Rate of Sickling of Red Cells during Deoxygenation of Blood from Persons with Various Sickling Disorders

By Samuel Charache and C. Lockard Conley

Sickled erythrocytes are rapidly removed for the circulation, an occurrence that accounts for the hemolytic aspects of sickle cell disease. Anemia is a usual result but is often asymptomatic and is not the principal cause of the high morbidity and reduced life span of affected persons. Many of the clinical manifestations of sickle cell anemia have been attributed to the increased blood viscosity and impaired blood flow caused by deformation of deoxygenated erythrocytes. Red cells of asymptomatic persons with sickle cell trait (A-S) require a lower partial pressure of oxygen for production of sickling than do red cells of patients with sickle cell anemia; presumably physiologic oxygen tensions are not sufficiently low to cause intravascular sickling of red cells of A-S heterozygotes. When persons with sickle cell trait are exposed to conditions in which there is regional or general hypoxia, symptoms of sickle cell disease may occur. Persons heterozygous for hemoglobin S and also for hemoglobin C or thalassemia (S-C, S-Thal) have diseases that usually are less severe than sickle cell anemia; red cells of these persons sickle at intermediate oxygen tensions. Nevertheless, correlations between severity of the clinical disorders and measurements of blood viscosity and of sickling in vitro have not been very precise; in fact, the primary role of the sickling phenomenon in the pathogenesis of clinical manifestations has been questioned.

This study was designed to define further the relationships between clinical status, sickling of red cells and viscosity of blood. Time is an important determinant of the occurrence of sickling, an observation recorded by early students of the phenomenon. Consideration of the velocity of blood flow through deoxygenated areas suggests that the rate at which sickling occurs has greater physiologic relevance than the degree of sickling finally achieved after prolonged exposure of blood to low oxygen tensions. Therefore, comparative studies were made of the kinetics of sickling during deoxygenation of blood from persons with various sickling disorders.

METHODS

The persons studied were patients from the Hematology Clinic and their relatives. In each case a characteristic electrophoretic pattern of hemoglobin was obtained on filter paper at pH 8.6 and on agar gel at pH 6.2. Fetal hemoglobin was measured by an alkali-denaturation technic. Sickling of red cells was demonstrated in sealed preparations.

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of blood mixed with 2 per cent sodium metabisulfite. Diagnoses were supported by family studies. All of the patients with sickle cell-thalassemia were Negroes; in each case a non-sickling parent or child was demonstrated to have a value for hemoglobin A2 exceeding 4.6 per cent. Hemoglobin A2 was measured spectrophotometrically after elution from a starch block on which electrophoretic separation had been achieved. The Caucasian patient with sickle cell-hemoglobin D disease has been described previously; the hemoglobin D was identified as D\textsubscript{Punjab}. The J-S heterozygote is a member of a family being described elsewhere; the hemoglobin J has been designated J\textsubscript{Baltimore}. Information pertaining to the S-F heterozygotes is contained in other reports.

Blood was collected in Ehrlenmeyer flasks, using 0.13 M EDTA (disodium salt) as an anticoagulant (0.3 ml. EDTA/10 ml. blood). The specimens usually were stored for 16-18 hours at 4 C. before studies were performed. In five experiments the rheological characteristics of blood stored in this manner did not differ from those of fresh blood. At the time of study, the uncapped flask was mechanically rotated in room air until the blood became bright red. The microhematocrit value was then determined, and the volume of packed red cells was adjusted to the desired value by addition or subtraction of autologous plasma.

To achieve deoxygenation, 2.5 ml. samples of blood were placed in Carrel flasks (3.5 cm. diameter) which were capped with vaccine bottle stoppers. Four to six flasks were mounted in a shaking incubator maintained at 37 C. with only the necks of the flasks protruding above the water level. Gas mixtures that had been bubbled through water at 37 C. were blown into each flask at a constant rate of flow (0.5 liter/flask/min.) and allowed to exhaust through an 18-gauge needle. The flasks were shaken 113 times per minute. Three gas mixtures were used, with oxygen in proportions by weight of 0, 3 and 20 per cent respectively; each contained 5 per cent CO\textsubscript{2} and the remaining gas was nitrogen. The approximate partial pressure of CO\textsubscript{2} in the flasks was 35 mm. Hg and that of oxygen 0, 20 or 140 mm. Hg. On two occasions the pH of blood treated in this manner was 7.46 as measured with a Beckman pH meter.

After the desired period of exposure to the flowing gas mixture, blood was removed from the Carrel flask with a greased syringe. About 0.25 ml. was injected into a solution of 10 per cent formalin in 0.85 per cent NaCl solution for determination of the proportion of sickled cells. The shape of red cells was unchanged after storage in formalin-saline for many weeks. Samples were coded to prevent bias in counting, and 200 erythrocytes were enumerated using an oil immersion objective. Only cells with sharp projections or a long axis more than 3 times the length of the short axis were considered sickled. "Filamentous" forms were more easily identified than "holly leaf" forms which sometimes resembled crenated cells. Virtually all red cells of patients with sickle cell anemia appeared sickled in sealed preparations with sodium metabisulfite; but 100 per cent sickling was never encountered in blood exposed as long as 2 hours to gas containing 0 per cent oxygen, although simultaneous measurements of oxygen saturation demonstrated at least 90 per cent desaturation. To determine the reproducibility of sickle cell counts a single deoxygenated sample containing predominantly holly leaf forms in formalin-saline was divided into six coded aliquots, and these were examined among an equal number of other coded samples. The mean and standard deviation of the counts was 52 ± 2.5 sickled cells/100 erythrocytes.

