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Mechanics of Freezing in Living Cells and Tissues

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Well into modern times, man's interest in biological freezing appears to have been concerned primarily with his defense against it. The vast literature concerning low temperature in biology has been predominantly directed toward the therapy of clinical and experimental cold injury without particular concern for the basic mechanisms of ice formation. Within the last 20 years, the preservation of food by freezing has stimulated much general interest in the potential usefulness of low temperature, but still surprisingly little basic work has been undertaken on the mechanisms involved. The majority of the earlier investigations into biological freezing have been made by a few scattered workers primarily interested in the preservation of tissue cells and microorganisms. The principal impetus to low-temperature biological research came with the availability of liquefied gases in the latter part of the 19th century, after which an infinite variety of tissues and organisms were subjected to freezing and thawing. Primary interest, however, invariably centered about the question of survival, and attempts to construct a picture of the mechanism of freezing and freezing injury were predominantly deductive. To the modern student of this subject, it is somewhat reassuring to find that the major part of the literature on biological freezing is fully as contradictory and confusing as that of any other growing field, indicating that its principles are neither totally self-evident nor without challenge.

In very recent years, the appearance of the biophysicist and the growing appreciation of the basic physical nature of biological phenomena has resulted in a reappraisal of much of the earlier work and the development of new evidence which has led to a considerable clarification of the mechanism of freezing in cellular materials in terms of simple and consistent physical phenomena. The extensive work of Luyet (*1*), with his particular attention to ultra rapid freezing and "vitrification," and the work of Lovelock on the mechanism of slow-freezing injury and glycerol protection (*2–4*) have done much to insert a foundation of theory under the wide-spread but empirical applications of freezing. Sufficient of the principles underlying freezing, freezing injury, and the means for its control are now available so that a reasonably well-integrated picture of the process can be hypothesized.

The existing and potential applications of freezing, both as a means of preservation and as a vehicle for the suspension and study of transient phenomena, are legion. This article is an attempt, not to accumulate in detail the many existing techniques applicable to special problems or to review the literature in its entirety, but to present an integrated hypothesis of the mechanism of biological freezing and the known means of preventing otherwise inevitable damage, with the hope of making more immediately recognizable the potential merit of the biological solid state as a unique and useful research tool.

Physical Principles of Ice-Crystal Growth

Relatively unspectacular for the biologist, perhaps, but nonetheless essential to an understanding of the rules of freezing and thawing, are the general concepts of ice-crystal nucleation and growth. It is these two phenomena that will interact to determine crystal size, a factor of obvious significance in any biological freezing. Only by understanding the factors governing ice-crystal development can we hope to influence them for our own ends. It may perhaps make the following dissertation on crystallization more palatable to the biologist if I point out that the single most important and fundamental concept in biological freezing is that, regardless of the mysterious complexity of the biological matrix, freezing represents nothing more than the removal of pure water from solution and its isolation into biologically inert foreign bodies, the ice crystals. All the biochemical, anatomical, and physiological sequelae of freezing are directly or indirectly the consequences of this single physical event.

The ultimate crystal size is immediately dependent on the crystal nucleation rate and the crystal growth rate. Both of these rates are temperature dependent, and their control is primarily a problem in heat exchange which, in turn, depends upon the thermal and geometric characteristics of the specimen, the temperature and characteristics of the coolant, and the nature of the specimen-coolant interface. All materials possess definite crystal nucleation and growth coefficients which can be changed by altering the composition of the material. In addition, once crystallization has been completed, there remains yet another factor, recrystallization, the growth of large crystals at the expense of smaller, which can radically alter the state of affairs even after the solid state has been achieved.

As will be evident from the more detailed discussion to follow, the various factors influencing crystal development are themselves somewhat controllable, rendering the peculiar problems of biological freezing in turn more amenable to discipline.

Crystal nucleation. A crystal nucleus is an aggregation of molecules that may grow to form a larger crystal. Two mechanisms for the formation of nuclei are presumed to exist (*5, 6*). The first, homogeneous nucleation, results from random fluctuations in the density and configuration of water molecules. A nucleus is said to be of critical size when

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it has an equal chance of growing or diminishing. In thermodynamic terms, this is the size at which the free energy available from the spontaneous transformation is equal to the surface free energy of the nucleus. Under these conditions, the free energy of the nucleus will diminish with either the addition or subtraction of molecules; it is in unstable equilibrium and must grow or vanish. The radius of a nucleus of critical size is directly proportional to the liquid-crystal interface energy and inversely proportional to the liquid-crystal free energy difference. This makes the size of a critical nucleus strongly temperature dependent. At temperatures near the melting point, the critical size becomes very large, approaching infinity at the melting point. As the temperature is reduced below the freezing point, the critical size becomes smaller. Generally, the probability of homogeneous nucleation is very low until rather extreme degrees of supercooling are attained, when the probability rises sharply within the range of a few degrees to very high values. This sharp rise in nucleation rate effectively limits the temperature to which supercooling can be carried. This maximum supercooling temperature, designated by Turnbull (*6*) as $(\Delta T_{-})_{max.}$, generally bears a fixed relationship to the absolute melting temperature, T_0. For water, the experimentally determined ratio of $(\Delta T_{-})_{max.}$ to T_0 is 0.14, placing the limit to which water may be supercooled at −39°C (*7*).

