**King Saud university Pharmacology dept.**

**College of pharmacy Practical Biochemistry**

**(224 PHL)**

***Lab No. (1)***

**Types of biochemical experiments:**

1. **Chemical method**

* It is used for a qualitative purposes “ to identify the presence or absence of a substance”

1. **Colorimetric method**

* It is used for a quantitative purposes “ to measure the concentration of a substance”

**Instrument used in Colorimetric method:**

Spectrophotometer

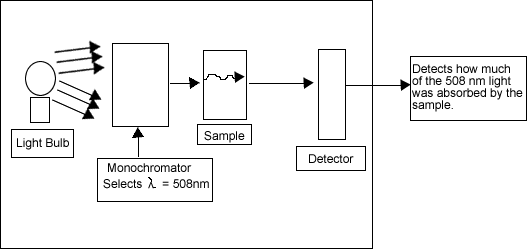


Diagram of spectrophotometer composition

**Types of Specimens used in the laboratory experiments:**

1. Blood
2. Urine
3. Cerebrospinal fluid (CSF)
4. Saliva

**Factors affects the results and validity of the specimen:**

1. **Diet:** Dietary constituents may temporarily alter the concentrations of analytes in blood significantly. e.g. a carbohydrate containing meal is likely to increase blood glucose concentration.
2. **Drugs:** Many drugs influence the chemical composition of blood. Sometimes it may be possible to delay starting treatment, or to stop it temporarily, to enable essential investigations to be performed.

BLOOD Analysis

**Blood** is a tissue that circulates in the closed system of blood vessels*.*

# **Functions of Blood :**

* Respiration, Supply of [oxygen](http://en.wikipedia.org/wiki/Oxygen) to tissues (bound to [hemoglobin](http://en.wikipedia.org/wiki/Hemoglobin), which is carried in red cells) .
* Nutrition, delivering of nutrients such as [glucose](http://en.wikipedia.org/wiki/Glucose), [amino acids](http://en.wikipedia.org/wiki/Amino_acids), and [fatty acids](http://en.wikipedia.org/wiki/Fatty_acids)
* Excretion of waste products such as: CO2, urea.
* Defense mechanisms, e.g. [white blood cells](http://en.wikipedia.org/wiki/White_blood_cells) (WBC)
* Regulation of body water, MOA: when the body lost a lot of water→ high concentration of solutes in the blood→ solutes will attract water from salivary glands→ making dry mouth→ feel thirsty
* Regulation of acid base balance.
* Regulation of body temperature.

# **Compositions of Blood :**

1. **Cellular elements:** RBCs, WBCs, Platelets
2. **Plasma:**
3. Water
4. Solids: **a- Diffusible constituents**
   * Anabolic, synthetic: simple precursors which form complex end products. e.g. glucose, amino acids.
   * Catabolic, degradative: result from break down of complex molecules e.g. urea, uric acid, creatinine

# **b- Non diffusible constituents** e.g. albumin, globulin, fibrinogen.

# **Purpose of blood analysis:**

1) Diagnostic: Any change in normal constituent, or presence of abnormal constituent indicates a disease state .

2) Prognostic.

**Preparation of plasma and serum samples :**

- If whole blood is allowed to clot and the clot is removed, the remaining fluid is called **Serum.**

**Serum = Plasma – Blood clotting factor.**

**Preparation of Serum Sample :**

1. Obtain venous blood using empty centrifuge tube.
2. Allow the blood to clot.
3. Remove the clot.
4. Centrifuge.
5. Transfer the clear serum to a clean specimen tube using Pasteur pipette. Do not disturb the clot.

**Preparation of Plasma Sample :**

1. Put some anti coagulant in a clean dry centrifuge tube.
2. Add the venous blood and mix.
3. Centrifuge
4. Transfer the top clear plasma to a clean specimen tube.

**Anti-coagulants :**

**Anti-coagulants** are chemicals which prevent clotting of blood.

**1. Heparin:** Is the one that least interferes with chemical tests. It is present in body tissues in a concentration less than that required to prevent coagulation of blood.

