SYLLABUS

1. Hematology.
    a. RBC and WBC counting
    b. Differential leucocyte count.
    c. Clotting time.
    d. Bleeding time
2. Estimation of haemoglobin.
3. Determination of blood groups
4. Separation of plasma proteins (Group Experiment).
5. Determination of total iron binding capacity.
6. Pulmonary function tests, spirometry and measurement of blood pressure.
7. Separation of isoenzymes by electrophoresis (Group Experiment).
8. Histology of connective tissue, liver and/ brain - permanent slides.
9. Case studies (Renal clearance, GFR, ECG).

1. HEMATOLOGY

AUTOMATIC METHOD

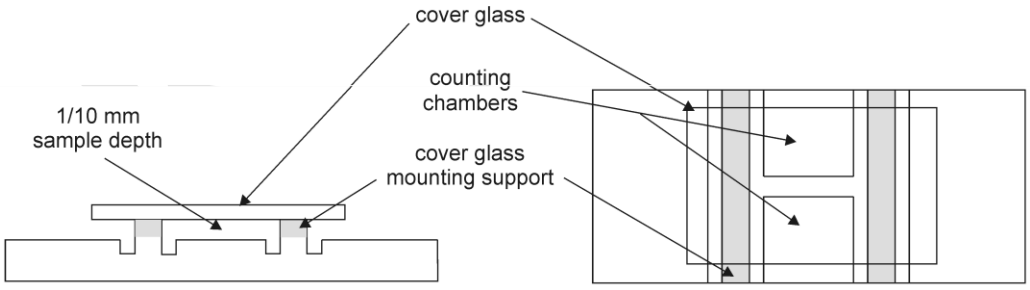
Complete blood count performed by an automated analyzer that counts the numbers and types of different cells within the blood. It aspirates a very small amount of the sample through the narrow tubing. Within this tubing, there are sensors that count the number of cells going through it, and can identify the type of cell; this is called flow-cytometry. For detection light detectors are used as well as the measurement of electrical impedance. One way the instrument can tell what type of blood cell is present is by size. Other instruments measure different characteristics of the cells to categorize them.

MANUAL METHOD

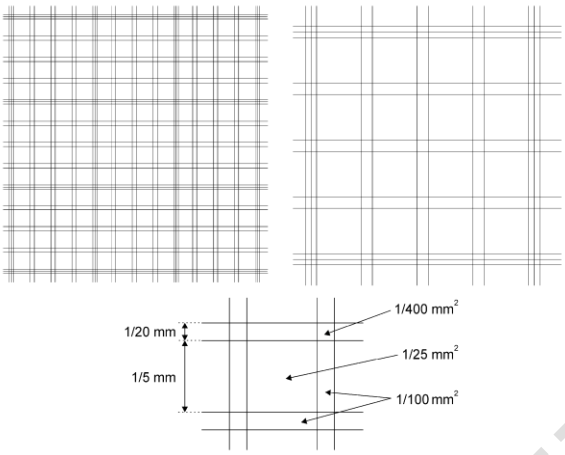
This measurement is made with a microscope and a specially ruled chamber (hemocytometer) using diluted blood.

The hemocytometer consists of a thick glass microscope slide with a rectangular indentation that creates a chamber. This chamber is engraved with a grid of perpendicular lines. The device is carefully crafted so that the area bounded by the lines is known, and the depth of the chamber is also known. It is therefore possible to count the number of cells or particles in a specific volume of fluid, and thereby calculate the concentration of cells in the fluid overall. Several types of hemocytometers are used; we use Türk counting chamber, engraved with two straight and one "H" shaped deep moats, which enclose the cover glass mounting supports and separate the two engraved areas. Each counting surface has an area of 9 mm2. The depth between the cover glass and the surface of the slide is 1/10 mm. The grid of the counting chamber consists of perpendicular lines situated at 1/5 mm and 1/20 mm respectively. The perpendicular lines delimit squares and rectangles where the blood cells are counted:

- small squares with the area of 1/400 mm2 for red blood cell count,
- big squares with the area of 1/25 mm2 for white blood cell count,
- rectangles with the area of 1/100 mm2 for platelet count.

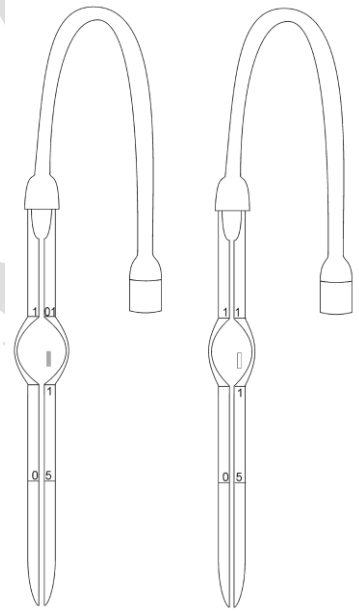


The hemocytometer – lateral and top view



The grid pattern of the Türk chamber

Diluting pipettes (Potain's pipettes) are capillary (very thin) pipettes with a mixing chamber. In the mixing chamber there is a mixing bead, colored in red for the red blood cell pipette and in white for the white blood cell pipette. On the capillary part of the pipette are marks for 0.5 and 1 µl, and above the mixing chamber is a mark for 101 µl on the red cell pipette and for 11 µl on the white cell pipette. A rubber tube with a mouthpiece is attached to the top- end of the pipette.



Diluting (Potain) pipettes for red (left) and white (right) blood cell count

A. i. RED BLOOD CELL COUNT

MATERIALS

- hemocytometer,
- diluting pipette for red blood cells,
- diluting fluid (Hayem's solution; Na2SO4 5 g, NaCl 1 g, sublimate 0.5 g, distilled water ad 200 g),
- light microscope,
- for blood sample collection: cotton balls, alcohol, sterile needles, rubber gloves.

PROCEDURE

Cleanse the hemocytometer and cover glass with a piece of cotton saturated with alcohol and let air dry. Prepare the hemocytometer by placing the cover glass on its mounting supports.

Note:

To avoid risk of infection, wash your hands with soap and water before and after doing any blood tests! When manipulating blood samples from another person, use disposable rubber gloves! Dispose of the used needles in special containers!

Cleanse the tip of the finger with a piece of cotton saturated with alcohol. Let the finger air dry. Using a sterile, disposable needle, quickly make a single puncture in the top of the cleansed finger (hold firmly between the thumb and forefinger) deep enough so that blood flows freely from the wound. Wipe off the first drop of blood with a piece of cotton; when a second drop has accumulated, proceed with the filling of the pipette. Do not fill the pipettes with blood until sufficient blood has welled up on the fingertip since these pipettes have a very small bore and blood clots extremely easily in them. The finger must not be squeezed.

Place the pipette tip just within the drop of blood. Suck up a continuous column of blood in the tube to the 0.5 mark on the pipette by using the mouthpiece. Wipe the excess blood from the tip of the pipette. Immerse the pipette tip in the red blood cell diluting fluid (Hayem's solution); and while holding the pipette vertically, suck the diluting fluid exactly to the 101 mark. Dilution should be done very quickly and precisely to prevent clotting of the blood and to insure accuracy. Thus a 200-fold dilution is obtained (the volume of the mixing chamber is 101 µl–1 µl=100 µl, containing 0.5 µl of blood).

Close the ends of the pipette with your thumb and middle finger and shake the pipette for 3 minutes.

Throw away the first two drops from the pipette (they come from the capillary part of the pipette and contain only diluting fluid) and with the third drop fill the hemocytometer: let the drop fall near the cover glass and the chamber will fill due to capillarity. The excess fluid will flow in the moats.

Place the hemocytometer in a light microscope and examine the sample using low-power objective (10 x) and weak light. Count the red blood cells in 80 little squares considering the cells inside the squares and from two sides.

RESULTS

Report the number of red blood cells as cells/mm3. First the average number of red blood cells in a little square must be calculated: N (total number of found red blood cells)/80 (number of squares). This must be reported to the volume corresponding to a little square: area*height of the chamber. Last the results must be corrected with the dilution of the blood.

$$NBRC = \frac{N}{NSq * Volume * Dilution} - \frac{N}{NSq * Area * Height * Dilution}$$

$$NRBC = \frac{N}{80 * \frac{1}{400} * \frac{1}{10} * \frac{1}{200}} = \frac{N}{80} * 400 * 10 * 200 = N * 10000$$

DATA INTERPRETATION

Normal range:

- males: 4.7–5.2*106/mm3
- females: 4.2–4.7*106/mm3
- newborn, young children: 5.5-6*106/mm3

Higher values – polycythemia:

- physiological: gender, age, high altitude, effort
- pathological: chronic bone marrow, lung or heart diseases

Lower values – anemia

- excessive bleeding
- decreased red blood cell production
- increased red blood cell destruction

A. ii. WHITE BLOOD CELL COUNT

MATERIALS

- hemocytometer,
- diluting pipette for white blood cells,
- diluting fluid (Türk's solution: glacial acetic acid 0.5 ml, gentian violet 1% 1.5 ml, distilled water ad 150 ml),
- light microscope,
- for blood sample collection: cotton balls, alcohol, sterile needles, rubber gloves.

PROCEDURE

Cleanse the hemocytometer and cover glass with a piece of cotton saturated with alcohol and let air dry. Prepare the hemocytometer by placing the cover glass on its mounting supports.

Note:

To avoid risk of infection, wash your hands with soap and water before and after doing any blood tests! When manipulating blood samples from another person, use disposable rubber gloves! Dispose of the used needles in special containers!

Cleanse the tip of the finger with a piece of cotton saturated with alcohol. Let the finger air dry. Using a sterile, disposable needle, quickly make a single puncture in the top of the cleansed finger (hold firmly between the thumb and forefinger) deep enough so that blood flows freely from the wound. Wipe off the first drop of blood with a piece of cotton; when a second drop has accumulated, proceed with the filling of the pipette. Do not fill the pipettes with blood until sufficient blood has welled up on the fingertip since these pipettes have a very small bore and blood clots extremely easily in them. The finger must not be squeezed.

