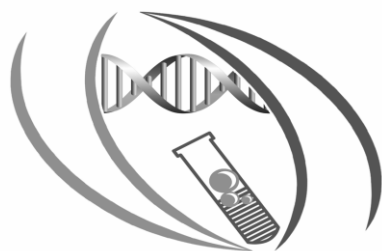


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**Practical Note
EXPERIMENTAL ENZYMOLOGY
(BCH 322)**

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Experiment 1

1. Methods of Enzyme Assay

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

1.1Objective:

To study the different methods for determining enzyme activity.

1.2Introduction:

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

1.3 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 α -ketoglutarate, to form pyruvate and glutamate under controlled condition (37°C) and $\text{pH } 7.4 \pm 0.05$

Alanine + α - ketoglutarate \rightarrow Pyruvate + glutamate

1.4 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.

1.5 Materials:

Test Tubes

Pipettes

Cuvettes

Water bath

Stop watch

Spectrophotometer

0.2M L-Alanine

2.0mM α -Ketoglutaric acid

100 mM Phosphate buffer at $\text{pH } 7.4 \pm 0.05$

1.0 mM 2,4-Dinitrophenylhydrazine in 1 N hydrochloric acid

0.5 N Sodium hydroxide

Alanine transaminase

1.6 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 ± 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 CO₂ Can be stored at room temperature.

4. ALT standard (40IU/L):

Solution of Sodium pyruvate in 100 mM Phosphate buffer at pH 7.4 ± 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.

1.7 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.

1.8 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	0.1 ml	-	-
ALT Standard	-	0.1 ml	-
Sample	-	-	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)			
5 ml 0.4M NaOH	5 ml	5 ml	5 ml
Mix, and return to 37 °C for exactly 5 minutes. Read absorbance of all tubes at 546 nm against blank.			

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

1.9 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

1.10 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

1.11 Method:

LDH Reagent	3 ml
Pre-warm at 37°C for 3 minutes and add Sample	0.1 ml

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 $\Delta A/\text{min}$

1.12 Results:

Tube	time	absorbance 340 nm
B	1 minute	
C	2 minute	
D	3 minutes	

Calculations:

$$\Delta A_1 = (C - B) / 1$$

$$\Delta A_2 = (D - C) / 1$$

$$(\Delta A_2 + \Delta A_1) / 2 = A$$

$$U/L = \Delta A / \text{min} \times 3.1 \times 10^6 / 6.22 \times 10^3 \times 1 \times 0.1$$

$$= \Delta A / \text{min} \times 4984$$

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

$$U/L = \Delta A / \text{min} \times \text{total volume} \times 10^6 / \epsilon \times d \times \text{sample volume}$$

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

1.13 Discussion and Conclusion:

1.14 References:

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

Experiment 2

2 Purification of acid phosphatase from wheat germ

2.1 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.

2.2 Introduction:

Acid phosphatases (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 phosphatases. 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

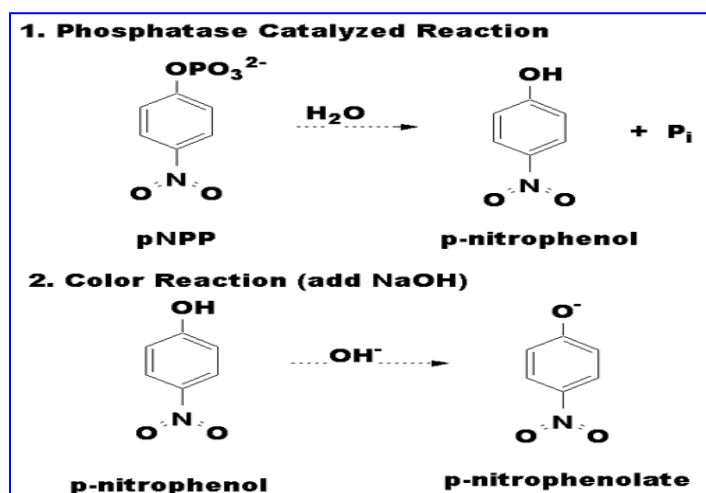
properties of the many proteins in a crude mixture, is taken to bring about fractionation of the proteins from one another. A procedure leading to a homogenous protein from a natural source

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.

2.3 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

MnCl₂, 1M

Sodium acetate buffer, 1M (pH 5.7)

Sodium acetate buffer, 0.05M (pH 5.7)

(NH₄)₂SO₄, saturated (pH 5.5)

BSA standard, 3mg/ml

EDTA, 0.2M (pH5.7)

EDTA, 0.005M (pH5.7)

Methanol, prechilled to -20°C

MgCl₂, 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, CuSO₄. 5H₂O, 10%NaOH

Na₂HPO₄, 0.006M

2.4 Method:

2.4.1 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 MnCl_2 for every 100ml fraction I (100ml fraction I \rightarrow 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 remainder 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 remainder 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°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 6-8°C

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 remainder. 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 remainder 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 remainder of the fraction.

