Studies on Abnormal Hemoglobins

I. Their Demonstration in Sickle Cell Anemia and Other Hematologic Disorders by Means of Alkali Denaturation

By Karl Singer, M.D., Amoz I. Chernoff, M.D. and Lily Singer, M.S.

RECENTLY, Pauling and associates demonstrated that hemoglobin obtained from sickle cell anemia erythrocytes differs electrophoretically from normal hemoglobin, the former moving as a positive ion and the latter as a negative one under certain experimental conditions. Evidence was also presented that the red cells in sickle cell anemia contain only the pathologic hemoglobin, whereas the erythrocytes of individuals with the sickle cell trait (sicklemia) show a variable mixture of both the normal and abnormal pigments.

Prior to this discovery, differences between fetal and adult hemoglobin were known to exist. The work of von Körber in 1866 and von Krüger in 1887 established that normal adult hemoglobin denatures more rapidly than fetal in an alkaline medium. It seemed, therefore, of interest to determine whether alkali denaturation procedures could be used to differentiate the abnormal sickle cell hemoglobin from the normal adult compound. In an attempt to devise a method which would yield consistent results, we examined many different buffer systems and alkaline mixtures at various hydrogen ion concentrations and temperatures. Finally, a simple technic was developed whereby an alkaline reagent destroyed normal hemoglobin completely within one minute. When this procedure was applied to hemoglobin solutions prepared from sickling erythrocytes, a fraction, relatively resistant to denaturation, was encountered regularly in sickle cell anemia but not in the typical trait. Two patients lacking a significant degree of anemia but with other manifestations of sickle cell disease, also showed an abnormal pigment. Similar pathologic hemoglobins were found in individuals with the Mediterranean syndromes and in 2 instances of hereditary spherocytosis. Furthermore, in the non-hereditary disorders, alkaline resistant hemoglobins were noted in such conditions as untreated pernicious anemia, chronic regenerative anemia, leukemia, and malignant disease of the marrow.

Material and Methods

All standard hematologic data were obtained with methods mentioned in previous publications.

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This work was carried out with the aid of a grant from the United States Public Health Service and the support of the Hulda B. and Maurice L. Rothschild Foundation for Scientific Research, and the Edna S. Klapman Fund. The Department is also supported in part by the Hematology Research Foundation and the Michael Reese Foundation, Chicago, Ill.
The diagnosis of sickle cell anemia was based on the presence of a marked or moderate anemia, increase of the reticulocyte count and elevation of the serum bilirubin, in addition to a compatible clinical picture and a positive sickling test. The finding of sickled cells in the blood film was taken as corroborative evidence for the existence of this disorder. Most individuals considered to represent instances of the sickle cell trait had entirely normal hematologic values except for the positive sickling phenomenon. Several patients with sickleemia, however, suffered from severe anemia due to other mechanisms, and it was in this latter group that the results of the denaturation procedure were of particular interest. The sickling tendency was demonstrated in all cases by means of the "rapid test," using sodium metabisulfite as a reducing agent. Comparative studies in our laboratory have established that this reagent gives the same reliable results as those previously described using B. subtilis cultures.

The diagnosis of the Mediterranean anemia syndromes was based on the presence of target cells, ovalocytes, basophilic stippling and decreased saline fragility in patients with mild, moderate or severe anemia and the familial occurrence of these anomalies. Some of the blood specimens used were sent to us within twenty-four hours after being drawn, through the courtesy of several other laboratories. Comparative tests on fresh and two day old blood have shown no essential difference in the results of the denaturation procedure.

In addition to these two types of hereditary hemolytic syndromes, several cases of typical familial spherocytosis, chronic aregenerative anemia, pernicious anemia, as well as numerous anemias associated with iron deficiency, infection, hemorrhage, malignancy, hepatic and renal disease, etc., were examined. The diagnosis in the cases of pernicious anemia and of chronic aregenerative anemia was verified by repeated marrow studies.

Normal values for the denaturation tests were established with blood specimens from laboratory personnel and from patients free of any hematologic abnormalities. Since fetal hemoglobin also shows a decreased rate of alkali denaturation, a series of normal infants up to 36 months of age was tested in order to establish the age limit beyond which the occurrence of a resistant pigment, demonstrable by our method, must be considered an abnormality.

