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J Clin Invest. 1995;**95**(6):2573-2580. <https://doi.org/10.1172/JCI117958>.

Research Article

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KCl Cotransport Activity in Light versus Dense Transferrin Receptor-positive Sickle Reticulocytes

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Abstract

A subset of sickle cells becomes K^+ -depleted and dehydrated before or soon after leaving the bone marrow. These young cells may be identified in blood as transferrin receptor-positive (TfR+) dense reticulocytes. KCl cotransport, which is normally active in young erythroid cells with a maximum at pH 6.8, is a candidate pathway for K^+ depletion of sickle reticulocytes. In this investigation, KCl cotransport activity was evaluated in young, TfR+ cells which had become dense in vivo and in age-matched cells which had retained normal hydration. Sickle erythrocytes were first separated into three primary density fractions, with care taken to preserve the in vivo hydration state. After normalization of intracellular hemoglobin concentration with nystatin, the cells were incubated at 37°C for 20 min at pH 6.8 and 7.4. Before and after incubation, each primary fraction was separated into four secondary density fractions. The percentage of TfR+ cells in each secondary fraction was measured and a density distribution for TfR+ cells was determined for each primary fraction before and after incubation. The density shift during incubation was a measure of KCl cotransport. TfR+ cells from the denser primary fractions II and III had significantly more density shift than TfR+ cells from the light fraction I. Although the shifts were larger at low pH, differences between primary fractions were also observed at pH 7.4. These data indicate that the cells which become dense quickly in vivo have more KCl cotransport activity than those which remain light in vivo, and support this pathway as a primary mechanism for dehydration of young sickle cells. (*J. Clin. Invest.* 1995, 95:2573-2580.) **Key words:** sickle cell anemia • potassium • erythrocyte • reticulocyte • transferrin receptor

Introduction

An important element in the pathophysiology of sickle cell disease is the presence of dense, dehydrated red blood cells (RBC).¹ These cells exhibit a wide range of rheologic, morpho-

logic, and membrane abnormalities (1), and their higher intracellular hemoglobin concentration greatly favors the formation of sickle hemoglobin (HbS) polymer (2, 3). Included in this population are cells which became abnormally dense either before or soon after release from the bone marrow, as indicated by the presence of reticulum and transferrin receptors (TfR) (4). Dense TfR+ sickle cells contain little or no fetal hemoglobin (HbF) and are markedly K^+ -depleted (4). However, the dehydration pathway(s) responsible for their formation in vivo, and their contribution to the hemolytic and vasoocclusive aspects of sickle cell disease, are poorly understood.

The possible routes for K^+ efflux from young sickle cells include KCl cotransport and the deoxygenation- and Ca-dependent pathways (5). Lew et al. (6) have proposed a complex series of cellular events initiated by a deoxygenation-induced leak of Ca into reticulocytes, followed by Ca-dependent K^+ efflux, intracellular acidification, and finally activation of KCl cotransport. This model is based on the in vitro requirements for deoxygenation and Ca^{++} to induce dehydration of light reticulocytes, as well as the pH-dependent KCl cotransport activity of young cells (7).

Many studies have examined the cation transport properties of density fractionated sickle cells. However, since each fraction is heterogeneous with respect to age, interpretation is complicated by the presence of maturation-dependent transport pathways, especially KCl cotransport. It has been possible to study reticulocyte-enriched light cells, but this fraction may contain both "fast track" cells (for which insufficient time had elapsed to become dense) and cells with relatively stable hydration. Since reticulocytes comprise only a small percentage of the denser fractions, it is more difficult to study the properties of young cells which became dense in vivo. In particular, it is of interest whether KCl cotransport activity is greater or more readily activated in the dense young cells compared to the light young cells.

In this investigation, RBC were separated into three fractions corresponding to their in vivo density. Each fraction was incubated under the appropriate conditions in the presence of nystatin, so that all postnystatin mean corpuscular hemoglobin concentration (MCHC) values were equivalent. Next, the fractions were incubated at either pH 7.4 or at lower pH values known to activate K^+ efflux through the KCl cotransport pathway. After activation, the cells were separated into four density fractions and the number of TfR+ cells in each fraction was determined by flow cytometry. TfR+ cells which were dense in vivo had a greater tendency to become dense in vitro after rehydration and low pH activation of KCl cotransport. At pH 7.4 there was no tendency for the TfR+ cells which had been light in vivo to change density, but even under these conditions there was a small, but significant, increase in the density of TfR+ cells which had been dense in vivo.

Methods

Buffers (mM). PBSKG (134 NaCl, 5 KCl, 1.4 NaH_2PO_4 , 8.6 Na_2HPO_4 , 11 glucose, pH 7.4, 290-295 mOsm/kg); HBK (140 KCl, 20 Hepes,

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Received for publication 16 November 1994 and in revised form 27 January 1995.

1. **Abbreviations used in this paper:** DIOA, ([dihydroindenyl]-oxy)alkanoic acid; DS, density score; HbF, fetal hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RBC, red blood cells; TfR, transferrin receptor; WBC, white blood cells.

J. Clin. Invest.

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0021-9738/95/06/2573/08 \$2.00

Volume 95, June 1995, 2573-2580

0.1 EDTA, 10 glucose, pH 7.4 at 37°C, 290–300 mOsm/kg); HBIS (135 NaCl, 20 Hepes, 5 KCl, 1 Na₂HPO₄, 1 CaCl₂, 1 MgCl₂, 10 glucose, 290–300 mOsm/kg, pH as required), HBIN (same as HBIS except Cl⁻ replaced with NO₃⁻). For Hepes buffers, the pH given in the text is the value at 37°C.

Blood collection. Informed consent was obtained under a protocol approved by the University of Cincinnati Institutional Review Board and blood was drawn into heparinized vacutainers. Subjects (genotype β^S/β^S) had not been transfused for at least 4 mo and were clinically stable. Blood was kept on ice during transport and processing was started within 2 h of phlebotomy.

