Monovalent Cation Transport in Irreversibly Sickled Cells

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ABSTRACT Using discontinuous density gradients of Stractan II, we have separated sickle cell blood into discrete subpopulations of reticulocytes, mature discoid cells, and irreversibly sickled cells (ISCs). We have measured active and passive fluxes of monovalent cations in mature discoid cells, ISCs, and normal control cells, also separated upon density gradients. These measurements revealed a decreased active cation transport in ISC-rich populations. However, parallel measurements of Na, K-ATPase activity showed normal ouabain-sensitive ATPase activity in ISCs. Passive permeability to external Rb was also normal in ISCs.

The observation of depressed pump activity in intact ISCs, contrasted with normal ATPase activity in ISC membranes, suggests the presence of factors in the intact cell which inhibit the active transport of Na and K in ISCs.

INTRODUCTION

During attempts to understand how the primary molecular defect in sickle cell anemia becomes expressed at the clinical level, investigators have become increasingly interested in the irreversibly sickled cell (ISC).[1] This cell is defined morphologically by its failure to return to the biconcave disk shape in the presence of oxygen. Although it has not been possible to demonstrate a direct correlation between the proportion of ISCs in the blood of a given patient and the frequency or severity of painful crises (1, 2), evidence exists to support the idea that ISCs may be clinically important. First, Serjeant et al. (3) have shown a direct relationship between the percentage of circulating ISCs and shortened erythrocyte survival. Second, Hahn et al. (4) have found that ISCs become undeformable much more rapidly than discoid sickle cells upon deoxygenation. These authors suggest that the propensity of ISCs to become rigid in transit through areas of low oxygen tension might cause them to initiate episodes of capillary obstruction.

The rapid rate of hemoglobin S polymerization which induces the abrupt onset of indeformability in ISCs can be attributed to their abnormally high hemoglobin concentration. This, in turn, is thought to result from water loss accompanying K efflux in excess of Na influx during a prolonged period of deoxygenation (5). In fact, ISCs have been found to exhibit reduced levels of total cations and cell water (6). As Glader and coworkers have already emphasized, cellular dehydration may exert other effects upon the properties of ISCs. They have found that the morphological change which is characteristic of irreversible sickling (apparently a membrane abnormality not simply related to hemoglobin concentration) appears to require K loss and cellular dehydration (7). Furthermore, even with adequate oxygenation there appears to be an adverse effect upon ISC deformability simply from the increased viscosity of the highly concentrated hemoglobin inside the cell (8).

Because this phenomenon of cellular dehydration may be important in the genesis of ISCs and their clinical effects, we have investigated major aspects of cell water control in sickle cells. Erythrocyte water content is governed primarily by monovalent cation content. In turn, the steady-state concentrations of monovalent cations reflect a dynamic balance between passive leakage and active transport of the ions involved, as modulated by the initial ion concentration. The abnormal cation concentrations found in ISCs may represent an altered steady state resulting from one or more changes in the components of the leak and pump network. Therefore, we have measured

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[1] *Abbreviations used in this paper*: BSKG, buffered saline with potassium and glucose (7.808 g NaCl, 0.373 g KCl, 2.302 g $Na_2HPO_4.7H_2O$, 0.194 g $NaH_2PO_4.H_2O$, and 2.0 g glucose, made up to 1 liter, adjusted to pH 7.4, and 290–295 mosmol/kg if necessary); ISC, irreversibly sickled cell.

passive and active fluxes of monovalent cations in isolated populations of ISCs and mature discoid sickle cells to identify any components of the cation control mechanism which function abnormally. We have found that the passive influx of ^{86}Rb is normal in both ISCs and non-ISCs. However, permeability to internal K is abnormally elevated in both populations. Furthermore, although high internal Na concentrations might be expected to stimulate active transport in ISCs (9), these cells actually exhibit a Rb transport deficit of ≅40% from normal activity. This decreased pump activity in intact ISCs, contrasted with normal ATPase activity in ISC membranes, suggests the presence of factors in the intact ISC which inhibit the active transport of Na and K.