Viscosity was estimated with a falling ball viscometer similar to that described by Allison. Steel balls in diameter were allowed to fall through a column of fluid within an open-end glass tube of internal diameter 0.125 ± 0.0003.َ

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*Dubnoff Metabolic Shaking Incubator, Precision Scientific Corp., Chicago, Ill.
†Puritan Compressed Gas Corp., Baltimore, Md.
‡HiVac Silicone Grease, Dow Corning Corp., Midland, Mich.
§Fafnir Bearing Co., New Britain, Conn.
||Fischer-Porter Co., Warminster, Pa.
A single glass tube was used; it was fixed in a vertical position in a water bath at 37 C., and samples were injected into the lower end through a thin polyethylene tube. Before blood was introduced from the syringe, the glass and polyethylene tubes were thoroughly rinsed with the gas mixture to which the blood had been exposed. The ball was raised with a magnet to a position 1 cm. above a line etched on the glass tube. The magnet was removed, and a stopwatch was employed to record the time required for the ball to fall between the first etched line and another 10 cm. below it. The ball rapidly attained constant velocity; the distance above the first line at which the ball was released did not affect the measurement if it was 1 cm. or more. This type of viscometer was selected because of the small blood sample required (1 ml.) and because of the ease with which the partial pressure of gases could be maintained during measurements. The dimensions of this instrument differ from those of a conventional falling sphere viscometer. Therefore, the viscosity of a series of sucrose solutions of known relative viscosity was measured with the device. The measurements (fig. 1) demonstrate that the viscometer is sensitive to changes in viscosity above an observed relative viscosity of 1.5; above this value the relationship between published and observed relative viscosities is linear. Almost all measurements on deoxygenated blood samples were made within this linear range. Blood is a non-Newtonian fluid, and because shear rate could not be controlled, data are recorded as the time in seconds required for the ball to fall 10 cm., rather than as units of absolute or relative viscosity. The steel balls used in these experiments were selected because they were "perfect" spheres, but they varied slightly in diameter, and the time for different balls to fall through distilled water varied between 10 and 11 seconds. Therefore, the same ball was used in each series of experiments; replicate measurements of the viscosity of water from day to day agreed within 0.2 seconds. Because the rate of fall was at times influenced by barely visible bubbles, the viscosity of each blood sample was measured repeatedly to ensure that three consecutive determinations were in agreement. Results were considered satisfactory when differences did not exceed 0.5 seconds for a time of fall of less than 1 minute, 1 second for a time of fall of less than 2 minutes, and 5 seconds for times greater than 2 minutes.

Hemolysates were prepared from red cells washed 3 times with 0.85 per cent NaCl solution; carbon tetrachloride was used as a hemolyzing agent. Hemolysates were not dialyzed. The proportions of various hemoglobins were measured after electrophoretic separation and elution from starch blocks; hemoglobin F was estimated by the alkali-denaturation method; and the proportions of hemoglobins S and D in the S-D patient were estimated after electrophoresis on agar gel as previously described. The solubility of deoxygenated hemoglobin in 2.24 M phosphate buffer was determined by the method of Itane, with the modification that the final solution of ferrohemoglobin was filtered once through filter paper (Whatman #42) before conversion to carboxyhemoglobin. In studies of the minimum concentration of hemoglobin required for gelation, hemolysates were diluted with distilled water. Two ml. samples were exposed to 0 per cent oxygen in Carrel flasks for 15 minutes; no sample was deoxygenated more than once. Gelation was considered to have occurred when more than 15 minutes was required for the ball of the viscometer to fall through the 10 cm. path. Hemoglobin concentration was determined only after hemolysates were reoxygenated.

The oxygen saturation of blood was measured by the method of Nahas, but Triton X-100 was used as a hemolyzing agent. Mean corpuscular hemoglobin concentration (MCHC) of oxygenated blood was calculated employing the hematocrit value obtained in Wintrobe tubes centrifuged for 30 minutes at 3000 rpm in an International Centrifuge, Model SBV-1, and the hemoglobin concentration determined with a Beckman spectrophotometer, Model B, using the cyanmethemoglobin method and commercial standards. Mean corpuscular sickle hemoglobin

*Acuglobin, Ortho Pharmaceutical Co., Raritan, N. J.
Fig. 1.—Viscosity of solutions of sucrose. Relative viscosity (viscosity of sample/viscosity of water) of a series of sucrose solutions was measured with the falling-sphere viscometer and related to the known relative viscosity of sucrose solutions.

Results

Effect of Deoxygenation on Viscosity of Blood from S-S Homozygotes and from A-S Heterozygotes

Blood specimens from a patient with sickle cell anemia and from a person with sickle cell trait were adjusted to a hematocrit value of 30 per cent. Four 2.5 ml. samples from each specimen were deoxygenated with a gas mixture containing 0 per cent oxygen; in identical fashion other samples were deoxygenated with 3 per cent oxygen. At 15-20 minute intervals, the contents of successive flasks were withdrawn and the viscosity was measured (fig. 2A). Viscosity of S-S blood increased almost threefold, approaching a maximum within 15 minutes during exposure to 0 per cent oxygen and within 60 minutes on exposure to 3 per cent oxygen. In contrast, the viscosity of A-S blood increased only slightly after 60 minutes of exposure to 0 per cent oxygen, and it did not change during exposure to 3 per cent oxygen. In a similar experiment, the viscosity of blood from a normal subject was not altered during exposure to either gas mixture.

