# PROGRESS IN HEMATOLOGY

Volume XIII

*Edited by*

Elmer B. Brown, M.D.

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GRUNE & STRATTON
*A Subsidiary of Harcourt Brace Jovanovich, Publishers*
New York London
Paris San Diego San Francisco São Paulo
Sydney Tokyo Toronto

Grune & Stratton, Inc.
111 Fifth Avenue
New York, New York 10003

*Distributed in the United Kingdom by*
*Grune & Stratton, Inc. (London) Ltd.*
*24/28 Oval Road, London NW 1*

Library of Congress Catalog Number 56-58463
International Standard Book Number 0-8089-1615-7
Printed in the United States of America

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# INTRODUCTION

Volume XIII of *Progress in Hematology* extends the series into the second quarter of a century. It is the result of the editor's conviction that the continuing fragmentation of hematology into multiple subdisciplines, with concepts and terminology that are beyond the comprehension of most generalists, requires effective interpretative reviews to allow us to stay abreast of the advances in hematology. Those looking only for the immediate payoff of information applicable to a busy practice may be disappointed in a number of chapters that examine more basic information awaiting application. For the student of hematology, however, whether he or she is in training, in the academic whirl as a teacher or investigator, or is the thoughtful clinician who is aware that better new concepts must be brought to bear on many longstanding problems—this volume should help explain some areas where progress is taking place.

Once again, the editor is most grateful to the dedicated authors of these reviews for sharing their enthusiasm and expertise in explaining their topics and meeting stringent time restraints.

Elmer B. Brown, M.D.

# Contents: Volume XIII, 1983

Robert M. Bookchin, M.D.
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# Red Cell Membrane Abnormalities in Sickle Cell Anemia

Sickle cell anemia, the first "molecular disease," due to the homozygous inheritance of the amino acid substitution $\beta 6$ glutamic → valine in the $\beta$-chains of hemoglobin (Hb), has thus far remained resistant to "molecular attack" at its source. Although genetic modifiers, such as those determining red cell content and distribution of fetal Hb,[1] may contribute to wide clinical variability, the basic pathophysiology of sickle cell anemia must ultimately be traced to the effects of the abnormal Hb in the "SS" red cells. Nevertheless, the presence of a high proportion of HbS in the cells somehow results in structural and functional abnormalities of their membranes, which may contribute to the two major clinical features of sickle cell anemia—hemolysis and widespread microvascular occlusion.

The red cell membrane, once viewed by hematologists as a mysterious sac to enclose hemoglobin and its supporting enzymes, is beginning to yield to structural analysis. Membrane physiologists, formerly content with using red cells simply as a convenient tool to study the biophysics of ion transport, are now drawn to look at cell shape changes in the microscope and to join biochemists in the search for structural–functional correlations. Here studies of pathophysiology and physiology become mutually enlightening, because the answers to how sickling, HbS, or other Hb abnormalities lead to structural and functional disturbances of the red cell membrane should improve our understanding of both the normal and abnormal states. Meanwhile, the lack of success in finding an effective anti-sickling agent directed against the HbS molecules has led therapy-oriented workers to ask whether prevention of membrane abnormalities, or membrane control of the cell environment, might alleviate the underlying disorder.

The earliest suspicion of abnormalities in sickle cell anemia (SS) red cell membranes arose from observations of cell morphology. With fully oxygenated SS blood, most of the red cells appear as normal biconcave disks, but a variable proportion (5 to 40%) appear spindle- or cigar-shaped or, less frequently, long, shrunken, and wrinkled. Many of these elongated forms resemble smooth versions of bi-pointed deoxygenated sickle cells, and since their abnormal shapes persisted with full oxygenation, they were termed

This work was supported by grants HL-28018 and HL-21016 from the NIH and from the MRC.

