TEST EVALUATION  
Pete Rainey, M.D., Ph.D.

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Hemoglobin Electrophoresis

METHOD

An aliquot of hemoglobin held in an appropriate support medium is subjected to an electric field. The hemoglobin molecules carry an electric charge and migrate under the influence of the field at a rate determined by the strength of the field, the size and shape of the molecules, the porosity of the support, and the net charge on the molecules. The net charge is determined primarily by the amino acid composition of the molecule and the pH at which the electrophoresis is carried out. Since all functional hemoglobins differ little in size and shape, if the conditions of the electrophoresis (voltage, pH and support medium) are held constant, the rate of migration will be proportional to the net charge on the molecule. Thus, hemoglobin molecules, whose structural differences result in differences in net charge at a given pH, may be separated and characterized by their relative mobilities. They may also be quantitated after separation. Hemoglobinopathies may be detected either by the finding of a band with an unusual mobility or by the appearance of abnormal amounts of normal hemoglobins.

THEORETICAL CONSIDERATIONS

There are several ways in which the net charge on a hemoglobin molecule may be altered. In the minor hemoglobins A2 and F, this involves a change in the subunit structure from α2β2 to α2δ2 or α2γ2, respectively. The abnormal hemoglobins H(β4) and Barts (γ4) also have different subunit structures. The most frequent cause of a change in net charge is a mutation which results in the substitution of a charged amino acid for an uncharged one, or vice versa. The replacement of a negatively charged glutamic acid by an uncharged valine in hemoglobin S is the best known example of such an alteration. A more subtle effect is seen where substitution of a hydrophobic amino acid for a hydrophilic one near an ionizable group results in suppression of ionization and a change in the group's pK. (The inverse of this may occur when a charged amino acid replaces an uncharged one in a hydrophobic region of the molecule; here, suppression of ionization may allow the substitution to occur without a significant change in net charge.) In addition, other types of mutation may result in the addition or deletion of one or more charged amino acids.

Another mechanism of charge alteration involves a change in the oxidation state of the heme iron from +2 to +3. This may add up to four positive charges. In some cases, this change may be accompanied by the formation of a coordination complex with negatively charged chloride at the oxygen binding site. This will result in a hemoglobin with no net change in charge, but which no longer binds oxygen.

The binding of other charged compounds to hemoglobin also affects the net charge. This phenomenon appears to be the foundation for citrate agar electrophoresis (3). Apparently, negatively charged citrate in the buffer binds to the 2,3-DPG binding site. Intrinsic charge differences appear to be minimized at the slightly acid pH.
employed and most hemoglobins comigrate with hemoglobin A. However, those hemoglobins with altered affinity for 2,3-DPG are separated with the relative mobilities paralleling the relative affinities.

PRACTICAL CONSIDERATIONS

At a moderately alkaline pH, all hemoglobins carry a variable net negative charge and move toward the anode (+) at a speed proportional to the charge. The relative mobilities are in general independent of the support medium and can be remembered by the mnemonic "Hemoglobin is a funny globin, some days even crazy" (4). This corresponds to relative mobilities H>I>A>F>G=S=D=E=C. Hemoglobins A2 and O, with mobilities similar to E and C, are missed by this mnemonic.

Alkaline electrophoresis of hemoglobin has been carried out using many different supports. Of these, starch gel electrophoresis appears to give the best resolution, but is cumbersome to carry out and difficult to quantitate. Cellulose acetate electrophoresis has generally become the method of choice because it is cheap, fast, and convenient, and lends itself to quantitation by densitometry after staining. It should be noted that many factors affect dye uptake and densitometric quantitation after staining should be considered to provide only rough estimates of the relative amounts of hemoglobin. Accurate determinations require elution before quantitation, or, better yet, chromatographic separation of larger amounts of hemoglobin.

Cellulose acetate electrophoresis is unable to resolve hemoglobins S, D and G from one another, nor hemoglobins A2, C, E and O. Citrate agar electrophoresis (discussed above) gives distinct mobilities for hemoglobins S, C, and F and is often used in conjunction with cellulose acetate. The mnemonic "cleverly separates A from F" (5) describes the relative position of the bands moving from anode to cathode. All other major hemoglobins comigrate with hemoglobin A in this system.

Many amino acid substitutions may occur which produce no change in net charge. In many cases, such changes have little or no effect on function and may never be suspected. In other cases, there may be functional consequences. Substitutions in the regions of contact between the α and β chains or in the heme binding pocket may result in hemoglobins with increased or decreased oxygen affinity which are electrophoretically indistinguishable from hemoglobin A.

Similarly, substitutions in different parts of a protein chain may have the same effect on net charge. Such hemoglobins will be structurally different, but electrophoretically indistinguishable. Some hemoglobins with letter names (e.g., hemoglobin D) in fact represent families whose individual members differ structurally but not electrophoretically. These members receive further designations (e.g., hemoglobin D. Punjab) to specify unique structures. Functional differences may vary considerably. When they result in variants which are readily differentiated clinically, the molecules will have distinct names (e.g., hemoglobins D and G).

Because of limitations such as those described above, hemoglobin electrophoresis is useful only as a screening technique. It may suggest a specific diagnosis, but does
not confirm it. Should an abnormal electrophoreogram be found which is consistent with one of the more common hemoglobinopathies, and the clinical presentation, other hematologic findings, ethnic background, and family history are also consistent, it is often unnecessary to proceed further. If discrepancies are present, or the electrophoreogram is consistent with more than one interpretation, further studies are indicated. These studies may encompass a wide variety of techniques. The reader is referred to the general references (1,2) for further discussion.

REFERENCES


4. Roche J, personal communication.

5. Rainey P, personal fabrication.