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Biophysical and Biological Factors Determining the Ability to Achieve Long-Term Cryobiological Preservation[1]

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Abstract

The BESTCapsule will maintain appropriate biological specimens for decades or centuries at cryogenic temperatures in the living state. Maintenance at temperatures below ~ -140° C is not a problem. No ordinary chemical reactions in aqueous solutions can occur . The only source of damage will be the slow accumulation of physical damage to DNA from background ionizing radiation. But this source of damage should not become serious in less than a millennium. Rather, the main problem in cryopreservation is to devise procedures for cooling the biological specimens to -196° C and returning them to normal temperatures without inflicting lethal injury.

Regardless of the cell type, there are certain encompassing biophysical factors and constraints that determine whether they will survive or die during freezing and thawing. Superimposed on these may be special biological factors that apply to specific cell types. This paper will emphasize the former and give illustrative examples of the latter.

A central purpose of the BESTCapsule is to maintain appropriate biological specimens for decades or centuries at low temperatures in the living state. To achieve such long-term survival, the temperatures have to be maintained below ~ -140° C. At temperatures above about -80° C, viabilities decline over weeks to months (Mazur, 1966). However, below about -140° C, the exceedingly high viscosity and lack of thermal energy prevents chemical reactions in aqueous solutions. The only known source of damage becomes the slow accumulation of physical damage to DNA from background ionizing radiation. A typical median lethal dose for cells is 500 rad. Since background radiation is about 0.1 rad/yr, the average cell ought to survive some 5,000 years at or below -140° C without being irreversibly damaged (Mazur, 1974). There is inferential evidence to support this assumption. Whittingham et al (1977) have shown that mouse embryos at -196 °C will survive more than two years of exposure to gamma radiation that is nearly 100- times background. The main problem in cryopreservation, thus, is not long-term storage; it is to devise procedures for cooling the biological specimens to -196° C and returning them to normal temperatures without inflicting lethal injury. [In the laboratory -196° C is a convenient cryogenic temperature because it is the temperature of liquid nitrogen].

DISCLAIMER

The choice of the types of specimens to preserve in the BESTCapsule depends on (1) the expected use to which they would be put in the future, and (2) whether cryopreservation procedures exist or can be devised for that cell type. With respect to item (1), a prime representatives of advanced organisms might be early embryos since they could be used to re-create the full individuals if, in the case of mammals, suitable surrogate mothers are available. Presumably, their cells could also be used in cloning procedures that are likely to evolve from Wilmut's recent cloning of sheep, and, of course, they would be a source of DNA. With respect to the item (2), it is possible now to cryopreserve early embryos from most mammalian species investigated. But that is not currently the case with embryos from advanced non-mammalian species such as insects, fish, and birds. However, hope for eventual success stems from the fact that embryos from the fruit fly *Drosophila* have now been successfully cryopreserved (Steponkus et al, 1990; Mazur et al., 1992b). Other candidates for cryogenic preservation might be reproductive tissues from higher plants and microorganisms. Many types of them (but not all) can be cryopreserved now.

Regardless of the cell type there are certain encompassing biophysical factors and constraints that determine whether they will survive or die during freezing and thawing. Superimposed on these may be special biological factors that apply to specific cell types.

The most important of the general biophysical factors is that cells must be cooled in such a way as to avoid the formation of more that trace amounts of intracellular ice. There are two diametrically opposed approaches to doing so. The first, classical, approach is to cool cells slowly enough so that the chemical potential of the water in their cytosol remains in near equilibrium with the chemical potential of the water in the solution that surrounds them (Mazur, 1984). The latter decreases during freezing as progressive ice formation in the extracellular medium removes water from the unfrozen solution and causes it to progressively concentrate. Above certain temperatures, even though external ice is present, the cell water remains unfrozen and supercooled, apparently because the plasma membrane prevents its nucleation by blocking the passage of external ice. The supercooled cell water possesses a higher chemical potential that that of the water in the external solution and ice. As a consequence, water flows out of the cell osmotically and freezes only when it comes in contact with the external ice. The dehydration resulting from this osmotic efflux lowers the chemical potential of the cell water. If cooling is slow enough, the lowering is sufficient to keep the cell water in near chemical potential equilibrium with the external ice. The important point for cell survival is that the cooling rate must be sufficiently low so that the cell and external water become equilibrated before the temperature has dropped low enough to permit the cell contents to become nucleated or "seeded" by the external ice (Mazur, 1984).

