
بسم الله الرحمن الرحيم

kingdom of Saudi Arabia

Ministry of Higher Education

king Saud University

College of Science

Biochemistry Department

Practical Note
METABOLISM
(BCH 447)

Prepared By

Dr. Abdurrahman M. Al-Senaidy
Dr. Abdulaziz A. Al-Jafari
Dr. Wajahatullah Khan
Dr. Samina H. Haq
Dr. Nikhat J. Siddiqi
Dr. Haifa R. Almubarak
Dr. Jane Kagwa
Mr. Mohammad A. Ismael
Mr. Zaki M. Almaghrabi
Mr. Fahad A. Alsaloom

TABLE OF CONTENTS

1. GENERAL LABORATORY AND SAFETY GUIDELINES 1

1.1 REGULATIONS 1
1.2 PRECAUTIONS 1
1.3 PERSONAL CLOTHING 2
1.4 EQUIPMENTS 2
1.5 GLASSWARE HANDLING 2
1.6 ACIDS AND BASES 2
1.7 LABORATORY NOTEBOOKS 2
1.8 GUIDELINES TO BE FOLLOWED 3

2. GUIDELINES FOR PREPARING LABORATORY REPORTS 3

2.1 ABSTRACT SECTION: 3
2.2 INTRODUCTION SECTION: 4
2.3 MATERIALS AND METHODS SECTION: 4
2.4 RESULTS SECTION: 4
2.5 DISCUSSION SECTION: 4
2.6 CONCLUSION SECTION: 4
2.7 REFERENCES SECTION: 4
2.8 ARTICLE WITHIN A JOURNAL: 4
2.9 BOOK CHAPTER OR ARTICLE WITHIN A BOOK: 5
2.10 COMPLETE BOOK: 5

3. ISOLATION OF GLYCOGEN FROM LIVER 5

3.1 INTRODUCTION 5
3.2 OBJECTIVE 6
3.3 MATERIALS AND METHODS 6
3.3.1 Chemicals 6
3.3.2 Solutions 6
3.3.3 Materials 6
3.3.4 Equipments 6
3.3.5 Glassware 6
3.3.6 Preparation of solutions 6
3.4 PROCEDURE 7
3.5 RESULTS 7
3.6 DISCUSSION 8
3.7 QUESTION 9
3.8 REFERENCE: 9

4. ENZYMATIC HYDROLYSIS OF GLYCOGEN AND DETERMINATION OF GLUCOSE 10

4.1 INTRODUCTION 10
4.2 OBJECTIVE 11
4.3 THEORY 11

4.4 METHODS AND MATERIALS 12
4.4.1 Chemicals 12
4.4.2 Equipments 12
4.4.3 Glassware 12
4.4.4 Preparation of solutions 12
4.5 PROCEDURE (SEE TABLE AT THE END) 13
4.6 RESULTS 14
4.7 DISCUSSION: 15
4.8 QUESTION 1: 16
4.9 REFERENCE: 16

.5 ESTIMATION OF ARGINASE ACTIVITY IN LIVER EXTRACT 17

5.1 INTRODUCTION 17
5.2 PRINCIPLE 17
5.2.1 Objective 17
5.3 MATERIALS AND EQUIPMENT 17
5.4 METHOD: 18
5.5 RESULTS 19
5.6 QUESTIONS: 20
5.7 REFERENCES: 20

6. CELL FRACTIONATION – ISOLATION OF MITOCHONDRIA FROM RAT LIVER 21

6.1 INTRODUCTION 21
6.2 THEORY 22
6.3 OBJECTIVE 23
6.4 ORGANELLE ASSAYS 23
6.5 MATERIALS AND METHODS 23
6.5.1 Materials 23
6.5.2 Chemicals 24
6.5.3 Equipment 24
6.5.4 Glass ware 24
6.6 PART 1: CELL FRACTIONATION AND PREPARATION OF MITOCHONDRIA BY DIFFERENTIAL CENTRIFUGATION 24
6.6.1 Protocol for Liver Fractionation 24
6.6.2 Isolation of Nuclei 25
6.6.3 Isolation of Mitochondria 25
6.7 PART 2A: ASSAY OF SUCCINATE DEHYDROGENASE IN THE MITOCHONDRIAL FRACTION 26
6.7.1 Preparation of Reagents 26
6.7.2 Succinate Dehydrogenase Assay (Mitochondria) 26
6.8 PART 2B: DETERMINATION OF PROTEIN CONTENT IN THE MITOCHONDRIA: 28
6.8.1 Bradford protein assay 28
6.8.2 Principle 28
6.8.3 Equipment 28
6.8.4 Procedure 28
6.8.5 Reagents 29
6.8.6 Assay 29

6.8.7 Specific Activity 29
6.9 RESULTS 30
6.10 DISCUSSION 31
6.11 LITERATURE CITED 32

7. TRIGLYCERIDE DETERMINATION 33

7.1 INTRODUCTION 33
7.2 PRINCIPLE 33
7.3 REAGENT COMPOSITION 33
7.4 MATERIALS REQUIRED 34
7.5 PROCEDURE 34
7.6 CALCULATIONS 34
7.7 REFERENCES 35

8. HDL-CHOLESTEROL DETERMINATION 36

8.1 INTRODUCTION 36
8.2 PRINCIPLE 36
8.3 REAGENT COMPOSITION 36
8.4 STORAGE AND STABILITY 36
8.5 SPECIMEN COLLECTION AND STORAGE 37
8.6 MATERIALS REQUIRED 37
8.7 PROCEDURE 37

9. LIPASE ASSAY(USING TURBIDIMETRIC METHOD) 39

9.1 INTRODUCTION 39
9.2 PRINCIPLE 41
9.3 REAGENT COMPOSITION 41
9.4 REAGENT PREPARATION 41
9.5 REAGENT STORAGE AND STABILITY 41
9.6 SPECIMEN COLLECTION STORAGE 41
9.7 MATERIALS REQUIRED 42
9.8 PROCEDURE 42
9.9 PROCEDURE NOTES 42
9.10 CALIBRATION 42
9.11 CALCULATIONS 42
9.12 RESULTS: 44

10. ESTIMATION OF GLUTATHIONE IN PLASMA 46

10.1 INTRODUCTION 46
10.2 OBJECTIVE FOR THE EXPERIMENT 46
10.3 ASSAY PRINCIPLE 46
10.4 METHODS AND MATERIALS 47
10.5 PREPARATION OF REAGENTS: 47
10.6 MATERIALS REQUIRED: 48
10.7 PREPARATION OF GLUTATHIONE STANDARD CURVE.: 49
10.8 PREPARATION OF BLOOD SAMPLE FOR GSH DETERMINATION. 49
10.9 CALCULATION OF GLUTATHIONE CONCENTRATION: 50

10.10 RESULT AND DISCUSSION: 51
10.11 REFERENCES: 51

11. UREASE ACTIVITY COMPARISON OF THE ACTIVITIES OF UREASE FROM DIFFERENT SOURCES 52

11.1 INTRODUCTION: 52
11.2 THEORY: 52
11.3 OBJECTIVE: 52
11.4 MATERIALS AND METHOD: 52
11.4.1 Material: 52
11.4.2 Chemicals: 52
11.4.3 Equipments: 53
11.4.4 Glassware: 53
11.5 PREPARATION OF SOLUTIONS: 53
11.6 PROCEDURE: 53
11.6.1 Part 1. Preparation of urease extracts. 53
11.6.2 Part 2: Comparison of activities of the two enzyme extracts. 54
11.7 TITRATION: 55
11.8 RESULTS 56
11.9 DISCUSSION: 57
11.10 QUESTION: 58
11.11 REFERENCES 58

12. D- XYLOSE ABSORPTION TEST 59

12.1 INTRODUCTION 59
12.2 OBJECTIVES 59
12.3 PRINCIPLE 59
12.4 MATERIALS AND METHODS 60
12.5 METHOD 60
12.6 ESTIMATION OF D-XYLOSE IN URINE 60
12.7 RESULTS 61

Introduction

This practical course is designed to train you to carry out some of the most common experimental procedures in biochemistry and metabolism. You will also gain some experience with various equipments commonly used in biochemistry. Before conducting each experiment, it is essential that you read this Laboratory Manual. It gives you the background information and an outline of the methodologies to be carried out. The Metabolism laboratory practical, similar to all laboratory courses, is a study of different methods in this area. Hence reading the manual in advance will help you to understand the basic concepts and philosophy behind each experiment. We expect you to participate enthusiastically in the actual lab work and not merely watch your lab partners do things for you. This practical course will provide you with an opportunity to learn valuable skills in the field of biochemistry so take full advantage of it!

1. General Laboratory and Safety Guidelines

Safe practices in the biochemistry laboratory are of great significance. Students must adopt safe and skillful methods while working in the lab. To achieve this goal the student must obtain the prerequisite knowledge of properties of materials present in the lab and must be acquainted with different hazards and harmful effects associated with their improper handling.

1.1 Regulations

1- Throughout your stay in the laboratory you must wear safety goggles.
2- Immediately inform your instructor in case of any accident.
3- Do not eat, drink, chew, or smoke in the laboratory.
4- Do not depart from the lab leaving an experiment unattended. If you need to leave the lab you must inform your instructor before leaving the lab.
5- After finishing the experiment turn off all the equipments, clean your work bench and reshelf all the equipments or chemicals.
6- Not sticking to these rules will result in instant removal from the lab.

1.2 Precautions

1- You must come to the lab with a serious awareness of personal liability and utmost consideration for others in the lab.
2- You must acquaint yourself with safety equipment location, acid-base neutralizing agents, eye wash, fire extinguisher, emergency shower, broom & dustpan and broken glass container.
3- You must listen carefully to all the instructions given by your instructor. If you are unsure of anything, always ask your instructor.
4- You must immediately clean all chemical spills.

5- While handling the chemicals you must wear gloves otherwise some chemicals may result in skin irritation.

6- While handling all electrical and heating equipments extra precautions must be taken to prevent shocks and burns.

7- Do not handle any broken glassware with your bare hands; use a broom and a dust pan to collect the pieces.

8- You must wash your hands with soap after finishing the experiment.

1.3 Personal clothing

Selection of clothing for the laboratory is generally left to the discretion of the student. However, due to the harmful nature of some chemicals, it is in the best interest of the student to wear proper and suitable clothing. You must wear a lab coat to help keep clothes protected. Open toed shoes must not be worn because they cannot protect you against chemical spills. Long hair should be tied back to avoid interference with motion or observation.

1.4 Equipments

Equipment must be placed in a safe and secure manner. Hot plate must be placed in safe location and kept away from the edge of the bench to reduce chances of body contact.

1.5 Glassware Handling

Glassware in the lab is generally delicate and fragile, and if not handled properly, may cause serious injuries. Do not use any chipped or broken glassware. After finishing the experiment, all glassware must be cleaned and kept back at the proper place.

1.6 Acids and Bases

In metabolism lab experiments you will be using different acids and bases. Hence care must be taken to avoid skin contact. While handling these chemicals, avoid eye and face contact. In case of acid or base contact with your skin, wash it with large amount of clean, cold water and inform your instructor immediately. For your own protection, neutralize acid or base spills before cleaning them up.

