POLYMERIZATION IN ERYTHROCYTES CONTAINING S AND NON-S HEMOGLOBINS

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ABSTRACT We analyzed the effects of protein and water nonideality and of erythrocyte heterogeneity on the polymerization of hemoglobin S in cells where there were significant amounts of non-S hemoglobins, sickle trait (AS), and SC disease. For AS erythrocytes, the calculated predicted results were in good agreement with measured polymer formation as previously reported (Noguchi C.T., D.A. Torchia, and A.N. Schnechter, 1981, *J. Biol. Chem.* 256:4168–4171). Throughout much of the physiologically relevant oxygen saturation region, polymer was not formed in AS erythrocytes. Measurements of polymer formation in SC erythrocytes as a function of oxygen saturation using ^{13}C NMR are reported here and also are in good agreement with the calculated predicted results. As in sickle (SS) erythrocytes, polymer can be detected in SC erythrocytes in the region above 60% oxygen saturation. The increased polymer formation in SC erythrocytes as compared with AS erythrocytes can be explained in terms of hemoglobin composition and concentration in SC erythrocytes, with the concomitant increase in the proportion of dense cells. These findings provide a basis for understanding the pathophysiology of sickle cell and of SC disease, in contrast to benign sickle trait, in terms of intracellular polymer formation.

INTRODUCTION

The polymerization of deoxygenated hemoglobin S has been studied in cell-free solutions as well as in the intact cell. Detailed biochemical and biophysical investigations of cell-free solutions have led to a comprehensive thermodynamic description for polymerization of hemoglobin S (Minton, 1977; Hofrichter, 1979; Gill et al., 1980; Sunshine et al., 1982). The extremely strong concentration dependence of the polymerization process demonstrates the nonideal behavior of concentrated protein solutions. We have previously used this thermodynamic description to predict the behavior of polymer formation in homozygous sickle (SS)[1] erythrocytes as a function of oxygen saturation (Noguchi et al., 1980; Noguchi and Schechter, 1981). Hemoglobin nonideality could account for the general behavior of polymer formation as a function of oxygen saturation for homogeneous SS erythrocytes, which was measured using ^{13}C NMR. However, the effect of heterogeneity in intracellular hemoglobin concentrations was not explicitly considered in that analysis. Furthermore, the analysis, which ignores the contribution of water nonideality, could not explain our previous ^{13}C NMR measurements of polymer formation as a function of oxygen saturation for sickle trait (AS) erythrocytes (Noguchi et al., 1981).

The recent addition of the nonideal behavior of water in the solvent to the hemoglobin nonideality in the thermodynamic description for hemoglobin S polymerization greatly improved the fit of the calculated prediction to the experimentally measured solubility of cell-free hemoglobin S solutions in the presence of oxygen (Gill et al., 1980; Sunshine et al., 1982). When applied to fractionated subpopulations of SS erythrocytes with a narrow range of intracellular hemoglobin concentrations, we found that the consideration of water and protein nonideality in the analysis could also predict the behavior of polymer formation as a function of oxygen saturation (Noguchi et al., 1983). This thermodynamic description and the density profile for a whole populations of SS erythrocytes were used to predict the amount of polymer in the whole blood sample and was in excellent agreement with the values measured using ^{13}C NMR (Noguchi et al., 1983).

Here we have used the thermodynamic analysis for hemoglobin S polymerization, including protein and water nonideality, to quantitate hemoglobin S polymerization in AS erythrocytes and in erythrocytes from individuals with SC disease. These results are compared with ^{13}C NMR measurements of polymer formation in SC erythrocytes and our previously reported ^{13}C NMR measurements in AS erythrocytes (Noguchi et al., 1981). We show that, within the framework of hemoglobin S polymerization, the behavior of polymer formation as a function of oxygen

[1] *Abbreviations used in this paper:* SS, homozygous for sickle cell anemia; AS, sickle trait (heterozygous); SC disease, double heterozygous for hemoglobin S ($\alpha_2\beta_2^{6Glu\rightarrow Val}$) and hemoglobin C ($\alpha_2\beta_2^{6Glu\rightarrow Lys}$); SS erythrocytes, sickle erythrocytes; AS erythrocytes, sickle trait erythrocytes; SC erythrocytes, SC disease erythrocytes.

saturation provides a general explanation for the clinical severity of SC disease in comparison with the benign clinical course of sickle trait (Bunn et al., 1977).

