An Assessment of the Prospects for Suspended Animation Through the Application of Natural Cold Tolerance Strategies

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Contents

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Chapter 1

Introduction

The very idea of "cold" seems contrary to life and is suggestive of death. As with many of the other extremes of seemingly inhospitable conditions, however, nature has adapted to the force of cold as an environmental pressure, and has established life in virtually all Earth temperature zones. Moreover, the adaptations that have evolved to protect different forms of life from cold have led in some cases to the beneficial use of low temperatures. In order to survive through harsh times of low food supply, lack of water, and cold itself, many species use cold to effect the reduction or virtual cessation of their metabolisms.

The existence of these abilities in advanced form, though, is very limited, and is completely absent in any of the large mammals.[1] It has become clear that the adaptations necessary for advanced cold tolerance are complex and extensive, often involving subtle changes at the cellular and molecular level. The inevitable question has come up as to whether or not these mechanisms could be mimicked in unadapted biological systems in order to reap the "enormous potential of low temperatures for the almost unlimited preservation of labile materials which, under normal circumstances, have a very limited life span" [*Franks*, 1985, p. 20]. In its most advanced form — the artificial application of these adaptations to accomplish cryopreservation or induced hibernation of humans — this hypothetical process has come to be known as "suspended animation."

Although the field has come into considerable disrepute through human cryopreservation's having been taken for granted in many science fiction works, and through the publicizing of its excessive and unscientific extrapolation into the commercial freezing of cadavers for future resuscitation, the study and applica-

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[1]Bears do not exhibit true hibernation; they simply go through an extended sleep.

tion of defenses against, and uses of, cold have wide scientific interest and several legitimate justifications for their growing importance. Firstly, there is scientific curiosity about the survival adaptations of Earth's plants and animals in general, and about cold as one of the fundamental biological forces; an increased knowledge of its effects could lead to new insights in other areas, such as a further understanding of the evolutionary effects of an ice age. Important uses of cold preservation techniques in medicine have also been suggested, including cell, tissue, and organ storage, and even whole-body applications during the imminent health care crisis [*Sternberg et al.*, p. 11]. Lastly, prospective preservation of specimens of endangered species and genotypes for agriculture, and uses in prolonged space travel are also possible incentives.

Despite these encouraging potential benefits, there are still major barriers obstructing success; the relatively small-scale cryopreservation of organs and even tissues is posing large problems. Nevertheless, modern research is continually extending current understanding of the underlying principles behind cold damage and cold tolerance of biological systems at the level of cells, tissues, and entire organisms. By identifying the essential biochemical bases behind natural cold survival strategies, and by applying this knowledge to other, larger systems such as that of a complete human, scientists may eventually be able to mimic natural cold tolerance and succeed in developing a procedure for artificial suspended animation.

Chapter 2

Principal Obstacles to Suspended Animation

In order to understand the natural adaptations which allow certain organisms to become cold tolerant, one must be aware of the fundamental obstacles to survival in suboptimal temperatures. The principal effects of cold which prevent any organism from simply moving somewhere cold and temporarily "slowing down" or stopping metabolic processes divide quite naturally into the two categories of "chill injury" and "freeze injury." In general terms, the first comprises the forms of damage which are due solely to a lowering of temperature, while the second includes the added effects of water undergoing the phase change from liquid to crystalline solid.

2.1   Water

That this classification of cold damage is based on the properties of water is not by chance. The H_2O molecule is the most common among life on Earth; there are very few metabolic or bio-synthetic reactions in which water does not serve as a reactant or product. Since water also undergoes a change of state in the middle of Earth's temperature range, it is a significant factor in any form of natural thermoregulation, especially for organisms adapted to survive through low temperature extremes; consequently, the properties of water are also of prime concern in any study of the artificial induction of hypometabolic states.

To begin with, some of the very basic properties of water are altered with changes in temperature. For instance, the increase in water's viscosity as it is
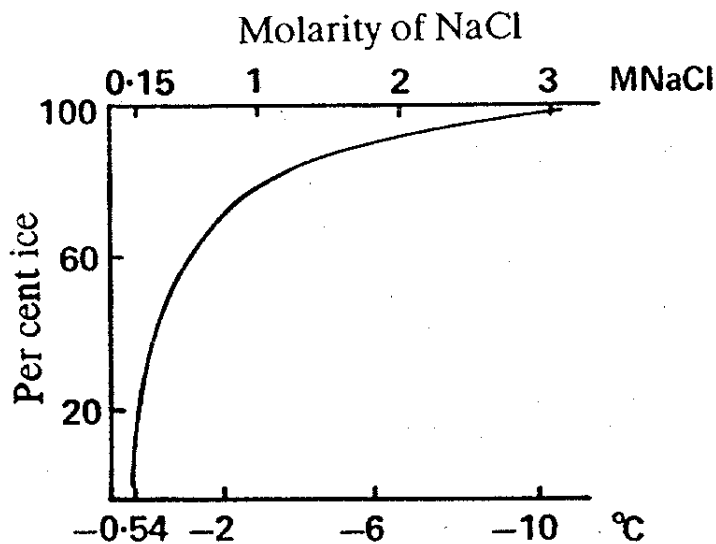
Figure 2.1: Degree of (simple) freeze concentration of a 0.154 M NaCl solution (isotonic saline). From [*Franks*, 1985, p. 58.].

chilled produces changes in its ionic activity and diffusion rates, which are significant in any biological system. A noticeable increase in the pH of neutral water also occurs with lowered temperature as a result of the decreasing dissociation constant [*Storey and Storey*, 1988, p. 53]. As well, the strength of hydrogen-bonds is especially thermolabile, and the energy changes resulting form cooling can have considerable effects on water, enzymes, membranes, and other macro-molecular complexes.

These causes of chill injury are augmented by those of freeze injury as temperatures are lowered below the freezing point. Firstly, water's unique characteristic of expanding below 4°C can result in physical stress and damage as ice is formed. This, coupled with the sharp points and edges of the growing crystals, can easily damage or rupture membranes, and makes survival of any intracellular ice impossible. In addition, the phenomenon of "freeze concentration" makes freezing very harmful even if it only occurs outside cells; as ice forms, the concentration of solutes in the remaining liquid water increases dramatically (see Figure 2.1), and the high concentrations, especially of salts, can lead to injury or death.

Another property of water in its solid phase that can produce harmful biological effects is its ability to recrystallize. "Recrystallization," or, as it is sometimes called, "ripening," is the disappearance of many small ice crystals during rewarm-
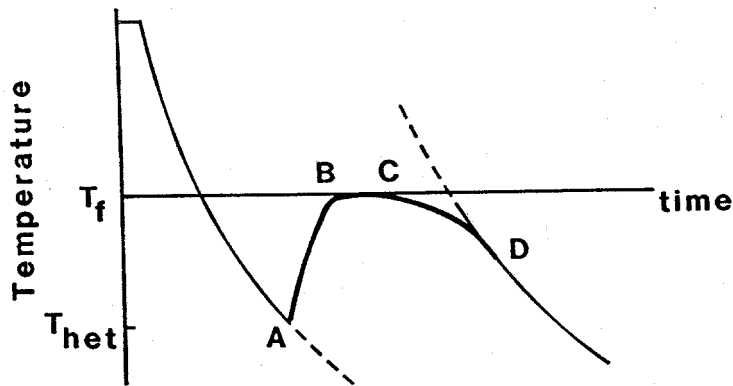
Figure 2.2: A typical cooling curve; A is the nucleation temperature (i.e. the water has undercooled to A); AB corresponds to recalescence (the release of latent heat); C corresponds to the onset of non-equilibrium freezing; freezing is complete at D. Recrystallization would occur between B and D. The bold line corresponds to freezing (0°C). (After Franks, 1982c.) From [*Franks*, 1985, p. 47].

ing to yield large ones. This rewarming can occur naturally during the process of freezing, as, after the first occurrence of ice, temperature rises slightly due to the latent heat given off by the phase change (see Figure 2.2). In biological tissue, the large crystals resulting from recrystallization can cause additional structural damage over that done by initial ice formation.

