University of Baghdad
College of Pharmacy
Department of Pharmacology
& Toxicology

Practical Physiology
2nd Class
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1st Course

EXPERIMENT 2

ESTIMATION OF HAEMOGLOBIN

Assist. Lec. Maryam Rasheed Abd

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Learning Objectives

On completion of this experiment, you should be able to:

1. Describe the structure, synthesis, and functions of hemoglobin.
2. Name the types and derivatives of hemoglobin.
3. Indicate normal levels of hemoglobin.
4. Name the common causes of increased and decreased levels of hemoglobin.
5. Determine the hemoglobin level by the Sahli's acid hematin method.
6. Name the advantages and disadvantages of this method.
7. Name other methods of estimation of hemoglobin.

Introduction

Hemoglobin (Hb) is a complex protein molecule inside RBCs that transports oxygen (O_2) and carbon dioxide (CO_2) to and from cells of the body. Each RBC has approximately 280 million Hb molecules. One gram of Hb carries 1.34 ml of O_2.

Structure

Hemoglobin is a globular molecule (Molecular weight=66,700 Daltons) composed of 4 globular protein subunits - each subunit has one heme group and a globin chain (Figure 1-a).

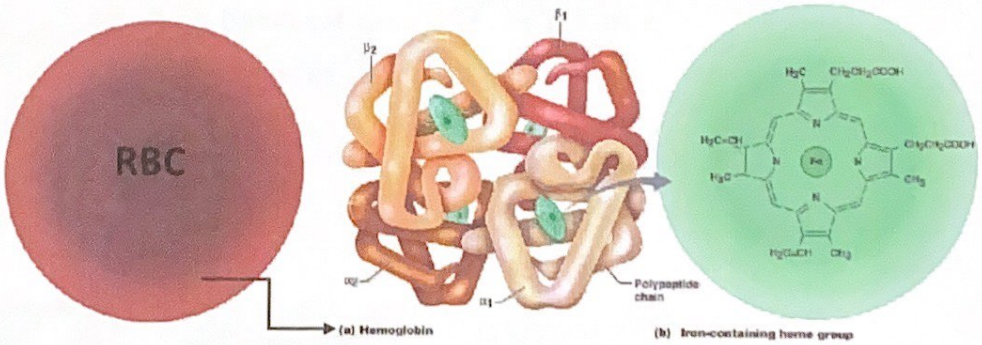


Figure 1: Structure of Hemoglobin.

- **Heme:** The heme is an iron containing porphyrin pigment, called iron protoporphyrin IX (iron PPIX). The porphyrin nucleus has four modified pyrrole subunits interconnected to each other via methine bridges (–CH=). Protoporphyrin is derived from porphyrin via the substitution of the outer hydrogen atom in pyrrole rings with four methyl groups (–CH3), two vinyl groups (–CH=CH2), and two propionic acid groups (–CH2-CH2-COOH). Each heme group contains an iron atom (ferrous, Fe^{+2}) (Figure 1-b). Each heme subunit can carry one molecule of O_2 bound to the central ferrous iron. Thus, each Hb molecule can carry four molecules of O_2.

- **Globin:** Globin is a protein substance that consists of four polypeptide ***globulin chains*** that are connected together. Each polypeptide globulin chain is attached to a heme moiety (Figure 1-a).

∴ Hemoglobin =**1 globin** (4 globulin) +**4 heme** groups

Types of Hemoglobin

Normal Hemoglobins

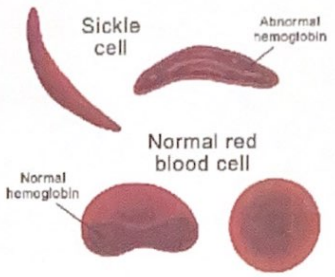
Adult hemoglobin:

