The Influence of Temperature on Red Cell Deformability

By Joseph R. Williamson, Maureen O. Shanahan, and Robert M. Hochmuth

This study was undertaken to examine the influence of temperature on physical properties of red cell membranes. Red cells adhering to cover slips were subjected to fluid shear stress in a rotating disc apparatus for 1 min or for 10 min at temperatures ranging from 2°C to 50°C. They were fixed while subject to shear stress by addition of glutaraldehyde and then processed for examination and photography by reflected-light microscopy. Cell dimensions were obtained with a computerized planimeter. At shear stresses under 2 dynes/sq cm, cells changed shape from biconcave discs to tear drops, the dimensions of which were influenced very little by temperature or duration of shear stress. Above 2 dynes/sq cm, filamentous processes or "tethers" developed at attachment points of cells to cover slips. Tether length and the percentage of cells possessing tethers increased markedly with increasing temperature and duration of shear stress. At approximately 48°C, a dramatic change occurred over a narrow temperature range such that cells were markedly elongated and irregularly deformed by a shear stress of 1 dyne/sq cm or less. These observations demonstrate that elongation of human red cells subjected to fluid shear stress in a rotating disc system is markedly influenced by temperature as well as by magnitude and duration of shear stress. They also indicate that significant increases in red cell membrane fluidity occur between 2°C and 24°C–37°C and again between 48°C and 50°C.

The ability of red blood cells to deform and change shape permits them to pass through capillaries of much smaller diameter than that of the red cell itself and is an important determinant of the life span of circulating red cells.1–3 The capacity to deform is determined by a number of variables including the fluidity and elasticity of cell constituents as well as cell shape. Although a number of physiologic and pathophysiologic factors influence deformability characteristics of red cells in vivo and in vitro, in only a few instances is there a clue to the mechanism involved. Furthermore, the structural elements which limit red cell deformability under physiologic conditions are still unknown.

Most in vitro studies of red cell deformability have been performed at room temperature, at 37°C, or at much higher temperatures, e.g., 50°C–55°C. At the latter temperatures, red cells are transformed into spherocytes, they exhibit budding, and they undergo fragmentation.4–10 If they are warmed to 50°C and subsequently subjected to shear stress at room temperature or at body temperature, red cells are less deformable than normal, as manifested by decreased filterability, decreased packing during centrifugation, increased viscosity, and de-
increased membrane elasticity. They also manifest an increase in osmotic fragility and increased mechanical fragility.

On the other hand, there is a paucity of information regarding physical properties of red cells at low temperature. In this communication we report observations on the influence of temperature (range 2°C–50°C) on the deformability characteristics of red cells subjected to fluid shear stress in a rotating disc apparatus.

MATERIALS AND METHODS

Preparation of Red Cells

Venous blood from healthy young adult donors was collected in acid citrate dextrose solution (1.5 ml of 0.8% of citric acid, 2.2% sodium citrate, and 2.45% dextrose per 10 ml of blood), washed three times with 50 volumes of Krebs-Ringer phosphate buffer at pH 7.4 (without calcium or magnesium but containing 4 mM sodium citrate, to minimize cell clumping, and 10 mM glucose), and diluted to a hematocrit of 0.4% in the same buffer minus sodium citrate.

Rotating Disc Apparatus

The rotating disc apparatus shown in Fig. 1 has a 22-mm flat-topped, stainless-steel disc which rotates at constant angular velocity about an axis perpendicular to its surface. An 18-mm glass cover slip to which cells are allowed to adhere was positioned in a slight depression on the disc surface so that the top of the cover slip was flush with the rim of the disc. The stainless-steel shaft was connected directly to a variable-speed DC motor (Electro Craft Corp., Hopkins, Minn.). The angular velocity of the shaft (and disc), averaged over 60-sec time intervals, was measured with a magnetic counter. The disc and shaft were totally immersed in a buffer-filled chamber (6.5 cm high x 6.2 cm diameter) formed by a thin (0.16-mm) silver cylinder and a Plexiglas cap and base. The water jacket surrounding the silver cylinder was formed of Plexiglas and contained an ethylene glycol solution which was heated or cooled and pumped through a circulating bath (Forma Scientific, Inc., Marietta, Ohio) at temperatures which could be varied between 0°C and 90°C. Temperatures in the waterbath and in the disc reservoir were measured directly with YSI model 8430 thermistors (YSI Instrument Company, Yellow Springs, Ohio). Bulk fluid motion from the circulating bath and from disc and shaft rotation kept the temperature uniform in the waterbath and in the disc reservoir, respectively.