The dependency of water on nucleation for the formation of ice crystals is its final relevant property. Water that is below its melting point but still in the liquid phase because it lacks ice nuclei is said to be "undercooled," and the degree to which a particular body of water will become undercooled is called its "thermal hysteresis." The rate, or probability, of nucleation in a body of water is proportionally to its volume (see Figure 2.3). A completely pure, undisturbed, and minute volume of water has recently been shown to undercool to -40° C before freezing (this is called the homogeneous nucleation point), even though its melting point remains at 0°C [*Franks*, 1985, p. 29]. Undercooling can be dangerous for biological materials because it often ends in very sudden ice crystallization if the water is eventually nucleated. It may, on the other hand, also be used advantageously to achieve low temperatures without the complications of intercellular ice formation.
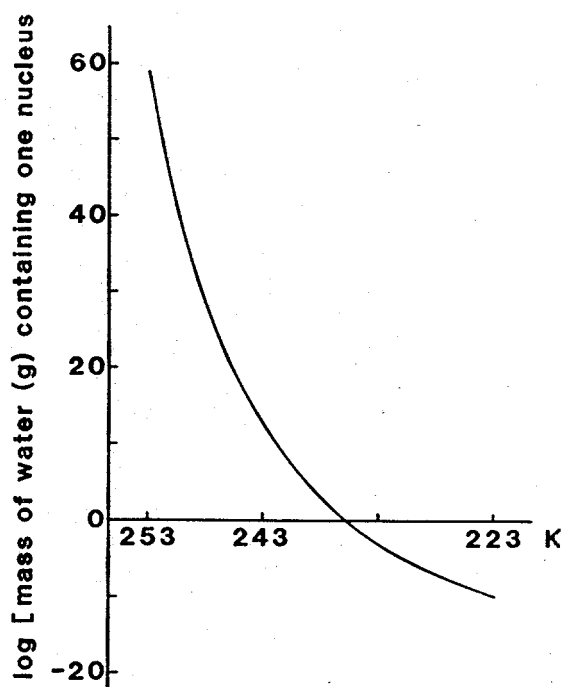
Figure 2.3: Mass of water likely to contain one critical size nucleus as a function of temperature, assuming homogeneous nucleation. From [*Franks*, 1985, p. 28].

2.2   Proteins and Membranes

The physical effects of cold apply not only to water itself, but to other, more complex, biological entities which are vital to cell operation, most notably the temperature-sensitive proteins and fragile membranes which are universal among Earth life. The performance of both proteins and membranes is very dependent on their structure, and both have very intricate and thermolabile structures. Consequently, any reduction in temperature — at least in any unprotected systems — can have profound repercussions due to reversible or irreversible damage to these fundamental building blocks.

The structural stability of proteins, with their three or four levels of organization, is a delicate balance between the forces of several different weak-bond interactions; in fact, the native form of the average protein, containing over 200 intrapeptide hydrogen bonds, is more stable than the denatured form by the energy of only a few hydrogen bonds.[*Franks*, 1985, p. 64] This marginal stability accounts for the phenomena of cold-induced dissociation and temporary denaturation, or, as a result of altered protein-protein interactions, the even more serious and usually irreversible aggregation of proteins. Added protein distortion can also occur as a result of ice crystal formation, and, more importantly, freeze concentration tends to put very large stresses on proteins, with the result of permanent denaturation or coagulation.

Even more complex than the structure and operation of individual proteins is the intricate organization of biological membranes. Temperature greatly affects all membrane-mediated processes, such as the maintenance of osmotic balance, provision of structure and protection from deformation, selective transfer and cell-cell communication, and specialized operations such as neural impulse transmission, which are carried out by cell ("plasma") membranes. Thylakoid membranes, which are instrumental in photosynthesis and respiration, are especially sensitive, and are among the first structures to be completely inactivated by cold.

The enzymes embedded in thylakoid and plasma membranes, as described by the fluid-mosaic model *Singer and Nicolson* [1972], are thought to undergo the same complex structural damage with low temperature as do free proteins (described above), resulting in changes in permeability and membrane action*Pringle and Chapman*; however, little has been shown conclusively.

Much better understood is chill injury done to the membrane in terms of its carbohydrate chains and the conformation, orientation, and mobility of its phospholipids [*Storey and Storey*, 1988, p.28]. There exists for any particular membrane a certain low temperature at which the lipids go through a phase change, becoming
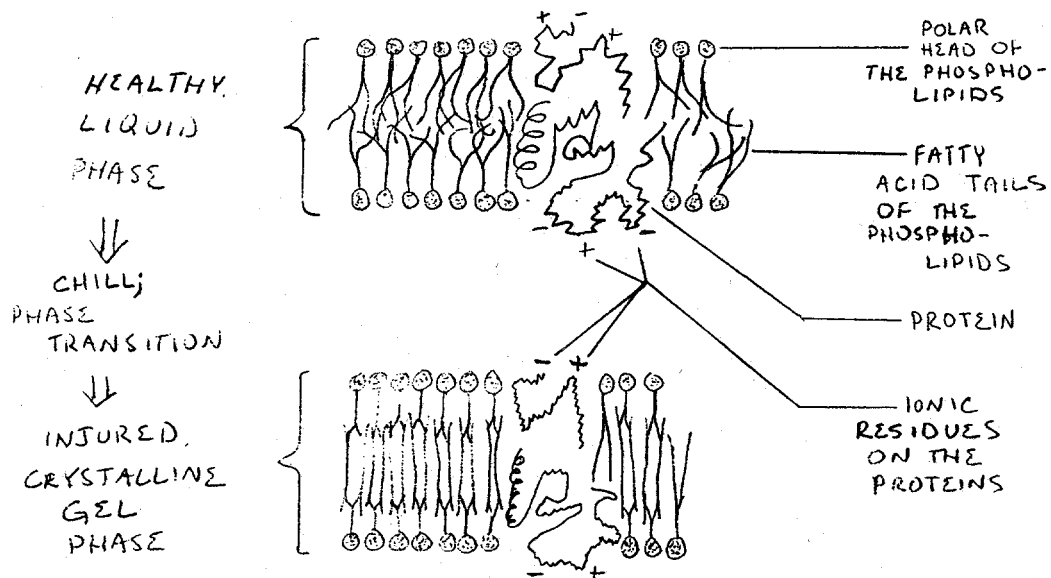
Figure 2.4: The chill-induced thermotropic phase change in a cell membrane. The phospholipids lose their normal fluidity (a) and become rigid and semi-crystalline (b) as they change to the gel phase. Proteins (shown here) and carbohydrate chains (not shown) simultaneously undergo thermotropic distortion, and thus lose their functional capabilities. After J. M. Wilson and A. C. McMurdo, "Chilling Injury in Plants," in Effects of Low Temperatures on Biological Membranes, eds. G. J. Morris and A. Clarke (London: Academic Press Inc., 1981), p. 149.

hexagonally semi-crystalline, and thus losing their vital diffusional freedom (see Figure 2.4). At the same time, the carbohydrate chains undergo thermotropism, becoming fully extended, and therefore no longer functional [*Pringle and Chapman*, p.97]. Naturally, this phase change causes a profound loss of membrane function, although it is generally reversible.

Freeze injury to membranes is a consequence of freeze concentration. As extracellular water freezes, the remaining extracellular solution becomes hypertonic and causes cell dehydration, thus concentrating intracellular solutes as well [*Grout and Morris*, p.152]. Consequently, the previously mentioned effects of freeze concentration occur not only to extracellular proteins, but to cell-bound enzymes in addition. If ice formation occurs rapidly, the membrane itself also suffers osmotic shock due to the rapidly changing osmotic gradient. The cell dehydration results in a considerable volume decrease, and thus in added membrane distortion, altered

membrane tensions, and lipid-lipid repulsion.

2.3   Metabolic Balance

Lastly, cold injury to live biological material may be caused by the physiological result of differential reaction rate changes. The estimated 100,000 different proteins in a single organism each have specific and differing reactions to thermal changes, and the effect of reduced temperature on enzyme-catalyzed reactions is not always Newtonian; it does not follow the Arrhenius curve. Thus, the unequal reductions of, and in some cases even increases in, biological reaction rates in suboptimal temperatures result in a disruption of cellular energetics and an increasing degree of metabolic imbalance. Because of the complexity of biochemical pathways, chains of reactions, and the reactions themselves, net production is far from being dependent solely on the slowest reaction step, and physiological imbalances can build up with time. One final form of such metabolic damage occurs when intercellular ice forms. In this case, cells can no longer carry out nutrient and gas exchange, and must survive on endogenous resources, or go short on fuel and other metabolites.

