

# Effects of Hemoglobin Concentration on Deformability of Individual Sickle Cells After Deoxygenation

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To assess the role of intracellular hemoglobin concentration in the deformability of sickle (HbSS) cells after deoxygenation, rheologic coefficients (static rigidity  $E$  and dynamic rigidity  $\eta$ ) of density-fractionated individual sickle erythrocytes (SS cells) were determined as a function of oxygen tension ( $pO_2$ ) using the micropipette technique in a newly developed experimental chamber. With stepwise deoxygenation,  $E$  and  $\eta$  values showed no significant increase before morphologic sickling but rose sharply after sickling. In denser cells, continued deoxygenation led to steep rises of  $E$  and  $\eta$  toward infinity, as the cell behaved as a solid. The  $pO_2$  levels at which rheologic and morphologic changes oc-

curred for individual SS cells during deoxygenation varied directly with the cell density. The extent of recovery in  $E$  and  $\eta$  during reoxygenation varied inversely with the cell density. These results provide direct evidence that the intracellular sickle hemoglobin (HbS) concentration of SS cells plays an important role in their rheologic heterogeneity in deoxygenation and reoxygenation. The elevations of  $\eta$  during  $pO_2$  alteration were greater than those of  $E$ , especially for the denser cells, suggesting the importance of the elevated dynamic rigidity in initiating microcirculatory disturbances in sickle cell disease.

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IT IS WELL KNOWN THAT the principal pathogenesis in sickle cell (HbSS) disease lies in the impairment of deformability of the sickle erythrocyte (SS cell)<sup>1-3</sup> due to the gelation of the sickle hemoglobin (HbS) after deoxygenation.<sup>4,5</sup> Thus, many investigators have studied the rheologic properties of HbS solutions,<sup>3,6</sup> SS cell suspensions,<sup>1-3,7</sup> and individual SS cells<sup>8-12</sup> with the aim of elucidating the rheologic abnormalities in sickle cell disease. These studies have shown that the viscoelastic coefficients of SS blood or SS cells are above normal even when fully oxygenated<sup>7-9</sup> and become further elevated with reductions in oxygen tension ( $pO_2$ ).<sup>3,11,12</sup>

In studying SS cell deformability, the micropipette aspiration technique<sup>8-12</sup> has the advantage of allowing the quantification of the rheologic properties of individual cells, rather than the average rheologic behavior of a cell population. A new experiment system we recently devised for the micropipette study of SS cells<sup>13</sup> has made it possible to measure the rheologic properties of a single cell under microscopic observation, while simultaneously altering and monitoring  $pO_2$  and maintaining the temperature at 37°C. Using this system, the viscoelastic coefficients of individual SS cells were determined as a function of  $pO_2$  by repeated aspirations of the same cell after controlled alterations of  $pO_2$ , thus allowing the tracking of the personal rheologic history of any given cell as a function of  $pO_2$ .<sup>13</sup>

The existence of dense SS erythrocytes has been regarded as an important determinant of the severity of sickle cell disease.<sup>14-16</sup> While there is evidence that the intracellular hemoglobin (Hb) concentration can affect SS cell deformability,<sup>5,10,12</sup> there is a lack of information on the direct relationship between the Hb concentration of individual SS cells and their rheologic behavior under controlled  $pO_2$  levels at body temperature. Our previous study on the relationship between the deformability of single SS cells and  $pO_2$  showed a considerable variation of the critical  $pO_2$  at which sickling and rheologic changes begin for individual cells.<sup>13</sup> It was suggested that this variability might be caused by the differences in Hb concentration among the SS cells in the unfractionated population.<sup>13</sup> In the present study, we fractionated the SS cells according to their density and applied our new micropipette system to study the effect of  $pO_2$  alteration on the viscoelastic parameters of individual density-separated SS erythrocytes. The aim was to elucidate the relationship

between  $pO_2$  and the rheologic properties of individual SS cells as a function of the cell density and, hence, the intracellular Hb concentration.

## MATERIALS AND METHODS

**Preparation of blood samples.** Blood was collected from nine patients with homozygous HbSS disease, with the approval of the Institutional Review Board, Health Sciences, Columbia University (New York, NY). Excluded from the study were patients in sickle cell crisis and those who had undergone transfusion therapy within the preceding 3 months. Fresh blood samples (8 to 10 mL) were drawn via venipuncture and centrifuged at 1,000g for 10 minutes, and the plasma and buffy coat were removed by aspiration. The red cells were washed three times in a buffered saline containing potassium and glucose<sup>5</sup> (BSKG; prepared by dissolving 7.808 g NaCl, 0.373 g KCl, 0.194 g  $NaH_2PO_4 \cdot H_2O$ , 1.220 g  $Na_2HPO_4$ , and 2.0 g glucose in 1,000 mL distilled water, pH 7.4, 290 mOsm/KgH<sub>2</sub>O) with the addition of bovine serum albumin (0.2%). Normal AA blood was drawn from healthy volunteers and prepared in a similar manner. AA cells, however, were not fractionated and used for comparison with SS cells in the oxygenated state.

Discontinuous density fractionation was performed with solutions of arabinogalactan polysaccharide<sup>17</sup> (Larex-L.O.; Larex International, Tacoma, WA). The Larex solutions, with discrete densities ranging from 1.077 to 1.153 g/mL in 0.015-g/mL increments, were prepared by diluting a stock Larex solution (adjusted to pH 7.4 and

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290 mOsm/KgH<sub>2</sub>O) with the BSKG solution. Densities of the solution were measured by using a digital densitometer (DMA 35; Anton Paar K.G., Gratz, Austria) at 20.0°C. Separation was achieved by 60 minutes of centrifugation at 28,000g and 20°C in a refrigerated superspeed centrifuge.

