Hemoglobin S Polymerization: Primary Determinant of the Hemolytic and Clinical Severity of the Sickling Syndromes

By Gary M. Brittenham, Alan N. Schechter, and Constance Tom Noguchi

We examined the extent to which the intracellular polymerization of sickle hemoglobin (HbS) can account for the severity of anemia and of vaso-occlusive manifestations in the various sickling syndromes. Polymer formation in sickle cell disease depends principally on the intracytoplasmic hemoglobin composition and concentration. In our studies, the polymer fraction in sickle red cells was determined from reported mean values for hemoglobin composition and mean corpuscular hemoglobin concentration (MCHC) in 12 groups of patients with sickle hemoglobinopathies (homozygotes for HbS, with and without coexistent \( \alpha \)-thalassemia or various forms of the hereditary persistence of fetal hemoglobin [HPFH], \( \beta^- \), \( \beta^+ \), and \( \delta \beta^- \)-thalassemia, and heterozygotes for HbS with HbA). The calculated HbS polymer fractions at full deoxygenation and at physiologic oxygen saturation values were closely correlated with mean blood hemoglobin concentrations. In addition, polymer fraction correlated with the ranking of the sickling syndromes by vaso-occlusive severity. We find that polymer fraction accounts for about 80% of the variability in hemolytic and clinical severity. The method of analysis presented here provides a quantitative and systematic means of assessing the role of polymer formation in the pathophysiologic manifestations of the sickling syndromes. Our results support the hypothesis that the intracellular polymerization of HbS is the primary determinant of the severity of both anemia and clinical symptomatology in the sickle hemoglobinopathies. © 1985 by Grune & Stratton, Inc.

THE PATHOGENESIS of the clinical variability of the sickling syndromes, those hemoglobinopathies in which one or two genes for Hbs \( (\alpha_2\beta_2^{\text{HbS-Val}}) \) are present, is incompletely understood, despite the precision with which their genetic basis is known.\(^1\)\(^2\) Other genetic abnormalities, such as \( \alpha^- \)- and \( \beta^- \)-thalassemia and the hereditary persistence of fetal hemoglobin (HPFH), may coexist with the sickle cell gene to give a variety of conditions of varying clinical severity (Table 1).\(^3\) Although these variations in severity have been characterized qualitatively and many factors have been identified that potentially alter the expression of the sickle hemoglobinopathies (eg, hemoglobin composition, the mean corpuscular hemoglobin concentration [MCHC], the mean corpuscular volume [MCV], the number of circulating irreversibly sickled cells, intracellular metabolic changes, membrane associated abnormalities, erythrocyte endothelial adhesion), no single factor nor any combination of these has been able to account for the pathophysiologic variability of these conditions. In this article, we examine the extent to which the intracellular polymerization of sickle hemoglobin can explain the degree of anemia and the clinical severity of the sickling syndromes shown in Table 1.

A relationship between the amount of HbS present within an erythrocyte and the oxygen tension at which gelation begins has long been recognized.\(^4\) It has been known for more than 25 years that HbS aggregates and the viscosity of whole blood increases within the physiologic range of oxygen tensions in sickle cell anemia but not in sickle cell trait.\(^5\)\(^6\) Recent \(^{13}\)C nuclear magnetic resonance (NMR) measurements have demonstrated the presence of HbS polymer within the physiologic range of oxygen tensions in erythrocytes from individuals with sickle cell anemia but not in cells from individuals with sickle cell trait.\(^7\)\(^8\) Intraerythrocytic HbS aggregates can be detected in cells with no morphologic abnormality.\(^6\) Earlier studies of erythrocytes from individuals with sickle cell anemia have also provided evidence for polymerization without deformation, using observation of rheologic properties at high oxygen saturations\(^9\) and ultrastructural examination immediately after deoxygenation.\(^10\) These studies of polymer in sickle erythrocytes\(^9\)\(^10\) led to the hypothesis that the intracellular polymerization of HbS, rather than sickling or morphologic deformation, is a principal determinant of the pathophysiology of sickle cell anemia.\(^1\)

Our recent studies of SS, AS, and SC cells have demonstrated that the amount of sickle hemoglobin polymer determined by direct \(^{13}\)C NMR measurements is quantitatively equivalent to that calculated from the intracellular hemoglobin composition and concentration using a theoretical analysis of hemoglobin polymerization. To examine the extent to which variations in HbS polymerization can account for variations in the pathophysiology of sickle cell disease,
we have calculated the proportions of HbS polymer in sickle erythrocytes and compared them with the severity of the hemolytic anemia and of the vaso-occlusive manifestations in 12 types of sickle hemoglobinopathies. This analysis provides a systematic and quantitative method for assessing the relative importance of the intracellular polymerization of sickle hemoglobin in explaining the clinical variability of these sickling syndromes.

