Monovalent Cation Composition and ATP and Lipid Content of Irreversibly Sickled Cells

By Margaret R. Clark, Richard C. Unger, and Stephen B. Shohet

Discontinuous Stractan gradients were used to separate heterogeneous populations of sickle cells into discrete subpopulations containing varying proportions of reticulocytes, mature discoid cells, and irreversibly sickled cells (ISC). The improved homogeneity of these preparations, together with an enhanced yield of ISC, allowed us to distinguish effects of cell maturation from those of irreversible sickling. With these cell preparations we have begun to define physical properties of ISC. We confirmed the marked abnormalities in cation composition of native ISC. Measurements of ATP in ISC did not substantiate prior reports of ATP deficits. Finally, no evidence for substantial loss of membrane lipids during the process of ISC formation could be demonstrated.

Although the molecular defect in sickle cell anemia has long been understood, the precise mechanism by which the polymerization of abnormal hemoglobin results in the clinical syndrome of sickle cell disease is not clearly defined. In an effort to extend understanding of the pathology of this disease, we focused our attention on a subpopulation of cells that appeared to be permanently damaged through their history of prolonged or perhaps repeated sickling. These cells, called irreversibly sickled cells (ISC), remain permanently deformed even when reoxygenation has broken down the hemoglobin polymers within the cell. Presumably because of ion and water loss during sickling, these cells have abnormally high hemoglobin concentrations (MCHC). Since the rate of HbS polymerization is strongly dependent upon hemoglobin concentration, hemoglobin inside ISC should be highly susceptible to polymer formation in oxygen-poor environments.

In experiments that measured the ability of cells to pass through small-pore filters after abrupt deoxygenation, Hahn et al. showed that ISC become undeformable within a fraction of a second after deoxygenation, whereas discoidal sickle cells from the same patient remain deformable for several seconds. These authors suggested that the rheologic vulnerability of ISC during deoxygenation may give these cells an important role in the clinical aspects of the disease. Although the proportion of ISC appears to be associated with increased hemolysis, there is no direct correlation between the proportion of ISC and frequency or severity of painful crises.
We were interested in defining the properties of ISC, with the idea that such information is essential for understanding the factors involved in their formation and ultimate fate. This knowledge might both clarify the clinical role of ISC and suggest methods of intervening in the process of ISC formation perhaps without having to prevent sickling altogether.

Because of their high hemoglobin concentrations, a major determinant of cell density, ISC can be separated conveniently upon density gradients. Using cells separated by centrifugation on Stractan II gradients, we measured concentrations of Na, K, ATP, and red cell lipids in order to assess the possible roles of abnormal ion transport, ATP depletion, and membrane fragmentation in ISC formation. In agreement with a recent report by Glader et al., we found that ISC had moderately elevated Na concentrations, drastically reduced K concentrations, and an overall reduction of total monovalent cations. In contrast to prior reports by Weed and Bessis and Glader et al., we found no consistent reduction of ATP in ISC. Furthermore, we were unable to find any significant deficit of membrane lipids in ISC, which might have been expected if membrane fragmentation during repeated sickle-unsickle episodes played an important role in their formation.

**MATERIALS AND METHODS**

Blood from normal controls and patients with sickle cell anemia was drawn into heparinized tubes just prior to separation on the density gradients. White cells were removed by repeated washing in phosphate-buffered saline containing potassium and glucose (BSKG) with aspiration of the buffy coat. In some experiments, white cells were removed by filtration of whole blood through a syringe filled with cellulose or cotton. Washed red cells were resuspended in BSKG to approximately 20% hematocrit and layered on top of discontinuous gradients of Stractan. This medium was prepared by the method of Corash et al. with slight modification. For the sickle cells four 2.5-ml portions of Stractan, generally with densities of 1.115, 1.110, 1.101, and 1.090 g/ml, were layered upon a cushion of Stractan with a density of at least 1.144 g/ml. This dense layer prevented packing of the ISC against the bottom of the tube during centrifugation. Control cells were separated on layers with densities of 1.096, 1.092, 1.090, and 1.088 g/ml, with a cushion of at least 1.115 g/ml. Gradients were centrifuged in a Beckman SW 27.1 rotor at 4°C for 30 min at 20,000 rpm. Centrifugation for 45 or 60 min did not alter the distribution of cells along the gradient. Tubes (17 ml) containing 10.5 ml total volume of Stractan solutions were used to separate 0.7-0.8 ml of cells per tube.