The rate of deoxygenation of blood of four persons, two with sickle cell anemia and two with sickle cell trait, was measured during exposure of blood to low oxygen tensions under the conditions of the preceding experi-
Fig. 2.—Alterations of (A) viscosity and (B) oxygen saturation of S-S and S-A blood during exposure to 3 per cent and to 0 per cent oxygen.

Deoxygenation was most rapid during the first 15 minutes; there was little change after 30-40 minutes. Increase in viscosity of S-S blood paralleled the decrease in oxygen saturation; in contrast, the viscosity of A-S blood had barely increased at a time when the blood was more than 90 per cent desaturated.

Hematocrit Value and Viscosity of Blood

Blood specimens from four normal persons and four patients with sickle cell anemia were adjusted to various hematocrit values. One portion of each
specimen was oxygenated by swirling in room air; another portion was deoxygenated by exposure to 0 per cent oxygen for 2 hours. The viscosity of the oxygenated samples displayed a curvilinear relationship to hematocrit value (fig. 3A). Viscosity of oxygenated S-S blood did not differ significantly from that of normal blood; an increase in hematocrit value from 20 per cent to 60 per cent caused the observed viscosity to be approximately doubled. Deoxygenation did not alter the viscosity of normal blood but caused a pronounced increase in viscosity of S-S blood, the increase showing a marked dependence on hematocrit value (fig. 3B). The observed viscosity of deoxygenated S-S blood at a hematocrit value of 60 per cent was approximately 50 times that observed at a hematocrit value of 20 per cent.

**Proportion of Sickled Cells and Viscosity of Blood**

Normal blood was mixed with compatible blood from a patient with sickle cell anemia. Various mixtures of A-A and S-S cells were prepared at each
Fig. 4.—Relationship of per cent sickled cells and hematocrit value to viscosity of blood.

of five hematocrit values. The proportion of S-S cells was evaluated from sealed preparations containing sodium metabisulfite. Samples of the blood mixtures were exposed to 3 per cent oxygen for 2 hours and the viscosity of each was measured. Viscosity of blood was approximately proportional to the product (per cent sickled cells) \( \times \) (hematocrit value)\(^2\) (fig. 4). The equation of the regression line is \( Y = 0.05 X + 21 \) (correlation coefficient \( r = 0.98, p < 0.001 \)).

Blood was obtained from another S-S patient on each of 4 days and was adjusted to a hematocrit value of 30 per cent. Portions of each sample were exposed to 0 per cent and to 3 per cent oxygen. Viscosity was measured after approximately 15, 30 and 60 minutes, and the proportions of sickled cells were estimated in specimens mixed with formalin-saline. The rate of increase of viscosity and the rate of sickling were calculated for each sample during the three time intervals (fig. 5). Viscosity measurements were quite reproducible from day to day during a period of clinical stability. Observed viscosity was proportional to per cent sickled cells; the equation of the regression line is \( Y = 0.05 (30)^2 X + 28 \) (\( r = 0.84, p < 0.001 \)). Rate of increase of
Fig. 5.—Changes in viscosity of blood during deoxygenation of specimens obtained from one patient with sickle cell anemia on 4 days. (A) Increase in viscosity of blood during exposure to 3 per cent and to 0 per cent oxygen. (B) Relationships between viscosity of blood and per cent sickled cells, and rate of change of viscosity and rate of sickling.

viscosity was proportional to rate of sickling; the equation of the regression line is $Y = 0.07 (30)^2 X$ ($r = .89, p < .001$).

Type of Sickling and Viscosity of Blood

Blood samples from 12 patients with sickle cell anemia were adjusted to a hematocrit value of 30 per cent and portions of each were exposed to 0 per cent and to 3 per cent oxygen for 15–60 minutes. Blood viscosity was measured, and the proportion of sickled cells and the predominant type of sickling (filamentous or holly leaf) were determined in formalin-saline prepara-
SICKLING RATE OF RED CELLS DURING DEOXYGENATION

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Fig. 6.—Relationship between viscosity of blood and per cent sickled cells in specimens from 12 patients with sickle cell anemia.

rations from each sample. For any value of observed viscosity, there was a range of proportions of sickled cells (fig. 6). Filamentous sickled cells were more common in samples of high viscosity, and holly leaf forms were predominant in samples of low viscosity. In some blood samples the proportion of filamentous forms increased with prolonged deoxygenation; in others, sickling was predominantly filamentous even in samples deoxygenated for a short time.

Viscosity of Blood and Clinical Status

The clinical status of 41 persons with sickling disorders was correlated with the rheological behavior of their blood during deoxygenation. Viscosity of 2.5 ml. samples of blood at a hematocrit value of 30 per cent was measured after deoxygenation with 3 per cent oxygen and with 0 per cent oxygen for various periods of time (table 1; fig. 7). Some of these data were analyzed by Student’s t test; the results appear in table 2.

