Liver Function Tests

(LFTs)

Learning Objectives

Up on the successful completion of this chapter, the students will be able to

- ✓Identify the pathology associated with liver
- ✓ Identify the laboratory tests aid in the diagnosis of liver disorders.
- ✓Perform different lab test related to LFT
- ✓Interpret the result related to the clinical syndromes

Introduction: Liver

- The liver is the largest internal organ of the body.
- It is a functionally complex organ that plays critical biochemical role in the metabolism, digestion, detoxification, and elimination of substances in the body.
- It is a relatively resilient organ that can regenerate when it has been damaged by some short-term injury or disease.

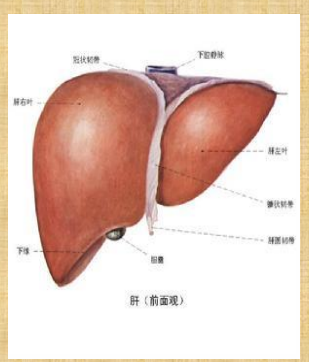
Introduction: Liver

- However, if the liver is damaged repeatedly over long period of time, it may undergo irreversible changes that permanently interfere with its essential functions.

- The weight of the liver is approximately
  - ✓ 3%-4% of its BW→ In carnivores
  - ✓ About 2% → In omnivores and
  - ✓ About 1%-1.5% → In herbivores.

Gross Anatomy

- It is located beneath and attached to the diaphragm, protected by the lower rib cage
- The liver is separated into a right and left lobe by the falciform ligament.
- The right is much larger than the left .



Gross Anatomy…..

- There is no known functional difference between the lobes, and blood flows freely between all areas of the liver.
- Unlike most organs, the liver is an extremely vascular organ that receives its blood supply from two sources:
  - ❖ The hepatic artery and
  - ❖ The portal vein.

Gross Anatomy…..

- The hepatic artery, a branch of the aorta, supplies oxygen-rich blood from the heart to the liver
- It is responsible for providing approximately 25% of the total blood supply to the liver.
- The portal vein supplies nutrient-rich blood (collected as food is digested) from the digestive tract.
- It is responsible for providing approximately 75% of the total blood supply to the liver.

Gross Anatomy…..

- The two blood supplies eventually merge and flow into the **sinusoids**, which drains between individual hepatocytes.
- Approximately 1,500 mL of blood passes through the liver per minute.
- The liver is drained by a collecting system of veins that empties into the hepatic veins and ultimately into the inferior vena cava

Gross Anatomy…..

The excretory system of the liver begins at the **bile** canaliculi.

This form intrahepatic ducts, where excretory products of the cell can drain.

Form the right and left hepatic ducts, which drain the secretions from the liver.

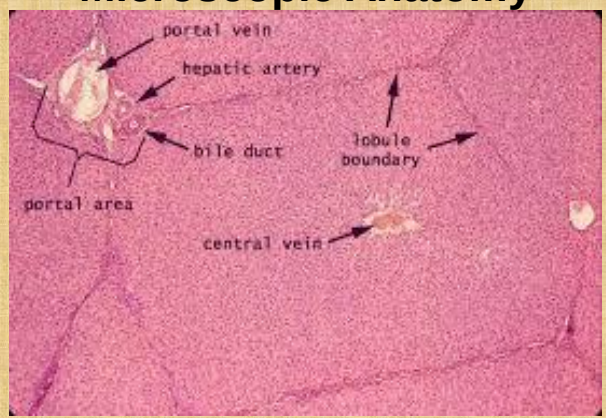
Merge to form the common hepatic duct

Which is eventually joined with the cystic duct of the gallbladder to form the common bile duct

Microscopic Anatomy

- The liver is composed of microscopic units called **hepatocytes**.
- They are the functional units of the liver and responsible for all metabolic and excretory functions performed by the liver.
- Each hepatocyte is roughly a six-sided structure with a centrally located vein (called the *central vein*) and portal triads at each of the corners.
- Each portal triad contains hepatic artery, portal vein and bile duct surrounded by connective tissue.

Microscopic Anatomy



Microscopic Anatomy

- The liver contains two major cell types: **hepatocytes** and **kupffer cells**
- The hepatocytes, making up approximately 80% of the volume of the organ
- These cells perform the major functions associated with the liver and are responsible for the regenerative properties of the liver.
- Kupffer cells are macrophages that line the sinusoids and act as active phagocytes.
- It is capable of engulfing bacteria, debris, toxins, and other substances flowing through the sinusoids.

Functions of Liver

- The liver performs four major Biochemical functions:
  - ➢ Metabolic Function
  - ➢ Storage
  - ➢ Detoxification and
  - ➢ Synthesis/secretion
- If the liver becomes nonfunctional, death will occur with 24 hours due to hypoglycemia.

Metabolic Functions of the Liver

- The liver is a large, chemically reactant pool organ that can
  - ☛Perform myriad of metabolic functions.
  - ☛Carried out rxn with high rate of metabolism.
  - ☛Share substrates and energy from one metabolic system to another.
  - ☛Process and synthesize multiple substances that are transported to other areas of the body.

Carbohydrate Metabolism

- In carbohydrate metabolism, the liver performs the following functions:
  - ☛Storage of large amounts of glycogen
  - ☛Conversion of galactose and fructose to glucose
  - ☛ Gluconeogenesis
  - ☛ Formation of many chemical compounds from intermediate products of carbohydrate metabolism
- ☛The liver is especially important for maintaining a normal blood glucose concentration.

Fat Metabolism

- Although most cells metabolize fat, specific functions of fat metabolism occur in the liver like:
  - ☛ Oxidation of fatty acids to supply energy for other body functions
  - ☛ Synthesis of large quantities of cholesterol, phospholipids, and lipoproteins
  - ☛ Synthesis of fat from proteins and carbohydrates

Oxidation of Fatty Acid

- To derive energy from neutral fats, the fat is first split into glycerol and fatty acids;
- Then the fatty acids are split by *beta-oxidation* into two-carbon acetyl radicals that form *acetyl coenzyme A* (acetyl-CoA).
- This can enter the citric acid cycle and be oxidized to liberate tremendous amounts of energy.
- Beta-oxidation can take place in all cells of the body, but it occurs rapidly in the hepatic cells.