Primary density fractionation. RBC were washed three times in cold PBSKG (5 min, 4°C, 1,500 g) and resuspended to 33% hematocrit in the same buffer. A stock stractan (arabinogalactan; Sigma Chemical Co., St. Louis, MO) solution was prepared by the method of Corash et al. (8) as modified by Clark et al. (9), and diluted with PBSKG to give the desired densities. Three-layer discontinuous stractan gradients (3.5 ml/layer) were prepared by sequential underlayering in 1.4 × 8.9 cm polyallomer tubes. The following densities were used: 1.073, 1.081 or 1.083, 1.094 g/cm³. The washed RBC suspension (1.5 ml) was layered on top of the gradient and the tubes were spun at 20,000 rpm for 45 min in a Ti41 (Beckman Instruments, Palo Alto, CA) rotor at 22°C. The interface between the buffer and the 1.073 layer, which contained only white blood cells (WBC) and/or platelets, was discarded. Three primary density fractions were isolated; I: 1.081/1.083 $\geq \rho \geq$ 1.073, II: 1.094 $\geq \rho \geq$ 1.081/1.083, and III: $\rho >$ 1.094. After dilution with excess cold PBSKG, each fraction was washed three times with cold PBSKG and the number of RBC in each fraction was counted (CBC5; Coulter Electronics Inc., Hialeah, FL).

Nystatin treatment. Because it was essential to conduct the density shift experiments on cells with equivalent MCHC and K⁺ concentrations, cells were treated with nystatin to adjust cellular K⁺ content. Theoretically, the MCHC achieved after permeabilization of the cell by nystatin should be a function of external cation (permeant species) and sucrose (impermeant species), and the cellular content of impermeant solutes such as organic phosphates and glutathione (10). When the different cell fractions were treated with nystatin in the same buffer (HBK + 25 mM sucrose), the resultant MCHC values were not the same (fraction I MCHC remained lower and fraction III MCHC higher than fraction II MCHC), presumably because of differences among the fractions in cellular organic phosphates or other impermeant species. Therefore, to produce equivalent final MCHC values, it was necessary to formulate the nystatin treatment solutions differently for the three fractions. To this end, additional sucrose was added to fraction I and additional KCl to fraction III, in amounts which were determined empirically as: fraction I: HBK + 50 mM sucrose; fraction II: HBK + 25 mM sucrose; fraction III: HBK + 25 mM sucrose + 50 mM KCl.

Before nystatin treatment an aliquot of cells was washed in HBIS, and a spun hematocrit and OD₅₄₀ were measured for calculation of the prenystatin MCHC. Treatment flasks were prepared in which 4 ml of the appropriate treatment buffer were mixed with 4.44 μ l of nystatin stock (30 mg/ml in DMSO, Sigma Chemical Co.) at 37°C, then placed on ice. 80 μ l of RBC from each primary fraction were washed once in the corresponding treatment buffer, resuspended to 400 μ l, and transferred to the appropriate treatment flask. The three mixtures were incubated for 20 min on ice with occasional swirling. After the incubation, the cells were transferred to 15-ml tubes, brought to room temperature, and washed seven times with the appropriate treatment buffer to which 1 mg/ml BSA was added. The washed cells were adjusted to ~40% hematocrit and placed on ice.

KCl cotransport activation. Just before activation, each primary fraction was washed twice in ice-cold HBIS 7.4 containing 1 mg/ml BSA, and resuspended to 40% hematocrit. In some experiments, the primary fractions were aliquotted and washed in either HBIS 7.4/BSA or HBIN 7.4/BSA. A spun hematocrit and OD₅₄₀ was obtained and the postnystatin MCHC calculated for each primary fraction.

1 ml of each incubation medium (e.g., HBIS 7.4/BSA and HBIS 6.8/BSA) was prewarmed to 37°C in 1.5-ml conical tubes. In some experiments, 0.1 mM ([dihydroindenyl]oxy)alkanoic acid (DIOA) was

added to selected incubations as a specific inhibitor of KCl cotransport (11). 50 μ l of cell suspension for each primary fraction was added to each incubation medium (20 μ l cells, final 2% hematocrit) to start the 37°C activation incubation. After 20 min, the cell suspensions were transferred to 14 ml of ice-cold HBIS 7.4. In experiments in which cells were incubated in HBIN, the first postincubation wash was in cold HBIN 7.4 and the remaining washes in HBIS 7.4. After two washes, the cells were transferred to 1.5-ml conical tubes and the hematocrits adjusted to ~40%.

Secondary density fractionation. Discontinuous stractan gradients for the secondary fractionation were prepared in 5 × 20 mm ultracentrifuge tubes (Beckman Instruments, Inc., Palo Alto, CA) and spun for 30 min at room temperature in an Airfuge microultracentrifuge (A-100 30° rotor; [Beckman Instruments, Inc.] 20 psig, 100,000 g). Layers were 1.086 (60 μ l), 1.094 (60 μ l), and 1.103 (60 μ l) g/cm³. In addition, there was a 20- μ l cushion with a density > 1.15 g/cm³. A few particles of blue dextran (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) were added to the 1.086 and 1.103 stractan to allow visual inspection of the gradients before loading the cells. 10 μ l of a 40% RBC suspension were layered on top of the gradient to give a total gradient volume of 210 μ l. Fractions were removed with the aid of a microtube fractionator (Beckman Instruments, Inc.) and had the following volumes (measured from the top of the original cell layer): fraction 1 = 40 μ l, fraction 2 = 60 μ l, fraction 3 = 60 μ l, fraction 4 = remainder. Each fraction was washed with 1 ml PBSKG, resuspended to 100 μ l, and stored overnight at 4°C.