**Mode of action:** Anti –thrombin i.e. :prevents the conversion of prothrombin to thrombin.

**Disadvantage:** High cost.

**2. E.D.T.A :** ( Ethylene di amine tetra acetic acid), **Not** used clinically.

**Mode of action:** Binds to Ca++

**Advantage:** prevents clumping of platelets.

***3.* Oxalates*:*** Na, K, Li, or NH4 salts are used, **Not** used clinically. .

* **Mode of action:** Forms insoluble salts with Ca++
* **Disadvantage:**

1- Inhibits Lactate dehydrogenase.

2- Na, K salts should not be used in determination of electrolytes as this lead to significant error.

**4. Na Fluoride***;* (enzyme poison)

* It inhibits glycolysis; therefore it is used in determination of blood sugar.
* It inhibits urease enzyme, therefore it is not used in determination of blood urea.

**5. Tri Na Citrate***:* Used mainly to determine E.S.R.

**6. Acid Citrate Dextrose Solution:** Used mainly to study platelets and to preserve blood.

**Deproteinization :**

**The purpose of deproteinization is to precipitate the proteins. This is necessary because:**

* proteins have a certain U-V absorption and could give false reading.
* Moreover proteins are colloids which make the solution turbid and difficult to read on the spectrophotometer.

- The precipitate can be used for determination of plasma proteins e.g. albumin, globulins.

- The protein-free clear supernatant can be used for determination of non-protein nitrogen compounds, glucose etc.

**Deproteinization Agents :**

**1. Trichloroacetic Acid &Tungestic Acid**

**Mode of action:** Proteins at a pH lower than their isoelectric pH become cations and are precipitated as insoluble salts of the acids.

**2. Zinc Hydroxide**

**Mode of action:** Proteins at a pH higher than their isoelectric pH become anions and are precipitated as salts of the heavy metals.

Ba,Cd, Cu hydroxides can also be used.

**4.Organic Substance :** remove water and extract some blood constituents e.g. ethanol, ether precipitate proteins and extract fat and cholesterol.

**Determination of Serum Glucose Concentration**

**Serum glucose is determined by two methods :**

1. Oxidation method (Enzymatic method)
2. Alkaline copper reduction method (Asatoor and King method)
3. **Determination of Serum Glucose Concentration**

**(Oxidation method)**

**Principle:**

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**Procedure:**

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**Calculation:**

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**Normal value:**

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# **Clinical significance:**

* **Hyperglycemia** is blood glucose level › 110 mg/dl

**Causes**: 1. Diabetes mellitus

2. Hyperactivity of thyroid, adrenal, pituitary gland

3. Increased secretion of growth hormone (Acromegaly)

4. Asphyxia causing acidosis and increased mobilization of

glucose from glycogen in the liver.

5. Exercise or stress causing increased secretion of adrenaline.

6. Pancreatic carcinoma or acute pancreatitis.

* **Hypoglycemia** is blood glucose level ‹ 70 mg/dl

**Causes:** 1. Overdose of insulin.

2. Hypoactivity of thyroid, adrenal, or pituitary gland.

3. Glycogen storage disease in which there is deficiency of

G-6-phosphatase therefore inability to produce glucose

from glycogen.

* **Renal Glucose Threshold** :
* Normally glucose present in the blood is filtered out in the renal glomeruli but reabsorbed back into the blood by the kidney tubules.
* The limit to the ability of the tubules to reabsorb glucose is 180 mg/ dl.
* If blood sugar level rises above this value glucose appears in the urine **(Glucosurea).**
* The blood glucose level of 180 mg/dl is therefore called the renal glucose threshold.

***Lab No. (2)***

1. **Determination of Serum Glucose Concentration (Alkaline copper reduction method)**

**Principle**:

* Glucose reduces the alkaline copper solution forming cuprous ions. The amount of cuprous formed is estimated colorimetrically by reacting with phosphomolybdic acid.
* The cuprous ions will be oxidized again to cupric and the molybdic acid will be reduced to molybdenum blue.
* The color intensity being proportional to the amount of glucose in the sample.