- **Hemoglobin A (HbA, also known as adult hemoglobin and hemoglobin A1):** About 97% of Hb in a normal adult is HbA. It consists of two alpha (α) chains (141 amino acid residues) and two beta (β) chains (146 amino acid residues), with the structural formula ($\alpha_2\beta_2$).
- **Hemoglobin A_2 (HbA_2):** Consists of two alpha and two delta chains. It has the structural formula of ($\alpha_2\delta_2$). The delta chains also contain 146 amino acids residues, but 10 individual residues differ from those in the beta chains. HbA_2 is found at low levels (2.5%) in normal human blood. It may be increased in beta thalassemia and in people with sickle-cell disease.
- **Glycated hemoglobin (glycohemoglobin, hemoglobin A1c, HbA_{1c}):** HbA1c is formed by the covalent binding of monosaccharides to HbA. The levels of HbA1c increase in poorly controlled diabetes mellitus. When diabetes is not controlled, the sugar builds up in blood and combines with Hb, becoming "glycated". HbA_{1c} has a glucose attached to terminal valine in each beta chain. Normal range for the HbA_{1c} is between 4% and 5.6%.

Fetal hemoglobin (HbF): Hb F is the major Hb in the intrauterine life and at term. Its structure is similar to that of HbA except that beta chains are replaced by gamma (γ) chains. The gamma chains also contain 146 amino acid residues, but have 37 that differ from those in the beta chain. HbF is normally replaced by adult hemoglobin soon after birth. The adult level of HbF is about 1%.

Abnormal Hemoglobins

Hemoglobin S (HbS): the alpha chains are normal but in each beta chain, one glutamic acid residue has been replaced by a valine residue. Hb S is present in sickle cell anemia.



Hemoglobin Complexes and Derivatives:

- **Oxyhemoglobin (oxyHb):** is the normal, oxygen-loaded form of Hb, in which iron is in the reduced (ferrous, Fe^{+2}) state. The Hb molecule when fully saturated with O_2 (four O_2 molecules combined with one Hb molecule).
- **Carbaminohemoglobin (CO_2Hb):** When CO_2 combines with Hb, carbaminohemoglobin (CO_2Hb) is formed. CO_2 combines with globin, not with heme.
- **Carboxyhemoglobin (COHb):** When Hb combines with carbon monoxide (CO), carboxyhemoglobin (COHb) is formed. CO competes with oxygen at the heme binding site. Hemoglobin's binding affinity for CO is much greater than its affinity for O_2, which consequently displaces O_2 on Hb and reducing oxygen carrying capacity of blood.
- **Methemoglobin (metHb):** When blood is exposed to some drugs, or oxidizing agents *in vitro* or *in vivo,* the ferrous (Fe^{+2}) iron of Hb is converted into ferric (Fe^{+3}) iron, forming metHb, which is dark bluish in color. When present in large amounts, it gives a dusky appearance to the skin.
- **Sulfhemoglobin (SulfHb):** It is formed by the action of some drugs and chemicals (e.g., sulphonamides), the reaction being irreversible.
- **Cyanmethemoglobin (hemiglobincyanide, HiCN):** It is formed by the action of cyanide on Hb. (Hemoglobin is metHb).

Functions of hemoglobin:

1. Transport and exchange respiratory gases (O_2 and CO_2). Each gram of Hb, when fully saturated, carries 1.34 ml of O_2.
2. Hb acts as a buffer in maintaining blood pH.

Normal Values

Adult males: 14–18 g/dL of blood

Adult females: 12–16 g/dL of blood

Note: **COHb** (< 1.5%, 9% in smokers), **metHb** (< 2%), **SulfHb** (undetectable)

Conditions that alter hemoglobin concentration

❖ **Conditions that decrease hemoglobin concentration**

Physiological	Pathological
- Pregnancy - Children - Women	- Different types of anemia. - After severe hemorrhage. - Hemolysis due to transfusion of incompatible blood, reactions to chemicals and drugs, bacteremia, and artificial heart valves. - Variety of systemic diseases e.g., leukemia, lymphoma, kidney disease, cirrhosis, hyperthyroidism, and systemic lupus erythematosus (SLE).

❖ **Conditions that increase hemoglobin concentration**

Physiological	Pathological
- High altitudes. - Newborns and infants - Excessive sweating (hemoconcentration)	- Haemoconcentration states of blood (due to loss of body fluid), e.g., severe burns, severe diarrhea, and vomiting. - Polycythaemia vera. - Chronic obstructive pulmonary disease (COPD) - Congestive heart failure (CHF).

