# Static and Dynamic Rigidities of Normal and Sickle Erythrocytes

## Major Influence of Cell Hemoglobin Concentration

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**Abstract.** Static and dynamic deformabilities of erythrocytes are important determinants of microcirculatory blood flow. To determine the influence of increased cellular hemoglobin concentration on these properties, we quantitated static and dynamic deformabilities of isolated subpopulations of oxygenated normal and sickle erythrocytes with defined cell densities using micromechanical manipulations of individual cells. The rheological properties measured to characterize static deformability were membrane extensional rigidity and bending rigidity. To characterize dynamic deformability of the cells, we measured the time constants for rapid elastic recovery from extensional and bending deformations.

The extensional rigidity of sickle cells increased with increasing cell hemoglobin concentration while that of normal cells was independent of the state of cell hydration. Moreover, sickle cells were found to exhibit inelastic behavior at much lower cell hemoglobin concentrations than normal cells. In contrast, the dynamic rigidity of both normal and sickle cells was increased to the same extent at elevated hemoglobin concentrations. Moreover, this increase in dynamic rigidity with increasing cellular dehydration was much more pronounced than that seen for static rigidity. Both the increased static and dynamic rigidities of the dehydrated sickle cells could be greatly improved by hydrating the cells. This suggests that increased bulk hemoglobin concentration, which is perhaps inordinately increased adjacent to the membrane, plays a major role in regulating the rigidity of sickle cells. In addition, irreversible membrane changes also appear to accompany cell dehydration in vivo, resulting in increased membrane shear rigidity and plastic flow. We expect that the marked increases in rigidity of dehydrated sickle cells observed here may have a major influence on the dynamics of their circulation in the microvasculature.

## Introduction

Both the static (time-independent) and dynamic (time-dependent) deformabilities of erythrocytes are important determinants of local flow rates and distribution in microcirculatory beds. Abnormal cell rheology can alter blood distribution and, in severe cases, even cause local occlusion of capillaries. It is generally recognized that the rheological behavior of erythrocytes in sickle cell disease is abnormal, especially for the oxygenated dense irreversibly sickled cell (ISC)[1] fraction and in states of reduced oxygen tension for all cell populations (1–6). However, the extent to which the rheology of the more nearly normal, less dense cell fractions are compromised by the disease is not known. Moreover, the relative contributions of intrinsic membrane and cytoplasmic properties to cellular deformability in sickle cells is also not known.

Almost all of the previous work on sickle erythrocyte properties has involved cell suspensions or single erythrocytes in fluid shear fields. Although these experiments provide good indications of relative changes in cellular deformability, they do not provide measures of the material properties of the cells independent of the experiment. Moreover, it is not possible to differentiate between static and dynamic deformation response of the cell to external forces in such fluid shear and suspension experiments. The static and dynamic rigidities would both be expected to significantly modulate the rate of entry of erythrocytes into small capillaries and influence the pressure drop

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1. *Abbreviations used in this paper:* ISC, irreversibly sickled cells; B, membrane bending modulus; $\mu$, membrane extension modulus; $\Delta t$, capillary entrance time; $\tau_e$, time constant for rapid elastic recovery from extensional deformation; $\tau_f$, time constant for rapid elastic recovery from bending deformation.

across small vessels of caliber less than the cell diameter. Thus, it is important to determine these properties for both sickle and normal erythrocytes. Moreover, since widely varying cellular hemoglobin concentrations are a characteristic feature of sickle cell disease, it is also important to correlate these measurements with that parameter. Finally, detailed evaluation of static and dynamic deformation responses might well provide insight into the relative roles of the membrane and the cytoplasm in altered cell rheology.

Cellular rigidity and deformability are descriptive terms which depend on a number of extrinsic and intrinsic cellular factors. The extrinsic factors are the surface area-to-volume ratio and cell conformation. The surface area-to-volume ratio simply defines a geometric constraint for cell deformation; that is, the cell can easily be deformed as long as the surface area is not required to increase or the cytoplasmic volume to decrease. The influence of cell conformation on deformation is subtle and not easily quantitated. Examples of conformation characteristics that affect deformation of a cell as it enters a capillary include length-to-breadth ratio and cell sphericity. The sufficiently redundant surface area-to-volume ratios of normal and sickle erythrocytes do not restrict cell deformation. Likewise, the conformational influence of the cell morphology appears to be minimal. Hence, extrinsic rigidity factors do not appear to be of primary interest in comparison to microrheological properties of normal and sickle erythrocytes. The crucial variables are intrinsic material properties. These include membrane extension and bending rigidities, time response of the membrane to extension and bending forces, recovery of the membrane after deformation, and cytoplasmic properties (cytoplasmic viscosity and yield stress).

When erythrocytes enter small capillaries, they are deformed by extension and folding. Such deformations cause: (*a*) membrane extension without change in surface area (i.e., a local square region of the membrane surface is extended into a rectangle without change in its area); (*b*) membrane bending or folding; and (*c*) shear and displacement of the cytoplasm. The simplest rheological approximation that has been successfully used to quantitate intrinsic rigidity properties of normal erythrocytes is based on the view of the cell as a liquid interior encapsulated by a viscoelastic solid membrane shell (7). In this model, the membrane extension and bending moduli, $\mu$ and B, characterize the static rigidities of the cell, and the time constants for rapid elastic recovery from extension and bending deformations, $\tau_e$ and $\tau_f$, characterize the dynamic rigidities of the cell. The dynamic rigidities of the cell limit the rate of entry into a small capillary. These rigidities are proportional to the static coefficients of rigidity for extension ($\mu$) and bending (B), multiplied by the respective material time constant ($\tau_e$ or $\tau_f$), and inversely proportional to the capillary entrance time ($\Delta t$), i.e., $\mu \cdot \tau_e/\Delta t$ and $B \cdot \tau_f/\Delta t$. Hence, not only does the static resistance to cell deformation affect cell transit through small capillaries, but the dynamic resistance can also be a significant impedance to cell transport through the microcirculation, since the flowing cells must enter the capillary during a brief interval ($\Delta t$). Another important rheological property is the "recoverability" of cell shape after the cell has been deformed at very slow rates. Observations of recovery in deformation experiments of long duration (e.g., a few minutes) evaluate the tendency for cells to retain persistent deformations on a time scale much longer than the capillary transit time. This is important because persistent deformations of the cell could be enhanced by polymer formation in regions of low oxygen tension and perhaps be involved in the production of ISCs.

Erythrocyte mechanical properties are readily accessible to measurement with the use of micromanipulation techniques. Aspiration by small micropipettes provides the requisite level of force ($10^{-6}$ dyn) to deform the cell such that both cell deformation and force can be quantitated. This approach has been widely used to determine properties of normal and abnormal erythrocytes (8–12). In the present study, we have used similar methods (described in Methods) to establish values for the rigidity properties of oxygenated normal and sickle erythrocytes as functions of cell density and hence cell hemoglobin concentration. Our intent has been to investigate the relative involvements of the membrane and the cytoplasm in the microrheology of erythrocytes, and to evaluate the extent of alterations in membrane material properties in sickle cell disease.

