ADVANCES IN MOLECULAR AND CELL BIOLOGY

Series Editor: E. EDWARD BITTAR
Guest Editor: JOHN S. WILLIS

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THERMOBIOLOGY

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CONTENTS

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PREFACE

"Italy is a geographical expression only," Clemens von Metternich wrote to the chancelleries of Europe in 1814, dismissing any thought of political reunification of the fragmented peninsula. Something of the same sort might be said of the expression "Thermobiology" today. Notwithstanding widespread studies and even several biological journals devoted to temperature, it is difficult to perceive a *field* of thermobiology as such. Interest in the effects of temperature on biological systems is fragmented into specific thermal ranges and often connected with particular applications: subzero cryobiology and preservation of cells and tissues or survival of poikilotherms, para-zero cryobiology and preservation of whole organs and survival of whole animals, intermediate ranges and physiological adaptation and regulation, high temperatures and use of heat for killing cancer cells, very high temperatures and limits of biological structure.

Yet it has not always been so, and there are good reasons why it need not remain so. General and comparative physiologists such as W. J. Crozier, H. Precht, J. Belehradek, F. Johnson, C. L. Prosser, and others have sought throughout this century to lay foundations for unified approaches to temperature in biological systems.

Recent findings also serve to suggest principles and processes that span the range of temperatures of biological interest. Microviscosity of

membranes is an issue originally of interest to low temperature biologists but with relevance to limiting high temperatures; conversely for protein structure. Certain "heat shock proteins" now appear to be responses to generalized stress, including low temperature.

Inevitably, the chapters of this book reflect the "zonal" character of thermobiology: two chapters (by Storey and Raymond) deal with protection against subfreezing temperatures; three (Hazel, membrane structure, Dietrich, microtubular structure, and Kruuv, cell growth) deal with the effects of and modulation to cool-to-moderate superfreezing temperatures, one (Willis) with modulation (of membrane ion transport) to moderate-to-high temperatures and two (Li, heat shock proteins and Lepock, proteins in general) with stressfully high temperatures. Explicit in each of these chapters, however, are principles and issues that transcend the parochialism of the temperature range under consideration.

The assistance of Marcus Fechheimer and Robert Wondergem in reviewing certain chapters is gratefully acknowledged.

John S. Willis
Guest Editor

ADAPTATIONS FOR FREEZING SURVIVAL IN ECTOTHERMIC VERTEBRATES

Kenneth B. Storey and Janet M. Storey

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I. STRATEGIES OF WINTER HARDINESS

Winter temperatures plunge below 0 °C over vast areas of our planet and organisms inhabiting these regions must have effective mechanisms for dealing with seasonal cold and with the accompanying limitation, or complete interruption, of food availability (Marchand, 1991). Some species use migration to relocate to warmer climates and escape winter altogether. Most others use some sort of avoidance strategy to shelter themselves from extreme low temperatures by retreating underwater or underground or even just beneath the leaf litter and snowpack. Other species endure the full rigors of winter with well-developed mechanisms for resisting severe cold stress.

Although some animals, mainly endotherms, remain active and continue foraging throughout the winter, most animals enter a state of suppressed metabolism during the cold winter months. Most do not eat, having accumulated large body reserves of lipids and carbohydrates during summer and autumn feeding, and most are relatively inactive. When also coupled with low body temperatures, this causes a general lethargy among most ectotherms. Other species show more aggressive forms of metabolic arrest that allow them to greatly extend the time that they can survive using only a fixed reserve of stored body fuels. Many insects enter a programmed, endocrine-mediated diapause that may suppress oxidative metabolism by 10- to 20-fold (Danks, 1987). Freshwater turtles, by hibernating underwater, take advantage of the metabolic suppression induced by anoxia/hypoxia with the result that the metabolic rate of submerged animals at 5 °C is only about 10% of the corresponding metabolic rate in air at the same temperature (Herbert and Jackson, 1985). Small mammals combine metabolic arrest with a profound drop in body temperature to reduce metabolic rate during hibernation to only 1-8% of the resting euthermic rate (Geiser, 1988).

In addition to the widespread use of metabolic suppression as a means of winter survival, many species must use additional protective strategies to deal with exposures to temperatures below 0 °C and the potential destruction wrought by the freezing of body fluids. Endotherms have a relatively low risk of freezing for even though small mammal hibernators allow their body temperature to fall to near 0 °C, or even to subzero values as in Arctic ground squirrels and some bats (Davis and Reite, 1967; Barnes, 1989), they are still regulating body temperature and make adjustments to increase their metabolic rate and alter the perfusion of body extremities when there is a risk of freezing. Ectotherms must use

different strategies and for many the choice is to hibernate in a site where temperatures do not normally fall below the freezing point of body fluids (Gregory, 1982; Ultsch, 1989). The sometimes observed winterkill of fish and frogs in ponds and streams shows the risk of this strategy to individuals in some winters even though the strategy may be an acceptable one for a population as a whole. Other ectotherms cannot avoid exposure to subzero temperatures; some face the full extremes of winter air temperatures but many others endure milder subzero exposures (lows of about -6 °C to -8 °C) while wintering in the leaf litter under the snowpack.

One of two strategies may be taken for enduring temperatures below 0 °C and these are generally termed freeze avoidance and freeze tolerance (Zachariassen, 1985; Storey and Storey, 1989; Block, 1990). Animals that avoid freezing use adaptations to lower the supercooling point or crystallization temperature of their body fluids to a value well below the anticipated minimum temperature of the hibernation site. For example, many of the invertebrates living under the snowpack supercool to -10 °C to -15 °C using mechanisms that include the seasonal elimination of potential ice nucleating sites from their bodies (e.g., evacuation of the gut, changes in hemolymph protein composition), shielding from contact with external ice (e.g., waterproof cocoons), and the addition of antifreeze (or thermal hysteresis) proteins to their body fluids (Zachariassen, 1985; Storey and Storey, 1989; Duman et al., 1991a, 1991b). When much lower temperatures must be endured, the supercooling point is pushed even further by the production of large quantities of polyhydric alcohols. For example, glycerol concentration in the body fluids of some insects can reach 2-3 M or 20-25% of the fresh weight to allow some species to supercool to as low as -55 °C (Ring, 1981; Storey and Storey, 1991). However, a supercooled animal is always at risk of spontaneous nucleation, and hence instant death from freezing, and this risk increases with decreasing temperature and increasing time of subzero exposure. Other species have developed the second strategy, freeze tolerance. These take control of the process of freezing so that ice formation occurs in a regulated manner at relatively high subzero temperatures. Ice is sequestered within extracellular or extra-organ fluid compartments only and intracellular freezing is prevented (Storey and Storey, 1988).

The present review focuses on various recent advances in our understanding of freeze-tolerance in animals. We have chosen, of necessity, to highlight only selected aspects of the molecular and cellular adaptations involved in this phenomenon and, in addition, focus primarily on

studies with vertebrate animals. The reader is referred to numerous other volumes and reviews for in depth discussions of other aspects of the phenomenon (e.g., Zachariassen, 1985; Ultsch, 1989; Marchand, 1991; Duman et al., 1991a, 1991b; Storey and Storey, 1988, 1992a, 1992b; Storey et al., 1996; Lee and Denlinger, 1991).

II. FREEZE TOLERANCE IN ANIMALS

The ability to withstand the formation of ice in extracellular fluid spaces has evolved in a variety of species from diverse phylogenetic origins including some reptiles and amphibians, many insects, some intertidal molluscs and barnacles, and various invertebrate microfauna (Aarset, 1982; Storey and Storey, 1988, 1992a, 1992b; Block, 1990). Among vertebrates, well-developed freeze tolerance that is ecologically relevant to winter hibernation has been confirmed for five species of terrestrially-hibernating frogs, one salamander species, and three species of turtles (Table 1). These all endure long-term freezing with stable ice contents of 45-64% of total body water and hibernate under conditions where natural freezing exposures are likely (Storey and Storey, 1992a). A new report indicates that a high altitude lizard *Sceloporus grammicus* from the Mexican mountains may also belong to this group; these lizards experience subzero temperatures in their natural environment and experimental tests showed that they endured freezing at -2.5 °C for 37 hours (Lemos-Espinal and Ballinger, 1992). Several other reptile and amphibian species are able to endure short-term freezing stress at high subzero temperature and with low ice contents, but these abilities are too underdeveloped to be used as a viable strategy of winter hibernation (Table 1).

Freezing places two main stresses on the cells of organisms: osmotic stress and ischemic stress. The solidification of pure water into ice crystals leaves behind an increasingly concentrated unfrozen solution and ultimately macromolecules may be damaged if dehydration or ionic strength reach extreme values. Ice formation inside cells is also highly damaging to subcellular architecture and microcompartmentation and, hence, is lethal for all freeze-tolerant animals. Therefore, freeze-tolerance in nature refers to the ability to withstand ice formation in extracellular fluid compartments and freeze-tolerant animals take steps to avoid the possibility of intracellular nucleation while controlling ice growth in extracellular spaces. Many freeze-tolerant animals have developed specific ice nucleating proteins that are added to blood or hemolymph

Table 1. Freezing Survival by Vertebrates: Species with Well-developed, Ecologically-relevant Freeze Tolerance versus Those that Endure Brief Freezing Stress

	Well-developed Freeze Tolerance	*Survive Short Freezing Exposures*
Amphibians	*Rana sylvatica*	*Ambystoma laterale*
	Hyla versicolor	*some aquatic ranids*
	Hyla chrysoscelis	
	Pseudacris crucifer	
	Pseudacris triseriata	
	Hynobius keyserlingi	
Reptiles	*Chrysemys picta marginata*	*Trachemys scripta elegans*
	Chrysemys picta bellii	*Thamnophis sirtalis*
	Terrapene carolina	*Podarcis muralis*
	Terrapene ornata	
	Sceloporus grammicus	

Source: Summarized from Storey and Storey (1992a).

during autumn cold hardening and serve to ensure that nucleation occurs in extracellular spaces (Wolanczyk et al., 1990; Duman et al., 1991a,b; Madison et al., 1991; Storey et al., 1991, 1992a), but ice nucleators in some species have also been traced to foreign bodies (e.g., microbes, fungi) in the gut or on the body surface (Bale et al., 1989; Tsumuki et al., 1992; Lee et al., 1995). However, the consequence of extracellular ice growth is intracellular dehydration; because ice excludes solutes from its matrix, the remaining extracellular solution becomes increasingly concentrated and this causes an osmotic outflow of water from cells. Cells shrink and ice grows until an equilibrium is reached when the osmolality of the remaining body fluids rises to a value whose melting point is equivalent to body temperature. Extreme dehydration can cause extensive physical damage to cells; membrane compression appears to be the first and most damaging effect of dehydration and can result in the irreversible breakdown of bilayer structure and a loss of membrane integrity (Mazur, 1970; Wolfe et al., 1986). Upon thawing this damage is seen as the inability of cells to regulate osmotic balance or ionic composition and by the leakage of macromolecules into the extracellular milieu. Thus, to prevent irreversible damage due to excessive cellular dehydration, most freeze-tolerant animals accumulate high concentrations of low molecular weight cryoprotectants, generally sugars or polyhydric alcohols. The colligative action of these molecules in solution limits the extent of cellular dehydration that can occur and serves to

maintain a critical minimum cell volume. In practice, most freeze-tolerant animals endure the conversion into ice of up to about 65% of total body water, but this limit may be reached at widely different temperatures depending upon the cryoprotectant concentrations maintained by each species or individual (Storey and Storey, 1988). Cryoprotectants also stabilize the structures of macromolecules against the stresses of dehydration or low temperature. Polyols are particularly effective in stabilizing protein structure whereas trehalose and proline interact with the polar head groups of phospholipids to spread and stabilize the bilayer structure of membrane lipids, thereby counteracting the compression caused by cell volume reduction (Rudolph and Crowe, 1985; Fink, 1986; Crowe et al., 1987). Cells surrounded by ice are also cut off from exogenous supplies of oxygen and metabolic fuels that are normally delivered by the circulation and must survive in this ischemic state for the duration of the freezing episode. Although the low body temperature of the frozen state ensures that metabolic rate is low, individual cells must still contain sufficient fermentable fuels to allow them to sustain a viable energetic state throughout the freezing episode (Storey and Storey, 1985, 1986).

Furthermore, freeze-tolerant animals experience a slowing and finally a cessation of vital signs during the freezing process and must possess mechanisms that permit the coordinated reactivation of functions such as heart beat, breathing, and skeletal muscle movement during thawing. These physiological events have begun to be characterized in freeze-tolerant vertebrates (Storey et al., 1996). In frogs, the freezing front moves from the periphery (after nucleation somewhere on the skin surface) inwards towards the core organs, progressively cutting off circulation and halting organ functions until finally heart beat is the last detectable vital sign to cease (Layne et al., 1989). The molecular mechanism(s) of the spontaneous cessation of vital signs are not yet known but might be related to cell volume changes that could, when cell volumes drop below specific values, inhibit specific cell functions such as contractile activity or neuronal excitability. During thawing in the wood frog *Rana sylvatica,* heart beat is the first function to be restored followed soon thereafter by blood flow to the skin, spontaneous breathing, and finally by skeletal muscle reflexes (Layne and First, 1991). Contraction strength of *R. sylvatica* gastrocnemius muscle varied inversely with freezing duration (0-96 hours at -2 °C) when muscles were removed from frogs 3 hours after thawing began (but not after 24 hours thawing) suggesting that early recovery of motor functions is linked to the reversal of one or more

time-dependent metabolic changes occurring during freezing (Layne, 1992). Analysis of the recovery process in freeze-tolerant box turtles, *Terrapene carolina,* showed a sequence of motor responses, from simple to complex, during thawing. The first response seen was a reflex retraction of an individual limb in response to poking. This was followed *somewhat later by coordinated retraction of limbs and head in response* to poking one limb, and finally by voluntary locomotion (Storey et al., 1993). These results show that although individual muscles can quickly regain their ability to respond to stimuli after thawing, the ability to coordinate muscle movements via the central nervous system requires a longer recovery time.

Some of the adaptations that support freeze tolerance, including ice nucleating proteins and cryoprotectants, have been extensively analyzed and reviewed. The reader is referred to several other publications for more in-depth analyses of these topics (Storey and Storey, 1988, 1991; Duman et al., 1991a, 1991b). The following discussions will focus mainly on some new advances in our understanding of natural freeze tolerance in amphibians and reptiles. In particular, several recent studies have investigated the relationships between cell volume regulation and freeze tolerance. The well-developed capacity to endure wide variation in body water content that characterizes all amphibians appears to have been the foundation for the development of freeze tolerance by terrestrially hibernating frogs.

III. CRYOMICROSCOPIC ANALYSIS OF FREEZING IN FROG LIVER

As explained above, cell volume regulation is probably the most critical factor in freezing survival (Lovelock, 1953; Mazur, 1984; Storey and Storey, 1988) with mechanisms that limit the extent of cellular dehydration and protect/stabilize macromolecules being of primary importance in the cryoprotection of cells. However, when entire tissues or organs are frozen, additional forms of freezing damage can occur. In a tissue, ice tends to form first in the lumen of blood vessels (or in liver in the sinusoids) and water is drawn out of cells to freeze in the vascular space (Rubinsky et al., 1987; Rubinsky and Pegg, 1988). The vascular expansion that results can do physical damage to delicate blood vessels and, upon thawing, capillaries are not functional if structural integrity has been lost (Rubinsky et al., 1987). Studies with mammalian tissues have

shown that this type of damage can be extensive and it is obvious, therefore, that freeze-tolerant animals must have developed natural solutions to this problem.

Ice formation in tissues can be visually examined using the technique of directional solidification to control the propagation of the freezing front through a tissue slice followed by cryomicroscopy to reveal the resulting effects on tissue ultrastructure. In this technique a glass microslide holding a tissue slice is cooled between predetermined high (above the tissue freezing point) and low (below the freezing point) temperatures by propelling the slide at a controlled rate between the high and low temperature bases of the stage (Rubinsky and Ikeda, 1985). This initiates a unidirectional propagation of ice through the tissue in a manner that should mimic the directional mode of ice penetration into an intact organ *in vivo*. In a recent study we applied these techniques for the first time to a freeze-tolerant animal, the wood frog *R. sylvatica* (Storey et al., 1992b). We compared the freezing behavior of liver slices from control, 5 °C-acclimated frogs with those from frogs given prior whole animal freezing exposure at -4 °C; this freezing temperature is easily endured by wood frogs and induces the synthesis of high levels of glucose as a cryoprotectant (Storey and Storey, 1984). Slices were exposed to controlled freezing *in vitro* on the directional solidification stage at either -7 °C, a survivable temperature for the frog, or at -20 °C, a temperature that is not experienced naturally. Subsequently, samples were flash frozen in liquid nitrogen slush and prepared for the scanning electron microscope. The results were striking. The normal structure of liver from control frogs, after flash freezing, is shown in Figure 1A; hepatocytes have dimensions of 20-30 μm and are interspersed with sinusoids of 10-20 μm. By contrast, when control liver slices were first frozen on the directional stage to -7 °C, hepatocytes appear extremely shrunken. Virtually no water appears to be left in the cells and huge extracellular ice crystals fill the sinusoids (Figure 1b). Indeed, it can be calculated (Rubinsky and Pegg, 1988) that only about 7% of initial cellular water would remain in control hepatocytes frozen at -7 °C. This same pattern of freezing is observed with mammalian liver or kidney slices (Rubinsky et al., 1987; Bischof et al., 1990) and is consistent with predictions, that is, ice forms first in the vascular space, water is drawn out of cells, cells shrink in size, and the vascular space expands and fills with ice.

However, when frogs were first given a natural freezing exposure at -4 °C, the subsequent images of liver slices frozen at -7 °C were very

different (Figure 1C,D). Ice again appeared in an expanded vascular space but hepatocytes were less shrunken and the cells were speckled with small intracellular ice crystals. These crystals, formed during immersion in the liquid nitrogen slush, are clear evidence of the presence of a substantial amount of unfrozen water in the cells at -7 °C. The different appearance of these cells compared with those from control liver can be traced to the presence of high levels of cryoprotectant; mean glucose content was 280 μmol/g wet weight (or 350 mM in cell water) in liver of the frogs exposed to -4 °C, compared with 1-5 μmol/g in controls (Storey et al., 1992b). The colligative effect of this amount of glucose would result in the retention of 18% of the initial cell water when cells are frozen at -7 °C.

These results provide the first visual evidence that the cells of freeze-tolerant vertebrates follow the pattern of cell and tissue freezing that has been observed in cryomedical studies with material from freeze-intolerant animals and clearly illustrate the importance of natural cryoprotectants in limiting cell volume changes during freezing. Furthermore, the images indicate the physical basis for the survivable range of freezing temperatures determined from whole animal studies. The limit in nature for *R. sylvatica* seems to be about -6°C to -8 °C, values well matched to the minimum low temperatures likely to be encountered under the snowpack. The micrographs revealed that cells from -4 °C-exposed frogs, containing high cryoprotectant levels, maintained substantial amounts of free intracellular water when frozen at -7 °C, whereas cells from control animals were virtually dehydrated, with no visible evidence of free water. However, when liver slices from -4 °C exposed frogs were frozen at -20 °C on the directional stage, the cells showed extreme dehydration and no indication of the presence of remaining intracellular ice as was seen in control slices frozen to -7 °C (Storey et al., 1992b). Thus, it is apparent that the cells of freeze-tolerant animals are adapted to resist the potential injuries caused by freezing, but only within the natural range of subzero temperatures experienced by the species.

As mentioned previously, freezing damage to mammalian tissues has been linked to vascular expansion; large amounts of ice forming in the lumen of blood vessels (or sinusoids) can damage the integrity of the surrounding microcapillary walls so that their function is compromised after thawing (Rubinsky et al., 1987; Bischof et al., 1990). The micrographs in Figure 1 show that this phenomenon also occurs during the freezing of tissue from freeze-tolerant animals, and undoubtedly also

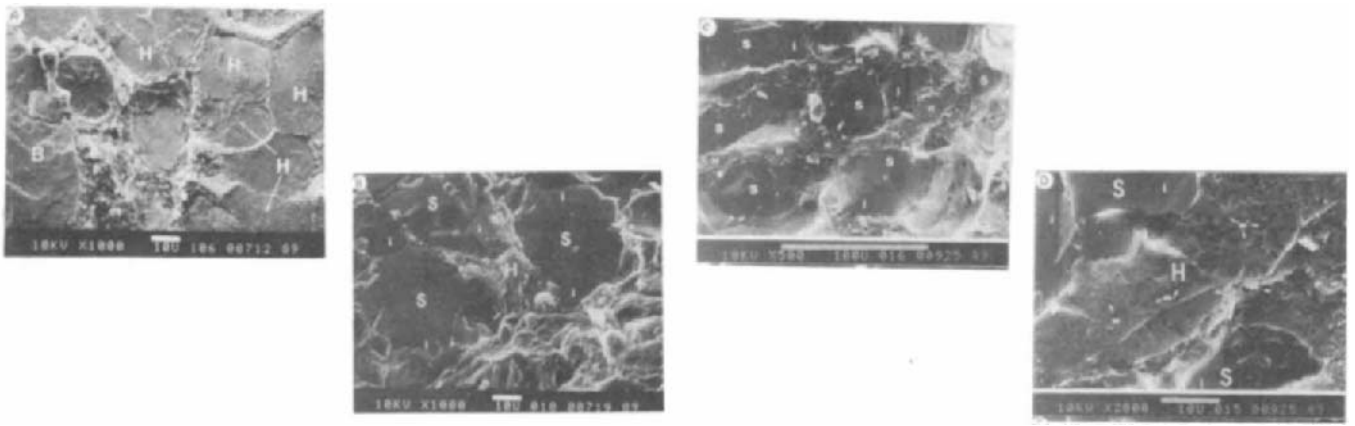

Figure 1. Scanning electron micrographs of liver slices from *Rana sylvatica*. (**A**) The normal appearance of liver from control 5 °C-acclimated frogs; slices were flash frozen in liquid nitrogen slush. (**B**) Liver from 5 °C-acclimated frogs frozen to -7 °C on the directional freezing stage. Note the much reduced volume of the hepatocytes and the large single ice crystals in the sinusoids. (**C**) Micrograph of liver from a frog that was given prior freezing exposure *in vivo* at -4 °C for 24 hours and then slices were frozen *in vitro* to -7 °C on the directional stage. Ice crystals are seen in the sinusoids, which are expanded relative to normal sinusoids, but hepatocytes are less contracted than in (*B*) and their granular appearance indicates the presence of intracellular free water remaining at -7 °C. (**D**) A higher magnification of (**C**) clearly showing intracellular ice crystals formed when the -7 °C tissue was flash frozen in the liquid nitrogen slush. H, hepatocytes; B, blood vessel; S, sinusoid; i, ice; w, water. Scale bars are 10 or 100 μm as show (from Storey et al., 1992).

occurs in vivo. Natural freeze tolerance must, therefore, include some mechanism(s) for preventing irreversible mechanical damage to the vasculature during freezing. Obviously, cryoprotectant synthesis is one such mechanism because this leads to a greater retention of water within cells. However, another mechanism has recently been indicated. This is the extra-organ sequestration of ice. A frozen frog contains a large amounts of extra-organ ice, much more than would be expected from the amount of liquid in these compartments normally. A large mass of ice fills the abdominal cavity and large flat crystals run sandwiched between the skin and skeletal muscles. Much of this ice appears to be the result of a net loss of organ water during freezing. Indeed, the liver of a frozen frog is noticeably smaller than the corresponding organ of a control animal and this was also seen when magnetic resonance imaging was used to examine freezing and thawing *in vivo* (Rubinsky et al., 1994). When quantified for frogs frozen slowly at -2.5 °C, organ water contents were found to decrease by 2.8%, 8.7%, 12.7%, 19.5%, and 24.2% for eye, brain, skeletal muscle, liver, and heart, respectively, compared with organs from unfrozen animals (Costanzo et al., 1992). By the evacuation of water from organs and its innocuous sequestration in extra-organ sites, the potential for damage due to excessive ice expansion within the microvasculature of organs would be greatly reduced.

IV. DEHYDRATION TOLERANCE IN FROGS: A PRECURSOR TO FREEZE TOLERANCE?

As illustrated in Figure 1, and apparent from measurements of up to 65% of total body water as extracellular ice, freezing places a severe dehydration stress on cells and organs of freeze-tolerant animals. How are freeze-tolerant animals able to endure such extensive cellular dehydration? Recent studies on wood frogs have provided new insights into this question. All amphibians have highly water permeable skin and because of this have evolved high tolerances for variations in body water content and the osmolality of body fluids as well as numerous strategies for resisting water loss that are matched to the lifestyle of different species (Shoemaker, 1992). In fact, in many ways, freezing is just another variant of water stress and predictably, therefore, some of the metabolic responses to freezing may have evolved out of pre-existing amphibian adaptations for dealing with cellular dehydration (Churchill and Storey, 1993; Storey et al., 1996). Two questions followed from this. Would the

hydration state of frogs influence their freezing behavior? Would dehydration alone, in the absence of freezing, trigger various metabolic events that are adaptive for freezing survival?

To begin to answer these questions, experiments were set up to analyze the effects of freezing and the influence of covering vegetation on the water relations of wood frogs. Earlier observations had shown that frogs frozen without insulating cover would soon "freeze-dry" to death (Storey and Storey, unpublished results). Furthermore, since frozen frogs cannot move and undoubtedly also cannot take up water from their frozen surroundings, it could be presumed that they would be unable to redress any net loss of body water over what could be weeks of continuous freezing during winter hibernation. Thus, one factor in long-term freezing survival should be the protection of the frozen animal from whole body desiccation. This turns out to be a function of the protective covering provided by the hibernation habitat. When frogs were placed in dry plastic boxes with no insulating cover and then held at either 1 °C or frozen at -2 °C, animals showed high rates of water loss. The mean rate was 0.32% of initial body water content lost per hour, or 7.7% per day, and was not significantly different for frozen versus unfrozen frogs or from values reported for evaporative water loss by other anuran species (Hillman, 1980; Shoemaker, 1992; Churchill and Storey, 1993). By contrast, wood frogs held in boxes filled with damp sphagnum moss showed no loss of water over 6.5 days at 1 °C and lost only 2.5% of total body water when frozen at -2 °C with this protective covering. The study also showed that wood frogs reached their vital limit between 50-60% of total body water lost (Churchill and Storey, 1993) and from this it can be calculated that a frog could endure only 7-8 days of dry conditions before dying. Thus, it is clear from these data that the choice of a protected and moist hibernation site is critically important for the winter survival of frogs particularly if animals must endure long bouts of freezing during which they cannot move to seek a more favorable microenvironment.

Hydration state also affected the supercooling and freezing characteristics of wood frogs. Fully hydrated autumn-collected *R. sylvatica* showed no significant supercooling and began freezing when body surface temperature fell to -0.8 °C, only slightly below the approximately -0.5 °C freezing point of body fluids (Churchill and Storey, 1993). However, frogs that were first experimentally dehydrated at 5 °C until they had lost either 25% or 55% of their initial body water supercooled to mean values of -2.6 °C and -4.8 °C, respectively, before freezing began. This effect of dehydration on supercooling point may be due to

two factors, a lack of moisture on the outer body surface so that inoculative freezing is inhibited and an increase in the osmolality of body fluids. The latter also underlies the reduced amount of body ice accumulated after 24 hours freezing at -2 °C: mean ice contents were 48.9%, 47%, and 20.5% of total body water for control, 25%, and 50% dehydrated animals, respectively (Churchill and Storey, 1993). Freezing also stimulated an increase in the levels of blood glucose and lactate in these animals, as has been well documented previously (Storey and Storey, 1984, 1986). Significantly, however, levels of both metabolites also increased as a function of dehydration; glucose was 7.2-fold and lactate 4.6-fold higher in the blood of 55% dehydrated frogs after freezing than in frogs that were not dehydrated before freezing exposure (Churchill and Storey, 1993). This not only indicated a synergistic interaction between freezing and dehydration stresses but also suggested that cryoprotectant biosynthesis might be stimulated by dehydration alone. Could the trigger for cryoprotectant production be, not freezing per se, but perceived cell volume changes resulting from extracellular ice formation?

Experiments then analyzed the effects of dehydration alone on metabolism in wood frogs by subjecting animals to controlled dehydration over silica gel desiccant at 5 °C. Five groups of autumn-collected frogs were compared: 5 °C controls, frogs dehydrated to approximately 25% or 50% of total body water lost, and 50% dehydrated frogs that were then rehydrated either partially (back to 25% dehydrated) or fully. Surprisingly, such extensive losses of total body water resulted in very little change in the water contents of individual organs (Churchill and Storey, 1993). Thus, even though the measured percentages of total body water lost were high, 28.5 ± 0.1% and 49.1 ± 2.3% for the two groups, the water content of liver fell only slightly from a mean control value of 90.9% to 87.8% and 87.4% in these two experimental groups. Heart and kidney showed no significant change in organ water content even when 50% of total body water was lost and the greatest effect seen was a drop from 82% to 71.4% water for skeletal muscle in 50% dehydrated animals (Churchill and Storey, 1993). This shows that organ water content is strongly defended and that water is lost first from extra-organ and plasma fluid spaces (note that bladders were evacuated before experiments began).

Figure 2 shows the corresponding effect of dehydration and rehydration at 5 °C on organ glucose levels in autumn-collected wood frogs. Data are expressed as nmol glucose/mg protein since organ protein contents (mg protein/g dry weight) showed virtually no variation during either dehydration or rehydration (Churchill and Storey, 1993). Dehydration

stimulated a strong increase in glucose content of all organs that resulted in maximal levels that were nine to 313-fold higher than the corresponding control values. Maximal amounts of glucose in gut, muscle, and kidney ranged from 165 to 404 nmol/mg, corresponding to values of 16-35 μmol/g wet weight. Glucose rose in brain, heart, and liver to 1,092, 1,409, and 1,263 nmol/mg protein, respectively (equal to 72, 43, and 127 μmol/g wet weight). This pattern of glucose accumulation during dehydration and glucose clearance during rehydration was correlated with inverse changes in liver glycogen reserves (Churchill and Storey, 1993). Lactate also rose significantly in four organs as a result of whole animal dehydration with peak values in organs of the 50% dehydrated or the partially rehydrated frogs and reduced levels in the fully rehydrated frogs; maximum net increases were 50.5 and 51 nmol/mg protein in heart and liver, respectively (Churchill and Storey, 1993).

These metabolic responses of autumn wood frogs to dehydration at 5 °C are virtually the same as the responses to freezing; the same result has also been found for another freeze tolerant species *P. crucifer* (Churchill and Storey, 1994). Freezing stimulates a massive glycogenolysis in liver that rapidly elevates glucose to levels of about 200 μmolg/gww in core organs and up to about 50 μmol/g wet weight in peripheral tissues such as skin and skeletal muscle (Storey and Storey, 1986 Storey; 1987a). This same hyperglycemic response to dehydration, in the absence of freezing, clearly suggests that cryoprotectant biosynthesis in frogs developed out of a volume regulatory response. Cell volume change is the critical signal and not the physiological stress, freezing or dehydration, that caused the volume reduction. Since cryoprotectant biosynthesis during freezing is triggered less than five minutes after nucleation (when whole animal ice content is still negligible), it is probable that the initial stimulus comes from peripheral receptor cells, most likely on the skin. These would experience a threshold level of water loss when freezing begins around them and then transmit a signal that triggers glycogenolysis in the liver. Glucose is then rapidly synthesized and exported into the circulation, to be delivered to and taken up by all other organs as freezing progresses. The signal involved in mediating this response has not yet been identified but appears to be hormonal. Furthermore, since freezing-induced glucose synthesis is inhibited by injections of propranolol, a β-adrenergic antagonist (Storey and Storey, 1996), it appears that the signal acts by triggering changes in intracellular cyclic AMP levels in the liver.