The quantitative definition of "slow enough" depends primarily on the water permeability of the cell (Lp) and its temperature coefficient or activation energy (Ea), and on the surface-to-volume ratio of the cell (A/V). The human erythrocyte, for example, has a very high Lp, a low Ea, and a high A/V, and as a consequence, "slow enough" is about 1000 ^{o}C/min. In contrast, a pre-implantantion mouse embryo has a 10-fold lower Lp, a 3-fold higher Ea, and a substantially lower A/V. As a consequence, "slow enough" for these embryos is about 0.5 C/min. For the majority of cells studied, the critical cooling rate lies between 1 and ~20 ^{o}C/min. If the cell has been cooled at or below the critical rate to below its nucleation temperature (-10 to -30^{o} C) and to a temperature at which it has lost most of its

freezable water, it can can then be cooled rapidly to -196 ^{o}C without inducing lethal internal ice. The transition temperature between slow and rapid cooling generally lies between -35 and -60 ^{o}C.

Although slow cooling as defined above is mandatory for the survival of cells by the classical approach, it is not sufficient, at least for most cell types other than microorganisms. Survival also requires the presence of molar concentrations of cryoprotective solutes. Most cryoprotective solutes are low molecular weight, relatively non-toxic, nonelectrolytes that are capable of permeating the cell. The most common are glycerol, dimethyl sulfoxide (DMSO), ethylene glycol, and propane diol. Protection is optimal when their concentration equals or exceeds 1 M, at least in cells that will tolerate such concentrations.

The cause of slow freezing injury and the basis by which these compounds protect against it is a matter of debate. One school, to which I subscribe, holds that the protection is primarily colligative; i.e., it depends more on the number of molecules present than on their specific chemical nature. More precisely, it depends on the molar ratio of the cryoprotectant to that of the endogenous solutes in and around the cell-- primarily electrolytes. There are three sub-categories to this school of thought. One, originally proposed by Lovelock (1953) is that injury results from the concentration of intra- and extra-cellular electrolytes that accompanies ice formation. In this hypothesis, compounds like glycerol protect by colligatively reducing the electrolyte concentrations at a given temperature. The second view, versions of which were proposed by Meryman (1974) and by Steponkus et al. (1983), is that damage is associated with the osmotic shrinkage of cells during freezing or with their re-expansion during thawing. The corollary is that intracellular cryoprotectants protect by reducing the extent of cell shrinkage at a given sub-zero temperature. The third view proposed by Mazur and colleagues (Mazur and Cole, 1989) is that damage occurs as cells are forced into contact with ice and with each other as progressive ice formation causes the progressive narrowing of the unfrozen channels in which they lie. In this hypothesis, substances like glycerol protect by keeping the unfrozen channels larger than would otherwise be the case. The three mechanisms are difficult to distinguish experimentally and are not mutually exclusive.

Another way to look at many of the biophysical events in cells is in terms of a series of osmotic volume excursions. The cells initially shrink rapidly when suspended in hyperosmotic solutions of cryoprotectant. If that cryoprotectant is permeating, they then reexpand at a lower rate, a rate that is proportional to their permeability to the cryoprotectant (Ps). As mentioned, they then shrink again during slow cooling, a process that is reversed during thawing. Finally they undergo osmotic volume excursions during the removal of the cryoprotectant, the direction and magnitude of which is dependent on the manner in which the removal is effected. If excessive, the volume excursions during this addition and removal can be injurious or lethal. The excursions can, however, be held to tolerable limits by adding and removing the cryoprotective solute in several steps of appropriate magnitude at appropriate intervals. "Appropriate" can be calculated if one knows the value of Lp and Ps. An example of such an analytical approach has been recently published by Gao et al. (1995) for human sperm, and we (Katkov and Mazur, unpublished) are applying a somewhat different approach to mouse sperm.

Most of the above discussion applies to the avoidance of intracellular freezing by slow equilibrium freezing (Mazur, 1984). There is, however, a diametrically opposite non-equilibrium route to avoiding internal ice and to cryopreservation. That route involves loading cells with sufficiently high concentrations of permeating glass-inducing solutes and cooling and warming them sufficiently rapidly to induce their interior to vitrify during cooling and remain vitrified during warming (Rall, 1987; Mazur, 1990). There are strong reciprocal interactions between the concentration of solute and the required cooling and warming rates. All, however, greatly exceed those required for slow equilibrium freezing. Concentrations must often exceed 6 M, and cooling and warming rates must often exceed 1000° C/min. In at least one case, the *Drosophila* embryo, they exceed tens of thousands of degrees/min (Steponkus et al., 1990; Mazur et al., 1992b).