## Methods

*Cell preparation.* Blood was drawn from three patients with homozygous sickle cell disease and two normal volunteer donors into acid citrate dextrose. To isolate cell populations whose densities (and therefore intracellular hemoglobin concentrations) were narrowly defined, erythrocytes were separated by using discontinuous Stractan density gradients (St. Regis Paper Co., Tacoma, WA) (13, 14). The density of the Stractan layers ranged from 1.088 to 1.139 g/ml in ~0.009-g/ml increments. Erythrocytes trapped between the interfaces of successive layers were removed with a Pasteur pipette, with care to avoid contamination with erythrocytes from the lesser density regions of the gradient. The isolated erythrocytes were washed three times with phosphate-buffered saline (PBS: 10 mM phosphate, pH 7.4, 290 mosmol/kg) and resuspended in PBS at a hematocrit of 5%. Cell hemoglobin concentration of isolated erythrocyte populations was determined from spectrophotometric measurements of hemoglobin as the cyanomethemoglobin complex and from spun hematocrits.

To obtain normal erythrocytes with elevated hemoglobin concentrations, erythrocytes from volunteer donors were dehydrated by using the antibiotic, Nystatin (E. R. Squibb & Sons, Inc., Princeton, NJ) (15). Treatment of cells with Nystatin in PBS solutions of varying osmolalities provided dehydrated cell samples from which two to three subpopulations of defined cell density and cell hemoglobin concentration could be isolated by Stractan density-gradient centrifugation.

*Measurement of membrane material properties.* Measurements of cell mechanical properties were made with the use of a multimicromanipulator microscope system. The system is centered around an inverted microscope (E. Leitz, Inc., Rockleigh, NJ) with several small micromanipulators mounted directly to the microscope stage. For each test, erythrocytes were injected in very small concentrations (0.05% hematocrit) into a microchamber with dimensions of $1 \times 2 \times 0.1$ cm on the microscope stage. The cell suspension was exposed to ambient air along two edges of the chamber. Cells were aspirated and/or ma-

neuvered by small, glass suction micropipettes attached to the micromanipulators. The pipettes were prepared from 1-mm glass tubes pulled to a needle point, then broken by quick fracture to obtain flat tips that had inner diameters in the desired range of 1–2 $\times$ $10^{-4}$ cm. The inner diameters of these pipettes were measured from the insertion depth of a tapered microneedle, which had been calibrated with the use of a scanning electron microscope. The pipettes were coupled by continuous water systems to micrometer positioned water manometers for zero pressure adjustment. Aspiration pressures were measured through the continuous water system by connection to digital pressure transducers with resolution of a few microatmospheres (a few dynes per square centimeter). All measurements were carried out at room temperature. The microscope was equipped with a long working length $\times$ 40 (0.6 NA) objective. Each micromechanical experiment was simultaneously recorded on video tape with the pertinent data (e.g., suction pressure and time) superimposed on the recording with the use of video multiplexing.

To evaluate the effect of rehydration on cell rigidity, we carried out mechanical tests on dense cells obtained from the bottom fractions of the Stractan gradient first in isotonic buffer (290 mosmol) and then in hypotonic buffer (154 mosmol). For accurate comparison, the tests were performed on the same cell, which was transferred by micromanipulation from one chamber containing isotonic buffer on the microscope stage to an adjacent chamber containing hypotonic buffer. The chambers were kept separate by an air gap where the transfer was made possible by insertion of each cell into a large bore pipette which was drawn through the air-water interfaces.

The membrane elastic moduli (shear and bending), viscoelastic recovery times (after cell extension or folding), and membrane yield after prolonged and/or excessive extensions were measured for normal and sickle cells of defined hemoglobin concentrations by using the experimental procedures and data analyses outlined below.

*Measurement of static rigidities.* The membrane extensional rigidity was derived from a static "loading" experiment. The procedure was to aspirate a cell normal to and near the center of its cross section. Fig. 1 shows the video recordings of pipette aspiration of a discoidal erythrocyte and an ISC in this type of experiment. The aspiration pressure was applied in increments of 50 dyn/cm$^2$ (loading phase) and each pressure was held for periods of $\sim$10 s such that the projection of the cell into the micropipette was observed to be stationary. After increasing the aspiration pressure to a level that resulted in a significant amount of the erythrocyte membrane being pulled into the micropipette (aspirated cell lengths of 2–3 $\times$ $10^{-4}$ cm), the aspiration pressure was decreased in increments of 50 dyn/cm$^2$ (unloading phase) and the decrease in the projection of the cell in the micropipette recorded. As the cells were released from the pipette, there appeared to be no adhesive contact between the cell and the pipette. In all instances, the cell moved freely out of the pipette. We deliberately used high concentrations of albumin in our suspending medium to minimize adhesion. Although we cannot totally rule out minimal influence of adhesion in these experiments, we do not believe it plays a significant part.

The membrane extensional rigidity was derived by relating the increase in the aspirated cell length ($L$) to the increase in applied suction pressure ($P$). Analysis (8) of this experiment has shown that $\mu$ is proportional to the derivative of the pressure with respect to length: $\mu \propto R_p^2 \cdot dP/dL$, where $R_p$ is the pipette inner radius.

The membrane bending rigidity was derived from measurement of the minimum pressure required to induce membrane buckling. During erythrocyte aspiration into the micropipette, when the suction pressure is sufficient to produce large aspirated lengths, the cell surface begins to wrinkle and eventually large folds occur, enabling the cell to move up the pipette until limited by the surface area and volume restrictions. For these experiments, a bent micropipette with inner diameter of $\sim$1.5 $\times$ $10^{-4}$ cm was used for cell aspiration to obtain a clear front face view of the cell surface and determine accurately the aspiration pressure at which the cell surface began to wrinkle. Fig. 2 shows the video recordings

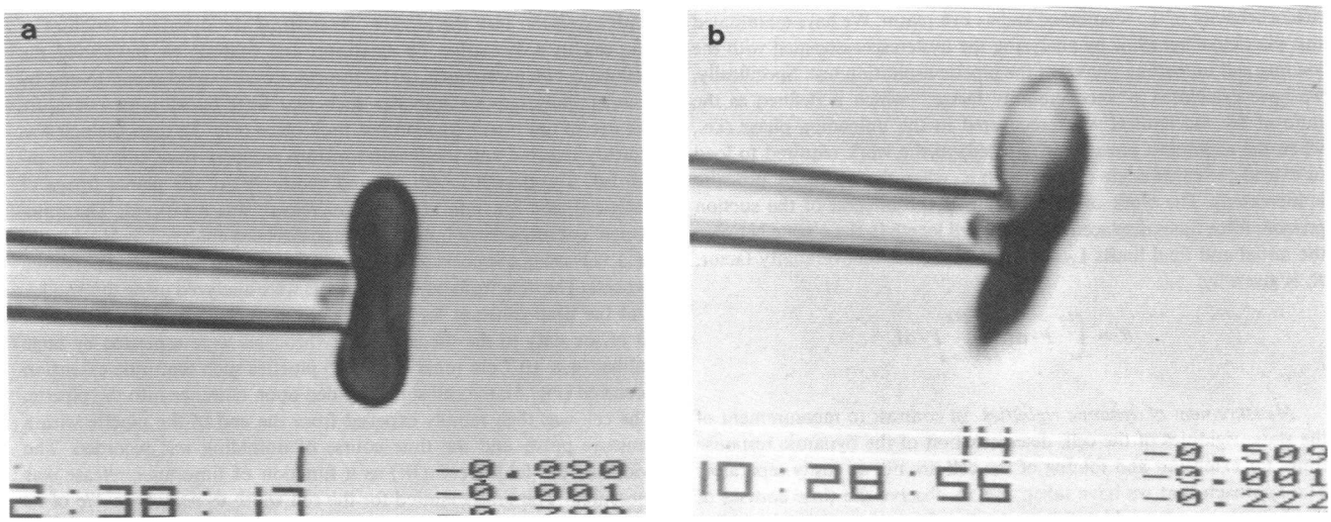
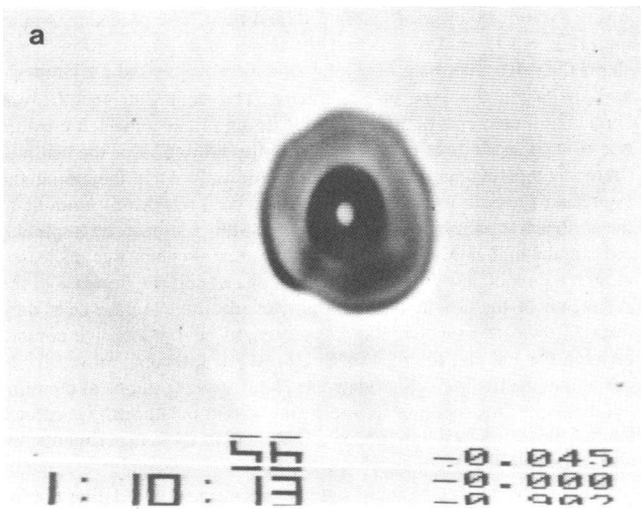



