
# EXPERIMENTAL ENZYMOLOGY (BCH 322)

## Lab Manual

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# Table of Contents

**1. SOME FACTORS AFFECTING POLYPHENOL OXIDASE ACTIVITY ..... 1**

**(INTRODUCTION TO ENZYMOLOGY) 1**

**1.1** OBJECTIVES ..... 1
**1.2** INTRODUCTION ..... 1
**1.3** MATERIALS ..... 3
**1.4** METHOD ..... 4
**1.5** RESULTS ..... 9
**1.6** DISCUSSION AND CONCLUSIONS ..... 11
**1.7** QUESTIONS ..... 12
**1.8** REFERENCE ..... 12

**1. METHODS OF ENZYME ASSAY.... 13**

**2.1** OBJECTIVE ..... 13
**2.2** INTRODUCTION ..... 13
**2.3** END POINT ASSAY ALANINE TRANSAMINASE (COLORIMETRIC\ENDPOINT ASSAY) 14
**2.4** PRINCIPLE ..... 15
**2.5** MATERIALS ..... 15
**2.6** PREPARATION OF SOLUTIONS: ..... 15
**2.7** PRECAUTIONS ..... 16
**2.8** METHOD ..... 16
**2.9** RESULTS ..... 18
**2.10** USE THE CO-ENZYME IN MEASURE THE ACTIVITY OF LACTATE DEHYDROGENASE 19
**2.11** METHOD: ..... 20
**2.12** RESULTS ..... 21
**2.13** DISCUSSION AND CONCLUSION: ..... 22
**2.14** REFERENCES ..... 22

**3. PURIFICATION OF ACID PHOSPHATASE FROM WHEAT GERM 23**

**3.2** INTRODUCTION ..... 23
**3.3** MATERIALS ..... 24
**3.4** METHOD ..... 26
**3.4.2** PART TWO: ASSAY OF ACID PHOSPHATASE ..... 29
**3.5** RESULTS ..... 31
**3.6** DISCUSSION AND CONCLUSION ..... 31
**3.7** REFERENCE ..... 32

**4. THE EFFECT OF INCUBATION TIME ON THE RATE OF AN ENZYME CATALYZED REACTION 33**

**4.1** OBJECTIVES ..... 33
**4.2** INTRODUCTION ..... 33
TIME COURSE OF THE REACTION ..... 34
**4.3** MATERIALS ..... 35
**4.3.1** EQUIPMENTS ..... 35
**4.3.2** PREPARATION OF SOLUTIONS ..... 35
**4.4** METHOD ..... 36
**4.5** RESULTS ..... 38
**4.6** DISCUSSION AND CONCLUSION ..... 39
**4.7** QUESTIONS ..... 40
**4.8** REFERENCE ..... 40

**5. THE EFFECTS OF ENZYME CONCENTRATION ON THE RATE OF AN ENZYME CATALYZED REACTION 41**

**5.1** OBJECTIVES ..... 41
**5.2** INTRODUCTION ..... 41
**5.3** MATERIALS ..... 42
**5.3.1** EQUIPMENTS ..... 42
**5.5** RESULTS ..... 44
**5.6** DISCUSSION AND CONCLUSION ..... 45
**5.7** QUESTIONS ..... 46
**5.8** REFERENCE ..... 46

**6. THE EFFECT OF TEMPERATURE ON THE RATE OF AN ENZYME CATALYZED REACTION 47**

**6.1** PART I: THE EFFECT OF TEMPERATURE ON THE RATE OF AN ENZYME CATALYZED REACTION 47
INTRODUCTION ..... 47
**6.2** MATERIALS ..... 48
**6.3** METHOD ..... 49
**6.4** RESULTS ..... 51
**6.5** DISCUSSION AND CONCLUSION ..... 52
**6.6** QUESTIONS ..... 53
**6.7** REFERENCES ..... 53

**7. THE EFFECT OF PH ON THE RATE OF AN ENZYME CATALYZED REACTION 54**

**7.1** OBJECTIVES ..... 54
**7.2** INTRODUCTION ..... 54
**7.3** MATERIALS ..... 55
**7.4** METHOD ..... 55
**7.6** DISCUSSION AND CONCLUSION ..... 58
**7.7** QUESTION ..... 59
**7.8** REFERENCES ..... 59

**8. THE EFFECT OF SUBSTRATE CONCENTRATION AND INHIBITOR (INORGANIC PHOSPHATE AND SODIUM FLUORIDE) ON THE RATE OF AN ENZYME CATALYZED REACTION 60**

**8.1** OBJECTIVES ..... 60
**8.2** INTRODUCTION ..... 60
**8.3** MATERIALS ..... 63
**8.4** METHOD ..... 63
**8.5** RESULTS ..... 65
**8.6** DISCUSSION AND CONCLUSION ..... 66
**8.7** QUESTIONS ..... 67
**8.8** REFERENCES ..... 67

**9. THE INHIBITION OF ACID PHOSPHATASE BY INORGANIC PHOSPHATE AND SODIUM FLUORIDE 68**

**9.1** OBJECTIVES ..... 68
**9.2** INTRODUCTION ..... 68
**9.3** MATERIALS ..... 71
**9.4** EQUIPMENTS ..... 71
PREPARATION OF SOLUTIONS ..... 71
**9.5** METHOD ..... 72
**9.6** RESULTS ..... 73
**9.7** DISCUSSION AND CONCLUSION ..... 74
**9.8** QUESTIONS ..... 75

**9.9** REFERENCE ..... 75

**10. MODIFICATION OF ACTIVE SITE CYSTEINE OF LACTATE DEHYDROGENASE** **76**

**10.1** OBJECTIVES ..... 76
**10.2** INTRODUCTION ..... 76
**10.4** METHOD ..... 77
**10.5** RESULTS ..... 79
CALCULATIONS ..... 80
**10.6** DISCUSSION AND CONCLUSION ..... 80
**10.7** REFERENCE ..... 81

# Experiment 1

## 1. Some factors affecting polyphenol oxidase activity

## (Introduction to Enzymology)

### 1.1 Objectives:

- To demonstrate activity of the enzyme polyphenol oxidase in crude extract prepared from potato.
- To demonstrate the chemical nature of the enzyme.
- To investigate the substrate specificity of the enzyme.
- To investigate the effects of various temperatures on the activity of the enzyme.

### 1.2 Introduction:

When we consider enzyme catalyzed reactions in the living cell, the reacting substances, upon which an enzyme acts, are termed the substrates. The substances produced as a result of the reaction are the products. Enzyme catalyzed reactions are mostly reversible and involve the formation of an intermediate enzyme-substrate complex.

$$E + S \rightleftharpoons ES \rightleftharpoons E + P$$

Enzyme Substrate Enzyme -substrate complex Enzyme Product

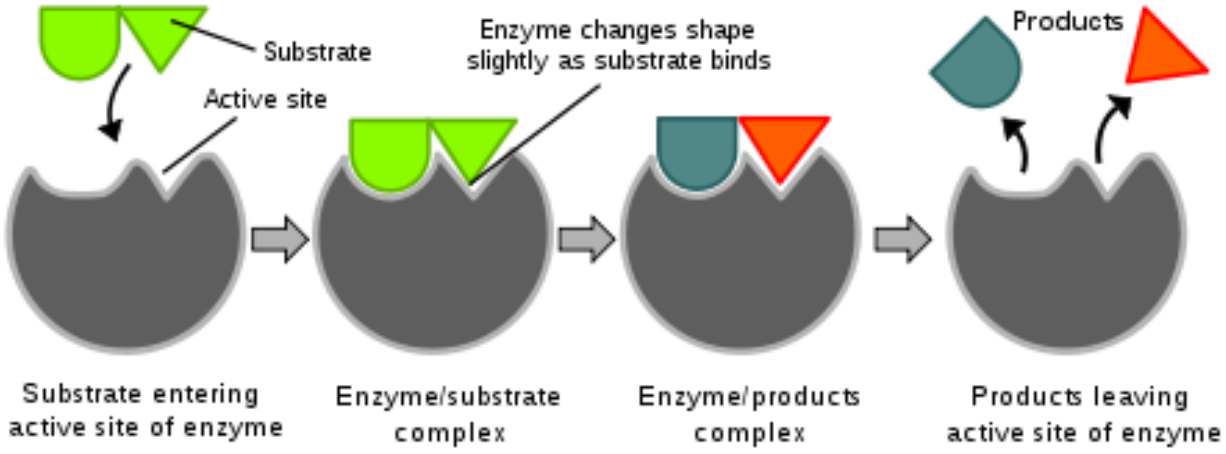
The formation of an enzyme-substrate complex increases the possibility for chemical reaction by:

1- Lowering the energy of activation, and

2- Reducing the element of chance in the collisions of molecules or ions.

The rate of reaction is accelerated through the catalytic action of the enzyme. A single enzyme molecule, even though it can react over and over, is only capable of combining with a given total number of substrate molecules per minute. This number known as the turnover number varies from enzyme to enzyme. Many enzymes have a high turnover number. For example, catalase has a turnover number of 5 million per minute. Thus enzymes are generally effective in relatively minute concentrations in the living cell.

The formation of enzyme-substrate complex is confined to relatively small areas of the enzyme molecule, known as active sites. The structure of a particular substrate may induce the enzyme to "mold" itself over the substrate. This may be illustrated schematically in the following way:

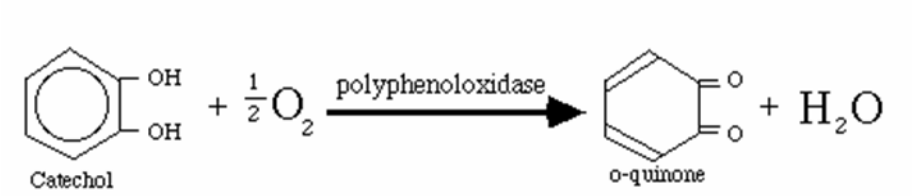



http://biochemistryquestions.wordpress.com/2008/07/15/induced-fit-model-of-enzyme-substrate

The "induced fit" hypothesis suggests that differences in the surface configuration (three-dimensional shape) of the active site are essential to specificity. In other words, only certain types of substrate molecule would be able to establish a close fit with a given type of enzyme molecule.

Because hundreds of reactions are simultaneously carried out in the living cell, it becomes difficult to study a single reaction in an intact living cell. However, it is possible to extract enzymes from cells and thus study enzyme catalyzed reactions in a test tube. In this experiment, a crude extract of the enzyme polyphenol oxidase will be prepared from the potato.

Polyphenol oxidase is a copper-containing enzyme with an optimum pH of 6.7. It catalyses the oxidation of di- and tri- hydroxyl phenol to the corresponding quinine.




This oxidation-reduction reaction is accompanied by a color change (quinines absorb light in the visible region of the spectrum). This reaction commonly occurs in nature and accounts for the "browning" of peeled potatoes and bruised fruits. You will familiarize yourself with the reaction catalyzed by the enzyme polyphenol oxidase, as it occurs removed from the intact living cell, i.e. in a test tube. This experiment is in four parts, corresponding to the four objectives listed on the first page.

## 1.3 Materials:

**0.01M catechol**

Dissolve 1.1g of catechol in 1 liter of distilled water. Adjust the pH to 6.0 with dilute NaOH to prevent auto oxidation. Keep this solution in a brown bottle in a refrigerator. (If the solution turns brown it is unfit for use.)

**0.1M NaF**

Dissolve 4.2g of sodium fluoride in 1 liter of distilled water.

**0.01M hydroquinone**

Dissolve 0.11g of hydroquinone in 100ml of distilled water.

**0.01M phenol**

Dissolve 94mg of phenol in 100ml of distilled water.

**5% trichloroacetic acid**

Dissolve 5g of trichloroacetic acid in 100ml of distilled water.

**5% trypsin**

Dissolve 5g of trypsin in 100ml of distilled water.

**Phenylthiourea**

A few grams of crystalline phenylthiourea should be available for the experiment.