The fractionated populations trapped between the interfaces of successive layers of the Larex solutions were collected by a puncture method. The five fractions, each of which covered the range of the 0.015-g/mL increment, had mean densities (g/mL) of 1.086, 1.100, 1.115, 1.130, and 1.146; they were referred to as fractions I, II, III, IV, and V, respectively. The mean corpuscular hemoglobin concentration (MCHC) of the density-fractionated erythrocytes was calculated from the cell density by using the linear correlation of Noguchi et al.<sup>5</sup> The resultant MCHC values (g/dL) were 26.6, 32.1, 38.1, 44.0, and 51.6 for fractions I through V, respectively. Each fraction was washed three times with an excess volume of the BSKG-albumin solution and resuspended in the same solution that had been equilibrated with a gas mixture (O<sub>2</sub>, 20%; CO<sub>2</sub>, 5%; N<sub>2</sub>, balance). The preparations and subsequent micropipette aspirations were completed within 12 hours of blood withdrawal. Furthermore, blood samples and prepared cell suspensions were stored at 5°C until shortly before use to avoid metabolic depletion of the cells, which is known to affect their deformability.<sup>18</sup>

**Micropipette aspiration tests under controlled pO<sub>2</sub>.** The system for micropipette aspiration, pO<sub>2</sub> alteration, and temperature control has been reported in detail elsewhere.<sup>13</sup> Briefly, a red cell suspension was loaded in a chamber of the experimental system containing a pO<sub>2</sub> electrode, a reference electrode, a thermocouple, and a fluid exchange system. The temperature of the medium solution was monitored and maintained at 37°C. The pO<sub>2</sub> was altered by replacing the medium in the chamber with another medium that had been deoxygenated to the desired pO<sub>2</sub> level. Continuous monitoring of the pO<sub>2</sub> in the chamber using pO<sub>2</sub> electrodes and an oximeter indicated that a new steady state was effected in 30 to 120 seconds. Using this system, we were able to change the pO<sub>2</sub> and continuously track a single cell without losing the cell or interrupting the microscopic observation.

Micropipettes with internal radii of 0.5 to 0.7 μm were prepared by the use of a micropipette puller. The micropipette was filled with the BSKG-albumin solution and connected hydraulically to a pressure control system.<sup>13,19</sup> The micropipette tip was manipulated for positioning at the smooth rim of an erythrocyte under microscopic observation. Round discoid cells were studied in each fraction, except for the heaviest fraction. In the heaviest fraction, because round discoid-shaped erythrocytes were very rare, we used oval-shaped cells (cells with an aspect ratio less than 1.5). These oval-shaped cells could be clearly differentiated from irreversibly sickled cells on a morphologic basis.

With the micropipette tip placed at the rim of the cell, a preset negative pressure was applied as a step function to induce an aspirational deformation. Depending on the pO<sub>2</sub> and the cell density, the applied pressure ranged from 2 to 350 mm H<sub>2</sub>O. After a predetermined period of time (usually 20 seconds for nonsickled cells and 60 to 120 seconds for sickled cells), the pressure was removed as a step function. After three to five aspirations on the fully oxygenated cells as controls, the pO<sub>2</sub> was modified by changing the medium, and the aspirations were repeated as soon as the new pO<sub>2</sub> level was stabilized. The pO<sub>2</sub> at which sickling occurred in the particular cell being studied was recorded as the critical pO<sub>2</sub>. Sickling was defined as a morphologic alteration of the cell with roughened surface and rim, usually with granulated cytoplasm or an elongated shape. Generally, the pO<sub>2</sub> was reduced to below 40 mm Hg even if sickling had already occurred. In some cells from the denser fractions, however, the reduction in pO<sub>2</sub> was stopped at 60 to 80 mm Hg because the cells had already become very rigid. After the last deoxygenation

experiment, the pO<sub>2</sub> was increased stepwise, and the aspiration tests were repeated. In the final step of reoxygenation with 20% oxygen (pO<sub>2</sub>, 142 mm Hg), the cells were exposed to this elevated pO<sub>2</sub> level for a sufficiently long period of time (more than 5 minutes) to ensure equilibration, and the aspiration tests were then performed. After completion of an experimental run on a single cell, a new cell suspension was reloaded in the chamber, and the same procedure was repeated from the beginning for another SS cell.

**Determination of rheologic coefficients.** The deformational entry of the SS cell was continuously recorded with a video recording system.<sup>13</sup> The pertinent data (time, aspiration pressure, pO<sub>2</sub>, and temperature) were recorded simultaneously on the videotape using a video multiplexer. The tape was played back through a dimension analyzer. The electrical output from the dimension analyzer, corresponding to the length of the aspirated cell tongue, was stored in a desktop microcomputer for numerical analysis.

Rheologic coefficients of the SS cells were derived from the constitutive equation introduced by Chien et al.<sup>19</sup> Once sickling had occurred, the SS cell often exhibited a persistent distortion during the relaxation process after its release from the micropipette.<sup>13</sup> Thus, the rheologic behavior of sickled cells during recovery deviated from that of an ideal viscoelastic material, and, therefore, we did not pursue the numerical analysis of the recovery phase and focused only on the deformation process. For the same reason, we used the nomenclature of static rigidity (E, dyn/cm), instead of elastic modulus, and dynamic rigidity (η, dyn × s/cm), instead of membrane viscosity, as proposed by Evans et al.,<sup>10</sup> to express the effective deformability of the cells. The value of E was determined from the aspiration pressure (P), the micropipette radius (R), and the maximal deformational entry of the cell into the pipette (Dpm) by fitting the experiment curve to the theoretical model, using the least squares method<sup>19</sup>:

$$E = \frac{P \times R}{(2 \times Dpm/R) - 1 + \ln(2 \times Dpm/R)} \quad (\text{for } Dpm/R > 1) \quad (\text{Equation 1})$$

The value of η was calculated as the product of E and the time constant of cell deformation (τ): η = E × τ (Equation 2). The dynamic rigidity η is defined for both the initial rapid deformation phase (phase I) and the subsequent slow phase (phase II).<sup>19</sup> However, because changes in the dynamic rigidity for phase II were similar to those in phase I, as reported for unfractionated SS cells,<sup>13</sup> only η in phase I was subjected to detailed analysis and is reported.