Quantitation of TfR+ cells. After the secondary centrifugation the number of cells in each density fraction was determined with an electronic counter (CBC5; Coulter Electronics Inc.). The cells in each fraction were reacted with mouse monoclonal FITC-conjugated anti-TfR (Dako Corp., Carpinteria, CA) and a matched idiotype control antibody (Sigma Chemical Co.). For each fraction, 40 μ l of cell suspension, with a hematocrit not > 2%, was placed in each of two 1.5-ml conical tubes. An equal volume of a 10:1 dilution of anti-TfR in PBS/1% BSA was added to one tube. An identical dilution of the idiotype control was added to the second tube. The mixtures were incubated at room temperature for 30 min with occasional mixing. After incubation, the cells were washed once in 0.5 ml of PBS/1% BSA, once in 1 ml of PBS, resuspended to 0.1 ml with PBS, and fixed with 0.2 ml of buffered paraformaldehyde (12).

The percentage of TfR+ cells in each secondary gradient fraction was determined by flow cytometry (EPICS C; Coulter Electronics Inc.), using gates to exclude cells that were outside the light scatter range of RBC. The cursor was set to give a small percentage of positive cells in the idiotype controls for each sample. This value was subtracted from the percentage of positive cells in the corresponding anti-TfR sample to obtain the corrected percentage of TfR+ cells.

A potential source of error is the presence of TfR+ WBC in the secondary density fractions, which could bias the density shift data. Since stress reticulocytes tend to be sticky and removal of WBC by standard filtration methods could remove small but important subsets of TfR+ cells, no attempt was made to quantitatively remove WBC in this study. However, when the cells were labeled with FITC anti-glycophorin A in a separate control experiment, at least 99.5% of the analyzed cells were erythroid in all prenystatin secondary fractions.

Data analysis. The number of TfR+ cells in each secondary fraction is equal to the total number of cells in that fraction (C_i) multiplied by the proportion of TfR+ cells in that fraction (F_i). The percentage of total TfR+ cells in each fraction (P_i) is obtained by dividing the number of TfR+ cells in that fraction by the total number of TfR+ cells summed over the four secondary fractions according to the following equation:

$$P_i = \frac{100 F_i C_i}{\sum_{n=1}^4 F_n C_n} \quad (1)$$

To characterize the entire density distribution in a secondary gradient with one number, the following density score (DS) was defined:

$$DS = \sum_{i=1}^4 i P_i \quad (2)$$

Table I. The Percentage of RBC in Each Primary Fraction, the Percentage of Each Fraction which Were TfR+, and the Percentage of the Total Number of TfR+ Cells which Were in Each Fraction

Experiment	RBC			TfR+			Total TfR+		
	I	II	III	I	II	III	I	II	III
	%			%			%		
1	3.9	66.3	29.9	14.9	3.1	0.6	20.6	73.0	6.4
2	2.8	59.0	38.2	29.7	4.6	0.9	21.4	69.8	8.8
3	3.9	71.5	24.5	4.3	3.2	1.4	6.0	81.7	12.2
4	1.8	62.8	35.4	6.4	3.5	1.5	4.0	77.3	18.7
5	8.0	47.5	44.0	27.5	5.7	0.5	42.7	52.5	4.3
6	4.0	22.0	74.0	25.1	21.9	1.8	14.0	67.3	18.6
7	5.2	52.4	42.4	20.7	2.4	0.4	43.0	50.2	3.3
8	21.8	58.1	20.1	32.9	2.4	1.1	81.6	15.9	2.5
9	11.5	50.5	37.9	14.5	1.3	0.2	69.5	27.4	3.2
10	7.6	49.8	42.5	30.9	2.2	0.4	65.0	30.3	4.7
11	14.1	72.5	13.4	5.9	2.9	0.8	27.3	69.1	3.5
12	18.6	70.1	11.4	7.2	2.0	0.8	47.3	49.5	3.2
Average	8.6	56.9	34.5	18.3	4.6	0.87	36.9	55.3	7.5
SD	6.5	14.1	16.8	10.8	5.6	0.50	25.6	21.4	5.9

A gradient with all the cells in fraction 1 would have a DS of 100, whereas a gradient with all the cells in fraction 4 would have a DS of 400. The change in density during incubation was quantitated by means of a delta density score (Δ DS), defined as the DS after incubation at 37°C minus the DS before incubation (i.e., postnystatin).

Results

Table I shows the results of the primary RBC fractionation for 12 experiments. The three fractions averaged 8.6% (fraction I), 56.9% (fraction II), and 34.5% (fraction III) of the total number of RBC. When the number of TfR+ cells in each fraction was expressed as a percentage of the total number of TfR, 36.9% were in fraction I, 55.3% in fraction II, and 7.5% in fraction III.

The three primary fractions had the expected differences in MCHC before nystatin treatment (Table II). There were no significant MCHC differences after treatment, demonstrating the success of the nystatin procedure at normalizing ion content and hemoglobin concentration. The corresponding density scores are shown in Table III. Before treatment with nystatin, the density scores correlated with the MCHC with a correlation coefficient of 0.87. After nystatin treatment, density scores were the same for all the fractions.

Fig. 1 A shows secondary gradient density distributions for all RBC from an experiment in which rehydrated RBC were incubated at three pH values: 6.8, 7.1, and 7.4. The prenystatin samples for each primary fraction (I, II, and III) have the expected differences in density distribution. In the corresponding postnystatin samples, the density distribution is normalized, so that each fraction begins the activation incubation on an equivalent basis. The lower panels of Fig. 1 A depict the density distributions of the three primary fractions after 20-min incubation at 37°C at several pH values. In general, the density distributions of the bulk red cell populations were stable over this incubation, with the exception of fraction I cells at pH 6.8. This light population of cells became more dense upon acidification, as anticipated from previous studies (7). However, the volume

regulatory behavior of the young, TfR+ cells was distinctly different from the whole cell population, as shown in Fig. 1 B. For these data, the density distribution of the TfR+ cells was computed by calculating the absolute number of TfR+ cells in a given secondary gradient fraction and dividing by the total number of TfR+ cells in all of the fractions of that secondary gradient. It is noteworthy that the prenystatin density distribution of the TfR+ cells in each of the three primary fractions (Fig. 1 B) closely approximates the distribution of the corresponding bulk cell populations (Fig. 1 A), indicating that the