Potatoes.

Homogenizer.

Cheesecloth.

Water baths at 37 and 70 ºC.

Container of crushed ice.

## 1.4 Method:

To detect and follow the progress of the reaction in this experiment a simple, qualitative method will be used. More sophisticated, quantitative methods of following enzyme catalyzed reactions will be introduced later in the course. In this experiment, record your observations according to the following scheme:

| **Degree of color intensity in test tube** | **symbol** |
|---|---|
| No color change (colorless) | – |
| Faint color (just detectable) | + |
| Definite color | ++ |
| Dark (deep) color | +++ |

The color should be brown, i.e. the color of the product (quinone). The intensity of this color will be proportional to the enzym's activity in the tube under observation.

**Preparation of enzyme extract**

1- Peel a small potato.
2- Cut a cube (2-4cms square) from the potato. Then cut this cube into small pieces and place them in a homogenizer.
3- Add 50ml of sodium fluoride solution (this inhibits other potato enzymes).
4- Grind the potato pieces for 1-2min in the homogenizer.
5- Pour the homogenate, through several layers of cheesecloth, into a 100ml beaker.

This is your crude extract containing the enzyme polyphenol oxidase.

Do not dispose of this enzyme extract until you have completed all parts of this experiment. Dispose of all waste (potato peel, cheesecloth, potato mash etc.) in the laboratory's waste container. It is absolutely essential to keep your glassware clean. Wash all your glassware thoroughly before and after each of the following experiments.

**1) Test tube enzymatic activity:**

a) Label three clean test tubes A, B and C.

b) Prepare each tube as follows:

Tube A: 15 drops of enzyme extract.
15 drops of 0.01M catechol solution.

Tube B: 15 drops of enzyme extract.
15 drops of distilled water.

Tube C: 15 drops of 0.0M catechol solution.
15 drops of distilled water.

c) Place all three tubes in a water bath at 37 ºC.

d) Shake each tube every 5 minutes to aerate, thereby adding oxygen to the solution.

e) Every 5 minutes, after shaking, hold the tubes up to the light and examine. Record the color in each tube, according to the scheme described in this page, in the table in the "Results" section. Continue for 25 minutes.

**2) Chemical nature of polyphenol oxidase:**

a) Label four clean test tubes A, B, C and D.

b) Prepare, and treat, each tube as follows:

Tube A: Add 15 drops of enzyme extract.
Add 15 drops of 0.01M catechol solution.
Shake tube and place in water bath at 37 ºC for 10 minutes.
Set tube aside as control with which to compare results of tubes B, C and D.

Tube B: Add 10 drops of enzyme extract.
Add 10 drops of 5% trypsin solution.

Shake tube thoroughly.

Place tube in a water bath at 37 ºC for 10 minutes.

Add 10 drops of 0.01M catechol solution.

Replace in the same water bath for 10 minutes.

Examine and compare with tube A.

Record your observations in the table in the "Results" section.

Tube C: Add 10 drops of enzyme extract.

Add 10 drops of 5% trichloroacetic acid.

Shake tube thoroughly and wait 5 minutes.

Add 10 drops of 0.01M catechol solution.

Place tube in water bath at 37 ºC for 10 minutes.

Examine and compare with tube A.

Record your observations in the table in the "Results" section.

Tube D: Add 15 drops of enzyme extract.

Add a few crystals of phenylthiourea.

Shake tube thoroughly and continue shaking it frequently during a period of 5 minutes.

Then add 15 drops of 0.01M catechol solution.

Place tube in water bath at 37 ºC for 10 minutes.

Examine and compare with tube A.

Record your observations in the table in the "Results" section.

Trypsin is a proteolytic enzyme in other words it hydrolyses the peptide bonds which link the amino acid residues, to denature and precipitate proteins, including enzymes. Phenylthiourea has a very strong chemical affinity for the element copper. It is able to bind with copper, even when the copper is attached to other chemical substances, as in the active site of polyphenol oxidase.

**3) Substrate specificity:**

a) Label three clean test tubes A, B and C.

b) Add 15 drops of enzyme extract to each tube.

c) Prepare each tube as follows:

Tube A: Add 15 drops of 0.01M catechol solution.

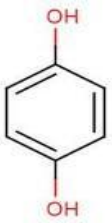
Tube B: Add 15 drops of 0.01M phenol solution.

Tube C: Add 15 drops of 0.01M hydroquinone solution.

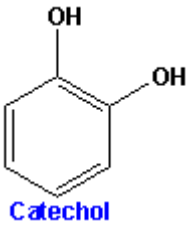
d) Shake the tubes gently and place them in a water bath at 37 ºC.

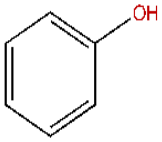
e) Examine the tubes after 5 minutes and after 10 minutes. Record the color in each tube, according to the scheme described on the page 3, in the table in the "Results" section.

The three compounds used as substrates in this part of the experiment are structurally related, as shown in the figure below. Each is capable of reacting with oxygen to form various colored products.




Hydroquinone




Phenol

**4) <u>Temperature and enzymatic activity:</u>**

a) Label three clean test tubes A, B and C.

b) Add 15 drops of enzyme extract to each tube.

c) Place each tube containing the enzyme extract, in a water bath for 10 minutes at the following temperatures:

Tube A: 0 ºC (in a container of crushed ice).

Tube B: 37 ºC.

Tube C: 70 ºC.

d) Add 15 drops of 0.01M catechol solution to each tube.

e) Shake each tube gently and quickly return it to its proper temperature condition.

f) Wait for 15 minutes. After this time, examine each tube, without removing it from its temperature condition, and record the color in each tube, according to the scheme described on page 3, in the table in the "Results" section.

## 1.5 Results:

### 1) Enzymatic activity

| Incubation time (minutes) | Degree of color intensity (Symbol: −, +, ++ or +++) | | |
|---|---|---|---|
| | Tube A | Tube B | Tube C |
| 0 | | | |
| 5 | | | |
| 10 | | | |
| 15 | | | |
| 20 | | | |
| 25 | | | |

### 2) Chemical nature of polyphenol oxidase:

| Tube | Treatment | Degree of color intensity (Symbol: −, +, ++ or +++) |
|---|---|---|
| A | Control | |
| B | Trypsin | |
| C | TCA | |
| D | Phenylthiourea | |

### 3) Substrate specificity:

| Substrate | Degree of color intensity (Symbol: −, +, ++ or +++) | |
|---|---|---|
| | 5 minutes | 10 minutes |
| Catechol | | |
| Phenol | | |
| Hydroquinone | | |

**4) Temperature and enzymatic activity:**

| Temperature (ºC) | Degree of color intensity (Symbol: −, +, ++ or +++) |
|---|---|
| 0 | |
| 37 | |
| 70 | |

In each of the above tables, the degree of color intensity may be considered proportional to enzymic activity.

## 1.6 Discussion and conclusions:

## 1.7 Questions:

1- In part 1 of the experiment, did you detect any reaction in tube B (enzyme extract and distilled water)? Give reasons why some reaction may be possible in this tube.
2- Can the enzyme be restored to an active state after TCA treatment (part 2)? Explain your answer.
3- What can you deduce about the specificity of polyphenol oxidase from part 3 of the experiment?
4- Explain the effects of temperature on the activity of polyphenol oxidase (part 4).
5- Suppose that two solutions of equal concentration were prepared, one of purified polyphenol oxidase and one of purified trypsin. Which solution, would you expect, would lose its enzymic activity first and why?

## 1.8 Reference:

Paum, S.J. and Bowen, W.R. (1972), Exercises in organic and biological chemistry, The Macmillan Company, 566 Third Avenue, New York 10022.

# Experiment 2

## 1. Methods of Enzyme Assay

1. End point assay: (colorimetric\endpoint assay) Alanine transaminase
2. Continuous assay: ( UV/ kinetics) Lactate dehydrogenase

### 2.1 Objective:

To study the different methods for determining enzyme activity.

### 2.2 Introduction:

All enzyme assays measure either the consumption of substrate or production of product over time.

Methods of quantitatively following enzyme reaction:

- Spectrophotometric methods
- Fluorescence methods
- Sampling methods
- Manometric methods
- Eletrode Methods
- Polarimetric Method

Enzyme assays can be split into two groups:

-Continuous assay , where the assay gives a continuous reading of activity

-Discontinuous assay, where the samples are taken, the reaction stopped then the concentration of . . . .

substrates/products determined.

Spectrophotometric methods:

1) Cases in which product absorbs but not the substrate.

e.g. Fumarate hydratase

2) The Co-enzyme NAD/ NADP have an absorption band at 340 nm in the reduced state

| Oxidized form | Reduced form |
|---|---|
| NAD | NADH |
| NADP | NADPH |
| FAD | $FADH_2$ |

Techniques for determine enzyme activities are 2 types (Spectrophotometer):

*Two point assay: the sample is incubation with the buffer substrate for a fixed period of time at the end of which the reaction is stopped and the amount of (P) or (S) used is measured (colorimetric techniques)

*Rate of reaction: assay changes are either measured at short intervals or are continuously

Enzyme classification:

1) Oxidoreductase
2) Transferase
3) Hydrolase
4) Lyase
5) Isomerase
6) Ligase

### 2.3 End point assay Alanine transaminase (colorimetric\endpoint assay)

An enzyme that catalyzes a type of reaction between an amino acid and α-keto acid.

•Specifically, this reaction (transamination) involves removing the amino group from the amino acid, leaving behind an α-keto acid, and transferring it to the reactant α-keto acid and converting it into an amino acid.

The enzymes are important in the production of various amino acids, and measuring the concentration of various transaminases in the blood is important in the diagnosing and tracking many diseases.

Alanine transaminase or ALT is a transaminase enzyme. It is also called: Serum glutamic pyruvic transaminase (SGPT) or Alanine aminotransferase (ALAT).

ALT is found in serum (at low level) but is most commonly associated with the liver

Function:

•It catalyzes the transfer of an amino group from alanine to a-ketoglutarate, to form pyruvate and glutamate under controlled condition (37°C) and pH 7.4 ± 0.05

Alanine + α- ketoglutarate → Pyruvate + glutamate

### 2.4 Principle:

- ALT catalyzes the reaction of L-alanine and α- ketoglutarate to form Pyruvate + glutamate
- The activity measure by following formation of pyruvate from alanine then react with 2,4-dinitrophenylhydrazine to convert 2,4-dinitrophenylhydrazone, then in alkaline pH can measure the absorbance at 546 nm.

### 2.5 Materials:

Test Tubes
Pipettes
Cuvettes
Water bath
Stop watch
Spectrophotometer
0.2M L-Alanine
2.0mM α-Ketoglutaric acid
100 mM Phosphate buffer at pH 7.4 $\pm$ 0.05
1.0 mM 2,4-Dinitrophenylhydrazine in 1 N hydrochloric acid
0.5 N Sodium hydroxide
Alanine transaminase

### 2.6 Preparation of solutions:

**1. ALT substrate:**

0.2 M L-Alanine, 2.0 mM α-Ketoglutaric acid, 100 mM Phosphate buffer at pH 7.4 $\pm$ 0.05. Also contains 0.2% v/v Preservative. Keep tightly capped and protected from contamination. Store at 2-8 °C.

**2. Color reagent:**

1.0 mM 2,4-Dinitrophenylhydrazine in 1 N hydrochloric acid , also contains 0.2% v/v preservative. Keep

tightly capped and protected from excessive exposure to direct sunlight. Store at 2-8 °C.

**3. Color developer:**

0.5 N Sodium hydroxide. Keep tightly capped and protected from excessive exposure to atmospheric $CO_2$ Can be stored at room temperature.

**4. ALT standard (40IU/L):**

Solution of Sodium pyruvate in 100 mM Phosphate buffer at pH 7.4 $\pm$ 0.05

equivalent to 40 IU/L of SGPT. Also contains 0.2% v/v Preservative. Keep tightly

capped and protected from contamination. Store at 2-8 °C.