"irreversibly sickled cells" (ISCs). Unlike deoxygenated SS cells whose multi-pointed shape distortions are produced by long bundles of polymerized HbS fibers, electron microscopy of oxygenated ISCs showed no intracellular polymer fibers, and their shapes were therefore attributed to persistent or permanent membrane changes.[2] The persistent shape abnormality of the ISCs suggested that these cells or their membranes, or both, were relatively rigid and nondeformable. Another feature of ISCs recognized early was an increased density associated with an increased cell Hb concentration,[3] a property which, in the light of our present understanding of the high concentration-dependence of HbS polymerization, should make these cells particularly prone to trapping in the reticuloendothelial system and more likely to initiate capillary occlusion. Because of their dramatic morphological changes, dehydration, and other features described in detail below, the ISCs are viewed as having the most extensive and advanced membrane alterations of the SS cell population, and functional abnormalities of SS cell membranes are often considered in terms of how they may contribute to the generation of ISCs. We will, therefore, focus first on the features of this advanced-stage membrane lesion.

## GENERAL PROPERTIES OF IRREVERSIBLY SICKLED CELLS

Apart from the morphology that defines them, the first distinctive features of ISCs were described by Bertles and Milner,[3] who observed that they were concentrated at the bottom of ultracentrifuged cell columns. The denser cell fraction, nearly all ISC, was found to have a normal mean cell Hb content but reduced mean cell volume, so that their increased intracellular Hb concentration (MCHC) must reflect dehydration. The high density provided a fairly simple means of separating ISCs from the rest of the SS cells for further study. It was also shown that the propensity for SS red cells to become ISCs was not related simply to their age (or time spent in the peripheral circulation) but to another aspect of heterogeneity, their relative proportion of fetal Hb (HbF). All SS cells are not created equal. As in other states of marrow erythroid stress, increased synthesis of $\gamma$ chains (of HbF) occurs in some cells but not others; thus, the increased HbF levels in SS red cells is not evenly distributed. ISCs were found to contain relatively little HbF, compared with non-ISCs; since fetal Hb admixed with HbS inhibits polymerization of the deoxy Hb, it would seem that those SS cells more prone to intracellular polymerization and sickling are more likely to become ISCs. By a variety of isotopic labeling techniques, the same investigators found that ISCs had a particularly rapid turnover in the circulation. They were not released from the bone marrow as such, but were formed relatively early in the circulatory life of SS cells, reaching a maximum in 4 to 7 days; they then tended to disappear from the circulation faster than mature non-ISCs, although their absolute numbers were maintained by continued formation.

The increased density and dehydration of ISCs are associated with a low total cation content of the cells. Tosteson et al. had first observed that the SS cell population as a whole had increased Na and decreased K concentrations,[4] but others have since found that the extent of the cation alterations, as well as a reduced cell water content, correlated with the numbers of ISC in the samples. Measurements on density fractions of SS cells[5,6] showed that discoid SS cells with normal density had normal or near-normal Na and K concentrations. In all except the lowest-density fractions, free of ISCs, there was a moderate increase in cell Na (to about 20 to 30 mEq/liter rbc), which continued to rise slightly with density (in terms of Na concentration in cell water). More dramatic was the

change in cell K, which fell progressively with increasing density and ISC proportions to a level of about 30 mEq/liter of shrunken cells. Since the fall in the K content was much greater than the rise in Na in the ISCs, their total cation content was quite reduced, consistent with the observed dehydration. The total calcium content of all SS cells is considerably increased[7-9] (as discussed in a later section), with the highest concentrations in the ISC fraction, particularly the most dense, shrunken cells, which may contain over 200 $\mu$moles Ca/liter rbc.[10]