**Alkali Denaturation Technic**

Essentially, the method employed consists of exposing a measured quantity of hemoglobin to the action of an alkaline reagent for an exact period of time. The denaturation process is then interrupted by means of a solution which simultaneously lowers the pH and precipitates the non-hemoglobin chromogens. After filtration, the unaltered hemoglobin is determined in a Beckman spectrophotometer at 540 μm and expressed as a percentage of the initial amount of hemoglobin.

**Reagents**

1. Exactly N/12 KOH or NaOH (pH of 12.7). Kept in refrigerator, in paraffin-lined bottles.
2. Precipitating solution: 800 ml. of 50 per cent saturated (NH₄)₂SO₄ plus 2 ml. of 10 N HCl.

An approximate 10 per cent hemoglobin solution is prepared from fresh oxalated or clotted blood obtained by venipuncture. The cells are washed once with normal saline, shaken for five minutes with 1.2 to 1.8 volumes of distilled water (depending on the degree of anemia) and 0.4 volumes of toluene (C.P.) and the mixture centrifuged at 3000 r.p.m.

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* We are indebted to Dr. W. Zuelzer, Detroit, Mich., Dr. C. V. Moore, St. Louis, Mo., Dr. L. K. Diamond, Boston, Mass., Dr. J. F. Suess, Pittsburgh, Pa., Dr. A. Soffer, Rochester, N. Y., and Dr. H. J. Sacks, Indianapolis, Ind. for their cooperation.

† Although we have used a Beckman spectrophotometer throughout this study, equivalent results have been obtained with both the Coleman junior spectrophotometer and the Klett-Summerson photoelectric colorimeter.
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for twenty minutes. The upper two layers are discarded, the clear red solution filtered, and adjusted to an approximate 10 per cent concentration by adding distilled water. The exact hemoglobin concentration is then determined.

One and six-tenths ml. of the alkaline reagent is placed in a serologic test tube and kept in a waterbath at 20 C. for several minutes. One-tenth ml. of the hemoglobin solution is then added, the pipet rinsed six times and the tube gently shaken for ten seconds. A stopwatch is started at the moment the hemoglobin is introduced into the denaturing medium. After exactly one minute, 3.4 ml. of the precipitating solution is added, the test tube inverted six times and the mixture immediately filtered through a double layer of filter paper.

With normal adult hemoglobin the filtrate is found to be colorless, whereas in the presence of resistant compounds a faintly brown to deeply red color may be seen. Thus, the results of this procedure are, usually, immediately apparent to the naked eye. However, we recommend the use of the spectrophotometer, since “colorless” filtrates may still give distinct readings (0.5 to 1.7 per cent of the original hemoglobin concentration). All determinations were performed at least in duplicate and the figures averaged. When values in the range of 1.7 to 2.4 per cent were found, the mean of six determinations was taken as the final result.

**TABLE 1.—Effect of Various Concentrations of Hemoglobin on the One Minute Denaturation Values**

<table>
<thead>
<tr>
<th>Normal Hemoglobin</th>
<th>Fetal Hemoglobin</th>
<th>Sickle Cell Anemia Hemoglobin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Concentration</strong></td>
<td><strong>1 Minute Den. Value</strong></td>
<td><strong>Concentration</strong></td>
</tr>
<tr>
<td>Gm.%</td>
<td></td>
<td>Gm.%</td>
</tr>
<tr>
<td>11.8</td>
<td>1.2</td>
<td>12.0</td>
</tr>
<tr>
<td>10.9</td>
<td>1.3</td>
<td>10.9</td>
</tr>
<tr>
<td>10.0</td>
<td>1.3</td>
<td>9.9</td>
</tr>
<tr>
<td>9.0</td>
<td>1.2</td>
<td>9.0</td>
</tr>
<tr>
<td>8.0</td>
<td>1.1</td>
<td>8.0</td>
</tr>
</tbody>
</table>

**Discussion of method**

The following aspects of the method deserve special comment.

1. Repeated washings of the erythrocytes prior to the preparation of the hemoglobin solutions did not influence the results of the denaturation procedure.