### 2.7 Precautions:

Color reagent: contains 1 N Hydrochloric acid which CAUSES BURNS. In case of contact, flush affected area with large amounts of water. Seek medical attention.

Color developer: contains 0.5 N Sodium hydroxide which is CORROSIVE. In case of contact, flush affected area with large amounts of water. Seek medical attention.

### 2.8 Method*:

1. Label 3 test tube (1 to 3)
2. Tube 1 is a Blank and 2-3 are same sample (duplicate)
3. Use the table as procedure

| | Blank | Standard | Sample |
|---|---|---|---|
| ALT Substrate | 0.5 ml | 0.5 ml | 0.5 ml |
| Pre-warm at 37 °C for 5 minutes and add: (using timed intervals) | | | |
| Distilled Water<br>ALT Standard<br>Sample | 0.1 ml<br>-<br>- | -<br>0.1 ml<br>- | -<br>-<br>0.1 ml |
| Mix, and incubate at 37 °C for exactly 30 minutes (use same timed intervals), and add: | | | |
| Color Reagent 2,4 dinitrophenylhydrazine ( DNPH) | 0.5 ml | 0.5 ml | 0.5 ml |
| Mix, and return at 37 °C for exactly 10 minutes, then add: (use same timed intervals) | | | |
| 0.4M NaOH | 5 lm | 5 lm | 5lm |
| Mix, and return to 37 °C for exactly 5 minutes. Read absorbance of all tubes at 546 nm against blank. | | | |

**5 ml**

Stability of endpoint reaction:

The Final color produced in the reaction should be measured within 60 minutes.

* (in accordance to manufactures protocol for the kit used

## 2.9 Results:

1- Absorbance at 546 nm for tube 2= --------

2- Absorbance at 546 nm for tube 3= --------

3- The mean =------

The data shown in the table below is used to convert absorbance at 546 nm into enzymatic activity in U/L of serum. Draw graph using the data in table with absorbance on the Y-axis and enzymatic activity in U/L on the X-axis. Record your result in the spaces provided below the table.

| ALT activity | Absorbance at 546 nm |
|---|---|
| 2.5 | 0.025 |
| 5.5 | 0.050 |
| 9 | 0.075 |
| 12 | 0.100 |
| 17 | 0.125 |
| 21 | 0.150 |
| 25 | 0.175 |
| 30 | 0.2 |
| 35 | 0.225 |
| 41 | 0.250 |

Absorbance of the sample at 546nm=

ALT activity of serum sample (from graph) = ……U/L

## 2.10 Use the co-enzyme in measure the activity of Lactate dehydrogenase:

An enzyme that catalyzes the conversion of lactate to pyruvate. (LDH)

This is an important step in energy production in cells. Many different types of cells in the body contain this enzyme. Some of the organs relatively rich in LDH are the heart, kidney, liver, and muscle. It is responsible for converting muscle lactic acid into pyruvic acid, an essential step in producing cellular energy.

Lactic acid dehydrogenase (LDH) is an enzyme that helps produce energy. It is present in almost all of the tissues in the body and becomes elevated in response to cell damage. LDH levels are measured from a sample of blood taken from a vein.

Generally, the upper limit of normal for adults is in the range of 200 units/liter.

Lactic dehydrogenase is present in almost all body tissues, so the LDH test is used to detect tissue alterations and as an aid in the diagnosis of heart attack, anemia, and liver disease.

Principle:

- UV light is often used, since the common coenzymes NADH and NADPH absorb
- UV light in their reduced forms, but do not in their oxidized forms.

NAD+ does not absorb Ultraviolet at 340 nm.

NADH strongly absorbs Ultraviolet at 340 nm.

- Rate can be measured as disappearance of reactant or accumulation of product.

If NADH product: increase the absorbance / min

If NADH substrate: decrease the absorbance / min

LDH activity and it can be followed

Lactate +NAD -------- pyruvate +NADH

The rate of NADH formation is indicated by the increase in absorbance at 340 nm

## 2.11 Method:

| | |
|---|---|
| tnegaeR HDL | 3 lm |
| Pre-warm at 37°C for 3 minutes and add Sample | 0.1 lm |

Mix and incubated at 37 °C for 1 minute, then read absorbance (at 340 nm against distilled water) every minute for 3 minutes) and determine ΔA/min

## 2.12 Results:

| Tube | emit | ecnabrosba 340 mn |
|---|---|---|
| B | 1 minute | |
| C | 2 minute | |
| D | 3 minutes | |

**Calculations:**

ΔA1 =(C-B) /1

ΔA2=(D-C) /1

(ΔA2+ΔA1)/2 =A

U\L=Δ A/min x 3.1 x 106 / 6.22 x 103 x 1 x 0.1

= ΔA/min x 4984

**Unit definition:**

One unit (U/L) is the amount of enzyme that will reduce one micromole of NAD per minute per liter of sample at specified temperature

U\L= ΔA/min x total volume x 106 / ε x d x sample volume

106 = Factor to convert milimole /ml to micromole/L

ε = molar absorptivity? of NADH at 340 nm (6.22 x 103)

d=Light path in cm

## 2.13 Discussion and Conclusion:

## 2.14 References:

Reitman, S & Frankel, S. (1957) Am J Clin Pathol., 28, 56-63.

# Experiment 3 to 5

## 3. Purification of acid phosphatase from wheat germ

### 3.1 Objectives:

1) To purify acid phosphatase from wheat germ as a rich source of the enzyme and for its availability.
2) To assay total enzyme activity at different purification steps.
3) To assay protein of different fractions and calculate specific activity.
4) To calculate the efficiency of the methods used for purification.

### 3.2 Introduction:

Acid phosphates (ACP) (orthophosphoric-monoester phosphohydrolases) are a group of enzymes that are widely distributed in nature and catalyze the hydrolysis of a variety of phosphate esters in an acidic environment to produce inorganic phosphate. ACP acts on a wide range of monoester of orthophosphoric acid either aliphatic such as glycerol-1-phosphate or aromatic like 4-nitrophenyl phosphate. It does not act on phosphoric diesters or triesters. $Mg^{++}$ ion act as an activator for the enzyme.. The hydrolysis of phosphate esters is an important process in energy metabolism, metabolic regulation and a wide variety of cellular signal transduction pathways. This hydrolysis reaction is often use to assay the activity of acid phosphates.The ACP optimum temperature and pH varies according to the tissue source e.g. ACP prostatic enzyme is labile and loses 50% of its activity after 1 hour at room temperature. Generally the ACP is unstable above 37◦C and pH>7. Acidification of the sample (pH <6.5) help to stabilize the enzyme. The ACP is found in cell organelles (lysosomes) and tissues.

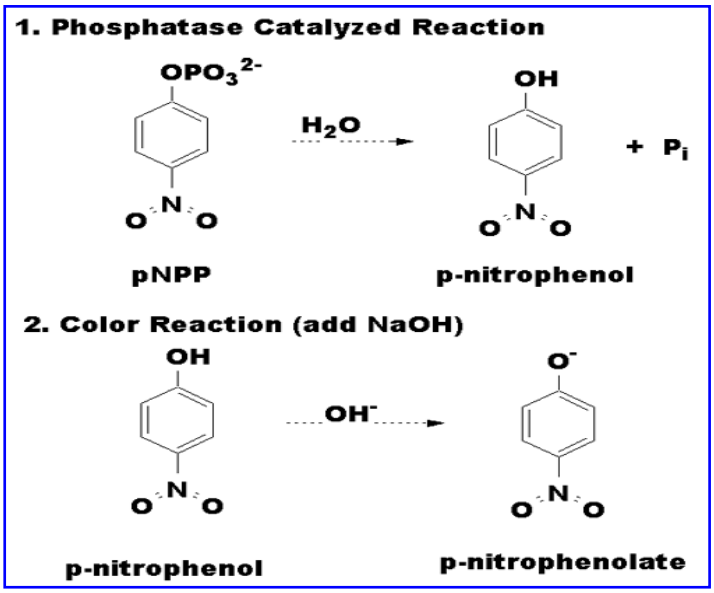
The highest source of ACP is prostate gland. Osteoclasts are rich source of ACP and serum ACP level is higher in growing children than adult. There is no single or simple way to purify all proteins. Nevertheless there are certain fundamental principles of protein purification upon which most fractionation procedures are based. The advantage, of small differences in the physical and chemical properties of the many proteins in a crude mixture, is taken to bring about fractionation of the proteins from one another. A procedure leading to a homogenous protein from a natural source

usually requires the sequential application of many techniques (e.g. solubility properties, adsorptive properties, chromatographic separation, heat denaturation and electrophoresis.) .

In this experiment the solubility properties has been used to purify Acid phosphates from wheat germ. The addition of salt such as ammonium sulphate results in precipitation of the protein. Step–by-step application of such technique to heterogeneous protein solutions often results in fractionation (purification) because of the differing degrees of solubilities among the proteins in the solutions.

Several methods can be used for determination of the purified protein e.g. Biuret test.

To measure ACP activity, a substrate called p-nitro phenol phosphate is usually used. This substance is colorless. However, it is hydrolyzed by acid phosphates to yield phosphate + nitro phenol. The nitro phenol is yellow. The amount of yellow color generated by the catalysis is a direct measure of the amount of nitro phenol produced and therefore, is an indicator of the enzyme activity.




After incubation of enzyme and substrate, the reaction is terminated by the addition of KOH.

### 3.3 Materials:

Wheat germ as a source of the enzyme

Distilled water

Cheesecloth (for filtration wheat germ)

filter paper

aluminum foil

scissors

thread and tape

dialysis bag

ice and plastic bucket

beakers(20ml,50ml,80ml,100ml)

volumetric flasks(100ml,250ml,500ml,1000ml)

plastic centrifuge tubes 15ml

spatulas and glass rods

measuring cylinders(50ml,100ml,300ml,500ml)

Pasteur pipettes

pipettes(0.05ml,1ml,2ml,5ml,10ml)

dark containers

test tubes and racks

thermometer

cuvettes

pH meter

balance

centrifuge

water bath

magnetic stirrer and magnetic hot plate

vortex

spectrophotometer

$MnCl_2$, 1M

Sodium acetate buffer, 1M (pH 5.7)

Sodium acetate buffer, 0.05M (pH 5.7)

$(NH_4)_2SO_4$, saturated (pH 5.5)

BSA standard, 3mg/ml

EDTA, 0.2M (pH5.7)

EDTA, 0.005M (pH5.7)

Methanol, prechilled to -20ºC

$MgCL_2$, 0.1M

KOH, 0.5M

PNPP, 0.05M

HCl conc. for adjusting the pH

NaOH 1M for adjusting the pH

Biuret reagent; sodium potassium tartar ate, $CuSO_4$. $5H_2O$, 10%NaOH

$Na_2HPO_4$, 0.006M

### 3.4 Method:

#### 3.4.1 **Part I:** Acid phosphatase isolation and purification:

Isolation of Acid phosphatase:

1. Suspend 50g of wheat germ in 200ml distilled water. Let the mixture stand for 30 min with occasional stirring.
2. Form a sac with two layers of cheese cloth and pass the suspension through it. Squeeze the residue as dry as possible. The material passed through the cheesecloth is the filtrate. Discard the dry residue inside the sac. Measure the filtrate volume.
3. Centrifuge the filtrate for 5min at 5000rpm .Decant the supernatant into a gradual cylinder and record its volume. Supernatant is known as the crude extract fraction I . Measure the Fraction I volume.
4. Remove and freeze a 1ml aliquot of fraction I for later assay of protein concentration and enzyme activity.

**Purification of Acid phosphatase:**

1. Place a plastic bucket, filled with ice, on a magnetic stirrer. Insert a 150ml beaker into the ice.
2. Transfer fraction I to the beaker and add 2ml of 1M MnCl2 for every 100ml fraction I (100ml fraction I→2ml$MnCl_2$)
3. Centrifuge for 5min at 5000rpm and collect the supernatant which is fraction II record the volume.
4. Suspend the pellet in 15 to 25 ml of 0.05M sodium acetate buffer (pH 5.7) by means of vortexing until the suspension appears uniform; remove undissolved protein by a 3 min centrifugation at 5000rpm. The pellet obtained at this point may be discarded. The supernatant is denoted as fraction III and its volume is recorded. Remove and freeze 1ml.