METHODS

Cell separation. Blood from 6 normal control subjects and 14 patients with sickle cell anemia was drawn into heparinized tubes. Leukocytes were removed by filtration of whole blood through cotton (10) and by repeated washing in buffered saline with potassium and glucose (BSKG) and aspiration of the buffy coat. Erythrocytes were resuspended to 20% hematocrit and layered on top of discontinuous gradients of Stractan II (St. Regis Paper Co., Tacoma, Wash.), prepared according to Corash et al. (11) with minor modifications (12). For separation of sickle cells, four layers of Stractan II, with densities of 1.115, 1.110, 1.101, and 1.096 g/ml, were placed upon a dense cushion of 1.144 g/ml to prevent packing of the cells against the bottom of the centrifuge tube. Normal control cells were separated analogously; densities of 1.101, 1.096, 1.092, and 1.083 g/ml were used on a cushion of 1.115 g/ml. The gradients were centrifuged at 4°C in a Beckman SW 27.1 swinging bucket rotor (Beckman Instruments, Inc. Fullerton, Calif.) at 20,000 rpm (52,000 *g* at tube center) for 30 min. Successive fractions, which had concentrated at the interfaces of the gradient, were collected with a Pasteur pipet and washed free of Stractan II by centrifuging three times from BSKG. Reticulocytes were counted in methylene blue-stained smears (13). ISCs were counted by phase-contrast microscopy of glutaraldehyde-fixed cells (3% glutaraldehyde in 0.05 M sodium phosphate, pH 7.4). Cells with a length:width ratio of at least 2:1 or with pronounced angular contours were designated ISCs.

ATPase assays. Erythrocyte membranes were prepared from the separated cells according to the method of Dodge et al. (14), with the following minor modification: instead of using sodium phosphate buffer for hemolysis and washing of the membranes, we used 20 mM Tris-HCl at pH 7.4 (23°C), with the addition of 1 mM EDTA.

The ATPase activity of the membrane preparations was defined as the rate of hydrolysis of adenosine 5′-[γ-^{32}P]-triphosphate (15). The assay system included 140 mM NaCl, 15 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 20 mM Tris-HCl buffer at pH 7.4 (23°C), and 50–120 μg membrane protein. Paired sets of samples were run with and without 0.1 mM ouabain for determination of the ouabain-sensitive portion of the ATPase activity. The reaction was initiated by the addition of 25 μl of 20 mM ATP (Sigma Chemical Co., St. Louis, Mo.), disodium salt, adjusted to pH 7.4, containing 25,000 cpm of [γ-^{32}P]ATP (16). Each reaction was run in triplicate in a final volume of 0.25 ml. After incubation at 37°C for 60 min, the reaction was terminated by the addition of an equal volume of ice-cold trichloroacetic acid solution (10 g/100 ml). Unreacted ATP was sequestered by the addition of 0.1 g of acid washed Norit A (American Norit Co., Inc., Jacksonville, Fla.) (17) suspended in 1 ml of 15 mM Na_2HPO_4 solution containing 1 g/100 ml bovine serum albumin (Sigma Chemical Co., fraction V). Subsequent centrifugation at 2,500 rpm for 15 min removed the charcoal-bound ATP. Radioactive inorganic phosphate released in the reaction was counted in aliquots of the supernate. Blanks to which membranes were not added were used to correct for phosphate contamination of the labeled ATP and for non-enzymatic hydrolysis. Total protein content of the erythrocyte membrane suspensions was estimated using the method of Lowry et al. (18). Residual hemoglobin in each membrane preparation was measured as the pyridine hemochromagen (19) and subtracted from the total protein to determine nonhemoglobin protein. For top, middle, and bottom fraction sickle cell ghosts, we found an average of 5.0, 5.8, and 6.7% of ghost-associated protein to be hemoglobin. ATPase activity was expressed as micromoles of inorganic phosphate per milligram of nonhemoglobin protein per hour.

Active and passive influx of ^{86}Rb. Influx assays were performed using cells from the bottom fractions of the gradient and from the second, rather than the top fraction. We chose second layer fractions to compare transport in ISCs to that in mature discoid cells. The top fractions, because of their high percentage of reticulocytes, would be expected to show abnormally high transport activity.

