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Cryopreservation of Mammalian Sperm: What We Ask Them to Survive

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Techniques for freezing bull sperm developed over the past 40 years have not yielded protocols for preserving sperm from other species. Recent advances in our understanding of cell membrane structure function and metabolism now permit alternative modes of investigation. These data will allow development of unique studies which should have a higher probability of yielding successful protocols for sperm from other species. In this review the authors will: (1) provide a general overview of cryopreservation; (2) review emerging concepts of membrane structure and the relationship of membrane composition to water and cryoprotectant movement; (3) emphasize how these parameters affect cell volume and surface areas; (4) focus attention on the concept that cryoprotectants will alter membrane structure and function in addition to their well-recognized effects on bulk solvent; and (5) emphasize the effect of the processing protocol on metabolic balance. These concepts are reintroduced in the context of the established and successful protocol for freezing bull sperm to illustrate the molecular responses that may be necessary to survive a freeze-thaw cycle.

Key words: spermatozoa, cryopreservation, cryoprotectant, membrane reorganization, membrane permeability, membranes, metabolism, metabolic balance, osmotic effects.

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Empirical studies over the past 40 years in the cryopreservation of sperm yielded techniques for freezing bull sperm, but left few clues and no specific technique for the preservation of other cells. This void is due, in part, to: (1) the dearth of methods to assess damage to subcellular compartments (and an appreciation of the importance of each); and (2) the slow realization of the uniqueness of bull sperm (generally treated as the prototype cell), and therefore, an absence of adequate cellular models upon which hypotheses can be framed.

Nonetheless, experiences over several decades have established that many biological systems can endure storage at ultra cold temperatures (eg, −196 C) *if* they can survive the passage to and from the storage temperature. A growing body of data identifies the major problems that the cell encounters. Temperature affects the "phase state" of the membranes, thereby altering their physical properties (Carruthers and Melchior, 1988b; Quinn, 1989). Extracellular ice crystallization results in a phase change in the external solvent, where the formation of the solid phase results in very large increases in the concentration of all other solutes in the remaining liquid (Mazur and Cole, 1989; Pegg and Diaper, 1989). The cell (interior, interface and

exterior) must respond to these changes within the finite time period allowed by the protocol (Mazur and Cole, 1989; Schneider and Mazur, 1984). Finally, thawing reverses the process, forcing the cell to retrace its path through the various phase changes (Schneider and Mazur, 1984; Schneider, 1986). Careful studies have identified an important requirement for developing an appropriate protocol for maximizing cell survival. A rate of temperature change must be identified that allows movement of water and cryoprotectant without either intracellular ice formation or irreversible membrane change. Explicit equations have been derived to calculate these variables for some cell types (Schneider and Mazur, 1984), but routine application of these concepts has been hindered by an absence of data establishing: (1) the relationship between cell structure (dictated by the composition of the various subcompartments) and function; and (2) cellular adjustments to solvent and solute movements.

In this review we will: (1) provide a general overview of the phenomenology of cryopreservation; (2) review emerging concepts of membrane structure and the relationship of membrane composition to water and cryoprotectant movement; (3) emphasize the effects of these parameters on cell volume and surface areas; (4) introduce the concept that the addition of cryoprotectants to cell suspensions will alter many aspects of cell structure and function in addition to their well-recognized colligative effects on bulk solvent; and (5) emphasize the importance of maintaining metabolic balance throughout the entire processing protocol.

The reader is alerted that extrapolations from model systems (where direct experimental tests can be conducted) to sperm (where studies now are difficult-to-impossible to complete because of the complexity of the system) are often made. We hope that such speculation will stimulate others to join us in the testing of the concepts we introduce.

What Does a Cryopreservation Protocol Do to Bull Sperm?

To begin this overview, we will examine a success story, the carefully optimized but empirically-derived protocol used to preserve sperm from dairy bulls. A highly successful, economically driven market has been developed and satisfied by: (1) development of processing procedures that employ a few specialists to package sperm; (2) selection of a limited number of sires for use, a process that was driven by intense selection for genetic improvement in milk production but also could have resulted in selection of males whose sperm successfully survive the protocol used by the industry; and (3) use of sperm that are thawed and inseminated in females (selected for milk production) by technicians highly skilled in the art. Before extrapolation and adaptation of this process to sperm from other species, a series of critical questions should be considered. Is the process ultimately intended for a few carefully selected males (eg, cattle, poultry or pigs), or will it be used for all males needing the technique (humans or horses)? Will the technique be used by a few specialists or by professional generalists that will employ cryoprotection as one part of their professional practice? Will the market be economically driven (applied agriculture), or is it emotionally driven (humans and companion animals), and how will this affect the protocol? Is the sperm cell of interest *really* similar to bull sperm? What are the unique features of reproduction in the species of interest (eg, sperm storage in the hen), and are these likely to impose special requirements on the sperm surfaces or structures?

An overview (Fig. 1) illustrates the relationships between time and temperature, with special emphasis on potential changes in cellular volume. Five distinct processing steps can be recognized: extension and cooling; penetrating cryoprotectant addition and packaging; freezing; storage; thawing and insemination. Each has its special relationship to membrane structure-function and cell metabolism. The first takes the cell through a temperature change that is known to alter the physical properties of all cell membranes (Carruthers and Melchior, 1988a,b; McElhaney, 1986; Quinn, 1989); the second demands a large volume change and rapid recovery (Fiser and Fairfull, 1989; Armitage, 1986; Armitage and Mazur, 1984; Schneider, 1986); the third further alters membrane structure and volume over a very short time period (Knox et al, 1980; Steponkus and Lynch, 1989; Schneider and Mazur, 1984); the fourth represents dormancy; whereas in the fifth, membrane recovery and expansion is required as the cell is removed from the storage diluent (Knox et al, 1980). *This graphic example causes one to marvel at successful survival rather than to despair over the difficulties of its implementation to the preservation of a variety of other sperm cell types.* This is especially true when placed in physiological context since ejaculated sperm, "destined" for deposition in the female tract and fertilization of the egg upon ovulation, instead has

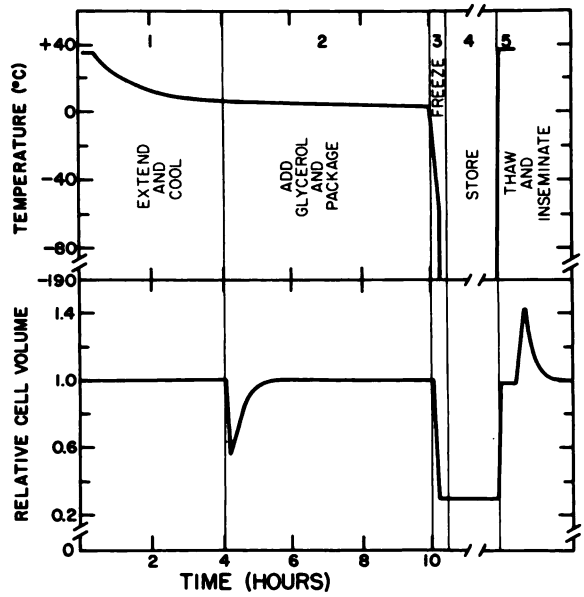

Fig. 1. Changes in temperature and cell volume associated with a cryoprocessing cycle for bull sperm. A time-event profile associated with a successful (but perhaps not optimum) cryopreservation process for bovine sperm is provided, with change in temperature plotted in the top panel and change in cell volume plotted in the bottom panel. This representation, adapted from a presentation by Schneider (1986) for embryos, divides the process into five stages (defined in text) and emphasizes both the dynamic state of the membrane and the short time-period over which the sperm are expected to respond to changes in their environment.

been captured, processed, and then returned on its way with the hope that a sufficient number of characteristics have been retained to allow efficient resumption of the process.

An intriguing aspect of such protocols is that rates of temperature change in one aspect of the overall process directly affect the range of temperature changes that can be used for another. It is well established that optimum cell survival requires that the rate chosen for freezing be paired with an appropriate rate of thawing (Mazur, 1984; Schneider, 1986). This is illustrated in Fig. 2, where rate of freezing (and resultant dehydration) leaves the cell in a tightly packed and restraining environment dominated by the effects of high solute and ice crystals; recovery requires plasticity of the cell. Recent advances in our knowledge of membrane structure now permit an analysis of this relationship.

