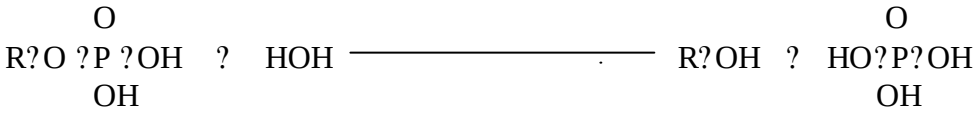
Experiment 7
(Lab Period 8)

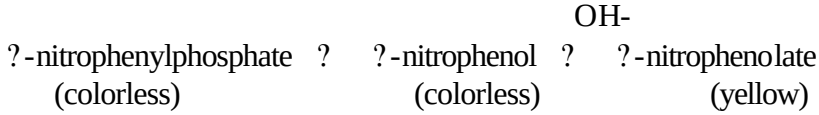
Quantitative Determination of Phosphatase Activity

Phosphatases are enzymes that catalyze the hydrolysis of organic-phosphate compounds, releasing inorganic phosphate from the rest of the molecule. The general reaction is:



Phosphatases serve a variety of functions in living organisms. Some help to digest food so that the smaller products can be absorbed or metabolized. Such phosphatases occur as secreted enzymes, as in the mammalian intestine. They also occur as dormant enzymes in seeds; during germination, they become active and mobilize stored food to be used by the emerging seedling. Other phosphatases occur in the lysosomes of phagocytic cells and help to digest particulate matter captured during phagocytosis. Still others are active in the cytoplasm and serve to recycle phosphorus in metabolism or to remove phosphate groups from proteins whose activities are regulated by the addition and removal of phosphate groups.

Many phosphatases are not very specific with respect to the compounds they use as substrates, and any one phosphatase will often be able to hydrolyze various organo-phosphates. The low specificity will allow us to utilize only one substrate to test the activity of two different phosphatases in this experiment. The test reaction that will be used to measure (assay) enzyme activity is hydrolysis of para-nitrophenylphosphate:



In this assay, the reaction is timed from the time of addition of enzyme to the time of addition of strong alkali (0.2 M NaOH). The reaction catalyzed by the enzyme is the first of the two shown in the diagram; this is the reaction we want to measure. Since both compounds are colorless we cannot do so directly, instead we add NaOH. The NaOH has two effects: (l) it converts p-nitrophenol to p-nitrophenolate and develops the yellow color, and (2) it stops enzyme activity. By measuring the quantity of -nitrophenolate we are in effect measuring the quantity of -nitrophenol that was present (the product) before the addition of NaOH. In performing this assay, measure as accurately as possible; the interpretability of your data will depend on the accuracy of your measurements of all the volumes of all the components of the reaction mixtures. The lab that you will perform consists of two parts. The first is a determination of the influence of phosphatase on the rate of the reaction and the second consists of an investigation of the influence of pH on the activity of phosphatase.

Rate of reaction catalyzed by phosphatase: In the first part of the experiment you will compare the rate of the reaction described above in the presence and absence of phosphatase. Therefore, you will be measuring the rates of both the catalyzed and uncatalyzed reaction which will allow you to determine the degree to which phosphatase increases the reaction rate.

In order to express the rate of reaction in units of moles of product produced per unit time you will be comparing the quantity of p-nitrophenolate to the amount of time that elapsed between enzyme addition and NaOH addition. To assay p-nitrophenolate you will use spectrophotometry since this solution absorbs light. The absorption spectrum has a peak at 405nm. Therefore, the first step will be to determine the relationship between Absorbance of light at 405 nm and the concentration of p-nitrophenolate (that is, you will determine E for this compound at 405nm using a standard curve as in experiment 1). Following the determination of E you will calculate reaction rate.

Influence of pH on phosphatase activity: For most enzymes, pH can influences the catalytic site directly by altering the charge of the protein in this region. The pH will also affect tertiary structure of the enzyme, and may also affect the ability of the enzyme to bind ("embrace") the substrate. As a consequence, the catalytic activity (i.e. the degree to which it increases the rate of the reaction) of all known enzymes is sensitive to pH, and each known enzyme exhibits its greatest activity at one pH--the enzyme's optimal pH.

In this experiment you will use two different phosphatases, wheat phosphatase and calf phosphatase. These two differ in their pH optima as they are used in very different organs. The calf phosphatase was purified from the intestinal lining; it is normally secreted into the intestines. The wheat phosphatase was purified from seeds; it is stored in lysosomes while the seed is dormant. From your knowledge (and your textbook), suggest which enzyme is the acid phosphatase (optimum activity in an acidic environment) and which enzyme is the alkaline phosphatase.

In this part, some students will work with the wheat enzyme, some will work with the calf enzyme, and some will receive both enzymes in one solution. Each of you will identify the enzyme(s) you received by determining the effect of pH on enzymatic activity.

NOTE: If necessary, review the directions for the use of the Spectronic 20 in Laboratory 1. All Absorbance readings today will be taken at 405 nm, the absorption peak of p-nitrophenolate (what color light does this compound absorb?). You could, of course, check the absorption spectrum of one of the solutions in Procedure A to be sure that this is the absorption peak.

Procedure

Part I Phosphatase activity

A - ?-nitrophenolate standard curve

l. Label 8 cuvettes: 0, 5, l0, 20, 30, 40, 50, 60. These labels indicate the concentration of p-nitrophenol in units of nmoles/ml.