After centrifugation, the cells were removed from the gradients with a Pasteur pipet and diluted with an approximately equal volume of BSKG. The cells were removed from the Stractan by centrifugation at 2000 g for 5 min and were washed twice in BSKG by centrifugation at 1000 g for 5 min. The cells were then resuspended to approximately 25% hematocrit in BSKG and samples were prepared for determination of red cell count, hematocrit, and hemoglobin on the Coulter S electronic counter or by manual methods. The cells were kept at 0-4°C throughout the separation and washing. Smears were prepared for reticulocyte counts, and cell samples were fixed in 3% glutaraldehyde in 0.05 M sodium phosphate, pH 7.4, for subsequent counting of ISC. For counting purposes, cells with a length to width ratio of 2:1, or with angular profiles, were designated as ISC. At this stage they had undergone resuspension and centrifugation in six changes of well-oxygenated medium, and no cells with morphology characteristic of deoxygenated sickle cells were ever observed.

For measurement of red cell Na and K concentrations, aliquots of the separated cell suspens-

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*BSKG: 7.808 g NaCl, 0.373 g KCl, 2.302 g Na₂HPO₄ · 7H₂O, 0.194 g NaH₂PO₄ · H₂O, 2.0 g glucose, made up to 1 liter with distilled water. The pH and osmolality were adjusted to 7.4 and 290-295 mosm/kg if necessary.*
sions were washed three times in isotonic MgCl₂ buffered to pH 7.4 with 10 mM Tris-HCl. The final hematocrit of the washed cells was measured using a microhematocrit centrifuge, and the cells were diluted into LiCl solution for determination of Na and K by flame photometry.

Red cell ATP was measured in Tris-borate extracts of washed cell suspensions using the luciferin-luciferase assay. Packed cell volume for calculation of ATP concentrations was measured in 6 of 11 experiments by hand-spun hematocrits and was measured using the Coulter counter in the other 5 experiments.

Lipid extracts were prepared from the washed cells according to the method of Rose and Oklander, and lipid phosphorus was measured using the method of Lowry et al. and cholesterol by the method of Zlatkis et al.

RESULTS

Sickle and normal cells were separated into four to six subpopulations on the Stractan gradients. Figure 1 is a photograph of a typical separation of sickle cells. The upper fraction from the gradient contained most of the reticulocytes, with 1%-10% reticulocytes in control samples and 15%-50% reticulocytes in top-layer sickle cell samples. Sickle cell fractions taken from the middle layers of the gradient provided samples composed almost totally of mature discoid cells, with less than 5% reticulocytes or ISC. The bottom layer contained 60%-85% ISC, and the remainder of cells in this layer were typically small, dense spherocytes rather than discoid cells. Usually, from 15 ml whole blood we could obtain approximately 0.5 ml of this ISC-rich fraction. Figure 2 shows illustrative photomicrographs of representative fractions.

Table 1 lists characteristics of sickle and control cells separated on Stractan gradients in a representative experiment. The range of hemoglobin concentrations and mean cell volumes in the sickle cell populations is much greater than that for the control cells, reflecting the heterogeneity of whole sickle cell blood.

