Physical limitations of the rapid freezing method

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[Plates 19 and 20]

Throughout the recorded history of biological freezing, it has generally been recognized that one may differentiate between rapid and slow freezing, although it has not always been realized how compelling is the distinction which can be drawn between the two on the basis of the markedly different distribution of ice crystals and the differing mechanism of cell injury which may result. In order, therefore, to discuss the physical limitations of rapid freezing, the term must be defined and differentiated from slow freezing. This paper thus becomes primarily a discussion of slow and rapid freezing from which the limitations of the rapid freezing method should become self-evident.

Possibly the single most important concept in biological freezing as well as the most difficult for the biologist to assimilate is the following: despite the collective and individual complexity of living cells, freezing in a biological matrix represents nothing more than the removal of all available water and its isolation into inert foreign bodies, the ice crystals. Freezing is a wholly physical phenomenon obeying known and simple laws and, tempting though it may be to imbue biological freezing with the same mysterious complexity that surrounds the cells themselves, it is quite unnecessary. The transfer of water out of solution into ice crystals is a very straightforward phenomenon and is, in effect, simply dehydration with the removed water sequestered locally in the tissue rather than entirely removed. From this dual event—dehydration with foreign body formation—stem all the physiological and biochemical events subsequent to freezing (Meryman 1956).

GROWTH CRYSTAL

The earliest form of a crystal is called a crystal nucleus. There are two kinds of nucleation in water. The first is called heterogeneous, or catalytic, nucleation. This is the deposition of water molecules in crystalline array on some foreign particle and is virtually the sole source of crystal formation under ordinary conditions. The second type is called homogeneous nucleation, and results from random fluctuations in density and configuration of pure water. The probability for homogeneous nucleation is virtually zero near the freezing point and remains so until about \(-40^\circ\) C, when the probability rapidly approaches one, thus limiting the supercooling of pure water to \(-40^\circ\) C.

Thus, by freezing slowly with the temperature rarely falling much below the freezing point, a few large ice crystals originating from catalytic sites are formed.

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If the rate of heat exchange is increased more catalytic sites are induced and the crystals increase in number, hence are smaller. If temperatures in the vicinity of \(-40^\circ C\) can be attained before any significant crystallization has taken place a dense shower of minute crystals appears through spontaneous homogeneous nucleation. This, in brief is the story behind the familiar fact that the faster the freezing the smaller the crystals.

This is not the whole story, however, since crystal growth itself must be considered. Every crystalline material has a definite rate of growth under given conditions. This rate is a direct function of temperature, that is, as the temperature is lowered, the growth rate slows. This relationship affects the ultimate crystal size for, with a severe reduction in temperature, the growth of existing crystals is curtailed, providing further opportunity for additional crystal nuclei to form. The introduction of certain organic solutes also has a marked effect in slowing the crystal growth rate, a fact of obvious practical value, as will be seen later.

An additional factor influencing the ultimate ice crystal size and one which is frequently overlooked is the phenomenon of recrystallization in the solid state. Intuitively, one is tempted to think of the frozen state as the solid state and therefore, within biological time, a static state. That this is far from true is demonstrated in figure 10, plate 19. This illustration reproduces electron micrographs of thin films of ice. These ice films have been prepared by allowing water vapour to fall upon a surface cooled by liquid nitrogen in a high vacuum. Such films fail to show crystalline patterns by X-ray diffraction and may be assumed to be either non-crystalline or very nearly so (Burton & Oliver 1936). When such films are permitted to warm, because of differences in vapour pressure between ice crystals of differing size and internal strain, larger crystals tend to grow at the expense of smaller. The very low temperatures at which this can take place are quite surprising. In a frozen biological medium, of course, each crystal is separated from its neighbour by an organic barrier and the rate of growth is substantially reduced. However, experimental data indicate that recrystallization may progress at a significant rate during the storage of biological materials at temperatures as low as \(-70^\circ C\).

Inasmuch as any consideration of rates of freezing must also entail some consideration of the rates at which heat is being removed, a brief examination of the kinetics of heat exchange in a solid will be profitable. Figure 11 shows three different stages of freezing into a sphere. Below each is drawn the temperature gradient within the solid. One can see that there is a steep gradient from the freezing boundary to the external ‘cold sink’ at all times—in other words, as soon as a particle freezes, its temperature drops rapidly as the freezing boundary proceeds beyond it. It is interesting to note that heat from the interior has equilibrated at the freezing temperature. This is the usual cause of the familiar plateau seen in most temperature records of freezing; the internal temperature has simply reached the freezing point in advance of the freezing boundary.

