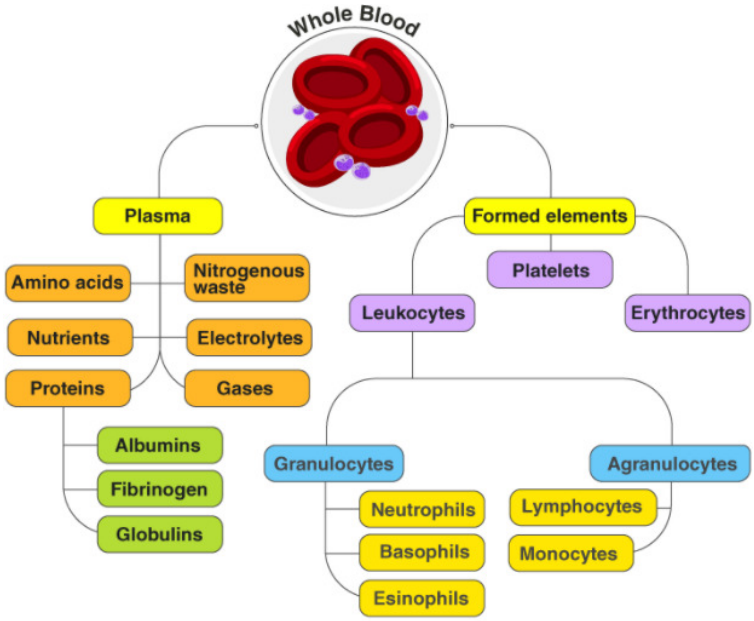
**Blood**

Blood is a fluid connective tissue that consists of plasma, blood cells and platelets. It circulates throughout our body delivering oxygen and nutrients to various cells and tissues. It makes up 8% of our body weight. An average adult possesses around 5-6 litres of blood.

**Composition of blood:**




Plasma

The liquid state of blood can be contributed to plasma as it makes up ~55% of blood. It is pale yellow in colour and when separated, it consists of salts, nutrients, water, amino acids and enzymes. Blood plasma also contains important proteins as albumin, fibrinogens, globulins and other components necessary for overall health. It also contains different nitrogenous waste, gases, and electrolytes. Hence, blood plasma transfusions are given to patients with liver failure and life-threatening injuries.

Formed Elements: Blood consist of different typed of cells known as formed elements of blood. These cells have their own functions and roles to play in the body. The blood cells which circulate all around the body are as follows:

Red blood cells (Erythrocytes)

RBCs are biconcave cells and without nucleus in humans; also known as erythrocytes. RBCs contain the iron-rich protein called haemoglobin, give blood its red colour. RBCs are the most copious blood cells produced in bone marrows. Their main function is to transport oxygen from and to various tissues and organs.

White blood cells (Leucocytes)

Leucocytes are colourless blood cells. They are colourless because it is devoid of haemoglobin. WBCs mainly contribute to immunity and defence mechanism.

There are five different types of White blood cells and are classified mainly based on the presence and absence of granules.

- Granulocytes: They are leukocytes, with the presence of granules in their cytoplasm. The granulated cells include - eosinophil, basophil and neutrophil.
- Agranulocytes: They are leukocytes, with the absence of granules in their cytoplasm. Agranulocytes are further classified into monocytes and lymphocytes.

Platelets (Thrombocytes)

- Thrombocytes are specialized blood cells produced from bone marrow.
- Platelets come into play when there is bleeding or haemorrhage.
- They help in clotting and coagulation of blood. Platelets help in coagulation during a cut or wound.

**Functions of Blood**

1) Fluid Connective Tissue: Blood is a fluid connective tissue composed of 55% plasma and 45% formed elements including WBCs, RBCs, and platelets. Since these living cells are suspended in plasma, blood is known as a fluid connective tissue and not just fluid.

(2) Provides oxygen to the cells: Blood absorbs oxygen from the lungs and transports it to different cells of the body. The waste carbon dioxide moves from the blood to the lungs and exhaled.

(3) Transports Hormone and Nutrients: The digested nutrients such as glucose, vitamins, minerals, and proteins are absorbed into the blood through the capillaries in the villi lining the small intestine.

The hormones secreted by the endocrine glands are also transported by the blood to different organs and tissues.

(4) Homeostasis: Blood helps to maintain the internal body temperature by absorbing or releasing heat.

(5) Blood Clotting at Site of Injury: The platelets help in the clotting of blood at the site of injury. Platelets along with the fibrin form clot at the wound site

(6) Transport of waste to the Kidney and Liver: Blood enters the kidney where it is filtered to remove nitrogenous waste out of the blood plasma. The toxins from the blood are also removed by the liver.

(7) Protection of body against pathogens: The White Blood Cells fight against infections. They multiply rapidly during the infections.

**Coagulation (blood clotting)**

It is a chemical reaction that leads to a fibrin clot.

Mechanism:

Step 1: Platelets release chemical factors such as thromboxane, PF3, ADP, serotonin, etc. These are called prothrombin activators.

Step 2: prothrombin → thrombin

Step 3: fibrinogen → fibrin

Coagulation is the actual formation of a blood clot. It results from a chemical "cascade" which begins with the prothrombin activators released by platelets. These chemicals cause the macromolecule prothrombin to break down into smaller units including **thrombin**. Thrombin acts on **fibrinogen**, a soluble polymer present in the plasma, and breaks it into monomers which re-polymerize into insoluble **fibrin**. The fibrin forms threads which knit the platelets and other cells into the clot.