1.7 Laboratory Notebooks

For all laboratory experiments use a bound notebook to keep record of all primary data and observations. You must organize your notebook every week before coming to the lab by writing the title of the experiment on a new page, with important equations or formulae from the lab manual, and all necessary calculations involving solution preparations, molar masses, etc.

Try to understand theoretical concepts and particular instructions given by your instructor before the experiment. The lab notebook must have a record of every experiment. The lab notes should be written in a manner that other people could understand them. Excellent note taking in the lab is an important skill that can be learned with little effort and practice.

1.8 Guidelines to be followed

1- Carry your notebook to the lab for each experiment.
2- Number all the pages in sequential order.
3- Use your notebook to record values directly and do not use loose scraps of paper.
4- Mention each measured quantity by its name and indicate the units.
5- Simply strike through the sentence and write the new sentence next to it, if you make a mistake in your notebook.
6- Tables are very useful to simplify the data entry; they should be prepared in advance before starting the experiment.
7- Do not depend on your memory and write down all observations for example color and phase changes, etc.
8- Last but not the least, you must write a brief conclusion of your experiment. It should address the objectives of conducting the experiment.

2. Guidelines for preparing Laboratory reports

The laboratory reports are major written assignments and should be written in the form of a scientific paper. The laboratory reports should contain the following sections:

- Title Page
- Brief Introduction
- Materials and Methods
- Results/Discussion
- References
- Appendix

All of the laboratory reports are expected to be well written, typed and preferably in English. Follow the following guidelines for each section to write a lab report.

2.1 Abstract section:

The abstract is a very short and yet thorough summary of the report so that one can get an idea about the experiment without reading your whole report. It should include the purpose of your experiment, the procedures you used to carry out the experiment, results you

obtained from the experiments, and your conclusions. The abstract should be no longer than a small paragraph (10-12 lines).

2.2 Introduction section:

This part should consist of any theoretical background information pertinent to understand your report. This section should be around 30-40 lines.

2.3 Materials and Methods section:

In this section you will write the material and methods you used to carry out the experiment (such as calculations, exact volumes, amounts, incubation times, etc.). You must also mention any modifications from the procedure mentioned in the manual.

2.4 Results section:

In this section of your lab report, you should report all your results that you get from your experiment. You should present them in a tabulated form so it will be easy for quick reference. You must number and label all the tables and figures (graphs, diagrams). This way it will be easy for you to refer to them in your discussion section. You should also include your sample calculations (if any) in the result section.

2.5 Discussion section:

In this section you are required to give a thorough description of what happened in the experiment. The discussion section is also where you interpret your results and make conclusions. You should refer to your tables and diagrams while explaining your results. You should compare your results to expected values (calculated or from the literature). Even if you obtained unexpected results, the discussion section is the section to justify or explain the reasons why you have obtained such results. Please remember how you interpret your results carries more weight than the results themselves.

2.6 Conclusion section:

The conclusion section is very similar to the abstract. It is just a quick overview of what was done and how. However, more importance is given to the results.

2.7 References section:

In this section you will provide an alphabetical listing (by first author's last name) of the references that you actually cited in the body of your report. Write all your references in the following format:

2.8 Article within a journal:

Koonin EV, Altschul SF, Bork P: BRCA1 protein products: functional motifs. Nat Genet 1996, 13:266-267.

2.9 Book chapter or article within a book:

Schnepf E: From prey via endosymbiont to plastids: comparative studies in dinoflagellates. In Origins of Plastids. Volume 2. 2nd edition. Edited by Lewin RA. New York: Chapman and Hall; 1993:53-76.

2.10 Complete book:

Margulis L: Origin of Eukaryotic Cells. New Haven: Yale University Press; 1970.

Experiment 1

3. Isolation of Glycogen from Liver

3.1 Introduction

Living organisms frequently contain stored carbohydrates which usually act as reserve materials. Those materials are stored in the form of polysaccharides such as the starch and insulin in plants and the glycogen of higher animals. The properties of many neutral polysaccharides are sufficiently different from those of other naturally occurring substances to permit their ready isolation. Thus when rat liver is homogenized in trichloroacetic acid (TCA) many high molecular weight compounds, such as proteins and nucleic acids are readily precipitated, while the polysaccharides remain in solution. Since polysaccharides are less soluble than sugars in solution. Since polysaccharides are less soluble than sugars in aqueous alcohol, glycogen can be separated from sugars and other water soluble compounds by precipitation with alcohol.

In general, animal glycogen is isolated from liver or muscle tissue. The condition of the laboratory animal from which the tissue is to be taken is important because the glycogen, especially liver glycogen, will vary in properties and yield according to

Glycogen is the main polysaccharide energy reserve in animals and is stored mainly in liver and muscle. When liver is ground up with trichloroacetic acid (TCA), the larger molecules such as proteins and nucleic acids are precipitated while glycogen remains in solution with sugars and other water–soluble compounds. The glycogen can then be separated from the other compounds by precipitation with aqueous alcohol in which it is less soluble.
The condition of the animal from which the liver is taken is important because the yield varies according to whether the animal is fed, fasted, ill etc. Therefore for a good yield the animal should be well fed before the liver is removed, the sample should be kept cold and the pH lowered with TCA.

3.2 Objective

To illustrate the method for isolating glycogen.

3.3 Materials and Methods

3.3.1 Chemicals

- Trichloracetic acid (TCA)
- Ethanol (95% v/v)
- Diethyl ether
- Sodium chloride

3.3.2 Solutions

- 5% TCA
- 10% TCA

3.3.3 Materials

- Liver (of well fed rat or other animal)
- Washed and dried sand
- Ice

3.3.4 Equipments

- Mortar and pestle
- Refrigerated centrifuge
- Water bath at 37°C.
- Glass rods

3.3.5 Glassware

- Two 20 ml beakers
- One 100 ml beaker
- 100 ml graduated cylinder
- 50 ml graduated cylinder

3.3.6 Preparation of solutions

- 10% TCA(w/v)
    - Dissolve 100 g of TCA in 1.0 litre.
- 5% TCA (w/v)
- Dilute the above solution 1:2
- 95% Ethanol (v/v)

Caution! TCA causes severe burns; wash accidental spills on skin with plenty of running tap water for a minute.

3.4 Procedure

1. Weigh about 5.0 g of cold liver **quickly** to the nearest 0.1 g, transfer to a mortar, cut into small pieces, grind with about 0.5 g of clean cold sand and 10%TCA (1 ml per g tissue).
2. Centrifuge homogenate at 3,000 rpm for 5min at 4°C. Pour off supernatant into a 50 ml graduated cylinder.
3. Rinse out mortar with 5% TCA (using same volume as for 10% TCA already used). Add this rinsing fluid to the centrifuge tubes containing residue from first centrifugation. Stir up residue and re-centrifuge for another 5 min. at 3,000 rpm. Discard pellet. Add supernatant to that already collected.
4. Record total volume; add twice this volume of 95% ethanol, slowly with stirring, to supernatant. Allow to stand while precipitate settles. If it does not, add a little NaCl and warm cylinder in water bath at 37º C.
5. Centrifuge suspension at 3,000 rpm for 3 min. Discard supernatant. Dissolve pellet in centrifuge tubes in 5 ml water and re-precipitate by adding 10 ml of 95% ethanol. Re-centrifuge and discard supernatant.
6. Stir up pellet with 3 ml 95% ethanol, re-centrifuge and discard

   supernatant. Now add 3 ml diethyl ether, stir up pellet, re-centrifuge and discard supernatant. This final pellet contains glycogen from the liver. Air -dry the glycogen in the tube and weigh it.

3.5 Results

Record total yield and glycogen content per 100 g liver.

3.6 Discussion

3.7 Question

Why are time, temperature and pH important in the initial stages of the isolation of glycogen, but not in the latter stages?

3.8 Reference:

1. Campbell, Mary; Farell Shawn. (2008). Biochemistry (6th ed.). Canada: Brooks/Crole.

2. The Biochemistry Department (2008). Laboratory Manual in General Biochemistry : University of Santo Tomas.

3. Johnson, C.R., Miller, M.J., Pasto, D.J. (1998). Experiments and Techniques in Biochemistry. United States of America: Prentice Hall Inc

Experiment 2

4. Enzymatic hydrolysis of glycogen and determination of glucose

4.1 Introduction

The structure of the glycogen molecule is fan-like; with long chains of glucose residues linked by 1, 4-glycosidic bonds, with 1, 6- links at the branch points. So the whole glycogen molecule has only one free reducing end, where the C_1 of a glucose residue is free (exposed). Thus the glycogen molecule is essentially non-reducing.

Hydrolysis converts glycogen from a non-reducing substance into reducing substances. Hydrolysis of the glycogen molecule with acid results in splitting of all its glycosidic bonds giving only glucose molecules as the product. Enzymes are more specific in the bond type they split. Thus salivary amylase (α-amylase) will randomly split only 1, 4- glycosidic bonds and produce a mixture of products consisting of glucose, maltose and maltotriose molecules. The increase in the number of reducing groups is determined using 3, 5-dinitrosalicylic acid (DNS) in alkaline solution.

The oxidation of carbohydrate or related compounds is the main source of energy for many organisms. The readily digestible carbohydrates of the mammalian diet include the starches, amylase and amylopectin, as well as glycogen. Amylose is a linear polysaccharides consisting of glucose units linked to one another in sequence by α-1-4 bonds. Amylopectin and glycogen are branching polysaccharides, in addition to α-1, 4 bonds, they have α-1, 6-glycosidic bonds at the branch points. The hydrolysis of these glycosidic bonds is catalyzed by either acids or enzymes, in the acid-catalyzed hydrolysis there is a random cleavage of bonds, with the intermediates formation of all the various possible oligosaccharides and with the final conversion of these oligosaccharides to glucose.

In the presence of amylases, which have been classified into two main groups, α and β according to the mode of their attack on the polysaccharide.

The amylases of animal origin are all α-amylases and in the digestive system are found in saliva and in pancreatic juice. A-amylases catalyze the rapid, random hydrolysis of internal α-1, 4 bonds. They do not hydrolyze α-1, 6 linkages, regardless of molecular size nor do they hydrolyze maltose. Thus glycogen is initially split by α-amylase action into branched dextrins of medium molecular weight and only small amounts of maltose are formed. Further action of α-amylase decreases the molecular weight of these dextrins yielding oligosaccharides. The final degradation products of the action of α-amylase on glycogen are glucose, maltose and isomaltose.

A second enzyme β-amylases which is widely distributed in plants and microorganisms, also catalyze the hydrolysis of glycogen. They catalyze the successive hydrolysis of the second α-1, 4 glycosidic bond from the free nonreducing ands of glucose chains, releasing maltose units. But β-amylases do not hydrolyze α-1, 6 bonds, nor do they hydrolyze α-1, 4 bonds of glucose chains beyond an α-1, 6 branch residues. Thus, after all the nonreducing end glucose chains have been trimmed back to the branch residues, the final products of the action of β-amylase on glycogen are maltose and the remaining limit dextrin.

