Intracellular Polymerization of Sickle Hemoglobin

EFFECTS OF CELL HETEROGENEITY

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ABSTRACT To determine the extent to which the broad distribution in intracellular hemoglobin concentrations found in sickle erythrocytes affects the extent of intracellular polymerization of hemoglobin S, we have fractionated these cells by density using discontinuous Stractan gradients. The amount of polymer formed in the subpopulations was experimentally measured as a function of oxygen saturation using ^{13}C nuclear magnetic resonance spectroscopy. The results for each subpopulation are in very good agreement with the theoretical predictions based on the current thermodynamic description for hemoglobin S gelation. We further demonstrate that the erythrocyte density profile for a single individual with sickle cell anemia can be used with the theory to predict the amount of polymer in unfractionated cells. We find that heterogeneity in intracellular hemoglobin concentration causes the critical oxygen saturation for formation of polymer to shift from 84 to >90%; polymer is formed predominantly in the dense cells at the very high oxygen saturation values. The existence of polymer at arterial oxygen saturation values has significance for understanding the pathophysiology of sickle cell anemia. The utility of these techniques for assessing various therapeutic strategies is discussed.

INTRODUCTION

The intracellular polymerization or gelation of hemoglobin (Hb)[1] S ($\alpha_2\beta_2^{6Glu\rightarrow Val}$) upon deoxygenation is the primary pathogenetic event in sickle cell disease (1). A vast amount of information is available about the kinetics of polymerization of concentrated deoxyhemoglobin S solutions (2, 3), including the role of protein nonideality in affecting the properties of these solutions (4, 5). We developed nuclear magnetic resonance (NMR) methods for examining polymerization inside the erythrocyte and we detected significant amounts of polymer in whole sickle cell blood under physiological conditions (6, 7). We also showed that the theory of protein nonideality explains these experimental results to a first approximation.

To do these analyses, we assumed explicitly that sickle erythrocytes were uniform in intracellular Hb composition and concentration (6). In actuality these two properties have complex distributions in whole blood from sickle cell patients (8–11). It is possible to separate cells by density and measure the distribution of intracellular Hb concentration (12, 13). This distribution, in particular the existence of very dense cells (including the "irreversibly sickled" or ISC fraction), has long been thought to be an important determinant of the severity of sickle cell disease. For this reason, it was important that we determine intracellular polymerization as a function of intracellular Hb concentration. In addition, recent progress in understanding the role of water nonideality in the polymerization process led to an improved thermodynamic formulation, which is in better agreement with the measured solubility of cell-free HbS solutions as a function of oxygen saturation (14–16).

We report here on the NMR measurement of intracellular polymer as a function of oxygen saturation in "uniform" subpopulations of sickle erythrocytes separated on discontinuous Stractan gradients (12, 17). We show that by using this new thermodynamic analysis and by explicitly considering the distribution of cell densities we can predict the behavior of polymer in unfractionated blood. Since intracellular polymer

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[1] *Abbreviations used in this paper:* BSKG, buffered saline with potassium and glucose; Hb, hemoglobin; ISC, irreversibly sickled cells; MCHC, mean corpuscular hemoglobin concentration.

is likely to be the primary determinant of sickle erythrocyte rheology, these results are relevant to understanding the pathophysiology of, and possible therapeutic approaches to, sickle cell disease.

METHODS

Blood specimens were drawn from individuals with sickle cell disease. The HbF level was determined by alkali denaturation. For cell gradients the erythrocytes were washed three times in buffered saline with potassium and glucose (BSKG: 7.808 g NaCl, 0.373 g KCl, 0.194 g $NaH_2PO_4 \cdot H_2O$, 1.220 g Na_2HPO_4, 2.0 g glucose, and deionized water to make 1 liter) (17), pH 7.4, 290 mosmol (as determined on an osmometer, model 2007, Precision Systems, Inc., Sudbury, MA) and layered on Stractan. The Stractan (St. Regis Paper Co., New York) was prepared using the method of Corash et al. (12) as modified by Clark et al. (17). The Stractan was adjusted to 290 mosmol, pH 7.4, by the addition of bovine serum albumin (3 g/100 ml solution) 0.15 M potassium-phosphate buffer, pH 7.4 (adding 10 ml to 90 ml solution), $MgCl_2 \cdot 6H_2O$ (1.6 mg/100 ml available water), glucose (200 mg/100 ml available water), and variable amounts of NaCl. Stractan solutions with densities ranging from 1.076 to 1.167 g/ml were made by dilution of the stock Stractan solution with BSKG. The discontinuous gradients were formed by layering the Stractan solutions into a 4 × 0.62-in. or 3.5 × 1-in. cellulose nitrate tube in order of decreasing density.

