Rheologic Impairment of Sickle RBCs Induced by Repetitive Cycles of Deoxygenation-Reoxygenation

By Gerard B. Nash, Cage S. Johnson, and Herbert J. Meiselman

The transformation of less-dense, discoidal homozygous sickle cells (HbSS) RBCs into dehydrated, rheologically impaired cells is believed to be an important factor in the pathophysiology of sickle cell disease. We investigated this process by subjecting the less-dense fraction of HbSS RBCs, which contains a low percentage of irreversibly sickled cells (ISCs), to cyclic deoxygenation-reoxygenation for 15 hours at 37°C. This incubation procedure caused cell shrinkage, shifts in intracellular Na and K content, and formation of ISCs that closely resembled endogenous ISCs found in sickle blood. The viscoelasticity of the treated cells was tested using micropipette techniques to measure the membrane shear elastic modulus (μ) and the time constant for extensional shape recovery (t_c); μ represents the “static rigidity” of the cells, and the product μ · t_c was taken as a measure of their “dynamic rigidity.” Density separation of the incubated cells showed that their rheologic impairment (ie, elevation of both static and dynamic rigidities) paralleled cellular dehydration and that the newly formed dense cells had viscoelastic characteristics very similar to those of endogenous dense cells. Rehydration by osmotic swelling tended to normalize the dynamic rigidities of dense cells but had no significant effect on their static rigidities. Thus, cellular dehydration contributes to the observed changes of viscoelasticity, although an irreversible alteration of membrane structure also appears to be involved. Dense ISCs could be formed without added calcium, implying that entry of external calcium is not an essential requirement for cellular dehydration; ISCs formed without calcium tended to be less rigid (ie, to have lower static and dynamic rigidities) than those formed with calcium. Our results indicate that the cyclic incubation procedure closely mimics RBC rheologic deterioration in vivo and thus suggest its usefulness as a model for investigating this phenomenon.

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that of normal erythrocytes. Rehydration of the dense cells returned the membrane viscosity (or dynamic rigidity) to normal levels, whereas such rehydration improved the membrane rigidity in one study but not in another. This hydration-related change in membrane viscosity could be related to reversible Hb/membrane interactions, since such interactions have been shown to occur in normal RBCs. However, shrinkage of normal RBCs does not influence their membrane rigidity, implying that the membrane rigidification of the sickle cell membrane, which parallels increased Hb concentration, is probably due to a qualitatively different mechanism. Calcium-mediated cross-linking of membrane proteins, accumulated oxidant damage, and increased binding of HbS to the membrane have been suggested as mechanisms for membrane structural alterations; none, however, have been proven to cause the observed mechanical alterations.

The potential clinical significance of the cellular rheologic abnormalities exhibited by dense HbSS erythrocytes, and the present uncertainties as to their origin, led us to investigate the effects of cyclic deoxygenation-reoxygenation on the hydration and mechanical properties of HbSS erythrocytes. Ohnishi showed that such a process leads to the formation of dense cells and ISCs even without ATP depletion; the presence of Ca in the incubation medium was essential for these changes. By extending these prior observations to include measurements of membrane viscoelasticity, cell morphology, hemoglobin concentration, and Na and K content, we wished to determine whether this deoxygenation-reoxygenation process accurately mimics the in vivo process of rheologic deterioration. We also tested whether Ca was a necessary component for the deterioration and whether cell hydration alone explains the mechanical alterations. Validation of this in vitro process is essential if it is to be used as a model for illuminating the in situ mechanisms responsible for the formation of ISCs and dense HbSS cells.