Some measurements of ISC composition, and their interpretation, are complicated by the increased cell Hb concentration and reduced cell water content. A relatively low intracellular pH observed in the dense ISC-rich fractions of SS cells with MCHCs over 40 g/dl[11] may be partly related to the increased negative charge density of Hb. Although the molar ratios of 2,3-diphosphoglycerate (2,3-DPG) to Hb in this dense SS cell fraction were reported to be slightly low (0.86),[11] if we express the concentration in (reduced) cell water, to judge cell metabolism rather than Hb function, even the bottom SS cell fraction had 2,3-DPG concentrations about 20 percent higher than those of normal red cells. When adenosine triphosphate (ATP) levels in the dense ISC-rich fraction of SS cells were referred to cell numbers, with about 0.9 mmoles per $10^{13}$ rbc, ISCs were said to be "grossly depleted of ATP."[12] Similar measurements expressed as concentration per volume cells, however, were normal, and in terms of ISC water content, slightly increased.[6] These concentrations are important in judging the "metabolic state" of the cells, particularly with reference to membrane functions. From a functional standpoint, ISCs are not "metabolically depleted."

## MEMBRANE STRUCTURE OF IRREVERSIBLY SICKLED CELLS

The abnormal shape of ISCs persists in their membrane ghosts after hemolysis[13] and even after the ghosts are extracted with the nonionic detergent Triton X-100, which solubilizes all of the integral membrane proteins and most of the lipids, leaving the so-called cytoskeleton[14] or membrane skeleton. In the present view, red cell shape is determined mainly by this network of proteins on the cytoplasmic membrane surface, primarily spectrin, band 4.1* and actin. Spectrin, a long, flexible, high molecular weight protein comprising about 75 percent of the cytoskeletal protein mass, appears to exist primarily as $\alpha_2\beta_2$ tetramers (molecular weight 920,000, about 200 nm long), formed by end-to-end association of two $\alpha\beta$ heterodimers. Current information[15] indicates that spectrin is attached to the membrane by a specific high-affinity association with ankyrin (mainly protein band 2.1), which in turn is bound to the major transmembrane protein, band 3. By separate interactions less well understood, spectrin, actin, and band 4.1 associate as part of the skeletal meshwork. The associations between these components are apparently non-covalent, and shape changes in the normal red cell membrane may involve primarily molecular rearrangements.

Protein analyses of ISC membranes by standard techniques of SDS-PAGE or by two-dimensional electrophoresis have revealed variable differences in the relative intensity

*Membrane protein band numbers refer to the standard nomenclature of Steck[115] applied to polyacrylamide gel electrophoresis patterns obtained with ghosts solubilized in sodium dodecylsulfate (SDS-PAGE).

of some protein bands, but no consistent qualitative differences from normal, that is, no unique proteins.[16] Several groups, however, have found evidence of altered arrangement or behavior of normal protein components in ISC membranes, such as increased formation of spectrin-rich disulfide-linked polymer during ATP and reduced glutathione (GSH) depletion in vitro[17] and an increased extent of protein cross-linking by dithiobis (succinimidyl propionate).[16] It should be kept in mind that ISCs are not metabolically depleted and these are findings of altered reactivity. Platt et al.[18] recently described a decrease in spectrin binding by inside-out vesicles (IOVs) prepared from SS membranes (particularly with those prepared from ISCs) compared with AA membrane IOVs, despite identical protein patterns on SDS-PAGE and less than 1 percent residual Hb content. This observation indicated a decrease in the number of functional spectrin binding sites, suggesting alterations in ankyrin or ankyrin-band 3 complex in these membranes.

Findings by Lux and John[19] that ISC-shaped ghosts become round in media of high or low ionic strength, under conditions that inhibit formation of spectrin–actin complexes, support the likelihood that their altered shape is stabilized by non-covalent interactions between rearranged cytoskeletal proteins rather than fixation by covalent cross-links. It remains to be determined whether there are structural chemical changes in ISC membrane proteins which underly or accompany these rearrangements.

