Blood

Blood definition: is the red fluid constituent of the body that flows through the vascular channels(artery, vein and capillary). The total volume of blood in a 70Kg adult is about 5.5 liters of 8% of the body weight.

Blood is one of the most common specimens studied in biochemical laboratories in search of blood disorders, metabolic disorders and infection.

Blood has two major components:

1. **The cellular component**: consists 45% of total blood volume, includes red blood cells(erythrocytes), white blood cells (leucocytes), and platelets (thrombocytes). The thrombocytes are the smallest (2-4µm), the erythrocytes are medium-sized (7-8 µm), and the leucocytes are the largest of all the cells with a wide range (8-20 µm) of size.

   The red blood cells constitute the highest number of formed elements in the circulation (5,000,000/ mm^3 of blood) followed by platelets (300,000/ mm3 of blood) and leucocytes (4000- 11000/ mm3 of blood).

The erythrocytes help in transport of gases, that is, they carry oxygen from the lungs to the tissues and carbon dioxide from tissues to the lungs. The leucocytes assist in the defense processes of the body. The platelets assist in stoppage of bleeding(hemostasis).

Development of blood cells is called hemopoiesis. In , hemopoiesis occurs principally in the liver ,and then in the spleen in pregnancy period. In adults, hemopoiesis occurs in the bone marrow.

2. Non- cellular component (plasma): consists 55% of total blood volume, plasma is the clear yellow fluid consisting of a soluble protein called fibrinogen. Plasma is the principle medium for transportation of materials from one part of the body to another through the blood vessels. In summary, plasma consist of*90% water, *inorganic materials(electrolytes e.g. Na,K,Ca,Cl,Fe,Mg and etc.) ,*(6-8%) proteins (albumin, globulin, thrombin, fibrinogen), *nutrients (sugars, lipids, hormones, enzymes), * waste products (urea, uric acid, creatinine).

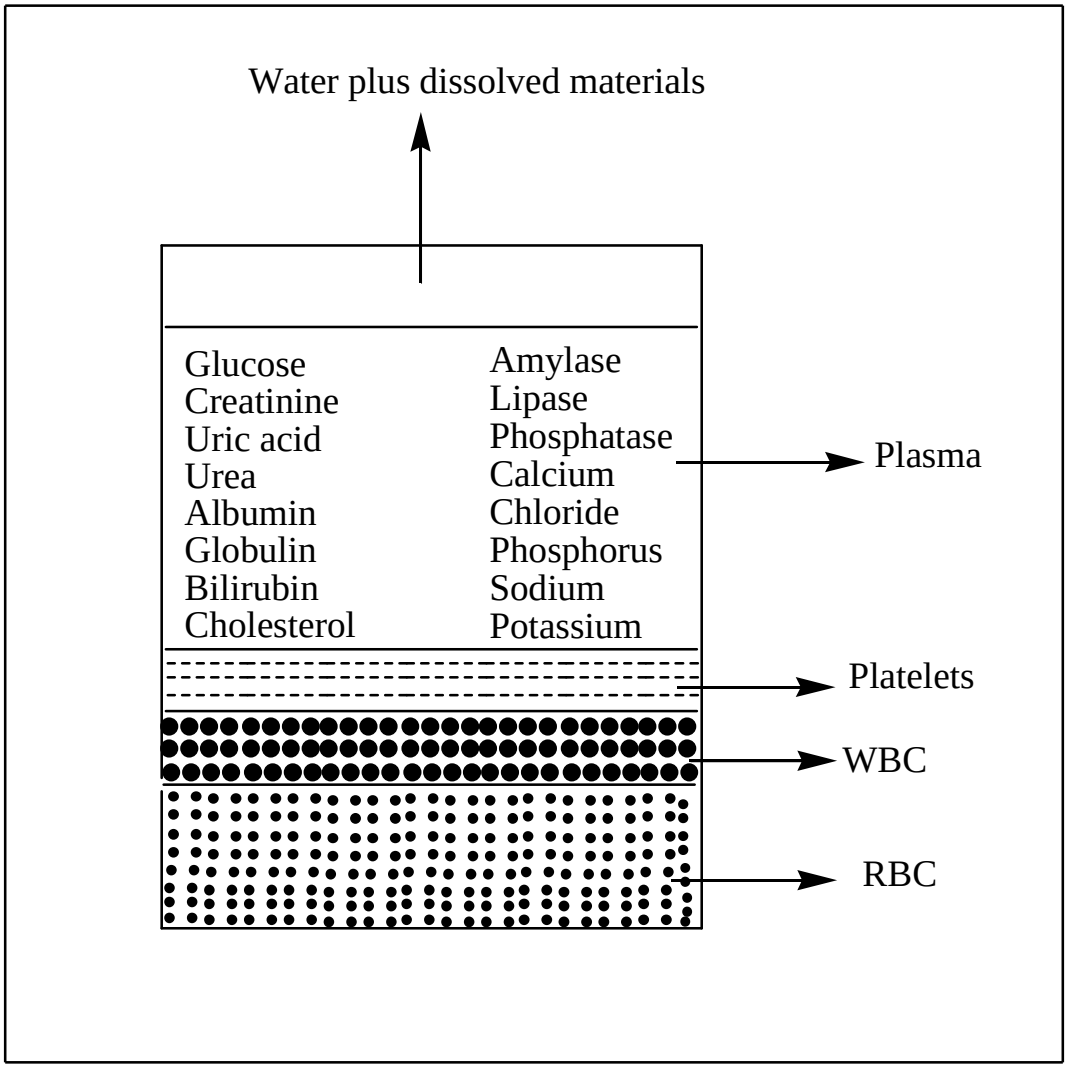
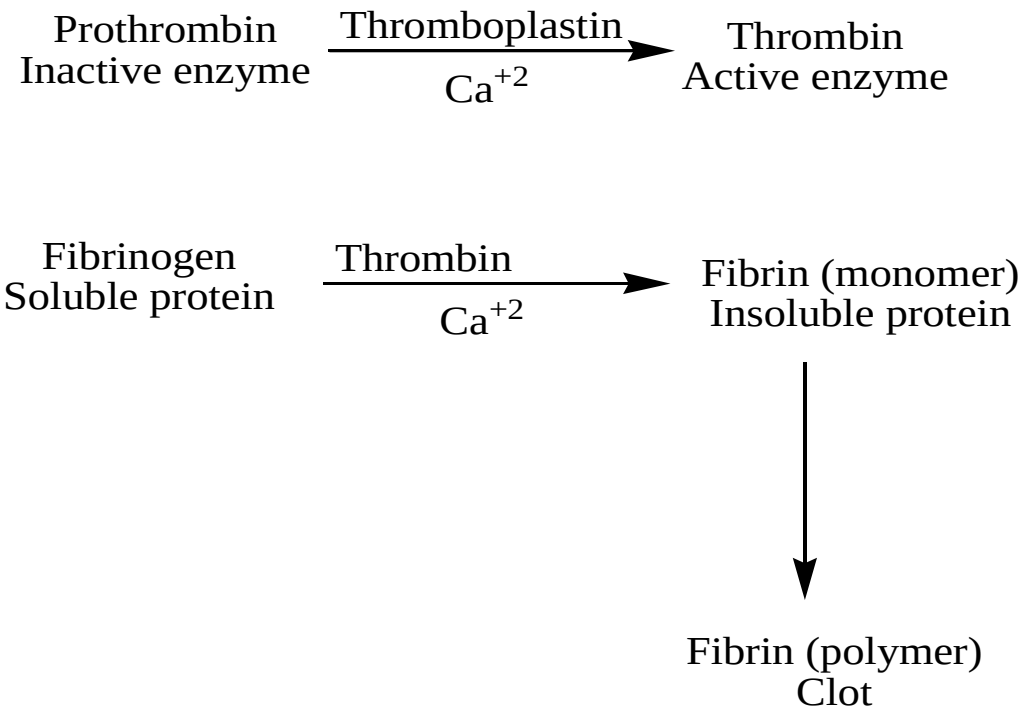


Fig. 1: Blood components

Blood coagulation (clotting)

Coagulation (thrombogenesis) is the process by which blood forms clots. Coagulation is highly conserved throughout biology; in all mammals, coagulation involves both a cellular (platelet) and a protein (coagulation factor) component.

Coagulation begins almost instantly after an injury to the blood vessel has damaged the endothelium lining the vessel. Exposure of the blood to proteins such as tissue factorinitiates changes to blood platelets and the plasma protein fibrinogen, a clotting factor. Platelets immediately form a plug at the site of injury; this is called *primary hemostasis*.*Secondary hemostasis* occurs simultaneously: Proteins in the blood plasma, called coagulation factors or clotting factors, respond in a complex cascade to form fibrin strands, which strengthen the platelet plug.



Procedure of blood collection

Venous blood is frequently collected while the blood may be taken from any prominent vein, a vein on the front of the elbow or for arm is almost universally employed. The arm should be warm. This improves the circulation. The arm is extended and a rubber tourniquet firmly

applied a few inches above the elbow. The skin over the vein is cleaned by rubbing over with spirit or ether. A well sharpened sterile hypodermic needle fixed onto a syringe can be held steady by a thump of the other hand of the operator.

When the needle enters the vein, the plunger is withdrawn slightly. If blood appears, the tourniquet is released. When the desired amount of blood has been drawn into the syringe, a small pad of wodsoaked with spirit or ether is placed on the arm where the needle was inserted, and the needle is withdrawn. This pad is held on firmly for a few minutes until bleeding stops.