Oxidation of Fatty Acid….

- The liver itself cannot use all the acetyl-CoA that is formed.
- Instead, it is converted into *aceto-acetic acid*, a highly soluble acid that passes from the hepatic cells into the extracellular fluid.
- Then transported throughout the body to be absorbed by other tissues.
- These tissues reconvert the aceto-acetic acid into acetyl-CoA and then oxidize it in the usual manner.

Synthesis of Ch, PL, and LP

- Liver is the principal organ in which cholesterol, phospholipid and lipoprotein are synthesized
- About 80% of cholesterol is converted into bile salts and secreted into the bile
- The remainder like phospholipid transported to body tissue everywhere in the body by the help of lipoprotein.
- Both of them are used by the cells to form membranes, intracellular structures, and multiple chemical substances that are important to cellular function.

Synthesis of Fat

- Liver also the primary organ where adipogenesis is undertaken
- Almost all the fat synthesis from carbohydrates and proteins occurs in the liver.
- Once it is synthesized, it will be transported to the adipose tissue by the help of lipoproteins to be stored.

Protein Metabolism

- The most important functions of the liver in protein metabolism include:
  - ☛ Deamination of amino acids
  - ☛ Formation of urea for removal of ammonia from the body fluids
  - ☛ Formation of plasma proteins
  - ☛ Inter-conversions of the various amino acids and synthesis of other compounds from amino acids

Deamination of amino acids

- Deamination is the removal of amino group from the AA (as free NH_3).
- It is required before Aa's can be used for energy or converted into carbohydrates or fats.
- A small amount of deamination can occur in the other tissues of the body, especially in the kidneys.
- But this is much less important than the deamination of Aa's by the liver

Formation of Urea

- Large amounts of ammonia are formed by the deamination process, and continually formed in the gut by bacteria.
- The liver removes ammonia from the body fluids by the process of urea formation.
- That is why, if the liver does not form urea, the plasma ammonia concentration rises rapidly and results in *hepatic coma* and death.

Synthesis of plasma protein

- Essentially all the plasma proteins, with the exception of part of the gamma globulins, are formed by the hepatic cells.
- The liver can form plasma proteins at a rate of 15 to 50 g/day.
- If half of the plasma proteins are lost from the body, it can be replenished in 1 or 2 weeks.
- With chronic liver disease (e.g., cirrhosis), plasma proteins, such as albumin, may fall to very low levels, causing generalized edema and ascites.

Bilirubin Metabolism

- When the RBCs have lived out their life span, they become too fragile to exist in the circulatory system
- Their cell membranes become rupture, and released hemoglobin
- This Hb will be phagocytized by tissue macrophages (also called the reticulo - endothelial system) throughout the body.

Bilirubin Metabolism….

- The hemoglobin is first split into globin and heme
- Then the heme ring is further breakdown to give free iron and biliverdin
- Free iron transported in the blood by transferrin, while straight chain of four pyrrole nuclei (Biliverdin) rapidly reduced to free bilirubin.
- The free bilirubin combines strongly with plasma albumin and circulate throughout the blood and interstitial fluids.

Bilirubin Metabolism….

- When passing in liver cells, it is detached from the plasma albumin to conjugated with glucuronic acid to form *bilirubin glucuronide* (water soluble).
- In these forms, the bilirubin is excreted from the hepatocytes by an active transport process into the bile canaliculi and then into the intestines.
- This bilirubin is further converted by bacterial action into highly soluble substance *urobilinogen*

Bilirubin Metabolism….

- Some of the urobilinogen is reabsorbed through the intestinal mucosa back into the blood.
- Some becomes altered to stercobilinogen and oxidized to form stercobilin and excreted along with faeces.
- About 5 percent is excreted by the kidneys into the urine after oxidized to urobilin

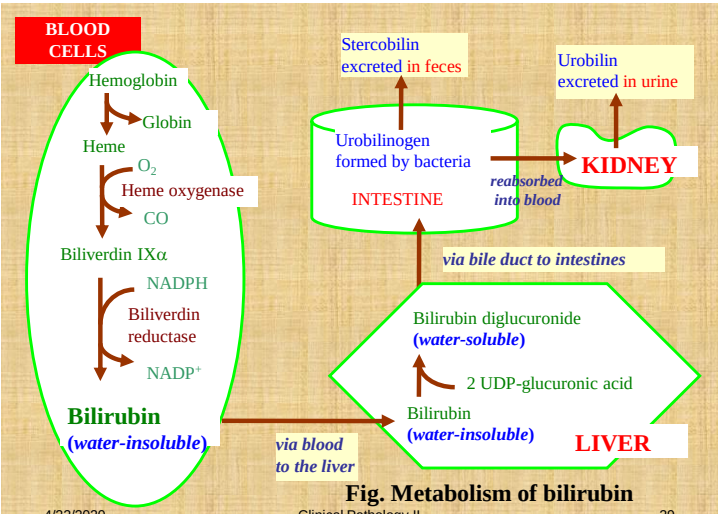


Fig. Metabolism of bilirubin

Storage Function

- The Liver is a storage site for Vitamins and Iron.
- The liver has a particular propensity for storing of vitamins
- It is also known as an excellent source of certain vitamins in need of body.
- The vitamin stored in greatest quantity in the liver is vitamin A
- But large quantities of vitamin D and vitamin B12 are normally stored as well.

Storage Function…..

- The liver also stores iron as Ferritin.
- Except for the iron in the Hb, by far the greatest proportion of iron is stored in the liver.
- The hepatic cells contain large amounts of a protein called *apoferritin*, which is capable of combining with iron in reversibly fashion.
- Therefore, when iron is excess in the body fluids, it combines with apoferritin to form ferritin and is stored in the hepatic cells and vice versa when needed elsewhere.

