Age-Related Changes in Deformability of Human Erythrocytes


The present study was designed to further the characterization of age-related changes in the deformability of human erythrocytes. The top (new) and bottom (old) 10% fractions of density-separated red cells from ten normal donors were subjected to graded levels of shear stress in a rheoscope. Measurements were made of steady-state elongation (cells tank treading in a state of dynamic equilibrium) and the time course of shape recovery following abrupt cessation of shear. In parallel with rheologic experiments, several physical and chemical properties were assayed to determine correlates of mechanical properties. These included mean cell volume, mean corpuscular hemoglobin concentration, type A, hemoglobin, glucosylation of membrane proteins, and membrane phospholipid and protein concentration. The microrheologic observations revealed that only about 90% of the old cells retained their capacity to tank tread. However, the tank-treading cells elongated less than their younger counterparts at corresponding levels of shear stress, thus demonstrating a reduced level of deformability. Further analysis of the data indicates that increases in membrane viscosity and elastic modulus along with a significant loss in excess surface area contribute to the limitation of the ability of the older cells to change shape.

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THE ROLE of erythrocyte deformability in vascular pathophysiology is a subject of intense interest currently. Investigations at both the macroscopic (whole blood or red cell suspensions) and the microscopic (cellular) levels seek not only to quantify deformability, but also to differentiate between normal and abnormal cell populations in this respect. The problem of detecting differences in deformability between two populations of red cells is complicated by the natural intrapopulation heterogeneity associated with in vivo aging. Hence, it is important to characterize the changes that occur with age in a normal cell population before attempting to assess potentially subtle disease-related changes.

Originating in the bone marrow, the normal human erythrocyte circulates for an average of 120 days. Its eventual removal from the circulation is generally believed to be a consequence of declining deformability, making it susceptible to sequestration in the spleen and other organs, wherein the erythrocyte must negotiate extraordinarily narrow passages. The critical determinants of the deformability characteristics of young or senescent erythrocytes have yet to be clearly sorted out. Changes in cell shape, in the viscoelastic properties of the membrane, and in the cytoplasmic viscosity are all potentially involved. The one aspect of erythrocyte aging upon which there is universal agreement is that the density (mass/volume) of the cells decreases with aging, these extreme fractions still contain a distribution of large and small cells that is as broad as the unfractionated cell population. Studies on density-fractionated cell populations have reported average cell volumes of 90 to 129 μm³ in the youngest (lightest) 5% to 14% fraction and 78 to 96 μm³ in the oldest (heaviest) 5% to 14% fraction. Even though the mean volumes of the light and heavy fractions indicate that volume decreases with aging, these extreme fractions still contain a distribution of large and small cells that is as broad as the unfractionated cell population.

There is a substantial range in cell volume data reported in the literature for human erythrocytes. Leif and Vinograd measured cell volume distributions electronically (Coulter counter, Coulter Electronics, Hialeah, Fla) and reported a skewed distribution that had a range from 45 to 170 μm³. Their data, along with other more recently reported data on mean cell volume, has a spread from 85 to 100 μm³ for unfractonated human red cells. Studies on density-fractionated cell populations have reported average cell volumes of 90 to 129 μm³ in the youngest (lightest) 5% to 14% fraction and 78 to 96 μm³ in the oldest (heaviest) 5% to 14% fraction. Even though the mean volumes of the light and heavy fractions indicate that volume decreases with aging, these extreme fractions still contain a distribution of large and small cells that is as broad as the unfractionated cell population. The differences in the reported cell volume data appear to be associated with centrifugation technique, the composition of the gradient medium employed, the size of the fractions isolated for measurement, and the technique of volume measurement (electronic, isotope dilution, photographic, micropipette aspiration).

