CHAPTER 40

BIOMEDICAL APPLICATIONS OF CRYOGENIC REFRIGERATION

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THE controlled exposure of biological materials to subzero (i.e., potentially freezing) states has multiple practical applications, which have been rapidly multiplying in recent times. Primary among these applications are the long-term preservation of cells and tissues, the selective surgical destruction of tissue by freezing, the preparation of aqueous specimens for electron microscopy imaging, and the study of biochemical mechanisms used by a multitude of living species to withstand the rigors of extreme environmental cold. Some of the applications are restricted to the research laboratory, but clinical and commercial environments are increasingly frequent venues for activities in low-temperature biology. The success of much of this work depends on the design and availability of an apparatus that can control temperatures and thermal histories. This apparatus can be adapted and programmed to meet the specific needs of particular applications.

This chapter briefly describes many of the principles driving the present growth and development of low-temperature biological applications. An understanding of these principles is required to optimize design of practical apparatus for executing low-temperature biological processes. Although this field is growing in both breadth and sophistication, this chapter is restricted to processes that involve temperatures below which ice formation is normally encountered (i.e., 32 °F), and to an overview of the state of the art.

PRESERVATION APPLICATIONS

Principles of Biological Preservation

Successful cryopreservation of living cells and tissues is coupled to control the thermal history during exposure to below freezing temperatures. The objective of cryopreservation is to reduce the specimen's temperature to such an extent that the rates of chemical reactions that control processes of degeneration become very small, creating a state of effective suspended animation. An Arrhenius analysis (Benson 1982) shows that temperatures must be maintained well below freezing to reduce reaction kinetics enough to store specimens injury free for an acceptable time (usually measured in years). Consequently, one of two types of processes is typically encountered: either the specimen freezes or it undergoes a transition to a glassy state (vitrification). Although both of these phenomena may lead to irreversible injury, most of the destructive consequences of cryopreservation can be avoided.

A change in chemical composition occurs with freezing as the water segregates in the solid ice phase, leaving a residual solution that is rich in electrolytes. This process occurs progressively as the solidification process proceeds through a temperature range that defines a "mushy zone" between the ice nucleation and eutectic states (Körber 1988). If this process follows a series of equilibrium states, the liquidus line on the solid/liquid phase diagram for a system of the chemical composition of the specimen defines the relationship between the system temperature and the solute concentration. The fraction of total water that is solidified increases as the temperature is reduced, according to the function defined by applying the lever rule to the phase diagram liquidus line for the initial composition of the specimen (Prince 1966). This relationship has been worked out for a simple binary model system of water and sodium chloride and has been used to calculate the thermal history of a specimen of defined geometry during cryopreservation (Diller et al. 1985). As explained later, the osmotic stress on the cells with a concurrent efflux of intracellular water results from chemical changes. The critical range of states over which this process occurs corresponds closely to the temperature extremes defined by the mushy zone. At higher temperatures there is no phase change, so osmotic stress does not exist. At lower temperatures the permeability of the cell plasma membrane is reduced significantly (as described via an Arrhenius function), and the membrane transport impedance is so high that no significant efflux can occur. Thus, the specimen's chemical history and osmotic response are coupled to its thermal history as defined by the phase diagram properties.

The property of a cell that dictates the response to freezing is the permeability of the plasma membrane to water and permeable solutes. The permeability determines the mass exchange between a cell and its environment when osmotic stress develops during cryopreservation. The magnitude of the permeability decreases exponentially with the absolute temperature. Thus, the resistance to the movement of chemical species in and out of the cell becomes much larger as the temperature is reduced during a freezing process. Since the osmotic driving force also increases as the temperature decreases, in general, the balance between the osmotic force and resistance determines the extent of weight transfer that will occur during freezing. At high subfreezing temperatures (defined generally by the mushy zone), the osmotic force dominates and extensive transport occurs. At low subfreezing temperatures, the resistance dominates and the chemical species are immobilized either inside or outside the cells. The amount of mass exchanged across the membrane is a direct function of the amount of time spent in states for which the osmotic force dominates the resistance. Thus, at slow cooling rates, the cells of a sample dehydrate extensively, and at rapid cooling rates, very little net transport occurs. The absolute magnitude of the cooling rate that defines the slow and rapid regimes for a specific cell depends on the plasma membrane permeability. A cell with high permeability requires a rapid cooling rate to prevent extreme transport. The converse holds for cells with low membrane permeability; they require prolonged high-temperature exposure to effect significant accumulated transport.

When very little transport occurs before low temperatures are reached, water becomes trapped within the cell in a subcooled state. Chemical equilibration is achieved with extracellular ice by the intracellular nucleation of ice. This phenomenon is referred to as **intracellular freezing**. In this process, a substantial degree of liquid subcooling occurs prior to nucleation, so the resulting ice

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structure is dominated by numerous very small crystals. Further, at low temperatures, the extent of subsequent recrystallization is minimal and the intracellular solid-state surface energy is high.

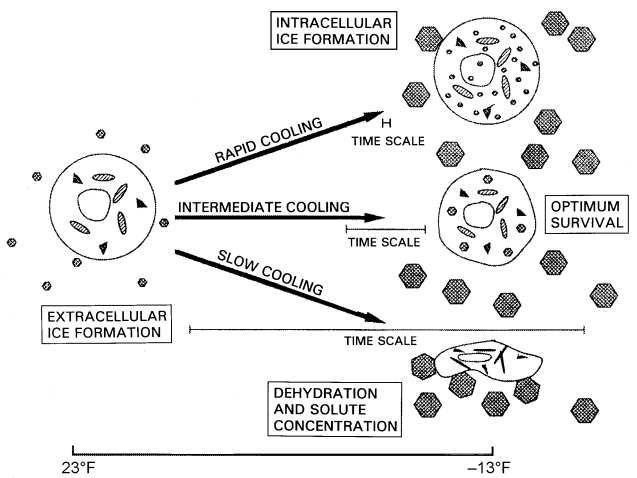
At slow cooling rates and at high subfreezing temperatures, both extensive dehydration of cells and an extended period of exposure to concentrated electrolyte solutions occur. Clear evidence shows that some combination of the dehydration and the exposure to concentrated solutes leads to irreversible injury (Mazur 1970; Meryman et al. 1977). Mazur (1977) has also demonstrated that freezing at cooling rates that are rapid enough to cause intracellular ice formation causes a second mechanism of irreversible cell injury. These processes are illustrated in Figure 1, which shows that each extreme of the cooling process during freezing produces a potential for damaging cells. Figure 1 also implies that an intermediate cooling rate should minimize the aggregate effects of these injury processes and define the conditions at which optimum recovery from cryopreservation can be achieved.