Whereas for most metals crystallization appears to be inevitable once $(\Delta T_{-})_{max.}$ has been attained, for some other substances the rate of nucleation apparently falls again if the temperature passes below this level before crystallization is completed (*7, 8*). The cause of this is not fully clear, although it seems probable that a rapid increase in viscosity may be responsible.

The second means for the formation of crystal nuclei is called heterogeneous nucleation, and is considered to be the result of catalysis by inclusions that presumably provide a substitute nucleus about which further crystal growth may take place. The probability for heterogeneous nucleation is quite poor near the freezing point, so that relatively small volumes (~10 milliliters) can be easily and repeatedly supercooled. The temperature to which any given sample can be repeatedly supercooled has been shown by Dorsey (*9*) to be quite reproduceable and has been called by him the temperature of spontaneous freezing (t_{sf}). These temperatures ranged, for different samples of pure water, to as low as −20°C and at no time was freezing initiated at a temperature higher than −3°C. From this evidence it appears that 0°C is not, for water, a consistent nucleation temperature. Supercooling is, then, usually necessary to initiate nucleation but, once having occurred, freezing can then proceed at the conventional freezing temperature. Since homogeneous nucleation is virtually impossible until extreme degrees of supercooling are obtained, heterogeneous nucleation is undoubtedly the dominant mechanism in all freezing of large volumes (*6*).

A reduction in the temperature of spontaneous heterogeneous nucleation (t_{sf}) with the addition of solutes has been shown by Dorsey (*9*), and also by Lusena (*10*), to be of the same magnitude as the reduction in freezing point. No attempt has been made to investigate the effect of solutes on the minimum temperature for homogeneous nucleation, $(\Delta T_{-})_{max.}$

Crystal growth. Companion to nucleation rates in determining crystal size is the velocity of crystal growth. Contrary to intuitive expectations, crystallization velocity, when considered independently from nucleation, does not increase with decreasing temperature but, as demanded by physical theory, crystal growth rates are reduced exponentially with decreasing temperature (*11*).

Thus, when freezing occurs in relatively large volumes (greater than 1 cubic centimeter) at or near the melting point, the principal and perhaps only source of nucleation will be heterogeneous from inclusions. If these are absent or if cooling rates are sufficiently rapid so that crystal growth from a few inclusions is insufficient to prevent supercooling, homogeneous nucleation becomes dominant at a lower temperature. The increase in the number of nucleation centers is further aided by the reduction at low temperatures of the crystal growth rate, encouraging the appearance of new nuclei rather than the growth of existing crystals.

A final landmark in the descending temperature scale is the glassy transformation temperature, roughly −130°C, below which ice-crystal growth cannot take place. The only well-documented demonstrations of noncrystalline ice have been obtained by the slow condensation of water vapor on a condenser surface held at liquid air temperature (*8, 12*). These experiments also demonstrated that nucleation and crystallization can occur at any temperature higher than this critical point inasmuch as glass ice, when warmed to −129°C, crystallized precipitously (*8*). The attainment of the glassy state through the supercooling of bulk water is extremely difficult if not impossible because of the ease and rapidity with which water is transformed into its low-energy crystal structure. However, once the sanctuary of the glassy transformation temperature has been reached, further change of state cannot occur regardless of the latent instability of the system.

Whereas the creation of pure glass ice is an extremely difficult procedure, it can be achieved without difficulty by the addition of certain compounds that are effective in reducing the crystallization velocity. Tammann and Buchner (*13*) determined the retardation effect of several compounds, and this investigation has been repeated and extended by Lusena (*14*). Both these authors demonstrate that relatively small amounts of certain alcohols, glycols, sugars, and proteins can exert considerable effect on the retardation of crystallization velocities, some reducing it by a factor of 10 at a concentration of approximately 5 percent and, for ethanol, by as much as a factor of 1000 at a concentration of 30 percent. The mechanism of this action is not clearly understood. It is highly unlikely that simple alteration of viscosity could account for the effect. A more plausible suggestion is that these compounds may act as impurities, included in the oriented structure of a growing crystal face by virtue of their bound and electrostatically associated water layers or as interfacial hydrates, creating an obstacle to the subsequent growth of that particular crystal face.

Crystal size. Ultimate crystal size will be inversely proportional to the population density when crystallization is complete. Crystal size is, thus, almost completely dependent on the number of nuclei formed. Since heterogeneous nucleation is temperature dependent, slight supercooling can increase nucleation from this source. If heat is removed from the specimen no faster than it can be supplied by a few growing crystals, the over-all temperature will fall to and remain at the freezing point, at which the probability of further nucleation is at a minimum. Under these conditions, a very few initial crystals can grow to completion without the formation of additional nuclei. If the removal of heat is rapid, new nuclei will be formed at a rate comparable to the degree of supercooling attained through the discrepancy between heat removal and the supply of latent heat and internal energy, which is a function of the specific heat of the material. Thus, with increasing rates of heat loss, increasing numbers of crystals are formed. When (ΔT_{-}) is reached, homogeneous nucleation is dominant. When the glassy transformation temperature is reached, crystallization ceases.