*Figure 1.* (*a*) Video micrograph of pipette aspiration of a discoidal, sickle erythrocyte from the light fraction (32 g/dl). The pipette inner diameter is $\sim$1 $\times$ $10^{-4}$ cm and the suction pressure is $\sim$400 dyn/cm$^2$. This experiment along with that shown in *b* are used to measure the extensional rigidity of the erythrocyte membrane. (*b*) Video micrograph of pipette aspiration of a sickle erythrocyte from a dense ISC fraction (44 g/dl). Here, the suction pressure is >2,000 dyn/cm$^2$ for a degree of extension equivalent to that shown in *a*, which demonstrates a significant increase in membrane stiffness.



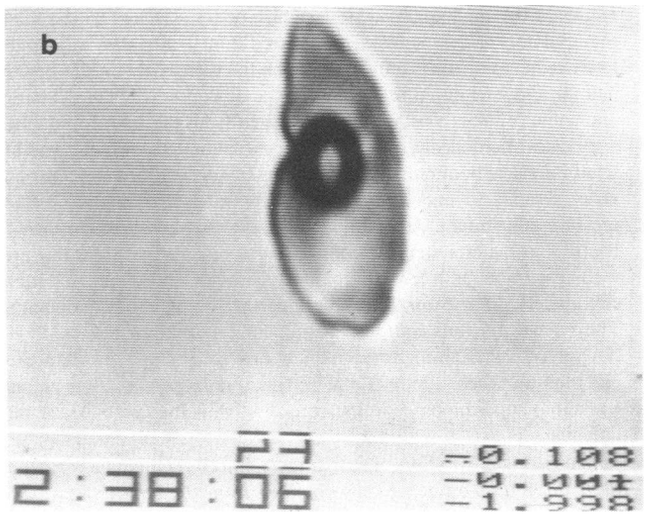




*Figure 2.* (*a*) Video micrograph of pipette aspiration of a discoidal, sickle erythrocyte from the light fraction as viewed from above the cell. The micropipette, $1.5 \times 10^{-4}$ cm inner diameter, appears as a dark ring in the center. Here the suction pressure, 450 dyn/cm$^2$, produced sufficient membrane extension to cause the cell surface exterior to the pipette to wrinkle. This phenomenon, called buckling, is used to determine the bending rigidity of the cell membrane. (*b*) Video micrograph of pipette aspiration of a sickle erythrocyte from a dense ISC fraction as viewed from above the cell. Here, the surface buckling first appears at pressures on the order of 1,000 dyn/cm$^2$. Hence, the bending rigidity of the sickle cell membrane is twofold greater than that of the discoidal cell from the light fraction shown in *a*.

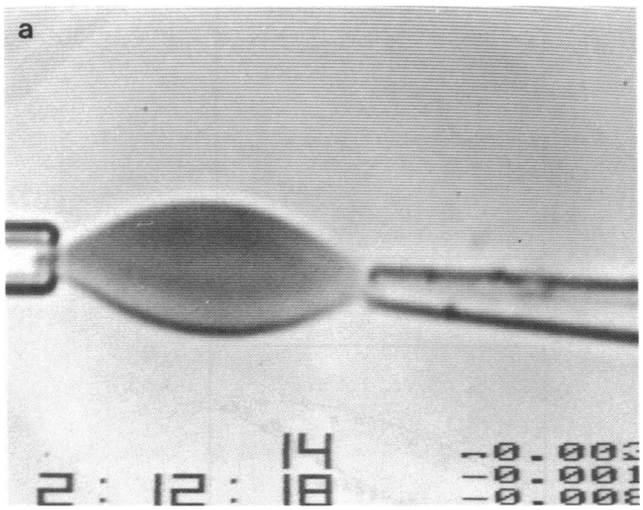
of a discoidal erythrocyte and an ISC being aspirated into a bent micropipette. B is proportional to the pressure, $\hat{P}$ at which the cell folds: $B = C \cdot R_p^3 \cdot \hat{P}$, where the coefficient, C, depends on the pipette inner radius ($R_p$) and is in the range of 0.005–0.012 (12).

Persistent deformation following the release of an erythrocyte extensionally deformed for prolonged periods of time is the result of plasticlike behavior of the membrane and/or cytoplasm. We have quantitated this plasticlike behavior by analyzing the hysteresis associated with the loading and unloading phases of the pipette aspiration test. Specifically, we have calculated a "recoverability factor," which is defined as the ratio of the mechanical work recovered in the unloading phase (i.e., when the aspiration pressure is reduced) to the work required to load or extend the erythrocyte membrane (i.e., when the aspiration pressure is increased). The work of deformation is the integral of the suction pressure force times displacement of the cell length in the pipette between the initial and final limits $L_0$ and $L_f$. Hence, the recoverability factor, $R$, is given by:

$$R \equiv \int_{L_f}^{L_0} P \cdot dL \Big/ \int_{L_0}^{L_f} P \cdot dL.$$

*Measurement of dynamic rigidities.* In contrast to measurement of the static rigidities of the cell, determination of the dynamic response times for extension and folding of the cell are not as easily separated. The approach that we have taken was to observe the time courses of rapid elastic recovery which followed cell deformations that were either predominantly extensional or bending in nature. In both these instances, elastic forces in the membrane drive the cell back towards its original shape. The rate of deformation recovery is limited by the viscous dissipation in the membrane and the cytoplasm. When the membrane static rigidity is essentially elastic, the time constants for viscoelastic recovery after deformation are essentially equivalent to $\tau_e$ and $\tau_f$ for dynamic response of the cell to applied forces. It would be preferable to measure the dynamic response of the cell to extension and folding directly through the application of instantaneous deformation forces; however, attempts to do this with micropipette aspiration have been unsatisfactory because membrane buckling may occur simultaneously with extension into the pipette. To separate the dynamic responses of the cell to deformation by extension and folding, we have used the following two procedures: (*a*) erythrocytes were extended end-to-end by diametrically opposed pipettes such that little buckling or curvature change of the membrane surface took place (Fig. 3); then the cell was quickly released and the length-to-width recovery time course was recorded. The overall length ($L$) and width ($W$) of the planar image of each cell as a function of time after release was measured. The time-dependent recovery of initial cell shape has been analyzed by Hochmuth et al. (11) using a viscoelastic model and is shown to follow an exponential process. The data from our experiments were analyzed using this model, and the time constant for extensional recovery derived from the best fit of the data to the theory. (*b*) Erythrocytes were aspirated by large caliber ($4 \times 10^{-4}$ cm inner diameter) pipettes such that little extension occurred (Fig. 4); the cell simply folded upon entrance into the pipette. The cell was then rapidly expelled from the end of the pipette with a pressure pulse, and the time course of unfolding was recorded. The width of the folded cell ($W$) as a function of time after release was measured. The time required for the cell width to recover to 1/e of its final width ($W_\infty$) was taken as a measure of the time constant for elastic recovery from bending.

