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Na^+/H^+ Exchange is Increased in Sickle Cell Anemia and Young Normal Red Cells

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Summary. Red cell volume regulation is important in sickle cell anemia because the rate and extent of HbS polymerization are strongly dependent on initial hemoglobin concentration. We have demonstrated that volume-sensitive K : Cl cotransport is highly active in SS whole blood and is capable of increasing MCHC. We now report that Na^+/H^+ exchange (Na/H EXC), which is capable of decreasing the MCHC of erythrocytes with $pH_i < 7.2$, is also very active in the blood of patients homozygous for HbS. The activity of Na/H EXC (maximum rate) was determined by measuring net Na^+ influx (mmol/liter cell · hr = FU) driven by an outward H^+ gradient in oxygenated, acid-loaded (pH_i 6.0), DIDS-treated SS cells. The Na/H EXC activity was 33 ± 3 FU (mean ± SE) (n = 19) in AA whites, 37 ± 8 FU (n = 8) in AA blacks, and 85 ± 15 FU (n = 14) in SS patients ($P < 0.005$). Separation of SS cells into four density-defined fractions by density gradient revealed mean values of Na/H EXC four to five times higher in reticulocytes (SS1), discocytes (SS2) and dense discocytes (SS3), than in the fraction containing irreversibly sickled cells and dense discocytes (SS4). In contrast to K : Cl cotransport, which dramatically decreases after reticulocyte maturation, Na/H EXC persists well after reticulocyte maturation. In density-defined, normal AA red cells, Na/H EXC decreased monotonically as cell density increased. In SS and AA red cells, the magnitude of stimulation of Na/H EXC by cell shrinkage varied from individual to individual. We conclude that Na/H EXC is highly expressed in SS and AA young red cells and decays slowly after reticulocyte maturation.

Key Words sodium-hydrogen exchange · red cells · sickle cell anemia · erythrocyte · reticulocyte · cation transport

I. Introduction

Studies from our laboratory have focused on the influence of cell age and inter-individual differences in the expression of volume-regulating transport systems in red cells of patients homozygous for HbS[1] (SS). We have previously reported that the K : Cl cotransport system, found predominantly in young red cells, controls the volume regulatory decrease (VRD) response to cell swelling [7, 9, 10] and is modulated by the deoxygenation-induced increase in Mg^{2+} concentration [8]. This transport system, under the influence of low pH or hyposmotic conditions, can alter MCHC and hence affect the rate of deoxygenation-induced HbS polymerization and probably the generation of dense cells. We have also studied the kinetics of activation of Ca^{2+}-activated, charybdotoxin-sensitive K^+ channels and demonstrated inter-individual differences in red cells from SS and AA donors [28]. No data exists, on the other hand, on transport systems which are activated when human red cells are shrunken. The present report examines the activity of Na^+/H^+ exchange (Na/H EXC) in SS and AA red cells, its cell age dependence and its response to cell volume reduction.

Na/H EXC is a transport system widely distributed in eukaryotic cells and implicated in many cellular functions, such as control of internal pH and cell volume, response to mitogens, growth factors and hormones, and Na^+ reabsorption by the kidney (*see* refs. [1, 19, 22] for reviews). The antiporter was first detected in nucleated *Amphiuma* red cells [4] and subsequently in dog, trout, and rabbit red cells [3, 15, 25]. We have recently demonstrated the presence of Na/H EXC in mature human red cells [13, 14, 26] by three independent lines of experimental evidence. First, elevation of red cell cytoso-

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[1] *Abbreviations:* HbA, normal adult hemoglobin; HbS hemoglobin S; DIDS, 4,4-diisothiocyanate-stilbene-2,2'disulfonic acid; DMSO, dimethyl-sulfoxide; V_{max}, maximal velocity; MOPS, 3-(N-morpholine-propane-sulfonic acid; Tris, (hydroxymethyl)-aminomethane; MCHC, mean corpuscular hemoglobin concentration; and FU, flux units, mmol/liter cell · hr.

lic calcium without a proton gradient stimulated a Na^+ influx inhibitable by amiloride [14]. Second, the imposition of an outward H^+ gradient in acid-loaded, DIDS-treated red cells markedly stimulated Na^+ influx partially blocked by amiloride [13]. Third, we recently investigated the kinetic properties and stoichiometry of Na/H EXC in red cells of normal subjects; it was established that the stoichiometric ration between net Na^+ influx driven by an outward H^+ gradient and H^+ efflux driven by an inward Na^+ gradient was 1 : 1, indicating that the antiporter was a fully coupled system [26]. Kinetic studies of Na/H EXC as a function of internal and external H^+ and Na^+ have also permitted us to establish the conditions to determine the apparent maximum rate (V_{max}) [26].

The present study was designed to investigate the expression of Na/H EXC in SS and AA red cells and the cell age dependence by determining the maximum rate (V_{max}) of Na^+ influx driven by an outward H^+ gradient. The results presented here indicate that antiporter activity is markedly increased in young SS and AA red cells, decays slowly after reticulocyte maturation, and exhibits large inter-individual differences.

II. Materials and Methods

A. Patient Material

Blood from patients followed in the Heredity Clinic at the Bronx Comprehensive Sickle Cell Center (Dr. H.H. Billett, Director) was obtained after informed consent and characterized as homozygous for hemoglobin S (SS) by two electrophoresis methods and a solubility test for hemoglobin HbS. Also studied were patients homozygous for hemoglobin A (AA) with high reticulocyte count secondary to the treatment of nutritional anemia.

B. Preparation of Red Cells

Red cells were studied the next day after preservation as previously reported [10]. Red cells were washed four times at 4°C with cold washing solution (CWS) containing 149 mM choline chloride, 1 mM $MgCl_2$, 10 mM Tris-MOPS, pH 7.4. A 50% cell suspension was made in CWS and hematocrit measured. A 50-μl sample of the cell suspension in CWS was lysed (1/50) with Acationox detergent (0.02% in double distilled water) for hemoglobin (Hb) (1/500 dilution) and Na^+ (1/50 dilution) determination. The initial MCHC was estimated by measuring the hematocrit and the optical density of Hb at 540 nm in a Perkin-Elmer Lambda 3 A spectrophotometer.

