Ion Transport Pathology in the Mechanism of Sickle Cell Dehydration

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Lew, Virgilio L., and Robert M. Bookchin. Ion Transport Pathology in the Mechanism of Sickle Cell Dehydration. Physiol Rev 85: 179–200, 2005; doi:10.1152/physrev.00052.2003.—Polymers of deoxyhemoglobin S deform sickle cell anemia red blood cells into sickle shapes, leading to the formation of dense, dehydrated red blood cells with a markedly shortened life-span. Nearly four decades of intense research in many laboratories has led to a mechanistic understanding of the complex events leading from sickling-induced permeabilization of the red cell membrane to small cations, to the generation of the heterogeneity of age and hydration condition of circulating sickle cells. This review follows chronologically the major experimental findings and the evolution of guiding ideas for research in this field. Predictions derived from mathematical models of red cell and reticulocyte homeostasis led to the formulation of an alternative to prevailing gradualist views: a multitrack dehydration model based on interactive influences between the red cell anion exchanger and two K+ transporters, the Gardos channel (hSK4, hIK1) and the K-Cl cotransporter (KCC), with differential effects dependent on red cell age and variability of KCC expression among reticulocytes. The experimental tests of the model predictions and the amply supportive results are discussed. The review concludes with a brief survey of the therapeutic strategies aimed at preventing sickle cell dehydration and with an analysis of the main open questions in the field.
I. INTRODUCTION

A. Sickle Cell Anemia: First “Molecular Disease”

Sickle cell anemia (SS) is an inherited disease whose origin and demographic roots may be traced to malaria endemic areas. The name derives from the peculiar sicklelike shapes that SS red blood cells (RBCs) acquire on deoxygenation. The first indication that this abnormal response resulted from a primary hemoglobin (Hb) abnormality was the observation that formation of sicklelike spicules on deoxygenation of RBCs from SS infants was absent or minimal until fetal hemoglobin (Hb F, $\alpha_2\gamma_2$) was replaced by adult Hb ($\alpha_2\beta_2$), at about 3–6 mo of age (272). In 1949, applying moving boundary electrophoresis to hemolysates from normal, sickle cell trait (AS) and SS subjects, Pauling et al. (221) showed that nearly all the Hb in the RBCs of (homozygous) SS patients had a mobility different from that of normal Hb (Hb A), and that heterozygous AS carriers had both types of Hb in their RBCs, ~40% of abnormal Hb S and 60% of normal Hb A. This crucial experiment identified a molecular disease and also explained its pattern of inheritance.

In one of the first applications of two-dimensional peptide mapping, Ingram (145, 146) identified the precise molecular difference between Hbs A and S as a single amino acid substitution on the $\beta$-chain of Hb, valine for glutamic acid in position 6. Epidemiological studies suggested that the persistence of the AS trait in malaria-infested areas resulted from the protection it afforded to AS carriers against severe malaria caused by *Plasmodium falciparum* (2, 3). Two general protective mechanisms had been considered in the literature: restricted parasite growth and enhanced immune retrieval of the parasitized RBCs. Much of the early work is conflictive and confusing, mainly because of the limitations of in vitro studies to reproduce the conditions in vivo (1). Recent work (9, 59, 135) strongly suggests that resistance in AS RBCs is caused by enhanced phagocytosis of RBCs infected with ring stage parasites in response to alterations of the membrane surface associated with increased hemichrome formation, aggregated band 3, elevated autologous IgG, and complement C3c fragments. Studies by a number of investigators (133, 224, 246), also in the early 1950s, characterized the functional abnormality of Hb S responsible for the sickle morphology: deoxygenated Hb S (deoxy-Hb S) tetramers spontaneously aggregate to form birefringent “tactoid bodies” composed of bundles of polymer fibers, which in turn formed a gel network. In the RBCs, these fiber bundles protrude into the plasma membrane generating elongated spiculated projections, a process described as “sickling.” For more detailed and complete historical backgrounds, with insightful anecdotical accounts, see chapters 11 and 12 in Bunn and Forget (54) and Serjeant’s historical review (240).

B. The Long Path From Molecular Origin to Pathophysiology

The wealth of information on the molecular, genetic, and epidemiological aspects of SS gathered in the 1950s and 1960s did little to help explain its pathophysiology. We briefly review here some relevant facts which illustrate the difficulties in connecting the basic molecular defect with clinical events. SS patients suffer chronic and acute organ failure and episodic pain attributable to vasoocclusive events in the microcirculation (18, 20, 172). SS RBCs exhibit marked heterogeneity of age, morphology, and hydration state (17, 98). A characteristic of the SS hematological profile is the presence of a highly dehydrated, dense, and rigid subpopulation of cells, many of which have a typical “elongated hard potato” appearance, which changes little on deoxygenation. These cells were termed “irreversibly sickled cells” (ISCs), since their abnormal shape persisted when the cells were fully reoxygenated in vitro, reversing all intracellular Hb polymerization; this indicated that their shape depended on membrane alterations, shown later to involve their cytoskeletons (209, 210). The “hyperdense” SS cells (e.g., $\delta > 1.118$ or MCHC $> 45$ g/dl), comprised mostly of ISCs, were shown to play a complex role in microvascular occlusion (172). Also, their proportion among circulating RBCs appeared reduced during painful vaso-occlusive crisis, consistent with their selective retention within blocked vascular domains (18, 20). However, although the proportion of circulating ISCs was found to correlate with the severity of the anemia (241), no significant correlation was found between the magnitude of the dense cell fraction and the frequency or severity of painful crises (19). The propensity of deoxy-Hb S to polymerize is much reduced in the presence of substantial fractions of Hb A or Hb F within the RBCs (255). RBCs from heterozygous AS subjects, with only ~40% Hb S, do not sickle in vivo at normal oxygen tensions, except in the hypertonic environment of the renal medulla, where their cell Hb concentrations are markedly increased by osmotic shrinkage. Persons with sickle trait have normal hematological profiles and are asymptomatic, except under the high stress condition of maximum exercise or in low $P_O_2$ conditions (high altitude or sudden decompression in aircraft) (54). Persons doubly heterozygous for Hb S and for hereditary persistence of fetal Hb (SP), have normal hematological profiles and no clinical evidence of sickling (54). The increased number of Hb F-containing RBCs (40, 84) in SS patients is most enriched among the normal-density SS cells and are selectively excluded from the densest cell fractions (17) (see Fig. 1). Thus Hb combinations that prevent sickling in vivo also prevent the development of the hematological and clinical manifestations of SS, including the formation of very dense, dehydrated RBCs,
despite the presence of substantial proportions of Hb S within the cells.

This brief overview emphasizes that in vivo sickling and generation of hyperdense SS RBCs are necessary conditions for the clinical manifestations of the disease and that the mechanisms by which sickling leads to the marked age and hydration heterogeneity of SS cells are among the most important pathophysiological issues in SS disease, and the specific subject of this review. Over five decades of intense research in many laboratories elucidated the complex links between the single molecular defect and the mechanisms of SS cell dehydration. As described below, important breakthroughs were provided by integrated approaches to cell physiology. In an era so dominated by “omics” and reductionist approaches, the development of this important connection between basic science and clinical application exemplifies the need for ongoing physiological expertise to bridge the gap between genes and molecules at one end and function or dysfunction at the other.

In this review we focus on the ideas which guided research at each stage, and on the puzzling and conflicting observations which led to the formulation of new questions and hypotheses; we point out how these formulations eventually led to the answers that represent the current consensus on the mechanisms of SS cell dehydration. We briefly survey the therapeutic strategies aimed at preventing SS cell dehydration and conclude with an overview of the open questions and future developments in the field.

For the benefit of readers not familiar with RBC physiology, the next section introduces some basic concepts and methodological considerations specific to RBC and reticulocyte homeostasis, which should help understand how SS RBCs become dehydrated in vivo.

II. BASICS OF RED BLOOD CELL AND RETICULOCYTE HOMEOSTASIS

Human RBCs are water-permeable sacs with the highest soluble protein concentration of any cell, ~7.2 mmol Hb/l cell water. A two-dimensional, spectrin-actin based, meshlike cytoskeleton, with highly structured links to integral membrane proteins embedded in the lipid bilayer, endows the RBCs their characteristic biconcave shape and flexibility (41, 66, 67, 209, 219, 220, 250). The high water permeability of the RBCs ensures their continued osmotic equilibrium in plasma so that they can shrink or swell only by the loss or gain of a fluid isosmotic with surrounding plasma. Since the plasma protein concentration is <1 mM, there is a powerful oncotic pressure driving water into the cells. To remain osmotically stable over their ~120-day circulatory life-span, mature RBCs evolved a strategy to maintain a nearly constant volume with minimal energy expenditure: their Na+ and K+ permeabilities are extremely low (10, 186) so that relatively few metabolically fuelled Na+ pumps per cell suffice to balance their cation gradient-driven “leaks.” As a result, the anion permeability was freed from limiting constraints, allowing the RBCs to evolve an extremely efficient CO2 ferry between tissues and lungs, based on high Cl− and HCO3− exchange (the Jacobs-Stewart cycle; Refs. 147, 148, 188) and electrodiffusional fluxes (130, 143, 144, 174). This high electrodiffusional anion permeability set the membrane potential very near the equilibrium potentials of Cl− and HCO3−, about −10 mV (110, 114, 138, 139, 176, 177, 267).