Experimental data have been obtained for the survival of a large number of cell types for freezing and thawing as a function of the cooling rate. Nearly without exception, the survival function follows an inverted V profile when plotted against cooling rate (Figure 2). This plot has been described as the survival signature of a cell; it illustrates the tradeoff between competing heat and mass transfer processes that govern the cryopreservation process. Solution concentration/osmotic effects lead to slow cooling rate injury. In this state there is adequate time for transport of water out of the cell before sufficient heat transport occurs to lower the temperature enough to drive the membrane permeability to nearly zero. Conversely, at rapid cooling rates the cell temperature is lowered so quickly that there is insufficient time for dehydration, and injury is caused by the formation of intracellular ice. The magnitude of the optimum intermediate cooling rate is a function of the magnitude of the membrane transport permeability. Higher permeabilities result in higher optimum cooling rates. Thus, the optimum thermal history for any cell type must be tailored for its unique constitutive properties.

For most cell types, the band width of cooling rates for optimum cryopreservation survival is small, and the highest achievable survival is unacceptably low. Fortunately, for practical clinical applications, the spectrum of working cooling rates can be broadened and the maximum survival increased by adding a **cryoprotective agent (CPA)** to the sample before freezing. Although a wide range of chemicals exhibit cryoprotective properties, the most commonly used include glycerol, dimethyl sulfoxide (DMSO), and polyethylene glycol. Numerous theories have been postulated to explain the action of CPAs. In simplest terms, they modify the processes of solute concentration and/or intracellular freezing (e.g., Lovelock 1954; Mazur 1970). Introducing CPAs to cell systems results in a major modification of the phase diagram for the system (Fahy 1980). In particular, the rate of electrolyte concentration with decreasing temperature may be reduced by nearly ten times, and the eutectic state depressed by as much as 108 to 144 °F. These consequences greatly extend the regime of the mushy zone during solidification (Cocks et al. 1975; Jochem and Körber 1987).

Although phase diagrams provide much information for understanding the possible states that may occur during the cryopreservation of living tissues, their interpretation is limited due to two major factors. First, the chemical complexity of living systems is far greater than the simple binary, ternary, or quaternary mixtures that are used to model their behavior. Second, and more importantly, the thermal data used to generate phase diagrams are usually obtained for near-equilibrium conditions. In contrast, most cryopreservation is executed under conditions far from the equilibrium state. For some situations, the goal is to maintain a state of disequilibrium; this domain includes vitrification methods that are applied to reach a solid glassy state that avoids ice crystal formation, latent heat effects, and solute concentration effects. In many cases, the degree of thermodynamic equilibrium reached for the low-temperature storage state may differ significantly between the intracellular and extracellular volumes (Mazur 1990). The equilibration can be controlled by manipulating the thermal boundary conditions of the cryopreservation protocol and by altering the system's chemical composition prior to initiating cooling. Many of the same chemicals used for cryoprotection may be added at higher concentrations to decrease the probability of ice crystal formation at subzero temperatures and elicit vitrification (Fahy 1988).

In addition to the thermal history of the interior of a specimen, the thermodynamic relations determining the release of the latent heat of fusion as a function of temperature in the mushy zone (Hayes et al. 1988) must be considered. A specimen of finite dimension has a distribution of thermal histories within it during the freezing process (Meryman 1966). The pattern assumed for modeling the evolution of latent heat during freezing has a large effect on the cooling rates predicted as a function of local position in a specimen. Consequently, the anticipated spatial distribution of cell survival as a con-



At slow cooling rates, dehydration is very large as water leaves the cell and freezes in an extracellular ice mass.
At rapid cooling rates, water is trapped in the cell and many small ice crystals form intracellularly.
At intermediate cooling rates, both deydration and intracellular freezing are moderated.

Fig. 1 Schematic of Response of Single Cell During Freezing as Function of Cooling Rate

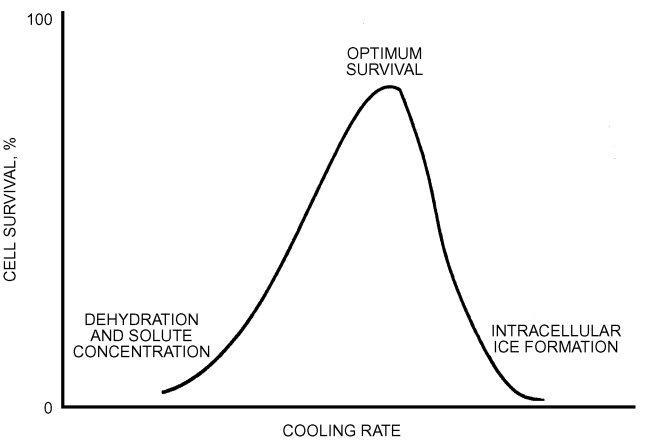


Fig. 2 Generic Survival Signature Indicating Independent Injury Mechanisms Associated with Extremes of Slow and Rapid Cooling Rates During Cell Freezing

sequence of the preservation process may depend strongly on the model chosen for the thermodynamic coupling between the system's thermal and osmotic properties. Hartman et al. (1991) applied this principle to evaluate how to choose the optimum location for a thermal sensor to record the most representative thermal history during the freezing of a specimen of finite dimensions. Hartman et al.'s analysis indicated that the geometric center of a sample is a poor selection for positioning the sensor. A position approximately one-third of the distance from the center to the periphery more accurately represents the integrated thermal history experienced by the mass during freezing.

