x-axis). Calculate how much undiluted enzyme is represented by the dilution used in the first 3 experimental tubes.

Time Course (Kinetic) Assay-Group 3

1. Prepare 9 test tubes and place 1.0 ml of 1 M Na_2CO_3 in all and 0.4 ml water in the first 8 tubes. Also add 1.0 ml pH 5 buffer, 1.0 ml of 2 x 10^{-4} M NPP, 2.0 ml of distilled water and 1.0 ml Na_2CO_3 in the last tube and use it as a blank to zero the spectrophotometer with it.

Tube number	1	2	3	4	5	6	7	8	9
ml pH 5 buffer	-	-	-	-	-	-	-	-	1.0
ml NPP stock	-	-	-	-	-	-	-	-	1.0
ml water	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	2.0
ml 1M Na_2CO_3	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

2. Prepare a 100 ml beaker containing 10 ml of pH 5 buffer, 10 ml of 2.0 x 10^{-4} M NPP and 20 ml of distilled water.

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3. Prepare a 1/2 dilution of the enzyme in a clean test tube.

4. At time 0 min, pipet 1.0 ml of diluted enzyme into the beaker, swirl immediately to mix the contents. Immediately pipet 3.6 ml of the reaction mixture into tube 1 and mix well. Return the beaker to 37°C water bath. Record A_{420} value for tube 1 using tube 9 as blank.

5. Continue taking 3.6 ml samples at 5 min intervals for 35 min and repeat the procedure as in step 4.

Incubation (min)	0'	5'	10'	15'	20'	25'	30'	35'	none
A_{420} nm	____	____	____	____	____	____	____	____	____
mM PNP	____	____	____	____	____	____	____	____	____

6. Plot mM of PNP produced (y-axis) vs. time of incubation (x-axis) on graph paper. Determine the slope of the linear portion of the graph (in fact, the whole assay may be linear).

Effect of Substrate Concentration-Group 4

1. Dilute 1.0 ml of the undiluted enzyme with 1.0 ml of water to get a two-fold dilution of the enzyme.

2. Prepare 8 test tubes and add the proper amounts of buffer, NPP, water and enzyme as outlined in the following table.

3. Incubate tubes at 37°C for 30 min.

4. After incubation, add 1.0 ml of 1 M Na_2CO_3 to all tubes. Record the absorbance value for each tube using tube 8 as blank. Plot mM PNP produced/30 min vs. mM of substrate.

5. Take the reciprocals of all the substrate concentrations and their corresponding O.D.s and construct a Lineweaver-Burk plot on graph paper. Determine the K_m and V_{max} of the enzyme-substrate complex from this graph.

Tube number	1	2	3	4	5	6	7	8
ml pH 5 buffer	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
ml NPP stock	0.1	0.2	0.5	1.0	1.5	2.0	2.5	0.0
ml water	2.8	2.7	2.4	1.9	1.4	0.9	0.4	2.9
ml 1/2 enzyme	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
	Incubate at 37°C for 30 min							
ml 1M Na_2CO_3	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
A_{420} nm	____	____	____	____	____	____	____	____
mM PNP	____	____	____	____	____	____	____	____

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Results of Enzyme Kinetics Lab Exercise

Name _____ Date _____ Section _____

Name of partners _____

Due in one week:

1. Fill in the blanks in the following tables:

(a) Construction of standard graph for PNP

Tube number	1	2	3	4	5	6	7
ml PNP added	2.0	2.0	0.0	0.0	0.0	0.0	0.0
ml water	0.0	2.0	2.0	2.0	2.0	2.0	2.0
ml 1M Na_2CO_3	1.0	1.0	1.0	1.0	1.0	1.0	1.0
mM PNP present	____	____	____	____	____	____	____
A_{420} nm	____	____	____	____	____	____	____
$\%T_{420}$	____	____	____	____	____	____	____