The fine homeostatic balance described above for normal RBCs, which is remarkably constant for most of their 120-day life-span, is markedly altered in SS cells, whose ion fluxes, ion content regulation, and hydration state become highly disrupted in the circulation.

A. Methodological Points

1. Density fractionation of SS RBCs

Density fractionation of SS cells, which allows the separation and subsequent study of RBCs in different hydration states, has become an essential tool for research on the mechanism of dehydration. The density distribution of SS cells is much more heterogeneous than that of normal RBCs (17), as illustrated in Figure 1. The usual method for simple analysis of RBC density distributions is centrifugation through Percoll or Percoll/arabinogalactan gradients (17, 69, 76, 98, 202), whereas for ion transport studies, isolation of SS RBCs by density fractionation on discontinuous arabinogalactan gradients is preferred (37, 76, 96, 216). Figure 1 provides a general guide of the distribution of each cell type as a function of density, and of their contributions to the heterogeneity expected within each density band. The cells that predominately in the lightest density ranges are reticulocytes and dehydration-resistant cells (DRCs) which represent a recently discovered (29), high-Na+, low-K+ group of cells that resist dehydration when K+-permeabilized in plasmalike media, as detailed below. In SS blood, F cells are distributed broadly among the light density fractions rich in reticulocytes and among the light discocytes, but are most enriched among denser and older discocytes. Hyperdense ISC occur almost exclusively among RBCs exceeding a density of 1.118 g/ml.

2. Ion fluxes

Ion fluxes in RBCs are usually expressed in units (or subunits) of moles per liter original cells per hour (mol · l original cells −1 · h −1). “Original” means that measured fluxes are referred to as 1 liter of normal, packed RBCs at
the beginning of an experiment. Because one liter normal RBCs contains a mean of 340 g Hb or \( \sim 10^{13} \) RBCs, fluxes are sometimes expressed in equivalent units of moles per 340 g Hb per hour (mol \cdot 340 g Hb\(^{-1}\) \cdot h\(^{-1}\)) or moles per 10\(^{13}\) cells per hour (mol \cdot 10\(^{13}\) cells\(^{-1}\) \cdot h\(^{-1}\)). To compare RBC fluxes with those expressed per unit area in other cell types, the following conversion applies assuming a mean RBC area of 155 \( \mu m^2 \) for human RBCs (273): 1 mol \cdot 1 original cells\(^{-1}\) \cdot h\(^{-1}\) is equivalent to \( \sim 2 \times 10^{-19} \) mol \cdot \mu m\(^{-2}\) \cdot s\(^{-1}\). With unidirectional cation fluxes in the order of 2–3 mmol \cdot 1 original cells\(^{-1}\) \cdot h\(^{-1}\) in normal RBCs, this is equivalent to a few hundred ions per \( \mu m^2 \) per second, an extremely low flux value among eukaryotes.

3. Use of ionophores

Much of the information about RBC \( Ca^{2+} \) and \( Mg^{2+} \) homeostasis was obtained using the divalent cation ionophore A23187 (77, 100, 102, 115, 194, 217, 229, 233, 237, 258, 262, 264). This important tool provided the state-of-the-art method to measure the activity of the plasma membrane \( Ca^{2+} \) pump (PMCA \( V_{\text{max}} \)) in intact RBCs and helped characterize the cytoplasmic \( Ca^{2+} \) and \( Mg^{2+} \) buffering properties of normal and SS RBCs (77, 96, 103, 193, 262). All these methods have been described in detail before (198) and detailed in a recent review (258); we note here only some of the unique ionophore properties used in studies of SS RBC \( Ca^{2+} \) homeostasis. Addition of the ionophore A23187 to RBCs generates an instant and remarkably uniform increase in divalent cation permeability (203, 204, 243–245). This uniformity results from a high partition coefficient of the ionophore in the RBC membrane, with high on-off rate constants, as shown by the instant redistribution of ionophore when ionophore-free RBCs are rapidly mixed into the suspension. Careful adjustments of three experimental parameters, hematocrit (Hct), extracellular \( Ca^{2+} \) concentration, and final concentration of ionophore in the cell suspension allow precise control of \( Ca^{2+} \) or \( Mg^{2+} \) fluxes, over a wide range of values. Addition of \( Co^{2+} \) in excess of \( Ca^{2+} \) in the media instantly arrests the ionophore-mediated \( Ca^{2+} \) fluxes, allowing exposure of uphill \( Ca^{2+} \) extrusion by the PMCA after a \( Ca^{2+} \) load and thus providing the basis for measurements of RBC PMCA \( V_{\text{max}} \) (44, 77, 230, 261). Although \( Co^{2+} \) is itself transported by the ionophore, its intracellular effects on the PMCA are those of a weak \( Mg^{2+} \) replacement (230). Finally, it is important to be aware that PMCA-saturating \( Ca^{2+} \) loads generate progressive and eventually irreversible ATP depletion because the rate at which a \( Ca^{2+} \)-saturated PMCA hydrolyzes ATP exceeds the glycolytic capacity of a large fraction of RBCs (192). Irreversibility results from the conversion of ATP to IMP, a three-enzyme process triggered by PMCA ATPase activity and can thus be prevented by inhibition of the PMCA (77, 263).

B. \( Na^{+}-K^{+} \) Fluxes in RBCs and Reticulocytes

The unidirectional fluxes of \( Na^+ \) or \( K^+ \) in mature normal RBCs are \( \sim 2–3 \) mmol \cdot 1 original cells\(^{-1}\) \cdot h\(^{-1}\), whereas in reticulocytes they may be 10- to 30-fold higher (10, 156–159, 232, 274). This traffic represents the instant pump-leak balance of these cells: the \( Na^+ \) pump, with its 3:2 \( Na^+ - K^+ \) stoichiometry, compensates for the combined net passive \( Na^+ \) and \( K^+ \) fluxes through all the individual passive transport pathways (197). Reticulocytes are the enucleated erythroid cells released from the bone marrow into the circulation where they differentiate into mature RBCs within 2–3 days. The maturation process involves
not only the loss of protein synthesis along with residual RNA, but also reduction or loss of many metabolic functions including a gradual decline in monovalent cation transport activity and the reduction or inactivation of selected transporters such as Na\(^+\) pumps and K\(^+\)-Cl\(^-\) cotransporters. Johnstone (155) showed that during maturation of reticulocytes, there was formation of exosomes containing proteins and lipids characteristic of the plasma membrane and that the transferrin receptor, known to be lost during red cell maturation, was contained in these exosomes. Intrinsic membrane proteins, such as the anion transporter, were retained, but there was no further information about other ion transporters. More recent studies on cation transport in rat reticulocytes (211) showed that their increased Na\(^+\) pump activity was considerably reduced during maturation, to a greater extent than their loss of membrane surface area, suggesting more specific loss of transport protein. Because reticulocyte volume decreases only by \(\sim 13\%\) during normal maturation (231, 232), there must be a coordinated and compensated reduction in pump and leak cation flux components (202). Circulating reticulocytes represent an extremely heterogeneous population of young cells, in a continuum of progressive maturation with decreasing Hb synthesis and cation turnover rates. Because of their larger volumes and incomplete Hb complement, normal reticulocytes are lighter than mature RBCs and can be enriched in the upper layers of density-fractionated RBC samples. Clearly, however, cation flux measurements in reticulocyte-rich RBC fractions represent mean values of broad distributions, from the low values of the mature RBCs to the much higher but less clearly determined upper values of the youngest reticulocytes. It is important to bear in mind the limitations this functional and cellular heterogeneity imposes on the interpretation of experimental flux measurements in reticulocyte-rich cell fractions, when only mean values are obtained.

C. Pathways of RBC Dehydration

Dehydration of human RBCs in vivo may result from the activation of one or more of three transporters expressed in their plasma membranes: 1) the Ca\(^{2+}\)-sensitive, small-conductance, K\(^+\)-selective channel (Gardos channel, IK1 or hSK4; Refs. 116, 137, 195, 269), also expressed in many other cell types (225); 2) a K\(^+\)-Cl\(^-\) cotransporter (KCC), regulated by internal pH and cell volume (46, 87, 107, 121, 181, 184), functionally active in reticulocytes and much less so in mature AA and SS RBCs; and 3) the Na\(^+\) pump (125, 227, 247). The three transporters differ considerably in their dehydrating modalities, potencies, and distributions among RBCs.