It is apparent, then, that crystal size is ultimately a function of the rate of cooling, and it is to the factors influencing this rate that we now turn our attention.

Rate of freezing. The rate of freezing is defined as the rate of advance of a freezing boundary in a linear direction through the medium. Since this rate and

the rate of cooling and nucleation are inseparably interdependent, we shall consider the rate of freezing as the controlling factor in crystal size. Experimentally, at intermediate rates of freezing, crystal size is found to be approximately inversely proportional to the rate of freezing (*15*). At very low rates approaching those obtained when single crystals are formed or at high rates producing incomplete crystallization, this relationship is obviously inapplicable.

Three physical characteristics of the specimen materially affect the rate of heat transfer and hence the rate of freezing: specific heat, thermal conductivity, and latent heat of fusion. In any nonsteady state, where temperature gradients are changing, specific heat and thermal conductivity become interdependent. As a thermal gradient flattens, internal energy is released at every point, but at a rate depending on its removal by conduction. The amount of heat to be lost depends, in turn, on the specific heat of the material. These two thermal constants (plus density, here of negligible importance) are often included in the single term, *thermal diffusivity*. An example of the significance of this relationship may be seen in the case of water and ice. While the ratio of thermal conductivities between the two is nearly 1/5, the ratio of specific heats is roughly 2/1, indicating that it will be about ten times easier to remove heat from ice as from water (neglecting convection).

The roles of thermal conductivity, specific heat, and latent heat of fusion in determining freezing rates may best be examined by considering separately the phases of a freezing material: the unfrozen interior, the frozen exterior, and the freezing boundary.

If the internal, unfrozen portion is above the freezing point, it will contain heat which must be lost prior to freezing. Since all this heat must pass across the freezing boundary, which is at a fixed temperature, the rate of heat loss from the interior will be wholly independent of coolant temperature or any factors outside the freezing boundary except as they affect boundary advance. The interior, in effect, "sees" only the freezing point as its temperature for eventual equilibration, much as water in a reservoir equilibrates at the height of the dam regardless of the drop beyond. The internal volume of a freezing material will, then, tend to approach equilibrium with the temperature of the freezing boundary. Whether or not it does so will depend on the efficiency of heat removal from the interior (thermal diffusivity) and on the rate of advance of the freezing boundary, which may or may not allow adequate time for internal equilibration.

Within the outer frozen portion of the specimen, a gradient exists between the external surface, at or near coolant temperature, and the freezing boundary at a fixed temperature. Not only must heat from the interior and the freezing boundary be transmitted across this gradient, but, as the interface advances, the distance increases, and the gradient flattens, necessitating the liberation of internal energy from the solid phase and the introduction, again, of thermal diffusivity as a factor.

From the freezing boundary itself originates latent heat of fusion which must pass to the coolant across the solid phase. It is now clear that specimen characteristics will influence the rate of freezing in the following manner: (i) the internal energy (dependent on specific heat) and the rate at which it may be removed from the unfrozen interior (thermal diffusivity), (ii) the amount and rate of production of heat from the freezing boundary (latent heat of fusion), and (iii) the efficiency of conduction of heat from these two sources through the frozen volume (conductivity) plus the removal of heat from the frozen volume itself (diffusivity).

The third major factor influencing rate of freezing is the geometry of the specimen (*15, 16*). A one-dimensional system, in which the freezing boundary advances as a plane front into a solid, shows rate of advance as a square root function of distance advanced. In the case of experimental models, using a dilute starch solution as the specimen, the diffusivity of the unfrozen starch solution is sufficiently poor so that, despite the decreasing rate of boundary advance, the unfrozen portion at no time reaches the freezing point before the arrival of the freezing boundary.

In a two-dimensional case, as exemplified by freezing inward from the periphery of a cylinder, the situation is vastly different from that of an advancing one-dimensional plane boundary. Here, as freezing progresses, the unfrozen portion is being reduced in volume, not linearly, but as the square of the radius, while the area of the freezing boundary is reducing only as a linear function of advance. In other words, as the freezing boundary advances into the cylinder, it receives from the interior and produces itself an exponentially decreasing quantity of heat to be removed to the outside. This, in effect, counterbalances the increasing distance from boundary to coolant. Experimental freezing curves show, for the cylinder, an initial rapid rate, stabilizing to a nearly linear rate of freezing until an acceleration is again observed as freezing is almost complete at the center. It is also interesting to note that, despite the poor diffusivity of the unfrozen interior, the rapid increase of surface-to-volume ratio, $2/r$, with boundary advance permits the interior of the specimen to approach temperature equilibrium with the freezing boundary prior to the arrival of the boundary. This is the cause of the familiar plateau at the freezing point obtained by thermocouple measurement from the interior of a freezing biological specimen.

