THE LIVER

It is a lobed organ lying in the upper right side of the abdomen beneath the diaphragm. It is the largest single organ in the body and chemically the most active. It weighs 1.44-1.66kg .It is connected to 2 blood vessels, i.e. the **hepatic artery** and the **portal vein**. The hepatic artery carries blood from the aorta whereas the portal vein carries blood containing digested nutrients from the entire GIT and also from the spleen and pancreas. These blood vessels subdivide into capillaries, which then lead to a **lobule**. Each lobule is made up to millions of **hepatic cells (hepatocytes)** which are the basic metabolic cells. Lobules are the functioning units of the liver.

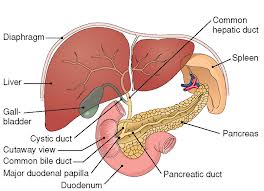
**Location of the Liver**

The liver is located **superolateral** (above and to the side) of the stomach. It is found in the abdominal cavity of the body, which is where many of the internal organs reside, and is **inferior** to (below) the lungs.



**.**

|  |
| --- |
| Liver |
| The Liver (shown in green) |



It is the largest internal organ in the body, and is second to the skin as the largest organ overall. It has a rubbery texture and is reddish-brown in colour.

**Shape and Sections**

The liver has a triangular shape, and is divided into four lobes. The left and right anatomical lobes are visible when viewing the liver from the front, while the **quadrate** and **caudate** lobes are visible when viewed from the underside. These lobes are divided further into smaller functional sections, called **lobules**. These lobules have various functions.

FUNCTIONS OF THE LIVER.

The liver is a complex organ which forms part of the reticuloendothelial system. It has got several roles it plays as explained below;

**Manufacture and secretion of bile.**

Bile is a yellow –green coloured fluid which contains cholesterol, a small amount of bilirubin and bile salts (cholic acid and chenodeoxycholic acid, conjugated with glycogen and taurine). The bile is transported through the hepatic ducts to the gall bladder where it is stored, concentrated and released into the duodenum where it is essential for the adequate absorption of **fats** and of **vitamin k**.

Much of the bile secreted into the intestines is reabsorbed in the liver and re-excreted as bile. Only a small amount of bile is normally excreted in faces.

**Metabolism and conjugation of bilirubin**

Bilirubin is an orange –yellow pigment derived from the catabolism of haem in the reticulo-endothelial (liver, spleen, bone marrow, thymus gland). The haem (iron porphyrin) of the haemoglobin molecule is first separated from the goblin and the porphyrin part is converted to biliverdin which is then reduced to bilirubin.

This bilirubin is unconjugated (indirect bilirubin) it is not soluble in water and so cannot be excreted in urine. It is bound to plasma albumin and transported in blood to the liver where the enzyme glucuronosyl transferase conjugates (joins) glucuronic acid to bilirubin forming bilirubin glucuronides (conjugated bilirubin). It is non-toxic and water soluble, therefore can be excreted in urine.

Bilirubin glucuronides are secreted into bile and later secreted into the upper small intestine. In the colon it is degraded then converted by bacterial action into compounds collectively known as urobilinogen/stercobilinogens which are excreted in urine /stool.

**Storage**

Stores glycogen and when required converts it to glucose (glycogenolysis) to maintain the blood glucose level. If the liver has used up all its glycogen stores and the blood glucose level is below normal, the liver is able to convert protein and fat into glucose (gluconeogenesis)

Stores **iron**, **vitamin A** and **B12**.

**Synthesis**

To manufacture plasma proteins i.e;**fibrinogen, albumin** and **globulin** (except **gamma globulin**) .The liver also produces transport proteins such as **transferrin** which binds and transports iron ,and heptoglobin which combines with free haemoglobin .**Alfa fetoprotein** is normally produced by the liver before birth.

Synthesis of blood clotting factors including **fibrinogen**, **prothrombin** and **factors** v, vii. ix. x. xi and xii ,**protein C** ,**protein S** and **antithrombin**

**Detoxification**

Detoxification of ammonia released from amino acid deamination by converting it to urea for excretion by the kidneys. The liver is also involved in the **oxidation**, **reduction**, **hydrolysis** or **conjugation** of other metabolic products, drugs, hormones and alcohol. Prolonged intake of alcohol seriously damages the liver cells causing **liver cirrhosis**

**Body defence mechanism**

Assists in the removal of worn out blood cells and micro –organisms from the body. The kupfer cells form part of the mononuclear phagocytic defence (reticulo endothelial system) of the body

**Hormone production and breakdown**,

Produces **thrombopoietin**, a glycoprotein hormone that regulate the production of platelets by the bone marrow, the liver breaks down/ inactivates hormones like **insulin**, **glucagon, sex hormones** etc. therefore helps to regulate the plasma hormone levels.

**Note:** currently there is no artificial organ or device capable of emulating all the functions of the liver (liver dialysis a treatment for liver failure only emulates a few functions .yet it is responsible for up to 500 separate functions usually in combination with other systems and organs .

**Carbohydrate metabolism**

Gluconeogenesis (the synthesis of glucose from certain amino acids, lactate or glycerol)

-glycogenolysis (breakdown of glycogen into glucose

-glycogenesis (the formation of glycogen from glucose). Muscle tissues can also do this

**The liver also performs several roles in lipid metabolism**

* Cholesterol synthesis
* Lipogenesis the production of triglycerides(fats)
* A bulk of lipoproteins are synthesized in the liver

**Red blood cell production**

In the first trimester, the liver is the main site of red cell production .by the 32nd week of gestation, the bone marrow has almost completely taken over the task.

The liver also produces insulin like growth factor 1(1GF-1), a poly peptide protein hormone that plays an important role in childhood growth and continues to have anabolic effects in adults.

The liver synthesizes angiotensinogen, a hormone that is responsible for raising the blood pressure when activated by rennin, an enzyme that is released when the kidneys sense low blood pressure.

The liver also functions as blood reservoir, being an expandable organ, large quantities of blood can be stored in its blood vessels. Its normal volume in the hepatic veins and that in hepatic sunuses is about 450ml .During cardiac failure with peripheral congestion the liver expands and 0.5 to 1 litre of extra blood is occasionally stored in the hepatic veins and sinuses due to high pressure in right atrium which causes back pressure in the liver.

LIVER FUNCTION TESTS (LFTS)

These are series of tests one can use to look for different analytes within a urine or blood sample. These are tests that measure various chemicals in blood made by the liver. They are tests that assess the state of the liver and the biliary system.

LFTS can give an idea of which disease to suspect but are not enough to make a diagnosis. They are just one of the series of tests done, to diagnose disease. If one is already diagnosed with a disease condition, LFTS can be help in monitoring the disease or treatment.

A rise or fall in the levels of substances tested may indicate damage or improvement to one`s liver. Some of the substances that may be looked for in one`s blood include **enzymes** and **proteins** made by the liver.

For example

**Alanine amino transferase (ALT**) and **Aspartate Aminotransferase (AST)** might be raised if the liver is **inflamed** or **injured**.

Alkaline phosphatase (ALP) and Gamma –Glutamyl transferase (Gamma GT) might be raised when there is **blockage** in the liver or bile duct or with a **high alcohol intake** or in bone disease

**Bilirubin**-it’s a chemical in bile and it’s a breakdown product of **haemoglobin** in the RES; bilirubin may accumulate in plasma (beyond normal values) if the **liver cells are damaged** and cannot conjugate bilirubin which can later be excreted by the kidney`s or if there is blockage to the flow of bilirubin. It can also rise if there is a problem with the gall bladder

**Albumin;** albumin is the main protein made by the liver and may be low in some cancers or in malnutrition. It **transports hormones**, **vitamins** and other substances throughout the body.

