Experiment 1 Comparison between Enzymes and non-biological Catalysts

Definition of enzymes: enzymes are biological catalysts. They greatly enhance the rate of specific chemical reactions that would occur very slowly.

Starch which the storage form of glucose in plant. Starch consist of

1- Amylose



1-4 α- glycosidic linkage

2- Amylopectin



1-6 α- glycosidic linkage

Contents of Saliva

- In animals, saliva is produced in and secreted from the salivary glands. It is a fluid containing
- Electrolytes: (2-21 mmol/L sodium, 10-36 mmol/L potassium, 1.2-2.8 mmol/L calcium, 0.08-0.5 mmol/L magnesium, 5-40 mmol/L cloride, 2-13 mmol/L bicarbonate, 1.4-39 mmol/L phosphate)
- Mucus. Mucus in saliva mainly consists of mucopolysaccharides and glycoproteins;
- Antibacterial compounds (thiocyanate, hydrogen peroxide, and secretory immunoglobulin A)
- Various enzymes. The major enzymes found in human saliva are alpha-amylase, lysozyme, and lingual lipase. Amylase starts the digestion of starch before the food is even swallowed. It has pH optima of 6.7-7.4. Human saliva contains also salivary acid phosphatases A+B, N-acetylmuramyl-L-alanine amidase, NAD(P)H dehydrogenase-quinone, salivary lactoperoxidase, superoxide dismutase, glutathione transferase, glucose-6-phosphate isomerase, and tissue protein. The presence of these things causes saliva to sometimes have a foul odor.

Healthy people produce about 1.5 L of saliva per day.

Amylase:- found in two forms

- 1- α -amylase (in saliva and pancreatic juice) which is endoglycosidase that attack starch randomly. Inactivated by the acidity of the stomach.
- 2- β-amylase (from plant origin) which is exoglycosidase cleaves maltose from the non-reducing end to produce β-maltose.

Principle:

When we want to measure enzyme activity either we measure the decrease in the substrate concentration or the increase in the product concentration.



Blue color

Hydrolysis of starch by Amylase	reaction with I_2
Starch	blue
Soluble starch	blue
Amylodextrin	purple
Erthrodextrin	red
Achrodextrin	colorless
Maltose	colorless

Hydrolysis of starch by non-biological catalysts

Acid Starch Glucose

Other uses of amylase in industry:

It is used in clarification of fruit juices. The turbidity present in natural beverages is due primly to the presence of starch and cellulose molecules too large to be completely soluble. Amylase hydrolysis these molecules to glucose which are more water soluble.

Reagents:

Starch 1% solution in 0.3% aqueous sodium chloride, freshly prepared; iodinated potassium iodide solution.

Dilute saliva 1ml: 10ml D.W, HCl 10% solution.

Method:

- 1- Transfer 1ml of distilled water to a test tube, 1ml of HCl solution to another test tube and 1ml of dilute saliva sample to a third test tube.
- 2- For each test tube prepare a series of 15 test tube, each containing2ml of pale yellow iodine solution.
- 3- Place the first and the third test tubes in a water bath at 38C°(check the temperature by the thermometer)
- 4- Place the second test tube in a boiling water bath.
- 5- Prewarm starch solution in the water bath for 5min at 38C°.
- 6- Add 5ml of starch solution to each of the three test tubes; stir the content with glass rode.
- 7- Record the time of addition of starch and at the end of each minute remove two drops reaction mixture using a test pipette, and add to one of the tubes of iodine solution.
- 8- Compare the color developed in the sample.
- 9- Draw the relationship between the color developed and the time required for the color to develop.

Lab report:

At the end of the experiment we notice the following differences:

Biological catalysis	Non-biological catalyst
α- amylase	HCl
Work under mild conditions	Work under drastic conditions
Required in small amount	Required in high concentration
High rate	Low rate
Highly specific	Unspecified
Protein in nature, has all	Not protein in nature, do not
chemical and physical properties	have chemical and physical
of protein	properties of protein

- 1- Plummer, D. An introduction to practical biochemistry. McGraw-HILL, london. 1978
- 2- Harvey, R and Champe, P. Lippincott biochemistry, london.2005

Result:

Draw the relation between the color developed and the time required for the color to developed

Time						
	Color					
	Enzyme HCl D.W					
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						
11						
12						
13						
14						
15						
16						
17						

Experiment 2 The hydrolysis of sucrose by yeast β -Fructofuranosidase and the determination of the produced sugars with Benedict quantitative method

Principle:

 β -Fructofuranosidase is a glycosidase found in yeast. It catalyses the hydrolysis of sucrose to glucose and fructose. The enzyme is also known as invertase or sucrase, but these names are no longer used.