After separation and overnight storage in BSKG at 4°C, the cells were resuspended in BSKG and incubated at 37°C for 15 min. Influx of Rb ions was determined by measuring the intracellular accumulation of ^{86}Rb added to the cell suspension medium. Approximately 10^6 cpm ^{86}Rb (ICN Pharmaceuticals Inc., Life Sciences Group, Cleveland, Ohio, or New England Nuclear, Boston, Mass. $^{86}RbCl$ in 0.1 M HCl, neutralized with 0.1 M NaOH) were added to paired samples with and without 0.1 mM ouabain. Cells were incubated at ≅10% hematocrit in a final volume of 1 ml. At times corresponding to 0, 15, 30, 45, and 60 min after addition of the ^{86}Rb, triplicate aliquots of cell suspension were diluted with 10 ml of ice-cold isotonic $MgCl_2$ buffered with 10 mM Tris-HCl, pH 7.4 (Tris-$MgCl_2$). These samples were washed three times in Tris-$MgCl_2$, and then decolorized with 0.5 ml H_2O_2. 10 ml of water was added, and the Cerenkov radiation from the ^{86}Rb decay was counted in a liquid scintillation counter. Supernatant samples were also taken immediately after addition of ^{86}Rb for determination of the specific activity of the external medium. The rates of influx of ^{86}Rb into cells in the presence and absence of ouabain were calculated from linear regression analysis of the ^{86}Rb influx data. Influx in the presence of ouabain was considered to represent passive influx. The increased influx without ouabain was considered to correspond to active pumping. Volume measurements for the influx experiments were made by spinning samples of cell suspension in microhematocrit tubes. Corrections for differences in packing between ISC and discoid fractions were applied on the basis of a separate series of three experiments in which we measured trapped medium in the packed cell column using [^{14}C]inulin as an extracellular marker. Trapped medium for discoid samples was found to be 4.2% of packed cell volumes; for ISC-rich samples it was 9.6%. The trapped volume did not vary proportionally with the percentage of ISCs, probably because the non-ISCs in the bottom fractions are mostly spherocytes, which also resist packing. Cell counts were also obtained, using the Coulter model S electronic cell counter (Coulter Electronics Inc., Hialeah, Fla.).

In some experiments ^{86}Rb transport into Na-loaded cells

was studied. For these experiments, separated cells at 2% hematocrit were incubated for 40 h in potassium-free phosphate-buffered saline (10 mM sodium phosphate, pH 7.4) containing 10 mM glucose. To retard bacterial growth, 100 U of penicillin and 100 g streptomycin (PennStrep, Grand Island Biological Co., Grand Island, N. Y.) were added to each milliliter of suspension. The medium was changed twice during the incubation to maintain a low K concentration. Active and passive influx of ^{86}Rb was then measured as in fresh cells. In one experiment, sodium loading was accomplished by storing whole blood with acid citrate-dextrose anticoagulant at 4°C for 9 days. The cells were separated on gradients, and the flux measurements were performed on the same day. Samples of Na-loaded cells were washed three times in isotonic Tris-$MgCl_2$ for determination of intracellular cation concentrations by flame photometry.

Passive influx of Na. Passive influx of Na was measured by the same methods used for Rb influx, except that a ^{22}Na tracer was used. Because the external concentration of Na was higher than that of K, we used $5-10 \times 10^6$ cpm of ^{22}Na (New England Nuclear carrier-free $^{22}NaCl$ in H_2O). Because only passive influx of Na was being studied, all samples contained 0.1 mM ouabain to suppress active efflux of Na.

Passive efflux of K. Passive efflux of K was measured in freshly separated sickle cells which had been washed three times in ice-cold Tris-buffered $MgCl_2$ (290 mosmol, 10 mM Tris-HCl, pH 7.4 at 0°C). Washed cells were resuspended in Tris-$MgCl_2$ containing 0.1 mM ouabain at 5–10% hematocrit and brought to 37°C during a 10-min preincubation period. From this point, suspension samples were removed at 15-min intervals over a 1-h period and were centrifuged for 1 min in a Beckman microfuge (Beckman Instruments, Inc.) to remove the cells. Aliquots of supernate were then added to equal volumes of an LiCl solution for subsequent analysis of effluent K by flame photometry (IL 443 flame photometer, Instrumentation Laboratory, Inc., Lexington, Mass.). During the course of the incubation, samples were also taken for determination of intracellular K and Na, and hematocrits were measured. To obtain a first-order rate constant for K efflux into the medium, we calculated the intracellular K concentrations for each time point from the total suspension K concentrations and the supernatant concentration for each time. A linear regression treatment of the log of the intracellular K concentrations vs. time yielded first-order rate constants which were independent of the internal K concentration (20). Corrections were not made for differences in packing of the various cell populations during hematocrit measurement. A test inclusion of this correction in a representative experiment showed it to have a negligible effect on the result; the rate constant for an ISC-rich sample relative to the control was increased by <2%.

