THE EFFECT OF VARIOUS COOLING RATES ON THE MEMBRANE ULTRASTRUCTURE OF FROZEN HUMAN ERYTHROCYTES AND ITS RELATION TO THE EXTENT OF HAEMOLYSIS AFTER THAWING

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SUMMARY

Human erythrocytes suspended in buffered isotonic saline were frozen to the temperature of liquid nitrogen at various cooling rates of 3, 140, 700, 1800, 3500, 8000 and 11 500 deg. C/min. The membrane ultrastructure in the frozen state and the extent of haemolysis after thawing were examined at each cooling rate. As the cooling rates increased from 3 to 3500 deg. C/min, the extent of lysis gradually decreased, but further increase in cooling rates in excess of 8000 deg. C/min resulted in an abrupt increase of lysis. Membrane-associated vesicles devoid of intramembrane particles (IMPs) were formed in the erythrocyte membranes frozen at cooling rates slower than 1800 deg. C/min. The frequency and size of these vesicles were highly cooling-rate-dependent and they were no longer formed in the erythrocyte membranes frozen at cooling rates faster than 3500 deg. C/min. Another membrane ultrastructural change associated closely with the formation of intracellular ice crystals appeared at cooling rates faster than 8000 deg. C/min. The membrane regions in direct contact with intracellular ice crystals were physically damaged and had an appearance resembling worm-eaten spots. The erythrocytes frozen at a cooling rate of 3500 deg. C/min exhibited ultrastructural integrity of the membrane by avoiding the membrane changes caused by either slow or fast freezing. It is suggested, from the close relation between membrane ultrastructure and the extent of haemolysis, that the ultrastructural integrity of membrane in the frozen state is important for avoiding haemolysis after thawing, and that the membrane ultrastructural changes caused by both slow and fast freezing were responsible for the lysis after thawing.

INTRODUCTION

Survival of frozen cells after thawing depends closely upon the cooling rates during freezing. For most cells examined, survival is low after either a slow or a fast cooling rate and becomes maximal at some intermediate cooling rate (Bank & Mazur, 1973; Mazur & Schmidt, 1968; Mazur et al. 1970; Rapatz, Sullivan & Luyet, 1968). The loss of survival in freeze–thawed cells has been correlated with the structural changes in the membrane (Levitt, 1966; Lovelock, 1953; Mazur et al. 1970; Meryman, 1968), but there are only a few studies that have been published regarding the membrane ultrastructural changes in relation to the occurrence of freezing injury.
In the present study, the effect of various cooling rates on the human erythrocyte membrane ultrastructure was investigated concurrently with the measurement of the extent of haemolysis after thawing. It was clearly demonstrated in this study that the membrane ultrastructure changed dramatically depending upon the cooling rate, and a close relation with the extent of haemolysis after thawing was seen.

**Materials and Methods**

**Materials**

Human erythrocytes were obtained from freshly drawn human blood and washed at least 3 times with 0.15 M NaCl in 0.005 M phosphate buffer solution, pH 7.4. Erythrocytes suspended in the buffered saline (haematocrit = 50%) were used for experimental material in this study.

**Freezing procedures**

For attaining various cooling rates, the following 3 specimens were prepared.
1. A droplet of cell suspension of 0.01 ml, which was placed in a small hole in an aluminium holder (1.5 mm thickness and 5 mm x 5 mm in size).
2. A droplet of cell suspension of 0.01 ml, which was placed on a copper holder (0.2 mm thickness and 5 mm x 5 mm in size).
3. A thin layer of cell suspension of 0.007 ml, which was prepared according to the 'sandwich method' of Nei (1973).

A cooling rate of 3 deg. C/min was attained as follows. A loaded aluminium holder was placed at the bottom of a thermoelectric bath (Sharp Co., model TE-262) kept at —2 °C. After equilibration of the temperature of the specimen to that of the bath, a small amount of ice at the tip of a pair of forceps was seeded into the specimen in order to disrupt super-cooling. After seeding, the temperature of the bath was set at —60 °C. This procedure resulted in a cooling rate of 3 deg. C/min between —2 and —60 °C. After the temperature reached —60 °C, the specimen was quickly immersed into liquid nitrogen.