The substrate sucrose is a non-reducing sugar, where as the products formed are both reducing sugar. Therefore the reaction can be followed by the estimation of the quantity of reducing sugar formed. Between the several methods which can be used for such estimation, Benedict quantitative method was utilized.

Benedict quantitative reagent is composed of:

- 1- Copper sulphate: to provide the oxidizing Cu^{+2} ions.
- 2- Sodium carbonate: to provide the alkaline medium necessary for the formation of the highly reactive reducing sugar 1-2 endediol.
- 3- Sodium citrate: combines with Cu carbonate to prevent its precipitation by forming a slightly soluble complex with cupric ions (Cu^{+2} ions). This complex dissociates slowly to give a sufficient supply of Cu^{+2} ions.
- 4- Potassium thiocyanate (KSCN): reacts with cupric ions to give Cu(SCN)₂
- 5- Potassium ferrocyanide ($K_4Fe(CN)_6$): prevents the re-oxidation of the formed cuprous thiocyanate (CuSCN) to cupric thiocyanate.

The reaction takes place as fallow:

1- The enolization of reducing sugar in alkaline medium to give a highly reactive reducing compound, which is 1-2 endediol.



- 2- Formation of cupric carbonaate $Na_2CO_3 + CuSO_4 \longrightarrow CuCO_3 + Na_2SO_4$
 - 3- Formation of sodium cupric citrate complex:



4- Ionization of sodium cupric complex



Sugar (cuprous thiocyanate, white ppt.)

Method:

A- Hydrolysis of sucrose by yeast β-Fructofuranosidase Prepare five tubes containing the following mixtures

Tube	1	2	3	4	5	
Sucrose 0.3M	10 ml	8 ml	6 ml	4 ml	2 ml	
D.W	0	2 ml	4 ml	6 ml	8 ml	
Buffer pH 4.5	6 ml	6 ml	6 ml	6 ml	6 ml	
	Pre	-incubate at	37C° for 5	min		
Yeast suspension	4 ml	4 ml	4 ml	4 ml	4 ml	
Incubate for 15 min						
1% NaOH	2 ml	2 ml	2 ml	2 ml	2 ml	
Final conc. Of sucrose	150	120	90	60	30	

Note:

The yeast must be added to each tube at a constant time intervals, i.e. tube 1 at time 0, tube 2 at 2min etc. This will enable the incubation time to be measured exactly and ensures that each tube is incubated for the same time. Incubate each tube for exactly 15 min. Stop the reaction by the addition of 2ml of 1% sodium hydroxide. This will be at 15 min for tube 1, 17 min for tube 2 and so on.

B- Determination of the hydrolyzed sucrose solution by Benedict method

- 1- Place the sugar solution of hydrolyzed sucrose from the previous experiment in burette.
- 2- Measure 5ml of Benedict quantitative reagent into 100 ml conical flask add approximately 1g of anhydrous sodium carbonate and few pieces of porcelain. Heat the mixture vigorously.
- 3- Run in the sugar solution slowly from the burette until a bulky white precipitate is formed. Continue the titration by adding the sugar solution drop by drop until the last trace of blue or green has disappeared.
- 4- Record the volume of the sugar required to titrate 5 ml of benedict reagent. This volume will be your titer number.

Note:

- 1- The end point must be determined while the mixture is still boiling. When the mixture is not boiling atmospheric oxidation occurs and the green color returns.
- 2- The addition of sodium carbonate to the titration mixture results in the liberation of CO₂, which prevents atmospheric oxidation.
- 3- If the mixture bumps or it becomes too concentrated during titration, remove it from the heater, boil 10 ml water in atest tube and add it to the reaction mixture. Heats the mixture until it boils again and continues the titration.
- 4- The tip of the burette must be over the mouth of the flask while mixture is titrated.

Calculation:

The concentration of the sugar in each tube can be calculated from the following sugar equivalent equation

M * V = M' * V'

The equivalents for a number of sugars are given as follow, but they only applied if the above conditions are strictly adhered to.