Mean cell volume data are coupled with surface area measurements to assess the influence of geometry on deformability. Because the red cell deforms at constant area, a cell with surface area in excess of that...
of a sphere of the same volume as the cell will be more deformable. Surface and volume measurements on individual cells that had been separated by density have demonstrated a decrease in both parameters with aging. Comparing the oldest 5% to 10% to the youngest 5% to 10%, Nash and Wyard found that the volume and surface area were reduced by 12% and 6%, respectively. Linderkamp and Meiselman used the same micropipette aspiration technique and reported that the oldest 5% had volume and surface area reduced equally by 20%. They concluded that the surface-volume ratio (S/V) was invariant with age, but observed that the older cells were more spherical than the younger cells. The latter observation is significant because it points out the inadequacy of S/V as an extrinsic indicator of deformability. This point is best understood by considering two spherical “cells” of different diameter. Since for spheres, S/V = 6/πd, the smaller sphere has a larger S/V, but neither has any excess surface. If the two spheres had membrane surfaces that could deform only at constant area, they would be equally undeformable, regardless of their differing S/V.

Two geometric parameters that do provide a measure of the influence of surface and volume changes on deformability are the excess surface area index (ESI) and the swelling index (SWI). The ESI is defined as the ratio of the actual cell surface area, S, to the area of a sphere of the cell’s volume, \( V \); it is equal to the “surface-area index” of Linderkamp and Meiselman. The swelling index is defined as the ratio of the spherical volume that could be enclosed by the surface area of the cell to the actual volume of the cell. From geometry:

\[
\text{ESI} = \frac{S}{(36\pi)^{1/3} V^{2/3}} \tag{1}
\]

and

\[
\text{SWI} = \frac{S^{1/2}}{(36\pi)^{1/2} V}. \tag{2}
\]

These two parameters are not independent, since it is easily shown that \( \text{ESI} = (\text{SWI})^{2/3} \). Here, the excess surface area index (ESI) or the excess surface area fraction (ESI, 1.0) will be used to assess the influence of geometric changes.

From Nash and Wyard’s surface and volume data, the excess surface area fraction (ESI, 1.0) decreased insignificantly (<3%) with aging. This agrees with the data of Shiga et al if one uses their reduction of membrane phospholipids with aging as an indicator of surface area loss. Linderkamp and Meiselman, on the other hand, reported data in which the excess surface area was reduced by 25% with aging in a comparison of the youngest and oldest 5% fractions.

The micropipette aspiration technique has been used to determine the shear modulus of elasticity of density-separated cells; slight increases with aging have been judged to be statistically insignificant. On the other hand, the membrane surface viscosity has shown a significant change with aging. By combining whole cell recovery time measurements with measurements of the elastic modulus, Linderkamp and Meiselman and Nash and Meiselman have reported increases in membrane viscosity of 76% and 52%, respectively. Further experiments by this group on red cell ghosts led to the interesting hypothesis that the increase in membrane viscosity with age may be due in part to hemoglobin–membrane interactions.

In the area of chemical changes, we note that Hochstein and Jain have given evidence of in vivo polymerization of membrane proteins consequent to radical-induced peroxidation of membrane lipids and have suggested this as another possible contributor to altered rheologic properties of the membrane. One would expect this kind of change to show up, if at all, in the membrane elastic modulus, but the above-mentioned micropipette data do not support the hypothesis. The recent review by Bocci provides a thorough survey of the chemical aspects of erythrocyte senescence. He concludes that proteolysis and natural desialylation of the membrane play only a minor role and that geometric and mechanical properties are the key factors in erythrocyte aging.

The present study was designed to further the characterization of age-related changes in the deformability of human erythrocytes. Our approach is basically microrheologic and focuses mainly on the dynamic response of normal erythrocytes to shear stress. We were especially interested in the capacity of the cells to “tank tread,” which is another important gauge of deformability under dynamic conditions. Density-separated cells were subjected to graded levels of shear in a rheoscope, and measurements were made of steady-state elongation and the time course of shape recovery following abrupt cessation of shearing. A separate study, which focused on the rate of membrane circulation or tank-treading frequency, has been published elsewhere. In parallel with these rheologic observations, assays were made of several physical and chemical properties in a search for correlates of rheologic behavior. These properties included mean cell volume (MCV), mean corpuscular hemoglobin concentration (MCHC), concentration of type A, hemoglobin, glucosylation of membrane proteins, and membrane phospholipid and protein content. The latter
were measured as an index of mean membrane surface area.

