HEMOGLOBIN (Hb) concentration plays a central role in the rate and extent of HbS polymerization in the red blood cells (RBCs) of sickle cell anemia (SS) patients.\(^1\) For this reason, two transport systems that can change the volume of young RBCs and hence the mean corpuscular Hb concentration (MCHC) in SS RBCs are of great interest: the K:Cl cotransport system, which controls the volume regulatory decrease response (VRD) in hypotonic or acid conditions,\(^4\) and the Na\(^+\)/H\(^+\) antiporter, which can produce a volume increase (VRI) in hypotonic or acid conditions.\(^5\) The activity of these transport systems is variable among SS individuals and changes with RBC age and density.\(^6\)\(^,\)\(^7\)

Fetal Hb (HbF) is also unequally distributed as a function of cell density and is enriched in those sickle cells of intermediate density (SS3 in our fractionation system),\(^1\) which are denser than discocytes (SS2) and are presumably older than both discocytes and the more dense SS4 cells (irreversibly sickled cells [ISCs] and other dense cells).\(^1\)\(^,\)\(^3\)\(^,\)\(^4\) HbF interferes with polymerization of deoxygenated HbS in vitro\(^3\)\(^,\)\(^5\) and reduces sickling (shape changes induced by deoxygenation) in HbS containing RBCs.\(^3\) The putative mechanism of enrichment is the preferential survival of F cells.\(^1\)\(^,\)\(^3\)

Recent work on the characteristics of transport systems involved in volume regulation of human RBCs will serve as the basis for our studies.\(^4\)\(^,\)\(^1\)\(^,\)\(^9\)\(^,\)\(^2\)\(^1\) Two aspects merit emphasis: (1) K:Cl cotransport activity appears to decrease sharply on reticulocyte maturation.\(^9\)\(^,\)\(^2\)\(^0\) The activity of mature RBCs is 10 to 20 times lower than that observed in young cells.\(^9\)\(^,\)\(^2\)\(^0\)\(^,\)\(^2\)\(^1\) (2) In contrast, the Na\(^+\)/H\(^+\) antiporter activity is 5 to 10 times higher in young cells but appears to decrease relatively slowly in SS RBCs with increased cell density.\(^1\)

We have previously demonstrated that in the density-defined discocyte fraction (SS2), which contains both half of all cells and half of all reticulocytes, some but not all of the cells are capable of cell volume regulatory decrease when swollen by exposure to hypotonic media or acid pH.\(^3\) In the present study we ask the following questions: (1) what percent of cells are capable of VRD response? (2) What are the characteristics of cells capable of VRD in terms of transport properties; specifically, do cells with high VRD response have high K:Cl cotransport? (3) Are cells capable of VRD homogeneous in cell age? (4) What is the HbF content of high VRD cells and can it be related to the formation of dense and ISC?

We describe here the presence and properties of the high VRD (H-VRD) and low VRD (L-VRD) SS RBCs using a novel method of isolation (hypotonic density-gradient centrifugation). We find that H-VRD cells consist of some but not all reticulocytes, and that L-VRD contain a smaller fraction of reticulocytes. H-VRD cells also have low HbF and high K:Cl cotransport activity, while exhibiting similar levels of glucose-6-phosphate dehydrogenase (G-6-PD) and Na\(^+\)/H\(^+\) antiporter activity when compared with that of L-VRD cells. We show that H-VRD SS cells can increase
their MCHC several grams per deciliter in less than an hour, a factor that favors HbS polymer formation. We suggest that the reticulocytes and young cells that exhibit strong VRD response may become dense cells and possibly ISCs.

MATERIALS AND METHODS

Patient material. Blood was drawn from 12 SS patients followed in the Heredity Clinic (Director Dr H. Billett) of the Bronx Comprehensive Sickle Cell Center (Bronx, NY) after informed consent and characterized by two electrophoresis methods and a solubility test for HbS. All patients included in the studies described here were homozygous for HbS (SS) by MstII digestion. Approximately half of the SS patients had concomitant α-thalassemia. Reticulocytes, HbF, and G-6-PD were measured on all 12 patients; transport studies were performed on nine patients; and F cells and F reticulocytes were determined for six patients, which were a subset of the nine used for transport studies.