## RESULTS

In the five fractions with defined densities, 24 cells were studied by micropipette aspiration. All cells exhibited morphologic sickling when deoxygenated below some critical pO<sub>2</sub> level. With further deoxygenation, some cells became so rigid that they did not respond to the maximal aspiration pressure (350 mm H<sub>2</sub>O) and behaved like solid bodies. In this state, the SS cell showed the characteristic roughened surface and granulated cytoplasm. In nonsickled SS cells, the aspiration pressure was applied for 20 seconds. In sickled SS cells, however, the duration of the micropipette aspiration varied from 60 to 120 seconds, depending on the extent of cellular hardening. When the cell could not be aspirated at all under the maximal negative pressure, we considered E and η as infinity, and the pO<sub>2</sub> level at which this first occurred is referred to as the solid-transition pO<sub>2</sub>.

**Rheologic properties in the control state.** The static rigidity (E) and the dynamic rigidity (η) of the SS cells before

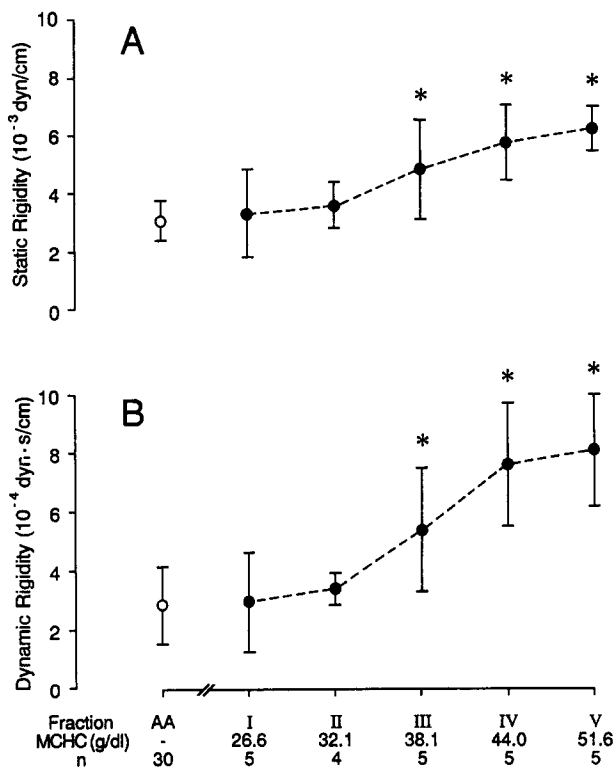


Fig 1. Rheologic properties of SS cells in the control, fully oxygenated state (before deoxygenation) as a function of cell fraction density and, hence, MCHC. The static rigidity  $E$  (A) and the dynamic rigidity  $\eta$  (B) of SS cells are plotted against fraction density. The values for unfractionated AA cells (open circles) are shown for comparison. Values are mean  $\pm$  SD. \* $P < .05$  ( $v$  AA cells).

deoxygenation are shown in Fig 1 for each fraction, together with those of the normal AA erythrocytes. In AA cells, the micropipette aspiration was performed on their rim to be more analogous to the site of aspiration for SS cells. The average  $E$  value of  $3.02 (\pm 0.74 \text{ SD}; n = 30) \times 10^{-3} \text{ dyn/cm}$  for our AA cells was slightly lower than those reported for dimple aspiration,<sup>19,20</sup> and this reflects the influence of the aspiration site.<sup>21</sup> Both  $E$  and  $\eta$  of SS cells increased with the rise in cell density (ie, MCHC). The  $E$  and  $\eta$  of fractions III to V of the oxygenated SS cells were significantly higher when compared with the control values in the AA cells ( $P < .05$ ).

The rheologic properties of the individual SS cells under oxygenated state showed marked heterogeneity (Fig 1), which is characteristic for sickle cell disease,<sup>5,14</sup> and the variance is considerable even in a given fraction. Therefore, the rheologic coefficients obtained during deoxygenation and reoxygenation for each SS cell were normalized by its own control values in the fully oxygenated state and are denoted as a static rigidity ratio or dynamic rigidity ratio. These normalized values are used for data presentation unless otherwise stated.

**Rheologic history of individual SS cells.** Figures 2 through 4 show the rheologic data of individual SS cells during deoxygenation and reoxygenation. The static rigidity

ratio ( $E$ ) and the dynamic rigidity ratio ( $\eta$ ) were tracked as a function of  $pO_2$ . To simplify the graphic presentation, only two typical examples from fractions I, III, and V are shown in each figure, but similar changes were observed for all of the cells studied. Each of the values for  $E$  and  $\eta$  at the initial stage and at the final stage was the mean of three to five measurements, and the measurements were highly reproducible, with coefficients of variation less than 5%.

In the least dense fraction I (Fig 2), the static rigidity ratio and the dynamic rigidity ratio did not show any significant change after deoxygenation, as long as the cells stayed unsickled. After the occurrence of sickling at the critical  $pO_2$ , however, the rheologic coefficients rose suddenly (10- to 20-fold for static rigidity ratio and 10- to 50-fold for dynamic rigidity ratio), and they continued to increase with further reductions in  $pO_2$ . These ratios gradually decreased with reoxygenation, but the values obtained after full reoxygenation were still 2 to 4 times higher than those in the initial oxygenated state. In this fraction, the cells resumed their smooth discocytic shape after full reoxygenation, but the static and dynamic rigidities did not completely recover to their control levels.

In the case of the moderately dense cells in fraction III, one cell (cell 2 in Fig 3) behaved like those in fraction I, except that the transition occurred at a higher critical  $pO_2$  (approximately 95 mm Hg) and that the magnitude of the increase was greater (280-fold for static rigidity ratio and 1,600-fold for dynamic rigidity ratio). The other cell in Fig 3 (cell 1) stayed unsickled until the  $pO_2$  was reduced to a critical level of 68 mm Hg, and then it assumed a solid-like state shortly after the sickling. With reoxygenation, the static and dynamic rigidity ratios decreased gradually, but they did not attain the control values. The morphology of these cells showed a partial recovery after reoxygenation, but they did not return to the round discoid shape.