MATERIALS AND METHODS

Preparation of RBCs

Blood samples were taken by venipuncture from ten hematologically normal volunteer donors (four males and six females), ranging in age from 26 to 67 years. The blood was collected into 10-mL vacutainers containing 1 mL of 3.8% sodium citrate and centrifuged at 1,000 g for ten minutes, which concentrated the red cells to a hematocrit of 80% in the donor’s plasma. Density separation was effected by short-duration, high-speed centrifugation of these concentrated (80% Hct) red cells at 10,000 g for 15 minutes (Beckman Microfuge, Palo Alto, Calif, model 11). Following centrifugation, the top and bottom 10% fractions were removed and designated as the young and old cells, respectively. The cells were then washed twice in isotonic phosphate-buffered saline ([PBS] 0.005 mol/L Kh2PO4 + Na2HPO4, pH 7.40, 290 ± 5 mosm/kg) plus 10 mmol/L dextrose. After the second wash, the cells were resuspended in the same buffer plus 20% donor plasma at a 20% Hct. Finally, these fractions were then diluted 1:1 by addition of suspending medium (see below) to a Hct of 2% and divided into three aliquots, each of which was subjected to an independent run in the rheoscope. All samples were tested within eight hours of venipuncture.

Suspending Medium

In order to achieve adequate elongation of erythrocytes and membrane tank treading at shear rates within the rheoscope’s operating speed range, it was necessary to increase the viscosity of the PBS buffer by the addition of dextran polymer (molecular weight 2 × 106; Sigma Chemical, St Louis). The dextran was dialyzed and freeze-dried to eliminate stomatocytic agents and dissolved in PBS buffer (less citrate + 10 mmol/L dextrose) in sufficient quantity to give a final suspension viscosity with 2% RBCs of 35 ± 1 cP at room temperature (22 to 24 °C). The same medium was used throughout the experiments. The salt concentration in the medium was adjusted to give an osmolality of 290 ± 5 mosm/kg, as determined by vapor pressure (Wescor 5100 C, vapor pressure osmometer, Logan, Utah).

Cone-Plate Rheoscope

Direct microscopic observations and video recording of red cells tank treading at various shear stress levels were accomplished by means of a counter-rotating cone-plate rheoscope (fabricated by K. Effenberger, Munich). The transparent cone-plate shear chamber had a nominal angle of 1.5° and was mounted on the stage of an inverted interference-contrast microscope (Diavert, E. Leitz KG, Wetzlar, FRG) with 100× objective. The microscopic objective was focused at the midplane of the gap at a radial distance of 1.5 to 2.0 mm from the center of rotation. A video camera (Dage-MTI 66 with Plumbicon tube, Michigan City, Ind), synchronized to 60 Hz strobe illumination (Strobex 236B, Chadwick-Helmuth Co., El Monte, Calif), transmitted images of the quasistationary cells to a videotape recorder (Sony Betamax SL, 5800, Tokyo) for subsequent analysis. The shear rate is practically uniform in the conical gap, and the applied shear stress, calculated as the product of shear rate by suspending medium viscosity, was set sequentially at values of 10, 20, 40, 60, 80, and 100 dynes/cm².

Indices of RBC Deformability

At each stress, six 35-mm photographs were taken of random groups of cells in the rheoscope gap. A typical field of stretched, tank-treading cells is shown in Fig 1. Enlarged 8-× 10-in photographs were placed on a magnetic digitizer board coupled to a Hewlett-Packard (Cupertino, Calif) 1,000 desktop computer. Cells having a sufficiently sharp boundary and not interfering with neighboring cells were manually traced with a cursor, thus converting the peripheral profile to digital form. At each stress, 30 to 50 cells were digitized. From the digital input, several geometric indices, such as length, width, circumference, area, ellipticity, and orientation of principal axes, were then computed. The percentage of cells not tank treading at each stress was determined by visual inspection and utilized as one index of deformability. For each of the tank-treading cells, the ratio of length to width of the quasiepipolar profile and stored. This ratio was the second selected index of cellular deformability. It should be noted that the length referred to here is not the true cell length, but the length of the cell image projected on the viewing plane. The projected length equals the true length multiplied by the cosine of an unknown angle of inclination. On the other hand, the measured width is a true width.