Detoxification Function

- The liver serves as a gatekeeper for substances absorbed by the GIT and those released into systemic circulation.
- The active chemical medium of liver is important to detoxify many drugs and other metabolites.
- In similar manner, several hormones secreted by the endocrine glands are either chemically activated or deactivated by the liver.
- It is also one of the major routes for excreting calcium by secreting into bile.

Synthetic and Secretion Function

- The liver forms large proportion of blood substances used in coagulation.
- It include fibrinogen, prothrombin, accelerator globulin, Factor VII, and several other important factors.
- Vitamin K is required by the metabolic processes for the formation of several of these substances.
- In the absence of vitamin K, the concentrations of all these decrease markedly, and prevents blood coagulation

Synthetic and Secretion…..

- Liver also play an important role in the synthesis myriad enzymes
- Among those, the most clinically useful include:
  - Aminotransferases (alanine amino-transferase [ALT] and aspartate aminotransferase [AST])
  - Phosphatases (alkaline phosphatase [ALP] and 5'neucleotidase),
  - δ glutamyltransferase (GGT), and
  - Lactate dehydrogenase

Assessment of Liver Function

What is the Purpose of LFTs?

- LFTs alone do not give full information to the clinician
- But in combination with a careful history and physical examination, can contribute to make an **accurate diagnosis of the specific liver disorder**.
- LFTs show abnormalities in response to
  - ✓liver inflammation
  - ✓liver injury due to drug, alcohol, toxins, microbes
  - ✓Liver malfunction due to blockage of bile flow
  - ✓Liver cancers etc…

Liver Function Test

LFTs are divided into

- True tests of liver function,
  - Such as serum albumin, bilirubin, and prothrombin time
- Tests that are indicators of liver injury or biliary tract disease.
  - Liver enzymes

Classification of liver functions test

Can be further classified based on the major functions of liver:

①**Excretion:** Measurement of bilirubin

②**Serum enzymes:** Transaminase (ALT, AST), alkaline phosphate(ALP), 5'-nucleotidase, LDH isoenzyme.

③**Synthetic function:** Prothrombin time, serum albumin.

④**Metabolic capacity:** Galactose tolerance test

Excretion : Bilirubin

- Bilirubin is the main bile pigment that is formed from the breakdown of heme in red blood cells.
- The broken down bilirubin travels to the liver, where it is secreted into the bile.
- Has two fractions:

▪**Conjugated:**
- Direct bilirubin
- water soluble
- excreted by the kidney.

▪**Unconjugated:**
- Indirect bilirubin
- Insoluble in water
- Bound to albumin in the blood.

Serum Bilirubin

- Normally, a small amount of conjugated bilirubin circulates in the blood.
- Serum bilirubin is considered a true test of liver function
- As it reflects the liver's ability to take up, process, and secrete bilirubin into the bile.

Laboratory diagnosis

- Total and conjugated bilirubin are measured by different techniques
- But unconjugated bilirubin is determined by subtracting conjugated from total bilirubin.
- The Jendrassik-Grof reaction and the Malloy-Evelyn method are the two common techniques for measuring bilirubin
- They use diazonium salt to produce a color reaction with bilirubin
- Bilirubin + diazotized sulfanilic acid → azobilirubin

Laboratory diagnosis

The Jendrassik-Grof method

- Performed at a pH of 13, measures the blue-colored product at 600 nm.
- The solution can be alkalinized using an alkaline tartrate solution.
- The alkaline reaction produces a more intense color than the equivalent reaction run at a neutral pH
- A sodium benzoate–caffeine mixture used as an accelerator and solubilizing agent for unconjugated bilirubin

The Jendrassik-Grof method…

- The individual fractions of bilirubin are determined by taking two aliquots of sample.
- One aliquot will react with the diazo reagent only and the other aliquot with the diazo reagent and an accelerator (caffeine -benzoate).
- The addition of the caffeine-benzoate will solubilize the water-insoluble fraction of bilirubin and will yield a total bilirubin value (all fractions).
- The reaction without the accelerator will yield conjugated bilirubin only.

The Jendrassik-Grof method…

- The reaction can be terminated by the addition of ascorbic acid by removing the excess diazo reagent.
- The final blue product is measured at 600 nm directly proportional to bilirubin concentration.
- Indirect (unconjugated) bilirubin may be calculated by subtracting the conjugated bilirubin concentration from the total bilirubin concentration.

The Jendrassik-Grof method…

- It has the following advantages over the Malloy-Evelyn method:
  - ☛ Not much affected by pH and heat changes
  - ☛ Insensitive to variation in protein concentration of the sample
  - ☛ Maintains optical sensitivity even at low bilirubin concentrations
  - ☛ Has minimal turbidity and less sensitive to interfering substances
  - ☛ Is not affected by hemoglobin up to 750 mg/dL

The Malloy-Evelyn method

- The diazotized sulfanilic acid reacts at the central carbon of bilirubin to split the molecule and forming two molecules of azobilirubin.
- It is performed at a pH of 1.3
- The azobilirubin produced is red-purple in color with a maximal absorption of 560 nm.
- The most commonly used accelerator to solubilize unconjugated bilirubin in this method is methanol, although other chemicals have been used.

Caution to Bilirubin Test

- Hemolysis may affect bilirubin measurement.
- Lipemia and proteinemia may falsely elevate bilirubin measurement.
- Bilirubin may be broken down by light or heat and should be protected from these environmental conditions

Hyperbilirubinemia

- Increased plasma concentrations of bilirubin occurs when there is an imbalance between its production and excretion
- Recognized clinically as jaundice
- It can be classified as
  - ✓ Prehepatic
  - ✓ Hepatic and
  - ✓ Posst Hepatic

Prehepatic (hemolytic) jaundice

- ✓ Results from excess production of bilirubin (beyond the livers ability to conjugate it) following hemolysis.
- ✓ Excess RBC lysis is commonly the result of autoimmune disease or hemolytic disease of the animal
- ✓ Laboratory Results: ↑ serum bilirubin (mostly unconjugated), ↑ urine urobilinogen, ↓ Hgb, ↓ Hct .