The needle is removed from the syringe and the blood transferred to an appropriate container, using minimum amount of pressure. The needle and syringe are immediately washed out with cold water to remove any remaining blood. The needle to prevent any infection must be stressed.

Blood samples

There are three biochemical samples (whole blood, serum and plasma).

Anticoagulants are used when either whole blood or plasma is required. Most of the anticoagulants remove calcium which is one of the factors required in coagulation process.

Anticoagulants definition: are compounds work to prevent coagulations (clotting) of blood. Also, called antithrombic or fibirinolytic or thrombolytic.

Types of anticoagulants:

1. EDTA (Ethylene diamine tetra acetic acid)

10% solution of dipotassium salt of EDTA, 0.1 ml of this is used / 5 ml of blood (Conc. 2 mg/ ml of whole blood).

2. Double oxalate

1.2% of ammonium oxalate + 0.8 % of potassium oxalate. Use 0.5 ml for 5 ml (Con. 2 mg / ml of whole blood).

3. Sodium citrate

0.106 M tri-sodium citrate in distilled water used in solution form and is not dried inside the container. 1 ml of this solution is used for 9 ml blood (the ratio of anticoagulant solution and whole blood comes to 1:9, which is ideal for coagulations studies).

4. Sodium fluoride-potassium oxalate

Used for blood sugar estimation (except in enzymatic method of sugar estimation). 1.2 % sodium fluoride + 5% potassium oxalate. 0.25 ml is used /5 ml blood (Caution fluoride is a poison)

5. Heparin

Limited to the determination of blood gases and electrolytes, 0.1-0.2 mg / ml of the blood.

Separation of serum and plasma

Serum

Non-anticoagulated blood yields serum, the fluid portion of clotted blood in order to obtain serum is collected in a plain tube without anticoagulant and allowed to clot for 30 min. at room temperature. When the clot seperates out , it is centrifuged 3000 rpm for 10 min. and serum be separation. Separation must not delayed much because prolonged contact of the cells with the serum will cause the release of the red cells constituents into the serum composition.

Plasma

Suitable anticoagulant should be mixed with the blood by gentle rotation. Excessive amount of anticoagulant should not be used. Separation of plasma should be done by centrifuging at low to moderate speed. If the plasma is not required immediately, the cells can be allowed to sediment out. The upper fluid is removed and finally cleared of any remaining cells by centrifugation. Plasma contains 90% water, in organic materials, 6-7% proteins, sugars, vitamins, lipids, hormones and enzymes, by products (urea, uric acid and creatinine).

Erythrocyte sedimentation rate

The erythrocyte sedimentation rate (ESR), also called a sedimentation rate or Westergren ESR, is the rate at which red blood cells sediment in a period of one hour. The erythrocyte sedimentation rate (ESR) is an easy, inexpensive, nonspecific test that has been used for many years to help detect conditions associated with acute and chronic inflammation, including infections, cancers, and autoimmune diseases. ESR is said to be nonspecific because(1) increased results do not tell the doctor exactly where the inflammation is in the body or what is causing it, and also (2) because it can be affected by other conditions besides inflammation. For this reason, the ESR is typically used in conjunction with other tests.

The ESR is governed by the balance between pro-sedimentation factors, mainly fibrinogen, and those factors resisting sedimentation, namely the negative charge of the erythrocytes . When an inflammatory process is present, the high proportion of fibrinogen in the blood causes red blood cells to stick to each other.

The ESR is increased in pregnancy, inflammation, anemia or rheumatoid arthritis,chronic kidney diseasesand old age.A very high ESR usually has an obvious cause, such as a marked increase in globulins that can be due to a severe infection. The doctor will use other follow-up tests, such as blood cultures, depending on the patient's symptoms. People with multiple myeloma (tumors that make large amounts of immunoglobulins) typically have very high ESRs even if they don't have inflammation.

In many of these cases, the ESR may exceed 100 mm/hour, and decreased in polycythemia, sickle cell anemia, hereditary spherocytosis, and congestive heart failure. It may be increased in kidney cancer. The basal ESR is slightly higher in females.

Stages in erythrocyte sedimentation:

There are 3 stages in erythrocyte sedimentation 1) Stage 1 : Rouleaux formation - First 10 minutes 2) Stage 2 : Stage of sedimentation or settling - 40 mins 3) Stage 3 : Stage of packin - 10 minutes , sedimentation slows and cells start to pack at the bottom of the tube

Normal values:

Note: mm/hr. = millimeters per hour.

Westergren's original normal values (men 3mm and women 7mm) it was confirmed that ESR values rise with age and generally higher in women. Values are increased in states of anemia, and in black populations.

Adults

ESR reference ranges in healthy adults:

Age (year)	20	55	90
Men	12	14	32
Women	18	21	23

Children

- Newborn: 0 to 2 mm/hr.
- Neonatal to puberty: 3 to 13 mm/hr., but other laboratories place an upper limit of 20.

Some interferences which increase ESR:

- increased level of fibrinogen, gamma globulins.
- technical factors: tilted ESR tube, high room temperature.

Some interferences which decrease ESR:

- abnormally shaped RBC (sickle cells, spherocytosis).
- technical factors: short ESR tubes, low room temperature, delay in test performance (>2 hours), clotted blood sample, excess anticoagulant, bubbles in tube.

Procedure:

Westergren method:

1. The Westergren tube is marked from 0 (top) to 200 (bottom).
2. Anticoagulant used is 3.8% trisodium citrate solution.1 part of anticoagulant is added to 4 parts of blood.

3. The mixture is drown into Westergren tube up to zero mark and the tube set upright in a stand with a spring clip on top and rubber at bottom.
1. The level of the top of the red cell column is read at the end of 1 hour .

Blood type

A blood type (also called a blood group) is a classification of blood based on the presence or absence of inherited antigenic substances on the surface of red blood cells (RBCs). These antigens may be proteins, carbohydrates, glycoproteins, or glycolipids, depending on the blood group system . The two most important ones are ABO and the RhD antigen; they determine someone's blood type (A, B, AB and O, with + and - denoting RhD status).

ABO blood group system

The *ABO system* is the most important blood-group system. The *O* in ABO is often called *0*(*zero*, or *null*) in other languages.

Phenotype	Genotype
A	AA or AO
B	BB or BO
AB	AB
O	OO

The ABO System Is of Crucial Importance in Blood Transfusion This system was first discovered when investigating the basis of compatible and incompatible transfusions in humans. The membranes of

the red blood cells of most individuals contain one blood group substance of type A, type B, type AB, or type O.

Individuals of type A have anti-B antibodies in their plasma and will thus agglutinate type B or type AB blood. Individuals of type B have anti-A antibodies and will agglutinate type A or type AB blood. Type AB blood has neither anti-A nor anti-B antibodies and has been designated the universal recipient. Type O blood has neither A nor B substances and has been designated the universal donor. The explanation of these findings is related to the fact that the body does not usually produce antibodies to its own constituents. Thus, individuals of type A do not produce antibodies to their own blood group substance, A, but do possess antibodies to the foreign blood group substance, B, possibly because similar structures are present in microorganisms to which the body is exposed early in life. Since individuals of type O have neither A nor B substances, they possess antibodies to both these foreign substances.

The ABO Substances Are Glycosphingolipids& Glycoproteins Sharing Common Oligosaccharide Chains The ABO substances are complex oligosaccharides present in most cells of the body and in certain secretions. On membranes of red blood cells, the oligosaccharides that determine the specific natures of the ABO substances appear to be mostly present in glycosphingolipids, whereas in secretions the same oligosaccharides are present in glycoproteins.

Rh blood group system

The Rh system is the second most significant blood-group system in human-blood transfusion. The most significant Rh antigen is the D antigen, the presence or absence of the Rh antigens is signified by the +

or − sign, so that for example the A− group does not have any of the Rh antigens.

Rh factor definition:

- Rhesus (Rh) factor is an inherited trait that refers to a specific protein found on the surface of red blood cells, The proteins which carry the Rh antigens are transmembrane proteins, whose structure suggest that they are ion channels. If your blood has the protein, you're Rh positive the most common Rh factor. If your blood lacks the protein, you're Rh negative.
- Although Rh factor doesn't affect your health, it can affect pregnancy. Your pregnancy needs special care if you're Rh negative and your baby's father is Rh positive.
- If you're pregnant, your health care provider will recommend an Rh factor test during your first prenatal visit. The Rh factor test is a basic blood test that indicates whether you're Rh positive or Rh negative.
- immunization against Rh can generally only occur through blood transfusion or placental exposure during pregnancy in women.

ABO Blood Types

There are four principal types: A, B, AB, and O. There are two antigens and two antibodies that are mostly responsible for the ABO types. The specific combination of these four components determines an individual's type in most cases. The table below shows the possible

permutations of antigens and antibodies with the corresponding ABO type ("yes" indicates the presence of a component and "no" indicates its absence in the blood of an individual).

ABO Blood Type	**Antigen A**	**Antigen B**		**Antibody anti-A**	**Antibody Anti-B**
A	yes	no		no	Yes
B	no	yes		yes	No
O	no	no		yes	Yes
AB	yes	yes		no	No

For example, people with type A blood will have the A antigen on the surface of their red cells . As a result, anti-A antibodies will not be produced by them because they would cause the destruction of their own blood. However, if B type blood is injected into their systems, anti-B antibodies in their plasma will recognize it as alien and burst or agglutinate the introduced red cells in order to cleanse the blood of alien protein and old age.