The rheologic changes were more prominent in the densest fraction V. The cells shown in Fig 4 were easily sickled at critical  $pO_2$  values of approximately 100 mm Hg or even higher, and both cells behaved as a solid body with only small decreases in  $pO_2$ . Thus, for this densest fraction, a hyperrigid state occurred at a higher  $pO_2$  level than for the less dense fractions. When reoxygenated, the cells had markedly higher rheologic coefficients at a given  $pO_2$  than those obtained during deoxygenation; the extent of this hysteresis was more prominent in these densest cells. The morphology of the densest cells also showed relatively little recovery during the period of observation after reoxygenation.

**Relation of morphologic and rheologic changes to MCHC.** The above-mentioned results indicate that the rheologic rigidification of individual SS cells is closely related to their morphologic change, and both alterations occur at the same levels of critical  $pO_2$ . Comparison of the results obtained from different fractions shows that the critical  $pO_2$  varies with the cell density above 1.100 g/mL, or MCHC above 32.1 g/dL (Fig 5). While the cells in the densest fraction were easily sickled at rather high  $pO_2$  values (approximately 100 mm Hg), cells from the least dense fractions were not sickled until deoxygenated to approximately 50 mm Hg (Fig 5A). The  $pO_2$  level for solid-transition shows a similar rela-

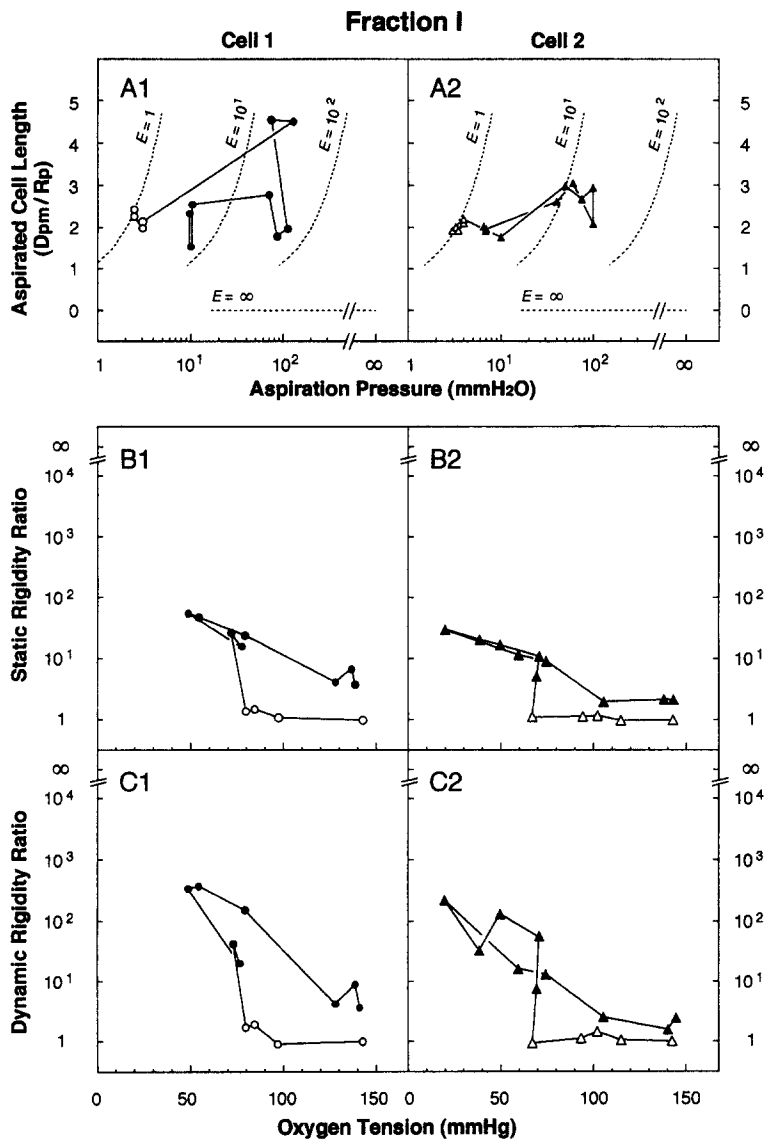


Fig 2. Rheologic coefficients and aspiration data of SS cells from fraction I (MCHC, 26.6 g/dL) during deoxygenation and reoxygenation. Two typical examples, represented by different symbols (circles for cell 1 and triangles for cell 2), are shown. Open and filled symbols represent the values before and after the occurrence of sickling, respectively. The aspiration data (relation between aspirated cell length  $Dpm/Rp$  and aspiration pressure  $P$ ) are shown in A1 and A2. Dotted lines are theoretical relation between  $Dpm/Rp$  and  $P$  derived from Equation 1 for static rigidity ratios 1,  $10^1$ ,  $10^2$ , and infinity. The static rigidity ratio (B1 and B2) and the dynamic rigidity ratio (C1 and C2) are shown as functions of oxygen tension. The rheologic coefficients showed no significant changes before sickling, but they rose sharply with the onset of sickling.