25 ml of Benedict's reagent is equivalent to 50 mg of glucose

53 mg of fructose

68 mg of lactose

74 mg of maltose

49 mg of hydrolyzed sugar

Since we used 5ml of Benedict reagent which is equivalent to 9.8 mg of hydrolyzed sugar

5ml benedict = 9.8 mg

5ml benedict = Titer no. ml

9.8 mg of sugar = Titer no. ml

X mg of sugar = 1 ml X mg of sugar/ ml = 1 *9.8 /titer No. *dilution factor Dilution factor= Final volume / Initial volume

- 1- Plummer, D. An introduction to practical biochemistry. McGraw-HILL, london. 1978
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Result:

Fill the table below and plot a relationship between the substrate and product concentration.

Tube no.	[S]	Titer number	[P]
	mM	ml	mg/ml
1			
2			
3			
4			
5			

Experiment 3 The determination of the optimum temperature of salivary Amylase

The optimum temperature: is the temperature at which an enzyme shows maximum catalytic activity. This temperature can be determined by following the increase and the decrease in the activity of salivary α -amylase. α -amylase catalyses the hydrolysis of α -1-4 linkage of starch with the production of maltose. The reaction is followed by measuring the increase in the reducing sugar, the product. The reagent used is 3,5-dinitrosalicylate which is reduced in alkaline medium to 3-amino-5-nitrosalicylic acid. The yellow color produced is measured at 540nm.



Materials:

- 1- phosphate buffer (0.1M, pH6.7)
- 2- Starch substrate (0.5% in phosphate buffer)
- 3- Sodium chloride NaCL (1%)
- 4- Sodium hydroxide NaOH (2N). Sodium potassium tartarate. Dissolve 150g of sodium potassium tartarate in 250ml distilled water.
- 5- 3,5- Dinitrosalicylic acid. Dissolve 5g of 3,5- Dinitrosalicylic acid in 100ml of 2N sodium hydroxide. Prepare the 3,5- Dinitrosalicylic acid by mixing (4) and (5) and complete them to 500ml with distilled water.
- 6- Amylase enzyme, dilute 1ml saliva to 100ml distilled water.

Procedure:

Since the goal of the experiment is to determine the optimum temperature for amylase, the same experimental steps will be done at the following temperatures: 0 C°, 10 C°, 20 C°, 37 C°, 40 C°, 60 C°, 70 C°. for each student prepare 3 tubes and add the following reagents.

Tube no.	1	2	3 (Blank)	
Substrate (ml)	2.5	2.5	2.5	
Starch 5%				
Buffer (ml)	1	1	1	
Phosphate				
	1	1	1	
Activator (ml)				
NaCL				
Pre h	eat at the chosen t	temperature for 10	0 min	
Enzyme (ml)	0.5	0.5		
D.W (ml)	-	-	0.5	
Incubate for 15 r	nin. at the chosen	temperature then	stop the reaction	
	by adding	2N NaOH		
Inhibitor (ml)	0.5	0.5	0.5	
NaOH				
The next step is t	to determine the a	mount of reducing	g sugar produced	
in each tube by using 3,5, dinitrosalicylate reagent.				
Reagent (ml)	0.5	0.5	0.5	
Heat the tube in boiling water bath for 5min. cool and read the				
absorbance of the colored product at 540nm.				

Note: all tubes must be cooled to the same temperature before reading on the spectrophotometer, since the extinction is sensitive to temperature change.

Results:

Plot a curve of the change in the initial reaction (V) against temperature.

What is the optimum temperature for the enzyme α -amylase?

- 1- Plummer, D. An introduction to practical biochemistry. McGraw-HILL, london. 1978
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- 3- Boyer, R. Concepts in biochemistry. John Wiley and sons, New york. 2002

Experiment 4 Estimation of lipase activity

Lipase is a pancreatic enzyme secreted into the small intestine. It catalyses the hydrolysis of triacylglycerols to free fatty acids and glycerol as follow:



Triacylglycerol

The release of fatty acids in the solution will cause decrease in the pH and the rate of the reaction may be followed by:

- 1- Noting the change of pH with time.
- 2- Titration the liberated free fatty acids with standard alkali using a suitable indicator
- 3- By continues titration using an automatic apparatus, (pH-state) which keeps the pH constant and at the same time plots a curve of titer number against time.