Cells which adhered to the disc surface were subjected to a fluid shear stress at the surface...
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(r_x) given by:
\[
\tau_x = 0.800 \mu r (\omega^2 / r)^{1/2}
\]

where \( \mu \) is the dynamic viscosity, \( \nu \) is the kinematic viscosity, \( \omega \) is the angular velocity in rad/sec and \( r \) is the distance from the center of the disc to the cell.\(^{16} \) It can be seen from this expression that the shear stress is 0 at the disc center and increases linearly with the radius. For a 1.8-cm disc (\( r = 0.9 \) cm) at room temperature (\( \mu = 0.01 \) dyne sec/sq cm and \( r = 0.01 \) sq cm/sec), an angular velocity of 256 revolutions per min will yield a fluid shear stress of 10 dynes/sq cm at the outer edge of the disc.

**Technique for Shear Stressing Cells and for Quantification of Cell Deformation**

In order to subject cells to fluid shear stress in the rotating disc apparatus, cells were first allowed to settle on and adhere to round glass cover slips 18 mm in diameter. This was accomplished by use of a cylindrical settling chamber. The procedure for cleaning and preparation of cover slips was as follows: Cover slips were (1) rubbed gently between two fingers (covered with finger cots) under hot running tap water, (2) rinsed in distilled water two times, (3) brought to a boil in 5 N sodium hydroxide, (4) rinsed in distilled water two times, (5) brought to a boil in 6 N hydrochloric acid, (6) rinsed thoroughly in distilled water, and (7) dried in an oven at 150°C. After cooling to room temperature, cover slips were coated with bovine albumin by immersing them in a solution of 0.05% albumin in Krebs-Ringer phosphate buffer for 5 min and then rinsed five times with the same buffer. The albumin-coated cover slips were then kept in buffer until used (the same day). A wet cover slip was centered on the disc and the cylindrical settling chamber placed over it. One milliliter of buffer (without Ca, Mg, or Na citrate) was added, followed by 1 ml of red cell suspension. The cells were mixed gently and allowed to settle for 5 min at room temperature. The settling chamber and holder were then mounted on the shaft of the rotating disc apparatus. Before removing the settling chamber, the fluid level outside was adjusted to the same level as that inside. The buffer chamber was then filled to the top, the cover was positioned, and rotation was initiated.

Experiments were terminated by introduction, through a port in the bottom of the disc chamber, of 30 ml of 4% glutaraldehyde in Krebs-Ringer phosphate buffer over a 15-sec period while the disc was still rotating. The final concentration of glutaraldehyde was 1%, and the osmolarity of the diluted fixative was 384 mosmols. The disc was rotated for an additional 3 min in the fixative and then stopped. (In separate experiments we ascertained that shrinkage due to fixation is less than 4%\( ^{18} \) ) The disc holder with cover slip in place was then washed thoroughly in distilled water. The cover slip was removed, dipped in liquid nitrogen, and dried in a desiccator under vacuum in the presence of phosphorus pentoxide.

Cover slips were examined with a reflected-light microscope, and photographs were taken of cells at the disc center (0 shear stress) and at distances from the center corresponding to shear stresses of 1, 2, 3, 4.5, and 6 dynes/sq cm. Tension produced in the red cell membrane by these fluid shear stresses was uniaxial in the direction of flow, and the relationship between shear stress and relative increase in cell body length was linear; at higher shear stresses tension in the cell membrane became biaxial, and the relationship between fluid shear stress and cell body length was nonlinear.\(^ {17} \) Shear stresses of this magnitude were well below estimates of maximum shear stress at the wall for both arteries and capillaries.\(^ {18} \)

Since temperature affects buffer viscosity which in turn influences shear stress (see equation (1)), distances from the disc center corresponding to a given shear stress were computed for each temperature. Photographs were taken on 4 in x 5 in sheet film at a magnification of 1000 and en-

