Notes

3

ESTIMATION OF HEMOGLOBIN

3.1 INTRODUCTION

Hemoglobin is the major constituent of the red cell cytoplasm, accounting for approximately 90% of the dry weight of the mature cell. It is comprised of **heme** and **globin**.

OBJECTIVES

After reading this lesson, you will be able to:

- describe the structure of hemoglobin
- list the function of hemoglobin
- explain the various laboratory methods for estimation of hemoglobin
- enumerate the advantages and disadvantages of each method
- discuss the normal value and interpretation of abnormal results

3.2 STRUCTURE OF HEMOGLOBIN

The Hemoglobin molecule is a tetramer consisting of two pairs of similar polypeptide chains called **globin chains**. To each of the four chains is attached **heme** which is a **complex of iron in ferrous form and protoporphyrin.**

The major (96%) type of hemoglobin present in **adults** is called HbA and it has 2 alpha globin chains and 2 beta globin chains ($\alpha_2\beta_2$). The gene that codes for the formation of α globin chains is located on chromosome 16 and that which

codes for the formation of β globin chains is on chromosome 11. In adults, a minor amount of HbA2 ($\alpha_2\beta_2$) is also present and constitutes less than 3.5%.

During embryonic and fetal life other different types of hemoglobins predominate. These include **Gower-1** (present in early embryonic life), **Gower-2 and Hb Portland**. After the eighth week of development, embryonic hemoglobins are replaced by **Fetal hemoglobin** ($\alpha_2\beta_2$). This remains the predominant hemoglobin until after birth and constitutes 50-90% of the total hemoglobin. After birth, it's concentration decreases to less than 2% by 30 weeks of age. HbA is then the predominant hemoglobin.

The structure and time of occurrence of various hemoglobins is shown in the table below.

Name	Structure	Present in
Adult Hb (HbA)	$\alpha_2\beta 2$	Adults
Fetal Hb (HbF)	$\alpha_2\gamma_2$	Fetal life
Hb Portland	$\zeta_2\gamma_2$	Embryonic life
Gower-1	$\zeta_2\varepsilon_2$	,,
Gower-2	$\alpha_2\varepsilon_2$	,,

Function of Hemoglobin Heme has the ability to bind oxygen reversibly and carry it to tissues. It also facilitates the exchange of carbon dioxide between the lungs and tissues. Thus, **hemoglobin functions as the primary medium of exchange of oxygen and carbon dioxide.**

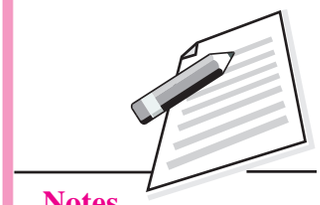
3.3 ESTIMATION OF HEMOGLOBIN IN THE LABORATORY

Various methods are available for estimation of hemoglobin in the laboratory.

I. Methods based on development of color. These are
   - Sahli's or acid hematin method
   - Cyanmethemoglobin method
   - Oxyhemoglobin method
   - Alkaline hematin method

II. Measurement of oxygen combining capacity

III. Measurement of iron content



3.4 METHODS BASED ON COLOR DEVELOPMENT

The **commonly used** methods are **Sahli's/ acid hematin** method and **Cyanmethemoglobin** method. The details of these methods are described below.

Sahli's/acid hematin Method

Principle: Blood is mixed with N/10 HCl resulting in the conversion of Hb to acid hematin which is brown in color. The solution is diluted till it's color matches with the brown colored glass of the comparator box. The concentration of Hb is read directly.

Equipment required

Hemocytometer which consists of

- comparator box which has brown colored glass on either side
- Hb pipette which is marked upto $20mm^3$(0.02ml blood)
- Tube with markings of Hb on one side
- glass rod
- dropper

Reagents required

N/10 HCl

Distilled water

Sample: Venous blood collected in EDTA as described earlier

Procedure

1. Add N/10 HCl into the tube upto mark 2g%
2. Mix the EDTA sample by gentle inversion and fill the pipette with 0.02ml blood. Wipe the external surface of the pipette to remove any excess blood.
3. Add the blood into the tube containing HCl. Wash out the contents of the pipette by drawing in and blowing out the acid two to three times. Mix the blood with the acid thoroughly.
4. Allow to stand undisturbed for 10min.
5. Place the hemoglobinometer tube in the comparator and add distilled water to the solution drop by drop stirring with the glass rod till it's color matches with that of the comparator glass. While matching the color, the glass rod must be removed from the solution and held vertically in the tube.