After layering 1–10 ml of washed cell suspension (~0.3 hematocrit) on the Stractan gradient, the tubes were centrifuged at 20,000 rpm for 45 min in a Beckman SW 28 or SW 28.1 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA). The fractionated cells populations were then collected from the 0.62-in. tubes by using a tube slicer (Tubs-200, Nuclear Supply, Bethesda, MD) or by pipetting from the 1-in. tubes. For determination of the average intracellular Hb concentration of each fraction, the hematocrit was adjusted to ~0.3 with BSKG and then the exact hematocrit was determined by centrifugation in a capillary tube. The corresponding Hb was determined spectrophotometrically from the concentration of cyanmethemoglobin prepared from the lysed cells. We previously found that this method is reproducible and is minimally affected by trapped plasma (18).

For ^{13}C NMR, SS erythrocytes suspended in Earle's balanced salt solution without bicarbonate and with 25 mM Hepes, pH 7.2, were equilibrated with gas mixtures of various oxygen content using a spinning cup tonometer (IL-237, Instrumentation Laboratory, Inc., Lexington, MA) as previously described (6). Cells were anaerobically transferred into an 8-mm NMR tube containing the same gas mixture, cells packed, and supernatant removed anaerobically. A different sample of cells was used for each gas mixture. Final packed cell volume was 0.65 ml and the total oxygen saturation was determined with the MBA-Micro Blood Analyzer (Advanced Products SRL, Milan, Italy) (19). Samples were stored on ice and the NMR measurements were completed within 18 h.

For each sample at the various oxygen saturations, natural abundance ^{13}C NMR spectra were obtained at 37°C using a Nicolet TT-14 spectrometer modified for experiments in solids as previously described (20). Proton scalar decoupled spectra (standard 90°-t sequence) of the fully oxygenated sample and fully deoxygenated sample were used to determine the absolute amount of free Hb remaining after deoxygenation. The amount of polymer in samples at intermediate oxygen saturations was determined from the proton-enhanced spectra for each sample. (Adamantane was used to determine the Hartman-Hahn condition for the proton-enhanced spectra.) The 100% oxygen saturation proton-enhanced spectra were used as a base-line correction for the other proton-enhanced spectra. The time required for each spectrum was 20–40 min.

Theoretical analysis. Following the approach of our previous studies, to calculate the amount of polymer formed at varying oxygen saturations we used the thermodynamic theory for gelation developed by Minton (based on the two-state model for gelation and the nonideal behavior of Hb at physiologic concentrations) (4) as modified by Gill et al. (15) to include the nonideal behavior of water. This is summarized by the equations of Sunshine et al. (16). For i species of Hb in a gelled mixture, the activity coefficient γ_σ of free hemoglobin in solution is related to the activity coefficient γ_σ^0 of free Hb in a pure deoxyhemoglobin S gel by

$$(\gamma_\sigma C_\sigma/\gamma_\sigma^0 C_\sigma^0)(a_w/a_w^0)^r = 1/\sum_j x_j e_j, \quad (1)$$

where C_σ is the solubility of Hb in the mixture, C_σ^0 is the solubility of pure deoxyhemoglobin S, x_i is the solution mole fraction of species i, and e_i is the relative tendency for species i to be incorporated into the polymer ($e = 1$ for pure deoxyhemoglobin S and $e = 0$ for HbF and the HbS/HbF hybrid (16, 21)). For intermediate oxygen saturations, we use the same parameters as previously described by Hofrichter for mixtures of deoxy- and carboxyhemoglobin S (14) with $e = 0.4$ for the single T-ligand state and $(0.4)^k$ for the k-ligand species. The value of e for R-state is 0. The original equation proposed by Minton (4),

$$(\gamma_\sigma C_\sigma/\gamma_\sigma^0 C_\sigma^0) = 1/\sum_j x_j e_j, \quad (2)$$

has been expanded by Gill et al. (15) to include the nonideal behavior of water by the addition of the ratio $(a_w/a_w^0)^r$ of the solvent activity, a_w, to the value at zero oxygen saturation, a_w^0. r is the ratio of moles of solvent to moles of Hb in the polymer phase. This ratio can be expressed as

$$(a_w/a_w^0)^r = \exp - \int_{C_\varphi}^{C_\sigma} \left(\frac{1/C\rho - \bar{v}}{1/C - \bar{v}}\right)\left(\frac{1}{C} + \sum_{k=2}^{6} B_k C^{k-2}\right) dC. \quad (3)$$

$C\rho$ is the concentration of Hb in the polymer phase (70 g/dl) and $\bar{v}$ is the partial specific volume of Hb. B_k are parameters obtained from sedimentation equilibrium data of Ross et al. (5) by expressing the activity coefficient for Hb as a function of concentration.