5. Place a plastic bucket filled with ice on a magnetic stirrer. Insert a 400ml beaker into the ice
6. Transfer the reminder of fraction II in the beaker and add slowly with gentle stirring, cold, saturated ammonium sulfate to fraction II. Add 54ml of ammonium sulfate for every 100ml of fraction II being processed:

   This brings the solution to 35% saturation in ammonium sulfate; the addition should be done slowly over a period of 5-10min to avoid denaturation of proteins which is indicated by the formation of off-white foam at the surface of the solution.
7. Continue stirring for 10-15 min after all the ammonium sulfate has been added.
8. Centrifuge for 5min at 5000rpm and collect the supernatant and measure its volume:
9. This supernatant is transferred to the 400ml beaker in the ice bath on the magnetic stirrer for a second fractionation with ammonium sulfate:

   It's added in the same procedure as mentioned above. This brings the solution to 57% saturation in ammonium sulfate.
10. Suspend the pellet from step 8 as step 4 after centrifugation the pellet may be discarded and the supernatant is fraction IV remove and freeze a 1ml aliquot then freeze the reminder separately.
11. Transfer the beaker from step 9 to a 70°C water bath and stir gently with a thermometer. Allow the solution to warm to 60$^{o}$C and maintain it at that temperature for exactly 2 min. After this heat treatment, transfer the beaker quickly into the ice water bath (placed in the freezer). Stir the solution with the thermometer until the temperature has dropped to 6-8C
12. Centrifuge at 5000rpm for 5min, collect the supernatant and measure its volume denoted as fraction V. Remove and freeze a 1ml aliquot then freeze the reminder separately.
13. Suspend the pellet obtained in step 12 in 20ml of cold distilled water. After the pellet has been evenly suspended centrifuge the solution for 5min at 5000rpm to remove undissolved protein.The obtained pellet may be discarded and the supernatant is denoted as fraction VI. Measure the volume of Fraction VI volume.The purification may be interrupted at this point and the preparation may be stored frozen at -20C.
14. Determine the protein conc. in fraction VI from the standard curve prepared as shown in the table below:

| Reagent | Tube Numbers | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| BSA (bovine serum albumin) standard, 3mg/ml(ml) | - | 0.2 | 0.4 | 0.7 | 1 | 2 | 3 | - |
| unknown(ml from all fractions | - | - | - | - | - | - | - | 1 |
| H2O (ml) | 3 | 2.8 | 2.6 | 2.3 | 2 | 1 | - | 1 |

a- Add 3 ml of Biuret reagent to each tube and mix. Let the tubes stand at room temperature for 30 min, then measure the absorbance at 540nm zeroing the instrument on water.

b- Construct a standard curve from the absorbances in tubes 2-7 and determine the concentration of the unknown samples from the fractions. Tube 8 is the unknown sample.

c- From standard curve in the result calculate the concentration for each fraction from 1 to 6.

15. To the solution obtained in step 13 add 0.1ml of 0.2M EDTA and 0.05ml of saturated ammonium sulfate for every 1ml of solution being processed.
16. To the solution obtained in step 15 add 1.75ml of prechilled methanol with gentle stirring for every 1ml of solution being processed. The methanol must be as cold as possible when added to the solution.
17. Centrifuge for 5 min at 5000rpm, collect the supernatant and determine its volume
18. Save the bulk of this supernatant by storage at -20C. Measure out a 20ml aliquot and place it in a dialysis bag (cut the appropriate length of dialysis bag and wash it in

19. distilled water then place it in distilled water over night, tie the bag from below with thread, pour distilled water into the bag to insure that there is no leaking then pour the sample into the bag and tie the bag from above, place the bag in a beaker filled with distilled water in a bucket filled with ice on a magnetic stirrer for 6 hours) The resulting dialyzed solution is denoted as fraction VII. Remove and freeze 1ml aliquot and freeze the reminder. Measure volume of Fraction VII volume .
20. Suspend the pellet from step 17 in 5ml of distilled, cold water
21. Centrifuge for 5min at 5000rpm. Collect the supernatant and re suspend the pellet in 5ml of cold distilled water.
22. Centrifuge the suspension for 5 min at 5000rpm. Collect the supernatant and discard the pellet.
23. Combine the two supernatants obtained in steps 20 and 21 this represents fraction VIII record the volume of fraction VIII. Remove and freeze a 1ml aliquot and transfer the reminder of fraction VIII to the dialysis bag . Measure volume of Fraction VIII.
24. Dialyze it for 6 hours as mentioned above.
25. Collect the dialyzed solution denoted as fraction IX. Measure its volume . Remove and freeze a 1ml aliquot and freeze the reminder of the fraction.

### 3.4.2 Part two: Assay of acid phosphatase:

To do the enzymatic assay we must chose one fraction from the nine which contain the highest concentration of the enzyme. To do this we will take the diluted fractions and undiluted fractions, do the Biuret reaction and calculation concentration from standard curve.

#### 3.4.2.1 (1) protein determination:

a-Thaw the saved 1mL aliquots of fractions I- IX from the enzyme purification and mix each fraction gently by inversion. Assay the fractions for protein concentration using the Biuret method. From each thawed fraction remove 0.1mL and diluted accurately with water according to the suggested dilution schedule shown in table above.

b-For Biuret method takes 1mL from each diluted fraction +2mL $H_2O$+ Add 3 ml of Biuret reagent to each tube and mix. Let the tubes stand at room temperature for 30 min, then measure the absorbance at 540nm zeroing the instrument on water.

c- Use the standard curve from step 14 to determine the concentration of the unknown samples from the fractions.

#### 3.4.2.2 (2) Enzyme Assay:

1- The enzyme fractions must be diluted so that the substrate (PNPP) will not be used up immediately but rather be converted to product (PNP) in a liner fashion during the 15 minutes of incubation.
2- The enzyme assay do for each diluted and undiluted enzyme fractions by this stapes:

a) Begin by adding 0.2mL of enzyme fraction to 0.9mL distilled water set up ten tubes. Tube 1 will be for a reagent blank; tubes 2 through 10 will be for the enzyme fractions I through IX.
b) To each of the 10 tube add 0.1mLof 1M sodium acetate buffer (pH=5.7) + 0.1mL of 0.1M $MgCl_2$.
c) Initiate the reaction by adding 0.1mLof (0.05M) PNPP, mix and incubation for 15 min.
d) After incubation stop the reaction by add4mL of KOH .
e) Measure the absorbance against a blank at 405nm.

## 3.5 Results:

## 3.6 Discussion and conclusion:

### 3.7 Reference:

1.Verjee Z.H.M. Isolation of Three Acid Phosphatases from Wheat Germ European J. Biochem. 9 (1969) 439-44

2. Switzer. R.L. , Clark. J. M .Jr. Experimental Biochemistry (2nd. Ed.) 1977, Freeman and company.

# Experiment 6

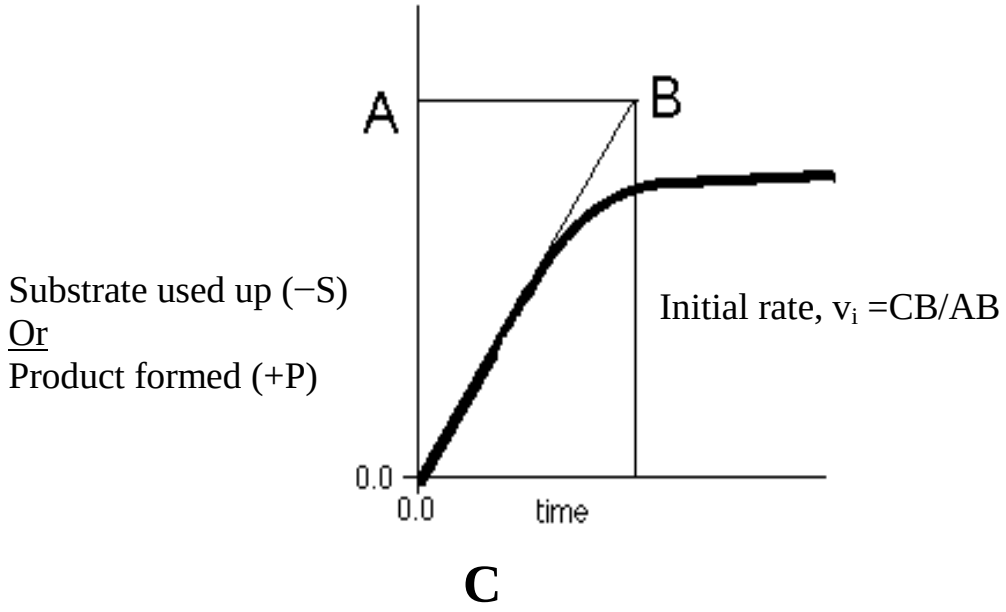
## 4. The effect of incubation time on the rate of an enzyme catalyzed reaction

### 4.1 Objectives:

1) To monitor the progress of an enzyme catalyzed reaction.
2) To determine the initial rate of the reaction ($v_i$).

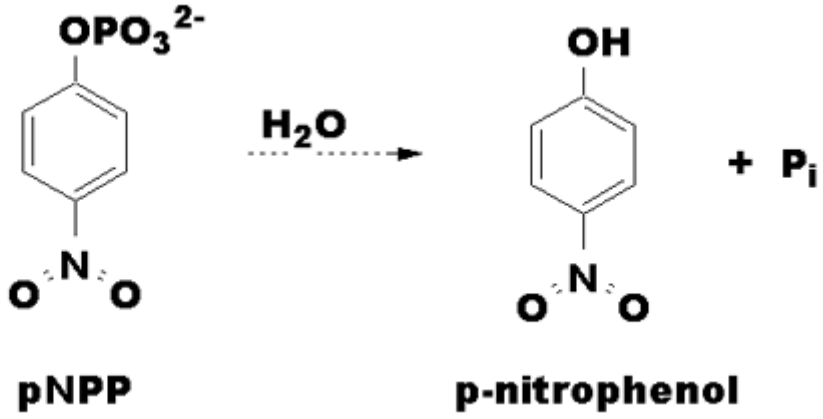
### 4.2 Introduction:

The progress of an enzyme catalyzed reaction may be followed by measuring either the quantity of substrate used up or the quantity of product formed and plotting against time. Typically, a curve of the following type is obtained:




The rate of the reaction is highest at time zero and decreases with increasing time, eventually falling to zero itself, where the above curve reaches a plateau. This usually occurs either when all the substrate is used up or when equilibrium is reached. The initial rate of reaction, $v_i$, measured as the tangent to the above curve at the origin (time= 0), is used in the study of enzyme kinetics and is affected by factors which include enzyme concentration, substrate concentration, temperature and pH.

For all of the above mentioned experiments, the acid phosphatase will be used. Under acid conditions, the enzyme catalyzes the hydrolysis of p-nitrophenyl phosphate (pNPP) to inorganic phosphate and p-nitrophenol (Figure-1). If base is added to the mixture after the completion of the reaction, the p-nitrophenol is converted to a colored form which absorbs lights at 405 nm. Assuming a path length of 1.0 cm and an extinction coefficient o f18.8 x $10^3$ liter $mol^{-1}$ $cm^{-1}$, you can use the absorbance at 405 nm to calculate the number of micromoles of p-nitrophenol released. Since this is a fixed-time assay that is stopped after 5 minutes, the velocity of the reaction (µmoles of p-nitrophenol/minute) can be easily computed.




**ACID PHOSPHATASE**
**pH=5.7**

Figure-1. The hydrolysis of p-nitrophenyl phosphate by acid phosphate. pNPP, p-nitrophenylphosphate.