6. Remove the stirrer and take the reading directly by noting the height of the diluted acid hematin and express in g%.

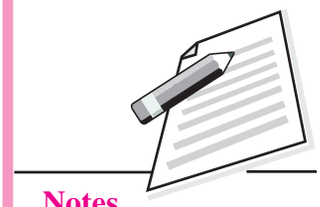
Fig. 3.1: Hb comparator box with brown glass on either side and tube with acid hematin solution in centre. The color of the solution is matched with the glass and the concentration of Hb is read directly

Advantages

- Easy to perform
- Quick
- Inexpensive
- Can be used as a bedside procedure
- Does not require technical expertise

Disadvantages

- Less accurate.
- All hemoglobins (oxyhemoglobin, sulphemoglobin) are not converted to acid hematin and hence the value of Hb obtained is less than the actual value.
- The color of acid hematin develops slowly.
- Color of acid hematin fades with time and dilution must be done exactly after 10 min when the color development is maximum
- Individual variation in matching of color is seen.



- If the matching point is passed, the whole procedure has to be repeated.
- Color of glass in the comparator box tends to fade with time .
- Lack of a true standard.

Cyanmethemoglobin method

This is the **internationally recommended method** for determining hemoglobin

Principle: Blood is diluted in a solution containing potassium cyanide and potassium ferricyanide. The latter converts Hb to methemoglobin which is converted to cyanmethemoglobin (HiCN) by potassium cyanide. The absorbance of the solution is then measured in a spectrophotometer at a wavelength of 540nm or in a colorimeter using a yellow green filter.

Equipment required

Hb pipette

Spectrophotometer

Reagents required

Drabkin's solution pH7.0-7.4 which contains

Potassium cyanide	50 mg
Potassium ferricyanide	200 mg
Potassium dihydrogen phosphate	140 mg
Nonionic detergent	1 ml
Distilled water	1 L

The solution should be clear and pale yellow in color. When measured against water as a blank in a spectrometer at a wavelength of 540 nm, the absorbance must be zero. **The solution is unstable if exposed to light and can be stored at room temperature in brown borosilicate bottles for several months.** However, if the room temperature is higher than 30°C, the solution should be stored in a refrigerator but brought to room temperature before use. The solution must **never be frozen.**

The pH of the solution must be checked every month.

Discard the solution if found to be turbid/if pH is outside range/ it's absorbance is not zero at 540 nm.

Do not pipette Drabkin's solution by mouth.

Sample: Venous blood collected in EDTA

Notes

Procedure

1. Take 5ml of Drabkin's solution in a test tube.
2. Mix the blood sample by gentle inversion and draw 0.02ml of blood into the Hb pipette. Wipe the outer surface of the pipette to remove excess blood.
3. Place the pipette into the tube containing Drabkin's solution and slowly expel the blood into the solution. Mix well and let it stand undisturbed for 5min.
4. Measure the absorbance of this solution at 540nm in a spectrophotometer after adjusting the OD at 0 by using Drabkin's solution as blank.
5. Calculate the hemoglobin concentration using a standard curve.

Advantages

- All forms of Hb except sulphemoglobin are converted to HiCN.
- Visual error is not there as no color matching is required.
- Cyanmethemoglobin solution is stable and it's color does not fade with time so readings may not be taken immediately.
- Absorbance may be measured soon after dilution.
- A reliable and stable reference standard is available from World Health Organisation for direct comparison.

Disadvantages

- Diluted blood has to stand for a period of time to ensure complete conversion of Hb.
- Potassium cyanide is a poisonous substance and that is why **Drabkin's solution must never be pipetted by mouth.**
- The rate of conversion of blood containing carboxyhemoglobin is slowed considerably. Prolonging the reaction time to 30min can overcome this problem.
- Abnormal plasma proteins cause turbidity when blood is diluted with Drabkin's solution.
- A high leucocyte count also causes turbidity on dilution of blood. Centrifuging the diluted blood can help overcome the turbidity.