On the basis of the simple rheological model of the erythrocyte as a liquid interior encapsulated by a viscoelastic membrane, $\tau_e$ for response of the cell to the extension is determined by the ratio of viscous dissipation to the rate of elastic work in extension; this is approximately given by



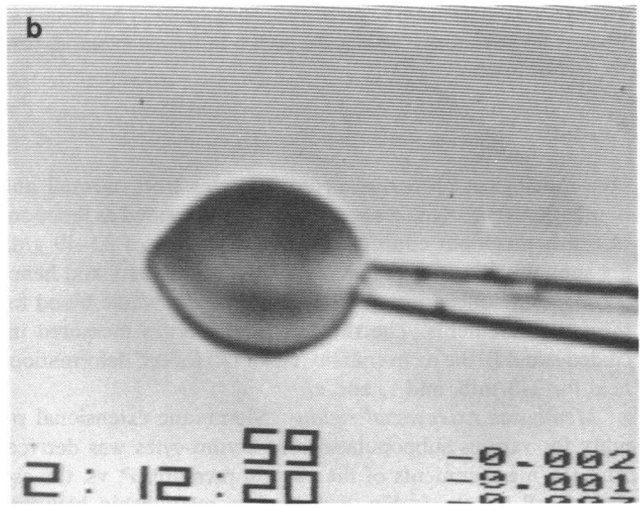
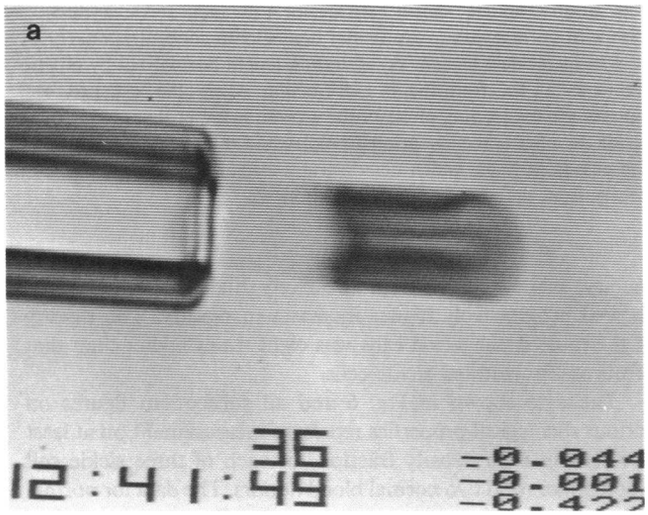




*Figure 3.* (*a*) Video micrograph of end-to-end extension of a sickle erythrocyte from the light fraction produced by diametrically opposed pipettes. The cell is shown just before release with the time given in the lower left-hand corner of the figure. (*b*) Video micrograph of the sickle erythrocyte shown in Fig. 4 *b* subsequent to release. The cell shown here has recovered to 90% of its final length. This experiment is used to determine the time constant for rapid elastic recovery from extensional deformations.

the relation, $\tau_e \sim (\eta_m + \bar{\eta}_{Hb} \cdot \delta)/\mu$, where $\eta_m$ and $\bar{\eta}_{Hb}$ are the viscosities of the membrane surface (dynes · second per centimeter) and the cytoplasm (dynes · second per square centimeter), respectively; and $\delta$ is the thickness of the erythrocyte. For times greater than $\tau_e$, membrane and cytoplasmic dissipation can be neglected and the forces are dominated by the static rigidity of the cell membrane. For times much less than $\tau_e$, viscous forces in the membrane and cytoplasm dominate and increase in inverse proportion to the time scale over which the deformation occurs. Similarly, the time constant, $\tau_f$, for cell folding without extension is determined by the superposition of viscous dissipation in the cytoplasm and the rate of elastic work of bending the membrane. This time constant can be approximately represented by: $\tau_f \sim (\bar{\eta}_{Hb} \cdot D)/(B \cdot C^2)$, where $D$ is the characteristic dimension of the erythrocyte (i.e., cell diameter) and C is the curvature of the fold. Again, for times greater than $\tau_f$,



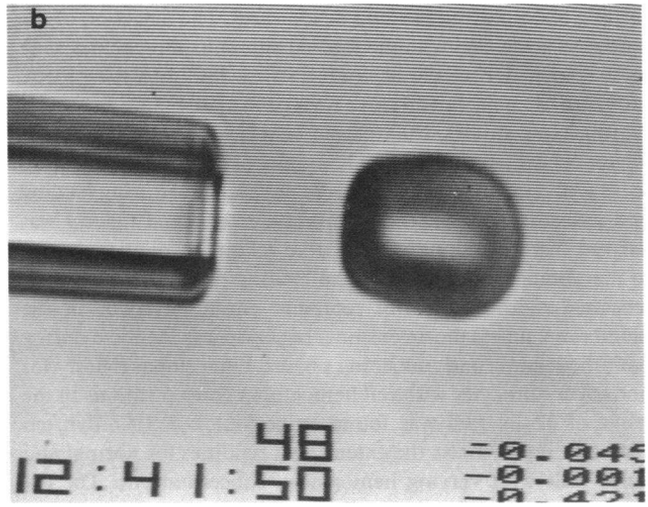




*Figure 4.* (*a*) Video micrograph of a sickle erythrocyte from the light fraction just after expulsion from a large ($4 \times 10^{-4}$ cm inner diam) micropipette. When the cell entered the large pipette, it simply folded without extension. The unfolding was observed and the time constant for rapid elastic recovery is measured by this technique. (*b*) Video micrograph of the same sickle erythrocyte shown in *a* as it recovers from the bending deformation.

folding of the cell is resisted by the static bending rigidity of the membrane, whereas for short times, viscous dissipation in the cytoplasm opposes the folding forces.

## Results

Five rheological properties of erythrocytes from normal and homozygous sickle cell disease blood were measured as functions of cell hemoglobin concentration in the range of 32–49 g/dl. Six subpopulations of cells with defined cell density and hence hemoglobin concentration were isolated from whole blood for these measurements. The rheological properties measured included $\mu$ and B, the recoverability factor ($R$) for cell deformations held for 2–3 min, and $\tau_e$ and $\tau_f$.