Place the pipette tip just within the drop of blood. Suck up a continuous column of blood in the tube to the 0.5 mark on the pipette by using the mouthpiece. Wipe the excess blood from the tip of the pipette. Immerse the pipette tip in the white blood cell diluting fluid (Türk's solution); and while holding the pipette vertically, suck the diluting fluid exactly to the 11 mark. Dilution should be done very quickly and precisely to prevent clotting of the blood and to insure accuracy. Thus a 20-fold dilution is obtained (the volume of the mixing chamber is 11 µl– 1 µl=10 µl, containing 0.5 µl of blood).

Close the ends of the pipette with your thumb and middle finger and shake the pipette for 3 minutes.

Throw away the first two drops from the pipette (they come from the capillary part of the pipette and contain only diluting fluid) and with the third drop fill the hemocytometer: let the drop fall near the cover glass and the chamber will fill due to capillarity. The excess fluid will flow in the moats.

Place the hemocytometer in a light microscope and examine the sample using

low-power objective (10 x) and weak light. Count the white blood cells in 25 big squares considering the cells inside the squares and from two sides.

RESULTS

Report the number of white blood cells as cells/mm3. First the average number of white blood cells in a big square must be calculated: N (total number of found white blood cells)/25 (number of squares). This must be reported to the volume corresponding to a big square: area*height of the chamber. Last the results must be corrected with the dilution of the blood.

$$NWRC = \frac{N}{NSq * Volume * Dilution} \quad \frac{N}{NSq * Area * Height * Dilution}$$

$$NWBC = \frac{N}{25 * \frac{1}{25} * \frac{1}{10} * \frac{1}{20}} = \frac{N}{25} * 25 * 10 * 20 = N * 200$$

DATA INTERPRETATION:

Normal range:

- adults: 6000-8000/mm3
- newborn, young children: 14000-20000/mm3

Higher values – leukocytosis:

- physiological: after effort, after meals, women: menstruation, pregnancy, childbed
- pathological: infection, inflammation, poisoning

Lower values – leucopenia: anaphylactic shock, viral infections, X-ray exposure.

B. DIFFERENTIAL LEUKOCYTE COUNT (DLC)

The leukocytes are also called white blood corpuscles (WBC) and formally known as white cells of the blood but these are not white colour, these are colourless. The white blood cells are protect our body against any diseases by fighting with infections (bacterial, viral, protozoan, parasitic etc.), antigens and also against malignancy. These are two types; Granulocytes (Neutrophil, Eosinophil and Basophile) and Agranulocytes (Monocyte and Lymphocyte). After staining the blood film with Leishman stain, the blood smear examine in the microscope under oil immersion objective (100X). Under oil immersion objective the leukocytes are seen as follows:

MORPHOLOGY AND FUNCTION OF LEUKOCYTES

Neutrophil

Neutrophils are round shape, 10 – 15µ in diameter. The cell contains cytoplasm and nucleus. The nucleus shows variable numbers of lobes, 2 – 7 lobes hence called polymorphonuclear leukocytes. The nucleus stain purple blue and the chromatin are coarse and ropy. The cytoplasm contains two types of granules – Primary granules and Secondary granules. When the cell stained with Leishman stain, only secondary granules are stained and these are violet colour granules, which are amphophilic. The cytoplasm takes pink colour.

Neutrophils are called first line defenses as they move first to fight the invading micro-organisms (bacteria etc.). Neutrophil with their enzymatic armory are superb killers. The activated neutrophils engulf the bacteria (phagocytosis) and released different enzymes into the phagocytic vesicles, which killed the bacteria and then digest it.

Eosinophil

Eosinophils are round shaped, 10 – 15µ in diameter. The cell contain cytoplasm packed with coarse brick red colour granules which takes acidic stain (eosin) hence called eosinophil. And the nucleus of the cell consists of 2 – 3 lobes. The nucleus stains purple blue colour and chromatin is course and ropy.

Eosinophil contains a Major Basic Protein (MBP) which damages the larvae of the parasites. There is one eosinophilic cation protein which probably neutralizes heparin anticoagulant. They have a property that prevents anaphylaxis (anti allergic action). They are motile and phagocytic. Chemotoxis is also shown by the eosinophils.

Basophile

Basophils are round shape, 10 – 15µ in diameter occasionally seen in the blood smear from a healthy person. The cell contain 'bilobed' or 'S' shaped nucleus stains purple blue and chromatin is coarse and ropy. The cytoplasm of the cell packed with course blue basophilic granules. On an average all granules are of equal size, which obscured the nucleus.

Basophils release the histamine resulting in immediate hypersensitivity reaction and also have role in inflammation. They contain heparin, protease and other mediators of inflammation. They are motile and phagocytic.

Monocyte

Monocytes are large, round, 10 – 20µ in diameter The cell contains a large kidney shaped, light purple blue stained nucleus and pale blue colour cytoplasm contain no granules. Some time few fine purple granules and vacuoles are seen in the cytoplasm.

The monokines secreted by monocytes stimulated T-cells, take part in inflammation, act as pyrogen and stimulate formation of acute phase proteins. They converted into macrophases and phagocytosis the microorganism, dead tissues etc.

Lymphocyte

In the blood circulation two types of lymphocyte are found, these are small lymphocyte and large lymphocyte. The small lymphocytes are believed to be the resting phase and when active they become large.

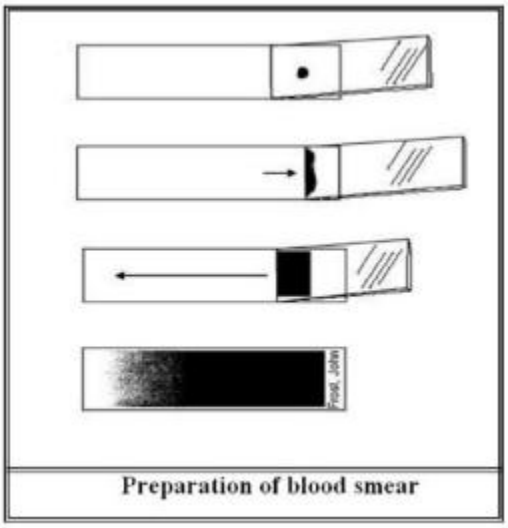
- **Small Lymphocyte :**

Small lymphocytes are small; round shaped, 8 – 10µ in diameter agranulocyte. The cell contains a large nucleus that occupied almost the total cell and scanty cytoplasm is seen. The cell contain cytoplasm takes blue colour and nucleus stains deep blue colour.

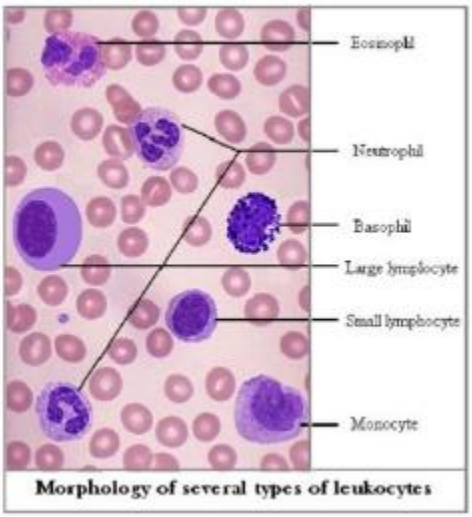
- **Large Lymphocyte :**

Large lymphocytes are round shaped, 10 – 18µ in diameter agranulocyte. They contain a round shaped deep blue nucleus with course and ropy chromatin and abundant clear blue cytoplasm with out any granules.

They are involver in the very important defense mechanism, called immunity. The B-lymphocytes are responsible for Humoral immunity and T-lymphocytes are responsible for Cell mediated immunity.



Preparation of blood smear



Morphology of several types of leukocytes

There are three major steps involved in differential cell count:

1. Preparation of blood smears

2. Staining of blood

3. Staining of smear

4. Microscopic examination

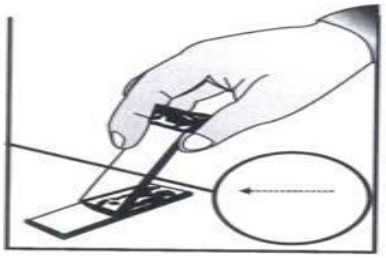


Fig. 7.1: Smear preparation

Preparation of Blood Smear:

Take a clean grease free slide. Obtain capillary blood directly from the finger or EDTA anti-coagulated blood. Place it on a corner of a slide. Take a spreader (Spreader is a slide with sharp edges).

Touch the edge of the spreader to blood drop on slide. Slightly push it backward so that, the drop is spread evenly to the edge of the spreader. Now, spread blood with the help of a spreader across clean grease free slide.

The angle between spreader and slide should be about 45°. With a quick movement, push the spreader towards the other end of the slide. Blood film should not be too thick. It should be 1 cm. from the edge of slide and 5 mm. in width.

Staining of Blood:

Blood cells have different structures, which take different stains. Some are basophilic; others are acidophilic while some cells accept neutral stain. Therefore, the stain used is combination of these three. Such stains are called as 'Romanowsky stain'.

The three different types of Romanowsky stains are commonly in use.

i. Leishman stain

ii. Giemsa's stain

iii. Wright's stain.

Each of the stain contains acidic stain, basic stain and buffer solution. Generally, methylene blue or touline are basic stains, and Eosin, Azure-I, Azure-II are the acidic stains in use.

It is advisable to stain a slide soon after preparation of blood smear.

Staining of Smear:

Leishman Stain:

Leishman stain is the most common and cheapest of all stain.

