Effects of 2,3-Diphosphoglycerate on the Mechanical Properties of Erythrocyte Membrane

By Richard E. Waugh

Investigations by Schindler et al. and Sheetz and Casalay have indicated that high (≈10 mmol/L) concentrations of 2,3-diphosphoglycerate (2,3-DPG) have a destabilizing effect on erythrocyte membrane and the membrane skeleton. We have investigated changes in the membrane mechanical properties that occur at elevated 2,3-DPG levels in both intact cells and ghosts. The membrane shear modulus, viscoelastic recovery time constant, critical force, "plastic" viscosity, and material relaxation time constant were measured by standard micropipette and flow channel techniques. Intact cells showed no change in mechanical properties at physiologic ionic strength and 2,3-DPG concentrations of about 20 mmol/L, except for an increase in membrane viscosity resulting from an increased cellular hemoglobin concentration that occurs when the 2,3-DPG concentration is elevated. At ionic strengths 20% below physiologic and 2,3-DPG concentrations of ≈20 mmol/L, decreases in membrane shear modulus and membrane viscosity were observed. In ghosts, no changes in these properties were observed at a 2,3-DPG concentration of 10 mmol/L and ionic strengths as low as 25% below physiologic, but a decrease in the force required to form tethers (critical force) was observed at physiologic ionic strength. The decrease in membrane shear modulus and viscosity of intact cells and the reduced critical force in ghosts are consistent with the results of other investigators. However, the difference in the effects of 2,3-DPG on ghosts and intact cells indicates that the effects of 2,3-DPG depend strongly on the conditions of the experiment. It appears unlikely that 2,3-DPG affects erythrocyte membrane material properties under physiologic conditions.

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THE erythrocyte membrane consists of a phospholipid bilayer containing many integral proteins, an extracellular glycocalyx, and a membrane-associated proteinaceous skeleton at the cytoplasmic surface of the bilayer. Mechanically, it is recognized that the bilayer behaves like a two-dimensional liquid. It resists changes in area elastically, but it can freely undergo constant area shear deformation (in-plane deformation), and particles in the bilayer are free to diffuse in the plane of the surface. The shear elasticity that the membrane exhibits is due to the presence of the membrane skeleton, which also acts to limit the rate at which some integral proteins diffuse in the surface. Recently, it was observed that the lateral mobility of integral membrane proteins was increased in erythrocyte ghosts containing elevated levels of 2,3-diphosphoglycerate (2,3-DPG) and adenosine triphosphate (ATP). Subsequently, Sheetz and Casalay observed that polyanions, notably 2,3-DPG and ATP, accelerated the rate at which isolated membrane skeletons dissociate in vitro. The dissociative effects of 2,3-DPG were found to be much stronger than the effect of ATP on the isolated skeleton, 2,3-DPG having a half maximal effect at 2.5 mmol/L and ATP having a half maximal effect at 8.0 mmol/L. These results supported the hypothesis that the increased mobility of integral proteins was due to a decrease in the stability of the cytoskeleton, or an increase in the rate of exchange of the associations among the membrane skeletal components. Because of the importance of the skeleton in maintaining proper mechanical function of the membrane, it was postulated that 2,3-DPG might also affect the mechanical properties of the membrane. This postulate was supported by the observations of Chasis et al. that ghosts with elevated 2,3-DPG concentrations fragmented more readily than control ghosts in the ektacytometer. It was suggested that these effects might serve an important physiologic function.

To test the effects of elevated concentrations of 2,3-DPG on the mechanical properties of erythrocyte membrane we have measured the intrinsic membrane material constants that have been defined to characterize the membrane mechanical behavior. We have performed these measurements on the membranes of both intact cells and resealed erythrocyte ghosts. The particular material constants that were measured are described and defined in the following section.

Definition of Material Properties

The mechanical behavior of the red cell membrane is complex. (For reviews see Evans and Hochmuth and Evans and Skalak.) Different constitutive relationships have been used to describe the material behavior, depending on the magnitude and duration of the applied forces. The resistance of the membrane to area changes is large, and the area of the membrane of each cell can be presumed to be constant during these experiments. The membrane resists in-plane extensions elastically. The maximum shear resultant in the surface, $T_s$, is related to the material extension ratio, $\lambda$, by the surface elastic shear modulus, $\mu$:

$$T_s = \frac{\mu}{2} \left( \lambda^2 - \lambda^{-2} \right)$$

When surface deformations are dynamic, the membrane exhibits viscous character. For rapid deformations within the elastic regime, the total shear resultant can be written as the...
sum of the elastic and viscous contributions:

\[ T_s = \frac{\mu}{2} (\lambda^2 - \lambda^{-2}) + 2\eta V_s \]  

where \( \eta \) is defined as the membrane viscosity coefficient for rapid deformation and \( V_s \) is the rate of surface shear deformation.