Whereas, during freezing, heat is rapidly conducted from the freezing boundary through an efficiently conducting ice layer, during thawing, the reverse is true with a very significant effect. The first drawing in figure 12 is a diagram of a frozen sphere which has been plunged into a warm bath but has not yet begun to thaw. Yet
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the gradient graph shows that the temperature of the interior is already beginning to rise. In the second drawing this has progressed further and by the time thawing has begun, in the third, the interior of the sphere has risen to the melting point. The solid phase has a sufficiently good heat conduction to permit the rapid distribution of heat throughout the solid before any appreciable thawing has taken place. The significance of this will become apparent in proceeding to the application of these elements of freezing to a biological matrix.

Figure 11. Sequence of freezing into a spherical specimen. The top row indicates the position of the freezing boundary. Below, the thermal gradients are shown. Of particular interest is the fact that the interior equilibrates at the freezing point well in advance of the arrival of the freezing boundary. Peripheral to the freezing boundary the gradient is very steep and, with an advancing boundary, the temperature of a particle falls rapidly immediately after freezing.

Figure 12. Sequence of thawing in a spherical specimen. This figure illustrates the striking difference between thawing and freezing as illustrated in figure 11. Heat is efficiently distributed throughout the solid material prior to thawing so that internal equilibration at the melting point occurs almost before any thawing has taken place.

Figure 13a to d. Slow freezing in tissue. Crystal nuclei develop in the extracellular spaces. As they grow, water is removed from within the cell and added to the crystal which eventually dehydrates the cell which has been crowded into narrow bands between the crystals. The cell is now bathed in a highly concentrated solution of its own solutes.

Freezing in Cells

When a cellular biological medium is frozen, a curious and most significant phenomenon is observed; when freezing is slow, crystal formation is exclusively extracellular. Figure 13 illustrates the manner in which the growing crystals

Description of Plate 19

Figure 10. The recrystallization of ice at low temperature. Electron micrographs of vacuum evaporated replicas made from ice films produced in high vacuum. The rapid growth of large crystals from essentially non-crystalline films takes place at surprisingly low temperature.
remove water from intra- and extracellular spaces, until the crystals have grown to a size perhaps many times that of the individual cells, while the cells are compressed between them in a concentrate of their own solutes. In figure 14a, plate 20, is a section of rabbit liver showing the results of slow freezing. The open holes are the ice crystals. Remarkable though this anatomical deformation may be, more remarkable yet is the histological recovery of these cells upon thawing as seen in figure 14b, plate 20. The magnifications are the same. The cells have reimbibed their water and appear apparently undamaged. In fact, as well demonstrated by experimental frostbite procedures, many tissues can even survive such treatment providing the exposure has not been of excessive duration, proving conclusively that extracellular ice crystal formation per se is not necessarily lethal. It has further been demonstrated by Lovelock (1953) that when ice crystal formation is, as here, slow and extracellular, injurious effects usually do not result from the mechanical presence of the ice crystals, but from dehydration and the concentration of electrolytes which inevitably results from the removal of water to form ice crystals. To repeat, in general, it is not the physical presence of ice crystals, but the concentration of electrolytes that injures cells when freezing is slow and the ice crystals extracellular. It should be noted that this discussion is directed toward the freezing of animal cells and does not generally apply to freezing in plant cells.

The tendency for ice crystals to form extracellularly is seen only when the rate of freezing is relatively slow, as, for example, when a specimen is placed in a conventional deep-freeze unit or even in air at dry-ice temperature. One should add that all naturally occurring freezing is slow. When the rate of cooling is increased by immersion in a liquid bath at dry-ice temperature or lower, the tendency for extracellular crystallization is overcome and crystals form at random throughout the material, hence are predominantly intracellular. Figure 14c, plate 20, shows a section of liver that has been rapidly frozen. The extracellular tendency has been overwhelmed, and one would rather expect that the presence of intracellular foreign bodies of such appreciable size would be intolerable.