The final general biophysical constraint concerns the rate of warming and thawing. If the vitrification approach has been followed, the answer is clear. Vitrified specimens must be warmed very rapidly. If they are not, the glass will devitrify (crystallize into ice) with lethal consequences. If cells have been subjected to slow, equilibrium, freezing, the answer is variable. In most cells systems, rapid warming (i.e., > 100° C/min appears superior to slow warming (~1-5° C/min). This is reasonable since higher warming rates expose the cells for shorter times to the concentrated solutes in the unfrozen channels. In some cell systems, however, the warming rate has little or no effect. In still others, notably mouse embryos and higher plant cells, slow warming may be superior to rapid. The probable explanation is that during slow freezing, additional cryoprotectant is driven into the dehydrating cells. If subsequent warming is too rapid, there is not sufficient time for the excess to diffuse out, and the cells swell osmotically to a damaging extent.

A wide variety of cell types can be adequately cryopreserved when frozen in ways that satisfy the above general biophysical constraints. The successes include many and perhaps most single cells including micro-organisms and many and perhaps most early mammalian embryos. But there are other types that can not yet be cryopreserved or require special considerations for their cryopreservation. Some like eggs of the fruit fly *Drosophila* (Mazur et al., 1992a) and the malaria mosquito *Anopheles* (Mazur et al., (1994) are so sensitive to chilling even in the absence of ice formation that they will not survive slow equilibrium freezing. In order to outrun the chilling injury they must be cooled at very high rates. Ordinarily such high cooling rates would cause instant death from intracellular ice. To prevent that, the embryos must be loaded with concentrations of glass-inducing agents that are sufficiently high (8 M) to induce vitrification during cooling and prevent devitrification during warming, and yet not so high as to cause death from chemical toxicity . The problem is compounded in these insect eggs by the fact that the native eggs are impermeable to the required cryoprotectants. Successful cryopreservation requires that ways be found to permeabilize them in an innocuous fashion. Fortunately, a way was found in the case of *Drosophila* eggs; namely, treatment with the alkanes hexane or heptane (Steponkus et al, 1990; Mazur et al. 1992b). Permeability barriers are also present in *Anopheles* mosquito eggs (Valencia et al, 1997). and zebrafish eggs (Hagedorn et al, 1997). Procedures for overcoming them are under investigation.

Some stages or species of mammalian germplasm present other related or unrelated difficulties. Porcine sperm and embryos are extremely chill sensitive. In the latter, the

sensitivity appears related to the presence of lipid droplets in the cytoplasm. Mammalian unfertilized eggs (oocytes) have proved to be much more difficult to cryopreserve than fertilized stages and early embryos. The difficulties may be related to the fact that late oocytes are locked in Metaphase II, the stage that possesses a well-defined mitotic spindle. These special problems are discussed in more detail in companion papers by Leibo and by Kasai in this volume. Mouse sperm present still other difficulties. They are highly susceptible to mechanical injury (Schreuders et al, 1996; Katkov and Mazur, 1997) and to injury from small degrees of osmotic swelling and shrinking (Willoughby et al, 1996).

With the exception of *Drosophila* embryos (which contain some 40,000 cells), early mammalian embryos, and small fetal tissues like rat pancreases (Rajotte and Mazur, 1981; Mazur and Rajotte, 1984), most multicellular tissues and organs have also proven to be difficult objects to cryopreserve. The problems lie at several levels. One problem is a matter of scale. Large objects like organs have small surface-to-volume ratios, and consequently require very low cooling rates to avoid intracellular freezing. But these very low cooling rates exacerbate damage from concentrated electrolytes and cell dehydration. Another problem is that one has to simultaneously preserve a variety of cell types that may differ in biophysical requirements such as cooling rate. Some cell types, such as capillary endothelium appear especially sensitive to freezing injury. A third problem is a topological one. When water flows out of a slowly frozen single cell, it flows to the true outside. When water flows out of the individual cells in a tissue or organ, it preferentially flows into spaces like capillaries within the tissue or organ. The result is that these interior spaces are forced to expand as much as seven-fold (Rubinsky and Pegg, 1988) with consequent damage to the cell-cell connections in the layer that forms the interior space (Armitage et al., 1995). One approach that is being pursued by investigators like Fahy and Ali (1997) is to try to avoid ice crystal formation entirely by inducing vitrification. The problems of doing so are analogous to those discussed above for *Drosophila*, with the added complication that tissues and organs are too large to be cooled and warmed at very high rates.

Clearly, in considering what types or cells, organisms, and tissues ought to be preserved in the BESTCapsule, one has to match what would be desirable from the point of view of potential interest to investigators far in the future with what is currently feasible cryobiologically.

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