Republic of Iraq
Ministry of Higher Education and
Scientific Research
University of Basrah
College of Veterinary Medicine
Department of Physiology,
Pharmacology & Biochemistry

Practical Biochemistry

Prepare and Compose
By
Prof. Dr. Nawras Abdelah Alwan

2022

بسم الله الرحمن الرحيم

صدق الله العلي العظيم

سورة القلـــــم الآيــة 1

Dedication

All praise is to Allah, today we will fold the days' tiredness and the errand summing up between the cover of this humble work.

To whom he strives to bless comfort and welfare and never stints what he owns to push me in the successful way who taught me to promote life stairs wisely and patiently.....

To the spirit of my dear father

To the spring that never stops giving, to my mother who weaves my happiness with strings ...from her merciful heart

To my mother

To whose love flows in my veins and my heart always remembers them .....

My brothers and sisters

& the person closest to me

Nawras A. Alwan

Introduction

The Practical Biochemistry book was prepared as an assistant book for second-year students who study biochemistry in the first and second semesters. It includes most of the practical biochemistry curriculum.

The first chapter includes basic biochemistry and methods of work in the diagnosis and differentiation of compounds. The second chapter includes clinical biochemistry, which deals with the study of the concentration of compounds. As for the third chapter, it contains the preparation of special solutions in practical experiments that the student studies.

Prof. Dr. Nawras Abdelah Alwan

MSc. Biochemistry

Ph.D. Clinical Physiology

List of Contents

Chapter One

Basic Biochemistry

Biochemistry

Is a science that deals with chemical compounds for living materials and chemical changes which occurs through different living processes or can be define as a chemical analysis for biological processes include metabolism (anabolism and catabolism).

Chemistry of carbohydrates

Are aldeyde or ketone derivatives of polyhydric alcohols or compounds that yield these derivatives on hydrolysis. Carbohydrates serve as a principle source of energy. There are classified in to:

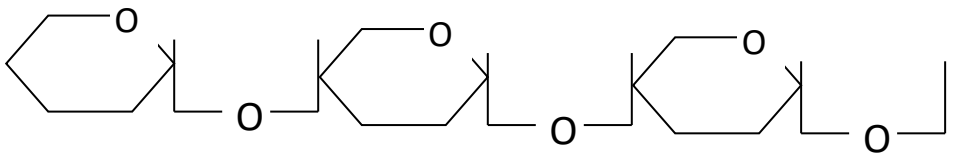
1-Monosaccharides: these kinds of saccharides can not be partion into simple picture (simple units) and have general formula ($C_nH_{2n}O_n$), for ex.: glucose, fructose and ribose.

2-Oligosaccharides: it is saccharides which contain from 2- 10 units from monosaccharide. ex.: maltose and lactose.

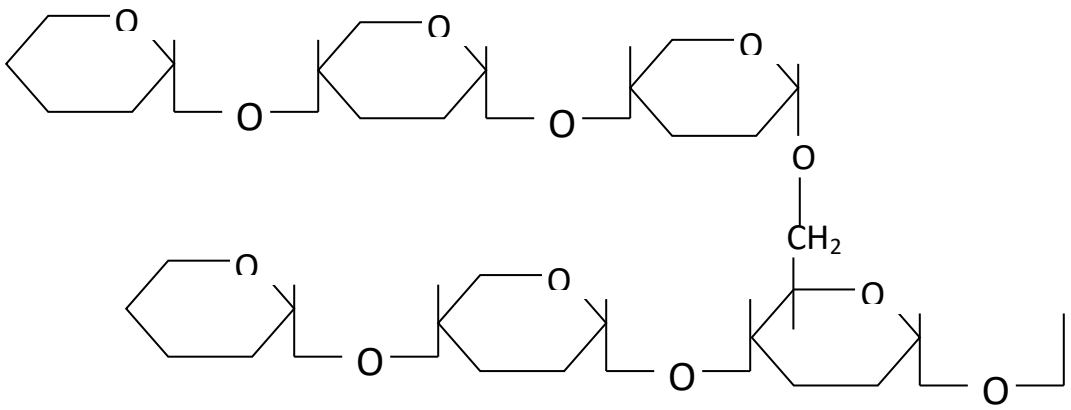
3- Polysaccharides: it is a saccharides which contain large number from monosaccharide which joined together by glycoside bonds, for ex.: starch, glycogen and dextrin.

Starch: It is polysaccharides consist from joined to chains known as:

1- **Amylose:** this chain consist from (1000-4000) units of glucose joined together by glycoside linkage type α(1,4).



2- **Amylopectin:** it is consist from joined two chains of amylose by glycoside bonds type α(1,6).



Colour reactions of carbohydrates

1-Molisch's test:

This is general test for all carbohydrates. It detects the presence the carbohydrates in a given solution.

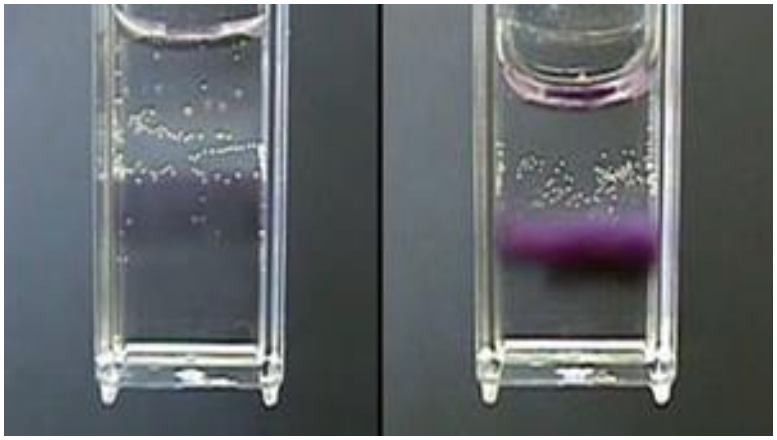
Principle: alcoholic α-naphthol forms furfural and furfural derivatives by conc. H_2So_4 acting on the sugar. This compound forms a reddish violet ring at the junction of the two liquids.

Method:

1- Put 2ml from sugar solution in test tube.

2- Then added 1-3 drops of α-naphthol to test tube and mix the solution well.

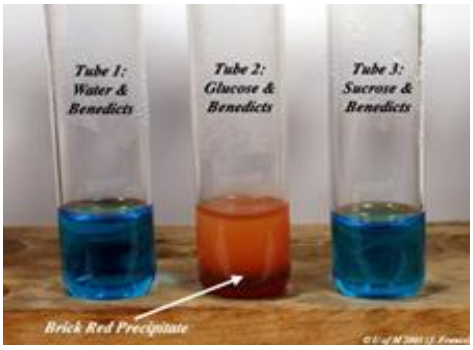
3- Added 2ml of concentration sulphuric acid from the side of the test tube to form violet ring at the junction of two layers.

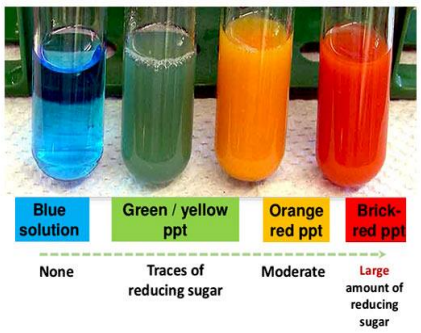


2- Benedict's test:

A carbohydrate with a free aldehyde or ketone group shows the reducing properties. These carbohydrates, in alkaline medium like sodium carbonate. These enediols reduce copper sulphate to cuprous hydroxide, which during the process of heating is converted into red cuprous oxide.

Method: Added 3ml of Benedict's reagent to test tube, add 0.5 ml of sugar solution. Mix well, boil the vigorously for 2 min. and then allow to cool (do not cool by immersing in cold water). A red, yellow or green ppt. develops depending upon the concentration of sugar present.





3- Barfoed's test:

This test is used to distinguish between monosaccharides and disaccharides. In this test the reduction is carried out in a weak acid medium.

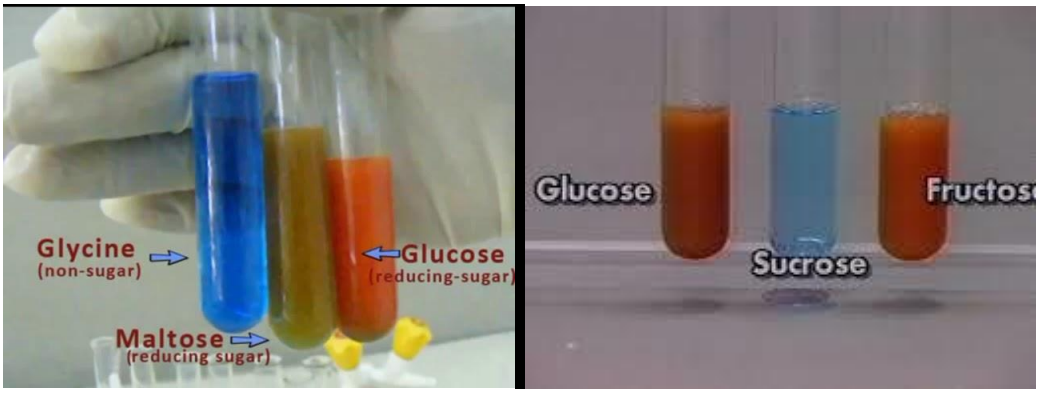
Methods:

1- Added 2ml of barfoed's reagent to 2ml of sugar solution

2- Mixture the solution well and then boiled for 3min. and cool it.

3- Observe red granular formation a long the side and at the bottom of test tube, these due to present monosaccharide (like: glucose, fructose).

Note : the monosaccharide react very fast with the barfoed's reagent, where as reducing disaccharide react very slowly because of presence of the weak acid but other prolonged heating the disaccharide may give a positive result.



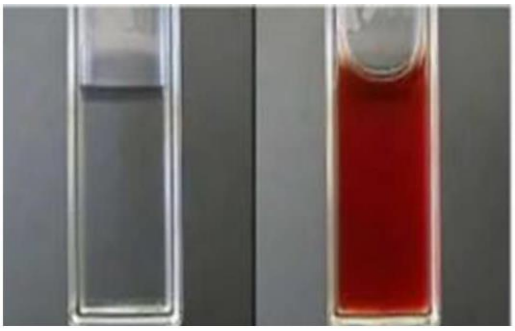
4-Seliwanoff's test:

This test of ketone sugar (like: fructose and sucrose), the keton group of ketose sugar is more reactive then aldehyde group of aldose and it reacts rapidly within 30 second of boiling to form furfural derivative. This then condenses with resorcinol to give a red colour.

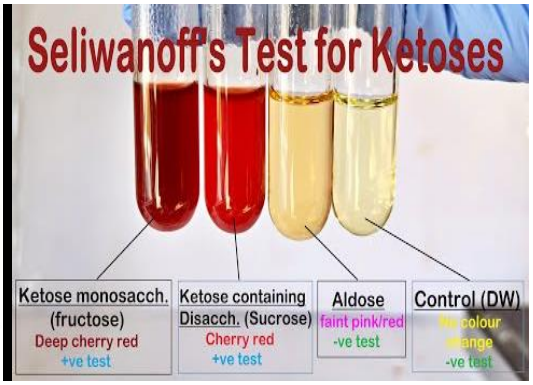
Methods:

1- Add 3ml of seliwanoff's reagent to 1ml of sugar solution and mix well.

2- Heat to boiling for 20-30 seconds, formation of red ppt. indicates the presence of fructose.



Negative Seliwanoff's test Positive Seliwanoff's test

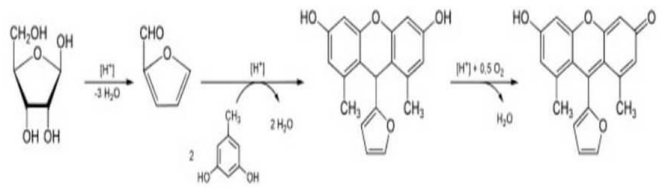


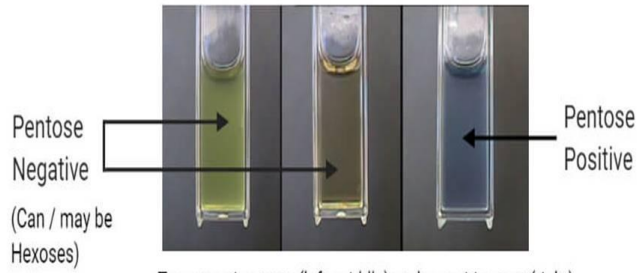
5- Bial's test:

This test specially for sugar contain five carbon atoms (like: ribose) which converted it to furfural and reaction with orcinol to production green solution.

Method: 1- Added 1ml of sugar solution to 1ml of bial's test

2- Mix the solution and boil for 5-10 min. and observe green colour solution.





Two negative tests (left, middle) and a positive test (right)

6- Iodine's test: It is a special test for polysaccharide for ex.: starch, dextrin and glycogen, the color of solution appear depend on amount of iodine adsorption on the surface of sugar.

Method:

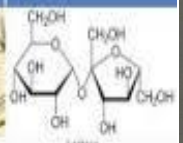
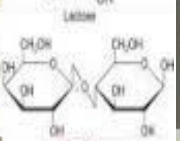
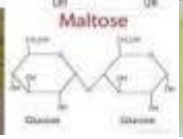
Add 1ml of iodine solution to 1ml of sample. A blue, red and purple colour in case of starch, dextrine and glycogen, respectively. In case of starch, blue colour disappears on heating and reappears on cooling, but red and purple in case of dextrin and glycogen do not reappear on cooling.

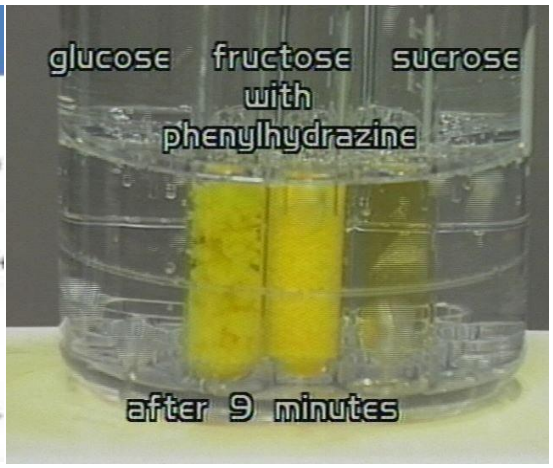
7- Osazone's test:

A solution of reducing sugar when heated with phenyl hydrazine formation yellow crystalline compounds called osazone are formed. In this test the glucose , fructose and mannose produce the same osazone, because of the similarities in their final four carbon molecules structures. Lactose gives powderpuff like, while maltose forms sunflower shaped osazone.

Method:

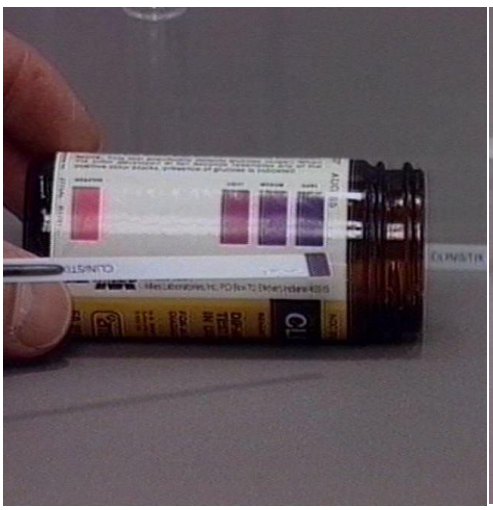
Mix 2ml of sugar solution with knife point of phenyl hydrazine hydrochloride and double the amount of sodium acetate crystals and add 5 drops of glacial acetic acid. Mix and warm a little to see that the solids are dissolve. And the boiling the solution for 20-30 min. allow the tube cool slowly without cooling it hurriedly under the tap to have better crystals. Examine the crystals under the microscope.

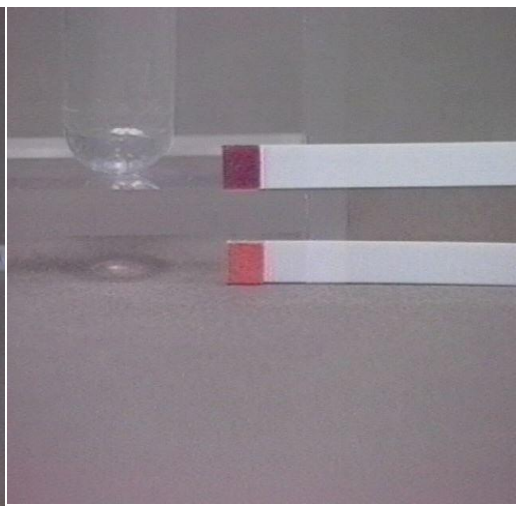
S. No.	Type of disaccharides	Osazone formation shape	Structure
1.	Glucose (Needle shape).		
2.	Lactose (Powder-puff or tennis ball shape).		
3.	Maltose (Sunflower shape).		



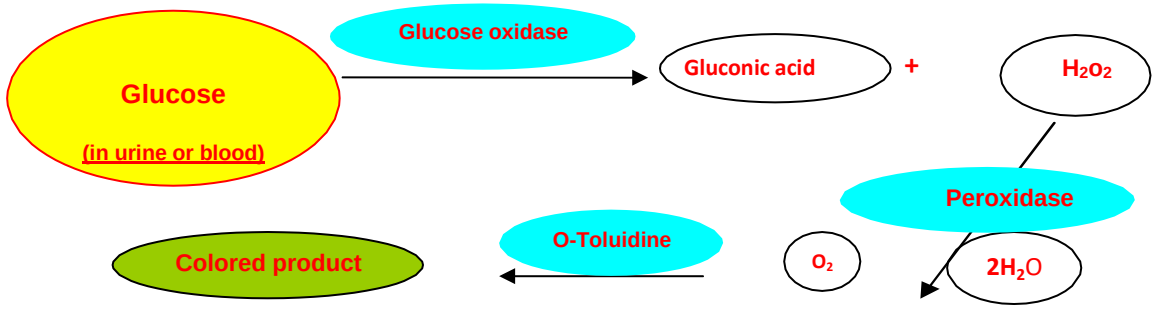
Biochemical test for glucose:

This method is commercially used in dipsticks used fortesting the presence or absence of glucose in urine or blood. The dipstick contains the enzyme glucose oxidase, peroxidase and o-Toluidine.





