Ektacytometric Measurement of Sickle Cell Deformability as a Continuous Function of Oxygen Tension

By Martin P. Sorette, Marc G. Lavenant, and Margaret R. Clark

In an effort to study the rheologic effects of small amounts of hemoglobin S (HbS) polymer in sickle red cells, we have used the ektacytometer, a laser diffraction couette viscometer, to measure sickle cell deformability as a function of oxygen tension. Sickle cell populations of defined intracellular hemoglobin concentration (MCHC) were isolated using Stractan density gradients and were resuspended in buffered polyvinylpyrrolidone solutions for deformability measurements. Using a gas-porous, hollow fiber gas exchange system to establish a linear gradient in oxygen tension, deformability was measured over a PO2 range of 76 to 0 mm Hg. Parallel spectroscopic determinations of oxygen saturation permitted determination of cell deformability as a function of oxygen saturation for each discrete MCHC population. From these measurements the level of oxygen saturation at which a loss in cell deformability was first detected could be defined. Then, using the data of Noguchi and Schecter, the amount of polymerized HbS in the cells at that defined level of oxygen saturation was estimated. The results of this analysis suggested that the quantity of polymer that caused a detectable loss in cell deformability increased with increasing MCHC. In addition, for MCHC above 30 g/dl, this represented a substantial fraction of the total HbS in the cell.

IT IS WIDELY ACCEPTED that the pathologic manifestations of sickle cell disease arise from abnormalities in blood rheology that ultimately derive from the formation of rigid hemoglobin S (HbS) polymer within the cells. Several different factors that influence blood rheology could potentially contribute to clinical problems: acute loss of deformability in red cells that develop polymer at low oxygen tension; chronically impaired deformability of red cells that have been dehydrated, possibly as a result of previous polymerization episodes; impaired capillary blood flow because of increased adherence of sickle red cells to the endothelial lining of the vessels; as well as factors other than sickle red cells that modulate the function of the microvasculature. Recently nuclear magnetic resonance (NMR) studies have provided evidence that sickle cells from the upper end of the distribution in mean cell hemoglobin concentration (MCHC) maintain polymeric HbS even at relatively high levels of oxygen saturation. On this basis it has been suggested that the continuous presence of polymer in circulating sickle cells may provide a dual hazard. First, polymerized red cells may have altered rheologic properties and a reduced ability to deform, and second, the residual polymer might itself impair the deformability of the red cell, reducing its ability to circulate through the microvasculature.

In an effort to evaluate the physiologic significance of this latter possibility, we have employed a means of measuring sickle cell deformability at low oxygen tension similar to those used by Johnson et al., Bessis et al., and Feo et al. By measuring the loss of cell deformability as a continuous function of oxygen tension, we have sought to determine the effect of a relatively defined intracellular concentration of HbS polymer on red cell rheology.

MATERIALS AND METHODS

After obtaining informed consent, as approved by the Human and Environmental Protection Committee of the University of California, San Francisco, blood samples were drawn into acid-citrate-dextrose (ACD) or EDTA. Patients were identified as being homozygous for HbS on the basis of hemoglobin electrophoresis and family studies performed by the Northern California Comprehensive Sickle Cell Center. To measure red cell deformability we used the ektacytometer, a laser diffraction couette viscometer that has previously been described in detail. For the present experiments the instrument was coupled to a gas exchange system similar to that described by Schmukler and Chien, which permitted the preparation of cell suspensions at defined oxygen tension (Fig 1). The gas exchange system was a bundle of 200 μm diameter gas-porous, hollow fibers (Celgard type X-10 MHF; Celanese Corp, Charlotte, NC), around which flowed a humidified mixture of nitrogen and oxygen at the desired oxygen tension. The fibers were sealed into a 40x5x2 mm piece of plexiglass housing with gas-tight compression fittings at the entry and exit ports. The red cells, suspended in a solution of 4% (wt/vol) polyvinylpyrrolidone (PVP) in isotonic buffered salt solution, were pumped through the fibers by a Harvard infusion pump (Model 600-000, Harvard Apparatus Co, Dover, MA) at a rate of 3.8 mL/minute. The suspension then flowed into a length of oxygen-impermeable tubing (Nylonflow pressure tubing 1/8 in, Polymer Corp, Reading, PA) immersed in a water bath at 37 °C, where it was held for varying periods to permit polymer formation. In normal operation a proportional metering valve was used to vary the proportion of oxygen to nitrogen in the gas exchange mixture over a chosen range as the cell suspension flowed through the gas exchange system. The fibers in which gas exchange occurred contained a cell suspension volume of 2.7 mL, and the full gradient of 76 mm Hg was established over a cell suspension volume of 30 mL. Once the oxygen gradient was established along the flowing cell suspension, the cells passed into a length of small diameter, gas-impermeable tubing, where they were equilibrated at 37 °C to permit polymer formation. The oxygen gradient was found to be maintained along the tube length for periods up to 90 minutes.