Hemoglobinometry

The term refers to measurement of the concentration (amount) of Hb in the blood. Various methods are available and can be grouped into the following categories:

1. Visual methods: This group includes Sahli (discussed later), Haldane, and Tallqvist methods, among others.

- ✓ **Filter paper method (Tallqvist Hemoglobin Chart):**

  This method is simple, inexpensive, rapid. A drop of blood, absorbed on a white filter paper is allowed to spread over the paper to form an even spot. As soon as the blood spot loses its gloss (before it dries), it is matched against a standardized color chart (Figure 2). The red color of blood corresponds to the amount of hemoglobin present.

  The method, though quick is rather inaccurate due to personal error. If the result indicates moderate to severe anemia, follow up with a more accurate test is required.

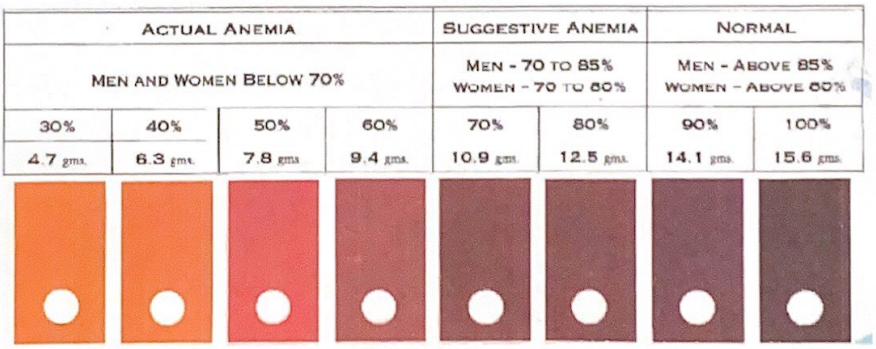


Figure 2: Hemoglobin scale (After Tallqvist)

2. Gasometric method

3. Spectrophotometric methods: The most important is the Hemiglobincyanide method.

✓ ***Cyanmethemoglobin (Hemiglobincyanide, HiCN) method:***

This method is the most accurate and most commonly used method of estimating Hb. In this method, all forms of Hb normally present in blood (oxyHb, reduced Hb, carboxyHb, and metHb) are converted into a stable compound— HiCN.

Principle: Blood is diluted in a solution (modified Drabkin's reagent) containing potassium ferricyanide and potassium cyanide. Potassium ferricyanide oxidizes the iron in heme to the ferric state to form methemoglobin, which is converted to hemiglobincyanide (HiCN) by potassium cyanide.

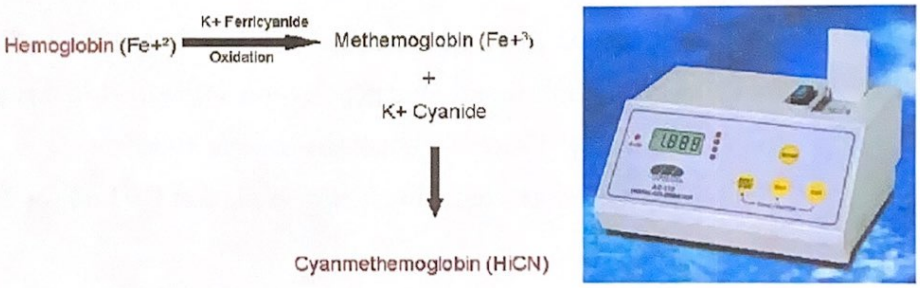


Figure 3: Cyanmethemoglobin (Hemiglobincyanide) method

HiCN is a stable-colored product, which in solution has an absorbance maximum at 540 nm, which can be measured via a spectrophotometer (Figure 3). Absorbance of the diluted sample at 540 nm is compared with absorbance at the same wavelength of a standard HiCN solution whose equivalent hemoglobin concentration is known.

4. Automated hemoglobinometry: The electronic hematology analyzer, along with cell counts, can also provide Hb concentration automatically.