Individuals with type O blood do not produce ABO antigens. Therefore, their blood normally will not be rejected when it is given to others with different ABO types. As a result, type O people are **universal donors** for transfusions, but they can receive only type O blood themselves. Those who have type AB blood do not make any ABO antibodies. Their blood does not discriminate against any other ABO type. Consequently, they are **universal receivers** for transfusions, but their blood will be agglutinated when given to people with every other type because they produce both kinds of antigens.

Blood group identification procedure:

It is easy and inexpensive to determine an individual's ABO type from a few drops of blood

1. Draw blood from an individual in normal saline solution.
2. taken three separated drops from the blood drawn and placed on the microscope Slide.
3. placed anti-A antibodies on the first drop and anti-B antibodies on the second drop and anti-D on the third.
4. Mix each drop of blood with anti added them separately using a wooden Stick.
5. Then note the agglutination process of red blood cells with the naked eye and then followed the process of hemolysis(decomposition of red blood cells) and you can know the blood group from the interaction between antigen and Antibody.

For example, if an individual's blood sample is agglutinated by the anti-A antibody, but not the anti-B antibody, it means that the A antigen is present but not the B antigen. Therefore, the blood type is A.

Hemoglobin

Hemoglobin (Hb) is the main constitute of erythrocytes, it is a conjugated protein carries oxygen from the lungs to the tissues, and carbon dioxide from the tissues to the lungs, it is made up of heme and globin. Hemoglobin forms an unstable, reversible bond with oxygen. In

its oxygenated state it is called oxyhemoglobin and is bright red. In the reduced state it is called deoxyhemoglobin and is purple-blue.

Each hemoglobin molecule is made up of four heme groups surrounding a globin group, forming a tetrahedral structure. There are four iron atoms in each molecule of hemoglobin, which accordingly can bind four atoms of oxygen.

Heme

The deep red, nonprotein, ironcontaining component of hemoglobi n that carries oxygen. Heme is aporphyrin with an iron atom at its center. One of the free valence electrons of the iron atom of heme isbound to the hemoglobin molecule, while the other is available for binding to an oxyge n atom. Ahemoglobin molecule contains four hemes.

Chemical formula: $C_{34}H_{32}FeN_4O_4$.

globin

The protein component of hemoglobin, made up of two alpha and two beta polypeptide chains. Changes in the amino acid sequence of these chains results in abnormal hemoglobins. For example, Hemoglobin S is a variant form of hemoglobin that is present in persons who have sickle cell anemia, a severe, hereditary form of anemia in which the cells become crescent-shaped when oxygen is lacking.

Hemoglobin is produced in bone marrowby erythrocytes and is circulated with them until their destruction when red blood cells die, the hemoglobin within them is released and broken down in the spleen, and some of its components, such as iron, are transported to the bone marrow by a protein called transferrin and used again in the production of

new red blood cells; the remainder of the hemoglobin becomes a chemical called bilirubin that is excreted into the bile which is secreted into the intestine, where it gives the feces their characteristic yellow-brown color.

What are normal hemoglobin values?

The hemoglobin level is expressed as the amount of hemoglobin in grams (g) per deciliter (dL) of whole blood, a deciliter being 100 milliliters.The normal ranges for hemoglobin depend on the age and, beginning in adolescence, the gender of the person. The normal ranges are:

- Newborns: 17 to 22 gm/dL
- One (1) week of age: 15 to 20 gm/dL
- One (1) month of age: 11 to 15gm/dL
- Children: 11 to 13 gm/dL
- Adult males: 14 to 18 gm/dL
- Adult women: 12 to 16 gm/dL
- Men after middle age: 12.4 to 14.9 gm/dL
- Women after middle age: 11.7 to 13.8 gm/dL

All of these values may vary slightly between laboratories. Some laboratories do not differentiate between adult and "after middle age" hemoglobin values.

What does a low hemoglobin level mean?

A low hemoglobin level is referred to as anemia or low red blood count. Lower than normal number of red blood cells is referred to as anemia and hemoglobin level reflects this number. There are many reasons for anemia.

Some of the more common causes are:

- loss of blood (traumatic injury, surgery, bleeding, colon cancer or stomach ulcer),
- nutritional deficiency (iron, vitamin B_{12}, folate)
- bone marrow problems (replacement of bone marrow by cancer)
- suppression by chemotherapy drugs
- kidney failure
- abnormal hemoglobin structure (sickle cell anemia or thalassemia).

What does a high hemoglobin level mean?

Higher than normal hemoglobin levels can be seen in people living at high altitudes and in people who smoke. Dehydration produces a falsely high hemoglobin measurement which disappears when proper fluid balance is restored.

Some other infrequent causes are:

- advanced lung disease
- certain tumors,
- a disorder of the bone marrow known as polycythemia

Hemoglobin estimation by sahli's method

Hemoglobinometer

an instrument that is used to determine the quantity of hemoglobin in the blood. It is based on comparison of the color of the tested blood, which is treated with hydrochloric acid, with the color of standards. It consists of two color standards and test tubes with two calibrations to determine hemoglobin in gram percent and percent (100 percent

corresponds to 16 gram percent; each gram corresponds to 6 percent). In many countries hemoglobinometers are used in which 100 percent on the scale corresponds not to 16 gram percent, but to 14.8 gram percent, 17.3 gram percent, and so forth. When blood is treated with a hydrochloric acid solution, the hemoglobin is converted to hematin hydrochloride, and the solution turns brown. The solution in the test tube is diluted by gradually adding distilled water until the color of the solution is the same as that of the standard. The quantity of hemoglobin is determined by reading the level of the solution on the scale of the test tube.

The amount of hemoglobin in the blood can be determined the chemical method (Drabkin method), which is based on the determination of iron in the hemoglobin molecule. Although this method is more accurate than the hemoglobinometer, it is not widely used because of their laboriousness.

Materials used

1. Sahli's haemoglobinometer.
2. Comparison tube and pipette (Hemoglobin pipette with rubber tubing and mouthpiece)
3. 2 Pasteur pipettes (one for HCl and one for distilled water)
4. 5. Glass rod to stir (stirrer)
5. Hydrochloric acid
6. Distilled Water

Procedure

1. Add hydrochloric acid (1: 10 diluted) to the haemometer tube (comparison tube) up to lowest graduation (0.02 gram)
2. Sterilize the fingertip with Isopropyl alcohol surgical spirit and allow it to dry
3. Using sterile lancet prick the finger tip
4. Wipe away first few drops of blood
5. Suck blood into the hemoglobin pipette (capillary pipette) up to 20 µL (avoid air bubbles coming into a tube)
6. Wipe the outside tip of pipette clean with tissue
7. Immediately transfer the blood to the comparison tube
8. Suck blood back into the pipette several times and blow out again into the tube (to mix blood with HCl)
9. Place the haemometer tube in the stand and allow for 10 minutes (during this period HCl lysis red cell and released hemoglobin on reacting with Forms a dark brown colored acid Hematin)
10. Now using a Pasteur pipette to add a few drops of distilled water and stir the contents with a glass rod .
11. Continue to add water drop by drop and stir the contents each time until the solution is just darker than the standard .
12. Carefully add one or two drops of water till the color exactly matches with that of the Standard and note the reading .
13. while taking the reading hold the haemoglobinometer against good daylight at arms length
14. The comparison tube represent 100 percent haemoglobin with reference to a standard which is 14. 8 g Hb / 100 ml of blood

Estimation of Blood Glucose

Glucose is a simple sugar which is a permanent and immediate primary source of energy to all of the cells in our body. . Glucose is classified as a monosaccharide because it cannot be broken down further by hydrolysis. It is further classified as a hexose because of its six-carbon skeleton and as an aldose, because of the presence of an aldehyde group on carbon 1. The aldehyde group condenses with a hydroxyl group so that glucose exists as a hemiacetal ring structure. This ring structure explains many of the reactions of glucose. The glucose in blood is obtained from the food that you eat. This glucose gets absorbed by intestines and distributed to all of the cells in body through bloodstream and breaks it down for energy.

The concentration of glucose in blood, expressed in mg/dl, is defined by the term glycemia. The value of blood sugar in humans generally ranges from (65 – 100) mg/dl. Blood sugar levels are regulated by the hormones insulin and glucagon which act antagonistically. These two hormones are secreted by the islet cells of the pancreas. When the blood glucose levels are high, insulin hormone secreted which causing liver to convert more glucose molecules into glycogen and when the blood glucose levels are low glucagon secreted and act on liver cells to promote the breakdown of glycogen to glucose and increases the blood glucose concentrations. Essentially blood glucose levels determine the time of secretion of these hormones.

The blood glucose level is easily changed under the influence of some external and internal factors such as body composition, age, physical activity and sex. Diabetes is a disease related by the abnormal metabolism of blood sugar and defective insulin production. So blood sugar levels are an important parameter for the study of diabetes. The level of glucose circulating in blood at a given time is called as blood glucose level. The blood glucose level varies at different time on various part of the day. Hypoglycemia is a possible side effect of diabetes medications in which blood glucose level drops below 65mg/dl. In people

with diabetes, the body doesn't produce enough insulin or respond to insulin properly. The result is that sugar builds up in the blood stream, damaging the body's organs, blood vessels and nerves. This condition in which too much sugar in the blood stream is called hyperglycemia.

The blood glucose analysis is used to detect both hyperglycemia and hypoglycemia.Glucose concentration may be determined in whole blood, plasma, or serum samples.