**Regulation and Requirements for Erythropoiesis** (the production of red blood cells)

The number of circulating erythrocytes in a given individual is remarkably constant and reflects a balance between red blood cell production and destruction. This balance is important because having too few erythrocytes leads to tissue hypoxia (oxygen deprivation), whereas having too many makes the blood undesirably viscous. To ensure that the number of erythrocytes in blood remains within the homeostatic range, new cells are produced at the incredibly rapid rate of more than 2 million per second in healthy people. This process is controlled hormonally and depends on adequate supplies of iron, amino acids, and certain B vitamins.

**Anaemia** (lacking blood) is a condition in which the blood has abnormally low oxygen-carrying capacity. It is a sign of some disorder rather than a disease. Its hallmark is blood oxygen levels that are inadequate to support normal metabolism. Anaemic individuals are fatigued, often pale, short of breath, and chilly. Common causes of anaemia include the following:

**1. An insufficient number of red blood cells.** The red blood cell count may be decreased due to blood loss, excessive RBC destruction, and bone marrow failure. Hemorrhagic anaemias result from blood loss. In acute hemorrhagic anaemia, blood loss is rapid due to severe stab wound. Slight but persistent blood loss due to haemorrhoids or an undiagnosed bleeding ulcer causes chronic hemorrhagic anaemia. Haemoglobin abnormalities, transfusion of mismatched blood, and certain bacterial and parasitic infections are possible causes.

**2. Low haemoglobin content.** When haemoglobin molecules are normal, but erythrocytes contain fewer than the usual number, a nutritional anaemia is always suspected. Iron-deficiency anaemia is generally occur due to inadequate intake of iron-containing foods and impaired iron absorption. The erythrocytes produced, called **microcytes**, are small and pale.

Pernicious anaemia is due to a deficiency of vitamin $B_{12}$, because of insufficient intake of food as meats, poultry, and fish containing $B_{12}$. Treatment involves regular intramuscular injections of vitamin $B_{12}$ or application of a $B_{12}$-containing gel (Nascobal) to the nasal lining once a week.

**3. Abnormal haemoglobin.** Production of abnormal haemoglobin usually has a genetic basis. Two such examples, thalassemia and sickle-cell anaemia, can be serious, incurable, and sometimes fatal diseases. In both diseases the globin part of haemoglobin is abnormal and the erythrocytes produced are fragile and rupture prematurely.

**In thalassemia,** one of the globin chains of haemoglobin is absent or faulty, and the erythrocytes are thin, delicate, and deficient in haemoglobin.

In **sickle-cell anaemia**, the disorder caused by the abnormal haemoglobin, haemoglobin S (HbS), results from a change in just one of the 146 amino acids in a beta chain of the globin molecule.

**What amino acid causes sickle cell anaemia?**

Sickle cell anaemia results from the single amino acid substitution of valine for glutamic acid in the beta-chain owing to a nucleotide defect that causes the production of abnormal beta-chains in haemoglobin S.

**Collection of blood**

Blood is taken for different kind of investigation. Generally, blood is taken three way (1) capillary blood (2) venous blood and (3) arterial blood.

Capillary blood: It is obtained from finger or thumb or heel (in case of infants). When just few drops are needed, this technique is used. For example, estimation of haemoglobin, detection of malarial parasite, for total leucocyte counts.

Venous blood: Most frequent collected blood is venous blood, which is taken from any prominent vein. A vein on the front of the elbow or forearm is generally chosen. Depending on the number of investigation 2-10 ml blood is taken using syringe.

**Preservation of blood:**

The collected blood is put in different vials for investigation. Some investigation are done with serum, some are done with whole blood. As time is required between the collection and conduct of the investigation, the whole blood is preserved using anticoagulant. Thus blood is prevented from clotting.

| Investigation | Anticoagulant | Mg of anticoagulant per ml of blood |
|---|---|---|
| Blood sugar test<br>and<br>Glucose tolerance test | Potassium oxalate<br>and<br>Sodium fluoride | 3 mg |
| Blood urea | Potassium oxalate | 2-3 mg |
| Blood counts<br>ESR (erythrocyte sedimentation rate) and PVC (packed call volume) | Salts of ethylene diaminetetraacetic acid (EDTA) | 2-3 mg |
| Some special investigation | Heparin | |
| For blood banking | Acid citrate-dextrose | |

For serum, the whole blood is kept undisturbed in vial for 20-30 minutes allowed to clot, then centrifuged. The clear pale yellow supernatant is called serum which is withdrawn by a Pasteur pipette. Serum is used for estimation of calcium, cholesterol, bilirubin, uric acid etc.

For plasma, the whole blood collected with anticoagulant is centrifuged immediately without waiting to get it coagulated. The supernatant obtained is called plasma.

**Blood sugar estimation:**

**Diabetes Mellitus:** It is a chronic disease due to disorder of carbohydrate metabolism, cause of which is either deficiency or diminished level of insulin.

Glucose is the principle sugar in blood. Estimation of glucose in blood has become a routine test in clinical biochemistry lab. For sugar test, blood is taken from vein and 1 mg of sodium fluoride and 3 mg of potassium oxalate is added as anticoagulant per ml of blood.

**Specimen for testing the Fasting blood sugar:**

The specimen of blood should be taken after 10-12 hours of the last meal. The normal fasting blood sugar is around **60-100 mg** per 100 ml of blood.

**Specimen for testing Post Parandial (PP) blood sugar:**

After taking the fasting blood, the patient is given a good breakfast containing 100 mg of carbohydrate. The blood specimen is taken 2 hours after taking the meal. The normal PP blood sugar level is between **120-160 mg** per 100 ml of blood.

**Interpretation of blood sugar test:**

If the fasting blood sugar value exceeds 100 mg/100 ml, it is indicative of **hyperglycaemia** and if glucose level falls below 60/100 ml, it is indicative of **hypoglycaemia**.