Method 2 has been adapted foe this experiment. The librated free fatty acids at different enzyme concentrations will be titrated with 0.05 N NaOH. Since we are using oils as substrates $CaCl_2$ is used as emulsifying agent for two reasons:

- 1- to increase the surface area
- 2- to decrease the surface tension, thus the oil drop is effetely attacked with the enzyme.

Materials:

- 1- Lipase (1g%)
- 2- Chloroform (10%)
- 3- Fresh oil as the substrate
- 4- Calcium chloride
- 5- Sodium hydroxide 0.05 N

Procedure

Prepare 6 tubs which contain the following:

Tube	1	2	3	4	5	B
Oil	2	2	2	2	2	2
Substrate						
(ml)						
CaCl ₂	1	1	1	1	1	1
		Ν	lix well	•	L	
D.W	8	6	4	2	0	10
Lipase	2	4	6	8	10	-
(ml)						
Incubate in a water bath 37C°						
Enzyme	0.02	0.04	0.06	0.08	0.1	-
concentration						

Titrate the liberated fatty acids with NaOH noting the time of the titration should not exceed 10 min.

Result

Draw a graph of enzyme concentration against ml of NaOH has taken. Is your curve hyperbolic or liner, comment?

- 1- Plummer, D. An introduction to practical biochemistry. McGraw-HILL, london. 1978
- 2- Harvey, R and Champe, P. Lippincott biochemistry, london.2005
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Experiment 5 Indirect estimation for aldehyde oxidase

Principle:

Milk contains the enzyme aldehyde oxidase which catalyses the oxidation of variety of aldehydes to acids. The reaction proceeds anaerobically and can be demonstrated by a suitable hydrogen acceptor such as methylene blue. The reaction is performed via an intermediate hydrated aldehyde as follow:



Methylene blue, which acts as a cofactor changes in color from blue to colorless, thus the progress of the reaction can be noted.

The reaction is conveniently carried out in a tube designed for the study of anaerobic reactions, Thunberg tube.

Materials:

- 1- methylene blue, 0.02g%
- 2- Fresh cows milk, different trade marks.
- 3- Neutral formaldehyde, 0.5 % (by titration with NaOH using ph.ph as indicator.

Procedure:

Prepare 4 Thunberg tubes. If the tubes are not available use ordinary test tubes covered with parafilm while the substrate is added by a syringe. Perform the following steps:

Tubes	1	2	3	4	
Fresh milk	5	5	5	5	
enzyme (ml)					
Boiling water bath	1 min	5min	-	-	
M.B	1	1	1	1	
Indicator (ml)					
Formaldehyde	1	1	1	-	
Substrate (ml)					
All tubes in water bath 40C°					
Note the time of complete decolorization					

- 1- Plummer, D. An introduction to practical biochemistry. McGraw-HILL, london. 1978
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Result

Tube	Time of	comment
NO.	declorization	
1		
2		
3		
4		

Which tube decolorize first?

Is methylene blue is the only substance that is reduced?

Experiment 6 Indirect estimation of lactate dehydrogenase

Lactic acid produced during anaerobic glycolysis can be converted to pyruvic acid with the aid of the enzyme lactate dehydrogenase when oxygen becomes available. The hydrogen acceptor NAD⁺ accepts the hydrogen atoms from the lactic acid and the pyruvic acid molecule results. Part of the produced pyruvic acid enters the citric acid cycle after being converted to acetyl coA. The remainder of the pyruvic acid is converted into glycogen.



In this experiment, yeast will be used as a source of lactate dehydrogenase. The reaction will be followed by allowing methylene blue dye to function in place of the natural hydrogen acceptor NAD⁺. As methylene blue is reduced it becomes colorless.