Composition:

i. Leishman stains powder – 0.15 gm.

ii. Methyl alcohol – 100 ml.

Leishman stain crystals are grounded in a glass mortar. This powder is first dissolved in few ml of methyl alcohol, and then the remaining quantity of alcohol is added, so that the entire volume becomes 100 ml. Pour the stain in a clean dry bottle, close it well. Do not open it or filter it within 3 weeks. After 3 weeks it is ready for use.

Blood films are placed in a staining tray. The dry blood film is then covered with stain. The stain should be evenly distributed over the entire slide. After 1 min. add distill water or buffer solution (Sodium- potassium phosphate buffer at pH 6.8) to the slide.

The distilled water / buffer solution should be carefully mixed with the stain. Keep it as it is for about 7 to 8 min. Then wash the slide with distill water to remove excess of stain. Air dry the film and observe under oil immersion objective of microscope.

Microscopic Examination:

First examine stained blood smear under, low power objective. Note the background colour and distribution of cells.

In an ideal staining smear, three zones can be identified:

1. Thick area or head of smear

2. The central area is body

3. At the end of smear is tail region

Choose the portion of the smear in the body region, slightly before the tail end.

Observe the slide under oil immersion objective by putting a drop of oil over the slide.

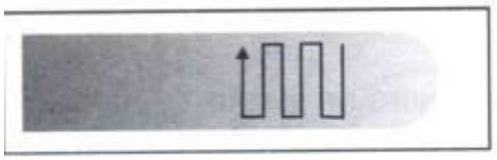


Fig. 7.2: Observation of smear

Examine the slide in tail region using OIO and move the slide as shows in Figure 7.2.

Count each type of white cell observed. Record the observations either on a piece of paper in a tabular form or on a cell counter. The cell counter has different keys for different types of WBC. Continue the counting till 100 cells are counted. This will give the average number of white cells.

Observation:

With Leishman stain, the cells are observed as:

1. Neutrophil:

Purple coloured nuclei with pink cytoplasm.

2. Eosinophil:

Cytoplasm is faint pink, nucleus is purple and granules are orange red.

3. Basophil:

Granules stain dark blue with purple nucleus.

4. Monocytes:

Pink cytoplasm with purple colour nucleus.

5. Lymphocyte:

Dark blue nucleus with light blue cytoplasm.

6. Platelets:

Violet coloured granules.

7. Red cells:

Pink colour.

Clinical Significance:

The basic use of DLC is to identify changes in distribution of white cells. These changes are related to specific infection, like bacterial, viral, parasitic, leukemic, etc. Different clinical terms are used for the different changes in normal values of the cells.

Normal Values

Cells	*In %*	*Per cu mm*
Neutrophil	60 to 70	3,000 to 7,000
Lymphocyte	25 to 35	2,000 to 3,000
Monocyte	2 to 6	100 to 600
Eosinophil	1 to 6	50 to 400
Basophil	0 to 0.5	0 to 50

Thus, Differential Leucocyte Count gives us the idea about distribution of different proportions of white cells.

C. BLEEDING TIME

AIM

To determine the bleeding time of a subject.

Requirements:

Sterile lancet, cotton, rectified spirit, filter paper, stop watch.

Procedure:

Duke's method: Sterilize the finger tip using rectified spirit and allow to dry. Make a sufficiently deep prick using a sterile lancet, so that blood comes out freely without squeezing. Note the time (start the stop-watch) when bleeding starts. Mop the blood by touching the finger tip with a filter paper. This is repeated every 15 seconds, each time using a fresh portion of the filter paper, till bleeding stops. Note the time (stop the stop-watch). It is seen that the blood stains on the filter paper get smaller to disappear finally when bleeding stops.

Discussion:

Bleeding time is the interval between the moment when bleeding starts and the moment when bleeding stops. Normal bleeding time (Duke's method) is ito 4 minutes. Bleeding time is prolonged in purpuras, but normal in coagulation disorders like haemophilia. Purpuras can be due to

1. Platelet defects - Thrombocytopenic purpura.
    a. Primary (Idiopathic) - Thrombocytopenic purpura
    b. Secondary - Thrombocytopenic purpura
2. Vascular defects - Senile purpura

Henoch Schonlein purpura

Platelets are important in preventing small vessel bleeding by causing vaso constriction and platelet plug formation.

Other method:

Ivy's method: Apply the sphygmomanometer cuff to the arm. Raise the cuff pressure and maintain at 40 mm of Hg. Under sterile conditions make a deep prick on the forearm just below the elbow. Bleeding time is noted as in Duke's method.

Normal value is 2 to 7 minutes

D. CLOTTING TIME

AIM

To determine the clotting time of a subject.

Requirements:

Fine capillary glass tubes of about 10 mm length, cotton, rectified spirit, lancet, stop watch.

Procedure:

Capillary tube method: (Wright's method)

Under sterile precautions make a sufficiently deep prick in the finger tip. Note the time when bleeding starts (start the stop watch). Touch the blood drop at the finger tip using one end of the capillary tube kept tilted downwards. The tube gets easily filled by capillary action. After about two minutes start snapping off small lengths of the tube, at intervals of 15 seconds, each time noting whether the fibrin thread is formed between the snapped ends. Note the time (stop the stop watch) when the fibrin thread is first seen.

Discussion:

Clotting time is the interval between the moment when bleeding starts and the moment when the fibrin thread is first seen.

Normal value is 3to 10 minutes.

Bleeding time and clotting time are not the same. Bleeding time depends on the integrity of platelets and vessel walls, whereas clotting time depends on the availability of coagulation

factors. In coagulation disorders like haemophilia, clotting time is prolonged but bleeding time remains normal.

Clotting time is also prolonged in conditions like vitamin K deficiency, liver diseases, disseminated intravascular coagulation, overdosage of anticoagulants etc.

Other method:

Modified Lee and White method:

Under aseptic precaution venepuncture is done and one ml. of blood is collected in each 3 small test tubes. Note the time when blood is taken. Keep the test tube in a water bath maintained at 37°c. Tilt the tubes every 30 seconds and see whether the blood is flowing. Repeat this till the tube can be inverted without the blood flowing out. Nore the time. Average value of the results in the 3 test tubes gives the clotting time.

Normal value is 2 to 7 minutes

2. ESTIMATION OF HEMOGLOBIN

Principle

When Blood is mixed with a solution of potassium cynide, potassium ferricyanide and Drabkin's solution, the erythrocytes are lysed by producing evenly disturbed hemoglobin solution. Potassium ferricyanide transforms hemoglobin to methemoglobin, and methemoglobin combines with potassium cyanide to produce hemiglobincyanide (cyanmethemoglobin). This way all types of hemoglobin present in blood are entirely transformed to a single compound cyanmethemoglobin. When the reaction is entire, absorbance of the solution is deliberate in a spectrophotometer at 540 nanometer. Hemoglobincyanide has a wide absorbance peak at this wavelength. The absorbance is compared with that of the standard hemiglobincyanide solution by using a formula to obtain the amount of hemoglobin.

Equipment

Spectrophotometer or photoelectric colorimeter

1. Pipette 5 ml
2. Sahli's pipette

Reagents

1. Drabkin's Solution
2. Cyanmethemoglobin standard solution with known hemoglobin value

Specimen

Blood obtained from skin puncture or EDTA-anticoagulated venous blood.

Procedure

1. Take 5 ml of Drabkin's solution in a test tube and add 20 µl of blood. This way, we will get the dilution of 1:25. Now mix the mixture and allow to stand for atleast 5 minutes. This time is adequate for transformation of hemoglobin to hemiglobincyanide.
2. Pour the test sample to a cuvette and read the absorbance of the test sample in a spectrophotometer at 540 nanometer or in a photoelectric colorimeter using a yellow-green filter. Also read the absorbance of the standard solution. Absorbance must be read against Drabkin's solution.
3. From the formula given below, the hemoglobin value is derived.

Hemoglobin in gm/dl = [Absorbance of test sample ÷ Absorbance of standard] x concentration of standard x [Dilution factor ÷ 100]

Preparation of table and graph: Result can be obtained quickly, if the table of graph is prepared which correspond absorbance with hemoglobin concentration. This is markedly acceptable when huge number of samples are daily processed on the same instrument.

For the preparation of a calibration graph, adulterate cyanmethemoglobin standards are commercially available. As another option, standard cyanmethemoglobin solution is diluted serially with Drabkin's solution. Concentration of hemoglobin (horizontal axis) in each dilution

is arranged against the absorbance (vertical axis) on a linear graph paper. A straight line connecting the points and passing through the origin is obtained. A table can be prepared relating absorbance to concentration of hemoglobin from the help of this graph.

Notes:

1. The hemiglobincyanide solution is stable so that delay in getting the reading of absorbance does not influence the result.
2. High TLC (total leukocyte cunt) (> 25,000/µl), abnormal plasma proteins (e.g. in Waldenström's macroglobulinemia, multiple myeloma) or lipemic blood (hypertriglyceridemia), can cause the error in results.

3. DETERMINATION OF BLOOD GROUPS

Objective

To understand the basic concept of Blood Grouping.

Principle:

It was in 1901, that Austrian-American immunologist and pathologist **Karl Landsteiner** discovered human blood groups. Karl Landsteiner's work helps to determine blood groups and thus opened a way for blood transfusions which can be carried out safely. He was awarded the **Nobel Prize in Physiology or Medicine** in 1930 for this discovery.

Death of the patient was the result in most cases before 1900, when blood transfusion was attempted. Blood transfusion was made much safer by the discovery of blood groups, as blood of the same ABO group could be chosen for each patient. However, there were still many cases of unexplained blood transfusion reactions. Biologists still went in search of these unexplained questions.