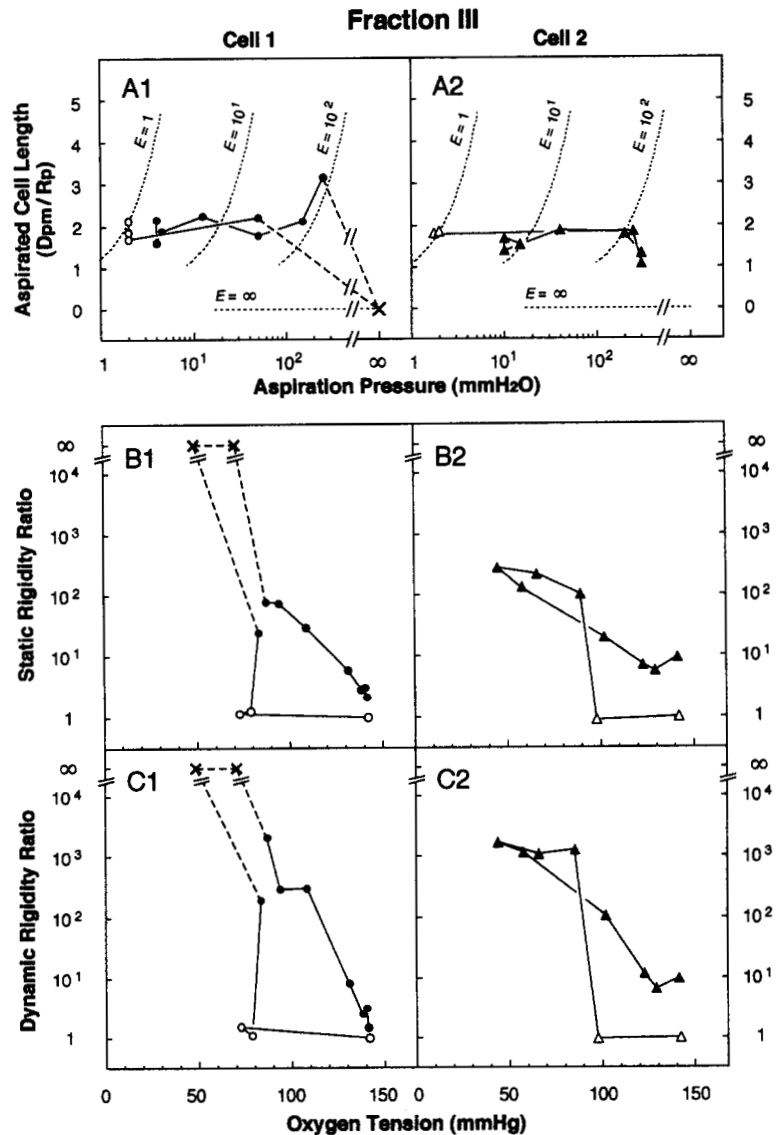
tion to MCHC (Fig 5B) as the critical  $pO_2$  for morphologic sickling (Fig 5A).

The static and dynamic rigidities after full reoxygenation often did not recover completely to the values obtained in the initial oxygenated state. The degree of incomplete recovery, or the rheologic hysteresis, was quantified by calculating a reoxygenation hysteresis index, which is the ratio of  $E$  or  $\eta$  at the final reoxygenation stage to the corresponding values at the initial oxygenated stage. This index for both  $E$  (Fig 6A) and  $\eta$  (Fig 6B) remained relatively constant for fractions I and II. For denser fractions, however, the recoverability became progressively poorer, and the reoxygenation hysteresis index rose to approximately 30 for  $E$  and approximately 70 for  $\eta$  in fraction V.

**Interrelation between  $E$  and  $\eta$ .** A crossplot between the static rigidity ratio and the dynamic rigidity ratio for all fractions under varying  $pO_2$  (Fig 7A) shows the relative changes of these two rheologic parameters during the modi-

fication of  $pO_2$ . When the values for both ratios were below approximately 10, the changes in static and dynamic rigidity ratios were essentially equal to each other. For larger changes, however, the increase in dynamic rigidity ratio became increasingly greater.

Because the changes in  $E$  and  $\eta$  with deoxygenation are functions of MCHC and there is a rheologic hysteresis, the crossplot has also been made for three fractions (I, III, and V) to show the paths of deoxygenation and reoxygenation (Fig 7B). The data for  $E$  and  $\eta$  from the least dense fraction I (dashed line) showed essentially the same relative changes with deoxygenation and reoxygenation: with deoxygenation to a  $pO_2$  of 40 mm Hg and then reoxygenation, the dynamic and static rigidity ratios rose to similar peak values and then returned along a path of reoxygenation very close to that of deoxygenation. In the heaviest fraction V (solid line),  $E$  and  $\eta$  increased beyond the turning points of the less dense fractions, and the increase of dynamic rigidity ratio became



**Fig 3.** Rheologic coefficients and aspiration data of SS cells from fraction III (MCHC, 38.1 g/dL) during deoxygenation and reoxygenation. Two typical examples, represented by different symbols (circles for cell 1 and triangles for cell 2), are shown in the same manner as those in Fig 2. Static and dynamic rigidity ratios showed no significant changes before sickling, but they rose sharply with the onset of sickling. Cell 1 assumed solid-like states, which are represented by X connected by dashed line.