Materials:

- yeast suspension
- 5% sodium lactate solution
- 0.1% methylene blue
- Water bath 37°C
- Boiling water bath

Procedure:

- Lable three clean test tubes as a, b and c as followed
- Make sure that you shake the bottle of yeast suspension before removing your sample

Test tube	Α	В	С			
Yeast suspension	2ml	2ml	2ml			
			Yeast suspension			
			Pre heated for 10			
			min in boiling			
			water bath and			
			cooled to 37°C			
			before being used			
Sodium lactate		10 drops	10 drops			
Methylene blue	1 drop	1 drop	1 drop			
Continue to add	methylene blue drop w	vise (mixing after each	n drop) until each			
S	solution becomes a uniform light blue in color					
Mix and place in water bath 37°C						
Observe the tubes at	Observe the tubes after 10 min. note any color changes and record your observations					

Results

Α	В	С

- 1- Plummer, D. An introduction to practical biochemistry. McGraw-HILL, london. 1978
- 2- Harvey, R and Champe, P. Lippincott biochemistry, london.2005
- 3- Boyer, R. Concepts in biochemistry. John Wiley and sons, New york. 2002

Experiment 7 The effect of hydrogen ion concentration on Catalase enzyme



Catalase is a common enzyme found in living organisms' red blood cells and yeasts are good sources of catalase. Its functions include catalyzing the decomposition of hydrogen peroxide to water and oxygen. Catalase has one of the highest turnover rates for all enzymes; one molecule of catalase can convert 6 million molecules of hydrogen peroxide to water and oxygen each minute. Catalase is a tetramer of 4 polypeptide chains which are at least 500 amino acids in length. Within this tetramer there are 4 porphyrin haem (iron) groups which are what allows it to react with the hydrogen peroxide. Its optimum pH is at a neutral level.

H_2O_2 ^{catalase}---> H_2O + O_2

Hydrogen peroxide is formed as a waste product of metabolism in many living organisms. It is toxic and must be quickly converted into other, less dangerous, chemicals.



Effect of pH on enzyme activity

www.biomedcentral.com/1471-2180/2/5/figure/F3

Principle:

Iodide anion I^{-2} is a weak reducing agent that will react with an oxidizing analytes to produce iodine

 $K_2I + H_2O_2 \longrightarrow I_2 + starch$

The iodine may be titrated with $Na_2S_2O_3$ to determine the H_2O_2 concentration

 $Na_2S_2O_3 + I_2 \longrightarrow Na_2S_4O_6 + 2I^-$

Materials:

- 1- hydrogen peroxide 0.005M
- 2- phosphate buffer of different values 3.8, 4.8, 6.8 and 8
- 3- Sulfuric acid 2N
- 4- Potassium iodide 10%
- 5- Sodium molybdate 1%
- 6- Sodium thiosulfate 0.005M
- 7- Starch 1%
- 8- Enzyme source 10% yeast and 1% blood collected in EDTA tube with ice cold distilled water, the solution must be kept cold through out the experiment.

Procedure:

Tube	1	2	3	4	
H_2O_2	15	15	15	15	
Substrate					
(ml)					
Selected	10	10	10	10	
buffer (ml)	рН 3.8	рН 4.8	pH 6.8	pH 8	
Pre-incubate th	he flask in ice	e bath for 5 mi	n		
Enzyme (ml)	1	1	1	1	
Incubate exactly for 5 min					
H_2SO_4	10	10	10	10	
Inhibitor (ml)					

<u>Note:</u> with each selected buffer make a blank instead of the enzyme add 1ml D.W.

The decrease in hydrogen peroxide is now determined as follow:

- 1- Add 10ml of KI and, 1ml of sodium molybdate 1% to the above mixture in the same flask.
- 2- Let the mixture stand for 20 min
- 3- Titrate the liberated iodine with 0.005 M sodium thiosulfate.
- 4- When the yellow color of the iodine begins to fade, add 3 drops of starch to act as an indicator.
- 5- Continue the titration until the purple color of the starch disappears.

Results:

Draw a curve of the pH against the titer no. ,enzyme activity

What is the optimum pH for the catalase.

Note:

Titrt. No. = Titer B – Titer T

- 1- Plummer, D. An introduction to practical biochemistry. McGraw-HILL, london. 1978
- 2- Harvey, R and Champe, P. Lippincott biochemistry, london.2005
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Experiment 8 Indirect estimation of succinic dehydrogenase

The citric acid cycle functions primarily to provide hydrogen atoms for the electrontransport chain. The hydrogen atoms are picked up from the citric acid cycle and carried to the electron transport chain by several coenzymes. FAD, one of these coenzyme. Transports the hydrogen atoms generated in citric acid cycle when succinic acid is dehydrogenated to form fumaric acid.