Although the overall lipid composition and quantity in SS cells and their ISC fraction did not differ significantly from similar-aged young normal red cells[6,20] there is considerable evidence of alterations in the membrane lipid organization. This was first detected as differences in the reactivity of membrane aminophospholipids of oxy-SS cells to a chemical probe.[21] With similar methods, evidence of phospholipid rearrangement was found during deoxygenation of SS discocytes, which was reversible on reoxygenation, and appeared permanent in oxy-ISC.[22] Further details of this same pattern of abnormalities was reported by Lubin et al.,[23] who exposed density-fractionated SS and normal red cells to non-penetrating phospholipidases, and found that deoxygenated SS discocytes and oxy-ISCs both showed an increase in phosphatidylethanolamine (PE), decreased phosphatidylcholine (PC), and the appearance of phosphatidylserine (PS) in the outer leaflet. These changes were recently correlated with evidence of an increased rate of transbilayer movement of PC in the membranes of SS cells during deoxygenation.[24] It is not yet known whether or how any skeletal protein rearrangements described above might be related to these lipid abnormalities, but the latter changes may have some direct pathological consequences: deoxygenated SS discocytes, oxy-ISC, and liposomes prepared with the corresponding outer-leaflet phospholipid composition were all shown to accelerate the blood clotting time in vitro[25] suggesting a possible contribution to the process of microvascular occlusion.

Evidence was presented that the distribution of negative charges on the surface of SS cells may be abnormal, based on clumping (rather than the normal even distribution) of cationized ferritin; these abnormalities might account for an observed increased adherence of oxy-SS cells (particularly ISCs) to cultured vascular endothelium,[26] which has been correlated with vasocclusive severity.[27] Other investigators, however, comparing the distribution of colloidal iron hydroxide on normal and SS ghost surfaces, found no evidence of differences in surface charge.[28] In a recent report, washed SS cells perfused through human umbilical cords, or through glass capillaries in which endothelial cells were cultured, showed minimal adherence, and no more than that of normal red cells. The possibility was raised that the observed increase in SS cell adherence may be due to plasma factors in SS blood,[29] and such plasma factors have, indeed, been found to play a major role in the observed adherence.[30]

## HEMOGLOBIN-MEMBRANE INTERACTIONS IN SICKLE CELLS

There has been much interest in the possible role of Hb in the structure and function of normal and abnormal red cell membranes. Native Hb binds to normal membranes under conditions of low ionic strength and pH ($< 7.2$); this binding increased with the net positive charge on the Hb ($HbA_2 > HbA$) and was disrupted at increased ionic strength or pH, suggesting an electrostatic type of interaction.[31] The nature and location of this type of binding was studied in detail by Shaklai et al.,[32,33] who measured hemoglobin quenching of a fluorescent probe embedded in the membrane bilipid layer, and found that at low ionic strength and pH (6.2), high-affinity binding sites for Hb were apparently located on or near the cytoplasmic surface of the transmembrane protein "band 3." Using similar fluorescent probes, Eisinger et al. recently developed methods to assess probe quenching by Hb in intact red cells (see below),[34] and found evidence of progressive binding or proximity between Hb and band 3 protein as intracellular pH was reduced below 7.2, to 5.9.[35] These findings suggest that such binding can occur in the ionic environment of the red cell, but that little or no binding takes place in the physiological pH range. This is not to say, however, that Hb has no effect on membrane structure. In a manner not yet clear, the state of aggregation of the membrane skeletal protein spectrin appears to be affected by the presence of Hb; the observed effects are probably not specific for Hb, and seem to be related to its surface charges.[36,37]

In SS red cells, Hb–membrane interactions may differ from normal in two ways: First, there is evidence of increased binding of native HbS to isolated membranes (at low pH and ionic strength), which might be partly due to the smaller net negative charge of this Hb.[31,38] But Shaklai et al.[39] found that after membranes were exposed to HbA or S at low ionic strength, much less of the HbS dissociated by raising the ionic strength to normal or by addition of glyceraldehyde-3-phosphate, which competes electrostatically with the Hb for membrane (band 3) binding sites; this raised the possibility of hydrophobic or irreversible binding of HbS to the membrane. Fischer et al.[31] observed that ghosts prepared by hypotonic lysis and washing of the dense, ISC-rich fraction of SS cells retained more Hb than ghosts from the lighter SS cells and also showed less binding of new Hb, but the state of the Hb bound to the ISCs was not determined.

