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Practical Note
METABOLISM
(BCH 447)

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Experiment 1

1. Isolation of Glycogen from Liver

1.1 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.

1.2 Objective

To illustrate the method for isolating glycogen.

1.3 Materials and Methods

1.3.1 Chemicals

- Trichloracetic acid (TCA)
- Ethanol (95% v/v)

- Diethyl ether
- Sodium chloride

1.3.2 Solutions

- 5% TCA
- 10% TCA

1.3.3 Materials

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

1.3.4 Equipments

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

1.3.5 Glassware

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

1.3.6 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.

1.4 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.

1.5 Results

Record total yield and glycogen content per 100 g liver.

1.6 Discussion

1.7 Question

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

1.8 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

2. Enzymatic hydrolysis of glycogen and determination of glucose

2.1 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.

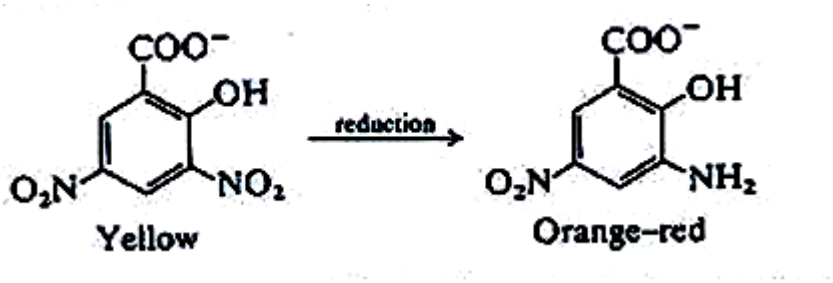
There are many methods for measuring the hydrolysis of glycogen and other polysaccharides, such as measurement of reducing sugars, or change of decreasing viscosity and the loss of capacity to give a blue color with iodine and finally the formation of split products.

2.2 Objective

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

2.3 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.



2.4 Methods and Materials

2.4.1 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

2.4.2 Equipments

- Boiling water bath
- Spectrophotometer

2.4.3 Glassware

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

2.4.4 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).

2.5 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.

2.6 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

2.7 Discussion:

2.8 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

2.9 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

3. D- Xylose Absorption Test

3.1 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.

3.2 Objectives

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

3.3 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.

3.4 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.

3.5 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 .

3.6 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:

3.7 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.

3.8 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?

3.9 References

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

Experiment (4)

4. Oral glucose tolerance test (GTT).

4.1 Objectives:

- Use OGTT in diagnosis of diabetes mellitus

4.2 Introduction

Serial measurement of plasma glucose before and after glucose is given orally should provide a standard method to evaluate individuals and establish values for normal and disease states. There are a number of factors that may affect glucose tolerance and that should be controlled or eliminated before such a test is performed; other conditions altering the glucose response need to be controlled during the test (Table 1). As a result, abnormal values may occur in the absence of diabetes mellitus, and under the same test conditions. When an oral glucose tolerance test is ordered, the following conditions should be met; omit medications known to affect glucose tolerance; perform the test in the morning after 3 d of unrestricted diet and activity; and perform the test after a 10-16 h fast. Plasma glucose should be measured fasting then every 30 min for 2 h after an oral glucose load. For adults, the recommended load is 75 g and for children, 1.75 g/kg, up to 75 g maximum.

Table 1;. FACTORS THAT AFFECT GLUCOSE TOLERANCE

1- Factors during the test affect glucose tolerance	
Posture	cigarettes
Nausea	time of day
Anxiety	activity
Coffee	amount of glucose ingested

2-Factors before the test that affect glucose tolerance

carbohydrate intake	propranolol
Time of pervious food intake	corticosteroids
GI surgery and malabsorption	Age

Thiazides	inactivity
Estrogens	weight
Di phenyl hydantoin	stress(surgery,infection)

- The National Diabetes Data Group has proposed a standard set of criteria for use in the diagnosis of diabetes mellitus. According to these criteria, no GTT is necessary is fasting plasma glucose is > 140 mg/dl, or 2-h postprandial value is > 200 mg/dl. Diabetes is confirmed if the 2-h plasma glucose and at least one other glucose. Value in a GTT are > 200 mg/dl. According to these criteria, diabetes mellitus in its early stages may not be identified but persons with abnormal results can be diagnosed with relative certainty. These criteria should be used for interpretation of glucose tolerance test result.
- An oral glucose tolerance test is not always necessary for the diagnosis of diabetes mellitus, particularly in a person with classic symptoms of diabetes mellitus (thirst, frequent urination, unexplained weight loss). The oral glucose tolerance test is indicated in the following situations:

1. Diagnosis of gestational diabetes mellitus.
2. Further evaluation of an individual with a borderline elevation of fasting or postprandial plasma glucose.
3. Risk counseling in an individual with a previously abnormal glucose tolerance test under suboptimal conditions (repeat results will often be normal).
4. Risk counseling in persons at high risk for the development of diabetes mellitus, but with normal fasting and postprandial plasma glucose values (a normal result, however, does not mean that diabetes mellitus will not develop in the future).
5. Evaluation of a patient with unexplained nephropathy, neuropathy, or retinopathy (abnormal results in this setting do not necessarily indicate a cause-and-effect relationship, and further studies will be needed to exclude conclusively other diseases).

5. Population studies for epidemiologic data.

TABLE. DIAGNOSTIC CRITERIA FOR DIABETES MELLITUS

.

1.Random plasma glucose ≥200 mg/dl(11.1 mmol/L)+ Symptoms of diabetes

2.fasting plasma glucose ≥126 mg/dl (7.0 mmol/L)

3.Tow- hour plasma glucose ≥200 mg/dl(11.1 mmol/L) During an OGTT

TABLE . CATEGORIES OF FASTING PLASMA GLUCOSE

1-Normal fasting glucose FPG<110 mg/dL

2- Impaired fasting glucose FPG ≥110 mg/dL and 126mg/dL

3- provisional diabetes diagnosis FPG≥126 mg/dL

4.3 Principle

- Glucose is produce a green color with o- toluidine in the presence of acetic acid and heat, with an absorption maximum at 630 nm .

4.4 Material and method:

1-Blood samples obtained at indicated (or suggested)time and plasma are separated .2- Oral glucose Tolerance Test (OGTT) is done as a following :

Omit medications known to affect glucose tolerance.

Perform in A.M. after 3 days of unrestricted diet and activity and after 10 h fast.

Measure fasting plasma glucose(sample A).

Give 100 g of glucose or equivalent orally .

Measure plasma glucose hourly for 2 h (sample B).

- **Glucose estimated by o-toluidine method**

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 glucose solution: 1.0g of glucose per 100 ml in 0.1 per cent benzoic acid (preservative) solution.
3. Working standard (0.5g/l) glucose solution: 5.0 ml of stock standard is diluted to 100 ml with distilled water.مراجعة الحسابات
4. Plasma sample .
5. Test tubes and test tube rack.
6. Spectrophotometer.
7. Water bath.

Label seven test tubes T_1 and T_2 (test A), T_3 and T_4 (test B) ,S (standard) and blank .Place the test tubes as shown below :

Test A (T_1 and T_2) 0.1ml of fasting plasma(sample A

Test B (T_3 and T_4) 0.1ml of Tow- hour plasma (sample B)

Standard (S1 and S2) 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 630 nm using a spectrophotometer and record the results in the table below.

4.5 Result and calculation:

Absorbance at 630 nm

Sample A		**Sample B**		**Standard**	

4.6 DISCUSSION:

4.7 References:

1. Tietz, N. W. (1987) .Fundamentals of Clinical Chemistry . W.B.Saunders. Philadelphia. third Edition .

2. Bishop .M; Fody. E and Schoeff .L.(2005) .Clinical chemistry .Principle , procedures and correlations .Fifth edition. Lippincott Williams &Wilkins.

Experiment (5)

5. Galactosemia

5.1 Objectives:

- To screen the galactosemia (initial test for galactosemia)

5.2 Introduction

Galactosemia is an inherited disorder marked by the inability to metabolize galactose .

Individuals with galactosemia are unable to metabolize galactose derived from lactose(milk sugar) to glucose metabolites

There are three forms of the disease:

- Galactose-1 phosphate uridylyl transferase deficiency (classic galactosemia, the most common and most severe form)
- Deficiency of galactose kinase
- Deficiency of galactose-6-phosphate epimerase

If an infant with galactose-1 phosphate uridylyl transferase deficiency is given milk, galactose-1 phosphate accumulate inside liver cells and causes hepatocellular damage and rapid liver failure. Other organs are also involved with this disease ,including the renal tubules and the eyes. Galactose-1 phosphate accumulation causes acute renal tubular failure and tubular loss of glucose ,phosphate,and amino acids. The loss of glucose in cooperation with the liver damage result in sever hypoglycemia .