Yeast will be used to supply the enzyme, and the reaction can be followed outside the body by using the artificial hydrogen acceptor methylene blue. The effect of malonic acid, a competitive inhibitor will also be demonstrated in this experiment.

Materials:

- Yeast suspension
- 5% sodium succinate solution
- 10% sodium malonate solution
- 0.1% methylene blue solution

Procedure:

Lable four small, clean test tubes a,b,c and d

Test tube	A (control)	В	С	D	
Yeast	2ml	2ml	2ml	2ml	
suspension					
Sodium		10 drops		5 drops	
succinate					
Sodium			10 drops	5 drops	
malonate					
Methylene	1 drops	1 drops	1 drops	1 drops	
blue					
Continue to add methylene blue drop wise (mixing after each drop) until each					
solution becomes a uniform light blue in color					
Mix and place in water bath 37°C					
Observe the tubes after 10 min. note any color changes and record your observations					

Results:

Test tube	Α	В	С	D

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Experiment 9 The effect of substrate concentration and time on the digestion of casein by trypsin

Principle:

Proteins are long chains of amino acids linked by peptide bonds. During hydrolysis these peptide bonds are broken. Depending upon the type of enzyme used and processing conditions, smaller chains of amino acids and different amino acid sequences are produced by the process.

Trypsin is a pancreatic enzyme (endopeptidase) which is secreted in the pancreatic juice as trypsinogen. Catalyzes the hydrolysis of peptide bound formed from the carboxyl groups of basic amino acids, L-Arginine and L-Lysine.



Casein which is the major protein of milk is digested with trypsin. The reaction can be followed by estimating the increase in the free amino groups using formol titration.

Formaldehyde reacts with the amino groups to form methylol compounds and the liberation of hydrogen ions. The solution will become more acidic. The amount of alkali required to neutralize the increase in acidity gives a measure of the amount of free amino groups formed from the protein digestion. Formaldehyde is also very effectively stops any further activity by reacting with the enzyme protein.



For an enzyme-catalysed reaction, there is usually a hyperbolic relationship between the rate of reaction and the concentration of substrate

substrate concentration

www.ucl.ac.uk/~ucbcdab/enzass/substrate.htm

Km

When the enzyme is saturaded with substrate the rate of the reaction is independent of the substrate concentration. In other words is zero order with respect to substrate. The velocity is dependent on the enzyme concentration when there is sufficient substrate to saturate the enzyme (first order)

- a- First order : at low substrate concentration the increase in velocity is almost linear in relation to substrate concentration.
- b- Zero order: The reaction rate is maximum when the enzyme is saturated with substrate.

Materials:

- Casein solution (1%, 2%, 4%, 6%, 8% at pH 8.5). Dissolve 4g casein in 90ml of water containing 2ml of 0.1N sodium hydroxide. Shake the suspension continuously and gently warm until dissolved. Adjust the pH to 8.5 with HCL and make up to 100ml with water.
- 2- Trypsin (4%). Curde trypsin prepared from pancreas is suitable.
- 3- Formaldehyde solution (40% w/v)
- 4- Phenolphthalein (0.255 in alcohol)
- 5- Sodium hydroxide (0.1N)

Procedure:

a- effect of substrate concentration

- 1- measure 5ml of formaldehyde into 5 small flasks.
- 2- Add one drop of phenolphthalein to each flask, the solution is colorless.
- 3- Titrate with 0.1N sodium hydroxide until the mixture is faintly pink. The color in all flasks should be the same.

The enzymatic reaction

Conical no.	1	2	3	4	5
Casein	10	10	10	10	10
(substrate) ml	1%	2%	4%	6%	8%
Pre-incubate in water bath 37C° for 10 min					
Trypsin	2	2	2	2	2
(enzyme) ml					
Incubate in water bath 37C° for 6 min					

- 1- Remove 5 ml of the mixture and add it to one of the five flasks containing formaldehyde.
- 2- Add one drop of phenolphthalein and titrate with NaOH until a faint but permanent color is obtained.
- 3- Repeat the same steps for each casein concentration.

Note: The final color at the end point should be the same in each case.

b- Effect of time

1- measure 5ml of formaldehyde into 5 small flasks.

2- Add one drop of phenolphthalein to each flask, the solution is colorless.

4- Titrate with 0.1N sodium hydroxide until the mixture is faintly pink. The color in all flasks should be the same.