In 1902, the fourth main type, AB was found by Decastrello and Sturli. It was the observations of Levine and Stetson in 1939, and Landsteiner and Weiner in 1940, that laid the foundations of our knowledge about the remaining major blood group- the Rhesus system. Once

reliable tests for Rhesus grouping had been established, transfusion reactions became rare! For this discovery Landsteiner was awarded the Nobel Prize in Physiology or Medicine in 1930.

The Components of Blood

The circulatory system distributes about 4-6 liters of blood to the adult human body. The blood mainly has 2 portions: **the Plasma** and **the Blood Cells**. Plasma is mainly composed of water, but contains different types of proteins and other chemicals such as: enzymes, glucose, fat particles, salts ,hormones , antibodies etc. It constitutes about 60% of the blood. Blood cells can be observed under a microscope on staining. The formation of blood cells occur in the bone marrow by the 'Hematopoietic stem cells'. They can be divided into 3 basic cell types:

Erythrocytes- Red Blood Cells (RBC):

As the name suggests, these red coloured cells give blood its red colour. (The word erythrocyte is from erythro-Gk.meaning red and Latin-cytos meaning cell.) 1 ml of blood contains approximately 5 million RBCs! The proportion of blood occupied by red blood cells is referred to as the hematocrit, and is normally about 45%. Mature RBCs are biconcave in shape, lack a Nucleus and many other organelles. They circulate in the system for about 120 days, carrying out their job, i.e., to supply oxygen.

This function is carried out by them most efficiently. since they are rich in an Iron-containing biomolecule called haemoglobin. Haemoglobin has high affinity for oxygen, thus binds to it and is transported from the alveoli (in lungs) to every part of the body. There is constant replenishment of RBCs in order to remove old cells that break down. (This process is carried out in organs like the liver, also producing by-products like bile pigments.) Millions of cels are released into the bloodstream from the bone marrow each day.

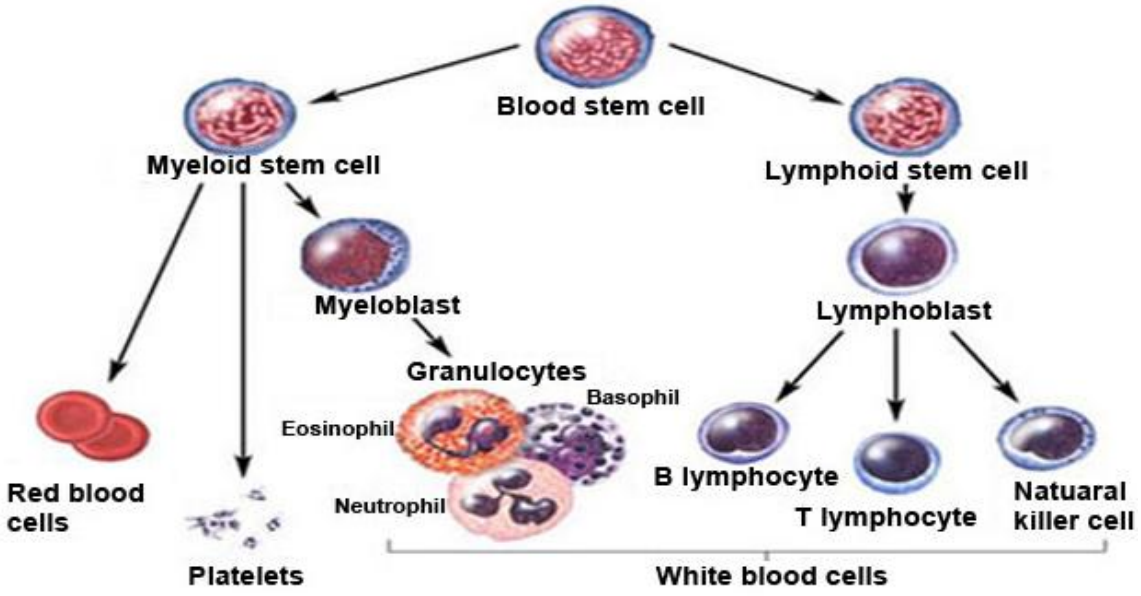
Leukocytes- White Blood Cells:

These cells are key players in our immune system. They are of different types such as neutrophils, lymphocytes, eosinophils, monocytes, basophils. Each of them have a variety of functions in our immunity. 1 ml of blood of an adult human contains about 4,000-11,000

leukocytes. Basically, they destroy and remove old or aberrant cells and clear cellular debris, as well as attack foreign substances and infectious agents (pathogenic entities).

Thrombocytes- Platelets:

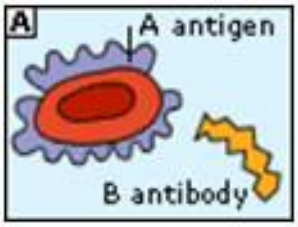
The coagulation or blood clotting process is taken care of by them. They act on clotting proteins like Fibrinogen, converting it into Fibrin. They create a mesh onto which RBCs collect and form a clot. This prevents excessive blood loss and also checks the entry of pathogens into the body. 1 ml of blood of an adult human contains about 200,000-500,000 platelets.



ABO blood grouping system:

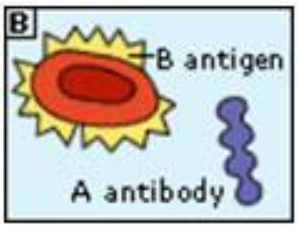
According to the AB0 blood group system there are four different kinds of blood groups: **A, B, AB and O** (null).

Blood group A



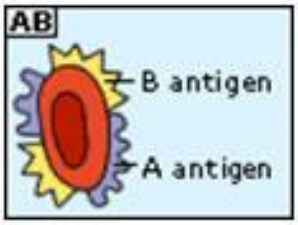
Blood group A: If you belong to the blood group A, you have A antigen on the surface of your red blood cells and B antibodies in your blood plasma

Blood group B



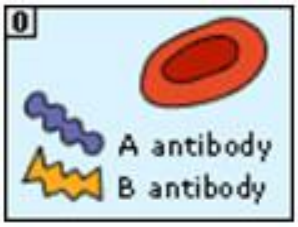
Blood group B: If you belong to the blood group B, you have B antigen on the surface of your red blood cells and A antibodies in your blood plasma

Blood group AB



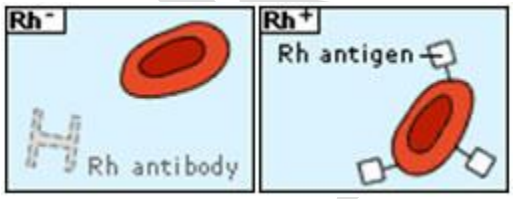
Blood group AB: If you belong to the blood group AB, you have both A and B antigens on the surface of your red blood cells but, neither A nor B antibodies in your blood plasma

Blood group O



Blood group O: If you belong to the blood group O (null), you have neither A nor B antigens on the surface of your red blood cells, but you have both A and B antibodies in your blood plasma

Rh factor

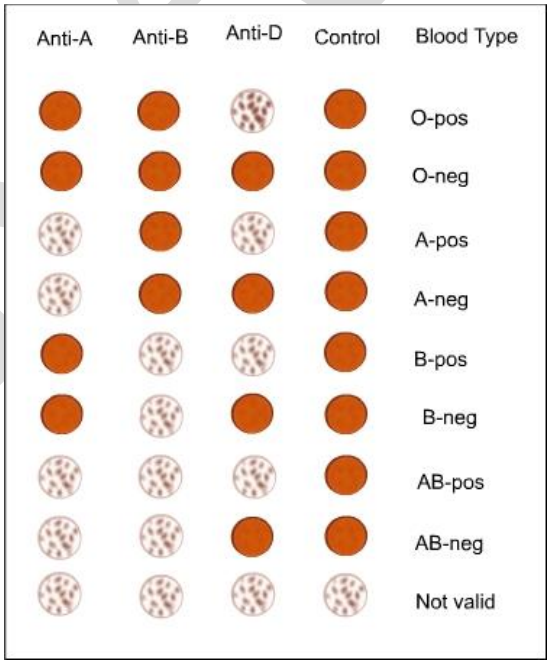


Rh (Rhesus) factor is found on the RBC's surface in most people. Like A and B, this is also an antigen and those who have it are called Rh+. Those who lack the antigen on the surface of RBCs are called Rh-. A person with Rh- blood does not have Rh antibodies naturally in the blood plasma. But a person with Rh- blood can develop Rh antibodies in the blood plasma if he or she receives blood from a person with Rh+ blood, whose Rh antigens can trigger the

production of Rh antibodies (as the immune system is triggered by the presence of an unknown antigen in the system). A person with Rh+ blood can receive blood from a person with Rh- blood without any problems.

Principle behind blood tests: ***Blood clumping or Agglutination observation.***

Compatibility between the blood groups of donor and recipient determines the success of a blood transfusion. The AB0 and Rh blood groups are looked at while conducting the test. In a diagnostic lab, Monoclonal antibodies are available for A, B and Rh antigen. Monoclonal antibody against Antigen A (also called Anti-A), comes in a small bottles with droppers; the monoclonal suspension being BLUE in colour. Anti-B comes in YELLOW colour. Anti-D (monoclonal antibody against Rh) is colourless. All the colour codes are universal standards. When the monoclonal antibodies are added one by one to wells that contain the test sample (blood from patient), if the RBCs in that particular sample carry the corresponding Antigen, clumps can be observed in the corresponding wells. A drop of blood is left without adding any of the antibodies; it is used as a control in the experiment. The monoclonal antibody bottles should be stored in a refrigerator. It is recommended to tilt the bottle a couple of times before use in order to resuspend the antibodies that have settled at the bottom of the bottle.



4. SEPERATION OF PLASMA PROTEIN

Introduction

Plasma contains over one hundred individual proteins, each with a specific set of functions and subject to specific variations in concentration under different physiologic and pathogenic conditions. They interact with virtually all body tissues or cells and they are intimately related to protein metabolism in the liver.

