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# Membrane transport of Na and K and cell dehydration in sickle erythrocytes

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*Abstract.* The cellular concentration of Hb S plays a central role in the kinetic of Hb S polymerization and cell sickling. Blood of patients with homozygous sickle cell (SS) anemia contains a variable fraction of cells which are markedly dehydrated and have increased Hb S concentration. Since a decrease in cellular Hb S concentration reduces Hb S polymerization and sickling, the study of the processes leading to sickle cell dehydration has important pathophysiological and therapeutic implications. Sickle cell dehydration is due to cellular loss of K and Cl. K loss in sickle cells can take place via either the $Ca^{2+}$-activated $K^+$ channel, or the K-Cl cotransport, or the combined effect of oxidative damage and deformation of the red cell membrane. Inhibitors of K transport through these pathways could be used to prevent dehydration of sickle cells in vivo, provided that they can be administered safely.

*Key words.* Cation transport; volume regulation; sickle cell; K:Cl cotransport; potassium efflux.

Blood of patients with sickle cell anemia is characterized by the marked heterogeneity in the distribution of erythrocyte densities[36]. Cells with low density and increased cation content reflect the increased number of reticulocytes and young cells. Cells of higher density are the expression of cell dehydration, one of the distinctive features of sickle cell anemia. Cell dehydration is due to a marked decrease in the K content of the red cells, as indicated in the table. The loss of cell K, Cl and water leads to a marked increase in the cellular concentration of hemoglobin S. This increase in cellular Hb S concentration has profound implications for the pathogenesis of SS disease, given the marked dependence of the kinetic of hemoglobin S polymerization on Hb S concentration[30]. Small increases in Hb S concentration will lead to a disproportionate reduction in the delay time for hemoglobin S polymerization and increased cell sickling. Therefore, considerable attention has been devoted to the cellular processes leading to sickle cell dehydration. The principal transport pathways for cations and anions of the human red cells are presented in figure 1. There is considerable interest in the study of their role in sickle cell dehydration, because this could represent a possible point of pharmacologic intervention for patients with sickle cell anemia. Any maneuver leading to decreased cellular concentration of Hb S would be associated with decreased Hb S polymerization and sickling. The theoretical arguments about the beneficial effect of decreasing hemoglobin concentration and MCHC have been confirmed in a limited clinical trial where cell swelling was induced by hyponatremia[93]. We will examine the principal abnormalities in Na and K transport described in SS cells and how they may contribute to dehydration of SS red cells.

Composition and characteristics of normal AA and density separated SS erythrocytes

| Cell composition | AA control (mmol/kg hemoglobin) | SS top | SS bottom |
|---|---|---|---|
| Cell $Na^+$ | 28.5 ± 4.2 | 37.3 ± 16.9 | 84.2 ± 23 |
| Cell $K^+$ | 296.7 ± 6.7 | 325.3 ± 15.4 | 78.1 ± 13.2 |
| Cell $Na^+$ and $K^+$ | 325.2 ± 8.5 | 362.7 ± 3.0 | 161.4 ± 9.0 |
| MCHC (g/dl) | 33.0 ± 1.1 | 32.4 ± 0.5 | 45.6 ± 2.3 |

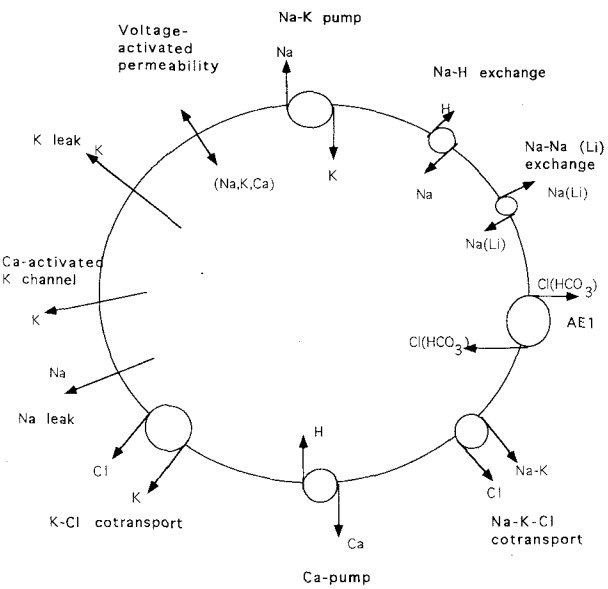



Figure 1. RBC cation and anion pathways in human erthrocytes.

## *Mechanisms leading to K loss and dehydration of sickle cells*

Two gradient driven systems for K transport are the major determinants of K loss in sickle cells, the K-Cl

cotransport and the $Ca^{2+}$-activated $K^+$ channel. However, two other mechanisms, the deoxygenation-induced Na and K fluxes and the effects of oxidation and/or deformation deserved attention for their possible role in promoting sickle cell dehydration.