MATERIALS AND METHODS

Sample Preparations

SC erythrocytes were prepared for the NMR measurements as previously described (Noguchi and Schechter, 1981). Cells were washed three times with Earle's balanced salt solution without glutamine and bicarbonate and with 25 mM HEPES at pH 7.2. Each sample at the different oxygen saturations was made by equilibrating 2 ml of the cell suspension with the appropriate gas mixture of oxygen and nitrogen using a spinning cup tonometer (IL-237; Instrumentation Laboratory, Inc., Lexington, MA) for 10–20 min. The sample oxygen saturation was determined with a MBA-Microblood Analyzer (Advanced Products SRL, Milan, Italy) (Rossi-Bernardi et al., 1977). The sample was transferred anaerobically into an 8-mm NMR tube specially fitted with a serum cap and flushed with the identical gas mixture. Cells were packed and the supernatant removed anaerobically. The final sample volume was 0.65 ml. The procedure started immediately after blood was drawn and completed within 24 h.

$^{13}C/^{1}H$ Magnetic Double Resonance

Natural abundance ^{13}C NMR spectra were acquired using a spectrometer (TT = 14; Nicolet Instrument Corp., Madison, WI) modified for high-power double-resonance experiments in solids (Sutherland et al., 1979). For each sample the probe containing an external $^{2}H_2O$ lock was tuned to 50Ω. Temperature was maintained at 37°C using a Dewar flask and nitrogen gas flow. For the standard 90°-t sequence, a 0.7 G (gauss) ($\gamma_2 H_2/\pi$ = 3 kHz) resonant field was used. For dipolar decoupling, a 13 G ($\gamma_2 H_2/2\pi$ = 55 kHz) resonant field was used. For cross-polarization the Hartman-Hahn condition ($\gamma_1 H_1 = \gamma_2 H_2$) was determined using adamantane. Nuclear Overhauser enhancement was suppressed during data acquisition. A 50-W ^{13}C transmitter (15.9 MHz) gave a 90° pulse in about 5 μs. The relative amounts of polymer was determined from the set of cross-polarization spectra. These values were converted to absolute amounts of polymer using the scalar decoupled spectra for the fully oxygenated and fully deoxygenated spectra as previously described (Noguchi et al., 1980).

Density Gradients

The erythrocyte profile for intracellular hemoglobin concentration was obtained by cell separation on a discontinuous Stractan density gradient as previously described (Corash et al., 1974; Clark et al., 1978). Cells were washed three times and suspended to three times the packed cell volume using phosphate-buffered saline containing glucose (Clark et al., 1978). Solutions of decreasing Stractan density (from 33 g/dl to 19 g/dl at 290 mosM) were layered into a 16 × 102-mm centrifuge tube. The cell suspension was layered on top of the gradient and the tube was centrifuged at 20,000 rpm at 5°C for 45 min in a rotor (SW 28.1; Beckman Instruments, Inc., Fullerton, CA). The tube was then sliced between layers to separate the various cell fractions and hemoglobin content of each fraction measured.

Numerical Analysis

Calculations were carried out with the use of a desk-top computer (4054; Tektronix, Inc., Beaverton, OR). The integral expression for $(a_w/a_w^o)^r$ (Eq. 4 below) was evaluated numerically. In solving Eqs. 2 and 3 (below), the solution mole fraction x_i for the various hemoglobin species other than $(\alpha_2\beta_2^S)$ [such as $(\alpha_2\beta_2^C)$, $(\alpha_2\beta_2^C)$, $(\alpha_2\beta^S\beta^C)$, $(\alpha_2\beta_2^A)$, or $(\alpha_2\beta^S\beta^A)$] is expressed terms of the solution mole fraction x_{ss} for $(\alpha_2\beta_2^S)$. x_{ss} is then varied until the condition expressed in Eq. 3 (or Eq. 4) is satisfied. The procedure for considering liganded hemoglobin S (i.e., the presence of oxygen) has been described by Hofrichter (1979). For the multiple liganded T-states of hemoglobin S, the e_i values used are e_{SS1} = 0.4 for a single liganded T-state and $e_{SSn} = (e_{SS1})^n$ for a T-state hemoglobin molecule with n-ligands bound with n = 2,3, or 4. e_i = 0 for R-state hemoglobin. For the hybrids $(\alpha_2\beta^S\beta^A)$ and $(\alpha_2\beta^S\beta^C)$, the same e_i value is used with $e_{SA} = e_{SC}$ = 0.4 (Bunn et al., 1982). For $(\alpha_2\beta_2^A)$ and $(\alpha_2\beta_2^C)$, the e_i value is zero ($e_{AA} = e_{CC}$ = 0).