After equilibration the cell suspension was pumped past an oxygen electrode (Model E 5046; Radiometer, Copenhagen) fitted with a high sensitivity rapid response membrane (Yellow Springs Instru-

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Submitted May 5, 1986; accepted August 13, 1986.

Supported by USPHS Grant No. HL 20895.

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0006-4971/87/6901-0060$03.0/0

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The oxygen electrode was calibrated using flowing PVP-buffer solutions that had been equilibrated with defined gas mixtures.

In these experiments, for each set deformability measurements it was important to know the actual concentration of deoxy HbS within the cells as a function of oxygen tension. To determine this we prepared cell suspensions along the oxygen gradient exactly as for the ektacytometric measurements, but after the equilibration period pumped them through a flow-through cuvette in a Beckman DU7 spectrophotometer (Beckman Instruments, Palo Alto, CA). As the cells flowed past the oxygen electrode and through the cuvette, repeated measurements of absorbance at 540, 560, and 576 nm were made for every 3.8 mm Hg change in \( pO_2 \). Using fully deoxygenated and fully oxygenated cell suspensions, a correction for light scattering by the cells was determined. The corrected absorbance data were then used to calculate the percentage of deoxyhemoglobin at each time point, using the equations of Benesch et al.\(^{13}\) Care was taken to ensure that the cell suspension volume between the oxygen electrode and the light path in the cuvette was the same as the corresponding volume in the parallel ektacytometric experiments. This obviated the need to correct for the spatial separation of the viscometer and the oxygen electrode when DI data were related to oxygen saturation. The measured values of oxygen saturation and MCHC were then used to estimate intracellular polymer content at the values of oxygen tension at which deformability changes occurred, using data from Noguchi.\(^{12}\) This involved taking interpolated values from the graph representation of their calculated curves for polymer fraction as a function of oxygen saturation for cells of various MCHC over the range of 24 to 48 g/dL. To permit use of this approach, which does not take into account the presence of hemoglobins other than HbS, only samples containing less than 10% HbF (<8%) were used in this analysis.

Whole sickle blood contains cells covering a broad range in MCHC, which has a large effect on HbS polymerization. Therefore we used density gradient centrifugation to isolate more homogeneous MCHC populations in which polymer content could be more closely defined. We used arabinogalactan (Stractan II; St Regis Paper Co, Tacoma, WA)\(^{14}\) to prepare discontinuous gradients, using six layers ranging in equal steps from 1.085 to 1.110 g/mL density, layered onto a cushion of at least 1.133 g/mL density. The gradients were centrifuged in a Beckman L3-50 ultracentrifuge (Beckman Instruments, Palo Alto, CA) for 30 minutes at 74,000 \( \times g \) at 20 °C. The separated cells were collected from the gradient interfaces using a Pasteur pipet and were washed three times in buffered saline containing glucose and potassium (135 mmol/L NaCl, 5 mmol/L KCl, 8.6 mmol/L NaH\(_2\)PO\(_4\), 1.4 mmol/L NaH\(_2\)PO\(_4\), and 11 mmol/L glucose, adjusted to pH 7.4) before use. MCHC was determined from a spun hemocrit and spectrophotometric measurement of hemoglobin as the cyanothrombemoglobin complex. Fetal hemoglobin was quantitated using isoelectric focusing techniques.\(^{15}\) For evaluation of sickle cell morphology, a double gas exchange system was constructed so that glutaraldehyde solutions and red cell suspensions could be added to identical DI \( pO_2 \). The effluent from both sets of fibers was then brought together to fix the cells without altering their oxygen saturation. The percent of sickled cells was determined from counts of 500 cells.