\*The mean shear stress at the wall in capillaries can be estimated from a simple force balance on a tube which states that the pressure drop times the area over which the pressure acts equals the shear stress at the wall times the area over which the wall shear stress acts. Thus, \( \Delta P \cdot \pi r^2 = \tau_w \cdot 2 \pi r L \) or \( \tau_w = \frac{\Delta P \cdot r}{2L} \), where \( \Delta P \) is the pressure drop, \( \tau_w \) is the wall shear stress, \( r \) is the capillary radius, and \( L \) is the capillary length. For \( \Delta P = 15 \) mm Hg, \( r = 5 \) \( \mu \)m, and \( L = 500 \) \( \mu \)m; \( \tau_w = 100 \) dynes/sq cm.
Fig. 2. Reflected-light photomicrographs (×1067) of red cells at various temperatures and shear stresses. (A) Unstressed red cells from the center of a disc after rotation for 1 min at 37°C. (B) Red cells subjected to a shear stress of 4.5 dynes/sq cm for 1 min at 2°C. Note the change in cell shape from biconcave discs in Fig. 2A to teardrop configurations. (C) Red cells subjected to 4.5 dynes/sq cm of shear stress for 1 min at 37°C. In contrast to cells shear stressed at 2°C (in Fig. 2B), many cells possess tethers of variable length. (D) Red cells subjected to 1 dyne/sq cm of shear stress for 1 min at 50°C. Cells are irregularly deformed and elongated, and tethers are much thicker and of less uniform diameter than those formed at 37°C. Many beads, droplets of hemoglobin, are present in the tethers.
Table 1. Body Length of Cells Subjected to Varying Shear Stress and Temperature for One Minute

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Shear Stress (dynes/sq cm)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4.5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>113 ± 15 (377)</td>
<td>121 ± 11 (388)</td>
<td>125 ± 11 (388)</td>
<td>130 ± 11 (244)</td>
<td>133 ± 13 (169)</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>113 ± 12 (316)</td>
<td>122 ± 12 (321)</td>
<td>128 ± 13 (265)</td>
<td>129 ± 13 (201)</td>
<td>135 ± 13 (133)</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td>122 ± 13 (300)</td>
<td>126 ± 13 (326)</td>
<td>132 ± 11 (252)</td>
<td>138 ± 13 (269)</td>
<td>142 ± 13 (186)</td>
</tr>
<tr>
<td>25</td>
<td></td>
<td>118 ± 10 (361)</td>
<td>124 ± 10 (391)</td>
<td>128 ± 10 (289)</td>
<td>131 ± 10 (341)</td>
<td>135 ± 12 (268)</td>
</tr>
<tr>
<td>37.5</td>
<td></td>
<td>110 ± 10 (387)</td>
<td>114 ± 10 (336)</td>
<td>119 ± 11 (343)</td>
<td>123 ± 11 (250)</td>
<td>127 ± 13 (164)</td>
</tr>
</tbody>
</table>

*Mean ± standard deviation of cell body length expressed as per cent of unstressed cell diameter.
†Number of cells measured.

RESULTS

Effects of Shear Stress and Temperature on Red Cell Morphology

Red cells deform from biconcave discs (Fig. 2A) to teardrop shapes (Fig. 2B) when subjected to shear stresses under 2 dynes/sq cm at temperatures below 45°C. Above 2 dynes/sq cm, a process or tether is frequently drawn out (Fig. 2C) from the cell at its point of attachment to the cover slip. At 48°–50°C, cells undergo a striking change in morphology compared to that seen at temperatures of 45°C or less; budding often is evident on cells not subjected to shear stress at the disc center, and even at shear stresses as low as 1 dyne/sq cm, the entire cell is irregularly deformed and markedly elongated (Fig. 2D).

Influence of Shear Stress on Cell Body Length

Between temperatures of 2° and 45°C, cell body length varied by no more than 10% for any given shear stress (Table 1). Nevertheless, cell body lengths were significantly longer at 25°C (p < 0.001) than at 2° or 45°C. Duration of shear stress did not influence cell body lengths which were as long after 1 min of shear stress as after 10 min (Table 2).

Table 2. Cell Body Length After Shear Stress of One-Minute and Ten-Minute Durations at 24°C

<table>
<thead>
<tr>
<th>Duration (min)</th>
<th>Shear Stress (dynes/sq cm)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>124 ± 9 (20)</td>
<td>133 ± 8 (20)</td>
<td>135 ± 8 (20)</td>
<td>139 ± 10 (20)</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>122 ± 9 (117)</td>
<td>128 ± 10 (89)</td>
<td>134 ± 10 (31)</td>
<td>130 ± 13 (39)</td>
</tr>
</tbody>
</table>

*Mean ± standard deviation of cell body length expressed as per cent of unstressed cell diameter.
†Number of cells measured.