When the membrane is forced to maintain a deformed geometry for a long time, the stresses in the membrane relax, and the surface begins to assume a new “equilibrium” geometry. The partial derivative of the log of the material extension with time is given by:

\[ \frac{\partial \ln \lambda}{\partial t} = \frac{1}{2} \frac{\partial \tilde{T}_s}{\partial t} \left[ \tilde{T}_s^2 + 1 \right]^{1/2} + \frac{\tilde{T}_s}{2\tau_c} \]  

where the dimensionless shear resultant \( \tilde{T}_s \) is the ratio \( T_s/\mu \), the characteristic time \( \tau_c \) is the ratio of \( \eta/\mu \), and \( t \) is time. The creep viscosity coefficient \( \eta_c \) characterizes the rate at which the material stresses relax.

In the flow channel experiment, cells that have adhered to a glass surface are subjected to a fluid shear force. If the force is large enough a strand of membrane (tether) is pulled from the cell body. The force at which tethers begin to form is called the critical force. Originally the critical force was thought to be related to the yield shear resultant of the membrane skeleton. However, recent evidence obtained in our laboratory indicates that tethers may not contain membrane skeleton. These observations support a view that tether formation involves stripping bilayer away from the cell. Therefore, the present results are reported in terms of the critical force, and not a yield shear resultant. Regardless of the molecular mechanism involved, the critical force provides a measure of membrane stability and resistance to fragmentation.

The rate at which the tether forms under forces exceeding the critical force is characterized by a viscosity coefficient. For historical reasons we will refer to this as the plastic viscosity of the membrane, even though tether formation may not actually involve plastic deformation of the membrane.

**MATERIALS AND METHODS**

**Intact cells**

Cells were obtained from healthy donors either on the morning of an experiment or on the evening before and kept in acid citrate dextrose plasma prior to preparation for the experiment. Cells were separated from the plasma by centrifugation, the buffy coat was discarded, and cells were washed twice in isotonc phosphate buffered saline (PBS) (130 mmol/L NaCl, 25 mmol/L Na2HPO4, 6.2 mmol/L KH2PO4, 10 mmol/L sodium azide, pH 7.4). The osmolarity of the solutions was measured by freezing point depression and fell within range, 290 to 305 mosm.

Elevation of the 2,3-DPG concentration was accomplished by the method of Duhm. Packed cells (0.25 mL) were brought to a total volume of 5.0 mL in an incubating buffer containing 10.0 mmol/L inosine, 10.0 mmol/L pyruvate, 50 mmol/L Na2HPO4, and 79.5 mmol/L NaCl (300 mosm, pH 7.4), and kept at 37 °C for two hours. To compensate for the decrease in internal pH due to the elevated concentration of impermeant anion after incubation, the cells were washed twice in phosphate buffered saline at high pH (pH 7.9 to 8.5) then suspended at high pH for measurement. Control cells underwent identical manipulations but were kept in PBS (pH 7.4) for the entire procedure. Cells not used for mechanical measurements were frozen at −70 °C until 2,3-DPG determinations could be made.

**Sucrose incubation to deplete cations**

In some control measurements, cells were preincubated in low ionic strength sucrose solutions to deplete cells of cations and increase the MCHC under physiologic conditions. Cells were washed twice in PBS then suspended at 5% hematocrit at 37 °C for 40 minutes in a solution containing 5 mmol/L NaCl, 3.0 mmol/L HEPES (pH 7.4), and 240 mmol/L sucrose. The osmolarity of the solution was 300 mosm. After 40 minutes cells were washed in PBS and suspended in PBS for measurements.

The concentration of 2,3-DPG within the cells was determined enzymatically (see Technical Bulletin No. 665, Sigma Chemical Co, St. Louis, Mo). All reagents were obtained from Sigma. Phosphoglycerate mutase was used to hydrolyse 2,3-DPG to 3-phosphoglycerate, and the amount of released phosphate was determined colorimetrically by the method of Fiske and Subbarow.

The mean cellular hemoglobin concentration (MCHC) was determined by standard techniques. A suspension of red cells was sampled for hematoctrit measurement using a capillary tube. An aliquot of the same suspension was taken for hemoglobin determination using Drabkin’s solution and absorbance measurements at 540 nm.