Table II. Intracellular Hemoglobin Concentration (g/dl) in the Primary Density Fractions before and after Treatment with Nystatin

Experiment	Prenystatin MCHC			Postnystatin MCHC		
	I	II	III	I	II	III
1	29.0	30.0	34.4	29.1	30.0	30.5
2	27.3	32.8	37.0	28.0	29.5	31.1
3	28.6	32.8	37.8	41.0	34.5	32.3
4	25.2	32.9	37.8	33.4	33.9	37.1
5	27.3	32.2	41.1	28.9	31.0	31.7
6	26.0	28.9	41.8	32.9	31.3	31.8
7	29.2	33.9	37.9	32.8	34.3	30.2
8	30.1	33.9	38.6	29.6	31.9	31.6
9	31.1	34.7	41.6	29.8	33.8	34.5
10	27.5	33.0	38.7	29.5	33.2	31.6
11	28.0	32.3	38.8	35.8	31.5	34.1
12	28.2	31.0	37.2	39.1	38.9	37.2
Average	28.1	32.4	38.6	32.5	32.8	32.8
SD	1.6	1.7	2.1	4.2	2.5	2.4
<i>P</i>	I vs II	II vs III	I vs III	I vs II	II vs III	I vs III
	<10 ⁻⁵	<10 ⁻⁷	<10 ⁻¹¹	NS	NS	NS

Table III. DS in the Primary Density Fractions before and after Treatment with Nystatin

Experiment	Prenystatin DS			Postnystatin DS		
	I	II	III	I	II	III
1				119	140	143
2	156	210	305	149	138	144
3	163	233	293	186	144	163
4	133	202	255	142	147	144
5				121	124	130
6				142	165	131
7	187	195	236	134	140	129
8	156	190	258	130	132	134
9	162	207	279		141	142
10	186	200	249	143	134	138
11	172	216	265	146	128	149
12	201	216	274	133	142	149
Average	168	208	268	140	140	141
SD	20	13	22	18	10	10
	I vs II	II vs III	I vs III	I vs II	II vs III	I vs III
P	<.0005	<10 ⁻⁵	<10 ⁻⁷	NS	NS	NS

dense TfR+ cells are not a light subset of the dense cell population. The postnystatin density distributions of TfR+ cells demonstrates that the nystatin procedure successfully rehydrated the dense TfR+ cells.

Upon incubation at 37°C for 20 min at pH 6.8, the TfR+ cells from fraction I (Fig. 1 B) exhibited a clear shift to higher density which was quite similar to that in the bulk cell population (Fig. 1 A), consistent with the fact that 33% of fraction I cells in this experiment were TfR+. After incubation, TfR+ cells from fractions II and III exhibited quite different density distributions from the bulk cell populations of these fractions.

There was a marked density shift at pH 6.8, with the majority of TfR+ cells moving from the lightest density level (postnystatin samples) to the second or third density level. At pH 6.8, TfR+ cells in fractions II and III exhibited a greater shift than those in fraction I.

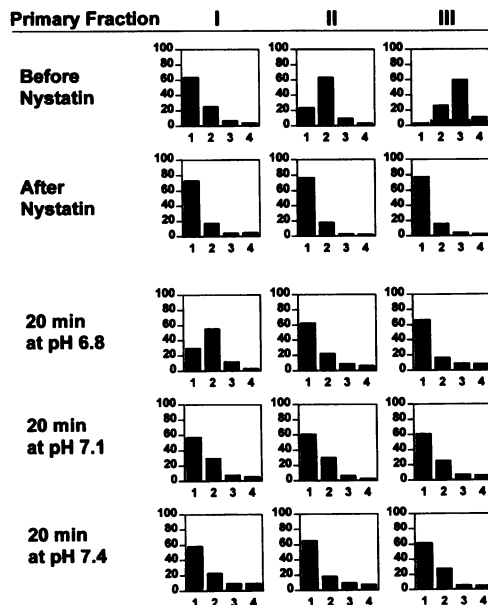
TfR+ cells from fractions II and III also differed from those in fraction I in that they exhibited density shifts at pH 7.1 and 7.4, as illustrated in Fig. 1 B. In most experiments, there was a distinct shift at pH 7.4 in the TfR+ cells from primary fractions II and III, but this did not occur in TfR+ cells from primary fraction I. The density changes which were observed at pH 6.8 and 7.4 are summarized in Table IV.

In summary, TfR+ SSRBC (RBC from patient with homozygous sickle cell disease) which were dense in vivo showed a greater density shift upon acidification than cells of comparable age which had been light in vivo. Furthermore, dense TfR+ cells (fractions II and III), when rehydrated, became dense even at pH 7.4, whereas less dense TfR+ cells (fraction I) remained stable.

To test the hypothesis that KCl cotransport activity was responsible for these shifts in density, incubations were performed under conditions in which the cotransporter was inhibited by Cl⁻ replacement by NO₃⁻ or by addition of DIOA. Results from a representative Cl⁻ replacement experiment are shown in Fig. 2, with panel A representing the distributions of all RBC and panel B that of TfR+ cells. Fig. 2 A demonstrates that the density shift of fraction I cells upon incubation at pH 6.8 was Cl⁻-dependent, as expected from previous work (7, 13). For the TfR+ cells in Fig. 2 B, density shifts at pH 6.8 in Cl⁻ media were greater for fractions II and III compared to fraction I. No change in density distribution was observed in any of the TfR+ cells in nitrate media at pH 6.8. At pH 7.4, a slight shift in density occurred in TfR+ cells of fraction III, which was not observed in nitrate media. Thus, the density shifts in rehydrated TfR+ cells upon incubation at 37°C at both pH 6.8 and 7.4 were Cl⁻-dependent.