A second type of membrane–Hb association in SS cells may involve denatured Hb derivatives. Although HbS is not considered a typical Heinz body-forming "unstable" Hb, multiple small inclusion bodies have been found in virtually all SS cells[40,41] that correlate with retention in their ghosts of relatively high amounts of denatured Hb exhibiting an absorption spectrum typical of hemichromes.[42,43] This property may be related to the relative instability of oxy-HbS on mechanical shaking or repeated deoxygenation and oxygenation, observed by Asakura et al.,[44] and to its increased propensity to surface denaturation.[45] In a survey of red cells from persons with several genetic variants of sickle cell disease, Kim et al.[41] found that high levels of both denatured Hb (in the ghosts) and inclusion bodies correlated with clinical severity of the sickling disorder, with a low proportion of HbF and absence of a functioning spleen, but not with the percentage of circulating ISCs. Similarly, Campwala and Desforges,[43] who examined ghosts from density-fractionated SS and normal red cells, found uniformly high levels of hemichrome in each SS fraction; but they found equally high levels only in the most dense (oldest) fraction of normal cells, which was thought to reflect accumulated oxidative damage to the Hb. These findings suggest that the polymerization tendency per se, which is greatest in the dehydrated ISCs, may have little to do with the increased denaturation of HbS and that

the latter may be an independent property of the Hb. The disproportionately higher levels of hemichrome in red cells from clinically severe sickling disorders (which also have higher proportions of HbS in the cells) could be due to the functional asplenia resulting from their increased sickling tendencies.

Although the terms *membrane-associated* and *membrane-bound* denatured Hb seem to be used interchangeably, it is not established whether these inclusions in SS cells (and the hemichrome retained in ghosts after hypotonic lysis and washing) are truly membrane-bound or floating freely in the cytoplasm and simply trapped in the ghosts. The difference could be of some relevance in terms of rheology and membrane structure and function in SS cells. Ultrastructural studies of cells containing the larger Heinz bodies occurring with unstable HbS or exposure to oxidant drugs suggests that they can be free within the cells;[46,47] if membrane attachment does occur, the binding seems to be non-covalent.[48]

The possible presence of native or denatured Hb, or both, associated with the membranes in SS cells led to suggestions that polymerization of membrane-bound HbS may contribute to structural and functional membrane abnormalities.[49] Electron micrographs of freeze-etched membranes of deoxygenated SS cells were described as showing polymer fibers fixed to the inner membrane surface,[50] but there is conflicting evidence as to whether the membrane plays a role in intracellular polymerization of deoxy-HbS. One report that intact or fragmented membranes accelerated polymerization[51] was not supported by a subsequent study, in which neither the kinetics of polymerization nor the equilibrium solubility of deoxy HbS was altered by addition of normal ghosts or inside-out membrane vesicle preparations.[52] Coletta et al.[53] measured the kinetics of HbS polymerization in individual SS red cells by observing the change in light scattering following laser photolysis of carboxy-Hb in the presence of dithionite; they found a wide range of delay times, which correlated well with the behavior of membrane-free Hb solutions whose concentrations and compositions were comparable to those in the cells. Eisinger et al.[34] recently developed techniques to study the membrane-Hb interface in intact red cells; the efficiency of resonance energy transfer from fluorescent probes imbedded in the lipid bilayer to heme acceptors, which is a measure of the proximity of Hb to the probes and its concentration close to the bilayer, was estimated using front-face fluorometry to determine the donor decay rates. When normal red cells were compared with density-fractionated SS cells by these methods,[54] the Hb concentration at the membrane-cytosol boundary or its proximity to the probes was considerably higher than normal for all SS cells and rose with increasing cell density and ISC count. Ghosts prepared by hypotonic lysis of the SS cell fractions, however, showed no more fluorescent quenching than normal ghosts, so the increased Hb in the boundary layer of SS cells could not be attributed to heme-containing material irreversibly fixed to the membranes. It would appear that in oxygenated SS cells there is an increased association of native Hb with the membrane, which may be due to the lower net negative charge of HbS and to the lower intracellular pH of SS cells, particularly the ISCs.[11] Upon deoxygenation and sickling, however, quenching of the membrane probes by Hb fell to about one-third of the oxy value for each density fraction of SS cells. When normal red cells were deoxygenated, a considerably smaller decrease in quenching was observed, which could be due to the increased net negative charge on deoxy Hb in the presence of organic phosphates. The decreased quenching for both SS and normal cells was reversible with reoxygenation. These findings indicate that deoxy HbS withdraws from the membrane bilipid layer to a greater extent than does normal deoxy Hb. Thus, the bulk of current evidence does not support any direct role of the membrane or membrane-bound Hb in the polymerization process.