* Blood clotting studies can also be done. Because the liver makes proteins that are needed for clotting, increases or decrease in these proteins can help indicate liver problems. **However, the most widely used test to check liver function are the albumin test and the bilirubin test. They measure how well the liver creates albumin a protein and disposes off bilirubin a waste product of the blood.**

CLINICAL SIGNIFICANCE OF LIVER FUNCTION TESTS:

LFTs are tests carried out on blood and urine for the diagnosis of and management of liver diseases. They are valuable in the following respects

* Establishing the presence or absence of liver disease
* Determining prognosis of a disease process
* Helps us to categorise liver diseases appropriately. The pattern of the blood results may help to say which disorder is causing the problem e.g. depending on which enzyme is raised
* Help in monitoring drug therapy
* Used as an entry point for antiretroviral therapy and its monitoring
* To monitor the activity and severity of liver disorders
* As a routine precaution after starting certain medicines to check that they are not causing liver damage as a side effect.
* The liver function tests are used to assess the functioning of the liver cells in all the metabolic reactions.

The malfunctions may be caused by;

Consumption of excessive alcohol and dietary toxins causes liver cirrhosis

Parasitic infection e.g. visceral leishmaniasis, hepatic amoebiasis, hepatosplenic schistosomiasis, hepatic hydatid disease

Infection with hepatitis, yellow fever, Marburg, Ebola, cytomegalovirus etc.

CLASSIFICATION OF LIVER FUNCTION TESTS.

It assists one to decide on an appropriate test to carry out which is a specific marker of a particular liver malfunction. Liver function tests are classified depending on the type of hepatic function examined as follows.

**Tests that measure secretion and excretion**

* These are tests which measure **hepatic uptake, conjugation** and **excretion of organic** **substances**. The common test used here is: Measurement of **serum /plasma bilirubin**
  + Qualitative detection of bilirubin, urobilinogen in urine
  + Hippuric acid synthesis test-detoxifying capacity of the liver Bromosulphthalein test

Among the above tests the common ones used to assess the liver function are the **serum bilirubin** and **urine bilirubin** and **urobilinogen** detection methods in urine

**Tests based on serum enzyme activity,**

1. Transaminases

-Aspartate Amino Transferase **(AST)**

-Alanine Amino Transferase **(ALT)**

2. Alkaline phosphatase **(ALP)**

3. Gamma- Glutamyl transferase **(Gamma GT)**

**Tests based on specific biochemical functions/synthesis**

1. Measurement serum/plasma total protein

2. Protein electrophoresis.

3. Measurement of serum/plasma albumin

(d) **Liver biopsy** –a small piece of liver tissue is taken for examination with a microscope for signs of damage or disease.

Liver biopsies are collected by a clinician and are of needle type

TESTS BASED ON ENZYME ACTIVITY

**What are enzymes?**

Enzymes are biological catalysts, protein in nature that speed up biochemical reactions in the body without themselves being destroyed or altered. Without enzymes to speed up biochemical reactions, life could not exist because the life of just one cell depends on the simultaneous occurrence of hundreds of chemical reactions that must take place rapidly.

Enzymes are catalysts that lower the activation energy of a chemical reaction and thereby increase the rate of reaction. (Enzyme catalyzed reactions often occur from one million to one hundred million times faster than the corresponding unanalyzed reaction). .

Enzymes are very specific with one or very few types of molecules called substrates.

**A substrate** is the reacting substance in the catalyzed reaction and is hydrolyzed to products

Enzymes are found in all tissues and fluids of the body catalyzing different reactions in different parts of the body.

**Enzyme activity**

It is defined as the amount of substrate the enzyme converts to a reaction product per unit time

For system Internationale, enzyme activity is defined as the amount of enzyme that under defined assay conditions catalyses the conversion of 1umol of a substrate per minute.

**Reference range for enzymes**

This refers to the range between the minimum and maximum value of a particular enzyme activity.

Properties of enzymes

* Most enzymes are **proteins**, which is one reason why they are affected by **heat, pH** and other factors that affect proteins.
* They are all biological catalysts. They speed up a reaction without being used up; this means they can be used over and over again.

A small amount of enzyme can affect the change of a large amount of chemical.

The reactions are reversible. Enzymes catalyze reaction in both directions (reversibility). e.g., starch..........................................>maltose. The enzyme amylase is specific to this reaction and thus catalyses it. It can catalyze it in both directions, which is the reaction from starch to maltose and also that of maltose to starch.

* Enzymes are highly selective (specific), catalyzing specific reactions only. This specificity is due to the shape of the active site of the enzyme involved i.e. they are selective for a single substrate or groups of similar substrates**, e.g., maltase reacts on maltose and sucrase on sucrose.**
* Enzymes have the ability to be controlled by effectors (activators, coenzymes, prosthetic group) whose concentration does not take part in the reaction.
* When enzymes enter chemical reactions they remain unchanged at the end of the reaction

**How do enzymes work? (Lock and key hypothesis)**

The first step involves the encounter of the enzyme with its substrate to form an enzyme -substrate complex. The part of the enzyme that binds with a substrate is called the **active site** (That part of the enzyme that has just the right shape and functional groups to bind to a specific substrate). The shape of the active site is complementary to the shape of the substrate; therefore, the substrate neatly fits into the active site of the enzymes. This determines the specificity of the enzyme because only the substrate that fits into the active site will be used in the reaction.

Because the substrate must fit in the active site, the mode of enzyme action is analogous to a **lock and key**.

The lock and key hypothesis explains how the substrate simply fits into the active site to form a reaction intermediate (the lock and key model of enzyme substrate binding assumes that the enzyme active site has a rigid structure that is precisely complementary in shape and charge distribution to the substrate)

Enzyme + substrate = enzyme –substrate complex

Enzyme-substrate (transition state). The substrate attaches temporarily to the active site by non-covalent forces (hydrogen bonds, ionic and hydrophobic interaction)

Enzyme-product complex

Enzyme + product

The fitting of the enzyme into the substrate explains the specificity of the enzymes

That is, a given enzyme is able to catalyse only a single reaction or at most a few reactions involving substrates with very similar molecular structure. At the end of the reactions, products are liberated into the solution, thus regenerating the original enzyme molecule. In this way one enzyme molecule can catalyze the transformation of many substrate molecules.

**Activation energy**

This is the energy required for a molecule to form an activated enzyme –substrate complex. Enzymes bind temporarily to reactants (substrates) and then catalyze the reaction. In doing so, they lower the amount of activation energy needed for the reaction to take place and to proceed faster. (Enzymes lower activation energy by changing the path by which the process occurs, providing a lower energy route for the conversion of the substrate into the product)

There must be an active site for substrate attachment and sufficient energy (activation energy), enough to overcome the energy barrier for the reaction.

FACTORS AFFECTING THE RATES OF ENZYME CATALYSED REACTION

These factors include:

* Temperature
* PH
* Concentration of enzyme or substrate
* presence of activators
* presence of inhibitors
* Time of incubation
* Glassware
* Light rays
* Reagents

Effect of temperature on enzyme activity

The activity of enzyme is strongly affected by changes in temperature. As the temperature of the solution containing the substrates and enzyme increase, the rate of reaction also increases (0 – 37oC). This occurs because as the temperature increases, the kinetic energy of the molecules also increases. As the molecules move around faster, there are more collisions, which result into more reaction between the enzyme and the substrate molecules. This effect continues up to the optimum temperature. Above the optimum temperature, the rate of reaction decreases as increasing kinetic energy causes the hydrogen bonds holding the tertiary structure of the enzyme to break. This causes the action site to change shape (denaturation). The substrate no longer fits in the active site and the rate of reaction decreases until all the enzyme molecules are denatured and rate of reaction is zero.

**Effect of PH on enzyme activity**

Each enzyme works within quite a small PH range, there is a PH at which enzyme activity is greatest **(Optimum PH).**

The PH changes affect the ionic charges on some of the **R - groups** present in the amino acids making up the enzyme molecule. Therefore, as a result of changing the PH, the tertiary structure will change and this will alter the active site and the substrate will no longer be able to fit in the enzyme molecule.