A. S-S homozygotes. Viscosity of blood from 14 patients with clinically severe sickle cell anemia increased most rapidly; in none of these cases did hemoglobin F exceed 7 per cent at the time of study. Rate of increase of viscosity of S-S blood during 15 minutes of deoxygenation differed significantly from that observed with blood from A-S, J-S, S-F and S-C heterozygotes,
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**Table 1.—Hematologic and Rheologic Data**

**CHARACHE AND CONLEY**
### Sickle Cell-hemoglobin D Disease

| G. B. | 24 | M | 32 | 15 | 8.7 | 45 | 14 | .14 | .67 | .40 | 38 | 38 | 45 | 44 |

### Sickle Cell-hemoglobin C Disease

| T. B. | 23 | F | 34 | 1.7 | 57 | 32 | 18 | .38 | .67 | .55 | 29 | 53 | 36 | 36 |
| R. D. | 24 | M | 38 | 1.0 | 48 | 36 | 18 | .10 | .05 | .34 | 27 | 52 | 38 | 38 |
| B. S. | 23 | F | 29 | 1.6 | 51 | 36 | 18 | .17 | .20 | .20 | 29 | 56 | 48 | 48 |
| C. E. | 21 | F | 33 | 1.0 | 45 | 36 | 18 | .70 | .10 | .43 | 24 | 51 | 33 | 33 |

| Mean | 34 | 2.0 | 1.5 | 53 | 34 | 18 | .78 | .26 | .45 | 27 | 53 | 39 | 39 |

### S-F Heterozygotes

| S. B. | 33 | M | 48 | 1.8 | 35 | 65 | 32 | 21 | .00 | .00 | 25 | 20 | 34 | 19 |
| J. G. | 26 | F | 36 | 1.6 | 28 | 72 | 32 | 23 | .20 | .05 | 37 | 28 | 54 | 34 |
| L. M. | 19 | F | 38 | 2.0 | 27 | 73 | 33 | 24 | .16 | .10 | 36 | 22 | 43 | 31 |
| L. D. | 8  | F | 38 | 1.7 | 31 | 69 | 33 | 23 | .20 | .05 | 33 | 24 | 47 | 33 |
| R. W. | 24 | F | 41 | 2.0 | 68 | 36 | 24 | .50 | .05 | 40 | 26 | 51 | 27 | 27 |

| Mean | 40 | 1.8 | 31 | 69 | 33 | 23 | .31 | .04 | .34 | 24 | 46 | 33 | 33 |

### Sickle Cell Trait

| W. H. | 27 | M | 46 | 1.3 | 1.0 | 41 | .10 | .00 | .00 | 25 | 24 | 26 | 24 | 24 |
| S. S. | 52 | M | 43 | 1.0 | 39 | .19 | .00 | .00 | 21 | 20 | 23 | 20 | 20 | 20 |
| J. R. | 33 | M | 42 | 2.2 | 34 | .16 | .00 | .00 | 26 | 26 | 26 | 26 | 26 | 26 |
| T. C. | 19 | M | 45 | 1.7 | 36 | .22 | .00 | .00 | 20 | 20 | 22 | 20 | 20 | 20 |

| Mean | 44 | 1.6 | 38 | .00 | .00 | 24 | 23 | 24 | 23 | 23 | 23 | 23 | 23 | 23 |

### J-S Heterozygote

| E. M. | 19 | M | 43 | 0.8 | 0.7 | 36 | 29 | 10 | .25 | .00 | .00 | 21 | 20 | 24 | 20 |

### Normal Men

| S. C. | 44 | 0.9 | 0.5 | 0 | 34 | 0 | >.5 | .00 | .00 | 28 | 28 | 28 | 28 | 28 | 28 |
| C. L. | 41 | 1.3 | 0.3 | 0 | 34 | 0 | >.5 | .00 | .00 | 26 | 26 | 26 | 26 | 26 | 26 |

| Mean | 43 | 1.1 | 0.4 | 0 | 34 | 0 | >.5 | .00 | .00 | 27 | 27 | 27 | 27 | 27 | 27 |

*Solubility of deoxygenated hemoglobin.*
and with the exception of one instance of S-C disease there was no overlap of values. Six additional patients with sickle cell anemia had had a relatively benign clinical course with few symptoms and at times little or no anemia. In two brothers with clinically mild disease (J. N., R. N.), rate of increase of viscosity of blood on deoxygenation was as rapid as in severe sickle cell anemia. In three other cases the rate of increase of viscosity during exposure of blood to 3 per cent oxygen was less than that encountered in any patient with severe disease. Of the three, two had proportions of hemoglobin F exceeding 15 per cent in their red cell hemolysates, but the hemolysate of the third (L. F.)* was indistinguishable from those of more severely affected patients. The brother of one of the patients with high proportions of fetal hemoglobin had clinically severe S-S disease, although his fetal hemoglobin was 21 per cent.

B. S-Thal heterozygotes. Five patients with sickle cell-thalassemia displayed striking heterogeneity of the clinical manifestations of their disease. One patient with 93 per cent hemoglobin S had anemia, jaundice and a clinical course as severe as that of typical sickle cell anemia. Of three patients with approximately 75 per cent hemoglobin S, one (S. B.) was severely anemic and recurrently symptomatic, while the others had anemia only during pregnancy. Another patient (L. Ha.), whose red cell hemolysate contained only 65 per cent hemoglobin S, repeatedly had hematocrit values over 40 per cent and the only symptom was recurrent renal hematuria. There was a good correlation between the clinical status of these patients and the rate of increase of viscosity of their blood.