In a three-dimensional case, freezing inward from the periphery of a sphere, the surface-to-volume ratio becomes $3/r$, and the shape of the freezing curve is similar to that of the cylinder but more rapid in terms of linear advance. Internal temperature equilibration at the freezing point is seen earlier and becomes complete when freezing has progressed only about half way to the center.

The same principles enumerated here for freezing apply equally to thawing. There is, however, one major practical difference: in general, the positions of the high and low diffusivity phases are reversed. Whereas in freezing, heat is released slowly from the internal, low-diffusivity material and rapidly removed through a good conductor, in thawing, heat is easily distributed throughout the internal, high-diffusivity solid but, as thawing proceeds, the heat is provided to the thawing boundary through an inincreasing layer of poorly conducting melt. This is strikingly shown in temperature records obtained from the two situations. In freezing, the boundary moves in rapidly, advancing well into the specimen before equilibration of the interior takes place, if at all. On thawing, heat is rapidly distributed through the volume of the high-diffusivity solid, which equilibrates throughout at the melting point, almost before any melting has taken place. The melting boundary then proceeds into the specimen at a much reduced rate compared with that of freezing. The significance of this difference will become apparent in later discussions of biological applications.

Recrystallization. In addition to the factors operating during the transition from liquid to solid and solid to liquid, there is one more aspect of crystallization that is of great importance in many applications of freezing to biology. This is recrystallization, the phenomenon of preferential growth in the solid state of large crystals at the expense of smaller ones. Recrystallization results primarily from surface energy differences between large and small crystals and to differences in free energy due to internal strain. Although in frozen biological media interfacial energy differences between the crystal and other phases, the intervening viscosity of solutions between crystals, and the complex influence of impurities seriously affect recrystallization rates, they are, nevertheless, still temperature dependent. The recrystallization of pure ice appears to obey the Arrhenius equation for rate processes (*17*).

At very low temperature, recrystallization is relatively slow, and equilibrium is approached while the crystals are quite small. At temperatures near the melting point, recrystallization is rapid, and crystals may grow to nearly visible size in less than an hour. The low temperatures at which significant changes in crystal size can occur are surprising. Electron microscope studies of recrystallization from a noncrystalline ice film show the development of crystals 1 micron long in 30 seconds at −70°C (*17*).

When a complex solution is frozen, each crystal will be surrounded by a layer of concentrated solute that will impede the diffusion of water molecules from one crystal to the next and reduce the rate of recrystallization. Although no figures are available for ice-crystal growth in biological solutions at very low temperatures, Luyet and Gibbs (*18*) describe the development of innumerable microscopic crystals into a single crystal filling an onion root tip cell after 2 hours' storage at −4° to −8°C.

With decreasing temperature, the rate of migratory recrystallization falls, approaching zero at the glassy transformation temperature. For pure ice, this temperature is in the vicinity of −130°C. (*8, 17*). For solutions containing several elements with differing glassy transformation temperatures, the resultant will be approximately the mean of all transformation temperatures corrected for relative concentration.

Freezing in Cellular Biological Systems

One of the most intriguing phenomena of freezing in tissue, or any viable cellular suspension, is the fact that with slow freezing, crystal nucleation is generally confined solely to the extracellular spaces (*19–21*), although Heard reports (*22*) intranuclear crystals in slowly frozen liver. Whether this tendency for extracellular crystallization results from an absence of heterogeneous nucleation sites within the cell or simply from minute differences in freezing point is unknown. It is nevertheless a fact that crystallization is wholly or predominantly extracellular until rather rapid rates of freezing are obtained and nucleation becomes general throughout the medium. It is on this phenomenon that we prefer to base the definition of rapid and slow freezing rather than on some particular, arbitrarily chosen, numerical rate. Subsequent references to slow freezing will indicate conditions under which only extracellular crystallization is obtained; the term *rapid freezing* refers to rates of cooling that are sufficiently high to cause intracellular crystal growth.

The mechanisms of injury by slow freezing and by rapid freezing are considered in detail in subsequent paragraphs. These are followed by an analysis of known means for the prevention of such injury.

Slow freezing. With the formation of an extracellular crystal nucleus and its subsequent growth, extracellular osmotic pressure is increased, and water is withdrawn from the cell and is ultimately added to the growing crystal. The increased concentration of the medium surrounding the crystal lowers the freezing point and hence the local temperature, permitting a lowering of the temperature in advance of the freezing boundary. New nuclei form here, effectively widening the freezing boundary without increasing the numerical density of crystals. The ultimate result is the development of a few large ice crystals that have incorporated all available free water and have relegated the saturated solution of electrolyte, carbohydrate, protein, and other cell constituents, with their "bound" water, to the crystal interfaces.