Chapter 3

Natural Examples of Cold Tolerance

Despite these seemingly insurmountable problems, there are a number of organisms that have developed means of resisting chill and freeze injuries, and are able to survive cold instead of avoiding it. Research has identified many of the strategies behind "freeze tolerance," "hibernation," and "freeze avoidance." and has begun to reveal the biochemical mechanisms behind these adaptations.

3.1 Freeze Tolerance

Species which incorporate the formation of internal ice as a part of their strategy to survive and make use of the effects of cold are said to be "freeze tolerant." The ice that forms, however, occurs only in extracellular fluid compartments, as no documented cases of survival after natural intracellular freezing exist. Recognized freeze tolerant organisms presently include plants, insects, intertidal invertebrates (barnacles, bivalves, gastropods, annelids, and nematodes), four species of frog, and (since recently) one species of turtle.[1]

In order to prevent or overcome the effects of freeze injury, freeze tolerant organisms have had to accomplish four general protective changes. These are (1)the control of extracellular ice formation in order to limit osmotic stress and permit the gradual shrinkage of membranes; (2) the control of cell dehydration and intracellular freeze concentration, and the prevention of intracellular freezing; (3) the stabilization of proteins and membranes against structural damage; and (4) the ability of tissues to survive long-term ischemia [*Storey and Storey*, 1988, p.67].

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[1]Smaller freeze tolerant organisms also exist, such as the microscopic *Rotifera* and *Tardigrada*.[*Storey and Storey*, 1988, p.32]

One adaptation used to control extracellular ice formation and render freezing non-injurious is the use of specific ice-nucleating proteins (INP's) in the hemolymph.[2] This has been identified as an important strategy in the freeze tolerance mechanisms of insects and intertidal mollusks,[3] and has several functions [*Storey and Storey*, 1988, p.40]. By efficiently acting as crystallization nuclei,[4] they cause extracellular nucleation to occur at relatively high (above -10°C) temperatures. This minimizes undercooling and ensures that ice formation rates are kept low. Also, by initiating ice formation at multiple sites, INP's promote the formation of very small crystals. These effects are important for preventing physical and osmotic cell membrane damage by ensuring that crystallization and the effects of freeze damage are gradual; this results in gentle concentration increases and the easing of osmotic shock, gradual cellular dehydration and membrane deformation due to the decrease in cell size, and a smooth metabolic transition from oxygen supply to anoxia.

As a result of such measures, the retarded freezing process has been found to take a total of 48 hours in the gall fly larva *Eurosta solidaginis Lee and Lewis* [1985] and 24 hours in the wood frog *Rana sylvatica Layne and Lee* [1987].

Another adaptation which has developed to limit the damage of extracellular ice is the use of "anti-freeze proteins" (AFP's)[5] which, by themselves, lower the freezing point relative to the melting point (i.e., induce undercooling). AFP's have been found in only three freeze tolerant species — two insects and one intertidal bivalve [*Storey and Storey*, 1988, p.41] — and, despite their seemingly contradictory functions, they were being used simultaneously with INP's. This paradox was resolved when it was pointed out *Knight and Duman* [1986] that AFP's were very effective in preventing the harmful phenomenon of recrystallization. Thus, the combination of INP's and AFP's induces gradual, even ice formation without extensive recrystallization during freezing. The chemical mechanism of AFP's, however, is, like that in INP's, still quite unclear. Recent studies have shown that AFP's also radically alter the morphology of growing ice crystals, causing parallel instead of dendritic needle growth *DeVries* [1983], which may be a factor in reducing physical freeze damage.

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[2]Both protein and lipoprotein INP's have been found *Duman et al.* [1985] and one from *Vespula maculata* has been purified *Duman et al.* [1984].

[3]INP's may also be used by frogs and / or turtles; it has not yet been shown either way.

[4]The mechanism behind INP catalysis of ice nucleation is not yet clear, although INP's have been found to be hydrophilic and their nucleating ability shown to be concentration dependent [*Storey and Storey*, 1988, p.40].

[5]These are also known as thermal hysteresis proteins, THP's.

As well as the complex polypeptide INP's and AFP's, freeze tolerant species synthesize a variety of simpler organic compounds which help to prevent the intracellular effects of freeze damage and stabilize protein and membrane structure. The first group of these low-molecular-weight solutes works solely through colligative effects. Firstly, accumulation of high intracellular solute levels colligatively reduces the melting point and thus inhibits intracellular freezing. Secondly, by making cellular fluid increasingly hypertonic, elevated concentrations of solutes considerably reduce the effects of cell dehydration as extracellular ice forms. This prevents the damaging phenomena of cell shrinkage and the intracellular freeze concentration of salts [*Storey and Storey*, 1988, p.69].

The compounds that accomplish this colligative protection in freeze tolerant organisms are usually sugars and sugar alcohols, although the use of amino acids and other intermediary metabolites is also common; the polyols glycerol, sorbitol, ribitol, threitol, and erythritol, the sugars glucose, sucrose, and trehalose, and the amino acids proline and alanine have all been found to increase radically in various freeze tolerant organisms during cold hardening. The sugars and polyols are produced from starch in plants and from glycogen in animals (see Figure 3.1), and the amino acids are thought to be synthesized *de novo*. Most insects predominantly use glycerol and sorbitol as a pair (see Figure 3.2), and frogs typically generate extremely high levels of glucose; blood glucose levels of *Rana sylvatica* increase from 1-5 μmol/mL to over 500 μmol/mL *Storey* [1985], and the concentration of sorbitol in *Eurosta solidaginis* similarly increases by more than 100 times, resulting in extracellular freezing at -8°C and survival to -40°C [*Franks*, 1985, p.135].

Some low-molecular-weight solutes have also been found to have important non-colligative functions as protein and membrane cryoprotectants. Sucrose and most other polyhydroxy compounds stabilize the native state of proteins and inhibit thermal denaturation (see Figure 3.3), albeit through unknown means. Trehalose also protects proteins, but is most important for its exceptional ability to inhibit the phase change in membrane phospholipids upon freeze dehydration *Crowe and Crowe* [1981]. The amino acid proline has a similar effect on membranes, though not as pronounced.[6]

Protection of proteins and membranes in freeze tolerant species is, in some cases, aided not only through the action of specific cryoprotectants, but also through

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[6]Trehalose is thought to work through hydrogen bonding with polar head groups on phospholipids; the mechanism of proline's cryoprotective ability is not understood. [*Storey and Storey*, pp.75-76]
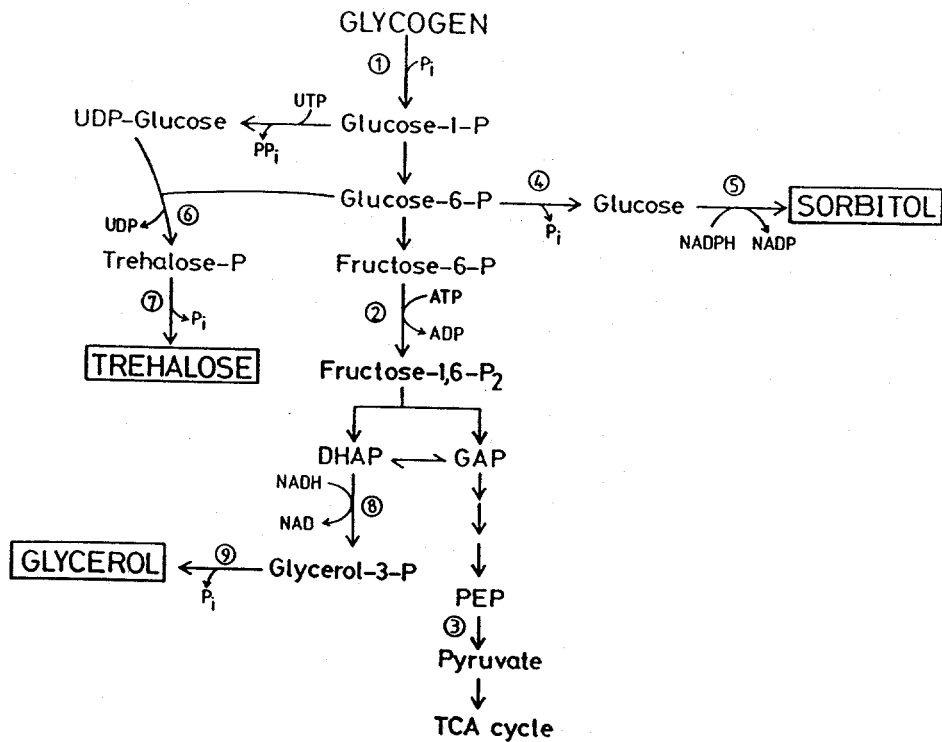
Figure 3.1: Biochemical pathways of the synthesis of major cryoprotectants in insects. Frogs and insects derive their low-molecular-weight cryoprotectants from glycogen. Encircled numbers represent various enzymes. Number (2) is the enzyme phosphofructokinase, which is known to become denatured with extreme cold. From K. B. Storey and J. M. Storey, "Animal Freeze Tolerance," p. 45.
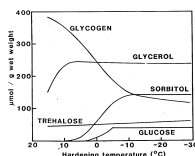