ANALYSIS AND RESULTS

The polymerization or aggregation of deoxygenated hemoglobin S can be analyzed within the framework of a two-phase system, a polymer phase in equilibrium with a solution phase (Minton, 1977). The concentration of free hemoglobin molecules is equal to the hemoglobin solubility C_σ (16 g/dl at 37°C for pure deoxyhemoglobin S) and the concentration of the polymer phase $C\rho$ is 69 g/dl (Hofrichter, 1979). For any gel of total hemoglobin concentration, C_T, the fraction of hemoglobin in the polymer phase, f_ρ, can be calculated using $f_\rho = C_\rho\,(C_T - C_\sigma)/C_T\,(C_\rho - C_\sigma)$ (Noguchi and Schechter, 1981). Studies of polymerization of cell-free hemoglobin solutions have led to the development of a thermodynamic analysis to explain the behavior of hemoglobin S solubility in solution upon the addition of ligands or other hemoglobins in mixtures with deoxygenated hemoglobin S (Minton, 1977; Hofrichter, 1979; Gill et al., 1980; Sunshine et al., 1982). For a mixture containing i types of hemoglobin molecules (for example, hemoglobin S and oxygen results in various liganded states of hemoglobin S) x_i is defined as the mole fraction in the solution phase of each i species. Assuming that the mixture has an ideal behavior and that long-range forces can be neglected, the hemoglobin solubility, C_σ, for this mixture is given by

$$C_\sigma/C_\sigma^o = 1\Big/\sum_i (x_i e_i), \qquad (1)$$

where e_i is the relative tendency for the i species to be incorporated into the polymer phase with e_i = 1 for pure deoxyhemoglobin S (see Materials and Methods [Minton, 1974]). C_σ^o is the solubility for pure deoxyhemoglobin S. Eq. 1 gives a linear relationship, for the ideal approximation, between $1/\Sigma_i\,(x_i e_i)$ and the solubility C_σ (Fig. 1 *a*) with $1/\Sigma_i\,(x_i e_i) = 2$ when $C_\sigma = 2C_\sigma^o$. Eq. 1 was used to predict the behavior of polymer fraction upon the addition of oxygen for hemoglobin S at 34 g/dl, an approximate mean corpuscular hemoglobin concentration of erythrocytes (Fig. 2 *a*) for this ideal approximation. At complete deoxygenation, ($C_\sigma = C_\sigma^o$), 0.7 of the total hemoglobin is polymerized. The calculated amount of polymer rapidly decreases with increasing oxygen saturation, going to zero at 0.35 oxygen saturation.

However, the nonideal behavior of hemoglobin becomes apparent as hemoglobin concentration increases (Ross, 1978) and in Eq. 1 it is necessary to replace the concentra-

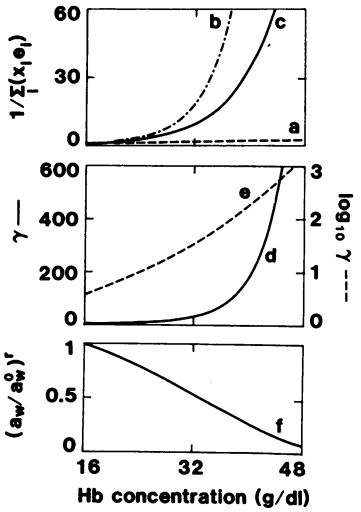

FIGURE 1 The behavior of $1/\Sigma_i(x_i e_i)$ as a function of the solubility of hemoglobin and the effect of hemoglobin and solvent concentrations on the theoretical analysis of hemoglobin solubility. The curves were calculated using the thermodynamic analysis for polymerization of hemoglobin S. *a* represents the ideal approximation, *b* includes the nonideal behavior of hemoglobin, and *c* includes the nonideal behavior of both hemoglobin and solvent. The solid line (—) in the center panel *d* represents the nonideal behavior of the activity coefficient γ as a function of hemoglobin concentration. The dashed line (---) *e* is the $\log_{10}$ of γ. In the bottom panel *f* represents the nonideal behavior of the solvent term $(a_w/a_{w_o})^r$ factored into the thermodynamic analysis as a function of hemoglobin solubility.