2.4.2 Part II: 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.

(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 H₂O+ 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.

(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 linear 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 0.1mL of 1M sodium acetate buffer (pH=5.7) + 0.1mL of 0.1M MgCl₂.

- c) Initiate the reaction by adding 0.1mL of (0.05M) PNPP, mix and incubation for 15 min.
- d) After incubation stop the reaction by add 4mL of KOH .
- e) Measure the absorbance against a blank at 405nm.

2.5 Results:

2.6 Discussion and conclusion:

2.7 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 3

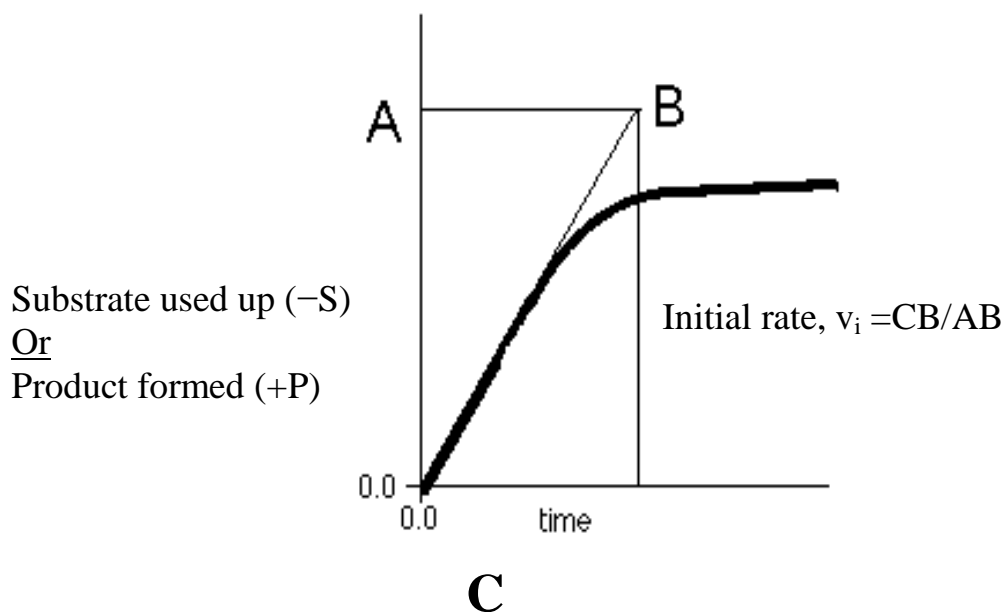
3) The effect of incubation time on the rate of an enzyme catalyzed reaction

3.1 Objectives:

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

3.2 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 of 18.8×10^3 liter $\text{mol}^{-1} \text{cm}^{-1}$, 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 ($\mu\text{moles of p-nitrophenol/minute}$) can be easily computed.

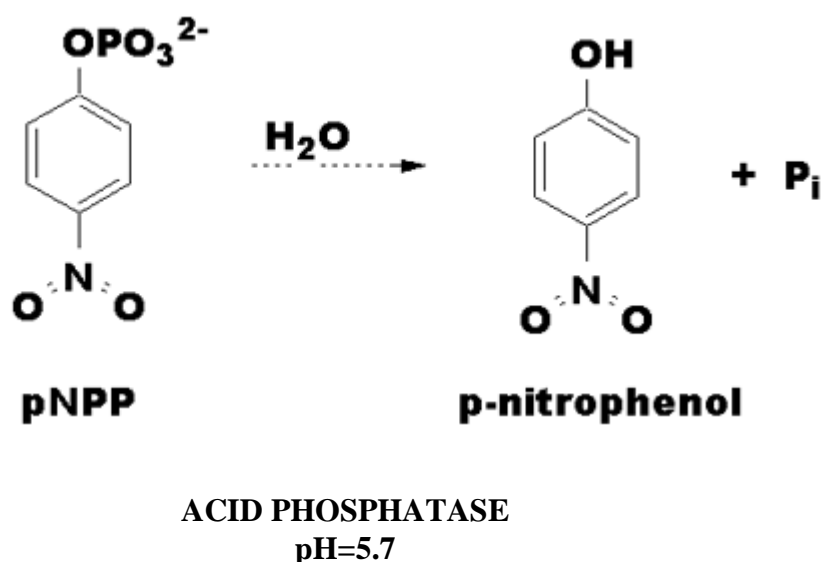


Figure-1. The hydrolysis of p-nitrophenyl phosphate by acid phosphatase. pNPP, p-nitrophenylphosphate.