**Method of estimation of blood sugar:**

**Principle:**

Reducing sugars in hot alkaline medium produce cnediols which are strong reducing agents that convert $Cu^{2+}$ ions $Cu^{+}$ ions which 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. Phosphomolybdic acid is added to this solution so that oxidation of $Cu^{+}$ to $Cu^{2+}$ is coupled with reduction of phosphomolybdic acid to molybdenum blue which can be estimated colorimetrically.

For that process, the proteins are to be removed to obtain a colourless filtrate. Otherwise the final colour development for colourimetric estimation will be interfered with.

**Requirements:**

1) Sodium sulphate-copper sulphate solution (isotonic solution)
2) Sodium tungstate solution
3) alkaline tartarate reagent
4) Phosphomolybdic acid (colour reagent)
5) Working standard glucose solution of concentration, 1.25 mg/100 ml (in isotonic solution)
6) Blood sample

**Procedure:**

Step 1: Place 0.1 ml of blood in a centrifuge tube. Add 3.8 ml of the isotonic solution and 0.1 ml of sodium tungstate solution to it. Gently mix the solution. Centrifuge the tube at 2000-2500 rpm. Collect the supernatant clear liquid.

Step 2: Label three test tube as T (test), S (standard) and B (blank). Proceed with the adding various solution in these tubes as follows:

| solution | Test tubes | | |
|---|---|---|---|
| | T | S | B |
| Test sample (obtained in Step 1) | 1.0 ml | 0.0 | 0.0 |
| Standard glucose solution | 0.0 | 1.0 ml | 0.0 |
| Isotonic solution | 0.0 | 0.0 | 1.0 ml |
| Alkaline tartarate reagent | 1.0 ml | 1.0 ml | 1.0 ml |

Cover the tubes with cotton plugs. Keep them in boiling water bath for 10 minutes.

Step 3:

Cool the test tubes to room temperature. Add 3 ml of the colour reagent to each of the above three tubes. Allow the tube to stand for 5 minutes at room temperature.

Step 4: At last, measure the absorbance of these three solutions at 540 nm.

**Calculation:**

Glucose present in blood sample = $\frac{OD_T}{OD_S}$ x dilution factor x conc. of standard glucose solution

$$= \frac{OD_T}{OD_S} \text{ x } 40 \text{ x } 1.25/100 \text{ ml}$$

**Regulation of blood sugar:**

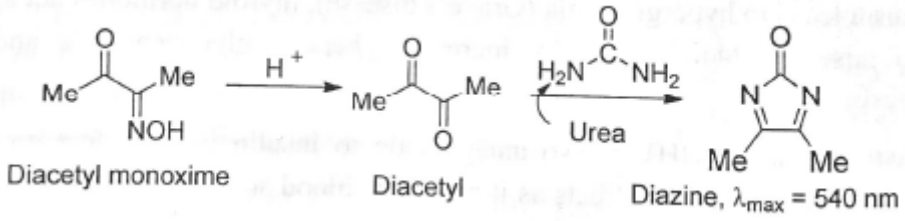
Blood regulation is the process by which the levels of blood sugar are maintained within the biological range. Insulin lowers the level of blood sugar whereas glucagon raises sugar level. A rise in blood sugar level stimulates insulin secretion and it lowers the sugar in several ways. It increases sugar uptake by tissues, promotes glycolysis for utilization of glucose, reduces glycogenolysis and gluconeogenesis in liver. When sugar level falls secretion of glucagon stimulated. It is antagonistic to insulin and increases blood sugar by enhancing glycogenolysis in the liver. Hormones like adrenaline, gluococorticoids also regulate blood glucose level. Growth hormone is also antagonistic to insulin in its effects on carbohydrate metabolism. It raises blood sugar by inhibiting the uptake and utilization of glucose by muscles.

**Blood urea**:

Urea is main end product of protein catabolism. It is formed in the liver and is excreted through urine. Urea represents about 45-50% of the non-protein nitrogen of blood and 80-90% of the total urinary nitrogen excretion.

Blood urea or serum urea is the most commonly performed to diagnose the kidney function. The normal value of blood urea is 15 - 40 mg/100 ml, but it may fall during pregnancy. It usually increases in any conditions which adversely affect normal kidney function. Renal function is affected by damage to the kidneys due to decreased blood flow or obstruction to the flow of urine.

**Principal:** Urea reacts with diacetyl monoxime in acidic conditions at nearly 100$^{o}$C to give a red coloured product which is measured colorimetrically at 540 nm. Thiosemicarbazide and ferric ions are added to catalyse the reaction and increase the intensity of colour. This method is linear only upto 300 mg % urea. For higher values if expected, the blood sample should be diluted.




**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 stirring. Make up the volume to 250ml with water. Keep in brown bottle at 4$^{o}$C.

2) Reagent B: Add 200 ml conc. $H_2SO_4$ to 800 ml water 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 $H_2O$ keep in brown bottle at 4$^{o}$C.

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

8) Working urea standard: Dilute 1 ml stock to 100ml with $H_2O$ so conc. is 1 mg/100ml

Procedure: 0.1 ml if serum/plasma is diluted to 10 ml. set up the test tubes as follows:

| solution | Test tubes | | |
|---|---|---|---|
| | T | S | B |
| Test sample (obtained in Step 1) | 1.0 ml | 0.0 | 0.0 |
| Standard urea solution | 0.0 | 1.0 ml | 0.0 |
| Water | 0.0 | 0.0 | 1.0 ml |
| acid reagent | 2.0 ml | 2.0 ml | 2.0 ml |
| Colour reagent | 2.0 ml | 2.0 ml | 2.0 ml |

Mix all the tube thoroughly. Keep in boiling water bath for exactly 30 mins. Then cool and take absorbance at 520 nm.