tion of hemoglobin, C_σ, with the activity $a_e = \gamma C_\sigma$ (Minton, 1977). The activity coefficient γ equals 1 at low concentrations and has a complex exponential dependence on hemoglobin concentration (Fig. 1 *d,e*). For hemoglobin solubility, Eq. 1 can be rewritten as

$$a_e/a_e^0 = (\gamma C_\sigma)/(\gamma_o C_\sigma^0) = 1\Big/\sum_i (x_i e_i), \qquad (2)$$

where γ_o is the activity coefficient corresponding to C_σ^0, the solubility of pure deoxyhemoglobin S. Eq. 2 gives a complex exponential dependence of $1/\Sigma_i(x_i e_i)$ on hemoglobin solubility (Fig. 1 *b*) with $1/\Sigma_i(x_i e_i) = 19$ when $C_\sigma = 2C_\sigma^0$. When Eq. 2 is used to predict the behavior of polymer fraction upon the addition of oxygen for hemoglobin S at 34 g/dl (Fig. 2 *b*), the polymer fraction decreases much more gradually with increasing oxygen saturation, becoming 0 at 95% oxygen saturation.

When in addition the nonideal behavior of the solvent is included in the thermodynamic description of hemoglobin S polymerization (Gill et al., 1980) the term $(a_w/a_w^0)^r$, representing the nonideal behavior of water in the hemoglobin S gell (Sunshine et al., 1982), is factored into Eq. 2 to give

$$(\gamma C_\sigma/\gamma_o C_\sigma^0)\,(a_w/a_w^0)^r = 1\Big/\sum_i (x_i e_i). \qquad (3)$$

a_w is the activity of water in the solvent, a_w^0 is the activity of water for pure deoxyhemoglobin S at C_σ^0, and r is the ratio

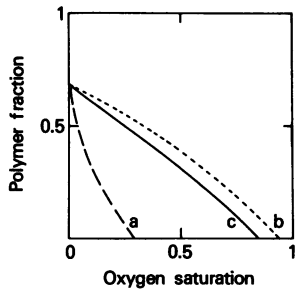

FIGURE 2 The amount of polymer formed as a function of oxygen saturation. Using the thermodynamic analysis, the amount of polymer was calculated for pure hemoglobin S at 34 g/dl. *a* represents the ideal approximation, *b* includes the nonideal behavior of hemoglobin, and *c* includes the nonideal behavior of both hemoglobin and solvent.

of moles of solvent water to moles of hemoglobin in the polymer phase. This ratio can be expressed as

$$(a_w/a_w^0)^r = \exp -\int_{C_\sigma^0}^{C_\sigma} \frac{1/C_\rho - \bar{v}}{1/C - \bar{v}}\left(\frac{1}{C} + \sum_{k-2}^{6} B_k C^{k-2}\right) dC, \qquad (4)$$

where $\bar{v}$ is the partial specific volume of hemoglobin and the B_k's are parameters expressing the activity coefficient for hemoglobin as a function of concentration (see Fig. 1 *d,e*). The behavior of $(a_w/a_w^0)^r$ is maximal when $C_\sigma = C_\sigma^0$ and decreases with increasing C_σ (Fig. 1 *f*). Comparing the results from Eq. 2 and 3, one sees that the rise in $1/\Sigma_i(x_i e_i)$ is slightly decreased as C_σ increases using Eq. 3 (Fig. 1 *c*) with $1/\Sigma_i(x_i e_i) = 10$ when $C_\sigma = 2C_\sigma^0$. The nonideal behavior of water and hemoglobin were used to calculate again the polymer fraction for hemoglobin S at 34 g/dl (Fig. 2 *c*). In the presence of oxygen, the predicted polymer fraction is slightly lower than the prediction calculated using Eq. 2, and goes to 0 at 85% oxygen saturation.