**NOTE:** PH of normal body is **6.8**. Most body enzymes wok with maximum efficiency at that PH with exception of a few enzymes like **pepsin** which acts best at **PH 2** (acidic stomach environment).

**Effect of substrate and enzyme concentration on enzyme activity**

**Substrate concentration**

The rate of reaction increases with increasing substrate concentration up to point X, above which any further increases in the substrate concentration produces no significant change in reaction rate. This is because the active sites of the enzyme molecules at a given amount are saturated with substrate. The enzyme – substrate complex has to dissociate before the active sites are free to accommodate more substrate.

Enzyme concentration

The rate of reaction increases with increasing enzyme concentration up to a point X, above which any further increases in the enzyme concentration produce no increase in the rate of reaction. At point X, all the substrate has been changed into products and there are no more substrates to fit in the available sites of the enzymes (Reaction becomes constant).

Effect of activators / cofactors on enzyme activity

Some enzymes require a second substance (activators) to be present in order to do their job.

Many enzymes consist of a protein **(apoenzyme)** and anon-protein **(cofactor)** components. The complex or the whole enzyme is known as a **holoenzyme**.

* Holoenzyme is the complete catalytically active enzyme together with its cofactor.

holoenzyme = apoenzyme + cofactor

Apoenzyme =is the protein component of the enzyme without any cofactor. These proteins are usually globular in nature, the secondary and tertiary protein structures of these enzymes and it is these structures that are disrupted by changes in temperature and PH.

Cofactor-is the non-protein component of the enzyme. It is a small organic or inorganic molecule that an apoenzyme requires for its activity.

**Effect of inhibitors on enzymes activity**

An inhibitor is a substance that diminishes or stops the rate of a chemical reaction.

An enzyme inhibitor is a substance that slows or stops an enzyme catalyzed reaction. These inhibitors block or distort the enzyme active site.

Inhibition of an enzyme reaction is either reversible or irreversible

**Reversible inhibition**

This implies that the activity of the enzyme is fully restored when the inhibitor is physically removed from the system. Reversible inhibitors do not react covalently with the enzyme.

Reversible inhibitors are classified as competitive and non-competitive

**Competitive inhibition**

The competitive inhibitor occupies the active site of the enzyme and prevents the substrate molecule from binding to the enzyme. The inhibitor and the substrate molecules have similar shape ie they are both complementary to the active site of the enzyme. The inhibitor therefore competes with the substrate for this same active site. Changing the proportions of these molecules changes the degree of inhibition. If the concentration of the substrate is increased enough the inhibition can be removed.

**Non-competitive inhibition**

Non-competitive enzyme inhibitors bind to the R groups of amino acids or perhaps the metal ion cofactors, however the binding is weak and the enzyme activity is restored when the inhibitor dissociates from the enzyme inhibitor complex.

These inhibitors do not bind to the active site but do modify the shape of the active site by binding elsewhere in the protein structure. **The entire three dimensional structure is needed to maintain the correct shape of the active site.**

**Note**: A non-competitive inhibitor is usually structurally different from the substrate. The attachment of the inhibitor on the enzyme distorts the shape of the active site so that the substrate can no longer fit properly into the enzyme active site.

**Irreversible inhibition**

Inhibitors react covalently with the enzyme, preventing the substrate from binding to this enzyme.

The inhibitor binds to one of the R groups of the amino acid in the active site. This inhibitor binding may block the active site binding groups so that the enzyme substrate complex cannot form.

* The activity of the enzyme is not restored when the inhibitor is removed.

Irreversible inhibitors which include **snake venoms** and **nerve gases** generally inhibit many different enzymes

**Time of incubation**

The substrate concentration falls with time as the product concentration increases. Timing is therefore important because the substrate and the product concentrations are changing all the time and it is one of these that is being measured. An accurate timer must be used to measure the incubation time of an enzyme and its substrate. (after the specified incubation time, all the substrate will be converted into products, therefore the reaction will not proceed any more).

**Glassware**

Glassware contaminated with traces of heavy metals or detergents can inhibit enzyme activity. The use of chemically clean glassware is essential. Cuvettes must be clean and their optical surfaces dry and free from scratches and finger marks.

**Light rays**

Ultraviolet light tends to inhibit enzyme activity while blue or red light tends to increase it, therefore samples for enzyme analysis must therefore be protected from direct light.

**Reagents** Test results can be seriously affected if reagents, particularly substrates and buffers are not prepared correctly. Precautions should be taken to avoid bacterial contamination of reagents.

STANDARD OPERATING PROCEDURES FOR CARRYING OUT LIVER FUNCTION TESTS BASED ON ENZYME ACTIVITY

**Measurement of transaminases**

These enzymes are involved in intracellular metabolism therefore alterations in serum enzyme activity indicates malfunction of the liver thus there are markers of cellular damage.

They catalyse a reaction that involves the transfer of an **amino group** from an **alpha amino** acid to an alpha keto acid compound resulting in the formation of different amino acid compounds. The process is called **transamination** and these enzymes include;

1. **Aspartate amino transferase (AST) formally known as serum glutamate oxaloacetate transaminases (SGOT)**

This enzyme is present in all tissues of the body of high metabolic activity. It is not organ specific. It occurs in the **kidney, liver, cardiac muscles** and **skeletal muscles**. It also occurs in relatively low concentrations in the **spleen, brain, pancreas** and the **lungs**. The enzyme is released in to circulation following an **injury or death of cells** and the amount of AST in blood is directly related to the number of damaged cells and the amount of time that passes between injury to the tissue and the test. Following severe cell damage, the blood AST level will rise in 12 hours and remain elevated for about 5 days. AST may be high in **liver diseases** but not specific. It is necessary because it is also an indicator of **myocardial damage** but **ALT is more specific for liver malfunction.**

Measurement of Aspartate amino transferase (AST) enzyme activity (Reitman-Frankel method)

**Purpose of the test.**

* Measurement of AST activity is performed to diagnose liver disease and myocardial infarction.

**Value of the test**

**-**It is used to diagnose **myocardial infarction**

-AST levels are used to differentiate liver disease conditions e.g. very high elevated levels are present in hepatocellular damage like in hepatitis. Small increase of AST is experienced in **obstructive liver disease**.

-used to determine the status of the liver cells before antiretroviral therapy is administered.

Principle

When AST is incubated at **37oC** for exactly **60 minutes** at a **pH 7.5** with a buffered substrate containing aspartate and alpha-ketoglutarate. AST catalyses the transfer of an amino group from aspartate to ketoglutarate, forming oxaloacetate and glutamate. The oxaloacetate reacts with **2, 4-dinitrophenylhydrazone (DNPH)** in alkaline medium to form **red brown colour** whose absorbance is measured in a colorimeter using a green filter or in a spectrophotometer at **505nm wavelength**.

**Method.**

Check the leaflet provided by reagent manufactures.

**Interpretation of results**

Reference ranges**: 4-12 u/l**

Very high AST activities usually accompany **liver disease** especially when there is hepatocellular damage e.g. in **hepatitis**. The higher the AST activity, the greater the degree of liver damage. In viral hepatitis, the serum or plasma AST is often raised before the patient appears jaundiced and remains elevated for as long as the virus remains active.

**Obstructive liver disease** is usually accompanied by a small or moderate AST rise especially in the early stages.

* AST is also raised in myocardial infarction
* AST being distributed in body tissues, many other diseases involving cellular damage may be accompanied by increases in AST levels e.g **severe bacterial infections, malaria, pneumonia, tumours** etc
* AST is also increased in muscle disorders and following **surgery, injury** or **blood transfusion**.
* AST is artefactually increased when haemolysis is present or if the blood has been stored unseparated.

**Sources of error**

High concentrations of **aldehydes, ketones** or **oxoacids** can stimulate elevated transaminase activity. Measurement against the sample blank instead of the reagent blank avoids the risk of finding such artefactuals

MEASUREMENT OF PLASMA OR SERUM ALT.

