The Influence of Oxygen Tension, Temperature, and Hemoglobin Concentration on the Rheologic Properties of Sickle Erythrocytes

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With the use of micromanipulation techniques, the shear modulus or "rigidity" $\mu$, the recovery time $t_r$, and the unfolding time $t_u$ for individual sickle cells have been measured at different oxygen tensions, temperatures, and cell densities. In these experiments, the partial pressure of oxygen was varied from 156 to 40 mm Hg and the temperature was controlled at 25°C or 37°C. Three mean cellular hemoglobin concentrations were studied: 29 g/dL, 33 g/dL, and 46 g/dL. The lighter cells (29 and 33 g/dL) exhibited at most a threefold increase in rigidity as the $p_O_2$ was decreased from 156 to 40 mm Hg. At 25°C, the densest cells (46 g/dL) also exhibited a threefold increase. However, at 37°C, the rigidity of these cells increased eightfold between 156 to 40 mm Hg. Compared with normal cells, this gives a rigidity that is 18 times larger. In contrast to the values for $\mu$, the values for $t_r$ and $t_u$ remained essentially unchanged (within the accuracy of the experiments) for the lighter cells and could not be measured for the densest cells.

The work presented here measured the specific changes in the rheologic properties of the sickle cell as a function of oxygen tension, temperature, and hemoglobin concentration. The rheologic properties studied included the membrane rigidity (or the "effective cell rigidity") when the cytoplasmic viscosity of the SS erythrocytes increased significantly, which occurred in the cells with a mean corpuscular hemoglobin concentration (MCHC) of 46 g/dL at 40 mm Hg), the viscoelastic recovery time, and the unfolding time. The objectives of this research were to (1) determine the changes in the rheologic properties exhibited by SS erythrocytes under physiologic conditions; (2) analyze the rheologic properties of SS erythrocytes as a function of $p_O_2$, temperature, and MCHC; and (3) infer the influence of polymer on the changes found in the rigidity of SS cells with decreases in $p_O_2$.

MATERIALS AND METHODS

Cell preparation. Blood was collected from healthy adults and from 14 adult patients (seven men and seven women) with homozygous sickle cell disease in heparinized (14 USP U/mL) Vacutainer tubes. The Duke University Comprehensive Sickle Cell Center determined sickle cell disease by electrophoresis on cellulose acetate followed by citrate acid electrophoresis. Patients in sickle cell crisis and those who had received transfusions within the past 3 months were excluded from this study. The average age of the patient group was 35.1 years (SD, 12.5 years; range, 18 to 65 years). All measurements were made within 24 hours of venipuncture. The cell preparation was performed at 25°C ($\pm 1^oC$). The blood was centrifuged at 670g for 10 minutes. The plasma and buffy coat, which contain the platelets and white blood cells, were removed by aspiration. The erythrocytes were then washed twice in a Heps-buffered saline solution (290 mOsm/kg, pH 7.4, 131.9 mmol/L NaCl, 4.7 mmol/L KCl, 2.0 mmol/L CaCl$_2$, 1.2 mmol/L MgSO$_4$, 7H$_2$O, 11.0 mmol/L Hepes acid, 9.0 mmol/L Hepes Na salt) and resuspended at an approximate hematocrit of 10% in Heps buffer. One milliliter of the red blood (RBC) cell suspension was layered on the top of a 10-mL density gradient to separate the cells according to their internal hemoglobin concentration (MCHC).