Although perhaps not part of the initial triggering of cryoprotectant output, volume decreases by liver cells themselves certainly occur during

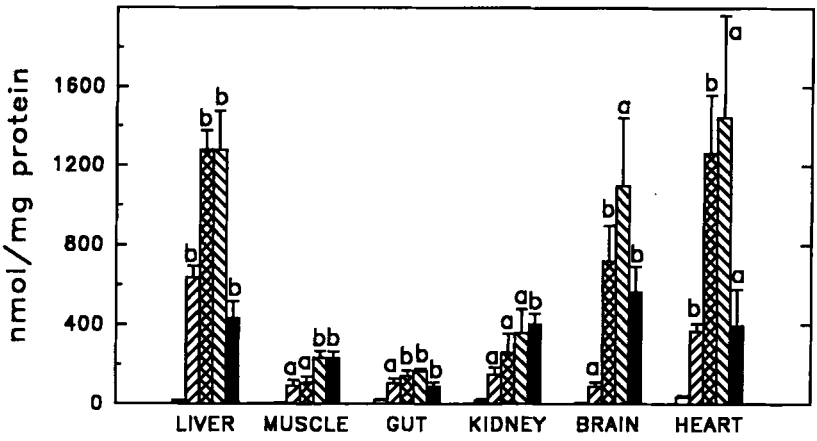

Figure 2. Effect of whole animal dehydration on glucose levels in six organs of autumn *R. sylvatica*. Frogs were dehydrated at 5 °C in closed containers over desiccant at a rate of water loss of 0.5% to 1% of total body water lost per hour and, after dehydration to 50% water lost, were rehydrated by placing animals in a tray of distilled water. Data are means ± SEM, n = 4. Bars are: □, control 0% dehydrated; ▨, 25% dehydrated; ▩,50% dehydrated; ▧, rehydrated to 25%; and ■, rehydrated to 0%. a, Significantly different from the corresponding control value, $p < 0.05$; b, $p < 0.005$. Data are from Churchill and Storey (1993).

freezing (Figure 1) and these may be key to sustaining glucose output from hepatocytes over many hours. Glucose levels in vertebrate blood are normally regulated within quite strict limits by the opposing actions of insulin and glucagon on liver glycogen metabolism as well as a direct effect (homeostatic control) of high glucose in inhibiting glycogen phosphorylase (Hers, 1976). Nonetheless, glucose levels in frozen frogs may reach 300 mM in liver compared with amounts of 1-5 mM in unfrozen animals. Some factor must permit continued glycogenolysis even as glucose rises to very high levels. Analysis of the kinetic properties of *R. sylvatica* liver glycogen phosphorylase showed that the enzyme exhibited similar sensitivity to glucose inhibition (dissociation constant = 12.5 mM) as does the mammalian liver enzyme (Risman et al., 1991). Furthermore, the isolated enzyme exhibited significant inhibition (60% to 95%) at glucose concentrations of 50 to 500 mM. The exceptionally high activity of phosphorylase in liver of wood frogs (activity was 12-fold higher in hepatocytes from *R. sylvatica* compared with *Rana*

pipiens (Mommsen and Storey, 1992)) probably accounts for the ability to retain significant phosphorylase activity even as glucose rises to very high levels. However, an additional stimulation of phosphorylase activity could come from cell volume signals. New studies with rat liver have documented the opposing effects on cell volume caused by insulin (cell swelling) and glucagon (cell shrinkage) and linked the regulation of proteolysis by these hormones to their effects on cell volume (Dahl et al., 1991). Another study has shown that incubation of hepatocytes under conditions that increase their volume (e.g., addition of amino acids or hypo-osmotic media) stimulates glycogen synthase activity, and hence glycogen storage, and that this effect is antagonized by the addition of glucagon (Baquet et al., 1991). Thus, liver cell shrinkage could be expected to promote continued liver glycogenolysis during freezing whereas the cell swelling that occurs when frogs thaw should facilitate cryoprotectant clearance and the reconversion of glucose into glycogen. In line with this, the data in Figure 2 for the rehydrating frogs shows that glucose levels remained elevated in frogs while they rehydrated from the 50% to 25% water loss value but that the sugar was cleared during further rehydration; this suggests that there is a critical cell volume associated with activating liver glycogen synthesis.

It is becoming clear, therefore, from these and other studies (see Watson, 1991 for review) that changes in cell volume can have many important metabolic effects on cells and that the actions of various extracellular stimuli may be exerted in whole or in part by their actions in altering cell volumes. Metabolic effects stimulated by glucagon in hepatocytes, increased glycogenolysis and reduced cell volume, may indeed be linked events whether or not hormones are present. Thus, in wood frogs the continuous decrease in cell volume that occurs as ice content builds up over many hours of freezing may be one of the most critical factors in stimulating and/or sustaining various metabolic events that protect cells while frozen (Storey et al., 1996). In addition to the glucose synthesis response by liver, other events that are volume-regulated might also include cryoprotectant uptake by other organs, the synthesis of specific stress-related proteins, and metabolic arrest mechanisms that improve ischemia tolerance while frozen.

V. CONTROL OF CRYOPROTECTANT SYNTHESIS

Three species of freeze-tolerant frogs produce glucose as their cryoprotectant: wood frogs (*R. sylvatica*), spring peepers (*Pseudacris crucifer*),

and chorus frogs (*P. triseriata); Hyla versicolor* accumulates glycerol instead (Storey and Storey, 1988). As discussed above, frogs do not maintain constant high glucose levels throughout the winter months as insects do with their polyol cryoprotectants but synthesize glucose in direct response to ice nucleation in body extremities. The wood frog shows several modifications of carbohydrate metabolism that allow it to rapidly produce large amounts of sugar within a few hours at subzero body temperatures. Some of these are quantitative modifications whereas other modifications allow cryoprotectant metabolism to respond to unusual triggers (freezing, cell volume change). Hepatocytes from *R. sylvatica,* for example, contained six times as much glycogen (up to 180 mg/g wet weight) and 12 times the glycogen phosphorylase activity as did cells from *R. pipiens* (Mommsen and Storey, 1992). The regulation of glycogenolysis in response to freezing has been extensively studied in wood frog liver (Storey and Storey, 1988; Crerar et al., 1988; Risman et al., 1991), but control over glycogen breakdown is not the only mechanism needed to ensure that glucose is produced and delivered to other organs. Two new studies address other aspects of this problem: the mechanism of inhibitory control over liver glycolysis that is needed in order to divert carbohydrate flux into glucose output, and the differences in glucose transport across frog cell membranes that are needed to ensure rapid export and import of cryoprotectant from cells.

Glycogen breakdown by glycogen phosphorylase yields glucose-1-phosphate (G1P) which is converted by a mutase reaction to glucose-6-phosphate (G6P). G6P sits at a branchpoint in metabolism and can have numerous fates in the cell including catabolism by glycolysis, oxidation via the hexose monophosphate shunt, reconversion to glycogen, and dephosphorylation to form glucose. To promote G6P channeling into this last fate, regulatory controls act to inhibit the other routes and, due to the magnitude of carbohydrate flux during cryoprotectant synthesis, these must exert powerful inhibition on the other pathways. Catabolism of G6P via glycolysis is regulated primarily at the phosphofructokinase-1 (PFK-1) reaction, the first committed step of the glycolytic pathway. In mammals, conditions that promote glucose efflux from liver (e.g., starvation, anoxia, catecholamine stimulation) inhibit glycolytic flux at the PFK-1 locus (Hue and Rider, 1987; Pilkis et al., 1987). The same is true in wood frog liver; an analysis of changes in the concentrations of glycolytic intermediates over the early minutes of freezing exposure showed rapid increases in liver G1P, G6P, and fructose-6-P (F6P), the substrate of PFK-1, concomitant with the rise in glucose output but no

significant changes in the levels of fructose-1,6-P_2, the product of PFK-1 (Storey, 1987b). Thus, it is apparent that glycolysis is blocked during cryoprotectant synthesis by inhibitory control on the PFK-1 locus. The mechanism of this inhibition was discovered only a decade ago for mammalian systems. At that time a powerful allosteric activator of PFK-1 was identified, fructose-2,6-P_2 F2,6P_2 (for review see Hers and van Schaftingen, 1982). This compound is synthesized by PFK-2 and activates PFK-1 in nanomolar concentrations (Figure 3; Hue and Rider, 1987). The activity of PFK-2 is, in turn, controlled by reversible protein phosphorylation of the enzyme (Hue and Rider, 1987; Pilkis et al., 1987). An extracellular signal, by activating cyclic AMP-dependent protein kinase, sets off a chain of intracellular phosphorylation events including the phosphorylation and activation of glycogen phosphorylase and the phosphorylation and inactivation of PFK-2 and glycogen synthase. Inactivation of PFK-2, as well as an activation of the oppositely-directed enzyme, fructose-2,6-bisphosphatase, leads to a rapid drop in F2,6P_2 levels which in turn results in a sharp drop in PFK-1 activity.

A new study has shown that wood frog liver exploits this same mechanism for inhibitory control over PFK-1 and glycolysis during cryoprotectant synthesis (Vazquez-Illanes and Storey, 1993). As Table 2 shows the levels of F2,6P_2 drop sharply in the liver of frozen frogs but rebound again when animals are thawed. Furthermore, freezing stimulated major changes in the kinetic properties of PFK-2. Thus, PFK-2 from liver of freezing-exposed frogs showed changes in kinetic properties including a 10-fold decrease in affinity for F6P, a decrease in enzyme maximal activity, and large changes in inhibitor constants (Table 2). All of these were reversed after thawing. The changes in the kinetic proper-

Table 2. Effect of Freezing and Thawing *In Vivo* on the Levels of Fructose-2,6-Bisphosphate and the Properties of 6-Phosphofructo-2-Kinase from *Rana Sylvatica* Liver

	Control	Frozen	Thawed
F2,6P_2, nmol/g wet weight	1.00 ± 0.12	0.23 ± 0.03[a]	1.08 ± 0.14[b]
PFK-2 properties			
V_{max}, μU/g wet weight	350 ± 12	239 ± 7.7[a]	229 ± 8.1[a]
K_m F6P, mM	0.11 ± 0.02	1.11 ± 0.04[a]	0.08 ± 0.02[b]
I_{50} PEP, μM	22 ± 1.3	53 ± 5.6[a]	30 ± 1.4[b]
I_{50} glycerol-3-P, mM	0.48 ± 0.06	28.6 ± 2.06[a]	0.99 ± 0.05[b]

Notes: [a] Significantly different from the corresponding control value, $p < 0.05$,
[b] Significantly different from the corresponding frozen value, $p < 0.05$.
Data from Vazquez-Illanes and Storey (1993).

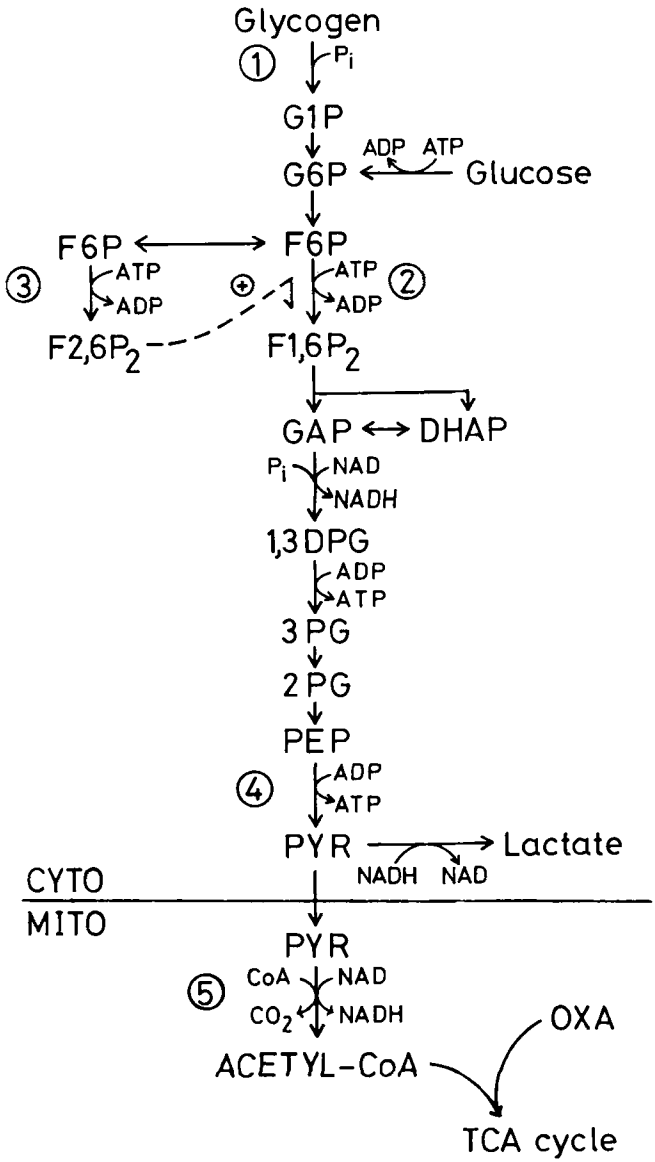

Figure 3. The glycolytic pathway showing the associated reaction of fructose-2,6-bisphosphate synthesis and the entry of carbohydrate into the tricarboxylic acid cycle. Key regulatory enzymes controlled by reversible protein phosphorylation are: (1) glycogen phosphorylase, (2) 6-phosphofructo-1-kinase, (3) 6-phosphofructo-2-kinase, (4) pyruvate kinase, and (5) pyruvate dehydrogenase.

ties of PFK-2 during freezing are consistent with the effects of protein phosphorylation on the enzyme, as occurs in mammals, and serve to produce a less active form of the enzyme. This would lead to a decrease in $F2,6P_2$ levels despite the fact that F6P substrate levels rise 10 to 20-fold in the liver of frozen frogs (Storey, 1987b). The decrease in $F2,6P_2$ content, in turn, reduces PFK-1 activity and, along with a probable protein phosphorylation of PFK-1 that may further inactivate the enzyme (Storey, 1987b), creates conditions where carbon flow into the triose phosphate section of glycolysis is blocked. Furthermore, freezing also stimulates the phosphorylation and inactivation of glycogen synthetase, ensuring unidirectional conversion of glycogen to glucose (Russell and Storey, 1995). Thus, in response to an external stimulus signaling that freezing has begun at peripheral body sites, protein kinase activity in liver is activated and targets at least three enzymes: glycogen phosphorylase, glycogen synthetase, and PFK-2. By activating the first and inhibiting the latter two a rapid increase in liver glycogenolysis occurs and is directed towards the production of glucose for export.

VI. GLUCOSE TRANSPORTERS

To rapidly raise cryoprotectant concentrations in the different organs of the freezing frog, an efficient mechanism for moving glucose across cell membranes is required. Cryoprotectants such as glycerol readily diffuse across cell membranes but glucose does not; the primary mode of glucose movement across cell membranes is carrier-mediated transport. Not surprisingly, then, freeze-tolerant frogs also show modifications of their glucose transport system that allow the rapid distribution of the sugar as a cryoprotectant.

The plasma membranes of vertebrate cells contain specific glucose transporter proteins that mediate glucose movement via facilitated transport. Five organ-specific isoforms have been identified in mammals (Pessin and Bell, 1992). Recent studies have assessed plasma membrane glucose transport in both the cryoprotectant-exporting organ (liver) and a cryoprotectant-importing organ (skeletal muscle) of wood frogs (King et al., 1993). As in mammals (Pessin and Bell, 1992), carrier-mediated glucose flux across frog plasma membranes proved to be stereospecific for the D-isomer and was effectively inhibited by cytochalasin B. However, a comparison of membranes from freeze-tolerant *R. sylvatica* with those from *R. pipiens* showed a much greater capacity for glucose transport in organs of the freeze-tolerant frog (Table 3; King et al., 1993).

Table 3. The Glucose Transport System in Frog Liver and Muscle Plasma Membrane Vesicles

	V_{max} n mol.mg protein^{-1}.sec^{-1}	$K_{1/2}$ mM	R_o pmol.mg protein^{-1}	K_d nM
Liver				
R. sylvatica	69 ± 18	48 ± 16	80 ± 7.1	269 ± 21
R. pipiens	8.4 ± 2.3	47 ± 21	17 ± 0.5	194 ± 60
	$p < 0.02$	N.S.	$p < 0.001$	N.S.
Muscle				
R. sylvatica	4.9 ± 1.0	39 ± 13	5.5 ± 1.5	71 ± 31
R. pipiens	0.6 ± 0.16	16 ± 7	4.4± 0.9	75 ± 13
	P < 0.01	N.S.	N.S.	N.S.

Notes: Data are means ± *SE*, $n = 3$ separate membrane preparations. Transport assays were performed at 10 °C for liver membranes and at 22 °C for muscle membranes. R_o is the number of transporters determined by cytochalasin B binding. K_d is the dissociation constant for cytochalasin B binding. The *p* values indicate significant differences between values for *R. sylvatica* and *R. pipiens*. Data from King et al. (1993).

The Vmax for glucose transport by liver and muscle plasma membrane vesicles was eightfold higher in both organs of *R. sylvatica* compared with R. pipiens. In the liver the greater transport Vmax for *R. sylvatica* appeared to result primarily from a much larger number of transporter sites in the membranes; thus, the number of transporter sites per milligram protein (R_o values), as determined by cytochalasin B binding, were 4.7-fold higher in *R. sylvatica* compared with *R. pipiens* liver membranes. Transporter activity in *R. sylvatica* liver membranes also appeared to be higher with estimates of the average carrier turnover numbers (V_{max}/R_o at 10 °C) being 862 ± 237 sec^{-1} for *R. sylvatica* versus 494 ± 135 sec^{-1} for R. pipiens. However, there was no significant difference in the affinity of liver transporters for glucose ($K_{1/2}$ values) between the two species.

In muscle the situation was somewhat different. The higher glucose transport rates in *R. sylvatica* muscle membranes were not due to a difference in transporter numbers; R_o values for cytochalasin B binding were the same between the species (Table 3). Instead, the freeze-tolerant frog showed a much higher carrier turnover number, 890 ± 244 sec-1 for *R. sylvatica* compared with 136 ± 45 sec-1 for *R. pipiens* (measured at 22 °C), indicating a difference in transporter activity for the two species.

These adaptations that modify the capacity for glucose transport across the plasma membranes of *R. sylvatica* organs would greatly enhance the capacity for distributing glucose throughout the body of the frog during freezing exposure. Liver glycogen, in amounts as much as

700 μmol glucose units/g wet weight, is rapidly broken down over the early hours of freezing exposure, transported out of the liver and delivered to other organs which accumulate organ-specific amounts of glucose ranging from 50 to 350 μmol/g wet weight (Storey and Storey, 1988). Such large movements of sugar, occurring at subzero body temperatures, require very high glucose transport capacities in both the exporting organ (liver) and the importing organs (such as skeletal muscle), and indeed, the data show that the wood frog, in comparison with the leopard frog, has made such adaptive adjustments. Recent studies have shown that wood frogs elevate glucose transport capacity seasonally, autumn-collected frogs showing a much greater capacity than summer frogs (King et al., 1995). Modification of glucose transport properties is one of a group of anticipatory adaptations for freezing survival that include other modifications such as liver glycogen content and greater liver phosphorylase activity that were discussed earlier.

Whether glucose transport capacity by wood frog membranes can also be further increased in the short term, as a direct response to freezing exposure, remains to be determined. Glucose transport capacity in mammalian skeletal muscle plasma membranes increases in response to insulin treatment or workload and in heart increases in response to these factors as well as increased glucose concentration (Zaninetti et al., 1988; King et al., 1989). These changes result from the translocation of transporters from an inactive microsomal pool. Heat stress also causes a translocation of glucose transporters in other cells (Widnell et al., 1990). Thus, both the capacity for efflux from liver and the uptake of cryoprotectant by other organs of the wood frog might also be further enhanced during freezing exposures by specific effects of hormones, high glucose concentrations, or temperature acting to stimulate the translocation of transporters from microsomal to plasma membrane sites.

VII. OXYGEN FREE RADICALS AND ANTI-OXIDANT ENZYME SYSTEMS

Our discussion to date has included new information on the mode of tissue freezing, the potential relationships between freeze tolerance and dehydration tolerance, and the mechanisms of cryoprotectant synthesis and distribution in freeze-tolerant animals. Freezing also has specific consequences for the energy metabolism of cells. Most importantly, natural freezing results in long periods of organ ischemia as a result of

the freezing of extracellular body fluids and the cessation of breathing and blood circulation. Freezing survival, then, depends on an ability to endure anoxia and ischemia using anaerobic pathways of energy generation to maintain viability in all cells and organs. This has been well-documented and discussed previously (Storey and Storey, 1984, 1985, 1986, 1988). However, another important consideration in ischemia tolerance has recently received much attention in the medical literature. This is the subject of reperfusion damage associated with the rapid reintroduction of oxygen and the resumption of oxidative metabolism. Such damage has been traced to a "burst" of reactive oxygen species generation when oxygen is first reperfused into an organ and has been identified as one of the most serious problems associated with the hypothermic storage of mammalian organ explants (Fuller et al., 1988; Ruuge et al., 1991; Eckenhoff et al., 1992).

Freeze-tolerant animals must undergo cycles of ischemia and reperfusion as part of natural freezing and thawing. It is highly likely, then, that one of the consequences of reoxygenation that they experience is an overgeneration of reactive oxygen species. We recently began studies to determine whether freeze-tolerant animals show adaptive changes to the normal antioxidant defense mechanisms that would enable them to better deal with oxygen free radical stress during thawing and reperfusion. Analysis of the adaptive mechanisms that occur naturally in freeze-tolerant animals may also prove instructive in the development of treatments for dealing with this problem as it occurs during the hypothermic or cryopreservation of mammalian organ explants.

Free radicals generated by a parietal reduction of O_2 pose a serious hazard to animal cells, particularly to membrane lipids, connective tissues, and nucleic acids (Harris, 1992). Antioxidant enzymes provide protection against the damaging effects of O_2^-, $\cdot OH$, H_2O_2, and organic peroxides. The three major enzymes involved are superoxide dismutase, catalase and glutathione peroxidase catalyzing reactions (1), (2), and (3), respectively:

$$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad (1)$$

$$2H_2O_2 \rightarrow O_2 + 2H_2O \quad (2)$$

$$ROOH + 2GSH \rightarrow ROH + H_2O + GSSG \quad (3)$$

(Halliwell and Gutteridge, 1985; Harris, 1992). The action of these enzymes in eliminating superoxides and peroxides limits the formation

of ·OH radicals, the most potent oxidant, which are generated by reactions involving iron (Haber Weiss reaction):

$$O_2^- + Fe^{3+} \rightarrow O_2 + Fe^{2+} \quad (4)$$

$$H_2O_2 + \rightarrow OH^- + \cdot OH + Fe^{3+}. \quad (5)$$

The importance of antioxidants to cellular metabolism has been illustrated in several ways including (as noted above) the damaging effects oxyradical overgeneration during the reperfusion phase following organ ischemia (Fuller et al., 1988; Ruuge et al., 1991; Eckenhoff et al., 1992). Other studies have correlated adaptive increases in the activities of antioxidant enzymes with situations where increased oxidative stress is predicted. Thus, the activities of mammalian lung antioxidant enzymes are enhanced during late gestation in preparation for the elevated oxygen pressure after birth (Clerch and Massaro, 1992; Frank and Sosenko, 1992). Antioxidant defenses also increase in brown adipose tissue of hibernating ground squirrels as a probable protection against the overgeneration of oxyradicals in the tissue during the arousal process (Buzadzic et al., 1990).

To analyze the role of antioxidant defenses in freeze tolerance we determined the effects of five hours of freezing exposure at -2.5 °C on the activities of antioxidant enzymes and the levels of glutathione in organs of garter snakes *Thamnophis sirtalis* (Hermes-Lima and Storey, 1993). For comparison, the effects of anoxic exposure (10 hours under nitrogen gas atmosphere at 5 °C) were also assessed. Although garter snakes are not among the most freeze-tolerant vertebrates, they do readily endure short-term freezing exposures (Churchill and Storey, 1992). As Figures 4a and 4b show, freezing exposure resulted in a significant increase in the activity of catalase in skeletal muscle and lung of garter snakes as well as an increase in muscle glutathione peroxidase activity. However, anoxic exposure did not affect the activity of either enzyme. By contrast, anoxia resulted in a significant increase in superoxide dismutase in liver and muscle, but enzyme activity was not affected by freezing exposure (Hermes-Lima and Storey, 1993). Activities of the secondary antioxidant defense enzymes, glutathione reductase and glutathione S-transferase, were largely unaffected by freezing or anoxia stresses except for a small decrease (27%) in glutathione S-transferase in liver of frozen snakes. Freezing exposure also had no effect on the glutathione status of snake organs, whereas anoxic exposure resulted in

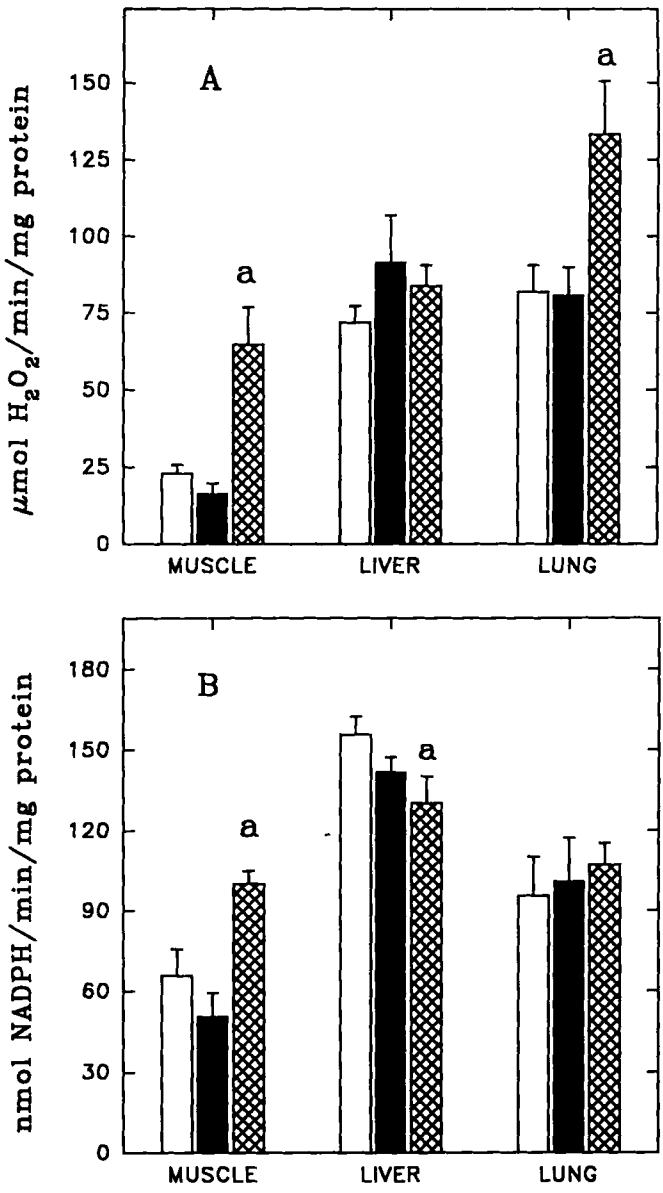

Figure 4. Effect of freezing or anoxia exposures on the activities of (**A**) catalase and (**B**) glutathione peroxidase in organs of garter snakes *Thamnophis sirtalis*. Open bars, control 5 °C acclimated snakes; filled bars, snakes given 10 hours anoxia exposure at 5 °C under 97.5:2.5% N_2/CO_2; hatched bars, snakes given five hours freezing exposure at -2.5 °C. Data are means $n \pm$ SEM, n = 3-5. a, Significantly different from the corresponding control value, $p < 0.05$ (from Hermes-Lima and Storey, 1993).

an increase in both reduced (GSH) and oxidized (GSSG) glutathione in skeletal muscle but did not alter the GSSG/GSH ratio (Hermes-Lima and Storey, 1993). Since an increase in the GSSG/GSH ratio is indicative of the overgeneration of oxyradicals (Ji and Fu, 1992), the lack of change in GSSG/GSH ratios during either experimental exposure suggests that an oxidative stress does not occur during the actual freezing or anoxic exposures. Therefore, this suggests that the activation of antioxidant enzyme systems during these stress exposures is an anticipatory response that prepares the animal to deal an overgeneration of oxyradicals at the termination of the freezing ischemia or anoxic insult. New studies have also examined the responses of antioxidant defense systems in wood frog organs to freezing and thawing (Joanisse and Storey, 1996).

VIII. SUMMARY

Natural freeze tolerance is well-developed in a variety of terrestrially-hibernating amphibians and reptiles as a mechanism of winter survival. Recent studies have investigated several new aspects of freeze tolerance in vertebrates. The technique of directional solidification coupled with cryomicroscopy has revealed new information about the physical process of freezing in organs. Micrographs of liver slices from the wood frog *R. sylvatica* showed that ice propagates through the vascular space of tissues and that as ice forms cells shrink in size and the vascular space expands. The studies also clearly confirmed the importance of the natural cryoprotectant, glucose, in limiting cell volume reduction during freezing. Thus, in the absence of the natural cryoprotectant, glucose, hepatocytes in liver slices frozen *in vitro* to -7 °C (a survivable temperature in nature) were virtually totally dehydrated whereas cells that were preadapted and contained high glucose showed the presence of substantial free intracellular water remaining at -7 °C. Other studies have compared the responses of wood frogs to desiccation versus freezing and found that metabolic responses to freezing (e.g., cryoprotectant synthesis by liver) are also stimulated when frogs are exposed to desiccating conditions, in the absence of freezing. Thus, some of the adaptations supporting freezing tolerance may be extensions of pre-existing amphibian mechanisms for dealing with wide variations in total body water content and the activation of responses such as cryoprotectant synthesis may be triggered and/or regulated by change in cell volumes. Studies have also further analyzed the regulation of cryoprotectant synthesis and

distribution in wood frogs. To promote glucose export, inhibitory control over liver glycolysis is focused on the PFK reaction via freezing-induced changes in the levels of the PFK activator, fructose-2,6-bisphosphate (F2,6P2), as well as changes in the activity of 6-phosphofructo-2-kinase that produces F2,6P2. The delivery of glucose to all organs as a cryoprotectant is also facilitated by changes in the carrier-mediated transport of glucose across wood frog plasma membranes. Compared with glucose transporters in an aquatic frog (*R. pipiens*), *R. sylvatica* plasma membranes showed both increased numbers and increased activity of glucose transporters in both the glucose-exporting organ (liver) and glucose-importing organs. Finally, studies have also examined the role of antioxidant defense systems in supporting freeze tolerance. Natural freezing survival involves long periods of organ ischemia while blood is frozen and circulation halted. In mammalian systems, oxygen free radical overgeneration during reperfusion has been identified as a key factor in tissue injury due to ischemia. An analysis of the effects of freezing exposure on antioxidant systems in garter snake organs has shown, however, that freezing induces changes in the levels of antioxidant enzymes (catalase, glutathione peroxidase) that should prepare organs to deal effectively with a burst of oxyradical overgeneration when perfusion resumes during thawing.

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RESPONSES OF MARINE FISHES TO FREEZING TEMPERATURES: A NEW LOOK AT COLLIGATIVE MECHANISMS

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I. INTRODUCTION

Many marine poikilotherms inhabit waters that are at or near the freezing point of seawater, but do not freeze. Invertebrates are in no danger of freezing because their body fluids are isosmotic or slightly hyperosmotic with respect to seawater. (The freezing point of a solution is a colligative property, and is therefore a function of the solution's osmolality.) However, the solutes employed intracellularly cannot be the same as those in seawater, in which the total ion concentration is approximately 1.0 M. Intracellular ion concentrations above ~0.2 M can interfere with many processes, such as enzyme activities, binding of ligands by enzymes, protein subunit assembly, protein solubility, interactions between phospholipid bilayers, and membrane fluidity (Somero 1992). To avoid these problems, marine invertebrates and some vertebrates rely on high concentrations of organic osmolytes to maintain an isosmotic condition (Yancey et al., 1982). Many of these osmolytes, such as amino acids and glycerol, are considered "compatible" (Brown and Borowitzka, 1979) because they have little effect on enzyme activities and other cellular processes. Others, such as urea, are disruptive at high concentrations, but can be moderated by the addition of counteracting solutes, such as some trimethylamines (Yancey et al., 1982).

Osmolalities of marine teleosts are typically only one-third those of isosmotic marine animals, due to lower concentrations of both ions and organic osmolytes (Holmes and Donaldson, 1969). Besides providing a more favorable environment for cellular processes, the ability to osmoregulate in these fishes allows optimum membrane potentials in nerve and muscle cells to be maintained despite changing environmental conditions. This, in turn, allows greater levels of activity than would be possible to a nonosmoregulating animal, such as the hagfish (reviewed by Ballantyne et al., 1987).

