Osmotic resistance of heat-damaged erythrocytes

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SYNOPSIS  Whole blood was heated for twenty minutes at 40°C., 45°C., 50°C., and 55°C. Changes in the osmotic resistance of the heat-exposed cells were then determined. The values obtained were plotted as lysis increments. This treatment revealed groups of cells of varying heat sensitivity. After heating to 50°C., cell groups were therefore prepared by partitioning cells between layers of a mixture of methyl- and butyl-phthalates of known densities. Three cell groups enriched in either oldest cells, cell fragments, or cell fragments and youngest cells were obtained. These groups subjected to serialosmotic lysis tests revealed that the densest, i.e., oldest fraction, had least thermal resistance. Fractionation and osmotic resistance studies provided clear evidence of some thermal damage to erythrocytes of median age, i.e., cells which would normally be expected to remain in the circulation for another two months. Some of the possible implications are discussed.

Burning injury always results in the direct exposure to heat of at least some of the circulating red cells. In severe injuries the morphological changes caused by exposure to heat are seen in the capillary blood. The extent of their fragmentation can even be used prognostically, as was shown by Topley (1958). Shen, Ham, and Fleming (1943) have shown that thermal injury was associated with an increased osmotic fragility which was most prominent early after the injury.

Both Ham, Shen, and Fleming (1948) and Kimber and Lander (1964) described three definite patterns of osmotic behaviour in the heat-damaged red cells: up to 24 hours after the injury the osmotic fragility was increased; the next 12 hours revealed a normal pattern, which at about 40 hours gave way to an increased osmotic resistance. The latter could be explained by the elimination of the oldest, presumably most heat-sensitive, cells.

Isaacs, Brock, and Minot (1925) first demonstrated the apparently different heat response of immature and mature erythrocytes and attempted to use this finding in the differential diagnosis of certain anaemias.

The possible clinical importance of differing heat sensitivities of cells of varying ages was indicated by Moore, Peacock, Blakely, and Cope (1946) who described three phases of blood loss: an immediate one, a second loss at about five days, and a late phase evident even seven weeks after the injury. Both Davies and Topley (1956) and Muir (1961) confirmed the late disappearance of the red cells.

Occasionally this may have been explicable by the development of an anaemia of infection.

Nevertheless the possibility of delayed effects of slight heat damage to some of the erythrocytes should be considered. A study of the osmotic behaviour of erythrocytes exposed to varying temperatures and analysis of the lysis curves obtained in terms of cell age groups seemed likely to offer some understanding of the problem.

MATERIALS AND METHODS

Pooled samples of normal blood either group A or group O were used to minimize the differences due to individual response.

HEATING  Two ml. amounts of blood were heated in a water bath at 40°C., 45°C., 50°C., and 55°C. ± 0.02°C. for 20 minutes and cooled by immersion into a water bath at 25°C. Heated samples were pooled as required.

LYSIS DETERMINATION  Fragility was determined on whole blood at a final dilution of 1 in 100 in buffered salines (Parpart, Lorenz, Parpart, Gregg, and Chase, 1947) corresponding to concentrations ranging from 0-10 to 1-50 g.% NaCl. The lysis occurring in 0-10 g.% NaCl was accepted as the 100% value. All tests were incubated at 30°C. for one hour. The tubes were then centrifuged and the extent of the haemolysis in the supernatant fluids was determined directly as oxyhaemoglobin in those tubes which showed extensive lysis.

Minor amounts of haemolysis were determined by microanalysis of the iron content of the haemoglobin, and the method used was based on those described by Connerty and Briggs (1962) and Fischl (1959). The

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method was calibrated with high purity iron (99-95%). Results were converted to oxyhaemoglobin which was accepted to contain 0-338% iron (Drabkin, 1965). The use of two different methods of lysis assessment required the determination of absolute rather than relative amounts of haemoglobin in the solutions. The oxyhaemoglobin method was therefore standardized by reference to a hemoglobin cyanide standard (B.D.H.).