Intrahepatic jaundice

- ✓ It is due to impaired uptake, conjugation or secretion of bilirubin.
- ✓ Reflects a generalized liver (hepatocyte) dysfunction.
- ✓ In this case, hyperbilirubinemia is usually accompanied by other abnormalities (the presence of biomarkers of liver function).

Posthepatic jaundice

- ✓ Caused by an obstruction of the biliary tree
- ✓ Accompanied by conjugated plasma bilirubin and bile acids accumulation in the plasma
- ✓ Characterized by pale colored stools (absence of fecal bilirubin or stercobilin), and dark urine (increased conjugated bilirubin)
- ✓ In a complete obstruction, urobilin is absent from the urine

Serum Enzymology

Enzymes

- Biological catalysis
- Very efficient –to increase the rates of reaction
- All are proteins- so liable to denaturation
- Specific to substrates
- Partly specific to tissues
- Assay by measuring its rate of specific reaction or activity catalyzed by the enzyme

Information from enzymes measurements

- Presence of disease
- Organs involved
- As a differential diagnosis
- Extent of disease-the more the damaged cells- the more leaked enzymes in blood
- Time course of the disease

Enzymes routinely measured in LFT

NAME OF THE ENZYME	Major Organ /tissue the enzyme available
Aspartate Amino Transferase (AST) or SGOT	Heart and Liver
Alanine Amino Transferase (ALT) or SGPT	Liver and Heart
Alkaline Phosphatase (ALP)	Liver, Bone, intestine and other tissues
γ glutamyl Transferase (γ GT)	Liver
5' Neuclutidase	Liver
Lactate Dehydrogenase (LDH)	Heart, liver, muscle, RBC

Measurement of enzyme activity

- Enzyme assays usually depend on the measurement the catalytic activity of the enzyme, rather than the concentration of the enzyme itself.
- Enzyme activity is expressed in International unit (IU)
- It corresponds to the amount of enzymes that catalyzes the conversion of one micromole (μmol) of substrate to product per minute

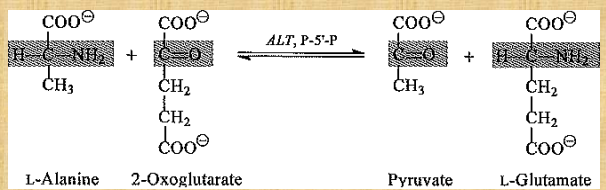
Transferase Enzymes

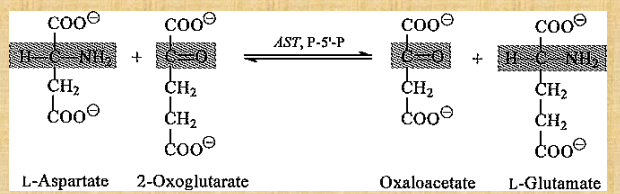
AST, ALT and GGT

Introduction to Transferases

- The aminotransferases constitute a group of enzymes that catalyze the interconversion of amino groups from aminoacid and α-ketoacids.
- Two enzymes having greatest clinical significance belong to this group:
  - Alanine Aminotransferase; (Alanine Transaminase or SGPT)
  - Aspartate Aminotransferase; (Aspartate Transaminase or SGOT)

Transaminases…..





Alanine transaminase (ALT)

- Alanine transaminase (ALT), also called Serum Glutamate Pyruvate Transaminase (SGPT)
- Source (Predominantly in liver, but it also found in heart, skeletal muscle and others)
- When a cell is damaged, it leaks this enzyme into the blood, where it is measured
- ALT rises dramatically in acute liver damage, such as viral hepatitis or paracetamol overdose
- Relatively sensitive and specific than AST for liver injury

Clinical Significance of ALT

- It reflect the hepatocellular pathology due to the releases of ALT linked with
  - ✓liver inflammation (hepatitis)
  - ✓Drugs overdose or toxicity
  - ✓Infections from Viruses or bacteria
  - ✓Alcohol
- It is commonly measured as a part of liver function test, to determine liver health.

Analytical Methodology of ALT

Cab be evaluated by two methods

1. Continuous Monitoring Method
2. Fixed End-Point Assay

Continuous Monitoring Method

- Coupled reaction at 37 ^{0} C or at room temp
- L-Alanine + α- oxoglutarate $\xrightleftharpoons{(ALT,\ P\text{-}5'\text{-}P)}$ pyruvate + L-glutamate
- Pyruvate + NADH + H $\xrightarrow{(LD)}$ Lactate + NAD^+
- Decrease in Abs at 340 nm (multi-point analysis).

Analytical Methodology…

- ***Notes:*** ***Coupled reaction*** refers to two or more rxn take place in the reaction cell and the product of the first reaction becoming the substrate for the second reaction.
- The second enzyme is provided by the reagent solution while the first enzyme is provided by the patient's sample.
- ***Continuous monitoring*** means taking multiple absorption readings to check the change in absorption is consistent over minutes.
- It is important to calculate the enzyme activity.

Calculating Absorbance Per Minute

- During ALT determination, the absorbance decreases over time
- The result is calculated as $-\frac{\Delta A}{Min} \times F$
- Where $F_{340} = -1746$
- So final activity is a positive number U/L which is a product of the above two

Continuous Monitoring Analysis

Example

- The following results for ALT were determined:

Time (min)	Absorbance
0	1.350
1	1.300
2	1.250
3	1.201

- Are the results progressing in the expected direction?

Answer: yes, they are decreasing

Continuous Monitoring Results of ALT analysis

- What is the Δ Abs for each minute?

Answer: -0.050/min; -0.050/min; -0.049 /min

Are the results consistent?