Reitman-frankel ALT method:

1. **Alanine amino transferase (ALT) formally called serum glutamate pyruvate transaminases. (SGPT)**

High concentrations of this enzyme occur in the liver and relatively low levels are found in the **heart muscle** and **kidney**, therefore its elevation is specific indicator of liver cell damage

**Value of test**

* Measurement of ALT activity is mainly performed to investigate liver disease. (Its elevation is specific indicator of liver cell damage).
* Increasingly, ALT is being measured to monitor for patients receiving retroviral drugs associated with hepatotoxicity such as **Neviraphine** and **Stavadine**.
* It is also used to monitor the course of other treatments like for **hepatitis, active post necrotic cirrhosis** etc.
* The test is used to differentiate between haemolytic and hepatic jaundice
* Used to determine the status of the liver before antiretroviral therapy is administered.

**Principle:**

ALT is incubated at **370c** for exactly **30** minutes in a PH **7.4** buffered substrate containing alanine and alpha ketoglutarate, ALT catalyzes the transfer of the amino group from alanine to ketoglutarate, forming glutamate and pyruvate

The pyruvate reacts with **2, 4-dinitro-phenylhydrazine (DNPH)** to form 2, 4 dinitrophenylhydrazone **(pyruvate hydrazone)** which in alkaline medium gives a red brown colour.

The intensity of the colour formed is directly proportional to the ALT activity in that solution (serum plasma) and its absorbance is measured in a colorimeter at **520nm** or spectrophotometrically at **505nm**.

**Note;** the method for ALT is not so different from that of AST by Reitman-Frankel. The difference is brought about by the substrates used in the two methods.

Normal reference range: **3-15 u/l**

Measurement of Alkaline Phosphatase (ALP) enzyme activity

ALP enzymes are non-specific zinc containing hydrolytic enzymes localised in **cell membranes** and **bones**. They display their maximum activity in the range of **PH 9.0-10.5**. ALP is practically present in all tissues of the body, especially in **intestinal epithelium, kidney tubules, bone (osteoblasts), liver** and **placenta**. It is normally released in to circulation when there is excessive cell damage or when the rate of synthesis increases.

**Value of the test**

Measurement of ALP activity is performed to investigate two groups of conditions:

* Hepatobiliary disease
* Bone disease associated with increased osteoblastic activity

In order to establish whether a raised serum or plasma ALP is of liver or bone origin, further tests are carried out for example **5-nucleotidase** or **gamma glutamyl transferase enzyme measurement** is done. These enzymes are also raised when increased levels of ALP are of hepatic origin.

ALP measurement is based on the hydrolysis of many **monophosphate substrates** by the enzyme to **inorganic phosphates**. There are a number of substrates which can be used in the assay, for example;

Di-sodium phenyl phosphate

B-glycerophosphate

4-nitrophenylphosphate

P-nitrophenylphosphate

Phenolphthalein monophosphate

Thymophthalein phosphate

Measurement of Alkaline Phosphatase (ALP) enzyme activity by Kind and Armstrong method

**Principle**

ALP hydrolyses Di-sodium phenyl phosphate to release phenol and sodium phosphate. The phenol reacts with 4-amino phenazone in presence of potassium ferricyanide in alkaline medium **(PH 9-10.5)** to give pink colour.

The intensity of the colour formed is directly proportional to the ALP enzyme activity in that sample and is estimated at **510nm** wavelength.

**Method**

Always follow the kit manufacturer’s instructions when performing the test.

TESTS BASED ON SECRETION AND EXCRETION

BILIRUBIN

It is an **orange –yellow pigment** formed in the liver by the breakdown of heamoglobin and excreted in bile.

Bilirubin circulates in the bloodstream in two forms:

**Indirect (or UN conjugated) bilirubin**. This form of bilirubin does not dissolve in water (it is insoluble). Indirect bilirubin travels through the bloodstream to the [liver](http://www.webmd.com/hepatitis/rmq-know-your-liver), where it is changed into a soluble form (direct or conjugated).

**Direct (or conjugated) bilirubin.** Direct bilirubin dissolves in water (it is soluble) and is made by the liver from indirect bilirubin.

BILIRUBIN METABOLISM

Bilirubin is an orange-yellow pigment derived from the breakdown of erythrocytes and other haem containing proteins such as **myoglobin** and **cytochromes**.

After 120 days, RBCs are broken down in the reticulo-endothelial system (liver, spleen, thymus gland and bone marrow). Haemoglobin is released and split into **haem (iron porphyrin)** and **globin**.

The globin is transported to **protein pool** for storage from where it is recovered for new amino acid synthesis. The haem (iron porphyrin) is split into **haem** and **porphyrin**.

The haem is transported to the **iron pool** where it is stored in two forms, the **ferritin** and **haemosderin**. Ferritin is a soluble **iron protein complex** while haemosderin is an **iron storage complex** that can be re-utilised for haemoglobin synthesis.

The porphyrin part is converted to **biliverdin a green substance** which is reduced by tissue enzymes to a yellow pigment called **bilirubin**.

This bilirubin is uncojugated (indirect bilirubin), it is not soluble in water so it cannot be excreted in urine.

It is bound to **plasma albumin** and transported to the liver where the enzyme **glucuronosyl- transferase** conjugates (joins) **glucuronic acid** to bilirubin forming **bilirubin glucuronides** (conjugated, direct or water soluble bilirubin).

This bilirubin is transported in bile to the gall bladder to be later secreted into the upper small intestine.

In the ileum and colon, bilirubin is deconjugated and is reduced by bacteria to various pigments and colourless chromogen called **urobilinogen** most of which (99%) are secreted in faeces as **stercobilinogens**.

A small amount of the reabsorbed urobilinogen (1%) is carried in blood through the liver and transported to the kidneys where it is excreted in urine.

When urobilinogen is exposed to air, it is oxidized to **urobilin**; therefore urine should be examined immediately.

**Note:** Normal urine contains small amount of urobilinogen **but no bilirubin**.

The conjugated bilirubin circulates in blood at low concentrations in normal conditions, however in disease conditions it is raised in blood **(hyperbilirubineamia)** leading to a clinical condition known as **jaundice/ icterus**. Accumulation of bilirubin in blood leads to yellowing of the **skin, sclera of the eye** and even **brain tissue (jaundice).** It becomes visible when the bilirubin level rises above twice the normal limit.

TYPES OF JAUNDICE

**Definition of jaundice;** It is where by bilirubin is raised in the blood circulation in higher amounts

Haemolytic jaundice (pre-hepatic jaundice)

This occurs following increased red blood cell breakdown leading to the formation of more bilirubin that what the liver can metabolise. The bilirubin that builds up is un conjugated type therefore cannot be excreted in urine. This type of jaundice can be caused by **malaria** **infection, HDNB, incompatible blood transfusion, certain drugs** like **sulphonamides** and **toxins from bacteria, snake venom** etc.

Hepatic jaundice (hepatocellular)

In this condition, the bilirubin is built up in plasma because it is not transported, conjugated or excreted by the liver because the **liver cells are damaged**. The bilirubin here is both unconjugated and conjugated and there will be increased amounts of **urobilinogen in urine**. The condition can be caused by **hepatitis due to viral infections, drugs, chemicals, bacterial infections** etc.

Post hepatic (obstructive) jaundice

This occurs following obstruction of the flow of bile in the small channels or in the main bile duct. This can be caused by gall stones or tumours closing the biliary tract. The bilirubin is conjugated, therefore it is found in urine.

Neonatal jaundice

This is commonly known as physiological jaundice and develops in new born babies in the following conditions:

- Haemolytic disease of New born that occurs within 24hrs after birth.

- When the baby is infected e.g**. septicaemia, congenital syphilis, toxoplasmosis** and **viral infections**.

- If the conjugation mechanism of the baby is fully developed.