2. The spectrophotometric readings obtained after denaturing normal hemoglobin solutions for one minute are probably not caused by a particularly alkali-resistant type of hemoglobin, since exposure to the alkaline reagent for twenty-four hours did not significantly reduce these values. The residual material gave a positive benzidine test in both the “one minute” and “twenty-four hours” filtrates. The benzidine test had to be modified, because of the interference of the \((NH_4)_2SO_4\) in the solution, by removing the latter with saturated BaCl_2. Prolonged centrifugation as well as precipitation with higher concentrations of \((NH_4)_2SO_4\) did not alter the results. Addition of sulfosalicylic acid to the acidified filtrate produced definite turbidity. Spectrophotometric analysis of this material was not feasible because of the small amounts available. Prolonged denaturation of fetal and other resistant hemoglobins leads to the same “residual” spectrophotometric readings as observed with normal pigment, probably due to the same material. Until more is known about this substance the results are reported without correction for this factor.

3. Numerous investigators using the method of alkali denaturation have pointed out the importance of adequately controlling not only the pH and temperature of the solution...
but also the concentrations of the reactants. Small variations in any of these factors may significantly alter the rate of denaturation. At a pH of 12.7, however, we have met with little difficulty in obtaining constant results.

Preparation of an exact 10 per cent hemoglobin solution was found to be unnecessary since varying the concentration from 9 to 11 per cent yielded essentially identical values. This was noted with solutions of both normal and abnormal hemoglobins. Examples of such experiments are presented in table 1. These studies were undertaken to rule out the possibility that small variations in the amounts of hemoglobin introduced into the unbuffered reagent could significantly alter the pH of the solution.

Temperature fluctuations from 19 to 21 C. have no appreciable influence on the results.

The advantage of the procedure employed in this study lies in its ability to denature normal hemoglobin completely within one minute, thus facilitating the detection of small amounts of more resistant pigments also present in the hemoglobin solutions. The use of a glycine-NaOH mixture at pH 12.7 is not necessary, since results with this buffer system were identical with those obtained with N/12 NaOH or KOH alone. At lower hydrogen ion concentrations (pH 11.7), the reaction is appreciably slower and much more difficult to control so that complete denaturation of the normal component requires a considerably longer period of time and makes identification of small quantities of abnormal pigment unreliable.

**Results**

1. Normals

   A. One hundred hematologically normal individuals above the age of three years were tested. Figure 1 shows the frequency distribution of the values found. Although the filtrate was always "colorless" to direct observation, spectrophotometric readings from 0.5 to 1.7 per cent of the original hemoglobin
concentration were still obtained. Statistical analysis showed a mean value of 1.0 per cent with a standard deviation, $\sigma$, of 0.32. Using $3\sigma$ as the "level of significance," a value of 2 per cent may thus be considered outside the range of normal, such a finding occurring by chance only once in 370 determinations.

B. Since fetal hemoglobin is alkali resistant, it was deemed necessary to determine the age beyond which this embryonic pigment is no longer demonstrable. Data in the literature\textsuperscript{15, 17, 19, 21} seem to indicate that fetal hemoglobin disappears completely within the first seven months of life. As can be seen from figure 2, with the procedure used in this study distinct amounts of resistant pigment were still noted in some healthy infants as late as two years after birth. This finding we attribute to the greater sensitivity of our method which removes all the adult hemoglobin from the solution before appreciably affecting the resistant fraction, thus permitting the detection of even small quantities of the latter. Since, as will be described below, abnormally denaturing hemoglobins are also detectable in a variety of hematologic disorders, evaluation of such results becomes feasible only after complete disappearance of the physiologically occurring resistant hemoglobin.

2. Sickle Cell Trait

Sixty carriers of the sickle cell trait were examined. Figure 3 demonstrates that the range of the one minute denaturation values was identical with that found in normals. The infants below the age of two years with a positive sickling test but no anemia are not included in this group because of the presence of fetal hemoglobin (figure 2).
3. **Sickle Cell Anemia**

Thirty-six cases of sickle cell anemia were investigated. The results are summarized in figure 4. In almost all instances the filtrates appeared distinctly colored by varying concentrations of unaltered hemoglobin. In those showing values below 2.5 per cent only a faint yellowish tinge was noticeable. The amounts of unaffected pigment, spectroscopically identified as oxyhemoglobin, showed a spread from 2.0 to 23.9 per cent. No clear-cut correlation was discernible with either the severity of the anemia or the reticulocyte count. It may be noted that only 3 of the 36 cases (11, 15 and 30) had relatively low one minute denaturation values (2.2, 2.0 and 2.2 per cent respectively) which were definitely but not strikingly outside of the normal range. In one of these cases (15), the highest reticulocyte count encountered in this series was present.