Next we examined the effect of DIOA on the density shifts

A (RBC)



B (TfR+)

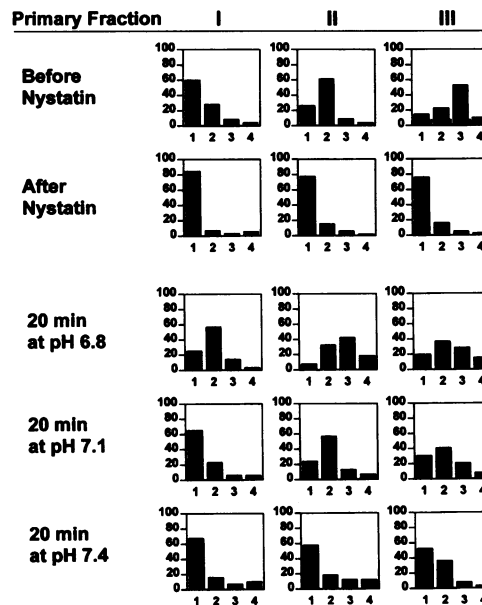
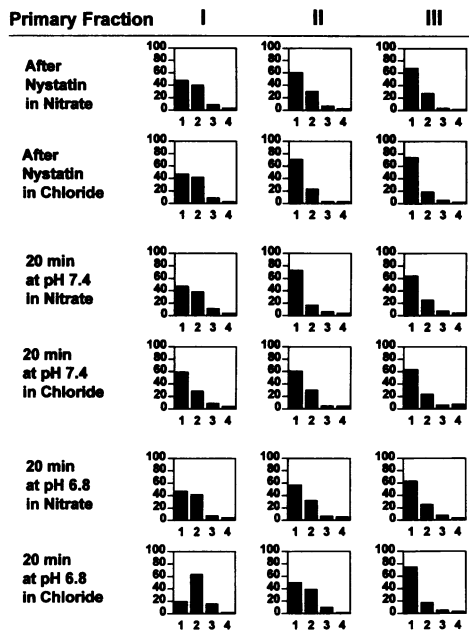


Figure 1. Sickle cells were separated into three primary density fractions and the MCHC normalized by treatment with nystatin. Each primary fraction was incubated for 20 min at the indicated pH and subjected to a secondary density fractionation. (A) Secondary density distributions for all cells; (B) secondary density distributions for TfR+ cells.

A (RBC)



B (TfR+)

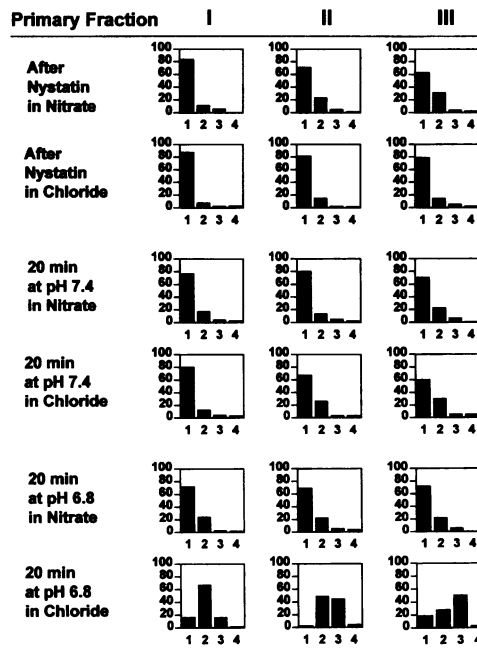


Figure 2. Sickle cells were separated into three primary density fractions, the MCHC was normalized by treatment with nystatin, and the cells were washed in buffer containing either chloride or nitrate. Each primary fraction was incubated for 20 min at pH 6.8 or 7.4 in buffer containing either chloride or nitrate. (A) Secondary density distributions for all cells; (B) secondary density distributions for TfR+ cells.

of incubated SSRBC. DIOA was present at 0.1 mM, which has been shown to produce 90% inhibition of KCl cotransport, measured as swelling-induced, Cl⁻-dependent K efflux (11). As shown in Fig. 3 B, incubation at pH 6.8 caused a marked density shift in TfR+ cells, especially from fractions II and III (compare postnystatin to 6.8 control). In the presence of DIOA, this acid-induced shift was substantially reduced in all fractions. Upon incubation at pH 7.4 (Fig. 4 B), TfR+ cells from fractions

II and III (though not from fraction I) also demonstrated a shift to higher density, which was inhibited by DIOA.

Discussion

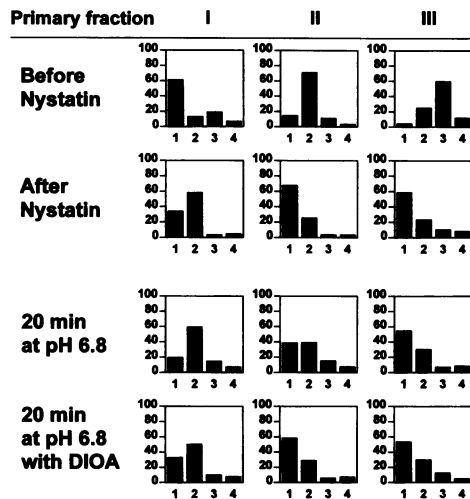
Since some of the pathways which potentially contribute to the increased density of sickle cells are age dependent, and since it is clear that each density fraction contains cells with a wide range of ages, it is important to distinguish age-defined cells for study. Young cells may be identified as either reticulocytes or TfR+ cells. Since TfR are shed during the reticulocyte stage of differentiation (14), TfR+ cells are a young subset of reticulocytes. Therefore, in the absence of nucleated red cells, all TfR+ erythroid cells in the circulation are reticulocytes, but not all reticulocytes are TfR+. Cell age can vary even within the TfR+ population, and age-dependent differences between light and dense TfR+ cells are possible. In the context of this study, the observed difference in KCl cotransport activity could potentially be due to younger TfR+ cells in the dense fraction. Within a TfR+ population, cells with higher TfR are younger than cells with lower levels. In this and a previous (4) study it was clear that, based on fluorescent intensity, the light cells exhibit a wide range of TfR and that the dense TfR+ cells tend to have a lower mean TfR level than the light TfR+ cells. Therefore, by this criterion, the dense TfR+ cells do not appear to be younger than the light TfR+ cells. The lack of density shift for any of the light TfR+ cells at pH 7.4, even those with the highest TfR signal, would further support the conclusion that the observed difference in KCl cotransport activity between light and dense fractions is not due to younger cells in the dense fraction. This analysis assumes that the complex process of TfR shedding (15) is the same for light and dense TfR+ cells.