**MATERIALS AND METHODS**

**Donor population and preparation of RBC suspensions.** Blood samples were obtained from patients with homozygous HbSS disease, with the approval of the University of Southern California Human Subjects Research Committee. Patients were defined by the usual hematologic criteria; those who had received transfusions within the prior 3 months were excluded. All patients were seen at the Comprehensive Sickle Cell Center, Los Angeles County-University of Southern California Medical Center, Los Angeles. Control samples were obtained from healthy adult laboratory personnel. Blood was collected by venipuncture into heparin (5 IU/mL), and measurements were completed within 24 hours of withdrawal. All cell preparations and measurements were carried out at room temperature (23 ± 1°C) unless otherwise specified. The basic HEPES buffer used to prepare cell suspensions contained HEPES (20 mmol/L), NaCl (140 mmol/L), KCl (5 mmol/L), MgCl2 (1 mmol/L), CaCl2 (2 mmol/L), NaH2PO4 (1 mmol/L), and glucose (5 mmol/L) with a pH of 7.4 and an osmolality of 300 ± 5 mosm/kg. Buffers with lower osmolalities (150 mosm/kg) were prepared by dilution of this basic buffer with distilled water. In selected experiments, the basic buffer was modified to obtain a Ca-free buffer (ie, without added CaCl2 but with 0.2 mmol/L EDTA).

Prior to the deoxygenation-reoxygenation process, the RBCs were density-fractionated to obtain a cell population with relatively low density and ISC content; the method used was based on that described by Ohnishi. Blood was centrifuged at 800 g for ten minutes, and a portion of the plasma was removed such that the hematocrit was increased to ~50%. Aliquots of 0.5 mL of this suspension were then layered onto preformed Percoll-Renografin density gradients and centrifuged at 1,500 g for seven minutes in a swing-out rotor. The gradients were formed from 56% Percoll (Pharmacia Fine Chemicals, Piscataway, NJ), 15% Renografin 76 (E.R. Squibb, Princeton, NJ), and 29% water (final pH = 7.6, 310 mosm/kg) by centrifugation in 16 × 100-mm polycarbonate tubes for 15 minutes at 13,000 g in an angle rotor. For HbSS blood, two major bands formed in these gradients: the top band contained the majority of the cells whereas the smaller bottom band preferentially contained ISCs (50.3% ISCs in the bottom fraction as compared with 3.9% in the top fraction, mean of six experiments). The two fractions were harvested separately, washed twice in large volumes of HEPES buffer, and finally adjusted to a hematocrit of ~40%. As compared with the top fraction, the RBCs in the bottom fraction had an elevated MCHC (43.9 ± 32.6 g/dL) and a greatly decreased internal Na + K content (70.2 ± 105.6 mEq/L cells). Discocytes in the bottom fraction tended to be thin and dehydrated in appearance whereas those in the top fraction had essentially normal morphology. The untreated HbSS bottom fractions were used only for these initial studies of cell morphology, MCHC and ionic content, and were not subjected to either cyclic deoxygenation-reoxygenation or to micropipette analysis. If normal HbAA blood was centrifuged on the preformed gradients, only one band was evident; this band had a position comparable to that of the top HbSS erythrocytes.

**Deoxygenation-reoxygenation treatment procedure.** A method based on that described by Ohnishi was used for the deoxygenation-reoxygenation process. Washed cells from the top fraction were diluted to ~5% hematocrit in HEPES buffer to which had been added 0.2 g/dL human serum albumin, penicillin, and streptomycin (100 IU/mL each) and an extra 5 mmol/L glucose. The suspension (15 mL) was placed in a 125-mL conical flask and swirled at 37°C for 15 hours, during which the sample was deoxygenated and reoxygenated every 15 minutes. This cycling was achieved by blowing water-saturated nitrogen over the suspension surface for ten minutes, then blowing water-saturated air for five minutes. The flow rate of the gases (1.25 SCFH) was controlled by a vertical rotameter (Model 7263, Matheson Gas Products, Cucamonga, CA), and the two gases were switched using a microprocessor-based timer (Model CD-03, Lindberg Enterprises, San Diego) and a three-way electric solenoid valve.

During the 15-minute deoxygenation-reoxygenation cycles, the suspension Po2 fell, on the average, to 28 mmHg and then rose to 130 mmHg. By the end of the 15-hour incubation, the medium osmolality had risen by 5 mosm/kg and the pH had fallen to 7.21 (average values). After the incubation, the suspension was centrifuged for 10 minutes at 800 g, and the cells were washed once and then resuspended at ~40% hematocrit in basic HEPES buffer. The incubated cells were then density-separated as described above to obtain new top and bottom fractions. For comparison, some HbSS samples were subjected to 24-hour incubation at 37°C with continuous exposure to nitrogen. In addition, some normal HbAA samples were incubated either for 15 hours using the abovementioned deoxygenation-reoxygenation procedure or for 24 hours under nitrogen.