Glucose + cupric cuprous + gluconic acid

Cuprous + phosphomolybdic acid cupric + molybdenum blue

**Procedure**:

1. Deproteinization:

Take the following in a clean and dry centrifuge test tube.

a) 0.1 ml of Na tungstate

b) 3.8 ml of isotonic Na2 SO4 / Cu SO4 solution

c) 0.1 ml serum

Mix well and centrifuge.

2. Transfer the clear supernatant to a clean and dry test tube using Pasteur pipette.

3. Take three more clean test tubes and label them ---- Test Standard Blank

Test ---- 1 ml of supernatant +1 ml of alkaline tartarate

Standard -------1 ml standard glucose + 1ml of alkaline tartarate

Blank------ 1ml of isotonic Na2SO4 CuSO4 soln +1 ml of alkaline tartarate

4. Mix well and close with cotton wool.

5. Put in a boiling water bath for 10 mins.

6. Cool under tap water.

7. Add 3ml of phosphomolybdic reagent to each tube. Shake well.

8. Add 3 ml of distilled water to each tube.

9. Mix well to remove all air bubbles. Let the tubes stand for 5 mins.

10. Measure the absorbance of T(Test) and S(Standard) against B( Blank) at

680nm.

**Calculation :**

Abs glucose sample

**Serum glucose (mg/dl)=** Abs glucose std X conc. of std ( 100 mg/dl )

**Normal Value :**

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***Lab No. (3)***

**Determination of Serum Total protein Concentration**

Plasma Proteins

**Classification**

**1) Simple Proteins:** They yield only α-amino acids on hydrolysis. e.g. albumin, globulin.

**2) Conjugated Proteins:** They are proteins combined with a non-protein substance called prosthetic group. e.g. nucleoprotein, phosphoprotein, metalloprotein, glycoprotein, lipoprotein etc.

**3) Derived Protein:** are derived from changes in original proteins. This includes denatured proteins.

* Plasma Proteins :

Contain both simple and conjugated proteins i.e. albumin, globulin, lipoproteins, glycoproteins, metalloproteins.

**Albumin Globulin**

1) Low molecular 1)complex molecule, high molecular weight. weight.

2) Soluble in half saturated solution 2) insoluble.

of ammonium sulphate. 3) 4 gm/dl . 3) 2.5 gm/dl

**Serum Total Protein can be determined by two methods :**

1. Kit method.
2. Biuret method (manual ).
3. **Determination of Serum Total protein Concentration**

**(Kit method)**

**Principle:**

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**Procedure :**

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**Calculation :**

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**Normal Value :**

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**Clinical Significance:**

* **High level of serum proteins is called Hyperproteinemia which could be due to:**

1. Dehydration.

2. Chronic inflammations e.g.: tuberculosis

3. Cancer.

4. Drugs e.g.: cortisone, oral contraceptives.

* **Low level of serum proteins is called Hypoproteinemia which could be due to:**

1. Severe hemorrhage.

2. Malnutrition.

3. Malabsorption.

4. High fever causing increased catabolism of proteins.

5. Acute or chronic hepatic insufficiency.

***Lab No. (4)***

1. **Determination of Serum Proteins (Biuret Method )**

* The determination is performed by estimating the total protein in the serum and that of albumin after separating the globulin by salt fractionation.
* The globulin is then calculated by the difference between total protein and albumin.

# **Principle :**

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Substances which contain two –C—NH2 groups joined together through a single carbon or nitrogen atom or those containing two or three peptide bonds give a blue or purple color with alkaline copper solution.

## Procedure:

**1)** **Total protein standard:** Pipette 6ml of sulphate-sulphite solution into a centrifuge tube. Add 0.4ml of standard serum, Invert to mix. Remove at once 2ml of the mixture into a test-tube labeled Total protein standard.

**2)** **Albumin standard:** To the rest of the mixture in the centrifuge tube add 3ml of ether. Stopper. Invert 20times to mix. **Do not shake vigorously or the protein will be denatured**.

Cap the tube and centrifuge for 5 minutes so that a firm globulin layer is formed. Tilt the tube and insert a Pasteur pipette into the clear solution below the globulin layer. Do not disturb the globulin film. Withdraw this solution into a clean test tube. Measure out exactly 2ml of this solution into another test tube labeled **Albumin standard**.