## Time course of the reaction.

The assay you are using is called a fixed-time assay because the reaction is stopped after 5 minutes and the velocity is calculated assuming that the relationship between product yield and time has been linear throughout. The object of this exercise is to demonstrate the validity of that assumption. Set up a series of identical enzyme reaction tubes each of which is allowed to incubate for a different period of time (zero through 30 minutes). The results should indicate how long the reaction is linear under the given conditions of substrate and enzyme concentration.

## 4.3 Materials:

Chemicals:

1.0M Sodium acetate buffer

0.1M Magnesium chloride

0.05M p-nitrophenyl phosphate

0.5M Potassium hydroxide

Stock solution of crude/ purified wheat germ Acid Phosphatase

### 4.3.1 Equipments:

pH meter

Water bath

Spectrophotometer

Glassware:

Test tubes

Pipettes

### 4.3.2 Preparation of Solutions:

(1)1.0M Sodium acetate buffer, pH 5.7: Dissolve 5.74ml of glacial acetic acid in 80ml of distilled water. Adjust pH to 5.7 by using 10M NaOH . Make up volume of buffer to 100ml using distilled water.

(2) 0.5M potassium hydroxide: Dissolve 28.1gm of KOH in 500ml distilled water.

(3) 0.1M Magnesium hydroxide: Dissolve 1.015gm of $MgCl_2.6H_2O$ in 500ml distilled water.

(4) 0.05M pNPP: Dissolve 1.68gm of pNPP in 100ml of distilled water

(5) Acid phosphatase (crude extract)from wheat germ: In a 250ml beaker kept in crushed ice transfer 50gm of wheat germ and add 200ml cold distilled water, mix and allow to stand for 30minutes with occasional stirring. Centrifuge the mixture at for 10 minutes at 3500 rpm. The supernatant is collected and stored in freezer

## 4.4 Method:

1. Prepare a series of seven reaction tubes labeled 0 through 30 minutes at 5-minute intervals (0, 5, 10 … minutes).
2. To each of these tubes add 0.5 ml of 1.0M sodium acetate buffer (pH 5.7), 0.5 ml of 0.1M $MgCl_2$, 0.5 ml of 0.05M p-nitrophenyl phosphate, and 5ml of distilled water.
3. Place all the tubes in a test rack situated in a water bath maintained at 37 ºC and let the temperature equilibrate for 5 minutes.
4. Add 0.5ml of the enzyme (appropriately diluted acid phosphatase) to the tube marked 0 minutes and immediately stop the reaction by adding 0.5ml of 0.5M KOH. This zero-time tube will serve as the blank against which all the others will be compared.
5. Add 0.5ml of the enzyme to the tube marked 5 minutes, mix, start the stopwatch, and let the reaction proceed for 5 minutes before adding the KOH to terminate the reaction.
6. Run all of the other reaction tubes in exactly the same fashion with the exception that each successive tube will be incubated for 5 minutes longer than the previous one (total reaction times to equal 0, 5, 10,…30 minutes). An efficient way to do this is to start each reaction at 2-minutes intervals, keeping an eye on the stopwatch and stopping each of the reactions at the appropriate time. It is helpful to prepare a schedule of events (Table 5-1) before you begin.
7. After all the reactions have been terminated, determine the absorbance at 405 nm for each sample. The zero-time sample should be used as the blank.
8. Repeat this experiment two more times.

Table 5-1. Schedule for time course experiment.

| Total incubation time (min) | Clock time (min) | |
|---|---|---|
| | Start reaction (add enzyme) | Stop reaction (add KOH) |
| 0 | 0 | 0 |
| 5 | 0 | 5 |
| 10 | 2 | 12 |
| 15 | 4 | 19 |
| 20 | 6 | 26 |
| 25 | 8 | 33 |
| 30 | 10 | 40 |

## 4.5 Results:

| Incubation time | Absorbance at 405nm |
|---|---|
| 0 (blank) | |
| 5 | |
| 10 | |
| 15 | |
| 20 | |
| 25 | |
| 30 | |

- Use the extinction coefficient ($18.8 \times 10^3$ liter $mol^{-1}$ $cm^{-1}$) for p-nitrophenol to calculate the micromoles of product released at each time point.
- Prepare a graph, plotting μmoles of p-nitrophenol released against time.

## 4.6 Discussion and conclusion:

## 4.7 Questions:

1. Is the time course linear throughout? If the time course is not linear, what are some factors that might contribute to the changed velocity at longer time period?
2. Is the 5-minute fixed-time assay valid for acid phosphatase? If not, how should it be changed?
3. Determine the initial velocity (v) for the acid phosphatase reaction from the slop of the linear part of the graph.

## 4.8 Reference:

G.Douglas Crandall, Selected exercises for the Biochemistry Laboratory, Chap.5, Oxford University Press, New York.

# Experiment 7

## 5. The effects of enzyme concentration on the rate of an enzyme catalyzed reaction.

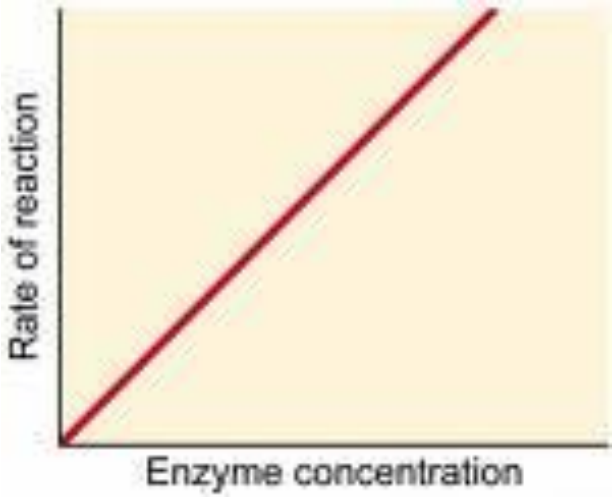
### 5.1 Objectives:

To establish the relationship between enzyme concentration and the rate of an enzyme catalyzed reaction.

### 5.2 Introduction:

This exercise illustrates the effect of increasing enzyme concentrations on reaction rate. You will perform a series of 5-minutes assays, in which a different enzyme concentration is added each time the reaction is initiated.

Provided that substrate remains in excess, the rate of an enzyme catalyzed reaction is directly proportional to increasing enzyme concentration. This relationship is shown in the figure below. The results should indicate the range of enzyme concentrations that yield a linear response.



## 5.3 Materials:

Chemicals:

1.0M Sodium acetate buffer

0.1M Magnesium chloride

0.05M p-nitrophenyl phosphate

0.5M Potassium hydroxide

Stock solution of crude/ purified wheat germ Acid Phosphatase

### 5.3.1 Equipments:

pH meter

Waterbath

Spectrophotometer

Glassware:

Test tubes

Pipettes

## 5.4 Method:

1) Label 7 test tubes (A, B, C, D, E, F, and G) and blank.
2) Pipette the following solutions as indicated in the following table:

| Tube no. | Buffer pH 5.7ml | $MgCl_2$ (ml) | Substrate (ml) | Dis. Water (ml) |
|---|---|---|---|---|
| A | 0.5 | 0.5 | 0.5 | 5.3 |
| B | 0.5 | 0.5 | 0.5 | 5.2 |
| C | 0.5 | 0.5 | 0.5 | 5.1 |
| D | 0.5 | 0.5 | 0.5 | 5.0 |
| E | 0.5 | 0.5 | 0.5 | 4.9 |
| F | 0.5 | 0.5 | 0.5 | 4.7 |
| G | 0.5 | 0.5 | 0.5 | 4.5 |
| Blank | 0.5 | 0.5 | 0.5 | 5.5 |

3) Place the tubes in the water bath at 37 ºC for 5 minutes.

4) Start the reaction by adding the enzyme at 2 minutes intervals as in the following table:

| Tube no. | Enzyme (ml) | Start the reaction (min.) | Stop the reaction (min.) |
|---|---|---|---|
| Blank | 0 | 0 | 0 |
| A | 0.2 | 0 | 5 |
| B | 0.3 | 2 | 7 |
| C | 0.4 | 4 | 9 |
| D | 0.5 | 6 | 11 |
| E | 0.6 | 8 | 13 |
| F | 0.8 | 10 | 15 |
| G | 1.0 | 12 | 17 |

5) Stop the reaction by adding 0.5 ml KOH after 5 min. as indicated in the previous table.

6) Read the absorbance at 405 nm against the blank.

## 5.5 Results:

| Tube no. | Enzyme concentration | Absorbance at 405nm |
|---|---|---|
| Blank | | |
| A | | |
| B | | |
| C | | |
| D | | |
| E | | |
| F | | |
| G | | |

● Use the extinction coefficient for p-nitrophenol to determine the micromoles of product produced in 5 minutes at each of the enzyme concentrations.

● Plot velocity against enzyme concentration (units/ml). Describe the shape of this curve and discuss the reasons for its shape.

## 5.6 Discussion and conclusion:

## 5.7 Questions:

1. What is the valid range of enzyme concentrations for the acid phosphatase assay?

## 5.8 Reference:

G.Douglas Crandall, Selected exercises for the Biochemistry Laboratory, Chapter 5, Oxford University Press, New York. 1983

# Experiment 8

## 6. The effect of temperature on the rate of an enzyme catalyzed reaction

Objectives:

1 To establish the relationship between temperature and the rate of an enzyme catalyzed reaction

2. To determine the optimum temperature for such a reaction

## 6.1 Part I: The effect of temperature on the rate of an enzyme catalyzed reaction:

### Introduction:

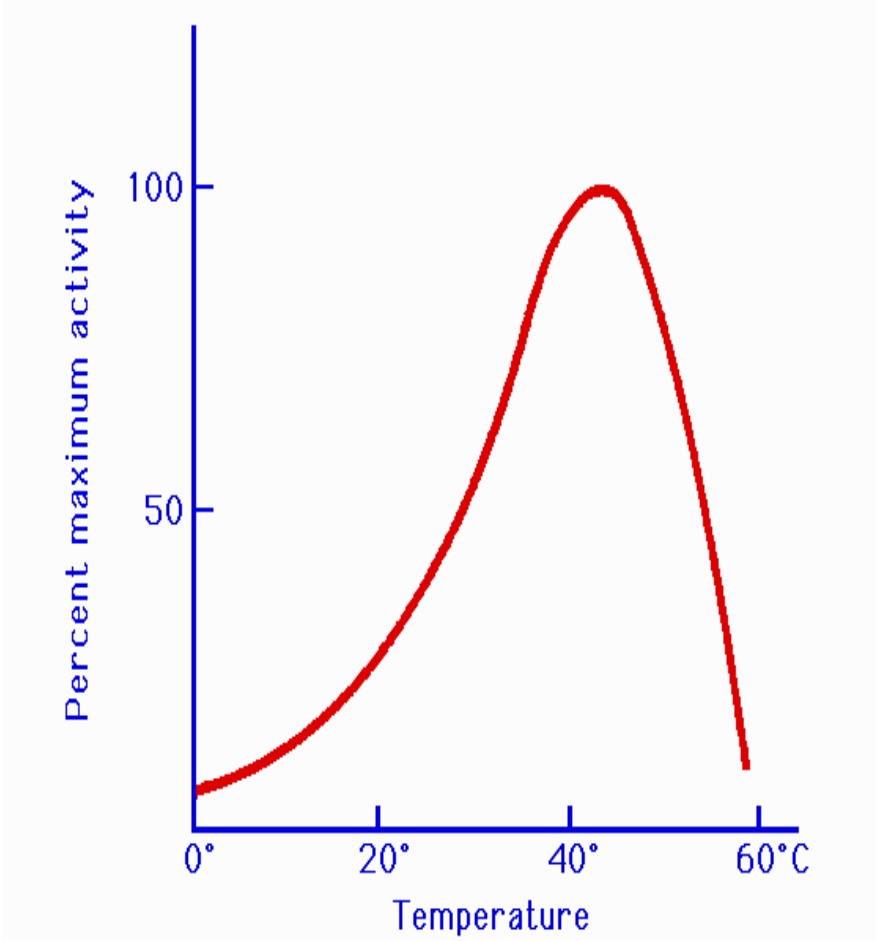
The rate of an enzyme catalyzed reaction is affected by changes in temperature. At low temperature ($0^oC$), the rate of reaction is low. As the temperature is increased, the rate of reaction increase until an optimum temperature is reached. Within this temperature range, the rate of reaction is approximately doubled for every 10 $^oC$ rise in temperature. With further rise in temperature, above the optimum temperature, the rate of reaction decreases due to denaturation of the enzyme protein and hence loss of activity.