*K-Cl cotransport*

This gradient-driven system couples the movement of K and Cl. Due to the high K content of the human red cells, net extrusion of K and Cl takes place via this system. K-Cl cotransport was described in sheep, dog and avian red cells (reviewed by Lauf et al.[65] and subsequently in cells of patients homozygous for hemoglobin C[10] and in sickle erythrocytes[11–16,19–21,37]. K-Cl cotransport is present in the reticulocyte-rich fraction of normal human red cells and is non active in mature cells[12,20,44]. The high activity of K-Cl cotransport in young normal red cells can also be used to isolate preparations enriched in reticulocytes[97]. Distinguishing features of this system are the *volume-dependence*, with activation by cell swelling and subsequent loss of K, Cl and water with volume regulatory decrease, *pH-dependence*, with optimum pH around 6.7–7.2 and inhibition at more acid or alkaline pH, *chloride-dependence* ($Br > Cl > I > NO_3 = SCN$) and *stimulation* by NEM (N-ethylmaleimide, a sulphydryl binding reagent). The system is not inhibited by 10 μM bumetanide (concentration sufficient to completely inhibit a related system, the Na-K-Cl cotransport) and is partially inhibited by high concentrations (1 mM) of another loop diuretic, furosemide[65]. K-Cl cotransport is inhibited by metabolic depletion and fluoride[87].

Detailed kinetic studies of K transport through the K-Cl cotransport in normal human red cells have provided evidence for a functional asymmetry of the system, with the transporter being prevalently in the outward facing conformation[61].

Rabbit erythrocytes have been used as an experimental model to study activation of K-Cl cotransport by cell swelling[2]. Volume changes rather than shape changes are involved in the turning on/off K-Cl cotransport in rabbit erythrocytes[51]. A phosphatase/kinase dephosphorylation/phosphorylation cycle mediates the response of K-Cl cotransport to volume changes[50]. The activation of K-Cl cotransport by cell swelling is markedly inhibited by the protein phosphatase (PP) inhibitor okadaic acid[52,63] or by a more specific inhibitor of PP-1 (calyculin a[98]). According to the model proposed by Jennings[50,52], the phosphorylated state of the carrier is probably the inactive form and dephosphorylation leads to activation of transport.

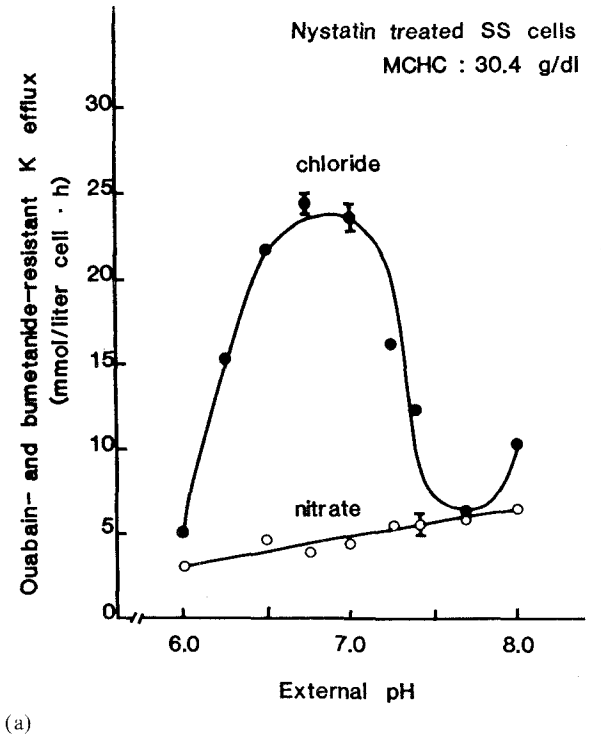
The K-Cl cotransport is inhibited by [(dihydroindenyl)oxy]-alkanoic acid (DIOA[39]). DIOA also inhibits the red cells anion exchange and cannot therefore be considered an absolutely specific inhibitor of K-Cl cotransport[39].

The coupling of K and Cl via K-Cl cotransport has been demonstrated by the fact that K can be transported against its concentration gradient down the chemical gradient of Cl, at constant membrane potential[14].