Internal pH was determined by measuring the ratio of internal to external chloride concentrations. The method was identical to the one described by Knauf et al with the exception that the cells were equilibrated at 37 °C rather than at 0 °C. 36Cl was used to determine the amount of chloride inside and outside the cells. Cell water was determined by wet and dry weights and corrections for trapped water were obtained by using tritiated inulin as extracellular marker. Given the internal and external chloride concentrations, the hydrogen ion concentration ratio (hence the difference between the internal and external pH) can be calculated from the Donnan equilibrium condition:

\[ \frac{[\text{H}^+]_{\text{int}}}{[\text{H}^+]_{\text{ext}}} = \frac{[\text{Cl}^-]_{\text{int}}}{[\text{Cl}^-]_{\text{ext}}} \]

or

\[ \text{pH}_{\text{int}} - \text{pH}_{\text{ext}} = \log_{10} \left( \frac{[\text{Cl}^-]_{\text{int}}}{[\text{Cl}^-]_{\text{ext}}} \right) \]

**Ghost preparation**

Resealed ghosts were prepared by a method similar to that of Schwoch and Passow. Cells were drawn and washed on the afternoon before an experiment and refrigerated overnight in isotonic phosphate buffered saline. Cells were suspended at 50% hematocrit in 165 mmol/L NaCl and cooled to 0 °C. Thirty-five milliliters (35 mL) of lysing solution (3.5 mmol/L acetic acid, 4.0 mmol/L MgSO4, 0.1 mmol/L phenyl-methyl-sulfonyl-flouride, and 1.0 mmol/L EGTA) were added to 3.5 mL of cell suspension. Care was taken to assure that the temperature remained at 0 °C (± 0.2 °C). After five minutes, 3.5 mL of resealing solution (2.0 mol/L KCl, 25 mmol/L TRIS) was added to the lysate. After five minutes at 0 °C the suspension was transferred to a 37 °C bath and incubated for 45 minutes. After incubation, the ghosts were washed in phosphate buffered saline (300 mosm, pH 7.4, unless otherwise noted) and suspended in the same buffer for measurements. All mechanical measurements were performed at 37 ± 1 °C.
Measurement of material properties

The surface elastic shear modulus was measured by micropipette aspiration after the method of Evans and La Celle. Cells were aspirated in the dimple region into a micropipette (0.9 μm ID). The length of the projection in the pipette, \( L_p \), was recorded as a function of the aspiration pressure, \( \Delta P \). Evans showed that the relationship between \( L_p \) and \( \Delta P \) is approximately linear when \( L_p > R_p \). Chien et al. have used the following linear approximation to Evans' original formulation:

\[
\frac{\Delta P R_p}{\mu} - C_1 \frac{L_p}{R_p} + C_2
\]

where \( C_1 = 2.45, C_2 = -0.63, R_p \) is the pipette radius, and \( \mu \) is the surface elastic shear modulus. The shear modulus was calculated from the measured data pairs (\( \Delta P, L_p \)) by linear regression to Equation 5.

The viscosity coefficient for rapid deformation was measured by the whole cell recovery technique developed by Hochmuth et al. Cells were allowed to settle onto a glass surface and adhere. The length-to-width ratio of the cells were measured exponentially with time and can be described by the following relationship:

\[
\frac{(L/W)_0 - (L/W)_\infty}{(L/W)_0 - (L/W)_\infty} = e^{-\frac{t}{\tau}}
\]

where \( (L/W)_0 \) is the largest ratio of length to width and \( (L/W)_\infty \) represents the length-to-width ratio in the completely relaxed state. The time constant, \( \tau \), is the ratio of the membrane viscosity coefficient to the membrane shear modulus: \( \tau = \eta/\mu \). The length and width of the cell were measured as functions of time, and the time constant was determined by nonlinear least squares regression with two free parameters, the time constant \( \tau \) and the relaxed length-to-width ratio, \( (L/W)_\infty \).

The cells were also tested using the flow channel technique. A thin (0.015 mm x 1.0 cm x 4.0 cm) channel was formed with a parafilm gasket between a slide and coverglass. The glass in the channel was pretreated with an 8.0% plasma solution. Cells were infused into the channel at a 1.5% to 2.0% hematocrit and allowed to settle on the surface of the channel and remain undisturbed for 30 minutes, during which time some of the cells became attached to the glass. A force was exerted on the cells by fluid moving through the channel. The flow was controlled by adjusting the pressure drop across the channel, and the pressure was monitored using a differential pressure transducer. The magnitude of the force was approximated by the product of the fluid shear stress at the wall times the projected area of the cell. To be consistent with previous flow channel studies, a value of 50 μm² was used for the projected area of the cell. This corresponds to a disc approximately 8.0 μm in diameter. When the force becomes large enough, a strand of membrane (tether) begins to form between the body of the cell and the attachment site. The rate of tether formation was measured as a function of the applied force. Two parameters were determined from the data: the critical force \( f_c \) is the force at which tether growth began to occur, and the plastic viscosity coefficient \( \eta_p \) provides a measure of how fast the tether forms when \( f_c \) is exceeded.

