SURVIVAL OF ERYTHROCYTES AFTER COOLING INTO LIQUID NITROGEN : RELATION WITH GLASS-FORMING TENDENCY ON COOLING AND THE TRANSITION FROM CUBIC INTO HEXAGONAL ICE ON REWARMING

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SURVIVAL OF ERYTHROCYTES AFTER COOLING INTO LIQUID NITROGEN : RELATION
WITH GLASS-FORMING TENDENCY ON COOLING AND THE TRANSITION FROM CUBIC
INTO HEXAGONAL ICE ON REWARMING

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Résumé : Si on représente le taux de survie de globules rouges refroidis
jusqu'à la température de l'azote liquide en fonction de la vitesse de
refroidissement lorsque la vitesse de réchauffement ultérieure est rapide,
celle-ci croît, décroît puis remonte aux plus grandes vitesses en présence de
suffisamment d'additif cryoprotecteur. Cette dernière remontée coïncide avec
la décroissance de la quantité de glace cristallisée dans la solution, aussi
bien en présence de diverses concentrations de 1,3-butanediol que de 1,2-
propanediol. Aux plus grandes vitesses, une survie élevée correspond à la
vitrification totale. Au réchauffement, si la solution était totalement
amorphe, de la glace cubique se forme d'abord. Elle se transforme ensuite en
glace hexagonale. Les dégâts du gel accompagnent ou suivent immédiatement
cette transition.

Abstract : If the survival rate of red blood cells cooled down to liquid
nitrogen temperature is plotted versus cooling rate, it increases, decreases,
then increases again at the highest cooling rates if the cryoprotectant
concentration is sufficient, when subsequent rewarming is fast. This last
increase coincides with the decrease of the quantity of ice crystallized in
the solution, in the presence of various concentrations of 1,3- butanediol or
1,2-propanediol. The high survival at the highest cooling rates corresponds to
total vitrification. On rewarming, if the solution was wholly amorphous, cubic
ice first crystallizes. It transforms afterwards into hexagonal ice. Freezing
damage parallels or follows immediately after this transition.

I - INTRODUCTION

Having determined among a variety of polyalcohols those which most favour the
wholly amorphous state in their aqueous solutions, even at low concentrations, it
was interesting to see whether or not the most efficient solutes were effectively
good cryoprotectants for cells. Therefore red blood cells were chosen as a model,
due to the simplicity of their structure. Their survival has been studied versus
cooling rate when they are cooled down to liquid nitrogen temperature and rewarmed
up to room temperature.
Survival curves of erythrocytes in buffered solutions containing various
concentrations of glycerol or 1,2-propanediol had been plotted for cooling rates
varying from 1 to 4 000°C/min (1). They were cooled in cylindrical straws of 0.5 ml.
They were rewarmed either slowly or rapidly. Rapid rewarming was obtained by
immersing the straws, initially at -196°C in a water bath at 37°C (warming rate of
about 5.000°C/min) and slow warming, by leaving the straw, initially at -196°C in
the air at room temperature (warming rate of about 100-200°C/min). Glycerol was
chosen because it is the most commonly used cryoprotectant and 1,2-propanediol
because it most favours the wholly amorphous state.

With 10, 15 or 20 % (w/w) glycerol (1) or 1,2-propanediol (Fig. 1) as the cooling rate is increased, the survival increases, passes through a maximum, then decreases. Such a curve with a maximum of survival is very common and has been observed for a variety of cells in the presence of different cryoprotectant (2-4). At the lowest cooling rates, as pure ice crystallizes out of the cells, too much water has enough time to flow out the cells in response to the resulting osmotic pressure. They are killed by an excessive shrinkage. At the highest rates, water does not have enough time to leave the cell and ice can crystallize in the diluted intracellular solution, killing the cells.
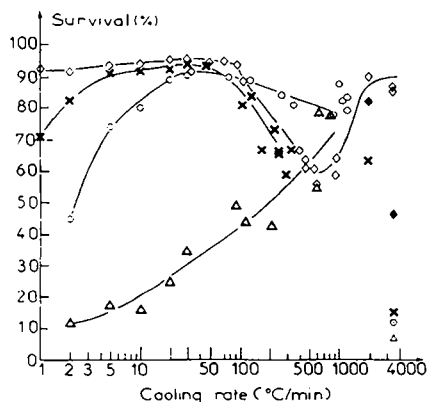

Fig. 1 : Survival (%) of red blood cells after cooling at different rates to -196°C in buffered solutions with △ : 10 %, ⊙ : 15 %, ✗ : 20 % and ◇ : 30 % (w/w) (hypotonic solution (1)) 1,2-propanediol and thawing by immersion of the straws into a 37°C water bath, and with 30 % (◆) (w/w) 1,2-propanediol (hypotonic) ; for a cooling rate of 2000°C/min, these straws were rewarmed in water at 37°C in the kleenex in which it had been cooled, and for a cooling at 3500°C/min, these straws were rewarmed in air at room temperature.

