We hypothesized that the deoxygenation-induced increase in cation permeability of sickle cells was related to mechanical distortion of the membrane by growing HbS polymer within the cell. To test this hypothesis, we determined the effect of deoxygenation on cation fluxes in sickle cells under conditions that restricted or permitted extensive growth of polymer, producing different degrees of membrane distention. Manipulation of suspending medium osmolality for density-isolated high and low mean cell hemoglobin concentration (MCHC) cells was used to regulate the extensional growth of polymer bundles and hence membrane distortion. For initially low MCHC cells, the deoxygenation-induced increase in both Na and K fluxes was markedly suppressed when the MCHC was increased by increasing the osmolality. This suppression corresponded to the inhibition of extensive morphologic cellular distortion. For initially high MCHC, ISC-rich cells, deoxygenation had minimal effect on K permeability. However, reduction of MCHC by a decrease in osmolality produced a concomitant increase in cation permeability and cellular distortion. These observations support the idea that the sickling-associated increase in membrane permeability is related to mechanical stress imposed on the membrane by bundles of HbS polymer.

ALTHOUGH polymerization of Hb S is a cytoplasmic event, it also has effects on the function of the red cell membrane. Perhaps the most dramatic example of this is the massive increase in membrane permeability to monovalent cations, as first described by Tosteson et al.1,2 In the 30 years since this important observation, the mechanism producing the permeability increase has not been elucidated. Because of the association between abnormal cation and volume regulation and the impaired rheology of sickle cells,3,5 clarification of this phenomenon is of considerable interest.

On the basis of previous observations that the magnitude of the permeability increase correlated with the proportion of morphologically distorted, sickled cells,6 we hypothesized that membrane distortion itself may be directly related to increased permeability. More specifically, we conjectured that mechanical stress on areas of the membrane that surround extended spicules of hemoglobin S polymer could produce local sites for increased cation flux. To test the relationship between morphologic distention and cation permeability, we made use of our earlier observation that the extent of sickle cell distention was related to the hemoglobin concentration inside the cell.7 We had shown that low MCHC discoid sickle cells assume highly spiculated shapes when deoxygenated in isotonic medium but undergo a relatively minor shape change when deoxygenated in a dehydrated state in hypertonic medium. Conversely, high MCHC irreversibly sickled cells (ISC) undergo little shape change when deoxygenated in isotonic medium, but form characteristic spiculated, sickled shapes when their MCHC is reduced by suspension in hypotonic medium. This difference in morphology may be related to the observations that the number of polymer domains formed during rapid deoxygenation (and presumably, rapid warming of chilled deoxygenated samples) varies with the concentration of HbS. Because polymerization depends on the concentration-dependent process of nucleation, low HbS concentration favors aligned polymer growth in a single or few domains, whereas high HbS concentration favors the growth of polymer in multiple, independent domains.8,9 By comparing the magnitudes of the deoxygenation-induced change in cation permeability in these two distinct morphologic states for each cell type, we hoped to determine whether cation permeability could be correlated with membrane distention.

MATERIALS AND METHODS

After informed consent was obtained from subjects with homozygous sickle cell disease, blood samples were drawn into either heparin or acid-citrate-dextrose (ACD). Red cells were separated from plasma, resuspended in buffered saline containing potassium and glucose (135 mmol/L of NaCl, 5 mmol/L of KCl, 8.6 mmol/L of Na2H PO4, 1.4 mmol/L of NaH2 PO4, 11 mmol/L glucose adjusted to pH 7.4 and 290 ± 5 mosm/kg), and were then separated to provide either a low MCHC (32 ± 0.5 g/dL), mature discoid cell population or an ISC-enriched population (39 ± 1.6 g/dL), using discontinuous Stracan density gradients as previously described.10 For measurements of permeability, the isolated cell populations were incubated at 37 °C, 5% hematocrit, for 4.5 hours in potassium-free, phosphate-buffered saline (PBS) (5 mmol/L of Na phosphate, pH 7.4, total Na concentration, 135 mmol/L). The medium also contained 0.1 mmol/L of ouabain to suppress active transport of Na and K, 10 mmol/L of glucose, and 100 U/mL of penicillin and 100 µg/mL of streptomycin (penicillin-streptomycin, Grand Island Biological Co, Grand Island, NY) to inhibit bacterial growth. The isotonic and hypotonic media contained 27 mmol/L of sucrose to minimize hemolysis, and the hypotonic solution contained additional sucrose to attain the desired osmolality without changing the Na concentration. However, the osmolality of the hypotonic solution was adjusted by reducing the NaCl concentration. For experiments using low MCHC cells, paired samples were incubated at 290 and 550 mosm/kg, whereas ISC-rich samples were incubated at 290 and 110 mosm/kg. To determine the effect of deoxygenation, identical pairs were incubated under room air and nitrogen atmospheres. To insure a well-defined starting point for the flux measurements, the spending media for deoxy samples were first deoxygenated by bubbling humidified N2 gas through them for 20 minutes while maintaining them at ice temperature. The cells were then added, and deoxygenation was continued by surface gassing for an additional