Figure 3.2: The cold-induced conversion of glycoen into sugars and sugar alcohols by *E. solidaginis* larvae. The pattern here of glycerol levels peaking early during acclimatization, followed by the production of sorbitol and glucose, is common among freeze tolerant insects. (After Storey et al., 1981). From [*Franks*, 1985, p. 136].
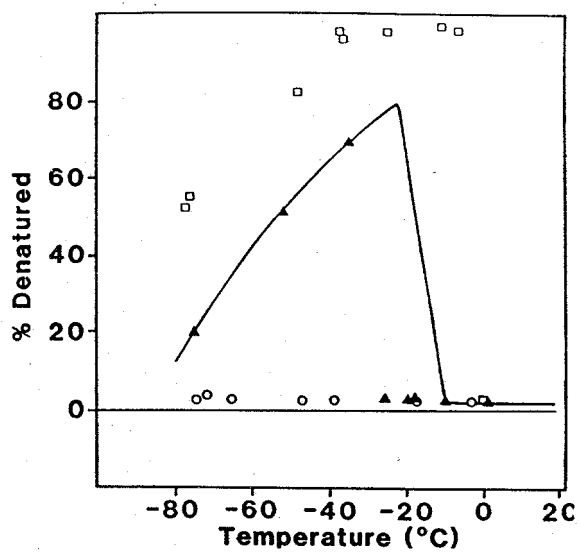
Figure 3.3: The effects of various additives on the *in vitro* freeze denaturation of a protein, chymotrpsinogen. Triangles represent of 0.1 M NaCl, squares 0.1 M urea, and circles 0.1 M sucrose. The drawn-out line represents the denaturation curve without any additives. From [*Franks*, 1985, p. 84].

temporary changes in composition. For instance, alterations in the amounts of various proteins and enzymes, and the synthesis of new protein isoforms, have been shown to occur in the cold hardening of some species [*Storey and Storey*, p.54]. Also, the modification of membrane composition, or "homeoviscous adaptation," is effected, especially in plants, to increase lipid fluidity at low temperatures and to prevent a phospholipid phase change. Many plants convert a substantial fraction of their cell membrane phospholipids to glycolipids, and *Chlorella ellipsoidea* also synthesizes specific fatty acids to protect plasma and chloroplast thylakoid membranes [*Franks*, 1985, p.116]. In some cases, lipids are completely removed from cell membranes to prevent chill or freeze damage *William et al.* [1981], but the manner in which these adaptations work is not yet clear. The combined effect of membrane cryoprotectants and homeoviscous adaptation is a substantial increase in membrane cold tolerance, and the capacity for the continuation of normal membrane activity at substantially reduced temperatures (see Figure 3.4).

Another adaptation of freeze tolerant organisms to reduce freeze damage is the increase in the amount of intracellular "unfreezable water," and, in plants, a reduction in the total amount of water.[7] Unfreezable, or "bound," water is that which is intimately associated with other molecules or macromolecular structures and cannot crystallize. Examples are H_2O involved in quaternary protein structure, hydration shells around solutes, water lining membrane pores, and water with such high solute concentrations that the viscosity retards — and functionally prevents — crystal growth [*Taylor*, 1987, p.19]. The increase of the ratio of unfreezable to freezable water is an important freeze tolerance strategy, used primarily by plants and insects, in which 20% of tissue water commonly becomes unfreezable.[8] Both low-molecular-weight solutes (chiefly polyols) and high-molecular-weight compounds (proteins and glycogen) have been identified as responsible for this phenomenon *Storey* [1981].

The end result of these adaptations is an impressive temperature tolerance for frozen organisms which end up containing only frozen and unfreezable water. Final ice contents vary considerably among different species and different temperatures; insects and marine invertebrates tolerate about 65% of body water as ice, with instances as high as 90% having been reported, while the range for freeze

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[7]Net dehydration would be lethal for frogs, which rely on a moist skin and damp conditions, and impossible for marine invertebrates, which are osmoconformers in a water environment. Freeze tolerant animals generally do not dehydrate because they can achieve sufficient protection through colligative protection and unfreezable water, which does cause added cell shrinkage. [*Storey and Storey*, p.69]
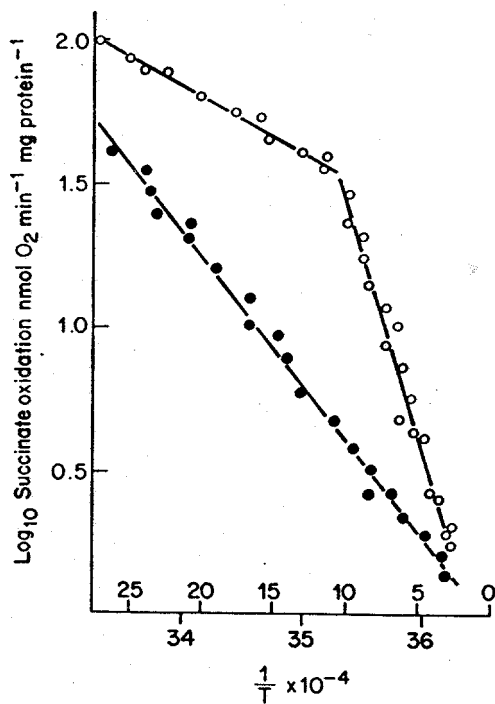
[8]E, p. 39

Figure 3.4: Typical Arrhenius plots for the respiration (specifically, succinic dehydrogenase activity) in plant mitochondria. Empty circles represent chilling sensitive plants and the filled in circles represent chilling resistant plants. The distinct change in slope of the upper graph caused by a phospholipid phase change in the membrane. From [*Franks*, 1985, p. 278].

tolerant frogs is 35% to 65% body water as ice [*Storey and Storey*, 1988, p.38]. Although freeze tolerant insects are usually acclimatized to withstand -25C or -30C, because of their contents of unfreezable water, species have been found that survive -70C, and some have even survived storage in liquid nitrogen (below -196C) [*Storey and Storey*, 1988, p.34].

Finally, all freeze tolerant organisms must have special adaptations to permit their cells to survive the abnormal conditions of isolation from extracellular fluid. Because individual cells are unfrozen and intact even in the organism's frozen state, their natural processes do not stop completely, yet all intercellular passageways are frozen, preventing any gas or nutrient exchange. To deal with this state of ischemia, marine invertebrates have become facultative anaerobes; the biochemical mechanism of this ability, however, is not properly understood. With the stimuli of cold and anoxia, marine invertebrates' metabolisms are depressed to 5% or 10% of aerobic rates *Storey* [1988]. Insects, too, have anoxia-tolerant cells, and severely depress their metabolic rates in the state of quiescence. In frogs, metabolism is thought to be somehow deliberately depressed beyond slow-down due to temperature by the high concentrations of glucose present in winter [*Storey and Storey*, 1988, p.66]. Frogs also have been found to exhibit anaerobic respiration when frozen and lacking lung, heart, or blood movement; after exhausting endogenous energy and fuel they begin to accumulate the anaerobic end products, lactic acid and alanine [*Storey and Storey*, 1988, p.66].