**Measurement of cellular viscoelasticity.** During passage through the microcirculation, the RBC acts as a viscoelastic solid; ie, under the influence of an applied force, the RBC deforms to a limited extent (dependent on its structural rigidity) at a limited rate (dependent on its viscous properties). In the present study, the viscolelastic behavior of the RBCs was characterized by measuring their time constant for shape recovery (tc) and membrane shear...
elastic modulus (μ). The microscope-video and micropipette systems were carried out at ambient oxygen tension (i.e., ~150 mmHg). In brief, the RBCs were diluted to ~5 x 10^6/mL and were allowed to settle for ten to 15 minutes and become point-attached to the surface of a glass microchamber. The remaining suspension was then removed and the chamber was flushed and finally filled with buffer containing 10% autologous plasma. A portion of the cell membrane was aspirated into a micropipette (ID 1.2 to 1.6 μm) positioned opposite the point of the cell's attachment to the chamber surface. The cell was extended and then released by slowly withdrawing the pipette. From video recordings of the shape recovery process, the change in ratio of cell length to width with time was measured; the essentially exponential recovery of cell dimensions was analyzed as described by Hochmuth et al.\(^2\) to determine tc. Each cell was then detached from the glass surface, and the same micropipette was used to measure the membrane elasticity: A small membrane portion was drawn into the pipette from the flat surface of the cell, the tongue length was measured for several increasing pressures, and the length-pressure data were analyzed to calculate the shear elastic modulus (μ).\(^5\)

The shear elastic modulus is a measure of the rigidity of the membrane and will determine, for example, the final degree of deformation attained when a force is applied to the cell (provided that the deformation is not constrained by geometrical factors such as the ratio of cell surface area to volume, e.g., ref 25). This membrane elasticity is also the driving force for the cell to recover its shape after the force is removed, with the rate of recovery limited by the viscous dissipation of the cell. The membrane of a normal RBC acts as a viscoelastic solid, and the time constant for shape recovery (tc) is determined by the quotient μ/η, where η is the membrane shear viscosity.\(^4\) Thus, the product μ · tc represents the membrane viscosity. For dense sickle cells, however, the relative contributions of membrane and internal Hb viscosities to the recovery process are somewhat unclear; furthermore, the behavior of these cells deviates from that of a simple viscoelastic body.\(^4\) Thus, Evans et al.\(^1\) used the term "dynamic rigidity" for the product μ · tc, and used it as a measure of resistance to dynamic deformation. We used the same nomenclature, with the results for cellular viscoelasticity expressed in terms of static rigidity (μ, dynes/cm) and dynamic rigidity (μ · tc, dynes.s/cm). Although both parameters may change with increasing MCHC and thus with increasing internal viscosity, they are still predominantly membrane characteristics; even at elevated MCHC levels, cytoplasmic dissipation should not meaningfully hinder cell recovery rates.\(^4\)\(^,10\)\(^,26\)

**Miscellaneous techniques.** Cell morphology was evaluated by light microscopy using RBC suspensions that had been equilibrated with room air before fixation with 1% glutaraldehyde. Distorted, boat-shaped cells (length/width ≥2) were classified as ISCs; these cells are a separate entity from sickled cells with sharp spicules. The hematocrit of cell suspensions was measured using the microhematocrit technique (13,000 g for seven minutes). Hemoglobin concentration (Hb, g/dL) was measured by the cyanmethemoglobin method, with 1% Triton X-100 (Bio-Rad Laboratories, Richmond CA) added to the reagents to ensure complete cell lysis. MCHC was calculated from the ratio of Hb to hematocrit. Solution osmolarities were determined by freezing point depression (Model 2007, Precision Systems, Sudbury, MA) and the partial pressure of oxygen (P(S0)2) was measured using a Radiometer Model PH71 system equipped with an oxygen electrode (Radiometer Company, Copenhagen). Na and K levels were measured using a flame photometer (Model 443, Instrumentation Laboratory, Lexington, MA); cells were washed twice in large volumes of choline chloride solution (300 mosm/kg, pH 6.8), their hematocrit was adjusted to ~40%, and they were then lysed by adding 5 vol 5% Triton X-100 in water. Background levels of Na and K in the Triton X-100 were subtracted from the final results, which are expressed as mEq/L cells.