RESULTS

When a gradient in oxygen concentration from 76 to 0 mm Hg was established along a flowing suspension of sickle cells and the cells allowed to equilibrate for 30 minutes, they showed the expected oxygen concentration-dependent reduction in the ability to deform under shear stress, as shown in Fig 2. The DI \( v \) \( pO_2 \) curves showed a constant, maximal DI
Fig 2. Variation in deformability signal (DI) as a function of oxygen tension for a Fraction 2 gradient population. As shown here, cell deformability decreased abruptly over a relatively narrow range in pO₂, producing a curve that essentially represented two intersecting lines. The point of intersection was used to define the pO₂ at which loss of deformability was first detected (pO₁). Reduction of the pH from 7.4 to 7.2 shifted this critical pO₂ to higher values, reflecting the pH-induced increase in HbS polymerization.

down to a critical pO₂ level, after which it decreased in essentially linear fashion. Thus, the intersection of the two linear portions of the curve was used to identify the pO₂ value at which a loss of deformability was first detectable (designated pO₁* in Fig 2). In addition, because this value was expected to vary with MCHC, we also used the pO₂ at which the DI fell to a value of 0.2 to characterize each curve. The data shown in this figure and in Figs 3 through 5 were obtained on cell populations collected from the interface between the first and second layers of Stractan (Fraction 2). Such density-separated cell populations displayed a sharper transition in deformability with oxygen tension than whole blood, as expected from their greater homogeneity (data not shown). It can be seen that these Fraction 2 samples in the various experiments did not give identical DI v pO₂ curves. Comparison of the oxygen dissociation curves for the samples showed that differences in oxygen affinity, presumably due to individual patient differences and differences in cell storage time, were the major source of variation.

A series of experiments was performed to determine the effect of important experimental variables and to define the optimal procedure to obtain reproducible, physiologically relevant data. As expected from the characteristics of the polymer equilibrium, several factors predictably affected the range of oxygen tension over which the cell deformability loss occurred. Reduction of the suspending medium pH from 7.4 to 7.2 caused a parallel shift of the DI v pO₂ curve to higher pO₂ (Fig 2), consistent with the increased Bohr effect of HbS, particularly below an extracellular pH of 7.4, and the corresponding enhancement of polymer formation.42 Most of the experiments employed paired deformability and oxygen saturation measurements at both pH 7.2 and 7.4 to permit comparison of the results with those of Noguchi et a17 and Noguchi17 on polymer content, since those experiments

Fig 3. Effect of suspending medium viscosity on the DI v pO₂ curve for a Fraction 2 gradient population. A decrease in viscosity below 19.5 cp at 37°C cp (4% PVP concentration) resulted in overall reduction in cell deformation and eventual blurring of the deoxygenation-dependent loss of cell deformability. (-----) 19.5 cp, (----) 12.5 cp, (———) 9.5 cp, (-----) 7.5 cp.

Fig 4. Comparison of DI v pO₂ relationship for continuous pO₂ gradient (continuous curve) and for measurements performed at fixed pO₂ values (open circles) over the same range for a Fraction 2 gradient population.

Fig 5. Relationship between the reduction in the DI signal and percentage of sickle cells for a Fraction 2 gradient population.
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were performed at pH 7.2. Some additional experiments were performed only at pH 7.4.

The position of the DI v pO2 curve also varied with MCHC, as shown by measurements using subpopulations of sickle cells isolated on density gradients (Fig 6). Again, the shift of the curves to higher pO2 with increasing MCHC was consistent with the known increase in polymer content with increasing MCHC for any given oxygen tension. The DI versus pO2 curves for unseparated cells (data not shown) were always broader than those for the gradient populations, reflecting the fact that the heterogeneous mixture of cells underwent polymerization and sickling over a broad pO2 range.

Another variable that influenced the position of the DI v pO2 curve was the viscosity of the suspending medium. A decrease in extracellular viscosity, achieved by reducing the concentration of PVP, resulted in a reduction in the proportion of deformable cells at progressively higher pO2 values. For higher MCHC cells there was also a reduction in the maximum attainable DI (Fig 3). We attribute this latter effect to the requirement for a threshold ratio of extracellular to intracellular viscosity to induce red cell deformation. A viscosity of 19.5 cp (4% PVP at 37°C) was chosen for subsequent experiments, since this viscosity showed clear-cut deformability differences among normal red cells from different density gradient subpopulations without introducing large variations among whole blood samples from different normal subjects. It was found that if the viscosity was reduced much below 19 cp, a substantial and varying proportion of cells from normal individuals would not deform, leading to a broadening of the range of normal isotonic DI values.