![Frequency distribution of one minute denaturation values in sickle cell trait](image)

**Fig. 3.**—Frequency distribution of one minute denaturation values in sickle cell trait expressed in percentage of 60 cases studied.

Patients 34 and 36 were infants aged 1 ½ and 1 year respectively, who exhibited the classical signs of sickle cell anemia. They are, therefore, included in this group, although their resistant pigment could still be interpreted as being merely due to the presence of the physiologically occurring fetal hemoglobin.

The great variations in the amounts of undenatured pigment observed in these patients with sickle cell anemia are of particular interest. These results cannot be explained on the basis of inherent errors of the method used. This is well illustrated in table 2 in which the denaturation values of 3 persons with sickle cell anemia, determined daily for five consecutive days, are listed. When repeated determinations were carried out during a six months period no essential changes occurred. Thus the one minute denaturation value of an individual with sickle cell anemia seems to remain relatively constant. Transfusions, how-
ever, tend to lower this figure temporarily in proportion to the quantity of normal blood given (Cases 2 and 7, figure 4).

Two cases listed in figure 4 require special comment. Case 22, the mother of Patient 21—a child with well established sickle cell anemia—is a 39 year old woman who apparently never developed obvious manifestations of sickle cell disease. Although her hemoglobin was 13.2 Gm. with 4.4 M. erythrocytes, her reticulocyte count was 2.8 per cent (normal level in our laboratory up to 1.5 per cent) in the absence of any gross bleeding. A few sickled erythrocytes and

FIG. 4.—One minute denaturation values in sickle cell anemia.

<table>
<thead>
<tr>
<th>Specimen</th>
<th>One Minute Denaturation Value on</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1st</td>
<td>2nd</td>
</tr>
<tr>
<td>Normal:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Case 1</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Case 2</td>
<td>1.1</td>
<td>0.9</td>
</tr>
<tr>
<td>Sickle Cell Anemia:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Case 7</td>
<td>13.6</td>
<td>12.5</td>
</tr>
<tr>
<td>Case 9</td>
<td>4.2</td>
<td>4.0</td>
</tr>
<tr>
<td>Case 10</td>
<td>5.3</td>
<td>5.5</td>
</tr>
</tbody>
</table>
target cells were seen in the stained film. There was decreased osmotic fragility with hemolysis completed at 0.16 per cent NaCl. Marrow examination showed an erythroid-myeloid ratio of 1:2. On two occasions her one minute denaturation value was 13.4 and 13.5 per cent, respectively. The possibility that this case may represent a compensated form of sickle cell anemia must be entertained. In order to classify the existing abnormality properly, further studies of the pigment metabolism, erythrocyte survival time, and electrophoretic pattern of the hemoglobin of this patient are needed.

Case 35 is a 22 year old male who has normal hemoglobin and red cell levels, 2 per cent reticulocytes, and many target cells but no sickled erythrocytes in the blood film. Examination of the marrow revealed an erythrocytic hyperplasia, the erythroid-myeloid ratio being 1.5:1. There was a slight hyperbilirubinemia (1.2 mg. per cent), while liver function tests were entirely normal. No evidence of bleeding was apparent. The one minute denaturation value was 4.3 per cent. As in the case mentioned above, this picture may be interpreted as being a compensated form of sickle cell anemia. A possible combination of a sickle cell trait with a Mediterranean syndrome, which has been reported recently, may also be considered, since in the latter disorder, abnormal hemoglobins may also be found.