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.

3.3 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

Equipments:

pH meter

Water bath

Spectrophotometer

Glassware:

Test tubes

Pipettes

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 $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 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

3.4 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 °C 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

3.5 Results:

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

- Use the extinction coefficient ($18.8 \times 10^3 \text{ liter mol}^{-1} \text{ cm}^{-1}$) 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.

3.6 Discussion and conclusion:

3.7 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 slope of the linear part of the graph.

3.8 Reference:

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

Experiment 4

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

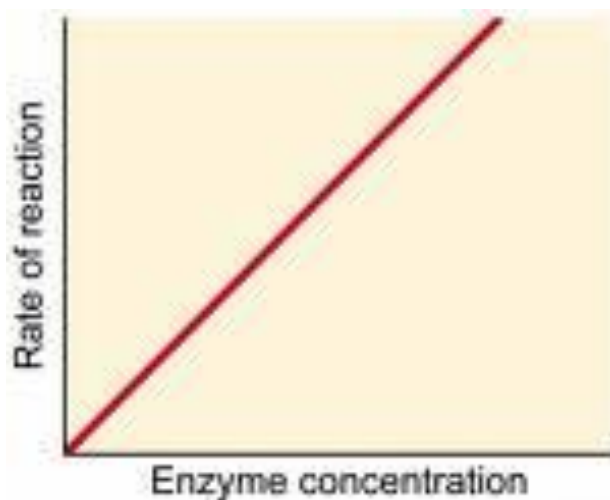
4.1 Objectives:

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

4.2 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.



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

Equipments:

pH meter

Waterbath

Spectrophotometer

Glassware:

Test tubes

Pipettes

4.4 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 °C 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.

4.5 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 curve (units/ml). Describe the shape of this curve and discuss the reasons for its shape.

4.6 Discussion and conclusion:

4.7 Questions:

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

4.8 Reference:

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

Experiment 5

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

5.1 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

5.2 Introduction:

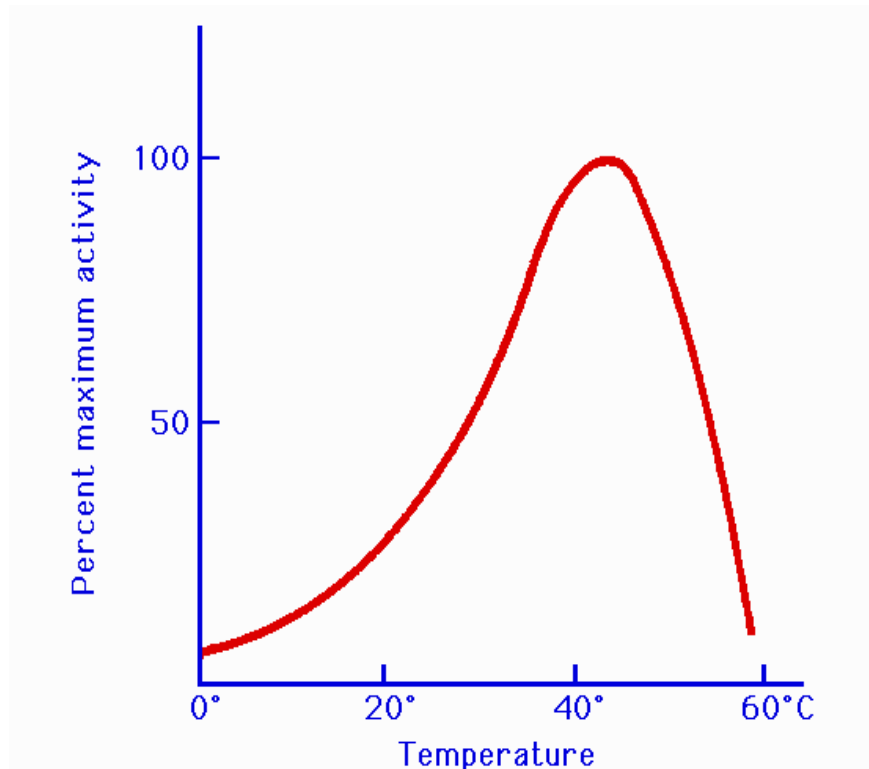
The rate of an enzyme catalyzed reaction is affected by changes in temperature. At low temperature (0°C), the rate of reaction is low. As the temperature is increased, the rate of reaction increase until an optimum temperature is reached. Within this temperature range, the rate of reaction is approximately doubled for every 10 °C 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 °C .

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:



5.3 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 $MgCl_2$

0.5 M KOH

5.4 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 (°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-nitrophenyl phosphate and 5ml of distilled water. Place the tubes in a water bath maintained at 0 to 4 °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.

