ABSTRACT
A mechanism for the geometric difference between young and old human red blood cells is proposed. It is assumed that the bone marrow produces a population of cells with a range of sizes and shapes, but only those cells which can pass through a theoretical 3.65μ cylindrical channel survive in the circulation. The important geometric parameters of an erythrocyte are the area and the volume. We suggest that as cells age they have a constant area but swell in proportion to their original volume. This hypothesis of aging was tested on 1,016 normal cells using the IBM 7040 computer. When the cells were theoretically swelled by approximately 15%, the population of cells which could still pass through the filter had a smaller mean area and volume and were more osmotically fragile than the same cells before swelling. Young and old cells were separated by centrifugation. The heaviest 10% of the cells (old cells) had a lower diameter, area, and volume and higher sphericity index than the lightest 20% (young cells). The calculated minimum filter diameter was not significantly different. The good agreement between the predicted and observed changes in cellular geometry between young and old cells provides supporting evidence that a cylindrical filter exists in the circulation and may play a role in determining the life span of the erythrocyte.

ADDITIONAL KEY WORDS
minimum cylindrical diameter  area and volume  theoretical aging  centrifugation

For years many investigators have been inquiring about the destruction of red blood cells. Theories to describe the early stages of red cell breakdown range from cellular fragmentation to physical trapping. Canham and Burton (1) recently demonstrated that the linear relation between the area and volume of single erythrocytes in a population of cells was evidence for a filtering network in the circulation. Their results indicated that somewhere in the circulation the red cell is required to squeeze through very narrow cylindrical channels. If the cell increased in volume so much that it could no longer get through these hypothetical channels, it would get trapped and eventually hemolyze. The hot-dog shape is the most suitable shape for the flexible red cell to adopt when it is required to navigate narrow channels (less than 4μ). Canham and Burton suggested that the filter was located in the spleen. The importance of the spleen in removing defective cells is well established (2, 3), but the function of the spleen in removing normal senescent cells is poorly understood (4). Ham (5) suggested that the spleen functions like a filter and the present paper provides a plausible explanation of this function. However, the evidence here does not directly assess splenic function.

The filtering experiments by Prothero and Burton (6) and Gregerson et al. (7) provide supporting evidence for the value of the calculated mean filter diameter of 3.33μ (1). The abrupt increase in viscosity for small tubes, reported by Dintenfass and Read (8), also suggests that the minimum cylindrical
diameter is an important dimension. The present paper will show how the relation of the geometry of the erythrocyte to a cylindrical filter can play an important role in determining the survival of red cells in the circulation.

Young and old cells\(^1\) can be separated according to their differences in osmotic fragility (9) or by their differences in density (10). Old cells are both osmotically more fragile and more dense. A review of the data regarding the geometry of young and old cells reveals that old cells are smaller in area, volume, and diameter and are more spherocytic (more osmotically fragile) (11, 12). Prankerd (13) reported that old cells contain 15\% less lipid, possibly accounting for the increased density associated with age.

We are proposing a biophysical mechanism for red cell aging in which the cells swell as they age, in proportion to their original volume. This implies a contradiction because old cells are smaller than young cells. However, a theoretical narrow cylindrical filter imposes a restriction on the circulating cells in such a way that the larger cells are selectively removed earlier, thus explaining the seeming paradox. The gradual increase in volume might result from an increased membrane permeability or to a decreased active transport. Tosteson and Hoffman (14) demonstrated that sheep cells with either high or low potassium content swell when the active transport mechanism is blocked. A change in either the membrane permeability or the active transport mechanism would lead to a new equilibrium volume (15). Zajicek (16) also made the assumption of a constant area with increasing volume as cells age. However, in his hypothesis each cell would be eliminated when it reached a constant critical volume, and not a volume dependent on the cell’s original volume as we propose. Ponder (17) conjectured that young cells were thin cells and gradually swelled with age, but he did not enlarge upon this hypothesis.

The part played by the changing geometry would complement the biochemical and biophysical changes reported in the literature for senescent erythrocytes (18-20) and might also explain some of the changes in cellular life span in disease.