The Na^+ concentration of the cell lysate was measured with a Perkin-Elmer 3030 B atomic absorption spectrophotometer using appropriate standards. The cellular Na^+ content (nmol/liter cells) was determined using the hematocrit of the cell suspension.

1. Modification of Cellular Na^+ Content

A modified nystatin method was used to reduce cellular Na^+ as previously described [6]. One ml of washed packed red cells was slowly added, while mixing, to 5 ml of cold loading solution (NLS) containing 140 mM KCl, 50 mM sucrose, 40 μl of a nystatin solution (5 mg in 1.3 ml of DMSO) for a final concentration of 35–40 μg/ml. The nystatin powder was kept at 4°C and protected from light and DMSO was added the day of the experiment. The cell suspension was incubated in the dark at 4°C for 20 min with periodic vortexing. Subsequently, the cell suspension was centrifuged, the supernatant discarded, the loading solution (without nystatin) renewed and incubated for an additional 10 min in the cold. Nystatin was removed by adding 5 ml of warm (37°C) nystatin washing solution (NWS) and incubating for 5 min at 37°C; NWS contained the same K^+ concentration as the loading solution, 50 mM sucrose, 10 mM glucose, 1 mM potassium phosphate buffer (pH 7.40 at 37°C), albumin 1 mg/ml. Nystatin removal was completed by four washes with NWS.

2. Modification of the Cellular pH

The cellular pH of nystatin-treated cells was modified by incubating the cells at 10% Hct in acid-loading solution for 10 min in a shaking water bath at 37°C [26]. The acid-loading solution (ALS) contained 170 mM KCl, 40 mM sucrose, 0.1 mM ouabain, 10 mM glucose, 20 mM Tris-MES, pH 5.8. The hypertonic media (around 360 mOsm) avoided cell swelling induced by chloride redistribution in acid medium. In this way the mean increase in red cell volume after acid loading was not greater than 5% of the original cells. After incubation for 10 min the pH_i was clamped by the addition of 1.0 ml of ALS containing 20 μl of 200 mM DIDS and 10 μl of 0.85 M methazolamide in DMSO to inhibit the anion exchanger of these drugs were 0.2 mM DIDS (1.75 mg/ml RBC) and 0.5 mM methazolamide. The cell suspensions were incubated in their respective ALS for an additional 30 min at 37°C. The pH of the cell suspension was measured; if the desired pH was not obtained then the ALS was replaced with fresh solution. The acid-loaded cells (pH_i 5.9–6.9) were washed four times with five volumes of cold (4°C) nonbuffered washing solution (UWS) containing 170 mM KCl, 0.15 mM $MgCl_2$, 0.1 mM ouabain, 10 mM glucose, 40 mM sucrose, and kept cold until flux measurement. For cells with pH_i over 6.9, UWS was made isotonic by reducing KCl to 140 mM and eliminating sucrose.

The pH of the loaded cells was measured by lysing 0.2 ml of packed red cells in 2 ml of nonbuffered Acationox (0.02%) and determining the pH of the lysate with a Radiometer pH meter at room temperature. The cell pellet was kept on ice ready for flux measurements. An aliquot of the cell suspension (50% hematocrit) was used for the measurements of intracellular Na^+ and Hb as previously described.

C. Measurement of the V_{max} of Na^+/H^+ Exchange

The antiporter activity was determined by measuring net Na^+ influx into acid-loaded cells after imposing an outward H^+ gradient [5, 13, 26]. Na^+ influx measurements were started by the addition of packed acid-loaded, Na^+-depleted cells (final hematocrit 1%) to Na^+ media with pH_o 6.0 or 8.0 which had been preincubated at 37°C. The Na^+ media contained 150 mM NaCl, 150-0

mM choline-chloride, 10 mM glucose, 0.1 mM ouabain, 0.01 mM bumetanide, 0.5 mM methazolamide, 40 mM sucrose, 0.15 mM $MgCl_2$ and either 10 mM Tris-MOPS (pH 8 at 37°C) or 10 mM Tris-MES (pH 6 at 37°C). At 1, 5 and 11 min transport was stopped by transferring a duplicate aliquot (0.2 ml) of the warm cell suspension into previously chilled Eppendorf tubes (1.5 ml) containing 0.7 ml of cold Na-free solution layered over 0.4 ml of dibutyl-phthalate (d = 1.04). The Na^+-free solution diluted the Na^+ concentration of influx media and reduced trapping of Na^+ in the cell pellet; it contained 80 mM choline-Cl, 80 mM KCl, 0.15 mM $MgCl_2$, 40 mM sucrose, Tris-MOPS (pH 7.4 at 4°C). The tubes were immediately centrifuged in an Eppendorf centrifuge at 12,000 × g for 15 sec; a longer time makes the subsequent lysing step more difficult. The supernatant was removed by aspiration, the tubes wiped to remove Na^+ contamination and then placed in plastic racks previously washed with distilled water. The bottom of the tubes were cut into 3-ml plastic tubes containing 1 ml of 0.02% Acationox to lyse the cells. The tubes were stirred and kept overnight at 4°C. Hemoglobin concentration was measured by dilution (1/50) 50 μl of the cellular lysate. Na^+ trapping was determined using cell-free solutions and zero time samples and was less than 1.0 μM Na^+. The cellular Na^+ (mmol/liter cells) was computed according to the following equation:

$$\text{Cellular } Na^+ \text{ (mmol/liter cells)} = \frac{Na_l \cdot OD_{cs}}{Hct \cdot OD_l \cdot 1000}$$

where Na_l = Na^+ concentration of the lysate, μM; OD_l = optical density of Hb from the lysate of the flux media sample; OD_{cs} = optical density of Hb from the lysate of the fresh cellular suspension; Hct = hematocrit of the fresh cellular suspension.

Net Na^+ influx was calculated from the slope of the regression line of cellular Na^+ content *vs.* time and expressed in mmol/liter cell · hr.