- Answer: Yes, the absorbance decreases consistently
- Average = -0.050 /min

Fixed End-Point Assay for ALT

Photometric Method

- ALT is coupled with 2,4 Dintrophenylhydrazine → Dintrophenylhydrazone (chromogenic product)
- Product measured photometrically at 340nm

Specimen for ALT

- Non-hemolyzed serum or plasma.
- Heparinized plasma
- < 2 day old samples
- Fasting specimen is preferred

Sources of Error in ALT

- Nonlinear results may occur from side reactions
- Hemolyzed samples can cause false positive
- Loss of activity if specimen is stored at room temperature
- Unstable analytical temperature (deviation from 37 ^{0} C
- Unstable photometer
- Substrate exhaustion due to high levels of enzyme activity
- Presence of ammonia will consume the NADH

Aspartate transaminase (AST)

- Aspartate transaminase (AST) also called Serum Glutamate Oxaloacetic Transaminase (SGOT)
- It is similar to ALT residing in liver parenchymal cells
- It is raised in acute liver damage, but is also present in red blood cells, skeletal muscle, heart, kidney, brain and pancreas pathology
- The ratio of AST to ALT is useful in differentiating causes of liver damage

Analytical Methodology of AST

- Continuous Monitoring Method
  - L-Aspartate + a-oxoglutarate $\xrightleftharpoons{\text{-(AST, P-5'-P)}}$ L-glutamate + oxaloacetate
  - Oxaloacetate + NADH + H+ $\xrightarrow{\text{–(MDH)}}$ L-malate + NAD^+
- Decrease in Abs. at 340 nm (multi-point assay)

Interpretation of AST

Elevated serum AST might be due to

- Muscle damage
- Hepatomegaly,
- Necrosis and hepatic inflammation
- Pancreatitis
- Drug over dose
- Intoxication

Specimen for AST

- Same as ALT

Analytical Errors for AST Activity

- Same to ALT

Gamma- Glutamyl Transferase (GGT)

- Found in endoplasmic reticulum (ER) of hepatocytes and cholangiocyts (bile duct epithelium)
- It is relatively more specific to the liver and more sensitive marker for cholestatic damage than ALP
- GGT may be elevated even with minor, sub-clinical levels of liver dysfunction.
- It can also be helpful in identifying the cause of elevated ALP (Alkaline phosphatase) is from liver or bone

GGT…..

- If ALP is high while GGT is normal, it suggests the source of the ALP is bone since GGT not produced in bone
- But the GGT level significantly affected by alcohol intake.
- Mostly associated with disease in the liver such as hepatobiliary obstruction and inflammation of this region
- Non hemolyzed serum and EDTA plasma can be used as a specimen

Analytical Methodology

- The most widely accepted substrate for GGT analysis is gamma-glutamyl-p-nitroanilide.
- The gamma glutamyl p-nitroanilide coupled with glycylglycine under the action of GGT releasing gamma glutamyl glycylglycine and p-nitroaniline.
- A p-nitroaniline is chromogenic product with a strong absorbance at 405 to 420 nm.

Analytical Methodology

The Reaction

Analysis of gamma-glutamyltransferase[1] (GGT) can be achieved by this coupled reaction at 37°C:

gamma-glutamyl-*p*-nitroanalide + glycylglycine $\xrightarrow{\text{GGT, pH 8.2}}$ gamma-glutamylglycylglycine + *p*-nitroaniline

Increase in absorbance at 405 nm is determined by continuous or endpoint monitoring.

The Phosphatase Enzymes

Introduction to Phosphatase

- The phosphatases include:
  - ❖Alkaline phosphatase (ALP)
  - ❖Acid phosphatase (ACP)
- It is an hydrolase enzyme catalyzes dephosphorylation reactions
- It is important for removal of phosphate groups from nucleotides, proteins, and alkaloids
- Unlike ALT and AST, It is not found in hepatocellular tissues.

Alkaline Phosphatase

- It is present in serum, liver, bone, intestinal mucosa, placenta, renal tubules and leucocytes.
- Since it is found in multiple organ, it has different iso-enzyme forms
- The activity in normal serum is predominantly of liver and bone origin.
- Those iso-enzyme can be differentiated by their heat stability, electrophoretic mobility and chemical inhibition

ALP Isoenzyme Characteristics

Characteristic	Name of Isoenzyme: Hepatic	Bone	Intestinal	Placental	Other
Heat Stability	Stable at 56°C for 30 minutes	Labile: disappears at 56°C within 10 minutes	Intermediate labile: disappears at 56°C within 15 minutes	Stable at 65°C for 30 minutes	Regan isoenzyme: most stable
Electrophoretic Order	Most anodic	Intermediate	Cathodic to bone fraction	Migrates with hepatic or bone forms	Renal isoenzyme: rare but most cathodic
Chemical Inhibition	Moderate inhibition by urea but low inhibition by L-phenylalanine.	Strong inhibition by urea but low inhibition by L-phenylalanine.	Strong inhibition by L-phenylalanine.	Resistance to urea but strong inhibition by L-phenylalanine.	Regan isoenzyme: Strong inhibition by L-phenylalanine.

ALP Isoenzyme Characteristics

Name of Isoenzyme	Hepatic	Bone
Heat Stability	Stable at 56^0 C for 30 minutes	Labile: disappears at 56^0 C within 10 minutes
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ALP Isoenzyme Characteristics…..

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Heat Stability	Intermediate labile: disappears at 56^0 C within 15 minutes	Stable at 56^0 C for 30 minutes	Regan isoenzyme: most stable
Electrophoretic Order	Cathodic -bone fraction	Migrates with hepatic or bone forms	Renal isoenzyme: rare but most cathodic
Chemical Inhibition	Strong inhibition by l-phenylalanine.	Resistance to urea but Strong inhibition by l-phenylalanine.	Strong inhibition by l-phenylalanine.

Test Method for Alkaline Phosphatase

- It is assayed by the Bessey Lowry and Brock method
- p- nitrophenyl phosphate + H_2O –(ALP, glycine buffer, Mg^{2+})→ p- nitrophenol (colorless) + PO4 $^{3+}$ →Yellow quininoid chromagen
- Subsequent addition of sodium hydroxide stops the reaction and converts the p-nitrophenol into yellow quininoid form.
- The intensity of the color is directly proportional to the amount of enzyme present in the sample and is measured at 400 nm.