The separation of RBCs into density fractions was achieved through discontinuous "Percoll" (Pharmacia, Uppsala, Sweden) gradients according to the method of Mackie et al. The gradients were successively layered, heaviest to lightest, in a 12-mL test tube. Separation was achieved by 30 minutes of centrifugation at 670g in an IEC Centrifuge (Needham, MA) with a swinging bucket rotor. The RBC band between each density gradient was removed through a syringe inserted in the side of the thin-walled polyallomer test tube at that band's particular height. The erythrocytes collected in each
band were washed and resuspended in the Heps buffer at an approximate hematocrit of 0.1% with 0.6% wt/wt human albumin. To check the density of cells from a particular band, a small sample of each fraction was layered on a range of phthlate ester oils with densities identical to the Percoll solution and spun in a 13,000g microhematocrit centrifuge (IEC MB) according to the method of Danon and Marikovsky modified as follows: the cells were spun only 5 to 10 minutes. The initial temperature of the phthlate ester oils was 25°C (±1°C) with a measured temperature increase of 3°C after spinning. The correlation given by Noguchi et al. was used to determine the MCHC from the cell density.

To minimize bacterial growth, 100 U/mL penicillin and 100 μg/mL streptomycin were added to all solutions. They were then filtered with a Gelman (Ann Arbor, MI) 0.2-μm filter. The osmolarities of the solutions were measured with a vapor pressure osmometer (Wescor 5100C, Logan, VT) and pH was measured by a digital pH meter (Beckman Model 3550, Irvine, CA). The temperature of the cell was controlled by water flow over the cell chamber. The temperature setting on the circulating water bath was calibrated using a thermocouple in the cell chamber. The temperature of the cells was controlled at 25°C or 37°C (±1°C).

The prepared cells from one density fraction were loaded into a flask on a rotating table where a saturated mixture of O₂, N₂, and CO₂ gases was swirled over the diluted erythrocyte suspension. After allowing 30 minutes for the cells to equilibrate to that particular pO₂, a syringe was inserted and a 0.5-mL sample was withdrawn and transferred to the glass chamber under the microscope. Part of the saturated gas mixture was directed past the two small openings in the glass chamber. An oxygen microelectrode (Microelectrodes Model MI-730 with Oxygen Meter Model OM-1, Londonberry, NH) in the erythrocyte suspension in the glass chamber measured the pO₂ throughout an experiment. The O₂, N₂, and CO₂ percentages were then changed, the remaining cells in the flask were equilibrated, and a new sample was loaded into the glass chamber under the microscope. For each density fraction, this procedure was repeated with varying O₂, N₂, and CO₂ concentrations. The gas concentrations used were 100% O₂, 21% O₂, 79% N₂, and trace CO₂ (air); 13.9% O₂, 80.9% N₂, and 5.3% CO₂ (representative of the gas concentrations in the lungs and the systemic arteries); and 5.3% O₂, 88.6% N₂, and 6.1% CO₂ (representative of the gas concentrations of typical arterial gases). The gas mixtures with CO₂ lowered the pH from 7.4 to 6.9.

**Measurement of viscoelastic properties.** To measure the rheologic properties of the erythrocytes, a micropipette was inserted into the chamber to manipulate the cells. These micropipettes were made by pulling Preiser Scientific 0.7 to 1.0 mm outside diameter pipettes (Charleston, WV) on a David Kopf Instruments Model 700 Vertical Pipette Puller (Tujunga, CA). The closed tip of the micropipette was snapped off by the methods of Merril and Ainsworth and Nash et al. The micropipettes were filled by boiling them under a vacuum in Heps buffer (290 mOsm/kg).

The micropipette was mounted in a De Fonbrune micromanipulator (Arenberg Ultrasonic Lab, Inc, Jamaica Plain, MA). The pressure inside the micropipette was controlled by a manometer connected to the micropipette by continuous water-filled tubing. The pressure in the micropipette was "zeroed" by adjusting the manometer's water reservoir level with a micrometer marked at 0.01-mm increments. Additional pressure could be applied by mouth or mechanical syringe to the reservoir chamber. A pressure transducer (Validyne Engineering Corp., Northridge, CA), connected to the pipette by a continuous water connection, measured changes in pipette pressure.

