Identification of Human Hemoglobins

C. A. J. Goldberg

Differences in the hemoglobins of the adult and the newborn have been recognized since 1866 (36). Rapid advances in this field, however, began in 1949, when Pauling and Itano (25) discovered that the hemoglobin of sickle cell disease differs from adult hemoglobin. Since that time, 8 hemoglobins have been identified, hemoglobin C (18) in 1950, hemoglobin D (14) in 1951, hemoglobin E (7, 17) and hemoglobin G (10) in 1954, and hemoglobin H (24, 28) in 1955. Hemoglobin I was described by Rucknagel and coworkers (30) in 1955, hemoglobin J by Battle and Lewis (2) in 1954. The hemoglobin listed as K by Allison (1) carries the designation J in the review of Itano (16), and is further described by Thorup (1, 16).

The heterogeneity of normal adult hemoglobin as manifested by its electrophoretic behavior was first described by Derrien (9) in 1953. Studies by Kunkel and Wallenius (19), and Shavit and Brener (31) supported this finding. Morrison and Cook (22) observed the heterogeneity of hemoglobin A by chromatography on an ion-exchange resin. More recently Cook and Morrison (8) have found hemoglobin F to be heterogeneous as well.

The alkali-resistant hemoglobin present in normal adults in very small amounts appears to be identical with fetal hemoglobin in amino acid composition according to studies made by Huisman et al. (12). Van der Schaaf and Huisman (34) have found that the alkali-resistant component present in some patients with sickle cell anemia differs from fetal hemoglobin in ultraviolet absorption spectra and amino acid composition.

Excellent reviews on the present status of the human hemoglobins by Chernoff (4, 5, 6) and Itano (16) have appeared in 1955.

From the William Pepper Laboratory of Clinical Medicine, University of Pennsylvania, Philadelphia, Pa.

Received for publication April 24, 1956.
Although electrophoresis at pH 8.6 is the most important single method for the identification of the various hemoglobins, a number of supplementary methods are required. The following methods will be discussed in this paper: electrophoretic separations at various H-ion concentrations, together with a method for quantitative measurement of the hemoglobins, chromatography, measurement of alkali denaturation, determination of the solubility of reduced hemoglobin, and sickling tendency.

**ELECTROPHORESIS**

Two methods for electrophoretic separation of hemoglobins will be described. Method I, in which an improved pressure-plate apparatus is used, is the method of choice. Method II is for use with apparatus in which the paper strips are suspended horizontally. Difficulties in separating hemoglobins by the conventional procedures in the latter have caused many workers to turn away from this method. However, it can be adapted for hemoglobin separations according to the technic described below.

**Factors Involved in Electrophoretic Separation of Hemoglobins**

The factors which influence the separation of hemoglobins are many. Those of particular importance may be listed in an equation as follows:

\[
d_a - d_b = F \frac{tRE}{Dr/2} \quad D = \frac{I}{q}
\]

where \(d_a - d_b\) = displacement of 2 different hemoglobins

\(t\) = time

\(R\) = resistance

\(E\) = field strength (potential gradient)

\(q\) = width of the paper in cm.

\(I\) = current in amperes

\(D\) = current density

\(r/2\) = ionic strength of the buffer system

\(F\) = factor (to include all other variables which are not of primary importance as f.i. temperature)

**Time and Field Strength.** The degree of separation of the hemoglobins is directly proportional to time and field strength. The optimal field strength for the methods described was found to be 4–6.5 v./cm. Experiments carried out over a time period of 15–20 hours at this field
strength will provide better patterns than experiments at higher potentials for a shorter time. However, the time should not exceed 20 hours, to prevent excess diffusion.

Frictional Resistance. The resistance to the flow of buffer through the paper should be high. This may explain why hemoglobin electrophoresis on compressed paper or on paper suspended over a ridge pole has been so much more satisfactory than on freely suspended horizontal paper strips, for buffer flow in the former is much less than in the latter. It is obvious therefore that the frictional resistance should be increased to obtain the satisfactory separation of hemoglobins on a free-hanging horizontal strip. This may be done in several ways. Paper may be compressed by means of a glass rod attached to the bridge by a strong rubber band (Fig. 1), or the paper strip may be cut to decrease the width of the paper hanging in the buffer solution (Fig. 2). A favorable buffer flow is obtained in the latter when the paper is cut down to one fourth of its original width. The frictional resistance may be increased further by the addition of nonelectrolytic solutes to the buffer system to increase the viscosity of the buffer. The effect of three substances was investigated: sucrose, dextran, and glycerol. As may be seen from Table 1, the effect of the addition of 3% dextran on the viscosity of the Veronal buffer slightly exceeds that of 25% sucrose. The effect of 5% glycerol is slightly less than that of 10% sucrose.