## OXIDATIVE DAMAGE TO SICKLE CELL MEMBRANES

The possibility of oxidative damage to red cells, explored previously in certain drug-induced and congenital Heinz-body hemolytic anemias and thalassemia, has more recently been considered and studied with SS cells. As discussed by Babior,[55] red cell damage by oxidizing agents involves (1) denaturation of Hb via methemoglobin to hemichromes and perhaps denatured globin, and (2) peroxidation of polyunsaturated fatty acids of membrane lipids, with generation of malonyldialdehyde (MDA), which can cross-link amino- or sulfhydryl-containing molecules. Red cell defenses against oxidation include enzyme systems for reducing metHb and maintaining NADH; vitamin E, which inhibits lipid peroxidation; superoxide dismutase to promote conversion of $O_2^-$ to $H_2O_2$; and catalase and the glutathione peroxidase/reductase enzymes to break down $H_2O_2$ (and maintain GSH).

A variety of abnormalities relevant to these systems have been described in SS cells. Chiu and Lubin found moderately decreased levels of vitamin E in both plasma and red cells of SS patients, associated with decreased intestinal absorption of the vitamin, and an increase in red cell glutathione peroxidase, which was thought to be compensatory.[56,57] SS cells showed an increased susceptibility to lipid peroxidation in vitro (estimated by MDA generation on exposure of cells to exogenous $H_2O_2$), which was normalized by addition of vitamin E[57] but was increased during deoxygenation only in the SS cells.[22] The latter response was thought to be related to the lipid reorganization in deoxy SS cells (described previously).

Hebbel et al.[58] reported that SS cells spontaneously generated twice as much superoxide, peroxide, and hydroxyl radical as normal red cells during in vitro incubation. The amounts of hydroxyl radical generated correlated with measured amounts of hemichrome material retained in the ghosts after hypotonic lysis, but not with the ISC count, reticulocyte count, or SS cell density. There is some evidence that the aging process in normal red cells involves accumulated oxidative membrane damage, and it is argued that acceleration of this process might play a role in the shortened survival of SS and other abnormal red cells. Jain and co-workers[59] found that membranes of dense, older red cells from normal rats showed increased amounts of fluorescent chromolipids and high molecular weight polymers and that these changes could be reproduced by addition of small amounts of MDA to unfractionated cells; exposure of normal (human) red cells to micromolar amounts of MDA produced cross-links between membrane aminophospholipids, and it appeared that some of the same cross-linked material was present in untreated membranes of dense SS cells.[60]

There is no question that exposure of red cells to sufficient exogenous oxidants causes damage to Hb and membrane and can result in red cell destruction. It is not yet clear, however, whether the increased amounts of oxidants generated endogenously in unstable Hb disorders or SS cells produce significant oxidative damage to the membrane or remain within the capacity of the cells' protective mechanisms.[55,61]