Sahli's Acid Hematin Method

Principle

The Hb present in a measured amount of blood is converted by dilute hydrochloric acid into acid hematin, which in dilution is golden-brown in color. The intensity of color depends on the concentration of acid hematin which, in turn, depends on the concentration of Hb. The color of the solution, after dilution with water, is matched against the standard golden-brown tinted glass rods by direct vision. The readings are obtained in g% (g/dL).

Apparatus and Materials:

- Sahli's Hemoglobinometer (Haemometer), which consists of (Figure 4):
  - **a.** Comparator.
  - **b.** Hemoglobin tube: a glass tube calibrated in g Hb % (2–24 g% or g/dL) in yellow color on one side, and in percentage Hb (10–140%) in red color on the other side. There is a brush to clean the tube.
  - **c.** Sahli's pipette or Hemoglobin pipette: marked at 0.02 ml (or 20 µL), no bulb.
  - **d.** Stirrer.
  - **e.** Pasteur pipette or glass dropper.
- Materials for a sterile finger prick
- Distilled water.

Figure 4: Sahli's Hemoglobinometer (Haemometer)

Reagents: Decinormal hydrochloric acid (N/10 HCl)

Procedure:

1. Clean the hemoglobinometer tube and pipette and ensure they are dry.
2. Review the Safety Alert in Lab 1.
3. Using the dropper, add 100 µL of N/10 HCl into the Hb tube (up to the lowest yellow mark of 2 g%), and set it aside.
4. Follow steps 1 through 9 of the "Blood Collection" section of Lab 2 to obtain a large drop of free-flowing blood. Draw blood by Sahli's pipette up to the 20 µL mark. Do not allow air bubble while drawing blood. Carefully wipe the blood sticking to the tip of the pipette.
5. Add the blood to the tube containing HCl by immersing the tip of the pipette to the bottom of the acid solution in the Sahli's tube and expulsion of the blood gently. Rinse the pipette few times by drawing up and blowing out the clear upper part of the acid solution till all the blood has been washed out from it. Avoid frothing of the mixture.
6. Withdraw the pipette from the tube by touching it to the side of the tube. Mix the blood with the acid solution by the flat end of the stirrer (by rotating and gently moving it up and down).
7. Put the Hb tube in the Sahli's comparator and let it stand undisturbed for 10 minutes. During this time, the acid ruptures the red cells, releasing their Hb into the solution (hemolysis). The acid acts on the Hb and converts it into **acid hematin** which is deep golden-brown in color.
8. Dilute the acid hematin solution with distilled water (add distilled water drop by drop with stirring) till its color matches the color of the comparator tinted glass rods.
9. Read the lower meniscus of the solution. Note the reading on the graduated tube. This is the haemoglobin level in g/dl. Some tubes also give level in percentage. To convert into g/dl, multiply the percentage with 0.146. Example:

   $10\% \times 0.146 = 14.6$ g/dL
10. Dispose of all used materials as described in Lab 1 & 2.

Note: Each time you compare the color, lift and hold the glass stirrer against the side of the Hb tube above the solution (rather than taking it out completely).

Observations

Compare your color matching with that of your work-partners and record the observations in the workbook. Take the average of 3 readings.

Report

Express the result as:

- Hb= ..... g%.
- Oxygen carrying capacity

✓ ***To calculate oxygen carrying capacity:*** *Knowing your Hb concentration, and that 1.0 g of Hb can carry 1.34 ml of O_2, calculate its oxygen-carrying capacity as ... ... ml O_2/dl.*

Advantages of Sahli Method

- The method is easy to perform and fairly quick.
- Its running cost is minimal since the instruments and reagents are inexpensive.
- It does not require electricity.

Disadvantages of Sahli Method

- Color matching is subjective (may vary from person to person).
- Acid hematin is not a stable compound and readings must be taken within the recommended time interval.
- The method estimates only the oxyHb and reduced Hb, other forms, such as carboxyHb and metHb are not estimated.
- After prolonged use the numbers on the graduated cylinder fade and are difficult to read.