5.5 Results:

Temperature($^{\circ}\text{C}$)	Absorbance at 405 nm
0	
10	
20	
30	
37	
50	
80	
100	

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

5.6 Discussion and conclusion:

5.7 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?

5.8 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 6

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

6.1 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.

6.2 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.

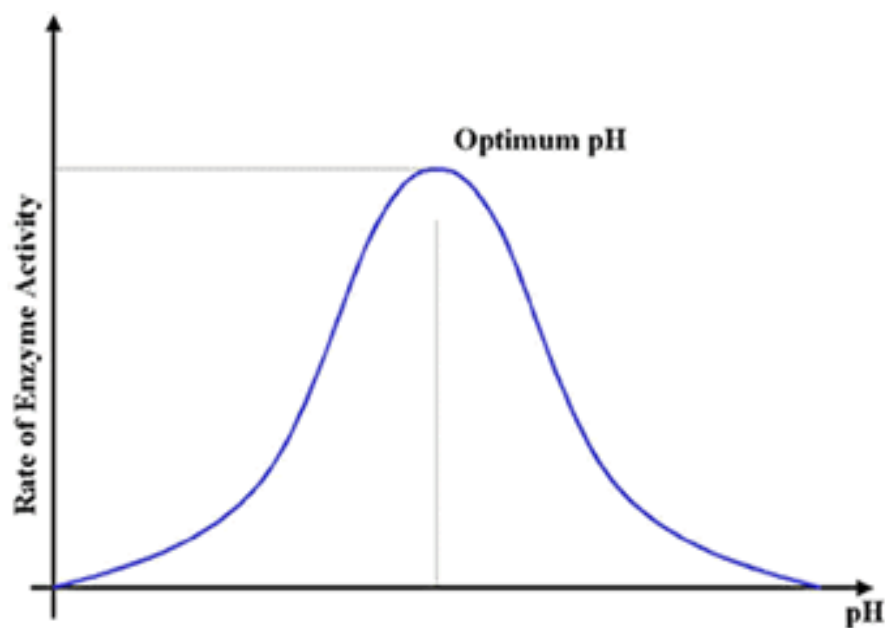
At higher or lower pH, the rate of an enzymatic reaction decrease.

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**6.3 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 $MgCl_2$

0.5 M KOH

6.4 Method:

1. Prepare 16 tubes labeled as follows:

(A₃ (blank), B₃, A₄, B₄, A_{4.5}, B_{4.5}, A₅, B₅, A_{5.5}, B_{5.5}, A₆, B₆, A₇, B₇, A₈, B₈).

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-nitrophenyl phosphate

5ml of distilled water

3. Place the tube in a test tube rack situated in 37°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).

6.5 Results:

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

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

6.6 Discussion and conclusion:

6.7 Question:

1-What is the optimum pH for acid phosphatase?

6.8 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 7

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

7.1 Objectives:

- 1) To establish the relationship between substrate concentration and the rate of an enzyme catalyzed reaction.
- 2) To determine the K_m and V_{max} of the enzyme for a particular substrate.

7.2 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.

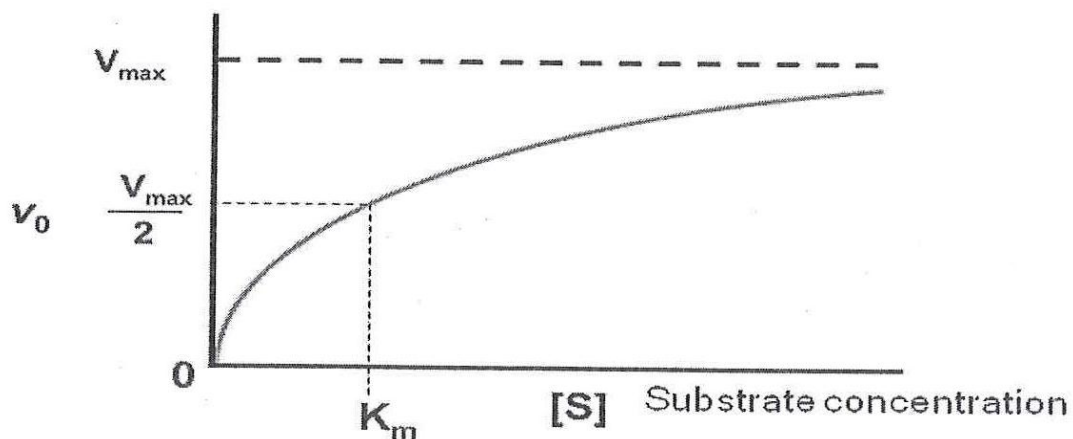
-At higher substrate concentration, the rate of reaction increases by