At subzero temperatures, however, low osmolalities in marine teleosts clearly present a danger. (The low osmolalities correspond to freezing points of about -0.5 ° C, whereas the temperature of seawater may be as

low as -1.9 °C.) Nevertheless, many fishes live their entire lives at these temperatures. Early attempts to explain this phenomenon (reviewed by Holmes and Donaldson, 1969) established that some fish exist in a supercooled state (and thus must completely avoid contact with ice) and that others have anomalously low freezing points. Gordon et al. (1962) found high nitrogen levels in the blood of a cold-acclimatized cod. Because the nitrogen was not precipitated by trichloroacetic acid (TCA), a common protein precipitant, they concluded that the nitrogen was due to a low molecular weight substance, which therefore must have been present at a high concentration.

II. ANTIFREEZE PEPTIDES AND GLYCOPEPTIDES

The problem of freezing resistance in fishes remained unresolved until DeVries and Wohlschlag (1969) reported the presence of several antifreeze glycopeptides in some antarctic fishes. Unlike most proteins, the glycopeptides were not precipitated by TCA. Later it became clear that the nitrogenous substance found by Gordon et al. (1962) was due to similar glycopeptides (Van Voorhies et al., 1978). Many different peptides with a wide variety of structures were subsequently identified (for reviews see Davies and Hew, 1990; Cheng and DeVries, 1991; Hew and Yang, 1992). The antifreeze peptides were found to act in a non-colligative way by adsorbing to ice crystals and thereby preventing them from growing (Raymond and DeVries, 1977). They thus have the unusual ability to prevent body fluids from freezing without significantly increasing their osmolality.

Much progress has been made recently in understanding how the binding process works. Observations of the effects of several different antifreeze peptides on the growth of large single crystals of ice indicated that binding was occurring on specific crystal faces (Raymond et al., 1989). A key feature, which is supported both by etching studies (Knight et al., 1992, 1993) and by molecular modeling (Wen and Laursen, 1992), appears to be lattice matching, in which the spacing of hydrophilic groups in the antifreeze peptides matches the repeat distance of oxygen atoms that lie on specific directions on specific ice crystal surfaces. However, if lattice matching alone were occurring, binding both parallel and antiparallel to these directions should occur with equal frequency. The above studies (see also Wen and Laursen, 1993; Madura et al., 1994) have shown that this is not the case, that is, there is a stereo effect in

which binding along one direction is preferred to binding along the opposite direction.

The rapid progress on the antifreeze peptides has tended to overshadow the fact that many marine fishes also increase concentrations of inorganic ions and organic osmolytes in response to cold. In some cases, these increases can make a greater contribution to freezing resistance than do the peptides. As will be shown below, they also lead to some additional and unusual physiological changes.

III. SERUM INORGANIC IONS

Serum ion concentrations in marine teleosts living in extreme cold environments tend to be higher then those in fishes inhabiting temperate environments. In the fishes of McMurdo Sound, Antarctica, where water temperatures remain near the freezing point of seawater throughout the year, serum sodium and chloride levels are typically 40% higher then they are in temperate water fishes (Dobbs and DeVries, 1975; Figure 1). Significantly lower chloride concentrations were found in closely related fishes in waters that were only a few degrees warmer (DeVries, 1971). In southern Alaska, fishes inhabiting deeper, colder waters had inorganic ion levels that were about 15% higher then those inhabiting shallower waters (Prosser et al., 1970; Figure 1). Sodium and chloride levels in Bering Sea fishes caught in early May (O'Grady and DeVries, 1982) were generally higher than those in temperate water fishes (Figure 1).

Similar patterns can be seen in single species that undergo seasonal temperature changes. Significant winter increases in serum sodium and chloride have been observed in two subantarctic fishes (Smith, 1972) and several subarctic fishes (Pearcy, 1961; Gordon et al., 1962; Umminger, 1969a; Duman and DeVries, 1975; Fletcher, 1977; Smith and Paulson, 1977; Raymond, 1993; Figure 1). The effect of temperature was also demonstrated by Duman and DeVries (1974) who showed that warm acclimation of several winter-caught fishes resulted in significant decreases in sodium and chloride levels.

Prosser et al. (1970) proposed that high inorganic ion concentrations in cold-acclimatized fishes serve to reduce the energy expended for osmoregulation, rather then to increase freezing resistance. This argument was based on cold water fishes that never experienced freezing temperatures. However, the finding of significantly higher inorganic ion concentrations in species that inhabited near-freezing waters (e.g.,

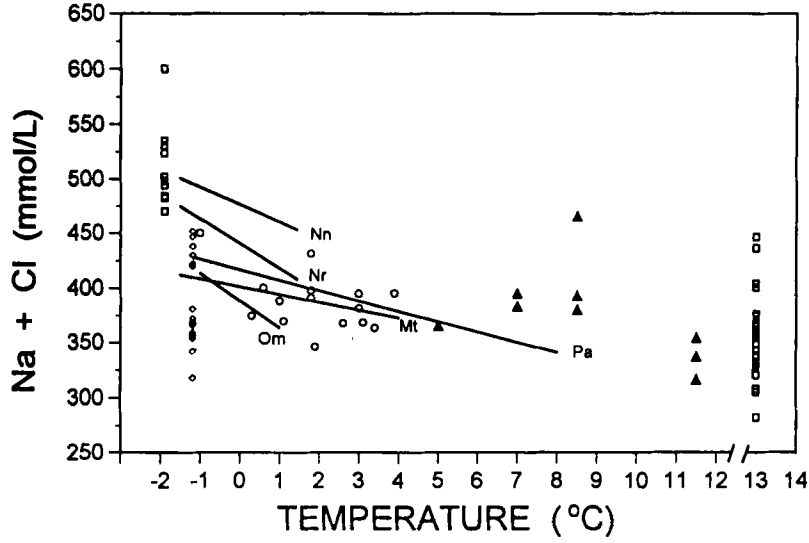

Figure 1. Serum sodium plus chloride ion concentrations in fish obtained at different environmental temperatures. □ (left), Antarctic (Dobbs and DeVries, 1975); ◊, Nova Scotia (Duman and DeVries, 1975); o Bering Sea (O'Grady and DeVries, 1982); ▲ southern Alaska (Prosser et al., 1970); □ (right), temperate water (Holmes and Donaldson, 1969). Lines show seasonal changes for several species. Nn and Nr, *Notothenia neglecta and N. rossii* (Smith, 1972); Om, *Osmerus mordax* (Duman and DeVries, 1974); Pa, *Pseudopleuronectes americanus* (Duman and DeVries, 1975); Mt, *Microgadus tomcod* (Gordon et al., 1962).

Dobbs and DeVries, 1975) led to the alternative hypothesis that these increases were of more use to the fish as a means of freezing resistance (O'Grady and DeVries, 1982). This is evident in Figure 1 where a relatively sharp increase in the concentration of sodium plus chloride occurs where temperatures approach the freezing point of the fishes body fluids. The increases in inorganic ion levels in these fishes 50-100 mMol/L (Figure 1), correspond to lowerings of the freezing points by about 0.1-0.2 °C. Although this amount may appear small, it can be enough to mean the difference between life and death to a fish that is living close to the freezing point of its body fluids.

Serum inorganic ion levels in the Antarctic fishes can be as high as 0.5 M, a level that is similar to those in marine elasmobranchs (Holmes and Donaldson, 1969). There may be some costs to achieving these high levels. High serum ion concentrations tend to increase intracellular ion

concentrations (Lange and Fugelli, 1965; Prosser et al., 1970), and this has been confirmed in the case of three Antarctic species (O'Grady and DeVries, 1982). In these species, muscle and liver tissues had about 175 mM potassium and 55 mM sodium, which are among the highest reported for vertebrates. The total ion concentrations in these fishes are in the range where interference in enzyme activities might be expected (Somero, 1992). High inorganic ion concentrations have also been cited as a cause of poorly mineralized skeletons in primitive fishes (Urist, 1963). The basis for this reasoning is that high ionic strengths reduce the activity of Ca^{2+}, and thus inhibit the precipitation of calcium salts. It is interesting in this regard that the skeletons of several Antarctic fishes (all of which have unusually high ion concentrations) are weakly mineralized (Eastman and DeVries, 1982). Potential limitations of inorganic ions, such as these, suggest that further increases in osmolality in cold water fishes might be better achieved with nonionic solutes.

IV. ORGANIC OSMOLYTES

Organic osmolytes are well-known in cold-acclimatized insects and amphibians (Storey and Storey, 1988; Lee, 1991). However, their existence in teleost fish has been viewed with some skepticism, because low freezing points that early reports had attributed to high osmolalities (Pearcy, 1961; Gordon et al., 1962; Raschack, 1969) were later shown to be due to low concentrations of antifreeze peptides. In addition, no osmolytes that occurred in significant concentrations could be identified. Gordon et al. (1962) found moderate concentrations of urea in two cods and a sculpin in eastern Canada, but found no evidence for several other common organic osmolytes. Increases in urea (Smith, 1972) and glucose (Umminger, 1969b) were found in some cold-acclimatized fishes, but in each case the concentration was too low to play a major roll in freezing avoidance. Unusually high osmolarities were reported in some greenling and smelt species in Alaska (Smith and Paulson, 1977), but these findings appeared to be inconsistent with the emerging antifreeze peptide story, and were not cited in the literature until recently.

A. Glycerol

The first organic osmolyte to be found in significant amounts in the blood of a cold-adapted marine teleost was glycerol. It was found in rainbow smelt, *Osmerus mordax*, from northern Japan and northern

Alaska (Raymond, 1992)and eastern Canada (Raymond, 1993), at serum concentrations between 200 and 400 mMol/L (Figure 2a). Glycerol increased serum osmolalities to more than 1,000 mMol/kg in winter-acclimatized Alaskan smelt, making these fish nearly isosmotic to the seawater. In these fish, glycerol's contribution to the freezing point depression (0.77°) exceeded the contributions of both sodium chloride (~0.64°) and antifreeze peptide (0.45°).

Glycerol was also found in surf smelt, *Hypomesus pretiosus japonica* (152 mMol/L), whitespotted greenling, *Hexagrammos stelleri* (131 mMol/L), and in another greenling, tentatively identified as *H. octogrammus* (23 mMol/L), all from northern Japan. These latter species were the same as those investigated by Smith and Paulson (1977), and thus confirmed, as well as explained, their reports of high osmolalities.

Despite these findings, glycerol does not appear to be widely distributed among arctic fishes. It was not found in a sculpin or two species of cod caught in -1,7°C water in Labrador (Gordon et al., 1962), and was present at only low levels (1-4 mMol/L) in Pacific herring, *Clupea harengus pallasi*, and the saffron cod, *Eleginus gracilis* caught in subzero temperatures in Japan and Alaska (Raymond, unpublished data). In fish caught by O'Grady and DeVries (1982) in the Bering Sea, serum osmolalities could be attributed almost completely to inorganic ions. Surprisingly, only small amounts of glycerol were found in another cold-acclimatized osmerid, the capelin (Raymond J.A., and Hassel A., unpublished data, Institute of Marine Research, Bergen, Norway).

B. Urea

Serum urea concentrations in cold-water fishes have been reported in the range 8-28 mM (Gordon et al., 1962; DeVries, 1971; Raymond, 1994). Although few comparative data are available, these values appear to be higher then they are in temperate water fish (Holmes and Donaldson, 1969;Wood, 1993). (Smith [1972] reported unusually high concentrations of urea in two subantarctic fish, but these values are questionable because they were referred to as small and unimportant.)

C. Trimethylamine Oxide

Trimethylamine oxide (TMAO) is common in muscle tissues of marine teleosts, but it has not previously been found in significant amounts in the blood (Norris and Benoit, 1945; Dyer, 1952; Forster et al.,

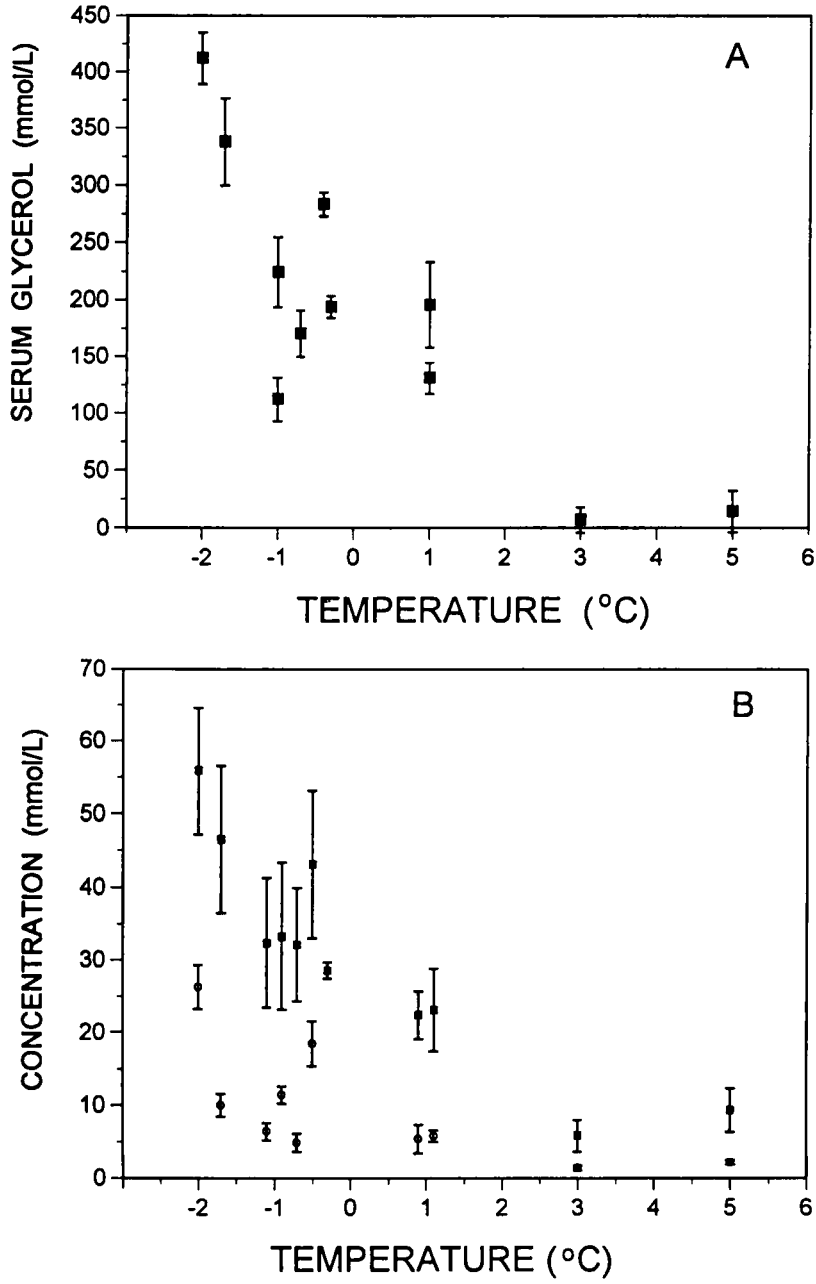

Figure 2. Serum organic osmolyte concentrations in rainbow smelt at different environmental temperatures. (A), glycerol; (B), TMAO (■) and Urea (o)

1958; Parry 1961; Gordon et al., 1962; Lange and Fugelli, 1965; Charest et al., 1988). Recent work has shown that TMAO occurs in the blood of winter-acclimated smelt at concentrations as high as 50 mMol/L (Raymond, 1994). In marine elasmobranchs, in which TMAO is a major osmolyte, serum TMAO concentrations are typically only one-half to one-fifth those in the muscle (Norris and Benoit, 1945; Yamada, 1967; Goldstein and Palatt, 1974; Robertson, 1975; Forster and Goldstein, 1976). The somewhat surprising result is that serum TMAO concentrations in winter-acclimatized smelt are comparable to those in marine elasmobranchs (~40-85 mMol/L) (Cohen et al., 1958; Forster and Goldstein, 1976).

TMAO was also found in the sera of cold-adapted Pacific herring and surf smelt, at concentrations roughly similar to those of the rainbow smelt (Raymond, 1994). Recently, significant serum TMAO levels have also been found in the capelin (Raymond and Hassel, unpublished data) and in some Antarctic nototheniids (Raymond and DeVries, unpublished data). However, other cold water species, such as cods and sculpins, appeared to have very little serum TMAO, in agreement with Gordon et al. (1962).

D. Other Osmolytes

Little information is available on free amino acids in the blood of teleost fishes. Modest values of ~10-15 mMol/L in rainbow trout (Kaushik and Luquet, 1979) and similar levels (7-18 mMol/L) in winter-acclimatized rainbow smelt (Raymond, 1992) suggest that amino acids are not an important contributor to the freezing point depression in fish. Although glucose is an important osmolyte in some amphibians, it has not been found to be osmotically important in cold water fishes. Reported concentrations in the blood are about 2 mMol/L in several antarctic fishes (DeVries and Lin, 1977), 5-7 mMol/L in rainbow smelt (Raymond, 1992), and less than 14 mMol/L in killifish (Umminger, 1969b). In some insects and amphibians, some sugar alcohols, such as sorbitol and threitol, occur as cryoprotectants (Storey and Storey 1988; Lee, 1991), but none of these were present in detectable amounts in the rainbow smelt, surf smelt, or greenling sera.

E. Seasonal and Latitudinal Variation

In fish that experience extreme seasonal temperature variations, concentrations of organic osmolytes, like those of inorganic ions, generally

increase in winter. Approximately 100% to 200% increases have been reported in winter in blood urea (Smith, 1972), blood glucose (Umminger, 1969b), and muscle TMAO (Hughes, 1959). Even more dramatic increases in serum glycerol, TMAO, and urea occurred in the rainbow smelt (Figures 2a and 2b), partly because these osmolytes, unlike those in the preceding examples, were nearly absent in autumn. Anecdotal confirmation of a seasonal change in the concentration of glycerol (a sweet-tasting compound) was provided by fishermen in Japan, Canada, and Alaska who all found that the fish had a sweeter taste in winter.

Among high-latitude species, there also appears to be a trend to elevated muscle TMAO levels with lower temperature. Significantly higher TMAO concentrations have been found in several arctic fishes (cod, haddock, and two species of flatfish) than in more southern populations of the same species (Shewan, 1951). Unusually high levels of muscle TMAO have also been found in four species of subantarctic fish (Oehlenschlager, 1991). The trend toward higher TMAO concentrations as lower temperatures does not apparently apply to temperate water fishes, because in some of these fishes TMAO levels appear to increase in summer (Hebard et al., 1982).

F. Bodily Distribution

Unlike antifreeze peptides, which are excluded from cells because of their size (Cheng and DeVries, 1991), organic osmolytes permeate the cells and thus tend to be more evenly distributed among different tissues. In smelt, glycerol and urea were roughly proportional to water contents of various tissues, with the exception that urea concentrations in the urine were reduced. TMAO was distributed less evenly, with relatively high levels in the muscle, lower levels in the blood and liver, and still lower levels in the urine. Relatively even distributions of urea and relatively uneven distributions of TMAO have also been found in other teleosts and marine elasmobranchs (e.g., Suyama and Tokuhiro, 1954; Goldstein and Palatt, 1974; Robberson, 1975; Sakaguchi and Murata, 1986; Charest et al., 1988). The mechanism by which TMAO gradients are maintained remains obscure.

G. Synthesis of Organic Osmolytes in Smelt

Rainbow smelt that had been starved during a two-week acclimation from fall to winter temperatures showed threefold increases in serum

TMAO and urea levels and a ninefold increase in serum glycerol concentration (Raymond, 1994). Increases in glycerol (from near zero to 60 mMol/L) were also seen in starved Canadian rainbow smelt that were cold-acclimated from late spring to winter temperatures (Raymond, unpublished data). These results appear to show that the smelt synthesize all three osmolytes in response to cold. TMAO synthesis is relatively uncommon in marine fishes, as most species appear to obtain it from their food (Hebard et al., 1982). Thus, one might ask whether the TMAO in the blood originated from the relatively large pool of TMAO already present in the muscle in the autumn. This seems unlikely, however, because muscle TMAO concentrations remained high during the two-week acclimation period, despite a significant loss of TMAO through the gills (see below). TMAO concentration also increased in the liver in cold-acclimated smelt. This is also consistent with TMAO synthesis, as the liver has been found to be a site of TMAO synthesis in several species (Baker et al., 1963; Augustsson and Strom, 1981). In fish that do synthesize TMAO, the pathway is thought to be phospholipid $\rightarrow$ choline $\rightarrow$ trimethylamine $\rightarrow$ TMAO, the latter step being catalyzed by trimethylamine oxidase (Van Waarde, 1988). Urea synthesis commonly occurs in teleosts via uricolysis, in which the uric acid is obtained from the degradation of purines (Forster and Goldstein, 1969). However, the ornithine urea cycle has also been found to be important in a few species (Mommsen and Walsh, 1989). As argmase activity in teleosts is often high (Cohen, 1976), conversion of dietary arginase to urea by arginase may occur in the absence of other ornithine urea cycle enzymes (Wood, 1993). In insects and frogs that produce glycerol in winter, glycogen has been identified as the source (Storey and Storey, 1988). Glycogen also appears to be a source of glycerol in the smelt, but it does not appear to be the only source, as serum glycerol levels remain high in starved smelt whose glycogen deposits have been depleted. Pyruvate, possibly from the degradation of protein, has recently been shown to be another source (Raymond, 1995).

H. Roles of Glycerol, TMAO, and Urea

In winter-acclimatized smelt, increased serum osmolality (due to increases in both inorganic ions and organic osmolytes) is clearly linked to freezing protection, because the increased osmolalities (a) were just enough to avoid freezing (Raymond 1992) and (b) were sharpest when temperatures fell below the normal (i.e., summer) freezing point of the fish's body fluids.

Why should the smelts need glycerol and other osmolytes when they have an antifreeze peptide? A problem with antifreeze peptides is that they inhibit freezing, but they do not lower the melting point. Thus, they cannot melt ice that might occur within a fish, such as through ingestion or spontaneous nucleation. Furthermore, some antifreeze peptides, such as the one found in Pacific herring (Raymond, 1989), may only retard ice growth rather then stop it. Antifreeze peptides of this type cannot provide long-term freezing protection. In contrast, glycerol and the other osmolytes actually lower the melting point, and thus can melt ice within a fish. A second problem with some of the antifreeze peptides is that they are excluded from cells because of their large size (Cheng and DeVries, 1991), and thus provide only limited freezing protection to cells. Organic osmolytes can pass through cell membranes with little resistance and thus protect cells as well as extracellular fluids. The main disadvantage of the organic osmolytes, especially the energy-rich glycerol, is that they are small enough to leak out of the fish.

TMAO and urea in the smelt probably also function as antifreeze osmolytes rather then nitrogen excretory products because of their seasonal variation, which is identical to that of glycerol, and because their levels in the urine are relatively low. A potential advantage of these two osmolytes is that they are energetically less expensive to produce than glycerol. An added benefit of all three osmolytes appears to be a reduced energy expenditure for osmoregulation, due to reduced ingestion of salt water (Raymond, 1993).

TMAO has a variety of roles in fish (Van Waarde, 1988). In muscle tissues, it acts as an osmoregulator, allowing the cells to maintain a relatively low total ion concentration, yet remain in osmotic equilibrium with the blood. This was clearly shown by Lange and Fugelli (1965) in a flounder that was transferred from fresh to salt water. Serum osmolality increased by 62 mM, almost entirely because of increases in Na^+ and Cl^-. The osmolality of muscle tissue water increased by a nearly equal amount, but with 73% of the increase coming from TMAO and other non-protein nitrogen compounds. A similar compensation mechanism might occur in rainbow smelt, where increases in serum ion concentrations in winter are accompanied by increases in muscle TMAO (Raymond, 1994).

Marine elasmobranchs and the coelacanth are characterized by high concentrations of urea and TMAO, with physiological concentrations typically at a ratio of ~2:1. TMAO has been shown to counteract the denaturing effects of urea at the same relative concentrations (Yancey et

al., 1982; Somero, 1992). The presence of both urea and TMAO in winter-acclimatized smelt invites a comparison, but it is unlikely that TMAO plays a similar role because urea concentrations are relatively low, and because the concentration ratio is approximately reversed. However, in view of the perturbing effects of high ion concentrations on enzyme activities, and the fact that intracellular ion concentrations in polar fishes are usually high (O'Grady and DeVries, 1982), it is possible that TMAO may serve to counteract the effects of high inorganic ion concentrations rather than high urea concentrations. Indeed, glycine betaine, a close relative of TMAO, and TMAO itself to a lesser extent, have been shown to provide partial protection against NaCl inhibition of some enzymes (Pollard and Wyn-Jones, 1979). The mechanism is not clear, but it is thought that these solutes help to stabilize proteins through their exclusion from the protein surfaces (Timasheff, 1992).

Another possible role for TMAO is that it may decrease the permeability of epithelial membranes (Daikoku, 1980). This would be of obvious benefit to smelts, elasmobranchs, and the coelacanth, all of which have TMAO and which must conserve other organic osmolytes, but direct evidence for such a mechanism is lacking.

V. PHYSIOLOGICAL RESPONSES OF RAINBOW SMELT TO HIGH OSMOLALITIES

The unusually high winter osmolalities or rainbow smelt result in osmoregulatory changes and problems of osmolyte conservation that are not experienced by other teleosts, or even by cold-hardy insects and frogs. These problems are not unique, however, as they are also experienced by marine elasmobranchs.

A. Water Balance

Because they live in a hyperosmotic environment, marine teleosts normally lose water osmotically and must replenish it by drinking. In rainbow smelt, winter increases in osmolyte concentrations resulted in significantly lower drinking rates (Figure 3), to the point where they closely resembled those of marine elasmobranchs (Raymond, 1993). This probably also resulted in lower energy requirements for osmoregulation, although the absence of glycerol production at above-zero tem-

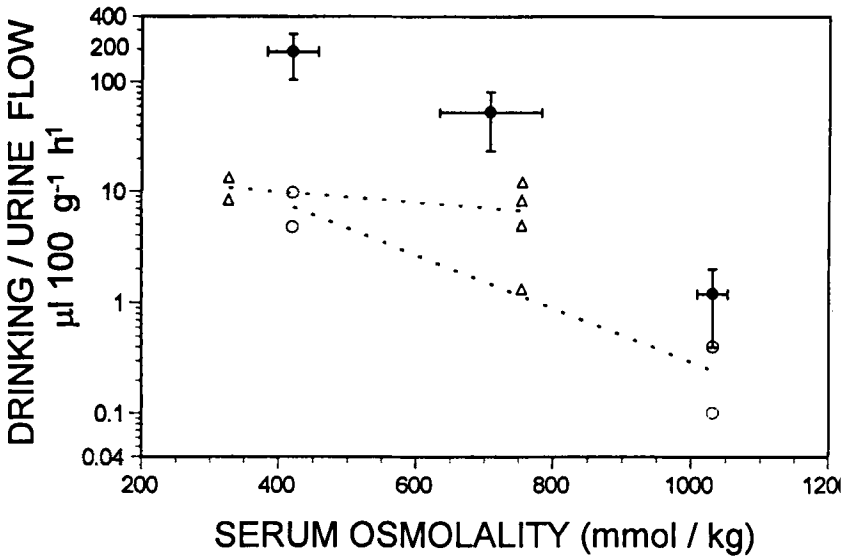

Figure 3. Drinking rate and relative urine flow in rainbow smelt as a function of serum osmolality. ●, drinking rate; O, Δ, relative urine flows in individual fish, averaged over first 12 hours, in seawater and brackish water , respectively. Dashed lines show changes in the averages of the marine and brackish water groups.

peratures (Figure 2) suggests that any energy saved was less than that lost through glycerol leakage.

Urine flow in rainbow smelt also appeared to be relatively lower at high osmolalities (Figure 3). This is consistent with the reduced drinking rate, because a major function of the kidney in marine fish is to excrete divalent salts ingested during drinking (Hickman and Trump, 1969).

B. Osmolyte Losses

Epithelial membranes are normally highly permeable to glycerol and other small uncharged solutes, and thus, high losses might be expected in a marine organism. Winter-acclimatized rainbow smelt lost glycerol at rates of 3-9 mg/100 g/h, the variation apparently being due to stress or level of activity, rather than to glycerol concentration (Raymond, 1993). These rates correspond to daily losses of between 3.8% and 13.4% of the fish's glycerol stores. From an energy standpoint, the losses correspond to about 0.5 kcal/100 g/day (assuming a caloric content of

3.7 kcal/g glycerol). This value appears to be comparable with published metabolic rates for polar fishes [ca 0.05 ml O_2/g/h at 0 °C (McDonald et al., 1987) × 4.8 kcal/l O_2 (Prosser, 1973) = 0.6 kcal/100 g/day]. These values indicate rather significant energy losses and may explain why smelt stomachs are almost always full in winter (Raymond, 1992). On a molar basis, the glycerol efflux rates correspond to 38-103 μmol 100 g^{-1} h^{-1}, a range that is comparable to the efflux of urea from marine elasmobranchs (20-60 μmol 100 g^{-1} h^{-1}; reviewed by Shuttleworth, 1988).

Because of an absence of comparative data in the literature, glycerol loss was measured in two marine catfish, *Arius felis*, that had been artificially loaded with glycerol. These fish lost glycerol at a pooled rate of 15.4 mg 100 g^{-1} h^{-1}. As discussed below, this rate may not be unusually high compared with those of the smelt because of the higher temperature, 22 °C, at which it was measured.

To determine the sites of glycerol loss, smelt were placed in a divided box in which the head and tail regions were separated by a membrane (Raymond, 1993). Stress from confinement in the box appeared to increase efflux rates in some of the fish but, in general, rates of glycerol loss from the head and tail regions were approximately equal. Glycerol was also detected on the gill and skin surfaces. These results, taken with the fact that the gills account for about 85% of the surface area in the head region, suggest that the loss of glycerol from the head region occurred largely through the gills. Losses by the tail region appeared to be due to evacuation of the gut, because the surface area in the tail region was far less then that in the head region, and because estimated losses through the urine were at most only a small part of the total loss. Other factors that seemed to imply a glycerol loss through the gut were high glycerol concentrations in the lower intestine and the occurrence of active feeding in winter populations. A possibly analogous situation occurs in the intestines of some antarctic fishes that contain high concentrations of low molecular mass antifreeze glycopeptides (DeVries, 1982). In these fish, the excretion of antifreeze with the gut contents represents an energetic loss, but it is one that appears to be necessary to prevent freezing in the intestine (Eastman, personal communication).

The occurrence of TMAO and urea in the blood of winter-acclimatized smelt raised the possibility that these osmolytes could also be lost through the gills. Fish placed in the divided box at -1 °C lost TMAO and urea from the head region, presumably mostly through the gills, at rates of 9 ± 4 and 8 ± 2 μmol 100 g^{-1} h^{-1}, respectively. Losses of TMAO and

urea in the tail region could not be measured accurately, but they appeared to be smaller than these values. These data suggest a total TMAO loss of 5-10% day^{-1}, a rate that is comparable to the 4-14% day^{-1} TMAO loss in marine elasmobranchs (Goldstein and Palatt, 1974), and to the above-mentioned glycerol loss in smelt. Urea losses from the head region of the smelt were consistent with the 1-4 μmol 100 g^{-1} h^{-1} losses reported for several marine teleosts (Sayer and Davenport, 1987) in view of the presumably higher urea concentrations in the smelt.

C. Membrane Permeabilities

Gill areas of the smelt, ~320 cm^2 100 g^{-1}, were found to be typical of sluggish polar fish (Kunzmann, 1990). Using this value and calculated skin areas, permeabilities of the total surface area in the head region to glycerol, P_g, were found to be 0.9-2.6 $\times$ 10^{-7} cm s^{-1}. These values are comparable to the permeabilities of skate gill epithelia to urea (~1 $\times$ 10^{-7} s^{-1}; Boylan, 1967).

The corresponding permeabilities for TMAO and urea were 2 $\pm$ 1 $\times$ 10^{-7} and 3 $\pm$ 1 $\times$ 10^{-7} cm s^{-1}, respectively. These values appear to be slightly higher then those for glycerol, as might be expected from their lower molecular masses. The permeability of smelt gills to urea at -1 °C appears to be about three times higher than it is for the skate at 15-20 °C (Boylan, 1967). If the measurements were made at the same temperature, an even greater difference in permeabilities would be expected. This suggests that the smelt gills are not specially adapted for conserving urea, as skate gills appear to be (Boylan, 1967).