Some cells in the least dense fraction of normal RBC, when placed into hypotonic conditions, exhibit a chloride-dependent volume decrease due to a ouabain- and bumetanide-resistant K

Table IV. ΔDS Calculated as the DS after Incubation (at pH 6.8 or 7.4) Minus the DS in the Appropriate Postnystatin Sample

Experiment	pH = 7.4			pH = 6.8		
	I	II	III	I	II	III
1	28	15	37	138	156	145
2	-18	30	56	86	139	144
3	-2	41	38	46	140	138
4	1	13	52	71	151	148
5	9	19	25	82	127	108
6	-1	1	30	102	124	150
7	0	1	21			
8	19	47	32	66	138	105
9		16	40			
10	-14	35	23			
11	-12	21	5	97	177	149
12	16	35	39			
Average	2.4	23	33	86	144	136
SD	14	15	14	28	17	19
P	I vs II	II vs III	I vs III	I vs II	II vs III	I vs III
	<.005	NS	<.0001	<.0002	NS	<.001

A (RBC)



B (TfR+)

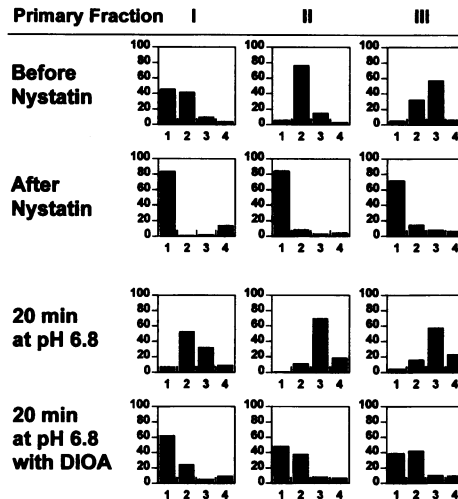


Figure 3. Sickle cells were separated into three primary density fractions and the MCHC normalized by treatment with nystatin. Each primary fraction was incubated for 20 min at pH 6.8 with or without DIOA. (A) Secondary density distributions for all cells; (B) Secondary density distributions for TfR+ cells.

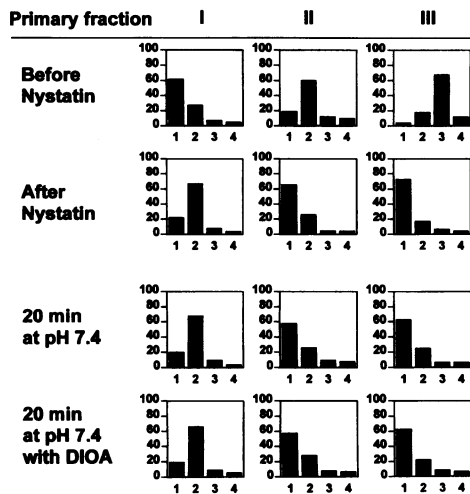
efflux (16) through the KCl cotransport pathway. A similar K efflux is also activated in the least dense fraction at low pH with maximum activity at pH 6.8 (16). This activity is not confined to reticulocytes, but does appear to be lost rapidly in young RBC (16, 17). Red cells containing HbS (SS), HbC (CC), or both (SC) have higher levels of this transporter (18), which appears to be dependent on substitution of a more positively charged amino acid at the $\beta 6$ or $\beta 7$ position. Cells containing HbC, which have only a moderate decrease in circulation lifespan, have activity which is the same as SS cells (19). KCl cotransport activity is inhibited when either SS2 (reversible discocytes) or reticulocyte-enriched AA cells are deoxygenated (20), an effect which has been attributed to increased cellular ionized Mg^{++} (20, 21).

Lew et al. (6) have proposed a model for the rapid dehydration of sickle reticulocytes in which an initial deoxygenation-dependent increase in intracellular Ca^{++} leads to activation of Ca^{++} -dependent K^+ channels, efflux of K^+ , and intracellular acidification. Finally, low pH activates KCl cotransport, which

causes rapid dehydration. Experiments to test this hypothesis (7) have focussed on the light reticulocyte-rich fraction of SSRBC. In these cells it was shown that at pH 7.4, deoxygenation and Ca^{++} are necessary to produce density increases. The absence of a density shift under oxygenated conditions is consistent with our findings, since oxygenated light TfR+ cells had stable density at this pH.

Brugnara et al. (22) have recently demonstrated the importance of the Ca^{++} -activated K^+ channel for the in vivo dehydration of sickle cells. Oral administration of clotrimazole, an inhibitor of this pathway, resulted in red cell density distributions shifted toward lower values. However, the effect of clotrimazole was greater in the older (postreticulocyte) cells, with little or no change in reticulocyte density (C. Brugnara, personal communication). These data imply that older cells, which have less capacity to pump out intracellular Ca^{++} than reticulocytes, are more sensitive to transient, deoxygenation-induced Ca^{++} influx. Since these older cells have little KCl cotransport activity, the Ca^{++} -activated K^+ channel is most likely the major route for

A (RBC)



B (TfR+)

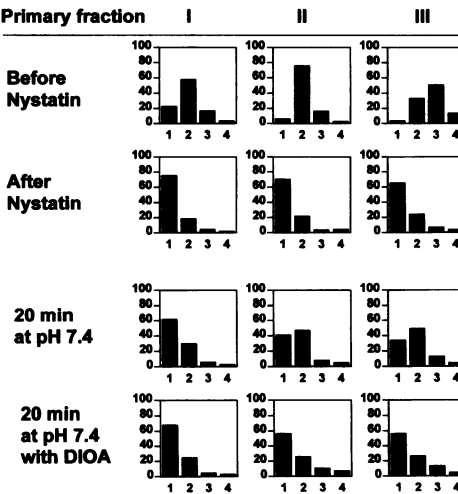


Figure 4. Sickle cells were separated into three primary density fractions and the MCHC normalized by treatment with nystatin. Each primary fraction was incubated for 20 min at pH 7.4 with or without DIOA. (A) Secondary density distributions for all cells; (B) secondary density distributions for TfR+ cells.