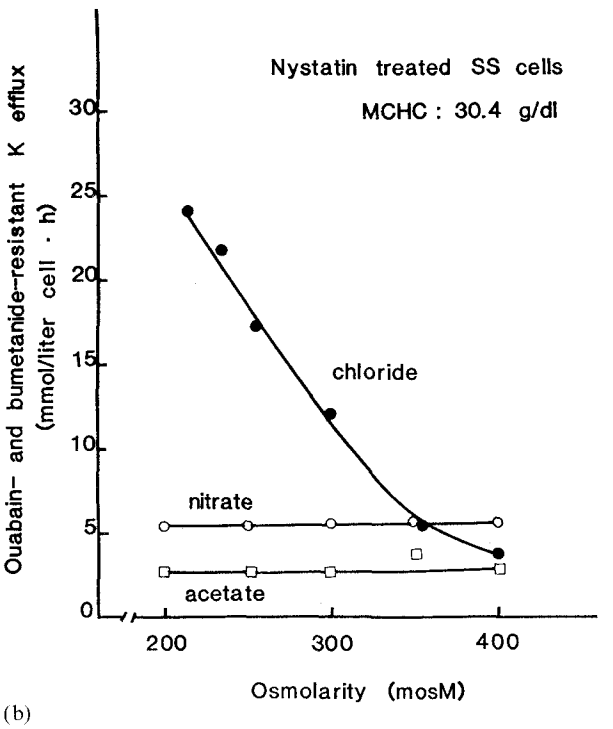
There have been multiple reports on the elevation of K-Cl cotransport in SS erythrocytes[11–16,19–21,37]. K-Cl cotransport activation leads to cell dehydration when SS cells are isosmotically swollen or are exposed to acid pH[16]. DIOA reduces K loss in SS cells[105]. K movement through the K-Cl cotransport of SS cells is inhibited when the intracellular $Mg^{2+}$ concentration is increased[13,21]. Figure 2A and B present data obtained in SS cells which demonstrate the chloride-dependence of the K-Cl cotransport, since K efflux through the system is inhibited when nitrate or acetate replace chloride. Figure 3A presents the dependence on chloride concentration of the K-Cl cotransport of SS cells, while figure 3B demonstrates that the K-Cl cotransport of SS cells is not affected by changes in membrane potential, as would be expected for an electroneutral K-Cl cotransport. All these data confirm a non-electrogenic and gradient-driven coupled 1K:1Cl transport via K-Cl cotransport in SS cells.

Since deoxygenation is associated with an increase in free cytoplasmic $Mg^{2+}$, K-Cl cotransport should be diminished in deoxygenated SS cells. However, $K^+$ efflux in deoxygenated SS cells is still partially DIOA-sensitive and this fraction is markedly increased at pH 7.0[85]. The K-Cl cotransport system is highly expressed in reticulocyte rich fractions of SS cells, and is highest in the fractions containing less Hb F[37]. K-Cl cotransport is lower in F cells probably because either these are more mature, older cells or the metabolic degradation/inactivation of the K-Cl cotransport proceeds faster in cells with relatively high Hb F content.

Increased activity of K-Cl cotransport is observed not only in SS cells, but also in CC, AC, AS and SC cells[15,86]. The presence of hemoglobin C induces significant changes in membrane transport and density of reticulocytes when associated with Hb S in SC disease. K-Cl cotransport is markedly increased in SC cells[19] and SC cells appear to be microcytic, hyperchromic, and severely dehydrated[5]. Reticulocytes in SC disease are found mostly in the densest fraction and their density can be decreased by maneuvers which inhibit K-Cl cotransport[67]. Recent studies have indicated that positively charged hemoglobin variants do not show an increased K-Cl cotransport unless the mutation is in the $\beta 6$ or $\beta 7$[86]. Since these Hb variants present only a moderate decrease in red cell life span and modest reticulocytosis, the increased K-Cl cotransport cannot be due simply to the presence of young cells. Possible mechanisms for these effects of hemoglobin on K-Cl cotransport are a reduction in the metabolic degradation of the system which normally takes place with red

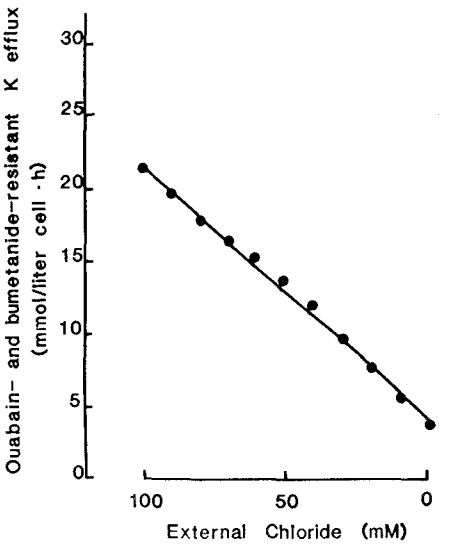



(a)




(b)

Figure 2. (a) The effect of external pH on the ouabain- and bumetanide-resistant K efflux from SS cells into NaCl and $NaNO_3$ media. (b) The effect of osmolarity on the ouabain- and bumetanide-resistant K efflux from SS cells into NaCl, $NaNO_3$ and Na acetate media. SS cells were treated with nystatin to obtain homogeneous distributions of hemoglobin concentration.