4. Mediterranean Anemia Syndromes

Figure 5 summarizes the data obtained in 12 cases of these disorders. It is now well recognized that all kinds of transitions exist between the severe Cooley anemia and the asymptomatic form of thalassemia, which can be identified...
only by special laboratory procedures.\textsuperscript{11-13} As can be seen from figure 5, all patients with severe or moderate anemia have definite pathologic one minute denaturation values. Individuals with minimal hematologic alterations show either normal or slightly elevated figures.

\begin{table}
\centering
\caption{Denaturation Values in Miscellaneous Hematologic Disorders}
\begin{tabular}{|l|c|c|c|c|}
\hline
\textbf{Diagnosis} & \textbf{Number of Cases} & \textbf{Positive Sickling Test} & \textbf{Abnormal Denaturation Values} & \textbf{Remarks} \\
\hline
Chronic iron deficiency & 11 & 4 & none & \\
Acute blood loss & 31 & 6 & none & \\
Anemia associated with chronic infections & 18 & 2 & none & \\
Anemia associated with renal disease & 13 & 1 & none & \\
Anemia associated with hepatic disease & 10 & 0 & none & \\
Anemia associated with generalized lupus & 2 & 0 & none & \\
Acquired L diaphoric spherocytic anemia & 3 & 0 & none & \\
Leukemias: & & & & Aged 4 and 49 yrs. \\
\hspace{0.5cm} (1) acute & 2 & 0 & 3.2 and 4.3\% & \\
\hspace{0.5cm} (2) chronic & 11 & 0 & 1 patient: 2.6\% & \\
\hspace{1cm} & & & 5 patients: 1.8-2.0\% & \\
Lymphoma (sarcoma, Hodgkin's disease, etc.) & 6 & 0 & none & \\
Carcinoma & 38 & 0 & 2 patients: 2.2 & Malignant cells demonstrable in marrow aspiration in both cases. \\
\hspace{2cm} and 2.5\% & & & Three of these patients also showed metastatic involvement of marrow. \\
\hspace{2cm} & & & Hgb. 7.6 Gm., RBC 2.7 M. \\
\hspace{2cm} & & & Age 69 yrs., sickle cell test positive. The other 2 patients had no anemia. \\
Multiple myeloma & 3 & 1 & 1 patient: 2.1\% & \\
Pernicious anemia: & & & & Aged 3\frac{1}{2}, 5 and 13 yrs. \\
\hspace{0.5cm} (untreated) & 2 & 0 & 1.8 and 2.2\% & \\
\hspace{0.5cm} (treated) & 4 & 0 & none & \\
Polycythemia & 4 & 0 & none & \\
Elliptocytosis & 4 & 0 & none & \\
Chronic regenerative anemia & 3 & 0 & 2.5, 2.7 and 5.0\% & \\
\hline
\end{tabular}
\end{table}

5. \textit{Hereditary Spherocytosis}

Seven patients with spherocytic hemolytic anemia were tested (figure 6). Six had a well-documented family history, while in one case (7, figure 6) no such data were available. However, the clinical and hematologic symptoms and signs,
the negative Coombs test and the response to splenectomy support this diagnosis. Patients 1 and 2, members of the same family, were the only ones with an elevated one minute denaturation value, established on three separate occasions. In the remaining subjects, the hemoglobin denatured normally. These results suggest that hereditary spherocytosis may be divided into two subtypes which can be differentiated by alkali denaturation, but are clinically indistinguishable.

6. Miscellaneous Hematologic Conditions

The denaturation test was also performed in 165 patients with a great variety of hematologic disorders. The results are summarized in table 3. In most of the conditions listed only normally denaturing pigments were observed. Some of the anemias (caused by iron deficiency, hemorrhage, etc.) occurred in individuals with the sickle cell trait and it was in these cases that the denaturation procedure was sometimes quite helpful in excluding sickle cell anemia.

Definitely elevated denaturation values were encountered in untreated pernicious anemia, chronic aregenerative anemia, acute and chronic leukemia and in malignant disease involving the marrow. In most instances the amount of resistant pigment is relatively small but, nevertheless, outside the normal range. In some cases, only traces of abnormal hemoglobin were found (values between 1.8 and 2 per cent).

Of particular interest was the detection of a resistant hemoglobin fraction in 3 children with chronic aregenerative anemia (pure red cell anemia). This syndrome is characterized by severe hypoplasia of the erythron but no apparent pathology in the production of granulocytes and platelets. Each of these patients require frequent blood transfusions to maintain hemoglobin levels of about 5 Gm. Whereas in 2 of these patients (aged 3½ and 5 years) the disease developed shortly after birth, the third, a 13 year old boy, experienced the onset of this malady within the past year. Recently, ACTH therapy caused a rise in the red cell count from 1.5 to 4.0 M. with a corresponding increase in hemoglobin from 4.6 to 12.8 Gm. in this latter patient. However, the quantity of abnormally denaturing hemoglobin (5.0 per cent) remained unaffected by this treatment.