D. Percoll-Stractan Density Gradients

Analytical and preparative isopycnic gradients were used as previously described [11, 16]. For preparation of density-defined classes of sickle cells [9], 0.5 ml of whole blood adjusted to hematocrit 50% (vol/vol) was added to 5.5 ml per tube of the gradient mixture of Percoll and Stractan prepared as described elsewhere [11]. The mixture was centrifuged and the cells aspirated by pipette from the formed gradient at previously established depths in the tube. Several tubes were prepared to obtain 0.5 to 1.0 ml of packed cells from each fraction. They were washed three times with isotonic saline and stored in autologous plasma on ice for shipping to Boston overnight. Density classes prepared by this technique are very sharply defined. This was demonstrated by centrifuging 50 μl of cells on an analytical gradient, which indicated that less than 5% of the cells exceeded the upper or lower density limits as described previously [16]. The reticulocyte count in the four density-defined fractions in SS blood has been reported previously [16].

E. Measurements of Volume-Sensitive Na^+/H^+ Exchange

Sodium-depleted, nystatin-treated red cells (1.5 ml) were incubated in 30 ml of loading solution containing 150 mM KCl, 10 mM Tris-MOPS, pH 7.4, 0.01 bumetanide, 0.1 mM ouabain; the cellular pH was clamped by adding DIDS and methazolamide as described in section B. Finally, the cells were washed three times with nonbuffered isotonic KCl. Net Na^+ influx was measured by incubating 0.1 ml of cells in 4 ml of three different Na^+ media containing: (*a*) 140 mM NaCl, 0.01 mM bumetanide, 0.1 mM ouabain, 0.5 mM neptazane, 20 mM Tris-MOPS, pH 7.4; (*b*) medium *a* + 100 mM sucrose and (*c*) medium *b* + 1.0 mM amiloride. Duplicate samples were taken at 0, 10 and 20 min and processed as described in section C. The net Na^+ influx was calculated per liter of fresh cells using the optical density of Hb at 540 nm of fresh cells to calculate the volume of cells in each cell lysate.

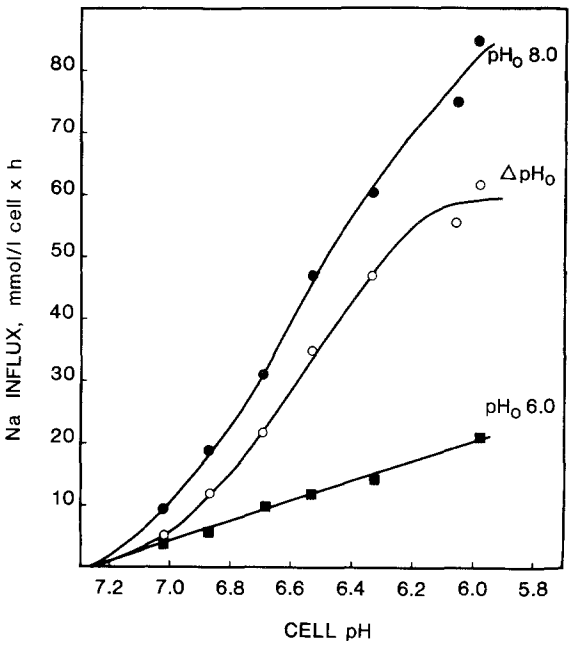

Fig. 1. Activation of Na^+/H^+ exchange in HbA red cells. The exchange activity (Δ pH_o) was estimated as net Na^+ influx driven by an outward H^+ gradient. Na^+ influx at pH_o 6.0 (■–■) was subtracted from values at pH_o 8.0 (○–○). Initial cellular Na^+ content was less the 1.0 mmol/liter cell. External Na^+ was 150 mM (*see* Materials and Methods for other details)

III. Results

A. Apparent Maximum Rate of Na^+/H^+ Exchange in Whole AA and SS Blood

The dependence of net Na^+ influx on cellular H^+ concentration was investigated in acid-loaded, DIDS-treated cells as previously described [13, 26]; the influx incubation media contained 150 mM NaCl at pH_o 8.0 or 6.0 (Fig. 1). The reduction of cellular pH (pH_i) caused a sigmoidal increase in net Na^+ influx from media with pH_o 8 (filled circles) which reached a maximum rate (V_{max}) of 58.0 mmol/liter cell · hr between pH_i 5.8 and 6.1 in this subject. In a previous study [26] we showed that red cell Na/H