**Calculation:**

Urea present in blood sample = $\frac{OD_T}{OD_S}$ x dilution factor x conc. of standard urea solution

$= \frac{OD_T}{OD_S}$ x 100 x 1.00/100 ml

**Regulation:**

Glucocorticoids have an anti-anabolic effect and thyroid hormones have a catabolic effect on protein and thus tend to increase the blood urea nitrogen. Androgens and growth hormones have an anabolic effect and thus decrease the formation of urea.

**Interpretation:**

Normal blood urea in adults is 15-50 mg/100 ml. Increase in urea adversely affects the kidneys and damage the renal function. Very high plasma urea concentration accompanied by renal failure is called uremia. It is frequently categorised as pre-renal, renal and post renal.

**Bilirubin estimation:**

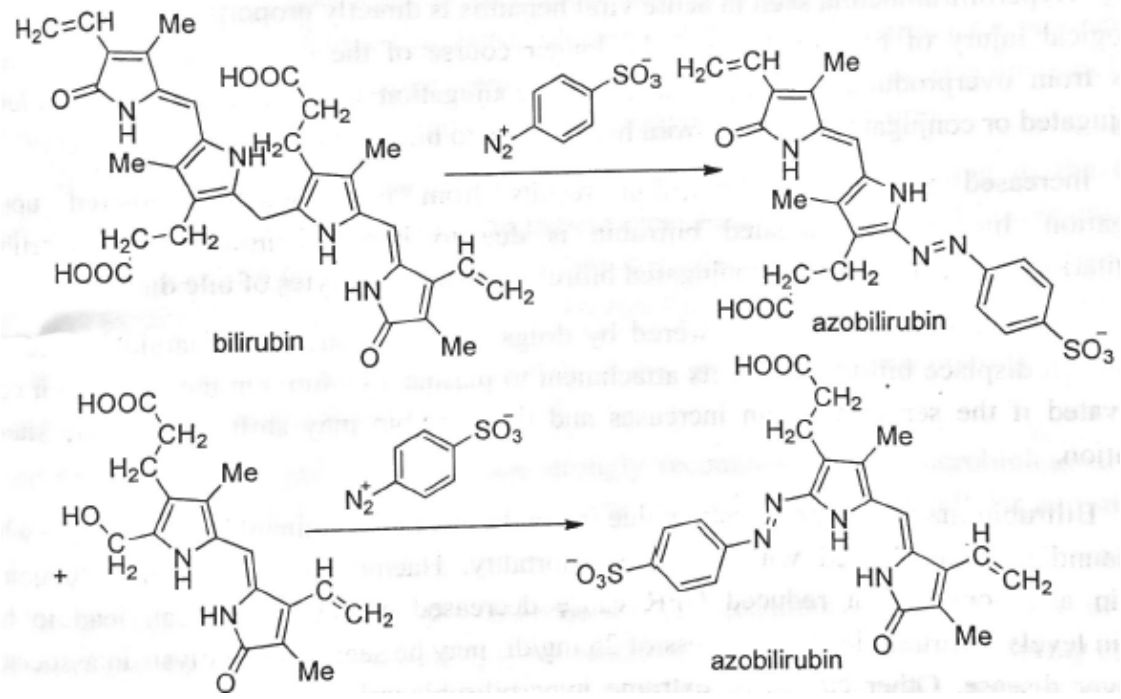
Bilirubin estimation is important to diagnose the condition of liver function. Bilirubin is formed by the reticuloendothelial cells of the liver from ferroporphyrin ring (Heme) obtained mainly from haemolysed red blood cells and also from myoglobin and iron containing enzymes. There are two forms of bilirubin, water soluble (conjugated or direct) and water insoluble (unconjugated or indirect) bilirubin.

The normal level of bilirubin in blood is 0.3-1.0 mg/dl. In case of jaundice the level of bilirubin is usually more than 2-0-2.5 mg/dl in serum.

**Principle (Van-den Bergh reaction):**

Bilirubin is determined qualitatively and quantitatively by means of diazo reagent. Diazotized sulphanilic acid (sulphanilic acid is treated with a mixture of sodium nitrite and hydrochloric acid) in presence of bilirubin forms a red compound, azobilirubin, which is measured colourimetrically.

When the reaction is carried out in aqueous medium, the water soluble conjugated bilirubin reacts to give so called direct van den Bergh reaction. When the reaction is carried out in methanol, the intramolecular hydrogen bonds are broken, thus both conjugated and unconjugated pigments react giving a measure of total bilirubin.



**Reagents:**

1. Diazo reagent: Make freshly before use the mixing 10 ml of solution A and 0.3 ml of solution B.

Solution A: 1g of sulphanilic acid and 15ml of concentrated HCl per litre in water.

Solution B: 0.5g of sodium nitrite/100ml in water. This solution should be kept in refrigerator renewed monthly.

2. Diazo Blank: 15 ml of conc. HCl/litre in water.

3. Methanol

4. Bilirubin standard: Dissolve 10 mg bilirubin in a minimum (about 5 ml) of 0.1N sodium acetate solution, as quickly as possible (since it is unstable in alkaline solution) and make volume (100 ml) 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).

**Procedure:** 0.2 ml if serum/plasma is taken and set up the test tubes as follows:

| solution | Test tubes | | | |
|---|---|---|---|---|
| | T | $T_C$ | S | $S_C$ |
| Test sample (obtained in Step 1) | 0.2 ml | 0.2 ml | 0.0 | 0.0 |
| Standard bilirubin solution | 0.0 | 0.0 | 0.2 ml | 0.2 |
| Water | 1.8 ml | 1.8 ml | 1.8 ml | 1.8 ml |
| Diazo reagent | 0.5 ml | 0.0 ml | 0.5 ml | 0.0 ml |
| Diazo blank | 0.0 ml | 0.5 ml | 0.0 ml | 0.5 ml |
| Methanol | 2.5 ml | 2.5 ml | 2.5 ml | 2.5 ml |

The solutions are mixed well and kept 30 minutes for total bilirubin (15 minutes for conjugated bilirubin) at room temperature. Absorbance of the solutions is measured at 530 nm. Absorbance of water is taken as blank.