Test Method

- It also assayed by Bowers and McComb modified method:
- 4-nitrophenyl phosphate + H_2O –(ALP, Mg^{2+}, pH 10.3)→ 4-nitrophenoxide
- Increase in Abs. at 405 nm in sphectrophotometer is proportional to the enzyme found in the specimen

Clinical Significance

- Elevations of ALP are of most diagnostic significance in the evaluation of hepatobiliary and bone disorders.
- ALP is found in very high concentrations in cases of extrahepatic obstruction
- Because bone is also a source of ALP, it may be elevated in bone-related disorders such as bone metastases, osteoblastic pathology and rapid bone growth during puberty.
- ALP is also found elevated in pregnancy due to its release from the placenta.

Sources of Error

Pre-analytic Errors

- Anticoagulants that remove Ca or Mg
- Not Fresh Sample
- In appropriate pH
- Hemolysis due to
  - Poor sample collection
  - Poor processing

Analytic Errors

- Substances that absorb light at 405 nm:
  - Lipids (lipemia)
  - Bilirubin

5'-Nucleotidase

- It is a phosphatase enzyme responsible for catalyzing the hydrolysis of neucleoside-5-phosphate esters.
- Its serum levels become significantly elevated in hepatobiliary disease.
- There is no bone source of 5NT, so it is useful in differentiating ALP elevations due to the liver from other conditions increased ALP concentrations (bone diseases, pregnancy, and childhood growth).

Methods of Analysis

- The substrates mostly used in measuring the activity of 5NT are AMP or inosine-5'-phosphate (IMP).
- However, these substrates are organic phosphate esters, also are hydrolyzed by other nonspecific (alkaline) phosphatases.
- The effect of ALPS on IMP is inhibited by P-glycerophosphate

Methods of Analysis…..

- Serum 5NT catalyzes the hydrolysis of IMP to yield inosine.
- Then it will be converted to hypoxanthine by purine-nucleoside phosphorylase
- Hypoxanthine is oxidized to urate and two moles of hydrogen peroxide under the influence of xanthine oxidase .
- The formation rate of hydrogen peroxide is measured by chromogenic system at specific WL of 510.

Synthetic functions:

1. Total plasma proteins/ albumin/ globulin/ A:G ratio

2. Formation of prothrombin by liver

Determination of Protein

- A healthy and functioning liver is required for the synthesis of serum protein.
- The measurement of serum proteins, therefore, can be used to assess the synthetic ability of the liver.
- Although these tests are not sensitive to minimal liver damage, they may be useful to assess the severity of hepatic dysfunction

Determination of Protein

- A decreased albumin level correlates well with chronic liver disease.
- Serum globulin levels are transiently increased in acute liver disease and remain elevated in chronic liver disease.
- The highest elevations are found in chronic hepatitis and post necrotic cirrhosis.

Determination of Protein

- Its determination includes measuring serum/ plasma level of total proteins, albumin, globulin and A:G ratio
- Since the majority of proteins found in plasma are synthesized by the liver,
- The lab result of the above mentioned bio-analytes indicated liver disorders.

Methods of Protein Analysis

- **Determination of Total Protein**
  - ☛Kjeldahl method
  - ☛Biuret Method
- **Determination of serum albumin**
  - ☛BCG
  - ☛BCP
  - ☛Electrophoresis

Methods for globulin determination

- The total globulin in serum can be determined by any one of the following way
  - ☛Globulin by difference (Total protein – albumin in serum sample)
  - ☛ Colorimetric methods using globulin reagent
  - ☛ Electrophoresis

Total Protein Analysis

- The total protein test is a rough measure of all of the proteins in the plasma.
- It can reflect nutritional status, kidney disease, liver disease, and many other conditions.
- But if total protein is abnormal, further tests must be performed to identify which protein fraction get involved and to made specific diagnosis.

Kjeldahl method

- In this method, an average of 16% nitrogen mass in protein is assumed to calculate the protein conc.
- The protein in the sample is subjected to acid digestion (H2SO4) with heat (340–360°C) to extract the nitrogen
- A catalyst, such as cupric sulfate and potassium sulfate are added to speed up and increase the boiling point respectively to improve the efficiency of digestion.

Kjeldahl method…..

- The H_2SO_4 oxidizes the C, H, and S in protein to CO_2, CO, H_2O, and SO_2.
- In the procedure all forms of nitrogen is converted to ammonium ion which can be separated by steam distillation
- A conversion factor of 6.25 is used for this techniques
- This method is not used in the clinical laboratory because it is time consuming and too tedious for routine use.

Biuret

- The Biuret reagent is made of (NaOH) and copper (II) sulfate (CuSO4), together with potassium sodium tartrate (KNaC4H4O6).
- This is the most widely used method
- Cupric ions (Cu^{2+}) in an alkaline medium react with at least two peptide bonds to form a violet-colored product.
- The reagent also contains sodium potassium tartarate that facilitate the reaction and maintain the solubility in alkaline medium

Biuret…..

- The chromogenic product formed by the reaction is measured at 540 nm.
- The color may varies from a pink to a reddish violet
- The color that is formed is proportional to the number of peptide bonds present and reflects the total protein level.
- Lipemia in the sample is an interference.