With 30 or 35 % (w/w) 1,2-propanediol, when the cooling rate is further increased, after a minimum, survival again increases to about 90 % (1) when subsequent warming is fast (Fig. 2). Such an increase is not observed with 30 or 35 % glycerol at similar cooling rates. It was suggested that this could be due to the higher glass-forming tendency of the 1,2-propanediol solutions : ice would have not enough time to crystallize both outside and inside the unshrunken erythrocytes.

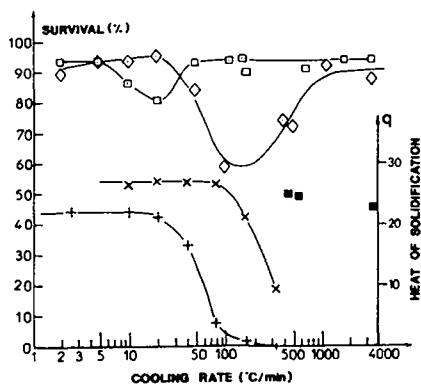

Fig. 2 : Comparison of the survival (%) of red blood cells after cooling at different rates to -196°C in isotonic (1) buffered solutions with 30 % (◇) or 35 % (▯) (w/w) 1,2-propanediol, and thawing by immersion of the straws into a water bath at 37°C,with the quantity of ice crystallized on cooling in aqueous solutions with 30 % (✗) or 35 % (+) (w/w) 1,2-propanediol.(▮) straws were warmed slowly by exposure to air at room temperature. The quantity of ice crystallized is represented by the number q of grams of ice whose solidification at 0°C would liberate the same amount of heat as from 100 g solution on crossing the corresponding peaks (ref. 5 p. 554). One obtains that heat in calories per 100 g of solution by multiplying q by 79.78 ; the values of q are given from ref. 6.

More recently, the survival has been compared with the quantity of ice crystallized on cooling, the conditions where damage occurs on rewarming have been studied not only for erythrocytes but also for other cells. Also, survival curves have been plotted in 1,3-, 2,3- and 1,2-butanediol due to their interesting physical properties (7). This is what is presented here.

II - SURVIVAL RATE AND QUANTITY OF ICE CRYSTALLIZED ON COOLING : CASE OF ERYTHROCYTES COOLED WITH 1,2-PROPANEDIOL

In fig. 2 , one sees that the increase of survival at cooling rates faster than that corresponding to the minimum, coincide with the decrease of the quantity of ice

crystallized in the aqueous solution, both with 30 or 35 % 1,2-propanediol. It is very likely that the 1,2-propanediol concentration is the same inside and outside unshrunken erythrocytes since a lot of time has been given for equilibration of this very permeable solute before freezing. The intracellular solution also contains solutes other than 1,2-propanediol. Nevertheless, the above coincidence suggests that the quantity of ice crystallized has the same variation inside unshrunken erythrocytes as in a solution of 1,2-propanediol concentration than the extracellular solution : the glass-forming tendency is the same inside the erythrocytes as when 1,2-propanediol is mixed with pure water. This also suggests that ice is always damaging when it crystallizes inside the cells on cooling, and that the high survival corresponding to the highest cooling rates corresponds to complete vitrification of the extracellular and intracellular solution.

Nei also observed a minimum and an increase of survival rate of erythrocytes cooled with glycerol, but at much higher cooling rates (8). He observed by microscopy that cell shrinkage is weak near the minimum of survival, and that no ice was visible inside or outside the cells at the highest cooling rates.