***Principle*:**



Clinical significance:

This test is used for qualitative determination of glucose in biological fluids (e.g. urine or blood) and subsequent diagnosis of "***Diabetes mellitus***".

Laboratory Exercise

Label 5 test tubes, one for each provided carbohydrate solution (glucose,fructose, lactose & starch) and perform the following tests:

1. **Molisch's test.**
2. **Fehling's test.**
3. **Benedict's test.**
4. **Osazone test.**
5. **Iodine Test**.

Chemistry of Proteins

The word "**protein**" is derived from the Greek word "***proteios***", which means "of primary importance". In fact, a protein plays an important role in all biochemical and physiological body processes; they act as enzymes, hormones, receptors, antibodies and are required for the structural integrity of cells.

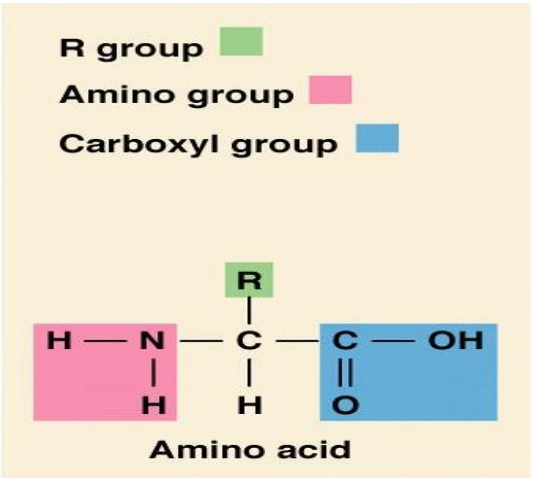
The aim of this practical session is to:

1- Obtain a simplified knowledge about protein structure.

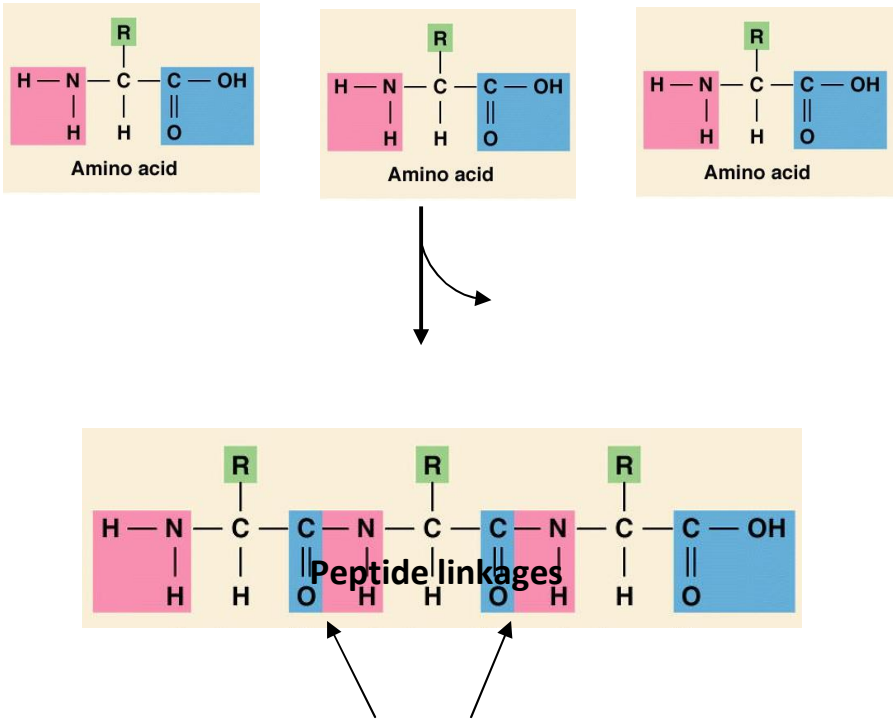
2- Practically apply this knowledge by performing some protein color andprecipitation reactions.

Protein structure

Proteins are organic compounds made of "**amino acids**" joined together by"**peptide linkages**".



These **pepetide linkages** are obtained by condensation reactions (removalof water) between carboxylic & amino groups of two adjacent amino acids.



Essential and non-essential amino acids:

There are 20 standard amino acids which differ in their side chain (**R**). Some of them are considered "**essential**" since they cannot be synthesizedin our body and must be therefore provided in the diet (e.g. tryptophan & phenylalanine), while others are "**non-esential**" and can be synthesized in the body (e.g. alanine & cysteine).

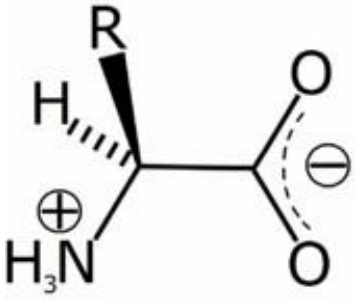
Functions of dietary proteins:

Proteins are necessary components in our diet. Through the process of digestion, proteins arehydrolyzed into amino acids that can be used for the synthesis of different body proteins (enzymes, hormones, antibodies,…etc), tissue repair and growth. Deficiency of proteins can cause general weakness, protein malnutrition diseases, and decreased resistance to infection.

Amphoteric nature of amino acids:

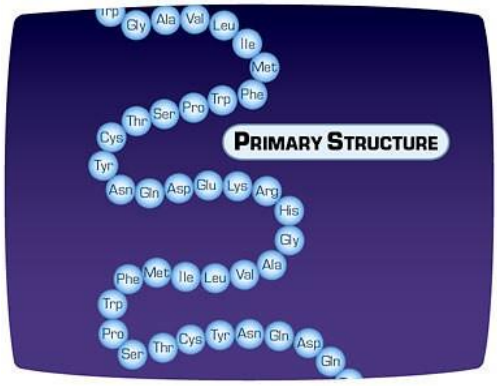
As amino acids have both an "**amino**" gp and a "**carboxylic**" gp, they are considered as both "**base**" and "**acid**", i.e. they are amphoteric. At a certain pH, the amino group can become protonated gaining a positive charge, and the acid group can become deprotonated gaining a negative charge. The resulting doubly charged ion is known as "**zwitterion**".

Zwitterion

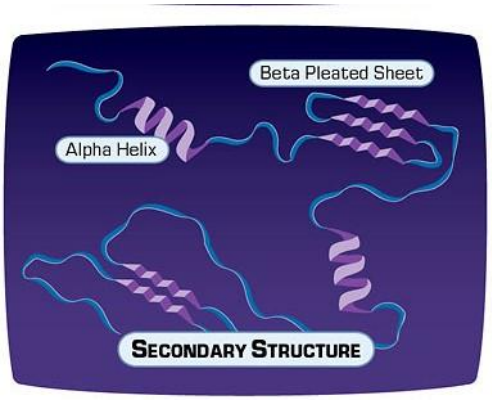


Aspects of protein structure:

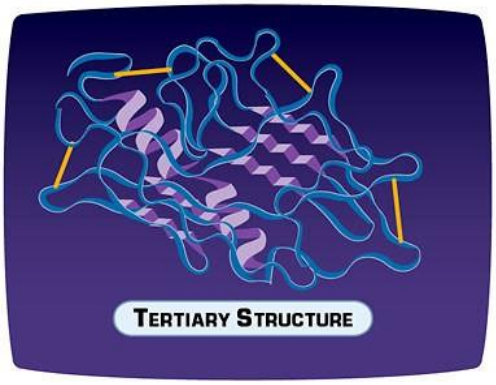
There are 4 aspects to describe a protein structure: 1°ry, 2°ry3°ry & 4°ry structures.



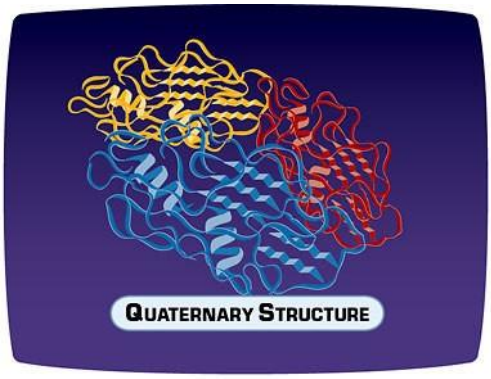
Amino acid sequence



Hydrogen bonding to give alpha helical or beta pleaded



Overall shape (globular or fibrous)



Assembly of protein subunits

Laboratory exercise

Using the provided solutions of **albumin** (egg white), **casein** (milk protein)and **gelatin** (animal collagenous material), perform the following:

A. General tests

B. Color reactions

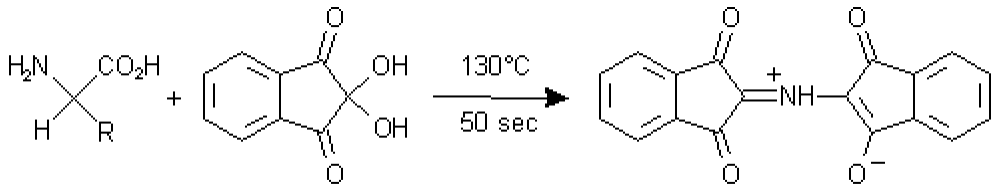
C. Precipitation reactions

A.General tests for proteins

1- Ninhydrin reaction:

Principle:

Ninhydrin reacts with amino acids in proteins at high temperature giving apurple colored complex.



Purple colored ninhydrin complex Amino acid

Ninhydrin is most commonly used as a forensicchemical to detect "**fingerprints"**, as amines left over from proteins sloughed off in fingerprints react with ninhydrin giving a characteristic purple color.

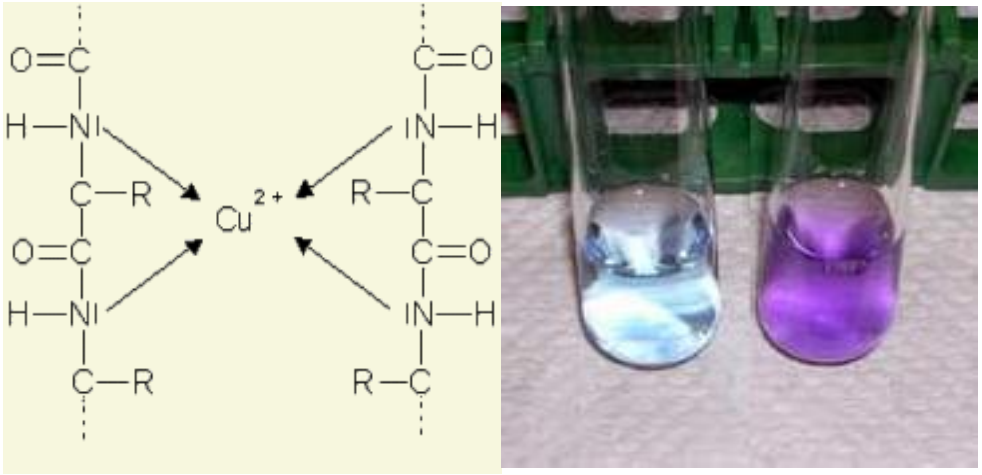
Procedure & observation:

-To 1 ml amino acid solution in a test tube, add 1 drop of ninhydrin.

-Put in a boiling water bath and observe the formation of a purple color.

2-Biuret test:

Principle: The biuret reagent (copper sulfate in a strong base) reacts with peptide bonds in proteins to form a blue to violet complex known as the "**biuret complex**". **N.B.** Two peptide bonds at least are required for the formationof this complex.



Biuret complex

Procedure & observation:

- To 2 ml of protein solution in a test tube, add 3 drops of 10% sodium hydroxide solution and 3-6 drops of 0.5% copper sulfate solution.

-Mix well; a blue to violet color is obtainedwith albumin, casein &gelatin.

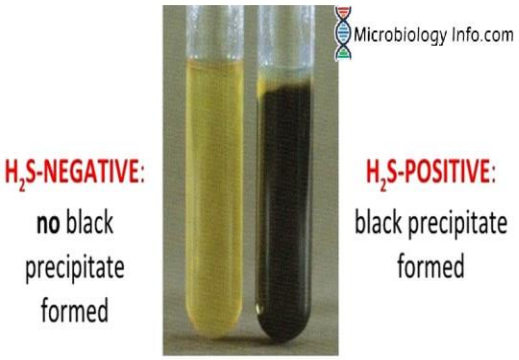
A. Color reactions of proteins

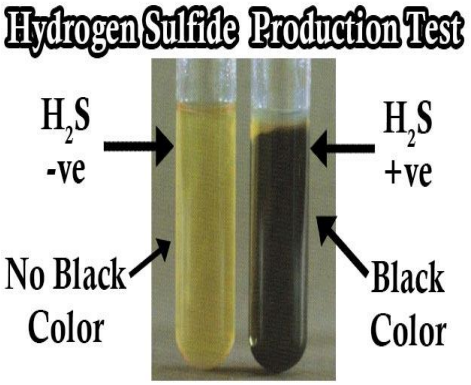
1. Reduced sulfur test:

Principle: Proteins containing sulfur (in cysteine and cystine) give a black depositof lead sulfide (PbS) when heated with lead acetate in alkaline medium.

Procedure & observation:

- To 1 ml of protein solution in a test tube, add 2 drops of 10% sodium hydroxide solution and 2 drops of lead acetate.
- Mix well and put in a boiling water bath for few minutes; a black depositis formed with albumin, while a slight black turbidity is obtained with casein due to its lower content of sulfur. Gelatin gives negative result.





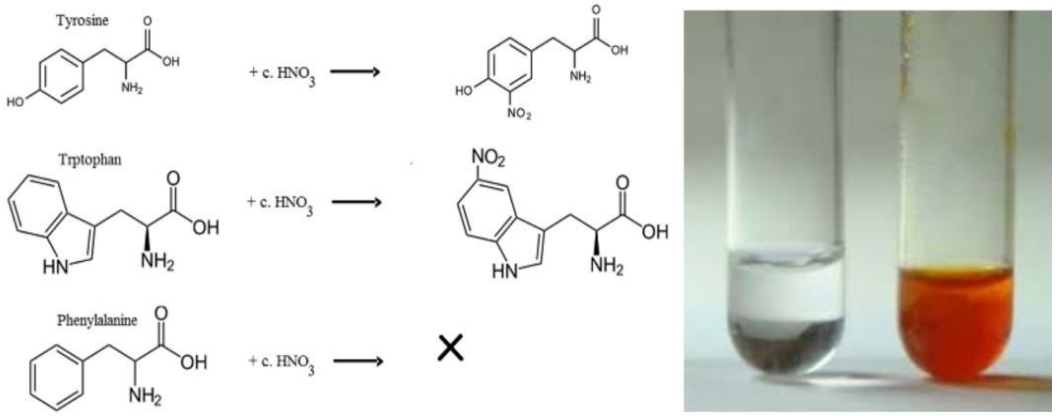
2- Xanthoproteic acid test:

Principle: Nitric acid gives a color when heated with proteins containing **tyrosine** (yellow color) or **tryptophan** (orange color); the color is due to nitration.

Procedure & observation:

- To 2 ml of protein solution in a test tube, add 2 drops of concentrated nitric acid.
- A white precipitate is formed and upon heating in a boiling water bath, it turns yellow with "**tyrosine**" and orange with the essential amino acid "**tryptophan**" indicating a high nutritive value.

Xanthoproteic test



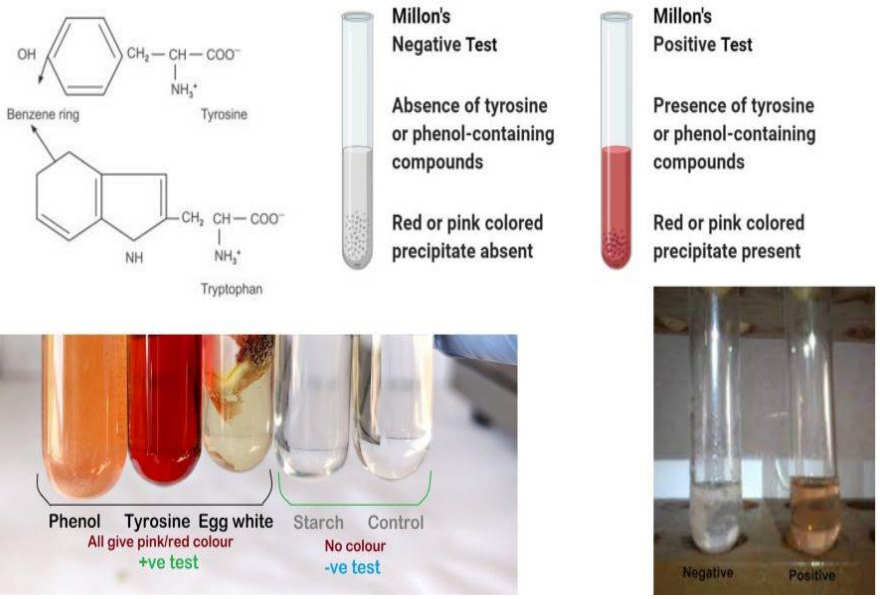
3- Millon's test:

Principle:

Millon's reagent (Hg/HNO_3) gives positive results with proteins containing the phenolic amino acid "**tyrosine**".

Procedure & observation:

- ❖ To 2 ml of protein solution in a test tube, add 3 drops of Millon's reagent.
- ❖ Mix well and heat directly on a small flame.
- ❖ A white ppt is formed with albumin and casein (but not gelatin); the pptgradually turns into brick red.



4-Hopkins-Colé test :

Principle:

Hopkins-Colé reagent (magnesium salt of oxalic acid) gives positive results with proteins containing the essential amino acid "**tryptophan**" indicating a high nutritive value.