**CELL FRACTIONATION** Cell age groups were obtained by fractionating the cells according to their densities after heating. Cells in their own plasma were subjected to a density fractionation based on that described by Danon and Marikovsky (1964). Thin-walled, open-ended glass tubing 12 × 0-2 cm. was coated with silicone fluid MS 1107 according to the makers' recommendations. Mixtures of di-methyl phthalate (SG 1:189) and di-n-butyl phthalate (SG 1:042) to give densities of 1:091, 1:093, 1:095, and 1:097 were used as the partitioning phase. The tubes were filled to a 1 cm. mark with the ester and to a 9 cm. mark with whole blood and flame sealed. Ten tubes were used for one density mixture. The tubes were centri-fuged at 4,000 g. for 15 minutes. The platelet and leucocyte layer was discarded by cutting the tubes at the appropriate levels. Fresh capillaries were filled with sufficient plasma to give a packed cell volume of 30% to 40%, if the desired cell group was transferred into it by capillarity. The tube contents were then mixed with an Astrup capillary magnetic bar and magnet.

By this method of fractionation only the cells within the sealed capillary end and those in direct contact with the ester mixture were lost.

**CALCULATION OF THE RESULTS** To obtain most information on the number of components contributing to a particular apparently sigmoid plot obtained from plotting the lysis values against the saline concentrations the data may be either differentiated graphically as was done by Ponder (1948) or the percentage differences in the lysis between each step in saline concentration may be plotted.

The latter method was described by Bolton (1949) and results in an "increment" curve. This method was chosen because of its simplicity and yet yielding adequate information.

**RESULTS**

Reproducibility of the method was tested by 12 replicate determinations on a normal sample of blood. Over the range of 0-25 to 50 g. % NaCl the standard deviation was ± 0-11-±0-27% haemolysis.

Table I summarizes the effect of varying temperatures on the osmotic resistance of erythrocytes. At 45°C., which under the conditions of heating gave a central blood temperature of 43°C. for 10 minutes, a slight decrease in osmotic resistance may be noted.

'Increment' plotting as shown in Fig. 1 reveals a slight skewness of the curve. Similar treatment of the post-50°C. data shows the two major components with at least two minor cell groups. One of these components falls within the normal fragility range, but is somewhat shifted to the right. When the blood was heated to 55°C. two major groups of osmotic behaviour became evident (Fig. 1). One of these comprised cells lysing in strongly hypertonic solutions.

The statistical significance of the different osmotic behaviour of the red cells at various temperatures was calculated by the t test. Table II shows that exposure to 45°C. only gave significant differences from the normal at 0-50 and 0-53 g. % NaCl. When the post-55°C. values were compared with those at 50°C. the differences were significant at all saline concentrations.

From this study it became evident that heating to 50°C. resulted in a number of cell groups which were likely to survive, if they occurred in vivo.

**TABLE I**

<table>
<thead>
<tr>
<th>NaCl (g. %)</th>
<th>Pre-heating (%) Haemolysis</th>
<th>Post 40°C (%) Haemolysis</th>
<th>Post 45°C (%) Haemolysis</th>
<th>Post 50°C (%) Haemolysis</th>
<th>Post 55°C (%) Haemolysis</th>
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<td>100</td>
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<td>100</td>
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<td>99-46</td>
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<td>2-60</td>
<td>7-44</td>
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FIG. 1. Mean 'increment' fragility curves of whole blood heated to 45°C, 50°C, and 55°C.