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, V_{max}

Figure A: Michaelis – Menten plot

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

$$V_i = \frac{V_{max} [S]}{K_m + [S]}$$

V_i = initial velocity

V_{max} = maximum velocity

$[S]$ = substrate concentration

K_m = Michaelis – Menten constant

K_m : is substrate concentration at half V_{max}

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

The K_m 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):

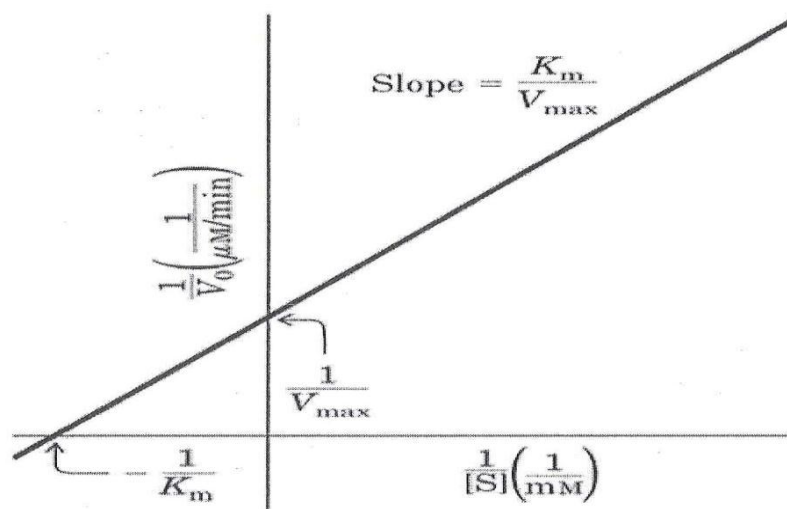
The Michaelis – Menten equation can be algebraically transformed into forms that are useful in the practical determination of K_m and V_{max} .

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

$$\frac{1}{V_i} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}} \cdot \frac{1}{[S]}$$

-By plotting $\frac{1}{V_i}$ against $\frac{1}{[S]}$,a straight line plot, the

Lineweaver – Burk plot, is obtained as shown below:



Both V_{\max} and K_m 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} .

7.3 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 $MgCl_2$
0.5 M KOH

7.4 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-nitrophenyl phosphate).

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

- Place the tubes in a test tube rack situated in a 37°C water bath and let stand for 5 minutes.
- 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.
- Determine the absorbance at 405 nm for each reaction mixture against the blank. (The tube containing no substrate should be used as the blank).

7.5 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 ($\mu\text{moles of p-nitrophenol/minute}$) for each substrate concentration.
- 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 K_m and V_{max} from the x and y intercepts.

7.6 Discussion and conclusion:

7.7 Questions:

1. Investigate the literature and see how your values for K_m and V_{max} compare with published ones for acid phosphatase.

7.8 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 8:

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

8.1 Objectives:

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

8.2 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).

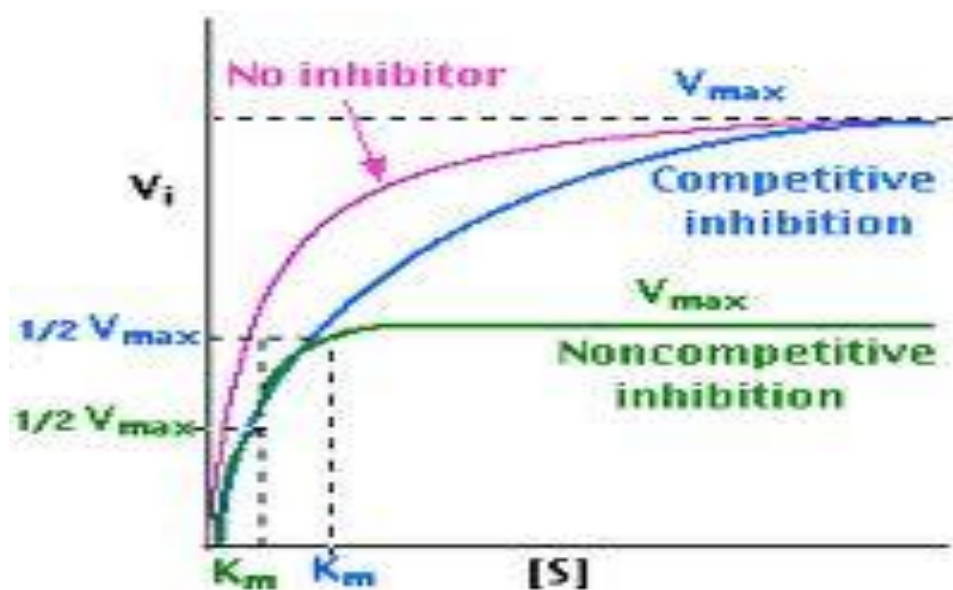


Figure 1 : A Michaelis-Menten plot for an enzymatic reaction performed in the presence and absence of competitive and noncompetitive inhibitor.