1. RBC calcium and the Gardos channels

At physiological intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\text{\(i\)}) levels of \(\sim 20–50\) nM, Gardos channels are inactive, but they can be activated when [Ca\(^{2+}\)]\text{\(i\)} levels are increased in pathological and experimental conditions. The [Ca\(^{2+}\)]\text{\(i\)} activation threshold of Gardos channels in intact RBCs is probably \(\sim 150\) nM (242, 264). The mean number of Gardos channels per RBC is not yet established, but the most reliable estimates are \(\sim 100–200\) (4, 48, 129, 137, 200, 205, 276). When the Gardos channels are maximally activated by a uniform, saturating Ca\(^{2+}\) load in the RBCs, flow-cytometry measurements show that during dehydration, the original volume distribution of the RBCs is conserved (205). This indicates that each donor’s RBCs do not differ much in the number or functional expression of their Gardos channels and that they all dehydrate fast when their channels are maximally activated. Unlike erythroid cells from other mammalian species, whose Gardos channel activity is lost during maturation (43), mature human RBCs show persistent Gardos channel activity. The single Gardos channel conductance is \(\sim 10\) pS in physiological conditions (128). The open-state probability of the channels at saturating Ca\(^{2+}\) levels has a peculiar temperature dependence. At 35°C it is very low (between 0.01 and 0.1); it increases sharply at 25°C and then falls slowly with decreasing temperature (127, 137). Thus, for experimental purposes, Gardos-mediated dehydration persists at low temperatures at which the PMCA is markedly inhibited (115, 201). In low-K\(^+\) media, activation of Gardos channels hyperpolarizes the cell (177), generating a favorable gradient for co-ion (Cl\(^-\) or HCO\(_3^{-}\)) exit via parallel voltage-sensitive pathways. The resulting net loss of KCl and KHCO\(_3\) is tightly coupled to osmotic-driven water loss, leading to cell dehydration. At saturated [Ca\(^{2+}\)]\text{\(i\)} levels for the Gardos channels, above 2 μM, the anion permeability becomes rate-limiting for net KCl loss (144, 173); at intermediate activation levels, either cation or anion permeabilities may limit the rate of dehydration. Dehydration via Gardos channels may be reduced by direct inhibition of the channels with charybdoxin (IC\(_{50}\) \(\sim 1.2\) nM) or clotrimazole (IC\(_{50}\) \(\sim 51\) nM) (5, 49), or indirectly, by inhibition of the anion permeability (12, 13, 50). The capacity of Gardos channels to mediate rapid RBC dehydration when fully activated far exceeds that of the other transporters. Gardos channel-mediated 42K or 86Rb fluxes may reach mean values of \(\sim 1\) mol \(\cdot\) l\(^{-1}\) \(\cdot\) h\(^{-1}\) (194). Such fluxes reflect mean permeability values on the order of \(2 \times 10^{-7}\) cm/s, equivalent to rate constants of \(\sim 10\) h\(^{-1}\) (111). At physiological Cl\(^-\) and HCO\(_3^{-}\) concentrations, full dehydration by maximally activated Gardos channels may take over 1 h, the rate-limiting anion permeability being on the order of \(2 \times 10^{-8}\)
cm/s, an order of magnitude lower than that through fully activated Gardos channels. When Cl\(^{-}\) and HCO\(_3\) are partially replaced by the more permeable SCN\(^{-}\) or HNO\(_3\) anions in experimental conditions, full K\(^{+}\) equilibration and dehydration may be attained within 3–4 min (111, 115, 259). It is important to bear this in mind as it illustrates the powerful restrictive effects of the Cl\(^{-}\) and HCO\(_3\) permeabilities on the speed and extent of RBC dehydration attainable during the brief intermittent periods of Gardos channel activation induced by sickling in the circulation, as discussed below. Under whole cell patch-clamp conditions, the anion conductance of RBCs under whole cell patch-clamp conditions is apparently much lower. The constitutive Ca\(^{2+}\) permeability represents a \(~1/10,000\) slippage through the anion exchange carrier rather than a separate conductive pathway (112, 113, 118, 173). This view is indirectly supported by the fact that Cl\(^{-}\) channels have thus far eluded detection in excised RBC membrane patches. However, certain kinetic and experimental difficulties (beyond the scope of this review) led Bennekou and collaborators (12, 13) to support the view that the Cl\(^{-}\) permeability, though still mediated by the same band 3 as the anion exchanger, is through a diffusional configuration of the protein. This group pioneered the use of anion permeability inhibitors as a promising new therapeutic strategy in sickle cell disease; their description of the anion permeability as a conductance is therefore included in section vD. In the present context, we would emphasize that it is only the magnitude, not the precise mechanism, of the anion permeability path that is an important determinant of the rate of dehydration induced by Gardos channel activation. Because the activity of Gardos channels is tightly controlled by [Ca\(^{2+}\)]\(_i\), it is necessary to review briefly the main processes involved in Ca\(^{2+}\) regulation in normal RBCs. Mature RBCs lack specialized Ca\(^{2+}\) accumulating organelles and Ca\(^{2+}\) signaling functions. Their total calcium content ([Ca\(_2\)]\(_i\)) is \(<5\) \(\mu\)mol/l original cells (30, 93, 134), of which \(<1\) \(\mu\)mol/l original cells can be extracted by treatment with Ca\(^{2+}\) ionophores in the presence of high Ca\(^{2+}\) chelator concentrations in the medium (30). This represents a maximal estimate, limited by measurement error, so that the true exchangeable Ca\(^{2+}\) is probably much lower. The constitutive Ca\(^{2+}\) permeability of RBCs (P\(_{Ca}\)) is extremely low, and Ca\(^{2+}\) ionophores have become essential tools in investigations requiring Ca\(^{2+}\) loading of the cells. Compared with other cell types, RBCs have minimal cytoplasmic Ca\(^{2+}\) buffering capacity (100, 260, 262). Cytoplasmic Ca\(^{2+}\) buffering can be approximated by the equation

\[
[Ca^{2+}]_i = \alpha[Ca_T]_i
\]

where [Ca\(^{2+}\)]\(_i\) is expressed in \(\mu\)mol/l cell water, \(\alpha \approx 0.2–0.3\) over a wide range of experimentally induced [Ca\(_T\)]\(_i\) values, and [Ca\(_T\)]\(_i\) is expressed in units of \(\mu\)mol/l original cells. Thus \(~20–30\%) of the total mobilizable RBC Ca\(^{2+}\) content is in ionized form in cell water. This is particularly important because it indicates that only minute net total Ca\(^{2+}\) gains are required for [Ca\(_T\)]\(_i\) to reach threshold activation levels of Gardos channels. Physiological [Ca\(^{2+}\)]\(_i\) levels (\(~20–50\) nM) represent the balance between passive Ca\(^{2+}\) influx and active Ca\(^{2+}\) extrusion, with a pump-leak turnover rate of \(~50\) \(\mu\)mol · l\(^{-1}\) original cells · h\(^{-1}\) (83, 206). As in many other cell types, active Ca\(^{2+}\) extrusion is mediated by a large-capacity (high V\(_{max}\)) ATP-fuelled, P-type pump, the PMCA (60, 257). In human RBCs, the PMCA is expressed with genomic isoforms 1 and 4 (60, 61, 234, 238, 239, 271). The mean V\(_{max}\) of the PMCA varies from 5 to 25 mmol · l\(^{-1}\) original cells · h\(^{-1}\), over two orders of magnitude higher than the mean pump-leak turnover rate of Ca\(^{2+}\) (77). Recent measurements revealed a large variation (over an order of magnitude) in PMCA V\(_{max}\) among the RBCs within individual blood samples (192). The distribution followed a broad unimodal pattern, with the lower V\(_{max}\) cells perhaps reflecting a declining Ca\(^{2+}\) extrusion capacity among aging RBCs. An important implication of this finding is that individual RBCs, comparably permeabilized to Ca\(^{2+}\), may reach balancing pump-leak steady states with [Ca\(^{2+}\)]\(_i\) levels below or above the activation threshold of Gardos channels, leading to markedly different dehydration responses. Deoxygenation of RBCs was also found to induce a \(~20\%) reduction in PMCA V\(_{max}\) in both AA and SS RBCs, with detectable effects on Ca\(^{2+}\)-induced dehydration of SS cells (96, 260).