Other experimental variables tested for their effect on the deformability-oxygen tension relationship included the cation composition of the suspending medium, the flow rate of the cell suspension through the gas exchange fibers, and the time period between gas exchange and deformability measurement. When cells were equilibrated in high-Na, low-K medium, the DI-pO2 curve was shifted to higher oxygen tensions as compared to the curve obtained for cells from the same sample that were equilibrated in a medium in which the Na and K concentrations were close to their intracellular concentrations. This difference was interpreted as a reflection of a net leakage of K ions out of sickled cells in the high Na medium, resulting in a loss in cell water and a concomitant increase in HbS concentration and polymer content. Therefore the buffer that minimized transmembrane cation gradients was used for the rest of the experiments.

The rate at which the cells were pumped through the gas exchange fibers affected the subsequent deformability of even fully oxygenated cells. If the flow rate was high (>4 mL/min), the maximum cell deformation was decreased. We surmise that this was likely the result of shear-induced fragmentation occurring as the cells entered or left the fibers. Calculation of Reynolds numbers for flow through the fibers at these rates indicates that turbulence was probably present, increasing the likelihood of cellular fragmentation. The delay time for polymer formation at low HbS concentration and at pH 7.4 was expected to be prolonged, particularly at higher levels of oxygen saturation. Therefore, we wanted to determine the maximum period for which the cells could be equilibrated at 37°C without undergoing damage and alteration in their deformability properties that could not be explained on the basis of HbS polymer formation. Variation of the equilibration period from 30 to 90 minutes had no significant effect on the DI v pO2 curve (data not shown). An equilibration period of 30 minutes was chosen for the study.

We performed experiments in which 10-mL aliquots of cell suspension were run through the system while a constant pO2 was maintained in the gas exchange system (Fig 4). Measurement of the DI at various fixed pO2 values along the gradient range showed that the DI-pO2 relationship obtained was very close to the continuous curve obtained using the decreasing oxygen gradient (when the continuous curve was adjusted for the spatial separation of the oxygen electrode and the ektacytometer). Therefore, we conclude that measurements performed using a decreasing gradient from 76 to 0 mm Hg oxygen provide accurate information about the proportion of sickle cells that can deform after equilibration at a given pO2 value.

Examination of cell morphology at various points along the pO2 gradient allowed us to define the relationship between the reduction in the DI signal and the percentage of sickle cells in a representative isolated cell population with an MCHC of 32 g/dL at pH 7.2 (Fig 5). We observed both characteristic elongated cells with spicules, and a larger

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**Table 1. Characteristics of Sickle Cell Gradient Subpopulations**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>MCHC (g/dL)</th>
<th>Reticulocytes (%)</th>
<th>ISC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>26.00 ± 0.72</td>
<td>10.95 ± 1.14</td>
<td>2.12 ± 0.29</td>
</tr>
<tr>
<td>2</td>
<td>29.49 ± 0.44</td>
<td>7.84 ± 1.09</td>
<td>2.95 ± 0.48</td>
</tr>
<tr>
<td>3</td>
<td>31.21 ± 0.47</td>
<td>5.59 ± 0.55</td>
<td>3.82 ± 0.46</td>
</tr>
<tr>
<td>4</td>
<td>33.20 ± 0.47</td>
<td>4.22 ± 0.55</td>
<td>5.95 ± 0.60</td>
</tr>
<tr>
<td>5</td>
<td>34.75 ± 0.37</td>
<td>2.83 ± 0.80</td>
<td>7.58 ± 0.93</td>
</tr>
<tr>
<td>6</td>
<td>39.48 ± 0.91</td>
<td>1.40 ± 0.19</td>
<td>12.59 ± 1.08</td>
</tr>
</tbody>
</table>

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**Fig 6. DI v pO2 curves for Fractions 1 to 4 of Stractan-separated sickle cells.** As the MCHC of isolated cell populations increased, the maximum DI decreased, and a deoxygenation-dependent loss in cell deformability occurred at progressively higher pO2. See Table I for characteristics of the cell populations.
A number of crumpled, or "holly leaf," cell forms, all of which were counted as sickled cells. We note that sickle forms appear in the fixed cell population prior to a point where there is a decrease in the DI signal, and that some discoid cells persist even at very low PO2.