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 in 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 uninhibited 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.

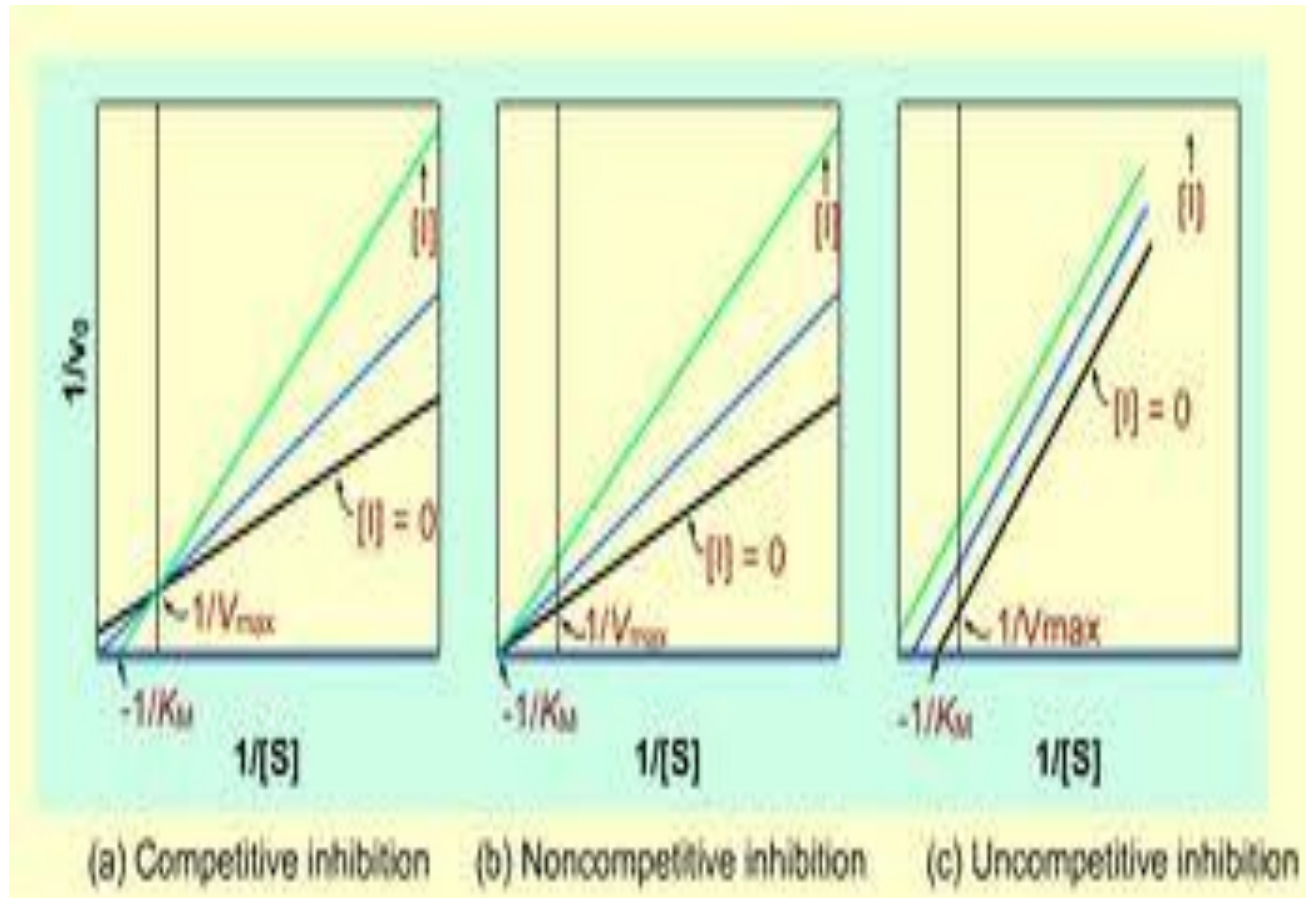


Figure 2. A Lineweaver-Burke plot for an enzymatic reaction performed in the absence of inhibitor and in the presence of increasing concentrations of competitive, noncompetitive and uncompetitive inhibitors.

The inhibition of acid phosphatase by inorganic phosphate.

Inorganic phosphate (P_i) 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.

8.3 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

Equipments:

pH meter

Waterbath

Spectrophotometer

Glassware:

Test tubes

Pipetts

Preparation of Solutions:

- (1) 0.005M K₂HPO₄: Dissolve 0.174gm of K₂HPO₄ in 200ml of distilled water.
- (2) 0.005M Sodium fluoride: Dissolve 0.05gm of NaF in 200ml distilled water.