A cooling rate of 140 deg. C/min resulted from the exposure of the loaded copper holder to liquid nitrogen vapour in a Dewar flask with distances of 2 cm from the surface of the liquid nitrogen.

A cooling rate of 700 deg. C/min resulted from immersion of 1 edge of the loaded aluminium holder into liquid nitrogen. During freezing, direct contact of the cell suspension with liquid nitrogen was avoided.

Cooling rates of 1800 and 3300 deg. C/min resulted from abrupt immersion of a loaded aluminium holder and a sandwich holder, respectively, into liquid nitrogen.

Cooling rates of 8000 and 11 500 deg. C/min resulted from rapid immersion of loaded aluminium and copper holders, respectively, into Freon 22 kept at —160 °C.

All the specimens were stored in liquid nitrogen until used.

**Measurement of cooling rates**

The cooling rates were determined by a 41-gauge copper constantin thermocouple fixed in the centre of the specimen suspension. The continuous recordings were performed with an oscillograph recorder (Yokogawa Electric Works Ltd. type 2901) or a recorder (Hitachi 056). The cooling rates were measured between 0 and —60 °C, except for the cooling rate of 3 deg. C/min which has been described already.

**Measurement of the extent of haemolysis**

The frozen specimens were rapidly immersed into buffered isotonic saline kept at 20 °C, and shaken to promote thawing. The warming rates in all the specimens lay between 7000 and 10 000 deg. C/min as measured between 0 and —196 °C.

The frozen specimens of 0.02 ml in total were thawed in 6 ml of buffered saline. They were divided into 2 aliquots, and 1 aliquot was sonicated for 20 s to achieve complete haemolysis.
Effect of cooling rate on membrane

Fig. 1. Percentage lysis of human erythrocytes frozen at cooling rates ranging from 3 to 11,500 deg. C/min, and thereafter thawed rapidly. The vertical lines show the extent of variation.

Both the freeze-thawed specimen, and the specimen freeze-thawed followed by sonication, were then centrifuged at 20,000 g for 30 min at 0 °C, and the amount of haemoglobin released into the supernatant was measured with a spectrophotometer (Hitachi 101). The extent of haemolysis was expressed as the ratio of the haemoglobin of the supernatant of the freeze-thawed specimen to the haemoglobin of the sonicated specimen.

Electron microscopy

From the frozen specimens, freeze-etching replicas were made as previously described (Fujikawa, 1978). The complementary freeze-etching replicas were also made according to the method previously described (Fujikawa, 1978).

Ultrathin sections were prepared as follows. The frozen specimens were freeze-dried in a freeze-etching apparatus (Jeol, Ltd type AFE 01). After the drying was completed, the specimens were sliced into small chips. They were fixed with OsO₄ vapour for 1 h at room temperature and transferred to absolute ethanol and then propylene oxide, and embedded in Epon, according to the method of Luft (1961). The grey-silver thin sections were cut with an ultramicrotome (Porter MT-1) using a diamond knife. After mounting on mesh, the sections were stained with uranyl acetate and lead citrate (Reynolds, 1963).

The replicas and thin sections were viewed with a JEM 100C electron microscope operated at a 100 kV accelerating voltage. The nomenclature of Branton et al. (1975) was used to designate the fracture faces, and the shadowing in all the freeze-etching electron micrographs used herein was from the bottom towards the top of the page.

RESULTS

Freezing human erythrocytes at cooling rates extending from 3 to 11,500 deg. C/min resulted in a U-shaped curve, when the extent of haemolysis was plotted against cooling rates (Fig. 1). Freezing at the very slow cooling rate of 3 deg. C/min caused nearly complete haemolysis. As the cooling rate increased from 140 to 3500 deg. C/min, the lysis gradually decreased to a minimum of about 50%. Further increase in cooling rate again resulted in the increase of lysis.
Fig. 2-6. Protoplasmic fracture faces of extracellularly frozen erythrocytes at cooling rates of 3 deg. C/min (Fig. 2), 140 deg. C/min (Fig. 3), 700 deg. C/min (Fig. 4), 1800 deg. C/min (Fig. 5) and 3500 deg. C/min (Fig. 6). An arrow in Fig. 3 shows an IMP-free patch of the edge of a PF and an exoplasmic surface (ES). Arrows in Fig. 4 show fused patches. EI, extracellular ice. × 70,000.
Table 1. Frequency and size of IMP-free patches on the protoplasmic fracture faces at different cooling rates