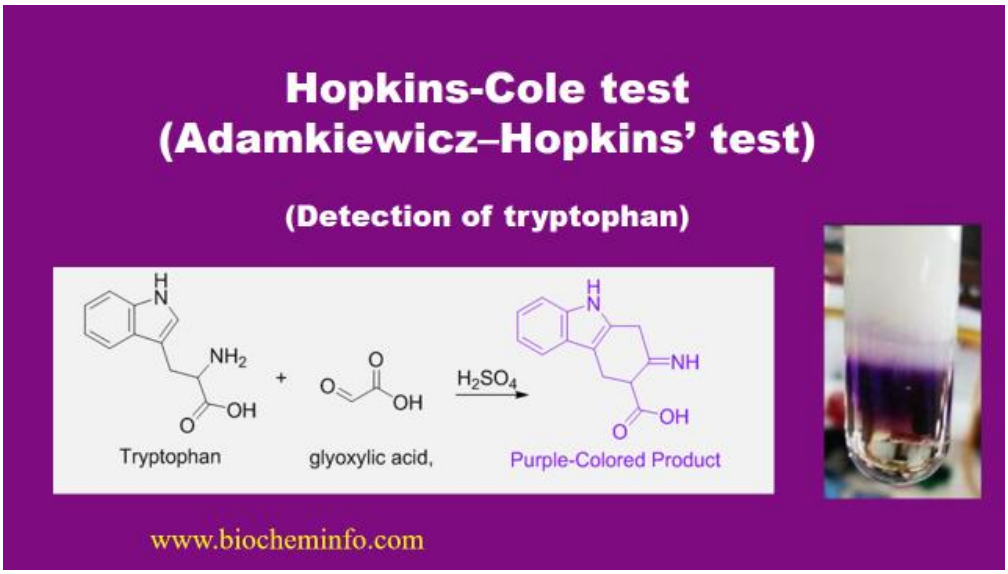
Procedure & observation:

-To 1 ml of protein solution in a test tube, add 1 ml of Hopkins-Coléreagent and mix well.

- Incline the test tube and slowly add 1 ml of concentrated H_2SO_4 on theinner wall of the test tube to form 2 layers.

- Put the test tube in a boiling water bath for 2 minutes.

- A reddish violet ring is formed at the junction between the 2 layers withalbumin and casein; gelatin gives negative results.



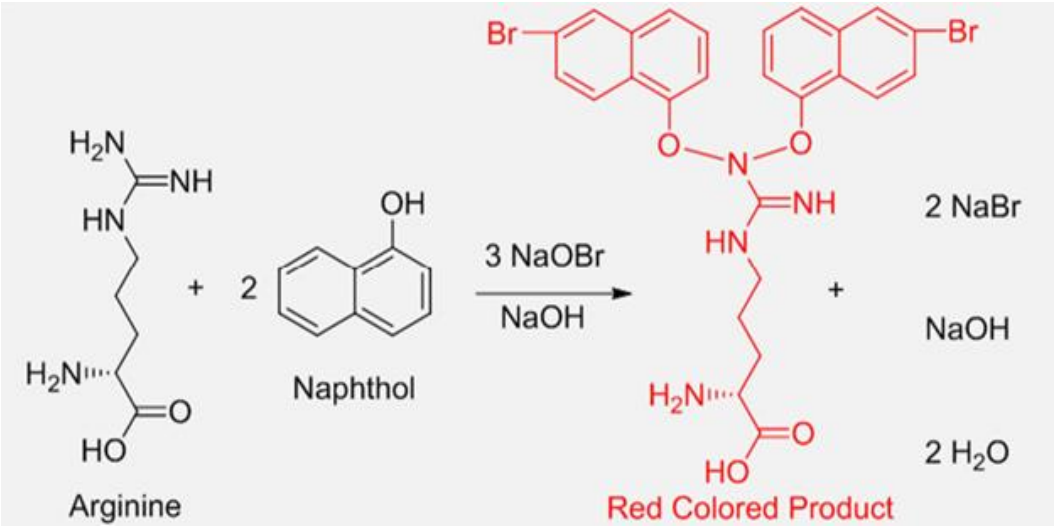
5-Sakaguchi Test: (Detection of Arginine)

The **Sakaguchi test** is a chemical test used for detecting the presence of arginine in proteins. It is named after the Japanese Food Scientist and Organic Chemist, Shoyo Sakaguchi (1900–1995) who described the test in 1925.

Principle:

Sakaguchi test is given by guanidinium compounds. The only amino acid containing the guanidine group is arginine. Therefore this test is specific for identification of arginine. Arginine reacts with α-naphthol in presence of an oxidizing agent such as bromine water or sodium hypochlorite to give a red coloured product. The other guanidine containing non-amino acid compounds also give this reaction.

Reaction:



Reagents:

1. Test solutions:

- Amino acids: 0.1 % solution of amino acids like glycine, arginine, lysine, tyrosine, etc.
- Protein solution: egg albumin in distilled water (around 10%).

- Guanidines: 0.1 % solutions of glycocyamine, methyl guanidine, and creatine.

2. NaOH: 40% (w/v).

3. α-Naphthol: 1% in alcohol.

4. Bromine water: Add a few drops of bromine to 100 ml water and shake. This should be done in a fume chamber. (Caution: Bromine can cause severe burns). **Or** use 10% sodium hypochlorite solution.

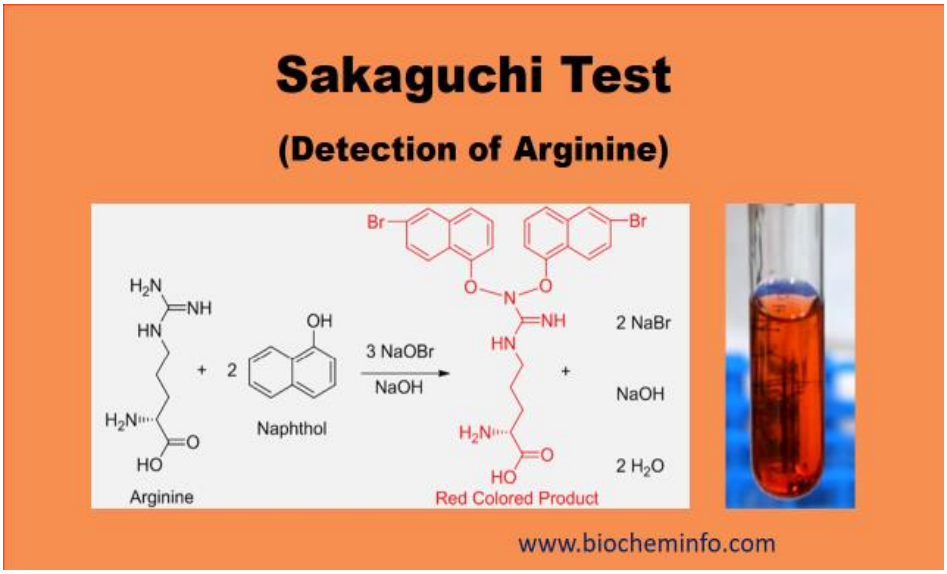
Procedure:

- Mix 1 ml of NaOH with 3 ml of the amino acid or test solution
- Then add 2 drops of α-naphthol.
- Mix thoroughly and add 4 to 5 drops of bromine water **or** add 1 to 2 drops of 10% sodium hypochlorite.
- Watch for the formation of red colour.

Result Interpretation:

Positive test: Formation of red colour indicate presence of arginine or a guaniginium compound.

Negative test: No formation of red colour indicate absence of arginine or a guaniginium compound.



Precipitation reactions of proteins:

1-Precipitation by heavy metals:

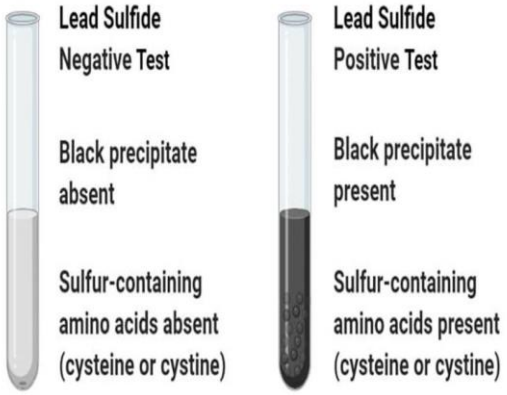
Principle:

Heavy metals (e.g. **Hg^{2+}, Pb^{2+}, Cu^{2+}**) are high molecular weight cations. The positive charge of these cations counteracts the negative charge of the carboxylate group in proteins giving a precipitate.

Procedure & observation:

-To 1 ml of protein solution in a test tube, add 1 drop of lead acetate;a white ppt is obtained.

-To 1 ml of protein solution in a test tube, add 1 drop of 10% coppersulfate; a blue ppt is obtained.



2-Precipitation by alkaloidal reagents:

Principle: Alkaloidal reagents (e.g. **tannate** & **trichloroacetate**) are high molecular weight anions. The negative charge of these anions counteracts thepositive charge of the amino group in proteins giving a precipitate.

Procedure & observation:

- To 1 ml of protein solution in a test tube, add tannic acid drop wise until abuff ppt is obtained.

-To 1 ml of protein solution in a test tube, add 1 ml of trichloroaceticacid (TCA); a white ppt is obtained.

N.B. Precipitation of proteins by heavy metals and alkaloidal reagents indicates the presence of both negative and positive charges and hence the **amphoteric** nature of proteins.

3- Precipitation by denaturation:

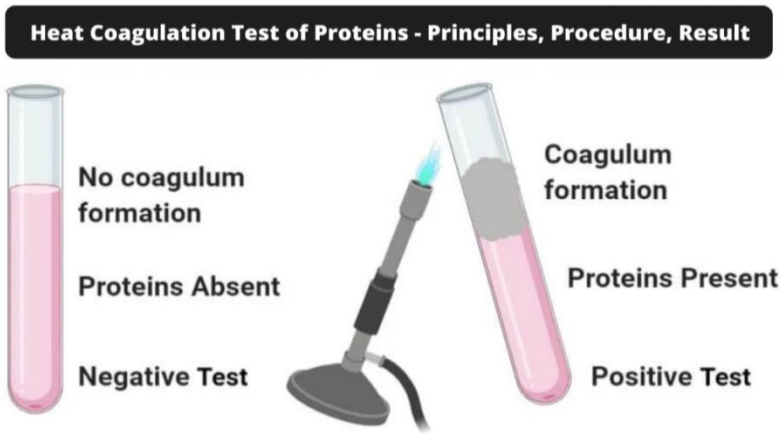
A-Denaturation by heat (heat coagulation test):

Principle: Heat disrupts hydrogen bonds of secondary and tertiary protein structure while the primary structure remains unaffected. The protein increases in size due to denaturation and coagulation occurs.

Procedure & observation:

-Put 2 ml of protein solution in a test tube, incline it and heat to boiling.

-A permanent clotting and coagulation is obtained with **albumin** only.



B- Denaturation by acids (Heller's test):

Principle:

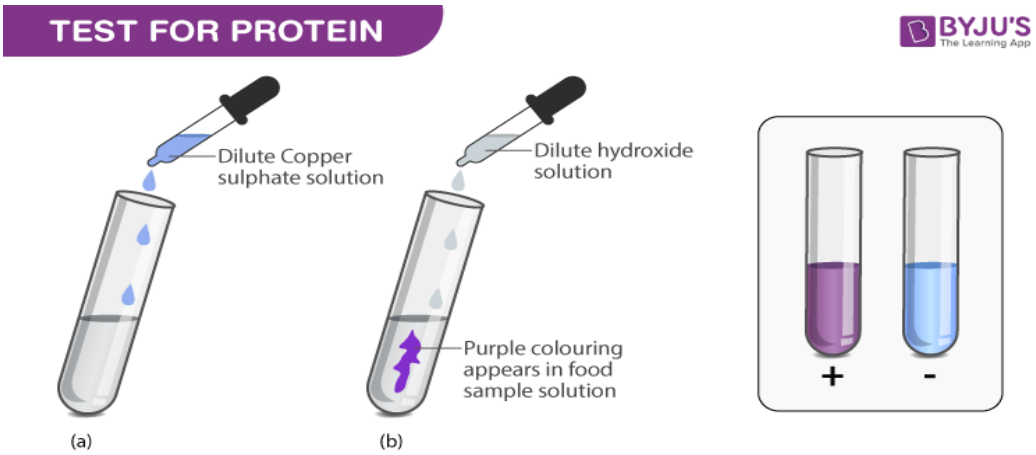
Nitric acid causes denaturation of proteins with the formation of a white ppt (this differs from the nitration reaction in "xanthoproteic acid test").

Procedure & observation:

- Put 2 ml of concentrated nitric acid in a test tube.

- Incline the tube and slowly add 1 ml protein solution drop wise to form alayer above the nitric acid layer.

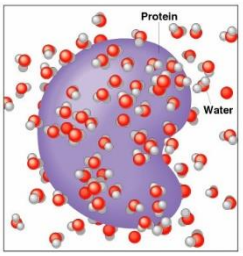
-A white ring is formed at the interface between the 2 layers.



4-Fractional precipitation by ammonium sulfate (salting out):

Principle:

Protein molecules contain both hydrophilic and hydrophobic amino acids. In aqueous medium, hydrophobic amino acids form protected areas while hydrophilic amino acids form hydrogen bonds with surrounding water molecules (**solvation** layer). When proteins are present in **salt** solutions (e.g. mmonium sulfate), some of the water molecules in the solvation layer are attracted by salt ions. When salt concentration gradually increases, the number ofwater molecules in the solvation layer gradually decreases until protein molecules coagulate forming a precipitate; this is known as "**salting out**".As different proteins have different compositions of amino acids, different proteins precipitate at different concentrations of salt solution.

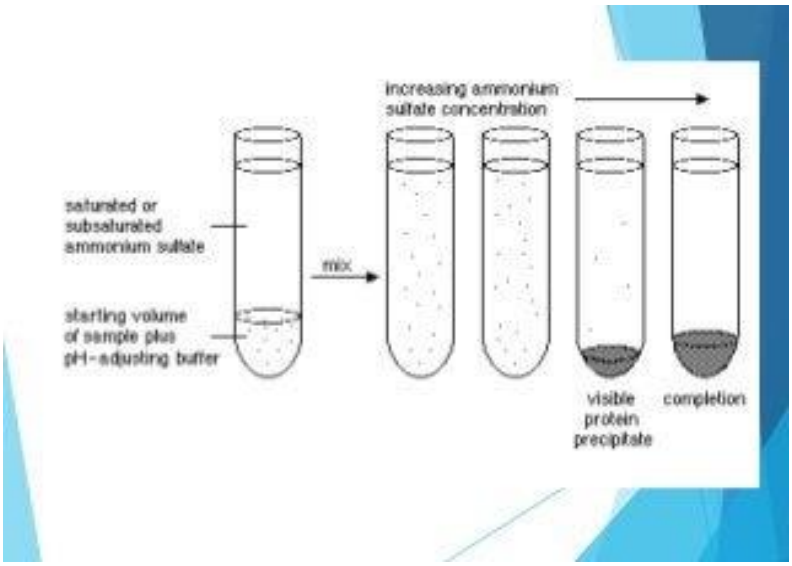


Procedure & observation:

❖To 2 ml of egg-white solution (containing both albumin & globulin), addan equal volume of saturated ammonium sulfate solution; globulin is precipitated in the resulting half saturated solution of ammonium sulfate.

❖Separate globulin by centrifugation and recover the clear supernatant.

- Add ammonium sulfate crystals gradually to the clear supernatant until full saturation occurs; another precipitate (albumin) is obtained.
- Separate albumin by centrifugation.



N.B.: The reason for the precipitation of globulin and albumin at different ammonium sulfate concentration could be that the solvation layer around globulin is looser and thinner than that around albumin. Therefore, globulin needs only half-saturated ammonium sulfate to loose its solvation layer while albumin loses its solvation layer in a fully saturatedammonium sulfate solution.

Laboratory exercise

1. Using the provided solutions of **albumin**, **casein** and **gelatin** perform the tests in the table below and write down your observations.

test	**Albumin**	**Casein**	**Gelatin**
Biuret test			
Reduced sulfur test			
Xanthoproteic acid test			
Heavy metal pptn			
Alkaloidal reagent pptn			
Heat coagulation test			
Heller's test			

2. Gelatin of high nutritive value? Why?

3. Observe the demonstration of fractional separation of albumin and globulin from egg white using ammonium sulfate (salting out).

Chemistry of lipids

The term "**lipids"** applies to a class of compounds that are soluble in organic solvents (e.g. alcohol and chloroform) and insoluble in water. Lipids are essential components of every living cell.

The **aim** of this practical session is to:

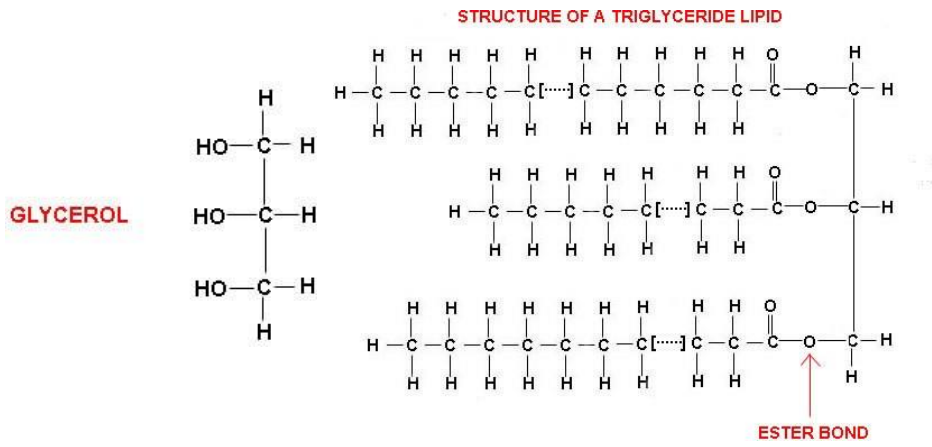
1. Obtain a simplified knowledge about lipids.
2. Perform different tests showing some lipid characteristics.

Classification of lipids

Lipids are classified into:

I. Simple lipids:

They are esters of fatty acids with various alcohols. They include **oils** and **Fats** which are esters of fatty acids with **glycerol** (i.e. triglycerides).

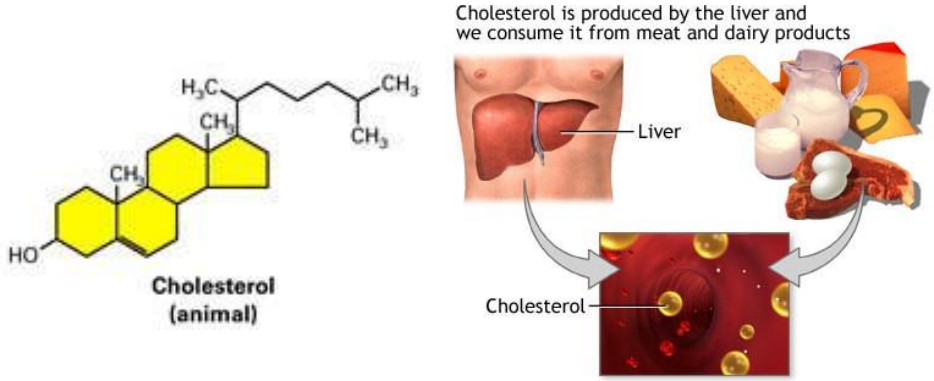


II. Complex lipids:

They contain (in addition to fatty acids and alcohols) additional groups as **phosphate** (e.g. phospholipids), **carbohydrates** (e.g. glycolipids) and **proteins** (e.g. lipoproteins).