Measurements of ATP. Erythrocyte ATP was measured in Tris-borate extracts of washed cell suspensions using the luciferin-luciferase assay system (21). ATP was expressed in units of micromoles ATP/per milliliter cells. This is a second-best alternative to expressing ATP on the basis of its concentration in cell water, because ISCs contain less cell water than discoid sickle and normal cells (6). However, because of limited sample, measurements of cell water were not feasible in these experiments. A sample correction for this effect, using mean corpuscular hemoglobin concentration differences to estimate the degree of dehydration, indicated that ATP in ISCs would be ≅10% higher relative to control values on a cell water basis than on a cell volume basis.

Statistical methods. Statistical evaluation was performed according to the methods described by Colton (22). Independent samples *t* tests were carried out using a Hewlett-Packard Co., Avondale, Pa., HP-55 calculator and program (23).

RESULTS

Measurements of ouabain-sensitive ATPase activity in membranes from normal controls and separated sickle cells revealed no abnormality associated with ISCs. The data summarized in Table I show an elevation of total ATPase activity and the ouabain-sensitive component in fractions from the top layers of the sickle cell gradients. However, both total and ouabain-sensitive ATPase activities of the ISC-rich bottom fractions were not significantly different from the activities

TABLE I
ATPase Activities in Membranes from Separated Sickle Cells

Population	Reticulocytes	ISC	*n*	Total ATPase*	Ouabain-sensitive ATPase*
	%	%			
Sickle cells					
Top fractions	31 (12–65)	5 (4–7)	9	1.03 (0.09)	0.52 (0.07)
Middle fractions	18 (3–37)	17 (2–44)	6	0.89 (0.11)	0.48 (0.08)
Bottom fractions	4 (2–5)	61 (40–76)	5	0.50 (0.02)	0.27 (0.06)
Unseparated normal controls	<0.5	—	11	0.51 (0.04)	0.30 (0.02)

Membranes were prepared from control cells and from sickle cells separated into three fractions using Stractan II gradients. ATPase activities were estimated by measuring the hydrolysis of [γ-^{32}P]ATP at 37°C catalyzed by the membrane preparations. Total ATPase activity was measured in the absence of ouabain; ouabain-sensitive ATPase activity is the difference in activity in the absence and presence of 0.1 mM ouabain.

* Micromoles of inorganic phosphate per milligram of nonhemoglobin protein per hour (SD).

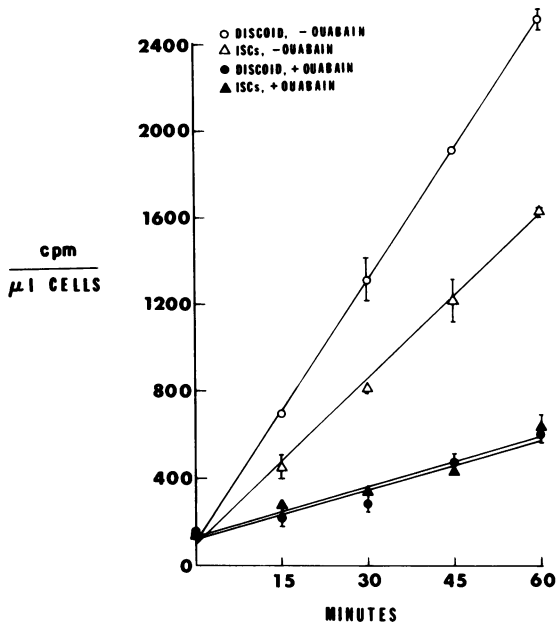

FIGURE 1 ^{86}Rb influx into separated sickle cells. Separated sickle cells were incubated at 37°C in phosphate-buffered saline containing 5 mM K and ^{86}Rb in tracer quantities. The lower lines connecting the solid symbols represent the influx of ^{86}Rb into cells in the presence of 0.1 mM ouabain; open symbols designate samples incubated without ouabain. The circles represent samples of mature discoid cells from the second fraction of the gradient; triangles represent the ISC-rich bottom fraction.

found for normal membranes. Because of the large quantities of cells required for this assay, we could not employ a pure mature discoid sickle cell reference population. We presume that they would have exhibited normal ATPase activities, and that the high levels of activity in the top and middle fractions were due to reticulocyte contamination (24, 25).