**Methods and Materials**

1. The minimum cylindrical diameter is twice the radius, \(r\), which satisfies the following equation: \(V = Ar^2 - 2\pi r^3/3\) (equation 1) (1). Both the volume, \(V\), and area, \(A\), are parameters of the equation. For each \(A\) and \(V\) there is a different \(r\), found by Newton’s method. (Although there are three real solutions to this cubic equation for the range of values of \(A\) and \(V\), only one is meaningful.)

2. A large number (1,016) of single erythrocytes from seven healthy subjects have already been photographed and measured to obtain the areas, volumes, and other geometric parameters (1). The areas and volumes were obtained by the theorem of Pappus. Although the mean volume is higher than that reported by the method of centrifugation, the importance of the data is the knowledge of the area and volume of individual cells rather than the averages of populations.

3. The 1,016 cells were considered to be one population of cells representing the full range of cellular ages. Each cell was allowed to swell theoretically by a percentage of its initial volume. The minimum cylindrical diameter was then recalculated from the new volume and the old area, using equation 1. If the cell was then too large to pass through a 3.65\(\mu\) channel without stretching its membrane, then the cell was eliminated from the population. At each degree of swelling, the means of the areas and volumes of the cells which could still pass through the 3.65\(\mu\) channel were determined. The size of the population diminished as the degree of theoretical swelling was increased. At 18\% swelling the population was reduced from 1,016 to 14.

Numerical calculations and statistical routines were done on the IBM 7040 at this university. Graphic results were obtained from a Calcomp plotter, model 563.

4. It is important to compare the effect of the theoretical aging process on erythrocytes with the changes in geometry measured experimentally. For this the geometry of young and old cells was studied using the hanging-drop technique described in detail previously (1, 21). Cells from one subject (male, age 26) were analyzed. Approximately 50 cells, from both the young and the old fractions of cells, were photographed on edge and measured. The two fractions were separated according to the method outlined by Danon and Marikovsky (10); a mixture of phthalate esters

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\(^1\)The age of an erythrocyte has been used to mean the time elapsed after the cell’s introduction into the circulation.
The computer plot of area versus volume for 1,016 red blood cells from seven normal subjects, aged 5 to 71. The straight line is for the 95% cut-off diameter, 3.65μ, determined by equation 1 in the text.

(immiscible with water) of known density was put with the whole blood in a microhematocrit tube. Cells were spun at approximately 11,000g for 30 minutes in a microhematocrit centrifuge in a cold room at 10°C. Slightly different mixtures of the esters were needed than were reported by Danon and Markovsky, (10), probably because we used human blood and centrifuged the cells in the cold room. (They studied the red cells of rabbits.) The old cells in the heavy fraction were hypersensitive to glass; they became crenated upon approaching glass and reverted to the biconcave away from the coverglass. Neither the use of sealing putty nor flaming the tip to seal the end altered this phenomenon. For this reason plastic coverslips were used and the problem was avoided. The young cells were not nearly as sensitive to the coverglass. The specific gravities of the mixed esters were approximately 1.090 and 1.102, which separated a young and old fraction representing approximately 20% and 10%, respectively, of the cell column.

Results

1. Figure 1 demonstrates clearly the linear area-volume relation. The normal areas and volumes were plotted by computer plotter and the 95% cut-off line (3.65μ) was drawn. (The same data were plotted differently before [1] because the plotter was not available at that time.) The unexpected linearity of the area-volume plot forms the fundamental evidence for the cylindrical filtering mechanism which is proposed. It must be emphasized that the diameter of the cylindrical filtering network fixes not only the slope but also the intercept of the straight-line relation of area and volume. In addition, there is the special significance of the 95% cut-off line, which coincides with the straight right margin of the data on the area-volume diagram. The left edge is not as linear.

The proposed aging process involves cellular swelling with no change in area. The location of a swelling cell on the area-volume graph moves to the right parallel to the volume axis. When it reaches the 95% line, the cell is eliminated from the circulation. This principle is presented schematically in Figure 2. Two groups of cells of different volumes are shown before and after swelling. The larger cells would move further toward the elimina-
A schematic interpretation of how the cylindrical filter can account for the decrease in area and volume when each cell swells. Theoretically any cell is eliminated from the circulation if its location on the graph falls to the right of the line.