The long-term membrane relaxation was measured using the method of Markle et al. Cells were suspended at dilute concentration in phosphate buffered saline (pH 7.4, ~300 mOsm) containing a carefully measured concentration of bovine serum albumin. Cells were aspirated into micropipettes in the dimple region. After a shear modulus measurement was performed the cell was held at a fixed pressure for 20 minutes. Exposure of the cell to light was minimized. After 20 minutes the cell was expelled from the pipette. A residual bump remained on the cell surface. The height of the bump was constant for periods of several minutes after expulsion. The height of the bump was measured within 1 minute after expulsion, as soon as the cell could be recaptured and positioned in the field of view. The ratio of the height of the bump in the pipette before release, \( Z_p \), to the height of the residual bump, \( Z \), gives a measure of the material relaxation (creep) coefficient, \( \eta_c \). The relationship between \( Z/Z_p \) and time for different values of \( \eta_c \) is given by the solution to Equation 3 and is not available in closed form.

RESULTS

Intact Cells

Effects of inosine-pyruvate-phosphate (IPP) incubation. The incubation of intact erythrocytes in IPP caused a dramatic increase in the concentration of 2,3-DPG within the cells. Typically, concentrations increased from about 2.0 to 4.0 mmol/L in control cells to postincubation levels of 17.0 to 24.0 mmol/L. The concentration of chloride within the cells decreased during the incubation because of the increasing concentration of impermeant anion. This causes a decrease in intracellular pH (calculable from the Donnan equilibrium) and an increase in the mean cellular hemoglobin concentration (MCHC) due to the loss of water that accompanies the loss of chloride. To avoid artifactual results due to the effects of changes in intracellular pH, cells with elevated 2,3-DPG were suspended in media with high external pH (7.9 to 8.5). The relevant data for solution osmolarity, MCHC, and intracellular pH are given in the tables summarizing the results of each mechanical test.

Shear modulus measurements. Three separate experiments are summarized in Table 1. Because of the strong dependence of the calculated value of the shear modulus on pipette radius and the uncertainty in measuring pipette diameters, valid comparisons between different cell populations can only be made within each experiment. Furthermore, because undetectable small particles lodged in the pipette tip during the experiments could change the effective radius of the pipette, measurements on control cells were performed at the beginning and the end of each experiment, and the average values were compared to ensure that the pipette had not been partially occluded during the course of the experiment. Any difference less than 10% in the value of \( \mu \) measured at the beginning and end of an experiment was considered satisfactory. In fact, for the data shown in Table 1, the difference was less than 3.0%.

Because cells with elevated 2,3-DPG have higher than normal MCHCs, the first experiment was performed at hypotonic salt concentration. A significant difference was observed between the moduli of cells loaded with 2,3-DPG by incubation in inosine-pyruvate-phosphate and cells incubated in saline (controls); \( (\mu_1 - \mu_2) = 0.0004, t \) test. Note
that this experiment (Table 1, experiment 1) duplicated (as closely as possible) the conditions of the experiment 2 in Table 2.

The second experiment was performed to see if the decrease in shear modulus was due simply to the change in salt concentration. No difference in shear modulus was observed in control cells over the range of salt concentrations used in the experiments.

The third experiment was performed to test for changes in shear modulus with 2,3-DPG concentrations at physiologic ionic strength. The cells with elevated 2,3-DPG levels were compared to two different control populations: one with a normal MCHC and one with an MCHC that had been elevated by preincubation in low ionic strength sucrose solutions. No difference in shear modulus was observed between cells with elevated 2,3-DPG concentrations and the controls.

In a fourth experiment (not listed in the table) the surface shear moduli of 2,3-DPG-loaded cells was found to be 0.0072 dyn/cm at 290 mOsm (15 cells, SD = 0.0014 dyn/cm) and 0.0054 dyn/cm at 220 mOsm (15 cells, SD = 0.0009 dyn/cm). These results are consistent with the results shown in Table 1, and indicate that the combination of elevated 2,3-DPG concentration and low salt concentration causes a reduction in the membrane shear modulus. The modulus for control cells at 290 mOsm was tested prior to the measurements on 2,3-DPG-loaded cells. The measured value was 0.0065 dyn/cm, SD = 0.0007, n = 10. Unfortunately, the pipette used in that experiment was lost before the last batch of control cells could be tested to check for partial occlusion of the pipette. Because of this, the results of this last experiment are inconclusive in themselves. Nonetheless, they are consistent with, and supportive of, the results given in Table 1.

