EXPERIMENTAL VARIATIONS IN SONIC FRAGILITY OF RED CELLS*

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The reaction of red cells to intense sonic and ultrasonic vibrations was studied by many workers following the development of the piezoelectric crystal and the magnetostriction oscillator. The original observers (1-10) attributed the hemolysis to rapid fluid movement as a result of cavitation. Slight physical-chemical changes in the cell were shown following subhemolytic exposures to ultrasonic vibrations, such as a small inhibition of cell respiration (11), an increase in viscosity and a decrease in cell volume (12), a slight decrease in the oxygen-combining power at high oxygen pressures (13), and an unchanged resistance to hypotonic saline (14, 15, 12). The suspension medium has an effect upon the rate of hemolysis (10).

Williams and Gaines (16) and Chambers and Gaines (17) using a sonic oscillator observed the laking of blood cells suspended in isotonic saline. By hemocytometer counts taken at regular intervals during the treatment, the following relationship was determined:

\[ N = N_0 e^{-kt} \]

where \( N \) is the number of uncytolized cells at time \( t \), and \( N_0 \) the original number of cells. The hemolysis was explained as the result of rapid alterations of tension and compression produced in the surrounding medium by the vibrations.

In the following experiments a further study of the kinetics of hemolysis by sonic oscillation was made. Also, the changes in sonic fragility brought about as a result of pretreatment of the cells were investigated.

Method

Blood was drawn by venipuncture from normal healthy subjects and used immediately for experimentation. The anticoagulant used was a two-to-one mixture of ammonium and potassium oxalate. Hemolysis was induced by a Raytheon 50 watt, 9 kc. magnetostriction oscillator having a laminated nickel rod attached to the diaphragm at the base of a stainless steel cup. One cc. of blood was diluted with 25 cc. of saline, and this was delivered directly into the cup and subjected to sonic vibration for varying periods of time. The suspension was centrifuged following oscillation and

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the supernatant liquid diluted one-to-four with distilled water. The hemolysis was expressed as a percentage of the reading obtained with a completely hemolyzed sample prepared by exposure to vibration for 7 minutes.

An output voltage of the oscillator was selected to produce approximately 50 per cent hemolysis in 30 seconds. At this output voltage, 60, a calculated maximum power of 2 watts is delivered to the sample in the cup. The output and hence the per cent hemolysis was affected by slight fluctuations of temperature in the cooling system of the magnetostriiction rod and chamber. To minimize this effect, water stored at room temperature was used for cooling the rod and chamber in all determinations to be described.

The density of the diluted supernatant liquid was determined with a Beckman spectrophotometer at a wave length of 505 mµ. Per cent hemolysis was then calculated as follows:

\[ P = 100 \times \frac{\text{Density of the partially hemolyzed sample}}{\text{Density of the completely hemolyzed sample}}. \]

To test the reproducibility of the technique, several aliquots from each of the blood samples of two subjects were exposed to sonic vibration for equal lengths of time. The average hemolysis in eight determinations for one subject was 52.6 per cent with a standard deviation of the mean of 0.35 per cent. The average for the other subject was 59.5 ± 0.36 per cent in nine determinations. From day to day, variations were somewhat larger. The mean and standard deviation of the mean for two subjects on 4 different days are as follows: (1) 57.4 ± 0.65 per cent hemolysis and (2) 57.3 ± 0.67 per cent.

The concentration of cells in the volume of blood added to the isotonic saline did not seem to affect the per cent hemolysis caused by a standard exposure to vibration. This is indicated by the following figures:

| Serum, cc | 0.75 | 0.50 | 0.25 | 0 |
| Cells, cc | 0.50 | 0.75 | 1.00 | 1.25 |
| Hemolysis, per cent | 50.3 | 50.7 | 50.7 | 49.9 |

Since the variations were within the error of experimentation, it was assumed that adjusted hematocrit ratios would not be necessary preceding each experiment.

Time-hemolysis determinations were made on the blood from five subjects (Table I). These experiments were followed by others in which the condition of the cells was varied by different types of treatment before they were exposed to sonic oscillation. The per cent hemolysis under these conditions was noted and compared to the normal for the blood cells of the same subject.

RESULTS

Destruction of erythrocytes by sonic means is logarithmic in rate. When the log of the number of cells, \( N \), remaining at the end of each time interval is plotted against the time of oscillation a straight line is obtained (17). If \( P \) equals the percentage of cells hemolyzed; 100, the total percentage of cells; and \( t \), time of oscillation in seconds, the first order reaction would be:

\[ \frac{dP}{dt} = k'(100 - P), \text{ or upon integration: } k = \frac{2 - \log (100 - P)}{t}. \]
The specific hemolysis rate constant, \( k \), is analogous to the reaction constant of chemical reactions or the "death rate constant" described by Rahn (18) and represents the slope of the logarithmic hemolysis curve, which should be constant in value for each time interval. This was found to be true (see Table I) in the data for five subjects listed in Table I.