*Membrane extensional rigidity.* Membrane extensional rigidity for various subpopulations of erythrocytes was derived from the measurements of the suction pressure, $P$, vs. the aspirated cell length, $L$. Fig. 5 shows the relationship between membrane tension and aspirated length during both the loading phase when the aspiration pressure was increased and during the unloading phase when the pressure was decreased, for a discoid sickle cell with a hemoglobin concentration of 32 g/dl (Fig. 5 *a*) and for an ISC with a hemoglobin concentration of 44 g/dl (Fig. 5 *b*). Two features that distinguish the response of these two cells with varying hemoglobin concentration to aspiration pressure are (*a*) much higher membrane tension was required to aspirate equivalent cell length of the dehydrated ISC into the pipette compared with the normally hydrated sickle cell, and (*b*) the curves of aspirated length vs. suction pressure were observed to be nearly the same during both the loading and unloading phases for the discoid sickle cell, while they were markedly different for the dehydrated ISC. For truly elastic behavior of the membrane, the curves during both phases of loading and unloading should be identical. The detection of the hysteresis associated with the loading and unloading phases such as that seen during the aspiration of an ISC into the pipette indicated that the membrane underwent plasticlike behavior. During these studies, truly elastic behavior was noted only for normal erythrocytes with cell hemoglobin concentrations of up to 38 g/dl. Because the curves were not identical during loading and unloading phases, the membrane extensional rigidity was derived from the slope of the curve relating aspiration length to membrane tension during the loading phase only. This extensional rigidity does not represent elastic behavior in the membrane, unless the extensional response was identical during both the loading and unloading phases. Extensional rigidity for normal erythrocytes with cell hemoglobin concentrations in the range of 32–34 g/dl was found to be $9\pm1.7 \times 10^{-3}$ dyn/cm (Table I). Fig. 6 shows the extensional rigidities for normal and sickle cells with varying hemoglobin concentrations. The extensional rigidity of normal cells was the same over hemoglobin concentrations in the range of 32–45 g/dl. On the other hand, extensional rigidity of sickle cells with elevated hemoglobin concentrations was markedly increased. Sickle cells with hemoglobin concentrations of up to 38 g/dl had normal values of extensional

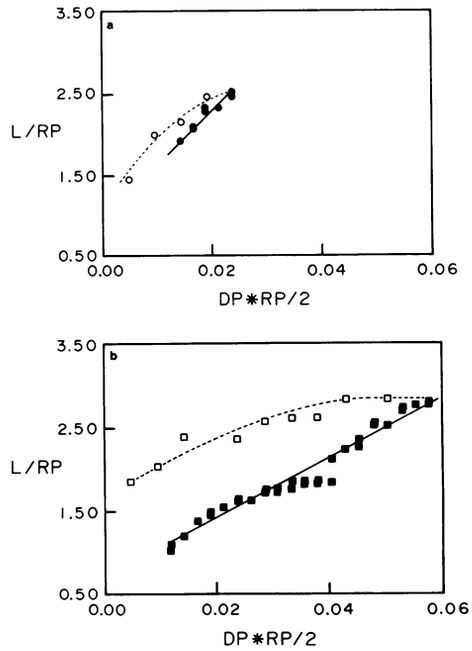



*Figure 5.* (*a*) Plot of membrane tension DP*RP/2 (dynes per centimeter, abscissa) vs. the aspiration length normalized by the pipette radius ($L$/RP) in the micropipette suction experiment. DP is the aspiration pressure; $L$, the aspirated cell length, and RP, the inner radius of the micropipette. The dark circles are the loading phase of the experiment; the slope of the straight line fit shown here gives the modulus of extensional rigidity for the cell membrane. The open circles are the unloading phase of the aspiration experiment; the difference between the loading and unloading phases represents the lethargic recovery of the cell membrane after extension for periods of time from 2 to 3 min. The data shown here are for a discoidal sickle erythrocyte with normal mean corpuscular hemoglobin concentration. (*b*) Plot of membrane tension vs. aspiration length normalized by pipette radius for an ISC from the 44 g/dl fraction. Again, the darkened squares are the loading phase of the aspiration experiment, which yields the membrane extensional rigidity and the open squares are the unloading phase which demonstrates a significant lethargy of recovery from the extensional deformation.

rigidity, while cells with progressively higher concentrations showed progressively higher increases in rigidity. The rigidity of the most dense sickle cells was eight times higher than that of normally hydrated sickle cells.

The data shown in Fig. 6 and all subsequent figures on various rheological properties represent measurement on at least five cells in each density fraction for each of three sickle cell blood donors and two normal blood donors. The data for normal cells at hemoglobin concentrations >42 g/dl represent cells isolated from whole blood, as well as those obtained by dehydration using the antibiotic Nystatin. There appeared to be no difference between the properties of dense normal cells and normal cells whose density was increased artificially.

*Table I. Normal Erythrocyte Mechanical Properties for Cells in 32–34 g/dl Fraction*

| Extensional rigidity ($\mu_0$) | Bending ridigity ($B_0$) | Recoverability factor ($R_0$) | Time constants for rapid elastic recovery: Extension ($\tau_e°$) | Time constants for rapid elastic recovery: Bending ($\tau_f°$) |
|---|---|---|---|---|
| *dyn/cm* | *dyn/cm* | | *s* | *s* |
| $9 \times 10^{-3}$ ($\pm 1.7 \times 10^{-3}$) | $1.8 \times 10^{-12}$ ($\pm 0.2 \times 10^{-12}$) | 0.85 (±0.05) | 0.12 (±0.03) | 0.25 (±0.09) |

Values are mean±SD.

*Reversibility of membrane deformation.* The plasticlike behavior of the membrane observed during the static aspiration experiment resulted in lethargic recovery of the deformation after the cell was released from the pipette. The extent of this plasticlike behavior depended both on the duration of the experiment and the magnitude of induced membrane extension. We quantitated this inelastic behavior by analyzing the hysteresis associated with the loading and unloading phases of the pipette aspiration test. Specifically, we calculated a "recoverability factor," which was the ratio of mechanical work recovered in the unloading phase to the work put in to extend the membrane during the loading phase. For normal cells, with cell hemoglobin concentration of 32–34 g/dl, the recoverability factor was between 0.85 and 0.9 for experiments of 2–3 min in duration (Table I). Fig. 7 shows the recoverability factor for both normal and sickle cells at varying intracellular hemoglobin concentration. The recoverability factor was close to unity for normal erythrocytes up to hemoglobin concentrations of 38 g/dl, but at higher concentrations it was markedly decreased. The sickle cells had reduced values for the recoverability factor beginning at appreciably lower hemoglobin concentrations in the range of 32–38 g/dl. When the mean values for the recoverability factor obtained for normal and sickle erythrocytes at different hemoglobin concentrations were compared with the t test, a statistically significant decrease was noted for sickle erythrocytes at both 32 g/dl ($P = 0.02$) and at 38 g/dl ($P < 0.001$). At higher hemoglobin concentrations, as with normal cells, sickle cells showed a marked decrease in recoverability factor values. The differences between the values for normal and sickle erythrocytes at these higher hemoglobin concentrations were not statistically significant ($0.3 < P < 0.5$).

*Membrane bending rigidity.* Membrane bending rigidity for various subpopulations of erythrocytes was also derived from the static aspiration experiments. Bending rigidity for normal erythrocytes with cell hemoglobin concentration in the range of 32–34 g/dl was found to be $1.8 \times 10^{-12}$ dyn-cm (Table I).

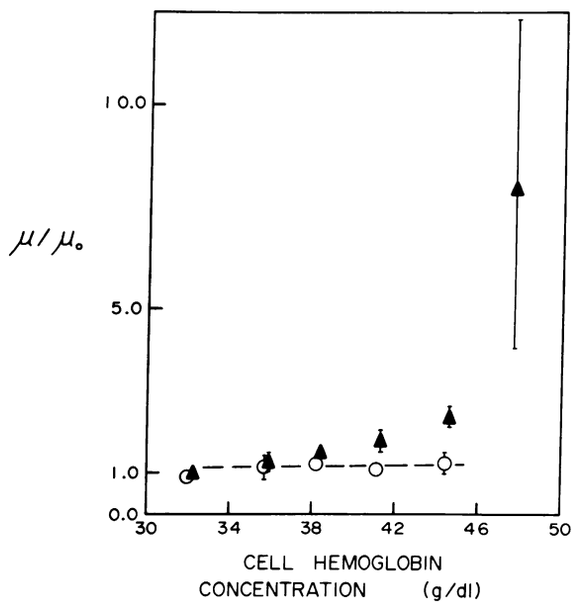



*Figure 6.* Ratio of the membrane extensional rigidity measured for cells in specific density fractions ($\mu$) to the average value of the extensional rigidity for normal cells in the 32 g/dl fraction ($\mu_0$). The open circles are the mean values of this ratio for normal erythrocytes and the darkened triangles are the mean values for sickle erythrocytes. The standard deviations are shown by brackets about the mean values and the characteristic behavior of normal cells is indicated by a hand-drawn, dashed line.