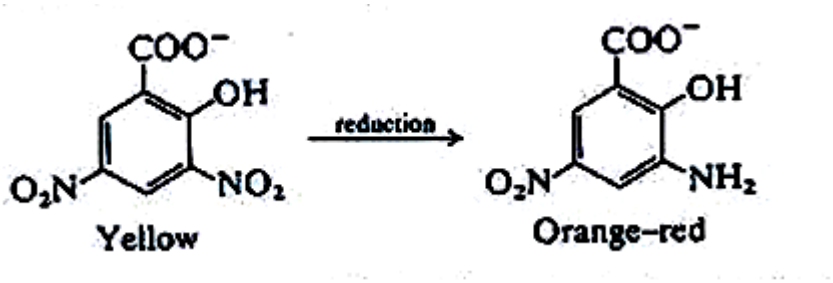
There are many methods for measuring the hydrolysis of glycogen and other polysaccharides, such as measurement of reducing sugars, or change of decreasing viscosity and the loss of capacity to give a blue color with iodine and finally the formation of split products.

4.2 Objective

To examine the polysaccharide nature of glycogen and show that hydrolysis increases the number of reducing groups.

4.3 Theory

Several reagents can be used to assay reducing sugars such as 3, 5 dinitrosalicylic acid in one of the compounds. In alkaline solution it is reduced to 3-amino-5- nitro salicylic acid, which is orange-red. Absorbance is determined at 540 nm.



Yellow Orange–red

4.4 Methods and Materials

4.4.1 Chemicals

- Glycogen isolated in the previous experiment
- Sodium dihydrogen phosphate (NaH_2PO_4)
- Sodium hydroxide
- Sodium chloride
- Sodium potassium tartrate
- 3,5-Dinitrosalicyclic acid (DNS)
- HCl

4.4.2 Equipments

- Boiling water bath
- Spectrophotometer

4.4.3 Glassware

- Small beaker
- Big test tubes (25 ml)
- Glass cuvettes

4.4.4 Preparation of solutions

0.02 M Na phosphate buffer, pH 6.9, containing 0.005 M NaCl (PS buffer):

1. Prepare 500 ml 0.04 M NaH_2PO_4 (MW 120). Dissolve 2.4 g in water and make up to 500 ml.
2. Prepare 250 ml of 0.04 M NaOH (MW 40). Dissolve 0.4g NaOH in water and make up to 250 ml.
3. 0.005 M NaCl (MW 58.5). Dissolve 0.2925g NaCl in a little water.
   To 500ml of the NaH_2PO_4 solution add 224 ml of the NaOH solution. Mix and measure the pH. If is less than 6.9 adjust by adding more NaOH solution. Add the NaCl solution and make up to 1 liter.

3, 5 -dinitrosalicyclic acid reagent (DNS):

2 M NaOH. Prepare 250 ml. Dissolve 20g in water and make up to 250 ml.

a) 3, 5- dinitrosalicyclic acid. Dissolve 10g in 200 ml 2M NaOH.
b) Sodium potassium tartrate. Dissolve 300g in 500ml water.

Prepare DNS reagent fresh by mixing A and B and making up to 1 liter with water

2M HCl: Take 16.7 conc. HCl in 100ml volumetric flask and make up to 100 ml with water.

1.2 M NaOH: Dissolve 4.8 g in water and make up to 100 ml.
Glycogen solution: Dissolve 32 mg glycogen in 4 ml phosphate buffer/ NaCl (8mg/ ml).

Saliva: Collect about 2 ml saliva in a small beaker. **Immediately before** use dilute 1: 20 with the buffer (Take 1ml saliva and add 19.0 ml buffer).

4.5 Procedure (see table at the end)

Label nine tubes 1 -9. Pipette 0.4 ml PS buffer in tube 1 and use as blank. Pipette 0.4 ml glycogen solution into each of the remaining tubes (2-9). Add 0.6 ml 2 M HCl to tube 9 and incubate in a boiling water bath for 30 min. Add 0.6 ml diluted saliva to each of the remaining tubes (1-8) and stand at room temperature. Immediately after adding diluted saliva to tube 2 stop action of the enzyme by adding 1 ml of DNS reagent. Stop the reaction in tubes 3-7 at 2 min. intervals and in tube 8 after 30 min in the same manner.

After tube 9 has been in water bath for 30 min. add 1 ml 1.2 M NaOH to neutralize HCl, then add 1ml DNS reagent.

Heat all the tubes for 5 min. in a boiling water bath. Cool by immersing in cold water. Add 8 ml water to each tube except tube 9. Add 7 ml water to tube 9. Read absorbance at 540 nm against the blank.

NB. Since the amylase content of saliva varies between individuals, in some cases it may be necessary to make different dilutions of saliva to get reasonable results. You may therefore have to repeat the experiment.

4.6 Results

Tube 9 contained the total glucose yield from complete hydrolysis of glycogen. Taking this as 100% conversion of glycogen to glucose, plot the percentage hydrolysis against time.

Tube No.	Time of hydrolysis (min)	Absorbance (540 nm)	percentage hydrolysis
2	0		
3	2		
4	4		
5	6		
6	8		
7	10		
8	30		
9	30		100

4.7 Discussion:

4.8 Question 1:

What would be the effect of substituting β-amylase for α-amylase?

Tube No.	PS Buffer (ml)	Glycogen Solution (ml)	Dilute saliva (ml)	2 M HCl (ml)	Time of Hydrolysis (min)	1.2.M NaOH (ml)	DNS Reagent (ml)	Water (ml)
1	0.4	-	0.6	-	30	-	1	8
2	-	0.4	0.6	-	0	-	1	8
3	-	0.4	0.6	-	2	-	1	8
4	-	0.4	0.6	-	4	-	1	8
5	-	0.4	0.6	-	6	-	1	8
6	-	0.4	0.6	-	8	-	1	8
7	-	0.4	0.6	-	10	-	1	8
8	-	0.4	0.6	-	30	-	1	8
9	-	0.4	-	0.6	30	1	1	7

4.9 Reference:

1. Landgrebe, J.A. (1993). Theory and Practice in the Biochemistry Laboratory: with Microscale and Standard Scale Experiments. California: Wadsworth Inc.

2. Johnson, C.R., Miller, M.J., Pasto, D.J. (1998). Experiments and Techniques in Biochemistry. United States of America: Prentice Hall Inc

Experiment 3

5. Estimation Of Arginase Activity In Liver Extract

5.1 Introduction

Arginase is one of the important enzymes in urea cycle which is the major disposal form of amino groups derived from amino acids and accounts for about 90% of the nitrogen-containing compounds of urine. Urea is produced by a set of enzymes present in the liver ,and then is transported in the blood to the kidneys for excretion. The arginase enzyme catalyses the final reaction in the urea cycle, the enzyme is present exclusively in the liver .Arginase catalyses the hydrolytic cleavage of the guanidino group of Arginine to regenerate ornithine and urea.

Arginine ↔ Urea + Ornithine

Mammalian liver arginase is activated by Co^{2+} and Mn^{2+} while ornithine and lysine are potent inhibitors.

5.2 Principle

The activity of the enzyme is determined by measuring the amount of urea produced ,urea is reacted with the reagent isonitrosopropiophenone and heated in boiling water, leading to the production of a red color compound which is measured spectrophotometrically at 520nm.

A urea standard curve is generated and the concentration of urea produced in the liver extract is determined from the standard curve.

5.2.1 Objective

Estimation of Arginase activity in liver extract.

5.3 Materials And Equipment

1- Arginine solution (0.5 mole/L) at pH 9.7 .
2- Perchloric acid 5% v/v, prepare by diluting the perchloric acid with water.
3- Manganase sulfate (4 mmole / L) freshly prepared.
4- Isonitrosopropiophenone reagent for urea estimation (2g/L) β-isonitrosopropiophenone containing 170 ml of conc. Sulfuric acid and 40ml phosphoric acid made up to 1L with water. (caution strong acids).
5- Standard urea solution (0.5 mmole/L).
6- Water bath at 37^0C.
7- Boiling water bath.
8- Marbles(to regulate boiling).
9- Aluminum foil.

10- Spectrophotometer.
11- Fresh rat liver.

5.4 Method:

One fresh rat liver is homogenized in a volume of cold potassium phosphate buffer (0.05 M, pH 7.4) equal to 3 times its wet weight. The homogenate is centrifuged in the cold for 1 minute at 8000 rpm. The supernatant contains the enzyme and must be kept cold. Dilute the liver extract 1:20 with ice-cold water and use this diluted extract for the enzyme .
Prepare a standard curve for urea by following the instructions in table 1.

Table 1.

Tube	Standard urea solution(0.5mmole/L)	Isonitrosopropiophenone reagent.	H_2O
A	0.2ml	1.8ml	--------
B	0.4ml	1.6ml	--------
C	0.6ml	1.4ml	--------
D	0.8ml	1.2ml	--------
E	1.0ml	1.0ml	-------
Blank	----------	1.0ml	1.0ml

Mix the contents of each tube ,heat in a boiling water bath with marble on top. The red color produced is sensitive to light so all the test tubes are wrapped in aluminum foil .After one hour remove the tubes ,transfer them to a beaker of cold water ,and cover with a black cloth. After allowing the tubes to cool for 15minutes , read the absorbance at 520 nm .Record results in table 3.

To estimate the arginase activity in the liver homogenate follow table 2.
Table2.

Tube	Arginine solution(0.5mole/L)	$MnSO_4$ solution (4mmole/L)
Test	1.0 ml	0.5 ml
Blank	1.0 ml	0.5ml

Mix the contents of each tube and equilibrate at 37^0C for 10 minutes then start the reaction in the Test tube by adding 0.5ml of diluted liver extract mix thoroughly incubate at 37^0C for 10 minutes ,stop the reaction by adding 5ml of perchloric acid .

In the blank the perchloric acid is added to the substrate and manganese solution before adding the liver extract (to inactivate the enzyme) and is incubated for 10 minutes at 37^0C.
The tubes are centrifuged to remove the precipitated protein , remove 1ml of the supernatant from both the Test and Blank tube and add to each 1.0ml of the isonitrosopropiophenone

reagent mix ,heat in a boiling water bath with marble on top .The test tubes are wrapped in aluminum foil ,after one hour they are removed from water bath, transferred to a beaker of cold water and covered with a black cloth .After they cool for 15minutes the Test absorbance is read against the blank at 520nm, and absorbance recorded in table 3.

5.5 Results

Table 3.

Tube	Absorbance at 520nm	Urea concentration mmol/L
A		
B		
C		
D		
E		
Test		

Plot the standard curve of urea and from the curve obtain the concentration of urea in the liver extract mmol/L .

Calculate the total arginase activity in the liver, and the arginase activity as micromoles of urea produced per gram of liver.

Concentration of urea in liver extract from curve --------mmol/L produced in 10 min.

Urea produced in 1minute = ------- /10 = -------mmol /min /0.5ml of liver extract.

Urea in 1ml of diluted liver extract = ------- x 2 = -------mmol/min/ml of diluted liver extract .