Our final general comments are directed to the strengths and weaknesses of current strategies for detecting cell damage associated with cryopreservation. Inspection of Figure 1 reveals that damage can be induced in one processing step but not necessarily expressed until the cell has undergone further treatments. Detection is further complicated by the fact that detailed analyses are difficult, if not impossible, to complete when sperm are at low temperature and in a semi-solid state. Therefore, it is often necessary to complete the entire processing procedure, analyze the cell suspension, and *then* attempt to assign the point of deleterious alteration of the cell so that the appropriate aspect of the processing protocol can be modified to ameliorate damage. This leads to the critical question of what should be used as an assay for cell damage. Sperm survival requires simultaneous solution of a multifactorial equation relating the probabilities of damages to all compartments. All sperm have: (1) a mitochondria-flagellar network (for metabolism and motility); (2) a nucleus (for

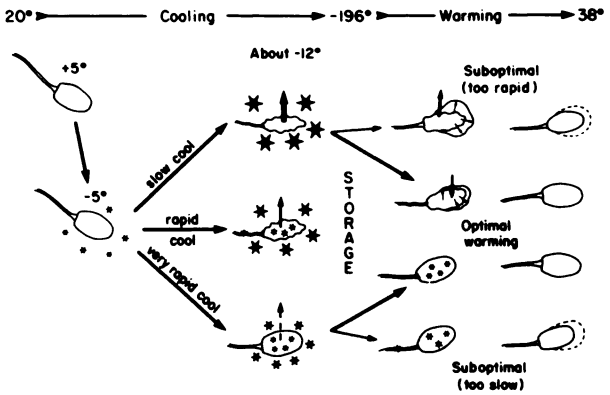

Fig. 2. Putative changes in sperm and extender during freezing and thawing. The effects of various cooling and freezing rates on formation of ice crystals and microcrystals (large or small stars) and the movement of solvents and penetrating solutes (heavy or light arrows) are shown. After an initial cooling and formation of extracellular microcrystals at about −5 C, cooling at variable rates affects both the rate of water movement out of the cell and the extent of intracellular ice formation. Thus, when the sperm arrives at −196 C, the intracellular and extracellular environments differ according to freezing rate. Damage can result if the thaw rate chosen is inappropriate. Extreme values are illustrated: (a) top right, where too rapid a thaw rate results in unbalanced rates of egress of cryoprotectant and influx of water (driven in turn by the rapid loss of the extracellular solid phase and a decrease in colligative properties of the surrounding solution); and (b) bottom right, where too slow a thaw rate results in recrystallization of microcrystals of intracellular water and resultant damage to cellular organelles. The optimal warming rate (the middle two paths on the right) depends on the cooling rate chosen and provides a mode of minimizing damages due to inappropriate rates of solute and water transport across the membranes and intracellular microcrystal formation. Figure used with permission from Amann and Pickett (1987) and related to a description of the cooling phase provided by Mazur (1984).

stable DNA storage); (3) an anterior head (for appropriate acrosomal activation); and (4) an equatorial segmental surface (for sperm binding to the egg). Species-specific features (eg, oviduct storage for chicken sperm) impose additional requirements. This demands: (1) a battery of assays, each directed to a specific compartment; (2) analysis after *in vitro* incubations intended to mimic the stresses imposed on the cell after removal from the cryoprotectant diluent; and (3) the development of assay protocols that simultaneously analyze single cells (rather than populations of cells) for damage to any one of the multiple compartments. The combination of unique spectroscopic probes of cellular compartments and flow cytometric analyses show promise (Graham, Kunze and Hammerstedt, manuscript in preparation), but this challenge has yet to be satisfied.

Proteins and Lipids and Their Integration Into Membranes

The various membranes (eg, plasma, mitochondrial, acrosomal) of the sperm cell are unique aggregates of lipids and proteins, assembled during spermatogenesis and modified during epididymal transit, storage, and ejaculation (Hammerstedt and Parks, 1987; Eddy, 1988). Membrane function is determined by the interactions of the various components, and any processing event that alters these interactions can be expected to alter the function (Aloia et al, 1988). The design of a cryopreservation protocol must begin with an appreciation of native membrane structure and then incorporate steps that minimize alterations. Study of membrane structure-function relationships is evolving rapidly, with many emerging concepts of direct relevance to cryopreservation. Our overview will introduce these concepts. The reader is referred to more specific reviews for details (Cullis and Hope, 1985; Jain, 1988; Hammerstedt, 1989). Several of these concepts have been discussed in detail by others in terms of the well documented "cold shock" phenomenon (see reviews by Watson, 1981; Watson and Morris, 1987 for details).

The original description of the fluid-mosaic model (Singer and Nicholson, 1972) contained many insightful comments on probable details of membrane structure beyond the often reproduced model of proteins penetrating, iceberg fashion, into a sea of lipid. The complexities that we are now able to describe in greater detail include layers of the membrane, membrane asymmetry, and the complex interactions of lipids and proteins that result in subtle but important alterations of membrane pores, enzymes, and receptors (Aloia et al, 1988). For our purposes, a cross-sectional view of the membrane (Fig. 3) is useful because it allows a schematic presentation of the spatial relationships of the membrane in terms of molecular dimensions. The concept is one of a succession of layers, where each layer has a unique lipid-protein distribution and potential relationship to membrane function and a unique susceptibility to alteration during a freeze-thaw cycle. Although the distribution of the components in each layer and their density on the surface has been difficult to establish, it is clear that all membranes (especially surface membranes) exhibit some level of complexity (Rao, 1987).

The next consideration is the role of alternative

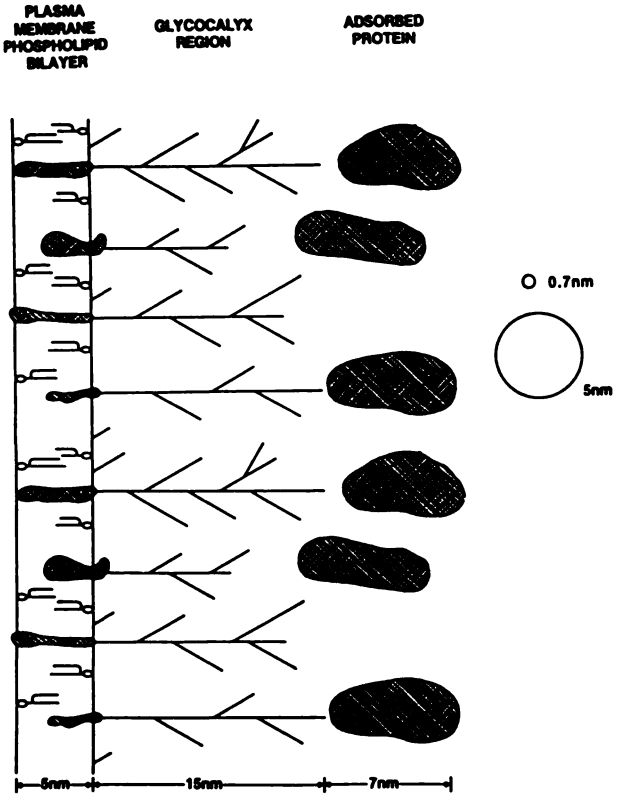

Fig. 3. Cross-sectional view of the plasma membrane. This schematic representation, used with permission from Hammerstedt (1990), illustrates the spacial relationships of the surface of the cell. The relative sizes of the soluble components that approach the membrane are shown (small sphere = 200 daltons and large sphere = 60,000 daltons). The approach of molecules to the bilayer is influenced by the frequency of appearance of the proteins that form the glycocalyx and the adsorbed protein coat.

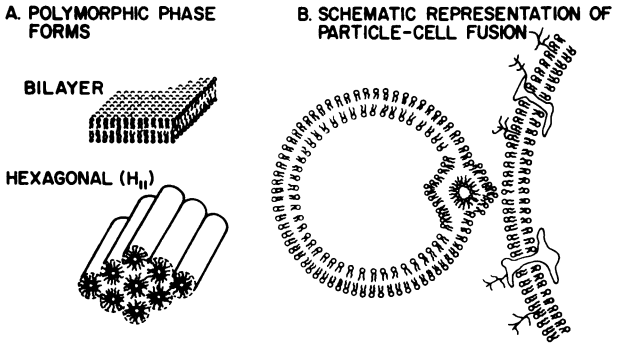

Fig. 4. Schematic representation of polymorphic phase forms observed in biological membranes. Two polymorphic phase forms are present in cell membranes (left panel). The bilayer form (planar configuration; head groups oriented to bulk water; hydrocarbon tails inward; continuous permeability barrier for polar molecules formed) is the most prevalent and the hexagonal II form (cylindrical form; head groups oriented inward; hydrocarbon tails outward; weak permeability barrier for polar molecules) is quite rare. In the right panel, a potential role for the minor form (hexagonal phase II) is illustrated as part of the membrane fusion events that may occur during the interaction of lipid vesicles and surface membranes of the cell.

forms of lipid aggregates within the bilayer (Houslay and Stanley, 1982; Cullis and Hope, 1985; Jain, 1988; Carruthers and Melchior, 1988a,b). In addition to the well-recognized bilayer lipid, which predominates in biological systems and is uniquely capable of providing a selective and controllable barrier to transbilayer movement of polar molecules, a minor aggregate form (hexagonal phase) is possible (Fig. 4, left). This latter form does not provide a permeability barrier and probably exists only transiently in biological membranes. Despite this fleeting existence, this form is believed to be very important in serving as a point-defect in the overall membrane structure with a special role in membrane fusion events (Ohki et al, 1988). Membrane fusion (either desired as a portion of events, such as the acrosome reaction, or as an undesired aspect of membrane disruption associated with processing) requires: (1) that the membranes involved be stressed to induce the tendency to undergo some form of rearrangement;(2) that the membranes be brought into proximity, as measured in molecular dimensions, to allow for interactions; and (3) that the stress be relieved to allow formation of an alternate membrane form. An example of this process is indicated in Fig. 4 (right).