K efflux. In contrast, sickle reticulocytes have higher Ca^{++} pump and KCl cotransport activities, and dehydration is more likely to be mediated by the KCl cotransport pathway.

The approach taken in our density shift experiments is fundamentally different than most previous studies. Rather than selecting a light population, which is enriched in reticulocytes but nevertheless heterogeneous, these studies examined an entire cohort of young TfR+ cells, including cells which became dense in vivo. If only light TfR+ cells had been selected for study, then about two-thirds of the TfR+ cells would have been eliminated, and differences in KCl cotransport among TfR+ cells in the three primary density fractions would have escaped notice.

We have shown that, when hydration is normalized, there is greater KCl cotransport activity in TfR+ cells which became dense in vivo when compared to TfR+ cells which were light in vivo. This implies that the cells destined to dehydrate the most quickly are more sensitive to a given degree of acidification of the cell interior, whether due to low extracellular pH or to transport-related H^+ shifts. Even at physiological pH, there is residual KCl cotransport activity in the rehydrated TfR+ cells from fractions II and III, whereas none was detected in TfR+ cells from fraction I. It may be that TfR+ dense cells represent a subset with extremely deranged cation transport, which become dense very quickly in vivo under mild activation conditions. The finding that the light TfR+ cells are quite stable at pH 7.4 during a short incubation would indicate that fraction I does not contain a significant number of TfR+ cells with KCl cotransport behavior such as seen in the denser TfR+ cells.

In these experiments, changes in the density distribution of TfR+ cells were determined after KCl cotransport was either stimulated or inhibited. This indirect methodology was necessary since the cells of interest are a small fraction of the total cells, especially in the denser fractions. The results show clear differences in the response of light and dense TfR+ cells to these conditions. These responses were blocked under experimental conditions which inhibit KCl cotransport, and the most straightforward interpretation is that the activity or regulation of this transporter differs in light and dense TfR+ cells. However, these experiments cannot eliminate the possibility that the activity of this pathway is the same in the three density fractions, with the variability in density shift due to differences in a compensating pathway, such as Na^+/H^+ exchange.

The activity of KCl cotransport has been shown to depend on the intracellular concentration of Mg^{++} (21). For sickle cells at pH 6.8, either removal or elevation of Mg with A23187 resulted in decreased activity of the transporter (21). At pH 7.4, low Mg increased activity, whereas elevated Mg was inhibitory (21). Normal density sickle discocytes have essentially normal Mg content, whereas dense SS cells ($P > 1.118$) have ~ 20% less (23). However, due to the decreased amount of 2,3-DPG present in dense cells, the concentration of free (ionized) Mg^{++} was found to be higher than in discocytes (23). In our studies, when the dense cells are rehydrated, the Mg concentration would be expected to decrease. However, the concentrations of Mg buffers, primarily ATP and 2,3-DPG, decrease proportionately, and it can be shown using published equations for Mg binding equilibria (24) that under these conditions, Mg^{++} levels stay the same or increase slightly. Therefore, it appears unlikely that the Mg^{++} concentration in the rehydrated heavy cells would change sufficiently to cause the observed increased KCl cotransport activity. Nevertheless, regulation of KCl cotransport by Mg^{++} is complex, and Mg^{++} levels have not been measured

for TfR+ cells. Therefore, it is possible that differences in the Mg^{++} concentration among the rehydrated TfR+ cell fractions could contribute toward the observed differences in KCl cotransport activity.

These data, together with information from previous studies, are consistent with the presence of multiple populations of sickle cells with different rates of dehydration. One population consists of those cells which become dense either before or very soon after leaving the marrow, while still TfR+. When a blood sample is taken, all or most of these cells are in the denser fractions. They have low HbF (4) and are K^+ depleted (4). When rehydrated, they quickly lose K^+ through KCl cotransport even when oxygenated and at physiological pH (this study), with the loss accelerated in low pH environments. Another population consists of young cells which are present in the light fraction but have the capacity to become dense quickly through activation of KCl cotransport by exposure to low osmolality or pH. Some of these cells may have become dense in vivo and therefore be present in the denser fractions, and it should be noted that in the present study not all of the rehydrated TfR+ cells in fractions II and III became dense when incubated at pH 7.4. It is clear, however, that not all sickle RBC become dense while very young. Our recent finding that dense TfR+ cells contain virtually no HbF, while non-TfR+ dense cells contain from 30 to 90% of the level in all unfractionated cells (4), suggests that substantial numbers of dense cells became dehydrated after the TfR+ stage. The reticulocyte labeling studies of Bertles and Milner (25) also support the concept of more gradual dehydration in a substantial number of sickle cells. Recent in vivo studies using the inhibitor clotrimazole (22) highlight the importance of Ca^{++} -dependent K^+ channels in the dehydration of the bulk sickle cell population (see above). A gradual increase in density may also occur in some cells via deoxygenation-induced K efflux and Na influx with unbalanced Na pump compensation (26).

In summary, it appears that the rate of sickle cell dehydration varies from extremely fast (TfR+ cells are present in the dense fractions), to fast (radiolabeled reticulocytes become dense over several days), to much slower (HbF-containing cells are enriched in the circulation). It will be important to ascertain the prevalence of cells in each category and the effect on this distribution of clinical treatments, such as HbF-augmenting therapy or transfusion.

Acknowledgments

This research was supported by grant RO1 HL51174 from the National Institutes of Health.