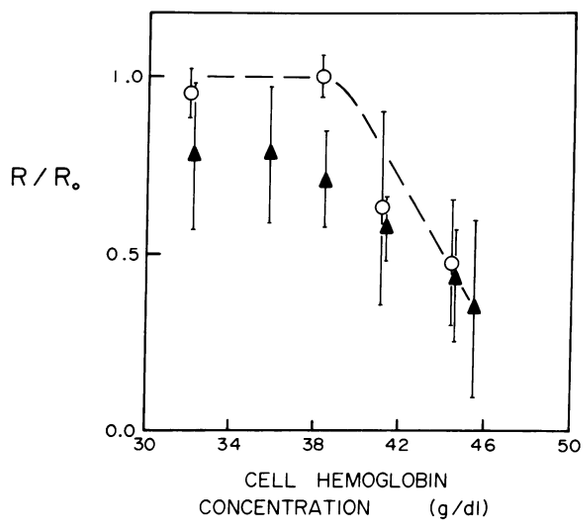



*Figure 7.* Ratio of the recoverability factor (following 2–3 min extensions) measured for cells in specific density fractions (R) to the average value of the recoverability factor for normal cells in the 32 g/dl fraction ($R_0$). The open circles are the mean values of this ratio for normal erythrocytes and the darkened triangles are the mean values for sickle erythrocytes. The standard deviations are shown by brackets about the mean values and the characteristic behavior of normal cells is indicated by a hand-drawn, dashed line.

Fig. 8 shows the bending rigidities for sickle cells as a function of hemoglobin concentration. The bending rigidity was essentially constant as the hemoglobin concentration increased. However, for the most dehydrated sickle cells with hemoglobin concentration in the range of 47–49 g/dl, the bending rigidity was increased ~50%.

*Dynamic response to extension.* The dynamic rigidities of normal and sickle cells were quantitated using two different experimental approaches, which enabled us to separate the dynamic responses of the cell to deformation by extension from that by folding or bending. In the first approach, to quantitate the dynamic response to extension, an erythrocyte was extended end-to-end by diametrically opposed pipettes such that little buckling or curvature change of the membrane surface took place; then, the cell was quickly released and the length to width ($L/W$) recovery time course was analyzed (Fig. 9). The time constant for extensional recovery (response) was derived from the best fit of the viscoelastic model to the observed data (7, 11). It can be seen from the data shown in Fig. 9 that the correlation between time course of recovery derived from the viscoelastic model and the observed data was good for both sickle cells with normal as well as elevated intracellular hemoglobin concentrations. The time constant for extensional elastic recovery for normal cells with hemoglobin concentration of 32–34 g/dl was determined to be 0.12 s (Table I). Fig. 10 shows the extensional recovery time constants for normal and sickle cells as a function of intracellular hemoglobin concentrations. The time constant for extensional recovery was strongly dependent on hemoglobin concentration for both normal and sickle cells. It increased gradually with increasing hemoglobin concentrations up to 38 g/dl, but above 38 g/dl the increase in time constant was dramatic. At 48 g/dl, the highest hemoglobin concentration studied, the time constant was 20 times higher than at 32 g/dl. There was no difference between the time constants for normal and sickle cells when measured at equal intracellular hemoglobin concentrations.

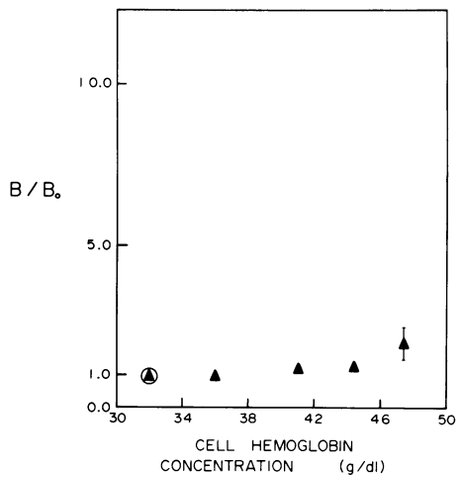



*Figure 8.* Ratio of the membrane bending rigidity measured for cells in specific density fractions (B) to the average value of the bending rigidity for normal cells in the 32 g/dl fraction ($B_0$). The open circle is the mean value of this ratio for normal erythrocytes and the darkened triangles are the mean values for sickle erythrocytes. The standard deviations are shown by brackets about the mean value.

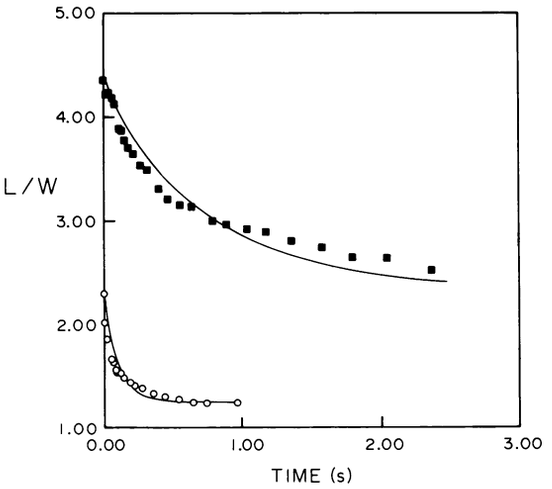



*Figure 9.* Time courses of rapid elastic recovery from end-to-end extension for two sickle erythrocytes. $L$ and $W$ are the length and width of the cell during the recovery process. The darkened squares are for an ISC from the 44 g/dl density fraction and the open circles are for a discoidal cell with normal mean corpuscular hemoglobin concentration. The solid curves are correlations with the simple surface rheological model for viscoelastic recovery which is used to determine the time constants for recovery from extensional deformation (11). Here, the dense ISC recovers more than a factor of eight times slower than the discoidal cell.

*Dynamic response to bending.* The dynamic response to bending was quantitated by aspirating the erythrocyte using a large diameter pipette such that the cell simply folded upon entrance into the pipette with little extension, then expelling the cell and recording the time course of unfolding of the cell (Fig. 11). Comparison of Figs. 9 and 11 shows that the time course of recovery during unfolding was clearly different from that observed during recovery from extensional deformation. The viscoelastic recovery from folding was not an exponential process. Hence, in order to compare similar levels of recovery between extension and folding experiments, we have quantitated the folding time constant by measuring the time when the cell width had recovered to within 1/e of its final width. $\tau_f$ for normal cells with a hemoglobin concentration of 32–34 g/dl was determined to be 0.25 s (Table I). The values of $\tau_f$ for normal and sickle cells at varying intracellular hemoglobin concentrations are shown in Fig. 12. The time constant for recovery from folding was also strongly dependent on hemoglobin concentration and was the same for both normal and sickle cells at similar hemoglobin concentrations.