In contrast to their normal ATPase activity, active cation transport in ISC-rich fractions was abnormally reduced. Fig. 1 illustrates this reduction in a representative experiment. The bottom layer used here contained 77% ISCs and 2% reticulocytes; the second layer contained mature discoid cells with only 6% ISCs and 3% reticulocytes. The difference in transport was associated with the presence of ISCs in the bottom layer, not with decreasing numbers of reticulocytes. Data from similar experiments are summarized in Table II. Active transport in ISC-rich fractions was nearly 40% below that in discoid sickle or normal cells. Comparisons of active and passive fluxes of second and bottom fraction sickle and normal cells were made using a two-tailed t test for independent samples (22). Only for active transport in the ISC-rich fractions was a significant difference found between sickle and normal cells ($P < 0.02$).

Actually, the usual expression of ion transport in units of ions per liter of cells per hour is not entirely appropriate in these circumstances where ISCs have decreased cell water and volume. Experiments in our laboratory have indicated that ISC-rich bottom fractions contain approximately the same amount of lipid as mature discoid cells from the same patient (26). Thus the transport activity per cell number should be proportional to the activity in terms of cell surface area. Therefore, in Table III we have compared the ^{86}Rb influx data for second and bottom fraction cells on the basis of cell number as well as cell volume. As expected, the deficit in transport by ISCs is somewhat greater when expressed in ions per 10^{12} cells per hour, than when expressed in ions per liter of cells per hour.

Although our previous studies had indicated that the ATP concentration in ISC-rich samples was not significantly depleted with respect to mature discoid sickle or normal cells (26, 27), we needed to ascertain that the overnight storage of separated sickle cells had not resulted in preferential loss of ATP from ISCs. Table IV summarizes data from an experiment in which ATP samples were taken immediately after cell separation and also after overnight storage at 4°C in BSKG, immediately before the Rb transport measurements were made. There was a modest increase in ATP concentration in fractions 2 and 5, presumably an effect of the high phosphate concentration in BSKG. Fractions 3 and 4, not used for the transport measurements, showed comparable increases in ATP. Fraction 1, which contained 18% reticulocytes, showed a 5% drop in ATP concentration, possibly because of the higher metabolic activity associated with younger cells. The absence of appreciable ATP loss from mature sickle cells during overnight storage indicates that cation transport in ISCs was not limited by ATP.

Because Mg-ATP is the actual substrate used for transport, Mg loss during overnight storage should also be considered as a source of pump inhibition. We have not measured Mg in ISCs, either before or after storage at 4°C in BSKG. However, we have measured changes in Na and K concentrations during overnight refrigeration, and the changes in ISCs did not exceed 15% of the total cation concentrations. Because membrane permeability to divalent cations is much lower than to Na and K, we do not think it likely that Mg depletion was the cause of decreased Rb transport in ISCs. However, on the basis of present information, this possibility cannot be totally discounted.

Because the abnormally increased levels of Na in ISCs (6) would be expected to result in stimulation of the active transport of Rb (9), we also measured Rb transport in sickle cells which were purposely loaded with Na. The data summarized in Table V show considerably higher rates of active Rb influx into Na-loaded sickle cells than into cells not subjected to this treatment (Table II). A comparison of transport activity in

TABLE II

^{86}Rb Influx into Separated Cells

Population	Reticulocytes	ISC	n	Rb influx	
				Active*	Passive*
	%	%			
Sickle cells					
Second fraction	6 (3–13)	6 (2–10)	6	0.274 (0.056)	0.066 (0.020)
Bottom fraction	2 (0–4)	62 (43–77)	6	0.174 (0.060)	0.080 (0.026)
Normal cells					
Second fraction	3 (1–9)	—	5	0.274 (0.048)	0.070 (0.030)
Bottom fraction	0 (0–1)	—	5	0.240 (0.028)	0.138 (0.042)

After separation into 4 to 5 fractions on discontinuous gradients of Stractan II, cells were incubated in buffered solutions containing 5 mM KCl and an ^{86}Rb tracer. Passive influx was defined as the influx in the presence of 0.1 mM ouabain. Active influx was defined as the difference in influx in the absence and presence of ouabain.