Plasma proteins perform a great many physiologic functions. They serve as transport molecules for lipids, hormones, vitamins, and metals. They help maintain osmotic balance and serve as enzymes, complement components protease inhibitors, or kinin precursors. They play an important role in hemostasis (as clotting factors), the regulation of cellular activity and function, and in the defense against infection (immunoglobulins). They contribute to the nitrogen needs of the body.

The key roles which plasma proteins play in bodily function, together with the relative ease of assaying them, makes their determination a valuable diagnostic tool as well as a way to monitor clinical progress.

Serum proteins have been separated into major fractions or groups for quite some time using solubility principles ("salting out" and ultracentrifugation techniques separating fractions (4.5s, 7s and 19s) based on molecular weight.

"Moving boundary" or "free electrophoresis" employing a fluid medium for protein separation was first introduced by Tiselius in 1937. He was able to resolve proteins into four major bands designated albumin, alpha, beta, and gamma globulin.

With the introduction of filter paper electrophoresis by Konig in 1937, the use of electrophoresis determinations became practical. The greater resolution of the method yielded five major fractions.

Procedures

Principle

The introduction of cellulose acetate in 1958 by Kohn made possible much more rapid analysis using a small sample volume. Other advantages of cellulose acetate over paper as a support media are its great tensile strength when wet, its almost pure and relatively uniform structure, minimal sample absorption, and its low affinity for dyes. Cellulose acetate also may be rendered almost crystal clear for easier quantitation. Agarose gels and polyacrylamide gels have become increasingly popular.

Electrophoresis is best used as a screening technique. Abnormalities may be further identified and evaluated by one of the immunological techniques.

The principle of electrophoresis is based on the fact that a charged particle placed in an electrical field will migrate toward one of the electrodes of the field depending on the

- electrical charge on the particle
- size of the particle
- strength of the electrical field
- nature of the medium used to support the particle during the migration process

The following lists would be applicable for protein electrophoresis using cellulose acetate as the support medium.

Supplies and Equipment

1. applicator
2. sample well plate
3. aligning base
4. cellulose acetate support medium
5. chamber and wicks
6. staining set
7. evaporation hood
8. densitometer

Reagents

- Barbitol Buffer pH 8.6

- Ponceau S stain
- 5% acetic acid
- Clearing solution: 125 mL of reagent grade methanol plus 50 mL DI water

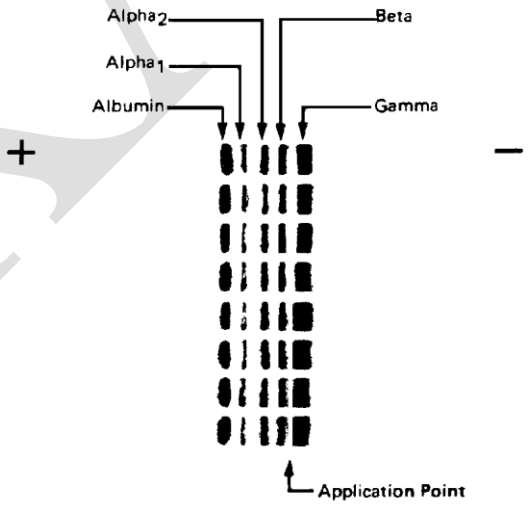
Specimens

- Fresh unhemolyzed serum is the specimen of choice. Plasma can be used but an extra peak will appear in the beta zone.
- Urine can be used, but usually concentration procedures must be done to assure the presence of protein in adequate amounts.
- CSF specimen. Concentration may be required.

Results

1. Relative Position of the Protein Fractions

The fastest moving band, and normally the most prominent, is the albumin band found closest to the anodic edge of the plate. The faint band next to this is Alpha1 Globulin, followed by Alpha2 Globulin, Beta and Gamma Globulins.



2. Calculations

Calculate from the integrator scale on the scan the relative percent of the various fractions in the sample.

Determine the total protein in the sample in grams per deciliter by a standard laboratory method.

Multiply the relative percent of each band times the total protein value. Divide this by 100. The result is the value in grams per deciliter for the various fraction.

Example: Total Protein = 8.2 gms/dL

Albumin = 59% (59/100) x 8.2 = 4.8 gms/dL

$Alpha_1$ = 5% (5/100) x 8.2 = 0.3 gms/dL

$Alpha_2$ = 8% (8/100) x 8.2 = 0.7 gms/dL

Beta = 10% (10/100) x 8.2 = 0.8 gms/d L

Gamma = 19% (19/100) x 8.2 = 1.6 gms/dL

Relative percent and grams per deciliter may be computed automatically using a computer accessory with the densitometer.

Expected values

	Mean (gm/dL)	S.D.	Range (+2 S.D.)
Albumin	4.27	0.32	3.63-4.91 gm/dL
$Alpha_1$	0.23	0.06	0.11-0.35 gm/dL
$Alpha_2$	0.91	0.13	0.65-1.17 gm/dL
Beta	1.00	0.13	0.74-1.26 gm/dL
Gamma	16	0.29	0.58-1.74 gm/dL

5. DETERMINATION OF TOTAL IRON BINDING CAPACITY

Summary and Principle

Most procedures for serum iron are based upon its release from combination, in the ferric form, with transferrin, the transport protein which binds iron at body pH. The released iron, after reducion to the ferrous state, is combined with one of several reagents to form a colored complex, which is quantitated colormetrically. Since only about one-third of the serum iron is normally bound to the gobulin, transferrin, the "unsaturated iron-binding capacity" (UIBC), or the additional quantity of iron that can be bound by the serum, is determined by saturating the transferrin with a known excess of iron. Unused iron is estimated by the same technique and UIBC calculated by the difference. The method presented is a modification of that reported by Persijn *et al*, using the chromogenic compound, Ferrozine. In addition to iron, copper is the only other trace metal found in serum reported to form a colored complex with Ferrozine. Neocuproine is therefore used in the color reagent to prevent copper interference. Iron is released from its combination with transferrin in acid medium, reduced to the ferrous form by hydroxylamine and reacted with Ferrozine to form a violet colored complex which is measured at 560 nm. A separate technique for serum unsaturated iron-binding capacity (UIBC) involves addition of a known excess of ferrous ions, which saturate available transferrin iron-binding sites. Excess (unbound) iron is then quantitated as described above, with UIBC being the difference in iron concentration between that added and that determined in the remaining excess. It follows that serum total iron-binding capacity (TIBC) is the sum of iron and UIBC

Reagents

Iron Color Reagent, Cat. No. FT715a 0371

A solution containing:

Ferrozine……7.8 mmol/L

Neocuproine…….14.4 mmol/L

Hydroxylamine Hydochloride ……220 mmol/L

Iron HA Buffer, Cat.No. F T715b 0 372

A solution containing:

Hydroxylamine Hydochloride……220 mmol/L in an acetate buffer. Also contains surfactants.

Iron TRIS Buffer, Cat. No. FT715c 0373

A solution containing: TRIS .....500 mmol/L in a buffer solution. Also contains surfactants.

Iron Standard (500 µg/dL), Cat. No. FT715d

Aqueous solution containing 500 µg/dL of ferric iron.

Reagent Preparation: The reagents and standard are supplied ready-touse.

Reagent Storage and Stability: Store HA buffer, TRIS buffer and standard at room temperature. Iron Ferrozine Color reagent should be stored at 2-8°C and protected from light. All reagents are stable until the expiration date on their respective labels.

Materials Required

Spectrophotometer, capable of absorbance readings at 550-570nm. Deionized or distilled water (Iron-free), Accurate pipetting devices, Cuvets, Interval Timer, Test tubes, Incubator block or Water bath (37°C)

Specimen Collection and Preparation: Serum is the preferred specimen since it avoids possible iron contamination from inorganic anticoagulants. Collection tubesshould be iron free and serum should be separated as soon asthe clot hasformed.

Sample Stability: Iron in serum is reported to be stable 4 days at room temperature and approximately 7 days at 2-8°C.

Interfering Substances: Hemolytic sera should be avoided. Young, et al list interfering substances which can affect the accuracy of iron values obtained. Bilirubin concentrations up to 15 mg/dL and copper concentrations up to 500 µg/dL will not interfere. This iron test is very sensitive to contamination, therefore, any glassware used must be iron free. We recommend using disposable laboratory materials when performing this test.

Test Procedure - Serum Iron

1. Pipet into cuvets the following volumes (mL) and mix well:
2. Incubate for 1 minute at room temperature.
3. Measure the absorbance (A1) of the Standard, Controls, and Sample against the Reagent Blank at 560 nm.
4. Add 0.1 mL (100 µL) Iron Color Reagent to each cuvet, mix well and incubate for 10 minutes at 37°C.
5. Measure the absorbance (A2) of the Standard, Controls, and Sample against the Reagent Blank at 560 nm. Read within 30 minutes.

	Reagent Blank (RB)	Standard (S)	Unknown (U)
Iron HA Buffer	2.3	2.3	2.3
Distilled Water	0.30	-	-
Standard	-	0.30	-
Sample (or controls)	-	-	0.30

Test Procedure - Iron Binding Capacity

1. Pipet into cuvets the following volumes (mL) and mix well:
2. Incubate for 1 minute at room temperature.
3. Measure the absorbance (A1) of the Standard, Controls, and Sample against the Reagent Blank at 560 nm.

4. Add 0.1 mL (100 µL) Iron Color Reagent to each cuvet, mix well and incubate for 10 minutes at 37°C.

5. Measure the absorbance (A2) of the Standard, Controls, and Sample against the Reagent Blank at 560 nm. Read within 30 minutes.