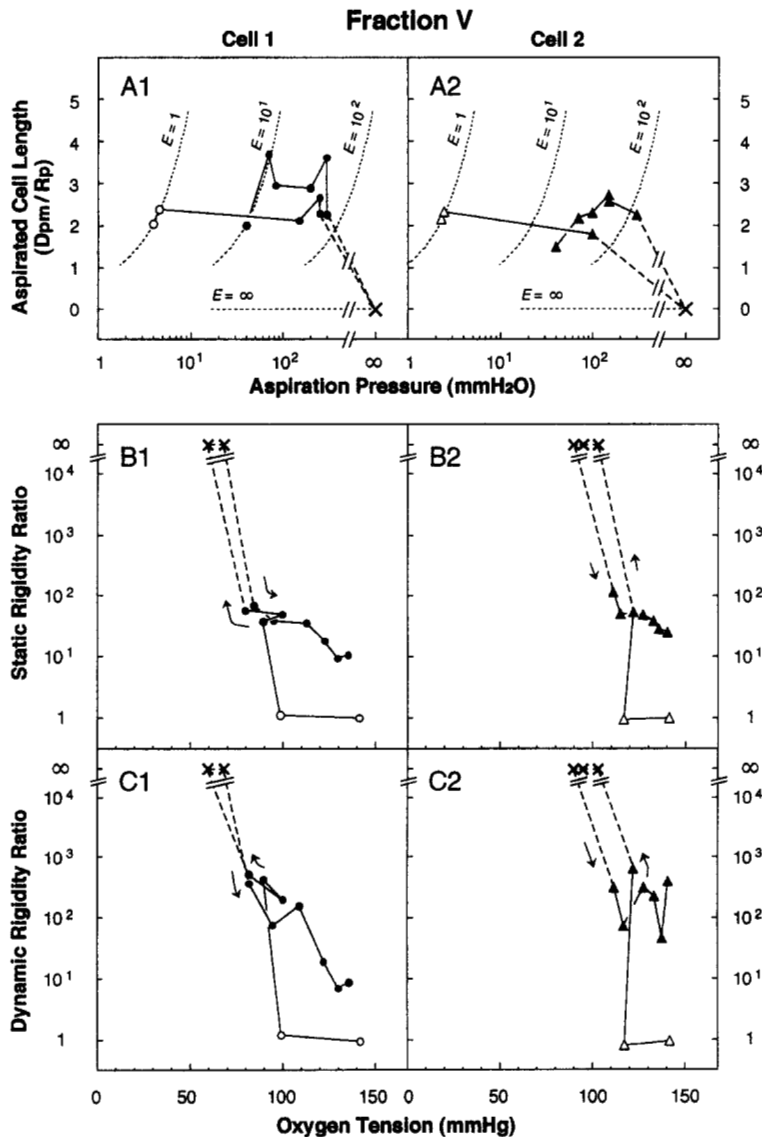
progressively greater than that of static rigidity ratio. At a  $pO_2$  of approximately 90 mm Hg, these rheologic parameters rose sharply toward infinity. With progressive reoxygenation,  $E$  and  $\eta$  resumed finite measurable values at a  $pO_2$  approximately 90 mm Hg, but the data points stayed much higher than the identity line, indicating relatively greater changes in  $\eta$  than  $E$ . After full reoxygenation, the data settled to the  $(E, \eta)$  point of (35, 70), which was far from the origin (1, 1) and from the final points of the less dense fractions, eg, (4, 5) for fraction I. The results from fraction III (dotted-dashed line) showed an intermediary profile between fractions I and V.

**DISCUSSION**

In a previous study on the effect of deoxygenation on unfractionated SS cells,<sup>13</sup> we found a sudden onset of rheologic alteration of individual SS cells with the occurrence of sickling. This sharp reduction in deformability with deox-

ygation has been reconfirmed in the present study using density-fractionated SS cells (Figs 2 to 4). One factor that could be responsible for this phenomenon is the shift in the relative contributions of the cell membrane and the intracellular fluid to the overall cell rheology.<sup>3</sup> The rheologic coefficients of individual cells determined by the micropipette technique before sickling represent primarily those of the cell membrane.<sup>19</sup> When the  $pO_2$  was reduced below a critical level, however, the extent of polymerization increased steeply, such that the rigidified intracellular fluid dominated the rheologic properties of the entire cell. Although plots of HbS polymer fraction against  $O_2$  saturation yield smooth curves with gradual changes,<sup>5</sup> the plot of polymer fraction against  $pO_2$  shows a much steeper rise with deoxygenation because of the S-shaped relation between  $O_2$  saturation and  $pO_2$ .<sup>22</sup>

With a few exceptions,<sup>15,23</sup> alterations in rheologic properties for both HbS solutions and SS erythrocyte suspensions



**Fig 4.** Rheologic coefficients and aspiration data of SS cells from fraction V (MCHC, 51.6 g/dL) during deoxygenation and reoxygenation. Two typical examples, represented by different symbols (circles for cell 1 and triangles for cell 2), are shown in the same manner as those in Fig 2. Static and dynamic rigidity ratios showed no significant changes before sickling, but they rose sharply with the onset of sickling. Both cell 1 and cell 2 assumed solid-like states, which are represented by X connected by dashed line.

have generally been reported to follow a continuously smooth curve, rather than a sudden transition. The continuous changes seen in SS cell suspension, in contrast to the sudden onset of rheologic changes of the individual SS cell at a critical  $pO_2$ , can be explained by the difference in critical  $pO_2$  values for individual SS cells in the population, as shown in the present study. Hence, a progressive reduction in  $pO_2$  leads to the increasing recruitment of SS cells, with lower intracellular Hb concentrations into the subpopulation that exhibits sickling and rheologic alteration, thus resulting in a continuous, smooth change in rheologic properties of SS cell suspensions.

With progressive deoxygenation below the critical level, some cells (mainly those from the denser fractions) exhibited a solid-like property as first reported in our previous study.<sup>13</sup> Such changes in the rheologic coefficients after deoxygenation are much greater than those reported by other investigators.<sup>12</sup> During and after reoxygenation, SS cell rheology

showed hysteretic behavior, the degree of which was density-dependent. Our data indicate that the rheologic coefficients of SS cells in fully oxygenated state, the critical  $pO_2$ , and the solid-transition  $pO_2$  all show positive correlations with the cell density for fractions II through V (Figs 1, 5, and 6), but the influence of cell density on SS cell rheology is not demonstrable between fractions I and II. Thus, intracellular Hb concentrations above 32 g/dL assume increasing importance in determining the rheologic behavior of SS cells after deoxygenation.

A comparison of the relative changes in the coefficients  $E$  and  $\eta$  after deoxygenation (Fig 7) showed that these two parameters rise in parallel when the changes are small (less than approximately 10-fold). When the changes are large, the rise in  $\eta$  is much steeper and becomes nearly 100-fold greater than the rise in  $E$  (Fig 7A). Furthermore, the SS cell rheology after reoxygenation exhibited a hysteretic behavior, especially for  $\eta$  of the denser fractions (Fig 6). It seems