Accumulation of galactose in the eye results in cataract formation.

A simple and inexpensive test used to the diagnosis of galactosemia is the urine reducing substance test by copper reduction method .Chromatography can be used to separate the sugars found in a urine sample .

If a positive test result is found ,more specific test would be necessary to determine the amount of galactose present.

5.3 Material

-thin layer chromatography (OR paper chromatography)

-standard galactose solution .

- patient urine sample .

- normal urine sample.

-Dinitrosalicylic acid reagent(DNS reagent is highly specific for reducing sugars) .Compsed of :

c) Sodium potassium tartrate. Dissolve 300g in 500ml water.
d) 3, 5- dinitrosalicyclic acid. Dissolve 10g in 200 ml 2M NaOH (warm).

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

- chromatography jar

-Solvent contains isopropanol: acetic acid : H_2O (3:1: 1)

5.4 Method

You will be provided with a silica gel plate. Place the plate on the bench with the silica gel side upward (handle the plate by the edges).
With a pencil draw a line about 2 cm from and parallel to the shorter edge of the plate. Draw the line gently so as not to break the surface of the gel. At equally spaced intervals mark the line at different places using the tip of the pencil on the silica gel plate. Carefully spot the

samples with a 5 µl pipette without making a hole in the adsorbant. Each sample should be on different mark. The spot on the silica gel should be no more than 2 mm in diameter.

Into the chromatography chamber, pour enough chromatography solvent to a depth of about 1 cm. Place the gel coated plate in the chamber . Cover the glass chamber tightly with aluminum foil. The solvent will rise up the silica gel by capillarity. When the solvent front is between 1 and 2 cm from the top of the gel, remove the chromatogram from the chamber and mark the position of the solvent front. Dry the plate in a stream of cold air in the fume cupboard.

Locate the sugars by spraying the paper with DNS reagent and heating briefly at 100^0C in an oven.

Note the colour and measure the R_f values.

The term "Rf" (relative flow) is used to express the performance of a solute in a given solvent system/support medium. The term Rf value may be defined as the ratio of the distance moved by a compound to that moved by the solvent. Rf value is constant for a particular compound, solvent system and insoluble matrix.

$$Rf = \frac{\text{Distance of migration of solute}}{\text{Distance moved by solvent}}$$

5.5 Calculation:

- Calculate the Rf value for each spot .

$$Rf = \frac{\text{Distance of migration of solute}}{\text{Distance moved by solvent}}$$

Sample	**Rf**
standard galactose solution .	
- patient urine sample .	
- normal urine sample	

5.6 Discussion and Conclusions:

5.7 References

1- Bishop .M; Fody. E and Schoeff .L.(2005) .Clinical chemistry .Principle , procedures and correlations .Fifth edition. Lippincott Williams &Wilkins.

2- http://www.nlm.nih.gov/medlineplus/ency/article/000366.htm

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5- Arneson,W and Brickell ,J.(2007).Clinical chemistry .F.A.Davis company

Experiment 6

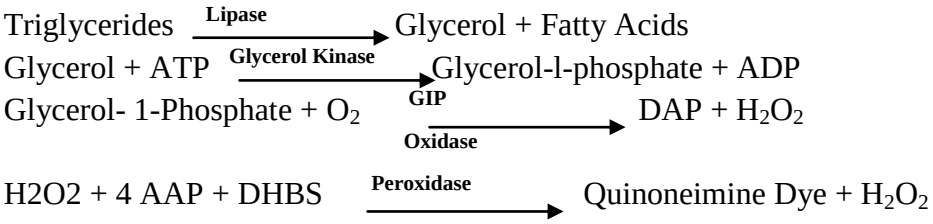
6. Triglyceride determination

6.1 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.

6.2 PRINCIPLE

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



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.

6.3 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.

6.4 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;

6.5 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.

6.6 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

6.7 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

7. HDL-Cholesterol determination

7.1 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.

7.2 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.

7.3 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.

7.4 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.

7.5 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.

7.6 MATERIALS REQUIRED

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

7.7 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.

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.