**RESULTS**

Cyclic deoxygenation-reoxygenation treatment for 15 hours with Ca caused a 14% increase of MCHC and a nine-fold increase in the number of ISCs in the unfractionated, treated cell population (Table 1). The morphology of the newly formed (ie, incubated) ISCs closely resembled that of endogenous ISCs, although the newly formed ISCs tended to have sharper, more pointed ends (Fig 1). Upon density separation of the treated cells, a new bottom fraction was evident, with a MCHC 29% greater, on the average, than the initial, untreated top fraction; the new top cells had a MCHC 7% greater than the initial, untreated top fraction (Table 1). Thus, during repeated cycles of deoxygenation-reoxygenation, the entire RBC population appears to shift to a slightly higher MCHC level, while a subpopulation of cells dehydrates much more extensively. These volume losses were associated with decreases in intracellular K that outweighed gains in Na. Consequently, the intracellular ratio of K to Na fell sharply, eg, from an average of 8.1 for the initial, untreated top fraction to 3.3 for the treated top fraction and to 0.8 for the treated bottom fraction. Deoxygenation-reoxygenation without Ca caused slightly less cell volume loss (12% without Ca v 18% with Ca) and slightly fewer ISCs to be formed (eightfold increase without Ca v 9.5-fold

<p>| Table 1. MCHC, Morphology, and Sodium and Potassium Content of HBSS RBCs Before and After 15 Hours of Cyclic Deoxygenation-Reoxygenation |
|---|---|---|---|---|</p>
<table>
<thead>
<tr>
<th>Cells</th>
<th>MCHC (g/dL)</th>
<th>ISC (%)</th>
<th>Sodium and Potassium Levels (mEq/L RBCs)</th>
<th>Na</th>
<th>K</th>
<th>K/Na</th>
</tr>
</thead>
<tbody>
<tr>
<td>With calcium (n = 6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial untreated top fraction</td>
<td>32.6 ± 2.6</td>
<td>3.9 ± 1.7</td>
<td>13.5 ± 6.8</td>
<td>92.1 ± 8.0</td>
<td>8.1 ± 2.8</td>
<td></td>
</tr>
<tr>
<td>Unfractionated treated cells</td>
<td>37.0 ± 2.0</td>
<td>35.4 ± 10.5</td>
<td>26.8 ± 5.0</td>
<td>56.6 ± 7.4</td>
<td>2.2 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>Top fraction</td>
<td>35.0 ± 2.2</td>
<td>13.7 ± 8.2</td>
<td>24.1 ± 7.6</td>
<td>71.1 ± 12.3</td>
<td>3.3 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>Bottom fraction</td>
<td>42.0 ± 0.9</td>
<td>51.4 ± 14.6</td>
<td>38.2 ± 6.9</td>
<td>28.0 ± 8.5</td>
<td>0.8 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>With without calcium (n = 2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial untreated top sample</td>
<td>31.2 ± 3.8</td>
<td>4.1 ± 1.0</td>
<td>10.2 ± 0.6</td>
<td>97.5 ± 0.1</td>
<td>9.5 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>Unfractionated treated cells (with calcium)</td>
<td>36.9 ± 0.4</td>
<td>39.0 ± 0.4</td>
<td>28.0 ± 0.3</td>
<td>55.0 ± 9.0</td>
<td>2.0 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>Unfractionated treated cells (without calcium)</td>
<td>35.0 ± 1.1</td>
<td>32.8 ± 8.6</td>
<td>37.9 ± 3.4</td>
<td>53.4 ± 5.6</td>
<td>1.4 ± 0.1</td>
<td></td>
</tr>
</tbody>
</table>

Data are mean ± SD for (n) donors. Calcium-HEPES buffer contained 2 mmol/L CaCl2; calcium-free HEPES buffer contained 0.2 mmol/L EDTA and no added calcium.
increase with Ca); thus, both effects were still evident even without added calcium (Table 1).