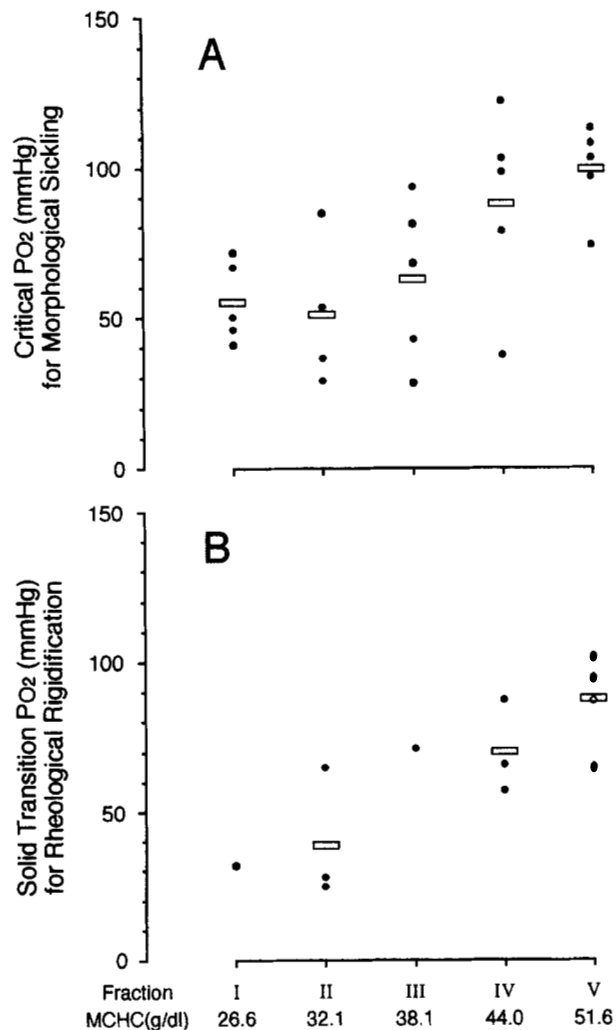


Fig 5. (A) Relationship between the critical  $pO_2$  and MCHC in SS cells. The critical  $pO_2$  is defined as the  $pO_2$  level at which sickling occurred in the cell observed under the aspiration test. Open rectangles denote the mean value for each fraction. There is a positive correlation between the critical  $pO_2$  and MCHC for fractions II through V. (B) Relationship between the solid-transition  $pO_2$  and MCHC in SS cells. The solid-transition  $pO_2$  is defined as the  $pO_2$  level at which the rheologic coefficients of the SS cell became nonmeasurable for the first time during the deoxygenation process. Because not all of the cells underwent the solid transition, the number of data points in B is fewer than in A. Open rectangles indicate the mean value for each fraction. There is a positive correlation between the solid-transition  $pO_2$  and MCHC for fractions II through V.

unlikely that this incomplete rheologic recovery is simply due to insufficient time allowed for reoxygenation of HbS. At the final state of reoxygenation, we exposed SS cells to 20% oxygen medium for a sufficiently long period of time to ensure equilibration. However, because we reoxygenated only to a  $pO_2$  level of 142 mm Hg, there is the possibility of the persistence of some polymerized HbS fibers. If very fine crystals or fibers of HbS persist and interact with the endosurface of the cell membrane,<sup>24,26</sup> they may increase the cellular rigidity. Another possible mechanism responsible

for this is that physical stress of repetitive pipette aspirations might lead to a cumulative derangement of the cytoskeleton protein organizations of SS cell membrane,<sup>27</sup> which might then cause the plasticity in the deformation and affect the measured rheologic coefficients of the tested cells. To assess or omit the possible effects of these factors, sickled SS cells must be subjected to repeated aspirations under rapid depolymerization, eg, by the use of carbon monoxide to assure complete melting of HbS polymers; this deserves future studies.

Rheologic behavior and morphologic characteristics of deoxygenated SS cells are dependent on the rate of deoxygenation.<sup>28,29</sup> The method used in the present study for altering  $pO_2$  in the experimental chamber did not allow us to complete deoxygenation and reoxygenation within a short period comparable with the mean circulation time in humans (less than 1 minute). Thus, red cells *in vivo* undergo more rapid cycles of deoxygenation and reoxygenation than the time course of the present study. Studies on the kinetics of HbS polymerization have suggested that most SS cells are not