References

1. Heibel, R. P. 1991. Beyond hemoglobin polymerization: the red blood cell membrane and sickle cell disease pathophysiology. *Blood*. 77:214-237.
2. Sunshine, H. R., J. Hofrichter, and W. A. Eaton. 1978. Requirements for therapeutic inhibition of sickle haemoglobin gelation. *Nature (Lond.)*. 275:238-240.
3. Brittenham, G. M., A. N. Schechter, and C. T. Noguchi. 1985. Hemoglobin S polymerization: primary determinant of the hemolytic and clinical severity of the sickling syndromes. *Blood*. 65:183-189.
4. Franco, R. S., R. Barker-Gear, M. A. Miller, S. M. Williams, C. H. Joiner, and D. L. Rucknagel. 1994. Fetal hemoglobin and potassium in isolated transferrin receptor-positive dense sickle reticulocytes. *Blood*. 84:2013-2020.
5. Joiner, C. H. 1993. Cation transport and volume regulation in sickle red blood cells. *Am. J. Physiol.* 264 (Cell Physiol 33):C251-C270.
6. Lew, V. L., C. J. Freeman, O. E. Ortíz, and R. M. Bookchin. 1991. A mathematical model of the volume, pH, and ion content regulation in reticulocytes.

- Application to the pathophysiology of sickle cell dehydration. *J. Clin. Invest.* 87:100–112.
7. Bookchin, R. M., O. E. Ortiz, and V. L. Lew. 1991. Evidence for a direct reticulocyte origin of dense red cells in sickle cell anemia. *J. Clin. Invest.* 87:113–124.
 8. Corash, L. M., S. Piomelli, H. C. Chen, C. Seaman, and E. Gross. 1974. Separation of erythrocytes according to age on a simplified density gradient. *J. Lab. Clin. Med.* 84:147–151.
 9. Clark, M. R., A. C. Greenquist, and S. B. Shohet. 1976. Stabilization of the shape of sickled cells by calcium and A23187. *Blood.* 48:899–909.
 10. Freedman, J. C., and J. F. Hoffman. 1979. Ionic and osmotic equilibria of human red blood cells treated with nystatin. *J. Gen. Physiol.* 74:157–185.
 11. Olivieri, O., D. Vitoux, D. Bachir, and Y. Beuzard. 1991. K^+ efflux in deoxygenated sickle cells in the presence or absence of DIOA, a specific inhibitor of the $[K^+, Cl^-]$ cotransport system. *Br. J. Haematol.* 77:117–120.
 12. Russo, V., R. Barker-Gear, R. Gates, and R. Franco. 1992. Studies with biotinylated rbc. 1. Use of flow cytometry to determine posttransfusion survival 2. Isolation using streptavidin conjugated magnetic beads. *Adv. Exp. Med. Biol.* 326:101–107.
 13. Brugnara, C., H. F. Bunn, and D. C. Tosteson. 1986. Regulation of erythrocyte cation and water content in sickle cell anemia. *Science (Wash. DC).* 232:388–390.
 14. Johnstone, R. M., M. Adam, J. R. Hammond, L. Orr, and C. Turbide. 1987. Vesicle formation during reticulocyte maturation: association of plasma membrane activities with released vesicles (exosomes). *J. Biol. Chem.* 262:9412–9420.
 15. Johnstone, R. M. 1991. Maturation of reticulocytes: formation of exosomes as a mechanism for shedding membrane proteins. *Biochem. Cell. Biol.* 70:179–190.
 16. Brugnara, C. and D. C. Tosteson. 1987. Cell volume, K transport, and cell density in human erythrocytes. *Am. J. Physiol.* 252 (Cell Physiol 21):C269–C276.
 17. Ellory, J. C., A. C. Hall, S. A. Ody, C. E. Poli de Figueiredos, S. Chalder, and J. Stuart. 1991. KCl cotransport in HbAA and HbSS red cells: activation by intracellular acidity and disappearance during maturation. *Adv. Exp. Med. Biol.* 307:47–57.
 18. Canessa, M., A. Spalvins, and R. L. Nagel. 1986. Volume-dependent and NEM-stimulated K^+ , Cl^- transport is elevated in oxygenated SS, SC, and CC human red cells. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 200:197–202.
 19. Olivieri, O., D. Vitoux, F. Galacteros, D. Bachir, Y. Blouquit, Y. Beuzard, and C. Brugnara. 1992. Hemoglobin variants and activity of the $(K^+ Cl^-)$ cotransport system in human erythrocytes. *Blood.* 79:793–797.
 20. Canessa, M., M. E. Fabry, and R. L. Nagel. 1987. Deoxygenation inhibits the volume-stimulated, Cl^- -dependent K^+ efflux in SS and young AA cells: a cytosolic Mg^{++} modulation. *Blood.* 70:1861–1866.
 21. Brugnara, C. and D. C. Tosteson. 1987. Inhibition of K transport by divalent cations in sickle erythrocytes. *Blood.* 70:1810–1815.
 22. Brugnara, C., B. Gee, C. Armsby, S. Kurth, M. Sakamoto, N. Rifai, S. Alper, and O. Platt. 1994. Inhibition of gardos channel and red cell dehydration by oral administration of clotrimazole in sickle cell disease. *Blood.* 84:220a. (Abstr.)
 23. Ortiz, O. A., V. L. Lew, and R. M. Bookchin. 1990. Deoxygenation permeabilizes sickle cell anemia red cells to magnesium and reverses its gradient in the dense cells. *J. Physiol.* 427:211–226.
 24. Bunn, H. F., B. J. Ransil, and A. Chao. 1971. The interaction between erythrocyte organic phosphates, magnesium ion, and hemoglobin. *J. Biol. Chem.* 246:5273–5279.
 25. Bertles, J. F., and P. F. A. Milner. 1968. Irreversibly sickled erythrocytes: a consequence of the heterogeneous distribution of hemoglobin types in sickle-cell anemia. *J. Clin. Invest.* 47:1731–1741.
 26. Joiner, C. H., O. S. Platt, and S. E. Lux IV. 1986. Cation depletion by the sodium pump in red cells with pathological cation leaks: sickle cells and xerocytes. *J. Clin. Invest.* 78:1487–1496.