For a population of erythrocytes containing different concentrations or mixtures of hemoglobins, the heterogeneity profile can be used in conjunction with the thermodynamic analysis to calculate the polymer fraction of the entire sample (Noguchi et al., 1983). For example, consider a population of erythrocytes with the same hemoglobin composition but with varying hemoglobin concentration. f_j represents the fraction of the population with a total hemoglobin concentration of C_j. Using the thermodynamic analysis for each j fraction, we calculated the polymer fraction, $f_{\rho j}$, and the oxygen saturation, y_{Tj}, as a function of the oxygen saturation y of the free hemoglobin phase ($f_{\rho j}$ and y_{Tj} can be expressed as $f_{\rho j}\,[y]$ and $y_{Tj}\,[y]$ to explicitly indicate their dependence on y). The polymer fraction f_ρ and oxygen saturation y_T for the entire population is given by

$$f_\rho(y) = \sum_j f_j f_{\rho j}(y)$$
$$y_T(y) = \sum_j f_j y_{Tj}(y). \qquad (5)$$

Using Eqs. 3 and 5 and information about the heterogeneous distribution of erythrocytes in whole blood, a prediction for the amount of polymer can be calculated based on the thermodynamic analysis described above. Such a calculation for polymer formation in AS and SC erythrocytes has been made using our previously reported measurements of the distribution in intracellular hemoglobin concentration for the respective populations (Bunn et al., 1982), see Table I. AS erythrocytes have 40% hemoglobin S and 60% hemoglobin A, and SC erythrocytes have 50% hemoglobin S and 50% hemoglobin C (Serjeant and Serjeant, 1972). In addition, the mechanism for hemoglobin polymerization of hemoglobins S and C mixtures is similar to comparable hemoglobins S and A mixtures (Bunn et al., 1982). The distribution in intracellular hemoglobin concentration for AS erythrocytes is similar to normal erythrocytes with an MCHC of ~32 g/dl (Bunn et al., 1982; Serjeant and Serjeant, 1972). SC erythrocytes have a distribution shifted to higher values of intracellular hemoglobin concentration with an MCHC of ~34 g/dl (Bunn et al., 1982; Fabry et al., 1982). (SS erythrocytes exhibit a broad distribution in intracellular hemoglobin concentration generally encompassing the lower and higher range found in both AS and SC erythrocytes, respectively (Noguchi et al., 1983; Fabry and Nagel, 1982).

We have used these data and the thermodynamic analysis to calculate predictions for the amount of polymer formed as a function of oxygen saturation. The results are shown in Fig. 3 *a, b*. Fig. 3 *c,* for comparison, is the behavior of polymer formation for a population of SS erythrocytes (Noguchi et al., 1983). As in the case for SS erythrocytes (Noguchi et al., 1980; 1983), polymer formation is maximal at complete deoxygenation for AS (Fig. 3 *a*) and SC (Fig. 3 *b*) erythrocytes and gradually decreases with increasing oxygen saturation. The contribution of cell heterogeneity in intracellular hemoglobin concentration is most apparent at the higher oxygen saturations, shifting the *x*-intercept to higher oxygen saturation

TABLE I
DENSITY PROFILE FOR AS AND SC ERYTHROCYTES*

Fraction number‡	MCHC§	Hemoglobin fraction	
		AS	SC
	g/dl		
1	<27	0.003	0.013
2	28.5	0.284	0.093
3	32	0.658	0.574
4	35	0.045	0.243
5	>37	0.010	0.064

*Data from Fig. 3 of Bunn et al. (1982).
‡Cells were separated by discontinuous Stractan gradients.
§For the theoretical calculations, the hemoglobin concentrations were normalized to give an MCHC of 31.9 g/dl for AS and 34.3 g/dl for SC.

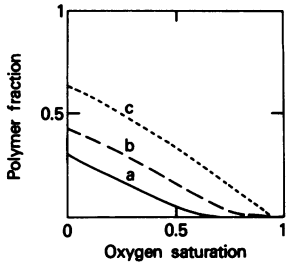

FIGURE 3 The theoretical analysis for polymer fraction as a function of oxygen saturation for AS, SC, and SS erythrocytes taking into account the nonideal behavior of hemoglobin and solvent. *a* represents the prediction for AS erythrocytes based on an average density profile for AS erythrocytes (see Table I) and *b* represents the prediction for SC erythrocytes based on an average density profile for SC erythrocytes (see Table I). *c* represents the prediction for SS erythrocytes for a single individual (Noguchi et al., 1983), but is close to average behavior of SS erythrocytes (Noguchi et al., 1980).

values. The two curves for polymer formation in AS and SC erythrocytes parallel one another. The larger values for polymer fraction in SC erythrocytes is a result of the greater percentage of hemoglobin S, the higher mean intracellular hemoglobin concentration, and the greater proportion of dense cells in SC erythrocytes compared with AS erythrocytes.