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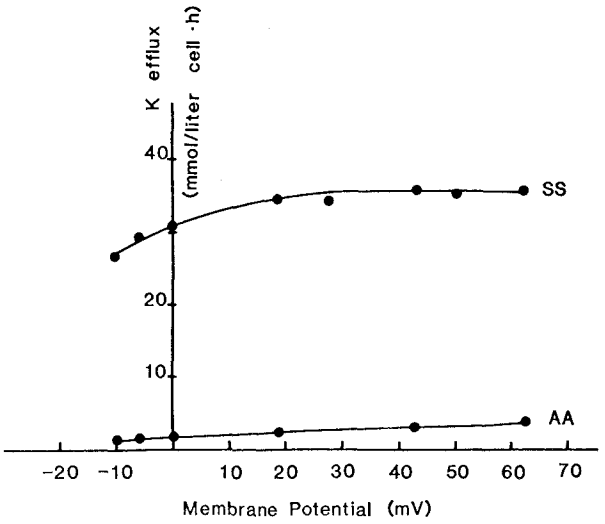


(b)

Figure 3. (a) Dependence on external chloride of the K-Cl cotransport of sickle cells. (b) The effect of membrane potential on the K-Cl cotransport of SS cells. SS cells were treated with nystatin to obtain homogeneous distributions of hemoglobin concentration.

cell maturation or an abnormal volume/pH regulatory cycle, possibly at the phosphatase/kinase level. The cytosolic protein concentration is the primary volume signal for the K-Cl cotransport of dog red cells[28]. Whether this mechanism could be different in cells containing positively charged Hb has not been established yet.

As in AA cells, the K-Cl cotransport of SS cells is inhibited by okadaic acid, indicating that PP-1 and probably a still undefined kinase are involved in the activation/inactivation of this transport system[88]. Whether this regulatory loop is altered by the presence of Hb S is not clear at this time. The lag time preceding activation of K transport by cell swelling and inhibition by cell shrinkage has been used to study the regulation of the K-Cl cotransport[50,52,63,98]. Preliminary data from our laboratory suggest that in SS cells the lag time for activation by hypotonic swelling is shorter and the lag

time for inactivation by cell shrinkage is prolonged compared to normal AA cells. Thus, the increased activity in SS cells could be attributed to differences in the regulation of the system.

Another important determinant of the activity of K-Cl cotransport is the oxidative damage of SS cells. There is ample evidence suggesting that K-Cl cotransport is markedly stimulated by a metabolite of dapsone[43], by acethyl-phenylhydrazine[17], and by a combination of NEM and oxidation[96]. Dithyothreitol (DTT) treatment of SS cells induces a significant reduction (20–30%) in the rate of $K^+$ transport via K-Cl cotransport. There are convincing data on the oxidative membrane damage present in SS cells[46]. Thus, another possible determinant of the increased activity of the K-Cl cotransport system in SS cells is the oxidative damage of the erthrocyte membrane.

*$Ca^{2+}$-activated $K^+$ channel*

Our understanding of the structure and function of voltage-gated $K^+$ channels has increased at a rapid rate[75], while much less is known about the structure of $Ca^{2+}$-activated $K^+$ channels. These channels have been divided into different subtypes according to the magnitude of their unitary conductances and sensitivity to insect toxins of the apamin and charybdotoxin (ChTX) families[66]. The $Ca^{2+}$-activated $K^+$ channel of red cells is also called the 'Gardos' pathway from the name of the Hungarian scientist who first reported the effect of $Ca^{2+}$ on $K^+$ permeability[40]. The Gardos pathway of red cells has been extensively characterized by patch clamp analysis[23,42,45] and as many as three types of unitary conductance (10–40 pS) have been described[23].

The $Ca^{2+}$-activated $K^+$ transport of normal human red cells containing hemoglobin A[18,107] and of sickle erythrocytes[84,107] has been found sensitive to inhibition by ChTX, a 37 amino acid toxin initially isolated from Leiurus venom. $Ca^{2+}$-activated $K^+$ transport is inhibited by ChTX with an $IC_{50}$ of $21 \pm 15$ pM in low ionic strength medium and $4.2 \pm 2.4$ nM in normal saline[18]. The channel is activated by micromolar concentrations of $Ca^{2+}$ ($K_{50} = 0.3$-2 μM) and is regulated by cAMP probably via phosphorylation[92]. A cytoplasmic protein (calpromotin) seems to play a role in promoting $K^+$ transport via the Gardos channel[77]. Binding studies with $^{125}$I-ChTX have indicated that normal AA red cells have $105 \pm 17$ binding sites per cell ($K_d = 75 \pm 19$ pM[18]). This estimate of the number of channels per red cell is similar to the values obtained in inside-out vesicles (100–200[3,70]) and is significantly different from the estimate of 1–3 channels per cell obtained in AA cells[107].