## MECHANICAL AND FLOW PROPERTIES OF SICKLE CELLS

The capacity of normal red cells (whose disk diameters are about 8 $\mu$m) to deform and squeeze through openings as small as 3 to 4 $\mu$m is critical to normal microcirculatory blood flow. Current questions center on the relative contributions of increased internal viscosity (e.g., due to cell dehydration and increased Hb concentrations), changes in cell surface-to-

volume ratio, altered mechanical properties of the membranes, and possible membrane—Hb interactions to abnormalities in whole-cell deformability. There is no question that even partial polymerization of intracellular HbS raises the internal viscosity of SS cells and reduces cell deformability. But fully oxygenated SS cells have also been shown to exhibit rheological abnormalities that are most severe in the dense ISCs.[62] There is also evidence of an increased tendency of (oxy) SS cells to aggregate[63] and possibly to adhere to vascular endothelium,[26] either of which could promote microvascular occlusion.

Various methods have been used to examine the rheologic and mechanical properties of red cells and their membranes. These techniques include measurements of bulk viscosity of cells suspended in plasma or buffers at varying hematocrits or shear rates, microsieving through polycarbonate filters with pore size 5 $\mu$m or less, and aspiration of part or all of the cells into micropipettes at measured pressures. In addition, special instruments have been designed to study the behavior and properties of cells under shear stress, such as the rheoscope, which permits microscopic observation of cell behavior during quantified shearing,[64] and the ektacytometer,[65] in which cell deformability is estimated by changes in laser diffraction patterns of cells subjected to laminar fluid stress. A variety of different or overlapping cell and membrane properties, often not precisely defined, are measured by these various techniques, and investigators have not agreed on which measurements or properties are most relevant for predicting whole-cell deformability and capillary flow behavior in vivo.[66,67] Under conditions of high shear stress in the ektacytometer, increased internal viscosity appears to dominate rheological behavior of ISCs, whereas any changes in membrane elasticity (rigidity), as detected by micropipette aspiration, would have greatest effects at low shear rates, that is, situations of slow flow.[66] By each type of measurement, the more dense, ISC-rich SS cell fraction is distinctly abnormal, even in the oxygenated state. Clark et al.[68] found that a marked decrease in deformability of oxy-ISC as measured with an ektacytometer could be nearly normalized by swelling them osmotically to normal MCHC values; artificially dehydrated cells were relatively nondeformable, and SS discocytes (with normal MCHC) behaved normally. These studies demonstrate a major role of dehydration per se in reduced SS cell deformability. On the other hand, Platt found that even after rehydration in vitro, ISCs subjected to rather high shear stresses in a cone-plate viscometer showed a persistent increased rate of hemolysis, indicating a mechanical fragility unrelated to cell Hb concentration.[69]

Studies using micropipettes with 2.6 to 3.2-$\mu$m bores, near the critical size allowing full aspiration of the cells, suggest that SS discocytes have normal membrane rigidity and cell deformability, but abnormalities seen with partial aspiration of ISCs[70] or full aspiration of both SS discocytes and ISCs[71] cannot be ascribed to specific cell or membrane properties. Specific measurements of membrane mechanical properties, which require use of smaller bore pipettes,[72] have yet to be done.

Smith et al.[73] recently distinguished populations of "hard" and "soft" ISCs based on their ability to pass through 3-$\mu$m Nucleopore filters. The hard ISCs, which comprised about 5 to 20 percent of the ISCs filtered, required higher pressure for aspiration into 3-$\mu$m micropipettes and were more resistant than the soft ISCs to aspiration into pipettes and filters with 2-$\mu$m bores. Interestingly, these two populations (derived from filtration of density-separated fractions containing over 80% ISCs) were not distinguishable morphologically, by mean cell volume, osmotic fragility, mean cell Hb concentration, or density gradient profiles. Therefore, the mechanical or deformation properties observed could not be attributed to differences in hydration or surface area and suggested intrinsic structural membrane alterations in the hard ISCs.