**NOTE:**

Unconjugated type of bilirubin predominates in this condition and sometimes it can be deposited on the brain a condition referred to as **kernicterus**

Total bilirubin and direct bilirubin levels are measured directly in the blood, whereas indirect bilirubin levels are derived from the total and direct bilirubin measurements.

When bilirubin levels are high, the [skin](http://www.webmd.com/skin-problems-and-treatments/picture-of-the-skin) and whites of the [eyes](http://www.webmd.com/eye-health/picture-of-the-eyes) may appear yellow ([jaundice](http://www.webmd.com/children/digestive-diseases-jaundice)). Jaundice may be caused by liver disease ([hepatitis](http://www.webmd.com/hw-popup/hepatitis)), blood disorders ([hemolytic anemia](http://www.webmd.com/hw-popup/hemolytic-anemia)), or blockage of the tubes (bile ducts) that allow bile to pass from the [liver to the small intestine](http://www.webmd.com/digestive-disorders/digestive-tract-15375)

Mild jaundice in newborns usually does not cause problems. But too much bilirubin **(hyperbilirubinemia)** in a [newborn](http://www.webmd.com/parenting/baby/default.htm) baby can cause [brain](http://www.webmd.com/brain/picture-of-the-brain) damage **([kernicterus](http://www.webmd.com/hw-popup/kernicterus))** and other serious problems. So some [babies who develop jaundice](http://www.webmd.com/hw-popup/jaundice-in-newborns) may need treatment to lower their bilirubin levels.

**Bilirubin excretion by the liver**

About 80% of bilirubin formed from haem each day arises from the red cells; 1g of haemoglobin gives rise to approximately 35mg of bilirubin. The remaining 20% comes from the red cells precursors destroyed in the bone marrow and from other haem proteins such as **myoglobin**, the **cytochromes** and others.

MEASUREMENT OF SERUM OR PLASMA TOTAL AND DIRECT BILIRUBIN

* It is performed to investigate the causes of liver diseases, jaundice, and to monitor a patient’s prognosis.
* Check [liver function](http://www.webmd.com/hepatitis/tc/what-the-liver-does-topic-overview) and watch for signs of liver disease, such as [hepatitis](http://www.webmd.com/hw-popup/hepatitis) or [cirrhosis](http://www.webmd.com/hw-popup/cirrhosis), or the effects of medicines that can damage the liver and here liver cirrhosis is where by the liver rots.
* Find out if something is blocking the bile ducts. This may occur if [gallstones](http://www.webmd.com/hw-popup/gallstones-8024), tumors of the [pancreas](http://www.webmd.com/digestive-disorders/picture-of-the-pancreas), or other conditions are present.
* Diagnose conditions that cause increased destruction of [red blood cells](http://www.webmd.com/hw-popup/red-blood-cell-erythrocyte), such as hemolytic [anemia](http://www.webmd.com/a-to-z-guides/understanding-anemia-basics) or [hemolytic disease of the newborn](http://www.webmd.com/hw-popup/hemolytic-disease-of-the-newborn).
* Help make decisions about whether newborn babies with [neonatal jaundice](http://www.webmd.com/hw-popup/jaundice-in-newborns) need treatment. These babies may need treatment with special lights, called **phototherapy**. In rare cases, blood transfusions may be needed

There are different methods used to measure serum or plasma bilirubin i.e.

**Van den Bergh reaction, Malloy** and **Evelyne, Powel and Jendrassik and Grof methods**. However the most commonly **reaction** used reliable and accurate methods are **Jendrassik-Grof** and **Van den Bergh methods**.

PRINCIPLE OF THE JENDRASSIK AND GROF BILIRUBIN

**Total bilirubin**

**Principle**

Sulphanilic acid is diazotized by the nitrous acid produced from the reaction between sodium nitrite and hydrochloric acid. Bilirubin in the serum/plasma sample reacts with the diazotized sulphanilic acid (diazo reagent) to form azo-bilirubin, caffeine an accelerator is added and gives a rapid and complete conversion to azo bilirubin. The pink acidic azo bilirubin is converted to blue azobilirubin by an **alkaline tartrate reagent** and the absorbance of the blue green solution is read in a colorimeter (orange filter) at **590nm** or spectophotometrically at **600nm**.+

Measurement of the azobilirubin in an alkaline medium removes turbidity and increases specificity.

The intensity of the azobilirubin (blue) formed is directly promotional to the amount of bilirubin present in the solution.

Bilirubin + caffeine + diazoreagent – acid azobilirubin

Acid azobilirubin + alkaline tartrate–alkaline azobilirubin

Note: in this method, only the measurement of total bilirubin is described

**NB. Conjugated (direct) bilirubin; this is measured in the absence of the caffeine benzoate catalyst and at an acid PH. Under these conditions, only the conjugated bilirubin will react. The reaction is terminated by ascorbic acid and alkaline tartrate is added.**

**Method**

Follow the kit manufacturer’s instructions**.**

Van den Bergh reaction for bilirubin estimation

**Principle**

Birirubin in serum reacts with diazotised sulphanilic acid forming an azo-bilirubin complex compound reddish violet in colour and its colour intensity is proportional to the amount of bilirubin in the sample and is measured at 540nm.

**Method**

Follow the kit manufacturer’s instructions

**QUALITATIVE ESTIMATION OF BILE PIGMENTS IN URINE**

The bile pigments are derived from the breakdown of the erythrocytes in the reticulo-endothelial system and they include: bilirubin, urobilinogen and urobilins.

BILIRUBIN

Normal urine does not contain bilirubin. It is only detected in urine when there are liver disorders in conditions like **hepatocellular** or **obstructive jaundice**. When bilirubin is raised in circulation **(bilirubineamia)**, it overshoots its renal threshold and is passed out in urine a condition called **bilirubinuria**. There are three methods used to detect bilirubin in urine namely:

* Fouchets method
* Ictotest tablet method
* Bilirubin tablet method

Fresh urine samples should be used and should not be exposed to fluorescent or day light to avoid bilirubin from being oxidized to biliverdin that cannot be detected by the available techniques**.**

**Fouchets method**

**The test is used to detect bilirubin in urine as an aid in the diagnosis of liver malfunction, for example in the following conditions:**

* **Cholestasis** (complete suppression of the flow of bile through bile channels) leading to the build-up of direct bilirubin in blood hence appearing in urine.
* Defective conjugation and or excretion of bilirubin for example in inherited disorders of conjugation and neonatal jaundice.
* Cholangitis (inflammation of the biliary tract)
* Pressure on the small bile ducts as in hepatitis or side effects of drugs.

**Principle**

Barium chloride reacts with the sulphate radicals in the urine to form a white precipitate of barium sulphate. Bilirubin if present gets attached to the precipitated barium sulphate. On addition of fouchets reagent, iron iii chloride oxidises bilirubin (yellow) to biliverdin (green). The intensity of the colour of the compound formed is directly proportional to the concentration of bilirubin in the urine.

**Specimen**

Fresh urine sample

**Reagents**

**1.** Barium chloride **(10%)**

Weigh 10g of barium chloride powder and dissolve in 100ml of distilled water.

2. Fouchets reagent

Dissolve 25g of trichloracetic acid in 50ml of distilled water.

Add 10ml of 10% iron iii chloride

Make up to 100ml with distilled water.

**Materials**

Reaction tubes

Filter paper or centrifuge

Pasteur pipettes

**method**

* Dispense 5ml of urine into a tube or universal bottle.
* Test the reaction of the urine, if alkaline acidify it with 1-2 drops of 33% glacial acetic acid.
* Add 2.5ml of 10% barium chloride reagent and mix well. A white precipitate will be formed.
* Filter or centrifuge the urine to get the precipitate that will contain the bilirubin if present.
* Unfold the filter paper to expose the precipitate or pour off the supernatant and remain with the deposit.
* Add 1 drop of fouchets reagent onto the precipitate on the filter paper or on the deposit in the tube.
* Wait for 30 seconds and report the results.