Clinical Biochemical finding in diabetes

1. Presence of large amount of glucose in urine.
2. Large volume of urine (Polyuria)
3. Polyphagia i.e. eats more frequently.
4. Increased catabolism of fat so there is increase in free fatty acid level in blood & liver.
5. Increased acetyl CoA is seen which further lead to increase formation of cholesterol & hence at formation of atherosclerosis.
6. Increased ketone bodies in blood & its appearance in urine leads to acidosis.
7. Increased catabolism of tissue protein for energy requirement lead to loss of weight & increased level of amino acid in blood which lead to more formation of urea by deamination of amino acid.

Types of samples of blood sugar:-

1) Fasting blood Sugar (FBS) : The blood sample is collected after the patient fasts for (9-12) hours or overnight.
2) Random Sample : Blood is collected at any time without prior preparation of the patient.

The normal fasting blood glucose varies from 65-100mg/dl and post prandial from 100-140 mg/dl. The appear limit increase with age and during pregnancy.

Hyperglycemia : Causes are :

1. Diabetes mellitus: It is a chronic disease due to disorder of carbohydrate metabolism, cause of which is either deficiency or diminished level of insulin resulting in hyperglycemia

(increased blood glucose level & presence of glucose in urine). Secondary metabolic defect is also seen, such as metabolism of proteins & fats.

Diabetes mellitus types:

a) Juvenile Diabetes or Type I Diabetes or Insulin dependent Diabetes Mellitus (IDDM) it is less frequent,Occurs before the age of 15 years.Due to less production of insulin from β cells of Langerhans (Pancreas)

b) Type II diabetes also called Non-insulin dependent Diabetes mellitus (NIDDM)it is more frequent in population. Occurs at middle age.β cell is degenerated to some extent but response to glucose load is seen.

1.
2. Hyperactivity of thyroid or adrenal gland.
3. Surgical removal of pancreas, pancreatitis, carcinoma of pancreas.
4. Drug induced e.g. : thiazide diuretics.

Hypoglycemia : When blood glucose falls below 60 mg/dl.

1. Most commonly seen due to over dosage of insulin in treatment of diabetes mellitus.
2. Hypothyroidism .
3. Insulin secreting tumors of pancreas – rare.
4. Severe exercise.
5. Starvation.
6. Chronic alcoholism
7. severe liver disease like – glycogen storage disorders

Collection of Blood Sample : Blood is usually collected from a vein and kept in a bottle containing sodium fluoride (Na F): potassium oxalate mixed at proportion of 1 : 3 Usually 4 mg of the mixture is required. Both substances act as anticoagulant and Na F prevents glycolysis in RBC's by inhibiting the enzyme 'enolase'.

Methods of Estimation : blood sugar can be estimated by a variety of ways

1. Enzymatic method : Measure only glucose in blood.

a. **Glucose Oxidase Method :** Glucose oxidase catalysis the oxidation of glucose to glucuronic acid and hydrogen peroxide. The hydrogen peroxide formed is broken down to water and oxygen by peroxidase in the presence of an oxygen acceptor which itself is converted to a colored compound, the amount of which can be measured calorimetrically. This method is highly specific for glucose and does not involve any other sugar or non-reducing substances.

$$\text{Glucose} + H_2O + O_2 \longrightarrow \text{Glucuronic acid} + H_2O_2$$

b. **Hexokinase Method**: In this

$$\text{glucose} + ATP \longrightarrow \text{glu-6-P} + ADP$$

$$G-6-P + NAD \longrightarrow \text{6phosphogluconate} + NADH + H^+$$

The rate of formation of NADH is followed spectrophotometrically at 340 nm.

2. **Reduction Methods**

Alkaline Copper Reduction Methods (Asatoor& King's method) This method utilizes the reducing property of sugar but other certain reducing substances such as glutathione, ascorbic acid, uric acid, creatine, etc. are also estimated by this method giving slightly higher values upto 20-30mg% than the normal blood sugar values.

Estimation of Blood Glucose by method of asatoor and King (modified)

Principle :

Reducing sugars in hot alkaline medium(copper sulphate) produce cnediols which are strong reducing agents that convert Cu^{++} ions to Cu^{+} ions combine with OH ion to form yellow CuOH which on heating gives red Cu_2O, Cu_2O produced is proportional to the amount of reducing sugar, Cu_2O reduced Phosphemolybdic acid to molybdenum blue which can be estimated colorimetrically.

Modified method gives values close of true glucose. This is achieved by diluting blood with isotonic ($CuSO_4$&Na_2SO) solution to prevent hemolysis of RBC. Protein must be removed before proceeding with the

determination, and this is carried out using sodium tungstate solution as the protein precipitant removed by centrifugation .

So method used is modification by Asatoor and King in 1954 and involves:

- Precipitation of blood proteins.
- Reduction of alkaline CuSo4 solution to cuprous oxide by glucose.
- Estimation of extent of reduction of blue colored complex by colorimetric measurement at 650 nm.

Reagents :

i. Isotonic solution : Na2SO4. 10H2O and $CuSO_4$. 5H2O
ii. Sodium tungstate 10%.
iii. Alkaline copper sulphate solution consists of two solutions:-

a) Dissolve 25g NaHCO3, 20gm anhydrous Na3CO3 and 18 g Pot. Oxalate in about 500 ml of water. Add 12 g of sodium potassium tartrate and make up the volume to 1 L.

b) Prepared by dissolving 1.3g of copper sulphate in 100ml of distilled water. Working solution is prepared by mixing equal volume of A and B.

iv. phosphomolybdic acid solution : To 35 g of molybdic acid add 5 g of sodium tungstate, and 200 ml of 10% NaOH. Boil for 30- 40 min to remove ammonia. Cool. dilute to about 350ml and add 125 ml of conc. (85%) phosphoric acid. Make volume up to 500 ml.
v. stock glucose Standard solution : dissolve 100mg glucose in 100ml benzoic acid.
vi. Working glucose standard solution : Dilute stock 1 ml to 100 ml so conc. Is 10 mg% with isotonic CuSo4 solution.

Procedure

1- Precipitation step:- in two centrifuge tubes, add 3.7 ml of isotonic (sodium sulphate+ copper sulphate) solution for each tube, then place (0.1ml) blood serum to the first tube(test) and (0.1ml) distilled water to the second (blank) and mix. Add 2 ml of sodium tungstate solution and mix. Centrifuge at 2000 rpm for 10 min Use clear supernatant for test.
2- Reduction step:- label 4 test tubes in the order given below:

	T	test blank	SB	B
T.Supernatant	1.0ml	-	-	-
B.Supernatant	-	1.0ml	-	-
Std. Glucose	-	-	1.0ml	-
Distilled water	-	-	-	1.0ml

3- Add (1ml) of alkaline copper sulphate in each test tube. Mix well. Plug the mouth of the tubes with cotton wool and put in boiling water bath for 10 min. After 10 min take out the tubes and cool them.
4- Color development step: add 3 ml of phosphomolybdic acid in all the test tubes. Then add 8 ml of distilled water to each tube. Mix, wait for 10 min, read O.D. at 650 nm.

Calculation :

$$\text{Blood glucose (mg/dl)} = \frac{\text{OD of Test}}{\text{OD of Std}} \times 80$$

Proteins

Blood is a complex mixture of cells suspended in a fluid medium, plasma. 90% of this fluid medium is water and the remaining 10% is dissolved proteins, minerals, glucose, etc. By far, the largest amount of the total solutes are the plasma proteins, collectively referred to as TOTAL PROTEIN. In serum, one of the plasma proteins, FIBRINOGEN, is missing, but it only constitutes 3-6% of the total protein in plasma. To standardize normal values, measurement of total protein has been confined primarily to serum samples. Serum proteins are important in maintaining the osmotic pressure and the pH of the blood. Liver produced fibrinogen and prothrombin proteins play a major role in coagulation.

Fibrinogen constitutes 0.2-0.4 g/dL of the protein in plasma. There is a small diurnal variation of total plasma protein of about 0.5 g/dL.

Serum total protein, also called plasma total protein or total protein, is a biochemical test for measuring the total amount of protein in blood plasma or serum. Protein in the plasma is made up of albumin and globulin. The globulin in turn is made up of α1, α2, β, and γ globulins. These fractions can be quantitated using protein

electrophoresis, but the total protein test is a faster and cheaper test that estimates the total of all fractions together.

- **Albumin** is made mainly in the liver. It helps keep the blood from leaking out of blood vessels. Albumin also helps carry some medicines and other substances through the blood and is important for tissue growth and healing.
- **Globulins** are made up of different proteins called alpha, beta, and gamma types. Some globulins are made by the liver, while others are made by the immune system . Certain globulins bind with hemoglobin . Other globulins transport metals, such as iron, in the blood and help fight infection. Serum globulin can be separated into several subgroups by serum protein electrophoresis. This test is often done to diagnose nutritional problems, kidney disease or liver disease, and If total protein is abnormal, you will need to have more tests will need to be done to look for the exact cause of the problem.

Normal Results

The normal range is 6.0 to 8.3 g/dL (grams per deciliter). The normal range of serum albumin is 3.7-5.3 g/100 ml. Serum globulin ranges from 1.8 to 3.6 g/100 ml. the A:G ratio is roughly 2:1 though it may range from 1.2:1 to 2.5:1. Normal value ranges may vary slightly among different laboratories.

What Abnormal Results Mean

Higher-than-normal levels may be due to:

- Chronic inflammation or infection, including hepatitis B or C
- Multiple myeloma dehydration

Lower-than-normal levels may be due to:

- Bleeding
- Liver disease
- Malabsorption
- Malnutrition
- Nephrotic syndrome

Methods of Determination

a) Kjeldahl Method
b) Folin-Ciocalteu
c) Refractometer
d) Biuret Method

The most widely used method is still the biuret method. (the name derives from the reaction between biuret and cupric ions in an alkaline medium.