**Interpretation:**

Low bilirubin is directly correlated with diabetes mellitus, metabolic syndrome and cardiovascular disease. High bilirubin indicates haemolysis, jaundice, hepatitis, drug toxicity etc. Normal serum bilirubin is less than 1 mg/dl. Hyperbilirubinaemia of more than 3 mg/dL results into clinical jaundice.

**Calculation:**

Bilirubin present in blood sample = $\frac{\text{OD of T} - T_C}{\text{OD of S} - S_C}$ x dilution factor x conc. of standard solution

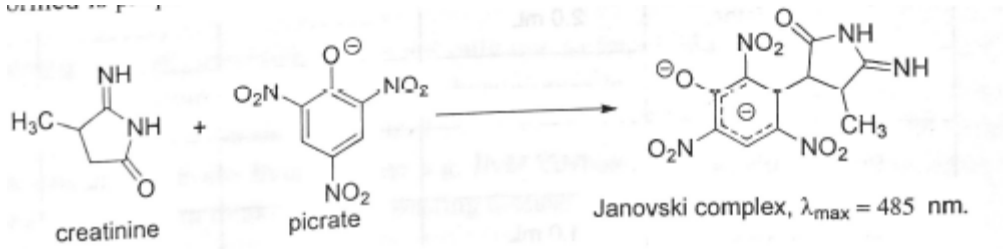
**Creatinine estimation**

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. 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 males: 0.7-1.4 mg/100 ml and Females: 0.4-1.2mg/100 ml

**Principle :** Creatinine in alkaline medium reacts with picric acid to form a red tautomer of creatinine picrate the intensity of which is measured at 520nm.




The two chief disadvantages with Jaffe's reaction are:

(1) Lack of specificity: Only 80% of the colour developed is due to creatinine in serum. Other non specific chromogens that react with picric acid are proteins, ketones bodies, pyruvate, glucose, ascorbate etc.(2) sensitive to certain variables like pH, temperature etc.

**Reagents:**

1) Picric acid – 0.04M (9.16g/L) (2) Sodium hydroxide - 0.75 (N) (3) Sodium tungstate - 10% (4) 2/3 (N) $H_2SO4$ (5) Creatinine standard stock – 100 mg% (6) Working standard – 3 mg%

**Procedure:** In a centrifuge tube, take 2ml of serum with 2 ml of distilled water. Add 2 ml sodium tungstate and 2 ml of 2/3 N sulphuric acid drop with constant shaking to precipitated proteins. Stand for 10 minutes and filter. Use protein free filtrate for test.

**Interpretation:**

Normal value of serum creatinine is 0-7-1.5 mg/dl (Male) and 0.4 -1.2 mg/dl (Female).

Creatinine is eliminated by glomerular filtration through kidneys and excreted in urine. So, high creatinine level in blood indicates the dysfunction of kidneys and abnormalities in renal function leads to renal diseases.

**Blood cholesterol estimation**:

Cholesterol is a fat-like substance that is found in all body cells. The liver makes all of the cholesterol 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 lipemia. High blood cholesterol is one of the major risk factors for heart disease.

Cholesterol like other types of lipid, is transported in blood as the complexes called lipoproteins. Lipoproteins are micelles of high molecular weight that transport non-polar lipids (triacylglycerols and esterfied cholesterols) via plasma. Each lipoprotein particle consists of an outer monolayer of protein (called apoprotein and polaripids contains phospholipid and unesterified cholesterol) and inner core of neutral lipids (triacylglycerols and esterfied cholesterols). The core neutral lipids are primarily inactive passengers whereas the apolipoproteins are mainly responsible for further metabolism of the particle by binding to specific enzymes or transport proteins on cell membrane.

Specimens of blood should be taken after the patient is kept for a week on normal diet and in the morning after fasting for 12 hours. Freshly separated plasma or serum is used. Heparin or EDTA is used as the anticoagulant.

**Principle:** Proteins are precipitated by and cholesterol extracted in an alcohol-ether mixture. The extract is evaporated and the residue dissolved in chloroform. Cholesterol reacts with acetic anhydride in presence of glacial acetic acid and sulphuric acid gives a highly conjugated green coloured compound. This reaction is called Liebermann-Burchard reaction. The acid catalysed dehydration of cholesterol and stepwise oxidation proceeds by sulphur trioxide to yield a highly conjugated carbocations which is responsible to give green colour.

**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 pure cholesterol 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 1500 rpm. 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'.

| solution | Test tubes | | |
|---|---|---|---|
| | T | S | B |
| Test sample (obtained in Step 1) | 5.0 ml | 0.0 | 0.0 |
| Standard cholesterol solution | 0.0 | 5.0 ml | 0.0 |
| Acetic anhydride-sulphuric acid mixture | 3.0 ml | 3.0 ml | 3.0 ml |
| Chloroform | 0.0 ml | 0.0 ml | 5.0 ml |

Mix and keep in dark for 15 min. Measure the absorbance of these solution at 560 nm. Set the photometer to 100% transmittance (or zero absorbance) with 'Blank'.