Preservation of Biological Materials by Freezing

Biological materials are primarily cryopreserved by freezing them to deep below freezing temperatures. Among clinical and commercial tissue banks, freezing is the predominant method for preservation. Following the discovery of the cryoprotective properties of glycerol (Polge et al. 1949) and other CPAs, procedures for cryopreservation were developed for storing a variety of cells and tissues. Table 1 summarizes the types of tissue frozen by standard cryopreservation techniques as of 1993.

A typical protocol for cryopreservation consists of the following steps:

1. Place specimen in an appropriate container.
2. Add CPA by sequential increments at reduced temperatures.
3. Cool to subzero temperatures.
4. Possibly induce extracellular ice nucleation followed by a controlled period of thermal and osmotic equilibration.
5. Cool through high subfreezing temperatures with the greatest degree of thermal control invoked for the entire process, then quench to storage temperature, usually in liquid nitrogen.
6. Store for extended periods.
7. Warm relatively rapidly by immersion in a heated water bath.
8. Serially dilute to remove CPA using nonpenetrating solutes to control the intracellular/extracellular osmotic balance.
9. Harvest the specimen for its intended application.

Table 1 Spectrum of Various Types of Living Cells and Tissues Commonly Stored by Freezing (as of 1993)

Tissue	Comments	References
Blood vessels	DMSO used for CPA; cooling rate < 2 °F/min.	Gottlob et al. (1982)
Bone marrow stem cells	DMSO is usual CPA; widely used in cancer therapy.	McGann et al. (1981)
Cornea	DMSO is usual CPA.	Armitage (1991)
Erythrocytes	Usual CPA is glycerol; high concentrations for slow cooling and low concentrations for rapid cooling; wide spread clinical use.	Turner (1970), Valeri (1976), AABB (1985), Huggins (1985)
Embryos		
Mouse	Many species of mammalian embryos have been cryopreserved successfully. The most common CPAs for these applications are glycerol and DMSO. 1,2-Propanediol is used for humans. Variations in the required thermal protocol and processing steps exist among the different species. Ice crystal nucleation is usually controlled by seeding.	CIBA (1977), Zeilmaker (1981)
Rat		Whittingham et al. (1972), Wilmut (1972)
Goat		Whittingham (1975)
Sheep		Bilton and Moore (1976)
Rabbit		Willadsen et al. (1976)
Bovine		Bank and Maurer (1974)
Drosophila		Wilmut and Rowson (1973)
Human		Mazur et al. (1992), Troundson and Mohr (1983)
Heart valves	DMSO is usual CPA; cooling rate of 2 to 3.5 °F/min.	Angell et al. (1987)
Hepatocytes	DMSO is usual CPA; cooling rate of 4.5 °F/min.	Fuller and Woods (1987)
Islets	DMSO used for CPA; cooling rate < 2 °F/min.	Rajotte et al. (1983), Taylor and Benton (1987)
Lymphocytes	DMSO is usual CPA; primary application is in clinical testing.	Knight (1980), Scheiwe et al. (1981)
Microorganisms	DMSO and glycerol used for CPAs.	James (1987)
Oocytes		
Hamster	Many species of mammalian oocytes have been cryopreserved successfully. The most common CPAs are glycerol and DMSO. Variations in the required thermal protocol and processing steps exist among the different species. Clinical applications in humans have been difficult to achieve.	Bernard (1991)
Mouse		Critser et al. (1986)
Primate		Whittingham (1977)
Rabbit		DeMayo et al. (1985)
Rat		Diedrick et al. (1986)
Human		Kasai et al. (1979), Van Uem et al. (1987)
Parathyroid	DMSO used for CPA; cooling rate of 2 °F/min.	Wells et al. (1977)
Periosteum	DMSO used for CPA; cooling rate of 2 °F/min.	Kreder et al. (1993)
Plants	Selected plants are cold hardy; some germplasm is cryopreserved.	Grout (1987), Withers (1987)
Platelets	Best success is with DMSO as CPA; high sensitivity to freezing and osmotic injury.	Schiffer et al. (1985), Sputtek and Körber (1991)
Skin	Both glycerol and DMSO used as CPA.	Aggarwal et al. (1985)
Sperm		
Animal	First mammalian cells frozen successfully. Broad applications for animals and humans using glycerol as CPA.	Polge (1980)
Human		Sherman (1973)

Details vary among individual tissue types; the references in Table 1 give sources of specific parameter values for individual tissues. The refrigeration requirements may vary considerably among different tissues, but basic principles and processes of the cryopreservation processes are generally consistent. The Bibliography and the references in Table 1 identify appropriate introductory references.

Most of the initial applications of cryobiology were in clinical, research, and nonprofit (e.g., the Red Cross) venues. More recently, however, the commercial sector has adopted cryopreservation methods (McNally and McCaa 1988). As the arsenal of practical cryopreservation methods has grown, the profit potential of freezing tissues for prolonged storage is being recognized and exploited. Thus, an added set of incentives and motives is driving the development of techniques that make use of challenging refrigeration schemes.

Preservation of Biological Materials by Freeze Drying

Freeze drying extends long-term storage at ambient temperatures without the threat of product deterioration. This process removes water from the specimen while it is frozen by sublimation. As a result, no thawing process occurs during rewarming. Thus, none of the decay processes associated with the presence of water in the liquid state are active. Freeze drying has been applied widely in the food processing industry where the product need not be rehydrated in the living state. Other applications, such as taxidermy, also avoid this stringent requirement. The list of biological materials frequently processed by freeze drying is extensive and encompasses various microorganisms, protein solutions, pharmaceuticals, and bone. Rowe (1970) reviewed the early state of the art of the physical and engineering aspects of freeze drying; more recently, Franks (1990) has reviewed the physical and chemical principles that govern the freeze-drying process from the perspective of achieving an optimal process design.

Figure 3 summarizes the processing steps for freeze drying (Franks 1990). In the figure, note the alternative process pathways from the native to the stored state. The path can be controlled by equipment design and operator intervention. The material initially is in the native state from which cooling is initiated. As subfreezing temperatures are reached, either the material remains subcooled in the liquid state or ice crystals form (either by spontaneous nucleation or by active seeding a substrate on which a solid phase may form). The material will vitrify if sufficiently subcooled. Ice crystals will grow in a nucleated material, with the rate of temperature change determining the structure and size distribution. Simultaneously, the solute becomes concentrated until the eutectic state is reached. At this state an additional solid phase may form, or the liquid solution may become supersaturated as the temperature is reduced further.