* (Counts per minute ^{86}Rb per liter of cells)/(counts per minute ^{86}Rb per liter of medium) per hour (SD).

the two sets of experiments indicates an average increase of 78% in second fractions which have been Na loaded. The bottom fractions showed an increase of 60% over untreated samples with Na loading.

Measurements of ATP concentrations after Na loading for 40 h in experiment 2 (Table V) gave values of 1.04 and 0.88 μmol ATP/ml cells for second and bottom fractions, respectively. These values were comparable to those obtained for fresh normal and sickle cell samples. Thus, ATP depletion during Na loading would not appear to be a problem in these experiments.

The passive influx of Rb was also increased in Na-loaded cells. The increase was more variable than that in active transport, but on the average, the second-layer fractions appeared more susceptible to an increase in ouabain-insensitive Rb influx than the bottom-layer fractions.

We measured the passive influx of Na into separated sickle and normal cells. Data from these experiments are shown in Table VI. The variation in Na influx was greater than that obtained in the corresponding Rb experiments. Because of the variability within each sample group, these data do not reveal any significant differences in Na permeability between the various groups. However, neither do they preclude the presence of a moderate increase in Na permeability for sickle cells.

Finally, we measured the passive efflux of K from freshly separated sickle cells. Fig. 2 illustrates the results from one of these experiments. In this figure we

TABLE III

Rb Influx Ratios for Bottom and Second Fraction Cells

	n	Influx ratio	
		Cell volume basis*	Cell number basis‡
Active influx			
sickle cells	4	0.65 (0.09)	0.58 (0.07)
control cells	4	0.89 (0.13)	0.82 (0.12)
Passive influx			
sickle cells	4	1.16 (0.15)	1.04 (0.14)
control cells	4	1.81 (0.24)	1.66 (0.26)

^{86}Rb influx into bottom and second fractions are compared with expression of fluxes on a cell volume basis vs. a cell number basis. The slight decrease in influx ratios on a cell number basis reflects the decreased mean cell volume in bottom fraction cells.

* (^{86}Rb Influx per liter of cells per hour – bottom fraction)/(^{86}Rb Influx per liter of cells per hour – second fraction)(SD).

‡(^{86}Rb Influx per 10^{12} cells per hour – bottom fraction)/(^{86}Rb Influx per 10^{12} cells per hour – second fraction)(SD).

TABLE IV

Effect of Overnight Storage in BSKG on ATP Concentrations of Separated Sickle Cells

	ATP			
	Before 4°C storage*	After 4°C storage*	Change in ATP	Ouabain-sensitive K influx‡
			%	
Fraction 2	0.97 (±0.03)	1.07 (±0.03)	+10.3	1.35
Fraction 5	0.84 (±0.01)	0.97 (±0.07)	+15.5	0.80
Fresh normal controls	1.01	—	—	—

Duplicate ATP extracts were prepared immediately after separation of cells and again just before initiation of transport measurements the following day.

* Micromoles ATP per milliliter of cells (range).

‡ Milliequivalents per liter of cells per hour.

TABLE V
^{86}Rb Influx into Na-Loaded Sickle Cells

Population	Reticulocytes	ISC	Internal Na*	Internal K*	Rb influx‡	
					Active	Passive
	%	%	*meq/liter cells*			
Experiment 1§						
Second fraction	9	49	78	26	0.454	0.116
Bottom fraction	6	82	99	6	0.286	0.116
Experiment 2‖						
Second fraction	16	6	37	32	0.468	0.178
Bottom fraction	4	80	66	5	0.290	0.130
Experiment 3¶						
Second fraction	22	12	28	49	0.548	0.156
Bottom fraction	3	78	33	21	0.208	0.088

Influx assays were performed as for Table II.

* Precision of multiple determinations was ±3 meq/liter cells.

‡ (Counts per minute ^{86}Rb per liter of cells)/(counts per minute ^{86}Rb per liter of medium) per hour.

§ Freshly drawn cells were separated on gradients, then incubated in K-free buffer at 37°C for 40 h.

‖ Whole blood drawn into acid citrate dextrose was kept at 4°C for 2 days, then separated and Na loaded at 37°C for 40 h.