Viscoelastic recovery. Measured values of the time constant for viscoelastic recovery for intact cells are tabulated in Table 2. Two experiments are summarized in the table. In experiment 1, cells with elevated 2,3-DPG concentrations are compared to control cells at physiologic salt concentrations. The time constant for recovery is longer at elevated 2,3-DPG levels. Note that in experiment 1, the increase in external pH overcompensated for the decrease in internal pH brought about by the elevated 2,3-DPG concentration. To see if this overcompensation might have affected the recovery time, measurements were performed on control cells suspended in a buffer with pH 8.5. For the controls at pH 8.5, r = 0.081s (SD = 0.030s, n = 15). This is not significantly different from the controls measured at pH 7.4 (P = 0.14). This indicates that the high pH was not the major factor causing the increased recovery time.

During the course of our studies several papers appeared indicating that the recovery time constant for an erythrocyte was increased at high MCHC. To check for the effects of increased MCHC in our measurements, experiment 2 was performed at reduced salt concentration to swell the cells and reduce the MCHC. Under these conditions no difference in the recovery time constant was observed between cells with elevated and normal 2,3-DPG concentrations.

Calculation of membrane viscosity. The membrane viscosity coefficient for rapid deformation is equal to the product of the recovery time constant and the membrane shear modulus. The coefficients calculated from the data in Tables 1 and 2 are given in Table 3. At physiologic salt concentrations there is an increase in the apparent membrane viscosity which is probably due to an increase in MCHC. At low salt concentration there is a reduction in membrane viscosity in proportion to the reduction in the membrane shear modulus. The data obtained from erythrocyte ghosts (see Tables 6 and 7) are also included for comparison.

Other viscosity coefficients. Because in our early experiments the viscosity coefficient for rapid deformation at physiologic ionic strength changed in the opposite direction

### Table 1. Shear Modulus of Intact Erythrocytes as a Function of 2,3-DPG Concentration

<table>
<thead>
<tr>
<th>Expt No</th>
<th>[2,3-DPG] (mmol/L)</th>
<th>Osmolarity (mOsm)</th>
<th>MCHC (g/dL)</th>
<th>pH&lt;sub&gt;a&lt;/sub&gt;</th>
<th>(μ) (dyn/cm)</th>
<th>SD</th>
<th>n</th>
<th>P (μ1 - μ2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.2 (control)</td>
<td>262</td>
<td>30.6</td>
<td>7.3</td>
<td>0.0063</td>
<td>0.0018</td>
<td>20</td>
<td>0.0004</td>
</tr>
<tr>
<td></td>
<td>21.3</td>
<td>242</td>
<td>34.4</td>
<td>7.2</td>
<td>0.0044</td>
<td>0.0009</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Control</td>
<td>242</td>
<td>37.1</td>
<td>—</td>
<td>0.0057</td>
<td>0.0014</td>
<td>22</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>298</td>
<td>34.5</td>
<td>—</td>
<td>0.0055</td>
<td>0.0018</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Control</td>
<td>292</td>
<td>—</td>
<td>—</td>
<td>0.0069</td>
<td>0.0010</td>
<td>19</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>292</td>
<td>46.0</td>
<td>7.2</td>
<td>0.0064</td>
<td>0.0009</td>
<td>16</td>
<td>0.04</td>
</tr>
</tbody>
</table>

*Elevated by preincubation in sucrose.

### Table 2. Viscoelastic Recovery Time Constant for Intact Erythrocytes With Elevated 2,3-DPG Concentrations

<table>
<thead>
<tr>
<th>Expt No</th>
<th>[2,3-DPG] (mmol/L)</th>
<th>Osmolarity (mOsm)</th>
<th>MCHC (g/dL)</th>
<th>pH&lt;sub&gt;a&lt;/sub&gt; (sec)</th>
<th>n</th>
<th>P</th>
</tr>
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<tr>
<td>1</td>
<td>19.7</td>
<td>301</td>
<td>41.0</td>
<td>7.8</td>
<td>0.107</td>
<td>0.054</td>
</tr>
<tr>
<td></td>
<td>4.7</td>
<td>301</td>
<td>36.4</td>
<td>7.2</td>
<td>0.067</td>
<td>0.029</td>
</tr>
<tr>
<td>2</td>
<td>19.1</td>
<td>242</td>
<td>36.9</td>
<td>7.2</td>
<td>0.065</td>
<td>0.036</td>
</tr>
<tr>
<td></td>
<td>1.4</td>
<td>242</td>
<td>32.1</td>
<td>7.3</td>
<td>0.065</td>
<td>0.034</td>
</tr>
</tbody>
</table>