The $Ca^{2+}$-activated $K^+$ channel of human red cells is inhibited by the $Ca^{2+}$-channel blockers nitrendipine ($IC_{50} = 130$ nM[31]), and nifedipine ($IC_{50} = 31 \pm 2$ μM[62]). While no mammalian $Ca^{2+}$-activated $K^+$ channels have been cloned so far, there is preliminary evidence of expression of this kind of channel from messages injected into oocytes[72]. A $Ca^{2+}$-activated $K^+$ channel has been expressed in Xenopous oocytes by injection of RNA obtained from cDNAs complementary to the *slo* locus of *Drosphila melanogaster*, which expresses an abnormal $K^+$ current lacking $Ca^{2+}$-dependence[1].

The Gardos pathway of sickle cells is heterogeneous, as indicated by our studies using charybdotoxin (ChTX) and $^{125}$I-ChTX as specific ligands for this system. Binding studies indicate that specific $^{125}$I-ChTX binding varies greatly among SS patients, from 1.45 to 15 fmol/$10^7$ cells (corresponding to 85–900 channels per cell compared to 60-120 channels per cell in AA control). Studies in density separated SS cells showed that cells from the least dense, top fraction have an increased number of binding sites but lower affinity for $^{125}$I-ChTX ($K_d = 105$–155 pM) whereas dense cells have fewer channels with higher affinity for $^{125}$I-ChTX ($K_d = 14$–22 pM). Thus, there are functional differences in the $Ca^{2+}$-activated $K^+$ channel between least dense and dense SS cells. The significance of these differences is not clear at the present time. Whether the differences in channel number are just a reflection of the presence of young cells and whether they correlate with propensity to dense cell formation has not been established yet.

Earlier work showed that generation of irreversibly sickled cells (ISC) under conditions of ATP depletion was dependent on the presence of external $Ca^{2+}$[53]. The possible involvement of the Gardos effect was advocated by Glader and Nathan in 1978[41]. However, they also showed that deoxygenation in the absence of ATP depletion did not affect the total red cell cation content. Experiments performed in SCN media, where the Cl permeability is not a rate limiting factor for $K^+$ transport, indicated that a $Ca^{2+}$-dependent $K^+$ loss takes place during deoxygenation and is associated with formation of dense cells[8]. However, these results did not allow to evaluate the role of the Gardos channel in SS cell dehydration under physiological conditions. Recent studies in SS reticulocytes have provided more convincing evidence for a role of the Gardos pathway in dehydration of SS cells[9]. Addition of external $Ca^{2+}$ reduced the magnitude of the deoxygenated Na and K fluxes. These fluxes are balanced in the absence of external $Ca^{2+}$ and, when $Ca^{2+}$ is added, a net $K^+$ loss can be described, which is inhibited by quinine, a low affinity inhibitor of the Gardos channel[9].

A $Ca^{2+}$-activated $K^+$ transport has also been described in mouse erythrocytes and in a transgenic mouse expressing human alfa and β-S-chains. Deoxygenation in mouse cells containing Hb S leads to $K^+$ loss, which is inhibited by ChTX, and to formation of dense cells[91].

*Effects of oxidation and shear stress*

Oxidative damage of the red cell membrane induced by oxygen free radicals increases K permeability[74,108]. Measurement of lipid peroxidation products in fresh SS cells indicates accumulation of thiobarbituric acid reactive substances (TBARS) and increased membrane lipid hydroperoxide (LOOH) similar to those measured in normal red cells after exposure to mild peroxidation[99]. Oxidative damage may render the sickle cell membrane more susceptible to mechanical stress and to conditions where the membrane is deformed. Marked mechanical deformation of AA cells produces a balanced $K^+$ loss and $Na^+$ gain in the absence of external $Ca^{2+}$[54]. When $Ca^{2+}$ is added to the external medium, there is a net $K^+$ loss, which seems to take place via the $Ca^{2+}$-activated $K^+$ channel[55].

Mild peroxidative damage has been shown to induce a deformation-induced leak pathway[47,80,100,101]. This leak pathway is reversible, independent from $Ca^{2+}$ and/or Cl, and leads to balanced movements of $Na^+$ and $K^+$ ($K^+$ loss = $Na^+$ gain). When medium pH is decreased below 7, and the cell exposed to shear stress, there is a net $K^+$ loss, which is not due to K-Cl cotransport[99]. When the osmolarity is decreased, in the presence of shear stress, there is also a net $K^+$ loss, which is inhibited by di-isothiocyanodisulfonyl stilbene (DIDS) (75%) and bromide (50%). Since the deoxygenation induced $K^+$ loss is also reduced by bromide[100], it is a possibility that deformation of mildly peroxidated normal red cells in hypotonic medium induces a leak pathway that mimics the increased $Na^+$ and $K^+$ leaks observed during deoxygenation. The unbalanced K loss induced by mild oxidative damage, and the effects of oxidation on K-Cl contransport and deformation induced-leak suggest that oxidative damage is an important determinant of cell dehydration in SS disease.