**MATERIALS AND METHODS**

**Hematologic and Clinical Data**

Hematologic and clinical data were compiled from published studies of series of patients with the types of sickling hemoglobinopathies listed in Table 1. We examined all the sickling syndromes with published reports of the data needed for the classification and calculations described below. Some sickling syndromes (eg, those with HbS and HbD, HbLepore, HbO, and HbS-thalassemia) could not be included because of the lack of clinical information or of relevant hemoglobin solubility data. If more than one study had been published, that describing the largest number of patients was chosen for Table 1.

### Table 1. Hematologic Data and Hemoglobin Analysis in Some Sickling Disorders

<table>
<thead>
<tr>
<th>Severity</th>
<th>Condition</th>
<th>N</th>
<th>Hb (g/dL)</th>
<th>Retics (%)</th>
<th>MCV (fl)</th>
<th>MCHC (g/dL)</th>
<th>MCH[HbS]C (g/dL)</th>
<th>HbA2 (%)</th>
<th>HbF (%)</th>
<th>HbS (%)</th>
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<tbody>
<tr>
<td>Asymptomatic</td>
<td>HbAS (African)</td>
<td>34</td>
<td>14.3</td>
<td>2.0</td>
<td>87.0</td>
<td>33.9†</td>
<td>13.7</td>
<td>2.4</td>
<td>0.8</td>
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<tr>
<td></td>
<td>HbS-γ-δ-HPFH (African)</td>
<td>9</td>
<td>14.0</td>
<td>—</td>
<td>86.0</td>
<td>33.3†</td>
<td>20.6</td>
<td>2.1</td>
<td>35.9†</td>
<td>62.0</td>
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<td>Mild</td>
<td>HbS-γ-δ-thalassemia (African)</td>
<td>3</td>
<td>11.4</td>
<td>2.9</td>
<td>74.0</td>
<td>32.7†</td>
<td>22.6</td>
<td>2.2</td>
<td>28.8†</td>
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<td></td>
<td>HbSS (Saudi Arab)</td>
<td>22</td>
<td>10.9</td>
<td>5.2</td>
<td>72.2</td>
<td>33.4†</td>
<td>23.2</td>
<td>2.1§</td>
<td>28.5#</td>
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<td>HbSS (Indian)</td>
<td>15</td>
<td>10.5</td>
<td>4.8</td>
<td>85.0</td>
<td>32.0§</td>
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<td>HbS-β-thalassemia (African)</td>
<td>39</td>
<td>10.3</td>
<td>4.0</td>
<td>71.0</td>
<td>31.2†</td>
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<td>15</td>
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<td>67.9</td>
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<td>Severe</td>
<td>HbSS-α-thalassemia (African)</td>
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<td>8.8</td>
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<td>71.2</td>
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<td>8.6</td>
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<td>HbSS-α-thalassemia (African)</td>
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<td>8.1</td>
<td>9.3</td>
<td>84.4</td>
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*± 1 SD.
†MCHC by Coulter counter.
‡Method unspecified.
§MCHC calculated from hemoglobin concentration and packed cell volume.
¶HbF by alkaline denaturation.
∥HbF by column chromatography.
#HbF by densitometry.

The reported MCHC by the proportion of each hemoglobin type. The syndromes were listed in the order of decreasing blood hemoglobin concentration.

Sickle cell anemia (HbSS) in individuals of African ancestry is a severe disease, associated with chronic hemolytic anemia, recurrent and painful vaso-occlusive crises, increased susceptibility to infection, chronic damage to a variety of organ systems, generally impaired growth and development, and a shortened life expectancy. The clinical impressions of the authors of the reports examined were used to group the sickling syndromes into three classes according to the severity of their vaso-occlusive manifestations: (1) asymptomatic, no increase in morbidity or mortality; (2) mild, not entirely free of vaso-occlusive manifestations but characteristically with fewer symptoms and a longer survival than are typical of the African form of sickle cell anemia; and (3) severe, clinically indistinguishable from the African form of sickle cell anemia. In the absence of an objective or established measures of the severity of vaso-occlusive complications, no attempt was made to compare the severity of the sickling syndromes within these three clinical classes. The severity of anemia (ie, the blood hemoglobin concentration) was not considered in the ranking of the vaso-occlusive manifestations.