The material is dried, either in the crystalline or vitreous state, by drawing a vacuum on the system at low temperature. Finally, the dried material may be stored at ambient temperatures, although the material is often stored at high subfreezing temperatures to minimize the probability of product deterioration by the activity of residual water. Production methods have been developed mainly by empirical experience and art. However, Franks (1990) argues for the need of a stronger scientific base to increase the process productivity and quality.

During freeze drying, many phase transitions either never occur or are precipitated at states far from equilibrium, and the slow kinetics of subsequent diffusion processes at low temperatures limit the system from moving toward equilibrium. Even if water crystallizes, the residual solutes are likely never to crystallize fully, if at all. As a supersaturated solution is cooled, the viscosity becomes so large that crystallization processes become undetectable. The so-called glass transition temperature is defined by the intersection of the liquidus curve on the phase diagram and an isoviscosity curve for which the mechanical properties of the material are glasslike. These states are illustrated on a state diagram, as shown in Figure 4. By definition, the state diagram does not represent a locus of the system's equilibrium states, but it provides a map of the temperature and composition combinations of defined kinetic properties (Franks 1985).

The intersection of the liquidus curve, when extended past the eutectic temperature t_e into the supersaturated region, and the glass transition curve defines a specific glass transition temperature t_g. This property depends on the combination of the solute concentration and composition. At states above the glass transition threshold, the material behaves as a viscoelastic medium, which is unacceptable for long-term storage. An important aspect of the state diagram

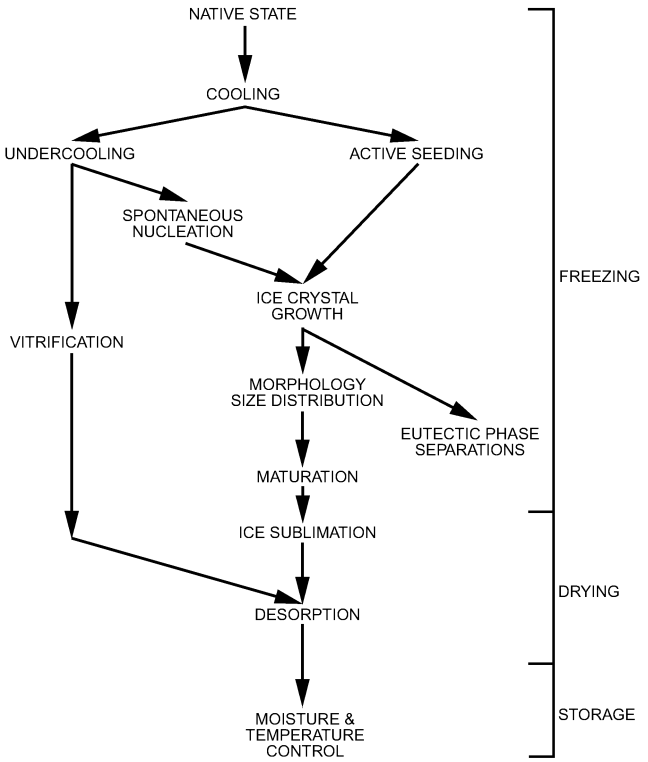


Fig. 3 Key Steps in Freeze-Drying Process

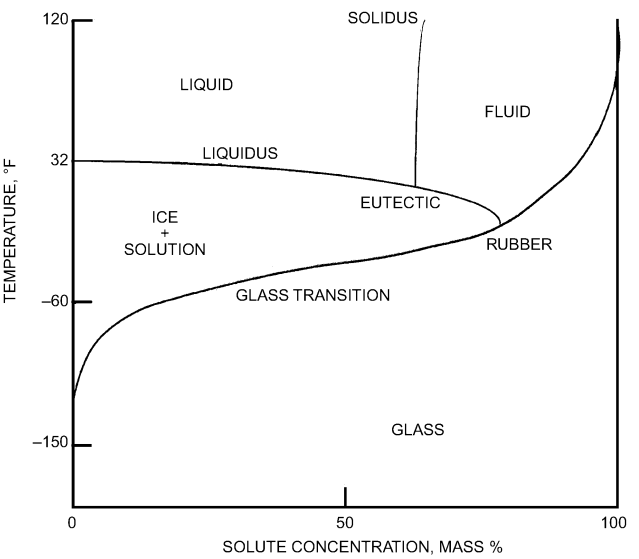


Fig. 4 Generic State Diagram for Aqueous Solution

is that the slope of the glass transition curve is very steep at high concentrations of solute. Consequently, the glass transition temperature t_g is well above 32 °F for a pure solute, thus providing for stable storage conditions. However, since small amounts of residual water in the system can significantly lower the glass transition temperature, it is important to check and control the moisture content of a freeze-dried product. To this end, Levine and Slade (1988) provided extensive data on the glass transition temperature and unfreezable water fraction of many molecular solutions of interest in the design of freeze-drying processes. It is most important that the water is removed from the material at a state temperature lower than the glass transition value at the local solute concentration value. If salts do not precipitate into a solid phase, then unfrozen water remains, which can affect the freeze-drying kinetics (Murase et al. 1991).

Preservation of Biological Materials by Vitrification

The vitrified state plays an integral, albeit partial and secondary, role in the preservation of tissues by freezing and by freeze drying. Vitrification may also be used as a storage technique in its own right. Because solidification is avoided, problems associated with the freezing concentration of solutes are avoided. In addition, there are no complications due to latent heat removal from the specimen at a moving phase front. However, water cannot achieve a vitreous state simply by cooling a solution of physiological composition. Therefore, vitrification is achieved only with the prior addition of high concentrations of solutes (i.e., CPAs) to alter the kinetics of the crystallization process and the locus of the liquidus curve and the glass transition curve on the state diagram. Although the CPA concentration that must be achieved before cooling is higher for vitrification than for freezing, the vitrification process produces no subsequent solution concentration phenomenon as does freezing. Therefore, if the higher initial CPA concentrations can be tolerated without injury above 32 °F where the addition occurs, vitrification may present a distinct benefit as an approach to long-term cryopreservation.