Enzymatic reaction

- 1- measure 50ml of the casein solution into a conical flask and place it in a water bath 37 C for about 10min
- 2- put about 7 ml of trypsin in a test tube and place in water bath 37C for about 10 min
- 3- after about 10min. add 5ml of the trypsin solution to the conical containing the casein and mix

- 4- after 2,4,6,8 min. remove 10 ml of the reaction mixture and add it to one of the conical flasks containing formaldehyde.
- 5- Add 5 ml of ph.ph to each flask and titrate with 0.1N NaOH untail a faint pink color but permanent pink color is obtained.

- 1- Plummer, D. An introduction to practical biochemistry. McGraw-HILL, london. 1978
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Result:

A-Plot the increase in titer number against the casein concentration

B- Draw a curve showing the relationship between time and activity. Calculate the initial velocity

Experiment 10 Enzyme inhibition

Non-competitive inhibition of salivary α-amylase

Types of inhibition

1- Competitive inhibition

Competitive inhibition occurs when substrate (S) and inhibitor (I) both bind to the same site on the enzyme. In effect, they compete for the active site and bind in a mutually exclusive fashion.



The degree of inhibition depends on relative concentration of the substrate and inhibitor. Almost the maximal velocity may be found in the presence of the inhibitor if the substrate concentration is high enough. example for such An inhibition the inhibition of the enzyme succinate dehydrogenase by malonic and maleic acids since acid maleic compete with the substrate (succinate) on the active site.

2- Non- competitive inhibition

If a reversible inhibitor can bind to the enzyme at a site that is distinct from the active site, it is described as a "noncompetitive inhibitor." In pure noncompetitive inhibition, the inhibitor binds with equal affinity to the free enzyme and to the enzyme-substrate (ES) complex.



The enzyme –substrate- inhibitor complex formed is unable to break down and the inhibition effectively occurs by reduction of the amount of the enzyme available. Increase of the substrate concentration has no effect on the degree of inhibition. Most noncompetitive inhibitors are not related chemically to the substrate and the same inhibitor may affect a number of enzymes. Example for such inhibition:

The reaction of Heavy metal ion Ag^{+1} and Cu^{+2} . The reaction of cyanide with iron prophyrin enzyme.

Principle:

 α –amylase catalyses the hydrolysis of 1-4 links of starch with the production of maltose. The reaction is followed by measuring the increase in the reducing sugar. The reagent 3,5-dinitro-salcylate is reduced in alkaline medium to 3-amino-5-nitrosalicylic acid. The yellow colour produced is measured at 540nm.

The experiment is preformed with an activator NaCL and with a noncompetitive inhibitor HgCL₂.

Materials:

- 1- Phosphate buffer (0.1M, pH 6.7).
- 2- Starch substrate with different concentrations (0.2%, .4%, .6%, .8% and 1% in phosphate buffer).
- 3- Sodium chloride NaCl (1% w/v)
- 4- Mercuric chloride
- 5- Sodium hydroxide NaOH (2N)
- 6- Sodium potassium tartarate. 150g of sodium potassium tartarate was dissolved in 250ml distilled water
- 7- 3,5-dinitrosalicylic acid. 5g of 3,5-dinitrosalicylic acid in 100ml of 2N NaOH. The dinitrosalicylate was prepared by mixing step 6 with step 7 and complete the volume to 500ml with distilled water.
- 8- Amylase enzyme was prepared by diluting 1ml of saliva to 25ml with distilled water.

Procedure:

For each starch concentration prepare 6 tubes and add the following

Tube	B	S+E+A	B	S+E+I	В	E+S
Starch	-	2.5	-	2.5	-	2.5
Substrate(ml)						
NaCl	1	1	-	-	-	-
Activator(ml)						
HgCl ₂	-	-	1	1	-	-
Inhibitor (ml)						
D.W	3	-	3	-	3	-
Amylase	-	0.5	-	0.5	-	0.5
Enzyme (ml)						
Incubate all tubes in water bath 37 C° for 10 min						
NaOH (ml)	0.5	0.5	0.5	0.5	0.5	0.5
Dinitrosalicylate	0.5	0.5	0.5	0.5	0.5	0.5
reagent (ml)						
Heat the tubes in a boiling water bath for 5 min						
Cool						
Read at 540nm						

- 1- Plummer, D. An introduction to practical biochemistry. McGraw-HILL, london. 1978
- 2- Harvey, R and Champe, P. Lippincott biochemistry, london.2005
- 3- Boyer, R. Concepts in biochemistry. John Wiley and sons, New york. 2

Results:

- A- Draw a plot of different substrate concentration and enzyme + substrate absorption and compare it with the behaviour of the enzyme with the activator and with the inhibitor.
- B- Draw the line-weaver Burk plot [1/S against 1/V] and show Km and the Vmax.