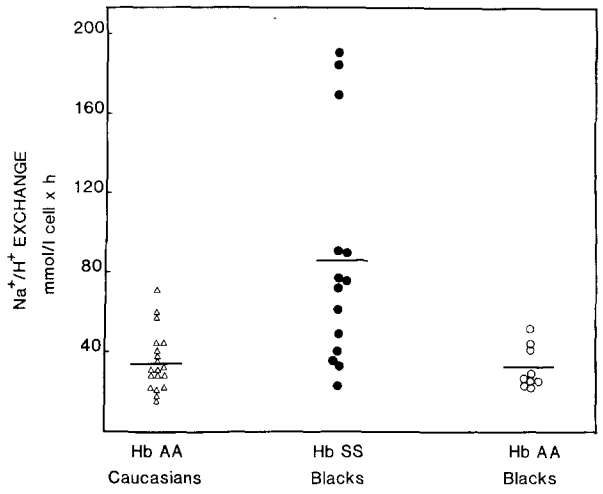

Fig. 2. Maximal rate of Na^+/H^+ exchange in whole blood of HbA and HbS subjects

exchange performs a 1 : 1 exchange of external Na^+ for external protons which is partially amiloride sensitive. For this reason, we chose to inhibit Na^+ influx stimulated by cellular acidification by increasing the external H^+ concentration. Thus when the acid-loaded cells were incubated in pH_o 6.0 Na^+ media (filled squares), Na^+ influx was significantly smaller and increased linearly with cellular acidification. The high value of Na^+ influx at $pH_i = pH_o$ also reflects changes in membrane permeability produced by covalent modification of the cell membrane by DIDS; at this pH_o, Na^+ influx was significantly lower in untreated cells (-DIDS) (7 mmol/liter cell · hr) than in DIDS-treated cells (25 mmol/liter cell · hr). Furthermore, acid pH_o inhibits Na/H EXC and therefore the subtraction of the Na^+ influx at acid pH_o provides a good determination of the passive movement of Na^+ induced by acid cellular pH and the new Cl_i/Cl_o distribution ratio. The activation of the antiporter can be accounted by the kinetic effects of H_i [13]. The difference between Na^+ influx in alkaline and acid media (ΔpH_o) represents the net Na^+ transport driven by an outward H^+ gradient ($H_i > H_o$) (i.e., Na^+/H^+ exchange). An external pH of 8.0 was chosen to maximize the outward H^+ gradient and reduce the inhibitory effects of H_o. The H^+-gradient driven Na^+ influx increased sigmoidally with increased H_i (Fig. 1, open circles) and reached V_{max} around pH_i 6.0. This is more likely to be an apparent V_{max} because the external Na^+ concentration was not saturating but a value less than three times the K_m (55 mm) [26]. However, for the sake of simplicity it will be named only V_{max}. The Hill plot of log v/V_{max}-v (Na^+ influx) *versus* log H_i was used to calculate the K_m of cellular H^+ required to activate Na/H EXC and the Hill coefficient (n_{app}). The K_m for H_i was 300 nM and n_{app} 2.8 which indicates the presence of strong cooperativity between H_i sites of the Na^+/H^+ exchanger.

Thus, to determine V_{max}, red cells (initial Na_i 0.4 mmol/liter cell) were acid loaded to pH_i 5.7 and incubated in 150 mM Na^+, pH_o 6.0, or pH 8.0 media. Intracellular Na^+ content increased linearly for 20 min at pH_o 6.0, but at pH_o 8.0 it was linear only for 10 min. The difference between net Na^+ influx at pH_o 8.0 and pH_o 6.0 represents the net Na^+ influx driven by an outward H^+ gradient (ΔpH_o). We previously showed [26] that ΔpH_o of Na^+ influx provided a better estimation of Na/H EXC than amiloride-sensitive Na^+ influx because it had a stoichiometric ratio of 1 : 1 with H^+ efflux driven by an inward Na^+ gradient while amiloride only partially (60%) inhibited Na^+ influx. From here on, 'Na/H EXC', will refer to V_{max}.

The within-assay reproducibility of Na/H EXC activity was determined by blinded measurements of duplicate samples from 10 subjects. The technical error was calculated according to the formula $(E^2/2N)$ where d is the difference in value between a split pair and N is the number of pairs. The absolute error was 3.3 mmol/liter cell · hr which is 12% of the mean value of Na/H EXC in this group (28.2 mmol/liter cell · hr). Measurement of the V_{max} of Na/H EXC was repeated one month apart in seven subjects. The mean values of both determinations were 40.0 ± 13.4 and 37.6 ± 13.4, respectively, and not significantly different.

Figure 2 depicts the V_{max} of Na/H EXC in red cells of 20 white AA subjects under the following conditions: Na_i 0.5–2.0 mmol/liter cell, Na_o 150 mM, pH_i 6, pH_o 8.0 and 6.0, 37°C. The mean value of the V_{max} of Na/H EXC was 33 ± 3 mmol/liter cell · hr but a large inter-individual variation was observed (6–63 mmol/liter cell · hr). No significant differences were observed between males and females in this study group. Also depicted in Fig. 2 are the Na/H EXC in red cells of 11 black AA individuals which are not significantly different from those observed in white AA individuals.

In 14 SS patients (all black), the mean V_{max} of Na/H EXC was 85.6 ± 15, double that in AA cells, a difference that was significant at the <0.001 level. A subset of three subjects have V_{max} over 160 mmol/liter cell · hr. The mean value of V_{max} of Na/H EXC excluding these three patients (59.3 ± 4.88, mean ± SE, $n = 11$) was still significantly higher ($P < 0.05$) than for normal subjects. Thus, the Na/H EXC in SS patients and in AA subjects exhibited considerable variation between individuals exemplified by a range between 23 and 183 mmol/liter cell · hr.

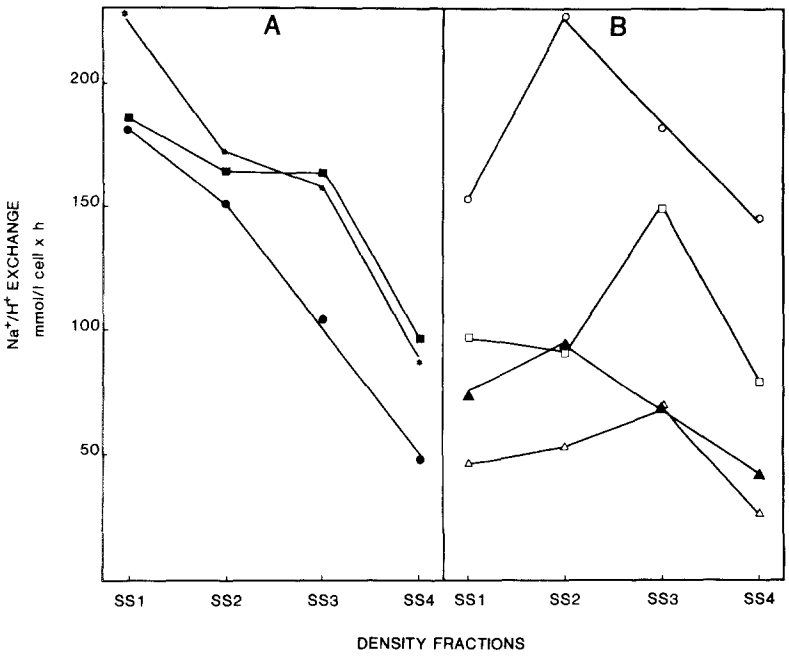

Fig. 3. Na^+/H^+ antiporter activity in density-defined fractions of seven subjects with SS cells. Values obtained in different subjects are indicated with different symbols. (*A*) Three subjects with maximum activity in SS1. (*B*) Four subjects with maximum activity in SS2 or SS3 in density-defined fractions