Analysis of Specific protein

- Proteins are separated on the basis of differences in their physicochemical properties such as:
  - ❖Size
  - ❖Charge
  - ❖Adsorption characteristics
  - ❖Solubility
  - ❖Heat-stability etc…

Methods for Determination of serum Albumin

- ❖ Dye binding
  - ☛ Methyl Orange
  - ☛ 2,4,HABA
  - ☛ BCG
  - ☛ BCP
- ❖ Electrophoresis

Dye binding

- A number of specific dyes have been used to bind with albumin and absorb light at a wave length different from the unbound or free dye.
- It include: Methyl orange, 2,4, hydroxyl-azobenzene benzoic acid, BCG (Bromcresol Green) and BCP (Bromcresol Purple)
- The first two dyes mostly subjected to pigment interference (bilirubin)
- The last two dyes are the recommended dyes since they are sensitive, specific and free from interference

BCG Methods

- The albumin's ionic charge at an acid pH (4.2, Succinate buffer) can bind to anionic dyes (BCG) to form complex
- In the procedure, the reagent dye is added in 3 tubes (One is free and two each containing 0.01ml of sample and standard respectively)

S. No	Reagents	Blank (B)	Standard (S)	Test (T)
1.	BCG Reagent	1.0 ml	1.0 ml	1.0 ml
2.	Albumin Standard	-	10 µl	-
3.	Serum	-	-	10 µl

- Mix well and allow the tubes to stand at RT for 10 min.
- Measure the O.D of all the tubes at 628 nm.

BCP Methods

- The procedure is similar to BCG
- The principle is the yellow BCP dye at PH 5.2 (acetate buffered solution) react with albumin.
- As a result the yellow colour of the unbound dye turns to green due to complex formation
- The increased in absorbance is measured at 603 and the light intensity absorbed at this spectrum is directly proportional to the concentration of albumin

Serum Protein Electrophoresis

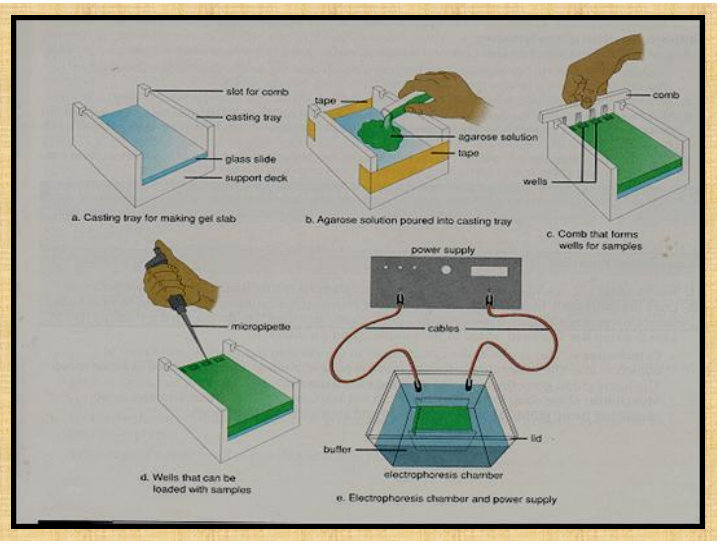
- It is the techniques that refers to the migration of particles under the influence of electric field
- It can be used to separate proteins on the basis of their size, shape or charge
- Serum samples are applied close to the cathode end of a support medium that is saturated with an alkaline buffer (pH 8.6).

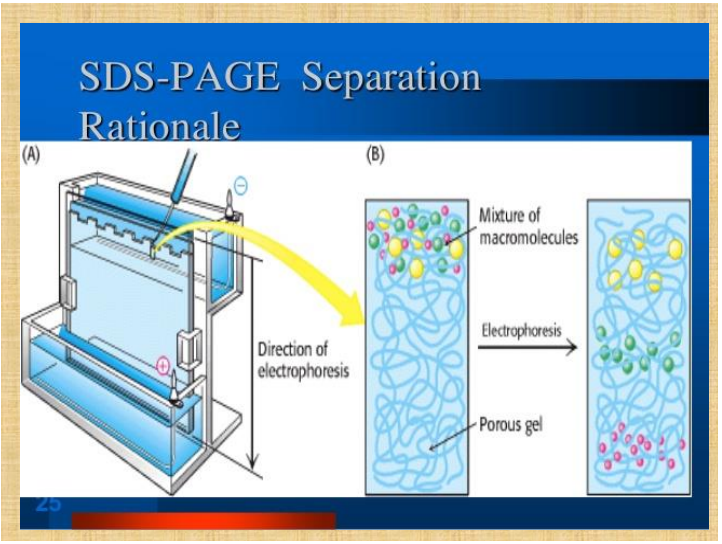
Electrophoresis…..

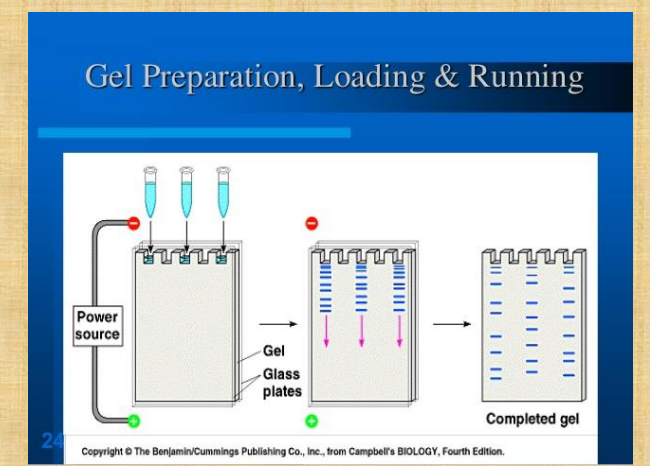
- The support medium is connected to two electrodes and a current is passed through the medium to separate the proteins.
- All major serum proteins carry a net negative charge at pH 8.6 migrate toward the anode.
- Using this methods, serum proteins appear in five distinct bands:
  - Albumin travels farthest to the anode, followed by α1-globulins, α2-globulins, β-globulins, and γ-globulins.
- The width of the band depends on the number of proteins present in the fraction.

Electrophoresis…..