Biuret Method

Principle :

All soluble proteins, on addition of a copper salt in alkaline solution (NaOH), give purple colored complex which is generally known as "biuret." The intensity of the violet colour that is formed is proportional tothe number of peptide bonds which, in turn, depends upon the amount of proteins in the specimen. This method uses a standardized alkaline copper tartrate solution. The reaction occurs in the peptide bonds of tripeptides or larger. The globulin in serum is precipitated by the addition of 28% sodium sulfite (salting out). Ether is added to help separate the precipitated globulin by centrifugation. The chemical determination of albumin is based on the formation of a purple colored complex (biuret) between alkaline copper sulfate and the albumin. Globulin is calculated by subtracting the measured albumin from the measured total protein.

Globulin = Total Protein - Albumin

Reagents

1. Biuret Reagent – 3 mg of copper sulphate is dissolved in 500 ml ofwater. 9 g of sodium potassium tartrate and 5 g of potassiumiodide are added and dissolved. 24 g of sodium hydroxide, dissolved separately in 100 ml of water is added. The volume is made up to 1liter with water. The reagent is stored in a well-stoppered polythene bottle.
2. sodium sulfite (28%): – dissolve (280g) of anhydrous sodium sulfite in (1L)of distilled water and kept at (37).

3. Standard protein solution :- – this is prepared by dissolving (0.5 g) bovine albumin in (100ml) distilled water .
4. Ether

Procedure :

1. In a centrifuge tube, take (0.5ml)of serum. Add (5.5ml) of 28% sodium sulphite. Gently rotate the centrifuge tube, so that the serum is evenly distributed in the solution. Add (1ml) of ether and gently shake it upside down slowly 20 times. Centrifuge for (10)min.
2. Label 4 test tubes (test of albumin, test of total protein, standard and blank), measure (5ml) of biuret reagent into each.
3. Put (2ml) of supernatant in <test of albumin> test tube, (0.2ml) serum with (1.8ml)normal saline in < test of total protein> with mixing, place (2ml) of standard protein solution into <standard> tube while in the <blank> tube, put (2ml) normal saline.
4. Mix well
5. Incubate at (37) C for (10)min.
6. Read optical density at 540nm.

Calculations

$$\text{Serum total proteins (g/100 ml)} = \frac{A_{total} - A_B}{A_{stad.} - A_B} \times 5$$

$$\text{Serum total proteins (g/100 ml)} = \frac{A_{alb.} - A_B}{A_{stad.} - A_B} \times 6$$

Since the colorimetric measurement of albumin is much simpler than that of globulin, the concentrations of total proteins and albumin are measured in serum, and globulin is obtained by difference.

Globulin = Total Protein - Albumin

Kidney function tests

Since the kidneys perform a multitude of functions, a single test cannot give information about the entire range of renal functions. A group of tests is required to evaluate the different renal functions. the results of

renal function tests may some-times be affected by extra-renal factors. Therefore, the results must always be interpreted in conjunction with the clinical picture.

The more important and commonly employed tests are:-

1) Blood urea
2) Serum creatinine
3) uric acid.

I- Urea Estimation & Blood Urea Nitrogen (BUN)

Urea is main end product of protein catabolism from deamination of amino acids. It is formed in the liver by urea cycle and is excreted by the kidneys through urine. Urea represents about 45-50% of the non-protein nitrogen (NPN) of blood and 80-90% of the total urinary nitrogen excretion.

Blood urea nitrogen varies directly with protein intake and inversely with the rate of excretion of urea.

The normal blood urea level is (15-45)mg/dl. It is slightly higher in males than in females. Elevated serum urea levels may be due to renal failure, acute upper gastroin hemorrhage, sustained hyperpyrexia. The term **uremia** is used to describe the presence of excessive amounts of urea and other nitrogenous waste in the blood.

Slight increases in urea (not more than three times the upper limit of the reference range) may occur when there is dehydration, diuretic therapy, gastrointestinal blood loss and any condition associated with increased protein breakdown such as malaria, typhoid, and surgical operations. Decreased serum urea levels could be due to pregnancy, low antidiuretic hormone secretion, low protein intake, severe liver diseases.Blood urea is represented as blood urea nitrogen (BUN). The (mg/dl) urea can be converted into BUN and vice versa by the following factor:

BUN= urea(mg/dl) × 0.467

And urea (mg/dl)= BUN× 2.14

Urea diffuses freely in and out of the red blood cells so its concentration both in cells and plasma is nearly the same. It is immaterial whether whole blood, plasma or serum is used for blood urea estimation.

Urea can be estimated by the following methods:

1- **Enzymatic using urease :**

a) Neseler's Method : Urea is converted to ammonia by the enzyme urease. Ammonia is made to react with Neseler's reagent (Potassium mercurie iodide) giving rose to a brown coloured compound which is read at 450nm the enzyme acts optimally at 55oC
pH 7.0 to 8.0 and is inhibited by ammonia and fluoride. Disadvantages are turbidity, colour instability and non linear, calibration beside susceptibility to contamination with NH3 from the laboratory and endogenous ammonia in the specimen.

b) **Berthelot Reaction :**

In this NH_3 reacts with phenol in presence of hypochlorite to form an indophenol which gives a blue coloured compound in alkali. Nitroprusside acts as a catalyst increasing the rate of reaction, intensity of colour obtained and its reproducibility.

c) In the urease/glutamate dehydrogenase method, glutamate

production from ammonia and 2.0 x 0- glutarate is measured by the absorbance change at 340nm. Owing to concomitant conversion of NADH+ to NAD+. Each molecule of urea hydrolysed causes the production of two molecules of NAD+.

2- **Kinetic Method:** GLDH method

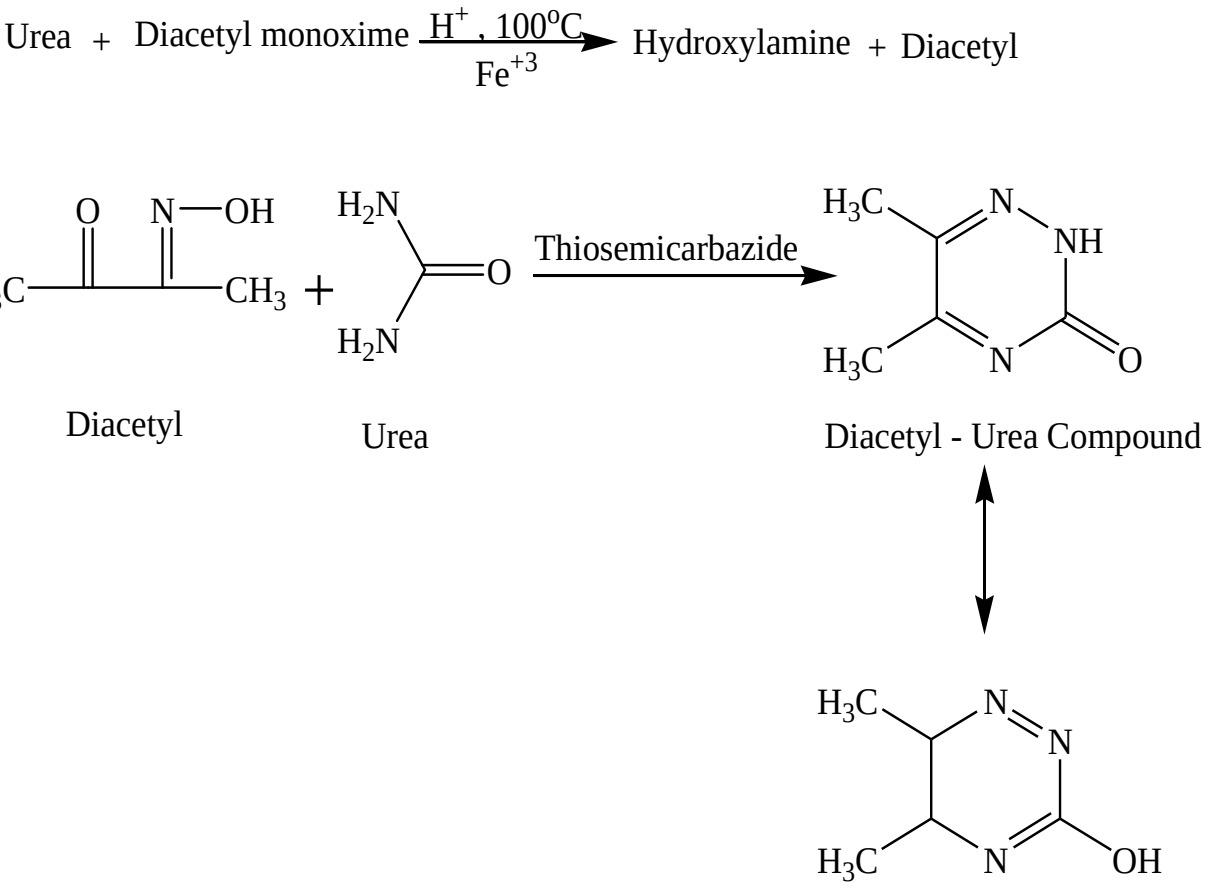
Urea is hydrolysed in presence of urease to produce ammonia and CO2 the ammonia produced combines with alfa oxoglutarate and NADH in presence of GLDH to yield glutamate and NAD+ The decrease in extintion due to NADH in unit time is proportional to the urea concentration.