*This patient's hemoglobin was "fingerprinted" by Dr. M. Naughton, Department of Biophysics, The Johns Hopkins Medical School. The pattern obtained was typical of hemoglobin S.
SICKLING RATE OF RED CELLS DURING DEOXYGENATION

Table 2.—Probability of Significance of Differences between Viscosity Measurements in Sickling Disorders

<table>
<thead>
<tr>
<th>Hemoglobinopathies</th>
<th>Rate of Increase of Viscosity during Deoxygenation Interval 10–20 min.</th>
<th>Observed Viscosity</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0% oxygen</td>
<td>3% oxygen</td>
<td>0% oxygen</td>
</tr>
<tr>
<td>SS-SC</td>
<td>&gt;.10</td>
<td>&lt;.01</td>
<td>&gt;.10</td>
</tr>
<tr>
<td>SS-SF</td>
<td>&lt;.01</td>
<td>&lt;.01</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>SS-AS</td>
<td>&lt;.01</td>
<td>&lt;.01</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>SC-SF</td>
<td>&gt;.10</td>
<td>&gt;.10</td>
<td>&gt;.10</td>
</tr>
<tr>
<td>SC-AS</td>
<td>&lt;.10</td>
<td>&lt;.10</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>SF-AS</td>
<td>&lt;.05</td>
<td>&lt;.10</td>
<td>&lt;.01</td>
</tr>
</tbody>
</table>

C. S-D heterozygote. A Caucasian patient with S-D disease was incapacitated by frequent episodes of severe pain in the back and extremities, sometimes associated with high fever and jaundice. Although he was only mildly anemic, he had had recurrent pulmonary infarctions, aseptic necrosis of the femoral capital epiphysis and an episode of hemiparesis. His red cell hemolysate contained only 45 per cent hemoglobin S. The viscosity of his blood increased on exposure to 3 per cent oxygen at a rate almost as rapid as that of S-S blood.

D. S-C heterozygotes. Four patients with S-C disease had a variable clinical course usually with little or no anemia. They were much less ill than most patients with sickle cell anemia. Red cell hemolysates contained from 48 to 57 per cent hemoglobin S. On deoxygenation of blood, the rate of increase of viscosity was between the rates observed with S-S and A-S blood.

E. S-F heterozygotes. Five persons were heterozygous for hemoglobin S and for hereditary persistence of fetal hemoglobin. One (S. B.) had aseptic necrosis of the femoral head; and another (J. G.), with mild hemiparesis, developed severe bone pain during an episode of bacterial pneumonia. None was anemic at the time of study nor gave evidence of a hemolytic disorder. Red cell hemolysates contained approximately 70 per cent hemoglobin S. Viscosity of blood during exposure to 3 per cent oxygen increased at a rate similar to that of A-S blood during the first 15 minutes.

F. A-S heterozygotes. Four asymptomatic and non-anemic persons with sickle cell trait had proportions of hemoglobin S ranging between 34 and 41 per cent. Viscosity of the blood did not increase during exposure to 3 per cent oxygen for 1 hour.

G. J-S heterozygote. This person, who was healthy and had neither anemia nor evidence of a hemolytic disorder, had 36 per cent hemoglobin S in the hemolysate. Viscosity of his blood did not increase during exposure to 3 per cent oxygen.

Solubility of Deoxygenated Hemoglobin and the Minimum Concentration of Hemoglobin S Required for Gelation of Hemolysates

The minimum concentration of hemoglobin at which gelation occurred after deoxygenation was determined in hemolysates from persons with hemoglobins S-S, S-D, S-C, S-F, A-S and J-S. Solubility of the deoxygenated
hemoglobin was measured in 2.24 M phosphate buffer. The hemolysates studied are listed in table 3 in the order of increasing hemoglobin concentration required for gelation. This sequence is the same as that obtained by listing the hemoglobinopathies in the order of their clinical severity. The concentration of hemoglobin required for gelation of the hemolysate was inversely related to the rate of increase of viscosity of blood during equilibration with 3 per cent oxygen. On the other hand, the solubility of deoxygenated hemoglobin was not linearly related to the concentration of the hemolysate required for gelation. In the group of hemoglobinopathies studied, clinical status was not directly related to the mean corpuscular sickle hemoglobin concentration, and there was little correlation between MCSHC and the rate of increase of viscosity of blood during exposure to 3 per cent oxygen.

**DISCUSSION**

The substitution of a valine for a glutamic acid residue in position 6 of the β chains of hemoglobin is the sole known primary abnormality in sickle cell anemia. All of the clinical manifestations of the disease presumably are attributable to this alteration in molecular structure. On deoxygenation, sickle cell hemoglobin tends to form elongate aggregates of low solubility, deforming the red cell and producing the sickling phenomenon. Hemoglobin S appears to be harmless and to function as satisfactorily as normal hemoglobin except under conditions in which the sickling deformation occurs. When intravascular sickling is prevented, persons with 70 per cent hemoglobin S in their red cell hemolysates display no evidence of anemia or disease, and the life span of their erythrocytes is normal. On the other hand, even A-S erythrocytes sickle and are rapidly sequestered from the circulation of hypoxic individuals.

The elevated levels of hemoglobin in serum of patients with sickle cell anemia suggest that some intravascular hemolysis occurs, a phenomenon that probably is related to the increased mechanical fragility of sickled cells. However, sequestration of sickled erythrocytes and their destruction by reticuloendothelial cells appears to be the principal hemolytic mechanism. Studies with Cr-labeled red cells have demonstrated the uptake of sickled cells by the liver and spleen; splenic aspirates contain a high proportion of sickled forms and sections of the liver show Kupffer cells engorged with masses of sickled erythrocytes. Often there is pronounced dissociation between the degree of anemia and the occurrence of symptoms. Many of the symptoms are readily explained by vascular stasis or occlusion caused by the high viscosity of blood containing sickled erythrocytes. The significance of changes in viscosity in relation to the sickling phenomenon and to the production of the clinical disorder previously has been emphasized.