†Cell length refers to the total length of a shear stressed cell including tether, if present. Cell body length refers to the teardrop-shaped portion of a shear stressed cell regardless of the presence or absence of a tether. For cells without tethers, cell length and cell body length are the same. Tether length refers to the tether or filamentous process only.
Effects of Shear Stress and Temperature on Total Cell Length and Tether Length

Between 2° and 45°C, total cell length varied less than 25% at shear stresses equal to or less than 2 dynes/sq cm for 1 min (Fig. 3, Table 3). At higher shear stresses of the same duration, cell length increased dramatically with increasing temperature. At 48°–50°C, the cells became highly deformable and were markedly elongated in most experiments by shear stress as low as 1 dyne/sq cm (Fig. 3). Since these studies were performed at temperature intervals of 10°–

Table 3. Effects of Temperature and Shear Stress for One Minute and for Ten Minutes on Cell Length

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Shear Stress (dynes/sq cm)</th>
<th>1 min</th>
<th>2 min</th>
<th>3 min</th>
<th>4 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4.5</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>113 ± 9† (70)†</td>
<td>122 ± 11 (70)</td>
<td>128 ± 13 (70)</td>
<td>145 ± 17 (50)</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>119 ± 9 (40)</td>
<td>131 ± 16 (40)</td>
<td>135 ± 18 (70)</td>
<td>177 ± 31 (80)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.05§</td>
<td>1.07</td>
<td>1.05</td>
<td>1.22</td>
</tr>
<tr>
<td>13</td>
<td></td>
<td>126 ± 13 (50)</td>
<td>137 ± 16 (50)</td>
<td>151 ± 23 (50)</td>
<td>192 ± 33 (50)</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>119 ± 13 (50)</td>
<td>135 ± 17 (50)</td>
<td>165 ± 31 (50)</td>
<td>296 ± 112 (50)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.95</td>
<td>0.99</td>
<td>1.09</td>
<td>1.54</td>
</tr>
<tr>
<td>24</td>
<td></td>
<td>131 ± 15 (110)</td>
<td>148 ± 20 (100)</td>
<td>162 ± 30 (80)</td>
<td>272 ± 85 (50)</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>143 ± 16 (120)</td>
<td>168 ± 37 (80)</td>
<td>323 ± 138 (50)</td>
<td>965 ± 379 (30)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.09</td>
<td>1.14</td>
<td>1.99</td>
<td>3.55</td>
</tr>
<tr>
<td>37</td>
<td></td>
<td>130 ± 10 (100)</td>
<td>149 ± 23 (80)</td>
<td>170 ± 61 (80)</td>
<td>563 ± 237 (60)</td>
</tr>
<tr>
<td>10</td>
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<td>147 ± 18 (60)</td>
<td>195 ± 61 (20)</td>
<td>336 ± 180 (40)</td>
<td>885 ± 308 (20)</td>
</tr>
<tr>
<td></td>
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<td>1.13</td>
<td>1.31</td>
<td>1.98</td>
<td>1.57</td>
</tr>
<tr>
<td>45</td>
<td></td>
<td>130 ± 14 (120)</td>
<td>152 ± 27 (90)</td>
<td>222 ± 70 (67)</td>
<td>636 ± 307 (30)</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>136 ± 18 (90)</td>
<td>181 ± 76 (60)</td>
<td>347 ± 242 (30)</td>
<td>874 ± 377 (20)</td>
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<td>1.05</td>
<td>1.19</td>
<td>1.56</td>
<td>1.37</td>
</tr>
<tr>
<td>48</td>
<td></td>
<td>126 ± 13 (120)</td>
<td>153 ± 31 (100)</td>
<td>265 ± 146 (60)</td>
<td>519 ± 235 (20)</td>
</tr>
<tr>
<td>10</td>
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<td>441 ± 142 (20)</td>
<td>475 ± 108 (20)</td>
<td>498 ± 178 (20)</td>
<td>799 ± 425 (20)</td>
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<tr>
<td></td>
<td></td>
<td>3.50</td>
<td>2.95</td>
<td>1.88</td>
<td>1.54</td>
</tr>
</tbody>
</table>