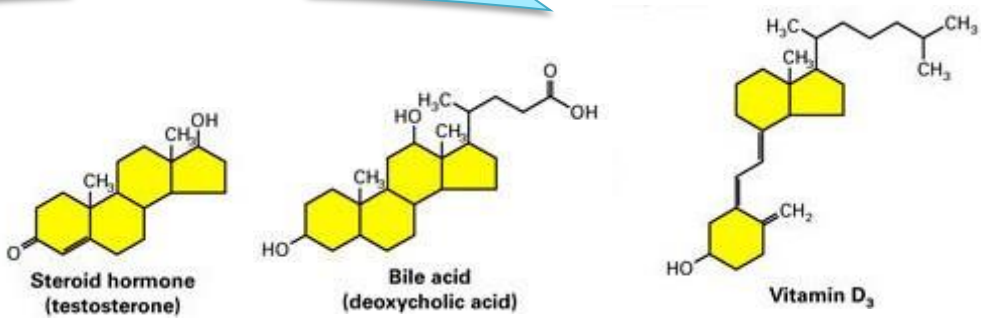
III. Precursor and derived lipids:

They include fatty acids, cholesterol, steroid hormones, fat-soluble vitamins (vitamin A, D, E & K) and eicosanoids, which include prostaglandins, leukotrienes and thromboxanes.



Biological functions of lipids:

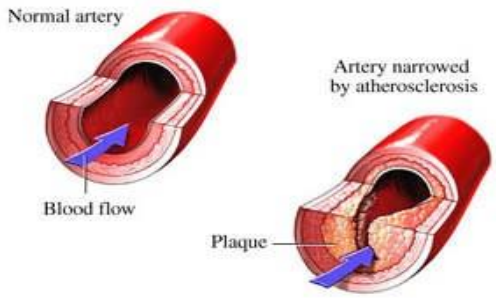
1. They provide energy storage and metabolic fuels.
2. Cholesterol is a precursor of steroid hormones, bile acids (which helpin digestion of dietary fats) and vitamin D.



3. They act as functional and structural components of the cell membrane.

4. Lipoproteins (e.g. LDL & HDL) are a mean for transporting lipids in blood.

5. Imbalance in lipid metabolism can lead to major clinical problems such as obesity and atherosclerosis.



Practical

1. **<u>Test for solubility of lipids in polar & non-polar solvents</u>:**

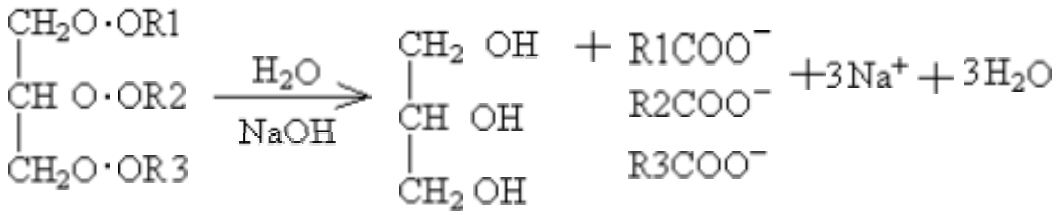
– Add about 2 ml of the provided **oil sample** to 5 ml of **water** in a test tube and try to mix oil with water. By shaking, oil and water mix initially; however, they gradually separate out to form 2 layers.

– Repeat the experiment using **chloroform** instead of water.

– Oil dissolves in the organic solvent "**chloroform**" but not in water because oils contain long hydrocarbon tails which are hydrophobic innature.

2. Saponification of oils:

Saponification of oils means the hydrolysis of triglycerides in the oil in presence of an alkaline medium (e.g. NaOH) into glycerol and fatty acids with the production of sodium salt of fatty acids or "**soap**".



– Place 5 ml of the provided oil sample in a 250 ml beaker.
– Add 15 ml of alcoholic NaOH solution.

Oil + alcoholic NaOH

boiling water bath

soap

- Cover the beaker with a watch glass and heat on a boiling W.B.

– Stir the reaction mixture from time to time using a glass rod until a semi-solid mass is formed.

– Put a small amount of this mass in a test tube containing about 5 ml of water and shake well; a **froth** is obtained indicating the presenceof **soap**.

– In another test tube, prepare a soap solution by dissolving a small amount of the formed soap in water. Add few drops of $CaCl_2$ or $MgSO_4$ to this solution and shake well; note the formation of insoluble Ca or Mg soaps without any froth. This explains why soap is not effective in hard water which contains calcium and magnesium;for soap to be effective, it must be soluble in water.

$$2\ RCOO^- + Ca^{2+} \longrightarrow (RCOO)_2Ca + \text{ppt}$$

3. <u>Detection of cholesterol in food extracts</u>:

–**Salkowski test:** to 2 ml of the provided chloroformic extract of canned food, add an equal volume of concentrated sulfuric acid; a yellow to brick-red color is formed indicating the presence of cholesterol.

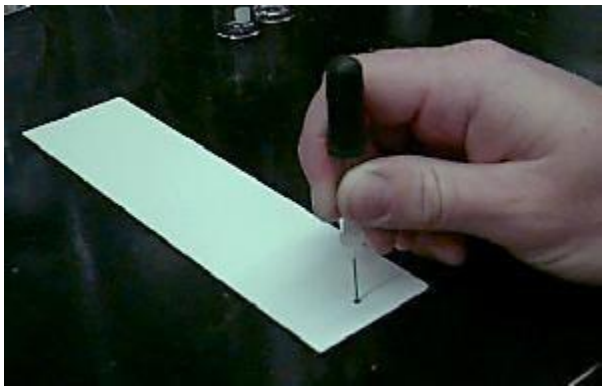
–**Liebermann-Burchard test**: add 10 drops of acetic anhydride and 2 drops of concentrated sulfuric acid to 2 ml chloroformic extract of canned food; a bluish-green color is formed indicating the presence of cholesterol.

N.B. Colors are due to sulfonation, acetylation and rearrangementin cholesterol molecule.

4. <u>Detection of cholesterol in a lipid mixture by thin layer chromatography:</u>

Thin layer chromatography (**TLC**) is based on the separation of a mixture of compounds as they migrate (with the help of a solvent

system) through a thin layer of adsorbent material. When using a non-polar solvent system, polar compounds are held back by the adsorbent while non-polar compounds advance further.



– With your pencil, draw a line about 1 cm from the bottom edge of a silica gel chromatography sheet.

– On this line, apply 2 spots: the first for the lipid mixture and the second for cholesterol as a reference. N.B. mark with your pencil "**M**" for the mixture and "**C**" for cholesterol.

– Put the TLC sheet vertically in a beaker containing 10 ml of a non-polar solvent system composed of petroleum ether : diethylether : glacial acetic acid (70:30:1).

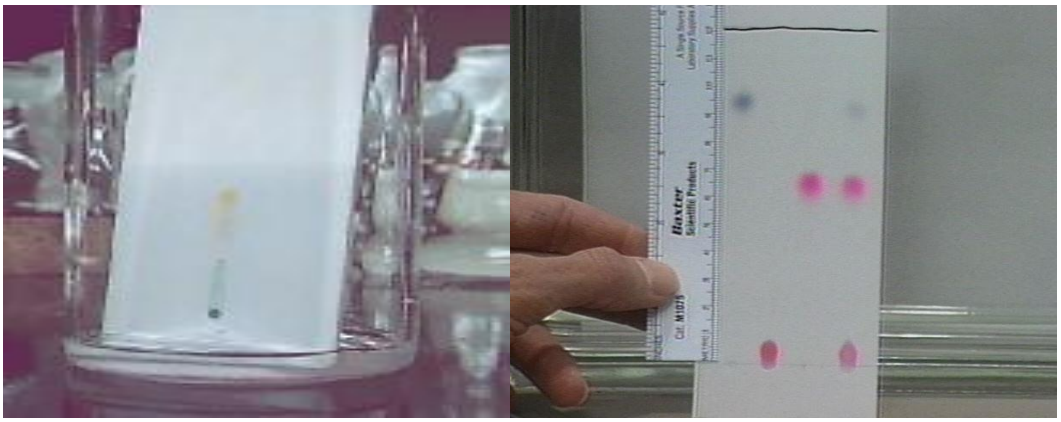
– Cover the beaker immediately with aluminum foil.

– Allow the solvent to rise to the appropriate level (about 1 cm from the upper end of the sheet).

– Remove the TLC sheet from the beaker and immediately draw a line with your pencil at the **front line** (level reached by the solvent).

– Allow the solvent to evaporate and place the TLC sheet vertically in another beaker containing few crystals of iodine; cover the beaker with a watch glass, iodine will sublime and colorize the lipid spots with a yellow color.

– Remove the TLC sheet and encircle spots with a pencil.
– Record the Rf value for each spot.



$$Rf = \frac{\text{Distance traveled by the spot}}{\text{Distance traveled by the solvent}}$$

– Note that if the lipid mixture contains cholesterol, a spot with thesame Rf value of reference cholesterol is obtained.
– Comment on the polarity of compounds in the separated spot

Laboratory exercise

Student Name: ……
Student number: ……

1. Test for solubility of lipids in polar and non-polar solvents:

Theprovided lipid is soluble in
……..……
because…….……
……..……

2. Saponification of oils:

What is the chemical nature of soap ?
…….……

3. Detection of cholesterol in food extract:

Salkowski test:
……

Liebermann-Burchard test:
……

Comment:……

4. Detection of cholesterol in a lipid mixture by TLC:

Rf value of cholesterol spot =
……

Does the mixture contain cholesterol ? ……

Chemistry of enzymes

Enzymes are proteins that act as catalysts for biological reactions. Enzymes, like all catalysts, speed up reactions without being used up themselves. They do this by lowering the activation energy of a reaction. All biochemical reactions are catalyzed by enzymes. Since enzymes are proteins, they can be denatured in a variety of ways, so they are most active under mild conditions. Most enzymes have optimum activity at a neutral pH and at body temperature.

Enzymes are also very specific – they only act on one substrate or one class of related substrate molecules. The reason for this is that the active site of the enzyme is complementary to the shape and polarity of the substrate. Typically, only one kind of substrate will "fit" into the active site.

In this experiment, we will work with the enzyme amylase. This enzyme is responsible for hydrolyzing starch. In the presence of amylase, a sample of starch will be hydrolyzed to shorter polysaccharides, dextrins, maltose, and glucose. The extent of the hydrolysis depends on how long it is allowed to react – if the starch is hydrolyzed completely, the resulting product is glucose.

You will test for the presence or absence of starch in the solutions using iodine (I_2). Iodine forms a blue to black complex with starch, but does not react with glucose. If iodine is added to a glucose solution, the only color seen is the red or yellow color of the iodine. Therefore, the faster the blue color of starch is lost, the faster the enzyme amylase is working. If the amylase is inactivated, it can no longer hydrolyze starch, so the blue color of the starch-iodine complex will persist.

You will also test for the presence of glucose in the samples using Benedict's reagent. When a blue solution of Benedict's reagent is added to a glucose solution, the color will change to green (at low glucose concentrations) or reddish-orange (at higher glucose concentrations). Starch will not react with Benedict's reagent, so the solution will remain blue.

Effect of Enzyme Concentration

During catalysis, the first step is the substrate (S) binding to the enzyme (E), giving an enzyme-substrate complex (ES). This is an equilibrium reaction, and will be favored by a high concentration of enzyme and/or substrate. After the substrate is bound, the reaction takes place, and then the product is released.

$$E + S \longrightarrow ES \longrightarrow E + P$$

Effect of Temperature

All reactions are faster at a higher temperature. However, enzyme-catalyzed reactions become slower or stop if the temperature becomes too high, because enzymes become denatured at high temperatures. Therefore, enzymes have an optimum temperature that corresponds to maximum activity. (At higher or lower temperatures, the activity of the enzyme is lower.) The optimum temperature is usually around body temperature (37°C).

Effect of pH

Each enzyme has an optimum pH. Above or below an enzyme's optimum pH, its activity is lower. The optimum pH of a particular enzyme corresponds to the pH of its natural environment. For many enzymes, this corresponds to pH values of around 7. For pepsin, which is active in the stomach, the optimum pH is 2 (the pH of the stomach).

Trypsin, which is active in the small intestine, has an optimum pH of 8 that matches the pH of the small intestine.

Effect of Inhibitors

Inhibitors are substances that slow down or stop enzymes. **Competitive inhibitors** are molecules that are very similar to the substrate, so they can bind to the enzyme but cannot react. They compete with the substrate for the active site of the enzyme.

Noncompetitive inhibitors are molecules that are not similar to the substrate and therefore do not bind to the active site. They do, however, bind to a different location on the enzyme and change the shape of the active site so that the substrate can no longer bind.

Irreversible inhibitors form covalent bonds to the enzyme and therefore cannot be removed.

Procedure

Important: read the entire procedure and make sure you understand it before you start this experiment. Before you start each part of the experiment, make sure you will have enough time to complete it. Advance planning is very important for this experiment.

Preparation:

Constant temperature water baths will be needed for this experiment. The laboratory has a large water bath that can be set to 37°C, and everyone will use this water bath. You will also need a low temperature and a high temperature water bath, and you can make your own. For the low temperature bath, use a 250-mL or 400-mL beaker, fill it about halfway with tap water, and add some ice to the water. The temperature of this bath when it comes to

constant temperature should be between 0 and 5°C. To make the high temperature water bath, fill a 250-mL or 400-mL beaker about two-thirds full and heat it until it boils. (You can heat it on a hotplate or on a Bunsen burner.) When it boils, you can reduce the heat, but keep it boiling. The temperature of this bath should be close to 100°C.

You will need some **1% starch solution** for each part of the experiment. Shake up the bottle of starch, and collect about 50 mL of the solution in a small beaker.

You will also need some of the **iodine reagent** for each part of the experiment. If dropper bottles of the iodine reagent are available, obtain one of them. If there are no dropper bottles, collect a small amount of this reagent (about 3-5 mL) in a small beaker, and get a clean dropper that you will only use for this iodine solution (to avoid contamination).

You can prepare your own fresh **amylase** solution by collecting about 1-2 mL of your own saliva in a small beaker. (You will need to spit politely into the beaker.) After you have collected 1-2 mL of saliva, add about 50 mL of water to the saliva and mix well. For health reasons, you should work with your own saliva solution (not someone else's). If a commercial amylase solution is available, you may use it instead of saliva. Collect about 40 mL of the solution in a small beaker.

You will also need a **white spot plate** and some **clean droppers**. It is important that you rinse a dropper well with deionized water before using it on a new solution to avoid contamination. If remnants of one solution on a dropper are accidentally transferred to a different solution, your tests will be inconclusive.

Reference tests:

Test for starch

Transfer a few drops of starch to one of the wells in the spot plate. Add one drop of the iodine reagent. Starch and iodine should react to give a deep blue-black complex.

Test for glucose

Place 3 mL of 1% glucose solution in a test tube. Add 2 mL of Benedict's solution and heat for 3-4 minutes in a boiling water bath. The reaction should produce a red-orange solid.

We will be using visual tests to evaluate the activity of the enzyme in each part of the experiment. You will collect samples of the enzyme-starch mixture at different times and add a drop of iodine reagent to each sample. The resulting color will tell you roughly how much starch has been hydrolyzed. When the enzyme activity is high, it won't take very much time to hydrolyze the starch. When the enzyme is slowed down or is inactive, the blue-black color will be seen for a longer time. You can assess the relative enzyme activity as follows:

Iodine test for starch	**Amount of starch remaining**	**Enzyme activity level**
Dark blue-black	All	None (0)
Blue	Most	Low (1)
Light brown	Some	Moderate (2)
Gold	None	High (3)

Part 1: Effect of Enzyme Concentration

1. Label five test tubes 1-5. Place 4 mL of 1 % starch in each of the first four test tubes. Place 4 mL of amylase solution in the fifth tube. Place all of the tubes in the 37°C water bath for 5

minutes. Obtain 5 clean droppers and label them 1-5. (To avoid contamination of these solutions, you will use a separate dropper for each mixture.)

2. Tube 1 will be your control and it will not contain any enzyme. Remove the tubes from the water bath momentarily, and quickly add 3 drops of the warmed amylase solution to tube 2, add 6 drops amylase to tube 3, and add 10 drops amylase to tube 4. Mix the tubes quickly by shaking them **gently**, and immediately put them back in the 37°C water bath. Record the time at which you added enzyme. Go on **immediately** to the next step.

3. Transfer four drops of each reaction mixture (tubes 1-4) using a separate clean dropper for each to separate wells on a spot plate. (Keep the test tubes in the water bath.) Add one drop of the iodine reagent to each. Record your observations. Use the visual color reference table to assess the enzyme activity. After you have recorded your observations, rinse off the spot plate.

4. Repeat step 3 after the mixtures have had five minutes of reaction time. Repeat step 3 after the mixtures have had ten minutes of reaction time.

5. In the test tubes where hydrolysis occurred, the presence of glucose can be confirmed with Benedict's reagent. Add 3 mL of Benedict's reagent to each test tube and put them in the boiling water bath for 3-4 minutes. The appearance of a green to orange or red precipitate indicates the presence of glucose. If the solution remains blue with no solid, then no glucose is present and no hydrolysis of the starch has taken place.

6. Make a graph of the enzyme activity vs. the amount of amylase solution using your results at 10 minutes.

Part 2: Effect of Temperature

7. Place 4 mL of 1 % starch solution in each of three clean test tubes. Place 4 mL of amylase solution in 3 separate clean test tubes (so you will have a total of six test tubes: 3 containing starch and 3 containing enzyme).

8. Place a starch tube and an amylase tube in the 37°C water bath. Place one tube of each in an ice-water bath, and one of each tube in a boiling water bath. Keep the tubes in their baths for 10 minutes to allow them to reach the temperature of their baths.