	Reagent Blank (RB)	Standard (S)	Unknown (U)
TRIS Buffer	2.2	2.2	2.2
Distilled Water	0.60	0.30	-
Standard	-	0.30	0.30
Sample (or controls)	-	-	0.30

Quality Control: Two levels of control material with known Iron content, determined by this method or an Iron Ferrozine procedure should be analyzed each day of testing

RESULTS

1) Values are derived by the following equation:

$$\text{Serum Iron (µg/dL)} = \frac{A^2\text{-}A^1 \text{ Unknown}}{A^2\text{-}A^1 \text{ Standard}} \times 500$$

Iron Binding Capacity

$$\text{a. Excess Iron (µg/dL)} = \frac{A^2\text{-}A^1 \text{ Unknown}}{A^2\text{-}A^1 \text{ Standard}} \times 500$$

b. UIBC (µg/dL) = 500 (the total iron added in µg/dL) - ExcessIron (µg/dL)

c. TIBC (µg/dL) = Serum Iron (µg/dL) + UIBC (µg/dL)

NOTE: Samples having Iron values greater than 1000 µg/dL are diluted 1:2 (1+1) with distilled water, the assay repeated and results multiplied by the dilution factor 2.

Expected Values

Serum Iron	**Serum TIBC**
Male: 65 - 170 µg/dL	250 - 450 µg/dL
Female: 50 - 170 µg/dL	250 - 450 µg/dL

Child: 50 - 120 µg/dL	250 - 400 µg/dL
Infant: 40 - 100 µg/dL	100 - 400 µg/dL
Newborn:100 - 250 µg/dL	-

For conversion to SI Units: µg/dL x 0.179 = µmol/L. It is recommended that each laboratory establish its own range of expected values, since differences exist between instruments, laboratories and local populations.

6. PULMONARY FUNCTION TESTS

Pulmonary Function Tests:

- Spirometry
- Lung Volumes
- Diffusion Capacity
- Maximal Voluntary Ventilation (MVV)
- Maximal Inspiratory Pressure (Pi max)
- Maximal Expiratory Pressure (Pe max)
- Arterial Blood Gas (ABG)
- Walking Oxymetry
- Bronchochallenge Tests

INDICATIONS:

Pulmonary Evaluation:

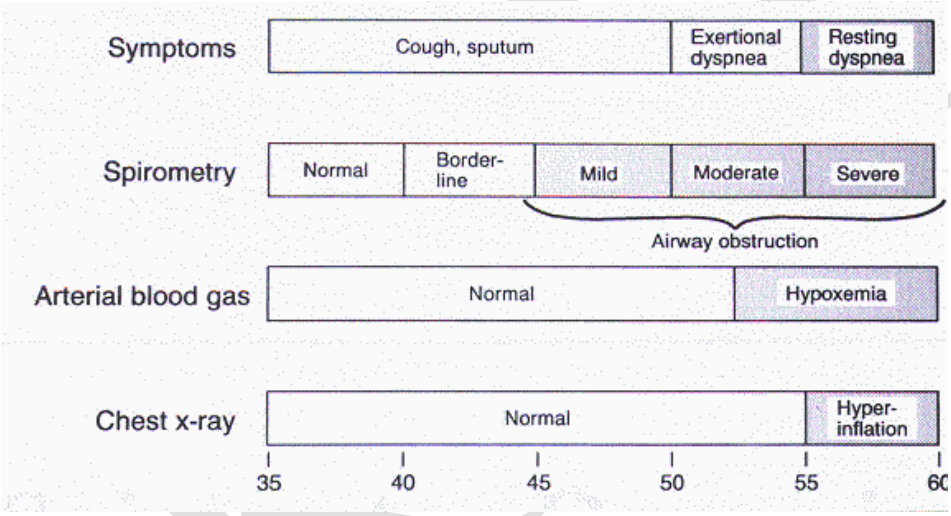
- Presence of impairment

- Type of Pulmonary dysfunction
- Quantification of impairment in known disease
- Monitor the progression of known disease
- Monitor the treatment response of known disease

Preoperative Assessment:

- Estimate the risk for postoperative complications (operability)
- Tolerance for lung resection (resectability)

Disability Evaluation



LUNG VOLUMES & CAPACITIES:

Tidal Volume (VT): The volume of air entering the nose or mouth per breath (500 ml).

Residual Volume (RV): The volume of air left in the lungs after a maximal forced expiration (1.5L).

Expiratory Reserve Volume (ERV): The volume of air that is expelled from the lung during a maximal forced expiration that starts at the end of normal tidal expiration (1.5L).

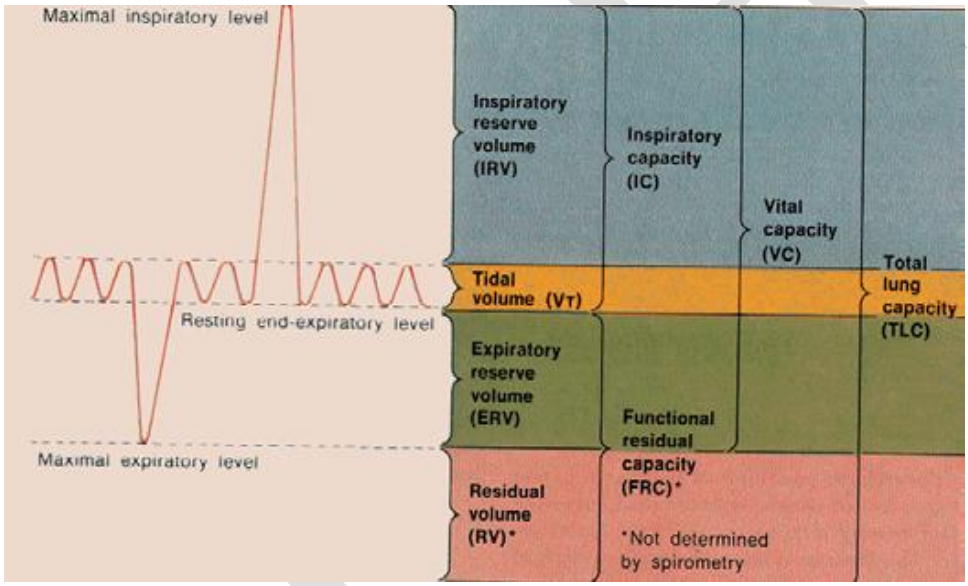
Inspiratory Reserve Volume (IRV): The volume of air that is inhaled into the lung during a maximal forced inspiration starting at the end of a normal tidal inspiration (2.5L).

Functional Residual Capacity (FRC): the volume of air remaining in the lungs at the end of a normal tidal expiration (3 L).

Inspiratory Capacity (IC): The volume of air that is inhaled into the lung during a maximal forced inspiration effort that begins at the end of a normal tidal expiration (VT+IRV=3L).

Vital Capacity (VC): The volume of air that is expelled from the lung during a maximal forced expiration effort starting after a maximal forced inspiration (4.5L).

Total Lung Capacity (TLC): The volume of air that is inhaled into the lung after a maximal inspiration effort (5-6 L).



Spirometry:

Measures the lung volume change during forced breathing maneuvers:

Forced vital capacity (FVC)

Forced expiratory volume in the first second (FEV-1)

Obstructive Lung Diseases:

- Emphysema & Chronic Bronchitis
- Cystic Fibrosis
- Asthma
- Bronchiectasis
- Some Interstitial Lung Disease: (combined)

Pre and Post Bronchodilator Spirometry:

 Goal: to evaluate the reversibility of the airway obstruction.

 Technique : repeat the spirometry after the treatment with bronchodilator.

 Criteria: required two criteria at the same time:

200 ml and 12% (both) change in either FEV-1 or FVC

 Patient with Reversible Airway Obstruction responds to treatment with:

- **Bronchodilator (short & long acting)**
- **Steroid inhaler**

Spirometry:

 Detects the obstructive lesions in the major airways.

 Characterizes the lesion:

A-Location of the lesion:

- Intrathoracic
- Extrathoracic

B-Behavior of the lesion during rapid inspiration and expiration:

- Fixed
- Variable

 Variable Intrathoracic Lesion:

Examples: Tracheomalacia & Intratracheal tumor.

 Variable Extrathoracic Lesion:

Examples: Vocal cord paralysis, Goiter, and Tumor

 Intra or Extrathoracic Fixed Lesion:

Examples: Tracheal stenosis & surgical stricture, and

7. SEPARATION OF ISOENZYMES BY ELECTROPHORESIS

THE ISOENZYME PROFILE OF LACTATE DEHYDROGENASE

Assaying the serum levels of lactate dehydrogenase (LDH) activity combined with the results of other clinically important enzyme assays (GOT, SGOT, CRK) provides an excellent tool for the physician to make decisions in cases accompanied by tissue damage or with a change in membrane permeability. In a myocardial infarction within 12 hours the LDH level of serum begins to rise, reaching its maximal value after 48-72 hours. The enhanced enzyme level is proportional to the damage of the heart muscle tissue and in serious cases its elevation could even be three-fold. The elevation of serum LDH activity might accompany other diseases (e.g. anemias, tumors, liver diseases) as well, so it is important to know from what tissue the LDH was released into the bloodstream. The characterization of the LDH isoenzyme profile helps to solve this problem.

The LDH molecule itself is a tetramer, composed of two types of subunits encoded by different genes. From the combination of the H(heart) and M(muscle) type of subunits in tetramers, five variants can be obtained: H4(LDH1), H3M1(LDH2), H2M2(LDH3), H3M3(LDH4) and M4(LDH5).

While LDH1 and LDH2 can be found mainly in the heart muscle and in erythrocytes, liver and smooth

muscle cells contain mostly the LDH5 isoenzyme.

All LDH isoenzymes catalyze the following reversible reaction:

Pyruvate + NADH+H+ == Lactate + NAD+

The different isoenzymes show different enzyme kinetic behaviour due to their different amino acid compositions, which also provides the possibility to separate the isoenzymes by electrophoretic methods.