III - SURVIVAL OF ERYTHROCYTES IN 1,3-BUTANEDIOL SOLUTIONS WHEN SUBSEQUENT REWARMING IS FAST

1,3-butanediol was chosen since the glass-forming tendency on cooling, and stability of the wholly amorphous state on rewarming of its aqueous solutions, are almost as high as those of 1,2-propanediol solutions for the same water content (9). The toxicity of 1,3-butanediol for erythrocytes is very low. With 35 % 1,3-butanediol in the buffered solution (10), the hemolysis reaches only 3.5 % ten hours after the addition, when the cells are maintained at 0°C (10).
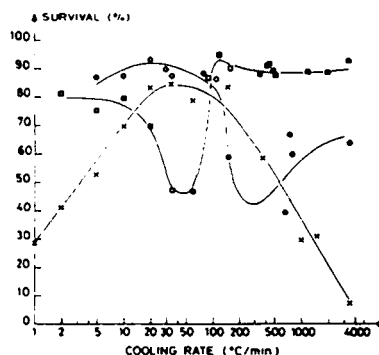

Fig 3 : Survival (%) of red blood cells after cooling at different rates to -196°C in isotonic phosphate-buffered solutions with 20 % (✕), 30 % (⊙) and 35 % (⊡) (w/w) 1,3-butanediol and thawing by immersion of the straws into a 37°C water bath.

Survival curves of erythrocytes in 1,3-butanediol solutions are given in fig. 3. With 20 % alcohol, only the classical peak of survival is observed. With 30 or 35 % of this solute, the minimum and the subsequent increase of survival are observed as with 1,2-propanediol. But mainly with 35 % solute, the minimum occurs at higher cooling rates with 1,3-butanediol, in agreement with its smaller glass-forming tendency.

In fig. 4, the increase of survival at the highest cooling rates (10) here also coincide with the decrease of the quantity of ice crystallized in the corresponding 1,3-butanediol aqueous solution (9). If it was merely due to chance, it would be very unlikely that this coincidence would occur for two concentrations of two different solutions.

Fig. 4 : Comparison of the survival (%) red blood cells after cooling at different rates to -196°C in isotonic buffered solutions with 30 % (⊙) or 35 % (⊡) (w/w) 1,3-butanediol, and thawing by immersion of the straws into a water bath at 37°C with the quantity of ice crystallized on cooling in aqueous solutions with 30 % (△) or 35 % (▽) (w/w) 1,3-butanediol. For the units of q, see the comments in Fig.2 legend. The values of q are given from ref. 9.
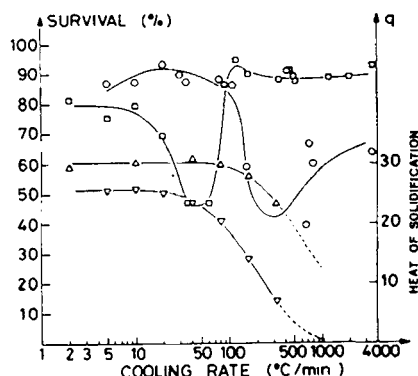
IV - SURVIVAL, WARMING RATE AND TRANSITION FROM CUBIC INTO HEXAGONAL ICE

It is much more difficult to impede ice crystallization on rewarming than on cooling. The cooling rates necessary to avoid ice crystallization on cooling are respectively about 300, 1000, 500 to 1000 and 4000 or 5000°C/min, with respectively 30 or 35 % 1,2-propanediol or 1,3-butanediol in water (Figs. 2 and 4). The warming rates necessary to avoid crystallization on rewarming are much higher : 7.5 x 10^8°C/min with 35 % 1,2-propanediol and 2.7 x 10^{12} °C/min with 35 % 1,3-butanediol.

However, a warming rate of about 5000°C/min (straws rewarmed in a water bath at 37°C) is sufficient to insure a high survival of the erythrocytes after cooling at 4000°C/min with 30 % or 35 % 1,2-propanediol or 35 % 1,3-butanediol. Through, in the absence of shrinkage on cooling the polyalcohol cannot concentrate inside the cells. This suggests that ice has crystallized inside the cells on rewarming, but that, when it crystallizes only on rewarming, it is not necessarily damaging. Indeed Rall et al (11) observed innocuous ice crystallization on rewarming cells of mouse embryos. If erythrocytes are rewarmed at only 100-200°C/min (straws rewarmed in air) damage occurs on rewarming (figs. 2 and 5).
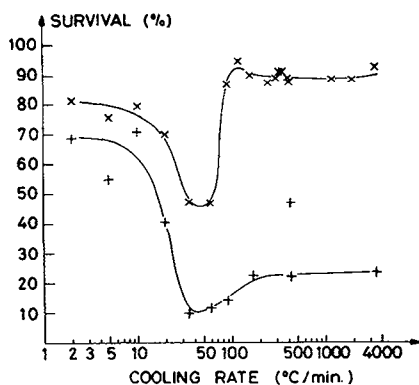

Fig. 5 : Survival (%) of red blood cells after cooling at different rates to -196°C in an isotonic buffered solution with 35 % (w/w) 1,3-butanediol and thawing by immersion of the straws in a 37°C water bath (✗) or by exposure to air at room temperature (+).