D. Conservation Mechanisms

Unlike insects and frogs that use organic osmolytes to avoid freezing death in winter (Storey and Storey, 1988; Lee, 1991), glycerol-bearing fishes live in an aqueous environment and remain active in winter. Both factors expose the fish to losses of osmolyte. This is not a unique problem, however, as it is also faced by marine elasmobranchs which must conserve nearly equal concentrations of urea. Although losses of glycerol by smelt are comparable to losses of urea by elasmobranchs, the mechanisms by which the two osmolytes are conserved appear to differ. Urinary losses, for example, are low in the elasmobranchs due to a highly efficient reabsorption of urea in the kidney tubule (reviewed by Henderson et al., 1988), but in the smelt, they appear to be low due to reduced urine flow during the period of glycerol production.

Epithelial tissues, such as gills, normally have a high permeability to urea (P_u). In elasmobranchs, the gills are the main site of urea loss, but their P_u is unusually low compared with those of other epithelial tissues. The underlying mechanism is not fully understood. In contrast, the permeability of smelt epithelia to glycerol, P_g, is not unusually low compared with other tissues (Table 1), and so a special mechanism to reduce permeability does not appear necessary. Less of a reduction is needed in P_g than in P_u because membranes are naturally less permeable to glycerol than to urea (Table 1). This is due in part to the fact that glycerol has a higher molecular mass and lower lipid solubility than does urea (Kotyk, 1977).

Low temperature also reduces P_g, and probably provides a further contribution to glycerol conservation in the smelt. Decreases in P_g by a factor of approximately 3 per 10 °C have been reported (for references see Raymond, 1993). This amount alone is enough to account for the higher glycerol loss from the catfish. In winter, the skins of some northern fishes undergo increases in thickness (Burton and Fletcher, 1983) or collagen content (McBride et al., 1960). Such phenomena, should they also occur in smelt, would likely provide a further decrease in permeability.

An additional conservation mechanism may be a sluggish lifestyle in winter (a behavior that is consistent with observations of winter-acclimatized smelt held in large aquaria), as it would minimize water exchange at the skin and gill surfaces where much of the glycerol loss occurs.

Losses of other osmolytes, such as TMAO and urea, would also be reduced by low temperature and a sluggish lifestyle. In addition, TMAO and urea, unlike glycerol (Raymond, 1993), appear to be conserved by the kidney, as their concentrations in the urine were lower than those in the blood. Reabsorption or urea and TMAO from the urine are well

Table 1. Permeability of Epithelial Tissues and Membranes to Urea and Glycerol

	Permeability $X\ 10^{-7}\ cm\ s^{-1}$		
Tissue	*Urea*	*Glycerol*	*Reference*
Skate gills	0.75-1		Bylan (1967); Payan et al. (1973)
Smelt gills and skin		0.9-2.6	Raymond (1993)
Rabbit gallbladder	890	180	Wright and Pietras (1974)
Toad urinary bladder	14	2.3	Wright and Pietras (1974)
Frog choroid plexus	120	69	Wright and Pietras (1974)
Human red cells	2387	0.58	Naccache and Sha'afi (1973)
Algal cell walls	1.3	0.032	Collander (1954)

known in marine elasmobranchs (reviewed by Henderson et al., 1988). Reabsorption of urea from the urine has also been observed in some teleosts (Curtis and Wood, 1991: Wright et al., 1992), although in *Lophius*, both TMAO (Forster et al., 1958) and urea (Brull and Nizet, 1953) are concentrated in the urine.

VI. SUMMARY

Cold-adapted teleost fishes face a dilemma: they require freezing points that are as low as that of seawater, but must achieve this without greatly changing the intracellular environment. These fishes have developed a variety of mechanisms to solve this problem, each having its own advantages and disadvantages. Some species appear to exist in a supercooled state, but they can survive only by avoiding ice. Increased levels of inorganic ions appear to be the most straightforward means of lowering the freezing point, but above about 0.2 M, they interfere with many cellular processes. Antifreeze peptides avoid this problem because they have little effect on osmolalities, but they can not melt ice that has already formed, and they are too large to enter cells. Organic osmolytes solve each of the above problems, but because they can easily pass through membranes, they are difficult to conserve. In the case of glycerol, the energetic cost appears to be high.

Finally, it should be noted that the occurrence of high ion concentrations and high osmolalities in some teleost species, as well as their osmotic effects on water balance, challenge some commonly accepted distinctions between teleosts and marine elasmobranchs. In Freezing environments where survival may hang on a thread, such distinctions appear to be dispensable.

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THERMAL ADAPTATION IN BIOLOGICAL MEMBRANES:

BEYOND HOMEOVISCOUS ADAPTATION

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I. INTRODUCTION

Membranes are macromolecular assemblages of lipids and proteins that: (a) simultaneously act as physical barriers to solute diffusion and yet catalyze specific exchange (transport) reactions between the compartments they separate, thereby defining the unique chemical compositions of the cytoplasmic and organellar compartments within a cell; (b) store energy as transmembrane ion gradients and regulate energy utilization in both time and space by selectively altering membrane permeability via the opening of voltage- (e.g., the voltage-gated Na^+, K^+ and Ca^{2+} channels of nerve and muscle cells) or ligand-gated (e.g., postsynaptic receptors for acetylcholine, glycine, etc.) ion channels; (c) provide an organizing matrix for the assembly of multicomponent metabolic and signal transduction pathways, which serves to both anchor (or in some cases solvate) and stabilize (by preserving active conformations and permitting the changes in volume and shape essential for function) integral membrane proteins (e.g., enzymes, receptors, and ion channels) and in some cases (e.g., mitochondrial electron transport and glycosylation reactions of the Golgi) to provide the physical substrate for membrane-associated enzymes; and (d) govern the transfer of information between cells and their immediate environment and between compartments within cells by (1) housing receptors and their associated coupling mechanisms (e.g., G-proteins) that respond only to specific stimuli, and

(2) supplying a reservoir of lipid precursors for the generation of lipid-derived second messengers. The structural matrix of biological membranes is a thin layer of lipid (primarily phospholipids) only two molecules thick, the phase behavior and physical properties of which are exquisitely sensitive to variations in temperature. In fact, no other class of biological molecule displays a greater diversity of structure over such a narrow range of temperatures within the physiological range. Consequently, changes in body temperature pose a serious challenge to the maintenance of physiological function in poikilotherms, for thermotropic perturbation of membrane structure can adversely affect membrane function (Hazel and Williams, 1990). However, membrane-mediated processes are so central to life that the remodeling of membrane architecture is a nearly universal and apparently essential component of acclimation (within the lifetime of an individual) or adaptation (over evolutionary time) to extremes of environmental temperature. By exploiting the structural diversity of membrane lipids, poikilotherms can fashion membranes with physical properties appropriate to the prevailing ambient temperature, and, in this way, restore membrane function following thermal challenge. Original observations in *Escherichia coli* (Sinensky, 1974) indicated that the fluidity of the membrane interior is, in fact, defended as growth temperature changes, a phenomenon Sinensky called homeoviscous adaptation (HVA). The extent of fluidity compensation has since been widely adopted to assess the efficacy of thermal adaptation in biological membranes (Cossins and Sinensky, 1986; Cossins and MacDonald, 1989).

The objectives of this essay are: (a) to establish why the restructuring of membrane lipid composition promotes the maintenance of physiological function when growth temperature is varied; (b) to briefly review the patterns and mechanisms of temperature- induced change in membrane lipid composition; (c) to critically evaluate the role of homeoviscous adaptation in the thermal adaptation of biological membranes; and (d) to explore alternatives to HVA as explanations for the observed patterns of thermotropic membrane restructuring.

II. EFFECTS OF TEMPERATURE ON MEMBRANE STRUCTURE AND FUNCTION

Changes in temperature perturb membrane organization and function by modifying the rates of molecular motion and the conformation of phos-

pholipid acyl chains, thereby determining the *phase state,* the *fluidity* or microviscosity (reflecting the *rate* of acyl chain motions), and the molecular *order* (reflecting the *extent* rather than the rate of molecular motions) of lipids in biological membranes.

A. Temperature and the Phase State of Membrane Lipids

Phospholipids can assume different phase organizations depending on both the temperature and the chemical composition of the phospholipid itself. Since transitions between phases can be driven by changes in temperature alone, temperature will determine the particular phase or combination of phases that coexist in a membrane. Three major phase types (each occurring in a variety of subtypes) have been described in phospholipid mixtures of biological origin; these include lamellar, hexagonal, and cubic phases (Figure 1). Because only lamellar phases preserve the barrier properties of membranes, they dominate membrane structure throughout the biological range of temperatures. Hexagonal and cubic phases can, however, form at the upper extremes of the biological temperature range and may play a role in membrane fusion (Siegel, 1986).

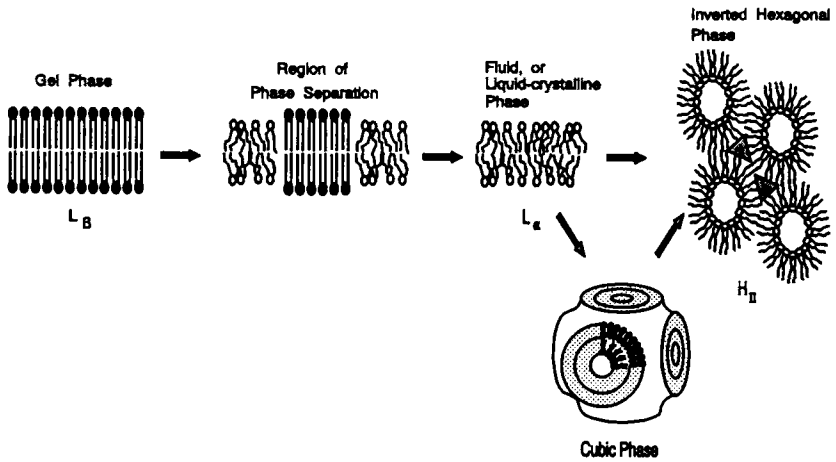

Figure 1. Effects of temperature on the phase state of phospholipids. Arrows in all cases indicate a phase change induced by a rise in temperature. Redrawn from Hazel (1993).

At physiological temperatures, membrane lipids are present predominantly in a relatively disordered, fluid, lamellar liquid-crystalline (or L_α) phase, characterized by high rates of phospholipid acyl chain mobility (Casal and Mantsch, 1984). Although rotations about carbon-carbon single bonds are both common (3-7 *gauche* rotamers per acyl chain (Mendelsohn et al., 1989)) and mobile (propagating up and down the length of the acyl chains), the liquid-crystalline phase is not as disordered as a liquid hydrocarbon due to the covalent attachment and parallel alignment of the acyl chains perpendicular to the plane of the membrane. In fact, a zone of constrained acyl chain motion extends 8-10 carbon atoms from the membrane surface into the bilayer interior before membrane order declines consistently toward the methyl terminus of the fatty acyl chain (Seelig and Seelig, 1980; Collins et al., 1990).

When temperature drops below the physiological range, phospholipid acyl chains adopt a highly ordered, all-*trans* conformation that packs tightly to form a "frozen," crystalline, or lamellar gel phase (most commonly the L_β phase; Figure 1). In this gel phase, acyl chains may be tilted ($L_{\beta'}$) or untitled (L_β) with respect to the bilayer normal, or the lamellae may be rippled (with the acyl chains tilted:$P_{\beta'}$); even phases in which phospholipids from opposite hemilayers interdigitate ($L_{\beta*}$) can be formed (Hazel and Williams, 1990). The restricted acyl chain mobility in the gel phase results in lower rates (compared to the fluid phase) of lateral phospholipid diffusion within the plane of the bilayer (diffusion coefficients of 5×10^{-10} cm^2/sec versus $4 \times 10^{-8} cm^2/sec$; (Wu et al., 1977)). Some disorder is, however, retained in the gel phase as evidenced by the occurrence of *gauche* conformers in 11% of the carbon-carbon single bonds at the C_{14}position of dipalmitoylphosphatidylcholine (Mendelsohn et al., 1989). Nevertheless, the fluid/gel or "chain-solidifying" transition (occurring at the temperature T_m) reduces the volume occupied per lipid molecule and the lateral pressure within the bilayer, while increasing both membrane order and bilayer thickness (Overath and Trauble, 1973).

Conversely, at temperatures above the normal physiological range, some lipids (most notably phosphatidylehtanolamine (PE)) assume hexagonal or cubic phases (Mantsch et al., 1981; Mariani et al., 1990). With rising temperature, both the conformational disorder and the *gauche* isomer content of the acyl domain continue to increase beyond the levels introduced at the chain-melting transition. As a consequence, the cross-sectional area of the phospholipid acyl domain eventually exceeds that of the headgroup and the molecular geometry changes from cylindrical

to conical. Conically-shaped phospholipids are more readily accommodated in the inverse hexagonal (H_{II}) than a lamellar phase, for in the H_{II} phase, phospholipid headgroups project into a central, water-filled channel, while the acyl chains extend radially to form hexagonally-packed lipid cylinders (Figure 1). Although formation of the H_{II} phase (occurring at Th) involves dramatic changes in the packing arrangement of membrane constituents, the enthalpy of the transition is only 5-15% of that associated with acyl chain melting, indicating both a lower energetic cost and a less cooperative transition (Seddon, 1990). The high degree of disorder accommodated in the H_{II} phase is manifest in both a marked reduction in the acyl chain order parameter (by a factor of up to fivefold) relative to the $L\alpha$ phase and the absence (or reduction) of a plateau region of constrained acyl chain motion (Sankaram and Marsh, 1989; Lafleur et al., 1990). Phospholipids such as phosphatidylcholine (PC), which possess a relatively large headgroup compared to their acyl chain volume, retain a cylindrical shape throughout the range of biological temperatures and are thus referred to as bilayer-stabilizing lipids. In contrast, conical phospholipids such as (PE), which possess a relatively small headgroup and a highly unsaturated acyl domain, assume the H_{II} phase most readily and are therefore considered to be bilayer-destabilizing lipids; nevertheless, even for PEs, the H_{II} transition occurs at relatively high temperatures (45-70 °C). However, dehydration of phospholipids causes Th to be lowered to within the physiological range, and since dehydration of apposing bilayers is an obligatory first step in membrane fusion, formation of the H_{II} phase may play a role in this process. Cubic phases, on the other hand, are difficult to demonstrate, but have been identified as intermediates in the $L\alpha$ H_{II} transition (Siegel and Banschbach, 1990).

Neither hexagonal nor cubic phases are likely to be permanent features of membrane organization since their formation destroys the barrier properties of a bilayer. Nevertheless, most biological membranes contain significant quantities of bilayer-destabilizing lipids and the physicochemical forces tending to drive these lipids into a nonbilayer phase are undoubtedly important features of membrane biology (Lindblom and Rilfors, 1989).

B. Temperature and the Phase State of Lipids in Biological Membranes

The diversity of lipid species present in most biological membranes broadens and reduces the cooperativity of the phase transition when compared to model lipid systems consisting of a single species of

phospholipid. For example, the gel/fluid transition extends over a temperature range of 15-20 °C in intact *E. coli* (Killian et al., 1992). The highest melting lipid species are the first to solidify as temperature drops, creating clusters of gel phase lipid dispersed in an otherwise fluid membrane. With continued cooling, the rigid domains enlarge at the expense of fluid regions, until ultimately all lipid species have "frozen." The temperature range between the onset and completion of the gel/fluid transition is called a region of phase separation since both fluid and gel phase lipids coexist in physically separated microdomains. Phase separations in biological membranes have been visualized directly by freeze-fracture electron microscopy (as the formation of particle [protein]-free domains of gel phase lipid with cooling due to the preferential partitioning of membrane proteins into fluid-phase lipids; Kameyama et al., 1980) and indirectly inferred from changes in the spectra of both electric spin resonance (ESR; Gordon and Mobley, 1984) and fluorescent probes (Illsley et al., 1988; Mateo et al., 1991; Parasassi et al., 1991) that preferentially partition into gel or fluid-phase lipids. Although the majority of membrane lipids are present in the fluid phase at normal physiological temperatures, a small but significant fraction may be routinely present in the gel phase. For example, from 10-15% of the lipids in brush border membranes of both human placenta (Illsley et al., 1987) and rabbit renal cortex (Illsley et al., 1988) are present in the gel phase at physiological temperatures and the proportion of gel phase lipids is even larger (≈25%) in plasma membranes of mammalian sperm (Wolf et al., 1990).

In addition, cholesterol at low concentrations of (< 20 md %) results in the formation of coexisting cholesterol-rich and cholesterol-poor domains in model membrane systems, and temperature modulates both the relative proportions of these domains and the interaction between cholesterol and other membrane lipids (Almeida et al., 1992). For example, cooling favors the formation of cholesterol-rich domains in human erythrocytes (Gordon and Mobley, 1984) and results in displacement of cholesterol from the headgroup region toward the bilayer interior in model membrane systems (Reinl et al., 1992).

Temperature-induced phase separations and transitions have been widely documented in membranes of homeotherms, microorganisms, and plants, but are much less common in multicellular poikilotherms, perhaps reflecting the relatively low temperatures to which the majority of the organisms studied have adapted and the preponderance of low-melting lipids in their membranes (for a review see Hazel and Williams, 1990). Discontinuities in Arrhenius plots of membrane physical proper-

ties, suggestive of phase separations and transitions, have, however, been consistently reported in membranes of warm-adapted poikilotherms such as the warm water fish *Channa punctatus* (hepatic and brain membranes; (Roy et al., 1992; Dutta et al., 1985) carp (sarcoplasmic reticulum; Ushio and Watabe, 1993)), and tropical copepods (Farkas et al., 1984, 1988). Phase transitions have also been detected by differential scanning calorimetry in hypothalamic membranes of the garden lizard (Durairaj and Vijayakumar, 1984).

C. Effects of Temperature on Lipid Order and Dynamics

Although less obvious than changes in phase state, temperature also modulates the rates and extent of molecular motions in membranes, which, in turn, determine the physical properties of the membrane interior. In fact, it is the accumulation of temperature-induced changes in molecular conformation, motion, and order within a particular phase that drives the thermotropic transitions documented in the previous sections. Rising temperature increases both the rate and extent of acyl chain motions, thereby increasing membrane fluidity and decreasing membrane order (refer to Figure 5). For example, the order parameter for a stearic acid spin probe declines monotonically from 0.862 at 2 °C to 0.638 at 40 °C in rat erythrocyte membranes (Whitesell et al., 1989), while in basolateral membranes of carp enterocytes, the polarization of diphenylhexatriene (DPH) fluorescence decreases nearly twofold between 0 °C and 40 °C, indicating a less restricted probe environment at higher temperatures (Cossins and Raynard, 1987). For reasons that are unclear, not all membranes accumulate disorder at a similar rate as temperature is changed, for example, rates of acyl chain motion in sarcoplasmic reticular membranes of winter flounder vary less with temperature than comparable preparations from rabbit muscle (Vrbjar et al., 1992). Nevertheless, thermal modulation of lipid dynamics within a given phase is a universal characteristic of membranes and most likely accounts for the positive correlation between temperature and the lateral diffusion coefficients of membrane constituents (Chen et al., 1990).

D. Thermal Perturbation of Membrane Structure and Function: The Consequences of Poikilothermy

The foregoing discussion leads to the expectation that any aspect of membrane structure or function sensitive to either the phase state or

physical properties of membrane lipids is likely to be significantly perturbed by a change in temperature. Consequently, because membranes perform so many vital cell functions, shifts in temperature pose a serious challenge to the maintenance of physiological activity in poikilotherms. These problems will be discussed within the context of the functional consequences of temperature-induced changes in the phase state and physical properties of membrane lipids.

Functional Consequences of Changes in Phase State

Membrane morphology is dramatically altered at the fluid/gel transition. As revealed by freeze-fracture electron microscopy, smooth-faced regions of crystallized lipid domain form at the onset of phase separation induced by cooling. For example, in *Acholeplasma*, integral membrane proteins, which are randomly distributed at a density of 200-400/μm^2 at teperatures above T_m, are excluded from the gel phase lipids and consequently cluster to attain densities as high as 5000/μm^2 at lower temperatures (James and Branton, 1973). Correlated with such structural changes, the activities of many membrane-associated processes experience a marked increase in temperature sensitivity on cooling (detected as a discontinuity in the Arrhenius plot) at a temperature that corresponds to either the onset or completion of the gel/fluid transition. Rates of membrane-associated enzyme activity are generally higher in the fluid than gel phase, suggesting that gel phase lipids constrain those motions of proteins required for catalytic activity. Cases where discontinuities in Arrhenius plots of enzyme activity correspond to the gel/fluid transition as detected by independent physical measurements include: adenyl cyclase activity in plasma membranes of rat hepatocytes (Livingstone and Schachter, 1980; Houslay and Gordon, 1982); succinate oxidation in rat liver mitochondria (Raison et al., 1971); glucose uptake across the apical membrane of both rat and rabbit enterocytes (Mutsch et al., 1983; Whitesell et al., 1989); and, the Ca^{++}-ATPase of carp sarcoplasmic reticulum (Ushio and Watabe, 1993). Although phase transitions are not common in membranes of multicellular poikilotherms (refer to section II.B), discontinuities in Arrhenius plots of membrane-associated activities have been reported in several warm-water fish, even though lipid phase transitions have not been independently demonstrated; selected examples include: mitochondrial respiration in both carp (van den Thillart and Modderkolk, 1978) and goldfish (Wodtke, 1976) liver; conduction velocity in the vagus nerve of carp (Harper et al., 1990); and

Na^+/K^+-ATPase activity in carp intestine (Behar et al., 1989). However, since there are many possible causes for breaks in Arrhenius plots (including the direct effects of temperature on protein structure and the K_ms of soluble enzymes), discontinuities in such plots do not constitute, on their own, definitive evidence for the involvement of a lipid phase transition. Nevertheless, abrupt changes in the temperature sensitivity of membrane- associated processes at the gel/fluid transition may well disrupt the integration of cellular metabolism. For example, the failure of mitochondrial oxidation to keep pace with glycolytic flux (not influenced by the phase state of membrane lipids) at temperatures below the T_m of mitochondrial lipids may lead to the accumulation of glycolytic end products at low temperature and injury in chilling-sensitive plants (Lyons, 1972).

Other aspects of membrane function are also perturbed at the gel/fluid transition. For example, the time required for prothrombinase assembly is increased by more than ninefold in gel- compared to fluid-phase lipids, due to the reduced rates of lateral diffusion of clotting factors (Xa and Va) in the gel phase (Goversriemslag et al., 1992). Anomalous permeability properties also frequently arise in the phase transition region, presumably reflecting packing defects at the boundaries between microdomains of gel 1=1 and fluid-phase lipids: both cation (Singer, 1981) and water permeabilities (Carruthers and Melchior, 1984) increase dramatically (30- to 130-fold) at the gel/fluid transition in membranes consisting of saturated PCs. Furthermore, in the cyanobacterium, *Anacystis nidulans*, the cold-induced loss of photosynthetic activity occurs at a temperature corresponding to the onset of phase separation in the plasma membrane, as evidenced by the leakage of both K^+ and amino acids (Murata, 1989). However, a general role for lipid phase transitions as a causative agent of chilling injury in plants has recently been challenged, based on the failure to detect phase transitions in lipid mixtures resembling those of spinach thylakoid membranes (Webb et al., 1992).

Functional Consequences of Altered Membrane Fluidity and Order

As previously discussed (refer to section II.C), changes in temperature can perturb rates of acyl chain motion, phospholipid conformation, and membrane order without inducing phase transitions or separations. Such temperature-driven modifications in lipid dynamics can, nevertheless,

significantly affect membrane function. Activities as diverse as Na^+/K^+-ATPase in lamb kidney (Harris, 1985), chloride transport in secretory granules of rat pancreas (Gasser et al., 1990), and low-density lipoprotein binding, uptake, and rates of degradation by rat hepatocytes (Kuo et al., 1990) are all directly correlated with membrane fluidity (as assessed by fluorescence polarization of DPH), as is the rotational mobility of spin-labeled sarcoplasmic reticular Ca^{++}-ATPase (Squier et al., 1988). Similarly, lysophospholipid acyltransferase activity in rat liver plasma membranes correlates negatively ($r = -0.867$) with the structural order parameter of the lipid bilayer (Momchilova et al., 1991). In addition, sodium current kinetics in rat brain neurons accelerate following supplementation with essential fatty acids, suggesting fluidity modulation of ion channel activity (Park and Ahmed, 1992). In contrast, increased membrane fluidity impairs the function of the PCP-NMDA receptor in rat brain membranes, presumably due to a loosening of the association between the subunits of the receptor complex (DePietro and Byrd, 1990). These data strongly imply that the physical environment provided by membrane lipids can influence the function of integral membrane proteins even in the absence of such major perturbations as a phase transition.

Besides influencing the activity, conformational state, and rates of motion of membrane proteins, the lipid environment also modulates interactions between proteins that occur within the membrane, such as those involved in transmembrane signal transduction pathways. For example, the rate constant for the activation of adenyl cyclase by the β_2-adrenergic receptor (which involves collision coupling between the hormone-occupied receptor and a G-protein/adenyl cyclase complex) varies more than 20-fold as a function of membrane fluidity in the plasma membranes of turkey erythrocytes (Hanski et al., 1979).

The barrier properties of both phospholipid vesicles and biological membranes also deteriorate exponentially (i.e., membrane permeability increases) with rising temperature (E_as range from 8-24 Kcal/mol; Galey et al., 1973), and leakage of K^+ from muscle fibers has been implicated as a cause of heat death in crayfish (Gladwell et al., 1975). The temperature dependence of membrane permeability appears to reflect primarily acyl chain mobility, since, in *Acholeplasma*, erythritol flux varies directly with the percentage of membrane lipid in the fluid phase (de Kruijff et al., 1973), and the water permeability of bovine tracheal epithelia is positively correlated with the fluidity of the apical membrane (Worman et al., 1986).

III. REQUIREMENTS FOR GROWTH AND FUNCTION AT DIFFERENT TEMPERATURES: MEMBRANE CONSTRAINTS

The sensitivity of membrane dynamics, structure, and function to variations in temperature precludes the possibility that any given membrane will perform optimally over as broad a range of temperatures as (a) successfully exploited by poikilotherms, or (b) encountered by many poikilotherms on a seasonal basis. It therefore follows that eurythermal poikilotherms, or those adapted to extreme thermal conditions, must moderate the acute effects of temperature on the phase state and physical properties of their membrane lipids, if physiological function is to be maintained at extremes of temperature. In other words, an *optimal* phase state or fluidity of the membrane may be required to support growth and/or physiological function, or at the very least, one or both of these parameters must be maintained within defined limits.

The notion that membrane physical properties or phase state may limit organismal growth has been most extensively studied by McElhaney and colleagues in the prokaryotic microorganism, *Acholeplasma laidlawii* (McElhaney and Souza, 1976; McElhaney, 1984a). *Acholeplasma* is ideally suited for such studies because it readily incorporates exogenous fatty acids into its membrane lipids, but lacks the ability to alter membrane lipid composition in response to variations in temperature. Although growth was not observed at temperatures below 8 °C even when the T_m of membrane lipids was below 0 °C, the minimum growth temperature varied widely with membrane lipid composition and, in *Acholeplasma* enriched with fatty acids characterized by relatively high T_ms, was clearly defined by the lower boundary of the gel/fluid transition. Inhibition of growth occurred when more than 50% of the membrane lipids were present in the gel phase, and growth ceased entirely when the proportion of gel phase lipids reached 90%, indicating that fluid phase lipids are required for growth. In addition, both the optimal and maximum growth temperatures (neither of which were determined by the upper boundary of the gel/fluid transition) were depressed in cells grown on low-melting point fatty acids, suggesting an upper limit to membrane fluidity that is compatible with normal membrane function. In fact, for cells grown on fatty acid mixtures containing perdeuterated palmitate (employed as a nonperturbing ^{2}H-NMR probe of membrane order), high rates of growth were restricted to a range of average molecular order parameters (S_{mol}: a measure of the time-averaged orientation between

the ^{2}H-C bond vector and the bilayer normal which can vary in magnitude from zero for a totally random orientation, to unity for an orientation perpendicular to the bilayer normal) between 0.140 and 0.177; order parameters either above or below this "optimal" range curtailed growth (Monck et al., 1992).

In summary, given the limited thermal range over which a designated set of membrane constituents will conform to the phase state and fluidity requirements for optimal membrane function, it follows that poikilothermic organisms, in order to function over a range of environmental temperatures, must restructure their membranes so that lipids of appropriate physical properties are matched to the prevailing thermal conditions.

IV. MECHANISMS OF THERMAL COMPENSATION: MEMBRANE REMODELING

The retailoring of membrane lipid composition is the most commonly observed cellular response to altered temperature. Poikilothermic organisms exploit the diversity in lipid structure to build membranes that exhibit phase behaviors and physical properties appropriate to their thermal circumstance. Phospholipids are the major structural lipids of biological membranes and can differ in the chemical composition of both their hydrophilic (i.e., headgroup) and hydrophobic (i.e., acyl chain) domains (Figure 2) as well as in the nature of the linkage between the hydrocarbon chains and the glycerol backbone. Variation in headgroup structure defines different classes of phospholipid (e.g., PC vs. PE or phosphatidylinositol (PI), etc.), whereas diversity in acyl chain composition defines unique molecular species within a phospholipid class (e.g., 16:0/18:1-PC versus 16:0/16:1-PC). Most phospholipids possess fatty acids esterified to both the *sn*-1 and -2 positions of glycerol, and are therefore called diacyl phospholipids. However, ether-linked alkyl chains are also common at the *sn*-1 position, and the presence of an α-β unsaturated ether linkage (alk-1-enyl) at the 1-position defines the important group of phospholipids designated as plasmalogens. In addition, some cellular membranes contain significant quantities (up to 50 mole %) of neutral lipids such as cholesterol. Conservative estimates of the number of different lipid species present in the erythrocyte membrane of mammals range from 150 to 200 (van Deenen, 1969), which is but a small fraction of the potential number of species that could, in fact, be

produced under different physiological circumstances. It is this chemical diversity that poikilothermic organisms exploit to fashion functional membranes within the constraints of the prevailing thermal environment. The versatility of lipid structure in adapting membranes to specific environments is no better illustrated than by the occurrence of tetraether lipids in thermo- and acidophilic archaebacteria (Figure 2). In tetraether lipids, two C_{40} isopranoid alkyl chains link together (via ether linkages) polar headgroups on opposite sides of the molecule. Monolayers of tetraether lipids are presumed to form stable membranes at elevated temperatures (65-85 °C) since the covalent attachment of the alkyl chains to both sides of the membrane minimizes thermal disruption of the membrane interior (Lanzotti et al., 1989).

Commonly observed patterns in the temperature-induced remodeling of biological membranes, although frequently differing in detail in an organism- and membrane-specific manner, attest to several ubiquitous mechanisms of thermal adaptation. These include: (a) a restructuring of the fatty acyl/alkyl chain and phospholipid molecular species compositions; (b) regulation of the phospholipid class composition; (c) shifts in the ratio of diacyl/plasmalogen phospholipids; and (d) variation in the cholesterol/phospholipid ratio.