The definitely abnormal denaturation values of 3.2 and 4.3 per cent in 2 patients with acute leukemia (aged 4 and 49 years) lend some significance to the frequency with which “borderline results” were observed in cases with well established leukemias and malignant disease of the marrow. These 2 leukemic patients showed a moderately severe degree of anemia (10.6 and 7.6 Gm., respectively) which was apparently not hemolytic in type (negative direct and indirect Coombs and trypsinized red cell tests).

In one patient, a 69 year old Negress with proven multiple myeloma and a

* Recently, another case of hereditary spherocytosis with a one minute denaturation value of 3.7 per cent has been encountered.
† These cases will be discussed in detail in another publication.
severe anemia (Hgb. 7.6 Gm. and RBC 2.7 M.), an elevated one minute denaturation value of 2.0 per cent was observed in the presence of a positive sickling test. Although such a finding may occur in sickle cell anemia, this case demonstrates that a positive denaturation test may also be obtained in instances of the trait complicated by malignant involvement of the marrow.

Although many more instances of hematologic disorders remain to be investigated, the findings thus far obtained signify that the appearance of alkali resistant hemoglobins is not confined to the hereditary hemolytic syndromes but may also occur in some acquired disorders.

**DISCUSSION**

Our studies clearly establish that an alkali resistant hemoglobin is regularly encountered in sickle cell anemia but is lacking in carriers of the trait. Abnormally denaturing hemoglobins were also found in the more fully developed Mediterranean syndromes and in one family with hereditary spherocytosis. Furthermore, such pathologic pigments were observed in 3 instances of chronic aregenerative anemia as well as in some cases of acute and chronic leukemia, myelophthisis anemia and pernicious anemia.

During the course of these investigations our attention was drawn to publications of several Italian workers who, using a technic different from our own, found that the hemoglobin in Cooley's anemia and in 3 cases of sickle cell anemia required a considerably longer period of time for complete denaturation than did the normal pigment. By means of the method described in the present communication, it has been shown, (1) that normally denaturing hemoglobins disappear completely within one minute; (2) that when abnormally denaturing pigments are demonstrable, a mixture of normally and abnormally denaturing compounds is always present; and (3) that the amount of the resistant hemoglobin varies considerably in different individuals with the same disease.

Our experiments were originally undertaken in order to determine whether the hemoglobin in sickle cell anemia, which has been shown to exhibit an abnormal electrophoretic behavior, would manifest a corresponding abnormal resistance to alkali denaturation. Pauling and his associates have convincingly demonstrated that sickling erythrocytes contain a hemoglobin (here designated as type S) that moves as a positive ion in the Tiselius apparatus in contrast to the normal compound (type N) which moves as a negative ion. In sickle cell anemia, these investigators found only the pathologic pigment (100 per cent type S) whereas in the trait cells a mixture of N and S hemoglobins was always present, the latter varying in concentration from 23 to 45 per cent. With the denaturation procedure, hemoglobin solutions prepared from sickle cell anemia cells reveal a resistant fraction in amounts from 2 to 24 per cent, but the trait

* Since this paper was submitted, Wells and Itano (J. Biol. Chem. 188:65, 1951) reported the occurrence of up to 20 per cent of an electrophoretically normal compound in hemoglobin solutions prepared from sickle cell anemia erythrocytes. The similarity between their findings and our results for resistant hemoglobin may be noted.
erythrocytes do not contain an abnormally denaturing pigment. From these data it must be concluded that no correlation exists between the electrophoretic and denaturation patterns of sickle cell hemoglobin. It should be pointed out that electrophoresis measures only one property of proteins, that is, mobility in an electric field. Molecules, contained in a protein fraction, which, on the basis of mobility, are apparently homogeneous, may show differences in shape, weight, size and chemical composition. Thus, the findings obtained by electrophoresis and denaturation are not contradictory but rather supplementary.