RESULTS

Erythrocytes were fractionated by using discontinuous Stractan gradients. The variation of intracellular Hb concentration with respect to Stractan concentration is shown in Fig. 1. A linear least squares fit of the intracellular Hb concentration measured for each fraction as a function of percent Stractan gives an r^2 value of 0.96. Such calibration curves are useful when the number of cells in the lighter or denser fractions is insufficient to determine the intracellular Hb concentration, or more generally, when only small amounts of sample are available.

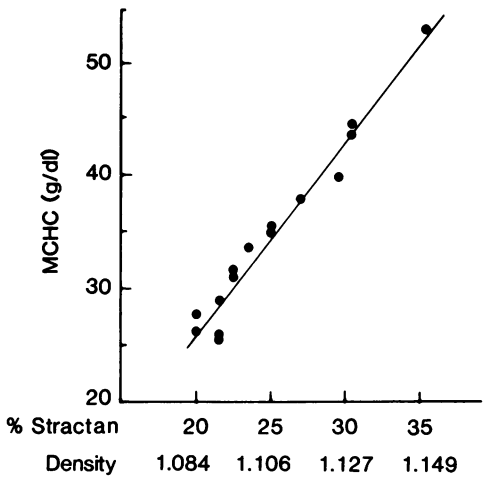

FIGURE 1 Intracellular Hb concentration vs. the average percent concentration Stractan between which the cells were collected. The MCHC of subpopulations of erythrocytes fractionated on discontinuous Stractan gradients was measured as described in the text. The straight line was obtained by linear regression analysis (r^2 = 0.96).

SS erythrocytes from four individuals with HbF levels varying from <2 to 6.3% were fractionated on Stractan gradients. Light fractions with a mean intracellular Hb concentration of 29.5 g/dl (<20% Stractan), intermediate fractions with a mean intracellular Hb concentration of 32.7 g/dl (20–23% Stractan), and dense fractions with intracellular Hb concentration of 41.7 g/dl (>28% Stractan) were chosen for measurement of the amount of intracellular polymer formed at different oxygen saturations using ^{13}C NMR (Fig. 2). (Cells from different individuals were not mixed.) At full deoxygenation the polymer fraction increased (from 0.6 to 0.8) as the intracellular Hb concentration varied from 29.5 to 42 g/dl. The polymer fraction decreased with increasing oxygen saturation for any given intracellular Hb concentration.

For comparison with these experimental results, amounts of polymer were also calculated using the modified thermodynamic description of gelation for solutions of HbS (see Theoretical analysis) at comparable concentrations (Fig. 2). The most striking feature of these calculations is that the theory predicts that for intracellular Hb concentrations <34 g/dl, no intracellular polymer should be detected above 84% oxygen saturation. However, for intracellular Hb concentrations at 42 g/dl or greater, polymer formation can occur even above 90% oxygen saturation.

Illustrated in Fig. 3 is an intracellular Hb concentration profile determined by a discontinuous Stractan gradient for an SS homozygous individual. The cells are distributed over a broad range (>20 g/dl) of intracellular Hb concentrations centering at 35 g/dl. The overall sample mean corpuscular hemoglobin concentration (MCHC) is 35.3 g/dl (with 6.5% HbF). (The peak in fraction 8 is indicative of a very broad tail