Polymer formation in AS erythrocytes experimentally measured by ^{13}C NMR was previously reported by us (Noguchi et al., 1981). These earlier results are illustrated in Fig. 4 *a*. Also included in Fig. 4 *a* is the curve for the calculated prediction for polymer fraction. For these calculations, the profile for the distribution in intracellular hemoglobin concentration reported for AS erythrocytes was scaled to fit mean corpuscular hemoglobin concentration of the samples used for the experimental measurements. The calculated predictions for polymer fraction in AS erythrocytes are in good agreement with the experimentally measured values.

The amount of intracellular polymer in SC erythrocytes was measured using ^{13}C NMR. These experimental results are illustrated in Fig. 4 *b*. For comparison, the predicted values for polymer fraction were calculated using the

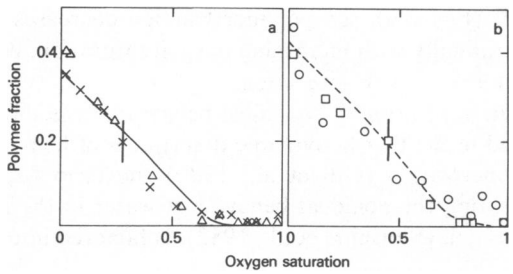

FIGURE 4 ^{13}C NMR measurements of polymer fraction as a function of oxygen saturation for AS and SC erythrocytes. The symbols represent the measured amounts of polymer using ^{13}C NMR. The curves represent the predicted behaviors for polymer fraction using the thermodynamic analysis with hemoglobin and solvent non-ideality. *a* is the result for AS erythrocytes and *b* is the result for SC erythrocytes.

thermodynamic analysis and the distribution profile for intracellular hemoglobin concentration. This calculated prediction is represented by the curved line in Fig. 4 *b*. Agreement is obtained between the calculated prediction and the experimentally measured result.

DISCUSSION

The utility of a thermodynamic description for polymerization or aggregation of hemoglobin S (Minton, 1977; Hofrichter, 1979; Gill et al., 1980; Sunshine et al., 1982) is that it provides a basis from which information obtained from cell-free solutions can be extrapolated to the cellular environment. Specifically, results obtained at complete deoxygenation or at hemoglobin concentrations <30 g/dl can be used to predict behavior at physiologic conditions such as the intracellular polymerization of hemoglobin S at 60 to 95% oxygen saturation (Noguchi et al., 1980). When the total hemoglobin concentration, C_T, exceeds the solubility of hemoglobin, C_o, the two-phase model can be used to determine the fraction of hemoglobin in the polymer phase, f_p (Minton, 1974). However, this information along with the solubility at complete deoxygenation (curve *a*, in Figs. 1 and 2) is not sufficient to predict the amount of polymer formed when oxygen is added. It is necessary to include the effect of hemoglobin nonideality, which markedly increases with increasing concentration (Fig. 1 *d,e*) (Minton, 1977; Ross et al., 1978). For example, at physiologic concentrations at 34 g/dl, the activity of hemoglobin is ~50 times the actually measured concentration (Ross et al., 1978). The greatest effect of nonpolymerizing hemoglobins on polymerization can be explained in terms of this nonideal behavior (curve *b*, Figs. 1 and 2), which together with the two-phase model, predicts the general behavior for the amount of polymer formed upon the addition of oxygen (Hofrichter, 1979).

Detailed agreement between the thermodynamic analysis and experimental results from cell-free solution studies of hemoglobin S polymerization in the presence of oxygen can be obtained by the additional consideration of the nonideal behavior of the solvent (Gill et al., 1980; Sunshine et al., 1982) reflected in the term $(a_w/a_w^o)^r$ (Fig. 1 *f*). This effect increases as the solubility C_σ increases (curve *c*, Fig. 1), further reducing the amount of polymer formed with increasing oxygen saturation (curve *c*, Fig. 2). The thermodynamic description for gelation now successfully predicts the behavior of intracellular polymer formation in subpopulations of SS erythrocytes with narrow distributions in intracellular hemoglobin concentration and in whole populations of SS erythrocytes (Noguchi et al., 1983).