Whereas the electrophoretic anomaly noted in the hemoglobin of sickling erythrocytes seems to be related to their ability to assume the characteristic change in shape, the functional significance of the abnormally denaturing fraction is not clearly discernible. No obvious relationship can be noted between the quantities of alkali resistant pigment and either the clinical severity of the anemia or the reticulocyte count.

Watson has pointed out that only a very small percentage of the red cells of the newborn exhibit the sickling tendency and has attributed this finding to the presence of fetal hemoglobin. Concomitant with the replacement of the embryonic pigment by the adult compound, the number of sickling cells increases. These observations seem to imply (1) that fetal hemoglobin is incapable of sickling and (2) that in the growing infant individual erythrocytes probably contain either fetal or adult hemoglobin but not a mixture of both.

At present, the evidence available permits the distinction of three different types of hemoglobin by means of electrophoresis and denaturation. Type N shows no abnormality with either method, type S is electrophoretically abnormal, and fetal hemoglobin (type F) is resistant to alkali denaturation (figure 7). The alterations in the hemoglobin molecules of type S and F are probably located in the protein moiety of the pigments.

The problem arises whether the alkali resistant hemoglobin fraction, regularly demonstrable in sickle cell anemia, represents a continued production of fetal pigment beyond the period during which the latter is physiologically detectable. Assuming that this resistant fraction is actually of the fetal type—and some evidence concerning this assumption will be given in the companion paper—one must postulate that the resistant pigment, in patients with the anemia, is always associated with S hemoglobin in the individual erythrocytes, since, under optimal conditions, all red cells can be induced to sickle. Thus, according to this hypothesis, all, or at least some sickle cell anemia erythrocytes would contain a variable mixture of S and F hemoglobins, similar to the arrangement in the trait cells which contain a mixture of the N and S compounds. The possible distribution of these different types of hemoglobin may be seen in the diagrams in figure 7.

On the other hand, the resistant fraction may represent a quite different type of hemoglobin. One may postulate that the electrophoretically detectable alterations in the hemoglobin molecule responsible for the sickling phenomenon can occur independently of those changes accounting for the increased resistance to alkali denaturation. Consequently, a hemoglobin molecule may show either
one of these aberrations or a combination of both. From the standpoint of this concept two types of S hemoglobin could be assumed to exist. Type $S_1$ would denature at a normal rate, but would differ from $N$ hemoglobin by its mobility in an electric field; type $S_2$ would show both abnormal mobility and increased resistance to denaturation, but would differ from $F$ hemoglobin in its ability to cause sickling. Trait cells would then contain a mixture of $N$ plus $S_1$ and at least some anemia erythrocytes, a mixture of $S_1$ plus $S_2$ (figure 7). Whether the alkali resistant fraction is distributed in all anemia erythrocytes or occurs only in some is unknown. This may become clear from a simultaneous study of the survival times and denaturation values of transfused sickle cell anemia cells.

Which of these possibilities is valid, cannot be decided at the present time. Only a combined quantitative study of the $S$ and $F$ hemoglobins by means of electrophoresis and denaturation procedures will bring about elucidation of these problems.

Our experience with almost one hundred individuals exhibiting the sickling phenomenon seems to indicate that an alkali resistant pigment is found only in the anemia but not in the trait. Since the amount of the abnormally denaturing hemoglobin is occasionally relatively small, it is conceivable that cases may be encountered without this fraction. In 2 instances we have found abnormal denaturation values with normal hemoglobin and red cell levels. As has been pointed out, however, these patients showed definite clinical and hematologic abnormalities not seen in the typical trait; these observations may, therefore, be interpreted as representing compensated or exceptional forms of sickle cell anemia.

The denaturation test promises to become an aid in differentiating sickle cell anemia from anemia due to other mechanisms occurring in carriers of the trait. It must, however, be kept in mind that the test is not specific for sickle cell anemia, since abnormally denaturing pigments may be found, physiologically, until the end of the second year of life, and pathologically, in certain hemato-
logic disorders referred to previously. Thus, interpretation of the results still requires careful consideration of the entire clinical picture.