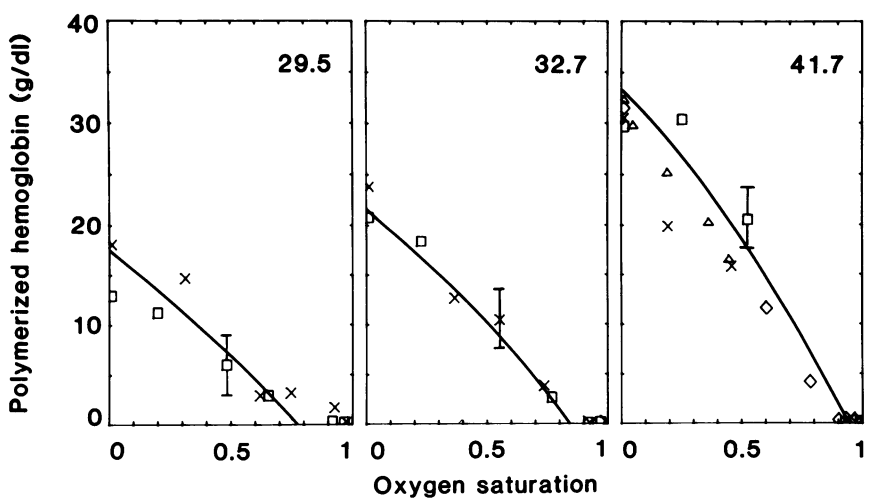

FIGURE 2 Polymerized Hb in sickle erythrocytes vs. oxygen saturation. Shown as the solid lines is the prediction using the thermodynamic theory (see text) for polymer formation in uniform populations of SS erythrocytes with MCHC values of 29.5, 32.7, and 41.7 g/dl, respectively. The symbols represent corresponding subpopulations of SS erythrocytes fractionated on discontinuous Stractan gradients with these MCHC values. ^{13}C NMR was used to measure experimentally the amount of intracellular polymer as a function of oxygen saturation in these subpopulations at each MCHC; the different symbols represent erythrocytes obtained from different individuals.

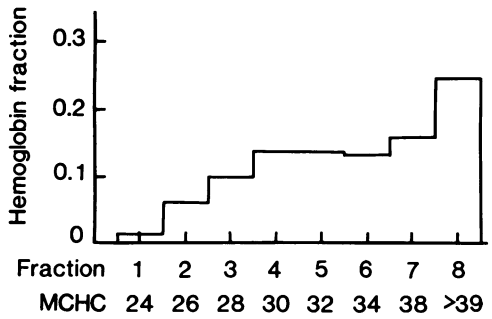

FIGURE 3 The density profile for sickle erythrocytes from a single individual. The fractions are arranged in order of increasing density. The MCHC values have been obtained from the corresponding percentage concentration of Stractan (see Fig. 1). The amount of Hb in each fraction was determined spectrophotometrically by cyanmethemoglobin analysis. The Stractan density gradient used 19, 21, 22, 23, 24, 26, 28 and 33%.

with respect to the distribution in intracellular Hb concentration and is consistent with a bimodal distribution in cell density [see for example reference 22].) The broad distribution in intracellular Hb concentration found in SS erythrocytes is in contrast with the distribution found in normal erythrocyte populations in which the majority of cells are within a range of 4 g/dl.

The intracellular polymer formation as a function of oxygen saturation was measured using ^{13}C NMR on the sample used to generate the data shown in Fig. 3. These results are illustrated in Fig. 4 (the line in Fig. 4 is the calculated prediction using the cell density profile as described below). At complete deoxygenation, 0.67 of the total Hb is polymerized. As the oxygen saturation is increased, the amount of polymer decreases, similar to the results shown in Fig. 2 for SS erythrocytes with "uniform" MCHC. The amount of intracellular polymer goes to zero at high oxygen saturation, in the region between 90 and 95% oxygen saturation. Using the modified thermodynamic theory for HbS gelation, we have assumed a homogeneous cell population and calculated the polymer fraction f_ρ using

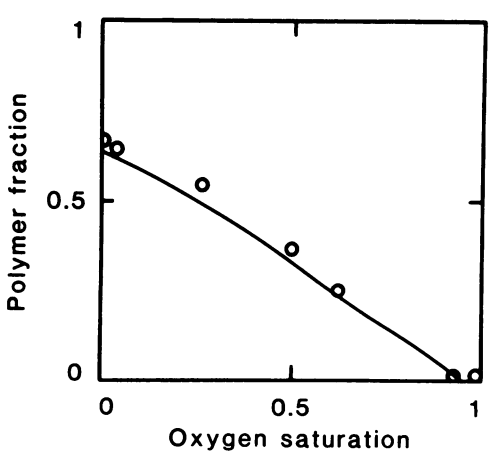

FIGURE 4 Intracellular polymer fraction in a whole population of sickle erythrocytes vs. oxygen saturation. The MCHC profile in Fig. 3 and the thermodynamic theory (see text) was used to predict the fraction of intracellular Hb polymerized as a function of oxygen saturation (solid line). The circles represent the actual ^{13}C NMR measurements of polymer fraction in the identical population of sickle erythrocytes described in Fig. 3.