*Duration of shear stress in minutes, i.e., 1 min or 10 min.
†Mean ± standard deviation of cell lengths (including tethers) expressed as per cent of mean diameter of unstressed cells.
‡Number of cells measured.
§Ratio of cell length at 10 min to cell length at 1 min.
12°C, additional data were obtained at 5°C intervals (between 2° and 25°C) at 6 dynes/sq cm to determine whether an inflection point or phase change could be demonstrated. As shown in Fig. 4, both total cell length and tether length increased gradually between 2° and 37°C.

When shear stresses of 1–2 dynes/sq cm were applied for a longer duration, e.g., 10 min, no appreciable increase in cell length was observed at temperatures of 45°C or less (Table 3). At 48°C, however, cell lengths were approximately three times as long as those observed after 1 min of shear stress. When higher shear stresses of 3 and 4.5 dynes/sq cm were applied for 10 min, the ratio of cell length at 10 min to that at 1 min increased dramatically between 13° and

Table 4. Per Cent of Cells With Tethers After One Minute and After Ten Minutes of Shear Stress

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Shear Stress (dynes/sq cm)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1*</td>
<td>0</td>
<td>0</td>
<td>24</td>
<td>48</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>35</td>
<td>81</td>
<td>86</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td></td>
<td>10</td>
<td>33</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>11</td>
<td>53</td>
<td>92</td>
<td></td>
</tr>
<tr>
<td>24</td>
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<td>6</td>
<td>29</td>
<td>54</td>
<td>88</td>
</tr>
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</tr>
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<td>19</td>
<td>78</td>
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<td>10</td>
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<td>1</td>
<td>34</td>
<td>90</td>
<td>95</td>
</tr>
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<td></td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

*Duration of shear stress in minutes.
†Per cent of cells possessing tethers. The number of cells in each group is the same as that in Table 3.
Fig. 5. Tether length as a function of temperature and shear stress for 10 min. Each point represents the mean of measurements from at least 20 cells, and the vertical bars represent 90% confidence intervals.

24°C, but did not increase further at higher temperatures. In general, total cell length and tether length increased with increasing duration and magnitude of shear stress as well as with temperature.

The percentage of cells with tethers was also markedly influenced by temperature as well as by magnitude and duration of shear stress (Table 4). From inspection of the table it is evident that no cells possessed tethers at the lowest temperature and shear stress conditions employed, while 100% of cells were tethered at the highest temperatures and shear stresses.

The critical influences of temperature and shear stress and their interactions on tether formation and growth are shown in Fig. 5. After 2 dynes/sq cm of shear stress for 10 min, mean tether length did not exceed 20% of unstressed cell diameter at 2° and 13°C. At 24°C, 37°C, and 45°C tethers were two to three times longer than those at 2° and 13°C, but did not differ significantly from each other. When the temperature was increased from 45° to 48°C, tether length increased sixfold! At 4.5 dynes/sq cm of shear stress for 10 min, tether length increased markedly between 2° and 24°C, but then reached a plateau and showed no further increase between 24° and 48°C.

Effect of Previous Heating to 49°C on Cell Deformability at Lower Temperatures

In agreement with observations of Rakow and Hochmuth, cells heated to 49°C for 1 min and then cooled to 25°C before shear stressing at 37°C for 1 min

<table>
<thead>
<tr>
<th>Shear Stress (dynes/sq cm)</th>
<th>Cell Body Length</th>
<th>Tether Length</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>4.5</td>
</tr>
<tr>
<td>Control cells</td>
<td>123 ± 7* (58)*</td>
<td>146 ± 11 (25)</td>
</tr>
<tr>
<td>Heated cells</td>
<td>111 ± 8 (74)†</td>
<td>120 ± 8 (31)</td>
</tr>
<tr>
<td>*Mean and standard deviation of cell body length and of tether length expressed as per cent of unstressed cell diameter.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>†Number of cells measured.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Effect of Previous Heating to 49°C on Cell Deformability at Lower Temperatures

In agreement with observations of Rakow and Hochmuth, cells heated to 49°C for 1 min and then cooled to 25°C before shear stressing at 37°C for 1 min