2. The KCC; dehydration-induced acidification

KCCs are expressed in a large variety of cell types, including erythroid precursors (121, 180, 181). In a pioneering study, Anderson et al. (6) detected transcripts for three of the four known KCC isoforms (KCC1, KCC3, and KCC4), and for two additional splicing isotypes, in RNA obtained from human erythroid cells derived from AA and SS blood. However, which of these KCC isoforms and alternative spliced variants participate in the functional expression of KCCs in human reticulocytes is still unknown. In normal and SS RBCs, KCC activity is substantially reduced during maturation but can be partially reactivated experimentally in mature RBCs by thiol-reactive agents such as N-ethylmaleimide (183) or by high hydrostatic pressure (131). In reticulocytes, most of the passive K\(^{+}\) traffic is mediated by KCC (46, 57, 132). KCC is a multiregulated transporter, influenced by the oxygenation state of Hb (120, 166), and stimulated by intracellular
acidiﬁcation, by cell swelling and to a lesser extent, by low intracellular Mg$^{2+}$ concentrations (53, 150, 152, 178, 182, 249). Its functional state appears to be regulated by phosphorylation and dephosphorylation of serine/threonine and tyrosine residues; it has not yet been determined whether these residues are located on the KCC transporter itself or on one or more regulatory proteins. Jennings and al-Rohil (150, 152) described indirect evidence that in rabbit RBCs, swelling activation of KCC occurs via inactivation of a serine/threonine kinase, and Bize and co-workers (23, 26) found that hypotonic swelling of RBCs was associated with increased activities of protein phosphatases (PP1 and PP2A), that PP1 increased in RBCs whose ionic strength was lowered at constant volume, and that PP1 appears to mediate part of urea-stimulated KCC activity. Activation of KCC follows dephosphorylation of a serine/threonine residue by PP1 (153, 249) and/or PP2A (23, 26). As pointed out by Joiner et al. (171), additional evidence of activation by some tyrosine kinase inhibitors (22, 80) and inhibition by others (101, 213) suggests multiple control points, including PP1 itself (25, 154). Proposed models of the biochemical activation pathways continue to be updated with the latest data (21), but the complex interrelations between the different aspects of activation or inhibition (changes in cell volume, pH, urea, oxygenation state, [Mg$^{2+}$], and phosphorylation/dephosphorylation) have yet to be determined.

KCC mediates a strictly coupled electroneutral transport of K$^+$ and Cl$^-$, independent of the membrane potential (151). Persistence of KCC activity in RBC samples may be constitutive, as in certain hemoglobinopathies (Hb C, Ref. 52), or associated with reticulocytosis, as with SS blood (46). In RBCs with active KCC, acidification may induce dehydration (37), whose extent may be limited by the inhibitory effect of cell shrinkage. Joiner et al. (171) have recently shown that acid activation of the KCC is abnormally exaggerated in SS reticulocytes and may thus easily overcome the intensity of the volume-regulatory “brake.” Inhibitory agents may act directly on the KCC or on its regulatory intermediates.

The driving force for RBC dehydration by K$^+$ permeabilization, as when mediated by Gardos channels or KCCs, is the outward electrochemical gradient of K$^+$. An important side effect of isotonic dehydration by KCl and KHCO$_3$ loss is cell acidification (188, 197). This effect was predicted by the Lew-Bookchin red cell model (188; Fig. 2) and was fully confirmed experimentally (111). Its mechanism is as follows. Within the RBCs, the positive charges on Na$^+$ + K$^+$ + Mg$^{2+}$, ~150 meq/l cell water, are balanced by negative charges on Hb$^-$, Cl$^-$, and HCO$_3^-$. To a gross approximation, Hb provides 50 meq/l cell water of negative charges, and Cl$^-$ + HCO$_3^-$ 100 meq/l cell water. The effluent from isotonically dehydrating cells contains ~150 meq/l KCl + KHCO$_3$ salts. Therefore, the concentrations of Cl$^-$ + HCO$_3^-$ in the effluent are higher than in the cell, and this necessarily dilutes their intracellular concentrations as dehydration proceeds. At approximately constant external concentrations of Cl$^-$ + HCO$_3^-$, intracellular dilution causes the [Cl$^-$]/[Cl$^-$]$_i$ and [HCO$_3^-$]/[HCO$_3^-$]$_i$ concentration ratios to increase. The parallel joint operation of the anion exchanger (AE1; Refs. 126, 175, 256) and CO$_2$ shunt in the RBC membrane, known as the Jacobs-Stewart mechanism (136, 147, 148), ensures rapid equilibrium given by

\[
\frac{[H^+]}{[H^+]} = \frac{[Cl^-]}{[Cl^-]} = \frac{[HCO_3^-]}{[HCO_3^-]},
\]

with cell acidification resulting from the increased inward anion concentration ratio. Cell acidification was also found associated with regulatory volume decrease processes in other cell types and was assumed to operate by the same mechanism as in RBCs (140).

3. The Na$^+$ pump

The Na$^+\text{-}K^+$ flux ratio through the Na$^+$ pump is 3:2 (117, 228). Because of the high anion permeability of RBCs and reticulocytes, electroneutrality is maintained mainly by anion efflux balancing the extra Na$^+$ efflux. If the Na$^+$ pump is stimulated by elevated internal Na$^+/K^+$ concentration ratios, the resulting increased NaCl efflux, if not compensated by changes in passive ﬂuxes, would induce cell dehydration (70, 123, 170). But with only ~400 copies of the Na$^+$ pump protein per cell (85, 125, 168), the effect is bound to be rather slow, as conﬁrmed with model simulations (36). Moreover, despite the elevated internal Na$^+/K^+$ in dense SS cells, their Na$^+$ pumps were found to be substantially inhibited (73). The inhibition was attributed to the abnormally high Mg/ATP ratio in these cells (217), as it could be partially relieved by increasing ATP levels towards the optimal ratio for the Na$^+$ pump (88, 103, 104). Na$^+$ pump ﬂuxes in dense SS cells were found to be inactivated even more by deoxygenation because Mg$^{2+}$ released from 2,3-diphosphoglycerate (2,3-DPG) elevated the Mg/ATP ratio further away from optimal (217). Thus Na$^+$ pump-mediated ﬂuxes may contribute only marginally to SS cell dehydration.

III. HOW DO SICKLE CELLS DEHYDRATE?

A. Early Findings and Puzzles

The relation between sickling and SS cell transport abnormalities was ﬁrst investigated by Tosteson and co-workers (265, 266, 268). They showed (268) that upon deoxygenation of fresh, heparinized whole blood, SS RBCs had a much larger gain of Na$^+$ and loss of K$^+$ than RBCs from normal controls. On reoxygenation, SS cells
gained K⁺ and lost Na⁺, indicating that the effects of deoxygenation were reversible. Since CO treatment of SS blood prevented both Hb S polymerization and Na⁺/K⁺ shifts on deoxygenation, the deoxygenation effect was strictly the result of intracellular Hb S polymerization. Further transport experiments with tracers of Na⁺/K⁺, and Cs⁺ indicated that sickling induced a reversible and poorly selective increase in the electrodiffusional cation permeability of the RBC membrane (265, 266). In retrospect, this seminal work provided the first demonstration of the sickling-induced permeability pathway that we currently designate $P_{\text{sickle}}$ (201). The nearly balanced Na⁺ gain and K⁺ loss during deoxygenation in vitro did not account for SS cell dehydration, and the question of how SS cells dehydrate was first formulated in the literature 12 years later, with the ground-breaking work of Bertles and Milner (17).

Although the distinct morphological features of ISCs were well known (the elongated potato-like shape mentioned above), Bertles and Milner (17) described some distinctive properties of this SS cell subpopulation which proved crucial and prescient of our current understanding of their origin. They showed that ISCs accumulated at the bottom of ultracentrifuged columns of RBCs, indicating their high density. Because their Hb content was normal, their high density reflected a dehydrated state. With various isotopic labeling techniques, they showed that ISCs were a relatively young SS cell subpopulation which, after release from the bone marrow as reticulocytes, was rapidly transformed into ISCs within 4–7 days, and then disappeared from the circulation faster than other mature, non-ISC RBCs. ISCs were therefore a subpopulation of SS RBCs in rapid circulatory turnover. The exclusion of most high Hb F-containing SS cells (whose polymerization was reduced or inhibited) from the hyperdense, ISC-rich cell fraction (Fig. 1) suggested that sickling was necessary for ISC formation.

Bertles and Milner’s pioneering work focused much of the subsequent research on the pathophysiology of sickle cell anemia on the mechanism of SS cell dehydration. However, in the absence of a clear understanding of the process, the relative young age and rapid turnover of ISCs remained a largely ignored, isolated observation. For the next two decades, research in the field was dominated by the notion of gradual dehydration (“gradualism”), with attention centered on the relative importance of the three...
transport systems potentially involved in SS cell dehydration: the Gardos channel, KCC, and the Na+/H1001 pump.

B. Discovery of the High Calcium Content of SS Cells

In 1973, Eaton et al. (89) and Palek et al. (218) independently reported that SS RBCs had a highly elevated total calcium content, which was highest in the densest SS cell fraction, rich in ISCs. Ca2+-uptake was found to be increased on deoxygenation, suggesting that Ca2+ was also permeable through P_sickle a finding subsequently confirmed in a variety of experimental conditions (31, 95). The Ca2+ link suggested that the Gardos channel might be the main dehydration pathway, and much research during the 1970s and 1980s explored experimental conditions that could generate ISCs in vitro by deoxygenation- and Ca2+-dependent processes (16, 33, 35, 46, 69–75, 97, 98, 122, 123, 160, 170, 214). However, rather than providing clear answers, the high calcium content of SS cells became a most confusing issue that took another decade to resolve. Why was the Ca2+ taken up during deoxygenation retained? Was the Ca2+ pump with its normal high-V_max somehow incapacitated? Did SS RBCs have much higher cytoplasmic Ca2+ buffering capacity than normal RBCs? Ca2+ was most elevated in the denser cell fraction, but was also substantially increased in SS cells with normal density (not dehydrated). What then prevented dehydration of these high-Ca2+ cells? Were their Gardos channels also inhibited?