**3) Total protein sample**  **4) Albumin sample**

Repeat all the above steps using serum sample instead of standard serum.

**5) Blank**: In a test tube labeled Blank add 2ml of sulphate-sulphite solution.

Now we have 5 test tubes in all **1) Total protein standard 2) Albumin standard**

**3) Total protein sample 4) Albumin sample 5) Blank**.

To each of these add 5ml of Biuret reagent

Place in a water bath at 37OC for 10 mins.

Read at λ540 nm.

**Calculations:**

Abs total protein sample

**Serum total protein (gm/dl)**= X conc. of std ( 3.5 g/dl )

Abs total protein std

Abs albumin sample

**Serum albumin (gm/dl)=** Abs albumin std X conc. of std ( 2 g/dl )

**Serum globulin ( gm/dl) =** Total protein – albumin.

**Normal Values**:

**Total protein**: 4.2 – 5.6 g/dl

**Albumin:** 2.3 –3.5 g/dl.

**Globulin:** 1.9 -2.1 gm/dl.

***Lab No. (5)***

Determination of Serum Non Protein Nitrogen Concentration

* The term non-protein nitrogen (NPN) includes the nitrogen from all nitrogenous substances other than proteins.
* The NPN could be measured as a group or individually.

**Major Constituents:**

Urea, uric acid, creatinine, ammonia etc.

**Importance :**

**Testing NPN in blood served as a test for kidney functions. Now it is replaced by determination of urea nitrogen because:**

(1) The NPN value is the result of many interfering and interacting factors. The route of elimination of various NPN compounds differs considerably. Some are excreted by glomerular filtrations only e.g. creatinine, uric acid is excreted by tubular excretion. Urea is excreted by glomerular filtration and then partially absorbed by the tubules.

(2) The increase of NPN is mainly a reflection of increase of urea nitrogen which normally makes up 45% of the total NPN.

# **Determination of Serum Urea Concentration**

**Urea** is the end product of protein metabolism in the body. It is synthesized in the liver from the (NH2) amino group resulting from deamination of amino acids.

**Principle:**

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**Procedure:**

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**Calculation:**

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**Normal value:**

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**Clinical Significance:**

* **Increase in blood urea nitrogen could be due to:**

**Pre-Renal Causes:** 1) Salt and water depletion.

2) Protein catabolism as in fever.

**Renal Causes:** 1)Glomerulonephritis .

2) Mercury poisoning.

3) Hyperparathyroidism.

4)Hypervitiminosis D.

Causes 3 and 4 cause increase in serum Ca and precipitation of Ca in the kidney tissue causing destruction of kidney tissue.

**Post-Renal Causes:** 1) Prostate enlargement.

2) Stones in urethra.

3) Tumor of the bladder.

All the above cause obstruction to urine flow producing back pressure on the kidney and kidney damage.

* **Decrease in blood urea nitrogen could be due to** : 1) Liver failure.

2) Malnutrition.

3) Over hydration.

4) Early stages of pregnancy.

# **2-Determination of Serum Uric Acid Concentration**

Uric acid is the end product of purine metabolism. It comes from endogenous metabolism of nucleoproteins and exogenously from food.

**Principle:**

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**Procedure:**

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**Calculation:**

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**Normal value:**

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**Clinical Significance**

**Increase in uric acid Hyperuricemia could be due to:**

1. Gout .

2. Toxemia.

3. Leukemia.

4. Age: menopausal women.

5. Drugs:Thiazide diuretics.

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**Decrease in uric acid Hypouricemia could be due to:**

1. Hepatitis.

2. Uricosuric drugs: salicylates, phenylbutazone.

3. Fanconi syndrome.

3-Determination of Serum Creatinine Concentration :

* Creatinine is the internal anhydride derived from dephosphorylation of creatine phosphate.

Creatine Creatinine + H2O

* Creatinine has no useful function and is eliminated in urine by glomerular filtration.
* It is not reabsorbed by the tubules to any significant extent, Therefore glomerular damage will decrease the rate at which creatinine is excreted.
* Creatinine clearance test can be used as a test for kidney function as its excretion parallels the glomerular filtration rate (G.F.R).
* A serum creatinine level over 2 mg/dl indicates **Renal Failure**.