*Effect of deoxygenation and sickling on $Na^+$ and $K^+$ transport*

In 1952 Tosteson et al.[102] showed that deoxygenation of SS cells was associated with $K^+$ loss and $Na^+$ gain. Subsequently, Tosteson et al.[104] demonstrated that this increased permeability took place through a diffusional pathway. The deoxygenation-induced fluxes in SS cells are different from those mediated by any known transport system because they are not inhibited by ouabain, furosemide, or replacement of Cl with $NO_3$ or $SCN$[6]. One important determinant of the deoxygenation induced $Na^+$ and $K^+$ movements is the morphologic deformation of the red cell induced by Hb S polymerization and sickling[76]. Conditions leading to marked morphology changes are associated with the largest increase in $Na^+$ and $K^+$ permeability, suggesting that deformation induced by the polymer spicule and deoxy fluxes are somehow associated. Recent evidence suggests that formation of Hb S spicules during sickling leads to uncoupling of the lipid bilayer from the red cell cytoskeleton[71]. This site of uncoupling could be involved in the deoxygenation-induced fluxes. The deoxygenation-induced fluxes are balanced in the absence of external $Ca^{2+}$. Their contribution to sickle cell dehydration is not direct, but can take place via the associated stimulation of the Na-K pump (see below). Addition of $Ca^{2+}$ results in a net $K^+$ loss with dehydration, which can be inhibited by antagonists of the $Ca^{2+}$-activated $K^+$ channel[8,9].

The characterization of the deoxygenation-induced fluxes has been carried out mostly in the absence of external $Ca^{2+}$. Roth et al.[94] demonstrated that the deoxygenation-induced $K^+$ efflux was pH dependent, with pH optimum around 7.6. The deoxygenation induced $Na^+$ and $K^+$ fluxes have been subsequently studied in great detail by Joiner[56,57]. His work shows that these fluxes are balanced and pH dependent, with a pH optimum around 7.4–7.5 and inhibition at pH values $<7$ and $>8$. The deoxy $Na^+$ and $K^+$ fluxes are activated when $O_2$ drops below 40–50 torr and are not chloride dependent. The deoxy-fluxes can be irreversibly inhibited by DIDS, with an $ID_{50}$ which is almost tenfold higher than to the $ID_{50}$ for inhibition of sulfate influx via the anion exchange protein (AE1). Use of DIDS inhibits the deoxy $Na^+$ and $K^+$ fluxes without affecting morphologic changes induced by deoxygenation. The effect of DIDS on the increased $Na^+$, $K^+$ and $Cl^-$ permeability induced by deoxygenation has been independently confirmed[26]. The deoxy-fluxes are not associated with increased permeability to erythrol, arabinose or mannitol, and therefore they seem to be limited to alkali metal cations[26]. The deoxygenation induced pathway is not selective for Li, Na, K, Rb or Cs, is linearly dependent on the concentration of Na or K, and is affected by changes in membrane potential[59]. All these properties are suggestive of ion movement via diffusion with, however, a restriction to monovalent or divalent cations.

*Generation of irreversibly sickled cells (ISC)*

ISC are defined as cells retaining a distorted shape even after the blood is equilibrated with 100% oxygen. The majority of these cells are markedly dehydrated and the persistence of the distorted shape can be attributed to the high concentration of Hb S, which leads to the presence of polymers even in the presence of 100% $O_2$[30]. Since the abnormal morphology persists in many ISC after they are lysed and hemoglobin S removed, an abnormal interaction among cytoskeletal proteins is also present in ISC[73]. Measurements of lateral and rotational mobility indicate that band 3 and glycophorin are irreversibly aggregated in dense sickle cells[29].

When SS cells are separated by density centrifugation, ISC are found mostly but not exclusively in the bottom densest fractions. However, dense fractions do not contain exclusively ISC. ISC have a markedly reduced $K^+$ content, increased $Na^+$ content and normal or slightly reduced ATP (Clark et al.[27] and table). Cell pH of ISC is reduced compared with normal AA and top SS cells[64], while the 2,3-DPG content is lower than in least dense SS cells and similar to AA controls[95]. Cell Hb F content is lower in ISC as compared with least dense SS cells[7].