The effect of these substances on the migration of the hemoglobins is a decrease in the total distance of migration with a negligible loss of resolution in pressure-plate types of apparatus. This effect is very beneficial when working at higher temperatures. The total distance of migration and the resolution of hemoglobins A, S, and C at 28° when 25% sucrose is added to the Veronal buffer is about the same as at 10° when Veronal buffer only is used (see Table 1). It is possible therefore to obtain satisfactory and comparable patterns of various hemoglobins at different temperatures by adding sucrose in amounts varying with temperature. Keeping the potential constant at 10° Veronal buffer may be used without any additional solute. At 20°, 10% sucrose may be added, and for temperatures around 30° the addition of 25% sucrose will result in satisfactory patterns.

Dextran proved to be less suitable in that it caused greater diffusion in the hemoglobin patterns. Little benefit was derived from the addition of dextran in amounts smaller than 3%. On the other hand, when 6% dextran was added to the buffer at a temperature of 28°, there was a complete loss of resolution. The effect of dextran cannot be ascribed to
Fig. 1. Paper attached to bridge. Fig. 2. Paper cut to decrease the width of hanging end.

Fig. 5. Separation of hemoglobins on freely suspended horizontal strips.

Plate 1
Fig. 6. Separation of hemoglobins on paper strips enclosed between pressure plates. Fig. 7. Galvanometer readings plotted against distance, yielding gaussian curves. Fig. 8. Gaussian curve showing distortion of a major peak. Fig. 10. Fetal hemoglobin shown by chromatography. Two completely separated zones.

Plate 2
Table 1. Displacement of Various Hemoglobins at 10° and 28° in Different Media, at 250 v., 16 Hr.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Solute added</th>
<th>Temp.</th>
<th>Relative viscosity</th>
<th>A (Displacement in mm.)</th>
<th>Hemoglobin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>28°</td>
<td>1.00</td>
<td>107 83 54</td>
<td></td>
</tr>
<tr>
<td>Veronal</td>
<td></td>
<td>10°</td>
<td>1.18</td>
<td>98 73 45</td>
<td></td>
</tr>
<tr>
<td>Veronal</td>
<td>10% sucrose</td>
<td>28°</td>
<td>1.82</td>
<td>62 47 32</td>
<td></td>
</tr>
<tr>
<td>Veronal</td>
<td>25% sucrose</td>
<td>28°</td>
<td>3.20</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Veronal</td>
<td>6% dextran</td>
<td>10°</td>
<td>1.00</td>
<td>70 51 29</td>
<td></td>
</tr>
<tr>
<td>Veronal</td>
<td>25% sucrose</td>
<td>10°</td>
<td>1.18</td>
<td>60 38 20</td>
<td></td>
</tr>
<tr>
<td>Veronal</td>
<td>1½% dextran</td>
<td>10°</td>
<td>1.82</td>
<td>32 24 13</td>
<td></td>
</tr>
<tr>
<td>Veronal</td>
<td>3% dextran</td>
<td>10°</td>
<td>2.00</td>
<td>58 44 30</td>
<td></td>
</tr>
<tr>
<td>Veronal</td>
<td>5% glycerol</td>
<td></td>
<td>1.11</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a* Measurements made in Ostwald viscosimeter.

*b* Experiments performed with the EC305 of the E-C Apparatus Company.

mere viscosity. When paper strips containing dextran buffer were stained with protein dyes, they retained a great deal more dye than those containing Veronal or sucrose-Veronal buffer. Hence, it may be assumed that there is interaction between the dextran and the paper, and perhaps between the hemoglobins and dextran as well.

When freely suspended horizontal strips were used for the separation of hemoglobins, the effect of the addition of sucrose to the Veronal buffer was not as satisfactory as it proved to be for the pressure-plate apparatus. Sucrose in concentrations of 25% or dextran in concentrations of 3% appeared to have a "drying" effect on the paper resulting, when dextran was used, in charring of the paper in the center, at the sample application site.