The next important concept on the molecular level is phospholipid asymmetry. The evidence for such arrangements, especially for plasma membranes, and implications of such features have been reviewed (Israelachvili et al, 1980; Kuypers et al, 1984; Gruner, 1985), and the salient points are summarized here. Formation of a stable bilayer with a radius of curvature expected for a cell demands that half the total phospholipid be allocated to each face of the bilayer (50% in both the inner and outer leaflets). However, each type of phospholipid has a preference for a specific orientation. Some [phosphatidylcholine (PC), sphingomyelin (Sph)] prefer an outward orientation while others [phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI)] orient inward. Arrange-

TABLE 1. Phospholipid Composition of the Plasma Membrane Overlying the Acrosome for Cauda Epididymal Sperm and Its Relationship to Lipid Asymmetry

Phospholipid Type	Preferred Distribution in Bilayer	Mole Percent in Recovered Vesicles		
		Ram	Boar	Rat
DPG	Inner	7	—	10
PE	Inner	15	28	31
PI	Inner	4	7	8
PS	Inner	2	3	10
Sum of the Inner Directed		28	38	59
PC + Sph	Outer	60	62	30
Lyso PC	Outer	123	—	—
Sum of the Outer Directed		72	62	30

Primary data for ram (Parks and Hammerstedt, 1985), rat (Agrawal et al,1988) and boar sperm (Nikolopoulou et al, 1985) were recalculated with regard to the propensity of the individual lipid classes to orient toward the inner vs. outer faces of the plasma membrane bilayer. Since rat sperm membranes have a phospholipid of unknown structure which could not be incorporated into this comparison, that total does not sum to 100%. A detailed discussion of other differences is provided in Agrawal et al (1988). See the text for a discussion of potential effects of these differences on membrane stability. Abbreviations used are: diphosphatidylglycerol (DPG); phosphatidylethanolamine (PE); phosphatidylinositol (PI); phosphatidylserine (PS); phosphatidylcholine (PC); sphingomyelin (Sph); and lysophosphatidylcholine (Lyso PC).

ments that force alternate orientations (examples to follow) could be predicted to have an effect on membrane stability. It is also important to note that each phospholipid species has a differential preference for bilayer vs. hexagonal aggregation states (Tilcock and Cullis, 1987; Quinn, 1989).

Data summarized in Table 1 illustrate the differences among species in the probability of inward vs. outward orientation of lipids, with the plasma membrane overlying the acrosome in bull, ram, and boar sperm having a potentially different display from that of the rat. The implications of such an arrangement, where phospholipid head groups are forced to orient toward the "undesired" membrane face, have not been evaluated for sperm. To date, no direct proof of such an orientation for intact sperm has been presented, but some support is provided by the analysis of isolated membrane vesicles (Hinkovska et al, 1986; Hinkovaska-Galcheva et al, 1989). These studies provide only tentative support because no proof has been presented that the vesicles removed from the sperm prior to analysis retained an outward orientation when they were released from the cell.

The protein-lipid interactions that occur within the membrane are extremely important for understanding cell function. Using results gathered from a variety of model systems and many different analytical methods, it is apparent that: (1) some lipids (often of the type that preferentially adopt the nonbilayer state) are preferentially clustered around integral membrane proteins (Quinn, 1989); and (2) the lipid environment around a protein influences functional properties of the aggregate unit (Carruthers and Melchior, 1988a,b). These factors are best studied in model membrane systems, where clear evidence for changes in the interactions of lipids and proteins as a function of temperature have been obtained (Carruthers and Melchior, 1988b). For each organism, the evolutionary process has resulted in selection of lipid constituents with physical characteristics that yield a liquid-crystalline array, where the acyl chains are relatively "disordered" (Carruthers and Melchior, 1988b). This membrane environment allows freedom of motion via diffusion for components in the membrane as long as the temperature is above a critical temperature. As the temperature is lowered below that point, ordering of lipids occurs with a resultant restriction of mobility far beyond that anticipated by single temperature effects on diffusional processes (Carruthers and Melchior, 1988b; Quinn, 19189). For bacteria, remodeling of the membrane is possible by replacing lipids with members whose aggregate physical properties provide the motional freedom needed at the new temperature of growth (Voelker, 1985; Carruthers and Melchior, 1988b; de Gier, 1989). Sperm, endowed with little biosynthetic capacity and evolved to exist at a relatively constant temperature, have no endogenous mechanism to match membrane properties to the requirements imposed by processing protocols. Such adjustments have to be incorporated into the protocol adopted for use (Foote, 1982; 1984).

When a biological membrane is cooled, a reordering of the membrane components is possible (Fig. 5). As the membrane passes through its critical temperature, the nonbilayer lipids that resided by the membrane proteins could aggregate together,

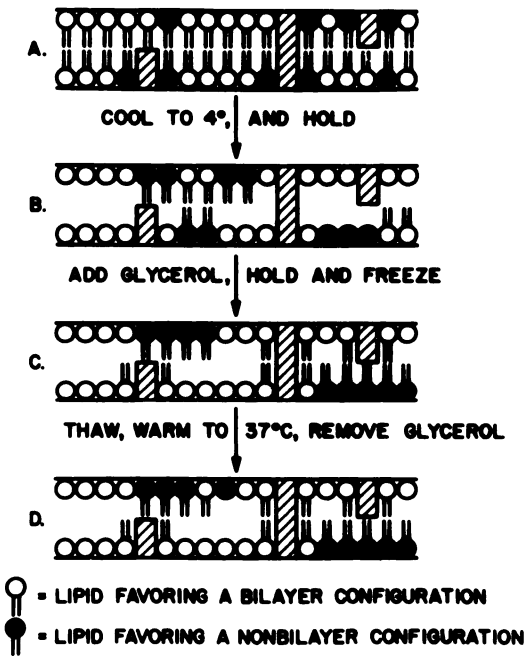

Fig. 5. Schematic representation of the possible effects of cooling and rewarming on the distribution of lipids around integral membrane proteins. State A represents the membrane at the time of collection, with the nonbilayer lipids preferentially associated with the integral membrane proteins (crosshatched blocks). Cooling results in a preferential clustering of the nonbilayer lipids into a "gel-rich phase" and a resultant formation of a system with bilayer lipids associated with the proteins (state B). Freezing results in increased ionic strength in the external solution and, if lipids are sufficiently mobile, further clustering of the lipids (state C). Rewarming yields a membrane system that may have alternative lipid partners for integral membranes (state D); any return to state A depends on sufficient lipid mobility to allow all membrane proteins to find their "original" partners. This figure is adapted from a more detailed presentation by Quinn (1989).

leaving the proteins with alternate lipid partners (Quinn, 1989). Further cooling and increased salt concentrations could encourage further assembly of the nonbilayer lipids into a gel-rich zone in the membrane. Rewarming would initially yield protein-lipid partners different from those entering the processing cycle. The original state, with its specific membrane properties, could be reestablished in a time-dependent process where diffusion allowed reassembly of the components into their original display. Others (Mazur, 1984) have linked the necessity for carefully paired rates of cooling and thawing to the extent of intracellular dehydration, but membrane alterations also could be the basis for the molecular memory recognized in these empirical studies.

Evidence to support the concept that the thermal history of a sample affects membrane features has been gathered for a few systems. Gorospe and Conn (1987) provided direct microscopic evidence that the movement of GnRH receptors of pituitary cells reflects the previous thermal treatment of the cell preparation. Muller and Krueger (1986) reviewed the evidence for alteration of membrane protein function by alteration of the lipid environment.