9. Read and record the temperature of the ice-water bath. Pour the amylase solution into the starch solution, mix, and put the tube back into the bath. Record the time.

10. Repeat step 10 for the 37°C bath. Repeat step 10 for the boiling water bath.

11. For each mixture, after 10 minutes of reaction have elapsed, transfer 4 drops of the mixture to a spot plate. Add 1 drop of iodine reagent to each sample. Record the color and activity level of the enzyme in each case. Graph the enzyme activity level vs. temperature.

Part 3: Effect of pH

12. Label four test tubes pH 2, 4, 7, and 10. In each tube, place 4 mL of the appropriate buffer (with the corresponding pH). Add 4 mL of amylase solution to each of the tubes. Get 4 additional clean test tubes, and put 4 mL of 1% starch solution in each tube. Place all 8 of these tubes in the 37°C water bath for about 5 minutes to allow the temperature to equilibrate. Rinse out four separate droppers.

13. Pour the contents of each starch test tube into a different amylase-buffer test tube. Mix them and return the tubes to the 37°C water bath.

14. After 15 minutes have elapsed, transfer 4 drops of each reaction mixture (using clean droppers) to a spot plate. Add 1 drop of iodine reagent to each. Record your observations and determine the enzyme activity level for each mixture. Rinse off the spot plate with deionized water.

15. Make a graph of the enzyme activity level vs. pH.

Part 4: Effect of Inhibitors

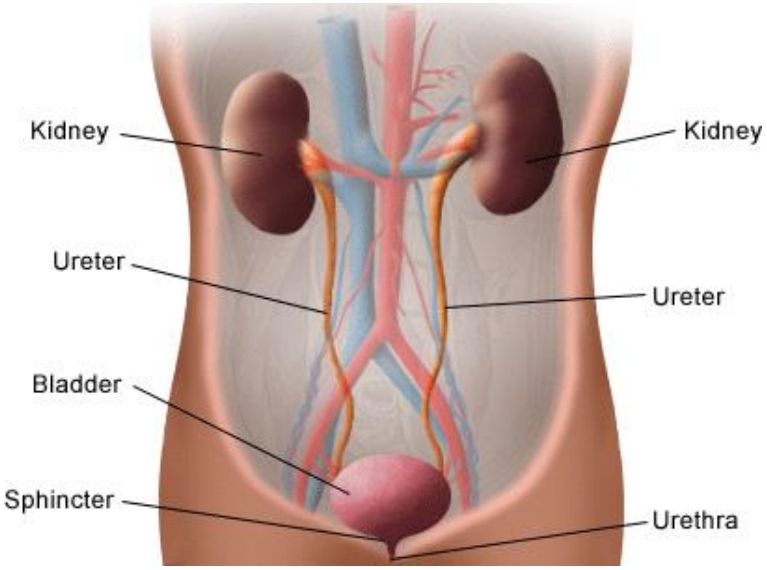
Place 4 mL of amylase solution in each of three test tubes. To the first tube, add 10 drops of 1 % NaCl solution. Add 10 drops of 95 % ethanol to the second test tube. Add 10 drops of either $AgNO_3$ or $Pb(NO_3)_2$ solution to the third test tube.

16. In each of three separate clean test tubes, place 4 mL of 1 % starch.

17. Place all six test tubes in the 37°C water bath for 5 minutes. When the 5 minutes is up, pour a tube of starch solution into each of the enzyme-inhibitor mixures. Mix each tube and return the tubes to the water bath for 15 minutes. Meanwhile, rinse out three droppers.After 15 minutes, transfer 4 drops of each mixture (using a clean dropper each time) to a spot plate. Add 1 drop of iodine reagent to each sample. Record your observations and the enzyme activity level in each case.

Urine analysis

"Urine" is a liquid produced by animals and humans through the kidneys, and is collected in the bladder and excreted through the urethra. Kidneys make urine by filtering out unwanted water, waste products, chemicals, sodium and potassium ions from the blood. Through a complex process, kidneys return an exact amount of sodium and potassium ions and some water to the blood stream so as to maintain a constant water and salt balance in the body.



Changes in the composition of urine occur very early in many "**diseases**", often before the patient is aware of any symptoms. For this reason and because urine can be obtained relatively easily, "**urine analysis**" was one of the first laboratory tests performed and related to diseases.

The **aim** of this practical session is to:

1. Obtain a simplified knowledge about routine urine analysis.
2. Perform simple chemical analyses on the provided urine sample.
3. Interpret results and comment on the case.

How can collection of urine samples:

The best sample for a routine urine analysis is a "**clean- catch**" or "**midstream**" sample collected after the external genitalia have been cleansed with an antiseptic solution. In this technique, the first portion of voided urine is discarded and the next portion is collected in a "**clean**" container (N.B. a sterile container is necessary if bacterial culture is requested). The container should be "**clearly labeled**" and urine must be analyzed soon after collection because most urine elements deteriorate at room temperature within one hour. A refrigerated specimen will retain its integrity only up to four hours.

Routine urine analysis

Urine analysis is a group of "**qualitative**" and "**semiquantitative**" analyses performed on urine samples. A routine urine analysis usually includes the following:

I. Physical examination
II. Chemical analysis
III. Microscopic examination

<u>I-Physical examination</u>:

Physical examination of urine includes observation of the **color**, **odor**,**turbidity**, and determination of **pH** and **specific gravity**.

Color

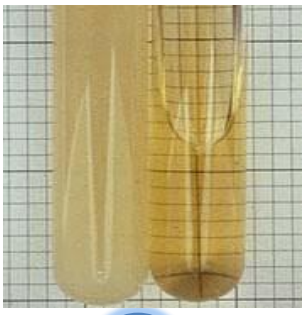
The normal colors of urine range from straw yellow to amber and are the result of normal metabolic end products such as **urochrome** and **urobilin**. Abnormal colors include red (due to presence of red blood cells or red pigments of beets), yellow-brown (due to presence of bilirubin), and orange (due to presence of some drugs); vitamin B complex can turn urine bright yellow.

Odor

The odor of urine is described as "**urinoid**". Diabetic ketoacidosis (in uncontrolled diabetes mellitus), starvation or a very low carbohydrate diet may cause the urine to have a sweet "**fruity**" odor due to the presence of acetone. Patients with urinary tract infections often have urine with a "**pungent**" odor.

Turbidity

Normal urine is clear. Turbidity can be caused by "**crystals**" or "**cells**" which separate after centrifugation of the urine sample. Crystals include amorphous urate, triple phosphate or calcium oxalate, and cells include pus cells or epithelial cells. Crystals and cells are detected by microscopicexamination of the sediment.



pH

Normally, freshly voided urine is acidic. Some foods (e.g. citrus fruits) and drugs (e.g. antacids) can affect urine pH. Alkaline pH may be noted in urinary tract infection due to alkaline fermentation by bacteria. In some cases, urinary pH is made acidic or alkaline by certain treatments to prevent the formation of certain types of kidney stones.

Specific Gravity:

Specific gravity measures urine density and is directly proportional to the amount of solutes present in urine. The normal urinary specific gravity ranges from 1.002 to 1.030. Decreased specific gravity occurs in case of diabetes insipidus and excessive fluid intake while increased specific gravity occurs in diabetes mellitus, severe vomiting or diarrhea. The specific gravity of urine is measured by a device called "**refractometer**" in which the extent of refraction of a beam of light depends on the concentration of solutes present in urine.

Refractometer

ll-Chemical analysis:

Different chemical tests are performed to detect **glucose**, **ketone bodies**, **protein**, **bilirubin** and **nitrite**.

Glucose: Glucose is normally absent or present in undetectable amounts in urine. When glucose level in blood exceeds the renal sugar threshold (160- 180 mg/dl) glucose starts to appear in urine. Presence of glucose in urine (glucosuria) is usually an indication of *Diabetes mellitus*.

Ketone bodies: When there is no adequate amount of carbohydrates in the diet, the body begins to utilize fatty acids to produce energy. When this increased metabolic pathway reaches a certain point, fatty acid utilization becomes incomplete and intermediary products occur in blood and urine. These products are the 3 ketone bodies: acetone, acetoacetate, and beta-hydroxybutyrate. Presence of ketone bodies in urine (ketonuria) is usually an indication of uncontrolled diabetes mellitus, starvation or a very low carbohydrate diet.

Proteins: Proteins are normally undetectable in urine. Presence of proteins in urine usually indicates kidney damage or glomerulonephritis.

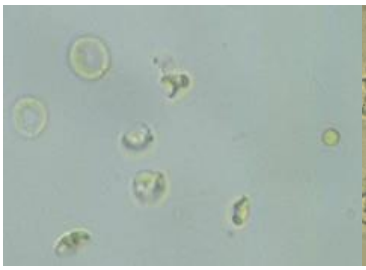
Bilirubin: Bilirubin is a yellow-brown breakdown product found in the bile. It results from normal heme catabolism. It is normally absent or present in undetectable amounts in urine. Most of the bilirubin produced is conjugated in the liver and excreted in the bile but a very small amount is reabsorbed from the intestine to the blood and is then excreted in urine. Elevated bilirubin in blood (and subsequent appearance in urine) indicates excessive bilirubin production due to increased destruction of RBCs (as in case of hemolytic anemia) or inability of the liver to adequately remove bilirubin dueto obstruction of bile ducts or a liver disease (e.g. liver cirrhosis or acute hepatitis).

Nitrite: In urinary tract infection (UTI), bacteria produce an enzyme that converts urinary nitrates to nitrites. Thus, the presence of nitrites in urine indicates UTI.

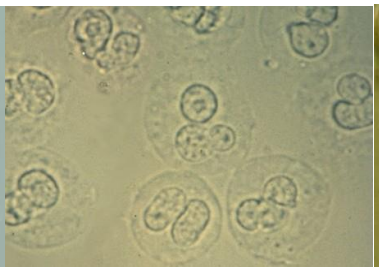
lll-Microscopic examination:

Microscopic examination of urinary sediment reveals very few or **no red or white blood cells or casts. No bacteria or parasites are** present and few crystals are usually normal. **"Red blood cells"** in urine may be caused by kidney stones or bladder tumor. "**White blood cells"** (pus) indicate a urinary tract infection.

"**Schistosoma eggs"** can indicate Bilharziasis. Excessive amounts of "**crystals**" or the presence of certain types of crystals can indicate kidney stones. Depending on the type, "**casts**" can indicate inflammation in the kidneys.

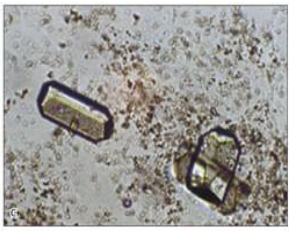


Red blood cells

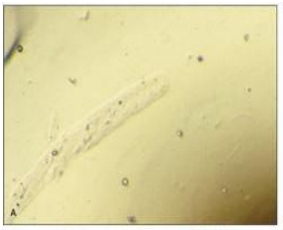


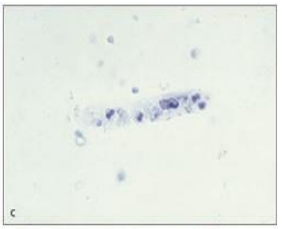
White blood cells

***Schistosoma hematobium* egg**



Urine crystals





Urine cast

Dipstick analysis

Dipstick analysis is an easy an convenient method for the detection of leukocytes, nitrite, protein, blood, ketone bodies, glucose and bilirubin in urine and for the determination of urine pH and specific gravity.

A "**dipstick**" is a paper strip with patches impregnated with chemicals that undergo a color change when certain constituents of urine are present in a certain concentration. The strip is dipped into the urine sample, and after the appropriate number of seconds, the color change is compared to a standard chart to determine urine constituents.

Practical

Using the provided urine sample, perform the following tests to detect **glucose**, **proteins**, **ketone bodies** and **bilirubin**:

Tests for glucose:

1. **Fehling's test:** in a test tube, add 1 ml Fehling A solution and 1 ml Fehling B solution to 2 ml urine sample. Mix well and put in a boiling water bath. A change in color indicates the presence of glucose.

2. **Benedict's test:** in a test tube, add 3 ml Benedict's reagent to 1 ml urine sample. Mix well and put in a boiling water bath. A change in color indicates the presence of glucose.

Test for ketone bodies:

Rothera's test: in a test tube, add 0.5 ml of saturated ammonium sulfate to 3 ml urine sample. Add 2-3 drops of ammonia solution and 2-3 drops 5% sodium nitroprusside and shake well. A permanganate-like color indicates the presence of ketone bodies.

Tests for proteins:

1. **Heat coagulation test:** in a test tube, put 2 ml urine sample. Incline the test tube and heat the surface. The formation of a coagulum indicates the presence of albumin.

2. **Heller's test:** in a test tube, add 2 ml of urine slowly to 2 ml of conc. HNO_3. A white coagulum is formed at the junction between the 2 layers indicating the presence of proteins.

Test for bilirubin:

Hay's test: The test depends on the surface activity of bilirubin as it lowers the surface tension of urine. Sprinkle a little of precipitated sulfur powder onthe surface of 2 ml urine. If bilirubin is present, sulfur powder will sink to the bottom of urine. If bile is absent, sulfur will remain on the surface of urine.

Laboratory exercise

Student Name: ……
Student number: ……

<u>Laboratory exercise</u>:

Using the provided urine sample N°(……..), perform the tests in the tablebelow illustrating your observations and results.

	Test	Observation	Result
Glucose	Fehling's test		
	Benedict's test		
Ketone bodies	Rothera's test		
Proteins	Heat coagulation test		
	Heller's test		
Bilirubin	Hay's test		

Chapter Two

Clinical Biochemistry

Principles of Instrumentation

Photometry: means "the measurement of light"

- If a substance can be converted to a soluble, colored material, its concentration may be determined by the amount of color present in the solution.
- Photometer & Spectrophotometer are instruments used for this type of measutment, in which a photocell or photomultiplier tube is used to detect the amount of light that passes through a colored solution from a light source.

Characteristics of Light

- Light is a form of electromagnetic energy that travels in waves.
- The wavelength of light is the distance between two beaks of the light wave, it is inversely proportional with its energy.
- Objects that appear colored absorb light at particular.

Table-1(wavelengths of various types of Radiation)

Types of radiation	Approximately wavelength
Gamma	< 0.1
X-rays	0.1-10
Ultraviolet	<380
Visible	380-750
Infrared	>750
radiowaves	$>25 \times 10^{7}$

Table –2 (the visible Spectrum)

Approximately wavelength	Color of absorbed light	Color of reflected light
400-435	Violet	Green–Yellow
435-500	Blue	Yellow
500-570	Green	Red
570-600	Yellow	Blue
600-630	Orange	Green blue
630-700	Red	Green

Spectrophotometer

Colorimetric determination of plasma/serum sugar level

In medicine, **blood sugar** is a term used to refer to the level of glucose in blood. Glucose, transported via the bloodstream, is the primary source of energy for the body cells. Blood sugar level (BSL), or serum glucose concentration, is tightly regulated in the human body so that its level remains within a certain limit (70 to 150 mg/dl) throughout the day.

The aim of this practical session is to:

1. Obtain a simplified knowledge about the regulation of BSL and itsclinical correlation to diabetes mellitus.
2. Recognize different methods used for the determination of BSL.
3. Determine the BSL in a serum sample of a fasting individual and comment on the case.

Regulation of blood sugar level

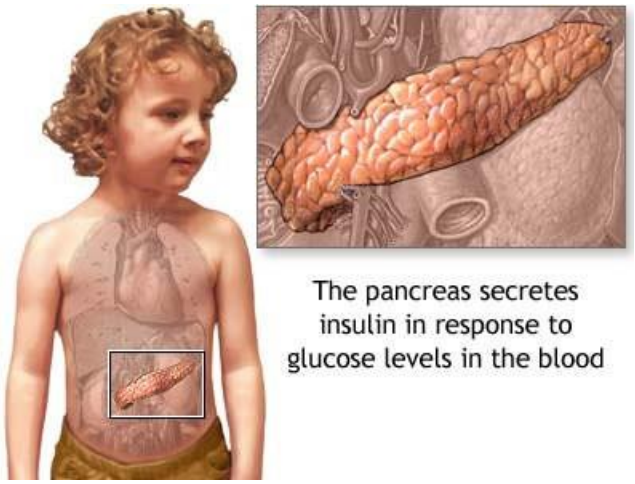
BSL is controlled by the following hormones:

1. **Insulin**: it is a polypeptide hormonesecreted from the beta cells of the islets of Langerhans in the pancreas; it lowers BSL causing hypoglycemia.

2. **Glucagon** (secreted from the alpha cells of the islets of Langerhans in the pancreas), **epinephrine (adrenaline)**, **corticosteroids** and **GH** raise BSL causing hyperglycemia.

Diabetes mellitus

The term "**diabetes**" is derived from a Greek word that means "excessive urine production", while the term "**mellitus**" is a Latin word that means a "sweet taste".



The pancreas secretes insulin in response to glucose levels in the blood

What's the *Diabetic mellitus*:

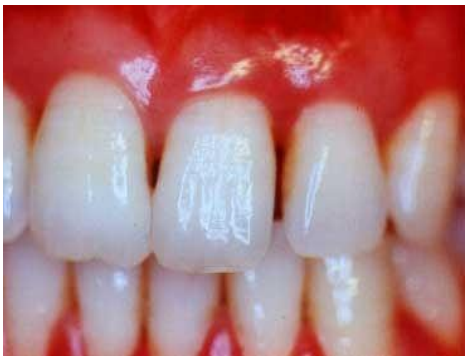
The name "*Diabetes mellitus*" comes from the Greek word , diabetes mean passage and mellitus mean honey sweet .the diabetic definition in medical term is any disorder accompanied by_a steady increased in the level of sugar level_far in excess of the normal value of the state of normal metabolism in the normal person. There are 2 primary types of diabetes:

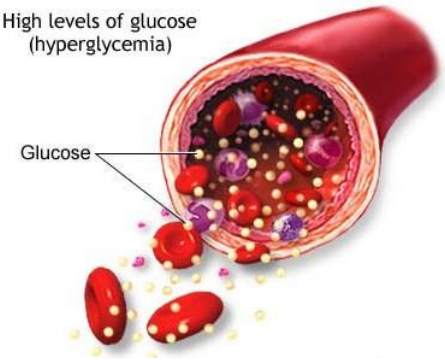
- Type 1 diabetes occurs when your immune system destroys the beta cells in the pancreas that create insulin. As a result, the body makes very little or no insulin of its own. People with type 1 diabetes must take insulin daily. Type 1 diabetes is sometimes called juvenile diabetes or insulin-dependent diabetes.
- Type 2 diabetes occurs when the pancreas does not make enough insulin, or the body cannot properly use the insulin it does create. Eventually, the pancreas may stop producing insulin altogether. Type 2 diabetes can affect people at any age. In both men and women, the more overweight an individual is, the greater the risk of developing type 2 diabetes.