B. Na^+/H^+ Exchange Activity of Density-Defined Hb AA and SS Red Cells

We determined the apparent V_{max} of antiporter activity in density-fractionated cells from seven SS patients to assess the influence of cell age. The whole blood Na/H EXC activity in these seven subjects was 99.8 ± 64 mmol/liter cell · hr. SS whole blood was divided into SS1 (rich in reticulocytes), SS2 (discocytes), SS3 (dense discocytes) and SS4 (very dense discocytes and irreversibly sickled cells). Two types of cell density-dependent patterns were observed (Fig. 3*A* and *B*). In some patients (Fig. 3*A*) the antiporter activity was highest in SS1 and monotonically decreased with density; in another four patients the Na/H EXC activity was the highest in SS2 or SS3. The mean ± SD in mmol/liter cell · hr was not significantly different in fractions SS1, SS2 and SS3 (Table 1); however, in comparison to mature AA cells, Na/H EXC activity was 4–5 times higher in the reticulocytes, discocytes and dense discocyte fractions (SS1–SS3) but only twice as high in the fraction containing very dense discocytes and irreversible sickle cells (SS4).

Na/H EXC was also studied in density fractionated Hb AA red cells of three subjects with nutritional anemia and high reticulocyte counts (Fig. 4). The antiporter activity was highest in the fraction with highest proprotion of reticulocytes (Fig. 4 and Table 1).

Table 1. Maximal rate of Na^+/H^+ exchange in density gradient fractionated HbS and HbA red cells

	Apparent V_{max} Na^+/H^+ exchange activity (mmol/liter cel · hr)				
	SS1	SS2	SS3	SS4	WB*
HbSS, n = 7					
Mean	138.5	136.7	130.0	75.8	99.8
SE	24.9	22.5	17.8	15.1	24.0
	AA1	AA2	AA3		
HbAA, n = 3					
Mean	143.9	114.5	52.8		
SD	70.4	48.0	—		

* WB = whole blood. SS and AA whole blood had reticulocyte counts of 10 ± 2% and 8 ± 2%, respectively.
The apparent V_{max} of Na^+/H^+ exchange was determined measuring net Na^+ influx driven by a H^+ gradient ($\Delta(pH_o\ 8 - pH_o\ 6)$ in cells acidified to pH_i 6.0.

C. The Effect of Cell Shrinkage on Na^+/H^+ Exchange Activity of AA and SS Red Cells

Since Na/H EXC [4, 18, 20, 21] can be activated even at physiological pH_i by cell shrinkage which elicits the Na^+ and water gain and the increase in cell volume described as volume regulatory increase (VRI) response, we investigated this property in SS and AA red cells. The response of Na/H

Table 2. The effect of cell shrinkage on amiloride-sensitive Na^+ influx into SS and AA red cells

	Isosmotic	Na^+ influx[a] (mmol/liter cell · hr) Hyperosmotic		Volume-sensitive	
			amiloride	ΔV	Δamiloride
	(1)	(2)	(3)		
HbS 1	6.0	8.7	5.5	2.7	3.2
2	14.0	16.0	10.0	2.0	6.0
3	9.0	17.8	10.0	8.8	7.8
4	4.2	8.4	5.0	4.2	3.4
5	23.0	31.8	14.0	8.8	17.8
6	54.0	89.3	59.0	35.3	30.3
7	35.0	38.0	37.0	3.0	1.0
8	39.3	35.0	29.0	4.3	6.0
Mean ± SD	23 ± 18	30 ± 26	21 ± 19	8.6 ± 11	9 ± 9
HbA	9.8	11.0	12.0	1.2	−1.0
	7.8	11.0	9.5	3.2	1.5
	8.8	10.4	3.9	1.6	6.5
	11.0	14.0	10.5	3.0	3.5
	4.7	4.9	4.4	0.2	0.5
	9.0	9.0	7.4	—	1.6
	9.6	9.8	9.6	0.2	—
	12.0	16.0	14.0	4.0	2.0
	9.8	8.1	10.0	—	—
Mean ± SD	9.1 ± 2	10.4 ± 3	9.0 ± 3	1.4 ± 1.6	3.1 ± 3

[a] The volume-sensitive component of net Na^+ influx was calculated as the difference between hyperosmotic and isosmotic media; (Δ2 − 1) the amiloride-sensitive component was estimated as the difference between hypertonic media with and without amiloride. (Δ2 − 3)

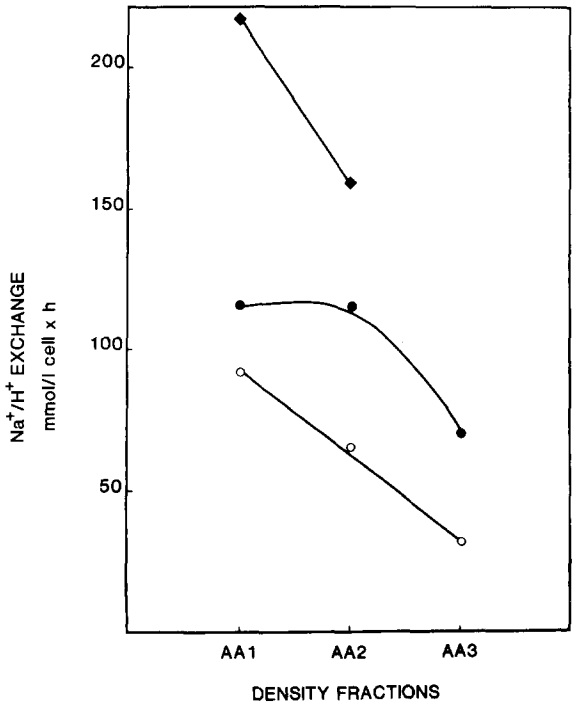

Fig. 4. Na^+/H^+ exchange activity in density-defined fractions of three subjects with HbA red cells and nutritional anemia. Reticulocyte counts 8 ± 2%

EXC to cell shrinkage (VRI) was determined by measuring net Na^+ influx into cells with pH_i 7.2 which were incubated in Na^+ media (140 mM NaCl, pH_o 7.4, 300 mOsm) or in Na^+ media + 100 mM sucrose (380 mOsm) with and without 1 mM amiloride (Table 2). Studies carried out in rat thymocytes have indicated that hyperosmolarity activates the transporter by decreasing the K_m for internal H^+ [19, 20].