**Calculation:**

Bilirubin present in blood sample = $\frac{\text{OD of T}}{\text{OD of S}}$ x dilution factor x conc. of standard solution

**Interpretation:**

1) Normal level of total serum cholesterol: 150-250 mg/dl

2) Hyper cholesterolemia – serum cholesterol >250 mg/dl

3) Hypo cholesterolemia – serum cholesterol < 100 mg/dl

4) Normal low density lipoprotein(LDL) cholesterol (bad cholesterol) <150 mg/dl

5) Normal high density lipoprotein(HDL) cholesterol (good cholesterol) > 40 mg/dl

An increase in serum cholesterol (hypercholesterolaemia) is found in diabetes mellitus, nephritic syndrome, obstructive jaundice, hypothyroidism, xanthomatosis and during ether anaesthesia. A decrease in serum cholesterol (hypocholesterolaemia) is found in hyperthyroidism, hepatocellular damage, anaemia (except haemorrhagic), acute infections, wasting disease, intestinal obstruction and terminal states of a variety of disease.

## Urine

### Physical appearance of Urine:

**Volume:**

A normal adult excretes daily from 1000 ml to 1800 ml of urine. The quantity depends on the water intake, external temperature, the diet and the in dividual's mental and physical condition. Increased urine volumes are observed in diabetes insipidus, diabetes mellitus and certain types of kidney diseases and decreased volumes are found in acute nephritis, fevers, diseases of the heart, diarrhea and vomiting. A volume more than 2000 ml in 24 h is termed Polyuria and less than 500 ml is termed Oliguria.

**Specific Gravity:**

The specific gravity of urine in 24 hours lies between 1.003 and 1.030 and varies according to concentration of solutes in the urine. The specific gravity of the urine varies with the food, water intake, and the activity of the individual. In chronic interstitial nephritis, the specific gravity is lowered. The specific gravity is increased in the excretion of abnormal substances such as albumin or glucose (e.g., diabetes mellitus).

**Colour:**

Normal urine is pale yellow. Reddish urine is due to the ingestion of naturally coloured foods (e.g., beetroot, blackberries). In fever, the urine may be dark yellow or brownish because of concentration. In liver disease, the urine may be green, brown, or deep yellow due to bile pigments. Blood or hemoglobin develops smoky to red colour. The urine is dark brown due to methemoglobin and homogentisic acid. Methylene blue gives the urine a green appearance. The urine is transparent. A turbidity is developed by precipitation of calcium phosphate. Strongly acid urine is pink due to the precipitation of uric acid salts.

**Odour:**

Fresh urine is normally aromatic. The odour is modified by the ingestion of certain foods or drugs. The ammoniacal smell of urine is due to the action of bacteria on urea. In ketosis, the odour of excreted acetone is detected.

**pH:**

The mixed sample of normal urine in 24 hours has a pH 6.0. Individual samples vary from 4.6 to 8.0. The urine is acid in high protein intake because excess phosphate and sulfate are formed in the catabolism of protein. The urine becomes alkaline on standing due to the conversion of urea to ammonia and loss of $CO_2$ to air. The acidity of urine is increased after strenuous muscular exercise (elimination of lactic acid), by ingestion of ammonium salts of strong acids. An alkaline urine may be produced by ingestion of sufficient $NaHCO_3$.

**Constituents of Urine:**

**Normal Constituents of Urine:** Human urine consists 95% water and 5% other substances like urea (9.3 g/L), Chloride(1.87 g/L), Sodium( 1.17 g/L) potassium(0.75 g/L), ammonia, proteins, hormones, creatinine, creatine, uric acids, phosphates, oxalate etc.

**Urea:**

Urea is the main end product of catabolism of protein in mammals. Its excretion is directly proportional to the protein intake. It consists of 80-90% of the total urinary nitrogen. In fever, diabetes, or excess adrenocortical activity, urea excretion is increased due to increased protein catabolism. Decreased urea excretion is due to decreased urea production in the last stages of fatal liver disease.

**Ammonia:**

Ammonia is formed by the kidney from glutamine or amino acids in acidosis. There is a high ammonia output in the urine in uncontrolled diabetes mellitus in which renal function is unimpaired.

**Creatinine and Creatine:**

Creatine is excreted by children and pregnant women and much smaller amounts in men. The excretion in men is 6% of the total excretion of creatinine. Creatinine is formed from creatine. The creatinine coefficient is the ratio between the amount of creatinine excreted in 24 hours and the body weight in kg. It is usually 20-26 mg/kg/day in normal men and 14-22 mg/kg/day in normal women.

**Uric Acid:**

It is the end product of the oxidation of purines in the body. It is not only formed from dietary nucleoprotein but also from the breakdown of cellular nucleoprotein in the body. It is slightly soluble in water and precipitates readily from acid urine on standing. Uric acid excretion is increased in leukemia, severe liver disease and various stages of gout. The concentrated urine on cooling forms a brick-red deposit which is mainly acid urate.

**Amino Acids:**

About 150-200 mg of amino acid nitrogen is excreted in the urine of adults in 24 hours. The infant at birth excretes about 3 mg of amino acid nitrogen per pound of body weight, and up to the age of 6 months the value reaches to 1 mg/pound which is maintained throughout childhood. The low excretion of amino acid nitrogen is due to its high renal threshold value. Increased amounts of amino acids are excreted in liver disease and in certain types of

poisoning. In cystinuria, 4 amino acids-arginine, cystine, lysine and ornithine are excreted in urine.

**Sulphates:**

The urine sulphur is derived from sulphur containing amino acids such as methionine and cystine and therefore, its output varies with protein intake. The urine sulphur exists in 3 forms. (a) Inorganic (sulfate) sulfur: This is the completely oxidized sulfur precipitated from urine (b) Ethereal sulfur (conjugated sulfates): It is about 10% of the total excreted sulfur. This includes the organic combination of sulfur excreted in the urine. It consists of the sulphuric esters of certain phenols. It forms no precipitate on addition of acidified $BaCl_2$. Some of the phenols are derived from putrefaction of protein in the large intestine (c) Neutral sulfur: These are un-oxidized sulfur and contained in cystine, taurine, thiocyanate or sulfides.