Continuous incubation under nitrogen for 24 hours caused, on the average, a 15% increase in MCHC (two experiments) and, in a single experiment in which these nitrogen-incubated HbSS RBCs were resubjected to density fractionation, a marked, new bottom density fraction was evident. These findings are thus in contrast to those of Ohnishi,22 who showed that continuous exposure to nitrogen for 15 hours caused no formation of dense cells. We also observed that irregularly shaped cells were formed following continuous nitrogen exposure for 24 hours, but that these RBCs tended to have several protuberances and did not closely resemble endogenous ISCs. Because of these observations, this noncyclic incubation procedure was not investigated further.

For normal HbAA RBCs, neither 15 hours of cyclic deoxygenation-reoxygenation nor 24 hours incubation under nitrogen caused significant cell shape changes or dehydration: MCHC decreased 1.5 ± 2.4% (mean ± SD, four experiments) after 15 hours of cycling or increased 3.0 ± 1.4% (two experiments) after 24 hours under nitrogen. The glucose levels in the incubation media (ie, 10 mmol/L) thus appear to have been sufficient to maintain RBC metabolism during these incubation periods; under these incubation conditions, cellular ATP levels do not decrease significantly.27

Table 2 compares the viscoelastic properties of density-separated incubated cells with those of density-separated HbSS RBCs from untreated blood. The data for endogenous cells are taken from a previous study carried out in this laboratory4 using identical measurement techniques and a similar, but not identical, group of patients. Although there is considerable heterogeneity in the RBC population from a single donor, averaged data for separated fractions should be comparable for different groups of sickle patients. We therefore used these earlier data4 for qualitative (but not statistical) comparisons, to assess whether the viscoelasticity values measured after the incubation procedure reproduce those found in untreated blood. Salient aspects of the data for incubated cells were: (a) The newly formed ISCs had static and dynamic rigidities essentially identical to those previously found for endogenous ISCs (<8% difference for both rigidities, Table 2); (b) the newly formed dense discocytes (ie, incubated discs from the bottom fraction) had elevated static and dynamic rigidities as compared with less-dense

Table 2. Viscoelastic Properties of Sickle Cells After Cyclic Deoxygenation-Reoxygenation Incubation: Comparison With Untreated Endogenous HbSS Cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>Static Rigidity (μ, 10^4 dynes/cm)</th>
<th>Dynamic Rigidity (μ - tc, 10^-4 dynes - s/cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISCs from bottom fractions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Incubated ISCs (n = 4)</td>
<td>12.0 ± 3.9</td>
<td>19.5 ± 10.0</td>
</tr>
<tr>
<td>Endogenous ISCs (n = 7)</td>
<td>12.9 ± 2.5</td>
<td>20.8 ± 6.2</td>
</tr>
<tr>
<td>Discocyes from top fractions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Incubated discs (n = 2)</td>
<td>6.7 ± 1.0</td>
<td>8.0 ± 2.7</td>
</tr>
<tr>
<td>Endogenous discs (n = 4)</td>
<td>6.2 ± 0.4</td>
<td>7.8 ± 1.4</td>
</tr>
<tr>
<td>Discocyes from bottom fractions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Incubated discs (n = 2)</td>
<td>10.7 ± 4.9</td>
<td>30.6 ± 17.3</td>
</tr>
<tr>
<td>Endogenous discs (n = 4)</td>
<td>7.1 ± 1.1</td>
<td>15.0 ± 6.0</td>
</tr>
</tbody>
</table>

Data for incubated cells are the mean ± SD of pooled data from (n) donors; calcium (2 mmol/L) was present in all incubation media. Data for endogenous cells are from Nash and colleagues4 and are the mean ± SD of the means from (n) donors. All data were obtained with RBCs suspended in 300 ± 5 mosm/kg buffer.
incubated discs from the top fraction; and (c) the newly formed dense discocytes also had greater rigidities than endogenous dense discs (Table 2). The deoxygenation-reoxygenation procedure had no deleterious effect on cellular viscoelasticity for cells that did not undergo marked dehydration. Incubated and endogenous discs from the top fractions had nearly identical values for both static and dynamic rigidity (Table 2).