Association Between Morphologic Distortion of Sickle Cells and Deoxygenation-Induced Cation Permeability Increase

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ten minutes, after which the incubation vessels were sealed. Because hemoglobin S does not polymerize at 0°C, we were thus able to deoxygenate the cells first and then rapidly initiate polymerization by transferring the cell suspensions to a 37°C water bath. The oxygenated samples were carried through the temperature changes in parallel with the deoxygenated samples. Initial samples were removed from the incubation mixtures before they were warmed to 37°C, and three additional samples were removed anaerobically at 90-minute intervals. These samples were separated by centrifugation, and the red cells were washed three times in isotonic Tris-buffered (10 mmol/L, pH 7.4 at 0°C) magnesium chloride. Both the initial supernatant incubation medium and washed cells were assayed for Na and K by flame photometry. Hemoglobin measurements on the incubation cell suspensions, together with hemoglobin and hematocrit determinations on the initial cell samples at incubation tonicities, were used to determine changes in intracellular cation concentration and content. Morphologic examination of cells removed anaerobically from the incubation vessels confirmed our prior observations (Fig 1) concerning the effect of MCHC on the morphology of deoxygenated sickle cells. The isotonic, initially discoid cells and the hypotonic ISC showed well-defined spicule formation, whereas the hypertonic discoid cells and isotonic ISC did not form extensive spicules.

Data analysis: Estimates of potassium efflux were obtained from measurements of potassium in both the incubation medium and the cells. Measurement of hemoglobin concentrations in the suspending medium throughout the experiment showed that hemolysis in the oxygenated samples was only 1% to 2%, and rose to 4% to 5% in the deoxygenated samples. No significant effects of changing the medium K concentration were observed. This contribution to the medium K concentration was insignificant in comparison to the total cation changes, so no correction for hemolysis was applied. Using the cation data, we calculated first-order rate constants for potassium efflux as described elsewhere. To evaluate the effect of MCHC on the deoxygenation-induced increase in Na permeability, we used linear regression analysis to determine the increases obtained by incubating cells at a given tonicity under an N2 or an O2 atmosphere. These deoxygenation-induced differences were then separately compared for low MCHC and ISC-rich cell populations at the two incubation tonicities, using a t test for independent samples.

RESULTS

Low MCHC sickle cells, when incubated in isotonic medium, showed the expected marked increase in ouabain-resistant K loss when deoxygenated (Fig 2). Increase of suspending medium tonicities to 550 mosm/kg caused an increase in intracellular K concentration, because of osmotically induced cell shrinkage, which resulted in an accelerated loss of K, even under oxygen. When these dehydrated cells were deoxygenated, K loss was further augmented. Under the conditions of these experiments, in which extracellular K concentration was near zero, the rate of K efflux was expected to depend on the intracellular K concentration. To test this expectation, we examined the relationship between the initial efflux rates and intracellular K concentration. Separate linear relationships were observed for oxygenated and deoxygenated cells (data not shown), suggesting that, as for ouabain-resistant K efflux from normal cells and K influx into deoxygenated sickle cells at extracellular K concentrations >5 mmol/L, K efflux from both oxygenated and deoxygenated sickle cells has a first-order dependence on intracellular K concentration. Therefore, we calculated first-order rate constants to facilitate comparison of membrane

![Fig 1. Scanning electron micrographs of low and high density sickle cells deoxygenated at different MCHCs: initially discoid sickle cells, deoxygenated at (A) low and (B) high MCHC; ISC-rich sickle cells deoxygenated at (C) high and (D) low MCHC.](https://www.bloodjournal.org/)

![Fig 2. Intracellular K concentration of low MCHC (32 ± 0.5 g/dL) sickle cells during incubation under N2 or O2 at 290 and 550 mosm/kg. Deoxygenated samples (solid symbols): oxygenated samples (open symbols): 550 mosm/kg medium (squares); 290 mosm/kg (circles). Bars indicate SEM.](https://www.bloodjournal.org/)
permeability in these cells that contained varying K concentrations. As shown in Table 1, the rate constant for oxygenated sickle cells was moderately increased when the cells underwent shrinkage in hypertonic medium. However, when the cells were deoxygenated, the K efflux rate constant was actually lower for the hypertonic cells than for the isotonic ones. The net effect of deoxygenation on the rate constant for isotonic, normovolemic cells was more than twice that for the hypertonic, shrunken cells, and this difference was highly significant, with a \( P \) value of .001 (Table 1).