<table>
<thead>
<tr>
<th>Cooling rates, deg. C/min</th>
<th>Mean* number of IMP-free patches/10 (\mu m^2)</th>
<th>Mean† diameter of IMP-free patches, nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0.5</td>
<td>1330</td>
</tr>
<tr>
<td>140</td>
<td>16.3</td>
<td>260</td>
</tr>
<tr>
<td>700</td>
<td>44.5</td>
<td>126</td>
</tr>
<tr>
<td>1800</td>
<td>95.5</td>
<td>89</td>
</tr>
<tr>
<td>3500</td>
<td>0</td>
<td>—</td>
</tr>
</tbody>
</table>

* Each mean is based upon 1000 \(\mu m^2\) areas.
† Each mean is based upon more than 2000 IMP-free patches, except for 3 deg. C/min. The mean for 3 deg. C/min is based upon 200 IMP-free patches.

The cooling rate of 3500 deg. C/min in which minimum lysis was attained was the fastest cooling rate used in this study that permitted extracellular freezing of erythrocytes. Faster cooling rates than 8000 deg. C/min resulted in intracellular freezing. (According to Mazur (1970), the critical cooling rate for permitting intracellular freezing of erythrocytes is approximately 5000 deg. C/min.)

Figs. 2–6 show the typical appearance of protoplasmic fracture faces (PF) of erythrocyte membranes frozen at cooling rates of 3, 140, 700, 1800 and 3500 deg. C/min, respectively. In all these cooling rates, erythrocytes were frozen extracellularly. In the erythrocytes frozen at a cooling rate of 3500 deg. C/min, the intramembrane particle (IMP) distribution on the fracture faces was normal, but in the erythrocyte membranes frozen at a slower rate than 1800 deg. C/min elevated patches devoid of IMPs were formed on the fracture faces. These patches frequently represented multilamellar fracture faces (Figs. 2–5), and also fused with each other to form larger patches (Fig. 4).

The frequency and size of these patches varied dramatically depending upon the cooling rates (Table 1). As the cooling rates increased, although the number of patches increased gradually up to a cooling rate of 1800 deg. C/min, their size decreased gradually. These patches were no longer formed on the erythrocyte membranes frozen at faster cooling rates than 3500 deg. C/min.

While the patches were elevated on the PF, there were depressed patches devoid of IMPs on the exoplasmic fracture faces (EF) (Figs. 7, 8). The frequency and size of these depressed patches on the EF were completely similar to those of the elevated patches on the PF at corresponding cooling rates. The prints of patches could also be recognized on the etched exoplasmic surfaces (ES) (Fig. 9), and occasionally such a
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patch was visible on the edge of an etched ES and a PF (Fig. 3). The complementary replica pairs proved that the elevated patches on PF and the depressed patches on EF were of a complementary structure (Fig. 10).

The thin section of freeze-dried erythrocytes revealed multilamellar structures associated with the membrane (Figs. 11–13). These structures could also be recognized as single (Fig. 11) or multilamellar bilayer vesicles (Figs. 12, 13) associated with erythrocyte membrane. The frequency and size of these bilayer vesicles, as revealed by thin sections, were closely similar to those of the IMP-free patches, as revealed by freeze-etching.

When intracellular freezing occurred at cooling rates faster than 8000 deg. C/min, another membrane change arose due to the formation of intracellular ice crystals. In the intracellularly frozen erythrocytes, altered membrane regions resembling ‘worm-eaten spots’ were observed on PF and ES after a considerable extent of etching (Fig. 14). The membrane regions of these worm-eaten spots corresponded to where intracellular ice crystals were formed in direct contact with membranes (Figs. 15 and 16). It was noted moreover that in freeze-fractured membranes before etching, such worm-eaten spots could not be observed on the fractured faces; they were covered by etchable ice crystals (Fig. 17). These results indicate that at the membrane region in direct contact with intracellular ice crystals, the path of fracturing was deviated extracellularly from the inner plane of the membrane, and removal of ice crystals from such regions by etching exposed the ES of the erythrocyte membrane that shows the worm-eaten spots.