Fahy (1988) presents an extensive summary of various constitutive properties of candidate CPAs for vitrification, as well as empirical data for the crystallization properties of solutes in aqueous solutions. Another important source of data for the design of vitrification processes is Boutron's research on thermal and glass-forming properties of solutions particularly relevant to cryopreservation. For example, Boutron (1993) deals with the glass forming tendency and stability of the amorphous (glassy) state of 2,3-butanediol in physiological solutions of varying chemical complexity.

In addition to modifying a system to be cryopreserved by adding a CPA, Fahy et al. (1984) have explored modifying the state behavior of tissues by cooling under high pressures. Pressures of up to 15,000 psi were used during cooling to reduce the melting temperature of water to about 16 °F and the homogeneous nucleation temperature to –65 °F, which is equivalent to the reduction in phase change state achieved by introducing a 3 molar concentration of a common CPA. However, limiting factors associated with thermodynamic properties and design of apparatus must be solved before this technique can be considered for practical applications.

The growth of submicroscopic (light) ice crystals, primarily during warming, has been hypothesized to be injurious to vitrified cells and tissues. Several approaches have been pursued to control this process. Rapid warming through the region of sensitive temperatures where crystal nucleation and growth are most probable is used to reduce the time of exposure to these processes (e.g., Marsland 1987). Problems with this technique have included ensuring a homogeneous temperature throughout the tissue and matching the hardware to the impedance properties of the specimen, especially for large organs composed of heterogeneous tissues. Alternatively, Rubinsky et al. (1992) have used biological antifreezes from polar fishes (which adsorb to specific faces of ice crystals to inhibit crystal growth) as a CPA constituent to reduce the susceptibility of mammalian tissues to injury. Accordingly, antifreeze glycopeptides have been added to the vitrifying solution to increase the post-thaw viability of vitrified porcine oocytes and embryos.

Vitrification of tissues and freezing cryopreservation have been most successful with physically small specimens (e.g., suspensions of isolated cells and small multicellular tissues). One of the major anticipated advantages for vitrification techniques is in processing whole organs for cryopreservation. To date this potential has not been realized, due in part to difficulty in solving engineering problems associated with the processing. The specimen must cool rapidly throughout to prevent significant numbers of ice crystals from forming in any portion of the tissue volume, which could then later propagate into other areas. Unfortunately, boundary conditions and heat transfer characteristics of relatively large organs do not allow such rapid cooling. The threshold cooling rates can be altered as a function of the tissue's chemical composition by adding a CPA: the most promising approach to resolving this limitation is likely to be chemical rather than thermal. Nonetheless, more effective control of the thermal boundary conditions could be beneficial.

The cooling process also produces a second problem that is in direct conflict with satisfying the threshold cooling rate requirement. As progressively larger temperature gradients are created within the specimen to boost the cooling rate, corresponding internal thermal stresses are generated. In the glassy state the elastic strength of the vitrified tissue can easily be exceeded, causing mechanical fracture of the tissue (Fahy et al. 1990). This phenomenon is obviously irreversible and totally unacceptable. Thus, cooling must be designed to reduce the temperature fast enough to avoid ice nucleation but slow enough to avoid mechanical fracture. Fortunately, some possible solutions to this quandary have been tested (e.g., annealing stages at appropriate thermal states) and hold promise for vitrification of large organs.

Preservation of Biological Materials by Undercooling

One option for cryopreservation in the undercooled state has found a limited range of applications. This technique avoids heterogeneous nucleation of ice crystals in subcooled water and maintains the storage temperature above the value at which homogeneous nucleation occurs (Franks 1988).

The undercooling method of storage is based on the fact that aqueous solutions can be cooled to temperatures substantially below the equilibrium phase change state without the nucleation of ice crystals. The temperature of spontaneous homogeneous nucleation for pure water is approximately –40°F. Thus, if externally induced heterogeneous nucleation can be blocked, a substantial window of subzero temperatures can be used for storage of biological materials. This approach avoids the injurious effects of ice formation and the freeze concentration of solutes as well as the need to add and remove chemical CPAs from the specimen, although the temperature range available is not low enough to ensure long-term storage without product deterioration. Because the physical basis of the undercooling process is much different from the alternative methods described previously, the strategy for developing effective storage is also substantially different.

The key to undercooled storage is the ability to control (prohibit) the nucleation of ice in the specimen. Although the homogeneous nucleation temperature is about 72 °F below the equilibrium freezing state, in practice it is difficult to reach even –4 °F due to heterogeneous nucleation by particulate matter in the specimen. Further, the presence of just a single ice crystal nucleus is adequate to feed the growth of ice throughout a large volume of aqueous medium. However, because heterogeneous nucleation occurs in the extracellular subvolume of a cell suspension, Franks et al. (1983) suspended the biological material in a medium of innocuous oil formed into microdroplets, thereby dispersing the bulk aqueous suspending solution. In effect, the material, such as cells, was suspended in a very thin film of aqueous solution, which dramatically

depresses the ice nucleating tendency of the extracellular matrix. By this method, living cells may be undercooled to nearly the homogeneous nucleation temperature (Franks et al. 1983). Subsequently, many different types of cells have been undercooled in water-in-oil dispersions to –4 °F or lower without injury (Mathias et al. 1985).

A similar approach has been developed for storing biochemicals. For example, an aqueous protein solution can be dispersed in an oil carrier formulated to form a gel, thereby trapping the biological material in very small isolated droplets in the inert matrix. Each of the microdroplets is unable to communicate with any neighboring droplets, thus preventing local ice nuclei from providing a substrate for ice growth in the material. Challenges of this process involve creating microdroplet dispersions for effective storage that recover when returned to ambient temperatures. The temperature must be precisely controlled to avoid both homogeneous nucleation by becoming too cold and accelerated product deterioration by becoming too warm. Typical storage temperatures are around –4 °F.