I. Enzyme kinetic methods to characterize the LDH isoenzymes.

A. Heat stability

The serum samples are incubated for 30 minutes at 57□ C and 65□ C in the presence of NADH. The LDH activities of the cooled samples are determined following each heat-treatment. A control sample held at room temperature presents the total amount of LDH activity. While the difference in the enzymatic activities between the control and the 57□ C treated sample gives the heat labile LDH activity, the enzyme activity measured from the 65□ C treated samples provides the heat stable LDH activity, which is elevated in myocardial infarction.

B. Substrate concentration dependence

This method is based on the fact that while the LDH5 isoenzyme is maximally active in the presence of 250 mM lactate, the optimal lactate concentration for the LDH1 isoenzyme is 10 mM, and there is a 50 % inhibition of LDH1 activity when it is assayed using 250 mM lactate as a substrate. By measuring LDH activity using both 10 and

250 mM lactate concentrations, the calculated ratio of the two activities helps to judge whether the sample contains LDH1 or LDH5 isoenzyme.

C. Substrate specificity

The LDH isoenzymes also show different substrate specificities towards pyruvate and 2-oxobutyrate as substrates. While LDH1 shows a higher specific activity when 2-oxobutyrate is used, the LDH5 isoenzyme is more active with pyruvate. The ratio of LDH activities measured in serum samples using the two substrates (HBDH/LDH) varies between 0.63-0.81 in normal cases. Ratios higher than 0.83 are characteristic for a myocardial infarction. Liver diseases are diagnosed when the ratio of enzyme activities is lower than 0.61.

II. Electrophoretic method for the separation of LDH isoenzymes:

Due to their different amino acid compositions the LDH isoenzymes can be separated by electrophoretic methods (agarose, cellulose-acetate,polyacrylamide can be used as a matrix). In

an electric field at pH 8.8 the LDH1 migrates at the fastest rate towards the anode, while the LDH5 is the slowest isoenzyme. After electrophoresis the LDH isoenzymes can be visualized by an activity staining process where the product of the enzymatic reaction is a water-insoluble dye precipitating in the gel where the LDH enzymes are located.

Practical part

I. We use method "C" (substrate specificity) to characterize the kinetic differences of LDH isoenzymes :

Assaying LDH activity is based on the fact that NADH has an absorbance maximum around 340 nm which cannot be found in the spectra of NAD. So when the enzyme assay is carried out using oxo-substrate and NADH, the time dependent decrease in the absorbance of the samples measured at 340 nm is proportional to the LDH activity (340=6220 M-1cm-1).

Solutions used:

- 50 mM phosphate buffer, pH7.5
- 8 mM NADH dissolved in phosphate buffer
- 10 mM pyruvate dissolved in phosphate buffer
- 100 mM 2-oxobutyrate dissolved in phosphate buffer
- LDH1 enzyme solution
- LDH5 enzyme solution

Measure directly into photometric cuvettes

	1	**2**	**3**	**4**	**5**
Phosphate buffer µl	1000	910	910	910	910
NADH µl	-	30	30	30	30
LDH1 µl		30	30	-	-
LDH5 µl	-	-	-	30	30
pyruvate µl	-	30	-	30	-

2-oxobutyrate µl	-	-	30	-	30

Use sample 1 as a blank. Start each reaction separately adding pyruvate or 2-oxobutyrate, preincubate for 2 minutes and then register the at 30 s intervals for 5 minutes. The enzyme activities are calculated from the linear part of the curves obtained by plotting the absorbance values versus time.

The HBDH/LDH ratios are calculated for both LDH1 and LDH5 enzymes

$$\text{Calculation: Activity (U/L)} = \frac{\Delta A \times V \times 1000}{d \times t \times 6.22 \times v}$$

where V is the total volume of the reaction mixture in cm3 and v is the volume of added enzyme in cm3, ΔA is the change in absorbance for time t (min) and d is the cuvette size (1 cm).

II. PAGE separation of LDH isoenzymes

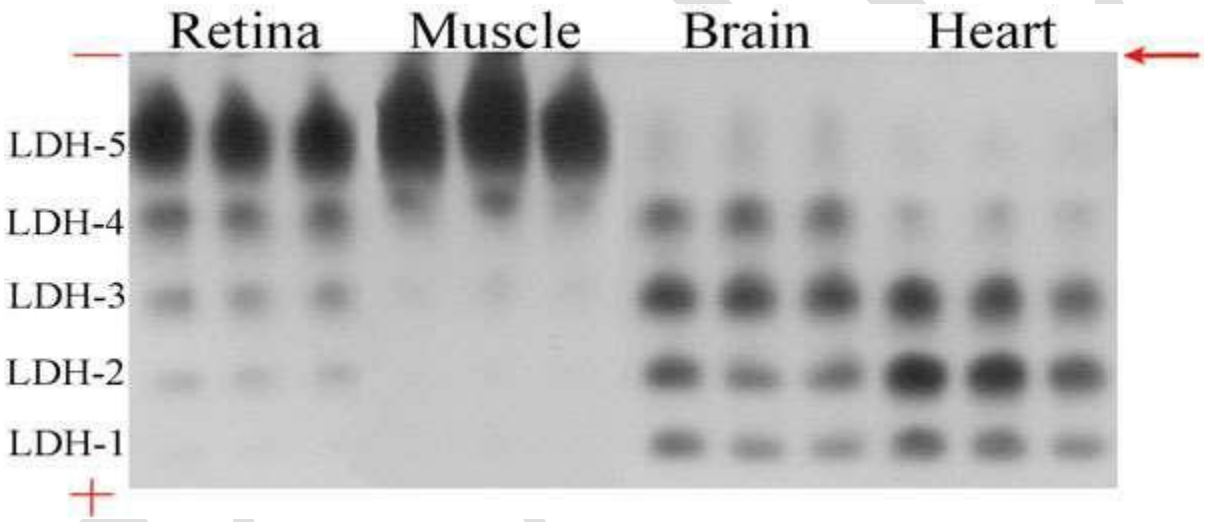
Materials:

- pre-cast 7.5% polyacrylamide gel slabs
- tank buffer (2.4 g Tris base, 11.6 g glycine/liter)
- samples made from liver, heart muscle, and kidney in sample loading buffer
- 1 M Tris- HCl, pH 8.0
- NAD, 10 mM
- tetrazolium-blue, 1 mg/ml
- phenazine-methosulphate, 1.6 mg/ml
- 1 M Na-lactate

In our experiments pre-cast 6.5% polyacrylamide slab gels will be used. The sample wells are rinsed out with the tank buffer and the gels are placed into the electrophoretic unit. Load 5 µl of each sample into different wells of the gel. Pour tank buffer into the reservoirs and connect the

electric cables. The positive pole is at the bottom. Turn the power source on and set the current at 12 mA. Run the electrophoresis for 90 minutes. Turn off the powerseparate the two plastic plates by prying them apart with a spatula and place the gel into the developing chamber which already contains the developer solution (H_2O 18.4 ml, 1 M Tris 4 ml, tetrazolium-blue 12 ml, phenazine-methosulphate 4 ml, Na-lactate 4 ml and NAD 1.3 ml). Incubate at 40 ^oC to develop color reaction for 20 minutes. In the color reaction NAD and lactate serve as substrates, phenazine-methosulphate is the primary electron acceptor and tetrazolium-blue is the final electron acceptor. Wash the gel with water

Electrophoretic profile of LDH isoenzymes obtained from different rat tissues



8. HISTOLOGY OF CONNECTIVE TISSUE, LIVER AND/ BRAIN - PERMANENT SLIDES

Introduction

Connective tissue is a term used to describe the tissue of mesodermal origin that that forms a matrix beneath the epithelial layer and is a connecting or supporting framework for most of the organs of the body. This lab will focus on the so-called connective tissue proper and cartilage; the next lab will focus on bone.

Overview of Connective Tissue

In contrast to epithelia, connective tissue is sparsely populated by cells and contains an extensive extracellular matrix consisting of protein fibers, glycoproteins, and proteoglycans. The function of this type of tissue is to provide structural and mechanical support for other tissues, and to mediate the exchange of nutrients and waste between the circulation and other tissues. These tissues have two principal components, an extracellular matrix and a variety of support cells. These two components will be the focus of this lab.

Most frequently, the different types of connective tissues are specified by their content of three distinguishing types of extracellular fibers: collagenous fibers, elastic fibers, and reticular fibers.

Ground Substance

The ground substance is an aqueous gel of glycoproteins and proteoglycans that occupies the space between cellular and fibrillar elements of the connective tissue. It is characterized by a gel-like viscous consistency and is polyanionic. The characteristics of the ground substance determine the permeability of the connective tissue layer to solutes and proteins.

Collagenous Fibers

Collagenous fibers consist of types I, II, or III collagen and are present in all types of connective tissue. Collagenous connective tissue is divided into two types, based upon the ratio of collagen fibers to ground substance:

- Loose (areolar connective tissue) is the most abundant form of collagenous connective tissue. It occurs in small, elongated bundles separated by regions that contain ground substance.
- Dense connective tissue is enriched in collagen fibers with little ground substance. If the closely packed bundles of fibers are located in one direction, it is called regular; if oriented in multiple directions, it is referred to as irregular. An example of regular dense connective tissue is that of tendons; an example of irregular dense connective tissue is that of the dermis.

Reticular Fibers

Reticular fibers are composed of type III collagen. Unlike the thick and coarse collagenous fibers, reticular fibers form a thin reticular network. Such networks are widespread among different tissues and form supporting frameworks in the liver, lymphoid organs, capillary endothelia, and muscle fibers.