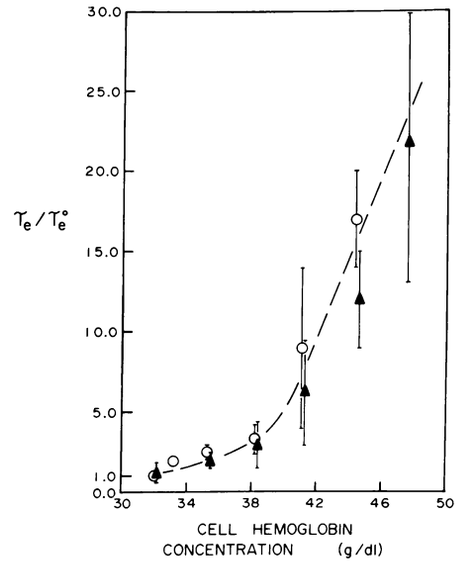



*Figure 10.* Ratio of the time constant for rapid elastic recovery from end-to-end extension measured for cells in specific density fractions ($\tau_e$) to the average value of the time constant for normal cells in the 32 g/dl fraction ($\tau_e^\circ$). The open circles are the mean values of this ratio for normal erythrocytes and the darkened triangles are the mean values for sickle erythrocytes. The standard deviations are shown by brackets about the mean values and the characteristic behavior of normal cells is indicated by a hand-drawn, dashed line.

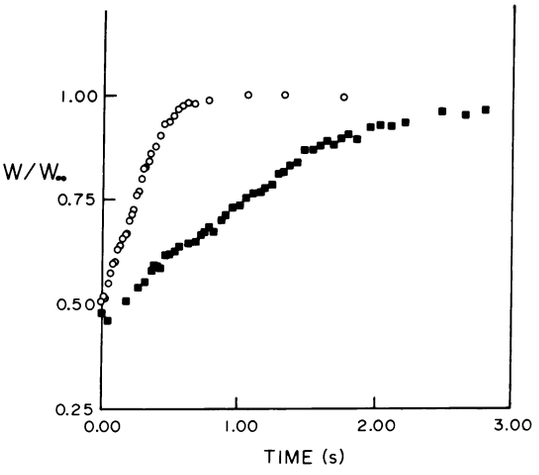



*Figure 11.* Time courses for rapid elastic recovery from cell folding without extension. $W$ is the width of the unfolding cell during recovery, and $W_\infty$ the final width of the unfolded cell. Again, the darkened squares are the data for an ISC from the 44 g/dl fraction and the open circles are data for a discoidal cell with normal MCHC. It is important to note that the dynamics of recovery from such bending deformations are clearly different than from extensional deformations as evidenced by the nearly linear time course for recovery in this unfolding experiment as contrasted with the exponential behavior shown in Fig. 9.

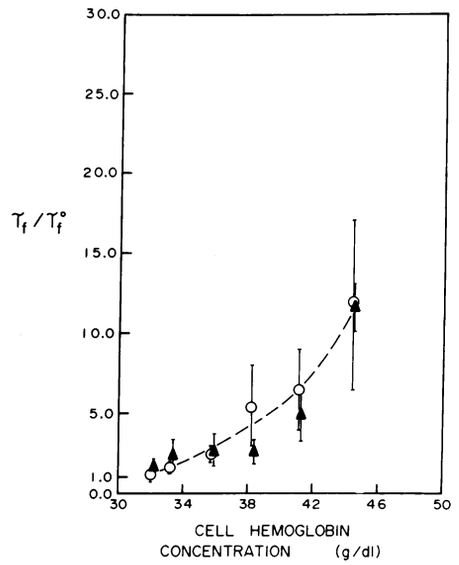



*Figure 12.* Ratio of the time constant for rapid elastic recovery from folding without extension measured for cells in specific density fractions ($\tau_f$) to the average value of the time constant for normal cells in the 32 g/dl fraction ($\tau_f^\circ$). The open circles are the mean values of this ratio for normal erythrocytes and the darkened triangles are the mean values for sickle erythrocytes. The standard deviations are shown by brackets about the mean values and the characteristic behavior of normal cells is indicated by a hand-drawn, dashed line.

The results described thus far on static and dynamic rigidities of sickle cells showed that the mechanical properties of the cell are strongly influenced by intracellular hemoglobin concentration and hence state of hydration of the cells. To critically evaluate the influence of state of hydration and to assess the reversibility of the effects, we studied the effect of rehydration on the mechanical properties of dense sickle cells. We measured the extensional rigidity and recoverability factor for a single Stractan fraction equivalent to a mean corpuscular hemoglobin concentration of 44.4 g/dl. The cells were first tested in isotonic buffer (290 mosmol), next in hypotonic buffer (154 mosmol), and finally back in isotonic buffer (290 mosmol). The results given in Table II, (normalized by the values of Table I) show that hydration is effective in reducing the static rigidity and lethargy of recovery of the dense cell to levels common to sickle cells in the light fractions. Subsequent dehydration back to the dense state caused the cell to stiffen and to exhibit its original lethargic recovery behavior. Similar results were obtained during the cell transfer experiments for four other erythrocytes from this density fraction. We also measured the time constants for unfolding of sickle cells from the two densest cell fractions (equivalent to 44.4 and 47.6 g/dl mean corpuscular hemoglobin concentration) first in the isotonic buffer (290 mosmol), followed by transfer of the same cell into hypotonic buffer (154 mosmol). These dynamic results (given in Table II) are consistent with the effect of rehydration on the static rigidity of the cell. The

*Table II. Effects of Rehydration on Sickle Erythrocyte Mechanical Properties*

| | Isotonic buffer (290 mosmol) | Hypotonic buffer (154 mosmol) | Isotonic buffer (290 mosmol) |
|---|---|---|---|
| Extensional rigidity $\mu/\mu_0$ (44 g/dl fraction) | 2.5 | 1.3 | 2.4 |
| Recoverability factor $R/R_0$ (44 g/dl fraction) | 0.45 | 0.81 | 0.29 |
| Time constant for rapid elastic recovery (bending) $\tau_f/\tau_f^\circ$ | | | |
| (44 g/dl fraction) | 8.6 | 1.1 | — |
| (47.6 g/dl fraction) | 42.0 | 2.2 | — |

rehabilitative effect on dynamic deformability is clearly evident and was a consistent feature of all 10 cells studied from these two density fractions. Another significant observation was that persistent deformations (bumps), which followed micropipette tests of extensional rigidity in the isotonic buffer, were reversed within a minute when the cell was released into the hypotonic medium.

## Discussion

Measurements of the rheological properties of defined cell density subpopulations of oxygenated normal and sickle erythrocytes have enabled us to show the marked influence of the state of cell hydration on a number of membrane and cellular properties that regulate the static and dynamic rigidities of these cells. We found that cellular dehydration significantly increases both the static and dynamic rigidities of the sickle cells.

The rheological properties measured to characterize the static rigidity of the erythrocytes included membrane extensional and bending rigidities and a "recoverability factor" to describe the inelastic behavior of the membrane after deformation. Membrane extensional rigidity was identical in various subpopulations of normal erythrocytes with increasing cell density, suggesting that extensional rigidity is invariant during normal erythrocyte aging, a finding consistent with those reported earlier by Linderkamp and Meiselman (16). However, in contrast to normal cells, the extensional rigidity of sickle cells increased with increasing cell dehydration. At least two possible explanations for this increase in extensional rigidity could be postulated. First, increased extensional rigidity is the result of increased association of sickle hemoglobin with the membrane at elevated hemoglobin concentrations inducing the formation of a membrane-associated gel of hemoglobin. Second, increased skeletal protein cross-linking as the result of calcium accumulation could provide the same elevation of extensional rigidity (17, 18). Our finding that extensional rigidity of the dehydrated sickle cells could be restored close to the values obtained for discoid normal erythrocytes by hydrating the cells, and that the rigidity is reverted to the original high value upon dehydration back to the initial state of hydration, favors the hypothesis that the formation of a reversible membrane-associated hemoglobin gel or polymer may contribute to the increased membrane rigidity. However, the observation that extensional rigidity could not be completely restored to normal levels by hydration suggests that permanent membrane skeletal changes should also be considered to play a role in the rigidification process.