7.8 RESULTS:

7.9 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)

8. Determination of ammonia in plasma

8.1 Introduction :

Excess ammonia exert toxic effects on the central nervous system. The major source of circulating ammonia is the gastrointestinal tract, where ammonia is derived from the action of bacterial proteases, ureases, and amine oxidases on the contents of the colcon as well as from the hydrolysis of glutamine in both the small and large intestines.

Under normal circumstances most of the portal vein ammonia load is metabolized to urea in hepatocytes in the Krebs –Henseleit urea cycle during the first pass through the liver.

Inherited deficiencies of urea cycle enzymes are the major causes of hyperammonemia in infants . Two other types of inherited metabolic disorders produce hyperammonemia ; namely ,those involving the metabolism of the dibasic amino acids, lysine and ornithine , and those involving the metabolism of organic acids such as propionic acid, methylmalonic acid, and isovaleric acid.

Of the possible acquired causes of hyperammonemia, the most common is advanced liver disease. Sever liver disease, whether acute(as in toxic or fulminant viral hepatitis and Reye's Syndrome) or chronic (as in cirrhosis),can lead to a significant impairment of normal ammonia metabolism .Hepatic encephalopathy in the cirrhotic patient can be precipitated by gastrointestinal bleeding ,which enhances ammonia production by bacterial metabolism of the blood proteins in the colon and subsequently increases blood ammonia levels.

Other precipitating causes of encephalopathy include excess dietary protein ,constipation ,infections ,drugs ,or electrolyte and acid-base imbalance.

When cirrhosis is accompanied by impaired venous drainage from the intestine into the liver via the portal vein ,collateral vessels shunt ammonia of intestinal origin way from the liver and into the general circulation, causing increased blood ammonia levels. npaired renal function may also accompany sever liver disease. As urine output decreases lood urea concentration increases, leading to increased excretion of urea into the intestine, here it is converted to ammonia.

Normal values

Adult male 15-45 µ g /dl venous

8.2 Principle

NH_3 + 2-Oxoglutarate + NADH+ H^+ $\xrightarrow{GLDH}$ L- + NAD^+

The Ammonia reacts with 2-Oxoglutarate, in the presence of L- Glutamate dehydrogenase and NADH, to produce L-glutamate. The resulting decrease in absorbance of NADH at 340 nm is proportional to the level of ammonia in the sample.

8.3 Precautions

1. Avoid ingestion of reagent as toxicity has not yet been determined.
2. Plasma specimens should be considered as infectious and handled appropriately.

- SPECIMEN

- PLASMA

Blood is collected from patients fasted at least 6 hours, using verified ammonia-free heparin as anticoagulant.

Donor should not clench fist during collection as muscular exertion often increases venous Ammonia levels. Since erythrocytes contain larger amounts of ammonia than plasma, hemolysis may increase results.

Heparin is the preferred anticoagulant because it reduces red cell ammonia production.

After drawing blood is placed in an ice bath and plasma is separated within 30 minutes. Ammonia in heparinized plasma are stable for about 3 hours stored in an ice bath.

- **Performance characteristics**

Linearity : 0.3 µg/ml - 20 µg/ml

8.4 Result:

8.5 References

1. Tietz, N. W. (1987) .Fundamentals of Clinical Chemistry . W.B. Saunders. Philadelphia. third Edition .

2. Arneson, W and Brickell ,J.(2007).Clinical chemistry .F. A. Davis company

Experiment (9)

9. Phenylketonurea

9.1 Objectives:

- Primary diagnosis for Phenylketonurea

9.2 Introduction:

Phenylketonuria (PKU) is a genetic disorder that is characterized by an inability of the body to utilize phenylalanine. In 'classic PKU' phenylalanine hydroxylase, is completely absent. This enzyme normally converts phenylalanine to tyrosine as a result ,Phenyl alanine accumulates in the blood and undergoes transamination to form phenyl pyruvic acid and its products as phenyl lactic acid and phenyl acetic acid are produced.

Phenyl acetic acid is conjugated with glutamine and excreted as phenyl acetyl glutamine in urine (responsible for "mousy odour" of urine). Also phenylalanine, and its catabolites, Phenyl pyruvic acid, and phenyl lactic acid are excreted in urine.

A normal blood phenylalanine level is about 1-2 mg/dl but in classic PKU, levels may range from 15 to 65mg/dl. Clinical features: Child is mentally retarded .

Accumulation of phenylalanine leads to defective "serotonin" formation.