Complications of *Diabetes mellitus*:

Diabetes mellitus can cause many **complications** that arise from the prolonged exposure of tissues to elevated glucose concentration. These include:

1. Renal failure.
2. Retinal damage.
3. Nerve damage.
4. Gangrene and amputation.
5. Concerning the field of **dentistry**, studies showed that patients with insufficient blood sugar control seem to develop gum disease more frequently and more severely than people who have good management of their diabetes, which is one of the leading causes oftooth loss among adults.





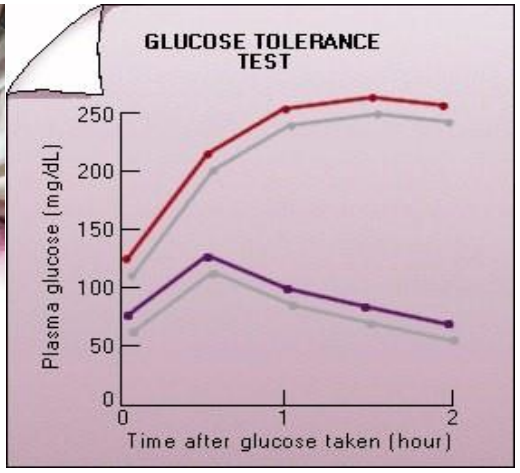
Diagnosis of *Diabetes mellitus*:

DM is diagnosed by demonstrating either of the following:

1. **Fasting BSL** at or above 126 mg/dl:

N.B. The normal fasting BSL is 70-110 mg/dl; therefore, values between 110 -126 mg/dl indicate impaired fasting BSL and prediabetes.

2. BSL at or above 200 mg/dl two hours after a standard oral glucose load in an **oral glucose tolerance test** (**OGTT**).



Fate of sugar in the body:

1- In the normal nutrition:

starch → higherdextrn → erythrodextrin → chrodextrine

higherdextrn → maltose

erythrodextrin → maltose

chrodextrine → maltose

maltose → maltose absorption in the mucous membrane of small intestine → glucose

1- converted to glycogen and stored in muscles and liver
2- converted to Co_2 and H_2O for liberation the energy
3- converted to Ketone bodies and amino acids and proteins
4- converted to fat and stored in adipose tissues.

2- In fasting case :

glycogen → glucose-6-P —(Glucose-6- phosphatase)→ glucose

Procedure:

After draw the blood and separation the serum or plasma , take 3 test tubes and marker as follow:

Addition	Blank	Standard	Sample
1-working solution	**1 ml**	**1 ml**	**1 ml**
2-standard	**---------**	**10 µl**	**-------**
3- serum or plasma	**----------**	**--------**	**10 µl**

And then mix and incubation the test tubes for 10 minutes at 37 ˚C and measure absorbance of sample (A sample) and standard (A standard) against reagent blank within 30 minutes.

Calculation:

$$\text{Glucose concentration (mg/dl)} = \frac{A_{sample}}{A_{standard}} \times 100$$

Expected values:

Adults (fasting)= 90-120 mg/dl

Children = 60-110 mg/dl

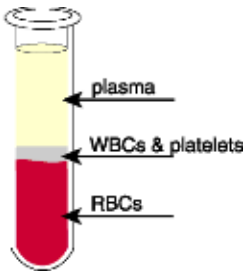
Newborns= 40-60 mg/dl

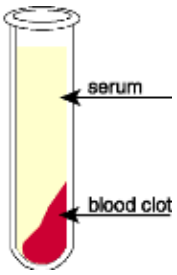
Colorimetric estimation of plasma/serum protein level

Blood, consists of 45% formed elements (cells) and 55% plasma. Plasma (the liquid portion of blood) consists mainly of water (about 90-92%), proteins, salts, oxygen, carbon dioxide, nutrients and waste.

"**Plasma proteins"** are of 3 major types: albumin, globulins and fibrinogen. They are all synthesized in the **liver**, with the exception of the gamma globulins which are produced by B- lymphocytes.

N.B. Plasma is obtained from blood in which an anticoagulant is added,while **serum** is obtained when no anticoagulant is added.





with anticoagulant **without anticoagulant**

Functions of plasma proteins:

1. Plasma proteins maintain blood osmotic pressure, pH and volume.
2. Albumin transports many substances in blood including hormones andsome drugs (plasma protein-bound drugs).
3. Gamma globulins (antibodies) fight infection.
4. Fibrinogen is necessary for blood clotting.

Normal value of plasma proteins: The normal value of plasma proteins in humans is **6 - 8 g/dl** (dl= 100 ml).

Clinical significance:

In some cases, the value of plasma proteins is lower than normal (below 6 g/dl); this is known as **hypoproteinemia** and can be caused by malnutrition, liver disease or severe burns. In other cases, the value of plasma proteins is higher than normal (above 8 g/dl); this is known as **hyperproteinemia** and can be caused by dehydration due to severe vomiting or diarrhea.

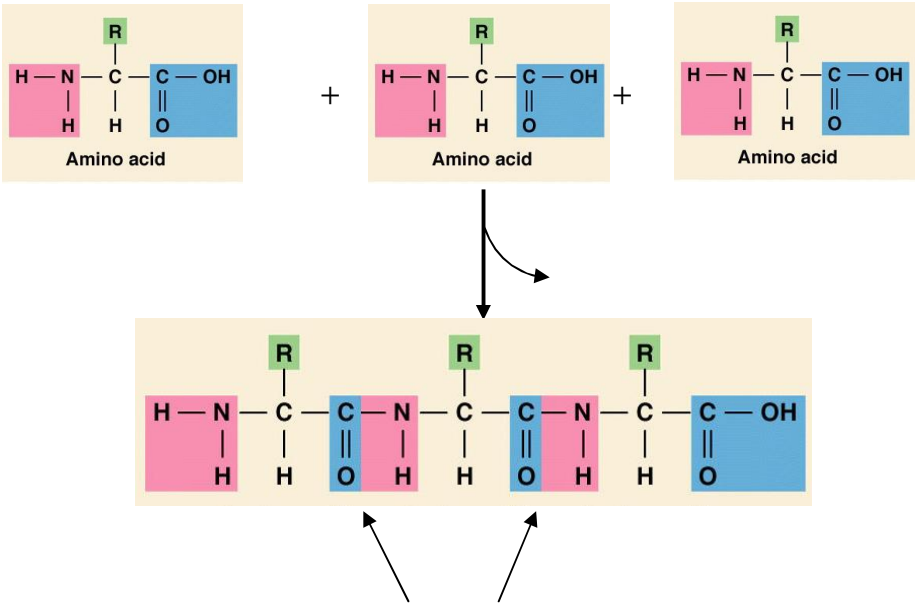
The **aim** of this practical session is to:

1. Estimate the concentration of proteins in a plasma sample using a colorimetric method, the "**Biuret method**".
2. Comment on the provided case.

Biuret method for colorimetricestimation of plasma proteins

Chemical structure of proteins:

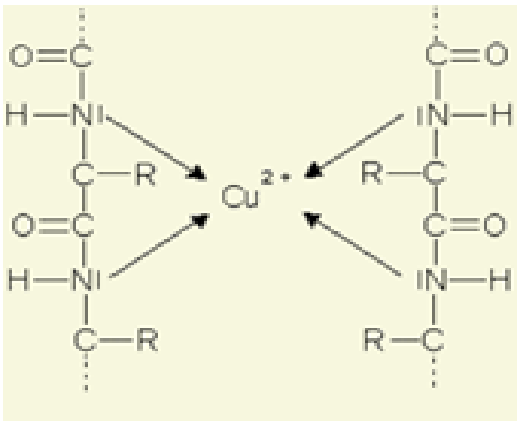
Proteins are polymeric compounds composed of "**amino acids**" joined together by "**peptide bonds**".



Peptide bonds

<u>Principle of the biuret method</u>:

- The biuret reaction is a method that can be used to determine the amount of protein in a solution.
- The biuret reagent (copper sulfate in a strong base) reacts with peptide bonds in plasma proteins to form a violet complexknown as the "**biuret complex**".
- **N.B.** Two peptide bonds at least are required for the formation of this complex.
- A **colorimeter** can be then used to measure the intensity of the color produced;
- the more protein present the darker thecolor.



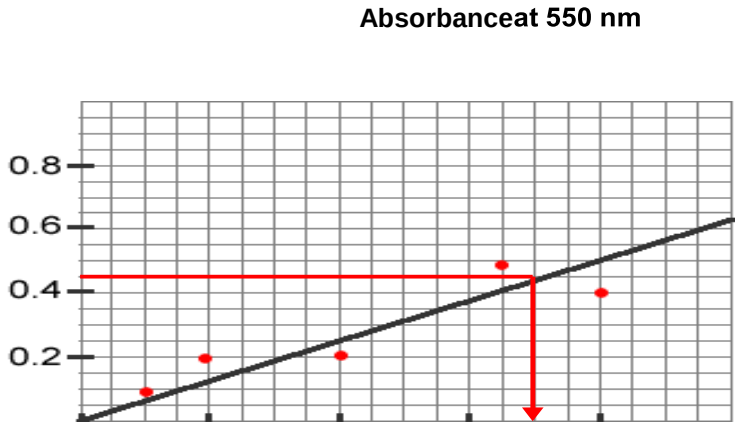
Biuret complex

- To estimate the concentration of plasma proteins, one of the two following methods can be used:

1. Performing the biuret reaction on a "**standard"** protein solution (i.e. of known concentration) and then applying the following equation:

$$C_{test} = C_{std} \times A_{test} / A_{std}$$

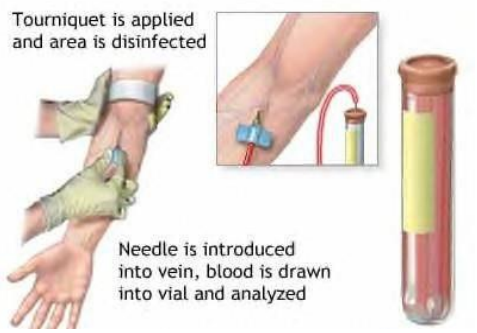
2. Performing the biuret reaction on a series of standard protein solutions and then constructing a "**standard curve**" by plotting the absorbance on the y-axis and the concentration on the x-axis. From this curve, the absorbance reading of any sample can be converted into its coresponding concentration.

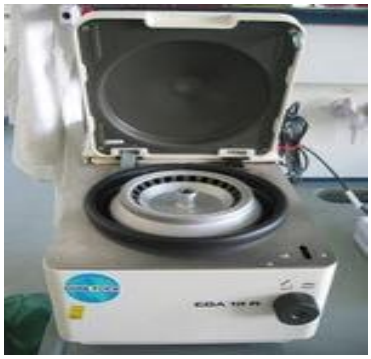


Practical:

1. Blood is drawn from a vein and transferred into a entrifuge tube containing an anticoagulant. In this case, blood will not clot and blood cells will settle to the bottom of the tube leaving plasma on the top.

2. Plasma is obtained by centrifugation of blood for 10 minutes.





• Determine the protein concentration in the provided plasma sample of patient **1, 2** or **3** using the **biuret method** as follows: In a clean dry test tube, add 0.5 ml of distilled water (blank) orplasma sample (test), then add 2 ml of biuret reagent.

	Blank	standard	Test
Distilled water	**0.5 ml**	**----**	—
standard	—	**0.5 ml**	**0.5 ml**
Biuret reagent	**2 ml**	**2 ml**	**2 ml**

Blue

violet

- Mix the content of each tube.
- Allow to stand for 15 minutes.
- Read the absorbance at 550 nm.
- Construct a "**standard curv**e" for plasma proteins using the values in the table below showing the absorbance reading of protein solutions of different concentrations.

Concentration (g/dl)	**Absorbance** (at 550 nm)
0	**0.00**
1	**0.07**
2	**0.14**
4	**0.28**
7	**0.49**
10	**0.70**
12	**0.84**

- Determine the concentration of plasma proteins (g/dl) in theprovided sample.
- **Comment** on the provided case.

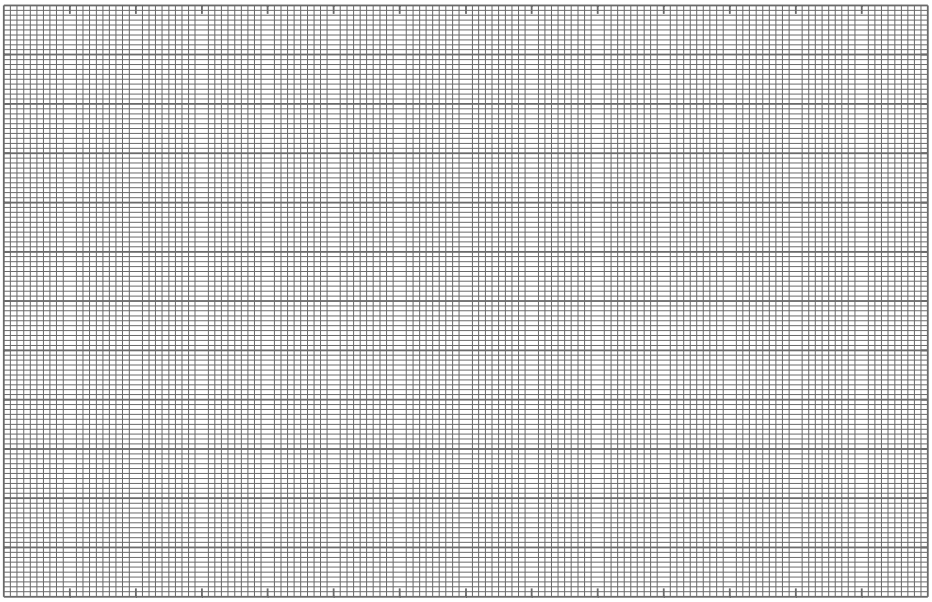
Laboratory exercise

Student Name: ……
Student number: ……

Laboratory exercise:

1. Determine the concentration of proteins in the provided plasma sample using the biuret method.

Results: Patient number ……..
A_{test} = …….
C_{test} = ……..



2. Write your **comment** on the case:

……

Colorimetric estimation of plasma/serum albumin level

Intended Use:

Spectrum Diagnostics albumin reagent is intended for the in- vitro quantitative, diagnostic determination of albumin in human serum on both automated and manual systems.

Background:

Albumin is the major serum protein in normal individuals. It maintains the plasma colloidal osmotic pressure, binds and solubilizes many compounds such as calcium and bilirubin. Elevated serum albumin levels are usually the result of dehydration. Hyperalbuminemia is of little diagnostic significance. Hypoalbuminemia is very common in many diseases including malabsorption, liver diseases. kidney diseases, severe burns, infections, cancer and some genetic abnormalities. In severe hypoalbuminemia (less than 2.5 g/dL), the low plasma oncotic pressure allows water to move out of the blood capillaries into the tissues causing edema.

Method:

Modified bromocresol green colorimetric method:

Assay Principle: Measurement of albumin is based on its binding to the indicator dye bromocresol green (BCG) in pH 4.3 to form a blue-green colored complex. The intensity of the blue-green color is directly

proportional to the concentration of albumin in the sample. It is determined by monitoring the increase in absorbance at 623 nm, or 578 nm.

Albumin + BCG $\xrightarrow{pH\ 4.3}$ Albumin-BCG Complex

Procedure:

	Blank	Standard	specimen
Reagent (R)	**2.5 ml**	**2.5 ml**	**2.5 ml**
Standard	**---------**	**10 µl**	**-------**
Serum/plasma	**-------**		**10 µl**

Mix , incubate for approximately 5 minutes at 20-25 ^{o}C. Measure absorbance of specimen ($A_{specimen}$) and standard ($A_{standard}$) against reagent blank within 60 minutes.

Calculation:

$$\text{Albumin concentration (g/dl)} = \frac{A_{sample}}{A_{standard}} \times 4$$

Expected Values

Adults

18 - 60 y	3.5 - 5.5 g/dL	(35 - 50 g/L)
>60 y	3.4 - 4.8 g/dL	(34 - 48 g/L)

Childern

14-18 y	3.2-4.5 g/dL	(32-45 g/L)
4d-14 y	3.8-5.4 g/dL	(38-54 g/L)

Newborns

0-4 day	2.8-4.4 g/dL	(28-44 g/L)

Colorimetric estimation of plasma/serum total cholesterol level

Cholesterol is a lipid sterol that is produced in and transported throughout the bloodstream in eukaryotes. Cholesterol is a critical compound used in the structure of cell membranes, hormones, and cell signaling. It is an essential component of animal cell structure in order to maintain permeability and fluidity. Cholesterol is a precursor for steroid hormones including the adrenal gland hormones cortisol and aldosterone, sex hormones progesterone, estrogens, and testosterone, and bile acids and vitamin D. Cholesterol is transported throughout the body within lipoproteins, which have cell-specific signals that direct the lipids they transport to certain tissues. For this reason, lipoproteins exist in different forms within the blood based on their density. These include chylomicrons, very-low density lipoproteins (VLDLs), low-density lipoproteins (LDLs), intermediate- density lipoproteins (IDLs), and high-density lipoproteins (HDLs). The higher the lipid content within a lipoprotein, the lower its density.

Cholesterol exists within a lipoprotein as a free alcohol and as a fatty cholesteryl ester, which is the predominant form of cholesterol transport and storage. High levels of cholesterol and cholesteryl esters (hypercholesterolemia) have been associated with cardiovascular disease such as atherosclerosis and heart disease, although lower levels

(hypocholesterolemia) may be associated with cancer, depression, or respiratory diseases.

Cell Biolabs' Total Cholesterol Assay Kit is a simple colorimetric assay that measures the amount of total cholesterol present in plasma, serum, tissue homogenates, or cell lysates in a 96-well microtiter plate format. The assay will detect total cholesterol (cholesteryl esters plus free cholesterol) in the presence of cholesterol esterase or only free cholesterol in the absence of the esterase enzyme. Each kit provides sufficient reagents to perform up to 192 assays, including blanks, cholesterol standards and unknown samples. Sample cholesterol concentrations are determined by comparison with a known cholesterol standard.