Using the conditions defined as described above, we obtained DI vs PO2 curves for 34 samples of density-separated sickle cells from 14 individuals, covering an MCHC range from 27 to 34 g/dL. These curves were characterized by two parameters: the PO2 at which the DI signal was first detectable, and the PO2 at which the DI signal fell to 0.2 (Fig 2). Then the parallel oxygen saturation measurements were used to determine the percent oxygen saturation at these PO2 values. These data, summarized in Fig 7, show that both the PO2 and oxygen saturation at which polymer formation interfered with cell deformability rose with increasing MCHC.

To relate the observed deoxygenation-dependent changes in sickle cell deformability to HbS polymer concentration, we used the estimates of Noguchi and her associates for polymer content at a given PO2 and intracellular HbS concentration. Using data only from samples containing less than 8% HbF, we have plotted the estimated polymer content resulting in the first detectable decrease in cell deformability as a function of MCHC (Fig 8). Since Noguchi's estimates were based on a suspending medium pH of 7.2 and since we were interested in the more physiologic pH of 7.4, we determined the DI as a function of oxygen saturation at both pH 7.2 and 7.4 and made the assumption that equivalent decreases in DI represented equivalent increases in polymer concentration at the two pH values. When the characteristic PO2 values were thus used to estimate polymer content at which a loss of deformability could first be detected, two observations emerged. First, the minimal detectable polymer increased with increasing MCHC. Second, in the higher MCHC cells it constituted a substantial fraction of the total hemoglobin inside the cells. Thus while it was 5% of total hemoglobin at 27 g/dL, polymer represented 35% at 34 g/dL at pH 7.2.

DISCUSSION

The present study provides new information concerning the rheologic effect of HbS polymer within red cells. While the physiologic interpretation of the results is limited by the fact that the threshold for loss of cell deformability depends on the selected experimental conditions, comparison with the rheologic effect of variations in MCHC and intracellular viscosity provides some perspective on the problem. The selected conditions for these experiments clearly distinguish oxygenated higher MCHC sickle cells from normal cells in their rheologic behavior, while oxygenated low MCHC cells are not distinct in their behavior. (This essentially repeats our previous observations for fully oxygenated, density-separated sickle cell populations. Moreover, deoxygenation results in a reduction in the ability of low MCHC sickle cells to deform and in an additional loss in the already reduced deformability of higher MCHC cells. The diffraction pattern generated by the ektacytometer at reduced oxygen tension indicates the presence of a double population of cells, deforming and nondeforming. Previously, it has been shown that the stably oriented, ellipsoidal pattern characteristic of red cells deforming under laminar shear stress requires a "tank-treading" motion of the membrane around the cell interior. Thus, the loss of cell deformability in the ektacytometer can be interpreted as a loss in the ability of the cell to undergo this dynamic, tank-treading motion. This is different from the ability of a cell to undergo gradual deformation from one stationary geometric form to another.

From the data presented in our cell morphology experiment, we have determined that the percent reduction in DI signal for a given percentage of sickle cells is greater than might be predicted from the linear decrease found with glutaraldehyde-fixed normal cells. There are at least two possible explanations for this result. First, reversibly sickled cells may orient in the shear field of the ektacytometer in a direction perpendicular to that of deforming cells, much like irreversibly sickled cells (ISC). Bessis and Mohandas have demonstrated that ISC produce a characteristic horizontal diffraction pattern that results from the perpendicular orientation of these cells to the direction of fluid flow, thus subtracting from the DI signal. A second possibility is that those cells that maintain a discoid appearance may contain sufficient HbS polymer to be partially or totally undeformable. However, recent studies suggest that the latter possibility is unlikely to contribute in a significant way to the interpretation of these data. Nash et al and Chien et al have noted that alterations in the viscoelastic properties of sickle cells were detected only in those cells that had undergone a morphologic change. Additional kinetic studies by Mozzarelli et al were also consistent with these findings. Those authors, using a measurement of the delay time for HbS polymerization to detect preexisting polymer, found that whenever polymer was present, the cells were morphologically sickled.