Glycerol was substituted in an effort to control evaporation (21, 27) while increasing the viscosity. It was used in amounts varying from 1 to 10 volumes per 100 volumes of Veronal buffer. The sharpest patterns, with the best resolution and least diffusion, were obtained with 5 volumes of glycerol per 100 volumes of Veronal buffer.

**Current Density.** The rate of separation of the various hemoglobins is inversely proportional to the current density. Hence, the current density should be kept as low as possible. It should not exceed 0.5 ma. per cm. of strip width. For hemoglobin separations on free-hanging horizontal strips, Whatman paper No. 1 is to be preferred to Whatman paper 3MM since it gives a current density half that obtained with the thicker paper at the same potential. Excessive current density will
manifest itself by a rapid rate of migration of the hemoglobins without any separation.

**IONIC STRENGTH AND BUFFER SYSTEMS AT pH 8.6.** The following buffers were investigated: (1) boric acid-potassium hydroxide, pH 8.6, 0.025M; (2) borate-phosphate buffer, pH 8.6, 0.017M; (3) borate-chloride-NaOH buffer, pH 8.7, 0.058M; (4) Michaelis buffer [as described for protein electrophoresis (35)] diluted 1:2 with distilled water pH 8.6, 0.05M; (5) barbital buffer (see under methods), pH 8.6, 0.06M.

Although separations may be obtained with the borate-phosphate buffer and the dilute Michaelis buffer, the barbital buffer is to be preferred since it causes less trailing and diffusion.

Since the rate of separation of the hemoglobins is inversely related to the ionic strength of the buffer, low ionic strength will be preferable. However, with lowered ionic strength, diffusion becomes an increasing problem. The optimal ionic strength should be determined for each buffer system. For the barbital buffer of pH 8.6, it is 0.06.

**pH.** Difficulties in separating hemoglobins by electrophoresis arise mainly from the fact that the isoelectric points of most hemoglobins differ only slightly, with the exception of that of hemoglobin H (Table 2).

Electrophoresis of the hemoglobins at pH 8.6 permits no distinction to be made between hemoglobins S and D, F and G, A and J, and I and H (Fig. 3). It has been recommended by Battle and Lewis (2) that electrophoresis be carried out also in a phosphate buffer at pH 7.8, which will result in separation of hemoglobins A and J (see Fig. 3). Furthermore, a third electrophoretic pattern carried out in a phosphate

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>6.87</td>
<td>.</td>
<td>High</td>
</tr>
<tr>
<td>S (B)</td>
<td>7.09</td>
<td>.</td>
<td>Low</td>
</tr>
<tr>
<td>C</td>
<td>7.30</td>
<td>.</td>
<td>High</td>
</tr>
<tr>
<td>D</td>
<td>.</td>
<td>.</td>
<td>High</td>
</tr>
<tr>
<td>E</td>
<td>.</td>
<td>.</td>
<td>High</td>
</tr>
<tr>
<td>F</td>
<td>6.98</td>
<td>Yes</td>
<td>High</td>
</tr>
<tr>
<td>G</td>
<td>6.98</td>
<td>.</td>
<td>?</td>
</tr>
<tr>
<td>H</td>
<td>5.6</td>
<td>.</td>
<td>Low</td>
</tr>
<tr>
<td>I</td>
<td>.</td>
<td>.</td>
<td>High</td>
</tr>
<tr>
<td>J</td>
<td>.</td>
<td>Moderate</td>
<td>?</td>
</tr>
<tr>
<td>K</td>
<td>.</td>
<td>?</td>
<td>?</td>
</tr>
</tbody>
</table>

*a* Isoelectric points obtained from the literature.

*b* Alkali resistance as determined by the method of Singer (see text).

*c* Ferrohemoglobin solubility, modified Itano method (see text).
buffer at pH 6.5 as suggested by Rucknagel et al. (30), will separate hemoglobins I and H, H being the only one to migrate anodically at this pH.