Cholesteryl esters can be quantified by subtracting the free cholesterol values from the total cholesterol value. Colorimetric measurement procedures are less costly but are subject to interfering substances and may require extraction steps and strong acids

Classical method = *Liebermann-Burchard*:

Involved extraction & hydrolysis. Uses sulfuric & acetic acids. Results in formation of a green color, proportional to the cholesterol concentration.

Three step process using a coupled reaction with cholesterol oxidase.

$$\text{Cholesterol} \xrightarrow{\text{Cholesterol-Esterase}} \text{Free cholesterol + Fatty Acid}$$

$$\text{Free cholesterol} \xrightarrow{\text{Cholesterol Oxidase}} H_2O_2$$

$$H_2O_2 + \text{Chromogen} \xrightarrow[\text{Peroxidase}]{\text{Horseradish}} \text{Colored Chromogen}$$

Factors affecting on cholesterol levels:

- Anything that affects HDL & LDL levels will affect cholesterol concentration-because these lipoprotein contain increased cholesterol
- Thyroxine level inversely affects cholesterol level
- hypothyroid associated with hyper cholesterol
- hyper thyroid associated with hypo cholesterol
- Estrogens
- documented that post-menopausal women have increased LDL cholesterol
- Pregnancy
- altered endocrine function resulting in increased cholesterol
- Others include hepatitis, nephrotic syndrome, emotional stress, and *Diabetes mellitus*
- Cholesterol level varies with age, sex, & diet

mg/dL< 200

Calculation:

$$\text{Cholesterol concentration (mg/dl)} = \frac{A_{\text{sample}}}{A_{\text{standard}}} \times n$$

n= 200 mg/dl of blood =concentration of standard

Colorimetric estimation of plasma/serum triglyceride level

Triglycerides (TGs): are essential fats (also called "lipids") transported in our bloodstream with cholesterol. They are called triglycerides because each molecule contains three fatty acids. TGs are the major source of energy used and stored by our bodies. They come from two sources—what we eat and what our liver makes. High blood TG levels can be genetic, or causedby diabetes, thyroid problems, kidney disease, or some medicines. Triglycerides (TG) are the main constituent of vegetable oil, animal fat, LDL and VLDL, and play an important role as transporters of fatty acids as well as serving as an energy source. TG are broken down into fatty acids and glycerol, after which both can serve as substrates for energy producing and metabolic pathways. High blood levels of TG are implicated in atherosclerosis, heart disease and stroke as well as in pancreatitis.

Principle: The concentration of serum triglyceride was measured by using a special chemical kit based on:

$TG + H_2O_2 \longrightarrow$ glycerol + fatty acids

Glycerol + ATP $\longrightarrow$ glycerol-3-phosphate + ADP

Glycerol-3-phosphate + $O_2 \longrightarrow$ Dihydroxy acetone + H_2O_2

$2H_2O_2$ + 4 aminoantipyrine + ADPS $\longrightarrow$ red quinon imine + $4H_2O$

Procedure of measurement:

The procedure of this kit is as:

Solution	Blank	Standard	Sample
Reagent	1 ml	1 ml	1ml
Standard	------	10µl	------
Serum	------	-------	10µl

After adding, mix and incubate the test tubes for 5 min. at 20°C or 10 min. at 37°C. Then read the optical density (OD) of standards and samples against blank at 520 nm. Calculated the serum TG concentration by the following formula:

$$\textbf{TG concentration} = \frac{A\ \text{sample}}{A\ \text{standard}} \times n = \textbf{mg/dl of blood}$$

n = **standard concentration= 200 mg/dl**

Colorimetric estimation of plasma/serum High-Density Lipoprotein Cholesterol level (HDL-C)

The principle role of HDL in the lipid metabolism is the uptake and transport of cholesterol from peripheral tissues to the liver through a process known as reveres cholesterol transport.

The principle:

This reagent is only used for treatment of specimens before the determination of HDL-C with a reagent for total cholesterol. Low density lipoprotein (LDL), very low density lipoprotein (VLDL) and chylomicrons from specimens are precipitated by phosphotungstic acid (PTA) and magnesium chloride. HDL-C obtained in the supernatant after centrifugation is then measured with a total cholesterol reagent

Procedure: Do not treat standard (vial R_2) enclosed in kit:

Solution	Macro-method	Micro-method
Specimen	**1 ml**	**0.5 ml**
Precipitant	**100 µl**	**50 µl**

Mix vigorously, let stand for 10 min. at room temperature and centrifuge for 15 min. at 3500-4000 RPM. with BIOLAB total cholesterol CHOD-PAP or equivalent: Let stand reagents and

supernatants at room temperature. Calibrate with standard enclosed in the kit or pre- treated series calibrator.

Solution	Blank	Standard	Sample
Reagent	1 ml	1 ml	1ml
Standard	------	25µl	------
Serum	------	-------	25µl
Distal water	25µl	------	-------

Mix well the test tubes and let stand for 5 min. at 37°C or 10 min. at room temperature. Record absorbance at 500 nm. (480-520 nm) against the reagent blank.

HDL concentration = $\frac{A_{sample}}{A_{standard}}$ X Standard concentration X1.1

1.1= standard remaining undiluted, 1.1 factor takes into account dilution of the specimen during the precipitation step.

Serum Low-Density Lipoprotein (LDL):

Serum LDL concentration can be calculated by the following equation: **LDL= TC- (HDL+TG/5)**

Serum Very Low-Density Lipoprotein (VLDL):

Serum VLDL concentration was calculated by dividing serum TG/5. **VLDL=TG/5**

Colorimetric estimation of plasma/serum urea level

Urea is the major end product of protein nitrogen metabolism. It is synthesized by the urea cycle in the liver and excreted through the kidneys. The circulating levels of urea depend upon protein intake, protein catabolism and kidney function. Elevated urea levels can occur due to renal impairment or in some diseases such as diabetes, infection, congestive heart failure and during different liver diseases. Determination of blood urea nitrogen is the most widely used screening test for renal function together with serum creatinine.

The concentration of urea in the blood serum represent mainly a balance between urea formation from protein catabolism and urea excretion by kidney. If kidney fail , blood urea Conc. Increase to high level and toxic condition known as (Uremia) will result.

In uremia, urea must be removed from the blood by clinical procedure called “Blood Dialysis “

Blood Urea Nitrogen (BUN)

—Sometime used as measurement of serum urea ½ Mwt of Urea is Nitrogen.

Normal range:

Serum urea (10-50) mg/dl

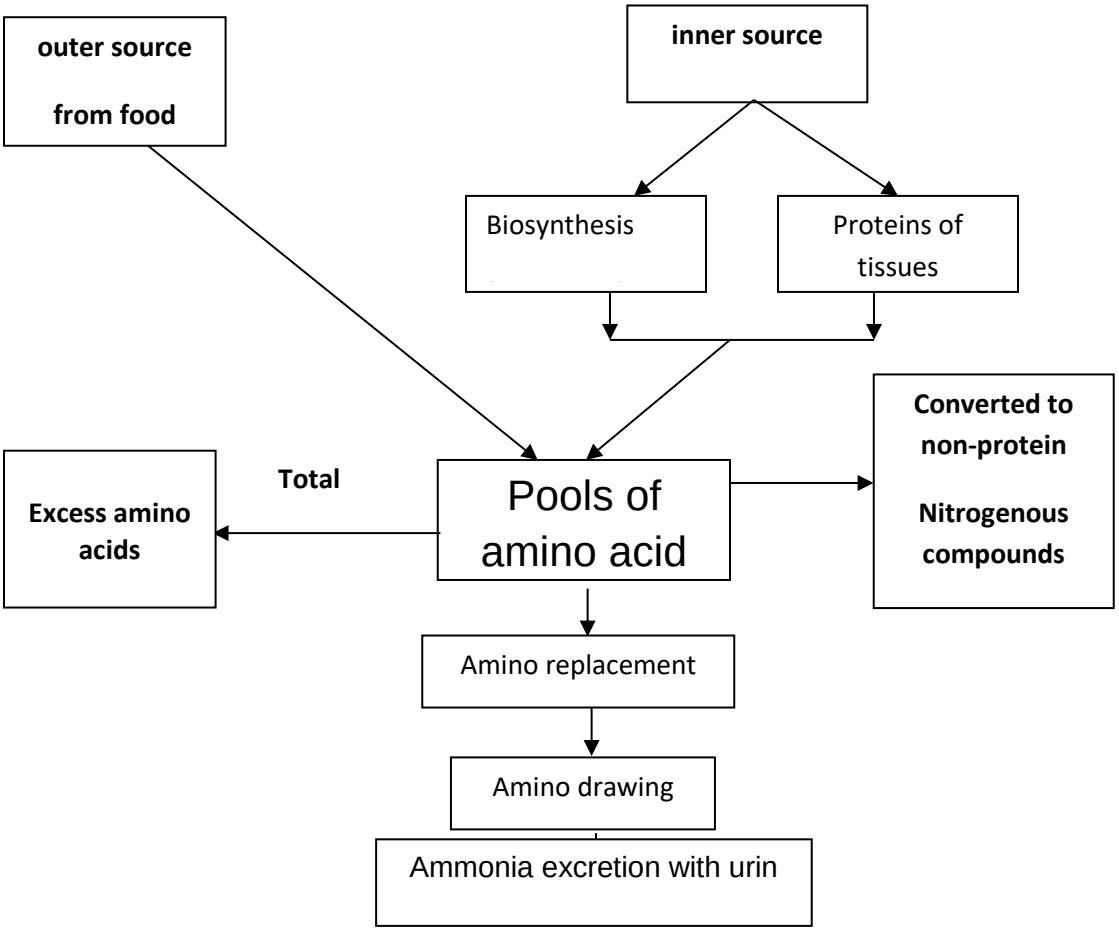
BUN (5- 25) mg/dl

Adults < 65 years = 15-50 mg/dl

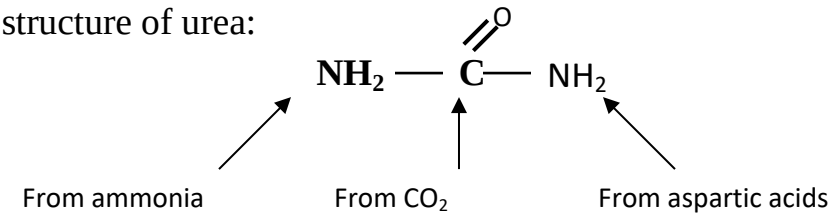
Adults > 65 years= < 70
mg/dlSerum urea normally varies depending on:

- Age (due to change kidney function)
- Sex (conc. are slightly higher in men)
- Diet (protein diet Urea)

Fate of protein inside the body:



Chemical structure of urea:



The urea synthesis in the liver from arginine (a.a.) by assessment of arginase enzyme . When the urea can't synthesis for any causes due to increase the level of ammonia in the body which is toxic compound and cause death.

The urea increase in the kidney diseases like:
1- chronic nephritis 2-Glomerular nephritis
3- Nephrosclerosis by close the urinary tract as a result of the presence of stone.
4- Prostate enlargement or swelling of the bladder and ureters.

Procedure:

After draw the blood and separation the serum or plasma , take 3 test tubes and marker as follow:

Procedure: Prefer as followed:

`Solution	Blank	Standard	Sample
Working Solution **Standard** **Sample (serum)**	1 ml ------- ------	1ml 10µl --------	1ml ------- 10µl
Mix, incubate the test tubes for at least 3min. at 37 °C or 5 min. at 20-25 °C			
Alkaline Reagent (R_3)	20µl	20µl	20µl
Mix and incubate for 5 min. at 37°Cor 10 min. at 20 to 25°C. Measure absorbance of specimen and standard against reagent blank within 30 minutes on wavelength 578nm.			

Calculation:

Serum Urea Concentration = $\frac{A_{specimen}}{A_{standard}}$ **X n = mg/dl of blood**

*n= 50 mg/dl of blood = concentration of standard

Expected values:

Adults < 65 years = 15-50 mg/dl

Adults > 65 years= < 70 mg/dl

Colorimetric estimation of plasma/serum amylase level

Amylase is an enzyme that helps digest carbohydrates. It is produced in the pancreas and the glands that make saliva. When the pancreas is diseased or inflamed, amylase releases into the blood. A test can be done to measure the level of this enzyme in a blood.

Amylase in serum arise mainly from the pancreas (P-amylase) and the salivary gland (S-amylase). Serum P- amylase activity is a more sensitive and more specific test than total amylase for the detection of acute pancreatitis.

Why the Test is Performed

• This test is most often used to diagnose or monitor acute pancreatitis. It may also detect some digestive tract problems.

The test may be done for

• Chronic pancreatitis

• Pancreatic pseudocyst

Chronic pancreatitis: Chronic pancreatitis is inflammation of the pancreas that does not heal or improve, gets worse over time, and leads to permanent damage.

Pancreatic pseudocyst: A pancreatic pseudocyst is a fluid-filled sac in the abdomen, which may also contain tissue from the pancreas, pancreatic enzymes, and blood.

Range of expected values:

Serum : 16-108 U/L

Urine: 0 - 14 U/Hour

low values in Serum is may due liver diseases and pancreatic insufficiency

Principle:

Amylase hydrolyzed p-nitrophenyl D-maltoheptoside (PNPG7) to P-nitrophenylmaltotriose (PNPG3) and maltotetrose . Glucoamylase hydrolyzes PNG3 to P-nitrophenylglycosie (PNPG1) and glucose. Then PNPG1 is hydrolyzed by glycosidase to glucose and P-nitrophenol which produce a yellow color. The rate of increase in Ab is measured at 405nm and is proportional to the amylase activity in the sample.

Materials:

Glassware:

1. Accurate pipetting devices.
2. Test tubes / rack
3. Timing device.
4. Heating block /bath (37 oC).
5. Spectrophotometer capable of reading at 405 nm (400-420 nm).

The cuvette compartment should be temperature controlled to maintain temperature (37 oC) during the assay.

Method:

Chemicals: SAMPLE 1

Amylase substrate (PNPG7):

1.0 ml Pre-warm at 37oC for 5 minutes and add:

Sample1 0.025 ml

Continue readings every 30 seconds for 2 minutes and determine ΔA/Min. Mix and incubate at 37oC for 90 seconds and read the absorbance at 405 nm against distilled water.

Results:

Absorbance AT 405

A 0 S
A 30 S
A 60 S
A 90 S
A 120 S

Calculations:

Amylase Activity in TEST (U/L) = ΔA/Min x 4824

ΔA/Min = (ΔA1+ ΔA2)÷2

ΔA1= (A 60 s – A30 s)+(A30 s-A0 s)

ΔA2= (A 120 s – A90 s)+(A90 s-A60 s)

Colorimetric estimation of plasma/serum phosphorus level

WHAT IS A SERUM PHOSPHOROUS TEST?

- Phosphorus is an important part of several of your body's processes. It helps with bone growth, energy storage, and nerve and muscle production. Many foods, especially meats and dairy products, contain phosphorus, so it's usually easy to get enough of this mineral in your diet.

- Most of your body's phosphorus is contained in your bones and teeth. However, some is in your blood. Your doctor can assess your blood phosphorus levels using a serum phosphorus test.

Hyperphosphatemia is when you have too much phosphorus in your blood. **Hypophosphatemia** is the opposite: having too little phosphorus. Various conditions, including liver disease and vitamin D deficiency, can cause your blood phosphorus level to become too high or too low.

A serum phosphorus test can be used to determine whether you have high or low phosphorus levels, but it cannot help your doctor diagnose the cause of your condition. The doctor will need to perform more tests to determine what is causing your abnormal serum phosphorus test results.

Clinical significance:

• Low Levels

Low phosphorus levels may be caused by a range of nutritional problems and medical conditions, including:

- lack of vitamin D
- not getting enough phosphorus in your diet
- malnutrition
- alcoholism
- hypercalcemia (high serum calcium levels)

High Levels

If your kidney function is impaired, excess phosphorus will likely build up in your bloodstream. Avoiding high-phosphorus foods, such as milk, nuts, beans, and liver can help you lower your phosphorus levels.

In other cases, high phosphorus levels may be caused by:

- certain medications, such as laxatives that contain phosphates
- dietary problems, such as consuming too much phosphate and/or vitamin D
- diabetic ketoacidosis (when your body runs out of insulin and begins to burn fatty acids instead)
- hypocalcemia (low serum calcium levels)
- hypoparathyroidism (impaired parathyroid glands, leading to low levels of parathyroid hormone)
- hyperparathyroidism (overactive parathyroid glands, leading to high levels of parathyroid hormone)
- severe burns

ESTIMATION OF PHOSPHORUS

Specimen
Serum.
Plasma must not be used. Anticoagulants may cause false low results.
Stability in serum: 7 days at +4°C
2 days at 20...25°C

Assay
Wavelength: 340 nm, Hg 334 nm
Optical path: 1 cm
Temperature: 20...25°C
Measurement: against reagent blank; one reagent blank per series is required

Pipetting Scheme

Pipette into cuvettes	Reagent blank	Sample or STD
Sample/STD	---	10 µl
RGT	1000 µl	1000 µl
Mix, incubate at least 1 minute at room temperature. Measure the absorbance of the sample and the STD against the reagent blank within 60 minutes (ΔA).		

Calculation of phosphorus concentration

$$C = 10 \times \frac{\Delta A_{Sample}}{\Delta A_{STD}} \quad (mg/dl)$$

or

$$C = 3.2 \times \frac{\Delta A_{Sample}}{\Delta A_{STD}} \quad (mmol/l)$$

Normal Values [3]
Inorganic phosphorus

Adults:	2.5-5.0 mg/dl	0.81-1.62 mmol/l
Children:	4.0-7.0 mg/dl	1.30-2.26 mmol/l

Colorimetric estimation of plasma/serum bilirubin level (Total & Direct)

To estimate the amount of bilirubin in serum.

➢ Bilirubin

It is a by-product of the breakdown of hemoglobin.

➢ Types of Bilirubin

- **Direct bilirubin**: Conjugated with glucoronic acid
- **Indirect bilirubin**: unconjugated, insoluble in water
- **Total bilirubin**: sum of the direct and indirect of bilirubin.

Notes: About 200 mg per day of unconjugated bilirubin are transported to the liver

Above about 2 mg/dl in the blood, leads to disease called Jaundice.