The cells were observed with a Leitz Diavert (West Germany) microscope equipped with a 40x, 0.65 numerical aperture (NA) long working distance Nikon (Japan) objective. Experiments were recorded on videocassette with a video camera (Dage-MTI, Michigan City, IN) mounted above the vertical eyepiece. Time and pressure were displayed on the video monitor (Setech-Carlson Electronics, Inc, New Brighton, MN) with a "data mixer" (Vista Electronics, La Mesa, CA). The camera image was recorded with a 1/4-inch videocassette recorder (Sony Corp of America, New York, NY). For data analysis, the tape was played through the video monitor that is equipped with a position analyzer (Vista Electronics).

The cells selected for micromanipulation had a shape that resembled the majority of the cells at a particular density and pO₂. The cells appeared fairly uniform because of the low fetal hemoglobin concentration (mean 3.2% ± 1.4%) and density separation. In general, all of the cells developed spicules and became increasingly more sickled as the pO₂ decreased. At low oxygen tensions (40 mm Hg), all of the cells tested exhibited spicules. Also, at low oxygen tensions (40 mm Hg), the cells at 46 g/dL exhibited plastic behavior.

The method of Evans and Waugh and Evans was used to measure membrane rigidity and determine a value for the elastic shear modulus for the membrane, μ. In this method, a small portion of the dimple region of the erythrocyte is aspirated into a micropipette that is approximately 1 μm in diameter. The radius of the micropipette, rₚ, was measured at the start of the experiment. The length of the cell projection into the pipette, Lₚ, was measured as a function of the aspiration pressure, P. A water manometer was used to increase the suction pressure in increments of approximately 1 mm H₂O (98 dynes/cm²). To allow the cell to reach its new equilibrium length, the length of the cell projection was measured approximately 30 to 60 seconds after each increment in pressure. Data were taken for each cell until it buckled or until it no longer responded to an increase in the aspiration pressure. Then, a value for μ was determined from the following equation:

$$\frac{P}{r_p} - \mu = 2.45 \left( \frac{L_p}{r_p} \right) - 0.63; \frac{L_p}{r_p} > 1.$$  

With the SS erythrocytes that were relatively rigid (i.e., MCHC = 46 g/dL, 40 mm Hg, 25°C and 37°C), the value of μ is no longer a measure of just membrane rigidity; rather, it is a measure of the overall or "effective" cell rigidity. There was a significant change that occurred in the sickle cells as the density increased from 33 to 46 g/dL at 40 mm Hg. The aspiration of the erythrocyte was no longer a function of the membrane properties alone, but rather a function of the membrane and the increasingly more rigid cell interior.

A value for the recovery time constant of the membrane was determined by the method of Hochmuth et al. in which an erythrocyte that is attached at a single point to the glass coverslip at the bottom of the chamber is extended by pulling it at a diametrically opposite point with a pipette that is approximately 1 μm in diameter. When the cell is released from the pipette, it recovers its shape within a characteristic time period, tᵣ. The recovery is driven by elastic forces in the membrane; however, the time rate of recovery is limited by the viscous energy dissipation in the membrane as well as the hemoglobin if the hemoglobin viscosity is approximately 100 times larger than normal.

The unfolding time yielded another measure of cell deformability. In this experiment, an RBC is aspirated into a 3.5 to 4.0-μm diameter pipette, then gently expelled with a pressure pulse. The width of the RBC was measured as the cell unfolded with time. The initial width was measured when the tail end of the cell first exited the pipette. Subsequent widths were measured every 1/60 to 3/60 of a second until the cell had completely unfolded and recovered its original shape. The inverse slope of the linear section of the unfolding data represents the "unfolding time," $$t_u = \frac{dW}{dt}$$, where W is the width of the cell at its midsection at any time t and
W∞ is the final width. The slope was determined by a linear regression analysis.