Urea concentration in 1ml of undiluted liver extact = ----- x Wt of liver x 3 x 20

Urea concentration in micromoles = -------- x1000 = ------- micromoles/min/ml .

Total activity present in liver = --------- x total volume of liver extract = -------micromole .

Arginase activity as micromoles per gram of liver = total activity in liver/ wt of liver(g).

5.6 Questions:

1- Although Arginine is present in many tissues ,urea synthesis occurs in the liver only, why?
2- What was the purpose of the Manganese sulfate ,and perchloric acid used in this experiment?
3- Deficiency in the liver Arginase activity leads to low levels of serum urea, but low levels of serum urea is not necessary associated with low arginase activity, explain.
4- What is the most important clinical complication of Arginase deficiency?

5.7 References:

- Advances in Clinical Chemistry by Oscar Bodansky.
- Internet link(http://www.fgsc.net/teaching/keenan.pdf)

Experiment 4 & 5

6. Cell Fractionation – Isolation of Mitochondria from rat liver

6.1 Introduction

Organelles are membrane-enclosed vesicles inside all eukaryotic cells that function in a variety of important cellular processes. In this CELL FRACTIONATION lab exercise, we will isolate mitochondria from rat liver by a technique termed differential centrifugation. We will then assay these fractions for succinate dehydrogenase, a specific marker enzyme for mitochondria. In this experiment we will learn the techniques of differential centrifugation and enzyme assays for succinate dehydrogenase.

Living organisms require a continuous supply of energy to maintain the many and varied functions characteristic of living matter. In most cases, this energy is obtained by the oxidation of metabolites which arise from the digestion of food. There are three ways that such oxidations can take place; by addition of oxygen, by removal of hydride, and by removal of electrons.

The oxidative process is initiated by the removal of two hydrogens from the substrate by a group of enzymes called dehydrogenases. The dehydrogenases transfer these two hydrogens directly to molecular oxygen to from hydrogen peroxide. However, most dehydrogenases will not react directly with oxygen but require intermediary enzyme systems. In the first step the substrate is oxidized by an enzyme that transfers a pair of hydrogen ions and electrons from the substrate to an electron acceptor. The electrons are then transferred by means of a series of coupled oxidations and reductions to a final electron acceptor, oxygen, which then interacts with the previously released hydrogen ions to form water. The series of enzyme-catalyzed reactions is called the electron-transport chain.

The dehydrogenases remove pairs of hydrogen atoms from substrate molecules. These enzymes consist of two parts, a protein [apoenzyme], which is different for each individual dehydrogenase, and a nonprotein, coenzyme, which is common to many dehydrogenases. The coenzyme nicotinamide adenine dinucleotide [NAD] contains as its active group nicotinic acid and adenine, ribose and two molecules of phosphoric acid. The coenzyme nicotinamide adenine dinucleotide phosphate [NADP] differs from NAD only by the possession of three molecules of phosphoric acid instead of two.

Oxidation of the substrate by the dehydrogenases thus involves the two hydrogen atoms removed from the substrate. Reoxidation of the reduced coenzyme is carried out by the flavoproteins. These flavoproteins act as hydrogen-transfer agents in a manner similar to NAD and NADP.

The action of tissue dehydrogenases was studied extensively by Thunberg, who found that certain dyes are highly colored in the oxidized form and are colorless in the reduced form. Some of these react readily with certain biological oxidation-reduction systems, and the occurrence and rate of the reaction is readily followed by observing the disappearance of the color of the oxidized from. The dye 2, 6-dichlorophenolindolphenol [DICPIP] is colored in the oxidized from and colorless when reduced. If the dye is added to a suspension of minced tissue, such as muscle, kidney or liver, it is rapidly reduced and loses its color, thus indicating the presence of dehydrogenases in the tissue extracts.

6.2 Theory

Cellular Fractionation

The arrangement of macromolecules within a cell is as important to cellular function as their catalytic activities. Cellular compartmentalization provides efficiency by bringing together related compounds (i.e. the enzymes of the mitochondrial electron transport chain), or by separating those compounds that can interfere with each other (i.e. lysosomal hydrolytic enzymes). Cellular compartmentalization is, in part, accomplished by various sub cellular organelles. In this lab exercise, we will isolate one of the sub cellular organelles viz., mitochondria from rat liver cells. The method we will use to separate the various organelles utilizes differential centrifugation to isolate components of different densities. With this technique, the heaviest or most dense organelles (i.e. nuclei) pellet in less time (and at less forces) than is required to pellet lighter organelles (such as mitochondria). First, a cell homogenate is made by breaking open the cell membrane. The homogenate is then centrifuged for a short period of time to remove cell debris and nuclei. The supernatant is then transferred to another tube and centrifuged longer to pellet the mitochondria.

For this type of fractionation experiment, which tissue we use, and the method of homogenization are dictated by the biological system. Some organs (i.e. liver) have one cell type that predominates, so are also well suited. Once a cell type is chosen (we will work with liver), it is important to obtain the organelles in a biochemically active, morphologically whole state. Homogenizers or blenders are used to break open the cells without damaging the organelles. Homogenizers have a precise clearance between the glass tube and the pestle, which breaks the cell membrane leaving the smaller organelle membranes intact. The homogenization buffer often includes sucrose to partially dehydrate the organelles keeping them intact.

No technique used to isolate organelles is perfect. It is very difficult to get pure unbroken preparations of any organelle. Techniques providing optimal isolation of one organelle may completely rupture another organelle. Thus methods are often used to measure the contamination of one organelle fraction with another. Analyzing each organelle fraction for organelle-specific marker enzymes can accomplish this.

6.3 Objective

This experiment consists of two parts viz,
Part 1: Cell fractionation and preparation of mitochondria by differential centrifugation.

Part 2: Assay of succinate dehydrogenase in the mitochondrial fraction.

6.4 Organelle Assays

We will evaluate the purity of our sub cellular fraction (in this case mitochondria) by analyzing the fraction for succinate dehydrogenase, the marker enzyme for mitochondria. It is scientifically well documented that some enzymes are located specifically within certain cell fractions.

6.5 Materials and Methods

6.5.1 Materials

- Ice bucket
- Centrifuge tubes (50 ml and 15 ml disposable)
- Test tubes (12 x 75 mm, 13 x 100 mm)
- Cuvettes for Spec 20
- Single-edged razor blades
- Micropipettes & tips
- Rat liver

6.5.2 Chemicals

- Glycine
- Sucrose
- NaOH
- Na2HPO4
- NaH2PO4
- Triton WR 1339
- Nitro blue tetrazolium
- Na-succinate
- sodium dodecyl sulphate
- Tris

6.5.3 Equipment

- Tissue homogenizers
- Refrigerated centrifuge with J2-21 rotor
- Spectrophotometer
- Electronic balance
- Water bath

6.5.4 Glass ware

- Pipettes & Graduated cylinders
- Test tubes
- Beakers

6.6 Part 1: Cell fractionation and preparation of mitochondria by differential centrifugation

6.6.1 Protocol for Liver Fractionation

In the following procedures, keep the tissues and fractions **ice cold** whenever possible.

1. Tissue Homogenization:
   A. Obtain a plastic weighing boat on ice containing pieces of rat liver.
   B. Use a razor blade or scissors to dice the liver into small pieces.
   C. Using an electronic balance, tare or zero an empty weighing boat.
   D. Use a spatula to add small pieces of liver to the boat until the mass is about one gm.
   E. Place the boat with your one gm of liver on ice. Return the remainder of the liver to the ice bucket.
   F. Mince the liver with a razor blade and transfer your one gm of liver tissue to a small glass (*Dounce*) homogenizer on ice.

G. Add ice-cold *Homogenization Buffer* (hypotonic solution of sucrose 0.25 mol/liter buffered with 20mmol/liter tris to pH 7) to the homogenizer.

H. Homogenize the tissue with up-and-down movement of the pestle until a homogeneous pink solution is obtained. Caution: Excessive grinding or heating can damage or inactivate sub cellular fractions.

I. Pour the homogenate into a plastic 50 ml tube labeled "H" (for homogenate). Dilute the final concentration of the Homogenate to 1.0 gram tissue/10 ml of cold *Homogenization Buffer* and keep on ice.

J. Rinse the homogenizer with tap water, and return to the instructor.

6.6.2 Isolation of Nuclei

A. Use a pipette to add about 8.0 ml of homogenate to a centrifuge tube. Save the remainder (about 2 ml) of the "H" fraction for later assay.

B. Place the tubes in opposite stalls in a centrifuge.

C. Centrifuge for 10 min at 600 x g to pellet the cell debris and nuclei.

D. Carefully remove the tubes from the centrifuge so as not to disturb the pellet.

E. Use a Pasteur pipette to transfer the supernatant (post-nuclear supernatant) to a clean centrifuge tube.

F. Re suspend (with a Pasteur pipette) the nuclear pellet in 4.0 ml of *Homogenization Buffer*. Transfer this nuclear fraction to a single 15 ml centrifuge tube labeled "N" and save on ice.

6.6.3 Isolation of Mitochondria

a. Place the tube containing the post-nuclear supernatant (from step 2E above) in a centrifuge.

b. Centrifuge for 20 min at 10,000 x g to pellet the mitochondria.

c. Carefully remove the tubes from the centrifuge so as not to disturb the pellet.

d. Use a Pasteur pipette to transfer the supernatant (post-mitochondrial supernatant, PMS) to a clean 15 ml centrifuge tube labeled "PMS". This PMS fraction includes the lysosomes, microsomes, and cytosol and some light mitochondria.

e. Re suspend the mitochondrial pellet (obtained in the previous step) in 4.0 ml of Homogenization Buffer.

f. Use a Pasteur pipette to transfer the re suspended mitochondria into one 15 ml centrifuge tube.

6.7 Part 2a: Assay of succinate dehydrogenase in the mitochondrial fraction

6.7.1 Preparation of Reagents

Note: Except 2% SDS, store all reagents on ice during the exercise. Dispense them into aliquots as indicated. Keep 2% SDS at room temperature.

1. **Buffer A**: 5 mM Na-glycinate, pH 8.5 + 100 mM sucrose
   As stock, make 100 ml of 1 M buffer (mol. wt. of glycine = 75); therefore take 7.5 g of glycine in about 70 ml water, adjust pH by drop wise addition of 10 N NaOH to pH 8.5. Make the volume to 100 ml. Freeze in 10 ml aliquots. Each day, thaw one tube. Dilute to 2 liter with water and add 68.4 g sucrose. This is Buffer A. Keep it ice-cold.
2. **Buffer B**: 200 mM Na-phosphate buffer, pH 7.4. Make 500 ml each of 0.2 M of NaH2PO4 and 0.2 M Na2HPO4. Add NaH2PO4 solution to Na2HPO4 solution to get pH 7.4. Store at 4°C.
3. **1% Triton WR 1339**: 2 g in 200 ml water. Store at 4°C.
4. **NBT Solution:** Dissolve 50 mg of NBT in 20 ml of water. Prepare fresh *everyday*. Keep on ice.
5. **Substrate B**: 100 mM Na-succinate, pH > 7. Na-succinate (formula wt. = 270). Add 5.4 g in 200 ml water. Store frozen at -20°C.
6. **SDS 2%**: 2% sodium dodecyl sulphate.
   Add 60 g of SDS to 3 liters of water. Store at room temperature.