Elastic Fibers

Elastic fibers contain the protein elastin, which co-polymerizes with the protein fibrillin. These fibers are often organized into lamellar sheets, as in the walls of arteries. Dense, regular, elastic tissue characterizes ligaments. Elastic fibers are stretchable because they are normally disorganized – stretching these fibers makes them take on an organized structure.

Cells of the Connective Tissue Proper

Although the connective tissue has a lower density of cells than the other tissues you will study this year, the cells of these tissues are extremely important.

Fibroblasts are by far the most common native cell type of connective tissue. The fibroblast synthesizes the collagen and ground substance of the extracellular matrix. These cells make a large amount of protein that they secrete to build the connective tissue layer. Some fibroblasts have a contractile function; these are called myofibroblasts.

Chondrocytes and osteocytes form the extracellular matrix of cartilage and bone. More details and chondrocytes can be found later in this laboratory; osteocytes will be covered in the Laboratory on Bone.

The macrophage is the connective tissue representative of the reticuloendothelial, or mononuclear phagocyte, system. This system consists of a number of tissue-specific, mobile, phagocytic cells that descend from monocytes - these include the Kupffer cells of the liver, the alveolar macrophages of the lung, the microglia of the central nervous system, and the reticular cells of the spleen. You will encounter each of these later in the course; for now, make sure you recognize that they all descend from monocytes, and that the macrophage is the connective tissue

version. Macrophages are indistinguishable from fibroblasts, but can be recognized when they internalize large amounts of visible tracer substances like dyes or carbon particles. Macrophages phagocytose foreign material in the connective tissue layer and also play an important role as antigen presenting cells, a function that you will learn more about in Immunobiology.

Mast cells are granulated cells typically found in connective tissue. These cells mediate immune responses to foreign particles. In particular, they release large amounts of histamine and enzymes in response to antigen recognition. This degranulation process is protective when foreign organisms invade the body, but is also the cause of many allergic reactions.

White fat cells are specialized for the storage of triglyceride, and occur singly or in small groups scattered throughout the loose connective tissue. They are especially common along smaller blood vessels. When fat cells have accumulated in such abundance that they crowd out or replace cellular and fibrous elements, the accumulation is termed adipose tissue. These cells can grow up to 100 microns and usually contain once centrally located vacuole of lipid - the cytoplasm forms a circular ring around this vacuole, and the nucleus is compressed and displaced to the side. The function of white fat is to serve as an energy source and thermal insulator.

Brown fat cells are highly specialized for temperature regulation. These cells are abundant in newborns and hibernating mammals, but are rare in adults. They have numerous, smaller lipid droplets and a large number of mitochondria, whose cytochromes impart the brown color of the tissue. The electron transport chain of these mitochondria is disrupted by an uncoupling protein, which causes the dissipation of the mitochondrial hydrogen ion gradient without ATP production. This generates heat.

Cartilage

Cartilage is a specialized form of connective tissue produced by differentiated fibroblast-like cells called chondrocytes. It is characterized by a prominent extracellular matrix consisting of various proportions of connective tissue fibers embedded in a gel-like matrix. Chondrocytes are located within lacunae in the matrix that they have built around themselves. Individual lacunae

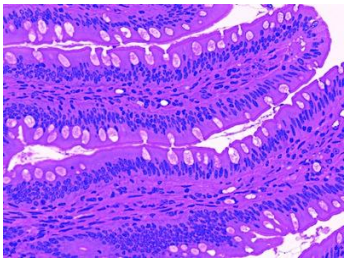
may contain multiple cells deriving from a common progenitor. Lacunae are separated from one another as a result of the secretory activity of the chondrocytes.

A highly fibrous, organized, dense connective tissue capsule known as the perichondrium surrounds cartilage. The fibroblast-like cells of this layer have chondrogenic potentiality, and are responsible for the enlargement of cartilage plates by appositional growth. Appositional growth involves cell division, differentiation, and secretion of new extracellular matrix, thereby contributing mass and new cells at the cartilage surface. It is in contrast to interstitial growth, in which new matrix is deposited within mature cartilage.

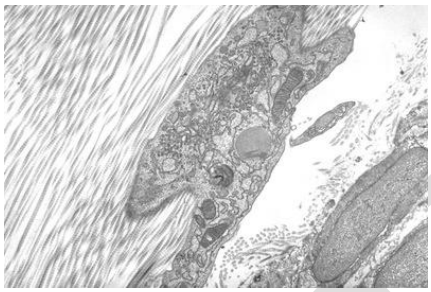
Three kinds of cartilage are classified according to the abundance of certain fibers and the characteristics of their matrix:

- Hyaline cartilage has a matrix composed of type II collagen and chondromucoprotein, a copolymer of chondroitin sulfates A and C with protein. Its high concentration of negatively-charged sulfate groups makes it appear intensely basophilic under H&E. This cartilage is found in the nose, tracheal rings, and where the ribs join the sternum.
- Fibrocartilage is distinguished by its high content and orderly arrangement of type I collagen fibers. It is typically located in regions where tendons attach to bones, the intervertebral discs, and the pubic symphysis.
- Elastic cartilage is characterized by the presence of abundant elastic fibers and is quite cellular. It is made up of type II collagen and is located in the auricle of the ear and the epiglottis.

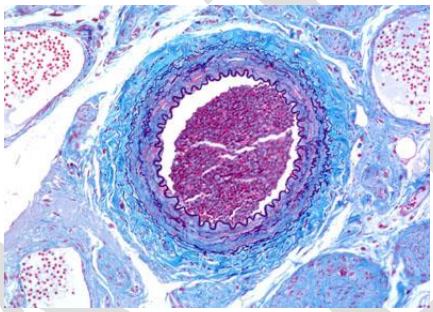
CONNECTIVE TISSUE



This is loose connective tissue. It provides metabolic support (blood vessels), immune support (white blood cells) and structural support.

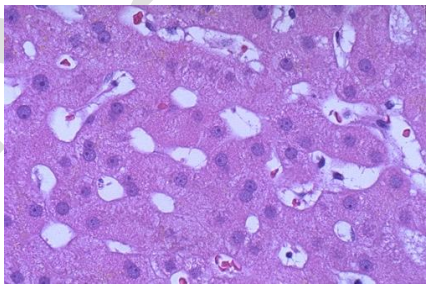


Fibroblast

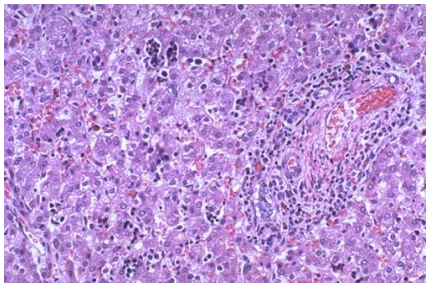


Elastic fibers. Allow wall of artery to expand and contract.

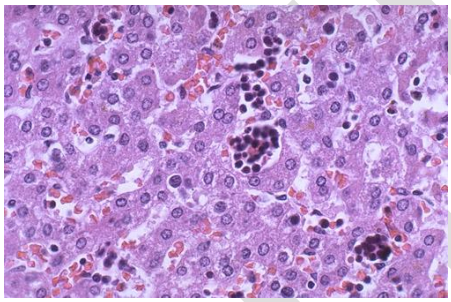
LIVER



At high power liver demonstrates a pattern of **cords of cells** with a nucleus and granular pink cytoplasm and some light brown lipochrome pigment. The sinusoids have scattered **Kupffer cells**, which have a macrophage-like function.

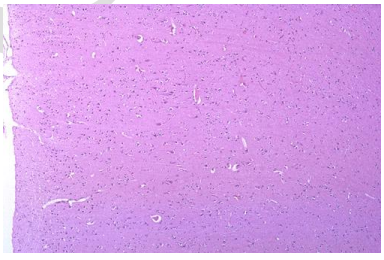


This is normal fetal liver at medium power demonstrating that at term there are **islands of extramedullary hematopoiesis** along with the liver cords. Note the **triad** to the right



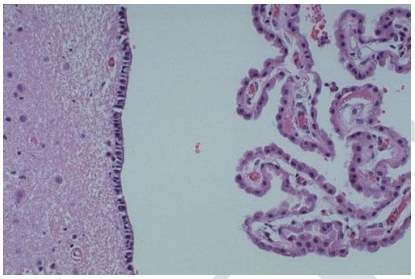
This is normal fetal liver at high power with **islands of extramedullary hematopoiesis** (mainly RBC precursors) along with the liver cords. In addition to albumin, the fetal liver produces alpha-fetoprotein.

BRAIN

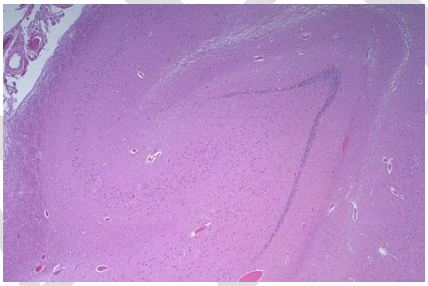


The cortex (gray matter) of the cerebral hemispheres is known as neocortex and has six indistinct layers. Beneath the pia-arachnoid there is an outer plexiform layer with nerve cells arranged horizontally. Next is the outer granular layer containing small pyramidal neurons. Next is the outer pyramidal cell layer with medium-sized pyramidal neurons. Below this is the inner

granular layer of larger pyramidal neurons. Beneath this is the inner pyramidal layer of larger pyramidal neurons. The innermost cortical layer is the polymorphous layer that lacks pyramidal cells. Beneath the cortex is the white matter.



Normal choroid plexus, with an **epithelial layer** around a **stroma with prominent blood vessels**. This is where the cerebrospinal fluid is formed. The **ependymal cells** lining the ventricle are present at the left.



The normal appearance of the hippocampus is seen here at low magnification. Hippocampus consists of "paleocortex" with three layers.