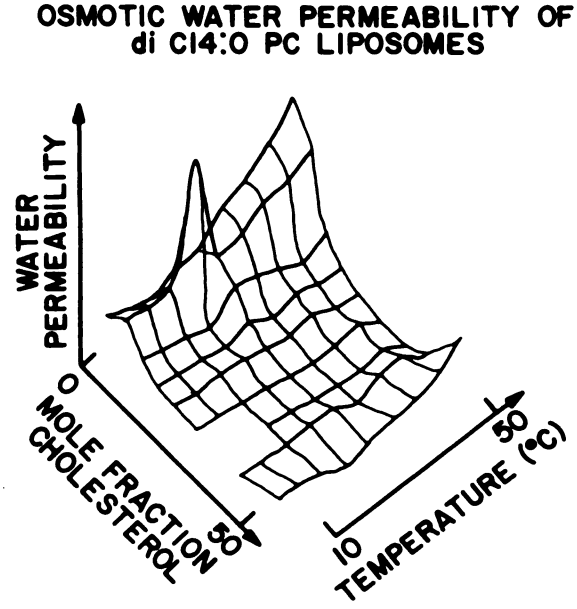

Fig. 6. Effect of lipid composition on water permeability of liposomes. Effect of temperature and lipid composition (increasing mole percent cholesterol in liposomes of dimyristoylphosphatidylcholine [di Cl_4:0 PC]) on water permeability as assessed by osmotically driven liposome swelling are illustrated. Figure adapted from one presented by Carruthers and Melchior (1988b).

Morris and colleagues (Morris and McGrath, 1981; Morris, 1982) established that the same features apply to liposome systems, where vesicle composition and rate of cooling affected the stability of the liposome during freezing from isotonic solutions. Morris and McGrath (1981) used cryomicroscopy to describe shape (and therefore, area) changes associated with freezing of liposome suspensions. Strauss and Ingenito (1980) found similar effects, especially when membrane proteins and cryoprotectants were included in the mixture. These data indicate that the previously unexplained features of freezing of intact cells may be elucidated by study of model liposomes.

Attempts have been made to relate total sperm lipid composition to propensity for survival from a freeze-thaw cycle (Poulos et al, 1973; Darin-Bennett and White, 1977; Hinkovska-Galcheva et al, 1989). The compilations are of value because they reaffirm the potential relationship of membrane properties to cellular damage, but they have not provided predictive power to direct design of alternative protocols. Since each membrane-bounded compartment has a unique lipid composition, and potentially a unique relationship to temperature change, achievement of our goal of protection of each individual compartment from damage will require evolution of a protocol that satisfies the needs of each subsystem.

Water and Cryoprotectant Movement Through Membranes

Inspection of Figure 1 reveals the dramatic movements of water and cryoprotectants that could occur in a sperm cell during processing. Unique but similar responses are likely to be seen for each spermatozoal subcompartment. Water transport has been studied in a variety of model and biological systems (de Gier, 1989; Verkman and Masur, 1988). It is now evident that the rate of water movement through membranes is affected by: (1) presence of "defects" in the membrane (acyl kinks in side chains of phospholipids introduced by unsaturated fatty acids; pores; and protein-lipid interfaces; Verkman and Masur, 1988; Carruthers and Melchior, 1988b); and (2) lipid composition and phase preference of the individual lipids (Carruthers and Melchior, 1988b; de Gier, 1989). A dramatic example of these effects is presented in Figure 6, where the results of a detailed study in a model liposome system of water transport, as a function of temperature and cholesterol:phospholipid ratio, are presented.

Cryoprotectants can be classified as penetrating (glycerol and dimethylsulfoxide) and nonpenetrating (lactose and trehalose) according to their ability to pass through the membrane. Both types can cause a dehydration of the cell by osmotically induced water egress, but they differ in their capacity to enter the cell and reside in cytoplasm and membranes. Less is known about cryoprotectant transport (see Levitt and Mlekoday, 1983). The process can be expected to proceed at a somewhat slower rate than for water, but with a similar dependence on membrane structure.

When considered in the context of the previous discussion of membrane structure, it is apparent that any change in membrane organization caused by lipid reorganization can affect the kinetics of water and cryoprotectant transport (de Gier, 1989; Mazur and Rajotte, 1981; Mazur et al, 1974; Mazur and Miller, 1976). Therefore, apparently simple questions of whether to add glycerol at 37 C or 4 C have a very large effect on the probability of success in surviving the large volume changes that are induced by this treatment (Fiser and Fairfull, 1989).

Cell Volume Changes and Their Implications for Survival

The review by Schneider (1986) and later studies by Shabana and McGrath (1988) provide an excellent example of measured changes in size of embryos and ova during passage through a freeze-thaw cycle. Since accurate volume and surface area measurements have not been made for the irregular shaped sperm, a discussion by analogy (Fig. 1) will be used to introduce several concepts. The first volume adjustment of the process cycle occurs in response to addition of cryoprotectant to cells in isotonic media. Its components are an initial fast shrinkage stage associated with osmotically driven egress of intracellular water, followed by a slower return to the original volume as the penetrating cryoprotectant enters. The second volume adjustment occurs when the extracellular water freezes. It reflects the outward movement of water in response to high concentrations of extracellular salts resulting from freezing of extracellular water. Thawing yields analogous but opposite volume changes. Since the organization of membrane components could change during the processing cycle, however, the kinetics of water transport may not be equivalent throughout.

The effects of the osmotic pressure gradients created during cryopreservation can be predicted theoretically. Addition of 1 M glycerol (approximating values currently used in semen preservation) should result in egress of osmotically responsive cell water to yield a cell at about 50% of its isotonic volume. Ram sperm, for example, have a cell volume of 35 μm^3, 40% of which is osmotically active (Hammerstedt et al, 1978); thus the cell volume before glycerol permeation could be about 60% of its isotonic value. As glycerol enters, the cell will return to its original volume. Volume adjustments of even greater magnitude will occur as the cells are exposed to hypertonic conditions during freezing because the solute concentrations will approach 3–5 Osm. This condition is reversed upon thawing, and the sperm should return to isotonic volume. A major change will occur when the sperm (equilibrated with glycerol) are transferred from the glycerol containing extender (1300 mOsm) to the reproductive tract (approximately 300 mOsm). A 4.3-fold expansion of osmoticaly active internal water will occur as water enters the cell more rapidly than the glycerol can exit through the membrane. As a result, the ram sperm must expand to 81 μm^3, a 2.3-fold overall increase in volume, before returning to its original isosmotic volume. How can the surface membranes accommodate this large reduction and expansion in volume?

The response to moderately hypertonic conditions could be accommodated by a closer approximation of the membrane systems as demonstrated by Jones (1971), but since the major space occupying structure in the head region is the nucleus, consisting of highly condensed nucleoprotein and chromatin crosslinked with disulfide bridges (Bedford and Calvin, 1974), a limit to such contraction exists. The acrosome and the intramembrane spaces in the anterior head might be capable of further reduction in volume, but substantial reductions in total cell volume could be accommodated only in the midpiece and tail regions.

No single experiment has directly evaluated these suggestions. However, reduction of the overall intracellular water of bovine sperm to less than 50% of its isotonic volume by exposure at ambient temperature to 700 mOsm NaCl resulted in a wrinkling (exvagination as observed by scanning electron microscopy) of the surface membranes in the head region (Williams and Hammerstedt, unpublished). In addition, Hammerstedt et al (1978) reported that bull sperm, but not ram and rabbit sperm, behaved as perfect osmometers in the hyperosmotic range of 300–700 mOsm. Hypertonic treatment of bull sperm (Bredderman and Foote,

1969) followed by estimation of cell volume by a particle size analyzer revealed a similar extent of shrinkage. Thus, species differences are apparent in the freedom of movement of water within sperm, and this could have important consequences during cryopreservation.

Excellent examples of cellular accommodation of the need for membrane surface adjustment are provided from the studies of Steponkus et al on cold adapted plant protoplasts (1989). In this system, capable of unique genetic expression and extensive biosynthesis, it is apparent that extra plasma membrane is stored via invaginations or intracellular vesicles. Sperm are incapable of such adjustments due to lack of biosynthetic capacity and the morphological reality that they possess minimal internal space to accommodate additional intrusions. The two reasonable possibilities are outlined in Figure 7. It is apparent that the layer concept (Fig. 3) of membrane structure is very important in the evaluation of the implications of either choice.