6.7.2 Succinate Dehydrogenase Assay (Mitochondria)

Mitochondria are the eukaryotic organelles that contain the enzymes of the citric acid cycle, the electron transport chain, and oxidative phosphorylation. The organelle is composed of an inner and outer membrane. The outer membrane is relatively permeable, allowing most molecules with molecular weights up to 5,000 Daltons to pass into the inter membrane space. The inner membrane, however, is very impermeable and allows only small uncharged molecules to penetrate into the matrix. The enzymes of the electron transport chain and oxidative phosphorylation are embedded in the inner side (matrix side) of the inner membrane. With the exception of **succinate dehydrogenase** (which is located within the inner membrane), all the enzymes of the citric acid cycle are located within the matrix.

Mitochondria can be prepared from a variety of eukaryotic tissues (both plant and animal) by differential centrifugation. They are pelleted immediately after removal of the nuclei. The presence of mitochondria in an isolated sub cellular fraction can be verified by assaying mitochondrial-specific (i.e., marker) enzymes. In these assays, care must be taken to choose

low molecular weight non-ionic substrates and dyes which are capable of penetrating both the outer and inner mitochondrial membranes.

The enzyme Succinate Dehydrogenase is an integral protein of the mitochondrial inner membrane. The major function of mitochondria is to generate energy (ATP) via oxidative phosphorylation. Succinate dehydrogenase, an FAD-containing enzyme, is involved in converting succinate to fumarate. In this assay, succinate is used as a substrate and nitro blue tetrazolium (NBT) as an artificial electron acceptor which changes to purple color when it accepts electrons. Thus, the formation of purple color is directly proportional to enzyme activity.

1. Label glass tubes (13 × 100 mm) and BLANK.
   Pipette into each of these tubes:
   0.2 ml Buffer B [200 mM Na-Phosphate buffer, pH 7.4]
   0.1 ml 2.5 mg/ml NBT
   0.1 ml 1% Triton WR-1339
   0.1 ml Substrate B [100 mM Na succinate, pH > 7]
2. When ready, add to each of the seven tubes 0.5 ml of the appropriate enzyme fraction. Add 0.5 ml Buffer A to the tube labeled BLANK. Note the starting time for each reaction.
3. Incubate all tubes at 37°C for 30 minutes.
4. Stop the reaction by adding 2.0 ml of 2% sodium dodecyl sulphate to each tube.
5. Read Absorbance at 630 nm in a spectrophotometer adjusted to zero with the blank.

6.8 Part 2b: determination of protein content in the mitochondria:

6.8.1 Bradford protein assay

The Bradford assay is very fast and uses about the same amount of protein as the Lowry assay. It is fairly accurate and samples that are out of range can be retested within minutes. The Bradford is recommended for general use, especially for determining protein content of cell fractions and assessing protein concentrations for gel electrophoresis.

The method described below is the "standard Procedure" with sensitivity to about 20 to 200 micrograms protein. Simply scale down the volume for the "micro assay procedure," which uses 1 ml cuvettes.

6.8.2 Principle

The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change. The assay is useful since the extinction coefficient of a dye-albumin complex solution is constant over a 10-fold concentration range.

6.8.3 Equipment

In addition to standard liquid handling supplies a visible light spectrophotometer is needed, with maximum transmission in the region of 595 nm, on the border of the visible spectrum (no special lamp or filter usually needed). Glass or polystyrene (cheap) cuvettes may be used; however the color reagent stains both. Disposable cuvettes are recommended.

6.8.4 Procedure

6.8.5 Reagents

1. Bradford reagent: Dissolve 100 mg Coomassie Brilliant Blue G-250 in 50 ml 95% ethanol; add 100 ml 85% (w/v) phosphoric acid. Dilute to 1 liter when the dye has completely dissolved, and filter through Whatman #1 paper just before use.

   The Bradford reagent should be a light brown in color. Filtration may have to be repeated to rid the reagent of blue components. "Homemade" reagent works quite well but is usually not as sensitive as the commercial product.

6.8.6 Assay

Assays are performed with 1 ml sample plus 5 ml reagent

1. Prepare standards containing 0-200 micrograms protein/ml (albumin or gamma globulin).
2. Prepare unknowns to estimated amounts of 20 to 200 micrograms protein per tube, same volume as the standard (1 ml).
3. (optional) Add 0.25 ml 1 M NaOH to each sample and vortex (This step is to be used only if the sample protein is not soluble in the dye-reagent).
4. Add 5 ml dye reagent and incubate for 5 min.
5. Measure the absorbance at 590 nm.

6.8.7 Specific Activity

Specific Activity relates the enzyme activity to the amount of protein in the sample. We can measure the amount of protein, and we can measure the enzyme activity. Combining the two will give us the specific activity. Specific activity is defined in terms of enzyme activity per mg protein in the sample (i.e., specific activity relates the enzyme units to the amount of protein in the sample).

6.9 Results

Calculate the following for the Results Section of the Lab Report:

Succinate Dehydrogenase Assay

1. Attach in the **Appendix** a table with all OD (absorbance) values recorded.
2. Calculation of absorbance (OD)/min/ml.
3. For example: If the activity of the H fraction = 0.05 OD/min and the volume of H that was tested was 0.25 ml then 0.05 OD/min/0.25 ml = 0.20 OD/min/ml of activity in the H fraction.

4. After protein determination, calculate the specific activity as follows:
5. Calculate the total protein (mg/ml) of each fraction
    a. For Example: If the volume of the H fraction assayed for protein = 0.05 ml (50 μl) and
    b. Protein (from standard curve)/ 0.05ml = 75 μg, then
    c. 75 μg protein/0.05 ml = 1500 μg/ml = 1.5 mg protein/ml for the H fraction.
6. Calculate the specific activity of succinate dehydrogenase (change in absorbance/min/mg protein) for each fraction.
    a. For Example using the above calculations for the H fraction:
    b. 0.20 OD/min/ml/1.5 mg protein/ml = 0.133 OD/min/mg protein = specific activity of SDH in Homogenate.

6.10 Discussion

The students are expected to discuss their results in this section. The following are expected to be discussed in this section.

Compare the specific activity of the various sub cellular fractions. In this regard, it is important to compare specific activities between different fractions (e.g., H, N, Mitochondria, etc.) within your experiment. How pure is each fraction? (As a rule of thumb, sub cellular fractions are considered "very clean" if the specific activity of the marker enzyme is 3 times higher than in other fractions.).

6.11 Literature Cited

Alberts, B., D. Bray, J. Lewis, M. Raff, K. Roberts, and J. D. Watson. 1989. How cells are studied.

Pages 135–198 (Chapter 4), *in* Molecular biology of the cell (Second edition). Garland Publishing, New York, 1218 pages.

Darnell, J., H. Lodish, and D. Baltimore. 1990. Principles of cellular organization and function. Pages 131–186 (Chapter 5), *in* Molecular cell biology (Second edition). W. H. Freeman, New York, 1105 pages.

Lehninger,AL, et al. 1993. *Principles of Biochemistry*, 2nd ed. Worth Publishers: NY. Chapter 18: Oxidative Phosphorylation and Photophosphorylation.

4. Karp, G 2002. *Cell and Molecular Biology*, 3rd ed. John Wiley. Section 18.6 & 18.7 (pp 757-759).

Novikoff, A. B., and E. Holtzman. 1976. Cell fractionation. Pages 28–31 (Chapter 1.2B), *in* Cells and organelles (Second edition). Holt, Rinehart and Winston, New York, 400 pages.

Bradford, MM. A rapid and sensitive for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry 72:* 248-254. 1976.

Experiment 6

7. Triglyceride determination

7.1 INTRODUCTION

Triglycerides are esters of fatty acids and are hydrolyzed to glycerol and free fatty acids. Triglyceride determinations when performed in conjunction with other lipid assays are useful in the diagnosis of primary and secondary hyperlipoproteinemia. They are also of interest in following the course of diabetes mellitus, nephrosis, biliary obstruction, and various metabolic abnormalities due to endocrine disturbances. Standard methods for the measurement of triglyceride concentrations involved either enzymatic or alkaline hydrolysis to liberate glycerol. This formulation makes use of the enzymatic hydrolysis and quantification since it is specific and not subject to interference by phospholipids.

7.2 PRINCIPLE

The enzymatic reaction sequence employed in the assay of Triglycerides is as follows:

Triglycerides $\xrightarrow{\text{Lipase}}$ Glycerol + Fatty Acids

Glycerol + ATP $\xrightarrow{\text{Glycerol Kinase}}$ Glycerol-l-phosphate + ADP

Glycerol- 1-Phosphate + O_2 $\xrightarrow[\text{Oxidase}]{\text{GIP}}$ DAP + H_2O_2

H2O2 + 4 AAP + DHBS $\xrightarrow{\text{Peroxidase}}$ Quinoneimine Dye + H_2O_2

The present procedure involves hydrolysis of triglycerides by lipase. The glycerol concentration is then determined by enzymatic assay coupled with Trinder reaction that terminates in the formation of a quinoneimine dye. The amount of the dye formed, determined by its absorption at 520 nm, is directly proportional to the concentration of triglycerides in the samples.

7.3 REAGENT COMPOSITION

Dissolve each of the following reagent vials with 15 ml of deionized water.

1. **Triglyceride reagent:**
   50 mM, Buffer pH 7.3, 3.3 mM ATP, 3.0 mM Magnesium salt, 0.7 mM 4-Aminoantipyrine, 0.8 mM 3, 5-Dichloro- 2-hydroxybenzene sulfonate, 7000 U/L Glycerol-l-Phosphate Oxidase, 0.01% Sodium azide, 200,000 U/L Lipase, 1000 U/L Glycerol kinase, 10,000 U/L Peroxidase.
2. **Triglyceride standard:**
3. Contains glycerol with surfactant to yield 200 mg/dl
   triglycerides as triolein. 0.1% Sodium azide is added as a preservative.

7.4 MATERIALS REQUIRED

Spectrophotometer capable of measuring absorbance at 520nm; Cuvettes; Pipettes capable of accurately measuring required volumes (1.0 ml, 2.0 ml, 0.01 ml, 0.02 ml); . Water bath set at 37° C; Timer; Distilled water;

7.5 PROCEDURE

1. Reconstitute Triglyceride Reagent as directed.
2. Label tubes: blank, standard, control, unknown, etc.
3. Pipette l.0 ml of reagent into all tubes.
4. Place all tubes in a 37° C heating block for at least 4 minutes.
5. Add 0.01 ml (10 µl) of sample to respective tubes and mix.
6. Incubate all tubes for five minutes at 37° C.
7. Zero spectrophotometer at 520 nm with reagent blank (range: 500–550 nm).
8. Read and record absorbance of all tubes.