¶ Same blood as Experiment 2, but kept in acid citrate dextrose at 4°C for 9 days, separated, and influx assay performed immediately.

have plotted the decrease in the natural log of the intracellular K concentration from its initial value as a function of time. Data from five such experiments are summarized in Table VII. In all experiments efflux rate constants for sickle cell samples were at least twice those obtained for normal cells. Top fraction sickle cells gave the highest rate constants, and frequently showed some curvature in a ln K vs. t plot, as can be seen in Fig. 2. Such curvature was absent in plots of data from middle and bottom sickle cell fractions and from all normal cell samples, including top, middle, and bottom fractions in an experiment in which normal cells were also separated on a gradient. ISC-rich fractions were not significantly more permeable in these experiments than mature discoid sickle cells. A two-tailed *t* test for independent samples yielded *P* values of 0.001 and 0.01 for comparison of the control with middle and bottom sickle fractions, respectively. The small difference in the means of middle and bottom fractions was not statistically significant.

TABLE VI
Passive Influx of Na into Separated Cells

Population	Reticulocytes	ISCs	*n*	Na influx*
	%	%		
Sickle cells				
Second fraction	7 (2–13)	3 (1–3)	3	2.84 (1.02)
Bottom fraction	2 (0–4)	50 (43–55)	3	3.14 (1.43)
Normal cells				
Second fraction	2 (1–7)	—	4	1.80 (0.65)
Bottom fraction	0 (0–1)	—	4	2.55 (1.11)

After separation on gradients, cells were incubated in buffered solutions containing 152 meq/liter Na and a ^{22}Na tracer for Na movements. The system also contained 0.1 mM ouabain to suppress active efflux.

* Milliequivalents per liter of cells per hour (SD).

DISCUSSION

For these transport studies we have used Stractan II gradients to separate heterogeneous sickle cells into discrete subpopulations of reticulocytes, mature discoid cells, and ISCs. In previous studies which employed unseparated sickle cells, a definition of specific cation flux characteristics of the various subpopulations was not possible. The gradient separation provides us with mature cell populations, whose pumping activity is not elevated by reticulocyte contamination (28, 29). This has allowed us to identify a decrease in the active monovalent cation transport in ISCs. Because Rb has been shown to be a good analogue for K in erythrocyte-cation transport (30), we conclude that this defect reflects a corresponding decrease in the ability of ISCs to transport K. In addition, we observed increased passive permeability to internal K from freshly separated sickle cells. In contrast to the pumping defect, this abnormality was not limited to ISCs, but was present in all sickle cell populations.

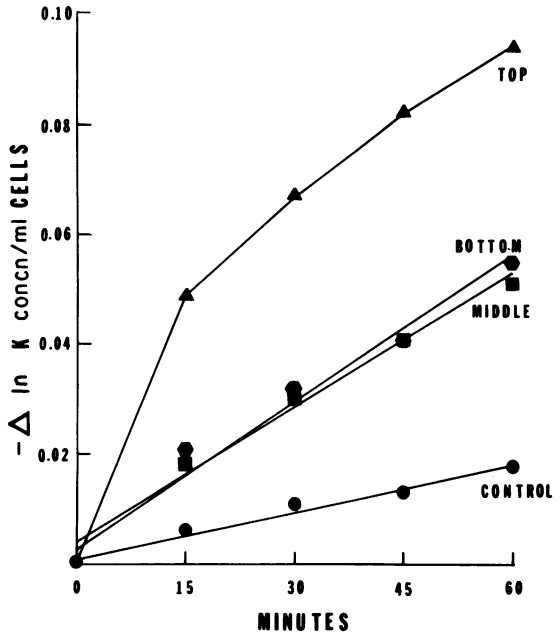

FIGURE 2 Passive K efflux from separated sickle cells. Immediately after separation on gradients, top, middle, and bottom fraction sickle cells were washed in Tris-$MgCl_2$, as were an unseparated control. Samples were incubated in Tris-$MgCl_2$ at 37°C, and effluent K in the suspension medium measured over a 1-h period. Data are plotted as the negative ln of the calculated intracellular K concentrations as a function of time. The first-order rate constant for K efflux is taken as the slope of this plot for each sample. The actual ion flux for a particular internal K concentration equals rate constant × concentration. Initial K concentrations were: Top—73.6, meq/liter cells; middle—72.9, meq/liter cells; bottom—22.8, meq/liter cells; and control—98.3 meq/liter cells.