3.2 Hibernation

Alternate strategies of tolerating cold have evolved which do not include the formation of internal ice: many organisms are able to lower their body temperature and survive in hypometabolic states without any water crystallization occurring. One such adaptation is that of torpor — the periodic and facultative reduction of body temperatures and physiological rates by endothermic animals in order to conserve energy or water [*Wang*, 1989, p.361]. This procedure is most advanced and probably the best understood in seasonal mammalian hibernators, which regularly become cooled to near-zero temperatures and have a series of specific adaptations that furnish them with an advanced ability for cold tolerance.

One key aspect of this special capability is the neurophysiological and neuroendocrinological control of the biochemical slowdown that accompanies hibernation. Hibernators never stop thermoregulating; instead, the specific areas of the hypothalamus that are involved in thermoregulation turn down the "set-point," or

"thermostat" (through an unknown mechanism) in the torpid state [*Wang*, 1987, p.358]. In order to keep functioning at low temperatures, this area of neural tissue acquires extra cold tolerance; cessation of its activity occurs at about 28C to 30C in non-torpid species, at 15C in those exhibiting shallow torpor, and at 5C in torpid hibernators *Wunnenberg et al.* [1986].

A relationship has been shown between the general metabolic depression of hibernation and that of normal slow wave sleep *Heller et al.* [1978]. The neurophysiological homology[9] of sleep, torpor and hibernation has accordingly been suggested, but it is presently unsubstantiated due to a lack of biochemical evidence [*Wang*, 1988, pp.6-7].

Several possible biochemical factors in this adaptation of inducing and maintaining hibernation have been proposed, although research on these "antimetabolic" substances is very fragmented. One such compound, serotonin, which is also involved in sleep regulation, is thought to facilitate the transition into hibernation through the inhibition of thermogenesis and the enhancement of heat loss [*Wang*, 1988, p.8]. In the Syrian Hamster *Mesocricetus auratus*, the brain serotonin turnover rate is 24 times higher in the torpid state than in the active state *Novotona et al.* [1975]. Endorphins (endogenous opioids), and especially the enkephalins, are also involved with sleep and have also been identified with the initiation and maintenance of hibernation *Beckman* [1986].

Another set of potential antimetabolic chemicals have been named "the antabolone" *Swan* [1963]. Found first in the brain of estivating lungfish, this peptide induces a 35% drop in oxygen consumption, a three degree temperature decrease, and shallow torpor when injected into cold intolerant rats. A similar substance was found in the hibernating thirteen-lines ground squirrel *Spermophilus tridecemlineatus* and caused similar effects in rats *Swan* [1981]. Antabolone has not, however, been isolated and its specific effects remain uncertain [*Wang*, 1988, p.34].

The concept of a specific chemical "hibernation induction trigger" has also been suggested, and several have been proposed. This mysterious molecule may be one of the endogenous opioids, but little has been conclusively shown *Oeltgen et al.* [1982].

In any case, the net effect of the neurological and chemical control in hibernators is a profound reduction in physiological activity. During torpor, heart and lung rates are depressed and blood vessels are constricted in order to sustain blood pressure. The heart rate drops to as low as 1/30th or less of the normal rate, and overall oxygen consumption falls by a factor of 100 or more [*Wang*, 1989, pp.368-

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[9]Evolutionarily speaking; as opposed to analogy.

369]. Parallel abatements occur in cellular activity. It is thought that the smaller hibernators actively inhibit their biochemical metabolisms, while for the larger ones, retardation due to cooling is sufficient; their overall metabolic rates follow a simple Arrhenius curve *Lasiewski et al.* [1967]. Generally, a 50% to 70% depression of mitochondrial respiration occurs during hibernation *Pehowich and Wang* [1984]. This is at least partially due to the loss of enzyme function with prolonged exposure to the cold and slightly acidic conditions of hibernation. For example, the catalytic activity of phosphofructokinase is significantly reduced *Hand and Somero* [1983], and other respiratory enzymes are thought to undergo similar, temporary damage, with the effect of halting glycolysis. As a result, energy during torpor and hibernation has been shown to come from fat hydrolysis instead of oxidative phosphorylation *Hand and Somero* [1983]; all glucose is saved for its protein-cryopreservation ability and for the rapid biochemical activity needed during arousal.

Another adaptation necessary for cold tolerance in hibernators is the increased resistance to membrane chill injury. Research has shown that the maintenance of high intracellular potassium ion (K^+) concentrations, and low intracellular sodium ion Ca^{2+} concentrations, is critical for survival [*Wang*, 1989, p.384]. Since the movement of these ions depends on both passive diffusion and ATP-driven active transport, and the rate of the latter is reduced more rapidly by cold than that of the former *Willis* [1986], the vital ionic balance is easily upset in non-hibernators. Hibernator cell membranes have somehow been able to develop an enhanced cold tolerance, and their ion pumps are affected less. Despite the complexity of the many ionic pathways involved, the ability of hibernators to keep their transmembrane balance intact is thought to be solely dependent on their superior ability in regulating intracellular Ca^{2+} concentrations as compared to their non-hibernating counterparts[*Wang*, 1989, p.384]. The reason for this fundamental difference, though is still unknown.

The hibernating species' solution to the other major biochemical barrier to membrane cold tolerance — the protection of proteins and phospholipids in membranes — is not well understood either. Hibernators do not accumulate significant concentrations of any cryoprotectants during winter, although their endogenous glucose does have protein-stabilizing effects. It seems that a prerequisite for successful deep hibernation is the ability to increase membrane lipid fluidity and prevent the normal crystalline phase change at low temperatures (see Figure 3.5). Although a large amount of research has been done to identify the specific developments that occur in hibernating species that accomplish this, almost nothing definitive has arisen [*Willis*, 1987, pp.301-303]. Homeoviscous adaptation is
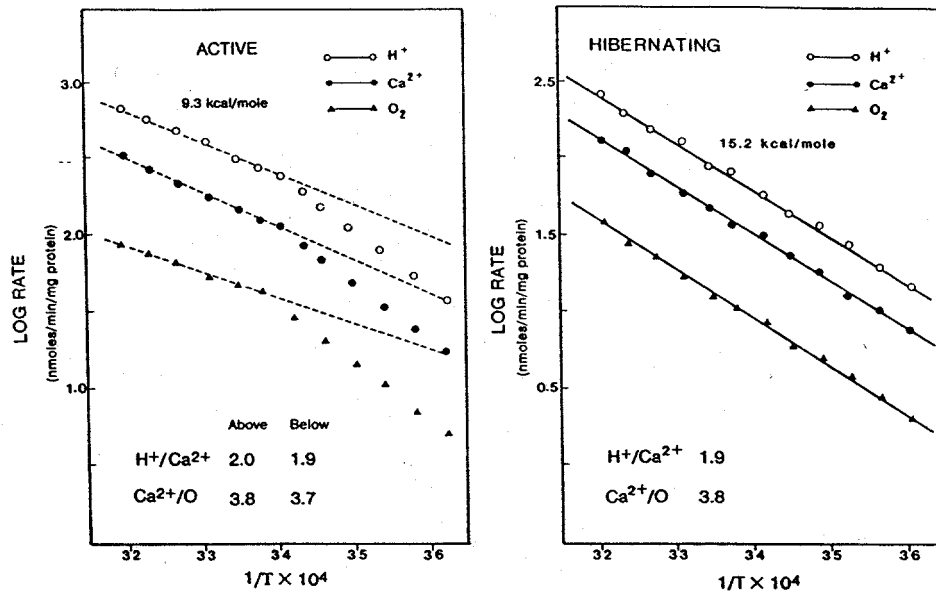
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Figure 3.5: Arrhenius plots of hydrogen ion efflux, calcium ion uptake and oxygen consumption in liver mitochondria from summer-active (left) and hibernating (right) Richardson's ground squirrels, *Spermophilus richardsonii.* The lack of a slope change in the values on the right suggests that the phospholipid phase change has been inhibited. From L. C. H. Wang, "Ecological, Physiological, and Biochemical Aspects of Torpor in Mammals and Birds," p. 386.

known not to occur in hibernators *Aloia and Raison* [1987], but "there remains the possibility that subtle changes in protein structure or local changes in lipid-protein interaction may account for the observed changes in Arrhenius kinetics" [*Wang*, 1988, p.33].

