Hemoglobin is a complex protein with a molecular weight of 64,958 and consisting of two separate pairs of globin chains, each chain being associated with one molecule of heme.

During early fetal life, hemoglobin Gower $\alpha_2^0 \epsilon_2$, hemoglobin Portland $\gamma_2^2 \beta_2$ and hemoglobin Gower II $\alpha_2^2 \epsilon_2$ are found in humans. Later during fetal life, hemoglobin F (HbF) $\alpha_2^0 \gamma_2$, HbA-$\alpha_2^2 \beta_2$ and HbA2-$\alpha_2^0 \beta_2$ are found.

The major hemoglobin in the erythrocytes of the normal adult is HbA. In addition, over 400 mutant hemoglobins are now known, some of which may cause serious clinical conditions, especially in the homozygous state or in combination with another abnormal hemoglobin. (1,2)

All inherited abnormal hemoglobinopathies seem to fall into one of three categories: (a) inherited abnormalities of the structure of one or more globin chains, such as HbS, HbC; (b) inherited abnormalities related to the rate of synthesis of one or more of the globin chains such as thalassemia syndromes; (c) failure of the normal switch from fetal HbF to adult HbA synthesis.

Hemoglobin electrophoresis is generally considered the best method for separating and identifying the hemoglobins. Practical approach to the diagnosis of hemoglobinopathies consists of the following tests: (1) electrophoresis in cellulose acetate - pH 8.6; (2) quantitative estimation of
HbA\textsubscript{2}; (3) quantitative estimation of HbF; (4) test for deoxyhemoglobin solubility; (5) citrate agar gel electrophoresis; (6) isoelectric focusing; and (7) globin chain electrophoresis. (3,4) In cases of thalassemia where diagnosis is difficult, determination of globin chain synthesis rates, chromatography, finger printing, amino acid sequencing is indicated. (5,6,7)

**Cellulose Acetate Electrophoresis (pH 8.6) (8,9)**

**Principle**

Hemoglobin molecules in an alkaline solution have a net negative charge which causes them to move towards the anode at a rate proportional to their negative charge.

Different hemoglobins, however, have different charges. Those with an electrophoretic mobility greater than that of HbA at pH 8.8 are known as the "fast hemoglobins:" these include Hb Barts, HbH, HbI, HbN and HbJ. Substitution of valine for glutamic acid in the beta chain HbS causes slower migration by reducing the negative charge. Substitution of lysine with its positive charge for glutamic acid, HbC, causes the slowest migration.

**Interpretation (10,11,12)**

The relative order of increasing electrophoretic mobility of the common hemoglobin bands from cathode to anode is as follows: HbA\textsubscript{2}; E=O=C; G=D=S; F; A; Bart's, N and H. (Fig. 1) Usually, HbA is preceded by a fainter band that merges
with HbA. This has been called A₃ and is believed to represent glycosylated hemoglobins.

It must be noted that hemoglobins A₂, C, O and E cannot be differentiated on the basis of electrophoretic mobility in this medium. Nor can hemoglobins D, G and Lepore be distinguished from HbS.

HbF is not seen unless it comprises 2% or more of the total hemoglobin.

A very slowly moving band that constitutes more than 15% of the total hemoglobin may be presumed to be HbC, O or E, since HbA₂ is rarely more than 10% of the total hemoglobin. HbC and HbO are usually about 40 to 45% of the total hemoglobin and virtually limited to patients of African descent, whereas HbE is usually about 30% of the total hemoglobin and limited to those of Southeast Asian ancestry.

When present, HbH constitutes approximately 5 to 15% of the total hemoglobin and most commonly seen in persons from Southeast Asia.

When present, hemoglobin Lepore constitutes only 10 to 15% of the total hemoglobin, whereas HbS, D or G constitutes 25 to 45% of the total hemoglobin.

Thus, by measuring densitometrically the proportion of these hemoglobin bands and by knowing the ethnic origin of a patient, a preliminary assessment of hemoglobinopathies can be made.

Combinations of hemoglobinopathies and thalassemias can also occur. Electrophoresis patterns, quantitative HbA₂ and quantitative HbF values are usually required to assess the type of hemoglobin abnormality and its severity.
Additional confirmatory tests are needed:

1. Solubility test if S band present

2. Hemoglobin stability tests for unstable hemoglobins, e.g., HbKöln, HbH.

3. Citrate agar electrophoresis at pH 6.2 is necessary to distinguish: (a) HbE, O and A₂ from HbC, (b) HbS from HbD and G.