Prevention of injury from slow freezing

Now let us consider these two kinds of freezing and examine means by which cell injury or destruction may be avoided. Following slow freezing, the injury results predominately from electrolyte concentration and is a biochemical process proceeding at a definite temperature-dependent rate. In view of this, one method of preventing injury might be to freeze slowly, then, before significant injury had developed, lower the temperature to one at which the rate of denaturation is

Description of plate 20

Figure 14. a, rabbit liver slowly frozen. The empty spaces represent the location of large extracellular ice crystals. b, a portion of the same tissue after being allowed to thaw. The cells have reimbibed the water released by the melting ice crystals and appear histologically intact. c, rapidly frozen liver. Left: The small holes scattered throughout the cells represent the location of intracellular ice crystals. Right: Following thawing the histology appears essentially normal save for rather pyknotic nuclei.
insignificant. This is, in effect, what is done when tumour samples are maintained for long periods of time in dry ice. The procedure is generally borderline, however, and is useful only because the survival of isolated cells fulfills the purpose. It should be noted in passing that this discussion applies only to well-hydrated cells; those which have a low water content and withstand dehydration will be unaffected by slow freezing.

The technique of slow freezing followed by a drop to low temperature where denaturation rates are slow is applicable only when destruction from electrolyte concentration is not immediate. In blood, haemolysis occurs rapidly when the salt concentration approaches 4-7%, well before freezing has been completed. The only way of preventing this rapid haemolysis is to reduce the amount of water removed to form ice and hence to reduce the salt concentration to an acceptable degree.

This is the basis of the glycerol technique (Smith 1954). Glycerol has the ability to attach to itself considerable water which is then unavailable to form ice but can still act as a solvent. Glycerol also passes freely through cell membranes. Therefore, the addition of glycerol to a specimen results in the binding of water and a reduction in the amount removed to form ice. The electrolyte concentration is therefore less and, if sufficient glycerine is added, is no longer lethal. The advantages of glycerine freezing are that the rates of freezing and thawing are relatively undemanding, and both procedures can be carried out easily under sterile conditions within a single vessel. Although for long storage temperatures should be maintained between −70 and −100°C, brief exposure to higher temperatures can be tolerated, so that the stored material can be transported in dry ice or can withstand brief refrigeration failures. The disadvantages lie primarily in the necessity for rather large concentrations of glycerol, 30 to 40% which may be toxic to many cells. Because of this, it is frequently necessary to remove the glycerol by dialysis following thawing. In the case of blood, glycerol is removed by washing the cells, which results in removal of the plasma and the preservation of washed red cells only. Both the introduction and the removal of the glycerol must be done carefully to avoid injury from osmotic causes. This means that the glycerolization and deglycerolization of larger fragments of tissue may become impractical because of the excessive time involved.

Prevention of injury from rapid freezing

If we are to attempt the preservation of life through rapid freezing, a wholly new approach is necessary, since the presence of intracellular ice crystals can be considered intolerable, and the only manner in which the cell may be spared their destructive force will be to freeze it so rapidly that the crystals will be so small as to be wholly innocuous. It is possible to freeze materials sufficiently rapidly to prevent such excessive crystal growth if the freezing is carried out in an extremely cold liquid such as liquid nitrogen or liquid air, and if the specimen is sufficiently small or, to be accurate, has a sufficiently high surface-to-volume ratio to permit the rapid heat exchange necessary. This is the prime limitation of the rapid freezing technique. In so far as no means is known to remove heat in large quantity
other than by conduction, sufficiently rapid freezing can only be achieved if the specimen is in the form of a film or a small droplet 1 or 2 mm or less in diameter.

Presuming that rapid freezing has been carried out successfully with the creation of ice crystals of such small size that mechanical damage is not done despite the intracellular location of the crystals, one must consider the circumstances necessary to preserve this situation during storage. There are two potentially lethal processes which can occur in the solid state. The first, as discussed earlier is the growth through recrystallization of ice crystals which may physically disrupt the cell. In addition to this, however, one should realize that a concentration of electrolytes similar to that following slow freezing has been created. Not all the water in a solution becomes ice during freezing. Much of the water is hydrogen bonded or adsorbed to organic molecules, where it may still be available as a solvent for cell solutes. Thus, even when a tissue is fully frozen, unfrozen water remains as a solvent for electrolytes and a vehicle for denaturation. The rate of such reactions will be a direct function of temperature. Thus, following rapid freezing, storage at very low temperature is mandatory in order that neither recrystallization nor denaturation may take place at a significant rate. For whole blood, temperatures below —80 and preferably below —100° C appear to be necessary for good storage survival over a protracted period of time.

Thawing a rapidly frozen material is possibly the greatest challenge in the whole procedure. At temperatures near the melting point, both recrystallization and denaturation can progress extremely rapidly. It is mandatory that the specimen be passed through this lethal temperature range as rapidly as possible. During thawing, as shown in figure 12, the internal temperature of the specimen rises to the melting point, the most lethal temperature which it can experience, and then must sit at this temperature while the melting boundary slowly progresses inward. Here again, small size and favourable geometry of the specimen are essential to success.