As a result of the uneven rates of ion motion and, presumably, many other cellular reactions, biochemical imbalances build up with time in torpid mammals. All hibernators are thus only able to survive for very restricted periods of time in hypometabolic states. Consequently, they hibernate in cyclical "bouts" of torpor (see Figure 3.6), lasting from a few hours to several weeks [*Wang*, 1989, p.364].
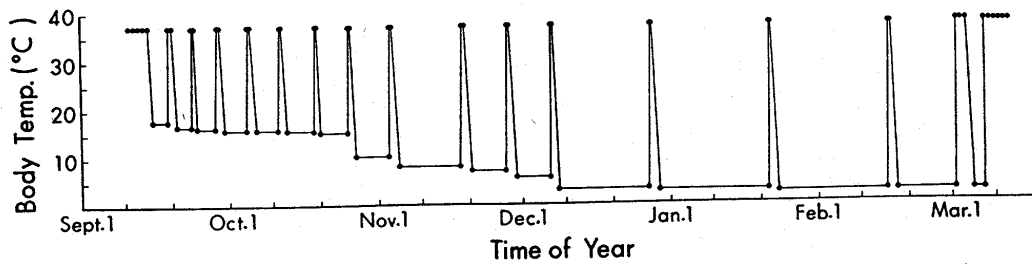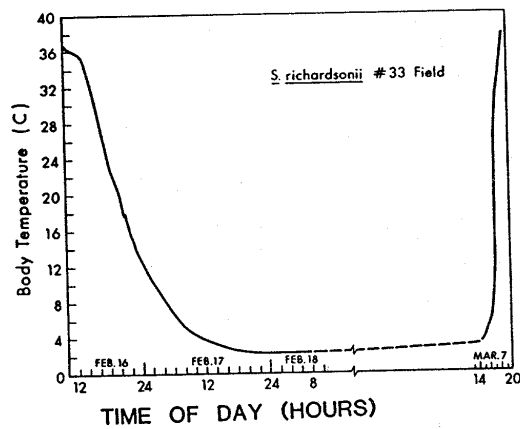
Figure 3.6: Body temperature (field) of a single hibernation bout and a winter hibernation in the Richardson's ground squirrel. Note the slow entry into the hibernation state (24 hours or longer) and the explosive arousal (of a duration of 5 hours or less). (After Wang, 1978.) From L. C. H. Wang (1989), "Mammalian Hibernation...", p. 4.

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3.3   Freeze Avoidance

These short bouts of hibernators, in comparison to the lengthy frozen states of freeze tolerant organisms, are a result of the relatively shallow temperature reductions effected by hibernators. In order to benefit from lower temperatures, and thus a less rapid build-up of chemical imbalances, without having to withstand intercellular ice, a third natural strategy of cold tolerance has developed. "Freeze avoidant" organisms are able to survive sub-zero temperatures without being freeze tolerant through the extensive use of undercooling. Such species are known to include plants, polar marine fish, nematodes, intertidal limpets, terrestrial gastropods, and vast numbers of terrestrial arthropods [*Storey and Storey*, p.56].

In order to overcome the effects of chill injury and to prevent the possibility of water crystallization, freeze avoidant organisms have developed four general adaptations. These are (1) the stabilization of proteins and membranes against structural damage; (2) the reduction of freezable water contents; (3) the elimination of potential ice nucleators; and (4) the reduction of both the melting point and the freezing point[10] of tissue water [*Storey and Storey*, 1988, p.31].

Since the first of these adaptations is common to freeze tolerant and freeze avoidant species, the methods used to accomplish it are also generally homologous. Freeze avoidant organisms increase concentrations of proline, trehalose, and polyhydroxy compounds in general, in order to stabilize membrane structure and inhibit thermal denaturation of proteins. Their membranes have also been found to undergo homeoviscous adaptation, similar to that of freeze tolerant organisms, in order to increase phospholipid fluidity and prevent the thermotropic phase transition from occurring.

Also similar to the adaptations of freeze tolerant species is the strategy among freeze avoidant organisms of greatly increasing quantities of unfreezable water, especially through high levels of low-molecular-weight solutes. Unlike freeze tolerant species, though, the adaptation of net dehydration is used extensively among freeze avoidant animals and plants. Also, in order to ensure intercellular and vascular freeze avoidance, many freeze avoidant organisms mobilize all fluids into very small volumes, which reduces the nucleation rate (see Figure 2.3), and, therefore, the freezing point. An example of this mechanism is the cold hardening of the arctic gastropod *Helix pomatia*. In response to the first signs of cell dehydration caused by intercellular ice, macromolecules (of molecular weight of 9×10^6

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[10]See the Glossary for the distinction between the two.

g/mol) in the hemolymph reversibly dissociate into their twenty component subunits. This immediately stops ice formation and, without affecting the melting point, promotes 10°C of undercooling by increasing protein-water interface area and "holding" tiny volumes of liquid between the protein subunits [*Franks*, 1985, p.124].

Other strategies of freeze avoidance include the seasonal elimination or inactivation of potential endogenous ice nucleators. The high levels of polyhydroxy compounds, especially glycerol, which are found in most hardened freeze avoidant organisms is thought to inactivate natural nucleation catalysts, and hence further decrease the freezing point [*Franks*, 1985, p.121]. The simple strategy of evacuating all food particles or other potential ice nucleators during hardening can also increase undercooling considerably; for example, by removing certain lipoproteins from its hemolymph in autumn, the stag beetle lowers its freezing point from -7C to -25C *Neven et al.* [1986].

The greatest effects of freezing point depression in freeze avoidant organisms are due to the extensive use of high solute concentrations and anti-freeze proteins. Unlike in freeze tolerant species, low-molecular-weight solute levels are highly elevated in the blood and interstitial fluid as well as the cytoplasm, in order to colligatively reduce melting and freezing points throughout the body. Glycerol is the most commonly used solute for this purpose among freeze avoidant species. For example, hardened organisms with glycerol comprising 15% to 25% of their fresh weight are not uncommon [*Storey and Storey*, 1988, p.31], and fractions of up to 30% have been found *Rickards et al.* [1987].

The use of AFP's among freeze avoidant organisms is probably the most universal adaptation to prevent ice formation, and it has been called "the key to successful freeze avoidance" [*Storey and Storey*, 1988, p.41]. Since 1964, many AFP's from fish and insects have been isolated and purified. All were found to be proteins or glycoproteins with large percentages of alanine [*Franks*, 1985, p.118]; however, the powerful effect of AFP's in inducing undercooling without affecting the melting point is not yet understood.

The combined result of the several freeze avoidance strategies for reducing the melting point and inducing undercooling is a greatly enhanced cold tolerance in the acclimatized state (see Figures 3.7 and 3.8). For example, the freeze avoidant tenebrio beetle ends up with internal melting and freezing points of -13C and -15C, respectively. Moreover, some species of woody plants in temperate North America can survive without their xylem water crystallizing down to a temperature of -50C, or 10C below the homogeneous nucleation point, while the water's melting point has only been reduced by 2C [*Franks*, 1985, p.117]. This startling

Month	M. contracta		U. impressa	
	Melting	Freezing	Melting	Freezing
	Temperature (°C)		Temperature (°C)	
February	−1.31	−10.3	−9.90	−21.5
June	−0.81	−3.8	−0.88	−6.2

Figure 3.7: Melting and freezing temperatures of the hemolymph from *Mercantha contracta* and *Uloma impressa* in their tender (June) and hardened (February) states. From [*Franks*, 1985, p. 120].