Additional supporting evidence that hemoglobin gel formation can affect membrane material properties comes from our findings that sickle cells exhibited an increased tendency to undergo plastic deformation during the aspiration experiments compared with normal erythrocytes, and that both normal and sickle cells exhibited inelastic deformation at elevated hemoglobin concentrations. The recoverability factor that measures the extent of the inelastic behavior was markedly reduced for dehydrated sickle cells, but could be restored to values characteristic of sickle cells with normal cell hemoglobin concentrations by hydrating the cells using hypotonic media. In addition, the persistent deformation which followed micropipette aspiration of the cells suspended in isotonic medium was reversed within a minute when the cells were released into hypotonic media. Both these findings suggest that a reversible hemoglobin gel adjacent to the membrane may be responsible for the inelastic behavior. In this regard, recent direct experimental support for increased association of sickle hemoglobin with the membrane is provided by the studies of Eisinger et al. (19) who have shown by resonance energy transfer measurements that the concentration of heme in the membrane boundary layer was greater than that in the cell interior. Moreover, the difference between the boundary layer and bulk cytoplasmic hemoglobin concentration was more pronounced as the cell density increased (20).

The dynamic rigidity of the normal and sickle cells, quantitated by measuring time constants for extensional recovery and recovery from bending, was also strongly dependent on the state of cell hydration. The two time constants were the same for normal and sickle cells at similar cell densities. This was in contrast to measurements of static rigidity in which the behavior of the sickle cells was different from that of normal cells. Moreover, increases in extensional and folding recovery times with increasing hemoglobin concentration were much more pronounced than those seen for either static extensional or bending rigidities. The time constant for extensional recovery of a sickle cell increased from 0.125 to 2.5 s as the hemoglobin concentration increased from 32 to 48 g/dl. However, the increased recovery times seen at elevated hemoglobin concentrations could be returned to normal values by hydration of the cells. This implied that increased hemoglobin concentration plays a dominant role in the dynamic behavior of the erythrocytes. The increase in the time constant for recovery from folding with increasing hemoglobin concentration appeared to follow closely

the measured increases in hemoglobin solution viscosity with concentration (21). This is to be expected since, during unfolding, the energy is predominantly utilized to displace the viscous hemoglobin solution in the cell interior. This is in contrast to the extensional recovery, during which dissipation in the membrane as well as the cell interior is involved.

Qualitative evaluation of the deformability properties of individual sickle erythrocytes by micropipette elastimetry has been carried out previously by La Celle (22) and Havell et al. (23). Both these investigators, using whole blood samples, reported that oxygenated discoid erythrocytes exhibited normal deformability, while irreversibly sickled cells had markedly increased membrane rigidity and reduced whole cell deformability. Nash and Meiselman (24) have recently reported that normally hydrated discoid sickle cells have normal values of elastic shear modulus and time constant for viscoelastic shape recovery, however the dehydrated sickle cells had normal time constant for shape recovery and the membrane shear modulus was twice normal. The results of these studies were interpreted by these investigators to reflect that altered rheological properties were predominantly a feature of irreversibly sickled cells with increased membrane rigidity. Clark et al. (25) studied the whole cell deformability of isolated subpopulations of irreversibly sickled cells using an ektacytometer, and concluded that cellular dehydration and consequent increase in internal viscosity, and not increased membrane rigidity, was the major determinant of the abnormal rheological behavior of these cells. Our studies, which represent the most comprehensive measurements of a number of rheological properties on defined density subpopulations of sickle and normal cells, clearly establish that altered rheological behavior of sickle cells is associated with major changes in both static and dynamic rigidities of the cells. Both altered membrane material properties and increased cytoplasmic viscosity are responsible for these observed changes. However, it appears that the effect of cytoplasmic hemoglobin concentration is predominant in this regard. In addition, the alterations in the rheological properties appear to be progressive and cumulative.

The significance of the measurements we have made can be put into proper perspective when they are viewed in the context of the recent work of a number of investigators regarding the marked heterogeneity of cell water content in sickle cell disease and the presence of a large percentage of dehydrated cells with elevated intracellular hemoglobin concentrations (14, 26–28). Erythrocyte density distribution, in whole blood of normal individuals, varies within narrowly defined limits. This implies that cell hemoglobin concentration is also tightly distributed. A majority of the cells (>90%) have hemoglobin concentration in the range of 29–35 g/dl, with <5% of the cells having hemoglobin concentration >37 g/dl, and <1% with a concentration >40 g/dl. In contrast, the density distribution of sickle cell blood is broadly distributed. It is not uncommon to find in blood from sickle cell patients up to 50% of the erythrocytes having hemoglobin concentrations >37 g/dl. We have routinely isolated between 5 and 10% of erythrocytes with a hemoglobin concentration in the range of 42–44 g/dl and 2–3% of cells with a concentration in the range of 46–48 g/dl from whole blood of sickle cell patients. Thus, the wide range of static and dynamic rigidity values we have measured represents the properties of erythrocytes circulating in the sickle cell patients.

The marked increases in static and dynamic rigidity of the sickle erythrocytes with elevated hemoglobin concentration will have major influences on the dynamics of circulation in the microvasculature. These include reduced rate of entry of erythrocytes into small capillaries as well as reductions in the flow velocity of the cells within the capillaries. Both these factors can significantly reduce oxygen delivery. In addition, increased static rigidity could result in mechanical trapping of these cells in the reticuloendothelial system, while increased dynamic rigidity could lead to prolongation of erythrocyte sojourn in the reticuloendothelial system, allowing for potentially deleterious increased time of contact with macrophages.

In addition to these possible consequences of increased rigidity, another aspect that needs to be considered is the increased probability of hemoglobin polymerization in these cells as a consequence of longer transit times through regions of low oxygen tension. Noguchi and Schechter (29) and Noguchi et al. (30) have recently shown that sickle cells with elevated hemoglobin concentrations can contain polymers even at arterial oxygen saturation values and formation of hemoglobin polymers in these already rigid cells can further retard the flow through capillaries and even cause vasoocclusion.

The finding that all the rheological properties of the dehydrated sickle cells can be nearly restored to the values characteristic of sickle cells with normal cell hemoglobin concentrations by cell hydration has implications for design of potential therapies for sickle cell anemia. Either reduction in the cell hemoglobin concentration by cell swelling or preventing the generation of dehydrated cells should lead to significant improvement in the rheological competence of circulating sickle cells.

From our current studies, it is clear that rheological properties of sickle cells are strongly influenced by the state of cell hydration. Cell dehydration appears to influence the static and dynamic rigidities of cells both by increasing hemoglobin concentration adjacent to the membrane (perhaps via a reversible membrane-associated hemoglobin gel), as well as by markedly increasing bulk cytoplasmic hemoglobin viscosity. In addition, irreversible membrane changes also appear to accompany cell dehydration in vivo, resulting in increased membrane shear rigidity and plastic flow. The rheological data we have obtained, coupled with the detailed knowledge of the state of cell hydration in various sickle cell patients, should enable us to better define the rheological consequences of the pathophysiology of this disease.

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