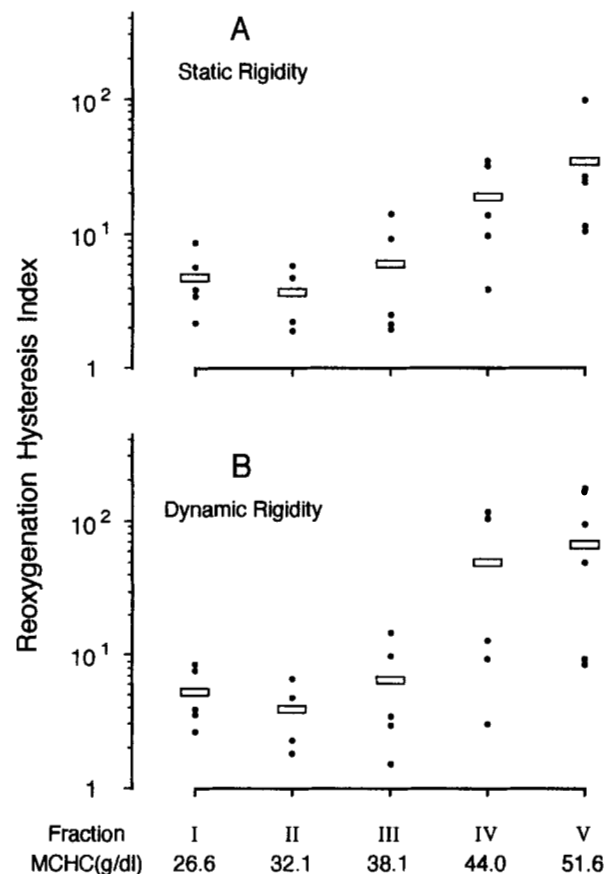
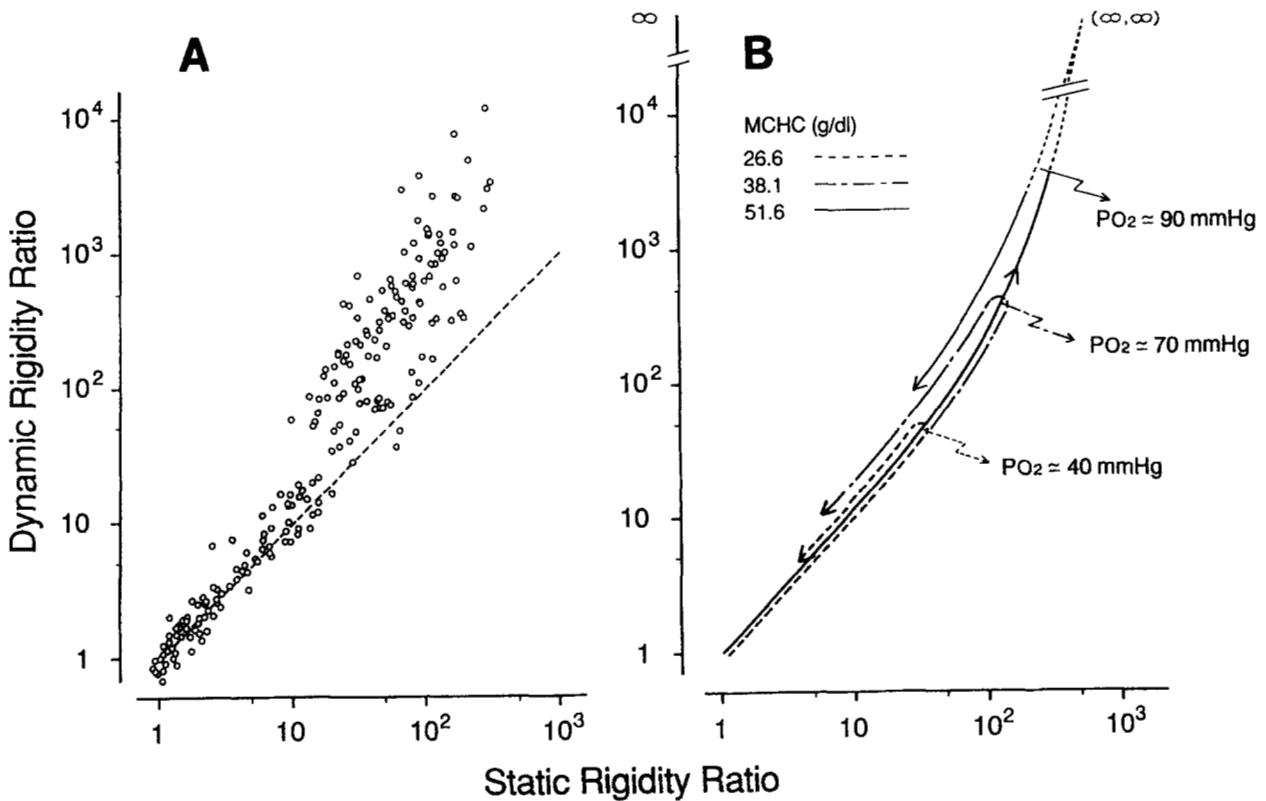


Fig 6. Relationship between the reoxygenation hysteresis index and MCHC in SS cells. The index, which gives an estimate of incomplete rheologic recoverability after reoxygenation, was calculated as the ratio of  $E$  (A) and  $\eta$  (B) at the final reoxygenation stage to their respective values in the initial oxygenated stage. Open rectangles represent the mean value in each fraction. The recoverability became progressively poorer with an increase in MCHC from fraction II to fraction V.





**Fig 7.** (A) Interrelation between static rigidity ratio ( $E$ ) and dynamic rigidity ratio ( $\eta$ ) of SS cells during deoxygenation and reoxygenation.  $E$  and  $\eta$  were normalized by their initial values before deoxygenation. The origin (1, 1) denotes the control (fully oxygenated) state. The diagonal dashed line denotes equal changes in the two ratios. (B) The average behavior of three fractions of SS cells during  $pO_2$  modification, showing the relationship between the static rigidity ratio  $E$  and the dynamic rigidity ratio  $\eta$ . The lines were drawn to fit the data points in A for fractions I (dashed line), III (dotted-dashed line), and V (solid line). The dotted lines connected to the solid lines for fraction V are drawn by extrapolation, where the rheologic coefficients could not be determined. The data points from the least dense fraction I showed linear changes up and down between the initial oxygenated state and a low  $pO_2$  of 40 mm Hg. In contrast, the data points from the densest fraction V exhibited a marked deviation from such a linear relation, and the terminal point after full reoxygenation was markedly higher than the origin. The data points from fraction III showed an intermediary pattern.

sickled in the steady-state circulation, mainly due to the shorter capillary transit time than the delay time before the onset of polymerization.<sup>22</sup> On the other hand, when deoxygenated very slowly, HbS polymerization can occur at  $pO_2$  levels higher than those in arterial blood.<sup>23</sup> Therefore, in consideration of the time factors involved in microcirculatory dynamics, our findings cannot be extrapolated directly to *in vivo* situations. Recently, there are several reports showing SS cell adhesion to microvascular endothelium.<sup>30-34</sup> Animal experiments using human red cells have suggested that the less dense SS cells are more adhesive than the denser SS cells.<sup>32-34</sup> Once any cellular adhesion has occurred, the microcirculation would inevitably be compromised, and local hypoxia would be worsened. This would cause more SS cells, irrespective of being adhered or not, to be exposed to lowered  $pO_2$  for a longer period of time and, hence, undergo rigidifications similar to those observed in this study. This would retard the deformational passage of SS cells through narrow vessels<sup>35,36</sup> and may cause vasoocclusive episodes.

In summary, we have shown that the rheologic properties of individual SS cells are strongly dependent on HbS concen-

tration. The critical  $pO_2$  for morphologic sickling and the  $pO_2$  for solid transition can be directly correlated with the HbS concentration above 32 g/dL. The hysteresis of rheologic coefficients during reoxygenation may play a pathophysiological role in that, once rigidified under given conditions, some SS cells may still be rigid even when reoxygenated on the arterial side of the circulation.

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