Table 4. Effect of Prior Heating of Red Cells to 49°C for One Minute on Body Length and Tether Length of Cells Subsequently Subjected to Shear Stress at 37°C

<table>
<thead>
<tr>
<th>Shear Stress (dynes/sq cm)</th>
<th>Cell Body Length</th>
<th>Tether Length</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>4.5</td>
</tr>
<tr>
<td>Control cells</td>
<td>123 ± 7* (58)*</td>
<td>146 ± 11 (25)</td>
</tr>
<tr>
<td>Heated cells</td>
<td>111 ± 8 (74)†</td>
<td>120 ± 8 (31)</td>
</tr>
<tr>
<td>*Mean and standard deviation of cell body length and of tether length expressed as per cent of unstressed cell diameter.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>†Number of cells measured.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Probability that the differences in cell body and tether length of heated and of control cells are due to chance alone.
RED CELL DEFORMABILITY

were much less deformable than normal (Table 5). Cell body length was significantly less than that of control cells shear stressed at 1 and 4.5 dyne/sq cm. In addition, although all cells possessed tethers after 1 min of shear stress at 4.5 dyne/sq cm, mean tether length of cells previously heated to 49°C was only one-third that of control cells.

DISCUSSION

These observations demonstrated that elongation of human red cells subjected to fluid shear stress in a rotating disc system is markedly influenced by temperature; in addition they confirmed and extended observations of other investigators on the effects of magnitude and duration of shear stress. Variations in temperature between 2° and 24°C had statistically significant effects on cell body length. The magnitude of these effects, however, was small compared to those on total cell length and on tether length. The decrease in body length of cells shear stressed at temperatures above 24°C may be related to formation of very long tethers; it is conceivable that enough cell membrane and contents were drawn into tethers to limit or reduce cell body size. Quantitative studies of cell body and tether dimensions will be required to determine whether or not this actually occurs.

Observations of tether length and of total cell length indicate that red cell deformability increases gradually between 2° and 24°–37°C and increases dramatically between 48° and 50°C. Since hemoglobin in solution behaves as a viscous Newtonian fluid with a viscosity (at concentrations extant in the red cell) four to five orders of magnitude less than the membrane viscosity estimated in the appendix, these temperature-related increases in cell deformability more likely reflect increased fluidity of cell membrane rather than of cell contents. We would suggest that raising the temperature from 2° to 24°C increases the kinetic energy of molecular constituents of the membrane, thereby loosening intermolecular associations to the point that they are overcome or pulled apart by shear stress of sufficient magnitude.

Several observations suggest that different mechanisms are responsible for increased membrane fluidity occurring between 2° and 24°C and that observed at 48°–50°C. First, the change at 48°C occurs over a very narrow temperature range, e.g., 2°–3°C, compared to the gradual change between 2° and 24°C. The latter observation is consistent with reports that red cell membrane lipid viscosity gradually decreases with increasing temperature with no sharp thermal transitions in this temperature range. One group has reported a phase change at 19°C; however, the validity of their observations has been challenged. Second, cells heated to 49°C and subsequently tested at 25°C or at 37°C demonstrate decreased elasticity and fluidity, manifested by shorter cell body lengths and shorter tether lengths, respectively (Table 5), and are less deformable than normal as shown by other investigators. Third, cells heated to 49°C undergo degenerative changes including sphering, budding, and fragmentation, none of which is observed at 24°C.

Several investigators have suggested that decreased deformability of cells heated to 50°C is caused by denaturation of membrane protein. Indeed, Jackson, et al. reported that a significant proportion of red cell mem-
brane protein is denatured at approximately 50°C, with some denaturation detectable beginning at about 45°C. They found no evidence of any phase change in extracted lipids in this temperature range.

The transient increase in cell fluidity we observed at 50°C is in sharp contrast to the decreased deformability reported by investigators who have heated cells to 50° or 55°C in the absence of shear stress and evaluated deformability later at room temperature or at 37°C. With the exception of data shown in Table 5, we subjected cells to shear stress while they were being heated. These observations and considerations suggest that, if cells are subjected to shear stress while a critical structural protein or other constituent is undergoing conformational changes which loosen its intermolecular associations with other membrane constituents, cell membrane material will flow more easily. When the cell is cooled, conformational changes and altered intermolecular associations of membrane constituents induced by heating may not be reversed, rendering the membrane less elastic and more viscous than normal. A good candidate for such a structural protein would be “spectrin”\(^2^7\) or “tektin,”\(^2^8\) a high-molecular-weight protein located on the inner surface of the membrane.\(^2^9\)\(^,\)\(^3^0\) Indeed, we have recently observed (unpublished data) that an isolated high-molecular-weight membrane protein, presumably spectrin, does aggregate in vitro at 49°C. These observations and interpretations lead to the inference that at physiologic temperatures red cell deformability characteristics are determined primarily by membrane proteins rather than by lipids.