$$f_\rho = C_\rho(C_T - C_\sigma)/[C_\sigma(C_\rho - C_\sigma)], \quad (4)$$

with C_T equal to the MCHC (Fig. 2). We have also calculated the polymer fraction using the density profile shown in Fig. 3 and the equation

$$f_\rho = \sum_i f_{C_i} C_\rho (C_i - C_\sigma)/[C_\sigma(C_\rho - C_\sigma)]$$

$$= \{C_\rho/[C_\sigma(C_\rho - C_\sigma)]\} \sum_i f_{C_i}(C_i - C_\sigma), \quad (5)$$

where f_{C_i} is the fraction of cells with intracellular Hb concentration C_i (Fig. 4). The sum is only over those cell fractions that contain polymer.

At complete deoxygenation, the theory predicts that 0.66 of the total Hb should be polymerized. The amount of polymer decreases with increasing saturation becoming zero at 0.87 for the uniform MCHC approximation and 0.95 for the actual heterogeneous cell population. When all the cells contain polymer, the uniform MCHC approximation is adequate for the polymer concentration. For this situation ($\sum_i f_{C_i} = 1$ and $\sum_i f_{C_i} C_i = C_T$), equation (5) reduces to equation (4). The Hb solubility increases with increasing oxygen saturation. When the solubility exceeds the intracellular Hb concentration of the lightest cell fraction, the homogeneous approximation breaks down. In this region [$(C_T - C_\sigma) < \sum_i f_{C_i}(C_i - C_\sigma)$], the uniform MCHC approximation (Fig. 2, *center* panel) underestimates the calculated polymer fraction and we begin to see the effect of cell heterogeneity (Fig. 4). The agreement between the measured polymer fraction and the theoretical prediction calculated using the heterogeneous intracellular Hb concentration profile is excellent.

DISCUSSION

The calculated amounts of intracellular HbS polymer, are in very good agreement with ^{13}C NMR measurements of polymer in sickle erythrocytes separated by discontinuous Stractan gradients with narrow ranges of intracellular Hb concentrations (Fig. 2). Furthermore, by using the cell density profile from a single

individual with sickle cell anemia (Fig. 3), we calculated the predicted amount of polymer as a function of oxygen saturation and found that it closely matches the polymer fraction in the whole cell population as measured by ^{13}C NMR (Fig. 4).

The two-phase model used in the theoretical analysis considers the HbS gel as a solution phase of free Hb molecules in equilibrium with a polymer phase. In the presence of oxygen, the thermodynamic theory as developed by Minton includes two components, oxygen and HbS, which behaves nonideally at physiological concentrations (4). This theory, recently modified by Gill et al. (15) to include the nonideal behavior of the solvent or water, results in a thermodynamic description for cell-free solution, which is in excellent agreement with corresponding data on cell-free solutions, particularly at high oxygen saturations (15, 16).

It is apparent from the theoretical calculations and experimental measurements that polymer formation in the heterogeneous whole population of the individual (MCHC = 35.7 gm/dl) shown in Fig. 3, goes to zero at 94% oxygen saturation (Fig. 4) rather than the lower value (87%) that would be predicted assuming a uniform cell population. The latter would be similar to the results for the uniform cell population in the center panel of Fig. 2. The difference between intracellular polymer formation in the whole cell population and intracellular polymer formation in a uniform cell population at the corresponding MCHC represents the contribution from cell heterogeneity.[2]

Although the amount of polymer is maximum at complete deoxygenation, significant amounts of polymer can be detected throughout much of the physiologically relevant oxygen saturation region, even at high oxygen saturations (>90%) in whole sickle erythrocyte populations (6). In fact, it is this observation which led us to suggest that abnormal rheology may occur on the arterial as well as the venous side of the circulation (1). The detection of polymer at high oxygen saturations is particularly interesting in view of the fact that individuals with sickle cell anemia usually have arterial oxygen saturation levels <90% and as low as 70% (23), where very significant amounts of polymer can be detected.