Preparation of Standard curve for Hemoglobin estimation by Cyanmethemoglobin method

In a laboratory which tests several samples in a day, it is more convenient to prepare a standard curve for Hb.

A WHO International reference HiCN standard is available commercially as 10ml sealed ampoules. This solution is stable for years. The exact concentration of Hb present in the solution is indicated on the label.

Preparation of standard curve

1. Make serial dilutions of the reference solution eg 1in2, 1in4, 1in8 and so on with Drabkin's solution. As the Hb present in the solution is known, the Hb concentration of each dilution will also be known.
2. Take the OD of each dilution in the colorimeter against a blank of Drabkin's solution.
3. Plot the OD against the Hb concentration on a linear graph paper. The absorbance is plotted on the vertical axis and the hemoglobin concentration on the horizontal axis.The points should lie in a straight line that passes through the origin.
4. From this graph a table of readings and the corresponding Hb value can be prepared. This is more convenient than the graph specially when a large number of readings have to be taken. After OD of the sample is taken, the corresponding Hb value can be directly read from the table.

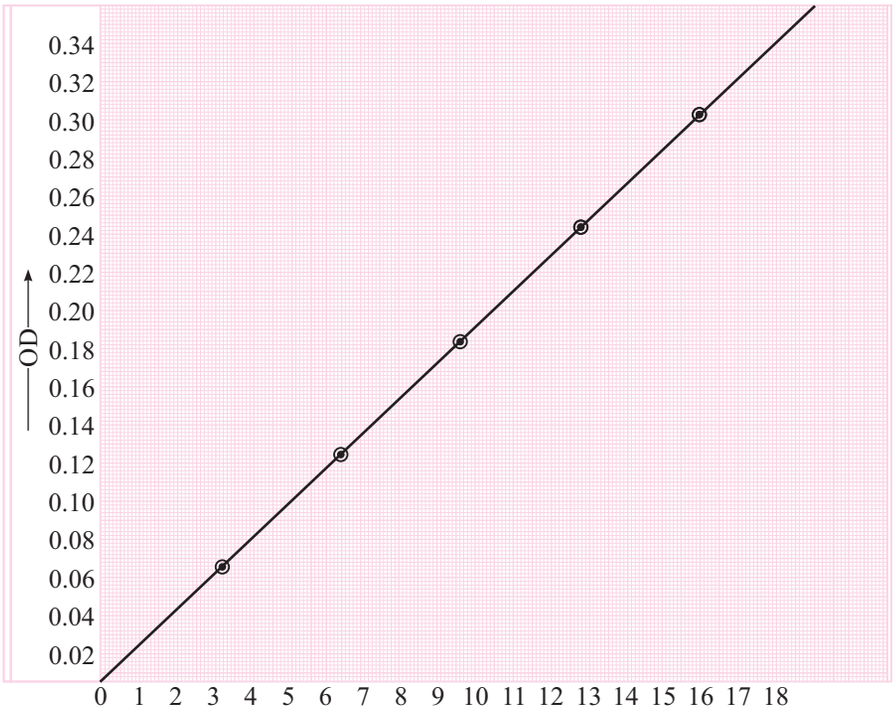


Fig. 3.2: Standard curve for Hb

3.5 OXYHEMOGLOBIN METHOD

Hb is converted to oxyhemoglobin by reaction with ammonia and the color of the solution is measured in a photocolorimeter**.**

The **advantages** of this method are that it is simple, quick and it's reliability is not affected by increased bilirubin level.

However, the **s**olution of oxyhemoglobin fades very quickly. It is not possible to prepare a stable standard.The method does not give satisfactory results in the presence of carboxyhemoglobin, methemoglobin and sulphemoglobin.

3.6 ALKALINE HEMATIN METHOD

Blood is converted to alkaline hematin by addition of alkali such as sodium hydroxide and the color measured in a colorimeter at 540 nm.