The deviation of fracturing as shown in Fig. 17 was a quite typical feature of intracellularly frozen erythrocytes, and not found in the extracellularly frozen cells despite the fact that they had conspicuous surface irregularity comparable to intracellularly frozen cells. It can be suggested that the deviation of fracture at the worm-eaten spots also reflects the occurrence of membrane alteration.

The frequency and size of the worm-eaten spots changed depending upon the cooling rate, which also meant that they depended upon the size of intracellular ice crystals (Table 2). As the cooling rate increased, although the number of worm-eaten spots increased, due to the increase of the number of intracellular ice crystals, the size of the worm-eaten spots decreased due to the decrease in the size of intracellular ice crystals.

Only the erythrocyte membranes frozen at a cooling rate of 3500 deg. C/min in the range extending from 3 to 11,500 deg. C/min kept their ultrastructural integrity by avoiding the membrane changes caused by either slow or fast freezing.

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Fig. 10. A complementary pair of fracture faces of an extracellularly frozen erythrocyte at a cooling rate of 700 deg. C/min. A, protoplasmic fracture face (PF). B, exoplasmonic fracture face (EF). x 90,000.

Figs. 11–13. Cross-sections of extracellularly frozen erythrocytes at cooling rates of 3 deg. C/min (Fig. 11), 140 deg. C/min (Fig. 12) and 700 deg. C/min (Fig. 13). x 100,000.
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Table 2. Frequency and size of worm-eaten spots at different cooling rates

<table>
<thead>
<tr>
<th>Cooling rates, deg. C/min</th>
<th>Mean* diameter of intracellular ice crystals, nm</th>
<th>Mean† number of worm-eaten spots /10 μm²</th>
<th>Mean‡ diameter of worm-eaten spots, nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>8000</td>
<td>410</td>
<td>20.1</td>
<td>131.6</td>
</tr>
<tr>
<td>11500</td>
<td>120</td>
<td>40.3</td>
<td>62.0</td>
</tr>
</tbody>
</table>

* Each mean is based upon 100 counts of intracellularly frozen erythrocytes.
† Each mean is based upon 1000 μm² areas.
‡ Each mean is based upon 1000 worm-eaten spots.

DISCUSSION

The membrane ultrastructure of human erythrocytes changed dramatically depending upon the cooling rates. The freezing of erythrocytes at cooling rates slower than 1800 deg. C/min resulted in the formation of IMP-free patches. These patches were elevated on the PF and depressed on the EF. Similar fracture patterns were obtained when abnormal lipoprotein (LP-X) vesicles that fused to erythrocyte membranes were freeze-fractured (Verkleij et al. 1976). Cross-sectional views of membranes indicated that these IMP-free patches corresponded to membrane-associated bilayer vesicles.

The formation of IMP-free vesicles was highly dependent on the cooling rate while slow freezing resulted in vesicle formation, rapid freezing did not. Moreover, using different washing times for cells did not change these results. Therefore, vesicle formation could be due to the structural change in erythrocyte membranes themselves during slow freezing.

It has been suggested that during slow freezing cells are exposed to a solution that is altered by the presence of extracellular ice crystals, and that the injury of cells by slow freezing is due to prolonged exposure of cells to these altered solutions (Levitt, 1966; Lovelock, 1953; Mazur, 1966; Meryman, 1968). The slower cooling rate means longer exposure of cells to the altered solution. The IMP-free vesicles were formed after exposure of erythrocytes to these altered solutions for a certain period of time. The critical cooling rate, which allowed the formation of IMP-free vesicles, lay between 1800 and 3500 deg. C/min. Moreover, prolonged exposure of erythrocytes

Figs. 14–16. Parts of intracellularly frozen erythrocytes at cooling rates of 8000 deg. C/min prepared by freeze-fracturing and followed by etching. Arrows show worm-eaten spots on the membrane caused by formation of intracellular ice. H, intracellular ice; CY, cytoplasmic region. Fig. 14, ×80,000. Figs. 15, 16, ×62,000.