The third index of deformability examined was characteristic time of shape recovery (tᵣ), derived from the time course of cell length following abrupt cessation of shearing. After establishment of steady shear flow in the rheoscope gap at a moderate shear stress of 20 dynes/cm², the rheoscope motor was switched off, bringing the flow in the gap to rest within 10 ms. Released from the distributed surface shear stress, suspended cells stop tank treading within 17 ms, and recover their natural biconcave shape over a period of about 600 ms. The shape recovery process was recorded on videotape and subsequently replayed frame by frame on a TV monitor. The start of the recovery was signaled on the monitor by an electrical impulse from the rheoscope motor. From this instant, the length of a selected cell’s image was measured every 16.67 ms on the monitor screen by means of a video caliper (Vista Electronics, La Mesa, Calif; model 305r). The length data were electronically transferred to an Apple II Plus microcomputer using an A/D converter (Mountain Computer, Denver). These data were finally fitted by a three-parameter (Lᵢ, Lᵣ, tᵣ) minimization of the mean square error to the simple exponential form

\[
\frac{L - L_i}{L_i - L_0} = e^{-\frac{t}{t_i}},
\]

where Lᵢ is the instantaneous length corresponding to time, t; Lᵣ is the initial length, ie, at the start of the recovery process; Lᵢ is final length.
(or diameter) of the discoidal cell; and $t_r$ is the characteristic recovery time. This process was repeated for ten different cells in each test aliquot. Further details of the method are given by Carroll.

**Physical/Chemical Assays**

Mean cell volume (MCV) and mean cell hemoglobin concentration (MCHC). MCV and MCHC in aliquots of the top and bottom 10% cell fractions were determined by Coulter Counter (model S Plus II, Hialeah, Fla).

Hemoglobin $A_{t}$. Hemoglobin $A_t$ (HbA,) levels were estimated by ion-exchange column chromatography using Isolab (Akron, Ohio) columns and reagents.\(^{15,17}\)

Red cell phospholipid-phosphorus. Aliquots of washed red cells (from the top and bottom 10% fractions of cells) were extracted with chloroform methanol (2:1) and washed by the method of Folch et al,\(^{18}\) and the phosphorus content of the extracted hydrolyzed phospholipids was determined by the method of Fiske and Subbarow.\(^{19}\)

Preparation of red cell membranes. Hemoglobin-free ghosts of washed red cells were prepared by the method of Dodge et al,\(^{20}\) for determination of membrane protein and for extraction of membrane phospholipids. Membrane protein content was determined by the method of Lowry et al,\(^{21}\) and phospholipid-phosphorus was determined by the method of Fiske and Subbarow,\(^{19}\) as described above.

Glucosylation of membrane proteins. Glucosylation of red cell membrane protein was assessed by a modification of the method of Schleicher and Wieland.\(^{22}\) Briefly, membrane preparations were hydrolyzed in 6 N HCl (for 24 hours) and eluted in a micropak MC H-10 column (30 cm x 4 mm) on a high-pressure liquid chromatography system (Varian 5000, from Varian Instrument Group, Walnut Creek, Calif). Standard furosine was prepared by hydrolysis (as above) of fructose-lysine. The identity of furosine was confirmed by gas chromatography-mass spectrometry. Elution of furosine was carried out by a gradient of phosphoric acid (9 mmol/L) and acetonitril 40%. Furosine was eluted at 3.63 ± 0.08 minutes over numerous experiments and was quantified by its absorbance at 280 nm. Five hundred picomoles of furosine gave an integrated area of 251,000 ± 28,000 units (mean ± SD). The concentration of furosine in the unknown samples was calculated from the areas under the peaks obtained for the furosine standards.

**Statistical Analyses**

Mean values and standard deviations for MCV, MCHC, HbA,, red cell phosphorus content, and membrane glucosylation were calculated for the young and old cell groups from all ten donors. For each aliquot sheared in the rheoscope (one donor, top or bottom fraction), the mean length-width ratio (L/W) at a particular stress was determined from a sample of 30 to 50 cells in that aliquot. These means were then averaged separately for the young and old fractions of the ten donors and plotted. Characteristic recovery times were calculated for ten cells from each test aliquot, and these values were averaged over ten donors. In all cases, the differences between the young and old cell groups were tested for statistical significance using a two-tailed paired t test.