Taking this into consideration, we suggest that the reduced deformation of sickle cells at low oxygen tension in the ektacytometer reflects the accumulation of an internal mass of polymerized HbS to a size sufficient to interfere with the tank-treading motion of the cell. It should be noted that in deoxygenation experiments such as those conducted here, polymer formation at higher oxygen tensions does not reach equilibrium during the period of experimental observation. At these intermediate oxygen tensions, the hemoglobin solution is effectively supersaturated. This probably explains
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![Graphs A, B, C, D showing relationships between variables.](image-url)
could be detected only when the oxygen saturation was
sufficiently low to cause morphologic change.25,26 It is reasonable
to anticipate that any deleterious effect of persistent polymer in
high MCHC sickle cells at higher PO2 would be more likely
to be exerted through its effect on the rate of polymer formation
than on the deformability of cells at high levels of oxygen saturation.

Fig 8. Estimated polymer concentration producing a loss in
cell deformability for cells of various MCHC. (A) pH 7.2 (B) pH 7.4.
The closed circle symbols designate the first detectable loss of
density, and the open circle symbols designate a decrease in
average DI to 0.2. The polymer fraction was estimated using the
data from Noguchi7 as described in Methods.

why the DI signal in our experiments fell sharply over a
narrow pO2 range.

Under the conditions of our studies of low and medium
density sickle cells, a decrease in sickle cell deformability
could be detected only when the oxygen saturation was
reduced to a level at which substantial concentrations of
polymer are expected to be formed. This finding is consistent with
the recent micropipette studies that demonstrated that
enough polymer must form within a cell to cause morpho-
logic change before deformability is impaired.26,27 It might
have been anticipated that micropipet measurements of
membrane deformation at the periphery of the cell would be
less sensitive than the ektacytometer to the presence of
polymer in the cytoplasm, but such a difference is not
apparent in these results. Thus, small amounts of polymer
may not interfere greatly either with the tank-treading or
viscoelastic deformation of the cells. In addition, our results
suggest that the minimum amount of polymer that interferes
with cell deformation increases with increasing intracellular
Hb concentration. This could arise because increasing intra-
cellular viscosity obscures the detection of the initial effects
do polymer. Alternatively, it may be that HbS polymeriza-
tion at low HbS concentration results in gradual growth of an
extended network of polymer originating from a few nuclea-
tion sites, whereas polymerization at high HbS concentra-
tions results in a rapid, multicenter growth of smaller
domains of polymer.29 It might be anticipated that the effect
of extended polymer structures would be more effective in
preventing cell deformation than a larger quantity of small,
independent aggregates.

Whatever the reason for the difference in the minimum
detectable quantity of polymer in cells of different MCHC, it
is of particular interest that the higher MCHC cells appear
less sensitive to polymer formation than the low MCHC
cells. It has been proposed that the small amounts of polymer
that appear to persist in high MCHC cells should have two
effects of potential clinical significance.9 First, the presence
of polymer would eliminate any delay in formation of poly-
derm of low oxygen tension. Second, the polymer might impair the cells rheologically so that they
would be less able to flow rapidly through hazardous areas of
low oxygen tension. Although their high intracellular viscos-
ity prevented us from studying this process in very high
MCHC cells, our results suggest that small amounts of
persistent polymer would have the least rheologic effect on
the cells in which it is most likely to be found. Thus, we would
anticipate that any deleterious effect of persistent polymer in
high MCHC sickle cells at higher pO2 would be more likely
to be exerted through its effect on the rate of polymer formation
than on the deformability of cells at high levels of oxygen saturation.

ACKNOWLEDGMENT

We are grateful to Drs Emily Smelzer and Shu Chien for
suggestions about the gas exchange system, and to Drs William
Lande, William Mentzer, and Stephen Embury for providing blood
samples for the study. We are also grateful to Dr Constance Noguchi
for providing information on her calculations of HbS polymer
content as a function of HbS concentration and oxygen saturation.
This is publication No. 76 for the MacMillan-Cargill Hematology
Research Laboratory.

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Ektacytometric measurement of sickle cell deformability as a continuous function of oxygen tension [published erratum appears in Blood 1987 Apr;69(4):1272]

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