With regard to the abovementioned density-related differences for discs, our prior study showed no meaningful effect of cell density on the static rigidity of endogenous discocytes, although a few stiff discs were detected. This discrepancy between the two types of dense discs (ie, incubated v endogenous discs from the bottom fractions, Table 2) probably arises from use of a more efficient separation procedure in the present study. That is, isolation of cells greater than a certain density was used herein, whereas isolation of the 10% densest cell was used in the earlier investigation. Nevertheless, the newly formed dense disc cells we tested still represented a heterogeneous group of cells, with their static rigidities distributed into two general categories: of the 16 cells measured, eight had a nearly "normal" mean μ value of 7.2 and the other eight had a μ value averaging 14.1 x 10⁻¹ dynes/cm. Thus, the dense fraction isolated herein appears to encompass an MCHC range wide enough to include discocytes with very different rigidities; an even finer differentiation on the basis of density would be needed to obtain a truly homogeneous cell population.

Figure 2 shows the effects of osmotic rehydration on the viscoelastic properties of the newly formed dense discocytes and ISCs; also shown, at 300 mosm/kg only, are data for the less dense discocytes from the top fraction. With regard to static rigidity (ie, μ), data in Fig 2 (upper panel) show that both cell types tended to have lower μ values in the 150-mosm/kg medium. However, the relatively small decreases observed (14% for ISCs, 22% for dense discs) were not significant (P > .10). Even when hydrated at 150 mosm/kg, the μ values of both the ISCs and the dense discs were greater (P < .01) than that measured at 300 mosm/kg for discs from the top fraction. Unlike these μ results, swelling of the dense cells in the lower osmolality medium caused marked, significant decreases of their dynamic rigidities (Fig 2, lower panel): a 37% decrease was observed for ISCs (P < .001) and a 62% decrease was observed for dense discocytes (P < .001). Thus, osmotic rehydration tended to normalize the dynamic rheologic behavior of these dense cells; ie, as compared with discs from the top fraction, the two-fold difference for dense ISCs and the nearly fourfold difference for dense discs observed at isotonic conditions were reduced to 30% or 45%, respectively, when these cells were tested at 150 mosm/kg.

The viscoelastic properties of ISCs formed with or without calcium are compared in Fig 3; also shown, as in Fig 2, are data for the less-dense discocytes from the top fraction. The newly generated ISCs formed without added calcium tended to have lower static and dynamic rigidities than those formed with Ca. This calcium effect was most pronounced for ISC located in the bottom fraction (Fig 3): μ was 19% less and μ · tcw was 28% less when calcium was omitted from the incubation medium. It is important to note that dense ISCs with static and dynamics rigidities significantly elevated (P < .01) above those for less-dense HbSS discocytes did form without addition of calcium (Table 1 and Fig 3). Moreover, the newly formed ISCs in the top fraction (Fig 3) underwent less change in viscoelastic properties than did those located in the new bottom fraction.

**Figure 2.** Effect of hypotonic rehydration on the static (upper panel) and dynamic (lower panel) rigidities of newly formed dense sickle cells from the bottom fraction. Data are shown for ISCs (●) and for discocytes (○) and are the mean ± SE of pooled data from two experiments; in each experiment seven or eight cells of each type were measured at each osmolality. Also shown are data (mean ± SE) for 16 incubated, less-dense (top) discocytes at 300 mosm/kg (○). Rehydration had only a slight effect on the static rigidities but significantly reduced the dynamic rigidities (P < .001).

**Figure 3.** Comparison of the static (upper panel) and dynamic (lower panel) rigidities of newly formed ISCs generated with (2 mmol/L) or without (no added Ca plus 0.2 mmol/L EDTA) of Ca. Data are for ISCs from the top (●) and bottom (○) density fractions and are the mean ± SE of pooled data from two experiments; in each experiment seven or eight cells of each type were measured. Also shown are data (mean ± SE) for 16 incubated, less-dense (top) discocytes (○). ISCs did form without added Ca but tended to have lower static and dynamic rigidities than when Ca was added.
DISCUSSION

The results demonstrate that cells closely resembling endogenous ISCs can be formed by repetitive cycles of deoxygenation-reoxygenation and that a new population of dense discocytes is also formed by this process. The new ISCs had similar shapes but somewhat sharper ends as compared with endogenous ISCs (Fig 1). In vivo, the pointed ends may be obliterated during multiple passages through the reticuloendothelial system, whereas orbital shaking during in vitro incubation is probably not vigorous enough to reproduce this phenomenon. The newly formed ISCs and dense discs originated from a preseparated population of ISC-poor, less-dense RBCs, yet they developed viscoelastic properties, MCHC, and Na and K levels representative of the dense cells found in untreated HbSS blood. Thus, a marked heterogeneity was introduced into the treated RBC population, with this heterogeneity closely resembling that which is characteristic of untreated, oxygenated sickle blood.3-5