ability is not understood, but it is clearly not due solely to colligative effects.
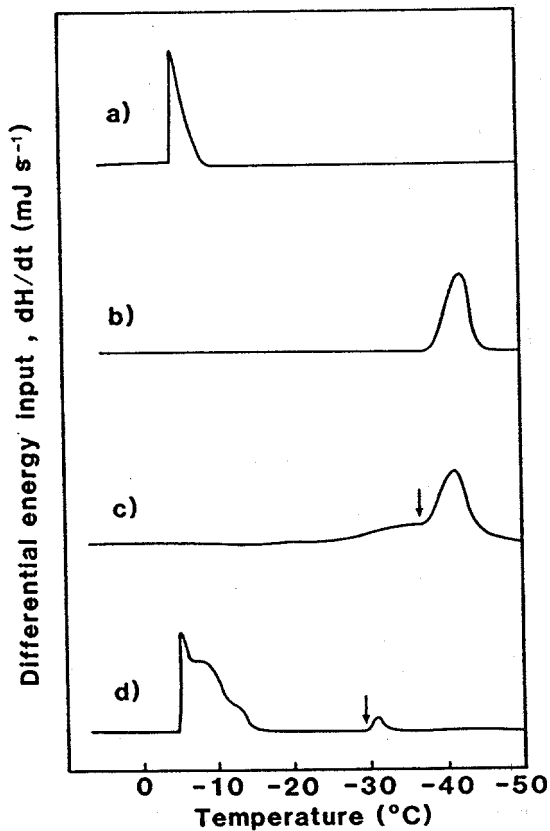
Figure 3.8: Differential thermal analysis of freezing exotherms (the latent heat released during crystallization) during cooling at a constant rate. (a) Bulk water (note the single, sudden crystallization exotherm after the undercooled, metastable water becomes nucleated); (b) Water dispersed in the form of a droplet emulsion in an inert oil (undercooling reaches the homogeneous nucleation point and crystallization is gradual and even due to the tiny size of the volumes of water); (c) Tissue water in fully hardened (freeze avoidant) peach flower buds (the first exotherm represents the formation of tolerated extracellular ice, while the smaller shows the lethal freezing of the remaining undercooled water, apparently not rendered unfreezable). Arrows in (c) and (d) represent the killing temperatures, both just before freezing exotherms. In (c) and (d), the melting points are about $-2°$C. From [*Franks*, 1985, p. 122].

Chapter 4

Applications and Conclusions

The research of natural cold tolerance strategies has revealed that adaptations are generally aimed at achieving a balance between the positive and negative effects of very low temperatures: the further that tissue is cooled, the less are the effects of metabolic imbalance, but the greater is the risk of ice formation. Thus, depending on the capabilities of various organisms, freeze tolerance, free avoidance, or hibernation can be used to attain optimum cold tolerance. In summarizing the general tactics that have been discovered in natural use and discussed in this paper, it is evident that nature has provided methods to overcome all of the recognized principal obstacles to suspended animation that were mentioned earlier, and also that many of the strategies are analogous among different phyla and approaches to cold tolerance. These include the use of polyhydric alcohols, sugars, and proline for colligative prevention of freeze damage as well as for non-colligative cryoprotection of complex molecules and structures; the use of INP's to control ice formation; the use of AFP's to reduce freeze damage or prevent freezing; the capability for ischemia tolerance; changes in water relationships in order to reduce the freezable water content; the reduction of potential endogenous ice nucleators; the ability to effect subtle changes in biomembrane structure or composition in order to prevent lipid phase changes and ionic depolarization of membranes; and the use of specific substances to inhibit metabolic rates both physiologically and biochemically.

Attempts to improve cold tolerance and to impose general hypometabolic states on unadapted organisms have been met with relatively limited success, considering the abundance of natural models available. Instances of inducing freeze tolerance since the pioneering work of Audrey Smith in the 1950's still only include the freezing of cells and very simple tissues or suspensions. Corneas, blood

components, sperm, erythrocytes, lymphocytes, nasal mucosa, intestinal tissue, parathyroid tissue, kidney tissue, spleen tissue, and teeth have been cryopreserved with great success by various groups [*Sternberg et al.*, p.6]. The use of glycerol is the main application of natural adaptations in these techniques. Lethal percentages of water as ice in these experiments have generally been found to be consistent with natural ice levels, or about 65% of body water [*Storey and Storey*, p.55]. Both groups of cold tolerant vertebrates — frogs and mammalian hibernators — use high glucose levels to combat cold injury, and this adaptation could well be very useful in attempts at cryopreserving mammalian organs, although problems with perfusing cryoprotectants, and studies of cooling and warming rates, seem to be presently dominating the field [*Jacobsen*, 1987, p.15].

Achievements in artificially inducing hibernation have been slightly more extensive, and, in fact, rats, hamsters and other small mammals can be artificially cooled to low above-zero temperatures, or even undercooled to colonic temperatures of -2.5C to -5.5C, with up to 100% survivorship after being artificially rewarmed and resuscitated [*Barnes*, 1989, p.1595]. Larger mammals, in the form of beagles, have been shown to successfully undergo such procedures, too, and have been cooled below 10C for over 70 minutes *Sternberg* [1987]. Because some of the smaller-scale adaptations (e.g. the cold tolerance of membrane ion-transport pumps and the membrane structure alterations possibly exhibited by hibernators) have not been mimicked yet, time in imposed hypometabolic states is very limited. The natural demonstration of time restrictions is an important lesson for the goal of suspended animation, and clearly indicates that any such state will be one of life and, albeit very slow, metabolic activity, not a state of "reversible death." The longest known duration of torpor is 76 days in bats *Twente et al.* [1986], and even freeze tolerant organisms are limited by time; *Eurosta solidaginis* larva survive twelve weeks frozen at -16C, but not one year *Storey* [1988].

The major reason why attempts at artificially producing cold tolerance have had so much difficulty adapting natural strategies is that an enormous amount of information concerning the detailed, biochemical means behind macroscopically apparent adaptations is still lacking. Thus, much of the contemporary research into cryopreservation and induced hibernation is still predominantly empirical, instead of being focused on the application of nature's millennia of experience. In not properly understanding the physico-chemical mechanisms of such vital cryoprotective substances as AFP's and low-molecular-weight protein and membrane stabilizers, much knowledge concerning their use and potential application is presumably also missing. Aspects of membrane lipid alterations remain "one of the most challenging tasks facing hibernation research," [*Wang*, 1988, p.26] and seem

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to be fundamental to improved cold tolerance, and thus the realization of suspended animation. A better understanding of such phenomena as the hibernating mammals' hypothalamic thermoregulatory and torpor-inducing mechanisms and their relation to sleep could also go a long way in promoting the success of artificial hibernation, since the use of a certain series of drugs (possibly opioids, an antabolone, serotonin, or the "hibernation induction trigger" initiated by the central nervous system) may be key in causing a general physiological slow-down. Also, "the biochemical mechanisms underlying metabolic depression are only beginning to be explored" [*Storey and Storey*, p.77], and would be equally vital in any form of suspended animation. The recognition of the importance and complexity of membranes, active transport pumps, and ions is still growing in the fields of freeze avoidance and hibernation [*Willis*, 1987, p.292], as well as freeze tolerance [*Storey and Storey*, p.75]. Lastly, considerable research is still needed into the reasons for the length of hibernation bouts, and the specific stimuli that trigger arousal.

Clearly, though, the incentives to mimic natural cold tolerance abilities for cells, organs, and organisms are only increasing with research. 18% to 31% energy savings were found due to the daily torpor of small rodents *Vogt and Lynch* [1982] and as much as 88% savings were estimated for seasonally hibernating Richardson's Ground Squirrels *Wang* [1979]. The savings achieved by freeze tolerant and freeze avoidant organisms during their depressed states are presumably even greater. The ability to become freeze avoidant was generally considered not to exist naturally among mammals, until very recently, when the arctic ground squirrel *Spermophilus parryii* was found to be undercooled in the hibernating state[1]*Barnes* [1989]; see Figure 4.1. At -3C, this state was estimated to give up to ten times the energy savings as are gained by normal hibernators in above-zero conditions *Barnes* [1989]. Other indications of homology between the various forms of cold tolerance have been provided by such examples as the two species of beetle which seasonally transform from freeze avoidance to freeze tolerance and vice versa *Horwath and Duman* [1984], and the insect *Pytho deplanatus* that undercools to -54C but is also freeze tolerant *Ring* [1982]. Growing incentives for the application of these powers to systems smaller than an entire organism include: the possible cryopreservation of organs — a *sine qua non* for any advances in transplantation [*Franks*, 1985, p.20] — and the storage of farm animal embryos for the development of "embryo banks" [*Polge*, 1977, p.11].