C. Discovery of Endocytic Inside-Out Vesicles

In the early 1980s, available evidence suggested two radically different alternatives to explain Ca2+ retention without immediate dehydration in SS cells: either a profound failure of both Ca2+ pumps and Gardos channels exclusive to intact SS RBCs (32, 33, 35, 187), or some sort of Ca2+ compartmentalization that rendered Ca2+ inaccessible to these targets on the plasma membrane (35). So strong was the general rejection of the idea of compartmentalization within RBCs at the time that not even its mention as a remote alternative was acceptable in submitted manuscripts. It took a multidisciplinary approach to resolve these options convincingly (34, 38, 199, 236, 275). Serial section analysis of SS RBCs clearly demonstrated the presence of intracellular compartments fully enclosed within the cells. Surprisingly, a much smaller number of fully enclosed vesicles was also found in a high proportion of normal RBCs. With the application of procedures that destroyed the permeability barrier of the RBC plasma membrane but retained intact vesicles inside, it was possible to show that these vesicles had ATP-dependent Ca2+-accumulating capacity, as expected from endocytic (inside-out) vesicles (EIOVs) with the PMCA pumping Ca2+ inwards (Fig. 2). X-ray microanalysis of the elemental composition of intravesicular and cytoplasmic compartments of cryosectioned SS cells showed clear distinctions: calcium was located exclusively in the vesicles, which also contained high Na+ and low K+, suggesting an endocytic origin, and high phosphate, consistent with intravesicular Ca2+ accumulation in the form of amorphous hydroxyapatite crystals (34, 38). The intracellular vesicles could be coated with specific PMCA antibodies, and vesicular uptake could be inhibited by vanadate, but not by thapsigargin (A. Muoma and V. L. Lew, unpublished observations). These results confirmed the endocytic origin of the Ca2+-accumulating vesicles and excluded the participation of residual, endoplasmic reticulum-derived organelles from earlier erythroid stages, which express thapsigargin-sensitive SERCA-type Ca2+ pumps (277) instead of PMCA-type pumps.

There had been earlier reports showing vesicular accumulation within RBCs of splenectomized persons (141). This had been interpreted as reflecting a continuous process of endocytic vesiculation in circulating RBCs, stabilized by extraction ("pitting") during passage through splenic sinusoids. Such an interpretation could also explain, at least in part, the unusually high accumulation of EIOVs in RBCs of SS patients because of their known functional asplenia (222, 223). In summary then, the high (micromolar) Ca2+ content of SS RBCs is restricted to EIOVs and results from a cumulative process initiated by increased Ca2+ influx through P_sickle. The resulting increase in [Ca2+]i stimulates the PMCA to accumulate Ca2+ in EIOVs while also extruding it through the plasma membrane. Ca2+ accumulation is thus the result of the prevailing balance between EIOV Ca2+ uptake and Ca2+ extrusion through the plasma membrane (Fig. 2). With hindsight, the long time it took to explain the significance of the high [Ca2+]i of SS cells represents a cautionary tale of “pride and prejudice” blocking the early consideration of unconventional alternatives.

D. Failings of “Gradualism”

With the PMCA and Gardos channels of SS RBCs now recognized as fully functional, the immediate question was whether the transient [Ca2+]i elevations during deoxygenation were sufficient to trigger Gardos channel activation and explain SS cell dehydration. When Ca2+ influx was uniformly elevated in AA cells to levels comparable to those in P_sickle-permeabilized SS cells (using Ca2+ ionophores), a small fraction of AA cells became rapidly dehydrated (264). This can now be explained by the wide distribution of PMCA activity found in AA cells (192). At low Ca2+ influx, only the cells with the weakest Ca2+ pump V_max activities would allow [Ca2+]i to reach or
exceed threshold activation levels of the Gardos channels. Results from different laboratories showed that applying single or cyclical deoxygenation to medium-density SS RBCs (discocyte fractions) generated variable extents of Ca\(^{2+}\)-dependent SS cell dehydration. However, the observations were difficult to relate to physiological conditions, raising some doubt as to whether Gardos-mediated dehydration played any role in vivo. This doubt was reinforced in 1986 by the advent of a contender dehydrator, KCC.

Hall and Ellory (132) and Canessa et al. (57) each reported a high level of KCC activity among light, reticulocyte-rich, and young SS RBC fractions. In detailed studies of the intracellular pH and volume regulatory properties of the KCC among different density fractions of SS cells, Brugnara et al. (46, 47) showed that stimulation of KCC-mediated \(^{86}\)Rb(K) fluxes by intracellular acidification was particularly steep in the lightest, reticulocyte-rich SS cell fraction, that inhibition by hypertonic-induced shrinkage, though present, appeared relatively weak, and that acidification of SS cells resulted in their dehydration. Thus the KCC of SS RBCs shared many of the characteristics of the KCC characterized in other species (86, 150, 152, 180, 181).

“Gradualism,” by which we mean the notion of gradual, progressive dehydration of all SS cells, from reticulocytes to ISCs, was the guiding idea behind all experimental designs until the late 1980s. It also pervaded the interpretation of all results, leaving an increasing body of experimental data unexplained. Although it was clear that both Gardos channels and KCC transporters had the potential to dehydrate SS cells, it was difficult to integrate the numerous reported experiments within a pathophysiological scheme that could generate the observed heterogeneity of age and hydration of SS cells. One problem was based on the uncertainty about whether \([\text{Ca}^{2+}]\) levels in deoxygenated SS cells ever reached Gardos channel activation thresholds. Another complexity was that Gardos-mediated dehydration alone could not explain the early observations (17) that the ISCs, the hyperdense, most dehydrated SS RBCs, appeared to be selectively generated from very young SS RBCs soon after their release from the bone marrow, because, except for the “dehydration-resistant cells” described below (DRCs; see sect. n°), all reticulocytes and RBCs are similarly sensitive to Ca\(^{2+}\)-induced dehydration. KCC-mediated dehydration alone also failed to explain ISC formation, particularly why ISCs should have the highest Ca\(^{2+}\) content of all SS cells and be relatively depleted of F cells. But the most important objection to exclusive KCC-mediated dehydration was the need to postulate circulatory acidification as the trigger, with no clear role for sickling-induced permeabilization.

E. Multitrack Dehydration as an Alternative to Gradualism

It became clear that although \(P_{\text{os}}, [\text{Ca}^{2+}]_i\), Gardos channels, and KCC symporters were important participants in SS cell dehydration, neither gradualism nor the idea of a dominant dehydration pathway would lead to an explanation of the well-characterized heterogeneity of age and hydration state of circulating SS cells. The specific issues that had to be addressed included the declining but still elevated proportion of reticulocytes observed among progressively denser SS cell fractions, the relatively young age of ISCs, and the exclusion of F cells from the hyperdense cell fraction. With large quantitative variations, these characteristics represent a consistent pattern among RBCs from all SS patients (Fig. 1).

A clear corollary of this formulation was that dehydration must not proceed uniformly and gradually from all SS reticulocytes to ISCs but rather at different rates in different SS RBCs, and fastest in the rapid-turnover ISCs. The questions then were whether this multitrack conditioning started at the reticulocyte stage or later, and by what mechanisms. At least for ISC formation, commitment to fast-track dehydration had to occur at a rather early maturational stage. By the mid 1980s, little was known about reticulocyte homeostasis, and it became clear that ignorance in this area was a major obstacle to progress. Our earlier experience with homeostatic modeling of Na\(^+\)-transporting epithelia (196) and of mature normal RBCs (188) generated predictions of such novel and unexpected behavior, subsequently confirmed (111), that it clearly exposed the limitations in our intuitive understanding of complex systems. This experience encouraged a similar effort to develop a mathematical-computational model of reticulocyte homeostasis encoding all the processes illustrated in Figure 2 (197).

The reticulocyte model (197) was developed with two important assumptions based on good experimental evidence: that the KCC, with intracellular pH and volume regulatory properties as described by Brugnara et al. (46) for the light reticulocyte-rich fraction of SS cells, constituted the main passive K\(^+\) transport pathway, and that the pump-leak, steady-state Na\(^+\)-K\(^+\) traffic across the reticulocyte membrane was at least one order of magnitude higher than in mature RBCs (232, 274). This formulation exposed a hitherto unsuspected need of high-capacity net Na\(^+\) and Cl\(^{-}\) entry pathways in reticulocytes to balance net Na\(^+\) efflux through the Na\(^+\) pump and net Cl\(^{-}\) efflux through KCC. A simple numerical example may help to illustrate this point. A reticulocyte with a KCC operating at a rate of 10 mmol · l original cells\(^{-1}\) · h\(^{-1}\) in steady-state will need a balancing K\(^+\) uptake of at least 10 mmol · l original cells\(^{-1}\) · h\(^{-1}\) through the Na\(^+\) pump. Because of its 3.2 Na\(^+\)-K\(^+\) stoichiometry, net Na\(^+\) extrusion by the pump will be at least 15 mmol · l original cells\(^{-1}\) · h\(^{-1}\). To
sustain a steady-state ionic balance and constant volume, such a reticulocyte will require net influxes of Na\(^+\) and Cl\(^-\) of ~15 and 10 mmol \cdot l \^{-1} \cdot h \^{-1}\), respectively. Thus, unlike with mature RBCs, a reticulocyte with a high expression of KCC would be highly vulnerable to rapid shrinkage in a low-Na\(^+\) or Na\(^+\)-free medium, a prediction confirmed by the experimental results (37). However, the nature of the Na\(^+\) and Cl\(^-\) entry pathways remains to be elucidated. Another important consideration to bear in mind is that because of their higher ionic traffic and similar volume, comparable transport perturbations will generate faster volume shifts in reticulocytes than in mature RBCs.