The optimum temperature is the result of the balance between the rate of an increase in the enzyme activity on the one hand and the rate of decrease due to denaturation on the other.

Most enzymes are inactivated at temperatures above 60 $^oC$ .

For most enzymes, the optimum temperature is at or above the temperature of the cells in which the enzyme is found in vivo.

The relationship between the rate of the enzymatic reaction and the temperature is shown in the figure below:




## 6.2 Materials:

Test tubes

Pipettes

Cuvettes

Water bath

Stopwatch

Spectrophotometer

Acid phosphatase enzyme

0.05 M PNPP

1 M sodium acetate buffer(PH 5.7)

0.1 M MgCl2

0.5 M KOH

## 6.3 Method:

1. One factor that is critical to the success of these experiments is the preparation and maintenance of water baths at different temperatures. Once a bath has been adjusted, its temperature should be continuously monitored and all temperature changes recorded. Baths prepared in the manner described in the following table:

**Table:**

Procedure for the preparation of water baths of different temperatures:

| Desired temperature ($^{o}$C) | Method of preparation |
|---|---|
| 0-4 | Ice plus tap water in an ice bucket |
| 10 | Tap water and ice |
| 20 | Tap water at room temperature |
| 30 | Thermostatted water bath |
| 37 | Thermostatted water bath |
| 50 | Thermostatted water bath |
| 80 | Hot tap water |
| 100 | Boiling water bath |

2. The effect of performing the reaction at different temperatures on the rate of reaction:

a. Label two assay tubes (A(blank),B) and into each of them pipette 0.5ml of 1.0M sodium acetate buffer (pH 5.7), 0.5ml of 0.1M $MgCl_2$, 0.5ml of 0.05M p-nitropheny1 phosphate and 5ml of distilled water. Place the tubes in a water bath maintained at 0 to 4 $^{o}$C and let the temperature equilibrate for

5 minutes.

c. Add 0.5 ml of enzyme to tube B and allow the reaction to proceed for 5 minutes before stopping

it with the addition of 0.5ml of 0.5M KOH . Tube A which serves as a reagent blank, should be treated in the same fashion except that 0.5ml of distilled water should be added to the reaction mixture instead of enzyme.

d. Place the tubes in a test tube rack at room temperature.

e. Repeat steps a through d using all the water bath temperatures described in the previous table.

When all of the reaction mixtures have returned to room temperature, determine the absorbance

at 405 nm of each experimental tube against its own blank tube A.

## 6.4 Results:

| Temperature($^{o}$C ) | Absorbance at 405 nm |
|---|---|
| 0 | |
| 10 | |
| 20 | |
| 30 | |
| 37 | |
| 50 | |
| 80 | |
| 100 | |

- Convert absorbance data to velocity data.
- Plot a graph illustrating the effect of different temperatures on the rate of the reaction.

## 6.5 Discussion and conclusion:

## 6.6 Questions:

1. Does the graph conform to your expectations? If not, why not?
2. What is the optimum temperature for acid phosphatase?
3. Why do you think it was necessary to prepare individual reagent blank to be run at each of the temperatures?

## 6.7 References:

- Crandall G.D. , (1983), selected exercises for the Biochemistry laboratory, Oxford university press, inc. New York
- Lehninger A.L. , (1993), principles of Biochemistry, Worth publisher,inc. , New York

# Experiment 9

## 7. The effect of pH on the rate of an enzyme catalyzed reaction

### 7.1 Objectives:

a) To establish the relationship between pH and the rate of an enzyme catalyzed reaction.

b) To determine the optimum pH for such a reaction.

### 7.2 Introduction:

The rate of an enzyme catalyzed reaction is affected by changes in pH.

Enzymes have an optimum pH or pH range in which their activity is maximal.

Optimum pH: is defined as the pH at which the rate of reaction is maximum.

At higher or lower pH, the rate of an enzymatic reaction decrease.

For most enzymes, the optimum pH lies in the range from pH 5 to pH 9

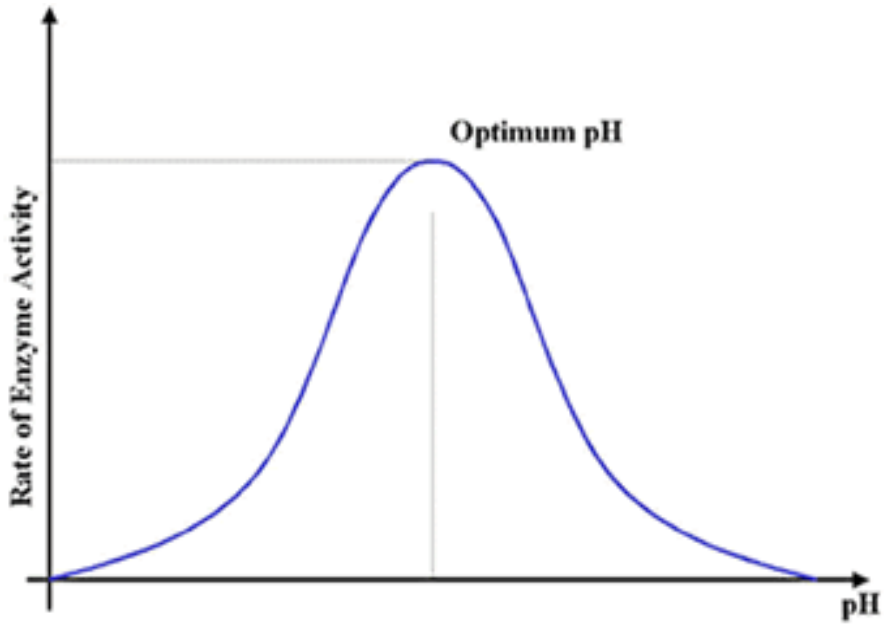
For the majority of enzymes, the relationship between the rate of an enzymatic reaction and pH takes form of a bell-shape. (Figure B)

The shape of pH activity curve is determined by the following factors:

1- Enzyme denaturation at extremely high or low pH

2- Effects on the charged state of the substrate or enzyme.

**Figure B**




### 7.3 Materials:

Test tubes

Pipettes

Cuvettes

Water bath

Stopwatch

Spectrophotometer

Acid phosphatase enzyme

0.05 M PNPP

1 M sodium acetate buffer of different PH

0.1 M MgCl2

0.5 M KOH

### 7.4 Method:

1. Prepare 16 tubes labeled as follows:
   ($A_3$ (blank), $B_3$, $A_4$, $B_4$, $A_{4.5}$, $B_{4.5}$, $A_5$, $B_5$, $A_{5.5}$, $B_{5.5}$, $A_6$, B6, $A_7$, $B_7$, $A_8$, $B_8$).
2. To each of these tubes add:

0.5ml of correspondingly pH sodium acetate buffer (note: there is a blank for each pH)

0.5ml of MgCl2

0.5ml of p-nitropheny1 phosphate

5ml of distilled water

3. Place the tube in a test tube rack situated in 37$^{o}$C water bath and let stand for 5 minutes.

4. Initiate each assay at 2-minute intervals by adding 0.5ml of the enzyme, run each reaction for 5 minutes, and stop it by adding 0.5ml of KOH. (Note: for the blank, the reaction is terminated by adding 0.5ml of KOH immediately after adding the enzyme).

5. Determine the absorbance at 405 nm of each experimental tube against its own blank (tube A).

## 7.5 Results:

| pH | Absorbance at 405 nm |
|---|---|
| 3 | |
| 4 | |
| 4.5 | |
| 5 | |
| 5.5 | |
| 6 | |
| 7 | |
| 8 | |

- Convert absorbance data to velocity data.
- Plot a graph illustrating the effect of different pH on the rate of the reaction.

## 7.6 Discussion and conclusion:

## 7.7 Question:

1-What is the optimum pH for acid phosphatase?

## 7.8 References:

- Crandall G.D. , (1983), selected exercises for the Biochemistry laboratory, Oxford university press, inc. New York
- Lehninger A.L. , (1993), principles of Biochemistry, Worth publisher,inc. , New York

# Experiment 10

## 8. The effect of substrate concentration and inhibitor (Inorganic phosphate and Sodium fluoride) on the rate of an enzyme catalyzed reaction

## Part A:

The effect of substrate concentration on the rate of an enzyme catalyzed reaction

### 8.1 Objectives:

1) To establish the relationship between substrate concentration and the rate of an enzyme catalyzed reaction.
2) To determine the Km and Vmax of the enzyme for a particular substrate.

### 8.2 Introduction:

The effect of substrate conc. on the rate of an enzyme catalyzed reaction when the enzyme is held constant is shown in figure A

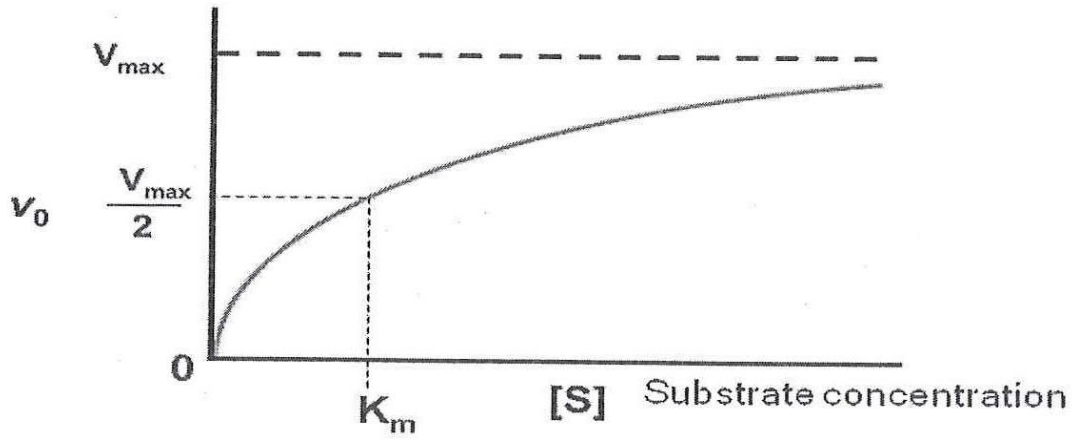
As the substrate level is increased, the velocity increases in a hyperbolic fashion.

-At relatively low concentration of substrate, the rate of reaction increase linearly with an increase in substrate concentration.

-At higher substrate concentration, the rate of reaction increases by smaller and smaller amounts in response to increase in substrate concentration.

-Finally, a point is reached beyond which there are only small increase in the rate of the reaction with increasing substrate concentration. This plateau is called the maximum velocity, Vmax

Figure A: Michaelis – Menten plot




The hyperbolic shape of this curve can be expressed algebraically by the Michaelis – Menten equation:

$$Vi = \frac{Vmax\ [S]}{Km\ [S]}$$

Vi = initial velocity

Vmax = maximum velocity

[S] = substrate concentration

Km = Michaelis – Menten constant

Km: is substrate concentration at half Vmax

( Km indicate the affinity of an enzyme for its substrate )

The Km can vary greatly from enzyme to enzyme, and even for different substrates of the same enzyme.