3- **Colorimetric Method :** Diacetyl Monoxime Method(DAM)

PRINCIPLE: Urea reacts with Diacetyl monoxime (DAM) at high temperature in an acid medium in the presence of ferric ions and

thiosemicarbazide. At high temperature, the DAM first decomposes to give hydroxylamine and diacetyle.The diacetyle then condenses with urea in the presence of thiosemicarbazide to give a purple (purple/red) color which is directly proportional to the urea present in serum or plasma. The absorbance of the purple (red) color produced is measured in a spectrophotometer at 520 (510-540) nm. . This method is linear only upto 300mg% urea. For higher values if expected, the blood sample should be diluted. The reaction formulas are described as bellow.

Thiosemicarbazide and ferric ions are added to catalyse the reaction and increase the intensity of colour. Thiosemicarbazide intensify the reaction and stabilize the red product to light.



SPECIMENS & MATERIALS

1. Mixed acid reagent (Ferric chloride + phosphoric acid + conc. sulfuric acid)
2. Mixed color reagent (Diacetyl monoxime + thiosemicarbazide)
3. Test sample.
4. Standard solution (5 mmol/l)
5. Distilled water
6. Test tubes
7. Pipette
8. Water bath

Caution: Concentrated solutions of formaldehyde and hydrochloric acid are highly corrosive and should not be pipetted by month. Use of a safety pipette is recommended

Reagents

1) **Reagent A :** Dissolve 5g of ferric chloride in 20ml of water. Transfer this to a graduated cylinder and add 100ml of orthophosphoric acid (85%) slowly with strring. Make up the volume to 250ml with water. Keep in brown bottle at 4oC.

2) **Reagent B :** Add 200 ml conc, H2SO4 to 800 ml water in 2L flask slowly with stirring and cooling.

3) **Acid Reagent :** Add 0.5 ml of reagent A to 1 L of reagent B. keep in brown bottle at 4oC.

4) **Reagent C :** Diacetyl monoxime 20g/L of water. Filter and keep in brown bottle at 4oC.

5) **Reagent D :** Thiosemicarbazide 5g/L of water.

6) **Colour Reagent :** Mix 67 ml of C with 67 ml of D and make up the volume to 1000 ml with D.H2O keep in brown bottle at 4oC.

7) **Stock urea standard :** 100mg/100 ml water.

8) **Working urea standard :** Dilute 1 ml stock to 100ml with DH2O so conc. is 1 mg/100ml.

Procedure :

1-prepare two big test tubes, label them, one for test (0.1 ml (serum/plasma) is diluted to 20 ml) the other for standard (0.1ml of standard urea solution is diluted to 20ml by distilled water).

2- set up three test tubes as follows :

B	**T**	**S**
2ml of D.W	2ml of test above	2ml of std. Above

3- to each tube add 4ml of reagent C

4- . Keep them in a boiling water bath for 20 minutes.

5- Remove from water bath and cool the tubes for 5 minutes.

6- Set the spectrophotometer at 520nm and measure the absorbance (A) of the tubes. Measurement should be taken within 30 minutes since the red color will fade after 2 hours.

Calculation :

$$\text{Blood urea (mg/dl)} = \frac{\text{O.D. Test.}}{\text{O.D. Std.}} \times 200$$

(To convert from mmol/l to mg/dl, multiply by 6. To convert from mg/dl to mmol/l, divide by 6) because the M.wt of urea is 60 g/mole.

Estimation Of Serum Creatinine

Creatinine is a waste product formed in muscle by creatine metabolism. Creatine is synthesized in the liver which then passes into circulation where it is taken up by skeletal muscle for conversion to creatine phosphate which serves as storage form of energy in skeletal muscles. Creatine and creatine phosphate are spontaneously converted to creatinine at a rate of about 2% the total per day. This is related to muscle mass and body weight. Creatinine formed is excreted in the urine. On a normal diet almost all creatinine in urine is endogenous(muscles and tissues){while urea amount depends on exogenous(diet proteins) metabolism}. Its excretion is fairly constant from day to day and has been used to check the accuracy of 24 hours urine collection. It is independent of urine flow rate and its level in plasma is quite constant.
Normal serum creatinine levels are:

REFERENCE VALUES

Serum or plasma:	
0,7 - 1,4 mg/dL = (61.8 – 123.7)µmol/L	Male
0,6 - 1,1 mg/dL = (53.0 – 97.2) µmol/L	Female
Urine: 15-25 mg/Kg/24 h	
10 - 20 mg/Kg/24 h =(88– 177)µmol/Kg/24 h	Male
8 – 18 mg/Kg/24 h =(71– 177) µmol/Kg/24 h	Female

It is higher in males since it is related to body size. Increased serum levels are seen in renal failure and other renal diseases in a manner similar to urea. Creatinine, however, does not increase with age and dehydration . It is also not affected by diet.

Creatinine clearance : is widely used to approximate glomerular filtration. You need a timed urine sample and a blood sample. The clearance of a substance: is the volume(ml) of plasma "cleared" of that substance per unit time (min).

Clearance (ml/min) = (conc. in urine) x (urine volume ml)

(conc. in plasma) X time of urine collection (min.).

In deciding how to "time" your collection, remember that you don't really need to collect urine for a full 24 hours. One group got more reliable results by a controlled collection over 4 hours, monitoring body position (kept them lying down) and hydration with body surface area measurement. Creatinine clearance is not a perfect measure of GFR, because some is not filtered and some is secreted into the proximal tubule. These fractions tend to cancel each other out in health, but when GFR drops below 30 mL/min, tubular secretion approaches or even exceeds the amount filtered at the glomerulus.

*Also, lots and lots of red meat (protein and creatinine-rich) can lead to overestimates (maybe 30%) in GFR in renal failure patients.

Reference range for creatinine clearance is 90-120 mL/min for young adults; values tend to fall by around 0.5 mL/year over age 20, worse for hypertensives .

* creatinine clearance is a pretty good estimate of glomerular filtration rate except at very low values, when tubular secretion of creatinine become proportionately greater.

Estimation of serum creatinine by Jaffe's Alkaline Picrate Method

Principle :

Creatinine in alkaline medium reacts with picric acid to form a red creatinine picrate. The time interval chosen for measurements avoids interferences from other serum constituents. The intensity of the color formed is proportional to the creatinine concentration in the sample. the intensity is measured at 520nm.

Creatinine + Picric acid + alkaline pH → 2,4,6 trinitrophenol

(Janovski's complex)

measured at 520 nm

The two chief disadvantages with Jaffe's reaction are:

- Lack of specificity :- Only 80% of the colour developed is due tocreatinine in serum. other non specific chromogens that react with picric acid are proteins, ketone bodies, pyruvate, glucose, ascorbate etc.
- Sensitivity to certain variables like PH temperature etc.

Reagents:

1) Picric acid – 0.04M (9.16g/L)
2) Sodium hydroxide – 0.75N
3) Sodium tungstate – 10%
4) 2/3 N H_2 SO_4
5) Creatinine standard stock – 100mg%
6) Working standard – 3mg%

Procedure :-

1- In a centrifuge tube, take 0.5ml of serum with 3.5 ml of distilledwater. Precipitate proteins by adding 0.5ml sodium tungstate and 0.5ml of(0.5 N) sulphuric acid drop by drop with constant shaking. Stand for 10 minutes, put the tube in the centrifuge for 10min, then filter.

2- Use protein free filtrate for test.

3- Make three test tubes as follows and add :

	B	**S**	**T**
Filtrate			2.5 ml
Standard	-	1.0 ml	-
D. Water	2.5 ml	1.5ml	-

1- To each tube add 2.5ml of sodium picrate solution(NaOH+ Picric acid).

2- Mix well Allow to stand for 15 min. then read absorbance at 520nm.

Calculation :

$$\text{Serum creatinine (mg/dl)} = \frac{\text{O.D. Test}}{\text{O.D. Std.}} \times \frac{\text{Amount of Std.}}{\text{Vol. of Serum}} \times 100$$

Conversion factor: mg/dL x 88.4 = µmol/L.

notes :

1- the Jaffe's reaction for measuring serum creatinine is not assensitive and reliable as method for urea. It is interfered with by Ketone bodies and glucose and hence false high values may be obtained in diabetes ketoacidosis.

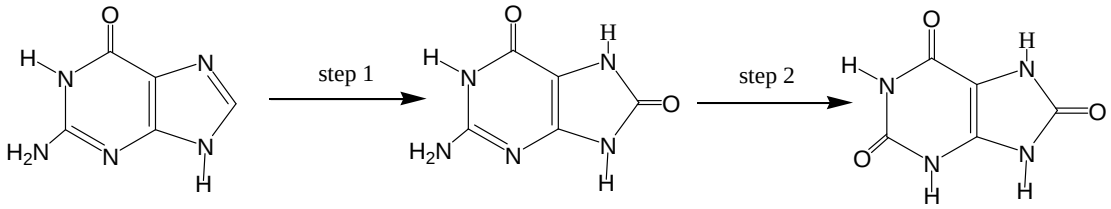
2- How serum creatinine is not significant. It is associated with muscle wasting diseases.

3- Creatinine stability: 24 hours at 2-8ºC in Serum or heparinized plasma.

4- In Urine: Dilute sample 1/50 with distilled water. Mix. Multiply results by 50 (dilution factor);you do not need protei precipitation and Creatinine stability: 7 days at 2-8ºC.