Preparation of H-VRD and L-VRD cells. All preparations (isolation of SS2 and separation into H-VRD and L-VRD cells) were performed on the same day as the blood was drawn. The cells were first passed through a Pall filter (Pall Biomedicals, Inc, Fejardo, Puerto Rico) to remove microclots and most of the white blood cells (WBCs). The discocyte fraction of cells was then isolated from SS cells by mixing 0.5 mL of packed SS whole blood with 5.5 mL of Percoll (colloidal silica coated with polyvinylpyrrolidone; Pharmacia Fine Chemical, Piscataway, NJ)-Larex (arabinogalactan; Consulting Associates Inc, Tacoma, WA) gradient mix prepared as previously described13 and centrifuging the mixture for 30 minutes at 38°C (gradient A). This procedure removes the remainder of the WBCs. The ionic composition of the gradient mix was millimolar: 5 glucose, 0.5 g% bovine serum albumin (BSA), 5 PO4 buffer at pH 7.4, and 100 NaCl with 4 KCl and 1 MgCl2. Cells from SS patients were separated into four density fractions12: SS1, which consists of reticulocytes and young cells with MCHC less than 33 g/dL (density <1.076); SS2, which contains reticulocytes, young RBCs, and discocytes with an MCHC between 33 and 37 g/dL (density 1.076 to 1.091); SS3, which consists of cells with an MCHC between 37 and 42 g/dL; and SS4, which consists of very dense discocytes and ISCs with MCHCs greater than 42 g/dL. The isolated SS2 cells have about the same percent reticulocytes as the lighter fraction, SS1, and have a smaller percent HbF than those in the denser fraction, SS3 (see Fig 4).

SS2 cells were isolated by pipette from isotonic Percoll-Larex density gradients (gradient A), washed three times with isotonic saline, resuspended in a hypotonic saline solution containing millimolar: 10 glucose, 0.5 g% BSA, 0.1 ouabain, 5 PO4 buffer at pH 7.4, and either 100 NaCl with 4 KCl and 1 MgCl2, or 100 NaNO3 with 4 KNO3, and 1 Mg(NO3)2, and incubated for 30 minutes at 38°C. At the end of 30 minutes the cells were washed with fresh hypotonic media, and then reconcentrated. The cells were then separated by density-gradient centrifugation at 38°C for a second time by mixing 0.5 mL of packed cells with 5.5 mL of hypotonic Percoll-Larex mixture (gradient B, containing either Cl− or NO3− as the anion), which has a different density profile than that used for preparation of SS2. This gradient mixture has a constant concentration of Percoll and a reduced level of Stractan to allow better separation of low MCHC cells. The procedure is illustrated in Fig 1. The second gradient mixture (gradient B) was prepared by adding 17.5 mL of Percoll in 32.5 mL of a solution containing

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**Fig 1.** Experimental method for isolation of H-VRD and L-VRD cells. (1) SS2 cells are isolated from sickle cell whole blood (SSWB) by density-gradient centrifugation using an isotonic continuous density gradient with physiologic concentrations of Na+, K+, and Cl− (Gradient A). (2) SS2 cells are incubated in hypotonic Cl− or NO3− media as described in the text at 37°C for 30 minutes. (3) The cells are then separated a second time on a hypotonic gradient with a different density profile (Gradient B) as described in Materials and Methods, which contains either Cl− or NO3−, into an H-VRD population which has an MCHC greater than that of cells incubated in NO3− and an L-VRD population that has simply swollen passively. (4) Finally, the separated cells are assayed for the properties discussed in the text.
millimolar: 4 KCl or KNO₃, 1 MgCl₂, or 1 Mg(NO₃)₂, and 5 PO₄ at pH 7.4 and adding 1 part of this mixture to 5 parts of the standard gradient mixture to give a final osmolarity of 220 mOsm. Cells were removed from the gradient by aspiration.

For the first series of studies the column of cells was divided into three equal parts: the top, middle, and bottom. For cation transport studies that required more cells, the column of cells was equally divided into top and bottom. Cells for cation transport were washed three times with isotonic saline to remove the gradient mixture, then twice with preservation solution, adjusted to an approximate Hct of 50, and packed in wet ice for overnight shipment to Boston. Density classes prepared by this technique are very sharply defined, as seen in Fig 1 (cells labeled SS2).

Reticulocyte, G-6-PD, and HbF determinations. Reticulocytes were determined by resuspending aliquots of cells in centrifuged plasma at Hct 50. Equal volumes of blood and new methylene blue reticulocyte stain were mixed and the samples were allowed to incubate at room temperature for at least 10 minutes, after which smears were made for counting. For G-6-PD determinations, an aliquot of whole blood and density-defined cells was washed three times with isotonic saline, adjusted to an approximate Hct of 50, and frozen at −135°C until they could be assayed. G-6-PD was measured using Sigma kit (345-UV, Sigma, St Louis, MO) for kinetic determination of enzyme activity at 340 nm. Complete lysis was assured by freezing in liquid nitrogen and thawing in water three times. Samples were spun in a microfuge before recording ultraviolet absorption to remove particulate matter. G-6-PD activity was recorded as U/g of hemoglobin where U is the amount of G-6-PD activity which will convert one micromole of substrate per minute. HbF was determined using Sigma kit (345-UV, Sigma, St Louis, MO) for kinetic determination of enzyme activity at 340 nm. Complete lysis was assured by freezing in liquid nitrogen and thawing in water three times. Samples were spun in a microfuge before recording ultraviolet absorption to remove particulate matter. G-6-PD activity was recorded as U/g of hemoglobin where U is the amount of G-6-PD activity which will convert one micromole of substrate per minute.