RESULTS

Three rheologic properties of sickle erythrocytes were measured as a function of cell density, pH, and temperature and then analyzed and converted to a function of polymer fraction. The three measured properties were the shear modulus of membrane elasticity, μ (or effective cell rigidity) for SS cells at 46 g/dL, 40 mm Hg, 25°C and 37°C, the recovery time constant, tR, and the unfolding time constant, tλ. A total of 400 cells were studied. Each data point represents from two to 16 cells.

Figure 1 shows the influence of pH on the shear modulus of membrane elasticity for sickle cells at different densities (29 g/dL, 33 g/dL, 46 g/dL) and at 25°C and 37°C. Normal erythrocytes (AA hemoglobin, 33 g/dL, 25°C) had a value for μ of 6.6 × 10⁻³ dyn/cm at a pH of 155 mm Hg. Nash et al15 found that the value for μ for AA cells showed no significant differences as the pH was reduced from 150 to 15 mm Hg. The sickle erythrocytes at 29 g/dL, 33 g/dL, and 46 g/dL, however, demonstrated significant differences (P < .05) in the values for μ at 25°C and 37°C as the pH was decreased from 156 to 40 mm Hg (100 to 40 mm Hg for the cells at 29 g/dL and 46 g/dL). The influence of cell density is also shown in Fig 1. The value for μ increased as cell density increased.

Figure 2 shows the influence of pH, density, and temperature on the recovery time constant, tR, of sickle cells. There is no data on the value of tR for SS cells at 46 g/dL since these cells were too stiff and could not be attached at a single point to perform the cell extension experiment. The general trends exhibited by the data show increases in the value for tR with increases in cell density and decreases in the value for tR with increases in the temperature in cells with 90% to 100% oxygen saturation (ie, 29 g/dL cells at 100 mm Hg and 33 g/dL cells at 150 mm Hg). The value for tR showed increasing trends as the pH was decreased. However, the increases were generally not significant (P > .05). Nash et al15 also saw no significant changes in the value for tR of discocytic SS erythrocytes with decreases in pH. However, Nash et al15 did find in sickled SS cells an increase in the half-time for membrane tongue growth with decrease in pH. Most of the SS cells in our experiments appeared discocytic at high pH, then became increasingly more sickled at the low pH. We did not observe any nonspiculated SS cells at low oxygen tensions.

Figure 3 shows the influence of pH, density, and temperature on the unfolding time constant, tλ. Normal AA erythrocytes (29 g/dL, 33 g/dL), at a pH of 150 mm Hg at 25°C and 37°C (33 g/dL), had a value for tλ that was very close to the value for tλ of sickle cells at the same density, pH, and temperature. Since the unfolding time is indicative of the
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Hb AA 33g/dL 25°C

HbSS 29g/dL 37°C

HbSS 33g/dL 37°C

HbSS 33g/dL 25°C

Hb SS 29 g/dL 37°C

HbSS 33 g/dL 25°C

Hb SS 33 g/dL 37°C

HbSS 46 g/dL 25°C

HbSS 46 g/dL 37°C

Fig 3. The unfolding time constant, dt/d\(\tilde{W}\) or \(\tau_u\), of SS erythrocytes (29 g/dL, 33 g/dL, 25°C and 37°C) and AA erythrocytes (29 g/dL, 33 g/dL, 25°C and 37°C) is shown versus \(P_{O_2}\). Each data point represents between five to 16 cells. Brackets correspond to standard deviations.

cells' overall deformability, this indicates that when sickle cells (33 g/dL) are at a \(P_{O_2} = 150\) mm Hg (corresponding to 90% to 100% saturation), their deformability is very similar to normal AA erythrocytes. Because sickle cells appear to be more adherent to each other, however, some delay before these cells unfold often occurred. The two sides of an individual sickle erythrocyte adhered together in the folded position. The values for the unfolding time in Fig 3 represent those cells that did not stick together before unfolding. Also shown in Fig 3, only the SS cells (29 g/dL and 33 g/dL) at 37°C exhibited an increasing trend in the unfolding time as the \(P_{O_2}\) was decreased.