Experiment 11 The effect of coenzyme Nicotinamide adinine dinucleotide as the coenzyme for lactate dehydrogenase

Principle:

Enzyme consists of the protein and a combination of one or more parts called cofactors. This enzyme complex is usually simply referred to simply as the enzyme.

Cofactors: A cofactor is a non-protein substance which may be organic, and called a **coenzyme**. The coenzyme is often derived from a vitamin. Another type of cofactor is an inorganic metal ion called a **metal ion activator**. The inorganic metal ions may be bonded through coordinate covalent bonds. The major reason for the nutritional requirement for minerals is to supply such metal ions as Zn^{+2} , Mg^{+2} , Mn^{+2} , Fe^{+2} , Cu^{+2} , K^{+1} , and Na^{+1} for use in enzymes as cofactors. Nicotinamide is from the niacin vitamin. The NAD⁺ coenzyme is involved with many types of oxidation reactions including lactate dehydrogenas.



Vitamin	Coenzyme	Function	
niacin	nicotinamide adenine	oxidation or hydrogen	
	dinucleotide (NAD ⁺)	transfer	
riboflavin	flavin adenine	oxidation or hydrogen	
noonaviir	dinucleotide (FAD)	transfer	
pantothenic acid	coenzyme A (CoA)	Acetyl group carrier	
vitamin B-12	coenzyme B-12	Methyl group transfer	
thiamin (B-1)	thiaminpyrophosphate	Aldehyde group	
	(TPP)	transfer	

Pyruvate forms a highly colored hydrazone with 2,4dinitrophenylhydrazine (DNP). An advantage for this reaction that compound other than pyruvate in the reaction mixture do not absorb at the wavelength used in the test. The amount of pyruvate converted to lactate can therefore be measured.

Materials:

- 1- Sodium phosphate buffer (0.1M, pH 7.4)
- 2- Sodium pyruvate. Dissolve 0.5g in 100 ml of phosphate buffer. The buffered substrate is prepared by diluting the above stock solution 1-50ml with buffer to give a concentration of 100mg/ml.
- 3- Reduced nicotinaminde adenine dinucleotide (10mg/ml). This is prepared in phosphate buffer just before use.
- 4- 2,4-dinitrophenylhydrazine (2mM). Dissolve 40 mg in 9 ml of concentrated hydrochloric acid and make up to 100ml with water.
- 5- Sodium hydroxide (0.4N).

6- Lactate dehydrogenase. Either serum of an appropriate dilution of commercial rabbit muscle enzyme is suitable.

Procedure:

Prepare 4 test tubes incubated in a water bath at 37C° as follow:

Tube	T ₁	T_2	Control	Blank	
Pyruvate	1	1	1	-	
Substrate					
(ml)					
Buffer (ml)	-	0.1	0.2	1.2	
NADH ₂ (ml)	0.1	-	-	-	
Enzyme (ml)	0.1	0.1	-	-	
Incubate for 15min at 37C°					
DNP (ml)	1	1	1	1	
Allow to stand in room temperature for 20 min. after the 20 min has					
been completed add.					
NaOH (ml)	10	10	10	10	
After 10 min read the colour developed at 510nm against the blank.					

- 1- Plummer, D. An introduction to practical biochemistry. McGraw-HILL, london. 1978
- 2- Harvey, R and Champe, P. Lippincott biochemistry, london.2005
- 3- Boyer, R. Concepts in biochemistry. John Wiley and sons, New york. 2002

Calculation:

Concentration of the unk. = $T_1abs. * control conc/control abs.$

T₂ abs. * control conc./control abs.

The amount of reacted pyruvate in the prescience of enzyme and cofactor= conc. Control – conc T_1

The amount of reacted pyruvate in the prescience of enzyme only

= conc. Control - conc T₂