Estimation Of Serum Uric Acid

Uric acid is the end product of purine metabolism in man formed by oxidation of Purine bases that circulates in plasma as sodium urate and is excreted by kidney. It is derived from the breakdown of nucleic acids that are ingested or come from the destruction of tissue cells; it is also synthesized in the body from simple compounds , Adenine and guanine are probably two of the most famous purines as they are DNA bases. Figure (1) shows how one of the four DNA bases, guanine, is metabolised into uric acid *via* the intermediate xanthine.



guanine xanthine uric acid

It is possible to treat gout with allopurinol a chemical which works by inhibiting xanthine oxidase the enzyme that produces uric acid from xanthine in step 2.

REFERENCE VALUES: Serum or plasma:

Women 2.5 - 6.8 mg/dL = 149 – 405 µ mol/L
Men 3.6 - 7.7 mg/dL =214 – 458 µ mol/L
Urine: 250 - 750 mg/24 h = 1.49 - 4.5 mmol/24 h

Hyperuricaemia :

- Seen in gout. Estimation is important in diagnosis and management of the disease.
- Renal failure due to decreased excretion. Blood urea also raised.
- Conditions of increased turnover of cells as in leukemia.
- Toxaemia of pregnancy.
- Diabetes Mellitues.
- Starvation.
- Drugs like diuretics.

Hypouricaemia :

- Liver diseases (where maximum synthesis of uric acid occurs) like cirrhosis.

- Renal disease that decrease renal tubular reabsorption .
- Drugs-uriconsuric in large does like salicylates.

methods of uric acid estimation :

1- Enzymatic Method: the enzyme uricase is used to oxidize uric acid to allantoin. The amount of uric acid oxidized is obtained by observing the decrease in O.D. at 293nm after the action of uricase.

2- Caraway's Method of Estimation:

Principle:- Phosphotungstic acid in alkaline medium oxidizes uric acid to allantion and itself gets reduced to tungsten blue which is estimated colorimetrically at 700mm.

Reagents :

(1) Sodium tungstate 10%.

(2) 2/3 N Sulphuric acid.

(3) Tungstic acid: Add 50ml of 10% sodium tungstate 50ml 2/3 N H_2SO_4 and a drop of phosphoric acid with mixing to 800ml water. Discard when cloudy. Store in brown bottle.

(4) Phosphotungstic acid: Stock-Dissolve 50g sodium tungstate in about 400ml of water. Add 40ml 85% phosphoric acid and reflux gently for 2 hours, cool, make volume to 500m. store in brown bottle. Dilute 1 to 1 for use.

(5) Na_3CO_3 10%.

(6) Standard uric acid solution stock-100mg%.

(7) Working uric acid solution-1mg%.

Procedure :

1- In a centrifuge tube pipette 0.6ml serum and add 5.4ml tungstic acid while

shaking. Centrifuge for 10min and process as follows.

	B	**T**	**S**
1. Standard uric acid (1mg%) ml.	-	-	1.0
2. Supernatant (ml)	-	3.0	-
3. D. Water (ml)	3.0	-	2.0
4. Na_3CO_3 (ml)	0.6	0.6	0.6
5. Phosphotungstate (ml)	0.6	0.6	0.6

Mix well stand at room temperature for 30 min. Read absorbance at 700 nm.

Precautions :

a) Serum must be used for test as certain substances in RBC like glutathione can also reduce and give false high colour.

b) Some uric acid estimation is carried down with the protein precipitate giving low results.

c) Lithium salts may be added to prevent, turbidity in the final coloured solutions.

d) Sometimes cyanide is added to increase the colour intensity.

Calculations:

$$\text{Serum Uric acid} = \frac{\text{O.D. Test}}{\text{O.D. Std.}} \times \frac{\text{Conc. of Std.}}{\text{Vol. of Serum}} \times 100$$

M.wt of uric acid is 168.11 g/mole.

Estimation of Serum Bilirubin

Biliburin is derived from the destruction of red cellsin the reticuloendothelial system. It is normally conjugated with glucuronic acid to make it more soluble, and excreted in the bile.
Serum bilirubin is in two types:

1- Conjucated or direct bilirubin: it is bilirubin glucuronide. It is water soluble.
2- Unconjucated or indirect bilirubin: it is albumin bound bilirubin, water insoluble, soluble in alcohol.

Both conjugated bilirubin and unconjugated bilirubin may be present in plasma. Conjugated bilirubin is water soluble. Unconjugated bilirubin is not water soluble and binds to albumin from which it may be transferred to other proteins such as those in cells membranes. It is neurotoxic, and if levels rise too high in neonates, permanent brain damage can occur.
Bilirubin excreted in urine as urobilinogen and in feces as stereobilinogen which is responsible for the brown color of feces. If bilirubin does not reach the gut, stools become pale in colour. Bilirubin in the gut is metabolized by bacteria to produce stereobilinogen. When high levels of conjugated bilirubin are being excreted, urine may be a deep orange colour, particularly if allowed to stand.

REFERENCE VALUES
Bilirubin Total
Up to 1.10 mg/dL=18.81 µmol/L
Bilirubin Direct
Up to 0.25 mg/dL=4.27µ mol/L

Any increase of bilirubin in the blood is an indication of Jaundice, which is yellow discoloration of the skin due to the presence of bilirubin in the plasma and is not usually detectable until the concentration is greater than about 1.1 mg/dl. there are three main reasons why bilirubin levels in the blood may rise:

1- *Haemolysis.* The increased haemoglobin breakdown produces bilirubin which overloads the conjugating mechanism.

2- *Failure of the conjugating mechanism within the hepatocyte.*
3- *Obstruction in the biliary system*

Principle: When bilirubin react with diazotized sulfanilic acid (Ehrlich's Reagent), bilirubin is converted to azobilirubin molecules which give a red purple colour in acidic medium, the intensity of which is read colorimetrically . Both conjugated and unconjugated bilirubin give purple azobilirubin with diazotized acid. Conjugated bilirubin can react in aqueous solution (Direct Reaction), whereas unconjugated requires an accelerator or solubilizer , such as methanol (Indirect Reaction-which gives total bilirubin).

Reagents :

1- Diazo reagent : Make freshly before use the mixing 10 ml of solution A and 0.3ml of solution B. Solution A : 1g of sulphanilic acid and 15ml of concentrated HCL per liter in water. Solution B : 0.5g of sodium nitrite/100ml in water. This solution should be kept in refrigerator renewed monthly.
2- Diazo Blank : 15ml of conc. HCL/liter in water.
3- Methanol
4- Bilirubin standard : Dissolve 10mg bilirubin in a minimum (about 5ml) of 0.1N sodium solution, as quickly as possible (since it is unstable in alkaline solution) and make volume with human citrated plasma (obtained from blood bank from outdated bottles. Plasma is left in sunlight for some hours before use to destroy bilirubin present). To be kept frozen in small fractions (for practical's methanol may replace plasma for making volume)

Procedure :

1- Label 4 test-tubes and proceed as follows :

	Control	**Standard**	**Total Bilirubin**	**Conj. Bilirubin**
Serum	-	0.2ml	0.2ml	0.2ml
Standard Sol. (10mg/dl	-	5ml	-	-

D. Water	1.8ml	-	1.8ml	4.3ml
Diaze Blank	0.5ml	-	-	-
Diazo reagent 0.5ml	-	-	0.5ml	
Methanol	2.5ml	-	2.5ml	-

2- Mix and allow to stand in dark for 30 min.

3- Read absorbance in next 10 min at 540nm using D. water as blank.

Calculation :

$$\text{Total S. Bilirubin (mg/dL)} = \frac{T(tot)-T(tot)c}{A\ STD} \times \text{Amount of std} \times \frac{100}{\text{Vol. of Serum}}$$

$$\text{Conj. S. Bilirubin (mg/dL)} = \frac{T(tot)-T(tot)c}{A\ std} \times \text{Amount of std} \times \frac{100}{\text{Vol. of Serum}}$$

Serum value of unconjugated bilirubin may be derived by subtracting the value of conjugated bilirubin from total bilirubin.

Conversion factor: mg/dL x 17.1 =µ mol/L.

Lipid profile: For lipid profile estimation following tests are performed

1- Total lipids : Principle lipids react with sulphuric acid, phosphoric acid and vanillin to form pink colour complex. Normal values 400-1000 mg/dl
2- Phospholipids : It is a fully enzymatic method which uses three different enzuymes- phospholipase D, Choline oxidase and per oxidase to developed colour which is measured at 500 nn. Normal range 160-270 mg/dl
3- Triglycerides :
4- Cholesterol :
5- HDL :
6- LDL : Cholesterol – HDL + VLDL
7- VLDL : TG/5

Estimation of Serum Cholesterol (Total)

Cholesterol is a fat-like substance that is found in all body cells. The liver makes all the cholesterol for the body needs to form cell membranes and to make certain hormones. The determination of serum cholesterol is one of the important tools in the diagnosis and classification of lipdemia. High blood cholesterol is one of the major risk factors for heart disease. Serum cholesterol varies from 150-240 mg/100 ml in healthy young adults. The level rises with age and may go up to 300 mg/100 ml in the elderly. An increase in serum cholesterol (hypercholesterolaemia) is found in diabetes mellitus, nephrotic syndrome, obstructive jaundice, hypothyroidism. An idiopathic hypercholesterolaemia of unknown actiology occurs in some families. Hypercholesterolaemia, from any cause, leads to atherosclerosis. A decrease in serum cholesterol (hypocholesterolaemia) is found in hyperthyroidism, hepatocellular damage, anaemia, acute infections, wasting disease, intestinal obstruction and terminal states of a variety of disease. Methods for cholesterol estimation:

1- Enzymatic method(cholesterol Oxidase)

2- Sackett's Method:

Principle : Proteins are precipitated and cholesterol extracted in an alcoholether mixture. The extract is evaporated and the residue dissolved in chloroform. By Liebermann-Burchard reaction, a green colour is developed and measured colorimetrically.