Preparation of the Hemoglobin Solution

Place 4 or 5 ml. of oxalated blood into each of two graduated centrifuge tubes of 15-ml. capacity and centrifugalize for 10 minutes. Withdraw the supernatant and add 10 ml. of saline to the packed cells. Stir gently and again centrifuge for 10 minutes. Withdraw the supernatant and wash the cells three more times with 10-ml. portions of saline (33). To a small portion of washed packed cells add 2 volumes of distilled water, and stir gently until hemolysis is complete. Centrifuge for 10 minutes. Use the clear hemoglobin solution for electrophoresis without delay. To the remainder of the washed packed cells in the graduated centrifuge tubes, add 2 volumes of barbital buffer pH 8.6, stir gently, and transfer the suspensions to two labeled freezer-storage tubes. Keep the samples frozen until needed (the minimal time is overnight). Hemolysis is effected by thawing. Thaw one tube either in the refrigerator or at room temperature. The tube may be warmed to body temperature, but should be cooled at once as soon as all ice has disappeared. Do not put the tube in warm or hot water! The sample is centrifuged for 10 minutes and the clear hemolysate is used for further testing.
Hemoglobin samples are apt to deteriorate rapidly; they should be kept in a refrigerator. The aqueous hemolysate deteriorates more rapidly than that buffered at pH 8.6. It should be used for electrophoresis on the day it is prepared. If electrophoresis cannot be done at once or is to be repeated, buffered hemolysate should be used. However, if hemoglobin H is present it will not be detected in the buffered hemolysate since it is destroyed by freezing (29).

Frozen samples of hemoglobin will keep for several months, however, refrigerated samples may deteriorate after one week.

**Method I. Electrophoresis Using a Pressure-Plate Apparatus**

**Apparatus**

The EC305 apparatus, manufactured by the E-C Apparatus Company, 23 Haven Avenue, New York, was used. It consists of two Plexiglas pressure plates arranged so that cooling water can circulate through the plates in close proximity to the filter paper strips. The buffer compartments are divided into three independent sections. This permits simultaneous separations in different buffer systems to be made if desired. A foam rubber pad between the top and bottom plates distributes the pressure uniformly and compensates for unevenness of the pressure plates or the paper. Local variations in buffer saturation of the paper are eliminated in this way. The electrodes consist of platinum strips separated from the paper by two baffles. Pressure is applied by means of an adjustable clamp attached to the center of the pressure plates. The power supply recommended is that supplied by the E-C Apparatus Company for use with the EC305 electrophoresis chamber.

**Reagents**

**Barbital Buffer pH 8.6.** Dissolve 20.6 Gm. of sodium barbital and 2.8 Gm. of barbital (diethylbarbituric acid) in carbon dioxide-free distilled water. Adjust the volume to 2 l. This buffer has an ionic strength of 0.06M.

**Barbital-Sucrose Buffer.** Add 500 Gm. of sucrose (or less as required) to the preceding before adjusting the volume to 2 l. (see text).

**Phosphate Buffer pH 7.8.** Dissolve 0.587 Gm. monobasic sodium phosphate (NaH₂PO₄·H₂O) and 6.50 Gm. of dibasic sodium phosphate (Na₂HPO₄) or 16.38 Gm. of dibasic sodium phosphate (Na₂HPO₄·12H₂O) in carbon dioxide-free distilled water. Adjust the volume to 2 l. The ionic strength of this buffer is 0.12M.

**Phosphate Buffer pH 6.5.** Dissolve 3.11 Gm. of monobasic potassium
phosphate (KH₂PO₄) and 1.49 Gm. of dibasic sodium phosphate (Na₂HPO₄) or 3.76 Gm. of dibasic sodium phosphate (Na₂HPO₄.12H₂O) in carbon dioxide–free distilled water. Adjust the volume to 1 l. The ionic strength of this buffer is 0.1M.

**Phosphate-Sucrose Buffers.** Add 250 Gm. (or less as required) of sucrose to the preceding before adjusting the volume to 1 l. (see text).

**Sodium Chloride Solution.** 0.9 Gm. per 100 ml. This solution is referred to as “saline” in the text.

**Hemoglobin Reference Solutions.** Solutions of hemoglobins A, S, and/or C.

**Preparing the Chamber**

Buffer solutions are poured in the three buffer compartments on one side of the apparatus. With the stopcocks on the leveling tubes open, some of the buffer is allowed to flow to the opposite side of the apparatus, to make sure that there is no airseal in any of the connecting tubes. The compartments on the other side of the instrument are now filled with buffer and the level is adjusted on both sides to about ½ inch over the lower baffle. The buffer levels are allowed to equilibrate for about 1 hour.