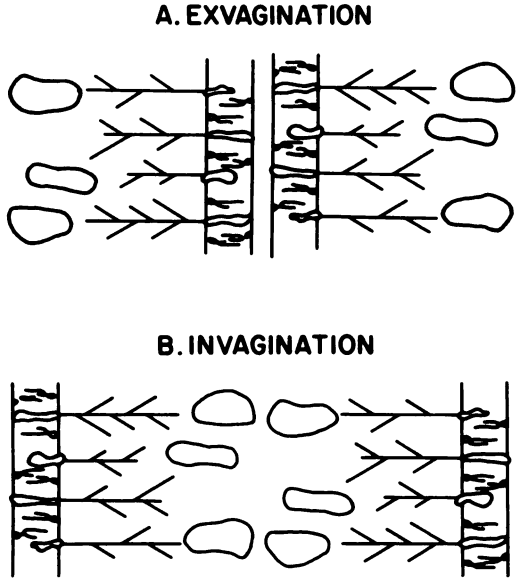

Fig. 7. Schematic representation of interactions that can occur if a sperm surface is folded inward or outward. Reducing cell water volume also causes a reduction in surface area of the cell. Since the membrane thickness (in terms of Fig. 2) cannot increase because of bioenergetic restraints, some mode of folding is necessary. The possibilities are a folding outward (exvagination; top panel) or a folding inward (invagination; bottom panel). The mechanism used will: (a) bring different surfaces together during the period that the cell has the reduced volume (inner halves vs. exterior halves of the bilayer); and (b) trap different amounts of water within the cytoplasmic side of the folds. Dimensions and description of the surface layers were presented in Fig. 2.

Accommodation via invagination (viewed as a minor possibility) would bring exterior layers together whereas exvagination would result in bringing the interior halves of the bilayer into close proximity. The latter choice brings inner membrane surfaces (without a glycocalyx region) close to one another, with the probability of unique forms of interaction for the sperm of various species (Table 1) resulting in varying extents of fusion as a result of cryopreservation-induced dehydration.

The observed exvagination of membranes is likely to have implications at the molecular level. Since the bilayer cannot become thicker (due to energetic considerations) and cannot reduce its surface area (because of head group interactions), a reduced internal volume (but constant surface area) could be accommodated by having the membrane fold upon itself (exvagination, invagination, creases). If this were the case, important questions that follow are: Where does the cell tuck the extra membrane to accomplish this temporary yet obligatory storage of surface until the cell regains its original volume at the end of the processing protocol? What if such folding results in molecular contact across bilayers? Will this result in fusion of the contiguous membranes? If so, will reswelling result in disaster if the residual membrane is not able to accommodate the original cell volume? Is one of the roles of lipid additives (eg, egg yolk) in extenders to provide lipids for membrane repair? Several studies have attempted to answer the last question (Foulkes, 1977; Evens and Setchell, 1978; Watson, 1976; Quinn et al, 1980), but no conclusion can be drawn because of the inability to distinguish (see Figure 3) between incorporation into the bilayer and simple adsorption into the glycocalyx region of the surface.

The volume expansion aspects provide a problem of a different order. Protoplast studies predict that the lipid bilayer cannot expand more than 2–3% without rupture (Steponkus and Lynch, 1989), so the large expansion upon dilution into glycerol free media must be explained in a different manner. Storage of excess surface membrane in a microscopically identifiable form has not been reported for sperm, so mechanisms involving mobilization of membrane should be rejected. Perhaps the solution involves a conversion of the middlepiece-tail from a cylinder to a sphere. A sphere has the highest volume to surface ratio of any geometrical shape whereas a cylinder has a much lower ratio. Thus, if a cylinder of fixed surface area were converted to a sphere, a considerable volume expansion could

be accommodated. Microscopic observations of a shape change involving a coiling of the tail within the membrane and a swelling of the midpiece/tail membrane towards a more spherical shape have been observed for bovine (Drevius and Erickson, 1966; Bredderman and Foote, 1969), ram (Duncan, 1988), and human (Jeyendran et al, 1984) sperm when the cells were exposed to hypertonic conditions. Ram sperm, for example, have paddle-shaped heads with cylindrical midpieces and tails, having a total surface area of about 140 μm^2, approximately half of which is found in the head (Duncan, 1988). Conversion of the 70 μm^2 membrane surface of the midpiece-tail region from a cylinder to a sphere would allow an increase in volume of about 50 μm^3. Such a geometric transition would easily accommodate the volume increase during the removal of the 1 M glycerol. Experimentation is needed to test these suggestions, but a linkage between morphological shapes of sperm from various species and their ability to tolerate osmotically induced volume adjustments is possible.

What Else Does Glycerol Do to the Cell?

Cryoprotectant addition (final concentration of 1–3 M) to cell suspensions alters colligative properties of bulk water to lower the freezing point, thus aiding in the survival of the cells (Mazur, 1984). Direct effects of cryoprotectants on the membranes of cells have not been considered, but should be significant since intramembrane concentration could exceed 1 mM if only 0.1% of the cryoprotectant resided in the membrane. These effects could be related to: (1) direct alteration of membrane bilayers (Hempling and White, 1984; Anchordoguy et al, 1987; Goodrich et al, 1988; Crowe et al, 1987); (2) interaction with bound proteins and glycoproteins (Armitage, 1986); and (3) induction of an increased bioenergetic demand. Thus, glycerol affects every zone of the membrane (Fig. 3) in addition to the aqueous compartments of the cell suspension.

Membrane bilayer structure is altered by glycerol, as reflected in an increased interdigitation of the nonpolar regions of the bilayer (O'Leary and Levin, 1984; Rudenko et al, 1984; Boggs and Rangaraj, 1985). This should alter permeability rates of water (essential to survival from freeze-thaw treatments) as well as specific and nonspecific transmembrane ion movements (Bashford et al, 1986). When the suspension freezes, extracellular ionic strength increases, and this (plus interactions with polyalcohols) can also alter permeability (Bashford et al, 1986; de Gier, 1989). Therefore, it is reasonable to assume that the response of each membrane type to cryoprotectant can dictate the extent of water and ion movement.

Electron microscopic studies established that glycerol addition to yeast causes alteration of membrane structure (Niedermeyer et al, 1977) and formation of gap-junction-like structures in rat prostate tissue (Kachara and Reese, 1985). Specific proteins are lost from erythrocytes during freezing, and these losses are changed by addition of glycerol (Ballas, 1981). If bound proteins are essential to sperm-egg or sperm-oviduct interaction, functionality will be lost by their removal during processing.

Cryoprotectants could be expected to have direct effects on bioenergetic imbalance via: (1) increased ATP demand (stimulated ion pump activity) by virtue of the anticipated permeability changes discussed above; and (2) metabolism of the cryoprotectant (best illustrated for glycerol). Substrate cycles for hexose metabolism in sperm have been studied in detail (Hammerstedt, 1983; Hammerstedt and Lardy, 1983)and the repeated phosphorylation and dephosphorylation of substrates results in a large ATP demand in the cell. Glycerol can be phosphorylated and dephosphorylated by ubiquitous glycerol kinase and general phosphatases. Molar concentrations of glycerol supply sufficient substrate, and the reduced ATP synthetic capacity at low temperatures (discussed in next section) will make the cell especially susceptible to increased ATP demands.

An interesting example of the effect of membrane perturbants on bioenergetic balance has been provided by the description of anesthetic effects on cell membranes and a resultant interaction with cell function (Bangham and Hill, 1986). The search for a molecular explanation of anesthetic action has been directed toward a detailed study of membrane effects without an equally detailed consideration of resultant alteration of reactions of the aqueous phase. Perhaps the study of cryopreservation of cells has suffered from an equally myopic approach, where the effects of cryoprotectants on the colligative properties of water have dominated the research and alternative points of interaction on the cell have not been adequately studied.

Relationship of Bioenergetics to Survival of Cells Under Stress

When a cell is in balance with its environment, metabolism is characterized by regulated and

balanced rates of ATP synthesis and consumption (Fig. 8, top). A unique ATP/ADP ratio for that metabolic state (alternatively represented as the energy charge; EC = [ATP] + 0.5 [ADP] / ([ATP] + [ADP] + [AMP])) results from the balance of ATP yielding pathways and ATP consumption. In sperm, the latter are probably dominated by ion pumping and motility since little biosynthesis occurs in these cells (Inskeep and Hammerstedt, 1985).