**Chlorides:**

These are excreted as NaCl and output varies with intake.

**Phosphates:**

The urine phosphates consist of sodium and potassium phosphates as well as calcium and magnesium phosphates. The most of the excreted phosphates is derived from ingested food which contains organic phosphates, e.g., nucleoprotein, phosphoprotein and phospholipids. Phosphates of food are not completely absorbed. Phosphate excretion is increased in certain bone diseases such as osteomalacia, wasting diseases of the nervous system and in renal tubular rickets. Marked increase of phosphate excretion is also observed in hyperparathyroidism and decrease in hypoparathyroidism and in infectious diseases.

**Oxalates:**

The amount of oxalate in the urine is low (20 mg/day) and found as calcium oxalate crystals in urinary deposits. The excretion of oxalate is increased by ingestion of fruits and vegetables containing high oxalates (spinach). Large quantities of oxalate are excreted in urine in inherited metabolic diseases. The oxalates present in urine are composed of partly unchanged ingested acid and partly oxidative products of other compounds.

**Minerals:**

The 4 cations of the extracellular fluid— sodium, potassium, calcium and magnesium—are present in the urine. Sodium content varies with intake. Urine potassium increases when the intake is increased or in excessive tissue catabolism. The excretion of potassium is affected by alkalosis. Sodium and potassium excretion are also controlled by the activity of the adrenal cortex. Calcium and magnesium are not completely absorbed and their presence in

the urine is low. But their presence in the urine varies in certain pathological states, particularly those involving bone metabolisms.

**Enzymes:**

Traces of many enzymes are excreted in urine including pancreatic amylase, pepsin, trypsin and lipase. The pancreatic amylase excretion is increased in pancreatic disease.

**Hormones and vitamins:**

Certain hormones (sex hormones) and vitamins (e.g., B, and C) are found in urine. The vitamin needs are assessed by studying the urinary output after test doses. The pregnancy test is also performed by the urinary sex hormones.

**Abnormal Constituents of the Urine:**

**Proteins:**

Normal adults excrete upto 150 mg proteins per day. Nephrotic syndrome is a clinical condition when kidney loses more than 3.5 gm proteins per day.

Proteinuria (albuminuria) is the presence of albumin and globulin in the urine in abnormal concentrations. The traces of protein (10-150 mg) present in normal urine cannot be detected by the ordinary simple tests. Pathologically, several proteins, such as serum albumin, serum globulin, haemoglobin, mucus, proteose, Bence-Jones proteins are found in urine.

**Physiologic proteinuria** occurs after severe exercise, after a high protein meal or as a result of some temporary impairment in renal circulation when a person stands erect. In 30-35% of pregnancy, there is proteinuria.

**Pathologic proteinuria** is marked in glomerulonephritis. The proteinuria increases with the increasing severity of the renal injury. Proteinuria also results in poisoning of the renal tubules by heavy metals like mercury, arsenic or bismuth.

**Hemoglobin** is also present as a result of hematuria due to hemorrhage from the kidneys or urinary tract, clotting may occur due to sufficient fibrinogen on passing of much blood.

**Mucus** is the term for an unidentified protein precipitated by acetic acid in the cold. It is mucin. The mucus is increased in infection of the bladder.

**Bence-Jones proteins** found in the urine are the peculiar proteins which are light chain fragments of globulins. Most commonly they occur in multiple myeloma and rarely in leukemia. They are precipitated when the urine warmed to 50-60°C and re-dissolved almost completely at 100°C and precipitated again on cooling.

**Glucose:**

Normal urine has reducing sugar concentration of 1-1.5 g/L. Of this glucose is 200-300 mg. In glucosuria when more than this quantity is found in urine.

Transient glycosuria is observed after emotional stress such as exciting atheletic contest. When blood glucose level is exceeded the range 160-180 mg/dL, glucose is excreted by kidney and renal glucosuria is observed. Physiological renal glycosuria is seen in pregnancy.

Many times, fructose, galactose, lactose pentose sugars are found in urine.

The presence of glucose must be tested by Benedict's test. But in case of pregnant women and lactating mother, the Osazone test must be performed for urine glucose to eliminate the lactose present in urine.

**Ketone bodies:**

Only less than 1 mg of ketone bodies are excreted in urine normally in 24 hours. Increased amount of ketone bodies are excreted in urine in starvation, diabetes mellitus, pregnancy, ether anesthesia, and some types of alkalosis. Excess fat metabolism may induce a ketonuria in many animals. Increased amount of ammonia is excreted in acidosis accompanying ketosis.

**Bilirubin and Bile salts:**

Bilirubin is found in the urine in cases of obstructive or hepatic jaundice. Bilirubinuria is accompanied by the excretion of bile salts. Bile salts may be excreted in urine without bile pigment in certain stages in liver disease. In excessive haemolysis, traces of bilirubin without bile salts are excreted in urine.

**Blood:**

In the injury of the kidney or urinary tract blood is excreted in the urine in addition to its presence in nephritis. Free haemoglobin is also found in urine after quick haemolysis, e.g., in black water fever (a complication of malaria) or after severe burns. This is known as haemoglobinuria. Presence of myoglobin in urine is observed in crush injuries and muscular disorder is known as myoglobinuria.

**Urinary Deposits:**

The commonest deposits are phosphates, oxalates and urates and are frequently seen in normal urine.

**Phosphates:**

They are usually found in alkaline urines. The commonest is ammonium magnesium phosphate which forms a characteristic crystal. A less common form is calcium hydrogen phosphate which forms long prisms. Amorphous calcium and magnesium phosphates may be

deposited from alkaline urines. The deposition of phosphates is due to a change in pH after the urine has been passed.

**Calcium Oxalate:**

This is found in acid urine but may be found in alkaline urine. The crystals are of two types—octahedra, dumb-bells. Calcium oxalate is insoluble in acetic acid.