**RESULTS**

The enlarged photographs of red cells subjected to graded levels of shear stress in the rheoscope were analyzed for (1) percentage of tank-treading cells and (2) steady-state elongation. At the lowest stress applied (10 dynes/cm\(^2\)), all of the young cells, but only ~88% (Fig 2) of the old cells, were capable of tank}

![](https://www.bloodjournal.org/content/8/3/276/F2.large.jpg)

**Fig 2.** Percentage of "old" erythrocytes tank-treading. Plotted points are means ± SD; numbers in parentheses indicate number of donors. Old cells were derived from the bottom 10% fractions. All young cells (top 10% fraction) were tank treading at all levels of applied stress.

![Projected length-width ratio of tank-treading cells as a function of applied shear stress. Plotted are means ± SD computed from means of eight or ten donors. Numbers in parentheses indicate number of donors at each stress.](https://www.bloodjournal.org/content/8/3/276/F3.large.jpg)
(66%) and the loss of membrane phospholipid (18%) in the old cells.

**DISCUSSION**

These rheoscopic measurements document a highly significant age-related reduction in steady-state elongation \((L/W)\) under shear and an increase in the shape recovery time \((t_c)\) of circulating red cells. Since these measurements of steady-state elongation were obtained under conditions of dynamic equilibrium, they can be influenced by the viscoelastic properties of the cells as well as by cell geometry, i.e., excess surface area. The 15% to 20% reduction in steady-state elongation of old cells is comparable to the increase in recovery time (25%) and the previously reported decrease in the rate of tank treading (-15%). The latter cellular characteristics, however, are thought not to be influenced appreciably by cell geometry. Thus, under the conditions of these experiments, differences in membrane viscoelastic properties of old and young cells are probably the major determinants of the differences observed in steady-state elongation as well as in shape recovery times.

The ability of red cells to tank tread in the rheoscope is relevant to red cell motion and deformation in capillary flow, where nonuniform surface tractions, due to asymmetry of shape and position as well as strong interaction with neighboring cells, also induce membrane rotation and cytoplasmic circulation. Furthermore, mathematical modeling of this fluid drop-like behavior has shown that the asymmetrical configuration and concomitant membrane rotation act to attenuate the hydraulic resistance to erythrocyte movement through the narrowest vessels. Evidence showing that erythrocytes with stiffened membranes, but still capable of tank treading, can circulate freely for hours suggests further that this capacity may be crucial to the erythrocyte’s passage through the spleen.

The characteristic shape recovery time, \(t_c\), is a function primarily of the viscoelastic properties of the red cell membrane. The Kelvin-Voigt viscoelastic solid, characterized by elastic and viscous elements acting in parallel, has been widely applied as a model of the red cell membrane, and for such a material, \(t_c\) is expressible as:

\[
t_c = \frac{\eta}{\mu},
\]

where \(\eta\) is the membrane surface viscosity (dyne s/cm) and \(\mu\) is the two-dimensional (surface) shear elastic modulus (dyne/cm). In this study, we found a mean \(t_c\) of 0.127 seconds for the youngest 10% cells and 0.152 seconds for the oldest 10%. Linderkamp and Meiselman have reported 0.099 and 0.148 seconds for their youngest and oldest 5% of separated cells, respectively. More recently, Nash and Meiselman gave 0.13 and 0.19 seconds, respectively. The comparison of the present results to these two reported results is remarkably good, considering the difference in technique (rheoscope v micropipette) and analysis (linear—equation 3, v nonlinear exponential recovery curve). Taken by itself, the increase in \(t_c\) with cell age says that membrane viscosity, \(\eta\), has increased more than membrane elastic modulus, \(\mu\). An additional independent determination of either \(\eta\) or \(\mu\) is therefore necessary to expose the change, if any, experienced by the other

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**Table 1. Physical and Chemical Properties of Young and Old Cell Populations**