The effect of cell shrinkage on the deoxygenation-induced cation leak was even more striking when the net uptake of Na was examined. Changes in intracellular Na were inversely related to those in intracellular K (Fig 3). As shown in Table 2, the net increases in Na content were only slightly larger for oxygenated cells in isotonic medium than in hypertonic medium. However, the cells that were deoxygenated in isotonic medium showed a markedly larger uptake of Na than did the cells shrunken in hypertonic medium (nearly fourfold). Again, the increase in Na uptake that was produced by deoxygenation was strongly inhibited by cell shrinkage to a highly significant extent (Table 2). Because the toxicity of the suspending medium was increased by adding sucrose, the shrunken cells would have been subject to a smaller transmembrane gradient of Na, and an increased intracellular chloride concentration would have been associated with a less negative membrane potential. Both of these effects, which would increase Na efflux, would be expected to decrease net Na uptake at an equal proportion in the oxygenated and deoxygenated cells. Thus, the observation that the effect of increased toxicity in suppressing Na uptake was much greater when the cells were deoxygenated suggests that this suppression was not simply due to an osmotically induced increase in Na efflux. The parallel reduction in K efflux from shrunken deoxygenated cells also provides support for an independent effect of cell shrinkage. When the cells were examined microscopically, it was found that the suppression of the deoxygenation-induced increase in cation permeability corresponded to the absence of highly spiculated and distorted cells in the hypertonic deoxygenated cell suspensions. Cells deoxygenated in isotonic medium showed

<table>
<thead>
<tr>
<th>Cell Sample</th>
<th>N</th>
<th>MCHC (g/dL)</th>
<th>( k_1 )</th>
<th>( k_2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low MCHC cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>290 mosm/kg</td>
<td>6</td>
<td>32.1 (.5)</td>
<td>.0225 (.0072)</td>
<td>.121 (.015)</td>
</tr>
<tr>
<td>550 mosm/kg</td>
<td>5</td>
<td>41.0 (.8)</td>
<td>.0406 (.0041)</td>
<td>.0824 (.039)</td>
</tr>
<tr>
<td>High MCHC cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>290 mosm/kg</td>
<td>6</td>
<td>38.9 (1.6)</td>
<td>.0540 (.0062)</td>
<td>.0783 (.0081)</td>
</tr>
<tr>
<td>110 mosm/kg</td>
<td>3</td>
<td>25.3 (3.2)</td>
<td>.113 (.020)</td>
<td>.232 (.057)</td>
</tr>
</tbody>
</table>

First-order rate constants were calculated from the measured ion fluxes and intracellular K concentrations. Values given are the mean for \( N \) experiments. SEM is given in parentheses. By \( t \) test for independent samples, \( *P < 0.001 \) and \( tP < 0.02 \).

![Fig 3. Changes in intracellular Na concentration of low MCHC (32 ± 0.5 g/dL) sickle cells during incubation under N₂ or O₂ at 290 and 550 mosm/kg. Deoxygenated samples (solid symbols); oxygenated samples (open symbols); 550 mosm/kg (squares); 290 mosm/kg (circles). Bars indicate SEM.](image)

Table 1. Effect of Deoxygenation on First-Order Rate Constants for K Efflux From Low and High MCHC Sickle Cells

<table>
<thead>
<tr>
<th>Cell Sample</th>
<th>( N )</th>
<th>MCHC (g/dL)</th>
<th>( k_1 )</th>
<th>( k_2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low MCHC</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>110 mosm/kg</td>
<td>3</td>
<td>25.3 (3.2)</td>
<td>.113</td>
<td>.232</td>
</tr>
</tbody>
</table>

Table 2. Effect of Osmotically Induced Shrinkage on Net Increase in Na Content of Low-Density Sickle Cells Incubated Under Nitrogen or Room Air