**Urates:**

They are usually found in acid urines. Uric acid separates into different forms including prisms, barrels, hexagons and needles which are always pigmented. Urates are re-dissolved on warming the urine. The cause of deposition of urates is the cooling of urine after it has been passed.

**Collection of urine:**

Urine specimens are collected in a variety of ways according to the type of specimen required, the collection site and patient type.

First morning specimen is the main choice for urinalyses and microscopic analyses.

Midstream clean catch specimens are strongly recommended for microbiological culture and antibiotic susceptibility testing because of the reduced incidence of cellular and microbial contamination.

Timed collection specimen may be required for quantitative measurement of certain analyte such as creatinine, urea, potassium, sodium, calcium, uric acid, amino acids, protein, oxalate etc.

Collection from catheters is often necessary. This is carried out using a syringe followed by transfer to a specimen tube.

To protect healthcare workers from exposure to the specimen and protect the specimen from to contaminants, leak proof cups should be used. Sterile containers are recommended for culture and sensitivity test of urine. Urine should be collected in sterile amber coloured containers for assay of light sensitive analytes such as urobilinogen and porphyrins.

**Preservation of urine**

There is bacterial overgrowth over the non-refrigerated and unpreserved urine, are not suitable for analysis and testing. Generally, a preservative is added to the collection container before the urine collection. Commonly used preservatives for chemical and urinalyses include tartaric acid, boric acid, chlorohexidine, thymol, sodium propionate or mixture of these. Preservation times are typically within the range of 24-72 hours.

**Formation of urine:**

Kidneys filter unwanted substances from blood and produce urine to excrete them. Generally, end products of metabolism of different substances and excess substances from diet are excreting. Kidneys maintain water and electrolytes balance. Urine formation involves three processes: glomerular filtration, tubular re-absorption and tubular secretion.

Glomerular filtration: Each kidney contains over one million tiny structures called nephrons. Each nephron has glomerulus, the site of blood filtration. As blood flows through the glomerulus, the glomerular hydrostatic pressure and osmotic pressure is created and promoting filtration. The filtrate includes water, small molecules and other dissolved substance such as urea, uric acid, creatinine, amino acids, glucose, Na+, K+ vitamins etc but large molecules like proteins, blood cells are cannot pass through filtration membrane. The amount of filtrate produced is 180 litres per day. About 99% of this filtrate is re-absorbed as it passes through the nephron and remaining 1% becomes urine.

Tubular re-absorbtion: When the filtrate exits the glomerulus, it flows into a duct in the nephron called the renal tubule. The filtrate is re-absorbed by diffusion, active transport, co-transport, and osmosis process from the tubules. The useful substances including water, electrolytes, organic nutrients such as glucose, amino acids, vitamins, hormones are selectively absorbed from the filtrate back into the blood in the proximal convoluted tubule (PCT). Only 60-70% of the filtrate reaches the Henle loop where water, sodium and chloride is re-absorbed, so that only 15-20% of the original filtrate reaches to the distal convoluted tubule (DCT) where more electrolytes are reabsorbed and only dilute filtrate entries the collecting ducts.

Tubular secretion: Tubular secretion occurs mostly in PCT and DCT where the unfiltered substances like antibiotics, toxins and excess substance like $H^+$, $K^+$ etc are secreted into tubule, combine with the remaining filtrate and become urine. The urine flows out of the nephron tubule to collecting duct. Ultimately it passes out of kidney through the renal pelvis, into ureter, and down to the bladder.

Average urine production in adult is about 1-2 litres per day depending on the state of hydration, activity level, environmental factors, weight and individual's health.

**Clinical analysis of urine:**

Dipstick test or reagent strip method: Dipstick, a thin plastic stick with at least 10 different chemical pads on it, is dipped in the small portion of collected urine. If certain substances are present, there will be a change in colour of the chemical pad. A dipstick test checks pH, glucose, ketones, protein, bilirubin, blood etc.

**pH:** This test is based on a double indicator system which gives a broad range of colours, usually orange to yellow (acidic) and green to blue (alkaline).

Glucose: Glucose is oxidized by aerial oxygen to produce gluconic acid and H2O2 in presence of glucose oxidase. Then peroxidase mediated the oxidation of chromogens by $H_2O_2$ that leading to colour.

**Ketone:** Acetoacetic acid, a ketone bodies, reacts with nitroprusside under alkaline medium producing a pink coloured complex.

**Protein:** This test is based on the principle of the protein error of indicators that means indicator changes its colour in presence of protein specially albumin. Generally, amino acid in a protein molecule accepts hydrogen ion from the indicator and so colour of the indicator is changed.

**Bilirubin:** This is based on azo-coupling reaction of bilirubin with diazotized 2,4-dichloroaniline in acid medium to produce an azo dye with colour pink to violet.

**Blood:** The urine trip test of blood is based on haemoglobin's pseudo peroxidase activity in catalysing a reaction between cumene hydrochloride and chromogens 3,3′,5,5′-tetramethylbenzidine produces a dark blue product.

**Benedict's test for sugar:** 5 ml of Benedict's solution is added to 8 drops of urine and heated to boiling for 3 minutes. A colour precipitate will be observed varying colour green to yellow and brown to reddish.

**Heat coagulation test for protein:** Heat the urine sample in a test tube and then 1-2 drops of acetic acid is added to it. On heating protein is coagulated and coagulation is increased further by the addition of acid. As a result urine becomes turbid.

**Microscopic examination:** Abnormal components like cells, casts, crystals, parasites, bacteria are detected using microscope. After centrifuge the urine sample, the sediment is taken on a glass slide and covered with cover slip. This slide is then examined under a microscope whether cells, casts, crystal are present or not.