TABLE II
COMPARISON OF 'INCREMENT' FRAGILITY CURVES BY THE T TEST

<table>
<thead>
<tr>
<th>NaCl = v. %</th>
<th>% Difference</th>
<th>t</th>
<th>P</th>
<th>NaCl = g. %</th>
<th>% Difference</th>
<th>t</th>
<th>P</th>
<th>NaCl = g. %</th>
<th>% Difference</th>
<th>t</th>
<th>P</th>
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<td>0.40</td>
<td>44.67</td>
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<td>0.14</td>
<td>0.36</td>
<td>0.7</td>
<td>0.45</td>
<td>9.98</td>
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<tr>
<td>0.42</td>
<td>69.61</td>
<td>23.8</td>
<td>&lt;0.001</td>
<td>0.30</td>
<td>0.44</td>
<td>0.28</td>
<td>0.8</td>
<td>0.47</td>
<td>14.70</td>
<td>3.98</td>
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<td>0.35</td>
<td>3.08</td>
<td>0.37</td>
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<td>20.37</td>
<td>4.72</td>
<td>&lt;0.001</td>
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<td>0.47</td>
<td>78.48</td>
<td>16.9</td>
<td>&lt;0.001</td>
<td>0.40</td>
<td>5.95</td>
<td>0.48</td>
<td>0.7</td>
<td>0.53</td>
<td>23.81</td>
<td>6.98</td>
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<tr>
<td>0.50</td>
<td>76.15</td>
<td>16.6</td>
<td>&lt;0.001</td>
<td>0.40</td>
<td>16.11</td>
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<td>&lt;0.001</td>
<td>0.45</td>
<td>15.07</td>
<td>1.23</td>
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<td>0.60</td>
<td>26.30</td>
<td>0.81</td>
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<tr>
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<td>1.43</td>
<td>0.2</td>
<td>0.47</td>
<td>4.78</td>
<td>2.18</td>
<td>&lt;0.05</td>
<td>0.70</td>
<td>30.24</td>
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<td>&lt;0.001</td>
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<tr>
<td>0.50</td>
<td>2.21</td>
<td>2.71</td>
<td>&lt;0.02</td>
<td>0.50</td>
<td>5.95</td>
<td>0.48</td>
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<td>0.75</td>
<td>32.91</td>
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<td>0.53</td>
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<td>&lt;0.02</td>
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</table>

To obtain some information on the age groups of the cells showing a particular response, cell fractionation was carried out after heating to 50°C and before subjecting the cells to varying saline concentrations.

The results of such an experiment are shown plotted conventionally in Fig. 2 and on the 'increment' basis in Figure 3. The top 12% and the bottom 22% fraction show opposite results. This top fraction consisted largely of fragmented cells and the behaviour of the top 22%, of which 10% consists of cells and not fragments, is of greater interest. This fraction clearly contains two cell populations besides the fragments of which one is apparently osmotically normal. The densest fraction had the least osmotic resistance.

DISCUSSION

The significance of these findings on the general heat susceptibility of all cells is that the normal cell population comprises cells of minimal sensitivity, some or all of the youngest, the old cells of maximal sensitivity, and a middle-aged group with some
FIG. 2. Fragility curves of heated cell populations (T = 50°C).

FIG. 3. 'Increment' fragility curves of heated cell populations (T = 50°C).
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variation in the individual heat response. For the patient the middle-aged group should be the most important and this osmotic study has shown that this group also is affected by a relatively low temperature such as could well be encountered.

A decrease in osmotic resistance is also a phenomenon associated with the normal process of ageing (Spear and Sass, 1964). The results of the study reported suggest that premature ageing cannot be the explanation, or at least not completely so, because different density groups show apparently a different response. Henriques (1947), in a study on the rates of thermally induced processes and their significance in epidermal heat injury, considered enzyme alteration as the primary change. This alteration corresponds to an activation energy of at least 150 Cals/mole and an entropy change of about 395 units. In skin this could be consistent with the reversible phase of heat injury.

In red cells which are incapable of oxidative phosphorylation reversibility is unlikely. The action of heat should therefore be considered in its effect on the rates of enzymic processes. Some enzymes of the glycolytic pathway show greater heat sensitivity than other, e.g., G-6PD (Cruickshank and Hershey, 1960). The red cell would therefore not be able to recover its energy reserves and its ATP content would become depleted. As the steady state level of erythrocyte ATP is low and there are no other significant energy reserves, its early exhaustion would lead to premature cell death or in vivo a steady rate of disappearance of such affected cells could be expected.

REFERENCES

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