The demonstration of an alkali resistant pigment in the Mediterranean syndromes adds another feature to the similarities between sickle cell anemia and thalassemia. Unlike the situation in sickle cell disease, where the trait and anemia are in most instances sharply demarcated, the Mediterranean disorders show all kinds of transitions from the most severe anemia to the asymptomatic form. Although the number of cases examined is too small to permit any definite conclusions, it is our impression that there exists a rough correlation between the severity of the hematologic findings and the elevations of the denaturation values. Asymptomatic cases with only a few target cells and decreased osmotic fragility usually show a normally denaturing hemoglobin.

The denaturation test may become valuable in differentiating thalassemia from iron deficiency anemia provided that the limitation of the procedure as a nonspecific method for demonstrating resistant hemoglobin is taken into consideration.

The finding of an abnormal hemoglobin in one family with typical hereditary spherocytosis, but not in others with equally classical symptomatology, also uncovers a new aspect of this disease. The association of a pathologic pigment and an abnormal shape of the erythrocytes in the same individual seems to furnish indirect evidence for the hypothesis that spherocytes, in familial hemolytic jaundice, originate in the marrow under genetic control. Splenectomy apparently had no influence on the production of the abnormal compound (Case 1, figure 6). That this splenectomized individual now exhibits normal hemoglobin and red cell levels is in keeping with the well-known fact that spherocytes, in familial hemolytic jaundice, are selectively destroyed by the spleen. Following this operation signs of increased hemolysis are no longer detectable. However, it has been convincingly demonstrated that after splenectomy, production of abnormal red cells continues, since, when transfused into normal recipients, these erythrocytes exhibit a shortened survival time. Thus, the presence of a pathologic pigment is again indicative of an intracorpuscular defect of the red cells.

Since all three hereditary hemolytic syndromes seem to show resistant hemoglobins, one must consider the previously mentioned hypothesis that in these disorders fetal hemoglobin production may persist beyond the physiologic age limit. An attempt to support this concept may be found in the second communication of this series. Persistent formation of fetal hemoglobin may also be assumed for the 2 children with chronic idiopathic regenerative anemia in whom the disorder developed shortly after birth. In the third patient, in whom the disease did not appear until the age of 13, this explanation does not seem a satisfactory one. One may, however, speculate that in conditions where the erythron is under severe chronic stress, a revival of the production of fetal hemoglobin may take place. The irregular appearance of resistant hemoglobins in small amounts in “acquired” diseases like leukemia, myelophtisisic anemia and pernicious anemia may then be explained in a similar fashion.
Although we are as yet not in the position to offer a well supported view on the significance of the observed alkali resistant hemoglobins in the various syndromes, we believe that this phenomenon is of some clinical importance. A positive denaturation test in the absence of the hereditary hemolytic syndromes may indicate a serious hematologic disorder.

SUMMARY

1. By exposing hemoglobin solutions to an alkaline reagent at a pH of 12.7 it was found that normal pigment is completely denatured within one minute. Thus even small amounts of more resistant hemoglobins, which may also be present in the solution, can be readily detected. Fetal hemoglobin, which is alkali resistant, may remain demonstrable until the end of the second year of life.

2. Alkali resistant hemoglobins were regularly encountered in sickle cell anemia (but not in the trait), in the more fully developed Mediterranean syndromes, and in 1 out of 4 families with hereditary spherocytosis. In addition to these hereditary hemolytic disorders, abnormally denaturing hemoglobin fractions were observed in 3 instances of chronic aregenerative anemia, and, irregularly, in patients with untreated pernicious anemia, acute and chronic leukemia and myelophthisic anemia. All other kinds of anemias were found to have only normally denaturing pigments.

3. Three definite types of hemoglobin are identifiable at present by means of electrophoresis and denaturation. These have been designated as type N (normal adult), type F (fetal), and type S (sickle cell hemoglobin). The hypothesis is advanced that the resistant fraction in the hereditary hemolytic syndromes may represent a continued production of fetal pigment beyond the physiologic age limit and the appearance of the abnormal hemoglobins in the "acquired" disorders may indicate a reactivation of such a mechanism. The implications of such an assumption for the distribution of the various types of hemoglobin in sickling erythrocytes are discussed.

4. The diagnostic significance of the denaturation test and its limitations are outlined.

REFERENCES

STUDIES ON ABNORMAL HEMOLOBINS. I.


Studies on Abnormal Hemoglobins: I. Their Demonstration in Sickle Cell Anemia and Other Hematologic Disorders by Means of Alkali Denaturation

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