RESEARCH APPLICATIONS

Electron Microscopy Specimen Preparation

Freezing has been adopted widely as a method of preparing specimens for examination by electron microscopy. The advantages of freezing are that it need not involve chemical modification of the specimen in the active liquid state and that the physical substructure of components may be preserved. Conversely, the cooling process may cause ice crystals to form, which would alter or mask the structure to be imaged and which could concentrate the solute locally and cause internal osmotic flows that would produce image artifacts. Thus, control of the thermal history during cooling is critical in obtaining a high-quality preparation for viewing on the microscope. Cooling rates of 2×10^5 to 2×10^6 °F/s or higher are desirable to minimize osmotic dehydration of cells and to avoid ice crystal nucleation and growth. Cooling removes heat from the surface of the specimen, and in most cases, the highest cooling rates occur at the boundary of the specimen. Thus the quality of preparation may vary significantly as a function of position, so the specimen should be mounted so that the dimension normal to the primary direction of heat transfer is as small as possible. Echlin (1992) presented a comprehensive summary of the cryoprocessing of materials for electron microscopy.

Bald (1987) analyzed factors that govern the cooling process during the cryopreparation of specimens. In each case, the objective is to cool the specimen as rapidly as possible. Three different approaches have been developed for cryofixation; these are classified as slamming, plunging, and spraying. Cooling by **slamming** is effected by mechanically driving the specimen and its mounting holder onto the surface of a cryogenically refrigerated solid block, which has a large thermal inertia in comparison with the specimen. The impact velocity of the specimen against the cold block is high to achieve as rapid a change in the thermal boundary conditions as possible. The drive mechanism is spring loaded to maintain continuous contact with the block following impact so that thermal resistance to the specimen is minimized.

The **plunging** technique uses a liquid rather than a solid refrigeration sink. As in slamming, the specimen is driven into a relatively large volume of cryogenic liquid. In common practice, the liquid is prepared in a subcooled or supercritical state so that heat transport from the specimen is not limited by a boiling boundary layer at the interface (Bald 1984). It is also important to eliminate a stratified layer of chilled vapor above the liquid through which the specimen would pass during plunging. Such a vapor layer would cool the specimen somewhat before contact with the liquid cryogen in the vapor medium, but because it has a relatively low convective coefficient the effective cooling rate is substantially reduced.

For the **spraying** method of cryofixation the specimen is held in a stationary mount, and a jet of liquid cryogen is directed onto the specimen. Heat is removed by a combination of evaporation and convection of the cryogen.

Analysis by Bald (1987) indicated that slamming is potentially the most effective method of rapid cooling for cryofixation. The velocity of the specimen during plunging must be 66 fps or greater to reach thermal performance levels characteristic of slamming. In general, it is easier to design apparatus to achieve the velocities required for satisfactory performance by spraying than plunging. Further, high plunge velocities are more likely to damage the specimen than are equivalent spray velocities. The most effective cryogen for both plunging and spraying is subcritical ethane.

After the temperature is reduced, further preparation for viewing on the electron microscope may involve mechanical fracture of the specimen, chemical substitution of one constituent such as water (Hunt 1984), or removal of a chemical constituent such as by vacuum sublimation of water (Linner and Livesey 1988; Livesey and Linner 1988; Echlin 1992). Sectioning and fracturing techniques are used to expose internal structure and constituents of a specimen. This approach to preparation is particularly appropriate at cryogenic temperatures, because biological materials become quite brittle and very little plastic deformation occurs that would alter the morphology. The exposed internal surfaces may be either imaged directly or modified by mechanical or chemical means.

Cryomicroscopy

Initial investigations that made use of cryomicroscopy were conducted in the early 1800s and have been pursued ever since. From its earliest adoption, cryomicroscopy made it possible to obtain useful information about the behavior of living tissues at subfreezing temperatures, but application has been limited primarily by the difficulty in controlling the refrigeration applied to the specimen.

Diller and Cravalho (1970) designed a cryomicroscope in which independently controlled refrigerating and heating sources controlled the specimen temperature and its time rate of change during both cooling and heating. Heating was produced by applying a variable voltage across a transparent, electrically resistive thick film coating deposited on the underside of a glass plate on which the biological specimen was mounted. The local temperature was monitored via a microthermocouple positioned in direct contact with the specimen, and this signal was applied as the input to the electronic control system. By miniaturizing the thermal masses of all components of the system, much higher rates of temperature change were achieved with this system than were previously possible (cooling rates approaching 2×10^5 °F/min). This system was cooled by circulating a chilled refrigerant fluid through a closed chamber directly beneath the plate on which the specimen was mounted.

Subsequently, the design was modified by McGrath et al. (1975) to eliminate the flow of refrigerant fluid from passing through the optical path of the microscope. Rather, heat was conducted away from the specimen via a thin radial plate that was chilled on its periphery by a refrigerant. This design is mechanically more satisfactory and offers a thinner working cross-section through the optical path, but the lateral temperature gradients are much higher. These two designs are known as **convection** and **conduction cryomicroscopes**, respectively (Diller 1988). The former has been adapted to allow for simultaneous alteration of the specimen's chemical and thermal environments (Walcerz and Diller 1991), and the latter has been commercially marketed with a computer control system (McGrath 1987).

These cryomicroscopes modulate the temperature where the specimen is mounted on the microscope to create the desired thermal history for an experimental trial. The dimensions of the specimen are limited by the field of view of the microscope optics, because the specimen is stationary during a trial. An alternative approach has been adapted to study the control of a different set of variables. In this

system a steady state temperature gradient is established across the viewing area of the microscope, and the specimen is moved in time through the gradient to produce the desired temperature history (Rubinsky and Ikeda 1985; Körber 1988). Advantages of this system are that specimens of macroscopic size may be frozen, as it has been adapted to controlled thermal preparation of specimens for electron microscopy (Bischoff et al. 1990); and the cooling rate applied to a specimen can be investigated as defined by the product of the spatial temperature gradient and the velocity of advance of the phase interface (Beckmann et al. 1990). A similar gradient stage was built by Koroush and Diller (1983) for analysis of solidification processes. This system included feedback control of the temperatures at the ends of the gradient to view a stationary specimen. Gradient cryomicroscopes have little application for cryobiology because of the limited range of cooling rates that can be achieved.