The time dependence of tether growth and the independence of cell body length changes in regard to duration of shear stress and the larger shear stresses required for tether formation than for cell shape (teardrop) change suggest that the two are independent phenomena. The readily reversible shape change of shear stressed cells from biconcave discs to teardrops very likely depends on elastic properties of the cell membrane.\(^3^1\) When a cell is deformed by shear stress, energy is stored in the membrane, probably in a protein (spectrin) meshwork; this stored energy is utilized in recovery of the original biconcave disc shape of the cell following removal of fluid shear stress. This interpretation is consistent with the observations of Rakow (personal communication) that the stress-elongation curve for point-attached cells exhibits no hysteresis during slow cyclic loading. This reversible shape change and the magnitude of the shear stresses involved are probably comparable to the reversible deformation of red cells during transit through the capillary bed.

On the other hand, the observation that only small portions of tethers are resorbed when shear stress is suddenly terminated,\(^1^9\) e.g., when fluid stops flowing over the cell, indicates that tether formation is a flow phenomenon.* Although some elastic recovery occurs on termination of shear stress, the remaining tether represents a net flow or permanent redistribution of membrane material and cell contents from cell body to tether. The bizarrely deformed red cells occurring in microangiopathic anemias\(^3^2,\)\(^3^3\) may very well constitute residua

\*A material is considered to flow if it continuously and irreversibly deforms when acted on by a constant stress (force/area).
of tether formation in vivo and subsequent fragmentation of red cells adhering
to or caught on diseased heart valves, small vessels, and thrombi. Indeed, since
the rate of flow of a material is a function of its viscosity, tether growth data
can be used to estimate red cell membrane viscosity as shown in the appendix.

APPENDIX

An Estimate of Membrane Viscosity From Tether Growth Data

The membrane is considered to be a two-dimensional incompressible material
which is isotropic in the plane of the membrane (i.e., in two dimensions)
and highly anisotropic in the third dimension. Since, over a wide temperature
range, a critical stress must be exceeded before membrane flow is initiated (i.e.,
before tethers are formed—see Fig. 3 and Reference 19), we assume as a first
approximation that two-dimensional flow of membrane material is governed by
the following equation, viz.

\[ T - T_0 = \eta (\Delta v / \Delta y); T > T_0. \]  

[2]

In equation 2, \( T \) is the membrane tension (with units in the cgs system of
dynes/cm), \( T_0 \) is the critical tension or yield tension which must be exceeded
before flow takes place, \( \Delta v / \Delta y \) (sec\(^{-1}\)) is some typical velocity gradient in the
plane of the tether where membrane flow occurs, and \( \eta \) is a constant of pro-
portionality. Note from equation 2 that \( \eta \) has units of dynes – sec/cm and will be
called the two-dimensional viscosity or surface viscosity. For a three-dimen-
sional material, the membrane tension in equation 2 would be replaced by a
shear stress (dynes/sq cm) and equation 2 would define what is often called a
“Bingham plastic,” In this case, the constant of proportionality would have the
units of three-dimensional viscosity (e.g., dynes – sec/cm). Now, let the over-
all rate of tether growth at a given fluid shear stress be denoted by \( \dot{\xi} \). This
growth is caused by two factors: flow of membrane material from cell to tether
and elastic stretch of tether material in the region where \( T < T_0 \). Thus, the
velocity gradient in the region of flow is

\[ \frac{\Delta v}{\Delta y} \leq \frac{\dot{\xi}}{d} \]

[3]

where \( d \) is a typical circumferential dimension in the region of flow. The cir-
cumferential and longitudinal dimensions are approximately equal in this
region. The combination of equations 2 and 3 immediately gives a lower limit
for membrane surface viscosity, viz.

\[ \eta \geq \left( \frac{T - T_0}{\dot{\xi}} \right) d. \]