Blood is a non-Newtonian fluid and viscosity cannot be defined in absolute terms without reference to the conditions of measurement. Viscosity of normal blood increases as the shear rate is reduced. Greenberg and his as-
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Sociates, employing an Ostwald-type viscometer, observed that viscosity of deoxygenated blood from patients with sickle cell anemia at a hematocrit value of 25 per cent was similar to that of normal blood at a hematocrit of 50 per cent; results obtained with the falling sphere viscometer used in the present study are similar. These observations, which appear to minimize the importance of changes in blood viscosity in sickle cell disease, probably have little relevance to the rheological properties of blood flowing through small blood vessels. Not only may shear rates in small vessels differ from those in simple viscometers, but the rigidity of sickled cells and their disproportionate length may prevent their passage through narrow and tortuous capillaries. Jandl and his associates demonstrated the relative impermeability to sickled erythrocytes of Millipore filter discs.

Distortion of suspended particles from discs into elongated rods, without change in volume, produces an increase in viscosity \( (\eta' - \eta) \) which is related to the initial viscosity \( (\eta) \), to the degree of asymmetry produced \( (\nu) \) and to the volume fraction occupied by the suspended particles \( (\phi) \):

\[
(1) \quad (\eta' - \eta) = \eta\nu\phi.
\]

A similar prediction of change of viscosity probably could be made for distortion of suspended discs into crescents. In our studies the observed increase in viscosity of blood undergoing sickling reveals a striking difference from this relationship in that the term corresponding to \( \phi \) is squared:

\[
(2) \quad (\eta' - \eta) = F\eta S H^2
\]

where \( S \) represents proportion of sickled cells, \( H \) is the hematocrit value and \( F \) is a proportionality factor. Deoxygenated sickle cells are not simple crescents but have filamentous processes of variable number and length. The presence of these filaments is undoubtedly a most significant factor in magnifying the effect on viscosity of changes in the volume fraction of suspended particles. The tendency of red cells to assume the “holly leaf” or “filamentous” forms is not constant. Presumably the term “\( F \)” in Equation (2) includes a variable corresponding to these differences. When blood specimens from a group of patients were studied at a fixed hematocrit value, viscosity was not precisely determined by the proportion of sickled cells, undoubtedly because of variations in the form of the sickled erythrocytes. The validity of the relationship shown in Equation (2) is supported by the observations of other workers who used different experimental conditions: blood viscosity was observed to be proportional to per cent sickle cells but not linearly related to hematocrit value.

Under the conditions of our experiments, there was a good correlation between the average clinical severity of the specific hemoglobinopathies studied and the rate of increase of viscosity of blood during exposure to 3 per cent oxygen. Nevertheless, among patients with sickle cell disease, clinical variations were encountered that could not be explained by measurable differences in the kinetics of sickling.

Intracorpuscular factors influencing the rate of sickling are complex and
the mechanism of the sickling phenomenon is not clearly understood. Sickling of red cells, gelation of hemolysates and the formation of insoluble precipitates on deoxygenation are all thought to involve the formation of "liquid crystals," anisotropic molecular aggregates with a state of internal order between those of liquids and of perfect crystals. Pauling and his associates proposed that there is a region on the globin of hemoglobin S that is complementary to another region on the surface of the molecule; under appropriate conditions, interactions occur between complementary sites on adjacent molecules, causing molecular alignment and aggregation. If non-S hemoglobin served only as an inert diluent in hemoglobin mixtures, the mean corpuscular sickle hemoglobin concentration should be a measure of the tendency of red cells to sickle, as proposed by Greenberg and his associates. In fact, hemoglobin F was observed to be almost incapable of interacting with hemoglobin S, but hemoglobins A and C were found to participate in the formation of mixed aggregates in hemolysates in red cells and in concentrated buffers. In the present study, hemoglobins D and J also were demonstrated to interact with hemoglobin S. The relative magnitude of the interactions, measured by determinations of the minimum concentration of hemoglobin S required for gelation of hemoglobin mixtures, is indicated by this sequence: D > C > J > A > F.

The rate of sickling is probably dependent on the strength of the interactions between hemoglobin molecules. When S-S blood was deoxygenated, sickling occurred promptly as soon as an adequate concentration of deoxygenated hemoglobin had been achieved. In contrast, an additional period of time was required before sickling occurred after deoxygenation of A-S blood. Rucknagel and Neel mention a similar observation. The magnitude of the rates of sickling determined in our experiments is a function of the method of deoxygenation employed. Rates of deoxygenation of blood in vivo may be much more rapid, and the data presented here must be interpreted with that understanding.

Presumably hemoglobins differ in their interactions with hemoglobin S because of differences in their molecular structure. Deoxygenation of hemoglobin has recently been shown to be associated with rearrangement of the α and β chains within the molecule, and it has been suggested that increased separation of the chains permits the complementary sites proposed by Pauling and his associates to approach within interacting distance of each other. By analogy with the myoglobin molecule, the approximate location of amino acid residues within the tertiary structure of hemoglobin is known, and the location of the abnormality of each of the hemoglobins of the present study has been identified (table 3). The substitutions that differentiate hemoglobins S, C and D from hemoglobin A are some distance from that which characterizes hemoglobin J; the interactions of hemoglobins C and D with S are similar, but differ from those of hemoglobins A or J. Available data are insufficient to explain the nature of the complementary or interacting sites. Fetal hemoglobin, which shows the least interaction with hemoglobin S, lacks β chains.
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The magnitude of hemoglobin interactions, as measured in vitro, was clearly related to the clinical status of the patients. Hemoglobin D_{Punjab} interacted strongly with hemoglobin S, and the S-D patient was incapacitated by manifestations of sickle cell disease although his hemolysate contained only 45 per cent hemoglobin S. Hemoglobins A and J_{Baltimore} interacted weakly with hemoglobin S, and A-S and J-S heterozygotes were healthy although some had almost as much hemoglobin S in their erythrocytes as the S-D patient. Hemoglobins F and S showed minimal interaction, and S-F heterozygotes displayed no evidence of a hemolytic disorder although 70 per cent of their hemoglobin was of the S variety.