Measurement of K+/Cl− cotransport activity. All transport studies were performed on the morning after the blood was drawn. All cells and fractions were loaded by a modified nystatin procedure to have 11.5 ± 2 Na⁺ and 95 ± 9 K⁺ mmol/L cell. K⁺ fluxes were determined as previously described by incubating cells at 2% (vol/vol) Hct in the following media millimolar: A: NaCl 140, pH 7.4; B: NaNO₃ 140, pH 7.4; C: NaCl 100, pH 7.4; and D: NaNO₃ 100, pH 7.4. All media contained millimolar: 0.1 ouabain (to inhibit the Na⁺ pump), 1 MgCl₂, or 1 Mg(NO₃)₂, 0.1 bumetanide (to inhibit Na⁺/K⁺/Cl− cotransport), 10 glucose, and Tris-MOPS at pH 7.4 at 37°C.

Cl−-dependent K⁺ transport was estimated by subtracting the flux in isotonic NO₃− media from that in isotonic Cl− media (A/B) or similarly in hypotonic media (C/D). The volume-dependent K⁺ efflux was estimated from the difference between hypotonic and isotonic NaCl (C-A) medium. The Cl− and volume-dependent K⁺ efflux was estimated from the difference between Cl− and NO₃− media (C-D). The NEM (N-ethylmaleimide)-stimulated efflux was estimated by adding 1 mmol/L NEM to media A and B. The NEM-stimulated Cl−-dependent K⁺ efflux was estimated from the differences in K⁺ efflux in Cl− (A-B) and NO₃− (B-D) media. Initial rates of K⁺ efflux were measured by sampling the efflux media in duplicate samples at 1, 5, and 30 minutes, which were centrifuged and the supernatant removed for K⁺ measurement by atomic absorption. The K⁺ efflux was calculated from the slope of the change in potassium concentration versus time and the Hct of the cells in the flux media.

Measurements of Na⁺/H⁺ exchange activity. Because the human antiporter is only partially amiloride sensitive, the activity was determined by measuring net Na⁺ influx into acid-loaded cells driven by an outward H⁺ gradient.12-14 RBCs were nystatin treated to reduce intracellular Na⁺ (Na⁺) content to 1.5 mmol/L cell water and then acid loaded to an intracellular pH of 6.0 by incubation in acid-loading solution and 50 μmol/L DIDS (4,4'-diisothiocyanate-stilbene-2,2'-disulfonic acid) to determine the maximum rate, as previously described.19 Na⁺ influx measurements were started by addition of packed acid-loaded, Na⁺-depleted cells (1% final Hct) to Na⁺ medium with pH 6.0 or 8.0, preincubated at 37°C in a shaking water bath. The difference between the Na⁺ influx at pH 8.0 to pH 6.0 was calculated as Na⁺ movement driven by an outward proton gradient. As previously described, this component is equal to that of H⁺ efflux driven by an inward Na⁺ gradient and therefore represents Na⁺/H⁺ exchange activity.20

Determination of F cells and F reticulocytes. HbF in erythrocytes was detected by incubating whole blood or density-defined RBCs with new methylene blue or brilliant cresyl blue for induction of reticular precipitation. Films prepared from this mixture were air dried, fixed in acetone-methanol (9:1 vol/vol), rinsed in phosphate-buffered saline and then in water, dried, and then incubated with staining solution containing anti-HbF antibodies conjugated to fluorescein isothiocyanate (anti-HbF-FITC) and 0.01% acridine orange. The preparations were incubated in moist chambers at 37°C for 2 hours. The acridine orange stained the precipitated reticulum of the reticulocytes bright orange, whereas the anti-HbF-FITC labeled the HbF bright green.20 This method permitted distinction and quantification of reticulocytes containing HbF (F reticulocytes) from those lacking it (5 reticulocytes). This is the most sensitive method for detection of intracellular HbF and is capable of detecting 3 pg of HbF per cell or less21 (normal cells contain about 30 pg of Hb).