**Results**

Immediate blue green colour around the fouchets reagent drop.........bilirubin present. The colour intensity being proportional to the amount of bilirubin present. (a blue colour is given by bilicyanin)

No blue green colour............bilirubin absent.

**Ictotest tablet test**

**Principle**

Bilirubin in the urine will react with the stable diazonium solid salt to produce a blue-purple azo-bilirubin. The intensity of the colour formed is proportional to the concentration of bilirubin in the sample. This method is specific for bilirubin.

Reagents

The tablet contains the following:

0.2mg p-nitrobenzene diazonium p-toluene sulphonate salt

100mg sulphosalicylic acid

20mg sodium carbonate

25mg boric acid

**materials**

white tile

ditilled water

Pasteur pipette

**Method**

Add 5 drops of fresh urine sample on to a white tile.

Place the ictotest tablet on the sample

Add 2 drops of distilled water on top of the tablet.

Wait for 30 seconds and read the results**.**

**Result**

A blue-purple colour development on the tablet indicates a positive test for bilirubin.

**Note:** false negative results occur if urine contains high levels of ascorbic acid.

False positive reactions may occur when the colour of urine is turned red due to certain drugs like phenazopyridine.

The tablet reagents are very sensitive and can detect 0.8-1.7umol of bilirubin which coincides with the lower limit of accepted pathological significance. Bilirubin is the only substance known to give the characteristic bluish purple colour with the reagent tablets.

**Precautions**

1. Make sure that the container for urine is absolutely clean and free from contaminants e.g disinfectants and detergents.

2. Recap the ictotest bottle tightly closed as soon as the tablet has been removed to avoid uptake of moisture.

**Bilirubin test strip method**

The test uses commercially prepared strips impregnated with reagent indicator and are available as multistiks**.**

**Principle**

Bilirubin in urine is coupled to 2,6-dichlorobenzene diazonium tetrafluoroborate in an acid medium to give a reddish violet azo-dye.

**Method**

* Read the instructions of the strip manufacturer before carrying out the test.
* Dip the impregnated end of the strip into the urine sample
* Remove the strip immediately and remove excess urine.
* Wait for a period of time as recommended by the manufacturer.
* Compare with the colour chat on the strip container
* Report results as recommended by the manufacturer.

UROBILINOGEN

Urobilinogen is formed as a result of bacterial action on conjugated bilirubin in the intestines. It is normal to find small amounts of urobilinogen from that reabsorbed from the intestines. Increase in urobilinogen is found in **liver disorders** and **conditions involving haemolysis**. The sample should be fresh and analysed immediately to avoid being oxidised to **urobilin (orange yellow pigment)** on exposure to light and air. If the delay in urine analysis is un avoidable, then a technique for detecting urobilin (Schlesinger’s test) is used, then the result is related to the concentration of urobilinogen present in the sample. If a 24 hour urine sample is to be used, it should be collected in a brown bottle.

**Note**

Freshly passed urine contains trace amounts of urobilinogen which is colourless, but on standing this is oxidised to urobilin an orange yellow pigment which contributes to the normal colour of urine together with other urochromes. When there is an excess of urochromes, the urine is **orange yellow** in appearance.

**Qualitative test for urobilinogen**

**Ehrlich’s test (Wallace and Diamond reaction)**

Urobilinogen reacts with 4-dimethylaminobenzaldehyde (Ehrlich’s reagent) to form a red condensation product. The colour intensity is proportional to the concentration of urobilinogen present in urine.

**Note;** urobilin will not give a positive reaction with this reagent. Porphobilinogen also gives the same colour reaction.

**Reagents**

**Ehrlich’s reagent**

**4-dimethylaminobenzaldehyde.....2g**

**Concentrated HCl...............20ml**

**Distilled water.......................80ml**

**Method**

* Label two test tubes (test T and control C )
* Add 10ml of fresh urine into each tube.
* Add 1ml of Ehrlich’s reagent to tube T and 1ml of 20% HCl to tube C.
* Mix and stand the tubes for 3-5minutes at room temperature.
* Check for colour development by looking down the tube held over a white surface.

**Results**

Faint red-pink colour...................urobilinogen in normal amounts

Distinct cherry red colour..............urobilinogen in excess

No red colour..........urobilinogen absent

Normal urine should give a faint red colour with Ehrlich’s reagent but not with 20% v//v HCl. Red colour with 20% v/v HCl may indicate contamination with dyes like methyl red.

**Note;** a similar **cherry red colour** is given by porphobilinogen ( an intermediate compound in haem synthesis which is produced in urine of patients with acute idiopathic porphyria, an inherited disease that affects the muscles and nerves.)

The colour due to urobilinogen is differentiated from that of porphobilinogen by use of sodium acetate, amyl alcohol or chloroform. After developing the colour with Ehrlich’s reagent, saturated sodium acetate solution is added which alters the PH, intensifying the colour given by urobilinogen and making it more soluble in the extracting solvent (amyl alcohol or chloroform) than porphobilinogen.

**Reagents**

1. Ehrlich’s reagent

2. Saturated sodium acetate (hydrated sodium acetate and distilled water)

3. Amyl alcohol or chloroform

**Steps followed to detect porphobilinogen**

To 5ml of fresh urine sample,

Add 5 ml of Ehrlich’s reagent,

Mix and stand for 3-5 minutes.

Add 10 ml of saturated sodium acetate solution,

Mix and stand for 2-3 minutes.

Add 1-2 ml of amyl alcohol or chloroform,

Mix well and allow to settle.

**Results**

Urobilinogen is soluble in the organic layer so any red colour remaining in the aqueous phase after extraction indicates a positive test for porphobilinogen.

No red colour in the organic layer........negative test for porphobilinogen**.**

Red colour in the organic layer........positive test for porphobilinogen

UROBILISTIX REAGENT STRIP TEST.

Urobilinogen reacts with Ehrlich’s reagent (4-dimethylaminobenzaldehyde) in an acidic buffer to form a red coloured compound. The colour changes from light orange to dark purple.

**Method**

* Read the strip manufacturer’s instructions before carrying out the test.
* Dip the reagent end of the strip into a fresh urine sample.
* Remove the strip immediately and remove the excess urine by taping against the rim of the container.
* Wait for a period recommended by the manufacturer and compare with the colour chat provided.
* Report the results as indicated by the manufacturer.

**Note:** the reagent strips are not specific for urobilinogen. They will react with some of the substances like p-aminosalicylic acid in a similar way like urobilinogen. Ehrlich’s test should be carried out on the sample to confirm the result.

UROBILIN

Urobilin is derived from urobilinogen on exposure to air and light and on addition of lugol’s iodine solution.

If urobilinogen test is negative, the urine should be examined for urobilin since all the urobilinogen may have been oxidised to urobilin.

**Schlesinger’s test**

**Principle**

Urobilinogen is oxidised to urobilin on addition of lugol’s iodine solution. The addition of zinc acetate leads to the production of a greenish-yellow fluorescent compound of zinc urobilin**.**

**Reagents**

**1. absolute ethanol**

**2. zinc acetate**

**3. tincture of iodine**

**Method**

* To 10ml of urine in a test tube, add a few drops of iodine.
* Into another tube, add 10 ml of ethanol and add 1g of powdered zinc acetate.
* Pass the solution from one tube to another until nearly all the powder is dissolved .
* Filter the mixture.
* View the filtrate from above. A greenish yellow fluorescence is present if urobilin is present.
* Normal urine should not give a detectable amount of fluorescence**.**

**TESTS BASED ON SPECIFIC BIOCHEMICAL FUNCTIONS/SYNTHESIS**

1. Measurement serum/plasma total protein

2. Protein electrophoresis.

3. Measurement of serum/plasma albumin

PROTEINS

Proteins are the chief nitrogenous constituents of the food we eat and of tissues of the body. They are known as building blocks of life.