High concentrations of hemoglobin S encountered in red cells of S-S homozygotes were generally associated with severe disease. Nevertheless, an occasional patient had a remarkably benign course, although the proportion of hemoglobin S in the hemolysate and the rheological behavior of the blood on deoxygenation were indistinguishable from those of more severely affected patients. Such clinical variations are presumably related to unidentified extracorpuscular factors. Accelerated blood flow would be expected to lessen arterio-venous oxygen differences and to shorten the time of exposure of red cells to reduced oxygen tensions. Physiologic variables, including rates of blood flow and of oxygen consumption, undoubtedly influence the manifestations of the disease. A relatively benign clinical course was encountered in some but not all S-S homozygotes whose hemolysates contained high concentrations of hemoglobin F. Fetal hemoglobin is heterogeneously distributed among the red cells in this disease; accordingly, the overall effect on sickling and blood viscosity cannot be predicted from the concentration of hemoglobin F in the hemolysate. In one of the clinically mild cases of sickle cell anemia, the rheologic behavior of blood was repeatedly different from that of severe cases although the level of fetal hemoglobin was not elevated. This observation, which is unexplained, suggests the possibility of additional intracorpuscular modifying factors.

The clinical severity of sickle cell-thalassemia was related to the rate of increase of viscosity of deoxygenated blood, and this was roughly correlated with the concentration of hemoglobin S in the hemolysate. Variations in the distribution of hemoglobins among the red cells may account for some of the heterogeneity of sickle-thalassemia, since it is known that hemoglobins A and F are not uniformly distributed in the erythrocytes in thalassemia.

Some clinical manifestations attributable to vascular occlusions are more common in certain heterozygous sickling disorders than in homozygous sickle cell anemia. Thus aseptic necrosis of the capital epiphyses of the humerus and femur occurs more frequently in patients with sickle cell-hemoglobin C disease or sickle cell-thalassemia than in those with sickle cell anemia. Viscosity of deoxygenated blood was found to vary with the square of the hematocrit value. This may possibly explain the peculiar susceptibility of relatively non-anemic heterozygotes to vascular lesions when local deoxygenation of blood occurs.

Other clinical manifestations are probably not primarily related to altera-
Table 3.—Date Pertaining to the Hemoglobins of the Patients Studied

<table>
<thead>
<tr>
<th>Hemoglobinopathy</th>
<th>Abnormality of the β Chain of Globin</th>
<th>Hb S in Hemolysate %</th>
<th>Initial Rate of Increase of Viscosity of Blood 3% Oxygen sec/min.</th>
<th>Minimum Concentration of Hemoglobin Required for Gelation of Hemolysate Gm./100 ml.</th>
<th>Minimum Concentration of Hemoglobin S Required for Gelation of Hemolysate Gm./100 ml.</th>
<th>Solubility of Deoxygenated Hemoglobin Gm./100 ml.</th>
<th>MCHC Gm./100 ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-S</td>
<td>Hb S: Valine for glutamic acid. Residue 6. Helix A.</td>
<td>95 (92–98)</td>
<td>.99 (.44–1.73)</td>
<td>19</td>
<td>18.0</td>
<td>.02 (.015–.025)</td>
<td>30 (27–34)</td>
</tr>
<tr>
<td>S-D</td>
<td>Hb D: Glutamine for glutamic acid. Residue 121. “corner region” GH</td>
<td>45</td>
<td>.40</td>
<td>19.5</td>
<td>8.8</td>
<td>.14</td>
<td>14</td>
</tr>
<tr>
<td>S-F</td>
<td>Hb F: γ chain for β chain</td>
<td>69 (65–73)</td>
<td>.04 (.00–.10)</td>
<td>24</td>
<td>16.8</td>
<td>.18 (.16–.20)</td>
<td>23 (21–24)</td>
</tr>
<tr>
<td>A-S</td>
<td>Hb S: Valine for glutamic acid. Residue 6. Helix A.</td>
<td>38 (34–41)</td>
<td>.00</td>
<td>30</td>
<td>11.4</td>
<td>.16 (.10–.22)</td>
<td>13 (12–14)</td>
</tr>
</tbody>
</table>
tions in blood viscosity. Leg ulcers occur in a number of unrelated chronic hemolytic anemias.\textsuperscript{59} The eunuchoid habitus of patients with sickle cell anemia perhaps is attributable to chronic anemia since preliminary data suggest that epiphyseal closure can be accelerated by vigorous transfusion therapy in children with the disease.\textsuperscript{60} The retinal vascular anomalies of sickle cell-hemoglobin C disease may reflect a unique interaction between the two abnormal hemoglobins, for similar changes are not observed in either S-S or C-C homozygotes.\textsuperscript{61,62}