In most, if not all, soft-tissue cells there is no gross membrane rupture by slow freezing. Even though it is frozen for long periods of time, upon thawing, the water is reimbibed by the cells, and their immediate histological appearance is often indistinguishable from the normal (*21, 22*). More remarkable still, many cells show complete recovery provided that the exposure has not been of excessive duration (*23*). It is therefore apparent that extracellular crystal formation is not per se a uniformly lethal event. The lethal factor, a direct result of crystal formation, is the exceedingly high concentration of electrolyte resulting from the removal of water from solution (*4, 24*). Since this is a biochemical factor, it shows both time and temperature dependency as well as species differences.

Not only is there temperature dependence because of the slowing of chemical rates with reduced temperature but, between 0° and about −10°C, because of both freezing point lowering and the variable degree of binding of water, only part of the freezable water is removed from solution. Lowering the temperature increases the amount of water frozen from as little as 50 to 60 percent at the freezing point to more than 90 percent at −10°C (*24, 25*).

A wide variability exists in the response of different tissues to freezing. While epidermal and muscle cells can withstand mild freezing for an hour or more (*23, 26*), erythrocytes seem to succumb almost instantly (*2*). Lovelock (*4*) considers −5°C as the lowest temperature to which mammalian cells may be slowly frozen and still survive. In any case, the increase in concentration, as temperature is lowered to about −10°C, has far more influence on the rate of biochemical injury than the opposing effect of lowered temperature in reducing biochemical rates.

A hypothetical action spectrum of the rate of injury with decreasing temperature might be expected to appear as follows. (i) With a fall of 1 or 2 degrees below the freezing point, only part of the water has been frozen out and the denaturation from electrolyte concentration is very slow or perhaps nonexistent. (ii) With a further reduction in temperature, more water is frozen out, and the resultant concentration becomes more acute, reaching its maximum somewhere between −5° and −10°C, with a corresponding rapid increase in the rate of production of injury. (iii) If the specimen survives this far, further decrease in temperature causes no further change in the degree of dehydration but simply results in a logarithmically decreasing rate of chemical denaturation, presumably reaching a standstill at the glassy transformation temperature somewhere in the vicinity of −100°C or below.

Reversing the process results in a logarithmic increase in rate of injury with increasing temperature until the vicinity of −10°C is reached. Here it appears that there may be some hysteresis of the action spectrum (*27*). That is to say, at −4°C, for example, 10 percent of the freezable water in a given system may have remained liquid during freezing because of some sort of adsorption or binding. However, once frozen, it will not necessarily return to the liquid state upon being raised again to −4°C. Thus, the degree of dehydration and the rate of injury therefrom may be far greater at temperatures between 0 and −10°C upon warming from a lower temperature than they were during the initial freezing.

Thawing can thus affect the slowly frozen tissue in two ways. (i) Exposure to high electrolyte concentration at high temperature will produce a high rate of injury, particularly if an action spectrum hysteresis such as suggested in the previous paragraph exists to a significant degree. (ii) A prolonged exposure at higher temperatures may permit the growth of ice crystals by recrystallization prior to actual melting, although, in view of the apparent inoffensiveness of the large extracellular ice crystals to begin with, their slow redistribution is probably equally innocuous.

Identical reasoning applies to the question of storage temperature. Crystal growth through recrystallization will presumably be of little consequence because of the extracellular position of the crystals. The deleterious effects of storage can be attributed primarily to the biochemical process of dehydration denatur-

ation. This, being temperature dependent, reduces in rate exponentially with temperature decrease, presumably reaching zero rate at the glassy transformation temperature.

It should be pointed out that there is at least one situation in which the phenomenon of extracellular growth with slow freezing does not occur. Cells that have been frozen under conditions known to be lethal do not show a preferential extracellular crystallization on a second freezing, but crystallize uniformly throughout with the growth of large, destructive intracellular crystals (*21*). (This is one reason for the familiar advice on frozen foods, "do not refreeze.") Whether this is simply a reflection of loss of viability and membrane permeability or of some more subtle effect of freezing per se has not been experimentally investigated.

Rapid freezing. When the rate of freezing becomes sufficiently rapid, the tendency for preferential extracellular nucleation is subordinated and nuclei appear uniformly throughout the specimen, forming crystals that are predominantly intracellular. The rates of freezing necessary to achieve this condition are quite high, producing crystals which are small and numerous. Experimental freezing in rabbit liver showed that, when freezing was rapid enough to introduce intracellular crystals, the crystal size had been reduced to about twenty microns (*21*).

Whereas, with slow freezing, the physical presence of the extracellular crystal appears to be of minor significance, an intracellular crystal created by rapid freezing cannot be dismissed so casually. If its size exceeds that of the cell which contains it, the result is obvious. The size which it can attain within the cell without exerting lethal trauma has not been determined, but it is clear from the few records of successful rapid freezing that the rate must be extremely high and the crystal size extremely small for the result to be completely benign (*28, 29*).

In the addition to the lethal potential of intracellular crystal growth, rapid freezing also creates a dehydration with the same potential for denaturation as that responsible for injury following slow freezing. This being the case, the same concern over rates of denaturation will also influence the choice of storage temperature. It is impossible, as yet, to state with assurance whether the limitation in the choice of storage temperature will be because of crystal growth or dehydration denaturation. In all probability this will involve a substantial species factor, in which cells resistant to dehydration will succumb principally to crystal growth and vice versa. In any case, this represents one of the restrictions of the rapid-freezing technique, in which storage can be conducted satisfactorily only at very low temperatures, certainly below $-50°C$, where crystal growth and biochemical denaturation are minimized.