Many studies have attempted to replicate in vitro the processes leading to the formation of dehydrated ISC. Much of the published work has focused on the role of the Gardos pathway in cell dehydration. Demonstration of the involvement of this pathway has relied on the effect of removal of external $Ca^{2+}$ or on the use of various inhibitors. However, the experimental conditions used are various, do not reproduce the phenomena occurring in vivo and are highly dependent on the methods used to impose deoxygenation of SS cells. The majority of experiments have been conducted in whole SS blood, rather than in the more relevant least dense, reticulocyte rich fraction. Most of the studies have not taken into account that the rate of deoxygenation is an important determinant of the morphologic deformation induced by sickling[4]. Slow deoxygenation leads to formation of classic sickled red cells, while fast deoxygenation leads to formation of mostly irregularly shaped, deformed cells. The results published so far can be summarized as follows:

a) *Cell dehydration and ISC formation are not observed during fast deoxygenation of whole SS blood in the absence of external $Ca^{2+}$.* Various studies employing relatively fast deoxygenation protocols[6,41,56,57] have demonstrated marked changes in $Na^+$ and $K^+$ content, with no changes in water and total cation contents. Only when the anion Cl was substituted by SCN, and the $K^+$ flux through the Gardos channel was not limited by the Cl permeability, was there a formation of dense cells upon deoxygenation which was $Ca^{2+}$-dependent[8].

b) *Formation of ISC and cell dehydration can be induced by continuous slow deoxygenation of whole SS blood in a $Ca^{2+}$-dependent manner.* When a slow progressive deoxygenation protocol is used (24 h), morphologic deformation of SS cells is maximized, and $Ca^{2+}$-dependent formation of dense cells can be demonstrated[49].

c) *Cyclic oxygenation deoxygenation leads to $Ca^{2+}$-dependent production of ISC.* Work by Ohnishi et al.[81-84] has indicated that ISC can be generated after exposure to deoxygenation-oxygenation cycles. This process is $Ca^{2+}$-dependent, inhibited by external $K^+$, and by various inhibitors of the $Ca^{2+}$-activated $K^+$ channel[81-84]. Other studies have indicated that 80% of light SS cells become denser over a 4-h incubation time when exposed to oxy-deoxy cycles (4 min and 30 s 95% $N_2$, and 30 s 95% air[48]). This process required the presence of external $Ca^{2+}$. When SS cells were exposed to deoxygenation cycles of 15 min, over 15 h, initial studies in two patients showing $Ca^{2+}$-independent formation of ISCs[79] were not confirmed in a larger set of patients which showed a marked $Ca^{2+}$-dependence of this process[78].

d) *Short term deoxygenation of SS reticulocytes is associated with $Ca^{2+}$-dependent dehydration.* Experiments in SS reticulocytes have shown that 30 min of deoxygenation in the presence of $Ca^{2+}$ induces cell dehydration, which can be blocked by quinine, a low affinity inhibitor of the Gardos pathway[9].

e) *Dehydration induced by cyclic deoxygenation of whole blood is DIOA-sensitive.* When the incubation pH is lower than 7.40 (7.20), exposure of SS cells to 30 min of deoxygenation leads to unbalanced K loss, which can be inhibited by DIOA[85]. This indicates that the K-Cl cotransport can be a mediator of dehydration during deoxygenation.

A comprehensive model which integrates the various transport pathways of the red cell membrane and other cellular variables, has been proposed by Lew and Bookchin as a way of examining possible mechanisms leading to dehydration of SS cells[38,68]. This integrated red cell model takes into account most of the variables affecting the human red cells and is therefore a thorough and rather comprehensive approach to defining the relative role of the various transport pathways, of cell volume and cell pH in determining ion content and volume of the human red cells. The various experiments and theories on generation of ISC and dehydration of SS cells have been tested with the aid of the integrated red cell model[69]. Two pathways seem to play a major role in formation of dense cells: the $Ca^{2+}$-activated $K^+$ channel and the K-Cl cotransport. The transient increase in free $Ca^{2+}$ induced by deoxygenation would lead to activation of the Gardos pathway, with loss of K, Cl, water and cytoplasmic acidification which in turn would activate the K-Cl cotransport with further K loss and dehydration.

The Na-K pump may also play a role in cell dehydration. Earlier work in normal human red cells has indicated that activation of the pump in cells with high $Na^+$ content leads to cell dehydration[24]. Work by Joiner and co-workers in SS cells and hereditary xerocytosis has provided further support to this hypothesis[60]. The deoxygenation fluxes would provide the bases for the increased cell $Na^+$ with subsequent dehydration due to the 3 Na/2 K stoichiometry of the Na-K pump, and net loss of $Na^+$. However, studies with the integrated red cell model indicate that this mechanism can account for only a small fraction of the dehydration of SS cells, since the reduction of $K^+$ content of SS erythrocytes far exceeds the observed increase in cell $Na^+$[68].