They are complex compounds of high molecular weight that yield amino acids on hydrolysis.

All proteins contain carbon, oxygen, hydrogen and nitrogen.

Most of them contain sulphur in addition and a few contain phosphorus

**Sources of proteins**

From foods like meat, eggs, milk, cheese, fish, cereals, vegetables etc.

Before proteins are absorbed they are broken down by proteolytic enzymes to their simplest constituents (amino acids) and it’s the amino acids that are absorbed into the various capillaries of villi for transportation into the liver

Proteins in body cells and tissues

The human body contains thousands of different proteins many of which are structural elements of cells or organised tissues and others are soluble in intracellular and extra cellular fluids as catalysts, transport agents, regulatory components, locomotion and other functions

Proteins analysed in routine laboratories are those in **blood, urine, CSF, amniotic fluid, saliva, faeces, peritoneal fluid** and **pleural fluids**. Their measurement in these physiological fluids provides important information for the diagnosis of many pathological and inherited conditions.

**Note:** proteins are synthesized by a common mechanism from a common pool of 20 amino acids .amino acids combine to form peptides or proteins

**Note; proteins are mainly synthesized in hepatocytes (liver cells) except immunoglobulins which are synthesized in lympho reticulo system.**

FUNCTIONS OF PROTEINS

Proteins demonstrate numerous biological functions

**Transport**

Proteins act as carries to transport other molecules e.g. **haemoglobin** is a protein that carries oxygen in the red blood cells. **Myoglobin** transports oxygen to the muscle cells, **thymoglobulin** binding protein transports thyroxin, **transferrin** transports iron from the liver to bone marrow etc

Albumin forms the largest proportion of plasma protein transport system. It carries various hormones, ions, vitamins, bilirubin, free fatty acids, calcium and many drugs enabling them to be soluble in plasma

Attachment to albumin renders many of the molecules inactive.

It maintains the osmotic pressure of the plasma. It has an osmotic effect that helps to maintain the blood volume.

**Storage**

Proteins serve as reservoir e.g. ferritin stores lron in the liver

**Motion**

Proteins have contractile and motile feature. Muscle contraction occurs when the filaments containing the proteins actin and myosin slide along one another. Our muscles including that most important muscle, the heart, contract and expand through the interaction of **actin** and **myosin** proteins.

The cells with cilia or flagella, microtubule which contain tubulin protein slide along each other facilitating movement. Sperm can swim because they have long flagellum made up of proteins.

**Structure**

Some proteins are structural components of cells or tissues e.g. **collagen** is a wide spread protein that provides structural frame work of intracellular tissue support in connective tissue, cartilage, bone and other tissues

Elastin is a stretchable support protein

Keratin is a tough protein of the finger nails and hair

**Hormones(regulatory proteins)**

Many of the hormones are peptides or proteins e.g. **insulin, growth hormone, adrenocorticotrophic hormone**. Hormones regulate metabolism.

**Clotting mechanism**

Proteins fibrinogen and thrombin are clotting factors.

**Immune mechanism**

Antibodies are proteins that help control the spread of infections and eliminate foreign materials from the body

Enzymes

The majority of the enzymes that have been studied are proteins. They catalyse

Biochemical reactions essential to life e.g**. pepsin, trypsin** etc.

**Growth substance**

Proteins may promote growth and regeneration of tissues in the embryo and adult.

Components of cell membrane

Proteins are important components of the cell membranes, including the receptors on the cell surfaces that control the passage or action of various chemicals that influence the cell.

* Maintaining level of body fluids (fluid balance in the body due to osmotic pressure created by plasma proteins).
* Provide energy in case of carbohydrates scarcity
* Nutrient proteins; serve as sources of amino acids for embryos and infants. Egg albumin and casein in milk are examples of nutrient storage proteins.

MEASUREMENT OF TOTAL SERUM/PLASMA PROTEIN

TOTAL PROTEIN MEASUREMENT

THE BIURET METHOD.

Principle;

The biuret method depends on the presence of peptide bonds in all proteins

The peptide bonds react with Cu2+ ions in alkaline medium to form a reddish violet colored product the absorbance of which is measured spectrophotometrically at **540nm** the intensity of the color produced is proportional to the number of peptide bonds that are reacting and therefore to the amount of protein present in the reaction system.

The biuret reagent contains sodium potassium fartrate to form a complex with cu2+ ions and maintain their solubility in alkaline medium (solution).

**NB:** amino acids and dipeptides doesn’t react the reaction and occurs with any compound containing at least two peptide bonds.

**Measurement of Albumin**

BCG METHOD

**Value of test**

Serum albumin is mainly increased to **investigate liver disease, protein energy malnutrition, disorders of water balance, nephrotic syndrome** and **protein losing gastrointestinal disease**.

**Principle**

Bromocresol green (GCG) is an indicator which is yellow between **pH 3.5-4.2** when it binds to albumin the color of the indicator changes from **yellow** to **blue green**. The absorbance of the color produced is measured calorimetrically at **590nm**. And the intensity of the color formed is directly proportional to the concentration of albumin present in the solution.

MEASUREMENT OF SERUM OR PLASMA TOTAL PROTEIN (BIURET METHOD)

**Note:** serum is obtained from clotted blood. It contains **globulins** and **albumin** but no fibrinogen. The fibrinogen has been converted to insoluble fibrin as the blood clots.

Measurement of total serum proteins (albumin and globulins) has a limited clinical value because albumin constitutes about 60% of the total serum proteins, therefore a rise or fall in total serum protein is mainly due to changes in albumin.however the methods will be discussed below.

**Value of the test**

It is the recommended manual colorimetric technique for measuring serum or plasma total protein to investigate many disease states including severe **protein deficiency, chronic liver disease** and **malnutrition.**

**Principle of the test**

Proteins are built up by amino acids joined together by a linkage of carboxylic group of one amino acid and an amino group of another amino acid. The linkage is a peptide bond (-CO-NH-) and it is this linkage that reacts with the alkaline copper solution giving rise to a blue purple colour whose intensity is directly proportional to the concentration of the proteins in the sample.

(The cupric ions in the reagent join the peptide bonds of the protein molecules in alkaline medium to form a **blue-violet coloured complex** whose absorbance is measured in a colorimeter using a yellow-green filter or in a spectrophotometer at 540nm wavelength. The absorbance of the complex formed is directly proportional to the concentration of the protein in the sample**.)**

Protein +copper ions in alkaline pH------protein-copper complex(blue-violet**)**

**Equipment**

Colorimeter

Reaction tubes

Pipettes

Timer

Water bath

**Reagents**

Biuret reagent-sodium hydroxide-----100mmol/l

-potassium iodide-----16mmol/l

-copper sulphate---------6mmol/l

-sodium potassium tartrate---16mmol/l

Blank reagent-sodium potassium tartrate----16mmol/l

-sodium hydroxide---------100mmol/l

Protein standard 6og/l (6.0g/dl)

**Reagent handling and preparation**

**Biuret reagent:** add 100mls of distilled water to one vial of biuret concentrate. Stable for 12 months when sealed and stored at 15 to 25oC protected from light.

Blank reagent: dilute the contents of blank reagent bottle with 200mls of distilled water, rinsing the bottle thoroughly. Stable for 12 months when sealed and stored at 15 to 25oC.

The standard is ready for use.

Blank reagent is used only for sample blanking if the sample is highly icteric, lipaemic or haemolytic. Do not use for routine samples

**Specimen**

Serum, EDTA plasma, heparinised plasma. Protein is stable in plasma for 72 hours from the time of collection when stored at 4oC. If longer storage is necessary, store frozen at -20oC.

**Method**

Label tubes B, S, T1, T2 etc depending on the number of samples to be tested.