The rapidity with which destructive ice crystals can grow in the solid state renders the thawing procedure equally, if not more, demanding than the freezing procedure. This is particularly true in view of the observations that both denaturation and crystal growth rates increase exponentially with temperature and that, as discussed previously, the kinetics of heat exchange during thawing are far less favorable than they are during freezing. The many experiments of Luyet (*1, 28*) and others (*26, 29*) demonstrate beyond question that very rapid thawing is essential to survival after rapid freezing.

One extreme subdivision of rapid freezing which has been extensively investigated by Luyet (*1*) is the attainment of cooling rates sufficiently rapid to avoid any crystallization whatsoever. This achievement, vitrification, is extremely difficult to attain, not only requiring the ultimate in small specimen size and favorable geometry, but the addition also of compounds designed to shrink cells and bind water. Inasmuch as Luyet's ultimate criterion of noncrystallinity has been optical isotropy as demonstrated by polarized light, crystals smaller than a few tenths of a micron may not have been detected in his apparently vitrified specimens. While it may be true that some organisms can withstand rapid freezing only if crystallization is wholly prevented, it has at least been shown that total vitrification is not a mandatory prerequisite for the survival of mammalian erythrocytes. X-ray diffraction studies of rapidly frozen blood show crystallization to be present, although survival of the cells is not affected (*29*).

If true vitrification were necessary for the survival of certain specimens, the upper limit for storage would then be sharply limited by the glassy transformation temperature, below which the vitreous state is stable and above which crystallization goes precipitously to completion in the form of numerous and extremely small crystals (*8*). The requirements for the thawing of vitreous specimens without permitting crystallization would be extremely stringent.

Practical Application of Freezing to Preservation

Slow freezing. As discussed in previous paragraphs, the principal cause of injury from slow freezing is not the physical presence of extracellular ice crystals, but the denaturation incurred by the dehydration resulting from the incorporation of all free water into ice. To prevent this injury, there appear to be only two alternatives. First, if we presume that the brief exposure to initial freezing can be tolerated, the temperature may be reduced immediately after freezing to very low, stabilizing temperatures. With some cells, such as erythrocytes, this is not practical since destruction is virtually immediate once dehydration has reached a critical level. With other cells that will survive slow freezing for brief intervals, this technique appears to have been successfully applied by the storage at dry-ice temperature of many organisms and tissue specimens in which the survival of isolated individual cells is acceptable.

A second alternative is to prevent a lethal degree of concentration by reducing the amount of ice that forms. This is the basis for the use of glycerine. Most sugars and glycols are strong hydrogen bonders and are efficient binders of water. One mole of glycerine will prevent approximately 3 moles of water from freezing. The addition of glycerine or any other efficient, nontoxic binder that will pass through cell membranes (*30*) to a biological solution reduces the amount of water available to crystallize and hence limits the degree of dehydration produced. As pointed out earlier, some dehydration is compatible with survival; hence it is unnecessary to tie up all the water. The relationships between glycerine, freezing temperature, salt concentration, and survival have been completely determined for erthrocytes by Lovelock (*3*). Not all cellular materials will withstand freezing even in the presence of glycerine. In many cases it is the glycerine itself which becomes toxic in concentrations sufficient to protect against freezing.

Even when immediate denaturation is prevented by reducing dehydration with glycerine, there is still a slow decay in viability, perhaps again the result of the glycerine itself which, because of its properties as a strong hydrogen bonder, may denature protein directly (*31*). This effect is, like any other biochemical process, temperature dependent and can be retarded by lowering the temperature; it eventually becomes negligible at temperatures around $-80°C$ or below.

With the glycerine method, the rate of thawing is of little significance since the crystal size has already approached its maximum and since dehydration denaturation has been prevented with glycerine. An excellent discussion of this technique, as well as an extensive review of the literature of low-temperature biology in general, has been prepared by Smith (*32*).

Rapid freezing. There are three re-

quirements for successful preservation by the technique of rapid freezing: (i) very rapid freezing, (ii) low-temperature storage, and (iii) very rapid thawing. Most of the failure of past efforts to obtain survival after rapid freezing can be attributed to insufficient recognition of one of these requirements. It is not enough that a specimen be plunged into liquid air; it must also be of such geometrical form that uniformly rapid freezing throughout the specimen is permitted. Luyet has attained this by the use of thin films on a cover slip or supported by a wire loop (*1, 28, 33*). In our laboratory, we have found the very small sphere formed by spraying from a rapidly oscillating jet to be more amenable to quantity production in the freezing of whole blood and other suspensions (*34*). In any case, the attainment of a favorable surface-to-volume ratio is of paramount importance. The upper limit of size in the freezing of whole blood appears to be a sphere of nearly 1.0 millimeter diameter. It should be pointed out, of course, that it is never possible to distinguish between the trauma of freezing and the trauma of thawing when survival is the criterion of success. It may well be the adverse heat-exchange relationships during thawing which are the real limiting factors in specimen size.