*Determinants of the increased cell $Na^+$ of dense sickle cells*

In the pump-leak model of Tosteson and Hoffman[103], the concept of active pumps working in parallel with diffusional leaks when the cell is at steady state was established. The model was demonstrated in LK and HK sheep red cells, where major differences are present in cation composition, pump, and leaks activities. In this model, the active extrusion of $Na^+$ and the inward transport of K via the Na-K pump balance the inward leak of Na and the outward leak for $K^+$. When the model was proposed, knowledge of the transport pathways was limited to the Na-K pump. All the other pathways were therefore considered as leak pathways. While more complex models can now be used to examine the steady state conditions of red cells, Tosteson-Hoffman's model can still be used to analyze the determinants of the high cell $Na^+$ of ISCs.

High cell $Na^+$ can be the result of: 1) a reduction in the activity of the Na-K pump, due to either a reduction in the number of pumps or an abnormality of a constant number of pumps; 2) an increased $Na^+$ leak; 3) both. All these possibilities are applicable to ISCs:

a) *Alterations in the Na-K-pump of Na-K ATPase.* This enzyme consists of two subunits, the $\alpha$-subunit with catalytic properties and the $\beta$-subunit, involved in the interaction with the red cell membrane and cytoskeleton. The Na-K ATPase is a small fraction of the total membrane proteins in the human red cells. Ouabain is a specific inhibitor of the Na-K ATPase. $^3$H-ouabain-binding studies of human red cells have provided estimates of the number of pumps per cell ranging from $228 + 28$[32] to $470 \pm 30$ with more pumps[58] in the least dense, reticulocyte rich fraction and fewer pumps[58] in the densest fractions[58]. The Na-K ATPase catalyzes the extrusion of $Na^+$ in exchange for $K^+$ (3:2 stoichiometry) and determines the high $K^+$, low $Na^+$ content of human red cells. The rate of $Na^+$ transport through the Na-K pump in normal human red cells is $4.9 \pm 0.9$ mmol/l cell $\times$ h.

$^{86}Rb^+$ influx mediated by the Na-K pump is abnormally low in SS dense cells whereas the red cell membrane ATPase activity is normal[25]. This reduction is not due to a change in the affinity for external $K^+$ of intact red cells, which was found normal in SS cells[89]. Recent studies on the $Mg^{2+}$ composition of SS cells[90] have shown that the reduced pump activity is due to an increased Mg/P ratio. When this ratio is decreased, the transport activity of the Na-K pump returns to normal values.

b) *Increased $Na^{2+}$ entry.* The Na-H exchange system moves $Na^+$ into the cell in exchange for $H^+$. In human red cells it was first described from the stimulatory effect on $Na^+$ influx of increasing internal $Ca^{2+}$ [33] and subsequently identified via the coupling of $Na^+$ influx and $H^+$ efflux[34]. In other cell types, but not in human erythrocytes, the Na-H exchange is responsible for volume regulatory increase following hypertonic shrinkage and for cell pH regulation. The system is functionally distinct from another pathway, the Na-Na or Na-Li countertransport[35]. Na-H exchange is functionally silent in human red cells, where it can be demonstrated only after extensive manipulations, involving inhibition of the anion exchange with DIDS and presence of a large pH gradient. Na-H exchange is more active in reticulocyte-rich fractions of human red cells[22]. Under normal conditions, influx of $Na^+$ via this pathway in human red cells is small. The Na-H exchange can be normal or increased in SS cells. The lack of volume-dependence of Na-H exchange in red cells and the fact that Na-H exchange is present mostly in SS reticulocytes suggest that the increased $Na^+$ entry of dense cells may not be due to Na-H exchange. However, cellular pH could decrease and lead to activation of Na-H exchange with increased Na entry in the kidney medulla or in the peripheral circulation when obstruction is present.

Increased $Na^+$ influx could also take place through increased leak, which is a characteristic of young cells[106], as a consequence of oxidation and deformation, or as a consequence of deoxygenation, via the deoxygenation-induced Na influx.

The mutation in $\beta$-6 of Hb S leads to substantial modifications in the properties of the erythrocyte. The changes observed in membrane transport are related to the decreased life span of the cell, with the presence of reticulocytes and young cells, to the oxidative damage imposed on the membrane by the presence of Hb S, to the polymerization of Hb S and the physical distortion of the membrane which accompanies sickling and to the interaction between Hb S and the cell membrane. The presence of altered membrane transport properties also affects the pathogenesis of the disease, on the bases of the associated cell dehydration and increased Hb S concentration.

Our better understanding of the determinants of dehydration in SS cells will eventually lead to new therapeutic stages aimed to prevent cell dehydration and reduce cell sickling in vivo. While the ultimate cure of sickle cell anemia may be gene therapy, prevention of cell dehydration could be an important and efficacious alternative, until these new technologies become available.

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