[Note: Final color is stable for sixty minutes at room temperature.
For spectrophotometers requiring more than 1.0 ml of reagent add 0.02 ml (20 µl) of sample to l.0 ml of reagent. After 10 minutes of incubation at 37°C add 2.0 ml of distilled water to all tubes, invert to mix, and read immediately at 520 nm.]

The reagent is linear to 1000 mg/dl Triglycerides. Samples with values above 1000 mg/dl should be diluted with water, re assayed and the results multiplied by the dilution factor.

7.6 CALCULATIONS

A = Absorbance

A (unknown)/A (standard) × Concentration of standard (mg/dl)

= Triglyceride value of unknown (mg/dl)

Example: 0.24/0.31 x 200 = 154.8 mg/dl

NOTE: To obtain the results in SI units (mmol/L) multiply the result in mg/dl by 10 to convert dl to liter and divide the value by 885, the molecular weight of triglycerides as triolein.

Example: 154.8 mg/dl x 10/885 = 1.75 mmol/L

7.7 REFERENCES

- Tietz N.W., Clinical Guide to Laboratory Tests, Second Edition W.B. Saunders Company, Philadelphia, USA 554-556, 1990.

- Naito, HK (1984): Disorders of lipid metabolism. In: Kaplan LA, Pesce AJ, eds. Clinical Chemistry, theory, analysis, and correlation. St. Louis: Mosby Company. 550-593.

Experiment 7

8. HDL-Cholesterol determination

8.1 INTRODUCTION

Cholesterol is a fatty substance found in blood, bile and brain tissue. It serves as a precursor to bile acids, steroids and vitamin D. The concentration of total cholesterol in serum has been associated with metabolic, infectious and coronary heart diseases. In the plasma, cholesterol is transported by three lipoproteins: high density lipoprotein (HDL-Cholesterol), low density lipoprotein (LDL-Cholesterol), and very low density lipoprotein (VLDL-Cholesterol). Castelli and co-workers have indicated that an inverse relationship exists between serum HDL-Cholesterol and the risk of coronary heart disease. The measurement of total and HDL Cholesterol and triglyceride provides valuable information for the prediction of coronary heart disease and for lipoprotein phenotyping. Our precipitating reagent uses the well established precipitating properties of polyethylene glycol 6000 at pH 10.0.

8.2 PRINCIPLE

When serum is reacted with the polyethylene glycol reagent, all the low and very low-density lipoproteins (LDL and VLDL) are precipitated. The HDL fraction remains in the supernatant. The supernatant is then used as a sample for cholesterol assay.

8.3 REAGENT COMPOSITION

We will be using the kit to check the HDL concentration in the blood serum. The kit comes with the following reagents and their composition is mentioned below.

1. HDL Cholesterol Precipitating Reagent:

20% w/v polyethylene glycol 6000 in glycine buffer at pH 10 (25°C). Store at room temperature (18-30°C).

2.Cholesterol Standard:

Cholesterol in alcohol 50 mg/dl stored at room temperature of 18-30°C tightly capped.

8.4 STORAGE AND STABILITY

Store the reagent set at room temperature (18-30°C) tightly capped. The reagent should be discarded if:

1. Sedimentation or turbidity has occurred.
2. The reagent does not meet stated performance parameters.

8.5 SPECIMEN COLLECTION AND STORAGE

1. Test specimens should be serum and free from hemolysis.
2. Patient must be fasting for at least 12-14 hours.
3. HDL in serum has been reported to be stable for seven days at 2-8°C.

8.6 MATERIALS REQUIRED

1. Enzymatic Cholesterol Reagent Set
2. Centrifuge
3. Test tubes/rack
4. Timer
5. Heating block
6. Spectrophotometer

8.7 PROCEDURE

1. Label tubes: control, patient, etc.
2. Mix equal amount of serum and HDL cholesterol precipitating reagent in the glass tube and mix vigorously, e.g. 0.2 ml serum + 0.2 ml HDL precipitating reagent.
3. Centrifuge for ten minutes at 1500-2000 x g.
4. Separate supernatant from precipitate. The supernatant fraction contains HDL.

Run the total cholesterol assay according to the instructions, but double the sample volume to compensate for the previous dilution. If the total cholesterol test phenol free requires a 0.025 ml (25 µl) sample, use 0.05 ml (50 µl) for the HDL determination. Keep original sample volumes for standards.

NOTE: If the supernatant is cloudy/hazy, the sample should be re-centrifuged. If the sample still remains cloudy, dilute the serum sample 1:1 with saline and test the sample again. However, in this case the final results must be multiplied by two because of the dilution factor.

8.8 EXPECTED VALUES:

Male HDL: 26-63 mg/dl

Female HDL: 33-75 mg/dl

The range of expected values may slightly differ due to the differences in the instruments, laboratories, and local populations.

8.9 RESULTS:

REFERENCES

- Tietz N.W., Clinical Guide to Laboratory Tests, Second Edition W.B. Saunders Company, Philadelphia, USA 554-556, 1990.

- Michel R. Langlois; Victor H. Blaton Historical milestones in measurement of HDL-cholesterol: Impact on clinical and laboratory practice Clinica Chimica Acta 2006, 369, 168-178

Experiment 8

9. LIPASE ASSAY(USING TURBIDIMETRIC METHOD)

9.1 INTRODUCTION

Lipase is defined as a group of enzymes, which hydrolyze the glycerol esters of long-chain fatty acids. The measurement of lipase activity in serum and other fluids evaluate the conditions associated with pancreas. Voget et al. (1963), proposed an olive oil emulsion in measuring the rate of change in turbidity over a specific unit of time. Later, Shihabi et al. modified the previous method and eliminated some interference.

In a normal diet about 25 to 50 percent of the caloric intake of man consists of lipids. These substances are the most concentrated form of food energy in our diet. When metabolized lipids produce about 9.5 Kcal per gram. For this reason animals tend to build up fat deposit as a reserve source of energy. In times of starvation, the body will metabolize these stored fats. Even so, some lipids are required by animals for bodily insulation and as protective sheath around some of the more vital organs. Lipids consist of fats and oils which are esters of glycerol and their differences are due to the differing fatty acids with which glycerol may be combined. Fats are made up of fatty acids which are for the most parts saturate, which oils are primarily composed of fatty acids portions which have greater numbers of double bonds. Fats are usually obtained from animals sources, while oils are commonly obtained from vegetable sources.

Hydrolysis of simple lipids is accomplished by hot alkali or lipases. Since the lipid materials are frequently insoluble in water hydrolysis in aqueous alkali is slow. The rate of hydrolysis is accelerated by the use of appropriate solvents. For example, alcohol or elhylene glycol.

From the sample equation

$$3\,R - C^{\bullet} - O - R + 3KoH \text{----------} > 3R - C^{\bullet} - oK + 3RoH$$

Triglyceride (Fat) Potassium hydroxide Potassium salt of fatty acid (soad) glycerol

One can see that 1 mole of alkali is consumed for every mole of ester saponified. Therefore the quantity of alkali consumed by complete hydrolysis of lipid sample is related to the number of ester bonds originally present in the sample.

The consumption of alkali by a particular lipid is commonly expressed in terms of the saponification number, which is defined as the number of milligrams of KoH consumed in the complete saponification of 1 g of fat or oil. The product of saponification of a neutral fat or oil is glycerol and a mixture of the salts of the component fatty acids. Fatty acids can be separated from glycerol by acidification of the saponified solution, followed by filtration or either extraction. Estimation of the average molecular weight of the fatty fraction

can be made from the neutralization equivalent of the sample. The neutralization equivalent is defined as the number of grams of acid required to neutralize 1 equivalent of alkali. In practice, the neutralization equivalent is determined by the titrating anhydrous organic acids with standard alkali and then using the equation

$$\text{N.E.} = \frac{\text{weight of sample in grams}}{\text{Volume of alkali in milliliters} \times \text{normality}} \times 1000$$

Mammalian metabolism of lipids begins with the breakdown of ingested dietary lipids by enzymes released from the pancreas into the digestive tract. These enzymes are known as lipases. They are belong to the general class of esterases. The simple esterases catalyze only the hydrolysis of esterases of simple alcohols. Lipases catalyze the hydrolysis of esters of glycerol but not hydrolyze simple esters. The substrates of lipases are diglycerides and triglycerides and the hydrolysis of monoglycerides by these enzymes is very slow.

A number of requirements are involved in the demonstration of optimum lipase activity. First, it is necessary to increase the effective concentration of the substrate. High-molecule weight di- and triglycerides are insoluble in aqueous solutions and are therefore unavailable to the enzyme. The effective concentration of these substrates is increased by emulsification with such agents as soaps, proteins or bile salts. Second, it is necessary to add calcium ions to precipitate the free fatty acids as they formed during hydrolysis. Third, the activity of lipase preparations is marked affected by the hydrogen ion concentration, pH, Optimum activity is obtained at pH 8-9. To maintain a favorable pH, an ammonium hydroxide-ammonium chloride buffer at pH 8 is used.

During the hydrolysis, hydrogen ions are produced. These hydrogen ions are consumed by the buffer, leading to formation of NH_4^+ ion and the weakening the buffer capacity. The buffer capacity is restored during hydrolysis by the occational adding ammonium hydroxide and keeping the system pink to phenolphthalein. The ammonium ion concentration is determined by titration of aliquots of the reaction mixture in alcohol-ether with potassium hydroxide. The alcohol-ether serves two purposes. First, to stop lipase activity and second, to increase the apparent pK of the phenolphthalein. Thus the addition of alcohol-ether to an aliquot of the lipase reaction mixture decolorizes the phenolphthalein and it is possible to titrate NH_4^+ with the stronger base to the usual pink colour of phenolphthalein.

Aims of Experiment

1. To identify unknown fat or oil by determining it saponification number.
2. And to investigate the lipase hydrolysis of that fat or oil and compare the lipase activity with alkali hydrolysis for the same fat or oil.

9.2 PRINCIPLE

Triglyceride + H_2O ⟶ mono + di-glycerides + fatty acids

Serum lipase hydrolyzes the olive oil emulsion. The decrease in turbidity at 400 nm (after incubation) is proportional to lipase activity in the specimen.

9.3 REAGENT COMPOSITION

1. Lipase Substrate:

0.8% (w/v) olive oil in alcohol.

2. Lipase Buffer:

69 mM Tris Buffer pH 9.0 (37°C), 10 mM Sodium Deoxycholate.

9.4 REAGENT PREPARATION

1. Add lipase buffer to a 50 mL Voget et al. (1963), proposed an olive oil emulsion in measuring the rate of change in turbidity over a specific unit of time. Later, Shihabi et al. (Year?/) modified it. Erlenmeyer flask. Add 25 mL distilled water and swirl to dissolve.
2. Pipette out 1 mL of well-mixed lipase substrate into buffer solution.

[Note: The absorbance of the emulsion prior to use must be greater than 1.0. Due to variations in regional temperatures, the absorbance may be less than 1.0. If this occurs, add 0.5 – 1.0 mL more substrate until absorbance is greater than 1.0.]

9.5 REAGENT STORAGE AND STABILITY

1. Un-reconstituted reagent should be stored at room temperature (18 - 30°C).
2. Reconstituted reagent is stable for seven days when refrigerated (2 - 8°C) and capped tightly.