RESULTS

Isolation of H-VRD and L-VRD cells from SS2 cells. We previously reported that some sickle cell discocytes (SS2) respond to swelling induced either by hypotonic Cl− or acid pH media by activating K⁺/Cl− cotransport, which produces a net loss of K⁺, CI−, and water and increases MCHC (increases RBC density, reduces cell volume).7 In the present study, we have selected hypotonic treatment because it elicits a more selective response of K⁺/Cl− cotransport. In contrast, acid-induced swelling modifies the activity of other ion transport systems (ie, Na⁺/H⁺ and CI-/OH− exchange) in addition to stimulating K⁺/Cl− cotransport. As indicated in Materials and Methods, density-defined SS2 cells were first isolated under isotonic conditions from whole blood of 12 SS patients and subjected to 30 minutes of hypotonic incubation in the presence of CI− or NO₃− followed by separation on hypotonic continuous density gradients in the presence of CI− or NO₃− (Fig 1). Thus, hypotonic incubation separates SS2 cells into two populations of cells: one that is capable of H-VRD response and another that has a small or nonexistent response and swells (L-VRD cells). The average MCHC of H-VRD cells exceeds that of L-VRD cells by 2 to 4 g/dL. To demonstrate that this response is chloride dependent, we determined the distribution of cells after exposure to hypotonic NO₃− media. We compared the distribution of cells found under hypotonic CI− conditions to the distribution of cells under hypotonic NO₃− conditions by densitometry (Fig 2) and determined the percent of cells that respond to hypotonic CI− by shrinking. We found that 60% ± 34%, n = 25 (mean ± SD, n = number of experiments, for 12 patients) of the cells in hypotonic CI− had a density (MCHC) greater
Fig 2. Density distribution of SS2 cells in a typical experiment involving hypotonic chloride and nitrate. Depth in the tube increases from the top on the left. The densitometer tracing is evaluated for 10 intervals as described. The MCHC indicated on the top was determined by using Pharmacia density marker beads and MCHC measurements of aspirated cells. The solid circles (●) represent the density of cells in hypotonic (220 mOsm) nitrate and the open circles (○) represent that in hypotonic chloride. In this example 60% of the cells in hypotonic chloride have an MCHC greater than 95% of the cells in hypotonic nitrate.

than 90% of the cells in hypotonic NO₃⁻. The average MCHC of SS2 cells in hypotonic NO₃⁻ is 27 ± 1 g/dL, which is comparable with that found for AA cells at the same osmolarity (220 mOsm); the MCHC of SS2 cells increases to 31 ± 1 g/dL in hypotonic chloride.

Activation of K:Cl cotransport results in the redistribution of reticulocytes and HbF. To determine if the SS2 cells that are capable of changing their density (volume) belong to a distinct population, we compared the distribution of several RBC characteristics after hypotonic incubation of SS2 cells and gradient separation in the presence of Cl⁻ and NO₃⁻. Three density classes with approximately equal numbers of cells in each class (top, middle, and bottom fractions) were isolated. The top (least dense) cells in the hypotonic chloride fractionation are more extreme representatives of L-VRD cells and the bottom (densest) cells are a subset of H-VRD cells. Fig 3A (solid bars) illustrates that reticulocytes are strongly enriched (79.3% ± 25.9%, [bottom - top]/bottom ± SD, P < 1.6 × 10⁻³, n = 9) while G-6-PD (Fig 3B, solid bars) is unchanged.

In contrast to the reticulocyte distribution, HbF is depleted in the bottom hypotonic chloride fraction (−72.5% ± 90.5%, [bottom - top]/bottom ± SD, P < .014, n = 13) (Fig 3C, solid bars). The behavior of F cells was studied in a smaller sample of six patients that was also used to study the behavior of F reticulocytes (as described below). In this smaller sample, the trend observed (F cells were depleted in the bottom of hypotonic chloride gradients) was consistent with that seen in the larger sample in which HbF was measured, but was not statistically significant (Fig 3D, solid bars, −40.2% ± 81.2%, P < .28, n = 6).

That the density redistribution of reticulocytes, HbF, and F cells is indeed driven by K:Cl cotransport can be demonstrated by comparing the results of experiments performed in hypotonic chloride (where K:Cl cotransport is active, solid bars) with those in hypotonic nitrate media (where K:Cl cotransport is inactive, open bars). In Fig 3, the bottom panels (delta [chloride – nitrate], hatched bars) depicts the chloride dependent response of the three SS2 subfractions. A positive value represents enrichment in the presence of Cl⁻ and a negative value represents depletion. Paired t-tests indicate that the difference between Cl⁻ and