Transformation of the Michaelis – Menten equation:

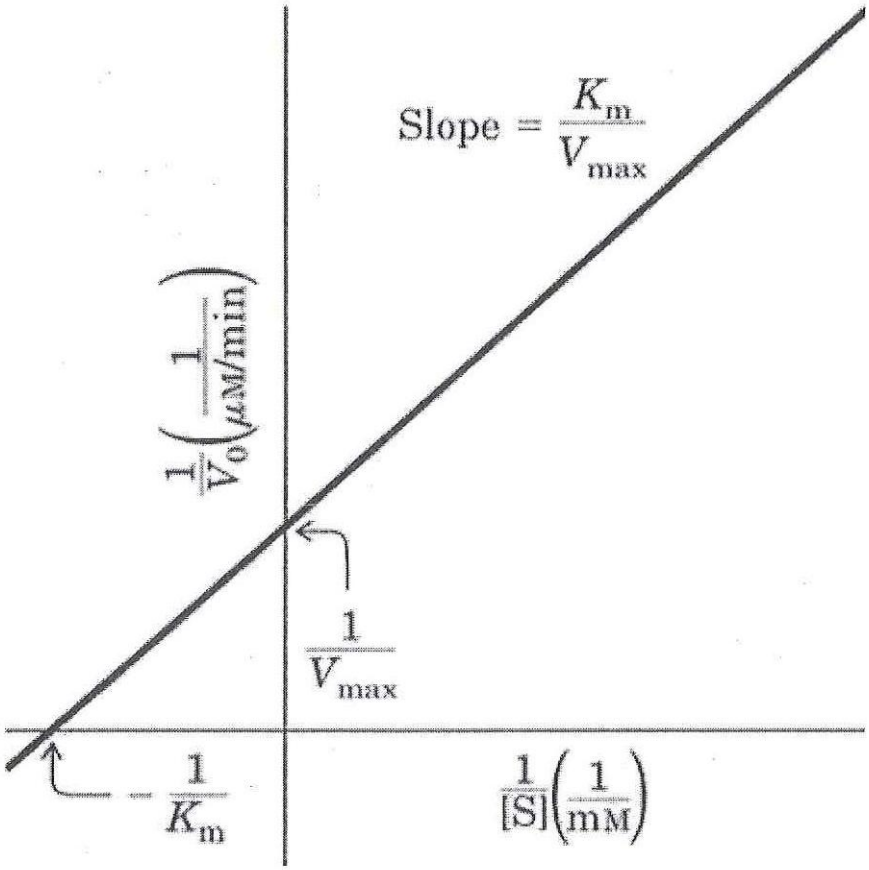
The double – Reciprocal plot(Lineweaver – Burk plot):

The Michaelis – Menten equation can be algebraically transformed into forms that are useful in the practical determination of Km and Vmax .

- One common transformation is derived simply by taking the reciprocal of both sides of the Michaelis – Menten equation to give Lineweaver – Burk equation:

$$\frac{1}{Vi} = \frac{1}{Vmax} + \frac{Km}{Vmax} \cdot \frac{1}{[S]}$$

-By plotting $\frac{1}{Vi}$ against $\frac{1}{[S]}$ ,a straight line plot, the Lineweaver – Burk plot, is obtained as shown below:



Both Vmax and Km can be obtained accurately from the intercepts of the straight line with the y-axis and x-axis.

The object of this experiment is to demonstrate the effect of performing the standard 5-minute assay in the presence of different substrate concentrations. The results should provide classic Michaelis-Menten data from which approximations of $V_{max}$ and $K_m$ can be made. Double-reciprocal plots of the same data should be done to arrive at even more exact values for $K_m$ and $V_{max}$ .

## 8.3 Materials:

Test tubes
Pipettes
Cuvettes
Water bath
Stopwatch
Spectrophotometer
Acid phosphatase enzyme
0.05 M PNPP
Different substrate concentration
1 M sodium acetate buffer
0.1 M MgCl2
0.5 M KOH

## 8.4 Method:

1. Prepare a series of substrate dilutions according to the protocol outlined in Tables I .
2. Set up eight assay tubes labeled according to the various substrate concentrations . To each of these tubes add 0.5 ml of 1.0 M sodium acetate buffer (pH 5.7), 0.5 ml of 0.1 M $MgCl_2$, and 5ml of distilled water.To each tube add 0.5 ml of the correspondingly diluted substrate (p-nitropheny1 phosphate).

Table I : Protocol for the dilution of substrate (p-nitropheny1 phosphate).

| Tube | 0.05M PNPP (ml) | Distilled water (ml) | Concentration of dilute PNPP (M) |
|---|---|---|---|
| A | 0 | 5.0 | 0 |
| B | 0.05 | 4.95 | 0.0005 |
| C | 0.10 | 4.90 | 0.0010 |
| D | 0.25 | 4.75 | 0.0025 |
| E | 0.50 | 4.50 | 0.005 |
| F | 1.00 | 4.0 | 0.010 |
| G | 2.5 | 2.5 | 0.025 |
| H | 5.0 | 0 | 0.050 |

PNPP=p-nitropheny1 phosphate.

3. Place the tubes in a test tube rack situated in a 37$^{o}$C water bath and let stand for 5 minutes.
4. Initiate each assay at 2-minute intervals by adding 0.5ml of the enzyme, run each reaction for 5 minutes, and stop it by adding 0.5ml of 0.5M KOH. Note that the final concentration of substrate in each reaction tube during the assay is 0. 0.05, 0.10, 0.25, 0.50, 1.0. 2.5, and 5.0 mM p-nitrophenyl phosphate.
5. Determine the absorbance at 405 nm for each reaction mixture against the blank. (The tube containing no substrate should be used as the blank).

## 8.5 Results:

| Tube number | Substrate concentration | Absorbance |
|---|---|---|
| B | | |
| C | | |
| D | | |
| E | | |
| F | | |
| G | | |
| H | | |

- Determine the amount of p-nitrophenol produced in 5 minutes for each substrate concentration.
- Calculate the velocity (µmoles of p-nitrophenol/minute) for each substrate concentration.
- Plot velocity against substrate concentration (mmoles p-nitropheny1 phosphate) in the standard manner of Michaelis and Menten. Determine $V_{max}$ and Km for acid phosphatase.
- Calculate the reciprocals of velocity (1/v) and substrate concentration (1/[S]) and present these data as a table.
- Prepare the double –reciprocal plot of Lineweaver and Burk and determine the Km and Vmax from the x and y intercepts.

## 8.6 Discussion and conclusion:

## 8.7 Questions:

1. Investigate the literature and see how your values for Km and Vmax compare with published ones for acid phosphatase.

## 8.8 References:

1- Crandall G.D. , (1983), selected exercises for the Biochemistry laboratory, Oxford university press, inc. New York

2- Lehninger A.L. , (1993), principles of Biochemistry, Worth publisher,inc. , New York

# Experiment 10 Part B:

## 9. The inhibition of acid phosphatase by inorganic phosphate and sodium fluoride

### 9.1 Objectives:

To determine the type of inhibition of acid phosphatase by inorganic phosphate and sodium fluoride.

### 9.2 Introduction:

There exist a number of molecular species which, in the presence of an enzyme and its substrate, have the effect of binding to the enzyme (or to the enzyme-substrate complex) and totally or partially inhibiting the reaction. In those cases where the binding is irreversibly, the reaction is inalterably inhibited and not subject to kinetic analysis. If the binding is reversible, however, the specific type of inhibition can be determined by kinetic analysis.

The three types of inhibition that can be clearly distinguished in this manner are competitive, noncompetitive, and uncompetitive. Experimentally, these are distinguished by amount of the inhibitor at ever-increasing concentrations of the substrate. When the inhibited reaction is compared with the normal reaction using the graphic analyses of Michaelis and Menten or Lineweaver and Burke, the type of inhibition is clearly indicated. In the case of competitive inhibition, high substrate concentrations wipe out the inhibitory effect and the $V_{MAX}$ for the inhibited reaction is identical to that for the uninhibited reaction (Figure 1).

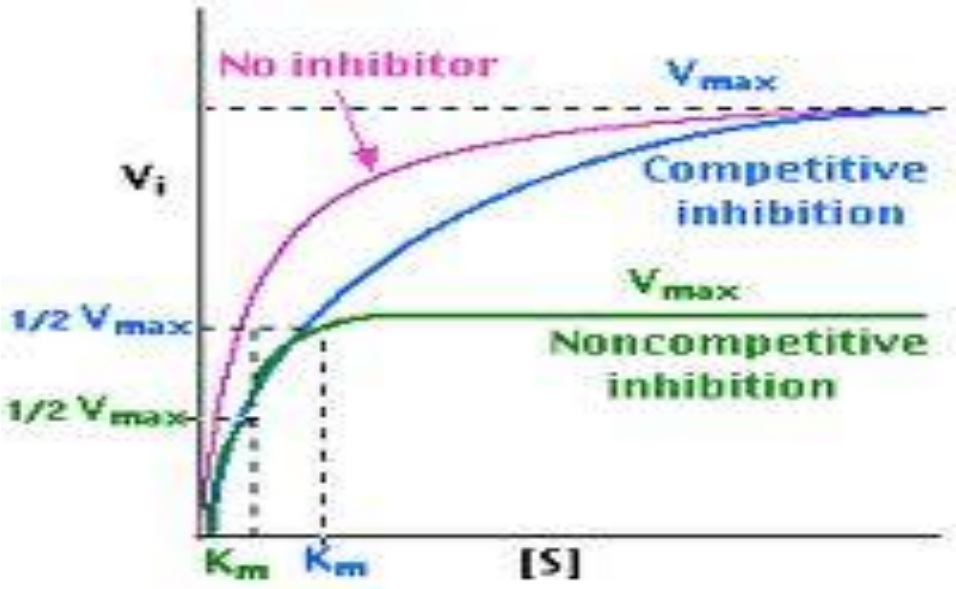



Figure 1 : A Michaelis-Menten plot for an enzymatic reaction performed in the presence and absence of competitive and noncompetitive inhibitor.

The $K_M$ of the inhibited reaction ($K_M$) is significantly higher than that of the reaction run in the absence of inhibitor, which indicates an apparent decrease I the affinity of the enzyme for its substrate. Noncompetitive inhibition yields the curve indicated in Figure 1, with a lower $V_{MAX}$ and a $K_M$ identical to the reaction in the absence of inhibitor.

Uncompetitive inhibition is characterized by a low $V_{MAX}$, a lower $K_M$ and a Michaelis-Menten curve similar to that of noncompetitive inhibition. The best way to distinguish the three types of inhibition graphically is to use the Lineweaver-Burk plot (Figure 2). Notice that in the case of uncompetitive inhibition the slope of the inhibited curve ($K_M$/ $V_{MAX}$) is the same as that of the noninhibited curve, whereas in the other two types of inhibition, the slope of the inhibited plot is greater.

In summary, it is relatively simple to distinguish the three types of reversible inhibition by comparing the Michaelis-Menten and Lineweaver-Burke kinetics in the presence and absence of the inhibitor.