The calculations based on the thermodynamic analysis have been extended to AS and SC erythrocytes (Fig. 3 *a,b*) based on previously published values for MCHC (31.9 and 34.3 g/dl, respectively), the proportion of hemoglobin S (40 and 50%, respectively), and the distribution profile for intracellular hemoglobin concentration (Bunn et al., 1982). The amount of polymer in AS erythrocytes (Fig. 3 *a*) is considerably less than that in SS erythrocytes (Fig. 3 *c*) and is insignificant throughout much of the physiologic range for oxygen saturation (Noguchi et al., 1981). The polymer fraction in SC erythrocytes (Fig. 3 *b*) parallels the behavior for AS erythrocytes but is ~0.13 greater up to 60% oxygen saturation. The increased polymer in SC erythrocytes is a direct consequence of the increased MCHC and increased percentage of hemoglobin S (Bunn et al., 1982; Serjeant and Serjeant, 1972; Fabry et al., 1982). An important feature of these calculations is that the polymer fraction for AS erythrocytes goes to 0 around 65% oxygen saturation in contrast to the polymer fraction for SC erythrocytes, which goes to 0 around 90% oxygen saturation. The tail at high oxygen saturations for SC erythrocytes reflects the contribution from cell heterogeneity in intracellular hemoglobin concentration, particularly the increased fraction of dense cells in comparison to AS erythrocytes. In fact, the calculations predict that polymer can be formed in SC erythrocytes up to 90% oxygen saturation, similar to SS erythrocytes (Noguchi and Schechter, 1981; Noguchi et al., 1983).

The present calculation for polymer fraction, which includes both protein and water nonideality, is shown in Fig. 4 *a* together with the earlier ^{13}C NMR data for AS erythrocytes (Noguchi et al., 1981). There is good agreement between the calculated prediction and the experimentally measured results. Shown in Fig. 4 *b* are the ^{13}C NMR measurements of polymer fraction for SC erythrocytes, which are in good agreement with the calculated prediction. These results demonstrate that the thermodynamic analysis can be applied to intracellular polymerization of AS and SC erythrocytes. Furthermore, hemoglobin polymerization in AS and SC erythrocytes is similar to polymerization in comparable cell-free solutions, and that crystallization of hemoglobin C does not contribute significantly to polymerization or aggregation in SC erythrocytes. In general, the intracellular hemoglobin concentration and the hemoglobin composition are the primary determinants of intracellular polymer formation.

Within the framework of hemoglobin S polymerization, the pathogenesis of SC disease can be explained in terms of the increases in MCHC, in the proportion of hemoglobin S, and in the proportion of dense cells in SC erythrocytes (Bunn et al., 1982; Serjeant and Serjeant, 1972; Fabry et al., 1982). This condition results in significant amounts of polymer formation throughout much of the physiologically relevant oxygen saturation region. In SS erythrocytes, deoxygenation resulting in hemoglobin polymerization can lead to increases in intracellular hemoglobin concentration. Cells appear to undergo irreversible increases in density when such changes occur repeatedly (Fabry and Nagel, 1982). However, cells that form polymer at complete deoxygenation do not necessarily give rise to dense cells as exemplified by AS erythrocytes whose density profile is analogous to AA erythrocytes. Other mechanisms besides those associated with hemoglobin S polymerization can

give rise to dense cells as in the case of AC and CC erythrocytes, where the density profile is also shifted to higher values (Bunn et al., 1982; Fabry et al., 1982). It is likely that the mechanism that gives rise to an increase in MCHC for AC and CC erythrocytes (AC and CC are benign phenotypes) is also responsible in part for the increase in MCHC in SC erythrocytes. In addition, the polymerization of deoxyhemoglobin S, which can increase the density of SS erythrocytes, may also be responsible for the large increase in the dense cell fraction in SC disease. In contrast, the absence of significant amounts of polymer formation in AS erythrocytes throughout much of the physiologically relevant oxygen saturation region (>60% oxygen saturation) would explain the benign course of sickle trait.

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