Fig 3. Distribution of reticulocytes, G-6-PD, percent HbF, and percent F cells in hypotonic chloride and nitrate density gradients. T, top; M, middle; B, bottom. The cells in the bottom fraction are the H-VRD-like population. All levels are normalized to the value of SS2 and averaged; the error bars are the standard error of the average. (A) and (B) in the upper panels show the effect of hypotonic chloride (●) or nitrate (○) on the distribution of reticulocytes (A, n = 13) and G-6-PD (B, n = 13) activity in three density fractions of SS2. The bottom panels (delta) show the paired difference between the values in Cl⁻ and nitrate. A positive delta value indicates enrichment in the H-VRD fraction. A negative delta value indicates that the property was depleted in the fraction cell population. (C) and (D) in the upper panels show the effect of hypotonic chloride (●) or hypotonic nitrate (○) on the distribution of HbF (C, n = 13) and F cells (D, n = 6) in three density fractions of SS2. The bottom row of panels shows the difference (delta) between the value in Cl⁻ and that in nitrate. In all cases, the top and bottom fractions were significantly enriched or depleted with respect to the control NO₃⁻ values when incubated in hypotonic Cl⁻ media.
NO\textsuperscript{−} values for the top cells (L-VRD-like) and bottom cells (H-VRD-like) are statistically significant in all cases ($P < .05$ to .005).

The distribution of cells found using hypotonic chloride gradients also differs from that found on physiologic, isotonic density gradients. Figure 4A illustrates the distribution of reticulocytes, G-6-PD, and HbF under isotonic conditions. SS cells with low density (MCHC) are enriched in reticulocytes and G-6-PD activity and low in HbF. The trends in percent reticulocytes and HbF seen under isotonic conditions are reversed in the presence of hypotonic Cl\textsuperscript{−}. This observation gives us the unique opportunity to estimate the effect of KCl cotransport on reticulocyte hydration. The movement of reticulocytes to the bottom fraction under hypotonic chloride conditions during the 1-hour period of the experiment implies that activation of K:Cl cotransport can induce an increase of MCHC of 1 to 3 g/dL × hour. Because this is not a kinetic experiment, this estimate represents a lower limit of the rate of dehydration.

In a separate experiment, SS2 cells were incubated for 1/2, 1, and 2 hours in hypotonic media: no further change in any of the properties was observed after the first half hour of incubation. This indicates that all changes described in this report were complete within the 30 minutes of incubation and the 30 minutes of separation by density-gradient centrifugation.

Transport properties of isolated H-VRD and L-VRD SS2 cells. To test the hypothesis that the cells which have migrated to higher density in hypotonic chloride gradients have arrived there due to their elevated K:Cl cotransport activity, NEM- and volume-stimulated chloride-dependent K\textsuperscript{+} efflux were measured in isolated H-VRD and L-VRD cells. These experiments are illustrated for a single patient in Table 1. Under isotonic conditions, there was no chloride-dependent K\textsuperscript{+} efflux in either fraction. The NEM-stimulated, chloride-dependent K\textsuperscript{+} efflux was 105% higher in H-VRD than in L-VRD. Exposure to hypotonic conditions showed a volume-stimulated, chloride-dependent K\textsuperscript{+} efflux that was 104% higher in H-VRD than in L-VRD. Figure 5A (top panel) illustrates variation among the seven patients of NEM-stimulated K:Cl cotransport of H-VRD (solid bars) and L-VRD (open bars). The difference in activity between these fractions (Fig 5A, delta, bottom panel) is positive in all cases. The mean values of H-VRD and L-VRD are shown in Table 2. Because of inter-individual variation in transport activity (Fig 5A, top panels), the differences between H-VRD and L-VRD cells were normalized before statistical analysis. Two approaches were used that yield results which are qualitatively very similar. In Table 2 the difference was normalized to the ion flux in SS2, using the following equation: (flux\textsubscript{H-VRD} − flux\textsubscript{L-VRD}) / flux\textsubscript{L-VRD} × 100.

### Table 1. CI-Dependent K\textsuperscript{+} Efflux From H-VRD and L-VRD Sickle Cells

<table>
<thead>
<tr>
<th>Media Composition</th>
<th>L-VRD Cells K\textsuperscript{+} Efflux\textsuperscript{a}</th>
<th>H-VRD Cells K\textsuperscript{+} Efflux\textsuperscript{a}</th>
<th>(H-VRD) − (L-VRD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>3.2 ± 0.2</td>
<td>4.6 ± 0.03</td>
<td>50</td>
</tr>
<tr>
<td>NaNO\textsubscript{3}</td>
<td>7.4 ± 0.17</td>
<td>7.1 ± 0.29</td>
<td>None</td>
</tr>
<tr>
<td>NaCl + NEM</td>
<td>13.4 ± 0.09</td>
<td>23.1 ± 0.4</td>
<td>—</td>
</tr>
<tr>
<td>NaNO\textsubscript{3} + NEM</td>
<td>10.3 ± 0.11</td>
<td>12.4 ± 0.3</td>
<td>—</td>
</tr>
<tr>
<td>Δ CI, NEM stimulated</td>
<td>7.3 ± 0.15</td>
<td>15 ± 0.03</td>
<td>106</td>
</tr>
<tr>
<td>NaCl</td>
<td>26.9 ± 0.4</td>
<td>51.7 ± 0.17</td>
<td>—</td>
</tr>
<tr>
<td>NaNO\textsubscript{3}</td>
<td>3.4 ± 0.2</td>
<td>3.7 ± 0.14</td>
<td>—</td>
</tr>
<tr>
<td>Δ CI, volume stimulated</td>
<td>23.6 ± 0.08</td>
<td>48.0 ± 0.04</td>
<td>104</td>
</tr>
</tbody>
</table>

Patient EM.