Excess of phenyl alanine in blood leads to excretion of this amino acid into the intestine. Here it competes with tryptophan for absorption. Tryptophan becomes subject to action of intestinal bacteria resulting in formation of indole derivatives which are absorbed and excreted in urine.

9.3 Materials:

1. Thin layer plates of silica gel: Prepare a slurry of silica gel G in 0.02 M sodium acetate and pour it onto the plate evenly. Dry the plate and activate it before use by heating at 150^0 C for 30 minutes.
2. Solvent -.butanol /acetic acid /H_2O 60/15/25 by volume

{or n-butanol :aceton :acetic acid :water(35:35:10:20)}

3. 0.2% ninhydrin in acetone

4. Microsyringe

5. Standard phenylalanine solution .

6. Patient urine sample

7. Normal urine sample

9.4 Method:

1-**Thin Layer Chromatography**

You will be provided with a silica gel plate. Place the plate on the bench with the silica gel side upward (handle the plate by the edges).

With a pencil draw a line about 2 cm from and parallel to the shorter edge of the plate. Draw the line gently so as not to break the surface of the gel. At equally spaced intervals mark the line at five different places using the tip of the pencil on the silica gel plate. Carefully spot the standard and sample solutions with a 5 µl pipette without making a hole in the adsorbant. Each sample should be on different mark. The spot on the silica gel should be no more than 2 mm in diameter.

Into the chromatography chamber, pour enough chromatography solvent to a depth of about 1 cm. Place the gel coated plate in the chamber. Cover the glass chamber tightly with aluminum foil. The solvent will rise up the silica gel by capillarity. When the solvent front is between 1 and 2 cm from the top of the gel, remove the chromatogram from the chamber and mark the position of the solvent front. Dry the plate in a stream of cold air in the fume cupboard. Locate the spot by spraying the plate with0.2% ninhydrin and heating the plate briefly at 100^0C in an oven. Note the colour and measure the R_f values.

The term Rf value may be defined as the ratio of the distance moved by a compound to that moved by the solvent. Rf value is constant for a particular compound, solvent system and insoluble matrix.

$$Rf = \frac{\text{Distance of migration of solute}}{\text{Distance moved by solvent}}$$

9.5 Calculation:

Calculate the Rf value for each spot .

$$Rf = \frac{\text{Distance of migration of solute}}{\text{Distance moved by solvent}}$$

Sample	**Rf**
standard phenylalanine	
- patient urine sample .	
- normal urine sample	

9.6 Discussion and Conclusions:

9.7 References :

1. Tietz, N. W. (1987) .Fundamentals of Clinical Chemistry . W.B. Saunders. Philadelphia. third Edition .

2. Chatterjea, MN.and Shinde ,R.(2007).Textbook of medical biochemistry. Jaypee Brothers medical publishers (p)LTD. New Delhi.

Experiment 10

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] .

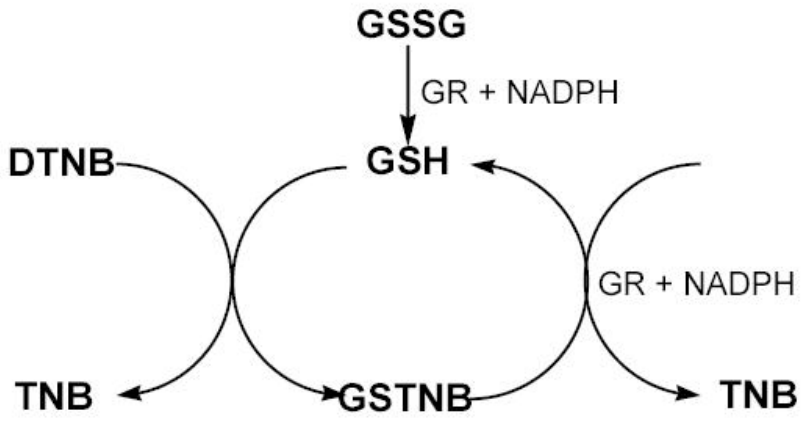


Figure 1: the principle of Glutathione assay

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 11

11. Estimation Of Arginase Activity In Liver Extract

11.1 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.

11.2 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.

11.2.1 Objective

Estimation of Arginase activity in liver extract.

11.3 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.

11.4 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.

11.5 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).

11.6 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?

11.7 References:

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