[4]

The quantity \( T - T_0 \) is estimated by making a simple force balance on the
tethered cell:

\[ T - T_0 \approx (\tau_s - \tau_{\sigma_0}) \frac{A}{\pi d}, \]

[5]

where \( \tau_s \) is the fluid shear stress acting on cell area \( A \) and \( \tau_{\sigma_0} \) is the critical shear
stress which must be exceeded before tether growth can take place. The substitution of equation 5 into equation 4 yields

\[
\eta \geq \left( \frac{T_s - \tau_{0}}{\ell} \right) \frac{A}{\pi}.
\]

Note that the quantity \(d\) cancels out. The results in Fig. 5 indicate that \(\ell \approx 0.12 \mu m/sec\) at 4.5 dynes/sq cm. Hochmuth et al.\(^9\) report a maximum value for \(\ell\) of approximately 0.2 \(\mu m/sec\) at 3.5 dynes/sq cm. As an approximation, therefore, let \(\ell = 0.15 \mu m/sec\) when \(T_s = 4\) dynes/sq cm. The critical stress for tethering, \(\tau_{0}\), is on the order of 2 dynes/sq cm (Fig. 3) and \(A = 50 \mu m^2\) (the surface area in contact with the fluid) for the human red blood cell. Substitution of these values into equation 6 gives the following estimate for surface viscosity \(\eta\):

\[
\eta \geq 10^{-2} \text{dyne sec/cm (poise cm).}
\]

The importance of surface viscosity and of a critical shear stress for tethering can be appreciated by considering what happens when a red cell becomes attached to a fibrin strand or an endothelial process within a vessel. If fluid shear stress acting on the cell exceeds the critical stress required for tether formation (\(\tau_{0}\)), then membrane material will flow from the cell at a rate proportional to \(\ell\) which in turn is proportional to \(\eta\) (equation 6). At any given shear stress \(\tau_s\) the rate of membrane flow into tether will depend on the values of \(\tau_{0}\) and of \(\eta\). If a portion of the tether breaks off, i.e., as a consequence of increased shear stress associated with a transient increase in blood flow, the remaining cell body will have a smaller surface area to volume ratio (since the surface area to volume ratio of tethers is much greater than for the remainder of the cell), and will therefore be less deformable than normal.

For purposes of comparison with three-dimensional red cell membrane viscosity estimates reported by others,\(^{21,22,34,36}\) the above value must be divided by a typical membrane thickness of \(\sim 100 \AA\) which gives a lower limit of \(\sim 10^4\) poise for three-dimensional or bulk membrane viscosity. (Note, however, that the value for surface viscosity is the meaningful one since flow is probably restricted to the two dimensions in the plane of the membrane.) The value of \(10^4\) poise for membrane viscosity is one to two orders of magnitude greater than estimates reported by Singer and Nicholson\(^{34}\) and by Peters et al.\(^{35}\) and is four orders of magnitude greater than those reported by Rudy and Gitler,\(^{21,22}\) Aloni et al.,\(^{36}\) and Solomon.\(^{36}\)

The estimates reported by Singer and Nicholson\(^{34}\) and by Peters et al.,\(^{35}\) which are in closest agreement with our own data, are based on rates of diffusion or migration of fluorescein-labeled surface proteins of hybridized cells and of erythrocytes, respectively. Peters et al. comment that most of the label in their experiments was on 100,000 molecular weight intrinsic membrane proteins which probably interact with the extrinsic protein spectrin on the inner surface of the membrane. They suggest that interaction of labeled intrinsic proteins with spectrin might interfere with their lateral mobility in the membrane. The same comments apply to the estimate reported by Singer and Nicholson.\(^{34}\)

Their estimate is based on the rate of mixing of labeled surface antigens of
hybridized cells in the experiments of Frye and Edidin. The estimates reported by Rudy and Gitler, by Aloni, and by Solomon are based on measurements of rates of diffusion and of fluorescence polarization of very-low-molecular-weight lipid-soluble markers which should be influenced very little by interaction with membrane proteins. In contrast to the above approaches, ours is the first estimate of membrane viscosity based on flow of membrane as a whole including extrinsic and intrinsic proteins.

ACKNOWLEDGMENT

We thank Professor Evan A. Evans of Duke University for many helpful discussions concerning surface viscosity.

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