**Theoretical Calculation of the Intracellular Fraction of HbS Polymer**

For each sickling syndrome, the intracellular hemoglobin polymer fraction was calculated as a function of oxygen saturation using the tabulated MCHC and fractions of hemoglobins S, F, A2, and A (Fig I). The methods for calculating the solubility of mixtures of hemoglobin have been developed within a thermodynamic for-
activity as a function of hemoglobin concentration (a, and (0.4) for the single-ligand T-state HbS molecule. The polymer phase and new hemoglobin activity of the solution phase can be expressed as

\[ a_s = \gamma C_s + (a_s^e/\Sigma x_j f_j)(a_s^w/a_s^e) \]

where \( \gamma \) is the activity of water; \( a_s^e \) is the activity of hemoglobin in the solution phase for a pure deoxyhemoglobin S gel; the new hemoglobin activity of the solution phase can be expressed as

\[ a_s^e = \gamma C_s^e, \]

Using this equation, we can calculate the hemoglobin solubility for any mixture of hemoglobins (provided the corresponding \( f_j \) values are known). The polymer fraction \( f_p \) can then be calculated from the total hemoglobin concentration \( C_t \) and the hemoglobin solubility \( C_s^e \)

\[ f_p = (C_t (C_s^e - C_s^e))/C_t (C_s^e - C_s^e) \]

where \( C_t^e \) is the hemoglobin concentration in the polymer phase. The thermodynamic description of gelation \( ^{11} \) is in excellent agreement with cell-free solution studies. \(^ {12,14} \) Furthermore, our most recent studies of intracellular polymerization on subpopulations of SS erythrocytes with relatively homogeneous MCHC values demonstrate the ability of the theory to predict intracellular polymer formation. \(^ {17} \) This analysis also allows us to account in detail for the measurements of polymer fraction in cells from patients with sickle cell trait or heterozygous for HbS and Hbc. \(^ {18} \)

HbS polymer fraction, blood hemoglobin concentration, and reticulocyte count could not be assumed to be normally distributed in the sickling syndromes; therefore, nonparametric analyses of correlation, regression, and prediction (Spearman rank correlation coefficient, \( r_s \)) were used to examine the relationships between these variables. To derive an equation expressing the relationship between mean hemoglobin concentration and polymer fraction, the nonparametric techniques of Adichie \(^ {22} \) were used for the estimation of the intercept, \( \alpha \), and those of Theil \(^ {22} \) for the slope, \( \beta \). The Spearman rank correlation coefficient was also used to determine the correlation between the ranked clinical severity and the calculated intracellular fraction of HbS polymer. The square of the Spearman rank correlation coefficient, \( r_s^2 \), was used to estimate the proportion of the variation in rank order of hemoglobin concentration, of reticulocyte count, or of clinical severity that could be attributed to the regression upon the rank order of polymer fraction.

**RESULTS**

The intracellular fraction of HbS polymer has been calculated as a function of oxygen saturation (Fig 1) for each of the sickling syndromes listed in Table 1. The values at 0% oxygen saturation, which require the fewest theoretical assumptions,\(^ {12,14} \) and at 70% oxygen saturation, which is within the physiologic range, are compared with mean levels of blood hemoglobin concentration for the sickling syndromes (Fig 2). The associations between hemoglobin concentration and the calculated polymer fraction at both oxygen tensions were determined using Spearman’s method. This analysis showed that the calculated fractions of HbS polymer were closely correlated with the mean blood hemoglobin concentrations in the sickling syndromes at both 0% \( (r_i = .93, P < 10^{-5}) \) and 70% \( (r_i = .89, P < 10^{-5}) \) oxygen saturations. Nonparametric linear regression analyses showed that hemoglobin concentration was inversely related to the calculated polymer fractions at both oxygen tensions by the expressions:

\[ y = -13.2x + 15.7 \] for 0% \( O_2 \) and

\[ y = -30.9x + 12.2 \] for 70% \( O_2 \).
for 70% O2. Thus, given the mean polymer fraction in a population with a specified sickling disorder, the expected mean hemoglobin concentration can be predicted from these results. The proportions of the variation in hemoglobin concentration explained by the regression analyses were 79% and 86% at 70% and 0% oxygen saturation, respectively.