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Table 4. Critical Force and Plastic Viscosity Coefficient (Intact Cells)

| [2,3-DPG] (mmol/L) | fC (10⁻³ dyn) | n | pHm | Avg | SD | n | P | Avg | SD | n | P |
|---------------------|---------------|---|-----|-----|----|---|---|----|----|---|---|---|
| 2.8                 | 1.5           | 4 | 7.2 | 1.00| 0.18| 17 |    |    |    |    |    | 0.0023 | 0.0014 | 16 |
| 22.0                | 2.3           | 4 | 7.4-7.6 | 0.98| 0.19| 31 | 0.80 | 0.0025 | 0.0017 | 31 | 0.58 |

Table 5. Residual Deformation (Intact Cells)

<table>
<thead>
<tr>
<th>[2,3-DPG] (mmol/L)</th>
<th>Z/Zm</th>
<th>Avg</th>
<th>SD</th>
<th>n</th>
<th>pHm</th>
<th>Z/Zm</th>
<th>Avg</th>
<th>SD</th>
<th>n</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
<td>0.6</td>
<td>6</td>
<td>7.1-7.3</td>
<td>0.58</td>
<td>0.10</td>
<td>12</td>
<td>0.15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20.2</td>
<td>2.0</td>
<td>9</td>
<td>7.4-7.6</td>
<td>0.52</td>
<td>0.13</td>
<td>15</td>
<td>0.15</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Ghost morphology and size. Morphologically, the ghosts were nearly all discocytes although there were usually a few echinocytes and cups. The number of echinocytes and cups varied from preparation to preparation, but these nondiscoidal cells were not used for measurements. We estimated the area of cells in the flow channel by taking the product of the length and width of the cells at zero applied force multiplied by π/4. It should be recognized that because of limited resolution, these measurements are potentially subject to significant errors in absolute magnitude, but they should provide reliable comparisons for the resting diameters of the different cell populations. The projected area of the 2,3-DPG-loaded ghosts was 60.6 μm², SD = 10.1 μm², n = 107. The projected area of control ghosts was 63.3 μm², SD = 9.4 μm², n = 59. These were not significantly different by the student t test (P = 0.08). Precise measurements of cell thickness were not possible, but no obvious difference in the thickness dimensions of the different cell types was observed.

There was no appreciable change in the viscoelastic recovery time constant under the conditions of our measurements (Table 6). The salt concentration of the 16 mmol/L 2,3-DPG samples was elevated because these ghosts were visibly swollen after formation and the higher salt concentration was needed to return them to their normal biconcave shape. (Phosphate was added to two of the samples at the suggestion of Dr M. Sheetz, who pointed out that the accelerative effects of 2,3-DPG on cytoskeletal dissociation that he has observed only occurred in the presence of phosphate.)

Table 6. Viscoelastic Recovery Time Constant for Erythrocyte Ghosts at Elevated 2,3-DPG Concentrations

<table>
<thead>
<tr>
<th>[2,3-DPG] (mmol/L)</th>
<th>[PO₄] (mmol/L)</th>
<th>(r) (sec)</th>
<th>SD</th>
<th>n</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>—</td>
<td>0.049</td>
<td>0.024</td>
<td>69</td>
<td>—</td>
</tr>
<tr>
<td>2.0</td>
<td>—</td>
<td>0.054</td>
<td>0.027</td>
<td>33</td>
<td>0.39</td>
</tr>
<tr>
<td>4.0</td>
<td>—</td>
<td>0.042</td>
<td>0.021</td>
<td>22</td>
<td>0.22</td>
</tr>
<tr>
<td>8.0</td>
<td>—</td>
<td>0.038</td>
<td>0.019</td>
<td>24</td>
<td>0.03</td>
</tr>
<tr>
<td>16.0*</td>
<td>—</td>
<td>0.049</td>
<td>0.019</td>
<td>27</td>
<td>0.95</td>
</tr>
<tr>
<td>8.0</td>
<td>1.0-3.0</td>
<td>0.052</td>
<td>0.024</td>
<td>33</td>
<td>0.52</td>
</tr>
<tr>
<td>16.0*</td>
<td>1.0-3.0</td>
<td>0.050</td>
<td>0.026</td>
<td>26</td>
<td>0.94</td>
</tr>
<tr>
<td>0.0*</td>
<td>—</td>
<td>0.052</td>
<td>0.023</td>
<td>15</td>
<td>0.65</td>
</tr>
</tbody>
</table>

*These measurements were performed at elevated salt concentration (~450 mOsm).
results are consistent with the results on intact cells at low ionic strength (Table 2) that 2,3-DPG does not affect the viscoelastic recovery time constant.