![Fig. 1. Separation of sickle cells on Stractan gradients. Sickle cells washed free of white blood cells were layered onto a discontinuous gradient (1 x 8 cm), density range 1.115-1.090 g/ml. This figure, distribution of cells along the gradient after centrifugation at 52,000 g (tube center) for 30 min. Interfaces between gradient layers indicated by the dotted lines; and the density p of each layer given in g/ml.](image-url)
Fig. 2. Photomicrographs of sickle cell sub-populations from a gradient separation. (A) Methylene blue-stained smear of top fraction, showing large numbers of reticulocytes. (B) Phase-contrast photomicrograph of glutaraldehyde-fixed cells from second gradient layer, showing predominance of discoid cells. (C) Phase-contrast photomicrograph of fixed cells from bottom layer, showing predominance of ISC.

Table 1. Characteristics of Separated Sickle Cells From a Representative Experiment

<table>
<thead>
<tr>
<th>Gradient Fraction</th>
<th>ISC (%)</th>
<th>Reticulocytes (%)</th>
<th>Total Cells (%)</th>
<th>MCV (cu μm)</th>
<th>MCHC (g/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sickle cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (top)</td>
<td>5</td>
<td>36</td>
<td>5</td>
<td>108</td>
<td>26.9</td>
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<tr>
<td>2</td>
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<td>31</td>
<td>23</td>
<td>105</td>
<td>30.3</td>
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<tr>
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<td>2</td>
<td>10</td>
<td>40</td>
<td>99</td>
<td>31.5</td>
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<td>5</td>
<td>2</td>
<td>18</td>
<td>90</td>
<td>32.7</td>
</tr>
<tr>
<td>5</td>
<td>8</td>
<td>&lt;0.5</td>
<td>6</td>
<td>83</td>
<td>34.6</td>
</tr>
<tr>
<td>6</td>
<td>26</td>
<td>&lt;0.5</td>
<td>6</td>
<td>82</td>
<td>35.9</td>
</tr>
<tr>
<td>7 (bottom)</td>
<td>67</td>
<td>&lt;0.5</td>
<td>3</td>
<td>79</td>
<td>36.1</td>
</tr>
<tr>
<td><strong>Normal cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>—</td>
<td>9</td>
<td>2</td>
<td>93</td>
<td>32.8</td>
</tr>
<tr>
<td>2</td>
<td>—</td>
<td>&lt;0.5</td>
<td>27</td>
<td>92</td>
<td>33.6</td>
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<tr>
<td>3</td>
<td>—</td>
<td>&lt;0.5</td>
<td>45</td>
<td>90</td>
<td>34.9</td>
</tr>
<tr>
<td>4</td>
<td>—</td>
<td>&lt;0.5</td>
<td>26</td>
<td>89</td>
<td>35.7</td>
</tr>
</tbody>
</table>

Washed cells were separated on discontinuous density gradients of Stractan II, density range 1.090–1.115 g/ml for sickle cells, 1.080–1.101 g/ml for normal controls. Each fraction represents pooled corresponding fractions from three identical gradients. MCV and MCHC in this case were obtained from the Coulter S electronic counter.
Measurements of intracellular Na and K concentrations showed marked abnormalities associated with the ISC-rich fractions. The data are summarized in Fig. 3, which shows the Na and K concentrations of each sickle cell fraction plotted as a function of the percentage of ISC in that fraction. A moderate increase in Na concentrations in ISC was accompanied by a sharp decrease in K concentrations, with overall decrease in Na + K and therefore cell water. In four top-layer fractions containing high percentages of reticulocytes, we found a modest decrease in K (73–78 meq/liter cells), accompanied by increased Na (22–44 meq/liter cells). These values are not included in Fig. 3.

Because ISC are dehydrated relative to the discoid sickle population, it would be more appropriate to measure cation concentrations in meq/liter cell water rather than meq/liter cells. This was not practical for our experiments because of limited quantities of sample and the inherent insensitivity of cell water measurements. The use of cell volume as a basis for concentration calculations is thus a second-best alternative. However, we can estimate the extent of dehydration in the ISC-rich populations from the increase in MCHC of these fractions. For the experiments in which cations were measured, the average decrease in cell water calculated by this method was 11%, with a mean deviation of 2.5%. Thus the true K and Na concentrations of ISC were about 11% higher.
relative to discoid cell concentrations than the values calculated on the basis of cell volume. Therefore the K deficit was somewhat less severe and the Na increase more severe than would be concluded from the data expressed in concentration per cell volume.