Table 1 classifies the sickling hemoglobinopathies by reported clinical severity. Two groups of patients were considered asymptomatic: heterozygotes for HbS and A (sickle cell trait)23,24 and heterozygotes for HbS and the Gγβ-thal form of the hereditary persistence of fetal hemoglobin (HPFH).25 By contrast, for the severe condition, Serjeant et al26 found no substantial difference in the clinical expression of the HbS-β-thalassemia (African) and the HbSS (African) genotypes, so these syndromes were classed together. Although HbSS-α-thalassemia (with two of the four α-globin genes deleted) appears to be milder in certain manifestations (eg, acute chest syndrome and chronic leg ulceration) than sickle cell anemia (African) with the full complement of α-globin genes, there is not a change with regard to other clinical manifestations.27,28 (Heterozygotes for α-thalassemia 2 [-α/αα] are intermediate.) The remainder of the sickle hemoglobinopathies listed in Table 1 were classed as mild. Sickle cell disease in both India29 and Saudi Arabia30-32 has been reported to be more benign than the African form. Prospective studies of infants with sickle cell anemia have now demonstrated much less morbidity and mortality in the first years of life in Saudi Arabia33 when compared to Jamaica.34 Pembrey et al35 found that HbS-β-thalassemia (Saudi Arabian) was also less severe than the African form. Hb S-β-thalassemia (African) is typically milder than the Hb S-β-thalassemia from Africa,36 and the small number of cases reported of HbS-γ-β-HPFH (African) and HbS-γ-δβ-thalassemia37 seem to be still milder.

High-calculated fractions of intracellular HbS polymer for any given oxygen saturation are associated with more severe clinical manifestations. Despite the small number of classes and groups, this relationship is highly significant at oxygen saturations of both 0% (r = .89, P < 10^-3) and 70% (r = .92, P < 10^-3); thus, variations in the proportion of HbS polymer account for about 80% of the variation in clinical severity. At oxygen saturation values greater than 70%, erythrocytes from individuals with sickle cell trait have no HbS polymer, confirmed by direct 3C NMR measurements.6 A similar result is obtained for the nonanemic, asymptomatic Hb S-γβ-HPFH genotype. By contrast, erythrocytes from individuals of the remaining genotypes listed in Table 1 all would be expected to contain significant HbS polymer within the physiologic range of oxygen saturations. Moreover, larger amounts of intracellular polymer are closely correlated with more severe hemolytic anemia (Fig 2) and a more severe clinical course (Table 1). We found a comparable, but inverse, correlation with reticulocyte levels (for 0% oxygen saturation, r = .93, P < 10^-3 and for 70% oxygen saturation, r = .96, P < 10^-3).

The severity of anemia in the sickling hemoglobin-
opathies is determined by the balance between erythrocyte destruction and production. The intracellular fraction of HbS polymer provides an index of the factors influencing red cell destruction but not of those affecting productive capacity. As a result, some variation in blood hemoglobin concentration among individuals with similar intracellular concentrations of HbS polymer is to be anticipated. Nonetheless, the range of the variation in blood hemoglobin concentrations in the sickling syndromes is generally no greater than that found in normal individuals. For example, in the 88 patients with sickle cell anemia (African) reported by Higgs et al\textsuperscript{28} the width of the 95\% confidence interval for hemoglobin concentration (5.6 to 10.0 or 4.4 g/dL) is the same as that derived from a representative sample of a natural population of adult males (12.7 to 17.1 or 4.4 g/dL).\textsuperscript{38}

**DISCUSSION**

Our results show that differences in the severity of hemolytic anemia in the 12 sickling hemoglobinopathies examined are closely associated with differences in the extent of the intracellular polymerization of HbS. The correlation between these variables is highly significant ($P < 10^{-7}$), despite the confounding influence of differences in the methods used to determine hemoglobin composition and MCHC. Regression analyses indicate that some 80\% or more of the variation in hemoglobin concentration in the syndromes in Table I can be attributed to variation in the calculated HbS polymer fraction. Studies of correlation cannot alone establish causation, but our results suggest that either HbS polymerization itself, or other factors closely associated with polymerization, are largely responsible for the anemia of the sickling disorders.

The results at 0\% saturation (Fig 2A) correlate directly with measurements on solutions of hemoglobin, however, the results based on extrapolations to 70\% oxygen saturation (Fig 2B) are more likely to be physiologically relevant, albeit subject to greater uncertainties because of the difficulties in ascertaining the $e$ values for non-S hemoglobins. While a full description of HbS gelation may be more complex\textsuperscript{39,40} than the theoretical approach used here, data from other studies of gelation\textsuperscript{7,8,41} suggest that at least the relative ordering of the calculated fraction of HbS polymer will be confirmed when direct measurements are made. (We calculate for hemoglobin SC disease, based on our recent solution and cell studies\textsuperscript{42} and values of 35 g/dL MCHC and 51.6\% HbS,\textsuperscript{43} a polymer fraction of 0.44 at 0\% saturation and 0.06 at 70\% saturation.\textsuperscript{18} These values, for an average blood hemoglobin of 11.3 g/dL,\textsuperscript{44} are in good concordance with the other data in Fig 2.) These studies can be expanded to include other mutant hemoglobins as data on the effect of the mutant hemoglobins on deoxyhemoglobin S solubility become available.