The purpose of this research was to test the validity of this hypothesis by imposing the aging process on the data for normal cells.

2. Table 1 presents the computed results of swelling the 1,016 normal cells by 18% in 2% increments. The first line in the table indicates the normal values before filtration. The second line shows the result of theoretical filtration without swelling. As the swelling increased, the mean area decreased, clearly indicating the preferential elimination of the larger cells. After an initially slight increase, the mean volume of the surviving population became less also. The sphericity index increased, meaning that the surviving population had an increased osmotic fragility. (The sphericity index has values from zero to unity and is a quantitative index of thickness or thinness [1].)

3. The results of the hypothetical aging mechanism are consistent with the study of the geometry of young and old erythrocytes. Table 2 provides the data for the geometrical parameters of young and old cells separated by the phthalate ester method of Danon and Markovsky (10). The young cells (top 2% of the hematocrit tube) are larger with respect to diameter, area, and volume and are less

TABLE 1

<table>
<thead>
<tr>
<th>Percent swelling</th>
<th>Area (μ²)</th>
<th>Volume (μ³)</th>
<th>Sphericity* index</th>
<th>Population size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before filtration</td>
<td>138.2 ± 17.8</td>
<td>105.8 ± 17.5</td>
<td>.782 ± .023</td>
<td>1016</td>
</tr>
<tr>
<td>After filtration</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0†</td>
<td>137.5 ± 16.9</td>
<td>104.6 ± 16.1</td>
<td>.781 ± .027</td>
<td>966</td>
</tr>
<tr>
<td>2</td>
<td>136.9 ± 16.3</td>
<td>105.6 ± 15.6</td>
<td>.790 ± .026</td>
<td>906</td>
</tr>
<tr>
<td>6</td>
<td>135.6 ± 15.4</td>
<td>105.8 ± 14.7</td>
<td>.798 ± .025</td>
<td>801</td>
</tr>
<tr>
<td>8</td>
<td>134.4 ± 15.1</td>
<td>105.8 ± 14.2</td>
<td>.805 ± .024</td>
<td>655</td>
</tr>
<tr>
<td>10</td>
<td>133.1 ± 15.1</td>
<td>105.4 ± 14.1</td>
<td>.812 ± .024</td>
<td>489</td>
</tr>
<tr>
<td>12</td>
<td>131.7 ± 15.4</td>
<td>104.7 ± 14.4</td>
<td>.817 ± .024</td>
<td>317</td>
</tr>
<tr>
<td>14</td>
<td>128.7 ± 15.8</td>
<td>102.4 ± 14.6</td>
<td>.824 ± .026</td>
<td>180</td>
</tr>
<tr>
<td>16</td>
<td>126.8 ± 16.4</td>
<td>101.2 ± 15.0</td>
<td>.829 ± .027</td>
<td>98</td>
</tr>
<tr>
<td>18‡</td>
<td>124.6 ± 16.0</td>
<td>99.5 ± 14.4</td>
<td>.835 ± .028</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>121.0 ± 17.3</td>
<td>96.0 ± 14.8</td>
<td>.840 ± .033</td>
<td>14</td>
</tr>
</tbody>
</table>

Values are means ± SD.

* 4.84 × Vol²/μ²/Area. † Only 95% of the 1,016 cells can pass the filter since 3.65μ is the 95 percent cut-off point. ‡ One cell became a sphere.
TABLE 2