Discussion of rapid freezing

Applications of rapid freezing have not as yet been very extensively investigated, its principal success at this stage being in the preservation of whole blood. With the addition of glucose to a concentration of 5%, loss of red cells may be reduced to 2 or 3% (Meryman & Kafig 1955). The glucose is believed to reduce the rate of crystal growth and thus to reduce the ultimate crystal size. For blood preservation, rapid freezing has one distinct advantage in that the whole blood is recovered. The clotting factors of the plasma appear unaltered. The platelets are morphologically unchanged although their functional integrity has not yet been investigated. More complex nucleated cells do not survive as well. Although successful transplants of both the Walker 256 and Ehrlich Ascites cells have been achieved with rapidly frozen material, there is a 100% mortality of bull spermatozoa.

The principal physical limitations of rapid freezing, small specimen size to facilitate rapid freezing and thawing and the need for low temperature storage, are limitations of the technique itself. However, having successfully recovered some biological organisms uninjured following rapid freezing, one must then ask why other viable entities fail to survive this experience. This is not at present an
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answerable question, but at least two observations which bear upon it can be made. First, it appears that it is a more primitive form of cell which survives rapid freezing most readily. The micro-organisms in particular appear to be very suitable for preservation by this technique, although of course many of them will survive conventional freezing even without glycerol. As was observed previously, any cell which can survive dehydration can survive freezing. Of mammalian cells which have been successfully preserved by rapid freezing, the erythrocyte is hardly a representative member, since it contains no internal structure or nucleus. The tumour cells which have been successfully frozen were tested by their ability to form new growth on transplantation, but no attempt at assay was carried out. It is probable that only isolated cells survive, but that these were adequate to perpetuate the tumour. There have not been sufficient investigations of rapid freezing in mammalian tissues to give a clear answer to the question of their suitability for this technique. If the answer is that more complex cells are more sensitive to intracellular crystals of a size which the erythrocyte can tolerate, this then becomes merely a question of the quality of the technique. Refinements of the procedure to permit the creation and maintenance of yet smaller crystals should provide a solution to a limitation of this nature.

The second observation which may well serve to eliminate certain cells as candidates for rapid freezing is the phenomenon of thermal shock. This term is used to describe cell injury which occurs as a direct result of a rapid change in temperature. This has been demonstrated to occur in spermatozoa at temperatures well above the freezing point. Such injury is suspected to be the result of differences in thermal expansion within the specimen (Lovelock 1955). It should be reiterated that this particular demonstration of thermal shock was made at temperatures above the freezing point. At lower temperatures where freezing takes place it is impossible to differentiate between injury due to freezing or thawing and any other independent effects of the temperature change per se. Although thermal shock may well be an important factor in limiting the applicability of rapid freezing, it cannot be experimentally demonstrated in the presence of freezing, and, until such a time as it can be, the term cannot be meaningfully used under these circumstances. One cannot ignore, however, the fact that spermatozoa are killed by a temperature change of only 2°C per minute at temperatures above freezing. This may well indicate an unusual and excessive sensitivity but, when the rate of temperature change is, as it is in rapid freezing, a matter of hundreds of degrees per second, even harder cells may be expected to succumb to an inability to adjust to this mechanical stress.

In summary, the limitations of the rapid freezing technique as it is used today can be enumerated as follows:

1. The rate of freezing must be extremely high in order to form very small ice crystals. Because of the unavoidable limitations of heat transfer this requires the fragmentation of the specimen into separate particles of small size.

2. Storage must be carried out at a low temperature without interruption. Even momentary rises to temperatures in the vicinity of −50°C or higher can be disastrous.
3. Thawing must be extremely rapid to prevent either the growth of ice crystals in the solid state or denaturation due to the electrolyte concentration produced by the freezing out of water. Here again, the limitations of thermal transfer require a very small specimen size, although the use of dielectric heating may reduce this problem to some extent.

4. A limitation in the ability of certain cells to withstand rapid temperature change is a problem of undetermined extent. The physical problem of differences in thermal expansion between various components of a cell may cause extremely rapid temperature changes to become mechanically destructive.

References (Meryman)

- 96°C for 3 min
- 85°C for 0.5 min
- 77°C for 1.5 min
- 80°C for 5.5 min
- 70°C for 0.5 min

Figure 10