**Procedure**

Strips of filter paper Whatman 3 MM, 18½ x 2½ inches, are dipped in buffer, blotted between layers of filter paper, and placed on a teflon sheet on top of the foam rubber pad in the chamber. Rest the ends of the paper on the end walls of the apparatus and do not immerse them in buffer at this stage. Apply about 10 μl. of hemoglobin solution to the center of the paper in a straight line, working swiftly. A mechanical applicator such as the EC412 (E-C Apparatus Company) or the Spinco applicator is very useful. Another, though less satisfactory, way is to apply the samples as spots. Several samples may be applied to a single strip of paper. One or more controls consisting of known hemoglobins should be included with each run. When all samples are applied, the ends of the paper are bent downward and immersed in the buffer solution. The strips are covered with a Teflon sheet. The lid is closed and the clamping device applied to yield maximum pressure. The clamps on the leveling tubes are closed and the circulation of tap water is started through the top and bottom plates. A current of 250 v. is applied (corresponding to about 6.3 v./cm. potential gradient) for 17 hours.
At the end of the experiment, the current is disconnected, and the lid is tilted carefully to drain off condensate which must not be allowed to come in contact with the paper strips. The Teflon sheet covering the paper strips is lifted off and the ends of the papers are trimmed to the size of the Teflon sheet on which they rest. The strips are allowed to dry in situ on the bottom Teflon sheet. Do not heat the strips.

**Method II. Electrophoresis Using a Freely Suspended Horizontal Strip**

**Apparatus**

Two types of apparatus were used; that described by Grassmann and Hannig (Bender and Hobein, Munich, Germany) and the apparatus manufactured by the A. H. Thomas Company (Philadelphia, Pa.). Strips of filter paper Whatman No. 1, 1½ x 16 inches, are used for either of these. The ends of the paper may be cut as described in Fig. 4, or the uncut paper may be compressed as shown in Fig. 2.

**Reagents**

**Barbital-Glycerol Buffer.** Add 50 ml. of glycerol to 1 l. of the barbital buffer described under method I.

**Phosphate-Glycerol Buffers.** Add 50 ml. of glycerol to 1 l. of the phosphate buffers described in method I.

**Preparing the Chamber**

Buffer is poured into the electrophoresis chamber with the bridge in place. Buffer levels are adjusted by means of a leveling tube, as described for the electrophoresis of serum proteins (35).

**Procedure**

Paper strips, cut as preferred, are dipped in glycerol buffer, blotted, and applied to the bridge. For compressed-paper strips, the glass rod is
lifted slightly and the damp paper is slipped underneath it. Before releasing the glass rod, the paper is gently pulled straight and taut. Care should be taken that the paper strip is compressed over its entire width, on both sides of the bridge. The bridge is placed in the chamber and the lid is closed. A direct current of 150 v. is applied, resulting in a potential gradient of about 6.3, when the A. H. Thomas apparatus is used. When the Grassmann apparatus is used, the voltage-regulator knob should be turned to its maximum (the maximum output of the Grassmann power supply is 115–120 v.). The stopcock on the leveling tube is left open. After 1 hour the hemoglobin samples are applied to the paper strips, at the center, by means of small applicator strips of 2 mm. width as described previously (35). The stopcock on the leveling tube is closed and the hemoglobins are permitted to migrate overnight. The optimal temperature for hemoglobin separations is 10–20°. At temperatures over 30° and below 5° separations are poor. At the end of the migration period, the current is disconnected and the bridge is lifted out of the chamber. The ends of the paper are gently blotted against the bridge and the patterns are allowed to dry at room temperature.

Interpretation of the Patterns

The hemoglobin zones are clearly visible on the paper strips. They may be tentatively identified by inspection and comparison with the reference hemoglobins (Figs. 5 and 6). To measure the amounts of each, the strips may be passed through a densitometer (Photovolt or its equivalent) at 2-mm. intervals, using a 420-μ filter. By plotting the galvanometer readings against distance, a tracing is obtained consisting of one or more gaussian curves. If more than one hemoglobin is present, the tracing may consist of two or more well-defined peaks (Fig. 7) or an extra component may appear as a distortion of a major peak (Fig. 8). In either event gaussian curves are completed for each component, and the areas under each peak are measured by means of a planimeter, as is done for similar tracings of serum proteins. The results may be expressed in relative percentages by dividing the area under each peak by the total area of all peaks combined and multiplying this figure by 100.