Experience with the rapid freezing of blood has shown that even the best attainable heat-exchange relationships are often not sufficient to permit 100-percent recovery of intact cells. Fortunately, of the several compounds mentioned previously which are effective in retarding crystallization, several are nontoxic as well as freely transportable through cell membranes. Dextrose, in particular, has been found to be extremely effective in improving the recovery of rapidly frozen erythrocytes.

There appears to be considerable limitation in the choice of the coolant in which the freezing is done. Adequate heat exchange can be achieved only through the use of a liquid coolant at very low temperature, virtually limiting the choice to liquefied gases. The most popular gas, nitrogen, is unfortunately one of the least efficient since, being easily available only at its boiling point, it can acquire heat from the specimen only by vaporizing; in so doing, it forms an insulating gas layer around the specimen. Other gases, particularly propane, methane, ethane, and ethylene, are far more efficient when cooled to very low temperatures because of their relatively high boiling points. The freezing of a thin film or small droplet in propane at −195°C appears almost instantaneous to the eye. However, the diffusion of the coolant into the specimen is extensive, and large amounts of coolant gas are evolved on thawing even after prolonged exposure of the frozen specimen to high vacuum. In the case of blood, no cells survive freezing in coolants other than liquid nitrogen or liquid air.

The assumption that it is the diffusion of toxic gas into the specimen which is responsible for its destruction is not entirely conclusive since there is also evidence that there may be such a thing as too rapid freezing. The use of centrifugal force to accelerate the evolution of gas during freezing in liquid nitrogen presumably improved the rate of freezing but resulted in decreased survival of erythrocytes (*34*). There are reports in the literature of a phenomenon which has been termed "thermal shock" wherein rapid change of temperature *above freezing* results in destruction (*32*). Lovelock (*35*) proposes that this is due to differential thermal expansion between structural components of the cell. It is wholly plausible that, on rapid freezing, the rapid temperature change *prior* to freezing becomes lethal if the rate of thermal expansion exceeds the rate of relaxation permitted by the modulus of elasticity of cellular components. It is unfortunately impossible to cool slowly first, then freeze, since, in order to avoid the dominance of heterogeneous crystal nucleation from catalytic inclusions, the specimen must be rapidly supercooled to near −40°C to initiate homogeneous nucleation before other crystal growth can take place. This supercooling can be accomplished only by extremely rapid heat exchange which may, in itself, be a source of thermal shock. Lecithin is reported to alleviate thermal shock (*32*).

Storage of rapidly frozen specimens will be successful only if carried out at a temperature below that at which ice crystals can grow to lethal size or denaturation proceed at an appreciable rate. For mammalian erythrocytes, the minimum useful storage temperature appears to be roughly −60°C (*34*). Above this temperature, hemolysis develops rapidly. Luyet (*36*) has shown that the rate of hemolysis increases continuously with temperature increase and that there is no discontinuity at any fixed temperature. It is impossible to interpret this initial data as indicating either crystal growth or denaturation as the primary cause of injury. Future studies of the storage of cells known to be resistant to dehydration may shed more light on this question. Regardless of the maximum storage temperature, further reduction effects rapid improvement in results because of the direct logarithmic relationship between temperature and the rate of both crystal growth and biochemical denaturation. Under any circumstances, storage in liquid nitrogen, at −197°C can be considered as essentially indefinite.

Thawing, following rapid freezing, has been shown to be a very demanding procedure. It seems highly doubtful that there is anything analogous to thermal shock which might place an upper limit on the rate at which heat could be exchanged in this direction. In any case, the problem of getting heat into a specimen is such that excessive rates are presently far from realizable. Heat exchange by conduction from a warm liquid appears to be the only immediately practicable procedure. However, the liquid cannot be warmer than the upper limits of biological temperature, generally around 50°C. This is very disadvantageous because, when the specimen is approaching equilibration at the melting point, it is in the most lethal temperature range; more than half the total heat to be exchanged is still represented by its latent heat of fusion, and the temperature differential is only about 50°C. Where the specimen is semisolid, as a tissue, a large volume of well-agitated warming bath is easy to provide. Where a specimen such as blood cannot be diluted or even excessively agitated, the problem of maintaining the maximum possible temperature without exceeding it becomes acute.

The use of means of thawing other than simple conduction have been explored. The nonuniformity of radiant heat makes it difficult to melt all the specimen without overheating what has already melted because of the relatively poor conductive redistribution of the heat put in during the short thawing period. It is estimated that, for blood, 1 second is certainly the upper limit for the total allowable duration of thawing. Microwave heating would appear to offer an excellent solution except for the unfortunate fact that the absorption of energy by water is several thousand times that by ice at currently available frequencies. With this technique, the problem of timing to thaw a specimen in 1 second without boiling it in the ensuing millisecond would be nearly insoluble, even assuming that all portions completed thawing at exactly the same instant. The successful development of a technique other than simple heat exchange by conduction would probably extend considerably both the size and species range amenable to rapid freezing.