It should be noted that data on the kinetics of HbS gelation at these intermediate oxygen saturation values are not yet available. Hence, with regards to pathophysiology of disease, the importance of the amount of polymer formed as measured by equilibrium techniques such as ^{13}C NMR spectroscopy vs. the time required for intracellular polymer to form cannot be determined without further detailed investigation. However, it seems likely that many or most cells in a sickle cell patient always have some aggregated HbS. Under these circumstances long delays for polymerization due to nucleation processes may not be significant and increases and decreases in the amount of polymer may be relatively rapid compared with circulation times. For the purpose of this study we neglected variation in 2,3-diphosphoglycerate, pH, HbF, and other factors, which also contribute to the heterogeneous cell distribution. However, we expect these factors to contribute only a minor effect in predicting intracellular polymer formation.[3]

The agreement between the calculated theoretical amount of polymer and the experimentally measured amount of polymer using ^{13}C NMR further demonstrates that membrane and other cellular constituents do not have a primary effect on the amount of polymer formed. These equilibrium studies complement the kinetic studies of Goldberg et al. (24) in which it was found that membranes did not significantly affect polymerization kinetics of HbS solutions beyond the effect due to excluded volume. Studying single cell kinetics, Coletta et al. (25) also concluded that gelation was not significantly altered by the erythrocyte membrane.

These thermodynamic analyses can be extended to other sickle syndromes. For example, the experimentally measured values of polymer formation in AS (sickle trait) erythrocytes, which we have previously reported (7), can now be explained on a theoretical basis (results not shown). In SC disease the thermodynamic theory would predict an enhanced intracellular polymerization of S in SC cells due to the increased proportion of S (as compared with AS cells) with the major effect due to the existence of a large number of cells with high intracellular Hb concentration (18, 26, 27). Although the existence and importance of dense cells in SC, as well as SS, has been recognized for some time, it is only now that several separation techniques allow for a quantitative assessment of these cells (18, 22, 26).

The precise factors or parameters that determine cell density or intracellular Hb concentration are un-

[2] The addition of the solvent nonideality term to the analysis we previously used (6) shifts the x-intercept for a homogeneous cell population of MCHC = 34 gm/dl from 95 to 84% oxygen saturation. Cell heterogeneity shifts the intercept to higher oxygen saturation values.

[3] The effect on polymer formation of the variations in Hb F concentration usually found in individuals with sickle cell disease is much smaller than that due to the variations in total Hb concentration. However, the uneven distribution of Hb F in SS erythrocytes may be responsible for the apparent overestimation of polymer calculated for the uniform dense cell population (Fig. 2, *right* panel).

clear. Fabry et al. (22) demonstrated that deoxygenation of SS erythrocytes shifts the cell density profile to higher values. Furthermore, it is believed that repeated or prolonged sickling of SS erythrocytes (or more precisely repeated intracellular polymerization) induces loss of intracellular potassium and an increase in intracellular Hb concentration. The mechanism of this is not well understood. The presence of a large dense cell fraction per se does not a priori result in HbS-like polymer formation and a disease state, as demonstrated by the shift to higher densities in AC or CC cells, which are relatively benign phenotypes (18, 27). The abnormally large fraction of dense cells associated with sickle cell anemia contains the majority of ISC (28). However, not all dense cells have abnormal morphology. Understanding the detailed mechanism by which polymer formation results in a change in cell density is critical in determining factors controlling manifestation of disease. The rheological properties of normal and dense cells with varying amounts of HbS polymer needs further study. It should be noted that membrane alterations in ISC do not appear to significantly contribute to the abnormal rheology of these cells (10) whereas the nonaggregated Hb in very dense cells can further increase viscosity, as compared with Hb in cells at 34 g/dl (29).

We have demonstrated elsewhere that there is a striking relationship between polymer formation and severity of disease in the various sickling syndromes (30). Using Hb level as an index of the severity of hemolytic anemia, we obtained an excellent correlation between Hb level and polymer formation (both at 0 and 70% oxygen saturation) based on the average hematologic parameters for various populations with 12 different sickle syndromes. The amount of polymer formed was also used successfully to rank sickle syndromes into groups that increased in clinical severity with increasing polymer formation. These analyses were done without explicit consideration of cell heterogeneity. We believe that quantitative analysis of intracellular polymer formation may be a useful tool in predicting disease severity, particularly when more information is available about the distribution profile for intracellular Hb concentration in each of the various sickle syndromes.

The information obtained from these studies should be useful for the evaluation of therapy. We have recently used the Stractan gradients to study two sickle cell patients receiving 5-azacytidine to increase γ-gene biosynthesis and HbF levels (31). We found that the proportion of dense cells was markedly reduced by this treatment. The reduction in the proportion of dense cells should dramatically reduce polymer formation, particularly above 84% oxygen saturation. Using these techniques, the effects of various agents designed to reduce intracellular polymer formation—by increasing cell volume, increasing HbF, inhibiting intermolecular contacts, etc.—can be compared as potential therapies for sickle cell anemia.

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