8.4 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 MgCl₂, 4ml of distilled water, and 1.0ml of 0.005M K₂HPO₄ or 5mM Sodium 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.
3. Place the tubes in a 37 °C water bath for 5 minutes.
4. 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.
5. Determine the absorbance at 405 nm for each sample, using the first tube (0 mM p-nitrophenyl phosphate) as the blank.
6. Repeat the experiment two more times.

8.5 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 ($\mu\text{moles of p-nitrophenol/minute}$) 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.

8.6 Discussion and Conclusion:

8.7 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?

8.8 Reference:

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

Experiment 9

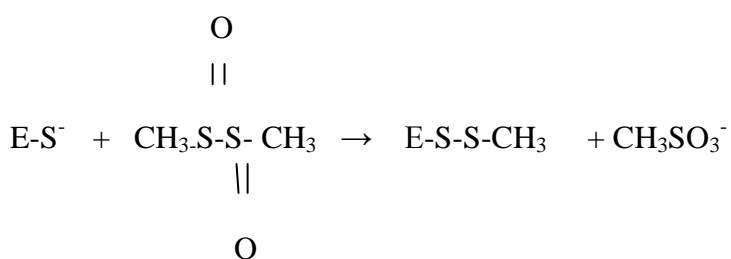
9.Modification of active site cysteine of Lactate Dehydrogenase

9.1 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.

9.2 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.

9.3 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
- 0.01MNAD⁺ in phosphate buffer

0.05M oxalate in phosphate buffer

pH meter

Waterbath

Spectrophotometer

Glassware:

Test tubes

Pipettes

9.4 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/ml in 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 label these A-1, A-2, A-5, etc. Also at 30 minutes 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.

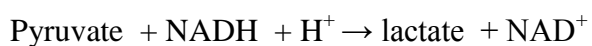
(3) Add 0.05ml MMTS to C (time 0) . After 15 minutes and 30 minutes remove 0.05 ml samples from C and label C-15 and C-30.

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:



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.

9.5 Results:

No.S.	tubeTo	absorbance at 340nm

Calculations:

- (1) Determine the specific activity of unmodified LDH in $\mu\text{moles/min/mg}$.
- (2) Draw a graph showing the loss of activity with time.

9.6 Discussion and conclusion:

9.7 Reference:

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

Experiment 10

10. Determination of ALT ,AST, ALP and LDH in serum

10.a Determination of ALT and AST in serum:

10.1.a Objective:

To determine the level of these enzymes in serum as a tool to study liver function.

10.2.a Introduction:

An alanine aminotransferase (ALT) test measures the amount of this enzyme in the blood. ALT is found mainly in the liver, but also in smaller amounts in the kidneys, heart, muscles, and pancreas. ALT formerly was called serum glutamic pyruvic transaminase (SGPT).

ALT is measured to see if the liver is damaged or diseased. Low levels of ALT are normally found in the blood. But when the liver is damaged or diseased, it releases ALT into the bloodstream, which makes ALT levels go up. Most increases in ALT levels are caused by liver damage.

The ALT test is often done along with other tests that check for liver damage, including aspartate aminotransferase (AST), alkaline phosphatase, lactate dehydrogenase (LDH), and bilirubin. Both ALT and AST levels are reliable tests for liver damage.

A) Estimation of ALT and AST in serum (GOT and GPT)

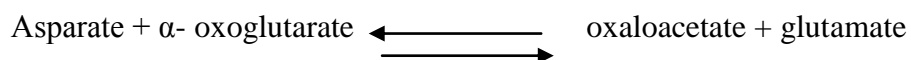
Transaminases (aminotransferases) are enzymes which catalyse the transfer of the α - amino group of an α - amino acid to the α carbon atom of an α - keto acid. The product of the reaction are a new α - amino acid corresponding to the original α - keto acid, and a new α - keto acid corresponding to the original α - amino acid.

Transamination reactions are generally reversible and they play an important role in the metabolism of amino acids in the body, Transaminases are present in almost all tissues both in the cytoplasm and in the mitochondria.

All transaminases contain pyridoxal phosphate (vitamin B6) as a prosthetic group.

The most important transaminases identified are glutamate-oxaloacetate transaminase (GOT) and glutamate- pyruvate transaminase (GPT)

The reaction catalysed by GOT is shown below:



The activity of these two enzymes are quite low in serum, whereas it is relatively high in such tissues as liver, skeletal muscle and kidney.

Measurement of SGOT and SGPT is useful in the clinical diagnosis of certain diseases. These enzymes will be increased in cases where cells containing large quantity of the enzymes are ruptured, thereby releasing their content into the blood stream.