In many cases, particularly where more complex organisms or tissue cells are to be frozen, it is mandatory that some additive be incorporated to prevent injury to the cell by rapid freezing and thawing. These materials are not always identical to those useful for slow freezing. Whereas the glycols, particularly glycerine, have been found superior for preventing injury during slow freezing, glycerine is nearly valueless in aiding the recovery of red blood cells after rapid freezing, while glucose and other hexose sugars, urea, and sodium citrate have provided sub-

stantial benefit (*34*). Luyet, on the other hand, found glycerine greatly superior to glucose or sodium chloride in protecting chick embryo heart from damage by rapid freezing (*37*). Three possible mechanisms of protection suggest themselves: (i) a viscosity increase, retarding diffusion of water during freezing and creating smaller crystals; (ii) "binding" of water, reducing the total amount of ice formed; and (iii) reduction in crystal growth rate, permitting the nucleation of additional crystals with an ultimate smaller size (*14*). Regardless of the specific additive, it appears essential that it penetrate the cell and, of course, that it be nontoxic. Some shrinkage of the cell also seems to be useful provided that this is not in itself deleterious.

Discussion

Slow freezing, where possible, is probably the more practicable technique. Crystals are allowed to grow extracellularly and dehydration denaturation is prevented with an additive, usually glycerine. The technique is obviously restricted to those specimens in which rather high glycerine concentrations are tolerated. Any situation in which extracellular crystals are mechanically injurious is also obviously incompatible. The introduction of glycerine into a specimen is relatively simple. Since glycerine exerts little or no osmotic effect, it should be used in an isotonic saline solution. Freezing should be very slow to prevent the accidental initiation of intracellular crystals and to permit the constant readjustment of osmotic pressures as water is frozen out of solution. Although the requirements for storage are not terribly demanding—any commercial freezing unit is satisfactory—for long storage, temperatures in the dry-ice range or lower are advisable to reduce slow decay to a minimum. Subsequent removal of the glycerine by dialysis is a time-consuming and exacting procedure but generally necessary following thawing.

Rapid freezing has the major disadvantage of requiring a division of the specimen into particles of extremely small size in order to achieve rapid heat exchange. Storage must be at least at dry-ice temperature and, for some materials, possibly much lower. Whereas temporary temperature increases above this range are tolerated by specimens that are slowly frozen with glycerine, even momentary rises are destructive to the rapidly frozen specimen. The technique and the equipment are not excessively demanding. Good quick freezing can be achieved by squirting a suspension from a syringe through a very fine-gage needle onto the surface of liquid nitrogen or liquid air. The needle and syringe should be rapidly oscillated back and forth to break up the droplet pattern. The frozen droplets may be subsequently thawed by sifting them into a warm saline solution.

The use of thin films is also a satisfactory method of obtaining rapid heat exchange. This method has the drawback, however, that the specimen cannot be removed from the film support since this requires fracturing the frozen film. All structures lying in the path of the fracture will be destroyed.

The use of some additive designed to shrink the cells and, more important, to pass into the cell and exert a specific effect through the binding of free water and the reduction of crystallization velocities is a useful device for improving the results of rapid freezing. In fact, in more complex organisms and tissue cells, it appears to be essential. A great deal of work on this subject has been reported by Luyet and his associates, who have demonstrated the survival of a wide variety of viable entities following rapid freezing and thawing, usually in the presence of ethylene glycol (*38*). Many of the recent achievements of rapid and of glycerine freezing, including the rapid freezing of whole blood, have been substantially antedated by modest reports from Luyet's laboratory, which, over the last several decades, has produced a wealth of valuable and important experimental data.

Slow freezing, with glycerine protection, has attracted considerable interest in the last few years, and its practicality and usefulness in many applications have been well demonstrated (*32*). Examples of the successful application of rapid freezing to living entities more complex than bacteria are few and isolated, stemming primarily from Luyet and his associates (*33, 39*). Instances of failure to obtain survival of mammalian cells after rapid freezing are also to be found (*40*), although it is not yet clear whether this failure resulted from injury by thermal shock, as suggested by the author, or is simply a confirmation of the difficulty of meeting the stringent heat-exchange requirements.

There are indications that the use of glycerine and related compounds may significantly extend the applications of rapid freezing while requiring lower concentrations of these additives than would be necessary with slow freezing. These two techniques, glycerine and rapid freezing, should not be considered as in any way independent or competitive since, in all probability, combinations of the two will greatly extend the range of each into areas otherwise denied to both.

With the advent of increasing information regarding the mechanism of freezing and freezing injury, the usefulness of low temperatures in biology should experience considerable development. Whether through the medium of slow freezing with glycerine, rapid freezing, or combinations of the two, low-temperature biology offers exciting potentialities for biological research, not only for the purpose of indefinite preservation, but to provide a true state of suspended animation for the study of transient phenomena which can in this way be interrupted and immobilized for biological eternity in the solid state.

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