\textsuperscript{a}K\textsuperscript{+} efflux units are in mmol/L cell × h = SEM. All cells were loaded by the nystatin procedure to contain 100 mmol K\textsuperscript{+}/L cell.

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Fig 4. Distribution of cells, percent reticulocytes, absolute percent reticulocytes, HbF, and G-6-PD activity in isotonic density gradients of SS whole blood. (A) Distribution of percent reticulocytes, percent HbF, and G-6-PD activity in RBC density fractions. The results represent the average and error bars are standard errors of values obtained in the nine patients used for transport studies. Reticulocytes (C), G-6-PD activity (●), and HbF (□). In this panel the percent cells expressing a property are the percent cells in each fraction. The difference in the percent HbF in SS1 and SS4 versus that in SS2 and SS3 is statistically significant. (B) Density distribution of cells and absolute reticulocytes of SS whole blood under isotonic conditions. The results are the average value and standard error for the nine patients used in the transport studies. (C), Percent cells in each density fraction; (●), the absolute percent of all reticulocytes in each fraction.

Fig 5. NEM-stimulated K:Cl cotransport and Na\textsuperscript{+}/H\textsuperscript{+} antiport activity in L-VRD and H-VRD cells from seven SS individuals. Transport activities were expressed in flux units millimolar cells × hour. (A) The upper panel shows the NEM-stimulated K:Cl cotransport activity of H-VRD (●) and L-VRD (□) cells. The bottom panel shows the difference between the H-VRD cells and the L-VRD cells (delta = H-VRD − L-VRD). The standard error of the mean of individual K:Cl cotransport measurements varied between 0.1 to 0.4 flux units (see also Table 1). Note that NEM-stimulated K:Cl cotransport was enriched (positive values) in the H-VRD cells in all cases. (B) The upper panel shows the Na\textsuperscript{+}/H\textsuperscript{+} exchange activity of H-VRD (●) and L-VRD (□) cells. For individual measurements of Na\textsuperscript{+}/H\textsuperscript{+} exchange the standard error of the mean varied between 1 and 4 flux units. The bottom panel shows the differences between the H-VRD cells and the L-VRD cells (delta = H-VRD − L-VRD) (□). Note that the Na\textsuperscript{+}/H\textsuperscript{+} antiport activity did not show a consistent pattern of enrichment or depletion between H-VRD and L-VRD cells.
The standard deviation for an unpaired average is large because of large inter-individual variation.

The effect of α-thalassemia on the distribution of reticulocytes was examined and we found that the ratio (ReticEsatron − Reticθ) was found to be 210% ± 140%, N = 13 for α-thalassemia trait (aa/−α) and 440% ± 520%, N = 10 for patients with the full complement of α genes; however, the result was not statistically significant (P < .15).

H-VRD cells have 25% less HbF than L-VRD cells (P < 5.5 × 10^-4, Table 2). This trend is the same as that observed in cells separated into three populations (Fig 3) and is in the opposite direction to that found in cells separated on hypotonic N_2_5^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^
Distribution of reticulocytes, F reticulocytes, and F cells in H-VRD and L-VRD RBCs. To test whether the unequal distribution of HbF between H-VRD and L-VRD cells reflects low K:Cl cotransport in F reticulocytes, the percent reticulocytes, F reticulocytes, and F cells were determined for six patients (Fig 6). The absolute percent reticulocytes in the bottom fraction of hypotonic chloride gradients (H-VRD-like cells) are enriched over the absolute percent reticulocytes in the bottom fraction of nitrate gradients by 21.7% ± 11.9% (chloride bottom - nitrate bottom) ± SD, \( P < 6.5 \times 10^{-3} \), n = 6, Fig 6A). The absolute percent F reticulocytes (percent F reticulocytes multiplied by percent reticulocytes multiplied by the number of cells, normalized to 100%) were enriched to the same extent that reticulocytes are enriched 25.4% ± 16.4% (chloride bottom - nitrate bottom) ± SD, \( P < .013 \), n = 6, Fig 6B). Another way of examining these data is to determine the percent of reticulocytes that are F reticulocytes; this value was constant (6.5 ± 0.8, mean ± standard deviation) in all density fractions studied. However, F cells are selectively depleted from the bottom fraction by -23.8%. These results indicate that the K:Cl cotransport activity of F reticulocytes and non-F reticulocytes are similar.