- After separation, the protein fractions are fixed by immersing the support medium in an acid solution (e.g., acetic acid)
- This helps to denature the proteins and immobilize them on the support medium.
- In the next step, the proteins are stained by a variety of dyes including Amido black, Coomassie blue etc... to visualized the band
- It can be also presented in graphical presentation by the use of densitometer

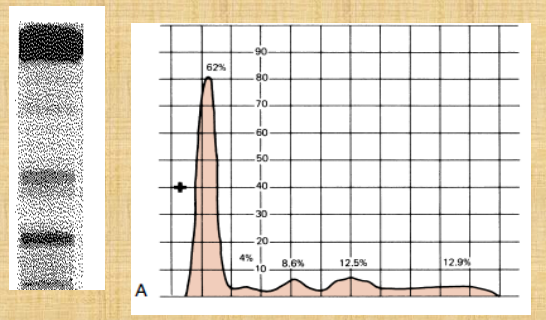






Separation of protein in band form

Graphical presentation using denstometer



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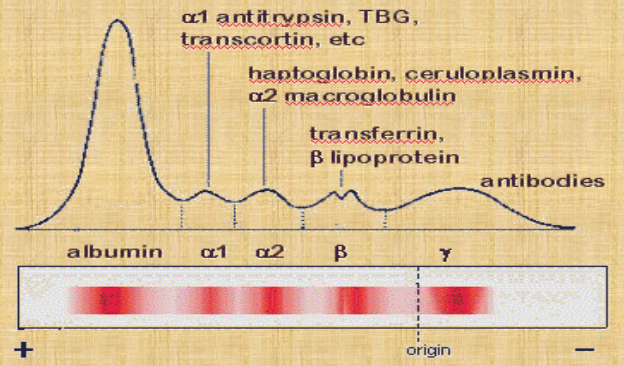

Methods for globulin determination

- The total globulin in serum can be determined by any one of the following way
  - ☛ Globulin by difference (Total protein – albumin in serum sample)
  - ☛ Colorimetric methods using globulin reagent
  - ☛ Electrophoresis

Colorimetric Methods

- Globulin can react with Globulin reagent (glyoxylic acid) in the presence of string acid.
- Globulin reagent is composed of Glyoxylic acid, copper sulphate, acetic acid and sulfuric acid.
- Copper sulphate in the reagent enhances the color formation

Procedure

	Blank	Standard	Test
Globulin reagent	1 ml	1ml	1ml
Glogulin std	-	10µl	
Serum	-	-	10µl

- Cover tubes, mix and heat at 100 ^{O}C for 5 min
- Cool in tap water for 3 min and remix
- Read the absorbance of all tubes against reagent blank

Specimen

- Serum and plasma may be used
- But the presence of fibrinogen in plasma makes the levels for total proteins 2 to 4 g/L higher than serum levels.
- A fasting specimen is not required but may be desirable to decrease lipemia.
- Total protein is stable in serum and plasma for 1 week at room temperature and for at least 4 months at –20° C

Clinical significance

➢ **Hypoproteinemia**
- Malnutrition and/or malabsorption
- Excessive loss as in renal disease
- GI leakage
- Excessive bleeding, severe burns
- Excessive catabolism
- Liver disease

➢ **Hyperproteinemia**
- Dehydration
- Monoclonal increase
- Polyclonal increases

Metabolic capacity

Galactose tolerance test

Basis:

- Galactose is a monosaccharide, almost exclusively metabolized by the liver.
- The normal liver is able to convert galactose into glucose.
- But this function is impaired due to intrahepatic disease and the amount of blood galactose and urine galactose become excessive.
- The liver can be assessed by measuring the utilization of galactose which is referred as galactose tolerance test.

Method :

- Oral galactose tolerance test
- IV galactose tolerance test

Oral galactose tolerance test

- The test is performed @ morning after a night fast.
- A fasting blood sample is collected which serves as a "control".
- 40mg galactose dissolved in a cup-full of water is given orally.
- Further blood and urine samples are collected at ½ hour intervals for 2-5 hours.

Result of OGTT

Normal or obstructive jaundice:

- The blood galactose returns to normal (17mg/dl) within one hour.
- 3gm or less of galactose are excreted in the urine within 3 to 5 hours

Intrahepatic jaundice:

- The blood galactose level continues to rise even after two hours
- The excretion amounts 4 - 5gm or more during the first 5 hours.

Prothrombin time (protime or PT)

- Prothrombin is a plasma protein that is converted into thrombin during blood clotting.
- Prothrombin is formed in the liver from inactive "preprothrombin" in the presence of vitamin K.
- Then Prothrombin also converted to thrombin

in presence of vitamin K

$$\text{prothrombin} \xrightarrow{Ca^{2+}} \text{thrombin}$$

Prothrombin time…..

- Prothrombin time is commonly increased in liver disease because the liver is unable to manufacture adequate amounts of clotting factor or disruption of bile flow
- However, a prothrombin time is not routinely used in the diagnosis of liver disease.
- Rather, serial measurements of prothrombin times may be useful
  - ☛ The progression of disease and
  - ☛ The assessment of risk of bleeding.

What is prothrombin time?

- The term prothrombin time is defined as a time required to take place clotting.
- It is referred as the function of prothrombin activity.
- A marked prolongation of the prothrombin time indicates severe diffuse liver disease and a poor prognosis
- PT is usually expressed in seconds. Normally it falls in 10 - 15 seconds.

Prothrombin time….

This test may be done:

- ✓When a case has a bleeding problem
- ✓To monitor those who is taking blood-thinning medicine
- ✓Before surgery to make sure a case will not bleed too much during the operation.

High PT values may occur during :

- ✓Taking blood-thinning medicines like warfarin
- ✓Severe liver disease
- ✓Inherited bleeding disorders
- ✓Vitamin K deficiency

Abnormally low PT values are usually not significant. However, may occur during:

- ✓Cancer
- ✓Blood clots
- ✓Taking certain medicines, such as birth control pills

THANK YOU

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"Ameseginalehu"

Reference Values of ALT

Serum or plasma reference ranges may vary with method

- Dog 10-109 U/L
- Cat 25-97 U/L
- Horse 3-21 U/L
- Cow 7-35 U/L
- Pig 22-47 U/L
- Shoat 15-44 U/L

Reference intervals

Species	GGT(IU/L)
Dog	1-10
Cat	2-12
Cow	6-18
Horse	6-32
Sheep	11-24
Pig	20-44

Serum levels increase in hepatobiliary obstruction, inflammation or in alcoholism