Bilirubin and jaundice

➢ **Jaundice** is caused by a build-up of **bilirubin (yellow color)** in the blood and tissues of the body.

➢ Jaundice is the discoloration of skin and sclera of the eye caused by high concentration of bilirubin.

➢ **The causes of jaundice may be classified as: Types of jaundice**

Pre-Hepatic Jaundice : Hemolytic disease

Hepatic Jaundice :-Cirrhosis of the liver, -Infective Hepatitis and-Neonatal Jaundice

Post-Hepatic Jaundice :Cholecystitis and 1-Pre-Hepatic Jaundice

Hemolytic disease (excess hemolysis)

- The production of un-conjugated bilirubin may exceed the conjugating capacity of the liver and hence the **serum levels of**

indirect (and of total)
bilirubin will be raised and that of direct in the upper normal range or just a little elevated.

- briefly ,Indirect bilirubin ---------- increased

Direct bilirubin---------- little increased

Total bilirubin ----------↑ increased excess hemolysis

Unconjugated bilirubin (in blood) upper normal range conjugated bilirubin (released to bile duct)

2-Hepatic Jaundice

Cirrhosis (in the absence of infection)

- Destruction of liver cells will lead to a reduced conjugating capacity with a:
  - Raised serum level of indirect (and of total) bilirubin,
  - with a low level of direct bilirubin

2-Hepatic Jaundice

Hepatitis (in the presense of infection)

- The conjugative capacity of the liver is approximately normal, but there is the inability to transport the conjugated bilirubin from the liver cells to the biliary system, and it will be regurgitated back into the blood.
- → **Hence:**
- The serum level of **unconjugated bilirubin** will be **normal**
- and that of conjugated (and total) bilirubin will be raised.

Neonatal Jaundice

- Conjugating enzymes in the liver are often absent at birth.
- →**Hence:**

- Raised serum level of indirect (and total) bilirubin is to be expected
- Low level of direct bilirubin.
  - The indirect bilirubin level will rise for the first few days after birth until the conjugating enzymes begin to synthesize.
  - If the conjugation process is delayed and the serum level of indirect bilirubin rises towards 20 mg/dl, an ultraviolet therapy or an exchange blood transfusion should be carried out owing to the danger of deposition of the insoluble unconjugated bilirubin in the basal ganglia of the brain leading permanent Brain Damage.

2-Hepatic Jaundice

3-Post-Hepatic Jaundice

Cholecystitis The bile duct is blocked.

- →**Hence:** The indirect bilirubin level is normal but conjugated bilirubin is regurgitated into the blood and excreted into the urine (raised conjugated and total bilirubin).
  - Bilirubin in serum is coupled with **diazotized sulphanilic acid** to form azobilirubin .
  - The intensity of the purple color that is formed is proptional to the bilirubin concentration in the serum.
  - The water soluble **conjugated bilirubin (direct bilirubin)** reacts easily with reagents such as diazotized sulphanilic acid. (with one minute)
  - while **the water insoluble unconjugated bilirubin (indirect bilirubin)** requires **methanol**, in order to accelerates the react with the diazotized sulphanilic acid.
  - In this experiment, the direct bilirubin is estimated in the absence of the solubilizing agent and then further bilirubin estimation in the presence of methanole will give the total bilirubin level.
  - The indirect or unconjugated bilirubin is then found by difference.

Method:

Label 4 tubes as TT (total test), TB (total Blank), DT (direct test), DB (direct Blank).

Total Bilirubin		Direct Bilirubin		
Test	**Test Blank**	**Test**	**Test Blank**	
0.5ml	**0.5 ml**	**1.0 ml**	**1.0 ml**	**Sulfanilic Acid Reagent**
0.02ml	**--**	**0.02 ml**	**--**	**Sodium Nitrite Reagent**
Mix and let stand for at least 1 min but no longer than 3min then add:				
0.05 ml	**0.05 ml**	**0.05 ml**	**0.05 ml**	**Sample**
After exactly 1 min . Read the absorbance of test and Test and Test Blank (of Direct bilirubin only) at 546 nm against distalled water . For Total Bilirubin add:				
0.5 ml	**0.5 ml**	**--**	**--**	**Methanol**
Mix and let stand for 5 min at room temperature and read the absorbance of test and test blank (of Total Bilirubin) at 546 nm against distilled water				

Calculation :

- **Concentration of direct bilirubin** = (abs. Test - abs. Test Blank) X 25m= mg /dl

- **Normal range:** Up to 0.5 mg/dl

- **Concentration of total bilirubin** = (abs. Test - abs. Test Blank)X 25 = mg /dl

- **Normal range:** Up to 1 mg/dl

- **Concentration of indirect bilirubin** = Conc of total bilirubin – Conc of direct bilirubin= mg /dl

- **Normal range:** 0.1-0.4 mg/dl

Kidney Function Tests

Objectives: Upon completion of lectures, students should be able to:

1. know the physiological functions of the kidney.
2. describe the structure and function of the nephron.
3. identify the biochemical kidney function tests with special emphasis on when to ask for the test, the indications and limitations of each kidney function tests.
4. interpret the kidney function tests properly.

Contents:

- **Functional units**
- **Kidney functions**
- **Routine kidney function tests (KFTs):**
  - **Serum creatinine**
  - **Creatinine clearance**
  - **Cockcroft-Gault formula for GFR estimation**
  - **Serum Urea**

Functional units :

- The nephron is the functional unit of the kidney
- Each kidney contains about 1,000,000 to 1,300,000 nephrons.
- The nephron is composed of glomerulus and renal tubules.
- The nephron performs its homeostatic function by ultra filtration at glomerulus and secretion and reabsorption at renal tubules.

Representation of a nephron and its blood supply:

Regulation of : - water and electrolyte balance.

- acid base balance. - arterial blood pressure.

- **Excretion of metabolic waste products and foreign chemicals.**
- **Hormonal Function: Secretion of erythropoietin & activation of vitamin D and activation of angiotensinogen by renin**
- **Metabolic Function: site for gluconeogenesis**

Kidney functions:

- Many diseases affect renal function.
- In some, several functions are affected.
- In others, there is selective impairment of glomerular function or one or more of tubular functions.
- Most types of renal diseases cause destruction of complete nephron.

Why to test the renal functions?

Routine KFTs include the measurement of :

- Serum creatinine (Cr).
- Creatinine clearance.
- Serum urea.

Both serum Cr and creatinine clearance are used as kidney function tests to :

- Confirm the diagnosis of renal disease.
- Give an idea about the severity of the disease.
- Follow up the treatment.

Serum creatinine (55-120 μmol/L in adult):

- Creatinine is the end product of creatine catabolism.
- 98% of the body creatine is present in the muscles where it functions as store of high energy in the form of creatine phosphate.
- About 1-2 % of total muscle creatine or creatine phosphate pool is converted daily to creatinine through the spontaneous, non enzymatic loss of water or phosphate.

Serum creatinine (55-120 μmol/L in adult):

- Creatinine in the plasma is filtered freely at the glomerulus and secreted by renal tubules (10 % of urinary creatinine).
- Creatinine is not reabsorbed by the renal tubules.
- Plasma creatinine is an endogenous substance not affected by diet.
- Plasma creatinine remains fairly constant throughout adult life.
- The glomerular filtration rate (GFR) provides a useful index of the number of functioning glomeruli.
- It gives an estimation of the degree of renal impairment by disease.

Creatinine clearance: Accurate measurement of GRF by clearance tests requires determination of the concentration in plasma and urine of a substance that is:

- Freely filtered at glomeruli.
- Neither reabsorbed nor secreted by tubules.
- Its concentration in plasma needs to remains constant throughout the period of urine collection.
- Better if the substance is present endogenously.
- Easily measured.

Creatinine meets most of these criteria:

- Creatinine clearance is usually about 110 ml/min in the 20-40 year old adults.
- It falls slowly but progressively to about 70 ml/min in individuals over 8o years of age.
- In children, the GFR should be related to surface area, when this is done, results are similar to those found in young adults.
- Clearance is the volume of plasma cleared from he substance excreted in urine per minute.
- It could be calculated from the following equation:

$$\text{Clearance (ml/min)} = \frac{U \times V}{P}$$

U = Concentration of creatinine in urine μmol/l , V = Volume of urine per min
P = Concentration of creatinine in serum μmol/l

Cockcroft-Gault Formula for Estimation of GFR

- As indicated above, the creatinine clearance is measured by using a 24-hour urine collection, but this does introduce the potential for errors in terms of completion of the collection.

- An alternative and convenient method is to employ various formulae devised to calculate creatinine clearance using parameters such as serum creatinine level, sex, age, and weight of the subject.

An example is **the Cockcroft-Gault Formula**:

$$\textbf{GFR} = \frac{\textbf{K} \times (\textbf{140} - \textbf{age}) \times \textbf{Body weight}}{\textbf{Serum creatinine (μmol/L)}}$$

Where K is a constant that varies with sex:1.23 for male & 1.04 for females.

The constant K is used as females have a relatively lower muscle mass:

- **It should not be used if:**

- Serum creatinine is changing rapidly
- the diet is unusual, e.g., strict vegetarian
- Low muscle mass, e.g., muscle wasting
- Obesity

Cockcroft-Gault Formula for Estimation of GFR: Limitations

Serum Cr is a better KFT than creatinine clearance because:

-Serum creatinine is more accurate.

-Serum creatinine level is constant throughout adult life

Creatinine clearance is only recommended in the following conditions:

- Patients with early (minor) renal disease.
- Assessment of possible kidney donors.
- Detection of renal toxicity of some nephrotoxic drugs.

Normal adult reference values: Urinary excretion of creatinine is 0.5 - 2.0 g per 24 hours in a normal adult, varying according to muscular weight.

- Serum creatinine : 55 – 120 μmol/L
- Creatinine clearance: 90 – 140 ml/min (Males)
  80 – 125 ml/min (Females)

A raised serum creatinine is a good indicator of impaired renal function, But normal serum creatinine does not necessarily indicate normal renal function as serum creatinine may not be elevated until GFR has fallen by as much as 50%

Serum Urea (2.5-6.6 mmol/L) in adult:

Urea is formed in the liver from ammonia released from deamination of amino acids. As a kidney function test, serum urea is inferior to serum creatinine because:

- High protein diet increases urea formation.
- Any condition of ↑ proteins catabolism *(Cushing syndrome, diabetes mellitus, starvation, thyrotoxicosis)* →↑ urea formation.
- 50 % or more of urea filtered at the glomerulus is passively reabsorbed by the renal tubules.

Normal values of Internal :

Sodium : 135 to 145 meq/L
potassium :3.5 to 5.5 meq/L
chlorides:100 to 110 meq/L
bicarbonate:24 to 26 meq/L
calcium :8.6 to 10 mg/dl
magnesium : 1.6 to 2.4 mg/dl
phosphorus : 3.0 to 5.0 mg/dl
uric acid : 2.5 to 6.0 mg/dl at ph 7.4
creatinine: 0.8 to 1.4 mg/dl

Colorimetric estimation of plasma/serum uric acid level

Uric acid: It is the final breakdown product of purine metabolism. It circulates in the plasma as sodium urate and is excreted by the kidney. It is derived from nucleic acid that are ingested or come from destruction of tissue cell.

Nucleic acid are of two types: Purine and Pyrimidine.

- The Catabolism of these Purine, Adenine and guanine produce uric acid.
- After breakdown of nucleic acid the uric acid formed is transported to the liver, blood then is filtered through the glomerular filtrate and appear in the urine.

Hyperuricemia:

Elevated levels of uric acid concentration, it can be due to increase urate formation or decrease excretion. Purine --- liver----xanthine ---uric acid--- blood urate --- kidney -- excreted in urine.

GOUT: Hyperuricemia can lead to disease condition called Gout. Gout is a clinical syndrome characterized by hyperuricemia and acute arthritis.

- Acute gout tats by deposition of sodium urate crystals which cause inflammation s/s pain and inflammation of joints.

Objective:

- **To know the uric acid level in the body.**
- **To diagnose a case of hyperuricemia**

Specimen:

- Serum is the best, heparinized plasma can be used.
- lipemic and increased bilirubin sample should be avoided.
- Also drugs such as thiazide and salicyclate cause elevation in uric acid.
- uric acid levels are effected by diet --- increased ingestion of red meat which is rich in nucleic acid purine.
- Urate concentration is higher in male then in female.
- Serum should be separated quickly as uric acid is related to cellular breakdown of RNA and DNA.

Method used:

a) Chemical method --- phototungstic acid method

b) Enzymatic ---- uricase methods.

Principle estimation of uric acid in blood serum:

a) Enzymatic estimation of uric uses a reagent containing two enzymes and a chromogen. Uric acid is oxidized by *uricase* to allantoin and hydrogen peroxide.

b) Uric acid + O2 + 2 H2O uricase allantoin + CO2 + H2O2

c) Hydrogen peroxide in the presence of *peroxidase* allows oxidative copulation of chromogens to yield a coloured compound suitable for the photometric determination.

Procedure:

addition	Sample	Standard	blank
Reagent	1 ml	1 ml	1 ml
Serum/plasma	20 µl	----	----
standard	----	20 µl	----
Distilled water			20 µl

Mix properly and incubate in the thermoblock for 2 min at 37°C. And then measure the absorbance of the sample and the standard at 550 nm against the blank

Normal Value:

- Male 3.4 ---- 7 mg /dl . Female 2.4 ----5 mg /dl URINE.

  250 – 750 mg /dl interpretation of result Causes of elevated level of uric acid

Chapter Three

Preparation Of Solutions

1-Molisch's reagent:

- Dissolve 3.75 g of α-naphthol in 25 ml of Ethanol 99%. This reagent should be prepared fresh.
- Concentrated sulphuric acid

2- Benedict's reagent:

One litre of Benedict's reagent can be prepared by mixing 17.3 grams of copper sulfate pentahydrate ($CuSO_4.5H_2O$), 100 grams of sodium carbonate (Na_2CO_3), and 173 grams of sodium citrate in distilled water (required quantity). Here, the copper(II) sulfate acts as a source of Cu^{2+} ions, the sodium carbonate provides an alkaline medium, and the sodium citrate forms complexes with the Cu^{2+} ions. Distilled water is used as a solvent.

The purity of Benedict's reagent can be checked by heating it in a test tube. No changes in the blue colour of the solution upon heating is an implication that the reagent is pure.

3- Barfoed's reagent:

Dissolve 13.3 g of copper acetate in 200 ml of distilled water and add 1.8 ml of glacial acetic acid to it.

Acetic acid: provide cupric ions and copper acetate: provides an acid medium.

4- Fehling's solution:

- Fehling A (CuSO4 dissolved in dil. H2SO4)
- Fehling B (sodium potassium tartarate dissolved in dilation of NaOH).

5- Tollen's reagent:

The preparation of Tollens reagent takes two steps.

Step 1: A few drops of dilute sodium hydroxide are added to aqueous 0.1 M silver nitrate.

$$2AgNO3 + 2NaOH \rightarrow Ag2O + 2NaNO3 + H2O$$

The above reaction precipitates a brown solid, silver(I) oxide.

Step 2: Next, an adequate amount of aqueous ammonia is added to dissolve brown silver(I) oxide.

$$Ag2O + 4NH3 + 2NaOH + H2O \rightarrow 2[Ag(NH3)2]OH$$

6- Osazone reagent:

Add 0.3 g of osazone mixture and five drops of glacial acetic acid to the test tube. Mix it well and warm the test tube gently in the water bath if required to dissolve all the elements. Keep the test tube in boiling water and observe the formation of crystals at various time points.

*Or:*0.5 g of phenylhydrazine hydrochloride and 0.1 g of sodium acetate.

Glacial acetic acid.

7-Iodine's reagent:

Dissolve potassium iodide (KI) in about 20-30 ml of distilled water. Add iodine and heat gently with constant mixing until iodine is dissolved. Dilute to 100 ml with distilled water.

8- Bail's reagent:

Consists of 0.4 g orcinol, 200 ml of concentrated hydrochloric acid and 0.5 ml of 10% ferric trichloride solution.

8-Preparation of carbohydrate/ amino acid solution:

Prepare 1% CHO/ amino acid solution in 100ml distilled water

9- Ninhydrin's solution:

Weigh 0.2 g. of ninhydrin and dissolve in 10ml of acetone or ethanol.

10- Biuret's reagent:

1-Dissolve 1.5 g of aqueous pentahydrate copper (II) sulfate and 6 g of potassium tartrate (Rochelle salt) in 500 ml of water.

2- We add to the previous solution 300 ml 10% (w/v) sodium hydroxide and complete the total volume with distilled water to 1 liter.

11- Xanthoproteic acid test reagent:

Concentrate of nitric acid.

12- Millon's test reagent:

Millon's Test Reagent: Mercuric and mercurous nitrates are dissolved in nitric acid and distilled water to make Millon's test reagent. In a 400 mL concentrate nitric acid solution, dissolve 160 grams of mercuric nitrate and 160 grams of mercurous nitrate. After that, 600 mL of distilled water is added to the reagent to make it 1000 ml.

13- Hopkin's Cole reagent:

1-Glyoxylic acid- It can be prepared by exposing glacial acetic acid to sunlight for a few days.

2- Concentrated H_2SO_4.

14- Sakaguchi's reagent:

1. NaOH: 40% (w/v).
2. α-Naphthol: 1% in alcohol.
3. few drops of 10% sodium hypobromite solution of bromine water.

References

- BCM210Practicals (1980). Charles Sturt university.
- Chawla (2003). Practical Clinical Biochemistry: Methods and Interpretations. Pp.: 1-100. ISBN 978-81-8061-108-7.
- Clinical Chemistry: Techniques, Principles, Correlations. (2010). 6th ed, Michael L. Bishop, Edward P. Fody, Larry E. Schoeff. C hapter 26. Pp: 557-577.

Republic of Iraq
Ministry of Higher Education and Scientific Research
University of Basrah
College of Veterinary Medicine
Department of Physiology, Pharmacology & Biochemistry

Prof. Dr. Nawras Abdelah Alwan

- Bachelor's in Veterinary Medicine and Surgery.
- MSc. in Biochemistry
- Specialization in Biochemistry from assistant lecturer to the degree of Professor
- Ph.D. Physiology
- I teach theoretical and practical Biochemistry for the 1st and 2nd stages in College of Veterinary Medicine – University of Basrah.

2022