Unlike the results on intact cells (Table 1), there was no effect of 2,3-DPG (at a concentration of 10 mmol/L) on the surface shear modulus of erythrocyte ghosts at either physiologic or reduced ionic strength (Table 7). However, there was a significant reduction in the force required to form tethers (Table 8). This is consistent with the observations of Chasis et al., who observed an increase in the rate of fragmentation of 2,3-DPG loaded ghosts as measured in the ektacytometer. Once the threshold was exceeded, the rate at which the tethers formed was not affected by 2,3-DPG, as indicated by the viscosity coefficients shown in Table 8.

**DISCUSSION**

The observations of Schindler et al. on the lateral diffusion of intramembrane proteins and the data of Sheetz and Casaly on the dissociation of erythrocyte membrane skeletons in vitro have provided strong evidence that a high concentration of 2,3-DPG is disruptive to the erythrocyte membrane skeleton. Because the membrane skeleton is the structural basis for the shear elasticity of the membrane and probably accounts for most of the membrane shear viscosity, changes in membrane material properties at elevated levels of 2,3-DPG were expected. Specifically, if a higher fraction of the skeletal proteins were to exist in a dissociated form, one would expect a decrease in membrane stability and possible reductions in the viscosity coefficients for viscoelastic recovery, tether formation, and creep. Consistent with these expectations, a reduction in shear modulus was observed in intact cells with elevated 2,3-DPG levels at subphysiologic salt concentrations, and a reduction in the critical force for tether formation was observed in resealed ghosts. The reduced surface shear modulus, coupled with observations that the viscoelastic recovery time constant did not change, indicate that the membrane viscosity coefficient for rapid deformation also is reduced at low salt concentration. However, no changes were detected in the surface shear modulus or the recovery time constant of ghosts, and no changes were detected in the critical force or the extent of residual deformation in intact cells. Furthermore, the reduction in shear modulus in intact cells was observed only at subphysiologic salt concentrations (240 mOsm) and did not occur under physiologic conditions.

These results demonstrate that lateral mobility is not simply related to the viscosity of the membrane composite as measured by the whole cell recovery experiments. We reach this conclusion in spite of the fact that the values of the membrane viscosity obtained by the two methods are quite similar. Koppel calculated a value of \(4.6 \times 10^{-4}\) dyn s/cm from lateral mobility measurements in mouse erythrocytes. This is very close to the values for normal cells obtained in the present investigation (Table 3). This agreement appears to be coincidental. In erythrocyte ghosts, Schindler et al. found that the lateral mobility of integral proteins was increased by a factor of 2.5 when the concentration of 2,3-DPG was increased from near zero to 12.5 mmol/L. In contrast, no change in the membrane viscosity of erythrocyte ghosts was detected in the present study at concentrations of 2,3-DPG up to 16.0 mmol/L. The fractional reduction in membrane viscosity observed in intact cells at low ionic strength and elevated (\(-20\) mmol/L) 2,3-DPG concentrations (Table 3, lines 1 and 3) is also significantly less than the fractional change in lateral mobility that Schindler et al. observed.

The reason for the discrepancy may be indicated by the results of the flow-channel experiments on ghosts. Preliminary immunochemical evidence obtained in our laboratory indicates that the tethers formed in the flow channel do not contain appreciable amounts of spectrin. That is, it appears that tethers consist primarily of a membrane bilayer that has been separated from the skeletal matrix. If this is so, the ease with which tethers form should depend on the “tightness” of the association between the bilayer and the membrane skeleton. The decrease in critical force that we have observed in ghosts may be indicative of a weakening of the skeletal-bilayer association and could account for the large increase in lateral mobility that has been observed. Weakening of the bilayer-skeletal interaction could also account for the increased rate of fragmentation of ghosts containing 2,3-DPG (10 mmol/L) that has been observed in the ektacytometer.