DISCUSSION

We have characterized the properties of SS cells with high VRD response to cell swelling to define its role in dense-cell generation in sickle cell disease. This study demonstrates (1) the function of K:Cl cotransport in determining RBC density and (2) that only a fraction of reticulocytes have high K:Cl cotransport activity that results in the capacity for extremely rapid change in density. These findings further expand our earlier studies of SS heterogeneity under isotonic conditions.6,27,28

A fraction of reticulocytes and discocytes (H-VRD) can rapidly increase density. Our results show that the SS2 fraction, which contains most of the discocytes and half of all reticulocytes, has a population of cells capable of rapid dehydration when challenged with either hypotonic or acid conditions. The rate of dehydration is unexpectedly large and can be estimated by comparing the final density of reticulocytes in hypotonic chloride and nitrate. From this comparison we conclude that H-VRD cells dehydrate as much as 3 g/dL × hour. Because SS2 contains about half of all reticulocytes found in whole blood, the percent H-VRD cells allows us to estimate that at least 45% of all reticulocytes are capable of rapid density changes. Are these reticulocytes at greater risk of dehydration? In the patient sample studied, the average percent dense cells in whole blood is 22%, which exceeds the percent H-VRD reticulocytes. Therefore, we conclude that while the high K:Cl cotransport of H-VRD cells puts them at risk of dehydration, other factors are also crucial at determining the final fate of these cells. One such factor is the cell's history of deoxygenation-related changes in cation content, and another factor may be level of Na+/H+ exchange activity that can counteract the decrease in volume.

The VRD response described here was elicited by hypotonic swelling; however, we have previously reported that acid-induced swelling also results in generation of denser cells. Recently Brugnarai et al9 reported increased density of SS RBCs after a 12-hour exposure to acid conditions. However, the cells that became denser were not characterized and the involvement of the K:Cl cotransporter was not demonstrated by control experiments in nitrate. More recently, Bookchin et al9 has also confirmed our observations that acid pH increases the density of SS RBCs after exposure to pH 7.0 for 30 minutes.

Cells with a large increase in density have high KCl cotransport activity. We have shown that K:Cl cotransport and Na+/H+ exchange are both very active in SS cells.6,11 In this study, we show that H-VRD cells have higher K:Cl cotransport, both volume-stimulated and NEM-stimulated, than L-VRD cells. In contrast, Na+/H+ antiport activity is not significantly different in H-VRD and L-VRD cells. A high Na+/H+ antiport activity may potentially counteract the shrinking effect of the K:Cl cotransporter if intracellular pH drops below 7.2.11 Hence, because the antiporter is equally active in both H-VRD and L-VRD cells, shrinkage in H-VRD cells will be less compensated than in L-VRD cells. That Na+/H+ antiport activity did not co-enrich with K:Cl cotransport further illustrates the heterogeneous distribution of transport activity among cells. We speculate that those cells with high K:Cl and high Na+/H+ antiport activity may be partially protected from density increase.

As has been demonstrated in this report and suggested previously,6,26,34 this transport system is capable of altering the MCHC of SS cells by several grams per deciliter per
hour under oxygenated conditions. However, we should note that high K:Cl cotransport is not solely a property of SS reticulocytes but is also a property of AA reticulocytes.\textsuperscript{6,7,12} The density increase induced by swelling may be involved in the normal increase in cell density that occurs during reticulocyte maturation; however, it may occur at a slower rate in vivo because, under our experimental conditions, the cells were exposed to hypotonic media for 1 hour.

The impact of high K:Cl cotransport on SS cells is different in SS than AA cells because in SS cells the elevation of MCHC will strongly affect both the rate and extent of polymer formation under deoxygenated conditions,\textsuperscript{2} thereby contributing to the pathophysiology of the disease. The volume-stimulated, chloride-dependent VRD response will be activated by low pH\textsuperscript{3,6} and decreased by the increased intracellular ionized Mg\textsuperscript{2+} produced by deoxygenation,\textsuperscript{9} conditions that are found in vivo in the kidney and ischemic tissue. Other pathways may also contribute to shrinkage of cells from sickle cell patients in both the oxygenated and deoxygenated states; we and others have demonstrated that a large portion of the deoxygenation-stimulated potassium efflux is chloride independent.\textsuperscript{11,22} This observation indicates that while part of the deoxygenation-stimulated K\textsuperscript{+} efflux may occur via K:Cl cotransport, other pathways play a major role.