When energy requirements are changed, the cell must be able to adjust the rates of ATP generation and consumption to establish a new ATP turnover rate reflecting the new situation. If generation and consumption rates change equally, ATP concentration (and EC) remains constant. Alteration in EC indicates a mismatch between ATP production and consumption. Data for bull sperm (Table 2) illustrate how decreased temperature lowers ATP consumption (by reducing motility?) more than ATP generation (represented by glucose consumption rate) with an increase in EC. Thus, for this cell type and situation, a decrease in temperature has not created a deficit in the "energy budget." Since the actual ATP cycle (Fig. 8, top) is complex, with each half composed of many individual reactions (Hammerstedt and Lovrien, 1983; Inskeep and Hammerstedt, 1985; Hammerstedt et al, 1988), its temperature response will be the summation of the temperature dependence of each individual step. Data for ejaculated bull and ram sperm (Table 3) clearly establish that sperm from various species can differ in the temperature sensitivity of their

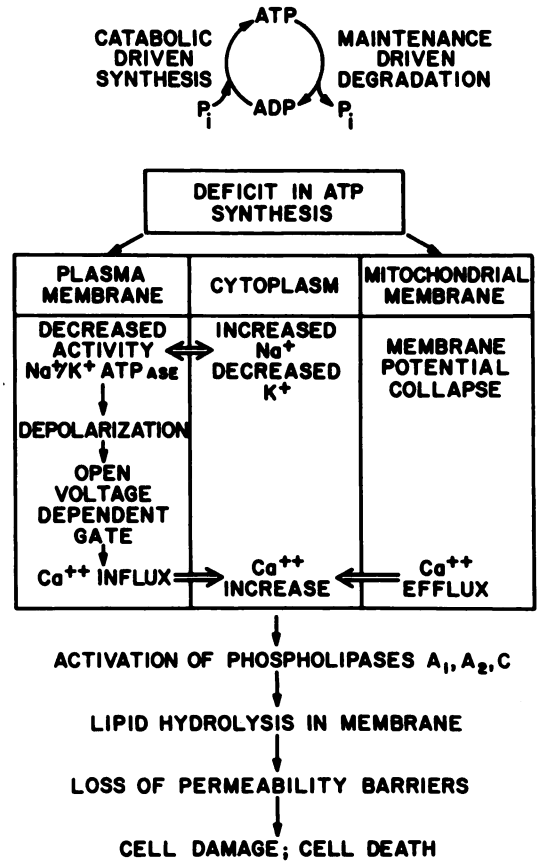

Fig. 8. Progression of events associated with unbalanced ATP synthesis-degradation. To maintain balanced ATP synthesis and degradation (top panel) when the temperature of incubation is altered, the rates of each half of the overall cycle must have the same temperature dependence. If this is not the case, a cascade of events (lower panel) is initiated whereby defective ion transport processes could result in collapse of the membrane potentials and increase in cytosolic calcium. This could be reversed by a timely elevation of cellular ATP. If the cell experiences a prolonged period of low ATP, processes such as activation of membrane phospholipases, hydrolysis of lipids and irreversible loss of membrane permeability, and cell damage and death can be initiated.

TABLE 2. Effect of Temperature on Metabolic Rate and Motility of Bovine Sperm

	Temperature (C)			
Metabolic Parameter	18	24	32	37
Glucose consumed*	0.14	0.33	1.03	2.37
Energy charge	0.81	0.75	0.77	0.74
Progressive motility†	9	20	42	58
Relative velocity†	0.8	1.4	2.3	3.1

Data taken from Hammerstedt and Hay (1980); details of statistical significance are presented therein.

*Expressed as micromoles consumed per h per 10^8 sperm.

†Motility of each preparation was recorded on videotape and evaluated by two different people unaware of sample treatment. Relative velocity of forward movement was based on an arbitrary scale of 0 = no movement; 4 = rapid movement.

TABLE 3. Efffect of Decreased Temperature of Incubation on Metabolic Parameters of Ejaculated Bull and Ram Sperm*

Metabolic Parameter	Bull	Ram
Total O_2 consumption	0.03	0.17
Total glucose consumption	0.14	1.00
Mitochondrial oxidation of pyruvate	0.10	0.50

Unpublished data of Hammerstedt and Hay for washed sperm suspensions incubated and analyzed using methods described in Hammerstedt (1975). Data are expressed as the ratio of metabolic rate at 22 C to metabolic rate at 37 C.

individual ATP generating pathways. Similar differences occur in ATP consumption pathways (Hammerstedt and Hay, 1980; Inskeep and Hammerstedt, 1985). These observations establish that a detrimental mismatch in ATP synthesis and degradation is not observed for bull and ram sperm, but the concept remains one that should be checked with sperm from other species.

Hochachka (1986) and Hochachka and Guppy (1987) elegantly outlined the energetics of cells that survive extreme environmental changes (eg, hypothermic responses of hibernators and anoxic conditions within diving seals). These systems survive by the capacity for effective metabolic coupling of the ATP cycle so that ATP consumption is slowed more rapidly than is ATP synthesis. For nonsurvivors, ATP concentrations drop and initiate an irreverisble set of reactions (Fig. 8, bottom). The inability to maintain ion balance results in an increase of cytosolic calcium which, in turn, activates phospholipases and proteases, and alters membrane structure. Such damage initiates irreversible sets of degradative steps that lead to loss of cell viability. In cryopreservation, bull sperm apparently maintain metabolic coupling when the temperature is decreased (Table 2), but perhaps sperm from other species cannot do so, especially if ATP demand is increased by metabolism of glycerol via substrate cycling reactions or cryoprotectant-induced increases in ion permeability discussed in earlier sections.

How Do These Concepts Fit Into the Processing Protocol?

The reader is directed to Figs. 1 and 3, as well as the primary data cited earlier, as we attempt to integrate existing concepts into our hypothetical cryoprocessing protocol. It is expected that future experimental results will invalidate some of the specific postulates. This prospect is welcomed since our objective is to stimulate alternative experimental tests in an area where contemporary techniques of membrane and cell biology are not being applied.

In the first step of the process, sperm are mixed with a diluent containing a buffer (citrate, TRIS or TES), divalent metal ions, glucose or fructose, and crude lipid (skim milk or egg yolk). Some variants contain a nonpenetrating cryoprotectant (lactose, PVP) and a detergent (eg, Orvus paste). The diluted sperm, plus all other components of the seminal plasma, are then cooled at a moderate rate to 4 C. Within a period of 30 min, the suspension passes through the critical temperature zone of 15–20 C, where the physical properties of the membrane change drastically. The following events should be considered: (1) buffers can chelate metal ions, altering ion-lipid head group interactions; (2) proteins bound to the surface can be altered (displacement by other components in the diluent or as a result of temperature-dependent equilibria); (3) sugars can bind to head groups, causing a partial immobilization of these components plus an alteration in the head group spacing; (4) lipid droplets may interact with the glycocalyx; (5) cooling could result in lipid segregation, with integral proteins entering into an altered lipid environment; (6) bioenergetic balance could be altered, as sperm stop flagellar action and catabolic processes adjust; and (7) ion balance could be altered, as the result of changes in pore structures and ion pumps in the membrane. Reviews (Watson, 1981; Watson and Morris, 1987) dealing with cold shock effects summarize data supporting many of these suggestions. Yet to be considered are the effects of male-to-male variations in the components of the seminal plasma, including, for example, sterol exchange proteins found in seminal plasma (Baker and Hammerstedt, unpublished data).

The next step brings the challenge of responding, in a period of seconds, to the water efflux caused by glycerol addition followed by the return to normal cell volume as the glycerol enters at a slower rate. Since the phase state of the membrane could be altered by cooling, rates of cryoprotectant permeability may differ greatly from those that the cell would experience if tested before cooling. These events should be considered: (1) as the cell contracts, inner surfaces of the membrane bilayer may come into direct molecular contact; (2) polyglycols may alter pore structures and ion transport; (3) bound glycoproteins could be displaced by the high concentration of glycols; (4) glycerol probably will enter *into* the lipid bilayer, causing a disordering of the acyl side chains; (5)) substrate cycling is possible, increasing the bioenergetic demand on the cell at a time when ATP synthesis capacity is minimal; and (6) the cells will be subjected to mechanical stresses as they are packaged for storage. Membrane related events are slow at lowered temperature (because of the reduced mobility of the various components), but the extended time spent at this step should allow many of these events to occur.

The next step, freezing, is critical to survival. The cells, probably affected by the first steps of the processing protocol, are cooled at a rapid rate (about 20 C/min). Within a minute, external crystallization of ice starts, and the cell membrane begins its tortuous path to the dehydrated storage state. As the various solutes rapidly rise in concentration in the remaining liquid phase, these events are possible in the next few minutes: (1) the cooling process is retarded by the heat of fusion as solidification occurs, and then the cooling process continues; (2) lipid segregation could continue and head group spacing may be altered; (3) hexagonal II phase formation may occur in the lipids; (4) pore structures could be changed even further; (5) proteins could be displaced as the salt and glycerol concentrations exceed 1 M; (6) osmotically driven contraction occurs over a period of seconds, perhaps driving the inner halves of the membrane bilayer together as the surface area adjusts to the change in cell volume; (7) bioenergetic metabolism essentially ceases; (8) solidification of external solvent induces cellular dehydration; and (9) the sperm become entrapped in small liquid channels of elevated solute concentration between the dominant ice crystals. Since sperm from various species differ in membrane composition, which influences transport rates, alternative solutions to the successful passage through these steps are necessary.

Storage brings a period of respite, since low temperatures result in minimal molecular motion. Alterations in this step are believed to be minimal, but direct proof of this is not available.