Fig. 17. Protoplasmic fracture face (PF) of an intracellularly frozen erythrocyte at a cooling rate of 8000 deg. C/min, prepared by freeze-fracturing without etching. Arrows show etchable ice. ×80,000.
to these altered solutions resulted in an increase of vesicle size. The freeze-etching micrographs indicated that the increase of size might result from the fusion of small vesicles.

Araki (1979) indicated that slow freezing of human erythrocytes resulted in the release of microvesicles after thawing. These released vesicles differed from erythrocyte membranes in their higher cholesterol content and lack of IMPs. The mechanism of microvesicle formation during slow freezing is unknown, but it is likely that such cholesterol-enriched and IMP-free vesicles may originate from the membrane-associated vesicles observed in this study. It is also interesting that such cholesterol-enriched and IMP-free vesicles were also released from the membrane by exposing erythrocytes to a concentrated NaCl solution at near sub-zero temperature without freezing (Araki, 1979).

One possible mechanism has been proposed for IMP-free vesicle formation in human erythrocyte ghosts. Elgsaeter, Shotton & Branton (1976) suggested that the conditions under which the spectrin meshwork contracts could cause bulk lateral flow of phospholipid molecules to form IMP-free vesicles. The formation of IMP-free vesicles during slow freezing might also reveal alteration in membrane protein structure.

If freezing injury of cells is caused only by the prolonged exposure of cells to altered extracellular solutions, then faster freezing of cells may result in a higher rate of survival. When intracellular freezing occurred at faster cooling rates, however, survival decreased abruptly in all the cells examined (Rapat et al. 1968). The same results were also obtained in this study. It has been suggested, therefore, that the injury of rapidly frozen cells is caused by factors different from slow freezing, and that the formation of intracellular ice crystals is the most likely factor causing injury to rapidly frozen cells (Mazur, 1966, 1970, 1977).

It has been indicated in this study that the formation of intracellular ice crystals in direct contact with the membranes brought about membrane ultrastructural changes resembling worm-eaten spots. It is difficult to confirm whether the worm-eaten spots reveal membrane ultrastructural change caused by formation of intracellular ice or are artifacts due to etching. It has been reported that etching causes partial membrane collapse (Pinto da Silva, 1973), and might result in structures resembling worm-eaten spots. However, occurrence of membrane alteration is shown by the fact that at the worm-eaten spots the path of fracturing is deviated out of the interior of the membrane. It is suggested that, in the worm-eaten spots, the molecular organization of the bilayer membrane is physically damaged by the formation of intracellular ice crystals that grew in direct contact with the membrane; and, as a result, fracture deviation occurred since hydrophobic membrane interior structure, which is the predominant pathway for fracturing in the membrane, was impaired in the region.

It is interesting to compare the relation between the membrane ultrastructure in the frozen state and the extent of haemolysis as a factor of cooling rate, since a close relation was found between them. The gradual decrease of lysis with the increase of cooling rate from 3 to 3500 deg. C/min, corresponded to the gradual decrease in IMP-free vesicle size, and haemolysis became minimal at 3500 deg. C/min, when the
Effect of cooling rate on membrane erythrocyte membrane kept its ultrastructural integrity by avoiding the formation of IMP-free vesicles. Rapid increase of lysis with a further increase in cooling rate corresponded with the formation of the worm-eaten spots.

It has been suggested by Mazur et al. (1970) that maximum survival (minimum lysis) is attained at the cooling rate which minimizes the time of exposure of cells to altered extracellular solutions and also prevents the formation of intracellular ice crystals. The optimum cooling rate for minimum lysis was a cooling rate in which erythrocyte membranes kept their ultrastructural integrity by avoiding the membrane changes caused by either slow or fast freezing. It is concluded from these results that membrane ultrastructural integrity is important for avoiding lysis after thawing, and that the membrane ultrastructural changes caused by slow or fast freezing are responsible for the increase of lysis after thawing.

The present study could, therefore, indicate the presence of a close relation between membrane ultrastructural change in the frozen state and the extent of haemolysis after thawing as a function of cooling rate. However, the mechanism of haemolysis in connection with the membrane changes is not clear as yet. Further studies involving membrane ultrastructural change during thawing are required for clarifying the precise relation between membrane ultrastructural change and the occurrence of haemolysis in freeze-thawed erythrocytes.

References


(Received 18 September 1980)