It gives a true estimate of Hb and is not affected by the presence of plasma proteins and lipids.

However, it is not used routinely as it is less accurate than the cyanmethemoglobin method and some forms of Hb such as HbF are resistant to alkali denaturation.

3.7 MEASUREMENT OF OXYGEN CARRYING CAPACITY

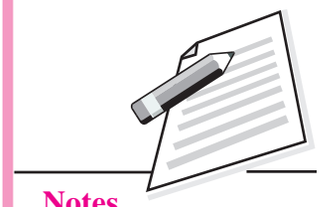
The oxygen combining capacity of blood is 1.34ml oxygen per gm of Hb. This is a measure of the function of Hb. It is not used routinely as it is not practical and gives results 2% lower than the other methods.

3.8 IRON CONTENT OF HEMOGLOBIN

Iron content can be measured and converted to Hb by using the formula 0.347 mg iron 100g Hb. It is however, impractical for routine use.

INTEXT QUESTIONS 3.1

1. Hemoglobin is comprised of ..... & .....
2. Hemoglobin molecule consists of two pairs of ..... chains
3. The predominant hemoglobin in adult is .....
4. Hemoglobin serves as primary medium of exchange of ..... & .....



3.9 DIRECT READING HEMOGLOBINOMETERS

Hemoglobinometers based on the cyanmethemoglobin/oxyhemoglobin method are available. The instrument has a built in filter and a scale and gives direct reading of Hb in g/dl. They are used in field surveys. It is imperative that the instrument is calibrated frequently.

Normal value of Hb

Males 15 ± 2 g/dl

Females 13.5 ± 1.5 g/dl

Significance of Hb estimation: Hemoglobin estimation is used as a screening test for detecting anemia. This is a frequently identified abnormality in our population. Anemia is not a diagnosis by itself and if detected, it's underlying cause must be ascertained. Hence, accurate Hb estimation is essential so that further tests can be done to ascertain it's cause and the patient treated accordingly.

WHO Cut Off Value below which Anemia is Present

Subject	Hb(g/dl)
Males	<13
Females	<12
Pregnant women	<11

INTEXT QUESTIONS 3.2

1. HbA is comprised of
   (a) $\alpha_2\beta_2$ (b) $\alpha_2\gamma_2$
   (c) $\alpha_2\delta_2$ (d) $\alpha_2\varepsilon_2$
2. In pregnant women anemia is present if Hb (g/dl)is less than
   (a) 12 (b) 10
   (c) 11 (d) 13
3. Cyanmethemoglobin method is preferred as
   (a) A reference standard is available for comparison
   (b) The color of the solution does not fade with time
   (c) No visual error is there
   (d) All of the above

WHAT HAVE YOU LEARNT

- The Hemoglobin molecule is a tetramer consisting of two pairs of similar polypeptide chains called **globin chains**. To each of the four chains is attached **heme** which is **a complex of iron in ferrous form and protoporphyrin**
- The major (96%) type of hemoglobin present in **adults** is called HbA ($\alpha_2\beta_2$).
- Hemoglobin functions as the primary medium of exchange of oxygen and carbon dioxide
- Various methods are available for estimation of hemoglobin in the laboratory. These are based on the estimation of hemoglobin by measurement of it's color
- The acid hematin or Sahli's method is easy to perform and gives quick results but is not recommended as it does not give accurate results
- The **Cyanmethemoglobin method** is the internationally recommended method for estimating hemoglobin which uses Drabkin's solution(a mixture of potassium cyanide and ferricyanide) to convert Hb to cyanmethemoglobin, the color of which is then measured in a spectrophotometer at 540nm and compared with a WHO reference standard
- Hb estimation is used as a screening test for detection of anemia

TERMINAL QUESTIONS

1. Describe the structure of Hemoglobin
2. List the functions of hemoglobin
3. Explain various laboratory methods for estimation of hemoglobin

ANSWER TO INTEXT QUESTIONS

3.1

1. Heme & globin
2. Globin
3. HbA
4. Oxygen & carbon dioxide

3.2

1. (a) $\alpha_2\beta_2$
2. (c) 11
3. d.