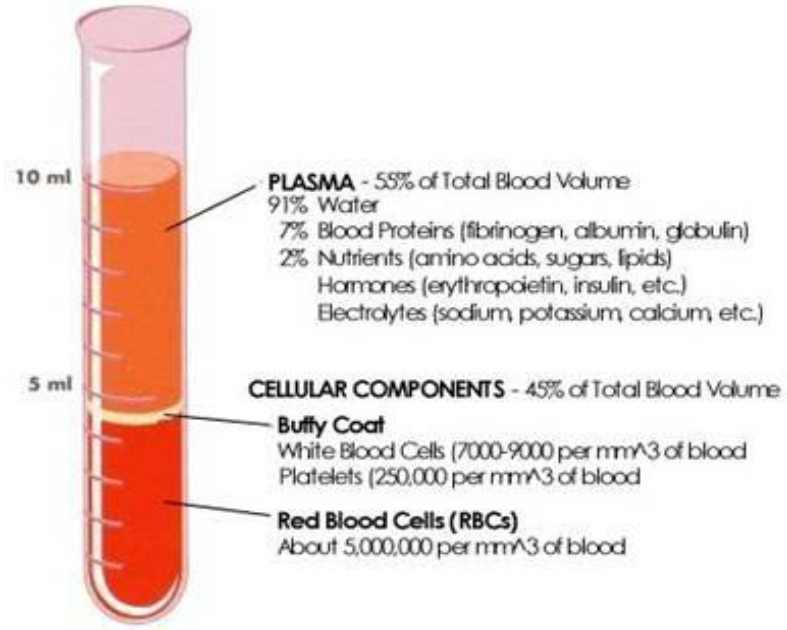
Practical lab 13
Hematocrit. Hemoglobin – identification, spectroscopy, crystalografic method (Teichman crystals)

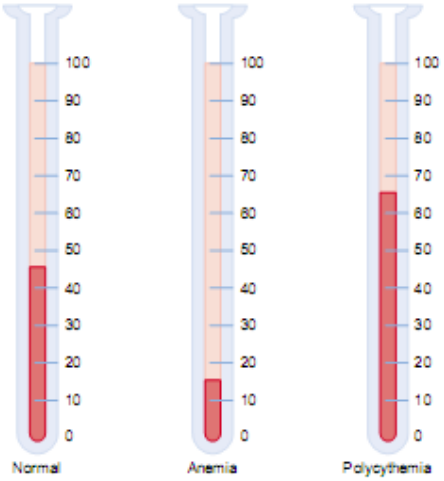
Hematocrit

Definition: The **hematocrit** (**Ht** or **HCT**) or **packed cell volume** (**PCV**) is the proportion of blood volume that is occupied by red blood cells.
Method and principle: blood cells are heavier than plasma, so they can be separated according to mass by centrifugation. Blood is sampled on anticoagulant and placed in a hematocrit tube of 100 mm in height. The blood is then centrifuged at 3000 rpm for 5-10 min, the result being (as you can see in the figure below) a red column, represented by packed red blood cells, the height of which represents the hematocrit and a transparent column represented by plasma. The buffy coat in-between represents white blood cells and platelets.
Reading: read the height of the red column on the tube; because the height of the tube is 100 mm, the value read on the tube represents a percentage value.



Normal values:

- Women – 38% (41% ± 6%)
- Men – 42% (46.5% ± 6)
- New-born -50%



A normal hematocrit (HCT) value defines the state of normocytemia, a smaller value – oligocytemia, a greater value – policytemia..

Variations of hematocrit:

1. With red cell count: - decrease in anemia, increase in poliglobulia
2. With place of sampling : venous blood has higher hematocrit than arterial blood, but lower than splenic blood.
3. With hydration status – hemodilution vs hemoconcentration
4. With erythropoiesis/ erythrolysis ratio

Amongst the erythrocyte parameters : hematocrit, hemoglobin, red cell count, we can establish the diagnosis of anemia. The type of anemia is established by taking into account the erythrocyte indices (MCV, MCH, MCHC)

MCV =mean cell volume of one RBC= HCT/red cell count = 80 -100fL
MCH= mean cell hemoglobin in one RBC= Hb concentration/ red cell count= 27- 32 pg
MCHC= mean hemoglobin concentration in all RBCs =hemoglobin conc/HCT= 320-360 g/L

With regard to these indices we can classify anemias into the following categories:

I. <u>normocromic, normocitic anemia</u>:
   - acute hemoraging
   - aplastic/hypoplastic anemia
   - leucemya
   - renal/ hepatic disease

II. <u>hipocromic, microcytic anemia:</u>
   - Iron deficient anemia
   - Cronic loss of blood
   - Hemolytic anemia
   - Cronic inflamation anemia

III. <u>normocromic, megalocytic anemia</u>:
   - Anemie through B12deficit
   - Anemie through folic acid deficit

HEMOGLOBIN

Hemoglobin (Hb) – major function in transporting blood gases.
Hemoglobin: a cromoprotein
Molecular weight = 68000da
has 4 subunits, each having 17000da :
- HEM (prosthetic group):
  - Fe2+
  - tetrapirolic nucleus (same as in chlorophyll and B12 Vitamin)
- GLOBIN (polypeptide)

α, β, γ, δ = types of polypeptide chains.

<u>Hb types</u>

	HbA	HbF	HbA_2
Structure	$\alpha_2\beta_2$	$\alpha_2\gamma_2$	$\alpha_2\delta_2$
%	96-98	0,5-0,8	1,5-3,2

The conversion from HbF to HbA occurs 3-6 month after birth.
65% of Hb is synthesized in erythroblasts
35% of Hb is synthesized in reticulocytes.