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[1]This was shown to be without the use of AFP's or elevated levels of low-molecular-weight solutes; the mechanism remains a mystery.
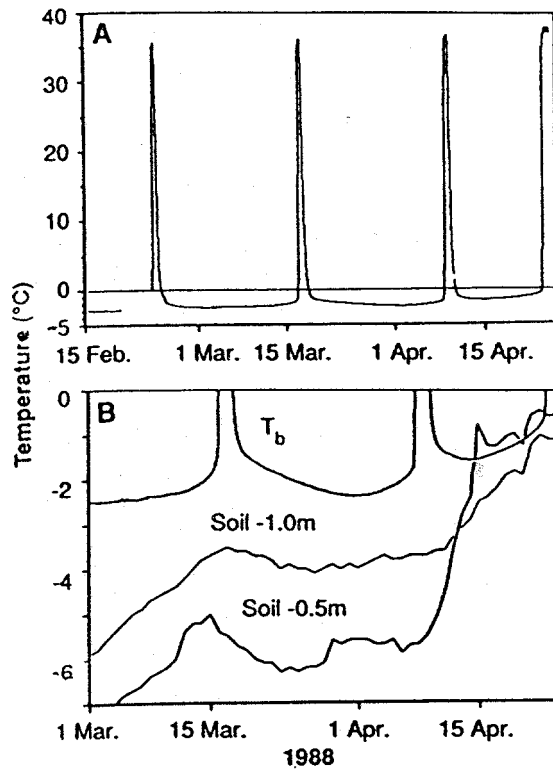
Figure 4.1: Body temperature (A) of a field hibernating female arctic ground squirrel *(Spermophilus parryii)* as indicated by an abdominal temperature-sensitive radiotransmitter; (B) with an expanded scale, abdominal temperature during the last three arousals from torpor and concurrent adjacent soil temperature. Core body temperatures drop as low as $-2.9°C$; this is the first freeze avoidant hibernator discovered. From Brian M. Barnes, pp. 1593, 1594.

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Current pragmatic methods of inducing hypometabolic states on different biological systems, however, make the field "still very much a recipe science" [*Franks*, 1985, p.192], and there are increasingly important reasons for scientists to turn to more analytical methods by applying natural adaptations, and especially those of mammalian hibernators. Comparative research on hibernator and non-hibernator mammalian cells has led to the conclusion that, upon isolation from the organism, "tissues form hibernators show little or no cold tolerance," suggesting that "the capacity for wide temperature tolerance persists in the genome of even conventionally cold-sensitive mammals, and that it is only the expression of this capacity that is altered" [, pp.287 and 304]. Conversely, studies with freeze tolerant organisms show that "freeze tolerance is a property of individual tissues, even when isolated from the whole animal" [, p.67]. These results suggest that the extensive homeoviscous adaptation and ability for survival of anoxia in freeze tolerant organisms will be much harder to mimic, while biochemical changes in hibernator cells are due solely to hormonal control. On the other hand, indications to suggest that effecting the chemical changes needed to induce extensive freeze tolerance may also be possible have been recently made by the artificial modification of membrane lipids to increase freeze tolerance *Steponkus* [1989]. Generally, though, research into natural freeze tolerance will only be harder than previously thought, while the route of induced hibernation is increasingly promising[*Biamond*, 1989, p.510].

New technology, such as the widespread ability to artificially synthesize polypeptides, and the invention and expanding use of the cryomicroscope [*Franks*, 1985, p.176], can increasingly aid in the understanding and use of cold tolerance adaptations. It has been just during the last decade that most of the biochemical mechanisms behind the necessary changes for cold tolerance have begun to be discovered, and, if research continues at the same rate, the remaining problems should begin to disappear. Now more than ever, the focus on and use of naturally evolved strategies for cold tolerance provides the most promising approach to achieve the goal of suspended animation.

Appendix A

Glossary

ACCLIMATIZATION: The (usually seasonal) adjustment of an organism's physiology to changes in environment, and, in this paper, to environmental temperature. The European equivalent of this North American term is "cold hardening."

ARRHENIUS PLOT: A graphical representation of the dependence of kinetic rate constants as a function of inverse temperature, useful in the identification of phase changes and the classification of kinetic mechanisms as simple or complex (i.e., simple Newtonian thermal relationships have a straight-line Arrhenius curve).

COLD TOLERANCE: A general term used to describe the ability to survive unusually low internal body temperatures instead of avoiding them through behaviour or thermogenesis.

COLLIGATIVE: Depending on the concentration of a solute only, not on the nature of the chemical species. Thus, the effect of many low-molecular-weight cryoprotectants is thought to be "non-specific," whereas that of antifreeze proteins, for example, is structure-dependent. The melting point of 1.0 L of a solution is depressed colligatively by 1.86C per mol of solute [*Franks*, 1985, p.120]. The freezing point is also colligatively depressed slightly further than the melting point (i.e. solutes lower the melting point and induce undercooling in a solution).

EXOTHERM: A distinct release of latent heat from a bulk substance undergoing an exothermic phase change. Modern techniques such as differential

scanning calorimetry sense exotherms externally, and can thus be used to detect phase changes of water or lipids in living tissue.

FREEZING POINT: Used in this paper to denote the "crystallization temperature" of a solution or tissue (i.e., as the temperature is lowered). The point at which water undergoes this phase change is dependent on the abundance of crystallization nuclei, and can be lowered independently of melting point. The effective freezing point of a body of water can be considerably below its melting point. (See MELTING POINT). The difference between a substance's melting point and freezing point is called its "thermal hysteresis."

HEMOLYMPH: The watery fluid counterpart of blood in certain invertebrates; it contains the cryoprotectants in hardened species.

HIBERNATION: A condition of torpor into which some animals lapse during winter. In this paper, however, it is used to denote "deep" or "profound" hibernation, in which body temperature falls to very low above-zero temperatures. The many different states of sleep, estivation, torpor, and hibernation have led to considerable confusion, as expressed by Russel (1975) in his title, "Cheirogaleids and Hibernation, Estivation, or Whatever." Lyman coined the phrase "deep hibernation" in 1948 for the state exhibited seasonally by small rodents. It is this form of torpor that is dealt with in this paper. (See TORPOR.)

HOMEOVISCOUS ADAPTATION: Bulk changes in the composition (e.g., and increase in the degree of unsaturation) of membrane fatty acids and lipids brought about by freeze avoidant and freeze tolerant species in order to maintain constant lipid (and possibly protein) fluidity at low temperatures. The term was coined by Sinensky (1974).

MELTING POINT: Used in this paper to denote the temperature at which ice will melt (i.e., as the temperature is rising). Melting point can be reduced by solutes. (See COLLIGATIVE). The melting point of pure water is 0C. The melting point of a solution cannot be below its freezing point, and, if crystallization nuclei are abundant, a body of water will freeze at its melting point. (See FREEZING POINT).

METASTABLE: Used to describe bodies of supercooled water or solution, because, if they are nucleated, they will spontaneously crystallize. A container of deeply supercooled water, for example, could be turned instantaneously

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into ice simply by being shaken or even tapped. Water becomes increasingly metastable with decreasing temperature.

NUCLEATION: The "seeding" of crystal growth. Cooled water needs some sort of rough surface to begin its crystallization phase change. As it is cooled below 0C, if no ice nuclei are initially present, it will become increasingly METASTABLE. Eventual ice nucleation can be either heterogeneous (catalyzed by a particle or surface acting as a nucleus) or homogeneous (water molecules, themselves, form nuclei sufficiently long-lived to catalyze crystallization due to random density fluctuations: -40C is the homogeneous nucleation temperature of pure water).

SUPERCOOLED / UNDERCOOLED: Used to describe a substance which is in the liquid phase even though it is cooled below its melting point. This occurs as a result of a lack of a crystallization nucleus (see NUCLEATION). Many animals supercool to some extent even without any hardening or protection.

THERMOTROPISM: Abrupt changes in phase or structure at specific "phase transition temperatures" undergone by various complex organic molecules.

THYLAKOID MEMBRANE: The inner reacting-surface membrane of mitochondria and chloroplasts, as distinguished from the outer ("cell") plasma membrane. The composition and functions of the two are quite distinct.

TORPOR: A broader term than hibernation, meaning the general state of a facultatively depressed metabolism in which the organism becomes relatively poikilothermic (its body temperature approaches that of the environment). Generally, though, when speaking of mammalian or avian torpor, it is used to mean a shallower temperature depression than that implied by "hibernation." (See HIBERNATION).

UNDERCOOLED: See SUPERCOOLED.

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