The volume-sensitive component of net Na^+ influx was calculated as the difference between the fluxes in hyperosmotic and isosmotic media. The amiloride-sensitive component was the difference between the fluxes in hypertonic media without and with amiloride. The individual values for the effect of cell shrinkage on whole blood of SS and AA individuals are presented in Table 2. Several individuals showed a marked increase in Na^+ influx upon incubation in hypertonic media which was inhibited by amiloride, while others lacked the volume-sensitive, shrinkage-activated Na^+ influx. A statistically significant increase in volume-sensitive flux was observed in SS samples (44.8 ± 40.8%, $P < 0.017$, paired, normalized difference) but not in AA (13.5 ± 18.4%, $P < 0.06$). Amiloride inhibition under hypertonic conditions was statistically significant for

SS (58.3 ± 37.4%, $P < 0.031$, ($flux_{(hyp)} - flux_{(hyp+amil)}$) but not in AA (26.4 ± 54.9%, $P < 0.19$). The percent increase in flux in hypertonic media was larger for SS (44.8 ± 40.8%) than for AA (13.5 ± 18.4%), but this increase was not statistically significant ($P < 0.053$).

IV. Discussion

We have recently investigated the kinetic properties and stoichiometry of Na/H EXC in red cells of normal AA subjects and established that the stoichiometric ratio between net Na^+ influx driven by an outward H^+ gradient and H^+ efflux driven by an inward Na^+ gradient was 1 : 1 [26]. Because a large fraction of the red cell Na/H EXC was found to be amiloride insensitive [26] as previously reported by Grinstein et al. [18] in rat thymocytes, we measured proton gradient-stimulated Na^+ influx instead of amiloride-sensitive flux. On the basis of that study, we have selected the optimal conditions (pH_i 6, pH_o 8, Na_i 2 mM, Na_o 150 mM) to estimate the maximal rate of this antiporter in SS and young AA red cells. When pH_i is clamped at 6.0 with DIDS, a very high Na/H EXC activity can be demonstrated. The mean value of the apparent V_{max} (33.0 mmol/liter cell · hr) in mature AA red cells of white and blacks (Fig. 2) is 5–10 times higher than of the Na^+ pump (V_{max} : 5.0 mmol/liter cell · hr). This indicates that this antiporter is potentially the most important Na^+ transport system present in the membrane of human erythrocytes. We also report here that a considerably higher level of expression of Na/H EXC can be demonstrated in SS whole blood, in normal Hb A density-defined cell fractions which were enriched in reticulocytes, and in density-defined cell fractions in which young red cells predominate. In this last category fall density-defined fractions isolated from sickle cell anemia patients and Hb AA individuals with high reticulocyte counts, which exhibited a V_{max} of Na/H EXC 5 to 10 times higher than mature red cells. Therefore, the low levels of activity in normal whole blood (which usually has less than 0.5% reticulocytes) may reflect decreased activity in older red cells. The wide range of variation of the Na/H EXC previously observed [26] in normal red cells (range 6 to 80 mmol/liter cell · hr) may therefore reflect individual differences in gene expression and/or in the rate of decay of the antiporter with cell age. Although cellular age appears to be an important factor in the determination of Na/H EXC activity in human red cells, inter-individual variations were also observed in SS and AA fractions enriched in reticulocytes (Figs. 3 and 4). Notably the Na/H EXC activity of 200 mmol/liter cell · hr (=nmol/min · 10^6 cells) observed in cell fractions enriched in reticulocytes of some subjects is similar to that previously reported for dog [25], *Amphiuma* [4] and trout [3] red cells and rat thymocytes [17].

In three SS subjects with very high activity (200 nmol/liter cell · hr) in the SS1 fraction, there was a monotonic decrease of Na/H EXC as cell density increased (Fig. 3*A*). In four other SS subjects, the SS1 fraction had a lower activity (99.0 mmol/liter cell · hr) and the maximum activity (120 mmol/liter cell · hr) was observed in the discocyte or dense discocyte fraction (SS2 or SS3). In all cases, the Na/H EXC activity in the SS4 fraction was the lowest and at least a half of the maximum activity (Fig. 3*A* and *B* and Table 1). These results suggest that, in some SS individuals, the discocyte or dense discocyte fractions might contain some very young cells and that density-defined fraction may be heterogenous in cell age. This would indicate that density-dependent fractionation of SS red cells does not yield homogeneous cell populations of progressively increasing age because other factors may contribute to increased cell density.

This study also provides evidence that the cell age dependence of Na/H EXC activity is different from that of volume-stimulated K : Cl transport. We previously reported [7] that the activity of Cl^-- and volume-stimulated K^+ efflux was highest in the discocyte fraction (SS2), while it was absent from dense discocytes (SS3) and irreversibly sickled cells (SS4) (Fig. 5). A possible interpretation of these findings is that the K : Cl cotransporter disappears upon reticulocyte maturation; in contrast, Na/H EXC appears to persist long after the disappearance of the reticulum.

The high concentrations of DIDS used in these experiments were needed to block 99.9% of the large number of copies of protein band 3 present in the red cell (1×10^6). However, we have previously demonstrated [14] that DIDS is not necessary for stimulation of amiloride-sensitive Na^+ influx if the red cells are acidified by elevation of the fixed anion content (ATP and 2,3-DPG).