Our theoretical calculations of the intracellular fraction of HbS polymer have been based on the simplifying assumption of a homogeneous population of erythrocytes. Most of the sickling syndromes listed in Table 1, however, have considerable heterogeneity in both hemoglobin composition\textsuperscript{44,45} and hemoglobin concentration.\textsuperscript{9,61} It is not known whether the pathologic manifestations of the sickling disorders related to polymer formation are the result of rheologic changes in all cells or in a subpopulation of cells that obstruct the microvasculature.\textsuperscript{38} In either case, the ranking would probably change little.

The severity of the vaso-occlusive manifestations in the sickle hemoglobinopathies has no objective measure that permits a relative ranking analogous to that provided by the hemoglobin concentration for the severity of anemia.\textsuperscript{1} Nonetheless, investigators have reported characteristic differences between groups of patients with different genotypes, and we wished to include this information in our analysis. The degree of anemia was not considered in ranking the severity of the vaso-occlusive manifestations. Descriptions of the course of individuals with sickle cell disorders have used the adjectives “mild” and “benign” to describe two distinct phenomena: (1) the presence of subgroups of individuals within a single genotype with less severe clinical manifestations in populations (usually of African ancestry) with typically severe disease\textsuperscript{49,51} and (2) the existence of populations (eg, in India\textsuperscript{29} and Saudi Arabia),\textsuperscript{30,31} whose members have a characteristically less severe disorder. The “mild” classification in Table 1 is used in this second sense to describe syndromes whose clinical course is typically less severe. Just as for the severity of anemia, variation in the severity of the vaso-occlusive manifestations of individuals with similar proportions of intracellular HbS polymer is to be expected. Nonetheless, by considering the clinical manifestations in all individuals of a given genotype, characteristic differences among the sickling syndromes, shown in Table 1, seem apparent and closely correlated with differences in the amount of intracellular HbS polymer under physiologic conditions.

Our approach provides a systematic and quantitative foundation for understanding differences between genotypes that have long been appreciated clinically and understood qualitatively. This method of analysis explicitly uses a theoretical framework to relate the types of non-HbS in the erythrocyte, the MCHC, and the MC[HbS]C to the intracellular polymerization of HbS, and in turn correlates the polymer fraction with the severity of both anemia and clinical symptomatology. Polymer formation as the primary determinant of pathophysiology has also been suggested by
other equilibrium and kinetic studies. Indeed, calculated delay times of sickling for several of these syndromes has been used to estimate how much inhibition of gelation might be necessary to obtain clinical benefit. At 0% oxygen saturation, predictions for the kinetics of polymerization would be ranked similarly to the amount of polymer formed and, hence, give an analogous correlation with disease severity. However, at physiologic oxygen saturations, kinetic data are not available for comparison. Determination of the intracellular polymer formation permits definition of the relative importance of factors that alter the gelation of HbS, e.g., changes in the distribution of hemoglobin concentrations, hemoglobin compositions, or other variables. The extent of HbS polymerization can thus also be used to evaluate proposed therapeutic approaches. For example, decreasing the polymer fraction (at 70% oxygen saturation) in patients with sickle cell anemia (African) from 0.15 to about 0.05 would be expected to result in a mild clinical state like that found in sickle cell anemia in India or Saudi Arabia.

Our results support the hypothesis that the intracellular polymerization of HbS is the primary determinant of the pathophysiologic manifestations of the sickling hemoglobinopathies. However, to fully explain the clinical differences between the sickling syndromes, further investigation is needed to clarify the importance of other factors, such as erythrocyte heterogeneity in individuals, the delay time of gelation, erythrocyte adherence to endothelial cells, intracellular metabolic changes, and membrane-associated abnormalities. Moreover, the relative contributions of these factors to the striking variability of clinical symptomatology in patients of the same genotype are at present uncertain, but will be examined by other studies, now in progress, to determine the extent of HbS polymerization in individual patients. Whatever the contribution of other factors, the close correlation between the intracellular polymerization of HbS and the anemia and symptomatology of the sickling hemoglobinopathies links the known clinical heterogeneity of the sickle cell disorders with recent genetic molecular and biophysical insights into their underlying pathophysiology.

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