**TABLE I**

*Specific Hemolysis Rate Constants \((k \times 10^4)\) during Various Time Intervals at a Vibration Frequency of 9 KC.*

<table>
<thead>
<tr>
<th>Subject</th>
<th>G.S.</th>
<th>H.R.</th>
<th>H.V.</th>
<th>B.R.</th>
<th>D.G.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>Anticoagulant</td>
<td>Anticoagulant</td>
<td>Anticoagulant</td>
<td>Diluted</td>
<td>Washed</td>
</tr>
<tr>
<td>O. V.</td>
<td>80</td>
<td>90</td>
<td>60</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>Temperature °C.</td>
<td>28</td>
<td>28</td>
<td>28</td>
<td>25</td>
<td>26</td>
</tr>
<tr>
<td>Time, sec</td>
<td>10</td>
<td>108</td>
<td>137</td>
<td>132</td>
<td>150</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>107</td>
<td>143</td>
<td>115</td>
<td>129</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>101</td>
<td>132</td>
<td>113</td>
<td>129</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>112</td>
<td>133</td>
<td>120</td>
<td>147</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>114</td>
<td>135</td>
<td>119</td>
<td>134</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>119</td>
<td>122</td>
<td>118</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>110</td>
<td>134</td>
<td>119</td>
<td>134</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>120</td>
<td>123</td>
<td>120</td>
<td>126</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>122</td>
<td>136</td>
<td>120</td>
<td>133</td>
</tr>
</tbody>
</table>

Average... 113 133 120 133 116
s. d. of mean... 2 2 3 3 1

O. V. indicates the output voltage of the oscillator at a plate voltage (P. V.) of 60. A value of \( k \) is the specific hemolysis rate constant calculated from the equation, \( k = \frac{2}{t} \cdot \log \left(\frac{100 - P}{P}\right) \).

The average \( k \) for the above determinations is 123 with a standard deviation of the mean of 4.

Average \( k \) values \((X \times 10^4)\) ranged from 113 to 133, with a mean of 123 and a standard deviation of the mean of 4.

The results of the determinations following pretreatment of the cells are shown in Tables II to IV.

1. Pretreatment for 1 hour by exposure to solutions of NaCl ranging in concentration from 0.35 to 2.0 per cent caused some hemolysis in the more dilute solutions and the remaining cells when tested for sonic fragility showed the changes given in Fig. 1 and Table II. In general hypotonic pretreatment increased the fragility and hypertonic treatment decreased the fragility with no change in concentrations between 0.6 and 0.9 per cent.
2. When cells were allowed to stand for 1 hour in contact with various concentrations of saponin ranging from 0.4 to 32.0 mg. per cc. of blood, and the remaining cells (see lower half of Table III for per cent hemolysis before oscillation) were subjected to oscillation, a decrease in the rate of hemolysis was noted. From Table III (upper) one may see that an initial small amount of saponin caused a decrease in hemolysis rate and increasing amounts caused no further change. From the time-hemolysis data in Table IV it is to be noted that the $k$ values do not remain constant but tend to decrease with longer exposure times.

3. Preheating of the blood was accomplished by immersing a test tube containing the desired amount of blood in a water bath and rotating manually until the blood reached 52.8 to 53.0°C., the procedure taking about 3 minutes. The blood was then immediately cooled to room temperature in a similar water bath and the standard procedure was used to obtain the time-hemolysis data shown in Table IV. A decrease in per cent hemolysis by sound of the remaining cells was found following preheating. Also, $k$ values do not remain constant for different times of exposures.

**TABLE II**

<table>
<thead>
<tr>
<th>Subject</th>
<th>M.L.</th>
<th>H.R.</th>
<th>N.M.G.</th>
<th>B.S.</th>
<th>R.C.</th>
</tr>
</thead>
<tbody>
<tr>
<td>O. V.</td>
<td>61</td>
<td>61</td>
<td>61</td>
<td>60</td>
<td>74*</td>
</tr>
<tr>
<td>Temperature, °C</td>
<td>27.5</td>
<td>27</td>
<td>28</td>
<td>27</td>
<td>28</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>NaCl, per cent</th>
<th>2.00</th>
<th>1.50</th>
<th>1.20</th>
<th>0.90</th>
<th>0.80</th>
<th>0.70</th>
<th>0.65</th>
<th>0.60</th>
<th>0.55</th>
<th>0.45</th>
<th>0.35</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>53</td>
<td>91</td>
<td>138</td>
<td>114</td>
<td>101</td>
<td>101</td>
<td>117</td>
<td>128</td>
<td>112</td>
<td>133</td>
<td>117</td>
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<td></td>
<td>59</td>
<td>81</td>
<td>114</td>
<td>113</td>
<td>111</td>
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<td>128</td>
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<td>64</td>
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<td>101</td>
<td>111</td>
<td>111</td>
<td>111</td>
<td>124</td>
<td>124</td>
<td>118</td>
<td>138</td>
<td>138</td>
</tr>
</tbody>
</table>