In contrast, thawing and insemination changes are dramatic, both in extent and rapidity. Within a minute, a reversal of the steps 1–3 occurs, but the cell clearly could differ from the unit that entered the process because of glycerol permeation into all elements of the cell. The melting of external ice crystals rapidly reduces solute concentrations and initiates an inward rush of water. As the temperature rises and the cell expands, these events could occur: (1) surface area of the sperm returns to normal; (2) lipids and proteins of the membrane regain mobility; (3) bioenergetic demand (ion balance, motility) increases; (4) integral membrane proteins redistribute within their lipid environment, with the pores, receptors, etc., potentially having changed properties; (5) surface components displaced during the processing must rebind; and (6) the glycerol is removed from the system (in cattle, after deposition into the female tract) with a resultant swelling of the cell and an eventual return to the original volume.

Concluding Remarks

Design of protocols for the cryopreservation of sperm from the various species should contain these elements: (1) a consideration of the sperm as an integrated set of membrane-bounded compartments, with an assay for evaluation of the functionality of each; (2) high concentrations of glycerol are likely to alter many aspects of the system in addition to the colligative properties of bulk solvent; (3) each and every compartment may have its own relationship to both temperature and potential interaction with the diluent, predisposition to osmotic swelling and resultant damage, and ATP requirements; and (4) solution to these potential problems will require successful development of a complex multifactorial equation (one factor for each subcompartment).

Solution to the problem will not be simple, but the path to success is becoming more apparent. Gamete research proceeds to a large extent as a derivative science, whereas advances in other areas (having the luxury of a relative abundance of investigators) are tested in the sperm-egg system. Careful comparisons will enrich the database, but the unique features of the gametes and their environments require careful interpretation and integration before data can be accepted. For example: (1) the roles of the various sperm subcompartments will become more clearly defined as appropriate assays are developed; (2) analytical procedures for isolating membranes from these compartments also are being developed, allowing distinctions to be drawn from compositional studies; (3) model membrane systems will continue to be intensively studied, with the roles and interactions of the components being characterized with regard to both individual and aggregate properties; and (4) an increased understanding of the events of fertilization and sperm-egg interactions will allow a more precise assessment of the effects of processing protocols on the desired endpoint of the process.

At this time, confusion exists about how to proceed. One reason for this is our tendency to consider all sperm alike, and therefore to seek a homogeneous mechanism for the biological events related to fertilization. Species differences are probable, but they are unlikely to be recognized unless care is taken: (1) to seek common mechanisms from a fundamental rather than a phenomenological

basis (eg, perhaps membrane destabilization is the important event in acrosomal activation, and therefore sperm systems could use either phospholipase or protease action to accomplish the desired goal); and (2) to remember to insert the species as a qualifier when describing data, especially in reviewing the literature.

The combination of growth in sister disciplines, rapid developments in the chemistry of lipid interfaces and surfaces, entry of newly trained investigators with unique perspectives on the problem to be addressed, and a greater appreciation of the rewards to be gained from development of successful protocols are providing the driving forces to guide research in cryopreservation in the next decade.

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References

Agrawal P, Magargee SF, Hammerstedt RH. Isolation and characterization of the plasma membrane of rat cauda epididymal spermatozoa. J Androl 1988;9:178–189.

Aloia RC, Curtin CC, Gordon LM. Lipid Domains and the Relationship to Membrane Function. New York: Liss, 1988.

Amann RP, Pickett BW. Principles of cryopreservation and a review of the cryopreservation of stallion spermatozoa. Equine Vet Sci 1987;7:145–173.

Anchordoguy TJ, Rudolph AS, Carpenter JF, Crowe JH. Modes of interaction of cryoprotectants with membrane phospholipids during freezing. Cryobiol 1987;24:324–331.

Armitage WJ. Osmotic stress as a factor in the detrimental effect of glycerol on human platelets. Cryobiol 1986;23:116–125.

Armitage WJ, Mazur P. Toxic and osmotic effects of glycerol on human granulocytes. Am J Physiol 1984;247:C382–C389.

Ballas SK. Red cell membrane protein changes caused by freezing and the mechanisms of cryoprotection by glycerol. Transfusion 1981;21:203–210.

Bangham AD, Hill MW. The proton pump/leak mechanism of unconsciousness. Chem Phys Lipids 1986;40:189–205.

Bashford CL, Alder GM, Menestrina G, Micklem KJ, Murphy JJ, Pasternak CA. Membrane damage by hemolytic viruses, toxins, complement and other cytolytic agents. J Biol Chem 1986;261:9300–9308.

Bedford JM, Calvin HI. The occurrence and possible functional significance of -S-S- crosslinks in sperm heads, with particular reference to eutherian mammals. J Exp Zool 1974;188:137–156.

Bredderman PJ, Foote RH. Volume of stressed bull spermatozoa and protoplast droplets, and the relationship of cell size to motility and fertility. J Dairy Sci 1969;28:496–501.

Boggs JM, Rangaraj G. Phase transitions and fatty acid spin label behavior in interdigitated lipid phases induced by glycerol and polymyxin. Biochim Biophys Acta 1985;816:221–233.

Carruthers A, Melchior DL. Effects of lipid environment on membrane transport. The human erythrocyte sugar transport protein/lipid bilayer system. An Rev Physiol 1988a;50:257–271.

Carruthers A, Melchior DL. Role of bilayer lipids in governing membrane transport processes. In: Aloia RC, Curtin CC, Gordon LM, eds. Lipid domains and the relationship to membrane function. New York: Liss, 1988b;201–225.

Crowe JH, Crowe LM, Carpenter JF, Aurell Wistram C. Stabilization of dry phospholipid bilayers and proteins by sugars. Biochem J 1987;242:1–10.

Cullis PR, Hope MJ. Physical Properties and Functional Roles of Lipids in Membranes. In: Vance DE, Vance JE, eds. Biochemistry of lipids and membranes. Menlo Park, CA: Benjamin/Cummings, 1985;25–72.

Darin-Bennett A, White IG. Influence of the cholesterol content of mammalian spermatozoa on susceptibility to cold shock. Cryobiol 1977;14:466–470.

de Gier J. Osmotic properties of liposomes. In: Benga G, ed. Water transport in biological membranes. Vol 1. From model membranes to isolated cells. Boca Raton, FL: CRC Press, 1989;77–98.

Drevius L-O,Erickson H. Osmotic swelling of mammalian spermatozoa. Exp Cell Res 1966;42:136–156.

Duncan AE. Studies of the effect of freezing rate on the cryosurvival of ram spermatozoa. PhD Thesis 1988; University of London.

Eddy EM. The spermatozoon. In: Knobil E, Neil J, eds. The physiology of reproduction. New York: Raven Press, 1988;27–68.

Evans RW, Setchell BP. Association of exogenous phospholipids with spermatozoa. J Reprod Fertil 1978;53:357–362.

Fiser PS, Fairfull RW. The effect of glycerol-related osmotic changes on post-thaw motility and acrosomal integrity of ram spermatozoa. Cryobiol 1989;26:64–69.

Foote RH. Cryopreservation of spermatozoa and artificial insemination: Past, present and future. J Androl 1982;3:85–100.

Foote RH. Buffers and extenders. What do they do? Why are they important. Proc X NAAB Tech Conf AI Reprod 1984;62–70.

Foulkes JA. The separation of lipoproteins from egg yolk and their effect on the motility and integrity of bovine spermatozoa. J Reprod Fertil 1977;49:277–284.

Goodrich RP, Handel TM, Baldeschwieler JD. Modification of lipid phase behavior with membrane-bound cryoprotectants. Biochim Biophys Acta 1988;938:143–154.

Gorospe WC, Conn PM. Membrane fluidity regulates development of gonadotrope desensitization of GnRH. Mol Cell Endocrinol 1987;53:131–140.

Gruner SM. Intrinsic curvature hypothesis for biomembrane lipid composition: A role for nonbilayer lipids. Proc Natl Acad Sci USA 1985;82:3665–3669.

Hammerstedt RH. Use of high speed dialysis to prepare bovine sperm for metabolic studies. Biol Reprod 1975;13:389–396.

Hammerstedt RH. Use of sperm as model for the study of metabolism. In: Lennon DF, Stratman FW, Zahlten RN, eds. Biochemistry of Metabolic Processes. New York: Elsevier, 1983;29–40.

Hammerstedt RH. Membrane models: Evolution from the fluid-mosaic standby. In: Basic Sciences Foundation of Medically Assisted Conception: An Agenda. Washington DC: National Academy Press, 1989;155–175.

Hammerstedt RH, Keith AD, Snipes WC, Amann RP, Arruda D, Griel LC Jr. Use of spin labels to evaluate effects of cold shock and osmolality on sperm. Biol Reprod 1978;18:686–696.

Hammerstedt RH, Hay SR. Effect of incubation temperature on motility and cAMP content of bovine sperm. Arch Biochem Biophys 1980;199:427–437.