Hem and Hb synthesis

I. Krebs cycle -> succinil coenzyme A (SucCoA)
II. SucCoA + glicin -> δ amino-levulinic acid (ALA)
III. 2 ALA ->Porfobilinogen (pirolic nucleus)
IV. 4 Pirols -> Protoporfirine IX (PP IX)
V. PP IX + Fe^{++} -> HEM (Fe^{++} is inserted in the center of PP IX)
VI. 4 Hem + polipeptide (globin) -> hemoglobin chain (α or β)
VII. 2 α chains + 2 β chains -> Hb A

Globin synthesis

- dimeric structure
- 2 polypeptide pairs

There are:

- 2 α chains having 141 aa
- 2 β chains having 146 aa.

Changing the sequence of aa leads to - type γ (146 aa, 37 different from type β)
- type δ (146 aa, 10 different)
- tip ε

The protein has an α helix spatial structure. The synthesis takes place in ribosomes, where the polypeptide α, β, γ, δ chains are formed.
The 2 dimmers (α_2 & β_2) form together with hem HbA. There are also A_1, A_2, A_3 types.
HbA_3 can be found in elder, having modified β chains.
HbF (fetal) has a high binding capacity for oxygen.
HbU (embryo type) – Gowers – has α_2, ε_2 chains.

Iron metabolism

Total iron amount in the body = 4±0,5g.
It is divided in the following compartments:

- hemoglobin – 67%
- iron stored as feritine and hemosiderin – 27%
- myoglobin –3,5%
- oxidative enzymes – 0,2%
- plasma

Iron absorption

Minimum requirements:

- newborn: 10 mg/24h
- children: 5 mg/24h
- young women: 20mg/24h
- pregnant women: 30mg/24h
- women after menopause: 10mg/24h
- men: 10mg/24h

Identification of hemoglobin

Principle:
comparing clorhemine obtained from the tested blood with the Sahli hemoglobinometer clorhemine standard solution.

Necessary material:

- Sahli hemoglobinometer consisting in:
  - 2 lateral tubes with the standard solution – brown color.
  - 1 central tube used for the test – it has a gradation from 10 to 140 Sahli units (100^{th} degree equals 16g Hb%)

- annexed: a capillary pipette for blood maneuvering (with a 20 μl sign) + 1 distilled water pipette + 1 glass stirring stick.
- HCl N/10 solution
- Distilled water

Protocol:
- Put in the test tube HCl N/10 until 10^{th} division.
- Add with the capillary pipette 20 μl blood.
- Brownish Clorhemine results.
- Dilute the resulted solution with distilled water till it gets the same color with the standard probe.
- Read the digits where the level has raised – meaning the HB amount in Sahli units.
- In order to transform it in grams, calculate as follows:

100.....16 g
read value…….....X g

X = 16 x read value / 100

Normal values:
men – 15-17g%
women – 13,5-15,5g%
newborn – 17-20g.

Hb Spectroscopy

Principle:
Spectroscopy is based on the difference between the absorbtion spectra of hemoglobin and its related compounds.

Necessary material:
- spectroscope with direct vision
- tubes
- hemoglobin & derived solutions
- crystals of sodium hydrosulfite
- saturated potassium fericianide.

Protocol:
- **oxyhemoglobin spectra** (OHb)

- diluted blood solution (2-3 drops in 20 ml distilled water)
- in contact with the air the blood turns bright red (Hb gets saturated with oxygen.)
- put the tube in front of the spectroscope
- the oxyhemoglobin absorbtion spectra has 2 bands:
  - a wide yellow one (577 nm)
  - a narrow green one (536 – 556 nm)

- **Reduced hemoglobin spectra**

- put in the HbO2 – 10 ml solution + a couple of crystals of sodium hydrosulfite.
- the color turns violet red, due to the reduced Hb.
- one can see a single green yellow band = Stokes band (530-548 nm)

- **Carboxihemoglobin spectra** (HbCO)

- Carboxihemoglobin is formed by the coordinative binding of CO to hem's iron. It is a reversible reaction. The affinity of Hb for CO is 210 times higher then for O2. If we breathe CO – intoxication– 40% carboxihemoglobin = lethal value.
- there are 2 bands:
  - A yellow one (564-579 nm)

- A green one (530-548 nm)

- both of them have a violet trend.

- **Met hemoglobin spectra**

- put 5-6 blood drops in 20 ml water.
- add 8-10 drops of saturated potassium fericianide solution. The solution gets dark brown due to the formed met hemoglobin.
- it has 4 absorption bands:
  - A narrow red one (620-630 nm) – specific for met hemoglobin.
  - 2 pale yellow green bands
  - a wide blue one (486-578 nm)
- met hemoglobin is improper for oxygen transport – a certain degree o hypoxia is reached.
  - Normally, erythrocytes contain 0,1-0,4 met hemoglobin;.

The crystallographic method (Teichman crystals)

Principle:
Hemoglobin treated with "arising" HCl (resulted from the reaction of NaCl + CH 3 COOH) -> clorhemine
Clorhemine can be identified as brownish diamond shaped crystals = Teichman crystals

Necessary material:
- a microscope slide, a glass slide cover, gas burner, microscope, blood, glacial acetic acid to which NaCl 1% has been added.

Protocol:
- Pour 1 blood drop on the slide.
- dry it by heating, but not over 40-45 degrees C – to avoid protein denaturation. Test it on the back of your hand.
- after add 1 drop of glacial acetic acid and NaCl 1% and cover it with the glass slide cover.
- heat it until it boils. Due to clorhemine the probe gets a brown color.
- cool it and put it under the microscope. It is suggested to cool it slower in order to get bigger crystals.

Discussion:
- the method is used in forensic medicine in order to identify blood stains.
- the crystals show the presence of blood without giving any information about the species.