* Plate voltage in this case = 80; in other experiments, 60.
### TABLE III

Rate Constants ($k \times 10^4$) for a 30 Second Period of Exposure to Sound Following Pretreatment of the Cells for 1 Hour with Saponin, Heating, Alcohol, and Ether

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>O. V.</td>
<td>25.5</td>
<td>24</td>
<td>23</td>
<td>26</td>
<td>27</td>
<td>28</td>
<td>25</td>
<td>21</td>
<td>24</td>
<td>23.5</td>
</tr>
<tr>
<td>Tem. °C</td>
<td>0.0</td>
<td>0.4</td>
<td>0.5</td>
<td>1.0</td>
<td>4.0</td>
<td>8.0</td>
<td>2.0</td>
<td>6.0</td>
<td>4.0</td>
<td>2.0</td>
</tr>
<tr>
<td>mg.</td>
<td>112</td>
<td>114</td>
<td>122</td>
<td>128</td>
<td>129</td>
<td>121</td>
<td>0.0</td>
<td>95</td>
<td>91</td>
<td>82</td>
</tr>
</tbody>
</table>

Per cent hemolysis before oscillation

<table>
<thead>
<tr>
<th>Value</th>
<th>4.0</th>
<th>1.2</th>
<th>4.2</th>
<th>50</th>
<th>7.7</th>
<th>12</th>
<th>7.9</th>
<th>No hemolysis</th>
<th>Hemolysis present but not measured*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Room temperature, approximate.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### TABLE IV

Rate Constants ($k \times 10^4$) for Various Time Intervals Following Pretreatment of the Cells with Saponin, and Heating

<table>
<thead>
<tr>
<th>Subject</th>
<th>H.V.*</th>
<th>B.R.†</th>
<th>D.G.§</th>
<th>P. I.*</th>
<th>H.G.*</th>
</tr>
</thead>
<tbody>
<tr>
<td>O. V.</td>
<td>62</td>
<td>69</td>
<td>60</td>
<td>65</td>
<td>65</td>
</tr>
<tr>
<td>Time, sec.</td>
<td>28</td>
<td>25</td>
<td>25</td>
<td>25</td>
<td>25</td>
</tr>
</tbody>
</table>

* Anticoagulant.
† Defibrinated.
§ Washed.

625
In a second set of experiments (Table III) blood was preheated to temperatures ranging from 44 to 60°, kept at this temperature for 3 minutes, and then cooled to room temperature. These determinations showed an increase in fragility at 50°, but a decrease both above and below this temperature, when the remaining cells (see Table III, lower) were subjected to oscillation.

4. Absolute diethyl ether or ethyl alcohol was added to the diluted blood in quantities ranging from 0.24 to 0.75 mg. ether per cc. of blood or 0.063 to 2.00 mg. alcohol per cc. of blood. These suspensions were allowed to stand 1 hour before
the remaining cells were subjected to vibrations. Both alcohol and ether seem to have an antihemolytic effect upon the red cell when it is traumatized in this manner (Table III). The effect increases with additional quantities of alcohol, but in hemolytic concentrations of ether (above 0.63 mg. per cc. of blood) the inhibition appears to be released somewhat and the rate of hemolysis approaches normal.

**DISCUSSION**

It is postulated that the differences in mean k values for the five subjects investigated (Table I) arise as the result of individual variations in membrane strength and thus a change in the resistance to hemolysis. There are fluctuations in temperature and output of the oscillator from day to day as was shown, but these were small in comparison with the differences observed. It should be noted that in one case blood was defibrinated by the glass bead method and in a second, washed cells were used, but these treatments did not change the resistance of the cells (Table I).

In isotonic saline the red cell has normal dimensions but in decreasing concentrations (19) progressive increases in volume and diameter are known to occur. The critical volume at which osmotic lysis occurs is that at which the surface area of the newly formed sphere equals the original disk and any further increase in volume causes stretching of the membrane (Ponder (19)). At this concentration the sonic fragility also appears to be increased as if the membrane had been weakened in the process of stretching.

In hypertonic solutions crenation occurs, the red cell shrinks, and the surface area is reduced. If the cell water is reduced to one-half its normal value, gelation of hemoglobin may take place (Ponder (19)) and the resulting rigidity protects the cell from hemolysis.