When ice crystallizes from a wholly amorphous solution, it is first cubic (7) (ice Ic) (12) then hexagonal (ordinary ice). Comparison of various X-ray experiments show that the speed of the transition from cubic into hexagonal ice is similar in pure water and in 1,2-propanediol solutions (1, 7, 13) and in 1,3-butanediol solutions, though a little slower in these last ones. Using an expression etablished by Dowell and Rinfret for pure water, one can deduce that the proportion x of hexagonal ice formed at a temperature T is related to the warming rate v by (1,11) :   x(T) = (1/v) exp(0.126 T - 26.5)   [1]
When T is in K and v in °C/min (unless this equation gives x>1 : in this case x=1, all ice has become hexagonal. T_m equals -13 and -16°C with respectively 30 and 35 % (w/w) 1,2-propanediol and -10.5 and -12.5°C with respectively 30 and 35 % (w/w) 1,3-butanediol in water. One finds x=1 at T_m for all these solutions at a low cooling rate of 200°C/min. At 5000°C/min at T_m , Eq.[1] gives respectively x=0.10, 0.07, 0.14 and 0.11 for these four solutions. Therefore almost no damage has occured

on fast rewarming where ice has remained mainly cubic, while more than half of the erythrocytes are hemolysed after slow rewarming where ice has enough time to become hexagonal.
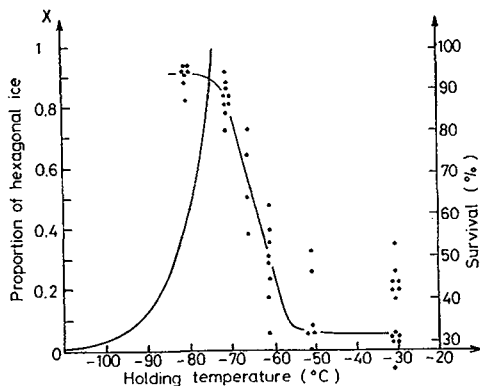
Fig. 6 : Survival (%) of erythrocytes (•) after fast cooling and fast rewarming from -196°C to various holding temperatures for 30 min and fast rewarming to room temperature (14). The other curve represents the proportion x of ice which has become hexagonal at the end of storage at the holding temperature.

To check whether this coincidence is more general, comparisons have also been made with other experiments. Nei (14) cooled erythrocytes rapidly in liquid nitrogen,

then rewarmed them rapidly to various temperatures where the cells were maintained for 30 min. before rapid rewarming to room temperature. In fig. 6 are represented both the survival rates of the cells and the quantity of hexagonal ice formed at the end of the holding temperatures deduced from the equation (10,11) :

$$x = 0.388 \times 10^{-12} \times t \times \exp(0.126T) \qquad [2]$$
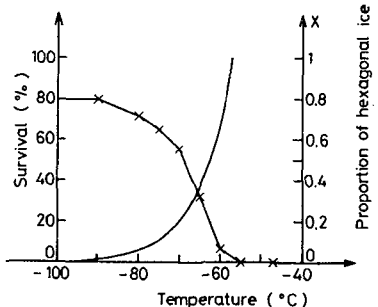
for the transformation at constant temperature.

Fig. 7 : Survival of eight-cell mouse embryos cooled slowly to -40°C and then rapidly to -196°C(**X**) as a function of the temperature T_{sr} at which slow warming to 2°C/min was converted to rapid warming at 500°C/min (11, 13). On the same figure is reported the proportion x of hexagonal ice formed at T_{sr}.

In the case of the experiments of Rall on embryos (Fig. 7) the transition from cubic into hexagonal ice coincides with the decrease in survival.

V - SURVIVAL OF ERYTHROCYTES AFTER COOLING WITH 2,3-BUTANEDIOL OR 1,2-BUTANEDIOL

2,3-butanediol has been tested because the wholly amorphous state of its aqueous solution can be easily obtained on cooling, but large amounts of ice and hydrate crystallize on rewarming. Untill now it is only the harmfullness of ice which has been studied. The question was : is hydrate crystallization also harmfull ? The toxicity of 2,3-butanediol for erythrocytes is low. When these are maintained at about 17-20°C with 30 % 2,3-butanediol, the hemolysis reaches 5 % after 10 hours. Survival curves have been drawn after cooling to -196°C at cooling rates from 1 to 3500°C/min with 20, 30 or 35 % (w/w) 2,3-butanediol. Unfortunately, survival was always very low, whether subsequent rewarming was fast (straws rewarmed in a water bath at 37°C) or slow (straws rewarmed in air). The hydrate crystallization always kills the cells.