Model simulations of the dynamic behavior of reticulocytes in response to various stimuli revealed a surprising intrinsic instability: a brief and transient acidification or K\(^+\) permeabilization episode, which would cause only a minor, brief, and fully reversible change in the homeostatic variables of the mature RBC model, triggered a slow but accelerating irreversible drift to a final hyperdense steady-state in reticulocytes (197). This response resulted from the intracellular pH sensitivity encoded for the KCC in the model (Figs. 2 and 3), since it did not occur when acid stimulation was removed from the kinetic definition of the KCC. Analysis of this response revealed an unsuspected positive-feedback loop (Fig. 3, right): any transient perturbation that lowered intracellular pH would stimulate the KCC; the resulting KCl and water loss would cause further dehydration and further acidification (by the mechanism described above as a side effect of dehydration by isotonic KCl loss of normal RBCs), thus perpetuating KCC stimulation, further KCl and water loss, and further acidification until the driving K\(^+\) gradient was dissipated. According to the model, immediately after the perturbation, the dehydration effect would be hardly detectable, but its intensity would build up over a day or two, depending on the KCC activity attributed to the cell. The delayed dehydration response also indicated that in SS reticulocytes with the KCC regulatory kinetics reported by Brugnara et al. (46), the inhibitory effects of shrinkage on KCC activity would be insufficient to prevent progressive cell dehydration.

F. Original Working Hypothesis on the Mechanism of Fast-Track Dehydration

The reticulocyte properties described above, if valid for at least a subset of SS reticulocytes, were consistent with a new hypothetic mechanism for fast-track dehydration and ISC formation in the circulation (190, 197). In principle, the triggering perturbation in vivo could be either sickling, followed by transient activation of Gardos channels with associated cell acidification, or local RBC acidification in the microcirculation. In addition, either event could also stimulate further dehydration in propor-
tion to its frequency. Based on these considerations, a working hypothesis was proposed for a fast-track mechanism of generation of ISCs directly from SS reticulocytes, as illustrated in Figure 3: sickling of SS reticulocytes in the circulation activates $P_{\text{sickled}}$. $P_{\text{sickled}}$ increases the permeability of the membrane to $\text{Na}^+$, $\text{K}^+$, $\text{Ca}^{2+}$, and $\text{Mg}^{2+}$ allowing increased net fluxes of these ions down their electrochemical gradients. Net $\text{Na}^+$ gain through $P_{\text{sickled}}$ is largely balanced by net $\text{K}^+$ loss with no immediate volume change (265, 266). Increased $\text{Ca}^{2+}$ influx would tend to elevate $[\text{Ca}^{2+}]_i$, exceeding the activation threshold of Gardos channels in some cells within each sickling episode, and thus triggering net KCl loss and dehydration with associated cell acidification.

Acidification, in turn, would stimulate KCC-mediated dehydration with intensity proportional to the level of expression and activity of KCC in each cell. Acid-stimulated KCC dehydration would operate continuously, both in oxy and deoxy conditions, thus speeding dehydration of high-KCC reticulocytes to become dense reticulocytes and eventually young ISCs. KCC activity may be additionally stimulated by high urea in the renal medulla and by kinase-mediated effects linked to the oxygenated state of the RBCs (119, 163, 248).

The hypothesized preference for a dehydration trigger dependent on sickling rather than acidification (Fig 3) derived from the need to account for the high $\text{Ca}^{2+}$ content of ISCs. However, at the time this hypothesis was being developed, there had been no investigations of the membrane-permeabilizing effects of sickling in light, reticulocyte-rich SS cell fractions. Guided by gradualist ideas, the general experimental approach had been to follow dehydration from the “normal” volume stage onwards, i.e., either with unfractionated SS cells in which discocytes dominate, or with density-fractionated discocytes. It was unknown whether $P_{\text{sickled}}$ was activated in reticulocytes, and there were reasons to doubt it. Because of the high-power dependence of Hb S polymerization on cell Hb concentration (90), reticulocytes, whose cell [Hb] was relatively low, had not been considered important participants in sickling or in sickling-induced effects. Hence, the starting point of an investigation of this hypothesis focused on the effects of sickling in reticulocytes (37).

G. Experimental Tests of the Fast-Track Dehydration Hypothesis

The experimental results (37) showed that deoxygenation of light, reticulocyte-rich SS cell fractions caused morphological sickling as well as substantial shifts in cell $\text{Na}^+$, $\text{K}^+$, and water content of the reticulocytes. $P_{\text{sickled}}$ in reticulocytes exhibited a number of distinctive features, absent in discocytes, the most surprising of which were powerful stimulation by heparin and inhibition by divalent cations, effects which remain unexplained. Sickling-induced features common to all samples tested were increased permeability to $\text{Na}^+$, $\text{K}^+$, and $\text{Ca}^{2+}$; generation of denser cell subpopulations due to net KCl loss in excess of NaCl gain, only in the presence of $\text{Ca}^{2+}$; and systematic reticulocyte enrichment from 50–60% to over 90% in the cell fractions that became denser on deoxygenation. As we have learned more recently, most of the nonreticulocytes in the light SS cell fractions were DRCs (Fig. 1, Ref. 29), so the observed enrichment indicated that the sickling effects in these fractions were exclusive to reticulocytes. Thus, despite their relatively low cell [Hb], deoxygenation-induced sickling produced more dramatic homeostatic responses in SS reticulocytes than in the discocytes, with measurable $\text{Ca}^{2+}$-dependent dehydration evident after a single deoxygenation episode. These results fulfilled a necessary condition for the hypothesis outlined in Figure 3.

The hypothetical mechanism of fast-track dehydration (Fig. 3) generated several predictions amenable to direct experimental tests. According to the hypothesis, reticulocytes present among denser cell fractions should represent rapidly dehydrating cells on the way to becoming ISCs. If the KCC-dependent positive-feedback loop participates in fast-track dehydration, then SS reticulocytes of intermediate density should be susceptible to further dehydration by acidification while oxygenated. Acidification of oxygenated SS discocyte-rich cell fractions, containing 2–20% reticulocytes, generated fractions of denser cells highly enriched (to over 80%) in reticulocytes (37). These results then confirmed that the reticulocytes present among SS discocytes represented cells with the properties expected from fast-track dehydrating cells.

Further insight into the participation of the KCCs and Gardos channels in multitrack dehydration could be obtained by testing the responses of F reticulocytes among the SS RBCs to acidification and deoxygenation. We would expect the dehydration responses to acidification of the high-F and low-F SS reticulocytes to be similar if they exhibited similar KCC activities. The experimental results fully confirmed that the KCC acid sensitivity was retained in the F reticulocytes (37). On the other hand, after deoxygenation, with sickling inhibited in the F reticulocytes, we would expect those reticulocytes that became denser to be largely depleted of Hb F. As illustrated in Figure 1, Hb F cells tend to accumulate in the dense discocyte fraction, whereas the hyperdense, ISC-rich fraction is virtually depleted of F cells. Partially protected from the effects of sickling, the SS F cells have the longest life-span (17, 106). Most of the F cells in the discocyte fraction represent mature F cells whose KCC activity (acid sensitivity) is lost; therefore, they should not be included among the SS discocytes made denser by acidi-
fication, an expectation fully confirmed by the experimental results (37).

From the theoretical analysis and the experimental results, a multitrack-dehydration picture emerged as follows: SS reticulocytes released from the bone marrow are invariably endowed with KCC expression. Despite their low cell [Hb], upon deoxygenation they are all exposed to sickling-induced, Ca$^{2+}$-dependent dehydration via Gardos channels. Those with high KCC activity are preferentially vulnerable to fast-track dehydration and ISC formation by the combined effect of intermittent Gardos channel activation during deoxygenation and continuously stimulated KCC activity; this explains the presence of acid-sensitive reticulocytes among discocytes on their way to becoming young ISCs. ISCs have the highest total Ca$^{2+}$ reticulocytes among discocytes on their way to becoming KCC activity; this explains the presence of acid-sensitive the combined effect of intermittent Gardos channel acti-

Those reticulocytes with lesser expression of KCCs dehy-
drate more slowly, almost exclusively via Gardos chan-
gles, and mature into KCC-silent, reticulum-free disco-
cytes and dense discocytes. Sickling-resistant F reticulo-
cytes are capable of dehydration by acidification, but this capacity is largely lost by the time they reach the density of discocytes and dense discocytes (Fig. 1). This multi-
track-dehydration hypothesis provided a satisfactory ex-
planation of the major heterogeneity features of SS cells and supplied a mechanism for fast-track dehydration that could finally explain the observations of Bertles and Mil-
ner (17). Multitrack dehydration now represents the general consensus on the origin of SS cell heterogeneity and on the alternative rates of SS cell dehydration (99, 105–
109, 212).