One set of unfolding experiments with SS erythrocytes was conducted with 100% \(O_2\) gas (\(P_{O_2} = 736\)) which yielded a value for \(\tau_u\) (33 g/dL, 25°C) of 0.70 s. This was not statistically different from the value for \(\tau_u\) for SS cells that were 100% saturated yet at a lower \(P_{O_2}\) of 156 mm Hg.

The influence of density on the value for \(\tau_u\) is similar to earlier studies. Increases in density resulted in increasing trends in the value of \(\tau_u\) for both AA and SS cells at a \(P_{O_2}\) of approximately 155 mm Hg. With small increases in density, from 29 to 33 g/dL, increases of 5% and 14% in the value for \(\tau_u\) were found for SS and AA cells, respectively (25°C, \(P_{O_2} = 155\) mm Hg). Evans et al. also found a small increase in the value for \(\tau_u\) for AA and SS cells in the 32 to 36 g/dL density range. Over a large density increase, 32 to 44 g/dL, Evans et al. found a 12-fold increase in the value for \(\tau_u\) in the AA and SS cells.

The data were converted from a function of \(P_{O_2}\), temperature, and density to a function of polymer fraction and density using oxygen saturation curves, Bohr effect equations, polymer fraction versus oxygen saturation curves, hemoglobin solubility data, and hemoglobin concentration data. The polymer fraction versus oxygen saturation curves were derived using SS cells at pH 7.2. Hemoglobin concentration data and hemoglobin solubility data were used to calculate the percent polymer at 0% saturation, \(P_o\), at pH 6.9, 25°C and 37°C. With the correct value for \(P_o\), the proper polymer fraction versus oxygen saturation curve was used to determine the polymer fraction at other saturation values. The rigidity, \(\mu\), was the only parameter that exhibited any significant changes. Thus, only the value for \(\mu\) as a function of polymer fraction is presented. Figure 4 illustrates this value as a function of polymer fraction and density. The SS cells with an MCHC of 29 g/dL did not exhibit any significant increases in \(\mu\) as the polymer fraction increased for 0 to 0.37. The SS cells with an MCHC of 33 g/dL exhibited a significant (\(P < .05\)) increase in \(\mu\) between 0 and 0.56 polymer fractions. However, between 0.14 and 0.56 polymer fractions, there was no significant change in rigidity. The rigidity in the SS cells at 46 g/dL exhibited a stronger relationship to polymer fraction. The value increased from 12 \(\times 10^{-3}\) dyn/cm at 0% polymer to 100 \(\times 10^{-3}\) dyn/cm at 80% polymer. The largest increase in rigidity was

Fig 4. The shear modulus of membrane elasticity, \(\mu\), of SS erythrocytes (29 g/dL, 33 g/dL, 46 g/dL, pH 6.9, 25°C and 37°C) is shown versus the polymer fraction. \(\mu\) Represents an "effective cell rigidity" in the SS cells with 0.60 to 0.80 polymer fraction. Each data point represents three to 14 cells (only one data point represents three cells). Brackets correspond to standard deviations.
found between 60% polymer and 80% polymer where the value increased from $40 \times 10^{-3}$ dyn/cm to $100 \times 10^{-3}$ dyn/cm.

DISCUSSION

The rheologic properties of SS erythrocytes are influenced by many factors, including cell density (MCHC), temperature, $pO_2$, and oxygen saturation. Previous studies of the rheologic properties of SS erythrocytes have focused on the function of MCHC, $pO_2$, or MCHC. The results presented here with regard to $pO_2$ and MCHC display trends similar to those reported in previous studies.

The data in this study and from Evans et al. show increasing trends in the value of $\mu$ for SS erythrocytes equilibrated with room air ($pO_2$, approximately 156 mm Hg) as the cell density increases from 30 to 46 g/dL. Evans et al. reported that SS cells at 46 g/dL, however, manifest a larger increase in the value for $\mu$. It is uncertain whether this result is due to increases in cell density alone or to polymer formation in combination with increased cell density.