Our observations raise the question of the in vivo importance of this antiporter. The physiological role of Na/H EXC working in parallel with an active Cl^-/HCO_3^- exchanger has been well established in kidney epithelial cells [23], *Amphiuma* [4], dog [18] and trout red cells [3], but the issue has not yet been addressed in human red cells. When Na/H EXC operates in parallel with the anion exchanger, a net NaCl and hence water gain can be induced. Studies carried out by Duhm and Becker [12] have demonstrated inward movement of Na^+-driven Na^+-carbonate ion pairs in exchange for bicarbonate; these authors postulated that the Na^+-carbon-

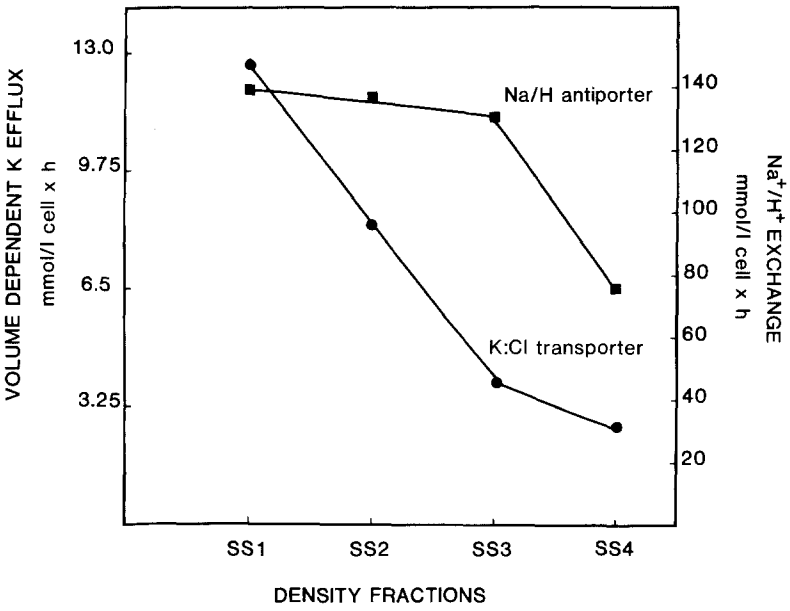

Fig. 5. Red cell density-dependence of the Na^+/H^+ exchanger and swelling-stimulated K : Cl cotransport in subjects with HbS [3]

ate ion pair could be involved in Na^+/H^+ countertransport, in H^+ secretion and in transport of chloride and bicarbonate [12]; further investigations should be performed to clarify the physiological role of this antiporter in circulating mature red cells.

It is worthwhile, nevertheless, to discuss some aspects of the possible behavior of the Na/H EXC in vivo. Because Na/H EXC interacts asymmetrically with internal and external H^+, a slight drop in pH_i will result in occupation of the H_i-regulatory site [2] and activate the system; in contrast, external acid pH will decrease the affinity of the external Na^+ sites (pK 7.4). However, several studies [21, 24] have shown that H_i can act as an allosteric activator and that this kinetic effect can override the effects of external H^+. Because the red cell is metabolically less active than the surrounding tissue, H^+ influx into the cells will be more rapid than red cell acid production, it is possible, therefore, that the Na/H EXC might play a role in vivo, when a small drop in intracellular pH occurs and an outward H^+ gradient is imposed as red cells approach the more alkaline pH of the pulmonary circulation. Wieth and Brahm [27] have calculated that in the resting state the capacity of the anion exchanger for transporting H^+ is very close to the limit of transit time (0.7 sec) of red cells in the pulmonary circulation. During physical exercise the situation is quite different because red cell transit time in the pulmonary circulation is reduced to 0.3 sec, which is too short to allow complete CO_2 equilibration. Wieth and Brahm, therefore, conclude that anion exchange becomes rate limiting for CO_2 transport when cardiac output is increased, a conclusion supported by two studies [27]. It is possible that under such circumstances, the cellular pH would become acid if an additional system, such as Na/H EXC, were not activated. On the other hand, because Na/H EXC has a different cellular pH dependence and an internal H^+ regulatory site, its activation may counteract the reduction of cell volume elicited by activation of K : Cl cotransport, producing a small Na^+ and water gain. Another potential circumstance in which this transport mechanism might be functional is during red cell transit through the renal medulla where high osmolarity is encountered and consequently red cell shrinkage ensues.

Several studies have established [19, 20] that the volume-sensitive Na/H EXC has a higher pK (pH 7.2) for activation than the acid-stimulated activity. For this reason we studied the effect of cell shrinkage on the Na^+/H^+ antiporter by measuring net Na^+ influx into cells at pH_i 7.2. The results show that a subset of sickle cell anemia patients exhibited a marked increase in Na^+ influx in hypertonic media, inhibitable by amiloride, demonstrating an active VRI response. As reported by Jennings et al. [21] in rabbit red cells, this particular component of Na/H EXC was fully amiloride-sensitive, in agreement with its ATP requirements [14].

If indeed the Na/H EXC is functional in vivo, it has the clear potential to modulate the expression of sickle cell anemia and deserves further study. Particularly because sickle cells are likely to spend longer times than normal cells under conditions of vascular stasis, low pH, and high osmolarity in different microcirculatory beds. The swelling response to shrinkage or acidification would have an anti-

sickling effect by lowering intracellular HbS concentration in those possessing sufficient activity of this antiporter.

We conclude that SS red cells have an increased Na/H EXC activity with significant inter-individual variation and a very different cell age dependence than the K : Cl cotransporter. In addition, the presence of an active VRI response due to this antiporter in a subset of SS patients, suggests that it has a potential role in modulating the tendency of SS cells to undergo sickling.