IV. DEHYDRATION-RESISTANT CELLS

Although the multitrack dehydration model offered a satisfactory explanation of the main characteristics of SS cell dehydration, new findings showed that among the light, reticulocyte-rich SS cells, there were RBCs with unusual characteristics not corresponding to any of the known SS cell types or stages. In a study of the Ca$^{2+}$-independent K$^{(86Rb)}$ transport properties of the light, reticulocyte-rich SS cell density fraction two distinct K$^{(86Rb)}$ pools were identified which differed in their K$^{(86Rb)}$ turnover rates (94): a rapid-turnover pool, inhab-
itble by high bumetanide concentrations, and a bumet-
anide-insensitive, slow-turnover pool, each corresponding to a separate RBC subpopulation. The rapid-turnover pool, which could be tracer-equilibrated within ~5 min, suggested the presence among light SS cells of RBCs with a high K$^{+}$ permeability and low K$^{+}$ content; the possible nature and origin of these RBCs were puzzling.

The mystery of the cells with the rapid-turnover pool of K$^{(86Rb)}$ was resolved with the discovery of DRCs (29). These cells were first recognized by their failure to dehy-
drate when exposed to the K$^{+}$-selective ionophore vali-

nomycin in low-K$^{+}$, plasmalike media, a property en-
abling their detection by flow cytometry and their isola-
tion by further density fractionation. They were found to represent between 10 and 60% of the light, reticulocyte-
rich fraction of SS cells (density $<1.091$) in different patients. Resistance to dehydration proved to result from Na$^{+}$/K$^{+}$ gradient dissipation, with substantial reversal of RBC Na$^{+}$/K$^{+}$ content ratios. (Ca$^{2+}$ + A23187)-induced K$^{+}$ permeabilization exposed similar fractions of DRCs as with valinomycin. K$^{(86Rb)}$ flux studies identified DRCs as the cells responsible for the bumetanide-sensitive, rapid-
turnover K$^{(86Rb)}$ pool. The morphology of DRCs showed elongated shapes reminiscent of ISCs, suggesting a possible origin from dense ISCs. Such an origin would mean that dense ISCs would become leaky to cations, dissipate their residual K$^{+}$ gradient while gaining more Na$^{+}$ than could be balanced by the Na$^{+}$/K$^{+}$ pump, eventually swelling and migrating to lower densities. Bottom-up migration was supported by the work of Holtzclaw et al. (142), who reinjected biotin-labeled dense SS discocytes and demonstrated their upward migration in vivo through the differ-
ent SS cell density levels, with eventual accumulation in the lightest density fraction. Together, these findings sug-
gest that SS DRCs may represent the senescent condition of ISCs and other dense SS RBCs before their removal from the circulation.

Nothing is known about the putative mechanism of bottom-up migration of ISCs, on their way to become DRCs. The conversion of a K$^{+}$-depleted, dehydrated RBC to a high-Na$^{+}$, swollen RBC must involve a sufficiently large Na$^{+}$ permeability increase for net NaCl gain to overcome Na$^{+}$/K$^{+}$ pump fluxes stimulated by the high Na$^{+}$/K$^{+}$ ratio of the cells. What activates such a perme-
ability pathway? The homeostatic environment within de-
oxygenated ISCs differs from those in normal RBCs or in SS discocytes (189). In the shrunken ISCs, the volume of cell water in the polymer water compartment (PWC) of deoxy-Hb S polymers may exceed that remaining in the cytosolic phase. Most small solutes partition within the PWC, whereas all soluble deoxy-Hb S at equilibrium with polymer, and all enzymes remain excluded from the PWC (27, 191). Activation of the Na$^{+}$ permeability pathway responsible for the ISC-DRC transition may thus result in some way from this special ISC environment, or simply from the dehydration itself.

Investigation of the origin and mechanism of forma-
tion of DRCs must be considered work in progress, in its early stages. DRCs were found in much smaller numbers among normal RBCs (29), and in increased amounts among RBCs from persons with β-thalassemia intermedia and with Hb E/β-thalassemia (28). Among SS cells they constitute the vast majority of nonreticulocytes recovered in the light, reticulocyte-rich fraction, accounting for most
of the RBC heterogeneity observed in this density fraction.

V. PROPERTIES OF \( P_{\text{sickle}} \)

The most profound gap in our knowledge of the mechanism of SS dehydration concerns the molecular nature of \( P_{\text{sickle}} \). It seems ironic that over half a century after the discovery of the “first molecular disease” we should remain ignorant of the molecular nature of the permeability pathway by which the basic Hb S abnormality leads to SS RBC dehydration and all its downstream effects. Despite this important gap, a number of important functional properties of \( P_{\text{sickle}} \) have recently been characterized.

A. Ion Selectivity of \( P_{\text{sickle}} \)

As noted above, \( P_{\text{sickle}} \) increases the permeability to Na\(^+\), K\(^+\), and Ca\(^{2+}\). Sickling was also shown to generate a reversible increase in SS RBC Mg\(^{2+}\) permeability, allowing net down-gradient Mg\(^{2+}\) movements (217); this proved to be an important basis for trials of Mg\(^{2+}\)-based therapy (39), as discussed below. The alkali metal cation selectivity of \( P_{\text{sickle}} \) was studied by Joiner et al. (169), who found no differences between Li\(^+\), Na\(^+\), Rb\(^+\), and Cs\(^+\); organic cations such as tetramethylammonium, tetraethylammonium, and N-methylglucamine were excluded from the RBCs. \( P_{\text{sickle}} \) is thus best characterized as a poorly selective permeability pathway for small, inorganic monovalent and divalent cations. Investigations of the effects of deoxygenation on electrodiffusional and self-exchange anion fluxes by Joiner et al. (164) showed no effects of deoxygenation on either flux component, whereas Clark and Rossi (74) reported a two- to threefold increase in tracer-Cl\(^-\) uptake by deoxygenated SS RBCs in the presence of DIDS. Thus anion movements through \( P_{\text{sickle}} \) are either absent or so small that they have no physiological significance. As noted above, SS reticulocytes and discocytes differ in some \( P_{\text{sickle}} \) properties. Particularly intriguing is the heparin sensitivity of \( P_{\text{sickle}} \) exclusive to reticulocytes: a deoxygenation pulse with the RBCs suspended in heparinized plasma dissipates their Na\(^+\)-K\(^+\) gradients much faster than in heparin-free serum (37). Also exclusive to reticulocytes is the inhibitory effect of divalent cations on \( P_{\text{sickle}} \) (not limited to Ca\(^{2+}\)) (37, 167).

B. The Stochastic Nature of the \( P_{\text{sickle}} \) Distribution

An important property of \( P_{\text{sickle}} \) is its stochastic nature, which becomes manifest when following the sickling responses of SS RBCs (193, 201). \( P_{\text{sickle}} \) permeabilization to Ca\(^{2+}\) was followed in SS discocytes using a method that allowed separation of RBCs whose [Ca\(^{2+}\)]\(_i\) levels were below or above Gardos channel activation during each sickling pulse (193). Removal of “activated” cells from a previous pulse had little effect on the fraction of activated cells in a subsequent pulse. The sickling-induced increase in Ca\(^{2+}\) permeability persisted for the duration of each sickling episode and was fully reversed on reoxygenation. These results, together with additional experiments in which the fraction of activated cells were studied as a function of the external Ca\(^{2+}\) concentration, showed that sickling is a stochastic event of random intensity among SS RBCs, capable of generating maximal Gardos channel activation in a small fraction of cells during each deoxygenation-sickling pulse. Consistent with the stochastic nature of \( P_{\text{sickle}} \), repeated pulses led to the progressive accumulation of dense cells, whereas single long pulses caused only an early production of a single small fraction of dense RBCs. At physiological extracellular [Ca\(^{2+}\)] levels, the mean Ca\(^{2+}\) influx increased from its normal value of ~50 \( \mu \text{mol} \cdot \text{l}^{-1} \cdot \text{h}^{-1} \) to as much as ~300 \( \mu \text{mol} \cdot \text{l}^{-1} \cdot \text{h}^{-1} \) (96). But it is important to bear in mind that mean flux values in deoxygenated SS cells provide no information about the wide range of \( P_{\text{sickle}} \) values induced in individual cells. Because \( P_{\text{sickle}} \) and [Ca\(^{2+}\)]\(_i\) may transiently reach very high values in some cells, Ca\(^{2+}\) effects previously considered of doubtful relevance may in fact participate in the pathology of dense SS cells. Prominent among these are activation of Ca\(^{2+}\)-sensitive proteases (62, 124, 149, 270) and cross-linking of membrane proteins (207) with consequent increase in membrane rigidity (209, 210).