In a previous report of increased active transport of Na and K in unseparated sickle cells, the authors suggested that this increase represented a compensatory response to increased passive permeability (31). We ascribe the abnormally high transport rates observed by those authors to the dominating effect of reticulocytes (28, 29). The passive permeability measurements from the same study are not directly comparable to ours because they were performed in isotonic sucrose solutions at low ionic strength. Loss of chloride under these conditions is accompanied by an increase in OH^- concentration inside the cell, leading to increased cation permeability (32). Thus we cannot say whether the substantial elevation of K efflux under those conditions bears any relationship to the abnormal K efflux which we observed.

By what possible mechanisms can reduced cation transport in ISCs be explained? Because metabolic depletion has been implicated in the generation of ISCs, a shortage of ATP to drive the cation pump should be considered. However, experiments in our laboratory have shown that ISCs contain sufficient ATP to make this an unlikely explanation for the pump deficit (26, 27). Our present results also dispell the possibility that reduced transport in ISCs results from artifactual ATP depletion during storage at 4°C.

Another reasonable possibility is that excess Ca may play some role. Ca is a potent inhibitor of active Na, K transport (33), and it also induces a specific permeability increase for internal K in normal cells (34). Several authors have reported elevations of Ca in sickle cells, particularly in ISCs (35, 36). Literature data concerning the effect of Ca upon Na transport (31) and passive K loss (37) in intact cells indicate that the passive permeability of the membrane to K is more sensitive to slight increases of intracellular Ca than is the Na, K pump. The quantitative data are thus consistent with the possibility that both the increased passive permeability we observed in all sickle cells and the reduced active transport found in ISCs could be the result of slight to moderate increases in Ca content in the various sickle cell populations. Our observations of normal ouabain-sensitive ATPase activity in ISC membranes, which were prepared and assayed in the presence of Ca-chelating agents, is also consistent with this hypothesis.

In this study of the monovalent cation flux properties of ISCs, we have found evidence for decreased active transport of K and Na. Several authors have reported instances of reduced Na, K pump activity in other disorders (38, 39), either as an absolute decrease in the rate of ion transport or as a rate of transport which was inappropriately low for the observed internal Na concentrations. However, the extent of pump suppression which we have observed in ISCs is considerably more severe than in these previous studies. There are other disorders in which erythrocytes have been shown to be dehydrated (38, 40), but in each instance there appears to be at least some absolute increase in Na,

TABLE VII
Passive Influx of K from Separated Sickle Cells

	n	$-k_o$*
		per h (SD)
Sickle cells		
Top	5	(0.058 [0.019])
Middle	5	0.037 (0.007)
Bottom	5	0.044 (0.005)
Unseparated		
Normal controls	5	0.014 (0.003)

* This is a first-order rate constant obtained from the linear regression analysis of the log of K concentrations remaining in the cells as a function of time. Top fraction value is bracketed because the ln K vs. t plots tended to show some curvature. Nevertheless, the lowest r value for these data was 0.94. For all middle, bottom, and control samples, r values were 0.985 or greater.

K pumping activity. ISCs present the hitherto unobserved combination of decreased cell water and decreased active transport of the cations which govern cell water content.

We cannot define the precise influence of reduced pump activity upon the cell water content of ISCs, because at this point it is difficult to quantitatively separate the effects of passive flux and active transport upon the total cation and water content of erythrocytes. It may be that the initial ion concentrations after reoxygenation dominate the subsequent fate of the cell, regardless of pump activity. As Glader and Nathan (41) have argued, a fixed stoichiometry of the active transport system may confer upon the pump an inability to counter a net loss of cations through increased activity. Under normal circumstances, an ≅50% excess of passive Na influx over passive K efflux is counterbalanced by an active transport ratio of three Na ions expelled for every two K ions pumped into the cell. If the passive fluxes are altered so that pump-independent K efflux exceeds pump-independent Na influx, increased pump activity with fixed stoichiometry cannot reverse the net efflux of total cations. It might even be possible that accelerated active transport would augment the cation loss. This possibility is supported by observations of Glader et al. (40), which showed that in vitro cation loss from desiccytes was curtailed by the addition of ouabain. To explore these relationships further, it would be useful to establish model systems which might allow definition of the critical regulatory parameters for cell water regulation.

The decreased capacity of ISCs to transport monovalent cations, which we have documented here, constitutes one more example of membrane defects induced during prolonged sickling. Defective ion transport, because of its possible influence upon cell water, and consequently the rheological behavior of ISCs, may play a significant role in this disease.

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