**Principle:**

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**Procedure:**

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**Calculation:**

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**Normal value:**

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***Lab No. (6)***

Determination of Blood Hemoglobin Concentration

* Hemoglobin is a conjugated protein, the non-protein part (prosthetic group) is heme, the protein part is globin.
* Hemoglobin is a chromoprotein and gives blood its red color.
* The most important property of hemoglobin is its ability to form stable complex with oxygen (oxyhemoglobin) which serves in O2 transport in the body.
* Iron is found in the ferrous form (Fe++) in both hemoglobin and oxyhemoglobin.
* Iron is in the oxidised form (Fe+++) in cyanmethemoglobin.

**Determination of Blood Hemoglobin Concentration**

**by Cyanmethemoglobin Technique :**

**Principle:**

Blood is treated with the Drabkin’s reagent which contains potassium ferricyanide, potassium cyanide, and NaHCO3.

# Hb + ferricyanide met-Hb

Met-Hb +cyanide cyan met-Hb

**Procedure:**

1) Take 2ml of Drabkin’s reagent(**Poison**) into a test tube.

2) Pipette 0.02 ml of blood sample . Blow it into the Drabkin’s reagent and mix well.

3) Incubate 3mins at room temperature.

4) Read at 540 nm against distilled water.

**Warning: Drabkin’s Reagent Contains Poison.**

**Calculation:**

Conc. of hemoglobin (g/dl)= 29.4 x A

**Normal value:**

14-18 g/dl in male .

12-16 g/dl in females .

**Clinical Significance :**

**A decrease in blood hemoglobin level is called** **Anemia**. It could be due to:

1) Decreased production of hemoglobin as in:

a) Protein deficiency

(b) iron deficiency

c) Vitamin deficiency: vit B12, Folic acid, Vit C etc

d) Leukemia.

2)Increased destruction of hemoglobin as in:

a) Hemolytic anemia.

b) Heavy metal poisoning: Lead.

c) Infectious diseases: malaria.

**An increase in blood hemoglobin level is called Polycythemia.** It could be due to:

(a) High altitudes .

(b) Congenital heart diseases.

(c) Emphysema.

**Determination of Serum Iron Concentration**

* The total amount of iron in an adult is 4-5gms/dl.

70-75% is in active form ( Fe+2 ) and 25% is stored in reticuloendothelial cells of liver and spleen as ferritin or hemosiderin ( Fe+3 ).

* Main site of absorption of iron is the duodenum and jejunum.
* **Physiologically active iron:**
  + Oxygen carrying chromoprotein e.g.: hemoglobin, myoglobin.
  + Enzymes: cytochromes, catalase, peroxidase.

**Principle:**

1) **Acidification:** transferrin-Fe+3 HCl transferrin + Fe+3

2) **Reduction:** Fe+3 +thioglycolic acid Fe+2

3) **Coloring:** Fe+2 +bathophenanthrolene pink color

**Normal Serum level**:

60-160 mcg/ dl

**Clinical Significance**:

**Increase in serum iron may be due to:**

1. Increased destruction of RBC as in hemolytic anemia.
2. Increase iron absorption.
3. Iron overload (blood transfusion).
4. Increased iron release from body stores e.g.: viral hepatitis.

**Decrease in serum iron may be due to:**

1. lack of sufficient intake.
2. Increase loss of iron as in hemorrhage, menstruation.
3. Pregnancy-increases demand on body stores.

**Determination of Serum Bilirubin Concentration**

Bilirubin is the major pigment present in human bile. It is the end product of destruction of hemoglobin.

* Hemoglobin destruction yields:
* Heme → Bilirubin
* Globin → Amino acid pool for reuse
* Iron → iron pool for reuse
* Iron free porphyrin → to be stored in reticuloendothelial cells of liver and spleen .