Cryomicrotome

The refrigerated microtome maintains tissue specimens at a subfreezing temperature in a mechanically rigid state so that very thin sections may be cut in preparation for viewing by electron microscopy. The degree of rigidity required is a function of the thickness of the specimen to be cut; thinner sections require greater rigidity, which is achieved by lower temperatures. Stumpf and Roth (1965) have determined that temperatures above –22 °F are adequate to obtain sections 1 μm thick, and temperatures below –94 °F facilitate the cutting of sections thinner than 1 μm. Thus, the apparatus must produce both a wide range of temperatures and accurate thermal control during the processing. The apparatus must also be designed to exclude environmental moisture that could contaminate the specimen, and to isolate the refrigeration apparatus from the sectioning chamber to minimize mechanical vibrations that could compromise the dimensional integrity of the delicate cutting process.

CLINICAL APPLICATIONS

Hypothermia

Although accidental hypothermia is the most widely encountered clinical condition of lowered body core temperature, induced hypothermia has been developed as a method of reducing the metabolic rate of selected organs, such as the heart and brain, during surgical procedures. This procedure is of particular benefit in neonatal patients whose blood vessels and surgical field are too small to effectively apply standard cardiac bypass procedures for maintaining peripheral circulation during surgery. If the temperature can be reduced to a suitably low level (54 to 68 °F), then it is possible to stop the heart and to pursue surgical procedures (in the absence of blood perfusion) without incurring irreversible injury. The period for which the body can be subjected to the absence of perfused oxygenated blood is a function of the hypothermic temperature and may last as long as an hour. These procedures require (1) the temperature of the organ to be within tolerances that limit tissue damage, and (2) the ability to lower and raise the temperature quickly to provide the maximum fraction of the low-temperature period for the surgical procedure. For example, Eberhart addressed the challenge of achieving a suitably rapid rate of cooling for the brain by perfusion through the vascular network with a chilled solution (Olson et al. 1985).

The most effective approach to cooling an internal organ is to circulate the blood through a heat exchanger outside the body. The blood is then perfused through the vascular system of the organ, which acts as a physiological heat exchanger. Weinbaum and Jiji (1989) demonstrated the efficacy of thermal equilibration between various components of the vascular tree and the local embedding tissue. Earlier procedures relied primarily on surface cooling to chill internal organs, which is significantly less effective than perfusion in most applications. The results of Olson et al. (1985) indicate that the brain can be very rapidly cooled to a hypothermic state by an infusion of cold arterial blood. However, when blood circulation was stopped for cardiac surgical procedures, a gradual but significant rewarming of the brain occurred due to parasitic heat flow from surrounding structures that had not been cooled. Thus, a combination of cold perfusion through the vascular system and surface cooling seems to provide the best control of the body core temperature during hypothermic surgical procedures.

Cryosurgery

In contrast with the previous applications in which the objective is to maximize the survival of tissues exposed to freezing and thawing, cryosurgery has the goal of selective total destruction of a targeted area of tissue within the body. Cryosurgery is applied to destroy and/or excise tissue that is either dead or diseased. It is usually one of several treatment alternatives and has risen and fallen in favor as a method of treating various types of lesions. In general, it has been most effective in treating lesions for which there is direct or easy external access to allow mechanical placement of a cryoprobe or the spray of a cryogenic fluid. The most commonly accepted uses of cryosurgery include the treatment of skin, mucosal, and gynecological lesions; liver cancer; and in cardiac surgery for treatment of tachyarrhythmias (Gage 1992). Other uses that have demonstrated efficacy but not such broad adoption are the treatment of hemorrhoids; oral, prostate, and anorectal cancer; bone tumors; vertigo; retinal detachment; and visceral tumors (Gage 1992).

Primary advantages of cryosurgery are that (1) it provides a bloodless approach to surgery, (2) in some applications it reduces the rate of death, and (3) the extent of destruction inside the affected area can be imaged with noninvasive methods (Gilbert et al. 1985). This latter process makes use of a continuous ultrasonic scan of the freezing zone to monitor the interface between the solid and liquid phases as it grows into the targeted tissue. Experimental evidence indicates that a close correlation exists between the extent of phase interface propagation and the boundary of the zone of tissue destruction (Rubinsky et al. 1990), and these results may be explained in large part by a model for the mechanism of destruction of the freezing process (Rubinsky and Pegg 1988). The model asserts that during freezing of tissue, ice forms preferentially in the vascular network. The ice also propagates through the vessels as the solidification front advances. The cells near the vascular network dehydrate due to osmotic stress, and this water then freezes in the vascular lumina. As a result, vessels may expand by as much as a factor of two (for electron micrographs, see Rubinsky et al. 1990) causing irreversible injury. Thus, the primary action of freezing in destroying tissue during cryosurgery may be by rendering the vascular system nonfunctional rather than by causing direct cryoinjury. Without an active microcirculatory blood flow, the thawed tissue will die rapidly.

Tissue is best destroyed by using a true cryogenic fluid, which is most commonly liquid nitrogen at –321 °F. The size of the probe and the flow rate of cryogen through it determine the volume of tissue that may be frozen. For example, a 0.4 in. diameter probe will produce in tissue an ice ball with a diameter as large as 1 in. (Dilley et al. 1993). Frequently, tumors exceed the capacity of a single probe, but at present, commercial multiprobe cryosurgery systems do not exist. As a result, multiple systems are used, which are hardware-intensive and compromise control over the freezing process (Onik and Rubinsky 1988). Thus, opportunities exist to improve cryosurgical apparatus.

Recent innovations have included operating the refrigerant system under vacuum, thereby creating liquid phase heat transfer with the active heat transfer surface of the probe, which has a considerably lower thermal resistance than a boiling interface (Baust et al. 1992). This approach to enhancing thermal performance is similar to that used to cool specimens for electron microscopy rapidly (Bald 1987).