In an effort to confirm previous reports\textsuperscript{13,14} that ISC are deficient in ATP, we measured ATP in separated sickle and normal cells. Data from these experiments are summarized in Fig. 4, which shows ATP concentrations in \( \mu \)moles/ml cells as a function of the percentage of ISC in each fraction. In just over half of these experiments, ATP concentrations were calculated using hand-spun hemocrits; in the remainder of the experiments we used mean cell volume data from the Coulter Model S cell counter. We found no differences in the results that would indicate a serious systematic error associated with one of these methods of determining cell volumes. As Fig. 4 shows, there was no evidence for a consistent decrease in ATP concentrations in ISC-rich fractions. Of 11 experiments only 3 showed a decrease of ATP in the bottom fraction, and this decrease was not simply related to the proportion of ISC. Because the ISC were dehydrated, the true concentration of ATP in \( \mu \)moles/ml cell water would actually have been about 10\% higher relative to the discoid cells, based on our estimate of cell water loss from the increase in MCHC. Thus the use of cell volume rather than cell water as the basis for ATP concentrations would, if anything, exaggerate rather than obscure any existent ATP deficiency in ISC.

The fractions containing more than 20\% reticulocytes are designated in Fig. 4 by triangular symbols. Many of these fractions had elevated levels of ATP, up to 25\% higher than mature cell fractions from sickle and normal blood. Control cells gave an average value of 1.01 \( \mu \)moles ATP/ml cells. There was no significant variation with position of these fractions along the gradient. Calculation of ATP in units of \( 10^{-16} \) moles/cell gave essentially the same picture (data not shown), except for a gradual slope downwards with increasing ISC because of declining mean cell volume in ISC-rich fractions. Again, only the three experiments that showed a pronounced drop in ATP/ml cells gave any indication
of a loss of ATP in ISC-rich fractions. Furthermore, because ATP utilization and synthesis are carried out by enzyme systems regulated by the concentration of ATP rather than the total amount of ATP in the cell, the cellular concentration of ATP is a more relevant measurement than the total cellular ATP.

Some authors have suggested that membrane fragmentation during unsickling constitutes a major route to ISC formation. Evidence for a reduction in the lipid content of ISC would support this hypothesis. We measured the lipid phosphorus and cholesterol content of lipid extracts from separated cells; Fig. 5 summarizes these experiments. Here the lipid data are calculated in terms of lipid weight per cell rather than concentrations, since once a red cell matures its membrane surface and lipid content appear not to change until senescence under normal circumstances. With the exception of high lipid content in some fractions containing a high proportion of reticulocytes (20%–30%) the cholesterol and lipid phosphorus content of the various cell fractions was constant. Because these studies included only four experiments, and because of the uncertainties in our measurements, the results do not exclude a variation of ±5% from the reference discoid cell populations. In a representative parallel experiment using separated normal cells, the cholesterol and lipid phosphorus de-
creased by 13% and 10%, respectively, from the top to the bottom of the gradient. Normal cells are not an optimal control, however, because there is a much wider range of cell age than in the sickle cell populations.

**DISCUSSION**

Previous studies of ISC employed high-speed centrifugation of whole blood to obtain top, middle, and bottom fractions with consecutively increasing proportions of ISC. In some cases the middle fraction was ignored and only top and bottom fractions compared. The deficiency of this experimental approach is that blood from patients with sickle cell anemia generally contains large numbers of reticulocytes and frequently elevated white blood cells. Both of these cell types will be concentrated in the top layer of centrifuged whole blood samples. Removal of white blood cells from whole blood before centrifugation circumvents the difficulty of removing these cells after they have been concentrated with the small population of top-fraction red cells. In addition, even if the white cells are successfully removed after separation, the remaining reticulocytes differ from normal mature cells in several respects, ATP and lipid content in particular. Reticulocyte-rich top fractions therefore are not an appropriate reference population for assessing possible deviations from normal function in ISC. A more suitable comparison would use young but mature discoid cells from the same patient or from control subjects with cells of a comparable age but otherwise normal. Such a reference population is available in the middle fraction of centrifuged whole sickle blood, but the effects of changing proportions of reticulocytes and ISC stand out much more clearly when data are available from five or six subpopulations, as was true of the experiments described here.