<table>
<thead>
<tr>
<th></th>
<th>Young (Top 10%)</th>
<th>Old (Bottom 10%)</th>
<th>(t)</th>
<th>Significance Level (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean cell volume† (MCV, (\mu m^3))</td>
<td>96.96 ± 3.30</td>
<td>94.69 ± 3.94</td>
<td>2.85</td>
<td>&lt;.025</td>
</tr>
<tr>
<td>Mean cell hemoglobin concentration (MCHC, g/dL RBC)</td>
<td>31.92 ± 0.39</td>
<td>32.74 ± 0.46</td>
<td>4.83</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Type A, hemoglobin (HbA,) (% of total Hb)</td>
<td>5.51 ± 0.63</td>
<td>7.17 ± 2.18</td>
<td>2.66</td>
<td>&lt;.050</td>
</tr>
<tr>
<td>Membrane glucosylation (nmol HMF/mg protein)</td>
<td>2.36 ± 0.92</td>
<td>3.91 ± 1.21</td>
<td>4.33</td>
<td>&lt;.005</td>
</tr>
<tr>
<td>Lipid-soluble phosphorus (mg Pi per RBC ( \times 10^{10} ))</td>
<td>3.99 ± 0.36</td>
<td>3.29 ± 0.32</td>
<td>10.15</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

All numerical entries represent mean ± SD calculated from the mean values of ten donors. HMF, hydroxymethylfurfural.

† Determined by Coulter counter.
mechanical property. Linderkamp and Meiselman estimated $\mu$ by micropipette aspiration and then calculated $\eta = t_e \cdot \mu$ (equation 4). They found a 16% increase in the mean $\mu$ from youngest to oldest 5% groups, a change judged to be statistically insignificant, whence they obtained $\eta = (0.54 \pm 0.15) \times 10^{-3}$ and $(0.95 \pm 0.22) \times 10^{-3}$ dyne-s/cm for their young and old cells, respectively—an increase of 76%. These investigators consequently concluded that increased membrane viscosity is predominant among age-related changes that might control splenic retention. This conclusion is consistent with that of Schmid-Schönbein and Gaethgens.

In contrast to the reported 12% to 26% decreases in MCV from youngest to oldest 5% cell fractions measured by micropipette aspiration, and the 13% to 29% decreases for comparable cell fractions estimated by hematocrit and cell count determinations, our electronic determinations showed only a 2.4% decrement between the youngest and oldest 10% fractions. On the other hand, our findings of an 18% decline in membrane phospholipids is well bracketed by the 8.5% to 26.0% decreases in membrane surface area measured by the micropipette technique and is consistent with reported 17% to 21% losses of membrane phospholipid and the 15% loss of membrane protein reported by Cohen et al. Surprisingly, Shiga et al measured no loss of membrane protein in the older cells, which is in conflict with the other findings cited.

The possibility that electronic measurements of erythrocyte volume can be influenced by cell deformability led us to seek an independent assessment of MCV. Using an isotope dilution technique with $^{57}$Co-EDTA as an extracellular space marker, we determined MCV in seven additional subjects and found a decrease of 11.1% $\pm$ 2.3% (SEM) from the youngest to oldest 10% fractions. Electronic measurements on the same cells revealed an average difference of only 1.1%. The isotope dilution data are more consistent with previously published data and suggest that our Coulter counter data underestimate the volume differences in old and young cells. We also measured membrane protein as well as membrane phospholipid in the top and bottom fractions of cells from three of these subjects. In each case, the fractional losses of membrane protein and phospholipids from the bottom 10% fraction of cells were virtually identical; the ratio of membrane phospholipid to protein in the top 10% fraction divided by the same ratio in the bottom 10% fraction was 1.03 $\pm$ 0.02 (SEM). Our finding that fractional losses of membrane phospholipid and protein from older cells are identical is consistent with the observations of Cohen et al and with their suggestion that membrane is removed from red cells in the form of lipoprotein. Thus, these findings attest to the validity of using red cell phospholipid content as an index of cell surface area and document a large decrease in excess surface area with aging.

To examine the degree to which the older cells have become more spherical in shape, we calculated the ratio of the ESI for the old cells (ESI$_o$) to that of the young cells (ESI$_y$):

$$\frac{ESI_o}{ESI_y} = \frac{S_o / V_o^{2/3}}{S_y / V_y^{2/3}}.$$  (5)

If it is accepted that membrane surface area is proportional to the measured amount of phospholipid per cell (Table 1), then the ratio $S_o/S_y$ is equal to the phospholipid ratio $3.286/3.986 = 0.824$. Using the Coulter volume data shown in Table 1, we then compute $ESI_o/ESI_y = 0.84$. This figure is considerably lower than the 0.92 indicated by the data of Linderkamp and Meiselman under isotonic conditions. If instead we take the volume ratio, $V_o/V_y = 1.124$, found by isotope dilution, the ratio $ESI_o/ESI_y$ becomes 0.89, which is in fair agreement with Linderkamp and Meiselman.