Saponin appears to decrease the sonic fragility of human red cells (Tables III and IV). This is true in samples in which saponin alone did not cause hemolysis. According to Ponder (19), shape transformations unaccompanied by volume changes occur in the following manner when saponin is the hemolytic agent. Crenation of the disk takes place first, followed by a crenated sphere, a glistening sphere, a prolytic sphere, and then hemolysis. These constitute a series of metastable forms with decreasing surface area. Since a decrease in fragility was determined in the above experiments, it is thought that two factors may be operating to produce these effects: (1) reduced surface area and diameter of the cell, and (2) crenation.

Several mechanisms may be operating to produce the effects observed following sonic oscillation of preheated cells. Below 50°C. there may be shrinking of the cell, resulting in a decrease in cell volume, due to the reduction in osmotic pressure (20–25). At 50°C. fragmentation has begun or is in process (20, 26–28) and it would appear that as the new cells or fragments are being formed they
are more fragile upon subjection to sonic oscillation. At 53°C, the division is complete and those cells not destroyed are microcytic in size. These smaller cells are apparently less easily damaged by the trauma, as was the case in hypertonic media. At 56 and 60°C, this is more pronounced and protein denaturation (15, 29) may have set in, which might lead to an increased rigidity of the cell wall.

Both saponin and preheating appear to affect the kinetics of the hemolysis. Values of \( k \) decrease slightly with time and it would appear that the cells are no longer behaving strictly according to a first order reaction. Perhaps two populations of cells are now involved, those which have been injured by the procedures and those which retain their normal reaction. This is shown in Table IV.

Alcohol and ether have an antihemolytic effect also. The mechanism of action of alcohol may be explained as follows: In the presence of 0.9 per cent sodium chloride alcohol makes an effective hypertonic solution for it is osmotically active in the presence of sufficient sodium chloride (30). Therefore, one is dealing with a crenated, smaller sphere and this type of cell is less easily destroyed by the cavitational disturbance. With ether an initial decrease in hemolysis was followed by a slight increase. However, with increasing amounts of ether the output of the oscillator decreased. Perhaps the ether affected the process of cavitation or the amount of gas dissolved in the liquid. If this is not responsible for the initial decrease it might be attributed to a decrease in cell volume (31). Microscopic observation showed crenation except in the smallest concentration of ether. In this case the cells were all spheroid and appeared to be protected. Alteration of the membrane; e.g., denaturation or coagulation of the proteins is a very possible factor. As hemolysis sets in, one might assume that the membrane had been weakened and hence an increase in hemolysis by trauma resulted.

One might think that the “remaining cells” are not representative of the whole sample since perhaps only the most hardy ones survived the pretreatment. However, it should be noted that many of the effects described took place in samples in which no hemolysis due to pretreatment was evident. A few examples are as follows:

1. A decrease in hemolysis by sound was demonstrated in hypertonic salt solutions in which there was no hemolysis before subjection to oscillation.
2. No hemolysis was evident in the less concentrated (less than 4 mg./cc. of blood) saponin solutions before exposure to sound, but the result showed a decrease in hemolysis by sound. (3) Alcohol caused no hemolysis, but the pretreated cells appeared to be more resistant to sound. (4) The effects of heating and the number of cells hemolyzed seemed to have no relationship to the number destroyed by sound (see Tables II and III).
In comparing the above results with the determinations made by others on the mechanical fragility of red cells by the glass bead method it would appear that the two are not the same. Shen et al. (32) showed an increase in mechanical fragility of cells exposed to 0.62 per cent sodium chloride. Results with sonic oscillation indicate that an increase in hemolysis does not appear until the concentration is reduced to below 0.60 per cent. Ham et al. (28) noted an increase in the mechanical fragility of cells heated to 52.8°C. Below this temperature he could not demonstrate an increase in fragility, as was done in the above experiments. Also, washing and defibrination did not appear to injure the cells in the few experiments attempted. Several investigators have found, however, that such procedures injure the red cell sufficiently to cause an increase in fragility to mechanical trauma.

**SUMMARY**

A magnetostriction oscillator has been used for determining the relative fragility of human red cells to 9000 cycle vibrations under some different environmental conditions. The destruction of the cells is a logarithmic function of time according to the equation

\[ k = \frac{2 - \log (100 - P)}{t} \]

Hypertonic saline solution, saponin, preheating, alcohol, and ether in sub-hemolytic concentrations decrease the fragility of human red cells subjected to sonic oscillation. Hypotonic saline, preheating to 50°C. for 3 minutes, and hemolytic concentrations of ether increase the fragility of red cells to 9 kc. vibrations. Following preheating of the cells, and in the presence of saponin the destruction deviates slightly from a true logarithmic rate of hemolysis.

**REFERENCES**