The relative percentages of various amounts of hemoglobin S added to hemoglobin A as calculated from electrophoretic patterns are in good agreement with the theoretical concentrations (Table 3). Similar studies of mixtures of hemoglobins A and F showed high hemoglobin F values in concentrations below 30 per cent of the total (Table 4).
Table 3. Recovery of Hemoglobin S from Mixtures of Hemoglobins A and S by Electrophoresis on Paper

<table>
<thead>
<tr>
<th>Hemoglobin</th>
<th>Theoretical (%)</th>
<th>Found from electrophoresis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>70</td>
<td>63</td>
</tr>
<tr>
<td>S</td>
<td>30</td>
<td>37</td>
</tr>
<tr>
<td>A</td>
<td>60</td>
<td>53</td>
</tr>
<tr>
<td>S</td>
<td>40</td>
<td>47</td>
</tr>
<tr>
<td>A</td>
<td>60</td>
<td>58</td>
</tr>
<tr>
<td>S</td>
<td>40</td>
<td>42</td>
</tr>
<tr>
<td>A</td>
<td>50</td>
<td>49</td>
</tr>
<tr>
<td>S</td>
<td>50</td>
<td>51</td>
</tr>
<tr>
<td>A</td>
<td>40</td>
<td>41</td>
</tr>
<tr>
<td>S</td>
<td>60</td>
<td>59</td>
</tr>
</tbody>
</table>

Table 4. Comparison of the Results of the 1-Minute Alkali Denaturation Test According to Singer, and the Relative % Obtained from Electrophoresis on Paper of Known Mixtures of Hemoglobins A and F

<table>
<thead>
<tr>
<th>Hemoglobin F content (%)</th>
<th>Alkali-resistant hemoglobin (%)</th>
<th>Total hemoglobin from electrophoresis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>70</td>
<td>69</td>
<td>77</td>
</tr>
<tr>
<td>60</td>
<td>56</td>
<td>62</td>
</tr>
<tr>
<td>60</td>
<td>60</td>
<td>..</td>
</tr>
<tr>
<td>50</td>
<td>50</td>
<td>46</td>
</tr>
<tr>
<td>40</td>
<td>40</td>
<td>42</td>
</tr>
<tr>
<td>40</td>
<td>40</td>
<td>38</td>
</tr>
<tr>
<td>31</td>
<td>29</td>
<td>35</td>
</tr>
<tr>
<td>21</td>
<td>21</td>
<td>32</td>
</tr>
<tr>
<td>21</td>
<td>22</td>
<td>..</td>
</tr>
<tr>
<td>11</td>
<td>12</td>
<td>26</td>
</tr>
<tr>
<td>11</td>
<td>11</td>
<td>27</td>
</tr>
</tbody>
</table>

FERROHEMOGLOBIN SOLUBILITY

The low ferrohemoglobin solubility of hemoglobin S distinguishes it readily from hemoglobin D which has a high solubility (see Table 2). The only other hemoglobin known at the present time to have a low ferrohemoglobin solubility is hemoglobin H.

Ferrohemoglobin solubility may be measured by a modification of the method of Itano (15).

Equipment

A Coleman Junior spectrophotometer or its equivalent is used. Cuvettes: Coleman # 6-310, 10 x 75 mm., used with # 6-108 adapter.
Reagents

PHOSPHATE BUFFER. Dissolve 16.9 Gm. monobasic potassium phosphate (KH₂PO₄) and 21.7 Gm. dibasic potassium phosphate (K₂HPO₄) or 17.7 Gm. dibasic sodium phosphate (Na₂HPO₄) in carbon dioxide-free distilled water. Adjust the volume to 100 ml.