It is also enlightening to consider these geometrical changes in terms of the excess surface area fraction (ESI-1), ie, the ratio of excess (or surplus) membrane area to that of a sphere of the same volume. According to Linderkamp and Meiselman, this fraction was 0.45 in their young cell population. Their finding of ESI = 1.34 for the old cells thus indicates a drop of 25% (from 0.45 to 0.34) in excess area fraction. A similar calculation with our data, but basing also on ESI-1 = 0.45 for young cells, gives decreases of 48% (volume ratio from Coulter data) and 65% (volume ratio by isotope dilution).

The MCHC measurements presented in Table 1 were also obtained by Coulter counter and thus show an increment (+2.5%) that is inversely proportional to the decrease in MCV, implying that hemoglobin is conserved. According to the Ross-Minton model for the viscosity of hemoglobin solutions, the 2.5% increment in MCHC would result in an increment of about 11% in cytoplasmic viscosity. Based on a value of 8 cP for the young cells at room temperature (corresponding to MCHC $= 31.9$ g/dL), we would expect the cytoplasmic viscosity of the old cells to be no more than 8.8 cP. If, however, we accept as more accurate the 11% decrease in MCV found by isotope dilution, the corresponding 11% increase in MCHC would result, according to the Ross-Minton model, in a cytoplasmic viscosity in the old cells of approximately 12.9 cP at room temperature—a 61% increase relative to the young cells. Even this much of an increase should produce a negligible impact on the measured $t_e$, as the shape recovery process is known to be dominated by
the viscoelastic properties of the membrane. The effect on tank-treading frequency is expected to be weak as well, since cytoplasmic dissipation, which is proportional to cytoplasmic viscosity, appears to account for less than half of the total dissipation in the tank-treading cell.29

The 66% increase in membrane protein glucosylation (relative to the youngest 10% group) is conspicuous and raises again the possibility that modifications in molecular configuration might alter membrane mechanical properties. Although glucosylation entails the binding of glucose molecules to certain protein groups within the membrane cytoskeleton, there is no evidence to our knowledge that this process affects either membrane elasticity or viscosity. Our study of erythrocytes in diabetes, described in a companion paper,29 supports this conclusion. While we observed significant differences in the extent of glucosylation of membrane proteins between diabetics and nondiabetics, the shear-induced responses of the diabetic erythrocytes were virtually identical to those of the control cells. Insofar as HbA1 is concerned, although the percentage of glucosylated hemoglobin was found to increase significantly with cell age, the fact remains that it represents only 7% of the total hemoglobin present in the old cells. Hence, even though the pure glucosylated hemoglobin may have a substantially greater intrinsic viscosity than nonglucosylated hemoglobin,31 its contribution to the effective cytoplasmic viscosity is bound to be minor. This is borne out by the studies of McMillan,32 who reported an insignificant increase in the viscosity of diabetic hemoglobin relative to normal.

Our glucosylation data on both hemoglobin and on membrane proteins do, however, attest to the effectiveness of the centrifugation technique used here in fractionating red cells according to age and are in agreement with those of Fitzgibbons et al.33 Nonenzymatic glucosylation is a time-dependent process, increasing monotonically with the duration of exposure to plasma glucose. The fact that the denser fraction of erythrocytes is more glucosylated means that those cells have been exposed to plasma glucose longer, ie, they must be older. Although one iron 59 tracer study1 casts doubt on the validity of age separation by density fractionation, these glucosylation data nevertheless constitute a reliable age marker and document that effective age separation was achieved in this study.

To summarize, our microrheologic observations indicate a significant impairment of RBC deformability with in vivo aging. Analysis of the results suggests that increases in both membrane viscosity and elastic modulus (stiffness) contribute to the loss of deformability. Significant loss (48% to 65%) of cell excess surface area is a geometric change that must be considered, along with the aforementioned changes in the intrinsic mechanical properties of the erythrocyte membrane, as a possible mechanism limiting cell deformability and contributing to splenic sequestration.

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Age-related changes in deformability of human erythrocytes

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