9.6 SPECIMEN COLLECTION STORAGE

1. Use fresh serum specimens. Hemolyzed specimens should not be used.
2. Lipase activity in serum is stable at room temperature for one week; sera may be stored for three weeks in the refrigerator (4-8°C) and for several months if frozen.

(Caution! Bacterial contamination of the specimens may result in an increase in lipase activity)

9.7 MATERIALS REQUIRED

Accurate pipettes (3.0 mL and 0.1 mL),
Timer
Test tubes/rack
Spectrophotometer with temperature controlled cuvette, heating bath (37°C) Serum controls

9.8 PROCEDURE

1. Reconstitute lipase reagent according to following instructions.
2. Label test tubes "blank", "control", "patient", etc.
3. Pipette 3.0 mL of reagent into appropriate test tubes and pre-warm at 37°C for at least five minutes.
4. Zero spectrophotometer with distilled water at 400 nm. (range: 390-420 nm).
5. Read and record absorbance of blank and place back in heating bath.
6. Using timed intervals, add 0.1mL (l00 µl) of sample to each tube, mix,
and read initial absorbance. Return each tube to heating bath after initial reading.
7. Exactly five minutes after the initial absorbance reading, using the same timed intervals, remove each tube from the heating bath and mix each tube. Read the absorbance of the blank and each sample tube against distilled water.

9.9 PROCEDURE NOTES

1. If the Absorbance of the "blank" is a negative value, consider it zero.
2. Elevated blank rates i.e. (0.005) and above may be caused by olive oil coating on cuvette surface. Periodically rinse with acetone followed by water flush.
3. Turbid samples should be diluted with distilled water (1:5). Multiply final answer by dilution factor.
4. Use fresh sera, when possible, for greatest accuracy.

9.10 CALIBRATION

The lipase activity in the sample is calculated based on the millimolar absorptivity of olive oil (3.15 in working solution).

9.11 CALCULATIONS

The enzyme activity is expressed in International Units. One International Unit (IU) is defined as the amount of enzyme that catalyzes the transformation of one micromole of substrate per minute under decreed conditions.

$$\text{Units/Liter} = \frac{\text{Corrected } \Delta\text{Abs /5 min}}{\text{Initial blank absorbance}} \times 1953.$$

Corrected Δ Abs./5min = Δ Abs test- Δ Abs blank

Example: (blank) Initial Abs. = 0.970.,
5 min. Abs. = 0.967 (if negative result, treat as zero)
Δ Abs blank = 0.970 - 0.967 = 0.003,
(test) Initial Abs = 1.300., 5 min. Abs. = 1.271,
Δ Abs/test a 1.300 - 1.271 = 0.029.
Corrected Δ Abs./5 min = 0.029- 0.003 = 0.026,
Therefore: 0.026/ 0.970 x 1953 = 52 IU/L 0.970

9.12 RESULTS:

RFERENCES

- Tietz, N.W. (Ed.), Fundamentals of Clinical Chemistry, W.B. Saunders Co., Toronto, 635 (1982).

- Kwon, D.Y. and Rhee, J.S. 1986. A simple and rapid colorimetric method for determination of free fatty acids for lipase assay. *J. Am. Oil Chem. Soc*.63:89-92.

Experiment 9

10. Estimation of Glutathione in Plasma

10.1 Introduction

Glutathione (gamma-glutamylcysteinylglycine or GSH) is a naturally occuring tripeptide whose nucleophilic and reducing properties play a central role in metabolic pathways, as well as in the antioxidant system of most aerobic cells.

GSH plays a critical role as a coenzyme with a variety of enzymes including, glutathione peroxidase, glutathione S-transferase and thiol transferase. GSH also plays major roles in drug metabolism, calcium metabolism, the g-glutamyl cycle, blood platelet and membrane functions. In addition, GSH is crucial to a variety of life processes, including the detoxification of xenobiotics, maintenance of the -SH level of proteins, thiol-disulfide exchange, removal of hydroperoxides and free radicals, and amino acid transport across membranes. Physiological values of intracellular GSH generally range from 1 to 10 mM.

Glutathione's three major roles in the body are:

- Anti-oxidant
- Blood Booster
- Cell Detoxifier

Glutathione deficiencies have been linked to many forms of cancer.

10.2 Objective for the Experiment

- To draw the standard Curve of Glutathione by given known amount of glutathione assay procedure using spectrophotometeric technique.
- To estimate the amount of glutathione in red blood cell sample.

10.3 Assay Principle

The principle of the assay is based on the oxidation of the reduced form of glutathione by the aromatic disulphide compound and 5,5-dithiobis-2-nitrobenzoic acid (DTNB) to form GSSG and the aromatic thiol,5thio-2nitrobenzoic acid (TNB). The yellow colour formed is measured as 412nm and is proportional to the amount of glutathione present in the sample. The assay principle was same as described as Beutler et al (1963)[1] .

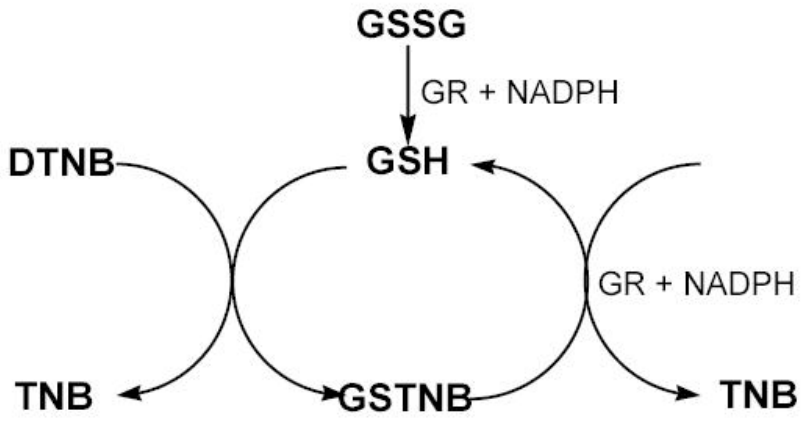


Figure 1: the principle of Glutathione assay

The enzymatic recycling method for quantitation of GSH and/or GSSG. GSSG, oxidized glutathione; GSH, reduced glutathione; GR, glutathione reductase; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TNB, 5-thio-2-nitrobenzoic acid; GSTNB, the disulfide product of reaction of GSH with DTNB.

10.4 Methods and Materials

- Glacial metaphosphoric acid,
- Disodium ethylenediamine tetra acetic acid(EDTA),
- Sodium chloride, sodium monohydrogen phosphate(Na_2HPO_4),
- 5,5-dithiobis-2-nitrobenzoic acid(DTNB), sodium citrate and glutathione.

All chemicals can be obtained from BDH(England), AVAONCHEM(U.K), WinLAB(U.K) and MERCK, Glutathione used as a product of BDH (England)

10.5 Preparation of Reagents:

1. **Precipitating Solution:**
   Dissolve 1.67g glacial metaphosphoric acid, 0.29g disdium ethylenediamine tetraacetic acid (EDTA) and 30g sodium chloride in 100ml of distilled water.

- **Note:** This solution is stable for approximately 3 weeks at 4^oC.

2. **Phosphate solution**:
   Prepare 0.3 Mol anhydrous Na_2HPO_4., by dissolving (42.6g) of anhydrous Na_2HPO_4 in 1 litre of bi-distilled water.
3. **DTNB reagent:**

Add 40mg of DTNB to 100ml of 1% sodium citrate solution.

Note: Precautions:

5-5'-Dithiobis(2-nitrobenzoic acid) (CAS 69-78-3) is irritating to the eyes, respiratory system and skin. In case of contact with eyes, rinse immediately with copious amounts of water and seek medical advice. Wear suitable protective clothing

10.6 Materials required:

Spectrophotometer- allowing light absorption measurements at 412 nm, from 0 - 2 absorbance units.

- Glass test tubes.
- Heparin coated test tubes
- Adjustable pipettes with disposable tips.
- Glass test tubes and vortex.
- Water bath kept within 22 - 37°C temperature range.

Tube No.	**GSH stock Solution (ml)**	**Phosphate Solution (ml)**	**Total Volume (ml)**	**GSH concentration (mg/dl)**	**GSH concentration (µg/ml)**
1	0.6	2.4	3	2	10
2	1.2	1.8	3	4	20
3	1.8	1.2	3	6	30
4	2.4	0.6	3	8	40
5	3	0	3	10	50
Blank	0	3	3	0	0
Sample1					
Sample2					

Table 1: Preparation of serial GSH concentration

10.7 Preparation of Glutathione standard curve.:

1. Prepare glutathione stock solution (10mg/dl) by dissolving 0.01g of glutathione in 100ml of phosphate solution and make 5 serial concentration of glutathione as described in Table 1. Keep two tubes as Blank.
2. From each 3ml final solution above , take 0.5ml solution in a separate test tube and add 2ml phosphate solution(3M)and 0.25ml of DTNB reagent. Make the volume to 3ml with double distilled water. Incubate the tubes at 37^0C for 10min. Read the absorbance at 412nm using spectrophotometer.
3. Plot the absorbance against glutathione concentration (µg/ml) in

Table 2: Glutathione standard curve data.

No of Tubes in duplicates	**GSH concentration (µg/ml)**	**Absorbance At 412 nm**	**Absorbance at 412nm (Mean)**
1	10		
2	20		
3	30		
4	40		
5	50		
Sample 1 Tube 1 Tube 2			
Sample 2 Tube 1 Tube 2			

10.8 Preparation of Blood Sample for GSH determination.

Best results are obtained with fresh blood samples

1. Collect the blood in heparinzed test tubes. Immediately shake the tubes and keep the blood at 4^oC .
2. Centrifuge at least 5 ml of whole blood at 600g at 4°C for 10 minutes.
3. The pellet contains the red blood cells and the supernatant is the plasma fraction.
4. Keep the supernatant (plasma) for glutathione assay. Discard the precipitate (erythrocytes).
5. Take 0.2 ml of plasma supernatant and add 1.8ml of deionized distilled water . Add 0.3ml of precipitating reagent (1) .

6. Centrifuge at 1200Xg for 10 min.
7. Add 2ml of Na_2PO_4(0.3M) to 0.3ml of above supernatant. Add 0.25ml of DTNB-Reagent (3) . Make up the volume to 3ml with tri-distilled water.
8. Incubate the above mixture for 10min at 37^oC.
9. Read the absorbance at 412nm using spectrophotometer.

10.9 Calculation of glutathione Concentration:

The glutathione concentration in the sample was calculated by plotting its absorbance on the standard curve and expressed as μg/ml of the plasma.

10.10 Result and Discussion:

1. What is the importance of Glutathione in our body?
2. Discuss the problems you encountered during this experiment?
3. Can you explain the assay basic principle?
4. What Precautions you must take while doing this experiment?

10.11 References:

Beutler E, et al.(1963) Improved method for the determination of blood glutathione. J Lab Clin Med 61: 882-890.