A. Acyl/alkyl Chain and Phospholipid Molecular Species Remodeling

Adjustments to the composition of the hydrophobic domain of membrane phospholipids are among the most commonly observed modifications in membrane structure to result from a change in growth temperature. Because this literature has been reviewed (Hazel, 1988; Hazel and Williams, 1990), the present discussion will focus only on selected examples of general tendencies in these data. An increased proportion of unsaturated fatty acids is the most commonly observed modification to membrane lipid composition as a result of growth at low temperature. Consequently, the unsaturation ratio (i.e., the ratio of unsaturated fatty acids (UFA)/saturated fatty acids (SFA) is negatively correlated with average cell or acclimation temperature, and among various membranes of fish tissues, the strength of the correlation varies in the rank order mitochondria > microsomes > synaptosomes. Elevated levels of polyunsaturated fatty acids (PUFA) are more likely to account for the increased unsaturation of membrane lipids in winter-active species, whereas in winter-quiescent animals, monoenes tend to accu-

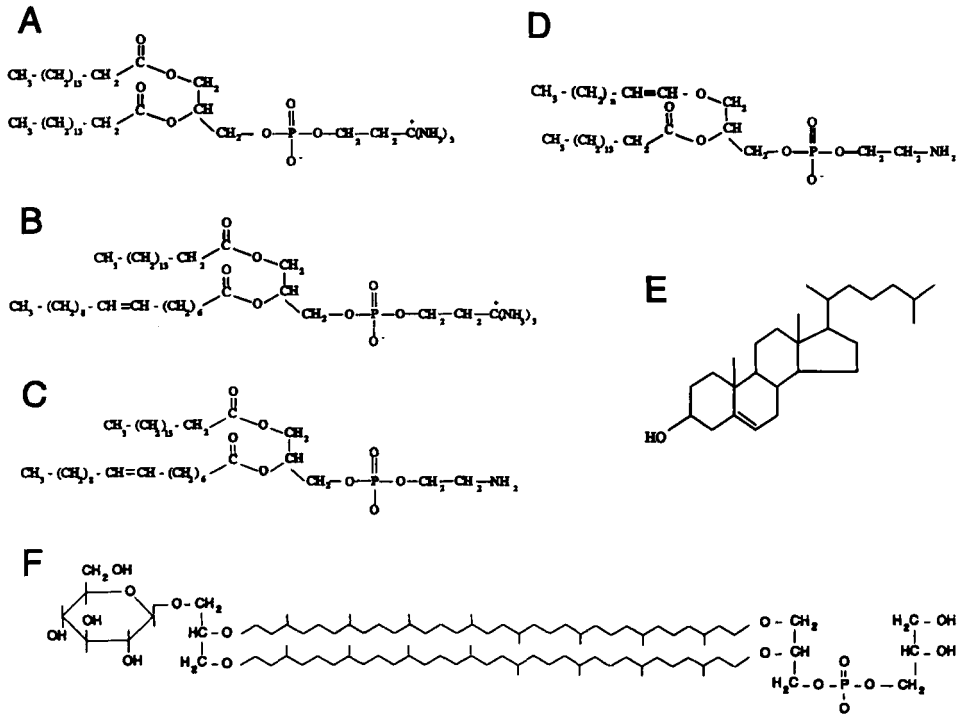

Figure 2. The structural diversity of membrane lipids. Lipids illustrated include two molecular species of phosphatidylcholine [PC] (**A**), dipalmitoyl phosphatidylcholine; (**B**), 1- palmitoyl 2-oleoyl phosphatidylcholine); **C**, 1-palmitoyl 2-oleoyl phosphatidylehtanolamine, [PE], (**D**), a PE plasmalogen; (**E**), cholesterol; and (**F**), diphytanylglycosylglycerol, a tetraether lipid.

mulate. In addition, interspecific comparisons among animals adapted to different thermal environments (e.g., Antarctic vs. temperate water fish) document similar temperature dependent patterns in acyl chain composition (Morris and Schneider, 1969; Patton, 1975).

There is little doubt that increased unsaturation of membrane lipids promotes survival at cold temperatures. For example, desaturase mutants of the cyanobacterium *Synechocystis* PCC6803 are more sensitive than wild type to low temperature photoinhibition of photosynthesis (Gombos et al., 1992). In addition, the transfer of a desaturase gene from the chilling-resistant *Synechocystis* into the chilling-sensitive *Anacystis nidulans* increases the tolerance of the latter to low temperature and lowers the T_m of plasma membranes in the transformed cells by 4-8 °C (Wada et al., 1990). Because *cis* double bonds introduce a kink of approximately 30° into the acyl chain, UFAs pack less compactly and possesses lower melting points than their saturated homologues. Consequently, the increase in membrane lipid order caused by an acute drop in temperature may be offset to a significant degree by the incorporation of unsaturates. Conversely, reduced levels of membrane unsaturation may contribute to survival at warm temperatures, for hydrogenation of thylakoid membranes in pea seedlings increases the resistance to heat stress (Vigh et al., 1989). Although induction of fatty acyl-CoA or phospholipid desaturase synthesis is a common mechanism for elevating membrane unsaturation at cold temperatures (Schunke and Wodtke, 1983; Hagar and Hazel, 1985), it is less straightforward to reduce membrane unsaturation at high temperatures. Both the biogenesis of SFA and the elimination of preexisting UFAs (either via selective turnover or dilution by cellular growth) are normally required to reduce membrane unsaturation (Jones et al., 1991). However, in the psychrophilic bacterium, Vibrio sp, *cis*-UFA are converted directly to *trans*-UFA (*trans*-UFAs exhibit T_ms intermediate between those of SFA and cis-UFA of equivalent chain length) as growth temperature rises, presumably due to the activation of a *cis/trans* isomerase, further underscoring the importance of the content of cis-UFA to the thermal adaptation of membranes (Okuyama et al., 1991).

In addition to the general level of acyl chain unsaturation, alterations in the stereospecific location of UFAs within a phospholipid, as well as the particular combination of fatty acids esterified to a common glycerol moiety (i.e., the molecular species composition of a phospholipid), may have a significant impact on membrane properties. In metazoans cold

acclimation is generally associated with increased proportions of long-chain, PUFA-containing and diunsaturated species of phospholipids (Hazel and Williams, 1990). Of particular interest, 1-monoenoic (18:1), 2-polyenoic species of both PC and PE accumulate at low temperatures in liver membranes of carp, suggesting that the pairing of long-chain PUFA with monoenes may be more central to the maintenance of an appropriate membrane architecture than the accumulation of PUFA alone (Fodor et al., 1995; Giorgione et al., 1995).

Branched-chain fatty acids (BCFA) may play a role similar to UFAs in the thermal adaptation of some microorganisms. For example, in several species of bacteria, the ratios of BCFA/SFA and *anteiso-/iso-*branched fatty acids rather than the content of UFAs increase at low temperature (Suutari and Laakso, 1992).

B. Restructuring of Membrane Polar Lipid (Headgroup) Composition

Membranes of cold-acclimated or cold-adapted animals are generally enriched in PE relative to PC (Hazel, 1988). Accordingly, the PC/PE ratio is positively correlated with growth temperature in metazoan poikilotherms and rises, on average, by 25% for a 20 °C rise in growth temperature. Furthermore, in an interspecific comparison between winter-active and winter-quiescent crayfish, only in the active species did the proportion of membrane PE increase during winter months (Pruitt, 1988). Similarly, the proportions of polar gangliosides (those containing from 5-7 sialic acid residues) are higher in brain lipids of cold-adapted poikilotherms than in birds and mammals (Rahmann et al., 1989), and among bacteria, glycolipid content increases over the entire thermal range of bacterial growth from psychrophiles to thermophiles (Fukunaga and Russel, 1990). Finally, among algae (Thompson, 1989a), *Acholeplasma* (Rilfors et al., 1984), and higher plants (Thompson, 1989b), increased ratios of monoglucosyldiglyceride/diglucosyldiglyceride (MGDG/DGDG) are a common feature of cold acclimation.

Although temperature-induced changes in lipid headgroup composition are varied and taxon-specific, the replacement of bilayer-stabilizing lipids (e.g., PC and DGDG) with nonbilayer- or H_{II} phase-promoting lipids (e.g., PE and MGDG) at cold temperatures is a common feature of thermal acclimation. Because H_{II} phase-forming lipids possess a relatively bulky hydrophobic domain, their presence in membranes increases both the lateral pressure and orientational order (Seddon,

1990), effects opposite to those generally associated with an increased degree of acyl chain unsaturation. The significance of temperature-induced changes in polar lipid composition to the thermal compensation of membrane structure and function remains to be established, but may reflect requirements for the maintenance of membrane dynamics appropriate to intracellular vesicle transport and fusion (refer to section V.C.2).

C. Altered Proportions of Diacyl versus Plasmalogen Phospholipids

The proportion of a given phospholipid class present in the plasmalogen (i.e., the alk- 1-enyl ether) as opposed to the diacyl form is frequently positively correlated with growth temperature (Hazel, 1988). This trend is particularly evident in the PE fraction of nervous tissue in fish, where, for example, the levels of PE plasmalogens rise from 7.7 to 15.2% in optic nerve lipids of 25 °C- compared to 15 °C-acclimated goldfish (Matheson et al., 1980). The greater resistance of ether linkages to chemical and enzymatic attack may enhance membrane stability at elevated temperatures. In addition, due, in part, to a closer proximity of the *sn*-1 and -2 chains in plasmalogen compared to diacyl lipids, ether-linked lipids tend to increase membrane order (Hermetter et al., 1989) and reduce permeability (Schwartz and Paltauf, 1977), both of which could contribute to the maintenance of membrane function at warm temperatures.

D. Modulation of Membrane Cholesterol

Because cholesterol exerts a profound effect on the physical properties of membrane lipids, it is a prime candidate to play a major role in the regulation of membrane structure and function during thermal adaptation. Cholesterol intercalates into the bilayer between phospholipids and orders fluid-phase lipids by interacting primarily with saturated acyl chains. Because cholesterol is anchored at the membrane interface by virtue of its hydrophilic 3B-hydroxyl group (Figure 2), but is shorter than most of the fatty acyl chains present in biological membranes, its ordering effect is largely restricted to the regions of the bilayer closest to the aqueous interface and there is little effect on the order of the bilayer interior (Yeagle, 1985); in fact, cholesterol incorporation actually creates free volume in the central part of the bilayer (Subcyznski et al., 1991). Consequently, the addition of cholesterol to fluid phase membranes both reduces membrane permeability and inhibits the activity of those enzymes (e.g., Na^+/K^+-ATPase and the sarcoplasmic reticular Ca^{2+}-ATPase) that

are sensitive to membrane fluidity (Madden et al., 1979; Yeagle, 1989). Based on these observations, cholesterol is expected to stabilize biological membranes and contribute to the thermal compensation of membrane structure and function to high temperatures. On the other hand, because cholesterol also disrupts the packing of gel phase lipids and causes the gel/fluid transition to broaden, or, in some cases, to disappear entirely (McMullen et al., 1993), cholesterol may act to reduce the impact of temperature change on membrane structure and function.

Although not a universal finding (e.g., Cossins, 1977), in the majority of cases (reviewed in Hazel and Williams, 1990) the cholesterol content of membranes is positively correlated with growth temperature, consistent with a primary role for cholesterol in stabilizing membranes at high temperatures. For example, the cholesterol/phospholipid molar ratio rises from 0.56 to 0.71 in synaptosomal membranes of 5 °C- compared with 25 °C-acclimated goldfish (Cossins, 1977). Cholesterol levels are also higher in erythrocyte membranes of mammals than fish (Sørensen, 1990). However, most of the studies reporting variations in membrane cholesterol with growth temperature predate the recent discovery that the vast majority (80-90%) of cellular cholesterol is present in the plasma membrane (Lange et al., 1989) and have focused on intracellular membranes, without assessing the extent to which these membranes may have been contaminated by other membrane fractions (particularly plasma membranes). This, coupled with the discovery that cholesterol interacts much less strongly with unsaturated (which comprise the vast majority of membrane lipids in poikilotherms) than saturated phospholipids (Finean, 1989), necessitates a critical reevaluation of the role of cholesterol in the thermal adaptation of biological membranes. However, a recent study employing gradient-purified plasma membrane fractions has reported a significant and positive correlation between membrane cholesterol content and growth temperature in the liver, kidney, and gills of rainbow trout (Robertson and Hazel, 1995), supporting a role for cholesterol in stabilizing membranes at elevated temperatures (Robertson and Hazel, 1996).

V. ADAPTIVE EXPLANATIONS FOR TEMPERATURE-INDUCED MEMBRANE RESTRUCTURING

A. The Concept of Homeoviscous Adaptation

The most commonly invoked explanation for temperature-induced remodeling of membrane lipid composition is that fine tuning of the

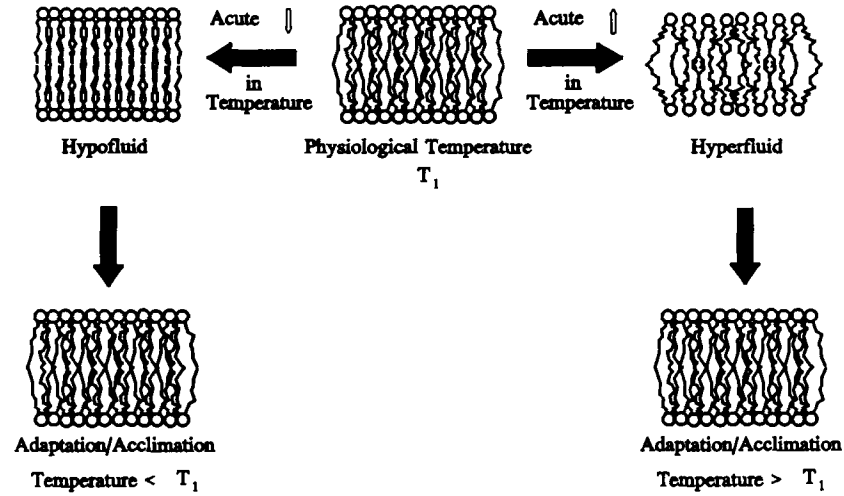

Figure 3. The concept of homeoviscous adaptation. Acute changes in temperature (illustrated by the horizontal arrows) will either reduce (in the case of cooling) or increase (in the case of warming) membrane fluidity, resulting in a less than optimal fluidity of the bilayer interior. The "optimal" membrane fluidity is then restored by the process of either thermal acclimation or thermal adaptation (indicated by the vertical arrows).

physical properties of a bilayer may result in the compensation of membrane function. Sinensky originally observed that although membranes of *E. coli* grown at 15 °C were more fluid (based on rates of motion of the electron spin resonance (ESR) probe methyl-12-nitroxyl sterarate) at any temperature than those of cells grown at 43 °C, fluidities were nearly equivalent when compared at the respective growth temperatures (i.e., in 43 °C-acclimated cells measured at 43 °C compared to 15 °C-acclimated cells measured at 15 °C). In fact, acute variations in membrane fluidity as temperature was varied between 15 °C and 43 °C exceeded by more than 100-fold the difference in fluidities between acclimated cells compared at their respective growth temperatures. Sinensky termed this defense of a similar membrane fluidity regardless of growth temperature homeoviscous adaptation (HVA; Sinensky, 1974). According to this hypothesis (Figure 3), optimal membrane function is restricted to a limited range of membrane fluidities: as temperature is raised acutely, fluidity increases beyond the optimal range and the membrane becomes hyperfluid; conversely, a drop in temperature results in a less than optimal fluidity. Persistent exposure to temperatures

either above or below those required to maintain optimal fluidity therefore necessitates (and perhaps initiates) acclimatory or adaptational alterations in membrane lipid composition that counteract and largely offset the direct effects of temperature on membrane fluidity.

Evidence in Support of Homeoviscous Adaptation

Due, in part, to the variety and range of motions displayed by lipid molecules (ranging from rotamer formation within an acyl chain, to wobbling and rotational motions of an entire molecule, to lateral diffusion within the plane of the membrane), it is not possible to define membrane fluidity with absolute rigor. No single technique for estimating fluidity is sensitive to the entire range of motions available to membrane lipids and, as a result, estimates of fluidity are biased by the type(s) of motion sensed. The steady-state fluorescence polarization of DPH and its derivatives has been used most widely in the comparative literature to assess membrane fluidity, but suffers the disadvantage that polarization values are more sensitive to changes in membrane order (a description of the static, time-averaged disposition in space of a membrane constituent) than lipid dynamics (rates of molecular motion). Nevertheless, steady-state polarization measurements do provide a useful indicator of relative changes in membrane properties and comparative conclusions drawn from such measurements have been verified by more sophisticated, time resolved polarization measurements, which permit estimates of membrane order and rates of probe motion to be clearly separated (Cossins and Raynard, 1987).

Support for homeoviscous adaptation as a means of redressing the thermal perturbation of membrane function experienced by poikilotherms is evident in both (a) *interspecific comparisons* of membrane fluidity among different species adapted (over evolutionary time) to dissimilar thermal environments, and (b) *intraspecific comparisons* between members of the same species acclimated (in the laboratory with temperature as the only manipulated variable) or acclimatized (to seasonal variations in a natural setting) to different temperatures. Evidence documenting interspecific homeoviscous adaptation in synaptosomal membranes of various fish species is illustrated in Figure 4 (Cossins and Prosser, 1978; Cossins et al., 1987). Note that membrane fluidity, when measured at a common temperature of 20 °C (Figure 4a), is highest (corresponding to the lowest polarization values) in synaptosomes of the Antarctic fish (of the genus *Notothenia*) and the Arctic sculpin, and

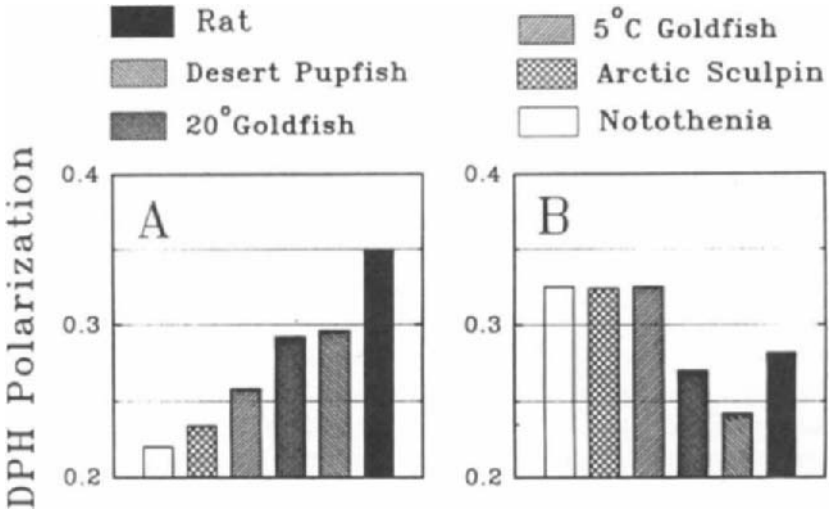

Figure 4. Evidence for interspecific homeoviscous adaptation derived from the fluorescence polarization of DPH in synaptosomal membranes of various species of fish and a homeotherm (the rat). Values in (A) were determined at a common assay temperature of 20 °C; values in (B) were determined at the respective cell or body temperatures. Data plotted were taken from Cossins and Prosser (1978) and Cossins et al. (1987). Redrawn from Hazel and Williams (1990).

lowest in the rat and desert pupfish, with values for the temperate goldfish being intermediate between these two extremes. Consequently, the rank order of membrane fluidities (Antarctic fish > (-1 °C) Arctic sculpin (0 °C) > goldfish (5-25 °C) > pupfish (34 °C) rat (37 °C)) is inversely correlated with body or habitat temperature, indicating that evolutionary adaptation to cold environments results in a significant fluidization of biological membranes. In contrast, membrane fluidities compared at the respective cell or body temperatures of each animal (Figure 4) are roughly equivalent, illustrating the phenomenon of HVA. Similarly, membrane lipids of Arctic copepods are more fluid than those of tropical species (Farkas et al., 1988), and both membrane order and rates of molecular motion (detected by Fourier transform infrared spectroscopy—FTIR) in sarcoplasmic reticular membranes of rabbit and winter flounder are nearly identical when compared at physiological temperatures (Vrbjar et al., 1992). The extent of fluidity compensation (or *efficacy* of HVA) can be assessed by comparing the difference in test temperatures required to produce equivalent membrane fluidities

with the actual difference in body or habitat temperature of the animals being compared. For example, the fluidity of rat brain synaptosomes at 28.5 °C is equivalent to that in synaptosomes of Antarctic fish measured at 0 °C, whereas the body or habitat temperatures differ by ≈37 °C, corresponding to a homeoviscous efficacy of 77% (28.5/37 × 100). In general, evolutionary adaptation to cold environments disorders membranes to an extent that nearly offsets the effects of lowered kinetic energy, as reflected in homeoviscous efficacies that are generally greater than 0.75 and frequently approach unity (Cossins and MacDonald, 1989).

HVA is also a common outcome of temperature acclimation in eurythermal temperate species. Acclimatory adjustments in the fluidity of plasma membranes isolated from hepatocytes of thermally-acclimated rainbow trout are illustrated in Figure 5. Note that an acute drop in body temperature from 20 °C to 5 °C orders the membrane to an extent indicated by a rise in the anisotropy parameter (derived from the fluorescence polarization of DPH) from point A to B (Figure 5, left). Subsequent acclimation to 5 °C fluidizes the membrane (as indicated by the leftward translation of the anisotropy curve on the temperature axis) to an extent represented by the interval from B to C. Since, in this case, fluidities (as reflected in the absolute values of the anisotropy parameter) are roughly equivalent in membranes of 20 °C- and 5 °C-acclimated trout when measured at the respective acclimation temperatures (Figure 5, right), perfect compensation of membrane fluidity is essentially realized. Although perfect compensation is not novel (other examples include: lymphocyte membranes of pinfish (Abruzzini et al., 1982); plasma membranes of thrombocytes and both T and B cells of channel catfish (Bly and Clem, 1988); thylakoid membranes of oleander (Raison et al., 1982); and membranes of *Bacillus stearothermophilus* (Esser and Souza, 1974)), more commonly the efficacies of intraspecific HVA range between 20-50% (e.g., in sarcoplasmic reticulum of carp (Ushio and Watabe, 1993), myelin and mitochondrial membranes of *Channa punctatus* (Roy et al., 1992), and more extensively reviewed in Cossins and Sinensky (1986) and Cossins and MacDonald (1989)), indicating that acclimatory compensations in membrane fluidity are frequently not sufficient to totally offset temperature-induced perturbations in membrane order. In addition, different membranes in the same animal, and in some cases the same tissue, may exhibit dissimilar efficacies of HVA. For example, mitochondria commonly display greater fluidity compensation (efficacy of HVA = 0.5-0.75) than other cellular membranes,

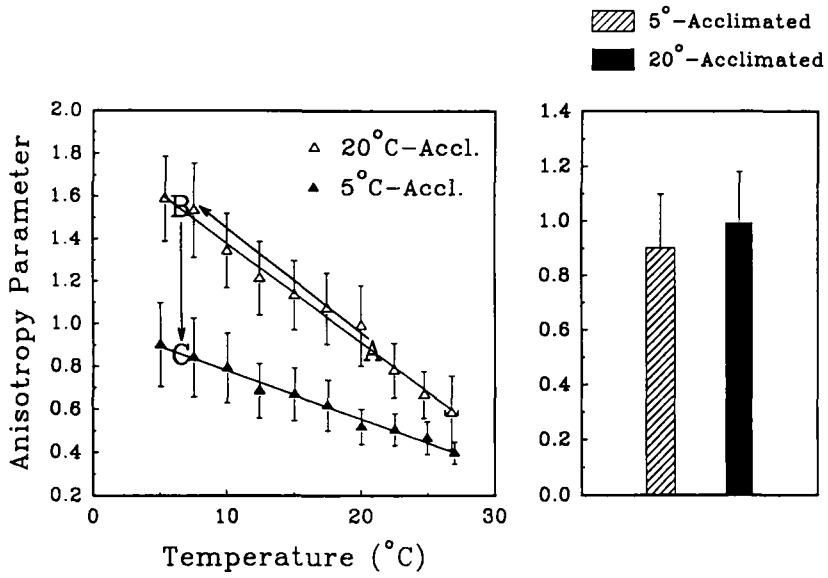

Figure 5. Intraspecific or acclimatory homeoviscous adaptation (based on anisotropy parameters derived from the fluorescence polarization of DPH) in isolated plasma membranes of hepatocytes from thermally-acclimated rainbow trout. Acute exposure of a 20 °C-acclimated trout to 5 °C results in a rigidification of the membrane corresponding to a rise in the anisotropy parameter from point A to B (left panel). Continued exposure to 5 °C results in acclimatory fluidization of the membrane corresponding to a reduction in the anisotropy parameter from point B to C. Fluidity compensation is nearly ideal as illustrated by the lack of a significant difference between fluidities determined at the respective acclimation temperatures (5 °C-acclimated fish assayed at 5 °C compared to 20 °C-acclimated fish assayed at 20 °C (right panel)). Data is redrawn from Hazel et al. (1992).

including microsomes, synaptosomes, or myelin (Cossins et al., 1980; Cossins and Prosser, 1982; Lagerspetz and Laine, 1984). Similarly, thermal compensation of membrane fluidity is evident in basolateral (efficacy of HVA = 0.5-0.75) but not brush border membranes of carp enterocytes (Lee and Cossins, 1990).

Interestingly, the capacity for HVA appears to be a basic cellular response since *in vitro* acclimation experiments employing either cell lines established in tissue culture (Tsugawa and Lagerspetz, 1990; Bols et al., 1992) or freshly isolated red blood cells (Dey and Farkas, 1992) demonstrate fluidity compensation varying from 34-100%. In addition,

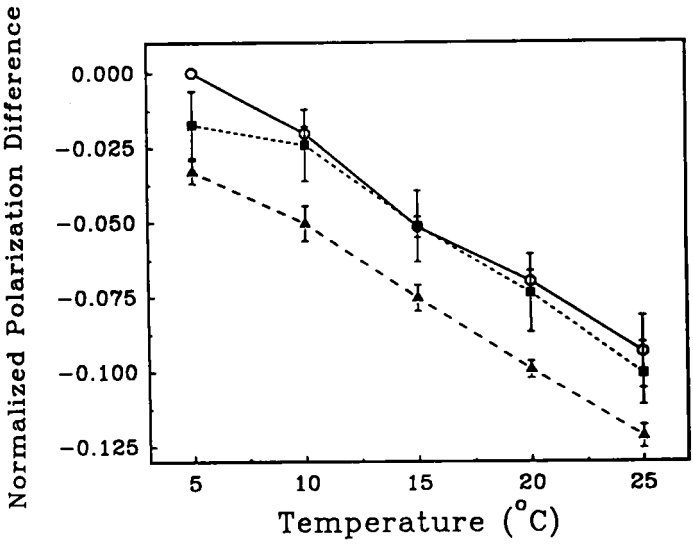

Figure 6. Fluidity adjustments in a purified plasma membrane fraction resulting from the *in vitro* incubation of hepatocytes isolated from 20 °C-acclimated rainbow trout for various periods of time at either 20 °C or 5 °C. Polarization values were determined for DPH in plasma membranes of: O, hepatocytes freshly isolated from 20 °C-acclimated trout; ■, hepatocytes of 20 °C-acclimated trout following six hours of *in vitro* incubation at 20 °C; ▲, hepatocytes of 20 °C-acclimated trout following six hours of *in vitro* incubation at 5 °C. Data from Williams and Hazel (1994).

in vitro acclimation protocols have established that significant compensation of membrane fluidity can occur within a few hours of temperature change (Dey and Farkas, 1992) as opposed to the much longer periods (days or weeks) generally required for the acclimation of both whole animal and tissue metabolic rates. Indeed, *in vitro* incubation of hepatocytes isolated from 20 °C-acclimated rainbow trout at 5 °C for only six hours fluidizes the plasma membrane sufficiently to offset 45-50% of the cold-induced increase in membrane order (Figure 6; Williams and Hazel, 1994).

The Significance of Homeoviscous Adaptation to the Thermal Compensation of Membrane Function

Robust correlations between membrane fluidity and the rotational mobility of Ca^{2+}ATPase (Squier et al., 1988) in addition to the activity

of Na^+/K^+-ATPase (Harris, 1985) suggest that the lipid environment of membrane-associated enzymes offers some resistance to the conformational changes required for catalysis. Fluidity compensation resulting as a consequence of thermal adaptation could thus contribute to the temperature independence of membrane function by regulating the activity of integral membrane proteins. Adaptational and acclimatory shifts in the thermal denaturation points of membrane-associated enzymes provide the strongest evidence in support of this view. For example, the LT_{50} (the temperature required to reduce enzyme activity by 50% in a 15-minute period) for Na^+/K^+-ATPase is nearly 3 °C lower in the more fluid synaptosomes of 6 °C (44.9 °C)- than 28 °C-acclimated goldfish (47.7 °C), a difference consistent with the observation that fluidizing the membrane by the addition of *n*-hexanol also reduced thermostability (Cossins et al., 1981). Furthermore, in a variety of both intra- and interspecific comparisons, differences in the LT_{50} s for synaptosomal Na^+/K^+-ATPase covaried closely with differences in the extent of HVA (as assessed by changes in DPH fluorescence polarization), suggesting a causal relationship between membrane fluidity and the thermal stability of membrane-associated enzymes (Cossins et al., 1986). Similarly, although chlorophyll denatured at a higher temperature in thylakoid membranes of warm- versus cold-acclimated oleander, the onset of thermal denaturation occurred at equivalent membrane fluidities in both acclimation groups (Raison et al., 1982). In addition, in an interspecific comparison among photoreceptor membranes of oxen, frog, and pike, both the thermostability of rhodopsin and membrane fluidity were similar in all species when measured at physiological temperatures (Tyurin et al., 1979). Finally, comparable increases in membrane fluidity induced either by low temperature acclimation or the application of n-hexanol reduce the heat resistance of ciliary activity in *Anodonta* gills equivalently (Lagerspetz, 1985).

Compensatory adjustments in membrane fluidity may also modulate the catalytic activity of membrane-associated enzymes. Higher rates of Na^+/K^+-ATPase activity in tissues of cold- versus warm-acclimated fish have, in a few instances, been correlated with higher membrane fluidities (Raynard and Cossins, 1991; Schwarzbaum et al., 1992), but a causal relationship between membrane fluidity and catalytic rate was not independently established. More commonly, fluidity modulation has been invoked, though not measured, to explain higher activities of membrane-associated enzymes in cold- versus warm-acclimated animals in those cases where protein (enzyme) titers do not increase following cold acclimation (e.g., the mitochondrial respiratory chain; Caldwell, 1969;

Hazel, 1972; Wodtke, 1981; and Na^+/K^+-ATPase; Smith and Ellory, 1971; Lagerspetz and Ellmen, 1984).

HVA, in the absence of direct measurements of membrane fluidity, has also been invoked to explain the temperature compensated permeability properties of biological membranes (Hazel and Schuster, 1976) and various aspects of temperature compensated neural function (Lagerspetz, 1974; Harri and Florey, 1979; Zecevic and Levitan, 1980; Zecevic and Pasic, 1983). Furthermore, in cold-acclimating goldfish, behavioral indices of neural function are strongly correlated with temporal changes in the fluidity of synaptosomal membranes (Cossins et al., 1977).

B. Limitations of Homeoviscous Theory as an Adaptive Explanation for Thermal Compensation in Biological Membranes

Since Sinensky originally proposed the concept in 1974 (Sinensky, 1974), homeoviscous adaptation has been the most extensively, and frequently the exclusive, theoretical paradigm applied to interpret both the pattern of temperature-induced change in membrane lipid composition and the extent of thermal compensation in biological membranes. The widespread acceptance of this perspective reflects both the weight of the evidence discussed in the previous section and the intuitive appeal of such a straightforward mechanism of cellular homeostasis. However, the efficacy of both acclimatory and evolutionary HVA varies widely, ranging from 10-100% for acclimatory HVA (Dey et al., 1993; Cossins and Sinensky, 1986) and at least one membrane system (the apical membrane domain of trout enterocytes) displays a significant inverse compensation of lipid order with thermal acclimation (Crockett and Hazel, 1995). Collectively these data indicate that there is no consistent relationship between either the direction or magnitude of HVA and the direction or severity of thermal stress, which argues against the general applicability of HVA as a paradigm of membrane adaptation. Furthermore, a careful analysis of the available data shows that some aspects of membrane adaptation are not consistent with HVA. Limitations to the general applicability of homeoviscous theory will be discussed in the context of specific observations that are difficult, or impossible, to explain in terms of HVA.

The Failure of Some Membrane Functions to Be Well Correlated with Membrane Fluidity

The complexity of the interactions between membrane constituents as they relate to specific membrane functions is no better illustrated than

by the work of Carruthers and colleagues on the reconstituted glucose transporter of human erythrocytes, which shows that membrane fluidity is a relatively unimportant factor in determining rates of sugar transport (Carruthers and Melchior, 1986). Although activity of the transporter increases markedly at the gel/fluid transition in bilayers of PC (a zwitterionic phospholipid), activity is unaffected by this transition in bilayers of phosphatidic acid, phosphatidylglycerol, and phosphatidylserine (all acidic phospholipids), suggesting that surface charge can stabilize the transporter against the most extreme changes in membrane fluidity (Tefft et al., 1986). In addition, activity of the transporter determined at 10 °C in gel phase bilayers of distearoyl (di-$C_{18:0}$) PC was identical to that observed in fluid phase bilayers of dimyrstoyl (di-$C_{14:0}$) PC at 60 ° C (Carruthers and Melchior, 1984) and greater than the activity supported by bilayers of dioleoyl (di-$C_{18:1}$) PC, even though the latter is unsaturated and has a lower melting point. Furthermore, both the enrichment and depletion of native erythrocyte membranes with respect to cholesterol reduced glucose transport (Yuli et al., 1981). Similar complexities also apply for the sarcoplasmic reticular Ca^{2+}-ATPase of rabbit skeletal muscle, which requires a minimal bilayer thickness (afforded by phospholipids with acyl chains of C_{14} or longer) rather than a unique membrane fluidity (Moore et al., 1981). In addition, ion-gating requirements of the acetylcholinesterase receptor include the presence of both cholesterol and a negatively charged phospholipid, in addition to a fluid membrane (Fong and McNamee, 1986). These selected examples illustrate the complexity of the variables that can significantly influence specific membrane functions and suggest that a focus confined to fluidity alone is too restrictive.