The effects of 2,3-DPG on the mechanical properties of intact erythrocytes under physiologic conditions appear to be minimal. The increase in membrane viscosity that we have observed is opposite to the behavior one would predict on the basis of the observations of Schindler et al., who measured an increase in the rate of lateral diffusion of membrane proteins at high concentrations of 2,3-DPG. (Increased rate of diffusion should correspond to decreased viscosity and more rapid viscoelastic recovery.) This result is almost certainly due to the increase in MCHC that occurs with increased 2,3-DPG concentrations and not due to the direct influence of 2,3-

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**Table 7. Shear Modulus of Ghosts at Elevated 2,3-DPG Concentrations**

<table>
<thead>
<tr>
<th>[2,3-DPG] (mmol/L)</th>
<th>Osmolarity (mOsm)</th>
<th>Avg* ± SD</th>
<th>Matched RBC Controls ± SD</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>300</td>
<td>0.0058 ± 0.0005</td>
<td>0.0060 ± 0.0003</td>
<td>0.37</td>
</tr>
<tr>
<td>10.0</td>
<td>300</td>
<td>0.0051 ± 0.0007</td>
<td>0.0056 ± 0.0011</td>
<td>0.40</td>
</tr>
<tr>
<td>0.0</td>
<td>225</td>
<td>0.0048 ± 0.0005</td>
<td>0.0055 ± 0.0005</td>
<td>0.23</td>
</tr>
<tr>
<td>10.0</td>
<td>225</td>
<td>0.0054 ± 0.0004</td>
<td>0.0053 ± 0.0008</td>
<td>0.80</td>
</tr>
</tbody>
</table>

*Each value represents an average of five cells.

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**Table 8. Flow Channel Results for Erythrocyte Ghosts**

<table>
<thead>
<tr>
<th>[2,3-DPG] (mmol/L)</th>
<th>((f_c)) SD</th>
<th>((f_n)) SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>1.05 0.27 15</td>
<td>0.0005 0.00164 0.0014 15</td>
</tr>
<tr>
<td>10.0</td>
<td>0.70 0.24 25</td>
<td>0.00178 0.0017 25</td>
</tr>
</tbody>
</table>

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This concentration was chosen because the increased fragmentation rate of ghosts in the ektacytometer observed by Chasis et al. was near-maximal at 10 mmol/L 2,3-DPG.
DPG. Two other laboratories have shown that high cellular hemoglobin concentration increases the viscoelastic recovery time constant of intact erythrocytes.\textsuperscript{19,20} The MCHCs in the present experiment are as high as the highest MCHCs in those studies. It is possible that the high MCHC in our experiments is masking a decrease in membrane viscosity caused by the elevated 2,3-DPG concentrations. Because of this, the change in membrane properties that we have measured at subphysiologic salt concentrations may be a more accurate reflection of the direct effects of 2,3-DPG on the membrane. It is surprising, however, that no effect of 2,3-DPG on the viscoelastic properties of ghost membranes was observed at any salt concentration.

A disturbing feature of the present results is the dissimilarity of the effects of 2,3-DPG on intact cells and ghosts. With the exception of the shorter recovery time constants for ghosts (indicative of lower membrane viscosity), the properties of the control ghosts were indistinguishable from the properties of the intact cells. However, none of the effects of 2,3-DPG on intact cells were observed in ghosts and vice versa. This indicates that the effects of 2,3-DPG depend strongly on the conditions of the experiment, and it emphasizes the danger of extrapolating results obtained in one membrane system to another.

The present observation of a change in the membrane shear modulus in intact cells at "low" salt concentration suggests there is a cooperative effect of high 2,3-DPG concentration and low ionic strength on the membrane properties. Such a cooperative effect could account, in part, for the dissimilar results obtained in the different membrane systems. The reduction of the shear modulus in intact cells was observed at a 2,3-DPG concentration of about 20 mmol/L and a salt concentration of about 240 mOsm. The concentration of 2,3-DPG in the ghost experiments (10 mmol/L) may have been insufficient to produce similar effects at the experimental salt concentration (225 mOsm). It is important to note that the changes in lateral mobility that were observed occurred at salt concentrations below 140 mOsm,\textsuperscript{1} and it is possible that changes in the membrane shear modulus of ghosts might occur under those conditions. On the other hand, the change in lateral mobility could be due to a completely separate effect, as indicated by the reduction in the critical force of 2,3-DPG loaded ghosts at physiologic salt concentrations.

The present results confirm the observations of early investigators that under some conditions 2,3-DPG acts to destabilize erythrocyte membrane. Our observations that the effects do not occur under physiologic conditions indicate that the effects of 2,3-DPG in erythrocytes are not physiologically important. However, the recent discovery of spectrin-like and ankyrin-like molecules in a variety of cell types raises the possibility that dissociative effects of 2,3-DPG could have importance in cytoskeletal regulation in other cells. The fact that the effects of 2,3-DPG are most evident in erythrocytes when the MCHC is low or in the absence of hemoglobin (in ghosts) suggests that the effect may be more important in cell types other than erythrocytes. Although the mechanism by which 2,3-DPG achieves its effect is not known, our observations suggest that it could play a role in regulating the interaction between membrane and cytoskeletal elements.

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Effects of 2,3-diphosphoglycerate on the mechanical properties of erythrocyte membrane

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