(b) Determination of pH optimum

Tube number	1	2	3	4	5	6	7
1.0 ml of buffer of **pH**	**2.0**	**4.0**	**5.0**	**6.0**	**8.0**	**10.0**	**7.0**
ml NPP stock	1.0	1.0	1.0	1.0	1.0	1.0	1.0
ml water	1.9	1.9	1.9	1.9	1.9	1.9	2.0
ml 1/2 enzyme	0.1	0.1	0.1	0.1	0.1	0.1	0.0
Incubate at 37°C for 30 min							
ml 1M Na_2CO_3	1.0	1.0	1.0	1.0	1.0	1.0	1.0
A_{420} nm	____	____	____	____	____	____	____
mM PNP produced	____	____	____	____	____	____	____

(c) Effect of enzyme concentration

Tube number	1	2	3	4	5	6	7	8	9	10
ml pH 5 buffer	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
ml NPP stock	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
ml water	1.9	1.9	1.9	1.9	1.8	1.6	1.4	1.2	1.0	2.0
ml 1/5 enzyme	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
ml 1/3 enzyme	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
ml 1/2 enzyme	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
ml undil. enzyme	0.0	0.0	0.0	0.1	0.2	0.4	0.6	0.8	1.0	0.0

	Continuation of table from previous page									
	Incubate at 37°C for 30 min									
ml 1M Na_2CO_3	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
A_{420} nm	____	____	____	____	____	____	____	____	____	____
mM PNP	____	____	____	____	____	____	____	____	____	____

(d) Time course (kinetic) assay

Tube number	1	2	3	4	5	6	7	8	9
ml pH 5 buffer	-	-	-	-	-	-	-	-	1.0
ml NPP stock	-	-	-	-	-	-	-	-	1.0
ml water	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	2.0
ml 1M Na_2CO_3	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Incubation (min)	0'	5'	10'	15'	20'	25'	30'	35'	none
A_{420} nm	____	____	____	____	____	____	____	____	____
mM PNP	____	____	____	____	____	____	____	____	____

(e) Effect of substrate concentration:

Tube number	1	2	3	4	5	6	7	8
ml pH 5 buffer	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
ml NPP stock	0.1	0.2	0.5	1.0	1.5	2.0	2.5	0.0
ml water	2.8	2.7	2.4	1.9	1.4	0.9	0.4	2.9
ml 1/2 enzyme	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Incubate at 37°C for 30 min								
ml 1M Na_2CO_3	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
A_{420} nm	____	____	____	____	____	____	____	____
mM PNP	____	____	____	____	____	____	____	____

Use extra sheets to answer the following questions and attach them to these pages.

2. Why must enzyme be added last to all reaction mixtures?

3. How can one determine that the λ_{max} of PNP is 420 nm?

4. You have determined the pH optimum of your enzyme. Discuss the effect(s) that raising the pH to 10 might have upon the structure and activity of the enzyme.

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Due in two weeks:

You are required to write a paper on Enzyme Kinetics lab exercises and submit a hard copy as well as an electronically copy of it within two weeks from the time you perform the experiments. Make sure you understand the Lab Report Format as given below. For the hard copy that you submit to your TA, include all your graphs (your standard graph, your experiment and those of other groups in the class and the Lineweaver-Burk graph) and a discussion of each graph in detail. The electronic copy submitted through the BlackBoard (see instructions on next page) is an exact duplicate of your report but it should not include any graphs. If you have any questions about the format of either report, ask your TA.

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Lab Report Format (For This Topic As Well As Others Throughout the Semester)

Any lab report that is called "a paper" should be typed in double-spaced format and should contain 5 parts. These parts should be clearly labeled:

Introduction: Provide some background information on the subject of the paper and then present the a null hypothesis for each experiment clearly.

Materials and methods: For the purpose of these experiments, assume your reader is familiar with general lab procedures so you don't have to describe them in detail. It suffices to include flow diagrams with some short descriptions directly within them.

Results: Show the results in figures (graphs or histograms) on a separate page and refer to it in the text: "As shown in Figure 1, the measurements indicate that...". Do not repeat the actual results in a table and also in a graph; you will lose points if you do so. The results need to be represented clearly only once. Keep the results section brief.