The Failure to Explain Some Aspects of Temperature-induced Alterations in Membrane Lipid Composition

There are two aspects of the temperature-induced restructuring of membrane lipids that are particularly difficult to explain by the classical HVA paradigm. The first of these is the preference for the accumulation of PUFA rather than monoenes displayed by most winter-active poikilotherms (refer to Section IVA). It is well-established that not all double bonds have an equivalent impact on the properties of a fatty acid and, surprisingly, that the number of double bonds in an acyl chain exerts only a minor influence on the physical properties of a phospholipid. For example, substituting oleic acid (18:1) for palmitic acid (16:0) at the *sn*-2

position of dipalmitoyl PC (to form 16:0/18:1-PC) reduces the melting point by 50 °C, whereas incorporation of a second double bond to form 16:0/18:2-PC lowers the melting point by an additional 22 °C, however, the presence of a third double bond in 16:0/18:3-PC actually increases the melting point slightly by 3 °C (Coolbear et al., 1983). In addition, Tm values for 16:0/16:1- and 16:0/22:6-PC do not differ significantly (-12 °C vs. -10 °C, respectively; Stubbs and Smith, 1984). Based on these observations, the most effective means of altering the physical properties of a membrane is to vary the proportion of SFAs, for the nature of the unsaturates present is of secondary importance, at least in terms of bulk phase physical properties. Thus, in terms of fluidity modulation, monoenoic fatty acids are predicted to be superior to PUFAs both with respect to the magnitude of the changes they produce in membrane physical properties and the lower metabolic cost of their production. Therefore, if HVA is the primary aim of temperature-induced membrane remodeling, then monoenes should play a more prominent role than they do in the restructuring process.The fact that they do not, implies that aspects of membrane architecture other than fluidity help to shape the nature of temperature-induced adjustments in membrane lipid composition.

The second compositional adjustment that is difficult to reconcile with a fluidity modulation hypothesis is the increased abundance of PE relative to PC in membranes of cold-adapted and cold-acclimated poikilotherms, for elevated proportions of PE commonly order rather than fluidize a membrane. Gel/fluid transition temperatures for PE are generally about 20 °C higher than those for PCs of similar acyl chain composition, due, in part, to (a) hydrogen bonding between the headgroups of PE but not PC; (b) the close packing permitted by the reduced steric bulk of the ethanolamine compared to the choline headgroup; (c) the expanded hydrophobic volume of PE relative to PC; and (d) and the greater hydration of PC than PE (Silvius et al., 1986). Consequently, some explanation other than HVA must be sought for the thermal modulation of PE/PC and MGDG/DGDG ratios in biological membranes. Since PC is a bilayer-stabilizing lipid, whereas PE destabilizes the lamellar phase (see Section IVB), modulation of the PE/PC ratio may well alter the phase behavior of the membrane, and, in particular, its proximity to the HII phase transition (refer to Section VC).

The Failure to Explain Specific Fatty Acid Requirements for Some Acclimatory Phenomena

Low temperatures are immunosuppresive to ectothermic vertebrates, due, in channel catfish, to an inhibitory effect on the function of helper

T cells (Bly et al., 1990). Immune responsiveness at low temperature can be partially restored following a period of cold acclimation, but the differential effects of temperature on T and B cell function cannot be attributed to an inability of the former to undergo fluidity compensation, since both cell types display nearly perfect HVA (Bly and Clem, 1988). Of particular interest, oleic acid (18:1*n*9) present in the culture medium can rescue ≈60% of the concancanavalin A-induced T cell proliferation normally inhibited at nonpermissive temperatures (temperatures < 17 °C), whereas linoleic acid (18:2*n*6) has no effect on either T or B cell mitogen-induced proliferation (Bly et al., 1990). Since linoleic acid is no less effective than oleate in fluidizing membranes, it is difficult to explain the fatty acid specificity of these immune rescue experiments in terms of fluidity modulation of membrane function.

The Uncoupling of HVA from Thermal Compensation of Membrane Function

The capacity for HVA and thermal compensation of membrane function are not always tightly linked. For example, higher rates of sodium pump turnover (measured as ouabain-sensitive K^+ -influx) in red blood cells of cold (3 °C) versus warm (20 °C) acclimated rainbow trout occur in the absence of increased numbers of pump sites and are correlated with an efficacy of HVA of 30-40% in red blood cell membranes, suggestive of fluidity modulation of sodium transport activity. However, a thorough seasonal analysis revealed HVA at times of the year when no sodium pump compensation could be demonstrated, which argues against a major role for membrane fluidity in controlling pump activity (Raynard and Cossins, 1991). This impression is reinforced by the contrasting responses to low temperature of two coexisting fish species in subalpine lakes of central Europe (Schwarzbaum et al., 1992). In the Arctic charr, *Salvelinus alpinus*, neither the activity nor the thermal stability of Na^+/K^+-ATPase in basolateral membranes of kidney were altered by cold acclimation in spite of substantial HVA (efficacy = ~78%); instead, a reduction in passive ion fluxes, which were 60% lower in cold (5 °C) versus warm (20 °C) acclimated fish, was the major acclimatory response. In contrast, the main adjustment to low temperature in the roach, *Rutilus rutilus*, was a fourfold increase in the density of pump sites, and adjustments in membrane fluidity (efficacy of HVA = ÷20%) were much smaller than those seen in *S. alpinus*. Thus, although cooling reduces the rate of active cation transport and the magnitude of cation gradients in both species, the

acclimatory adjustments responsible for the restoration of ion gradients at low temperature differ fundamentally between the species. In one case (*R. rutilus*) ion gradients are maintained at low temperature by an increase in transport capacity (i.e., the number of pump sites), while in the other case (*S. alpinus*) the passive dissipation of ion gradients is diminished by reductions in membrane permeability, and in neither case does HVA appear to play an essential role in the acclimatory process. These examples illustrate that thermal adaptation of membrane function must be viewed within the context of the full range of mechanisms that could, in fact, compensate for the acute effects of temperature change on the phenomenon being studied, which may well include responses in addition to, or other than, fluidity modulation.

C. Alternative Interpretations of the Temperature-induced Restructuring of Membrane Lipids

The failure of fluidity regulation to explain fundamental patterns of temperature-induced lipid remodeling in biological membranes of poikilotherms, coupled with the broad range of homeoviscous efficacies in various cell and membrane types, and the lack of a consistent mechanistic association between fluidity and various aspects of membrane function, suggest that some feature of membrane organization other than fluidity is subject to regulation. According to this view, homeoviscous adaptation may simply be a consequence of other homeostatic or acclimatory processes rather than an end unto itself. The remainder of this chapter will explore other aspects of membrane organization that may be subject to regulation as environmental temperature changes, in an attempt to identify general principles with broader applicability in an adaptive context than membrane fluidity.

Homeophasic Adaptation in Microorganisms

Since structural rearrangements are most extensive and functional perturbations likely to be most severe when the phase state of a membrane is altered, the adaptive significance of HVA may lie not in the fine tuning of membrane fluidity, but in its contribution to the preservation of a particular phase state of the membrane. Many microorganisms can grow and function normally with membranes of widely different fluidities (Russell and Fukunaga, 1990), but growth is frequently impaired when a critical proportion (in *E. coli*, 50%) of the membrane lipid is present in the gel phase

(McElhaney, 1984b). McElhaney has proposed the term homeophasic adaptation (HPA) to describe the pattern of thermal adaptation in microorganisms. HPA is viewed as a mechanism for extending the effective range of growth temperatures, for microbial membranes, unlike those of multicellular poikilotherms, generally contain sufficient quantities of SFA to form the gel phase at temperatures not far below the culture temperature. There are many examples among microorganisms of adaptive alterations in the phase behavior of membrane lipids following a period of growth at altered temperature. For example, in plasma membranes of *Anacystis nidulans*, the onset of phase separation is observed at temperatures of 5 °C and 16 °C in cells grown at 28 °C and 38 °C, respectively (Furtado et al., 1979); similar results have been widely reported among both microorganisms and plants (see Hazel and Williams, 1990).

A Dynamic Phase Behavior Model of Thermal Adaptation in Membranes

The extent to which the defense of an appropriate phase state constitutes a serious challenge to multicellular poikilotherms is difficult to assess. The scarcity (refer to Section IID) of phase transitions reported for membranes of multicellular poikilotherms implies that evolutionary adaptation has shaped membranes of sufficient fluidity to ensure that transitions to the gel phase occur only at temperatures well below the physiological range. Nevertheless, a broadened definition of HPA that encompasses dynamic rather than static phase behavior may be useful in interpreting temperature-induced changes in the membrane lipid composition of poikilotherms (Hazel, 1995). According to this view, the relationship between the temperature at which a membrane is functioning (T_a) and the temperatures of the gel/fluid and H_{II} phase transitions may be as important as the particular type of phase present (Figure 7). As illustrated in Figure 7, a rise in temperature decreases the interval between T_a and the H_{II} phase transition (T_h), while simultaneously moving the membrane further away from the gel/fluid transition (T_m); a drop in temperature has the opposite effects. Accordingly, thermal acclimation or adaptation, by altering the chemical composition of a membrane, modifies both T_m and T_h, so that the operational temperature (T_a) remains at a suitable interval above T_m yet below T_h. The proximity of the membrane to the H_{II} phase transition may be of particular importance since the H_{II} phase has been postulated as an intermediate in membrane fusion, and membrane fusion events are central to processes

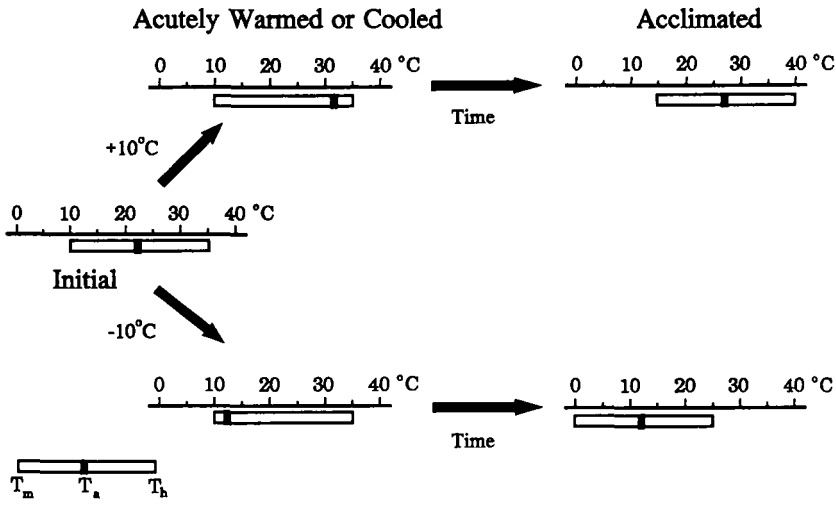

Figure 7. A dynamic phase behavior model of thermal adaptation in biological membranes. An acute rise or drop in temperature (indicated by vertical arrows in the figure) alters the relationship between the body temperature (i.e., the temperature at which the membrane is functioning (T_a) and the transitions to the gel (T_m) and H_{II} (T_h) phases (a rise in temperature decreases the interval between T_a and T_h while increasing the interval between T_m and T_a, whereas a drop in temperature has the opposite effects). Acclimation or adaptation to an altered temperature (indicated by the horizontal arrows in the figure) restores the "normal" dynamic phase behavior (the proximity of T_a to T_h and T_m) of the membrane.

of membrane traffic (e.g., exocytosis and endocytosis) in all cells. It is proposed that at physiological temperatures a membrane must be positioned close enough to the H_{II} transition (i.e., be sufficiently unstable) to permit the regulated fusion events associated with normal membrane traffic, yet be sufficiently stable to prevent these processes from occurring in an unregulated fashion. Accordingly, a drop in temperature is expected to compromise cell function by increasing the energy input required to attain T_h (i.e., increasing the temperature interval between T_a and T_h), and thereby inhibiting membrane traffic. Conversely, a rise in temperature, by decreasing the interval between T_a and T_h, is predicted to destabilize biological membranes, making unregulated fusion events more likely. In this regard, it is worth noting that membrane traffic is especially sensitive to changes in temperature. For example, the transport of cholesterol from its site of synthesis in the endoplasmic reticulum to the plasma membrane ceases at temperatures below 15 °C in a variety

of mammalian cell lines (DeGrella and Simoni, 1982; Kaplan and Simoni, 1985; Lange and Muraski, 1988). Further support for the dynamic phase behavior model of thermal adaptation in biological membranes is that lipid extracts from bacteria possess the capacity to assemble spontaneously into unilamellar structures at a critical temperature, frequently close to the growth temperature—temperatures above or below this critical temperature result in the formation of multibilayers. Furthermore, the spontaneous assembly of unilamellar membranes from the isolated lipids of neural tissue occurs only at the normal body temperatures of squid and rat (Ginsberg et al., 1991).

Finally, the dynamic phase behavior model can explain more of the experimental observations on temperature-induced alterations in membrane lipid composition than can fluidity based models. For example, the positive correlation between growth temperature and the PC/PE ratio in animal cell membranes can be explained in terms of dynamic phase behavior as an adaptation to restore (by the increased incorporation of bilayer-stabilizing phospholipids) the normal interval between the physiological temperature (T_a) and T_h, which is otherwise reduced as temperature rises. In fact, the balance between bilayer- stabilizing and destabilizing lipids may be especially important in regulating the dynamic phase behavior of a membrane. Indeed, Lindblom and Rilfors (1989) propose that some critical level of bilayer destabilizing lipids is required to clamp a membrane near the L_α/H_{II} transition under physiological conditions, so that localized regions of nonbilayer structure can form transiently in response to triggering mechanisms. The strongest evidence indicating that the dynamic phase behavior of biological membranes is subject to regulation is derived from recent experiments employing AD93 mutants of *E. coli* that lack the ability to synthesize PE (a bilayer-destabilizing lipid), which normally accounts for 70-80% of the phospholipids in parental strains (Reitveld et al., 1993). Cardiolipin (CL) and phosphatidylglycerol (PG), phospholipids which only form the H_{II} phase in the presence of divalent cations, replace PE in the AD93 mutants and these mutants (unlike the parental strain) display an absolute requirement for high concentrations of divalent cations. Furthermore, although the relative proportions of PG and CL in membrane lipids vary widely depending on the type (Ca^{++}, Mg^{++}, or Sr^{++}) and concentration of divalent cation, all cells displayed a bilayer-to- nonbilayer transition at approximately 10 °C above the growth temperature. These data indicate that membrane lipid composition is, in fact, regulated so as to maintain a specified propensity toward the formation of nonbilayer structures,

consistent with the concept of the regulation of dynamic phase behavior. In addition, recently described differences in the properties of PUFA and monoenes provide an explanation for the preferential accumulation of PUFA at low temperatures that is more consistent with a dynamic phase behavior model of thermal adaptation than a fluidity modulation model. Baenziger et al. (1992) propose that although monoenes are more than adequate for fluidity modulation, they may not pack sufficiently well to form a tightly sealed bilayer, particularly with respect to cation permeability. In contrast, because the multiple double bonds of PUFA increase both the cross-sectional area occupied by an acyl chain and membrane order in their immediate vicinity, simultaneously maintain the dynamic state of the bilayer and permeability characteristics compatible with biological function.

VI. CONCLUSIONS

The concept of homeoviscous adaptation has provided a useful theoretical framework in which to examine the thermal adaptation of biological membranes in poikilotherms. However, as emphasized by Cossins and MacDonald (1989), membrane function is influenced by many factors other than fluidity, and HVA should be viewed as but one of a broad repertoire of adaptive responses. The weight of available evidence suggests that the concept of homeoviscous adaptation does not adequately reflect the highly specific nature of lipid-protein interactions and cannot explain several consistently observed patterns in the temperature dependence of membrane lipid composition. Nevertheless, the generally similar effects of growth temperature on the membrane lipid composition of organisms from all kingdoms of life, coupled with the widespread occurrence of apparently compensatory, temperature-induced alterations in membrane fluidity provide compelling evidence that some attribute(s) of membrane organization (other than fluidity) is subject to physiological regulation. While it is unreasonable to assume that the regulation of any single membrane characteristic can account for the wide diversity in membrane structure and function and the variety of physiological contexts in which membranes must operate, a view of membranes emphasizing the dynamic phase behavior of membrane lipids, as proposed in this chapter, may provide additional insights into those properties of membranes that must be conserved in order for membrane function to be preserved at environmental extremes.

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COLD ADAPTATION OF MICROTUBULE ASSEMBLY IN ANTARCTIC FISHES

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I. INTRODUCTION

Cytoplasmic microtubules are a major filamentous component of the cytoskeleton of most eukaryotic cells. These versatile structures participate in many fundamental cellular processes, including the separation of chromosomes during mitosis and meiosis, the intracellular transport of organelles, nerve growth and regeneration, and the determination of cell shape (Dustin, 1984). At the structural level, cytoplasmic microtubules appear to be single, hollow cylinders (often termed *singlets* in contradistinction to the *doublet* microtubules of cilia and flagella) with maximal outer diameters of 30 nm and wall thicknesses of approximately 8 nm (Beese et al., 1987). Singlet microtubules are generally composed of 13 longitudinal rows of subunits, called protofilaments, but variation in protofilament number has been observed *in vivo* (Burton et al., 1975; Savage et al., 1989), *in vitro* (Pierson et al., 1978; Wade et al., 1990), and within individual microtubules (Chrétien et al., 1992). Biochemically, cytoplasmic microtubules contain a major protein subunit, the tubulin $\alpha\beta$ dimer, that forms the cylindrical wall and, in most cases, several microtubule-associated proteins (MAPs) that bind to and project from the wall (Dustin, 1984). The α and β tubulins of higher vertebrates are encoded by small gene families (ca. 6-7 functional genes for α and 6-7 for β), each member of which yields a structurally distinct polypeptide, or isotype (Sullivan, 1988).

Temperature, through its effects on the equilibria and kinetics of weak, noncovalent molecular interactions, plays an important role in the con-

trol of protein polymerization reactions. Microtubule assembly, for example, is an entropically driven process favored by high temperatures and mediated by the release of structured water from sites of intersubunit contact (Correia and Williams, 1983). It is not surprising, therefore, that the cytoplasmic microtubules of homeotherms (organisms, such as mammals and birds, that maintain stable, often supra-ambient body temperatures) are readily disrupted by small decreases in temperature below the physiological range of 35-39 °C. Furthermore, the formation of microtubules *in vitro* by the tubulins and MAPs of homeotherms is highly sensitive to thermal perturbation, with polymerization favored by temperatures near 37 °C and disassembly produced by low temperatures (0-4 °C). By contrast, many ectotherms (organisms whose body temperatures conform to the ambient temperature) are challenged to assemble microtubules at body temperatures that are generally well below those of homeotherms. What biochemical adaptations have evolved in cold-living ectotherms to offset, or compensate for, the destabilizing effect of low temperature on microtubule assembly? Partial, yet intriguing, answers to this question have emerged from analysis of cold adaptation of microtubule assembly in Antarctic fishes, the subject of this review.

From the standpoint of cold adaptation, the ectothermic fishes of the polar oceans, which now experience body temperatures as low as the freezing point of seawater (-1.86 °C), present biochemists with ideal experimental systems. The austral polar ocean has cooled since the start of the Oligocene approximately 38 million years ago, with Antarctic glaciation occurring late in the Miocene (7 million years before the present), and perhaps earlier (30 million years ago; Shackleton and Kennett, 1975; Kennett and Shackleton, 1976). Although in geological terms this oceanic cooling occurred recently, Clarke and Crame (1989) argue that the *rate* of cooling in the Antarctic, averaging 0.03 °C per 100,000 years (Eastman and Grande, 1989), was small relative to other oceanographic changes. Thus, the development of low environmental temperatures in the Antarctic has presented few, if any, evolutionary problems, and representatives of many marine taxa have independently developed, through mutation and natural selection, compensatory adaptations that maintain metabolic rates and equilibria at appropriate levels. The teleostean suborder Notothenioidei (comprising the families Nototheniidae (Antarctic rockcods), Channichthyidae (icefishes), Bathydraconidae (dragonfishes), Harpagiferidae (spiny plunderfishes), Artedidraconidae (plunderfishes), and Bovichtidae (thornfishes)), which is largely endemic to the Antarctic, provides numerous cold-adapted

species suitable to biochemical, physiological, and molecular-biological analysis.

What can we learn by analysis of cold adaptation of microtubule assembly in Antarctic fishes? First, investigations of this type should reveal specific structural alterations of tubulins and MAPs, including changes in primary sequence and/or posttranslational modification, that favor microtubule formation at low temperatures. Second, because some of these adaptations are likely to be located at sites of intersubunit contact, such studies will help to define the structural domains that contribute to tubulin-tubulin and tubulin-MAP interactions in *all* microtubule systems. Third, comparative analysis of cytoplasmic microtubules (and other cytoskeletal proteins) from cold-adapted fishes of distantly related lineages (e.g., Antarctic and Arctic) should expand our understanding of the repertoire of biochemical mechanisms and strategies that cold-living organisms employ to conserve protein polymerization reactions. Our objectives here are to review key findings from our laboratory on polymerization of microtubule proteins from Antarctic fishes, to place our results in the context of related work on microtubules from mesophilic and homeothermic organisms, and to suggest potential avenues for future experimentation. Limitations of space preclude consideration of the dynamics of Antarctic fishes microtubules and the drug-binding properties of their tubulins; the interested reader is referred elsewhere (Himes and Detrich, 1989; Skoufias et al., 1992).

II. MICROTUBULE FORMATION BY BRAIN TUBULINS FROM ANTARCTIC FISHES

To analyze thoroughly the mechanisms underlying cold adaptation of microtubule assembly, one must isolate tubulins and MAPs and evaluate their respective contributions in controlled polymerization reactions *in vitro*. In this section the purification and properties of brain tubulins from Antarctic fishes are described; isolation of MAPs, and their role in microtubule assembly, are discussed in Section V.

A. Purification of Brain Tubulin

We have developed methods for the rapid purification of tubulin (Detrich and Overton, 1986; Detrich et al., 1989) from the brain tissues of several Antarctic fishes (e.g., *Notothenia gibberifrons, N. coriiceps*

neglecta, and *Chaenocephalus aceratus*). We purify tubulin in two steps: (1) ion-exchange chromatography of brain high-speed supernatants on DEAE Sephacel to produce a fraction enriched in tubulin, followed by (2) one cycle of microtubule assembly *in vitro* to remove residual contaminating proteins. Figure 1 shows a sodium dodecyl sulfate (SDS) urea-polyacrylamide gradient gel that contains protein fractions that were obtained during preparation of tubulin from *N. gibberifrons*. The microtubules (lane 7) obtained by this method are composed of the α- and β-tubulins and are free of MAPs. Based on the sensitivity of the Coomassie stain, we estimate that these preparations contain greater than 98% α - and β-tubulins. Approximately 12 mg of tubulin can be obtained from 10 g of fish brain tissue in a period of 6-7 hours. Furthermore, the overall yield is 10-fold greater than that produced by an alternative method involving two cycles of temperature-dependent microtubule assembly/disassembly and phosphocellulose chromatography (Williams et al., 1985).

B. Temperature Dependence of Microtubule Assembly

We (Detrich et al., 1989) have evaluated the functional properties of the pure fish tubulins by turbidimetry (Gaskin et al., 1974), electron microscopy, and quantitative sedimentation (Johnson and Borisy, 1975). The principal results are presented in Figures 2-4 and Table 1.

Upon warming from 0 °C to 20-25 °C, solutions of fish brain tubulin form large polymers as judged by turbidimetry (e.g., Figure 2a). Polymerization follows a brief lag, during which microtubule nuclei are formed, and net growth continues to a steady-state in polymer mass, indicated by the approach to a plateau turbidity value. The resulting polymers are largely microtubules (Figure 3), but some polymorphic structures (e.g., sheets and microtubule-sheet hybrids) are also present. Progressive cooling of such microtubule solutions to several intermediate temperatures produces successive decreases in turbidity (Figure 2a, see arrows indicating times of temperature shifts), each of which approaches asymptotically a new, lower plateau. These observations demonstrate that, for temperatures within the range of 0-25 °C, brain tubulins from Antarctic fishes polymerize reversibly in response to thermal changes.

De novo polymerization of Antarctic fish tubulins *in vitro* also occurs at lower temperatures. Figure 2b, for example, shows microtubule assembly by *N. gibberifrons* tubulin at temperatures near (5 °C) or above (10 and 20 °C) the physiological range (-1.86 to +2 °C). At the lower

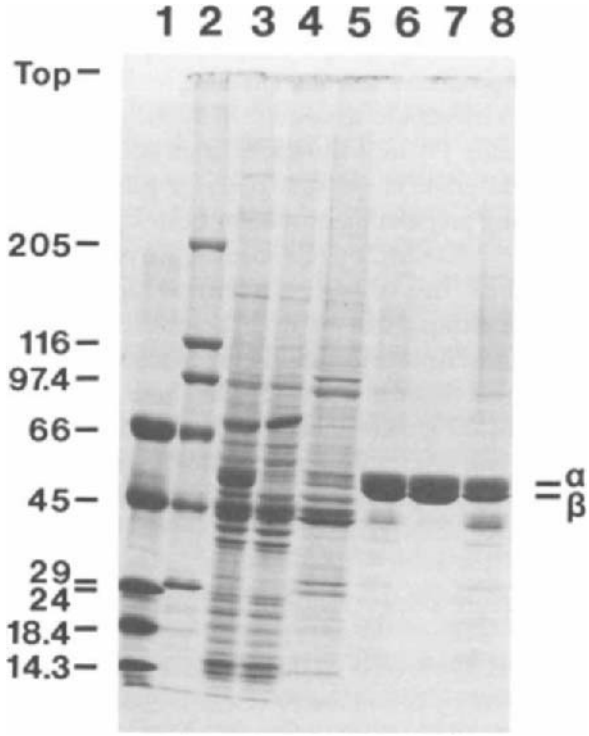


Figure 1. Purification of brain tubulin from an Antarctic rockcod, *Notothenia gibberifrons*. Tubulin was purified from brain tissue by DEAE ion-exchange chromatography and one round of microtubule polymerization as described previously (Detrich and Overton, 1986; Detrich et al., 1989). Protein fractions obtained at various stages of the purification were examined by electrophoresis on an SDS urea-polyacrylamide gradient gel (4-16% acrylamide, 1-8 M urea). Lanes: 1, low molecular-weight standards; 2, high molecular-weight standards; 3, high-speed supernatant (HSS) from brain; 4, proteins present in the flow-through fraction following application of the HSS to a column of DEAE-Sephacel; 5 and 6, proteins released from the ion-exchange resin by PMTG buffer (0.1 M Pipes-NaOH, 1 mM $MgSO_4$, 1 mM TAME, 0.1 mM GTP, pH 6.9 at 20 °C) containing 0.15 M and 0.40 M NaCl, respectively; 7 and 8, pellet and supernatant obtained by polymerization of sample 6 (20 °C, 20 minutes) followed by centrifugation (40,000 × g, 20 °C, 20 minutes) to collect the microtubule polymer. Electrophoretic migration was from top to bottom. The molecular weights of the standards (in thousands) and the positions of the tubulin chains (α and β) and of the top of the gel are indicated on the vertical axes. Reprinted from Detrich et al. (1989) with permission of the American Chemical Society.

temperatures, assembly occurs more slowly and requires greater tubulin concentrations to attain comparable final turbidities (polymer masses) (Himes and Detrich, 1989). Nevertheless, polymerization of Antarctic fish tubulin at low temperature is at least as efficient as microtubule formation by mammalian brain tubulin at high temperatures (e.g., 37 °C; see Section VI).

Quantitative analysis of the temperature dependence of the microtubule assembly reaction provides important information regarding the energetics that favor polymer formation. At a given temperature, the assembly of microtubules *in vitro* requires a minimal, or "critical," concentration of tubulin dimers (Gaskin et al., 1974; Johnson and Borisy, 1975). Below the critical concentration polymerization does not occur, whereas above it the extent of microtubule assembly is a linear function of the total tubulin concentration. We have determined critical concentrations for polymerization of tubulins from Antarctic fishes at temperatures between 0 and 20 °C by quantitative sedimentation (Detrich et al., 1989, 1992). Table 1 summarizes measurements for brain tubulins from *N. gibberifrons* and *N. coriiceps neglecta.* (Polymerization of egg tubulin will be addressed in Section IV.) The critical concentrations of the fish

Table 1. Temperature Dependence of Critical Concentrations for Polymerization of Antarctic Fish Tubulins

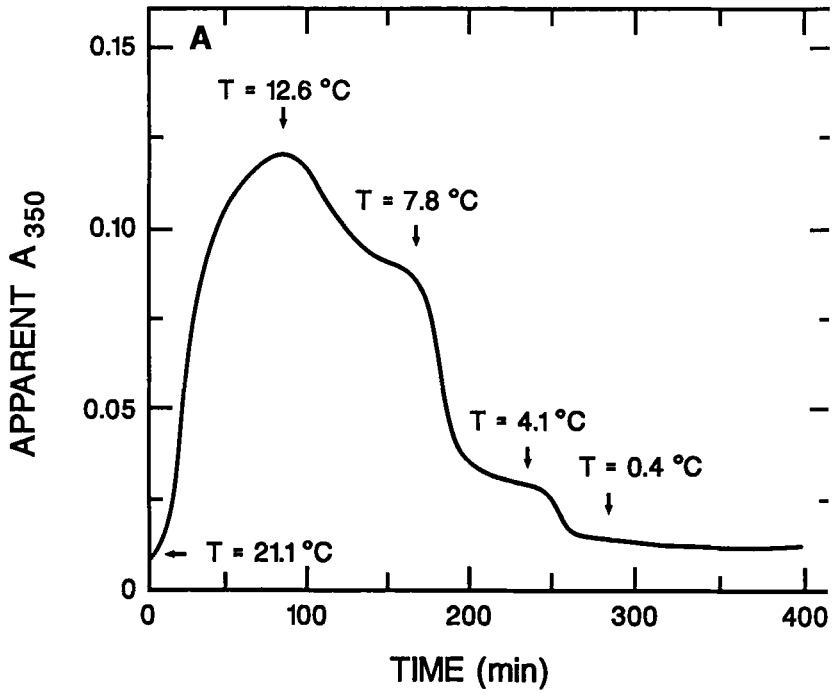
	Critical concentration of tubulin (mg/ml)		
Temp. (°C)	*N. coriiceps Egg*[a]	*N. coriiceps Brain*[b]	*N. gibberifrons Brain*[c]
0	—[d]	—	0.88
3	0.057	0.21[e] (±0.018)	—
5	—	—	0.26
6	0.047	—	0.16
8	—	0.11	—
9	0.021	—	—
10	—	—	0.12
11	—	0.062	—
12	0.016	—	0.086
15	0.0086	0.034	0.078
18	0.0024	0.025	0.020
19	0.0022	—	—

Source: Reprinted from Detrich et al. (1992) with permission from the American Society for Biochemistry and Molecular Biology.

Notes: [a] Twice-cycled tubulin from three different preparations.
[b] Once-cycled tubulin from three different preparations.
[c] Once-cycled tubulin from two different preparations; Detrich et al. (1989).
[d] —, Not determined.
[e] Mean (± S.D.), N = 2, one value from Detrich et al. (1989).

tubulins decrease from 0.87 mg/ml at 0 °C to 0.02 mg/ml at 18 °C. Under comparable solution conditions, the assembly of microtubules from pure mammalian brain tubulin at 37 °C requires protein concentrations in excess of 2 mg/ml (Williams et al., 1985). We conclude that brain tubulins from Antarctic fishes form microtubules *in vitro* at temperatures and protein concentrations that approximate the physiological.