Our results on ATP content of separated sickle cells provide an example in which a distinction between the effects of cell maturation and irreversible sickling was clarified by the availability of data from more than three fractions. Since the differences in ATP concentration were observed in the upper fractions, where the proportion of reticulocytes was changing, and not in the lower fractions, where the proportion of ISC was changing, we associated these changes with the process of cell maturation rather than with irreversible sickling.

Until an adequate study of a young but mature cell population is carried out, the possibility that ISC and discoid sickle cells may be relatively deficient in ATP for their age cannot be discarded. However, the constancy of ATP concentration in our separated normal cells, together with the data on the sickle cell populations, suggests that ATP levels drop with maturation of the reticulocyte to a discoid erythrocyte and remain constant until the cell becomes senescent. The limited data of Cohen et al. on ATP concentrations of centrifuged whole blood samples from normal subjects are also consistent with this view.

Glader et al. reported in an abstract that ISC are ATP deficient. This conclusion overlooks the problem of reticulocyte or white cell contamination in the top-layer fractions to which the ISC were compared. This view is supported by the fact that the ATP level reported for their ISC-rich bottom fraction was
CATIONS, ATP, AND LIPIDS IN ISC

comparable to the level we found for mature sickle and normal cells and to normal literature values. In addition, part of the decrease in ATP in ISC that they found came from expressing ATP in terms of content per cell rather than concentration per volume of cells or cell water. We feel this is a misleading way to look at the problem, since as mentioned previously ATP is normally regulated by concentration. Thus one would expect a small cell to have decreased ATP content relative to a larger cell of the same type. Ideally, one should measure ATP as its concentration in cell water. Because of limited sample and inherent difficulties in obtaining accurate values for cell water, we chose to measure concentration of ATP on the basis of cell volume as a reasonable compromise. Even so, as mentioned in Results, expression of ATP on a per-cell basis did not reveal a severe deficiency of ATP in ISC.

Weed and Bessis also reported that ATP levels were low in individual ISC, as measured after laser disruption of single cells in a medium containing firefly extract. We are uncertain about the reason for the difference between these findings and the current results, but there could have been problems with incomplete dispersal of the ATP from the viscous hemoglobin solutions within the laser-disrupted ISC.

Comparison of Stractan-separated sickle cells with normal controls showed that the abnormalities in Na and K concentrations previously reported for unseparated sickle cells can be attributed to the permanently distorted ISC. This conclusion is in agreement with the observations of Glader et al. on centrifuged sickle blood. Contrary to earlier conclusions, we found that ISC were not significantly depleted of ATP. This observation does not enlighten us about the role of ATP in generation of ISC; it indicates only that if ATP depletion is a requisite step for ISC formation, at some later time the cell is able to regenerate its ATP supplies.

Finally, in terms of lipid content, we found no difference between ISC and mature discoid cells from the same patient. Thus if membrane fragmentation and loss are involved in ISC formation, only a small amount of lipid is lost.

These studies demonstrate the value of employing density gradients rather than centrifugation of whole blood for the separation of sickle cells or similarly heterogeneous samples. Not only does one obtain several fractions suitable for clarifying the effects of cell age and cell pathology but also the recovery of the cells of interest is considerably more efficient using gradients. The results of this study reemphasize the fact that important consideration must be given to the presence of reticulocytes in studying properties of cells from any patient with shortened red cell survival.

ACKNOWLEDGMENT

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REFERENCES

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