The results presented in this study concerning the influence of $pO_2$ on the value of $\mu$ for SS erythrocytes differ from a portion of the results from Nash et al. who reported no change in the value for $\mu$ in SS discocytes, which resemble the normal AA discocytic cells in shape, as $pO_2$ decreased. With regard to sickled SS erythrocytes, however, they reported an increase in the value for $\mu$ as $pO_2$ decreased. The increases they found in their “sickled” SS cells are very similar to the increase in $\mu$ found in this study for SS cells at 46 g/dL, which, at low $pO_2$s, also appeared sickled. The SS cells observed in this study demonstrated increases in the value for $\mu$ with decreases in $pO_2$ not only for the cells at 46 g/dL, but also for those at 33 g/dL, which appeared discocytic in shape with spicules forming at low $pO_2$s. These increases in $\mu$ were influenced by cell density. The cells at the higher MCHCs showed greater increases in $\mu$, explainable by the greater polymer formation in the denser group of cells. The two groups of SS erythrocytes observed by Nash et al. demonstrated different traits; while the discocytic group showed no change in the value of $\mu$ with decreases in $pO_2$, the sickled group produced large increases in the value of $\mu$ with decreases in $pO_2$. Because MCHC was not analyzed, it is possible that the discocytic cells had a much lower density and contained more fetal hemoglobin. The presence of fetal hemoglobin could have decreased the amount of polymer formed at low $pO_2$s and, consequently, produced little or no changes in the value for $\mu$. Due to density separation and cell populations with low concentrations of fetal hemoglobin, all the cells in this study developed spicules at low oxygen tensions. In general, the cells in Fig 4 developed spicules when the polymer fraction reached 0.20 to 0.30, although the overall shape was more disc-like than sickled. As the polymer fraction increased, the cell became more sickled. The densest cells with polymer fractions of 0.60 and greater generally resembled the typical elongated sickle shape.

The cells with an MCHC of 46 g/dL showed a much larger increase in the value for $\mu$ than the cells with an MCHC of 29 and 33 g/dL. Because Evans et al. showed large increases in the value for $\mu$ in oxygenated SS cells with an MCHC above 40 g/dL, part of the large increase we found in the cells with an MCHC of 46 g/dL could be attributed to an increase in density and part to the formation of hemoglobin polymers as deoxygenation occurs.

This study showed a generally increasing trend in the values for $t_s$ of SS erythrocytes with decreases in $pO_2$. However, due to the large standard deviations and small sample sizes, there emerges no statistically significant changes in the value for $t_s$ as $pO_2$ is decreased.

When analyzing the results under physiologic conditions ($pO_2 = 40$ to 100 mm Hg, 37°C), the average density (33 g/dL) SS erythrocytes exhibited a threefold increase (over AA erythrocytes) in the value of $\mu$, while the densest (46 g/dL) SS erythrocytes demonstrated an 18-fold increase. This indicates that at 33 g/dL, some of the cell’s hemoglobin has gelled and, thus, some of the polymer is associating with the membrane. Even with a threefold increase in the value of $\mu$, the SS erythrocytes (33 g/dL) were still easily deformed and therefore would probably still be capable of traversing the microvascular system. The 18-fold increase in the value of $\mu$ in the SS cells at 46 g/dL indicates significant gelation of the hemoglobin and a large increase in cell rigidity. Accordingly, the 18-fold increase in the value of $\mu$ indicates that the densest 3% (MCHC ≥ 46 g/dL) of the SS erythrocytes in a sickle cell patient’s blood probably contribute most to blocked capillaries and "sickle crises.”

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RHEOLOGICAL PROPERTIES OF SICKLE ERYTHROCYTES

The influence of oxygen tension, temperature, and hemoglobin concentration on the rheologic properties of sickle erythrocytes

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