Where B-blank solution

S-standard

T1- sample1

T2- sample 2

Pipette in to the tubes as follows:

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | B | S | T1 | T2 |
| Distilled water | 20ul | ----- | ----- | ----- |
| Standard | ---- | 20ul | ------ | ----- |
| Sample 1 | ----- | ------- | 20ul | ------ |
| Sample 2 | ------ | -------- | ------ | 20ul |
| Biuret reagent | 1000ul | 1000ul | 1000ul | 1000ul |

Mix thoroughly and incubate for 10 minutes at 20-25oC.

Measure the absorbance of the sample and standard against the reagent blank at 546nm ( 530-570 nm).

Record the absorbance.

Calculate the protein concentration of the tests using:

absorbance of test

absorbance of standard x concentration of standard.

The method is linear up to 130g/l. Samples above this concentration should be diluted 1 + 1 with o.9% sodium chloride and reassayed. Multiply result by 2.

Note: when icteric, haemolytic or lipaemic sera are being used, a sample blank must be measured against water and the absorbance obtained subtracted from the absorbance of the sample. (Implying that if the sample is lipaemic, icteric or haemolysed another column for sample blank is added)

**Total protein normal reference range**

Adults..........66-87g/l (6.6-8.7g/dl)

Children up to 6 years........56-85g/l (5.6-8.5g/dl)

Neonates ..................53-89g/l (5.3-8.9 g/dl)

**Interpretation of results**

An increase or decrease in total plasma protein is either due to changes in all plasma fractions or one of them.

**The conditions that are associated with an increase in total plasma proteins are:**

* Haemoconcentration following shock, severe vomiting or diarrhoea.
* Increased globulin production associated with chronic infections such as TB, kalaazar (leishmaniasis), and tropical splenomegaly.
* The presence of abnormal globulins such as in **multiple myeloma, macroglobulinaemia** or **lymphoma.**
* Collagen vascular diseases and some forms of liver disease.
* Falsely increased values may be obtained if the specimen contains dextran from an intravenous infusion.

**Decreases**

A decrease in the total protein concentration level is more common and may be caused by:

* Low protein intake as in the severe forms of protein energy **malnutrition (kwashiorkor)**
* Malabsorbtion as in chronic pancreatitis, celiac disease and sprue.
* Loss of protein from the body in urine as in **nephritic syndrome**. Proteins can also be lost from the skin following **severe burns or from the bowel** as in **ulcerative colitis** and other forms of protein losing gastroenteropathy.
* Liver disease associated with a reduction in protein synthesis, although the total protein may be within the normal range because the albumin levels fall and the globulin levels rise at the same time.
* An increase in the body’s need for protein e.g. following surgery or serious tissue damage when protein is required for energy and repair, high protein demands lead to **hypoalbunaemia (low albumin levels)**

**Sources of error**

* Wrong wave length used for the determination
* Light source
* Temperature of incubation
* Incubation period
* Cleanliness e.g. of cuvettes used in measurement
* Bacterial contamination of reagents
* Reagent expiry
* Calibration frequency

**Advantages of the biuret method**

* Quick and simple to use especially when big batches of sample are to be analysed.
* The purple colour developed is stable.
* It is very sensitive.

MEASUREMENT OF SERUM OR PLASMA ALBUMIN BY BROMOCRESOL GREEN METHOD.

Albumin is synthesized entirely in the liver and is present in the plasma in greater concentration than globulins. It diffuses easily through damaged membranes and is more readily filtered out by the kidneys than most globulins because its molecules are smaller.

**VALUE OF THE TEST**

Serum or plasma albumin levels are mainly measured to investigate liver disease, protein energy malnutrition, disorders of water balance, nephritic syndrome and protein –losing gastrointestinal diseases**.**

**PRINCIPLE**

Bromocresol green is an indicator which is yellow between **pH 3.5-4.2**. When serum is added to the indicator, the indicator binds to albumin and its colour changes from yellow to blue-green. The absorbance of the colour produced is measured in a colorimeter using an orange filter (Iiford no 607) or in a spectrophotometer at 632nm wavelength.

Albumin + BCG pH 4.2---albumin-BCG complex (blue-green)

(Serum albumin binds with the Bromocresol green indicator in acid medium to form a green BCG complex, the amount of which is directly proportional to the albumin concentration present in the sample.)

**SPECIMEN**

The method requires serum. Serum albumin is stable up to 72 hours at 4oC. If longer storage from the time of collection is necessary store frozen at -20oC.

**REAGENTS**

Bromocresol green concentrate –bromocresol green (0.24mmol/l)

-succinate buffer

Standard-------------------------45g/l (4.5g/dl)

**Reagent handling and preparation**

Dilute the concentrate by adding 52mls of distilled water. The diluted material is stable for 12 weeks at room temperature up to 25oC.

Undiluted BCG will be stable up to the expiry date quoted when stored under the recommended conditions**.**

**Method**

Label tubes B, S, T1, T2 etc depending on the number of samples to be tested.

Where; B-blank solution

S-standard

T1- sample 1

T2- sample 2

Pipette into the tubes as follows.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | B | S | T1 | T2 |
| Distilled water | 10ul | ----- | ------ | -------- |
| Standard | ------ | 10ul | -------- | ------- |
| Sample 1 | ------ | ------ | 10ul | ------- |
| Sample 2 | ------- | ------- | ------- | 10ul |
| Reagent | 3mls | 3mls | 3mls | 3mls |

* Mix and leave for 5 minutes at 20-25oC.
* Measure the absorbance of the standard and the samples against the blank at 600-650nm wave length.
* Calculate the concentration of albumin in the sample using:
* Albumin concentration in g/l = sample absorbance
  + - * + Standard absorbance x standard concentration
* The method is linear up to 60g/l (6g/dl). Sample above this should be diluted with 0.9% sodium chloride and reassayed.

**Interpretation of results**

**Normal values**

Adults-----3.5-5.2 g/dl (35-52 g/l)

Paediatrics---3.8-4.2 g/dl (38-42 g/l)

Increases: serum or albumin levels are rarely raised except artefactually by prolonged venous stasis (hyper albuminea)

Decreases: many of the causes of low total protein levels are the result of hypoalbuminaemia especially the nephritic syndrome

Several parasitic infections cause a reduction in the synthesis of albumin.

**ELECTROPHORESIS**

In this technique separation of particles is due to movement of charged particles through an electrolyte (buffer) when subjected to an electric current.

If the particles are charged differently, they move in opposite direction, the positively charged particles to the **cathode** and the negatively charged to the **anode**.

The buffer should be kept at a fixed PH to ensure that the charge on the particles and therefore the rate of migration is stabilised.

* Electrophoresis is the migration of charged particles in an aqueous medium under the influence of an electric field

SERUM PROTEIN ELECTROPHORESIS

Electrophoresis is an analytical technique that can be used to separate and analyse a diverse range of ionized analytes.

It can be used to separate **proteins, nucleotides, peptides, amino acids, nucleic acids nucleosides organic acids, small cations** and **anions** in body fluid and tissues.

With electrophoresis, a sample of serum or urine is placed in a special paper that is treated with a gel .An electric current is applied to the gel, causing proteins in the sample to move across the gel to different points, the gel is then stained and placed in a special machine that produces a tracing that indicates the levels of proteins.

Proteins contain numerous fractions of similar mobility, therefore when subjected to electrophoresis; the sharpness of the separation depends upon the extent to which each fraction is homogeneous in its migration. Each protein carries a net charge which varies with the PH of its environment .Proteins contain many **ionisable (NH2) amino groups** and **(-COOH) carbonyl groups** therefore may be charged positively or negatively depending on the PH of the medium in which it is suspended ;i.e. behave as **ampholytes**

**Note;** **An ampholyte** takes a positive charge in a solution more acidic than its isoelectric point and takes a negative charge in an alkaline medium and moves towards the node.

**Note:** isoelectric point is the PH at which a molecule has no net charge and will not move in an electric field

Therefore in stable PH solution, proteins submitted to an electric current migrate to the position where the PH is equal to the isoelectric point