Procedure

Add the following to small test tubes: 1.8 ml. phosphate buffer, 20 mg. (approximately) sodium hydrosulfite, and 0.2 ml. of buffered hemoglobin solution (see under preparation of hemoglobin solutions). Mix and allow to stand for 15 minutes. A precipitate should form within seconds. Filter through Whatman filter paper No. 5, or its equivalent. Measure into the cuvettes: 3.8 ml. phosphate buffer, and 20 mg. (approximately) sodium hydrosulfite. Add 0.2 ml. hemoglobin filtrate. Mix by inverting twice. Measure absorbancy at 415 mμ.

Control

Measure into a graduated cylinder of 25-ml. capacity 20 ml. distilled water. Add 0.1 ml. hemoglobin solution. Rinse the pipet. Mix by inversion. Transfer about 4 ml. of the solution to a cuvette. Measure the absorbancy of the control solution as described for the unknown.

Calculation

\[
\text{Solubility} \% = \frac{A_{\text{unknown}}}{A_{\text{control}}} \times 100
\]

where \(A\) is the absorbancy.

Results

The solubility of hemoglobins A and F has been found to be 90 per cent or higher by this method. The solubility of S is very low. Figure 9 shows the slope of absorbancy readings for known mixtures of hemoglobins A and S, according to the method described.

WET SLIDES

In order to determine sickling of the erythrocytes, a drop of a suspension of red blood cells in a freshly prepared solution of sodium hydrosulfite (37) is placed on a microscope slide, covered with a coverslip and examined under a microscope after 15–20 minutes.
A drop of a suspension of red blood cells in saline is placed on a second microscope slide, covered with a cover slip and sealed in with Vaseline or Cello-seal. The slide is examined directly in 24 hours and 48 hours for the presence of sickle cells.

ALKALI RESISTANCE

This test is used to distinguish between fetal or alkali-resistant hemoglobins and others, especially A and G. Battle and Lewis (2) have described a slight increase in alkali resistance in their patients with hemoglobin J trait.

Two excellent methods for the determination of alkali resistance have been described (3, 32). The one most commonly used is that of Singer (32).

CHROMATOGRAPHY

A recently developed method for the identification of hemoglobins is chromatography on ion-exchange resins (13, 22, 26). A simple way for carrying out chromatography in flat Lucite cuvettes has been described by Huisman and Prins (13, 26). Hemoglobins A, S, C, E, and F have been separated in this way (11). The behavior of the other hemoglobins has not yet been described. Fetal hemoglobin migrates faster on the resin than hemoglobin A, thereby producing two completely separated

![Graph](image-url)
zones (Fig. 10). Hemoglobins S and C maintain the same relationship as in electrophoresis at pH 8.6 (Fig. 11).

OTHER METHODS FOR THE IDENTIFICATION OF HEMOGLOBINS

Other methods for identifying hemoglobins include salting-out curves, determination of isoelectric points, extinctions at different wavelengths, and determination of the amino acid content of the hemoglobin. These are less practical for routine studies than the methods described.

EXPERIMENTAL

Mixtures of normal adult hemoglobin with various amounts of fetal hemoglobin obtained from cord blood were prepared to correlate the results of the alkali denaturation test with those of electrophoresis. As may be seen, the results of the alkali denaturation test correspond closely to the calculated amounts. However, the electrophoretic results

Fig. 11. Chromatography of hemoglobins on ion-exchange resin IRC-50. Schematic diagram.
* = cotton.
are invariably too high, especially in the presence of small amounts of fetal hemoglobin (see Table 3). Better results would probably be obtained from diapositive readings of chromatographic patterns, as suggested by Huisman and Prins (13, 26), since in the chromatographic patterns two completely separated zones are obtained, whereas in the electrophoretic patterns fetal hemoglobin appears as a shoulder on the peak of adult hemoglobin.

Analyses of mixtures of hemoglobins A and S in varying amounts showed good correlation between the ferrohemoglobin solubility, electrophoretic patterns, and the calculated amounts. In calculating the relative percentages of each hemoglobin the entire area under each gaussian curve was measured, excluding the trail. No attempt was made to correct for losses due to trailing. Trailing increases with the age of the sample (23) and when buffers of pH 7.6 and less are used (20). Also, as the sample ages, the residue remaining on the application line increases. Presumably this is denatured hemoglobin. The greater amount of residue remaining on the site of application when samples of hemoglobin are suspended in water rather than in buffer of pH 8.6 is probably due to the more rapid denaturation of hemoglobin at a lower pH. Perhaps the absence of salts contributes. It was particularly noticeable in samples containing abnormal hemoglobins. It was further noticed that electrophoretic patterns of old buffered hemolysates were characterized by increased diffuseness of the hemoglobin zones (see Fig. 6).