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References

1. Aronson, P.S. 1985. Kinetic properties of the plasma membrane Na-H exchanger. *Annu. Rev. Physiol.* **47:**545–560
2. Aronson, P.S., Nee, J., Suhm, M.A. 1982. Modifier role of internal H^+ in activating the Na^+-H^+ exchanger in renal microvillus membrane vesicles. *Nature (London)* **299:**161–163
3. Baroin, A.F., Garcia-Romeu, F., Lamarrer, T., Motais, R. 1984. A transient sodium-hydrogen exchange system induced by catecholamines in erythrocytes of rainbow trout, *Salmo gairdeneri*. *J. Physiol. (London)* **356:**21–31
4. Cala, P.M. 1980. Volume regulation by *Amphiuma* red cells. The membrane potential and its implications regarding the nature of the ion flux pathways. *J. Gen. Physiol.* **76:**683–708
5. Canessa, M. 1989. Kinetic properties of Na^+/H^+, Li^+/Na^+, Na^+/Na^+ and Na^+/Li^+ exchanges in human red cells. *Methods Enzymol.* **173:**176–191
6. Canessa, M., Brugnara, C., Cusi, D., Tosteson, D.C. 1986. Modes of operation and variable stoichiometry of the furosemide-sensitive Na and K fluxes in human red cells. *J. Gen. Physiol.*, **87:**113–142
7. Canessa, M., Fabry, M.E., Blumenfeld, N., Nagel, R.L. 1987. Volume-stimulated Cl^--dependent K^+ efflux is highly expressed in young human red cells containing normal hemoglobin or HbS. *J. Membrane Biol.* **97:**97–105
8. Canessa, M., Fabry, M.E., Nagel, R.L. 1987. Deoxygenation inhibits the volume-stimulated, Cl^--dependent K^+ efflux in SS and Young AA cells: A cytosolic Mg^{2+} modulation. *Blood* **70:**1861–1866
9. Canessa, M., Fabry, M., Spalvins, A., Blumenfeld, N., Nagel, R.L. 1987. Activation of a K : Cl transporter by cell swelling in Hb SS red cells. *In:* Pathophysiological Aspects of Sickle Cell Vaso-occlusion. R.L. Nagel, editor. pp. 201–215. Alan R. Liss, New York
10. Canessa, M., Spalvins, A., Nagel, R.L. 1986. Volume-dependent and NEM-stimulated K^+Cl^- transport is elevated in SS, SC and CC human red cell. *FEBS Lett.* **200:**197–202
11. Corash, L.M., Piomelli, S., Chen, H.C., Seaman, C., Gross, E. 1974. Separation of erythrocytes according to age on a simplified density gradient. *J. Lab. Clin. Med.* **84:**147–151
12. Duhm, J., Becker, B.F. 1978. Studies on Na^+-dependent Li^+ countertransport and bicarbonate-stimulated Li^+ transport in human red cells. *In:* Cell Membrane Receptors for Drugs and Hormones. A multi-disciplinary Approach. R.W. Straub and L. Bolis, editors. pp. 281–299. Raven, New York
13. Escobales, N., Canessa, M. 1986. Amiloride-sensitive Na^+ transport in human red cells. Evidence for Na/H exchange system. *J. Membrane Biol.* **90:**21–28
14. Escobales, N., Canessa, M. 1986. Ca^{2+}-activated Na^+ fluxes in human red cells: Amiloride sensitivity. *J. Biol. Chem.* **260:**11914–11923
15. Escobales, N., Rivera, A. 1987. Na^+ for H^+ exchange in rabbit erythrocytes. *J. Cell. Physiol.* **132:**73–80
16. Fabry, M.E., Mears, J.G., Patel, P., Schaefer-Rego, K., Carmichael, L.D., Martinez, G., Nagel, R.L. 1984. Dense cells in sickel cell anemia: The effects of gene interaction. *Blood* **64:**1042–1046
17. Grinstein, S., Cohen, S., Rothstein, A. 1984. Cytoplasmic pH regulation in thymic lymphocytes by an amiloride-sensitive Na^+/H^+ antiport. *J. Gen. Physiol.* **83:**341–369
18. Grinstein, S., Goetz, J.D., Rothstein, A. 1984. 22 Na fluxes in thymic lymphocytes. I. Na^+/Na^+ and Na^+/H^+ exchange through an amiloride insensitive pathway. *J. Gen. Physiol.* **84:**565–584
19. Grinstein, S., Rothstein, A. 1986. Mechanisms of regulation of the Na^+/H^+ exchanger. *J. Membrane Biol.* **90:**1–12
20. Grinstein, S., Rothstein, A., Cohen, S. 1985. Mechanism of osmotic activation of Na^+-H^+ exchange in the rat thymic lymphocytes. *J. Gen. Physiol.* **85:**765–787
21. Jennings, M.L., Douglas, S.M., McAndrew, P.E. II. 1986. Amiloride-sensitive sodium-hydrogen exchange in osmotically shrunken rabbit red blood cells: Possible role of an intracellular pK_a shift in transport activation. *Am. J. Physiol.* **251:**C32–C40
22. Mahnesmith, R.L., Aronson, P.S. 1985. The plasma membrane sodium/hydrogen exchange and its role in physiological and pathophysiological processes. *Circ. Res.* **56:**773–788
23. Malnic, G., Giebisch, G. 1979. Cellular aspects of renal tubular acidification. *In:* Membrane Transport in Biology. G. Giebisch, D.C. Tosteson, and H.H. Ussing, editors. Vol. IV, pp. 299–355. Springer-Verlag, New York
24. Parker, J.C. 1986. Interactions of lithium and protons with the sodium-proton exchanger of dog red blood cells. *J. Gen. Physiol.* **87:**189–200
25. Parker, J.C., Castranova, V. 1984. Volume-responsive sodium and proton movements in dog red blood cells. *J. Gen. Physiol.* **84:**379–401
26. Semplicini, A., Spalvins, A., Canessa, M. 1989. Kinetics and stoichiometry of human red cell Na^+/H^+ exchanger. *J. Membrane Biol.* **107:**219–228
27. Wieth, J.O., Brahm, J. 1985. Cellular anion transport. *In:* The Kidney: Physiology and Pathophysiology. D.W. Seldin and G. Giebisch, editor. Raven, New York
28. Wolff, D., Cecchi, X., Spalvins, A., Canessa, M. 1988. Charybdotoxin blocks with high affinity the Ca-activated K^+ channel of Hb A and Hb S red cells: Individual differences in the number of channels. *J. Membrane Biol.* **106:**243–252

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