Hammerstedt RH, Lardy HA. The effect of substrate cycling

on the ATP yield of sperm glycolysis. J Biol Chem 1983;258:8759-8768.
Hammerstedt RH, Lovrien RE. Calorimetric techniques for metabolic studies of cells and organisms under normal conditions and stress. J Exp Zool 1983;228:459-469.
Hammerstedt RH, Parks JE. Changes in sperm surfaces associated with epididymal transit. J Reprod Fertil Suppl 1987;34:133-149.
Hammerstedt RH, Volonte C, Racker E. Motility, heat, and lactate production in ejaculated bovine sperm. Arch Biochem Biophys 1988;266:111-123.
Hempling HG, White S. Permeability of cultured megakaryocytopoietic cells of the rat to dimethylsulfoxide. Cryobiol 1984;21:133-143.
Hinkovska V, Dimitrov G, Koumanov K. Phospholipid composition and phospholipid asymmetry of ram spermatozoa plasma membrane. Int J Biochem 1986;18:1115-1121.
Hinkovska-Galcheva V, Petkova D, Koumanov K. Changes in the phospholipid composition and phospholipid asymmetry of ram sperm plasma membranes after cryopreservation. Cryobiol 1989;26:70-75.
Hochachka PW. Defense strategies against hypoxia and hypothermia. Science 1986;231:234-241.
Hochachka PW, Guppy M. Metabolic arrest and the control of biological time. Cambridge MA: Harvard Univ Press, 1987.
Houslay MD, Stanley KK. Dynamics of bilayer membranes. New York: Wiley, 1982.
Inskeep PB, Hammerstedt RH. Endogenous metabolism by sperm in response to altered cellular ATP requirements. J Cell Physiol 1985;123:180-190.
Israelachvili JN, Marcelja S, Horn RG. Physical principles of membranes organization. Q Rev Biophys 1980;13:121-200.
Jain MK. Introduction to Biological Membranes; 2nd Ed. New York: Wiley, 1988.
Jeyendran Rs, Van der Ven HH, Perez-Pelaez M, Crabo BG, Zaneveld LJD. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. J Reprod Fertil 1984;70:219-228.
Jones RC. Ultrastructure of mammalian spermatozoa: The effects of buffer concentration in fixatives for boar spermatozoa. Micron 1971;2:350-362.
Kachara B, Reese TS. Rapid formation of gap-junction-like structures induced by glycerol. Anat Rec 1985;213:7-15.
Knox JM, Schwartz GS, Deller KR. Volumetric changes in cells during freezing and thawing. J Biomech Eng 1980;102:91-97.
Kuypers FA, Roelofsen B, Berendsen W, Op den Kamp JAF, van Deenen LLM. Shape changes in human erythrocytes induced by replacement of the native phosphatidylcholine with species containing various fatty acids. J Cell Biol 1984;99:2260-2266.
Levitt DG, Mlekoday HJ. Reflection coefficient and permeability of urea and ethylene glycol in the human red cell membrane. J Gen Physiol 1983;81:239-253.
Mazur P. Freezing of living cells: Mechanisms and implications. Am J Physiol 1984;247:C125-142.
Mazur P, Cole KW. Roles of unfrozen fraction, salt concentration and change in cell volumes in the survival of frozen human erythrocytes. Cryobiol 1989;26:1-29.
Mazur P, Leibo SP, Miller RH. Permeability of the bovine red cell to glycerol in hyperosmotic solutions at various temperatures. J Membr Biol 1974;15:107-136.
Mazur P, Miller RH. Permeability of the human erythrocyte to glycerol in 1 and 2 M solutions at 0 or 20 C. Cryobiol 1976;13:507-522.
Mazur P, Rajotte RV. Permeability of the 17-day fetal rat pancreas to glycerol and dimethylsulfoxide. Cryobiol 1981;18:1-16.
McElhaney RN. Differential scanning calorimetry studies of lipid-protein interaction in model systems. Biochim Biophys Acta 1986;864:361-421.
Morris GJ. The response of liposomes to various rates of cooling to −196C: effect of phospholipid:cholesterol ratio. Cryobiol 1982;19:215-218.
Morris GJ, McGrath JJ. The response of multilamellar liposomes to freezing and thawing. Cryobiol 1981;18:390-398.
Muller CP, Krueger GRF. Modulation of membrane proteins by vertical phase separation and membrane lipid fluidity: Basis for a new approach to tumor immunotherapy. Anticancer Res 1986;6:1181-1194.
Neidermeyer W, Parish GR, Moor H. Reactions of yeast cells to glycerol treatment. Alterations to membrane structure and glycerol uptake. Protoplasma 1977;92:177-193.
Nikolopoulou M, Soucek DA, Vary JC. Changes in the lipid content of boar sperm plasma membranes during epididymal maturation. Biochim Biophys Acta 1985;815:486-498.
Ohki S, Doyle D, Flanagan TD, Hui SW, Mayhew E, eds. Molecular Mechanisms of Membrane Fusion. New York: Plenum, 1988.
O'Leary TS, Levin IW. Ramen spectroscopic study of an interdigitated lipid bilayer dipalmitoylphosphatidylcholine dispersed in glycerol. Biochim Biophys Acta 1984;776:185-189.
Parks JE, Hammerstedt RH. Developmental changes occurring in the lipids of ram epididymal spermatozoa plasma membrane. Biol Reprod 1985;32:653-668.
Pegg DT, Diaper MD. The unfrozen fraction hypothesis of freezing injury to human erythrocytes: A critical examination of the evidence. Cryobiol 1989;26:30-43.
Poulos A, Darin-Bennett A, White IG. The phospholipid-bound fatty acids and aldehydes of mammalian spermatozoa. Comp Biochem Physiol 1973;46B:541-549.
Quinn PJ. Principles of membrane stability and phase behavior under extreme conditions. J Bioenerg Biomemb 1989;21:3-19.
Quinn PJ, Chow PYW, White IG. Evidence that phospholipid protects ram spermatozoa from cold shock at a plasma membrane site. J Reprod Fertil 1980;60:403-407.
Rao KV. Role of Cell Surface in Development. Vol. 1, Boca Raton, FL: CRC Press, 1987.
Rudenko SV, Gapochenko SD, Bondarenko UA. Effect of glycerol on the capacitance and conductivity of bilayer lipid membranes. Biophysics 1984;29:245-249.
Schneider U. Cryobiological principles of embryo freezing. J In Vitro Fert Embryo Trans 1986;3:3-9.
Schneider U, Mazur P. Osmotic consequences of cryoprotectant permeability and its relationship to the survival of frozen-thawed embryos. Thereogenology 1984;21:68-79.
Shabana M, McGrath JJ. Cryomicroscope investigation and thermodynamic modeling of the freezing of unfertilized hamster ova. Cryobiol 1988;25:338-354.
Singer SJ, Nicholson GL. The fluid mosaic model of the structure of cell membranes. Science 1972;175:720-731.
Steponkus PL, Lynch DV. Freeze/thaw-induced destabilization of the plasma membrane and the effects on cold acclimations. J Bioenerg Biomembr 1989;21:21-41.
Strauss G, Ingenito EP. Stabilization of liposome bilayers to freezing and thawing: Effects of cryoprotective agents and membrane proteins. Cryobiol 1980;17:508-515.
Tilcock CPS, Cullis PR. Lipid polymorphism. Ann NY Acad Sci 1987;492:88-102.
Verkman AS, Masur SK. Very low osmotic water permeability and membrane fluidity in isolated toad bladder granules. J Membr Biol 1988;104:241-251.
Voelker DR. Lipid assembly into cell membranes. In: Vance DE, Vance JE, eds. Biochemistry of Lipids and Membranes. Menlo Park, CA: Benjamin/Cummings, 1985;475-502.

Watson PF. The protection of ram and bull spermatozoa by the low density lipoprotein fraction of egg yolk during storage at 5 C and deep freezing. J Therm Biol 1976;1:137-141.

Watson PF. The effects of cold shock on sperm cell membranes. In: Morris GJ, Clarke A, eds. Effects of low temperatures on biological membranes. New York, NY: Academic Press, 1981;189-218.

Watson PF, Morris GJ. Cold shock injury in animal cells. In: Bowler K, Fuller BJ, eds. Temperature and animal cells. Cambridge, UK. Company of Biologists Limited, 1987;311-340.

Future Meetings
American Society of Andrology

1991: Montreal, Canada

Testis Workshop: April 25-27
Postgraduate Course: April 27
Annual Meeting: April 28-30

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1992: Washington, DC

Postgraduate Course: March 26
Annual Meeting: March 27-30

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1993: Tampa, Florida

Postgraduate Course: April 15
Annual Meeting: April 16-18
(Dates are tentative)

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