**Acid Citrate Agar Electrophoresis (pH 6.2)** (13)

Electrophoresis of hemoglobin in an acid citrate agar gel aids in the identification of hemoglobins D, E, G and O on the basis of differences in charge and molecular configuration. This procedure complements cellulose acetate electrophoresis at an alkaline pH and permits ready separation of hemoglobins that migrate together on cellulose acetate.

**Interpretation**

The relative order of hemoglobin migration in citrate agar at an acid pH is as follows: HbF, A=D=E=G, O, S, C. (Fig. 2)

HbD and G migrates with HbA and thus separate well from HbS. HbE migrate with HbA and thus also is easily differentiated from HbC. HbO has a slightly lower cathodal migration than HbA and thus can be distinguished from HbC as well as from HbA, D, G, E.

HbA₂ mobility is the same as that of HbA.
Isoelectric Focusing (15,16)

Principle

Thin-layer isoelectric focusing is based on the migration of proteins to their isoelectric points (pI's) when placed on a stable pH gradient of an electric field. The pH gradient is maintained by a mixture of ampholytes incorporated in a polyacrylamide gel. Hemoglobins migrate according to their pI's and are separated as sharp bands with high resolution and sensitivity.

The greatest advantages of isoelectric focusing over conventional electrophoresis are: much better resolution between proteins having the same or similar migration by electrophoresis and the ability to detect small amounts of minor components. In addition, an abnormal hemoglobin can often be identified solely by isoelectric focusing rather than using several electrophoretic procedures, different pH's and complementary tests. Isoelectric focusing is a cost effective procedure considering the fact that up to 50 samples can be run on one plate with, usually, no accompanying tests necessary.

Disadvantages of isoelectric focusing in comparison to conventional electrophoresis are: the test is technically more complicated and densitometric quantitative analysis of hemoglobins is not an easy task due to small size of the bands and background opacity.

Interpretation

The relative positions of common hemoglobins from the acidic anodal end to the basic cathodal end are: H, Bart's, A, F, S, E, A2, C. (Fig. 3) The identity of each band is established by the reference to A, F, S, C and Bart's
control, measurement of the distance of the band from HbA and reference to the chart of known hemoglobin positions.

**Conclusion**

Hemoglobin electrophoresis is the most useful standard laboratory procedure for the detection and identification of abnormal hemoglobins. Different media and different buffers vary in efficiency and adequacy for all separations and screening purposes. The simplest and most popular routine methods employ cellulose acetate membranes at alkaline pH. Cellulose acetate electrophoresis has come into general use because it is easier and quicker to perform, provides sharp resolution of hemoglobin bands and permits densitometric quantitation and permanent storage of a transparent film.

Electrophoresis in citrate agar gel at acid pH is a useful procedure for further fractionation and separation of the hemoglobins that migrate together on cellulose acetate electrophoresis.

In recent years, thin-layer isoelectric focusing in polyacrylamide gels has been increasingly used in identifying variant hemoglobins. Isoelectric focusing may replace conventional electrophoresis in the routine laboratory investigation of hemoglobinopathies since this method is cost effective and offers sharp band resolution and sensitive detection of minor components.

Hemoglobin electrophoresis separates proteins according to charge. Thus, its principal limitation is its inability to detect amino acid substitutions that do not alter charge. Hence, structurally different hemoglobins such as unstable hemoglobins and hemoglobins with increased oxygen affinity will be electrophoretically indistinguishable. However, most
presently known hemoglobins are electrophoretically abnormal, and, despite the limitations, hemoglobin electrophoresis remains the method of choice for the screening, detection and identification of the abnormal hemoglobins.
REFERENCES


Fig. 3. Isoelectric focusing of samples obtained from a patient with HbSC and a normal adult, a normal newborn, and a newborn double heterozygous for hemoglobin S and C-phenylalanine.

Fig. 4. 2. Electrophoretogram of various hemoglobin samples on citrate agar.

Fig. 5. Electrophoretogram of various hemoglobin samples on cellulose acetate.

Fig. 6. Electrophoretogram of various hemoglobin samples on cellulose acetate pH 6.0-6.2.