Figure 4 presents a thermodynamic analysis of the critical concentration data. For these van't Hoff plots, we have assumed that the apparent association constant for microtubule elongation (i.e., the addition of a tubulin dimer to the end of a microtubule) is equal to the reciprocal of the critical concentration (Gaskin et al., 1974; Lee and Timasheff, 1977; Detrich et al., 1989, 1992). The co-linearity of data points for the two brain tubulins (open circles, open triangles) demonstrates that their polymerization reactions are energetically equivalent. From the pooled data, we obtain estimates for the apparent standard enthalpy and entropy changes for microtubule elongation (ΔH°_{app} = +26.5 kcal/mol, ΔS°_{app} = +121 eu) that are large and postitive. Thus, the assembly of microtubules from the brain tubulins of Antarctic fishes is strongly entropy driven.



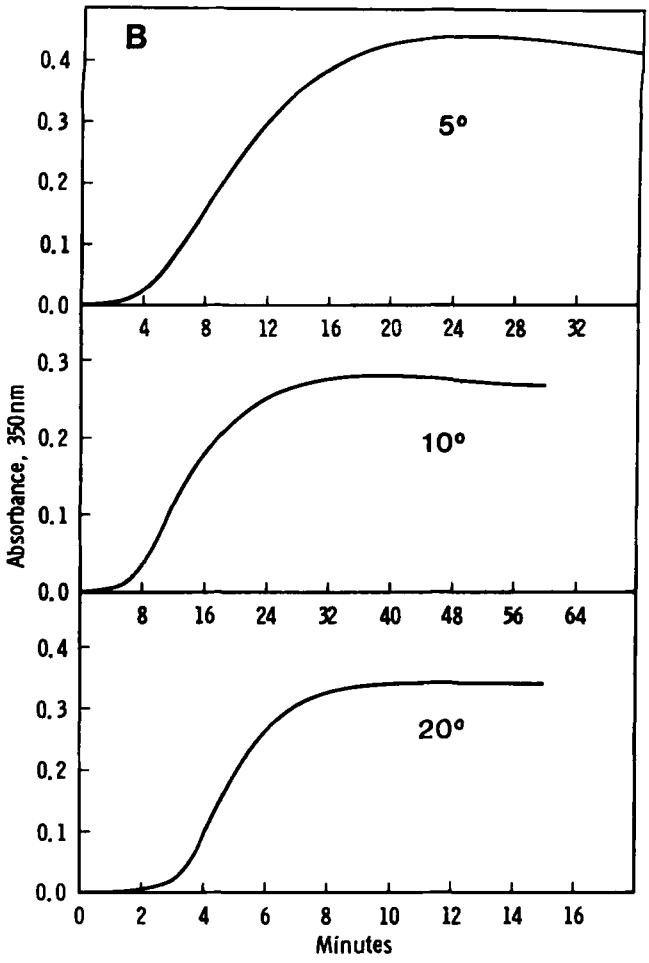


Figure 2. Polymerization of brain tubulin from *N. gibberifrons*. **A**, Reversible polymerization. A sample of purified tubulin (0.5 mg/ml) in PME buffer (0.1 M Pipes-NaOH, 1 mM $MgSO_4$, 1 mM EGTA, pH 6.82 at 20 °C) containing 1 mM GTP was warmed from 0 to 21.1 °C at zero time, and the turbidity generated by the sample was observed spectrophotometrically (apparent A_{350}). At intervals (see arrows), the temperature of the solution was reduced to the values indicated. **B**, Polymerization at near- and supra-physiological temperatures. Samples of tubulin from *N. gibberifrons* (in PME buffer containing 0.1 mM GTP and a GTP-regenerating system (Himes and Detrich, 1989)) were warmed from 0 °C to final temperatures of 5, 10, or 20 °C at zero time, and microtubule assembly was monitored by turbidimetry. Tubulin concentrations: 5 °C, 1.3 mg/ml; 10 °C, 0.96 mg/ml; 20 °C, 0.60 mg/ml. Reprinted from Detrich et al. (1989) and from Himes and Detrich (1989) with permission of the American Chemical Society

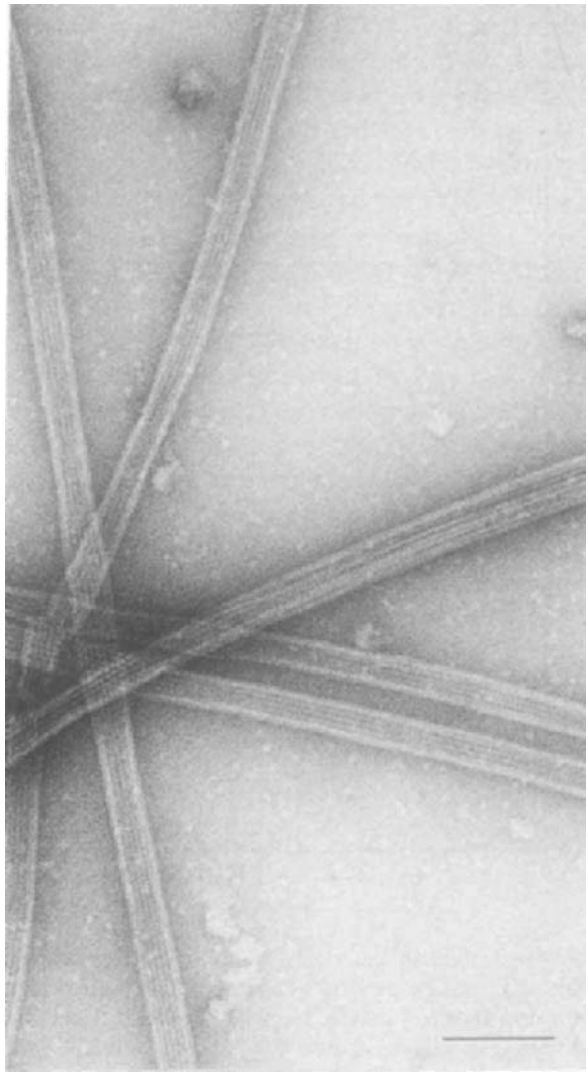


Figure 3. Electron micrograph of microtubule polymer assembled *in vitro* from an Antarctic fish tubulin. A solution of *N. coriiceps neglecta* tubulin (0.64 mg/ml in PME buffer (see legend to Figure 2) containing 1 mM GTP) was warmed from 0 to 20 °C at zero time, and a negatively stained specimen was prepared 30 minutes after the start of assembly. The protofilamentous substructure of these microtubules is readily apparent. The magnification bar represents 100 nm. Reprinted from Detrich et al. (1989) with permission of the American Chemical Society.

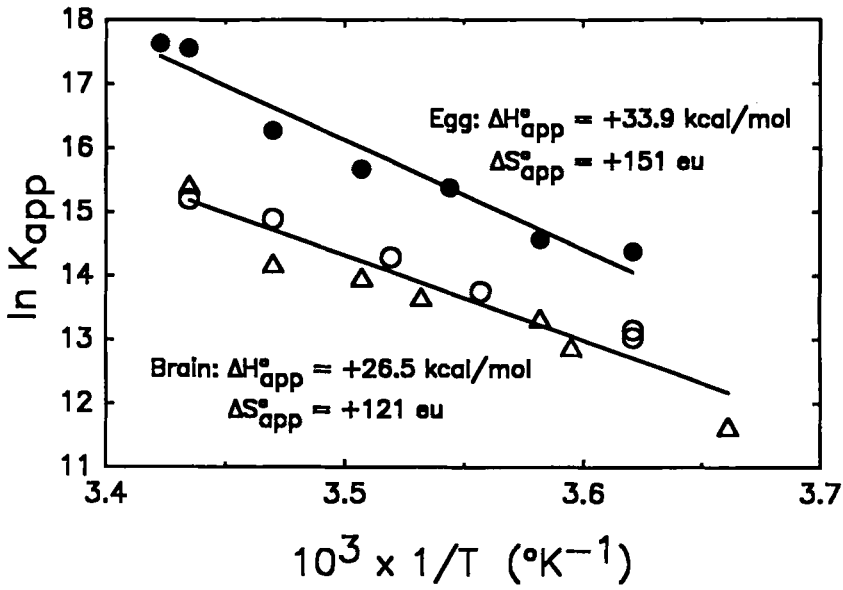


Figure 4. Polymerization energetics of tubulins from Antarctic fishes. In these van't Hoff plots, the natural logarithm of the apparent equilibrium constant for microtubule elongation by egg or brain tubulins (calculated from the corresponding critical concentration; see Table 1) is plotted as a function of the reciprocal of the absolute temperature. Symbols: open circles, *N. coriiceps neglecta* brain tubulin; open triangles, *N. gibberifrons* brain tubulin; closed circles, *N. coriiceps neglecta* egg tubulin. Both brain- and egg-tubulin data sets are fit well by straight lines (r = -0.975 and -0.953, respectively). Reprinted from Detrich et al. (1992) with permission of the American Society for Biochemistry and Molecular Biology.

C. Molecular Interactions Driving Polymerization at Low Temperatures

Noncovalent protein association reactions are mediated largely by four molecular interactions: hydrogen bonds, van der Waals bonds, electrostatic (ionic) bonds, and hydrophobic interactions. Only the latter two make positive contributions to the enthalpy and entropy changes of subunit association reactions (Ross and Subramanian, 1981). Thus, we hypothesize that the entropy-driven polymerization of Antarctic fish tubulins reflects a reliance on ionic bonds and/or hydrophobic interactions at sites of intersubunit contact. To estimate the relative contributions of these two interactions, we measured the critical concentration for polymerization of *N. gibberifrons* tubulin at 10 °C as a function of

Table 2. Salt Dependence of the Critical Concentration for Polymerization of *N. gibberifrons* Tubulin at 10 °C

[NaCl] (M)	Critical Concentration (mg/ml)[a]
0.0	0.041
0.1	0.094
0.2	0.16
0.4	0.34

Note: [a] Critical concentrations were measured in a polymerization buffer (0.1 M Pipes-NaOH, 2 mM $MgSO_4$, 1 mM GTP,1 mM DTT, 8 mM CaC_{l2}, 10 mM EGTA, pH 6.82 at 20 °C) supplemented with NaCl at the concentrations indicated (Detrich et al., 1989).

the concentration of a structure-making salt, NaCl (Detrich et al., 1989). High concentrations of NaCl strengthen hydrophobic interactions and weaken electrostatic bonds. As shown in Table 2, the critical concentration rose modestly, from 0.041 to 0.34 mg/ml, as the salt concentration was increased from 0 to 0.4 M. In contrast, NaCl at concentrations exceeding 0.25 M completely suppresses the polymerization of tubulins or total microtubule proteins from mammals (Olmsted and Borisy 1975; Himes et al., 1977). Thus, we suggest that hydrophobic interactions make proportionately larger contributions to the polymerization of Antarctic fish tubulins than they do to the assembly of mammalian tubulins.

III. STRUCTURAL FEATURES OF ANTARCTIC FISH BRAIN TUBULINS

Our ultimate objective is to determine the structural adaptations that favor the polymerization of Antarctic fish tubulins at low temperatures. Such adaptations may arise through changes in the primary sequences or posttranslational modifications of the α and/or β tubulins (or both), and they are likely to be located in structural domains that participate directly in, or modulate indirectly, contact between tubulin dimers. To address this problem, we have used a comparative strategy to identify biochemical features that are unique to the tubulins of the Antarctic fishes (e.g., Detrich and Overton 1986, 1988; Detrich et al., 1987; Detrich and Parker, 1993). Below we describe our recent studies of the subunit heterogeneity, primary sequences, and posttranslational modifications of tubulin subunits from Antarctic fishes and other organisms (temperate fishes and mammals).

A. Isoform Heterogeneity

The α and β tubulins from brain tissues of mammals and birds are heterogeneous proteins, each consisting of multiple isoforms[1] that can be separated on the basis of apparent molecular weight and isoelectric point (Field et al., 1984). Nevertheless, the isoform distributions of neural tubulins from mammals and birds are remarkably conserved. Does this conservation extend to the brain tubulins of Antarctic fishes? We have approached this question by comparing the subunit complexity of brain tubulins from Antarctic fishes and mammals by high-resolution isoeletric focusing (Detrich and Overton, 1986, 1988).

Neural tubulins from three Antarctic fishes and the cow are highly heterogeneous (Figure 5); each is resolved into approximately 20 distinct isoelectric variants. However, the focusing patterns of the fish tubulins, although similiar to each other, differ strikingly from the distribution of bovine isoforms. The isoelectric points of the Antarctic fish tubulins (~ 5.3–5.8) span a range that is more basic than that of the bovine chains (~5.2–5.6). The greater basicity of the Antarctic fish α tubulins is particularly conspicuous, but the major piscine β chains are also shifted to more alkaline pIs. Moreover, the basic character of the fish isoforms is expressed in the native tubulin dimer, which possesses 1-2 fewer net negative charges than does the bovine dimer (Detrich and Overton, 1986, 1988). Thus, the tubulin isoforms of Antarctic fish brain differ in their physicochemical properties from the neural tubulins of mammals. We suggest that the greater basicity of the fish tubulins is adaptively significant (see Section VI).

B. Primary Sequence of a Neural β Tubulin

To investigate the potential contribution of primary sequence variation to cold adaptation of microtubule assembly, we have initiated the cloning and sequence analysis of α- and β-tubulin cDNAs from the Antarctic rockcod *N. coriiceps neglecta*. As a first step, we have isolated and sequenced a 1.8-kilobase (kb) neural β-tubulin cDNA, Ncnβ1 (Detrich and Parker, 1993). This cDNA contains an open reading frame of 446 codons, 67 nucleotides of 5′ untranslated sequence, and 425 nucleotides of 3′ untranslated sequence beyond the stop codon (Figure 6).

The β tubulin encoded by Ncnβ1 (Figure 6) is most closely related to the neural β chains (classes I-IV) of other vertebrates (Sullivan, 1988; Wang et al., 1986; Monteiro and Cleveland, 1988). With respect to

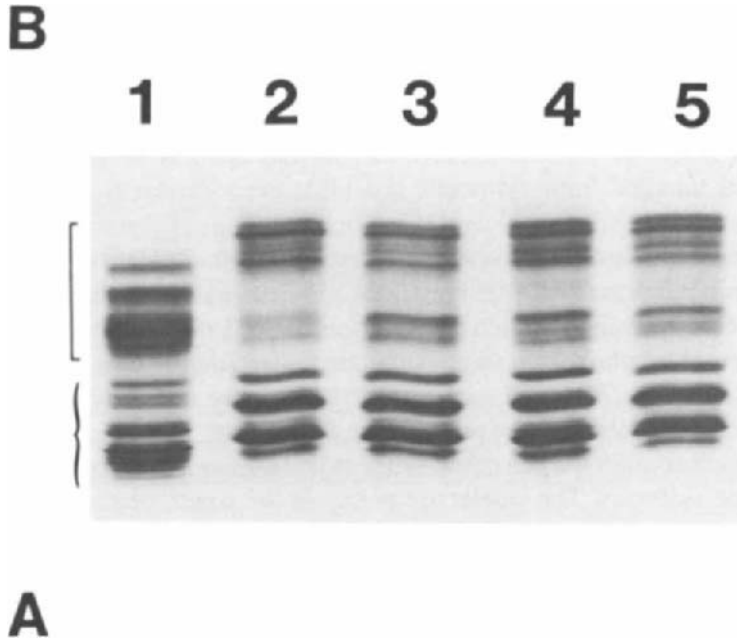


Figure 5. Heterogeneity of piscine and mammalian neural tubulins. Aliquots (25 μg) of tubulins from the cow (lane 1), from *N. gibberifrons* (lanes 2 and 3), from *N. coriiceps neglecta* (lane 4), and from *C. aceratus* (lane 5) were analyzed by high-resolution isoelectric focusing (cf. Detrich and Overton, 1986, 1988). The basic (B) and acidic (A) ends of the pH gradient are indicated. The square and curly brackets indicate the region of the gel occupied by the α and β tubulins, respectively. Reprinted from Detrich and Overton (1988) with kind permission from Pergamon Press Ltd.

β-chain isotypes of two homeotherms (mouse and chicken), the amino acid sequence homology of the Ncnβ1 polypeptide is 94.8% to class I, 95.0-95.7% to class II, 90.8% to class III, 95.1% to class IVa, 95.3-96.2% to class IVb, 90.7% to the nonneuronal class V, and 78.0-84.9% to the erythroid-specific class VI. The Ncnβ1 polypeptide is also strongly related (95.5%) to the class-II β tubulin of its close phylogenetic relative, the temperate amphibian *Xenopus laevis* (Good et al., 1989). Clearly, Ncnβ1 tubulin cannot be classified solely on the basis of overall primary sequence homology. However, joint consideration of nucleotide (not shown) and protein sequence homologies suggests that Ncnβ1 and its

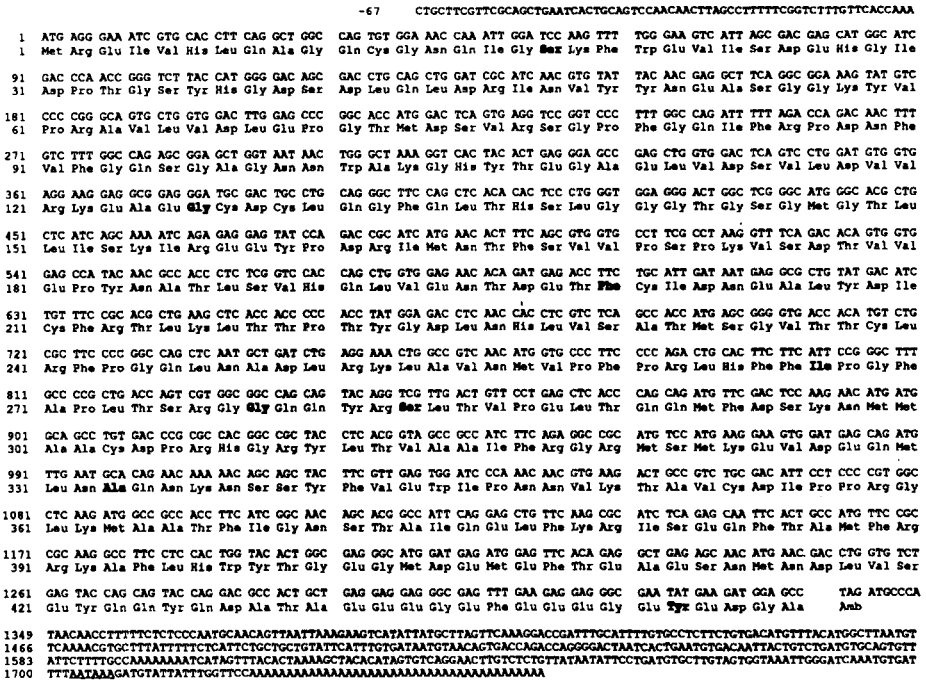


Figure 6. Nucleotide sequence of the Ncnβ1 cDNA and deduced primary sequence of the encoded β tubulin. Eight amino acid residues that differentiate Ncnβ1 uniquely from other vertebrate neural tubulins (classes I-IV) are shown in outline font. Nucleotide positions and amino-acid residues are numbered on the left. Amb indicates the amber translation termination codon, and the probable polyadenylylation signal is underlined. Reprinted from Detrich and Parker (1993) with permission from Wiley-Liss, In.

encoded β tubulin may be most parsimoniously assigned to the type II β-isotypic class (Detrich and Parker, 1993).

Of particular interest is the divergent carboxyl terminus (residues 431-446) of the Ncnβ1 chain (Figure 7). In higher vertebrates (e.g., mammals and birds), the carboxy-terminal region largely defines the six conserved isotypic classes of b tubulins (Sullivan, 1988). The carboxyl terminus of Ncnβ1, by contrast, violates this conservation; it is most closely, and almost equally, related to the carboxyl termini of two β-chain classes, II and IV. Comparison of conserved, coordinated residue replacements (Sullivan, 1988; Burns and Surridge, 1990) at other positions (residues 35, 57, 64, 170, 296, and 316) confirms the similarity of Ncnβ1 tubulin to both II and IVb (but not IVa) isotypes (Detrich and Parker, 1993). Therefore, interspecific conservation of β-tubulin isotypes, which is characteristic of higher vertebrates, may not apply to the more distantly related fishes.

Class	Organism	Tubulin	431	%Identity
I	mouse	Mβ5	E E E E D F G E E A E * E * E A	56
	chicken	cβ7	E E E E D F G E E A E * E * E A	56
II	mouse	Mβ2	D E Q G E F E E E E G * E D E A	62
	chicken	cβ1/2	D E Q G E F E E E G E * E D E A	75
	N. coriiceps	Ncnβ1	E E E G E F E E E G E Y E D G A	
IVb	mouse	Mβ3	E E E G E F E E E A E * E E V A	75
	chicken	cβ3	E E E G E F E E E A E * E E A E	69
IVa	mouse	Mβ4	E E * G E F E E E A E * E E V A	69
III	chicken	cβ4	E E E G E M Y E D D E E E S E Q G A K	58

Figure 7. Carboxy-terminal sequences of vertebrate neural β tubulins. Presented for comparison are the carboxy-terminal sequence of the Ncnβ1 polypeptide, beginning at position 431, and the corresponding isotype-defining sequences of neural β chains (classes I-IV) from higher vertebrates. Asterisks indicate single amino-acid gaps introduced to establish maximal sequence homology. For each pairwise comparison to the Ncnβ1 carboxyl terminus, sequence homology was calculated as percentage residue identity with respect to the longer sequence. For clarity in the presentation of classes I, II, and IV, the Ncnβ1/class-III alignment is shown in suboptimal register. However, the sequence homology value for the latter comparison (11 matches/19 residues × 100% = 58%) is calculated for its optimal alignment (i.e., single amino-acid gaps positioned after Ncnβ1 residues 435 and 437). β-isotype sequences for mouse and chicken are from Wang et al. (1986) and from Monteiro and Cleveland (1988), respectively. Reprinted from Detrich and Parker (1993) with permission from Wiley-Liss, Inc.

To identify potentially adaptive residue changes in Ncnβ1, we have compared its primary sequence to those of other β tubulins (Detrich and Parker, 1993). With respect to vertebrate β chains, the Ncnβ1 polypeptide contains several unique amino acid substitutions and an unusual, carboxy-terminal residue insertion (Figures 6 and 7). Three unique, but conservative, replacements are clustered near the center of the Ncnβ1 sequence (Ile for Met at position 267, Gly for Ser at 278, and Ser for Ala/Gly at 283). Furthermore, five of six total unique substitutions (including Ser for Ala/Thr at 18 and Gly for Ser/Asn/His at 126) are located in the amino-terminal two-thirds of the polypeptide. Replacement of tyrosine by phenylalanine at position 200, although conservative, is unusual in vertebrate β chains, where it occurs only in the erythroid-specific class VI. Substitution of alanine for a bulky aliphatic (Val/Ile) at position 333 is unique and nonconservative, and the tyrosine inserted at position 442 (Figures 6 and 7) is novel. We conclude that the Ncnβ1 chain is a class-II β isotype that contains several sequence changes of potential importance to its function at low temperature.

Multiple α- and β-tubulin isotypes are expressed in vertebrate brain (Sullivan, 1988), and we hypothesize that the cold-adapted phenotype of Antarctic fish tubulins results, at least in part, from a sequence variations common to some or all of these subunits. How many α- and β-tubulin isotypes are expressed in brain tissues of Antarctic fishes? Protein-chemical studies indicate that there are at least three distinct β chains. Direct sequencing of the β_1-electrophoretic variant[2] of neural tubulin from *N. coriiceps* neglecta (Figure 9a, lane 4) confirms the first 40 residue assignments of Ncnβ1 and reveals sequence heterogeneity at position 18, where both alanine and serine are detected (Detrich and Parker, 1993). Therefore, β_1 tubulin is composed of at least two isotypes, one of which corresponds to the cDNA described here. In addition, the neural β_2-electrophoretic species of Antarctic fish (Detrich et al., 1987) must correspond to the β_{III} genetic isotype (Banerjee et al., 1990). Preliminary evidence demonstrates comparable genetic heterogeneity of neural α tubulins from this fish (data not shown). Currently, we are pursuing cDNAs for all neural α and β tubulins so that we may catalogue the repertoire of primary sequence alterations of potential adaptive importance.

C. Posttranslational Modifications of Antarctic Fish Tubulins

The α tubulins of many organisms are subject to two posttranslational modifications, acetylation of lysine 40 and cyclic tyrosina-

tion/detyrosination at the carboxyl terminus (Sullivan, 1988). Acetylated and/or detyrosinated α chains are frequently found in subsets of microtubules that are stable to drug-induced disassembly (Sullivan, 1988; Bass and Black, 1990), but the connection appears not to be causal (Shulze et al., 1987; Khawaja et al., 1988; Webster et al., 1990). Using modification-specific antibodies (Gundersen et al., 1984; Shulze et al., 1987), we find that tyrosination of α tubulins from *N. gibberifrons* brain exceeds, and their acetylation equals, levels found in a chains from bovine brain (Skoufias et al., 1988). Thus, we infer that these two modifications are unlikely to contribute to cold adaptation of microtubule assembly in Antarctic fishes. Nevertheless, it remains plausible that unique posttranslational modifications of tubulin, as yet uncharacterized, possess adaptive significance.

IV. EGG AND BRAIN TUBULINS: EVALUATION OF THE MULTITUBULIN HYPOTHESIS

Two hypotheses have been advanced to explain the functional significance of multiple tubulin isoforms. The multitubulin hypothesis, originally outlined by Stephens (1975) and by Fulton and Simpson (1976), proposes that chemically distinct tubulins may differ in their polymerization properties or form microtubules with different functional characteristics. The regulatory hypothesis, presented by Raff (1984), argues that multiple tubulin genes, whose encoded isotypes are functionally interchangeable, have evolved unique regulatory sequences to place them under alternative programs of expression during development and differentiation. To test the multitubulin hypothesis, we have compared the polymerization efficiencies and structural features of tubulins from unfertilized eggs and from neural tissues of the Antarctic rockcod, *N. coriiceps neglecta* (Detrich et al., 1992).

A. Purification of Egg Tubulin

Our protocol for preparation of tubulin from eggs of *N. coriiceps neglecta*, which is based on that for brain tubulin, entails DEAE ion-exchange chromatography followed by two cycles of microtubule assembly *in vitro* (Detrich et al., 1992). Figure 8 shows an SDS-polyacrylamide gel containing protein fractions from the purification. Egg tubulin is removed selectively from a high-speed centrifugal extract

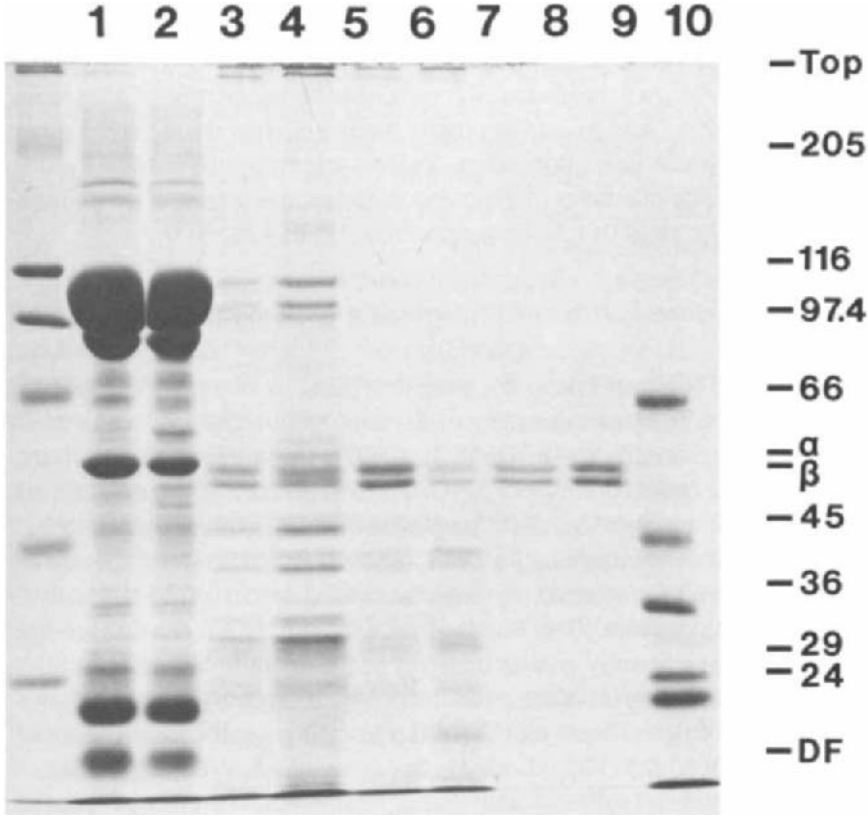


Figure 8. Purification of tubulin from eggs of the Antarctic fish, *N. coriiceps neglecta*. Tubulin was purified by DEAE ion-exchange chromatography and two cycles of microtubule assembly/disassembly *in vitro* (for details see Detrich et al., 1992). Protein fractions from the purification steps were examined by SDS-polyacrylamide gel electrophoresis on a 9% gel. Lanes: 1, high molecular weight standards; 2, egg high-speed supernatant (HSS); 3, proteins present in the non-bound fraction after application of the HSS to a column of DEAE-Sephacel; 4, tubulin-enriched fraction released from the DEAE-Sephacel column by high-ionic strength buffer; 5 and 6, supernatant and microtubule-containing pellet obtained from sample 4 following the first cycle of microtubule disassembly/assembly; 7, proteins removed from sample 6 by low-temperature centrifugation after depolymerization of microtubules; 8 and 9, supernatant and pellet obtained from clarified sample 6 after a second complete cycle of microtubule disassembly/assembly; 10, low molecular weight standards. The molecular weights of the standards (in thousands), the positions of the tubulin chains (α and β), and the top and dye front (DF) of the gel are indicated on the vertical axis. Reprinted from Detrich et al. (1992) with permission from the American Society for Biochemistry and Molecular Biology.

by the ion-exchange resin (compare lanes 2 (high speed supernatant) and 3 (unbound proteins)), is released from the resin by application of high ionic strength buffer (lane 4), and is progressively enriched during subsequent disassembly/assembly purification. Twice-cycled egg tubulin (lane 9) contains equimolar quantities of the α and β chains and is free of nontubulin proteins. The yield of tubulin ranges from 1.5 to 4 mg/100 g eggs.

B. Distinct Functional Properties of Egg and Brain Tubulins

We have compared the polymerization capacity of egg tubulin to that of brain tubulin by measurement of the temperature dependence of their critical concentrations (Detrich et al., 1992). Table 1 presents complete data sets for both tubulins. For *N. coriiceps neglecta* egg tubulin, critical concentrations range from 0.057 mg/ml at 3 °C to 0.0022 mg/ml at 19 °C. The critical concentrations for brain tubulins from the same species or from a second nototheniid, by contrast, are four- to 10-fold larger than those for egg tubulin. Thus, the intrinsic polymerization capacity of egg tubulin is significantly greater than that of the neural tubulin.

Van't Hoff analysis of the critical concentration data (Figure 4) shows clearly that egg and brain tubulins differ in their polymerization energetics (Detrich et al., 1992). Both the apparent standard enthalpy change (ΔH°_{app} = +33.9 kcal/mol) and the apparent standard entropy change (ΔS°_{app} = +151 eu) for elongation by egg tubulin exceed the values observed for brain tubulin (ΔH°_{app} = +26.5 kcal/mol, ΔS°_{app} = +121 eu) by approximately 25%. Thus, the polymerization of egg tubulin is under greater entropic control than are brain tubulins from the same, or related, species. We conclude that tubulins from two tissues of Antarctic fishes differ in their functional properties *in vitro*.