<table>
<thead>
<tr>
<th>MCHC (g/dL)</th>
<th>Isotonic Cells</th>
<th>Hypertonic Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial cell [Na]</td>
<td>22.1 (1.5)</td>
<td>28.2 (2.0)</td>
</tr>
<tr>
<td>mEq/10 g Hb</td>
<td>.688 (.047)</td>
<td>.688 (.049)</td>
</tr>
<tr>
<td>Net uptake of Na-mEq/10 g Hb</td>
<td>.046 (.014)</td>
<td>.037 (.016)</td>
</tr>
<tr>
<td>Oxy</td>
<td>.252 (.036)</td>
<td>.066 (.012)</td>
</tr>
<tr>
<td>Deoxy</td>
<td>.175</td>
<td>.029*</td>
</tr>
</tbody>
</table>

Paired samples of low-density sickle cells were incubated under nitrogen or room air at 290 or 550 mosm/kg. Effects of deoxygenation in causing increased Na uptake were compared for isotonic and hypertonic samples, using a \( t \) test for independent samples; a \( *P \) value of < .001 was found.

Net increase in Na content was expressed on the basis of cell Hb to take cell shrinkage into account.
CATION PERMEABILITY OF SICKLE CELLS

Fig 4. Changes in the natural log of intracellular K concentration of low (32 ± 0.5 g/dL) and high (39 ± 1.6 g/dL) MCHC sickle cells during incubation under N2 or O2 at various osmolalities. (A) Low MCHC cells. Deoxygenated samples (solid symbols); oxygenated samples (open symbols); 550 mosm/kg (squares); 290 mosm/kg (circles). Bars indicate SEM. (B) High MCHC cells; deoxygenated samples (solid symbols); oxygenated samples (open symbols); 110 mosm/kg (squares); 290 mosm/kg (circles).

DISCUSSION

In the present studies of sickle cells, a consistent relationship was found between the augmentation in cation permeability induced by deoxygenation and the distortion of the membrane by extended spicules of hemoglobin S polymer. When formation of these long spicules was inhibited because of high MCHC and high cytoplasmic viscosity, deoxygenation was much less effective in increasing membrane permeability. It might be argued that the experimental manipulations used to vary MCHC in any one set of experiments could have produced the differences in ion flux that were associated with these differences in cell morphology. However, the fact that we were able to show this in low MCHC cell populations by using hypertonic medium to inhibit the deoxygenation-induced leaks for both K and Na, and in high MCHC cells by using hypotonic medium to unmask the K leak, provides substantial support for the hypothesis that membrane distortion is necessary for the sickling-induced cation leak. Thus, although no one of the experiments would be sufficient to infer an association between the extent of cell distortion and increased membrane permeability, the consistency of this association under different conditions is significant. Furthermore, polymer formation alone cannot explain the deoxygenation-induced increase in membrane permeability, since the less deformed cells with high MCHC would have contained more polymer than the spiculated low MCHC cells.

Other investigators have suggested that mechanical stress imposed upon the sickle cell membrane by polymer spicules can disrupt other aspects of normal membrane function, such as maintenance of bilayer phospholipid asymmetry. In other studies, manipulation of suspending medium tonicity, similar to the approach used in the present study, showed a correspondence between the extent of membrane distortion and increase of lipid translocation, as well as loss of phospholipid asymmetry. Taken together, these observations support the hypothesis that the effects of HbS polymerization on both lipid organization and cation permeability are augmented or perhaps mediated by mechanical distention of the membrane.

Previously, it was noted that ISC-rich cell populations exhibited minimal effects of deoxygenation on disturbances of phospholipid organization. The present study shows that deoxygenation-induced cation permeability changes are also substantially reduced in the ISC-rich population. We attribute the damping of both of these effects to the inability of the highly concentrated hemoglobin within ISC to elaborate long spicules of hemoglobin polymer that mechanically stress the membrane. Recently, Embury et al reported that thalassemic sickle cells showed a smaller change in cation permeability upon deoxygenation than did nonthalassemic sickle cells. Subsequently, it was found that these cells also have an excess surface area-to-volume ratio. We speculate that this redundant surface area can accommodate polymer for-
mation with less membrane distention and may explain the smaller cation leak.

It has frequently been suggested that the deoxygenation-induced cation leak of sickle cells may play a role in the pathophysiology of the disease. The results of the present study suggest that it may not be necessary to inhibit hemoglobin polymerization completely, but that agents that limit large-scale growth of polymers may reduce the cation leak and hence cellular dehydration.

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Association between morphologic distortion of sickle cells and deoxygenation-induced cation permeability increase

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