Increased levels of SGOT and SGPT are found in cases of:

- 1- Myocardial infarction (accompanied with increase in CPK)
- 2- Viral hepatitis
- 3- Toxic liver necrosis
- 4- Cirrhosis
- 5- Malignant infiltration of the liver

-Normal levels:

Normal results may vary from lab to lab.

Alanine aminotransferase
4–36 units per liter (U/L) or 0.07–0.62 microKat/L

High values

- 1- Very high levels of ALT may be caused by:
- 2- Recent or severe liver damage, such as viral hepatitis.
- 3- Lead poisoning.
- 4- Drug reactions.
- 5- Exposure to carbon tetrachloride.
- 6- Decay of a large tumor (necrosis).

7- Shock.

Mildly or moderately high ALT levels may be caused by:

- 1- Mononucleosis.
- 2- Hepatitis. The ALT level in a person with hepatitis can be 20 times the normal value.
- 3- Alcohol abuse. People who drink excessive amounts of alcohol and take acetaminophen (such as Tylenol) can have high ALT blood levels.
- 4- Mildly elevated levels of ALT may occur in people who are growing quickly, especially young children.

Slightly high levels ALT levels may be caused by:

- Fatty deposits in the liver. Many medicines, such as statins, antibiotics, chemotherapy, aspirin, narcotics, and barbiturates. Long-term (chronic) diseases that affect the liver, such as cirrhosis.

10.3.a Material:

As shown in the provided kit.

10.4.a Method:

As shown in the provided kit.

10.5.a Result

10.6.a Discussion :

B: Estimation of ALP and LDH in serum**10.1. b. Introduction:****A) Alkaline phosphatase ALP:**

An alkaline phosphatase (ALP) test measures the amount of the enzyme ALP in the blood. ALP is made mostly in the liver and in bone with some made in the intestines and kidneys. It also is made by the placenta of a pregnant woman.

The liver makes more ALP than the other organs or the bones. Some conditions cause large amounts of ALP in the blood. These conditions include rapid bone growth (during puberty), bone disease (osteomalacia or Paget's disease), or a disease that affects how much calcium is in the blood (hyperparathyroidism), or damaged liver cells.

A test for alkaline phosphatase (ALP) is done to:

- 1- Check for liver disease or damage to the liver. Symptoms of liver disease can include jaundice, belly pain, nausea, and vomiting. An ALP test may also be used to check the liver when medicines that can damage the liver are taken.
- 2- Check bone problems (sometimes found on X-rays), such as rickets, osteomalacia, bone tumors, Paget's disease, or too much of the hormone that controls bone growth (parathyroid hormone). The ALP level can be used to check how well treatment for Paget's disease is working.

-Normal values(may vary from lab to lab).

Normal values(may vary from lab to lab).	
Adults:	30–126 units per liter (U/L) or 0.5–2.0 microkat/liter (mckat/L)
Children:	30–300 U/L or 0.5–5.0 mckat/L

Women in the third trimester of pregnancy have high ALP levels because the placenta makes ALP. Children normally have much higher ALP than adults because rapid bone growth is normal in children and bones make ALP.

- High values

- Very high levels of ALP can be caused by liver problems, such as hepatitis, blockage of the bile ducts (obstructive jaundice), gallstones, cirrhosis, liver cancer, or cancer that has spread (metastasized) to the liver from another part of the body.
- High ALP levels can be caused by bone diseases, such as Paget's disease, osteomalacia, rickets, bone tumors, or tumors that have spread from another part of the body to the bone, or by overactive parathyroid glands (hyperparathyroidism). Normal healing of a bone fracture can also raise ALP levels.
- Heart failure, heart attack, mononucleosis, or kidney cancer can raise ALP levels. A serious infection that has spread through the body (sepsis) can also raise ALP levels.

- Low values

- Conditions that lead to malnutrition (such as celiac disease) or are caused by a lack of nutrients in the diet (such as scurvy) can cause low ALP levels.

B) Lactic acid dehydrogenase LDH:

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.

LDH levels help diagnose lung disease, lymphoma, anemia, and liver disease. They also help determine how well chemotherapy is working during treatment for lymphoma.

Lactic acid dehydrogenase (LDH) is an enzyme that helps produce energy. Results may vary widely from lab to lab.

Normal LDH levels range from 100 units per liter (U/L) to 190 U/L.

10.2. b Material

As shown in the provided kit.

10.3. b Method:

As shown in the provided kit.

10.4.b.Result :

10.5.b Discussion:

10.6.b References:

- Practical Textbook of Biochemistry for MBBS Students by DM Vasudevan and SK Das, 2007, Jaypee Brothers Medical Publishers (P) LTD, New Delhi.
- <http://www.webmd.com>