The mechanical abnormalities of the newly formed dense ISCs and dense discocytes were similar (Table 2), even though these cells had obviously different shapes. The exact physiologic relevance of the ISC shape alteration is therefore uncertain, although ISCs did have abnormal viscoelastic properties even when located in the new top fraction where the less-dense discocytes behaved normally (Fig 3). The irreversible, elongated shape of ISCs has been considered to indicate that their membrane structure is permanently altered,28 and prolonged exposure to mechanical stress can lead to plastic deformation of the RBC membrane by a possible rearrangement of bonds between structural proteins.29 Thus, extreme and repeated deformations of the cell membrane, as imposed by sickling in the present experimental model, may directly cause the irreversible membrane distortion. However, why this irreversible shape change should happen to some dense cells and not to others is not clear; on the average, only 33% to 51% of cells in the new dense bottom fractions were ISCs (Table 1). Because the shape of ISC need not necessarily impair their in vivo flow behavior,30 the recent trend to consider the rheologic importance of the entire dense cell fraction (eg, refs 3, 31, and 32), rather than of the ISCs specifically, appears warranted.

The rheologic changes observed in the present study clearly parallel the degree of cellular dehydration and thus the increase of cell MCHC and density (shown in data for top and bottom incubated discs in Table 2 and for ISCs in Fig 3). In previous studies,4,5 hypotonic rehydration of endogenous dense cells normalized their dynamic rigidity; in one of these studies2 but not the other,4 hypotonic rehydration also normalized their static rigidity. In the present investigation, we again showed that rehydration returned the dynamic rigidity of dense, treated cells to a level close to that of less-dense discocytes, but that there was a lesser degree of improvement of their static rigidity (Fig 2). We therefore conclude that even after rehydration, some membrane structural abnormality gives rise to the residual elevation of static rigidity. The structural bases for these irreversible changes of membrane mechanical behavior remain obscure, as do the molecular causes of the permeability changes which give rise to the loss of water and K and the gain of Na and Ca; membrane distortion (ie, sickling) appears to be a necessary condition for cation permeability increases.13

With regard to the effects of extracellular Ca, it is of interest that Ohnishi22 showed that dense cells were not formed if the incubation medium did not contain Ca. Conversely, we observed that removal of Ca from the incubation medium had minimal effect on cell dehydration or ISC formation (Table 1), although ISCs formed without Ca tended to have lower static and dynamic rigidities than those formed with Ca in the incubation medium (Fig 3). Calcium enters the cell during sickling, and the accumulation of Ca in HbSS RBCs is well documented (eg, ref 19); this Ca is largely contained in cytoplasmic vesicles (12-14) and is believed to be unavailable for altering the structure and permeability of the cell membrane. However, transient, Ca-mediated inhibition of the K pump may occur during sickling,14 possibly in combination with a mechanical stress-induced increase in membrane permeability,15,16, both of these effects could contribute to the shifts of Na, K, and water that we observed. The formation of ISCs and dense discocytes without external Ca (Table 1) may arise from the latter stress-induced mechanism and may also be related to the movement of Ca between intracellular compartments during sickling rather than solely to the entry of Ca from the suspending medium.13,14

In summary, deterioration of the mechanical properties of HbSS cells, similar to that found in vivo, can be artificially induced in vitro by a cyclic deoxygenation-reoxygenation technique. This cyclic process closely mimics the in vivo process and offers the possibility of obtaining detailed information about the causes of the cellular alterations. The potential of this model for evaluation of new therapeutic approaches that modulate the process of irreversible RBC deterioration has already been demonstrated: Ohnishi22 showed that the antisickling drug cepharanthine inhibited ISC formation and, more recently, Ca-blockers were shown to inhibit dense cell formation.33 Based on our data, these agents22,33 also appear to have protected the RBCs from adverse rheologic changes.

ACKNOWLEDGMENT

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