Reagents

i. Alcohol-ether mixture – Ethyl alcohol (95%) and ether are mixed in a ratio of 3:1.

ii. Chloroform-This should be of a high purity and absolutely anhydrous.

iii. Acetic anhydride-sulphuric acid mixture-Acetic anhydride and conc.sulphuric acid are mixed in a ration of 20:1 just before use.

iv. Stock standard cholesterol solution 200 mg of chemically purecholesterol is dissolved in and diluted to 100 ml with chloroform.

v. Working standard cholesterol solution – 1 ml of stock standard cholesterol solution is diluted to 25 ml with chloroform. 5 ml of this solution contains 0.4 mg of cholesterol.

Procedure : Pipette 12 ml of alcohol-ether mixture in a dry centrifuge tube. Add 0.2 ml of serum slowly. Cork the tube and shake vigorously for one minute. Keep the tube in a horizontal position for half an hour. Centrifuge it at 1,500 r.p.m. for 5 minutes. Pour off the supernatant fluid completely in a small breaker, and evaporate it on a steam-bath or a hot plate. Make up to 5 ml with chloroform. Label the tube 'Unknown'. Label two other test tubes 'Standard' and 'Blank', and pipette respectively 5 ml of working standard cholesterol solution and 5 ml of chloroform the three tubes. Mix and keep in dark for 15 min. Adjust the wavelength of the photometer to 680 nm or put in a red filter. Set the photometer to 100% transmittance (or zero obsorbance) with 'Blank' and read 'Unknown' and 'Standard'.

Calculations

$$\text{Serum Cholesterol (mg/100ml)} = \frac{Au}{As} \times \frac{0.4}{0.2} \times 100$$

$$= \frac{Au}{As} \times 200$$

Bone Profile Testes

Calcium Determination

Calcium is required for cell function overall and for bone metabolism. Changes in calcium are used to assess bone function. Higher blood levels usually mean lower bone levels. Usually performed in conjunction with Phosphorous determinations. Calcium is present in serum in three forms – calcium bound to proteins,calcium bound to other organic substances, e.g. citrate, and ionized calcium.Most of the physiological functions of calcium depend upon the ionized fraction, but for routine work only total calcium in serum is estimated. A convenient and commonly used method is that of Clark and Collip*.

REFERENCE			**VALUES**
Serum	or		plasma:
Adults	8.5-10.5 mg /dL	=	2.1-2.6 mmol/L
Children	10 -12 mg/dL	=	2.5 - 3 mmol/L
Newborns	8 -13 mg/dL	=	2 - 3.25 mmol/L

Serum calcium varies from 9 – 11 mg/100 ml in healthy persons. The product of serum calcium and serum inorganic phosphorus is around 40 in adults and 50 in children. A change in the concentration of one is usually accompanied by an opposite change in the concentration of the other. Calcium is the most abundant and one of the most important minerals in the human body. Approximately 99% of body calcium is found in bones. A decrease in albumin level causes a decrease in serum calcium. Low levels of calcium are found in hypoparathyroidism, pseudohypoparathyroidism, vitamin D deficiency, malnutrition and intestinal malabsortion. Among causes of hypercalcemia are cancers, large intake of vitamin D, enhaced renal retention, osteoporosis, sarcosidosis, thyrotoxicosis, hyperparathyroidism. Clinical diagnosis should not be made on a single test result; it should integrate clinical and other laboratory data.

In rickets, the product of serum calcium and phosphorus decreases, usually bellow 30.

SAMPLES-

Serum or plasma: Separated from cells as rapidly as possible. Blood anticoagulants with oxalate or EDTA are not acceptable since these chemicals will strongly chelate calcium.

Principle:

Serum is treated with ammonium oxalate to precipitate calcium as calcium oxalate. The precipitate is washed with ammonia to remove excess oxalate and then treated with sulphuric acid to convert calcium oxalate into oxalic acid. The latter is titrated with standard potassium permanganate.

Reagents:

1. Ammonium oxalate, 4% - 4 gm of ammonium oxalate is dissolved in water and diluted to 100 ml.
2. Ammonia, 2% - 2 ml of conc. ammonia (S.G. 0.880) is diluted to 100 with water.
3. Sulphuric acid, 1 N-28 ml of conc. sulphuric acid is diluted to 1,000 ml with water.
4. potassium permanganate, 0.1 N-3.158 gm of potassium permanaganate is dissolved in water and diluted to 1,000 ml. This is allowed to stand for one week and then filtered through asbestos.
5. Potassium permanganate, 0.01 N-10 ml of 0.1 N potassium permanganate is diluted to 100 ml with water and standardized against 0.01 N sodium oxalate. 1 ml of 0.01 N potassium permanaganate is equivalent to 0.2 mg of calcium.
6. Sodium oxalate, 0.01 N-0.67 gm of sodium oxalate is dissolved in water. 5 ml conc. sulphuric acid is added. The volume is made up to 1,000 ml with water (1 ml of 0.01 N sodium oxalate = 1 ml of 0.01 N potassium permanganate.

Procedure:

Measure 2 ml of serum, 2 ml of water and 1 ml of 4% ammonium oxalate in a centrifuge tube. Mix thoroughly and allow to stand for at least half an hour. Mix again and centrifuge at 1,500 r.p.m. for 15 minutes. Pour off the supernatant fluid and drain the tube by keeping it inverted on a filter paper for a few minutes. Wipe the mouth of the tube

dry with a filer paper. Add 3 ml of 2% ammonia, shake, centrifuge and drain as before. Add 2 ml of 1 N sulphuric acid and shake vigorously. Keep the tube in a boiling water-bath shaking intermittently, until the precipitate completely dissolves. While the tube is still hot, titrate with 0.01 N potassium permanganate until a pink colour develops and persists for at least one minute. Note the volume of 0.01 N potassium permanganate used. Suppose it is x ml. For blank, take 2 ml of 1 N sulphuric acid and titrate it with 0.01 N potassium permanganate exactly as before, suppose the volume of potassium permanganate used in y ml.

calculation:

$$\text{Serum calcium (mg/100 ml)} = (x-y) \times \frac{0.2}{2} \times 100$$

$$= (x-y) \times 10$$

Conversion factor: mg/dL x 0.25= mmol/L.

Estimation of Serum Phosphorus (Inorganic)

Phosphorus is present in blood as inorganic phosphate and in combination with several organic compounds including carbohydrates, lipids and nucleotides. Inorganic phosphorus is present mainly in serum. Phosphorus is an essential mineral for tissue bone formation and is required by every cell in the body for normal function. Approximately 85% of the body phosphorus is found in bone and in teeth.

REFERENCE VALUES

Serum:

Children : 4.0 - 7.0 mg/dL = (1.3 - 2.2 mmol/L)

Adults : 2.5 - 5.0 mg/dL = (O.8 - 1.8 mmo/lL)

Low levels of phosphorus, can be caused by hypervitaminosis D, primary hyperparathyroidism, renal tubular disorders, antacids or malabsorption. High levels of phosphorus can be caused by diet, bone metastases, liver disease, alcohol ingestion, diarrhea and vomiting.

Method of Fiske and Subbarow*

Principle:

Serum is deproteinized with trichloroacetic acid. Protein-free filtrate is treated with acid molybdate which combines with phosphate to form phosphomolybdic acid. This is reduced by 1, 2,4aminonaphthol sulphonic acid to blue coloured phosphomolybdous acid (molybdenum blue). The intensity of the colour is measured colorimetrically.

Reagents:

1. Trichloroacetic acid, 10% - 10 g of trichloroacetic acid is dissolved in water and dilute to 100 ml.
2. Molybdate reagent – 25 gm of ammonium molybdate is dissolved in 200 ml of water and added to 300 ml of 10 N sulphuric acid. The volume is made up to 1,000 ml with water
3. Aminonaphtholsulphonic acid – 59.5 g of sodium bisulphate and 2g of anhydrous sodium sulphite are dissolved in water and dilute to 250 ml. 1 gm of recrystallized 1, 2, 4-aminonaphtholsuphonic

acid is dissolved in the above solution and dilute to 400 ml with water.

4. Stock standard phosphorus solution – 0.351 g of potassium dihydrogen phosphate is dissolved in water. 10 ml of 10 N sulphuric acid is added. The volume is made up to 1,000 ml with water. 5 ml of this solution contains 0.4 mg of phosphorus.
5. Working standard phosphorus solution – 5 ml of the stock standard phosphorus solution is dilute to 100 ml with 10% trichloracetic acid. 5 ml of this solution contains 0.02 mg of phosphorus.

Procedure :

Measure 9 ml of 10 % trichloracetic acid in a test tube. Add 1 ml of serum drop by drop with constant shaking. Filter. Transfer 5 ml of the filtrate to a tube labeled 'Unknown'. Pipette 5 ml of the working standard phosphorus solution into a tube labeled 'Standard' and 5 ml of 10% trichloracetic acid into a tube labeled 'Blank'. To each tube, and 3.6 ml of water. Mix after each addition. Let the tubes stand for 5 minutes. Read 'Unknown' and 'Standard' against 'Blank' at 680 nm or using a red filter.

Calculation :

Serum inorganic phosphorus (mg/100 ml)

$$= \frac{Au}{As} \times \frac{0.02}{0.5} \times 100$$

$$= \frac{Au}{As} \times 4$$

Conversion factor: mg/dL x 0.323 = *mmol/L.*