When electrophysiologists finally approach the study of \( P_{\text{sickle}} \) under patch clamp, they ought to bear in mind the probabilistic nature of \( P_{\text{sickle}} \) in each deoxygenation pulse before consulting their psychiatrist for the lack of reproducibility!

How could the stochastic behavior of \( P_{\text{sickle}} \) be interpreted? \( P_{\text{sickle}} \) must result from irreversible interactions between deoxy-Hb S polymers and membrane components that either generate a new transport pathway or modify a preexisting one. If the polymers interact in some way with the membrane cytoskeleton, they may induce the formation of oligomeric aggregates of band 3 dimers, which could function as a large ion channel (201), consistent with the observed inhibitory effect of stilbenes on \( P_{\text{sickle}} \) (161, 162). If the number of permeabilizing contacts between deoxy-Hb S polymers and the membrane in each deoxygenation pulse were small, random and variable from pulse to pulse in each cell, and if the permeabilizing structure remained stable for the duration of each deoxygenation episode, this could account for the observed stochastic behavior of \( P_{\text{sickle}} \), for the wide cell to cell variation in its magnitude, and for the constancy of \( P_{\text{sickle}} \) within each pulse (201). However, all such suggestions remain highly speculative at present, and much more
work will be needed to advance our knowledge of the molecular nature of \( P_{\text{sickle}} \).

VI. THERAPEUTIC STRATEGIES AIMED AT ION TRANSPORT TARGETS TO PREVENT SICKLE CELL DEHYDRATION

Each of the components outlined in Figure 3 participates in SS RBC dehydration and is therefore a potential therapeutic target. Following the sequence with which we analyzed the original hypothesis, we list the following specific targets: sickling (deoxy-Hb S polymerization), \( P_{\text{sickle}} \) (membrane permeabilization), Gardos channels, the electrodiffusional anion conductance pathway, KCC (K-Cl) and the Jacobs-Stewart mechanism (J-S). We will consider each of these in turn.

A. Antisickling Strategies

Deoxy-Hb S polymerization is directly responsible for \( P_{\text{sickle}} \) and is therefore a prime target. Of the many antisickling strategies considered in the past, only the use of the cytotoxic agent hydroxyurea proved both clinically successful and extensively applied (63–65, 251, 252). The rationale for its use was based on the enhancement of Hb F production it promoted in SS patients (226). However, hydroxyurea proved to have many additional effects other than enhanced Hb F production. Among many others, it increased the mean cell volumes in reticulocytes and mature SS cells (215), and this effect preceded the elevation in Hb F production (42), as did the clinical improvement in some patients. Thus, as originally suggested by Orringer et al. (215), the beneficial effects of this agent might be partly due to its direct effect on SS cell volume, the origin and nature of which remain unexplained.

Several other pharmacological agents capable of inducing Hb F production are at various stages of development, including butyrate derivatives, decitabine, a derivative of 5-azacytidine, and various combinations of these agents with hydroxyurea. Information about their mechanisms of action and current status is described in a recent overview (8).

B. \( P_{\text{sickle}} \) Inhibition

As discussed above, the molecular nature of \( P_{\text{sickle}} \) remains unknown. There is little doubt, however, that a specific \( P_{\text{sickle}} \) inhibitor would be well worth the investment of major effort. Promising future research lines in this area would be to attempt patch-clamping deoxygenated SS cells in cell-attached, or ionophore-perforated-patch configurations, to gain information about the charge-transfer properties of \( P_{\text{sickle}} \) or the development of a \( P_{\text{sickle}} \)-screening assay, to allow the systematic search for specific inhibitors. For such a search to be efficient, we need at least some preliminary clues about the molecular nature of \( P_{\text{sickle}} \) and its precise cation selectivity properties. Though not particularly enlightening, the only clues presently available are its differential properties in SS reticulocytes and discocytes considered above, and the inhibitory effect of stilbene disulfonates (161), suggesting band 3 protein participation in \( P_{\text{sickle}} \). Dipyridamole, a widely used antiplatelet agent, with limited side effects, was also shown to inhibit \( P_{\text{sickle}} \) (165), but no trials have been reported to date.

C. Gardos Channel Inhibition

After the discovery of the inhibitory effect of clotrimazole on Gardos channels by Alvarez et al. (5), its therapeutic potential was immediately recognized (49, 92). Brugnara and collaborators (81, 45, 235, 51) initiated a series of clinical trials of oral administration of clotrimazole with pharmacokinetic studies and obtained promising results [for a recent review, see Steinberg and Brugnara (253)]. Clotrimazole has many effects unrelated to Gardos channel inhibition (5, 15). A variety of adverse effects associated with oral administration of clotrimazole prompted a search for more potent and specific structural derivatives, leading to a novel Gardos channel inhibitor, ICA-17043, 10 times more potent than clotrimazole and with no effect on cytochrome P-450 (254). This agent is currently undergoing phase I/II clinical trials (253).

D. Anion Conductance Inhibition

As discussed above, although the mean value of the \( P_{\text{sickle}} \)-mediated cation fluxes elicited in each sickling episode is not particularly high, the \( \text{Ca}^{2+} \) permeability through \( P_{\text{sickle}} \) reaches sufficiently high values in a small fraction of RBCs for maximal activation of the Gardos channels (201). Because of the stochastic nature of \( P_{\text{sickle}} \), most (low-F) SS cells will eventually be exposed to transient periods of maximal Gardos channel stimulation in the circulation. During such periods, the extent of dehydration could be effectively reduced by blocking the anion conductance, thus reducing net KCl or \( \text{KHCO}_3 \) loss, a therapeutic strategy developed by Bennekou and colleagues (11–14). A novel family of anion conductance inhibitors, NS1652 (13) and NS3623 (12), appeared effective in reducing Gardos channel-mediated dehydration of SS cells and offers a promising new approach in SS therapy.
E. Control of KCC

No specific KCC inhibitors are currently available. Increased intracellular [Mg²⁺] and cell alkalization partially inhibit KCC in SS cells (53, 58, 179). Based on these effects and on the fact that $P_{\text{sickle}}$ permeabilizes SS cells to Mg²⁺ (217), Bookchin et al. (39) proposed a rationale for the possible benefits of controlled hypermagnesemia in the treatment of sickle cell anemia. By setting the plasma extracellular [Mg²⁺] levels above the electrochemical equilibrium of Mg²⁺ across SS cell membranes, deoxygenated SS cells would gain MgCl₂ and water, thus elevating $[\text{Mg}^2+]_i$ and $[\text{Cl}^-]_i$, with secondary cell alkalization due to reduction of the inward Cl⁻ concentration ratio. These effects should tend to reduce KCC activity and KCC-mediated SS cell dehydration. Additional beneficial effects, independent of KCC mediation, were expected from reduced Ca²⁺ influx through $P_{\text{sickle}}$ by competition with plasma Mg²⁺, from systemic vasodilatation, muscle relaxation, and inhibition of platelet aggregation. Anecdotal reference to beneficial Mg effects in preventing crisis in individual SS patients had been advanced many years before by Lehman (185). De Franceschi and collaborators performed the first systematic studies of oral Mg²⁺ supplementation on transgenic sickle mice (79) and SS patients (78). In both studies, the Mg²⁺ and K⁺ content of SS cells was substantially normalized, KCC activity was reduced, and SS cell density was lowered. Prolonged administration of oral Mg pidolate had the additional effect of reducing the frequency or extent of painful crises (78), thus confirming Lehman’s original observations. In experimental conditions it was possible to delay or block dehydration-induced cell acidification using anion exchange inhibitors (111). Such an effect would prevent the operation of the KCC feedback loop through the Jacobs-Stewart mechanism (Fig. 3). However, because of the multiple functions of the anion exchanger, this may prove a dangerous strategy even if suitable inhibitors became available.

VII. OPEN QUESTIONS AND THE DIRECTION OF FUTURE RESEARCH

Sickle cell anemia and related sickle cell disease syndromes have long been models for the investigation of RBC physiology and pathophysiology, and they remain an open field for extensive further investigation. At this stage, many of the fundamental issues underlying the mechanisms of sickle cell dehydration have been addressed, as well as the explanation of the age and volume heterogeneity of SS cells. Two major issues remain open in this area of research. The first concerns the molecular nature of $P_{\text{sickle}}$ addressed above. The second concerns the origin and mechanism of formation of DRCs. Do these cells represent the homeostatic condition of senescent ISCs, as the early observations suggested? By what mechanism do hyperdense ISCs become light (low density) DRCs? Could this be a common final path for discocytes and Hb F cells as well? Could DRCs from normal subjects also suffer a similar fate? Is the DRC condition an epiphenomenon or an essential part of the mechanisms of programmed RBC senescence and circulatory removal (7, 68, 208)?

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