Discussion: A brief essay of how your results reflect on the axioms and predictions. If your results contradict what other researchers have shown, i.e., what is in the textbook or lab manual, try to rationalize why your results are different. Was there something different with your assumptions, your equipment or your technique that would produce different results?

References: Cite authoritative sources to support your axioms and your reasoning. Use citations when referring to previously published ideas. The standard format is to put the author and year in parentheses, e.g., (Campbell, 1989) at the end of the sentence in the text. In the References section, list the references with a complete citation in alphabetical order. For example:

Campbell, N.A. and Reece, J.B. 2005. Biology. 7th Ed. Pearson, Benjamin/Cummings, 1231 pages.

Phillips, D.C. 1966. The three-dimensional structure of an enzyme molecule. Sci. Am. 215: 78-82.

The following are some more explanations by the Head TA, Jessica Ardis, for writing your report:

1. Aim for your introduction to be about one double-spaced page. You should have a little background information about enzymes and how they work. You do not have to explicitly state: "My first hypothesis is…" but you should introduce the questions that you sought to answer by doing the four experiments. You must cite references within the body of your introduction.

2. If you want to cite the lab manual, use Eby Bassiri for author, and University of Pennsylvania for publisher and cite the website of the Lab Manual in its entirety.

3. You must write about all four experiments even though your group did only one.

4. You do not need to show calculations for concentrations in the report.

5. Trend lines should only be shown on the standard graph and the Lineweaver-Burk plot. When you perform a linear regression, display the equation on the graph.

6. If your results do not make sense, speculate as to why they turned out the way they did in the discussion section. It is more important to be able to intelligently discuss results than it is for everything to have turned out perfectly.

Submitting Reports Electronically Using the Course BlackBoard

In addition to handing a hard copy of your papers to your TA, you will also be using the course's BlackBoard to submit a copy of each of your papers electronically. The first thing you need to do is to rename the file of your report according to the following format:

123sssWW-TYY-XxxxxxZ.doc

The meaning of each of the above abbreviations is as follows:
123=stands BIOL 123
sss=the lab section you are attending
WW=Name of the exercise (where WW=EK for Enzyme Kinetics Exercise, WW=ID for PCR-ID Exercise, and WW=TN for pGLO Mutagenesis Exercise)
T=Term (S for the Spring term or F for the Fall term)
YY= The last 2 digits of the current year
Xxxxxx=the first 6 letters of your last name; note that the first letter is upper case and the other 5 letters are lower cases
Z= the first letter of your first name in upper case
doc=Word document extension (please do not use the newer Word file extension)

For example, if Kimberly Stratowsky were submitting her Enzyme Kinetics Report and were attending Bio 123 section 104 during the Fall term of 2014, she would name her file: 123104EK-F14-StratoK.doc

Now log in to BlackBoard and click on the "Assignments" button on the left panel. Next click on the appropriate report to get a page that allows you to read the instructions, follow them and attach your 123sssWW-TYY-XxxxxxZ.doc report under "2. Assignment Materials". Finally click the submit button to send your report. If you need any help with the submission process, your TA will be happy to help you. Please do not leave submissions to the last minute so you have time to alleviate any problems if they arise. Reports not submitted by the due time will lose points no matter what the excuse is.

Grading Criteria

Your TA uses quite a number of criteria to grade your reports. These include quality of reasoning and clarity of exposition, correct interpretation of data and conclusions, ability to employ the hypothetico-deductive approach and quality of presentation (even neatness, spelling, grammar, etc).

The lab report is an individual effort. You will necessarily share results (data) in the lab, but any similarity in writing and reasoning between lab reports will be investigated. We have the ability to use software that can unequivocally show if a student's paper or parts of it have been copied from another source and is not totally the effort of the student. Be prepared to produce documentation of your work (lab notebook, rough draft, worksheets, etc.). Please note that any plagiarism will be dealt with severely.