**H-VRD cells are heterogeneous in cell age.** Many H-VRD cells are young cells, as demonstrated by a strong enrichment of reticulocytes. This is consistent with our\textsuperscript{6,21} and others\textsuperscript{12} previous observation of the marked age dependence of the K:Cl cotransporter in HbS and HbA RBCs. However, two observations indicate that H-VRD cells are not exclusively reticulocytes and young cells, nor do all reticulocytes and young cells belong in the H-VRD population: (1) On the average, approximately 21\% of all reticulocytes in SS2 have either a small or nonexistent volume response. (2) Only a small enrichment of the cell age marker G-6-PD activity is found in the H-VRD population. These observations demonstrate that the K:Cl cotransport activity of reticulocytes is heterogeneous, and this may arise by two possible mechanisms: (1) when cells enter the circulation they may have varying levels of K:Cl cotransport activity; or (2) the rate of decay of K:Cl cotransport activity may vary from cell to cell. The mechanism of loss of transport activity is unknown, but it may be due to changes in the biochemical processes (ie, phosphorylation and dephosphorylation) involved in regulation\textsuperscript{13} or it may be due to loss of transport sites pari passu with the reduction in cell membrane area and shedding of membrane proteins that takes place during reticulocyte maturation.\textsuperscript{14}

**Distribution of F reticulocytes and F cells is different in H-VRD and L-VRD cells.** The percent HbF in H-VRD cells was consistently lower than in L-VRD cells. It has been suggested by several investigators that F cells are on the average older than the other SS RBCs due to the inhibition of HbS polymerization by HbF.\textsuperscript{13,23} Therefore, any preparative procedure that relies on a volume change via a cell age-dependent transporter (such as K:Cl cotransport) would be expected to yield proportionately fewer cells with high HbF. We analyzed the distribution of F reticulocytes under the influence of hypotonic chloride conditions and found that both F and non-F reticulocytes have an equal probability of migrating to higher density under the influence of these conditions. In contrast, HbF and F cells are concentrated in L-VRD. Therefore, although the distribution of K:Cl cotransport for F reticulocytes is similar to that of other reticulocytes, F cells have a less active volume-stimulated K:Cl cotransport, possibly because they are on the average older cells, or, alternatively, because K:Cl cotransport decays more rapidly in F cells.

Concomitant a-thalassemia and sickle cell disease results in a milder clinical course: hemoglobin parameters are improved,\textsuperscript{3,5} survival is prolonged,\textsuperscript{17} and the percent of dense cells is reduced.\textsuperscript{12,26} Embury et al\textsuperscript{14} have reported a correlation between cation fluxes under deoxygenated conditions and the a-globin gene number. Our flux studies involved too few patients in each a-globin gene class to allow such analysis; however, the enrichment of reticulocytes in the H-VRD fraction was nearly twice as large for patients with all four a-globin genes as for patients with a-thalassemia trait. This trend was not statistically significant, but is consistent with the greater percent dense cells found in patients without a-thalassemia.\textsuperscript{12} A simple correlation between dense cells and H-VRD cells would not be expected because the percent dense cells reflects a dynamic balance between the rate of production and the rate of destruction.

The interaction of K:Cl cotransport and polymerization thus appears to be part of a vicious cycle whereby destruction of dense and irreversibly sickled leads to a young RBC population, which is characterized by high activity of transport proteins that are normally absent or inactive in the normal mature human RBC. Our results are in agreement with the early proposal of Bertles and Milner,\textsuperscript{7} which was recently restated by Bookchin et al,\textsuperscript{18} that at least some reticulocytes may become ISCs rapidly. In the study reported here, we further dissect this phenomenon by finding that only a fraction of reticulocytes in SS2 (79\%) can dehydrate rapidly, and that the capacity for becoming dense is also retained in a significant fraction of older RBCs. We also show that the presence of a K:Cl cotransport-mediated system that is stimulated by both hypotonic and acid conditions results in the capacity for generating denser cells.

In summary, we have identified a subset of SS reticulocytes and discocytes that are capable of volume reduction when swollen. This subset of cells (H-VRD) has a high percent F and non-F reticulocytes, a low percent HbF, coupled with high K:Cl cotransport activity and average Na\textsuperscript{+}/H\textsuperscript{+} exchange activity. K:Cl cotransport is unequally distributed among reticulocytes with 79\% of all reticulocytes in SS2 exhibiting high K:Cl cotransport; however, the majority of H-VRD cells (80\%) are not reticulocytes, but are discocytes that retain high K:Cl cotransport. Our findings suggest that dense SS cells (and possibly ISCs) might be generated shortly after egress from the marrow from the subpopulation of SS cells with high K:Cl cotransport.

**ACKNOWLEDGMENT**

The helpful discussions of T.L. Fabry and skillful technical assistance of Fanya Schonbuch are gratefully acknowledged.
VOLUME REGULATION

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Rapid increase in red blood cell density driven by K:Cl cotransport in a subset of sickle cell anemia reticulocytes and discocytes

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