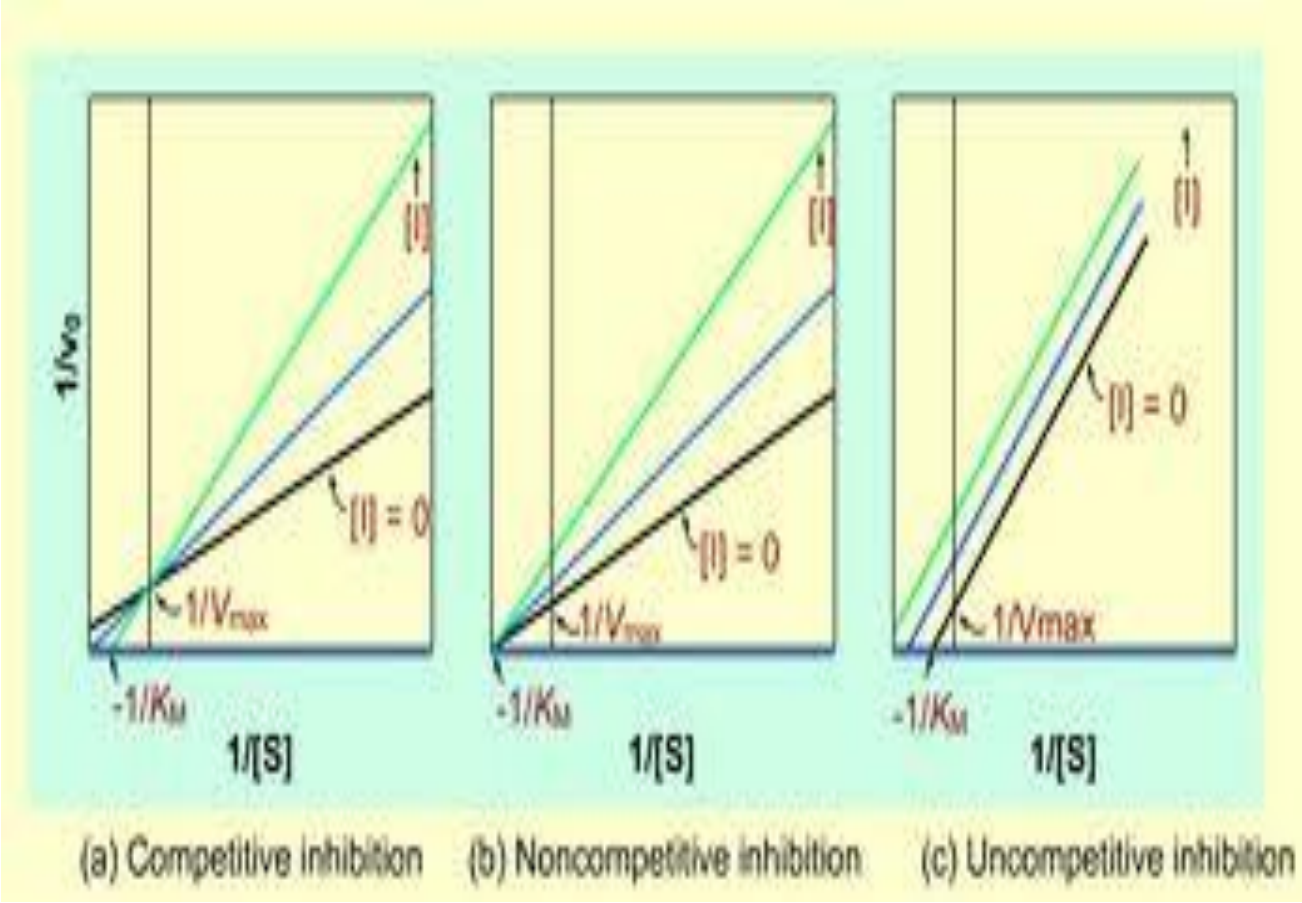



Figure 2. A Lineweaver-Burke plot for an enzymatic reaction performed in the absence of inhibitor and in the presence of increasing concentrations of competitive, noncompetitive and uncompetitive inhibitors.

The inhibition of acid phosphatase by inorganic phosphate.

Inorganic phosphate (Pi) and sodium fluoride are inhibitors of acid phosphatase and it is your task to determine whether they are competitive, noncompetitive, or uncompetitive inhibitor. The kinetics for the uninhibited reactions must be compared with those of reactions run in the presence of the inhibitor. The setup is basically the same as in the experiment for the effect of substrate concentration on reaction velocity, except that a constant amount of

phosphate (1.0 mM $K_2HPO_4$) or sodium fluoride (5mM) will be present in each reaction tube. Run the reactions as before and compare Michaelis-Menten and Lineweaver-Burke plots in

the presence and absence (kinetic properties II) of the inhibitor. Determinations of $V_{MAX}$ and $K_M$ will determine the specific mode of inhibition.

### 9.3 Materials:

Chemicals:
1.0M Sodium acetate buffer
0.1M Magnesium chloride
0.05M para-nitrophenyl phosphate
0.5M Potassium hydroxide
0.005M Potassium hydrogen phosphate
5mM Sodium fluoride
Stock solution of crude/ purified wheat germ Acid Phosphatase

### 9.4 Equipments:

pH meter
Waterbath
Spectrophotometer
Glassware:
Test tubes
Pipetts

### Preparation of Solutions:

(1) 0.005M $K_2HPO_4$: Dissolve 0.174gm of $K_2HPO_4$ in 200ml of distilled water.
(2) 0.005M Sodium fluoride:Dissolve 0.05gm of NaF in 200ml distilled water.

## 9.5 Method:

1. Use the same set of substrate dilutions prepared for the experiment on effect of substrate concentration on rate of enzyme catalyzed reaction.(0 to 0.05 M p-nitrophenyl phosphate).
2. Prepare eight reaction tubes labeled in accordance with substrate concentrations to be used. To each tube add 0.5 ml of 1.0 M sodium acetate buffer (pH 5.7), 0.5 ml of 0.1M $MgCl_2$, 4ml of distilled water, and 1.0ml of 0.005M $K_2HPO_4$ or 5mM Sodium

3. fluoride . To each tube add 0.5ml of the appropriate diluted substrate (p-nitrophenyl phosphate). Note that each tube contains a different substrate concentration and the identical inhibitor concentration.
4. Place the tubes in a 37 ºC water bath for 5 minutes.
5. Begin the reaction in each assay tube at 2-minute intervals by adding 0.5 ml of the enzyme, let the reaction proceed for 5 minutes, and then stop them by adding 0.5ml of 0.5M KOH.
6. Determine the absorbance at 405 nm for each sample, using the first tube (0 mM p-nitrophenyl phosphate) as the blank.
7. Repeat the experiment two more times.

## 9.6 Results:

| Tube | Concentration of dilute PNPP (M) | Absorbance at 405nm | | |
|---|---|---|---|---|
| | | Without inhibitor | With inorganic phosphate as inhibitor | With sodium fluoride as inhibitor |
| A | 0 | | | |
| B | 0.0005 | | | |
| C | 0.0010 | | | |
| D | 0.0025 | | | |
| E | 0.005 | | | |
| F | 0.010 | | | |
| G | 0.025 | | | |
| H | 0.050 | | | |

● Determine the amount of p-nitrophenol produced in 5 minutes for each substrate concentration.

● Calculate the velocity (μmoles of p-nitrophenol/minute) for each substrate

● Plot velocity against substrate concentration (mmoles p-nitrophenyl phosphate) in the standard manner of Michaelis and Menten. Determine $V_{MAX}$ and $K_M$ for acid phosphatase.

● Calculate the reciprocals of velocity (1/v) and substrate concentration (1/[S]) and present these data as a table.

● Prepare the double-reciprocal plot of Lineweaver and Burk and determine the $V_{MAX}$ and $K_M$ from the x and y intercepts.

● Prepare Michaelis-Menten and Lineweaver-Burke plots that compare the inhibited reaction with the uninhibited reaction.

● Determine the $K_M$ and $V_{MAX}$ in the presence of phosphate and sodium fluoride.

## 9.7 Discussion and Conclusion:

## 9.8 Questions:

1. Determine if phosphate and fluoride are a competitive, noncompetitive, or uncompetitive inhibitor? Justify your answer.
2. Discuss the difference you find
3. Investigate the literature to determine how your results compare with those of previous workers.
4. What do you think would happen if you ran this same experiment substituting ATP for the inorganic phosphate?

## 9.9 Reference:

G.Douglas Crandall, Selected exercises for the Biochemistry Laboratory, Chap.5, Oxford University Press, New York. 1983

# Experiment 11

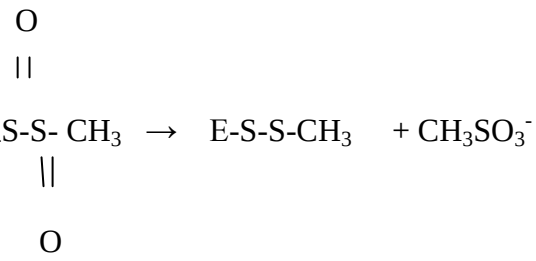
## 10. Modification of active site cysteine of Lactate Dehydrogenase

### 10.1 Objectives:

(i)To determine the time course of chemical modification of the enzyme .
(ii) To provide evidence that the reagent is modifying the active site of the enzyme.

### 10.2 Introduction:

The reagent methyl methane thiosulphonate (MMTS) is used to thiomethylate proteins in order to investigate the role of thiol groups in catalysis. This reagent reacts with the free –SH groups according to the equation :




Lactate dehydrogenase is one of the proteins that is susceptible to thiomethylation and this leads to an enzyme with altered catalytic properties compared with the native enzyme. This reaction would increase the understanding of the catalytic role of the thiol group.

### 10.3 Materials:

0.05 potassium phosphate buffer,pH 7.4
0.02M sodium pyruvate in phosphate buffer
3mg/ml NADH in phosphate buffer
1M methyl methane thiosulphonate
0.2mg/ml LDH in 0.1M phosphate buffer
0.01M$NAD^+$ in phosphate buffer

0.05M oxalate in phosphate buffer

pH meter
Waterbath
Spectrophotometer
Glassware:
Test tubes
Pipettes

### 10.4 Method:

**Modification of LDH**

1. Take two test tubes and label them A and B. Add 0.5ml of samples of LDH solutions (0.2mg/mlin buffer) to both the tubes. Add 0.5ml of buffer and keep at 27°C.

2. Remove 0.05ml samples from tube A &B and add them into 0.95ml of cold buffer on ice and label these samples A-0 and B-0.

3. To the original solution A now add 0.05ml of MMTS solution (1M) and note the time (time 0). Also add 0.05ml of water to solution B.

4. After 1,2,5,10,20 and 30 minutes , remove 0.05ml samples from solution A and add to 0.95ml of cold buffer on ice and lable these A-1,A-2, A-5, etc. Also at 30minutes remove 0.05ml from tube B into 0.95ml of cold buffer on ice to give B-30.

   The effect of dilution into cold buffer (2 above)

Protection of enzyme from modification by MMTS using substrate or substrate analogues
If the MMTS is reacting with a cysteine at the active site then performing the modification in presence of substrates or substrate analogues will protect the active site and reduce the rate of modification. For LDH this may be achieved by using $NAD^+$ and oxalate as protecting agents. Oxalate is acting as an analogue of the normal substrate.
(1)Set up Tube C:-
Tube C : 0.5ml LDH + 0.05ml $NAD^+$ 0.05ml oxalate +0.04ml buffer
(2)Remove a 0.05ml sample into 0.95ml of cold buffer (see 2 above) and label as C-0.

(3) Add 0.05ml MMTS to C (time 0) . After 15 minutes and 30 minutes remove 0.05 ml samples from C and label C-15 and C-30.

Ideally this experiment should be done at the same time as the normal modification experiment .

**Assay of LDH activity**

The assay of the enzyme activity of all the samples that have been taken has to be done in order to measure the effect of the MMTS under the various experimental conditions.

This assay depends on recording the decrease in absorbance at 340nm as NADH is oxidized according to the equation:

Pyruvate $+ NADH + H^+ \rightarrow$ lactate $+ NAD^+$

Assay conditions are as follows:

(1)2.6ml buffer + 0.1ml NADH + 0.1ml sodium pyruvate (0.02M) + 0.2ml LDH solution.

(2)Use 3ml of buffer for the blank cuvette. Set recorder at 30mm/min.

(3) Add all the reagents except the enzyme , mix well and record the O.D.

The O.D. should be about 0.8. Check that the absorbance remains constant over about 15 seconds. Now add the LDH solution and record the reaction over a suitable time period to allow an accurate measurement of the initial rate.

## 10.5 Results:

| S.oN. | ebutTo | absorbance at 340nm |
|---|---|---|
| | | |
| | | |
| | | |
| | | |
| | | |

## Calculations:

(1) Determine the specific activity of unmodified LDH in µmoles/min/mg.

(2) Draw a graph showing the loss of activity with time.

## 10.6 Discussion and conclusion:

## 10.7 Reference:

Bloxham, D.P. & Wilton, D.C. (1977)Biochem.J.**161**, 643-651.