Densitometer readings of electrophoretic patterns of patients with sickle cell traits invariably showed minor peaks in the "trail" about halfway between hemoglobin S and the application line. This area is

<table>
<thead>
<tr>
<th>Patient</th>
<th>Hemoglobin</th>
<th>Unstained (%)</th>
<th>S&lt;sub&gt;a&lt;/sub&gt; (%)</th>
<th>S&lt;sub&gt;b&lt;/sub&gt; (%)</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M.B.</td>
<td>A</td>
<td>69</td>
<td>31</td>
<td>32</td>
<td>68</td>
</tr>
<tr>
<td>S</td>
<td>69</td>
<td>31</td>
<td>32</td>
<td>68</td>
<td></td>
</tr>
<tr>
<td>S.</td>
<td>A</td>
<td>59</td>
<td>31</td>
<td>32</td>
<td>67</td>
</tr>
<tr>
<td>S</td>
<td>59</td>
<td>31</td>
<td>32</td>
<td>67</td>
<td></td>
</tr>
<tr>
<td>B.</td>
<td>A</td>
<td>64</td>
<td>31</td>
<td>32</td>
<td>68</td>
</tr>
<tr>
<td>S</td>
<td>64</td>
<td>31</td>
<td>32</td>
<td>68</td>
<td></td>
</tr>
</tbody>
</table>

* S<sub>a</sub> designates the protein peak in the S area closest to hemoglobin A.

* S<sub>b</sub> designates the protein peak in the S area closest to the site of application.
indistinguishable by visual inspection from the rest of the trail and its significance, if any, is unknown.

Patterns from three patients with sickle cell traits were stained with Amido Black 10B and readings of these patterns at 600 m\(\mu\) were compared with the readings of the unstained patterns at 420 m\(\mu\) (Table 5). In each instance the protein peak in the area of normal adult hemoglobin was narrower than the unstained peak of hemoglobin A. In the area of hemoglobin S, two protein peaks were observed.

**DISCUSSION**

The identification of abnormal hemoglobins should not depend on electrophoresis at pH 8.6 only. Hemoglobins D and S, F and G, J and A, and H and I show similar rates of displacement when subjected to electrophoresis at this pH. Furthermore, the rate of migration of hemoglobins A and F at this pH is so similar that separation is possible only under the most favorable conditions. Other methods must be used therefore to fully identify the hemoglobins. The distinction between hemoglobins S and D may be made by means of sickling tests and ferrohemoglobin solubility. Hemoglobins F and G differ in resistance to alkali denaturation, hemoglobin F being resistant while G is not. Hemoglobins A and J may be separated by electrophoresis in phosphate buffer at pH 7.8. Hemoglobins I and H may be separated by electrophoresis at pH 6.5. At this pH hemoglobin H migrates anodically while I migrates very slowly toward the cathode. They may be further differentiated by their difference in ferrohemoglobin solubility, that of hemoglobin H being low.

**SUMMARY**

Practical physical and chemical procedures available for the identification of hemoglobins have been discussed. These include electrophoresis, chromatography, alkali denaturation, and ferrohemoglobin solubility. The conditions essential for satisfactory electrophoretic separations have been considered and two procedures for paper electrophoresis at pH 6.5, 7.8, and 8.6 using apparatus for compressed paper strips and for freely suspended horizontal strips are presented. A method for the determination of ferrohemoglobin solubility also has been described.

**ADDENDUM**

Since this manuscript was submitted for publication, an article has appeared by O. A. Thorup et al. (*Science* 123, 889, 1956) in which a hemoglobin J is described that differs in many respects from the abnormal
hemoglobin described by Battle and Lewis (2) and referred to in the preceding pages. The official designation of the letter J has been given to the abnormal hemoglobin described by Thorup. This hemoglobin is found to migrate electrophoretically between hemoglobins A and I in buffers varying in pH from 6.5 to 8.6. It is not resistant to alkali denaturation, and it has a solubility in reduced form which exceeds that of hemoglobin A.

REFERENCES