Other problems in the design of cryosurgical equipment remain to be solved. For example, parasitic heat leakage along the probe stem to the cold tip extends the active surface capable of causing tissue damage away from the area designed for destruction. This leakage is particularly compromising to the surgical procedure for treating malignant diseases in locations other than on the body surface (Onik and Rubinsky 1988). The simple and convenient interchangeability of probe tips with various geometries and thermal capacities would enhance the flexibility of cryosurgical apparatus. Further, the increasing incidence of sexually transmitted diseases dictates the need for a cryosurgical probe that may either be effectively sterilized (Evans 1992) or be disposable (Baust 1993).

REFRIGERATION HARDWARE FOR CRYOBIOLOGICAL APPLICATIONS

In general, two classes of refrigeration sources have been adapted successfully to biological applications: vapor compression cycle cooling and boiling of liquid cryogens. Also, two types of thermal performance standards may be required of these refrigeration sources. As indicated in the previous sections, the thermal history during cooling is very often a critical factor in determining the success of a cryobiological procedure. The refrigerating apparatus must achieve a critical cooling rate within a specimen and regulate the cooling rate within specified tolerances over a designated range of temperatures. If the refrigeration apparatus is designed for general applications, these criteria will be demanded for a large variety of procedures.

A second important performance standard is the minimum specimen temperature that can be maintained in the system. Many biological applications depend on continuously holding the specimen at a temperature below a value at which significant process kinetics may occur. Of most importance are (1) control of the nucleation of ice or other solid phases in vitrified materials, and (2) limitation of recrystallization of small ice crystals that form during cooling. Many cryopreservation procedures require that the specimen be warmed from the stored state as rapidly as possible to avoid the above phenomena for which the kinetics are most favorable at higher subfreezing temperatures. For long-term storage of biological materials, temperatures below –184 °F are generally considered to be safe from the effects of devitrification and crystal growth. This state pushes the limits of refrigeration that can be produced by mechanical means, however.

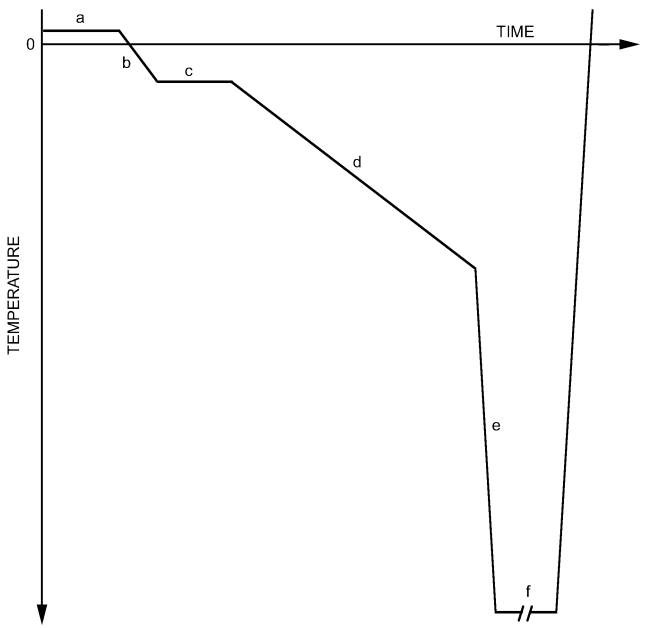


Fig. 5 Generic Thermal History for Example Cryopreservation Procedure

An example of a generic cooling, storage, and warming protocol for cryopreservation is shown in Figure 5. The protocol is divided into seven steps. The first (a) consists of adding a cryoprotective agent at a temperature slightly above freezing. This operation is usually executed with the specimen held in a constant-temperature circulating bath. The mixing and osmotic equilibration process may occur in several serial steps and last for half an hour or longer. The specimen is then immersed into a second constant-temperature bath held at a high subfreezing temperature (such as 14 °F). The cooling rate during this process (b) is uncontrolled, being governed by the inherent heat transfer characteristics of the container and the refrigerant fluid. This constant-temperature holding period (c) enables nucleation of ice in the specimen at a predetermined thermodynamic state and provides time for release of the latent heat of fusion and for osmotic equilibration between the intracellular and extracellular volumes. Subsequently, the specimen is placed into a controlled-rate refrigerator (d) and during which the temperature is reduced at a rate that maintains a balance between an acceptable osmotic state of the cells and avoids intracellular ice formation. As discussed earlier, the absolute magnitude of this cooling rate depends on the properties of the subject cell, and it may vary over several orders of magnitude for different specimen types. When the specimen reaches a temperature where kinetic rate processes approach zero (e.g., –112 °F), the specimen may be plunged (e) into a liquid nitrogen bath for long-term storage (f). Finally, the specimen is warmed and thawed by removing it from the refrigerator and immersing it directly in a water bath (g).

In practice, many variations exist on the cryopreservation scheme shown in Figure 5. One of the most frequent simplifications is to eliminate one or more of the steps (b through d). Whether such a simplification is acceptable depends on the sensitivity of the specimen to variations in the thermal history. This sensitivity is a function of the properties of the cells, the physical geometry of the specimen and its packaging for cryopreservation, and chemical modifications performed during step (a).

As the scientific basis for understanding and designing optimal protocols for processes in cryobiology has been strengthened, the specificity and sophistication of the associated refrigeration apparatus has likewise progressed. Therefore, considerable opportunity for improvements in cryobiology hardware remains. The 1980s witnessed the founding of many new commercial ventures with the objective of exploiting this potential. A common theme was an effective link to the scientific and/or medical community to ensure that equipment was designed to address the needs of the customers.

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Primary literature: The main English-language sources for general literature on cryobiology are found in two archival journals: *Cryobiology* and *CryoLetters*. In addition, the *Bulletin of the International Institute of Refrigeration* provides a timely listing of the world literature in low-temNSperature biology. Other references are distributed among a large number of journals that are either more general or are oriented toward specific physiological or applications areas.

Monographs: Numerous monographs have been written on the principles and applications of low-temperature biology. In general, these have been edited works in which contributing authors provide a series of expositions in focused areas of expertise. Over the last forty years, they have appeared rather consistently. A list of selected monographs follows:

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