2. Add 0.lM NaOH to these cuvettes as indicated in Table A.

3. Add 60 ?M ?-nitrophenol to the cuvettes as indicated in Table A. Mix the contents of each tube well.

Table A

Tube	Volume 0.1M NaOH	Volume 60?M ?-NP
0	6 ml	0 ml
5	5.5 ml	0.5 ml
10	5 ml	1 ml
20	4 ml	2 ml
30	3 ml	3 ml
40	2 ml	4 ml
50	1 ml	5 ml
60	0	6 ml

4. Read the Absorbance at 405 nm of each solution, as follows.
   a. Set the wavelength to 405 with the wavelength control knob.
   b. Place cuvette 0 in the sample holder and adjust the Transmittance to l00 %.
   c. Place cuvette 5 into the sample holder, read its A_{405}.
   d. Repeat until A_{405} has been recorded for each solution, working in order of increasing concentration of ?-nitrophenol.

B - Effect of enzyme on the rate of hydrolysis

l. Obtain an enzyme solution and keep it in ice until you are ready to add it to your reaction cuvettes. Work relative to a partner so that one student uses wheat phosphatase and the other uses calf phosphatase.

2. Using the buffer appropriate to your enzyme, prepare 10 cuvettes as indicated in Table B but DO NOT YET ADD ENZYME. (Be careful not to introduce any enzyme into cuvettes S0 - S4; any ?-nitrophenol formed in these tubes should arise by spontaneous (i.e., uncatalyzed) hydrolysis of ?-nitrophenylphosphate, and a little catalyst can have a significant effect.) Mix the contents of cuvettes S0 - S4 by closing each cuvette with Parafilm and inverting the cuvette two or three times.

Table B

Uncatalyzed Reaction

Tube	Buffer	6 mM ?-NPP	Water	Time of .2M NaOH addition
S0	2 ml	1 ml	1 ml	OT
S1	2 ml	1 ml	1 ml	10 min
S2	2 ml	1 ml	1 ml	20 min
S3	2 ml	1 ml	1 ml	30 min
S4	2 ml	1 ml	1 ml	40 min

Catalyzed Reaction

Tube	Buffer	6 mM ?-NPP	Enzyme	Time of .2M NaOH addition
E0	2 ml	1 ml	1 ml	OT
E1	2 ml	1 ml	1 ml	10 min
E2	2 ml	1 ml	1 ml	20 min
E3	2 ml	1 ml	1 ml	30 min
E4	2 ml	1 ml	1 ml	40 min

3. Place your cuvettes in the 37^{o}C water bath and allow them to warm to 37^{o}C for about 5 min.

4. Note the time. Now add enzyme to cuvettes E0 - E4 and mix the contents of each cuvette. The time at which you begin to add enzyme is "OT."

5. At l0-min. intervals, remove two cuvettes (one S and one E cuvette), add 2 ml NaOH per cuvette, and mix the contents.

NOTE: If you do not add the NaOH at the precise time intended, record the time at which addition was made. The results are equally useful and valid.

6. Using distilled water in a reference cuvette, read the A_{405} of each reaction mixture.

Part II Determination of optimal pH

l. Obtain an unidentified enzyme solution and keep it on ice.

2. Prepare nine cuvettes as indicated in Table C but DO NOT YET ADD ENZYME.

Table C

Tube	Buffer pH (2 ml each)	6 mM ? -NPP	Enzyme	0.2 M NaOH (at 30 or 40 min.)
0 (Blank)*	None	1 ml	None	2 ml
3	3	1 ml	1 ml	2 ml
4	4	1 ml	1 ml	2 ml
5	5	1 ml	1 ml	2 ml
6	6	1 ml	1 ml	2 ml
7	7	1 ml	1 ml	2 ml
8	8	1 ml	1 ml	2 ml
9	9	1 ml	1 ml	2 ml
10	10	1 ml	1 ml	2 ml

***Add 3ml of the MILLIQ-water in tube 0(Blank).**

3. Place the cuvettes in the 37°C water bath and allow them to warm to 37°C for about 5 min.

4. Note the time. Now add enzyme to each of the numbered cuvettes and mix.

5. After 30 min. remove the cuvettes from the water bath and stop the reaction by adding 2 ml NaOH per cuvette. Mix as soon as possible after adding the NaOH.

6. Using tube 0 as the reference cuvette, read A_{405} of each reaction mixture.

Calculations

Part I

A - Standard curve.

Cl. Plot A_{405} as a function of ?-nitrophenol concentration.

C2. Draw as straight a line as possible (a "line of best fit") through the points, with the origin (coordinates 0,0) as the eighth point.

C3. Using any two convenient points on the straight line, calculate the slope of the straight line; the units of the slope will be:

E = Absorbance/Concentration(nmoles/ml)

B - Effect of enzyme on rate of hydrolysis.

Cl. Convert each A_{405} reading to concentration (nmoles/ml) as follows:

C = A / E (see experiment 1)

Units are: Abs/[Abs/(nmoles/ml)] = nmoles/ml

C2. Plot product concentration (Y-axis) as a function of time (X-axis).

C3. Draw a line of best fit and calculate the slope for both types of reaction—spontaneous (uncatalyzed) and enzyme-catalyzed. The units of each slope will be: (nmoles/ml)/min. This is the rate of reaction for each type of reaction.

C4. To determine the effect of the enzyme on the rate of reaction, calculate:

Rate of catalyzed reaction / Rate of spontaneous reaction

Part II

Cl. Plot concentration (Y-axis) as a function of pH (X-axis).

Lab Report

A lab report is due for this experiment. It should follow the usual format and address the two main questions:

1. To what degree does the addition of enzyme increase the rate of reaction?

2. What is the optimal pH of your enzyme's catalytic activity? Which phosphatase were you given?