Knowledge of the pathogenesis of the manifestations of sickling disorders suggests approaches to therapy. As little as 30 per cent hemoglobin F in each red cell prevents sickling under physiologic conditions, but at present, methods for inducing production of hemoglobin F are not known. When more than 20 per cent of the hemoglobin of patients with sickle cell anemia was converted to methemoglobin, erythrocyte survival was prolonged; but initial attempts at therapy with sodium nitrite were unsuccessful.\textsuperscript{63} Carbon monoxide inhibits sickling when combined with hemoglobin\textsuperscript{64} and its therapeutic application has been suggested;\textsuperscript{65} adequate data pertaining to its clinical use are not available. Possibly other modifications of hemoglobin can be induced that will lessen the tendency of red cells to sickle. Acceleration of blood flow without a proportional increase in oxygen consumption might be accomplished by administration of agents such as triiodothyronine;\textsuperscript{66} the reduced arterio-venous oxygen differences and shortened exposure of red cells to hypoxic conditions should lessen the tendency of erythrocytes to sickle. Attempts to increase oxygen tensions within the circulation by administration of high concentrations of inspired oxygen caused suppression of erythropoiesis but did not lessen hemolysis in patients with sickle cell anemia.\textsuperscript{67} Since sickling is pH dependent,\textsuperscript{7} efforts were made to lessen intravascular sickling by administration of sodium bicarbonate; however, a protracted increase in the pH of the blood was not achieved.\textsuperscript{68}

Transfusional therapy of sickle cell disease presents special considerations. If the hematocrit value is elevated without a marked reduction of the proportion of cells capable of sickling, the viscosity of deoxygenated blood is greatly increased. Accordingly, except for correction of life-threatening anemia, transfusions may be potentially harmful. When the hematocrit value is not extremely low, transfusions probably should be employed primarily to dilute the sickle cells rather than to correct anemia. Anderson and his associates\textsuperscript{47} observed that a major decrease in symptoms of patients with sickle cell anemia was achieved by the use of exchange transfusions sufficient to maintain the proportion of sickling cells at 75–85 per cent. In our own experience, recurrences of symptoms of sickle cell disease were completely and indefinitely prevented as long as the proportion of sickling cells in the patient’s blood was kept below 60 per cent.

**Summary**

The clinical severity of various sickle hemoglobinopathies was directly related to the rate of increase of viscosity of blood during deoxygenation.
The principal determinants of the rate were the concentration of hemoglobin S in the red cell and the degree of interaction between hemoglobins when more than one major type was present. There was a close correlation between the rate of change of viscosity of blood under the conditions employed and the minimum concentration of the deoxygenated hemolysate required for gelation. Hemoglobin D_punjab showed the most marked interaction with hemoglobin S and hemoglobin F the least, accounting for the striking clinical differences between S-D and S-F heterozygotes. Clinical status of the patients and rate of increase of viscosity of partially deoxygenated blood were not closely correlated with the percentage of hemoglobin S in the hemolysate, the mean corpuscular sickle hemoglobin concentration, or the solubility of the hemoglobin. The viscosity of deoxygenated blood was directly related to the proportion of sickled red cells, but filamentous forms were associated with a greater viscosity than holly leaf forms; accordingly, viscosity could not be predicted precisely from the percentage of sickled erythrocytes in a blood specimen. The viscosity of blood containing sickled erythrocytes was approximately proportional to the square of the hematocrit value, an observation of relevance in explaining the frequent occurrence of vascular occlusive manifestations in patients with certain heterozygous sickling disorders without appreciable anemia. The data of this study support the concept that all of the clinical manifestations of sickle cell disease are attributable to the consequences of intravascular sickling of erythrocytes. Clinical differences between patients whose red cells appear to be similar probably can be explained by extracorpuscular factors influencing rates of sickling.

**SUMMARIO IN INTERLINGUA**

Le severitate clinic de varie hemoglobinopathias a falciformation esseva relationate directemente con le proratas del augmento de viscositate del sanguine durante le deoxygenation. Le principal determinantes del proratas esseva le concentration de hemoglobina S in le erythrocytos e le grado de interaction inter hemoglobinas quando plus que un typo major esseva presente. Esseva constatate un nette correlation inter le proratas del alteration in le viscositate del sanguine sub le conditiones empleate e le concentration minime del deoxygenate hemolysato requirite pro le gelation. Hemoglobina D_punjab monstrava le plus marcate interaction con hemoglobina S; hemoglobina F monstrava le minus marcate tal interaction. Iste factos explica le frappante differentias clinic inter heterozygoticos S-D e S-F. Le stato clinic del patientes e le proratas del augmento de viscositate de partially deoxygenate sanguine non esseva nettement correlationate con le procentage de hemoglobina S in le hemolysato, le valor medie del concentration de hemoglobina falciformatori corpuscular, o le solubilitate del hemoglobina. Le viscositate del deoxygenate sanguine esseva directemente relationate con le proportion de falciformate erythrocytos, sed formas filamentose esseva associate con un plus grande viscositate que altere formas. Per consequente, le viscositate non poteva esser predicite a base del pro-
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percentage de falciformate erythrocytos in un specimen de sanguine. Le viscositate de sanguine continente erythrocytos falciformate esseva approximativamente proportional al quadrato del valor del hematocrite. Iste observation es de signification in explicar le frequente occurrenzia de manifestaziones de occlusion vascular in patientes con certe heterozygotic disordines falciformatori sin grados appreciabile de anemia. Ie datos de iste studio supporta le conception que omne Ic manifestationes clinic de morbo de cellulas falciformes es attribuibile al consequentias del falciformation intravascular de erythrocytos. Differentias clinic inter patientes con erythrocytos apparentemente simile es probabilemente explicable per factores extracorpusicular que exerce un influentia super le intensitate del falciformation.

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SICKLING RATE OF RED CELLS DURING DEOXYGENATION


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Rate of Sickling of Red Cells during Deoxygenation of Blood from Persons with Various Sickling Disorders

SAMUEL CHARACHE and C. LOCKARD CONLEY