Attempts have also been made to measure freezing damage in the presence 1,2-butanediol, since this solute favours cubic ice crystallization (9) and seems innocuous. Unfortunetely, 30 % (w/w) of this solute is toxic, and the erythrocytes are already hemolysed before freezing.

VI - CONCLUSION

Traditionally, cryobiologists use glycerol or dimethylsulfoxide for cryopreservation of cells. Studies of ice crystallization in water solutions have allowed us to find two new cryoprotectants, 1,2-propanediol, and 1,3-butanediol. With these solutes high survival rates of erythrocytes are obtained after cooling into liquid nitrogen, not only in the classical case where shrunken cells are surrounded by ice, but also in the case of complete vitrification. The interesting properties of 1,3-butanediol have been found too recently to have, untill now, any other applications. On the other hand, since our studies, 1,2-propanediol has proved to be very interesting for the cryopreservation of other cells. Using this solute, Renard obtained survival rates close to 100 % with mouse or rabbit embryos (15, 16), even at stages of development considered till now as being difficult to cryopreserve. He applied his method to the bank of mouse embryos of the Pasteur Institut in Paris. In some cases, he could relate survival to the physical properties of the solutions (17). The technique has been extended to humans embryos by Testart (18). Babies have been born in 1986 from embryos cryopreserved in liquid nitrogen with 1,2-propanediol at the hospital of Clamart, near Paris. Using this cryoprotectant the rate of pregnancies is 3 times that using glycerol or dimethylsulfoxide (first babies born in Australia) (19). It is comparable to that without freezing. 1,2-propanediol can be used for clinical preservation of erythrocytes (20). Using a solution containing a mixture of 1,2-propanediol, dimethylsulfoxide, acetamide and polyethylene glycol, Rall and Fahy succeeded in cryopreserving mouse embryos by complete vitrification (21).

The encouraging results suggest that the study of glass-forming tendency and stability of the wholly amorphous state would perhaps be the way to find how to preserve the major organs of man and mammals by total vitrification.

At least in the case of erythrocytes the survival of unshrunken cells seems directly related to the glass-forming tendency on cooling. It is not related to the stability of the wholly amorphous state on rewarming, but damage seems correlated to the transition from cubic into hexagonal ice. Since the warming rates to avoid this transition are smaller than those necessary to avoid any intracellular crystallization in unshrunken cells, warming rates about 2000 to 5000°C/min would be sufficient to rewarm vitrified tissus or organs without damage, by using perhaps a microwave oven. This shows that the study of the transition from cubic into hexagonal ice is very useful. In the case of erythrocytes, damage comes just after : it may be correlated to the subsequent growth of the hexagonal ice crystals. Therefore a more detailed study including the determination of the size and shape of the crystals versus temperature could be also useful (22).   30 % 1,2-butanediol is toxic, but perhaps the use of this solute in ternary systems, or other solutes favoring cubic ice could be promising. The stability of the wholly amorphous state certainly plays a role in the case of shrunken cells which can be rewarmed at rates much lower than 2000°C/min. In their intracellular medium containing very high concentrations the warming rate necessary to avoid any ice crystallization can be much lower than that necessary to avoid the transition from cubic into hexagonal ice. 2,3-butanediol hydrate is damaging. Another method for cryopreservation may be to find innocuous hydrates. 2,3-butanediol may itself be interesting in ternary systems impeding at least partially its hydrate crystallization.

Acknowledgement

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COMMENTS

J. PEREZ

When amorphous water-alcohol is transformed into crystalline phase, damage of living cells is generally explained in terms of either osmotic pressure difference or mechanical effects induced by change in density. If the damage is due to the cubic-hexagonal ice transformation how to understand the mechanism of damage ?

Answer :

No relation has even been observed between damage due to intracellular ice crystallization and change in density. When damage occurs at the transition from cubic into hexagonal ice or just follows it, it could be due either to the consecutive growth of hexagonal ice crystals larger than cubic ice crystals, or to the sharper shapes of hexagonal crystals. These are only hypothesis till now the mechanism of this damage has not been observed experimentally.