Geometric Parameters of Young and Old Cells Separated by Ultracentrifugation

<table>
<thead>
<tr>
<th>Sample</th>
<th>Top 20% of cells</th>
<th>Bottom 10% of cells</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample size</td>
<td>55</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>Diameter (μ)</td>
<td>8.35 ± 0.48</td>
<td>7.86 ± 0.51</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Coeff. of variation</td>
<td>±5.7%</td>
<td>±6.6%</td>
<td></td>
</tr>
<tr>
<td>Area (μ²)</td>
<td>148.9 ± 15.9</td>
<td>133.1 ± 16.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Coeff. of variation</td>
<td>±10.7%</td>
<td>±12.2%</td>
<td></td>
</tr>
<tr>
<td>Volume (μ³)</td>
<td>112.9 ± 16.0</td>
<td>98.8 ± 14.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Coeff. of variation</td>
<td>±14.1%</td>
<td>±14.9%</td>
<td></td>
</tr>
<tr>
<td>Sphericity index</td>
<td>0.758 ± 0.019</td>
<td>0.777 ± 0.026</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Coeff. of variation</td>
<td>±2.6%</td>
<td>±3.3%</td>
<td></td>
</tr>
<tr>
<td>Minimum cylindrical diam. (μ)</td>
<td>3.27 ± 0.14</td>
<td>3.23 ± 0.13</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>95% Cut-off (μ)</td>
<td>3.559</td>
<td>3.493</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± sd. P value derived by t-test of significance between the means. Subject was a 26-year-old male.

spherocytic than the old cells (bottom 10% of the tube). The shift of mean sphericity from 0.758 to 0.777 corresponds to a shift in osmotic fragility of 0.02% NaCl. Although this is only a slight change, it is significant and is similar to the change reported by Prankerd (13).

The one parameter which did not change significantly between the young and old cells was the minimum cylindrical diameter. An examination of the area-volume graphs for the young and old cells (Fig. 3) reveals the same linearity of the right edge of the data as Figure 1. Although there are only 50 cells in each age group, it seems enough to provide good supporting evidence for the cylindrical-channel filtering theory.

Discussion

The experimental results of the geometric difference between young and senescent erythrocytes agree with those of other investigators (11, 12) and also with the results of the proposed aging mechanism. In the experimental study, the difference between the two age groups was approximately 15% with respect to both area and volume. There are three possible explanations for the discrepancy between the model and experiment. Firstly, the mechanism of aging was imposed on normal cells of all ages and should have been imposed on young cells only. (Unfortunately, we do not have enough data to do this.) Secondly, the geometric difference between young and old cells was determined for only one subject and using one technique. Thirdly, other mechanisms operating at the same time might affect the geometry of aging erythrocytes. For example, we did not consider the effect of the stiffness of the membrane. Because a large cell must undergo relatively greater shape changes than a small cell when navigating the circulation one might envisage the large cell to be more mechanically fragile and hence more susceptible to damage and lysis. In this way also, large cells would be preferentially eliminated from the circulation. From this experiment it is possible to approximate the swelling an average cell can tolerate before its own corpulence results in its being trapped and removed. We feel that between 10% and 20% swelling could explain the geometric differences between young and old cells.

Zajicek's proposed model (16) for the aging of red cells was based also on the cell's constant area with an increasing volume. However, he considered that a cell would reach a critical volume independent of the cell's original volume. By his mechanism there is no way of selectively removing the larger cells, and thereby accounting for the decreased cellular area and volume with age. We proposed that the cell's area determines what that critical volume will be and is therefore
The area-volume graph for the young and old human red blood cells separated by the method of Danon and Marikovsky (6). Although the old cells are significantly smaller with respect to area and volume, both the young and old cells have the same 95% cut-off line.

The proposed filtering mechanism might assist in the understanding of hereditary spherocytosis, a disease of the blood characterized by small, spherocytic red cells. The bone marrow might produce a normally sized population which has the defect of cells with leaky membranes (23) causing an equilibrium volume higher than normal. Only the smaller cells which can tolerate a greater percent of swelling could still pass the cylindrical filter (probably located in the spleen). The success of splenectomy in such cases might be explained by the removal of the filter. The remaining filtering system (the reticuloendothelial system outside the spleen) might not have such a narrow filter and therefore permit the circulation of a greater percent of the cells produced. We have just begun the study of the geometries of cells for subjects with and without hereditary spherocytosis, with and without splenectomies.

References
Difference in Geometry of Young and Old Human Erythrocytes Explained by a Filtering Mechanism
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