Experiment 10

11. Urease activity Comparison of the activities of urease from different sources

11.1 Introduction:

Enzyme activity is influenced by many factors such as pH, temperature, concentration of substrate and of enzyme, the presence of salts, inhibitors etc. The source of the enzyme is another factor which may affect activity of an enzyme.

Urease is a highly specific enzyme widely distributed in plant tissues. It was the first enzyme to be isolated in crystalline form in 1926. Summer (1926) purified the enzyme from jack bean meal.

Urease is very widely used in clinical laboratories for quantitative determination of urea in blood or urine. The amount of ammonia formed is titrated to give a measure of the amount of urea.

11.2 Theory:

Urease catalyzes the following reaction:

$$NH_2 - CO - NH_2 + H_2O \rightarrow 2\ NH_3 + CO_2$$

For every **one** mole of urea hydrolysed down, **two** moles of ammonia (base) are formed. The amount of ammonia formed is titrated with acid to give a measure of the amount of urea.

11.3 Objective:

Part 1. Preparation of urease extracts form soya beans (or jack beans) and water melon seeds.

Part 2. Determination of the activity of urease in soya beans (or jack beans) and water melon seeds.

11.4 Materials and Method:

11.4.1 Material:

Soya beans (or jack beans)
Water melon seeds

11.4.2 Chemicals:

Urea
Tris (hydroxymethyl) methylamine

Concentrated HCl (36% w/v)
Glycerol
Mercuric chloride.
Methyl red
Ethanol

11.4.3 Equipments:

Blender
Centrifuge and tubes
Stop clocks

11.4.4 Glassware:

Flasks (50ml)
Pipettes
Burettes (10ml)
Graduated cylinders (100ml)

11.5 Preparation of solutions:

0.05M Tris buffer pH 7.2:

Dissolve 6.06g tris (hydroxymethyl) methylamine in 950 ml water. Add 4.3 ml 36% (w/v) HCl and make up to 1 litre with water. Check pH.

50mg/100 ml urea (0.05%):

Dissolve 50mg urea in 30ml tris buffer. Make up to 100 ml with the buffer.

2% Mercuric chloride:

Dissolve 2g mercuric chloride in 50 ml water and make up to 100 ml with water.

0.04% methyl red indicator:

Dissolve 40mg methyl red in 50 ml ethanol and make up to 100ml with ethanol.

11.6 Procedure:

11.6.1 Part 1. Preparation of urease extracts.

Blend 5 g of soya beans or jack beans with 35 ml of tris buffer mixed with 15 ml glycerol. Pour suspension into centrifuge tubes. Centrifuge for 5 min. at 5,000 rpm. Transfer supernatant to a graduated cylinder and return residue to blender, wash it with 15 ml buffer mixed with 5 ml glycerol. Blend and transfer suspension to centrifuge tubes. Wash residue

out as much as possible from blender with 15 ml of buffer. Centrifuge for 5 min as before. Add supernatant to the graduated cylinder. Record total volume and store in a flask in a refrigerator until the solution (WHICH CONTAINS A CRUDE EXTRACT OF UREASE) is needed.

Prepare urease extract from the water melon seeds in the same way.

11.6.2 Part 2: Comparison of activities of the two enzyme extracts.

To determine the activity of urease in the crude extracts, the extract is mixed with urea, the substrate, and incubated at room temperature for 15 min. The reaction is stopped by addition of mercuric chloride. The quantity of ammonia formed is titrated with hydrochloric acid.

Label five 50ml flasks from number 0 (blank) to 4. Pipette the reagents shown in the table below into the flasks, in the order and quantities indicated. The flasks are now ready for addition of urea which will start the reaction.

Reagent	**Flask Number**				
	0	1	2	3	4
Tris- HCl buffer (ml)	7	2	2	2	2
Urease extract (beans, ml)	-	5	5	-	-
Urease extract (melon, ml)	-	-	-	5	5
HgCl2(ml)	0.5	-	-	-	-

It is important that for each flask the enzyme acts on the urea for exactly 15 min so that the results may be compared.

Add **2.5 ml of (0.05%) urea solution** to flask "0" (blank) and immediately start the timer. Mix the contents of the flask.

Then add and mix the same quantity of urea to each of the flasks 1-4 at half-minute intervals, as shown in the table below.

After 15.5 minutes add 0.5ml of 2% $HgCl_2$ to flask "1" to stop the reaction. Then add the same quantity of $HgCl_2$ to each of the flasks 2-4 at half-minute intervals, as shown in the table below.

Flask Number	0	1	2	3	4
Time for adding urea(minutes)	0	0.5	1.0	1.5	2.0
Time for adding $HgCl_2$ (minutes)	-	5.5	6.0	6.5	17.0

At this stage the flasks contain a total volume of 10ml.

Notice that for each flask there is a 15 min. interval between adding urea and adding $HgCl_2$.

11.7 Titration:

Add two drops of 0.04% methyl red indicator and titrate with 0.025 M HCl to the first pink colour. Record the volume of the acid used in the table below.

11.8 Results

Flask Number	0	1	2	3	4
Vol. HCl (ml)					

Subtract the volume of HCl for flask 0 from that of flasks 1, 2, 3 and 4.

Average volume of HCl for flask 1& 2 = ml

Average volume of HCl for flask 3&4 = ml

Calculate the activity of each urease extract as μmoles of urea hydrolysed per minute using the formula:

$$\text{Activity} = \frac{\text{Average volume HCl x molarity of HCl x } 10^6}{10^3 \text{ x } 2 \text{ x } 15}$$

($\frac{\text{Average vol. HCl x molarity}}{10^3}$ = moles HCl

Moles NH_3 produced = moles HCl

For every **one mole urea** hydrolysed **two moles NH_3** produced.

Reaction time is 15 min).

11.9 Discussion:

11.10 Question:

Suggest reasons for the differences in activities in the two enzyme preparations.

11.11 REFERENCES

- Polacco JC, Havir EA. Comparisons of soybean urease isolated from seed and tissue culture. J Biol Chem. 1979, 254 (5):1707–1715.

- Polacco JC. Nitrogen metabolism in soybean tissue culture: I. Assimilation of urea. Plant Physiol. 1976, 58 (3):350–357

Experiment 11

12. D- Xylose Absorption Test

12.1 Introduction

The small intestine can be studied in two parts ,the upper small intestine and the lower small intestine, Vitamin B12 absorption is the best test for the lower small bowl, while D- xylose absorption test is considered the best test for the upper small intestinal function. Impaired absorption of D- xylose occurs in conditions where there is flattening of the intestinal villae and this results in abnormally low urinary excretion of the test does of D-xylose.

In adults , the standard oral dose is 25 g after which the urinary output during the next five hours is 5.8 g (about 25% of the dose) in normal subjects.

In children, a 5g dose of D- xylose is usually satisfactory and the normal output in the urine is 25 % of the dose. In babies the xylose dose should not exceed the amount of glucose normally taken in one feed. Using the 25g dose , 5hours excretion of less than 2.5g occurs in patients with gluten sensitive enteropathy (coeliac disease),in patients with other non-gluten sensitive enteropathies (idiopathic steatorrhea) and in tropical sprue .The test is of diagnostic value since an output so low is rare in any other condition .In children the test is most useful in the differential diagnosis of celiac disease and cystic fibrosis. D-xylose absorption is impaired in the former but is not affected in the later. Treatment of coeliac disease with a gluten free diet improves D-xylose absorption but it remains low normal .In case of impaired renal function the D-xylose level in a 5- hours urine sample is low ,which can lead to false diagnosis of coeliac disease.

12.2 Objectives

a) To test the function of the upper small intestine.
b) To learn the technique of D-xylose estimation .

12.3 Principle

D- xlose is a pentose which produces a brown color with o- toluidine in the presence of acetic acid and heat, A brown complex will be formed with a maximum absorption at 475 nm which is used for the estimation of xylose. Hexoses also reacts with O- toluidine but produce a different complex with an absorption peak at 622 nm, this ensures that interference with glucose is minimum.

12.4 Materials and Methods

1. O- toluidine reagent: Dissolve 3g of thiourea in 1900 ml of glacial acetic acid, and add 100 ml of clear (light amber) O- toluidine. The reagent should be stored in a dark bottle at room temperature.
2. Stock standard D- xylose solution: 1.0g of D- xylose per 100 ml in 0.1 per cent benzoic acid (preservative) solution.
3. Working standard (0.5g/l) D- xylose solution: 5.0 ml of stock standard is diluted to 100 ml with distilled water.
4. Urine specimen (may be kept for a week at 4^0 C without deterioration).
5. Test tubes and test tube rack.
6. Spectrophotometer.
7. Water bath.

12.5 Method

The patient/volunteer should keep an over night fast, in the morning empties the bladder and discards the urine. Before breaking the fast , 25g of D-xylose in 250ml water is taken by mouth. The D-xylose can be made palatable with lemon concentrate with no interference with the test. The patient /volunteer should then drink water at one and two hours after drinking the D-xylose solution .All urine passed during the next five hours is collected .

12.6 Estimation of D-xylose in urine

Dilute the urine samples (a and b provide) to one liter with water and mix thoroughly . Then make further 1 in 10 dilutions of these urines with water. Label six test tubes T_1 and T_2 (test a), T_3 and T_4 (test b) ,S (standard) and B (blank) .Place the test tubes as shown below :

Test a (T_1 and T_2) 0.1ml of urine a diluted 1 in 10 .
Test b (T_3 and T_4) 0.1ml of urine b diluted 1 in 10 .
Standard (S) 0.1ml of the working standard.
Blank (B) 0.1ml of distilled water.

To each tube add 7.0 ml of O-toluidine reagent .Mix the contents of each tube thoroughly ,cover with aluminum foil and place them in a boiling water bath for ten minutes .Remove the tubes from the bath and cool them under a tap for 1-3 minutes. Measure the absorbance of each tube against a distilled water blank at 475nm using a spectrophotometer and record the results in the table below:

12.7 Results

Tube	**Absorbance at 475nm**
Test 1	
Test 2	
Test 3	
Test 4	
Standard	

The optical density of the standard should be about 0.2 .
Use the following formula for the estimation of D-xylose in urine .

$$\text{D-xylose} = \frac{\text{Mean abs of Test}}{\text{Mean abs of Std}} \times 0.5 \times 10 \text{ (dilution factor)}$$

Since that was estimated in 0.1ml of urine thus in 1ml of urine

$$\text{Urine D-xylose} = \frac{\text{Mean abs of Test}}{\text{Mean abs of Std}} \times 5 \times 10$$

Account for the urine dilution done after collection of urine and then multiply by the total volume of urine collected to determine the total amount of D-xylose excreted and compare with the minimum normal excretion of 5.8g.

Test $_a$ ---------g D-xylose .
Test $_b$ ----------g D-xylose.

12.8 Questions

1- Do either of the patients suffer from celiac disease, and if so which one?

2- In the function of the upper small intestine normal in these patients?

3- How was the interference of other reducing sugars minimized in your test?

12.9 References

Wooton, I.D.P. (1974) Microanalysis in Medical Biochemistry .Churchill Livingstock,London, p.249.