**Types of Bilirubin:**

1. **Conjugated or soluble or direct bilirubin:** In the liver bilirubin is conjugated with glucouronic acid which make it water soluble.
2. **Unconjugated or insoluble or indirect bilirubin:** before conjugation in the liver, bilirubin is lipid soluble.
3. **Determination of Serum Total Bilirubin Concentration :**

**Principle:**

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**Procedure:**

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**Calculation:**

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**Normal value:**

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**2-Determination of Serum Direct Bilirubin Concentration :**

**Principle:**

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**Procedure:**

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**Calculation:**

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**Normal value:**

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**Clinical Significance:**

**Hyper bilirubinemia is called Jaundice**

**Causes of Jaundice:**

1. **Pre-Hepatic Jaundice:** there is an increase of indirect bilirubin due to:
   1. incompatible blood transfusion
   2. hemolytic anemia
2. **Hepatic Jaundice** can be due to:
3. - Decreased glucouronyl transferase as in physiologic jaundice of newborn.

- There is increase in indirect bilirubin (lipid soluble) which can cross the blood brain barrier and produce encephalopathy.

1. - Hepato-cellular damage as in viral hepatitis, toxic hepatitis, cirrhosis.

- There is increase in both direct and indirect bilirubin.

1. **Post-Hepatic Jaundice** can be due to:

* Obstruction of the common bile duct by stone tumor, inflammation.
* There is increase mostly of direct bilirubin.

***Lab No. (7)***

**Determination of Serum Lipids Concentration**

* **Lipids** are fatty oily or waxy substances of animal or plant origin which are insoluble in water but soluble in non-polar solvents such as ether, chloroform, benzene etc.

# **Chemistry:**

* Many lipids are esters of fatty acids and glycerol.

**There are four forms of lipids present in plasma :**

**1-** **Fatty Acids:** These are straight chain compounds of varying lengths. They may be:

**a) Saturated :** palmitic acid, stearic acid.

**b) Unsaturated:** oleic acid, linoleic acid.

**Fatty acids may be :**

**a)** Esterified with glycerol (glycerides) .

**b)** Non esterified fatty acids (NEFA) are carried in plasma and bound to albumin.

**2 –Triglycerides (TG)** **:** They consist of glycerol esterified with three fatty acids.

**Two classes of lipoproteins are important for transport of triglycerides:**

**a)** **Chylomicrons:** for transport of triglycerides from GIT to the liver.

**b)** **VLDL(very low density lipoprotein)**: for transport of triglycerides from liver to cells.

**3-Cholesterol:**

* It has a steroid structure.
* 2/3 of the plasma cholesterol is esterified with fatty acids to form cholesterol esters.
* It is transported in plasma bound to lipoproteins.
* Cholesterol is synthesized in the liver.

**4- Phospholipids**: They are complex lipids resembling triglycerides but containing phosphate and a nitrogenous base.

**Sampling for Serum Lipids :**

1) Patient must be fasting for 14-16 hrs.

2) Normal weight constant for 2 weeks.

3) No treatment to lower lipids.

4) Investigation for hyperlipidemia is done about 3 months after myocardial infarction or major surgery.

5) The blood sample should not be heparinized as heparin is a lipid clearing factor.

1. **Determination of Serum Total Lipids Concentration**

**Total serum lipids include** NEFA, TG, Phospholipids, hormones,

cholesterol ester, glycolipids, fat soluble vitamins .

**Principle:**

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**Procedure:**

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**Calculation:**

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**Normal value:**

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1. **Determination of Serum Triglycerides Concentration**

**Principle:**

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**Procedure:**

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**Calculation:**

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**Normal value:**

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1. **Determination of Serum Cholesterol Concentration**

**Principle:**

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**Procedure:**

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**Calculation:**

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**Normal value:**

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**Clinical Significance:**

**Increase in serum total lipids Hyperlipidemia, triglycerides Hypertriglyceridemia, and cholesterol Hypercholesterolemia** could be due to:

1) Hypothyroidism.

2) diabetes mellitus.

3) chronic alcoholism.

4)oral contraceptives.

5) biliary obstruction.

6) stress.

7) nephrotic syndrome.

**Decrease in serum total lipids Hypolipidemia, triglycerides Hypotriglyceridemia, and cholesterol Hypocholesterolemia** could be due to:

1) Hyperthyroidism.

2) malnutrition.

3) malabsorption.

4) liver disease.