C. Structural Correlates

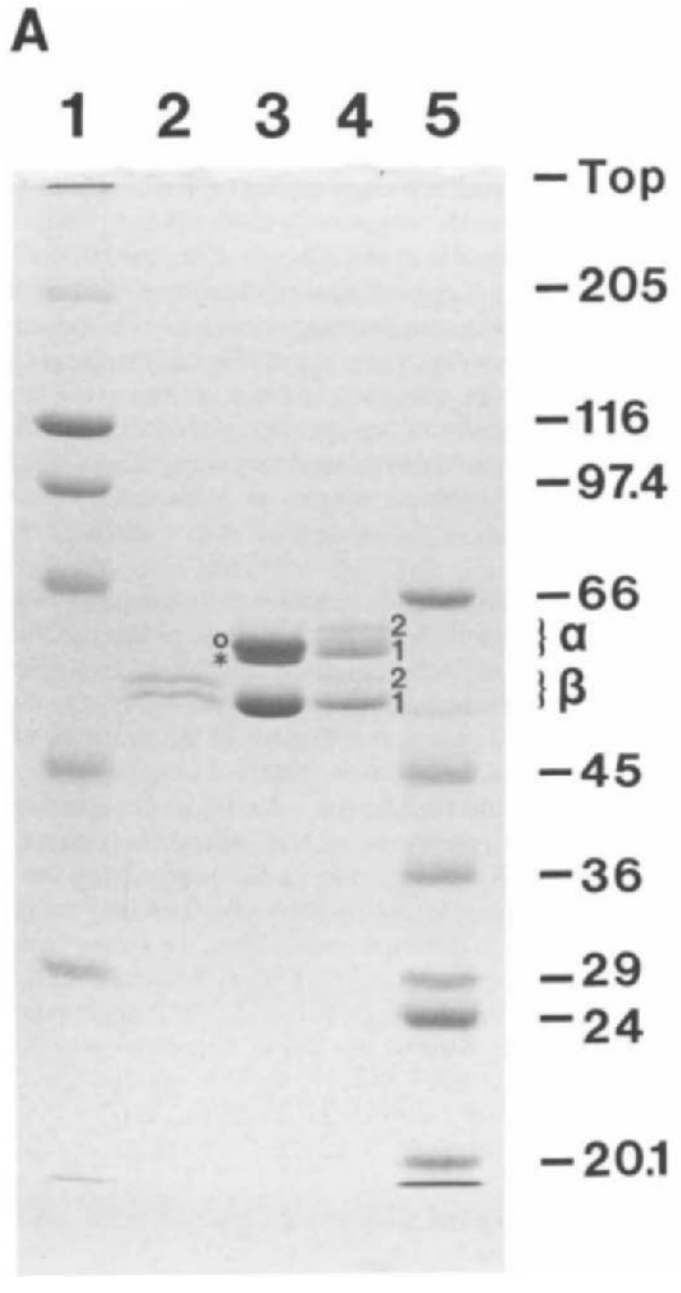
Our challenge now is to relate the distinct functional properties of egg and brain tubulins to specific structural features, either genetic or post-translational in origin, of their respective tubulin isoforms. As an initial step toward this goal, we have compared egg and brain tubulins electrophoretically and have generated peptide maps of their major α- and β-tubulin variants (Detrich et al., 1992).

Our first important observation is that egg tubulin contains fewer chemical variants than does brain tubulin. After reduction and car-

boxymethylation, brain tubulin from *N. coriiceps neglecta* migrates on SDS-polyacrylamide gels (Figure 9a, lane 4) as four components that differ in relative abundance and in electrophoretic mobility: α_1 (major), α_2 (minor), β_1 (major), and β_2 (minor) (Detrich et al., 1987, 1992). Identical treatment of egg tubulin (Figure 9a, lane 3) yields two prominent α-tubulin variants (α_{fast} and α_{slow}) with mobilities distinct from those of the brain α components. A β-chain species (operationally designated β_1) that comigrates precisely with brain β_1 tubulin is also present in egg tubulin, but the β_2 variant is absent (Detrich et al., 1992). Additional chemical heterogeneity of egg and brain tubulins is revealed by electrophoretic techniques (isoelectric focusing, two-dimensional electrophoresis) that possess greater resolving power (Figure 9b). Nevertheless, egg tubulin appears to be composed of but six α and two β variants, whereas brain tubulin isoforms (approximately 20 total) are much more diverse (Figures 5 and 9b). The low complexity of egg tubulin may reflect the expression of fewer tubulin isotypes in oocytes, the absence (or reduced activity) in eggs of posttranslational modification systems present in brain, or both.

Further insights emerge from comparative peptide mapping of tubulin subunits. Figure 10 presents isoelectric peptide maps of α tubulins from *N. coriiceps neglecta*, from the temperate dogfish, and from the cow. The patterns of tyrosine-containing peptides from the two egg α chains differ clearly from the peptide distributions of the neural α tubulins. Particularly striking is the apparent absence in egg α tubulins of a prominent subset of acidic peptides (pIs ~ 3.2-3.6, probably carboxy-terminal in origin) that are common to all brain α subunits. (Interpretative caution is warranted, however, due to the trivial possibility that egg α tubulins contain comparable acidic peptides that lack only the tyrosine required for detection in our experiment.) Thus, the greater basicity of the egg α chains (apparent pIs ~ 5.68-5.90 vs. 5.65-5.84 for α chains from *N. coriiceps neglecta* brain; Detrich et al., 1992) might result from reduction of the acidic character of a few of their component peptides. By contrast, β_1 tubulins from *N. coriiceps neglecta* eggs and from dogfish brain yield similar peptide patterns (data not shown), consistent with our previous observation that many β_1 tubulins share conserved structural features (Detrich et al., 1987).

We conclude that egg and brain tubulins from Antarctic fishes differ in their intrinsic polymerization capacities and therefore possess functional specificity *in vitro*. These functional distinctions correlate with structural differences between the α-tubulin isoforms of egg and brain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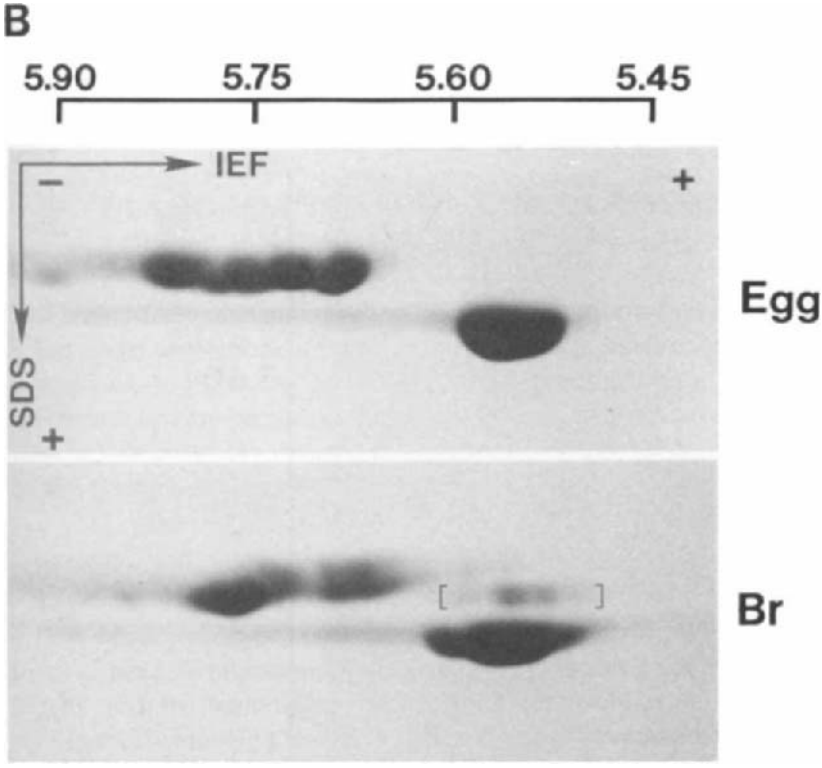

Figure 9. Heterogeneity of egg and brain tubulins from *N. coriiceps neglecta*. **A**, Analysis by SDS-polyacrylamide gel electrophoresis. Tubulins were reduced, carboxymethylated, and electrophoresed on an 8% gel (Detrich et al., 1992). Lanes: 1, high molecular weight standards; 2, unmodified egg tubulin; 3, carboxymethylated egg tubulin; 4, carboxymethylated brain tubulin; 5, low molecular weight standards. The molecular weights of the standards, the locations of carboxymethylated α- and β-tubulin variants, and the top of the gel are indicated on the vertical axis. The positions of brain α_1, α_2, β_1, and β_2 tubulins are indicated to the right of lane 4, and the locations of egg α_{fast} (asterisk) and α_{slow} (open circle) variants are noted to the left of lane 3. **B**, Two-dimensional electrophoretic analysis. Samples of egg tubulin (Egg, 15 μg) and of brain tubulin (Br, 20 μg) were focused in the first dimension, then electrophoresed in the second dimension (Detrich et al., 1992). Only the central regions of the second-dimension slab gels are shown, and the focusing (IEF) and electrophoretic (SDS) dimensions are indicated. The gels are aligned with respect to a common pH gradient (basic end on left), a portion of which is shown on the horizontal axis (top). Putative neural β_{III} isoforms are enclosed by the square brackets. Reprinted from Detrich et al. (1992) with permission from the American Society for Biochemistry and Molecular Biology.

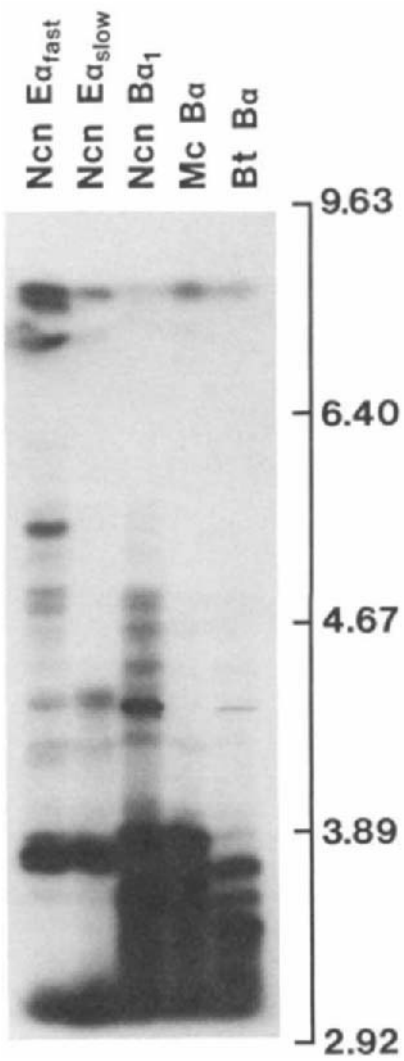

Figure 10. CNBr-generated peptide maps of α tubulins from an Antarctic fish, a temperate fish, and a mammal. Subunit preparations were digested by CNBr, and the resulting peptides were labeled with ^{125}I by the Chloramine T reaction, separated by isoelectric focusing, and visualized by autoradiography (Detrich et al., 1992). Samples: Ncn $E\alpha_{fast}$ and Ncn $E\alpha_{slow}$, α_{fast} and α_{slow} tubulins from *N. coriiceps neglecta* eggs; Ncn $B\alpha_1$, α_1 tubulin from *N. coriiceps neglecta* brain; Mc Bα, total brain α tubulin from the temperate dogfish, *Mustelus canis*; Bt Bα, total brain α tubulin from the cow, *Bos taurus*. The pH gradient of the focusing gel is shown on the vertical axis. Reprinted from Detrich et al. (1992) with permission from the American Society for Biochemistry and Molecular Biology.

and with compositional differences in their α- and β-subunit pools. Thus, our results provide strong evidence in support of the multitubulin hypothesis.

V. ROLE OF MAPS IN MICROTUBULE ASSEMBLY AT LOW TEMPERATURES

The brain MAPs of mammals promote microtubule formation at 30-37 °C when added to solutions of pure mammalian tubulin (Sloboda et al., 1976; Murphy et al., 1977). Do the MAPs of Antarctic fishes perform a similar function at low temperatures? To address this question, we have examined microtubule assembly systems reconstituted from pure fish tubulins and MAPs (Detrich et al., 1990; Detrich, 1991).

A. Purification of Brain MAPs

We have employed a modification (Detrich et al., 1990) of the taxol-dependent procedure of Vallee (1982) to isolate microtubules and MAPs from the brain tissues of Antarctic fishes. Figure 11 shows that microtubules prepared from *C. aceratus* (lane A) contain tubulin and a complement of presumptive MAPs (~15% of the total microtubule protein). Sedimentation of the associated proteins does not occur in the absence of taxol, nor do the proteins pellet from brain extracts that have been depleted of tubulin prior to incubation with taxol (Detrich et al., 1990). Therefore, these proteins are associated specifically with microtubules. Nearly quantitative displacement of the MAPs is obtained when the taxol-stabilized microtubules are exposed to elevated concentrations of NaCl; MAPs are recovered in the supernatant fraction (lane B) and tubulin in the microtubule pellet (lane C) following centrifugal resolution of the NaCl-treated sample. The yield of microtubule protein obtained by this method, ~1 mg/g of brain tissue, is identical to that reported by Vallee (1982) for calf brain cerebral cortex.

The protein compositions of brain MAP fractions from *N. coriiceps neglecta, N. gibberifrons, C. aceratus* (Figure 11, lane B), and other Antarctic fish are quite similar. The major MAP is a protein of unusually high apparent molecular weight (M_{ra} ~ 410,000-430,000, depending upon the fish species), but there are also clusters of proteins in the molecular weight ranges 220,000-270,000, 140,000-155,000, 85,000-95,000, 40,000-45,000, and 32,000-34,000 (Detrich et al., 1990). Some

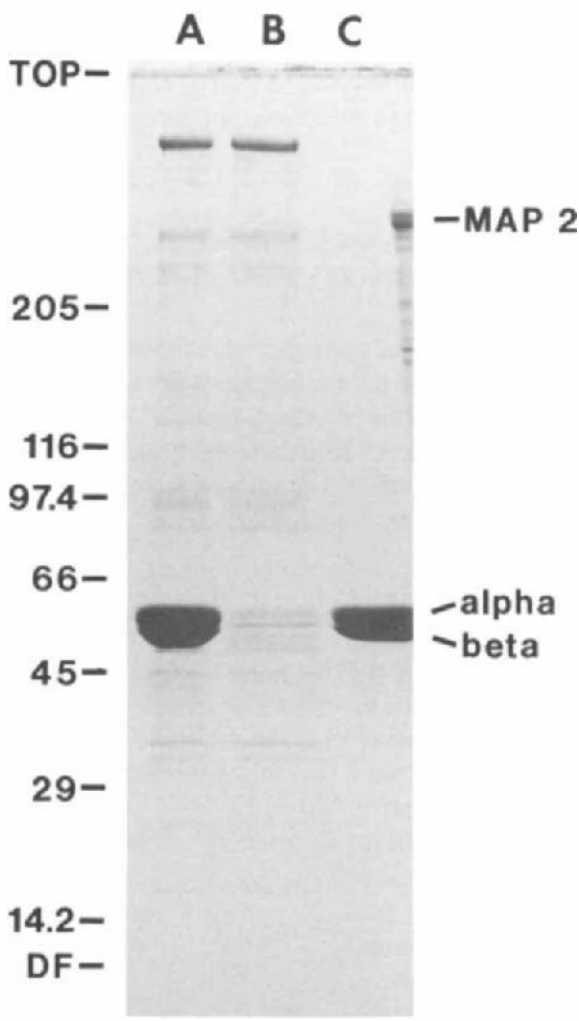

Figure 11. Taxol-dependent purification of microtubules and MAPs from brain tissues of the icefish C. *aceratus*. Samples from different steps of the purification were electrophoresed on an SDS urea-polyacrylamide gradient gel (4-16% acrylamide, 1-8 M urea) (Detrich et al., 1990). Samples: **A**, microtubules from taxol-dependent assembly; **B** and **C**, MAP-containing supernatant and tubulin-containing pellet, respectively, obtained by exposure of sample A to elevated ionic strength followed by centrifugal resolution. Electrophoretic migration was from top to bottom. The molecular weights of standards are given in thousands on the vertical axis, and the positions of the tubulin chains (α and β), bovine MAP 2 (M_{ra} ~ 275,000), the dye front (DF), and the top of the gel are indicated. Reprinted from Detrich (1991) with permission from Springer-Verlag, Berlin, Heidelberg.

of these proteins are probably functional homologues of the high molecular weight MAPs 1 and 2 (M_{ra} ~ 270,000-350,000), the tau proteins (M_{ra} ~ 55,000-62,000), and the low molecular weight MAPs (M_{ra} ~ 30,000-35,000) of vertebrate homeotherms (reviewed by Olmsted, 1986; Matus, 1988).

B. MAP-Mediated Assembly

Figure 12 shows the polymerization at 0 °C of *N. gibberifrons* brain tubulin in the presence of MAPs from *C. aceratus* (Detrich et al., 1990). Identical results are obtained for homospecific tubulin/MAP combinations (Detrich, 1991). Both the initial rate and the final extent of assembly increase with increasing concentrations of the MAPs (curves 3-7). Using thin-section and negative-stain electron microscopy, we found that the products of the assembly reactions are microtubules whose walls are decorated with numerous thin filamentous projections of ~25 nm. These microtubules depolymerize upon addition of the antimitotic drug podophyllotoxin (50 μM) or calcium ion (10 mM). At the subcritical concentration (0.67 mg/ml; cf. Table 1) employed in this experiment, tubulin alone fails to polymerize (curve 1), and a solution containing only MAPs develops little turbidity (curve 2). Clearly, some component or components of the MAP preparations is able to promote the assembly of microtubules from Antarctic fish tubulin at physiological temperatures.

To identify the assembly-stimulating MAPs, we collected MAP-induced microtubules (from an experiment with *C. aceratus* proteins) by sedimentation and examined their proteins by electrophoresis (Detrich et al., 1990). Seven proteins with apparent molecular weights between 60,000 and 430,000 cosediment with microtubules, albeit in small quantities. These proteins do not sediment from samples containing MAPs alone and therefore must be specifically attached to microtubules. We conclude that the seven cosedimenting proteins are bona fide MAPs that promote the formation of microtubules at low temperatures.

C. Polymerization of Heterologous Tubulin/MAP Combinations

To determine if the Antarctic fish MAPs contain significant adaptations that contribute to microtubule formation at low temperatures, we

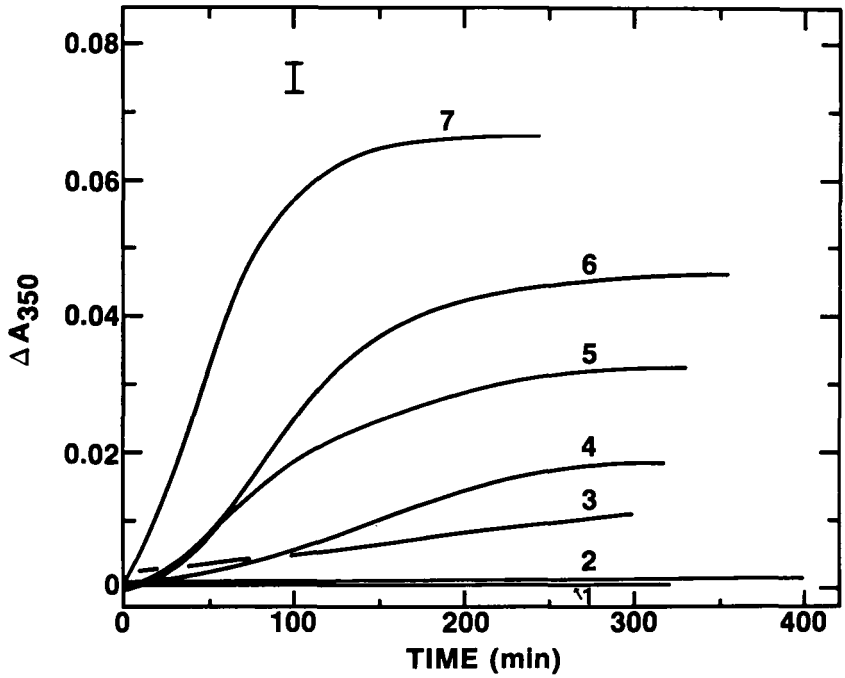

Figure 12. MAP-induced assembly of *N. gibberifrons* brain tubulin at 0 °C. Polymerization of tubulin (0.67 mg/ml) in the presence of increasing concentrations of MAPs from *C. aceratus* was monitored at 0 °C by turbidimetry (ΔA_{350}). Assembly was initiated at zero time by adding GTP (1 mM) to buffered solutions of the proteins lacking the nucleotide (see Detrich et al., 1990). Samples: 1, *N. gibberifrons* tubulin alone, 0.67 mg/ml; 2, *C. aceratus* MAPs alone, 0.17 mg/ml; 3-7, tubulin (0.67 mg/ml) containing MAPs at final concentrations of 0.07, 0.12, 0.18, 0.24, and 0.45 mg/ml, respectively. The bar in the upper left-hand corner indicates the approximate extent of experimental uncertainty in these results. Reprinted from Detrich et al. (1990) with permission from Wiley-Liss, Inc.

have examined the polymerization of heterologous combinations of tubulins and MAPs from these fishes and from mammals. We were surprised to find that bovine MAPs, when added to subcritical concentrations of *N. gibberifrons* tubulin, also stimulate the assembly of microtubules at 2 °C (Detrich et al., 1990). On a weight basis, both fish and bovine MAPs induce equivalent extents of microtubule formation at low temperatures. Bovine proteins corresponding to MAP 2A, MAP 2B, a MAP 1 species, and several proteins with lower molecular weights (probably including some tau proteins) are incorporated into the hybrid microtubules. Therefore,

mammalian MAPs, like the MAPs of Antarctic fishes, are able to promote microtubule assembly at temperatures near 0 °C. Reciprocal combinations containing bovine tubulin and Antarctic fish MAPs, by contrast, polymerize only at the elevated temperatures (33 °C) necessary for assembly of homologous MAP/tubulin systems from mammals. Furthermore, this assembly is cold-reversible, which demonstrates that the fish MAPs do not stabilize microtubules by the end-capping mechanism of mammalian stable tubule only polypeptides (STOP; Margolis et al., 1986). We conclude that MAPs from the cold-adapted fishes and from the mammal function similarly at both low and high temperatures. The MAPs of antarctic fish do not possess *special* capacities to promote microtubule assembly at low temperatures or to prevent cold-induced microtubule disassembly.

VI. DISCUSSION

In their pioneering study, Williams et al. (1985) proposed that cold-living ectotherms have overcome the destabilizing effects of cold temperatures on microtubule assembly through evolution of modified tubulins that possess large association constants. Their results, and our own, provide strong support for this hypothesis. We find that pure tubulins from two tissues of Antartic fishes from microtubules efficiently at temperatures as low as 0 °C. Piscine MAPs further enhance the assembly reaction at physiological temperature, but mammalian MAPs can be substituted with equivalent effect. For Antarctic fishes, then, cold adaptation of microtubule assembly must largely reflect modifications to the tubulin dimer. Such modifications are likely to include unique changes in primary sequence and/or in posttranslational modification, examples of which may be inferred from our structural studies.

A. Functional Adaptation of Antarctic Fish Tubulins

For both brain and egg tubulins of Antarctic fishes, polymerization is driven strongly by increases in system entropy (see also Williams et al., 1985). Their respective standard enthalpies (+26.5 and +33.9 kcal/mol) and entropies (+121 and +151 eu) are significantly larger than those found for pure tubulins from organisms with higher body temperatures (see Detrich et al., 1989 for a complete discussion). Microtubule elongation by porcine brain tubulin, for example, is characterized by an

enthalpy change of +6.3 kcal/mol and an entropy change of +44.4 eu (Robinson and Engelborghs, 1982), whereas sea urchin egg tubulin yields estimates of ΔH°_{app} ~ +11-16 kcal/mol and ΔS°_{app} ~ +61-79 eu (Detrich et al., 1989). Although comparisons are limited, the energetic trend suggests that entropic control of microtubule assembly increases with decreasing body temperature. Greater entropic control over, and the minimal salt perturbation of, the polymerization of Antarctic fish tubulins suggest that cold adaptation of microtubule assembly is based, at least in part, on an increased dependence on hydrophobic interactions relative to other bond types. Cold adaptation of actin filament formation, by contrast, reflects a greater reliance on polar bonds (Swezey and Somero, 1982). Thus, at least two adaptive strategies conserve cytoskeletal polymer formation at low temperatures (Detrich, 1991).

One important adaptive consequence of the interspecific differences in polymerization thermodynamics of tubulins is that they conserve the critical concentration for microtubule assembly. Measured *in vitro at physiologically relevant temperatures*, critical concentrations for tubulins from cold-adapted Antarctic fishes, from several temperate invertebrates (sea urchins, clams), and from mammals cluster in the range 0.36-2.5 mg/ml (Herzog and Weber, 1977; Suprenant and Rebhun, 1983, 1984; Detrich et al., 1985, 1989; Williams et al., 1985). Thus, organisms from disparate thermal regimes are able to form microtubules efficiently within the normal limits of their body temperatures.

B. Structural Determinants of Tubulin Polymerization at Low Temperatures

What are the molecular interactions and structural features that enable the tubulins of Antarctic fishes, unlike those of mammals, to polymerize efficiently at cold temperatures? In general, functional adaptation might result from an increased reliance on polymer-stabilizing interactions, from a reduction in destabilizing interactions, or from both. Constituting the former category, we suggest, are primary sequence changes that increase the hydrophobicity of tubulin domains involved in interdimer contact. In the latter we place reduction of electrostatic repulsion between tubulin dimers, mediated most likely by posttranslational mechanisms. We consider each proposal in the context of our current understanding of the three-dimensional organization of the tubulin dimer.

To a first approximation, each monomer of the native tubulin $\alpha\beta$ heterodimer folds to give large amino-terminal and smaller carboxy-ter-

minal globular domains (roughly two-thirds and one-third of each subunit, respectively).[3] The globular domains are linked by an exposed, protease-sensitive connecting segment (Mandelkow et al., 1985; Sackett and Wolff, 1986), and each monomer is terminated by a short, extended, and highly acidic "tail" (Sackett and Wolff, 1986). The strongest of the *inter*dimer contacts that drive microtubule assembly occur longitudinally between adjacent tubulin dimers within a protofilament (Erickson and Pantaloni, 1981) and are formed by the small carboxy-terminal domain of α tubulin and the large amino-terminal domain of the β subunit (Kirchner and Mandelkow, 1985). It is in these domains that we hypothesize that polymer-stabilizing interactions have been enhanced by increases in contact-surface hydrophobicity. (Lateral contacts between tubulin dimers of adjacent protofilaments also contribute to microtubule formation but, because structural remodeling of these regions appears not to be required for microtubule cold adaptation (Skoufias et al., 1992), they will not be considered here.) Opposing the favorable longitudinal contacts are the negatively charged carboxy-terminal tails, which project into the solvent (Breitling and Little, 1986; Sackett and Wolff, 1986) and are thought to generate electrostatic repulsion between tubulin dimers (Serrano et al., 1984; Bhattacharyya et al., 1985; Sackett et al., 1985). Thus, charge reduction in the tails, if present, should also be functionally adaptive.

Does the primary sequence of Ncnβ1 tubulin suggest adaptation via increased hydrophobicity? Most of the unique residue replacements (five of six) that we have detected in Ncnβ1 tubulin are found in the amino-terminal domain that participates in longitudinal interdimer contacts. It is noteworthy that the clustered triplet of substitutions (Ile^{267}, Gly^{278}, and Ser^{283}) is contiguous to β-chain residues (positions 241-256) that become occluded upon polymerization (Arévalo et al., 1990). Thus, we propose that these three residue positions (267, 278, and 283) define a portion of the surfaces that form longitudinal interdimer bonds. However, no clear physicochemical pattern emerges from consideration of the residue substitutions and insertions present in the Ncnβ1 sequence. A slight tendency towards increased polarity (e.g., Ser for Ala at position 18, Ser for Ala/Gly at 283, Tyr insertion at 442) is offset by reciprocal changes (Gly for Ser at 126, 278). Phe^{200}, Ile^{267}, and Ala^{333} increase, maintain, and decrease, respectively, the hydrophobicity of the Ncnβ1 chain relative to other vertebrate β isotypes. Perhaps the key to functional interpretation of the sequence alterations of Ncnβ1 resides in coordinated substitutions/insertions located in the complementary carboxy-terminal globular domain of α tubulin. Thus, our hydrophobicity-enhancement

hypothesis remains provisional and must be tested further by structural analysis of other α- and β-tubulin isotypes from Antarctic fishes.

We have previously suggested that reduced electrostatic repulsion between the tubulin dimers of Antarctic fishes, mediated by a decrease in the acidic residue content of the carboxy-terminal tails of the α and β chains, might contribute to microtubule formation at cold temperatures (Detrich and Overton, 1986, 1988; Detrich et al., 1987). However, the Ncnβ1 polypeptide does not, relative to vertebrate isotypes I-IV, contain reduced numbers of glutamyl and/or aspartyl residues in the primary sequence of its carboxyl terminus. Thus, charge reduction, if operative as an adaptive mechanism for this class-II β chain (and perhaps other neural tubulins), most likely would involve a decrease in the extent of posttranslational polyglutamylation (cf. Alexander et al., 1991; Rüdiger et al., 1992) of its carboxy-terminal tail. Alternatively, the charge status of other α- and/or β-tubulin isotypes may be of greater adaptive significance. Partial elimination of interdimer repulsion would be expected to unmask, or strengthen, the hydrophobic interactions that dominate microtubule assembly, thereby contributing to the increased entropic control over polymerization that is demonstrated by the tubulins of Antarctic fishes. Clearly, experimental manipulation of the carboxy-terminal charge status of Antarctic fish tubulins, coupled with quantitative assessment of the polymerization capacities of the products, should be pursued to evaluate conclusively the role of charge reduction in cold adaptation of microtubule assembly.

C. Functional Specificity of Egg and Brain Tubulins

Many tubulin isotypes copolymerize indiscriminately, in either normal or unusual cellular contexts, to yield functional microtubules (reviewed by Joshi and Cleveland, 1990; Murphy, 1991; Detrich et al., 1992; Ludueña, 1993). Such observations have fostered the view that tubulin isotypes are, in general, functionally interchangeable. Our comparison of egg and brain tubulins from *N. coriiceps neglecta*, by contrast, provides a compelling demonstration that distinct, singlet-forming tubulins from a single species can differ in their polymerization capacities. We suggest that the greater polymerization efficiency of egg tubulin may result from reduced charge-charge repulsion conferred by decreased (or absent) carboxy-terminal polyglutamylation (Detrich et al., 1992). Consistent with this possibility, the egg α subunits apparently lack several acidic peptides that are prevalent in brain α chains and probably repre-

sent glutamylated derivatives of their carboxyl termini. However, compositional differences in the α- and β-isotypic pools of egg and brain may also contribute to functional specialization.

A second notable example of functional specificity involves tissue-specific β-tubulin isotypes from the chicken. Murphy and his colleagues have shown that differences in the kinetics and energetics of microtubule formation by brain and erythrocyte tubulins of the chicken are attributable to their divergent β subunits (Rothwell et al., 1986; Murphy et al., 1987). The erythrocyte β isotype is both more basic and more hydrophobic than are the β tubulins of brain (Murphy et al., 1987). Thus, Murphy's results and our own (Detrich et al., 1989, 1992) argue strongly that the function of the tubulin dimer may be adjusted to specific cellular requirements by alteration of subunit charge and hydrophobicity.

D. MAPs and Cold-Stable Microtubules

Antarctic fishes also possess MAPs that might contribute to the energetics of microtubule formation at low temperatures. Using a microtubule affinity protocol, we have isolated MAPs from fish brain that resemble neural MAPs from other vertebrates. Upon addition to subcritical concentrations of fish tubulin at 0 °C, the fish MAPs enhance both the initial rate and the final extent of assembly in a concentration-dependent fashion. Thus, Antarctic fish MAPs promote microtubule formation by the same mechanisms that operate for mammalian MAPs at higher temperatures: they stimulate microtubule nucleation and enhance elongation by stabilizing the formed polymer (Murphy et al., 1977). It is unlikely, however, that the fish MAPs possess a *special* capacity to promote microtubule assembly at cold temperatures. Bovine MAPs, at concentrations comparable to the fish MAPs, are equally effective in stimulating the polymerization of fish tubulin at 0 °C, and fish MAPs enhance microtubule formation by bovine tubulin at high temperature (33 °C). This functional interchangeability suggests that mammalian and fish MAPs have similar microtubule-binding domains and occupy similar sites on the microtubule lattice.

Of the proteins comprising the MAP fractions of Antarctic fishes, the most prominent possesses an unusually large apparent molecular weight (M_{ra} ~ 400,000). Although MAPs of comparable size are not present in mammalian or avian neural tissues, Strömberg et al. (1989) recently found a large heat-labile MAP (M_{ra} ~ 400,000), as well as a heat-labile

MAP 2 cognate, in neural microtubule preparations from a northern cold-adapted fish, the Atlantic cod *Gadus morhua*. We suggest that the largest MAPs of the austral and boreal fishes are structurally, and perhaps functionally, homologous. It is surprising, therefore, that the cod MAP fraction demonstrates microtubule-binding activity, promotes the formation of microtubules by bovine tubulin at 30 °C, yet fails to stimulate polymerization of cod tubulin at like temperature (Fridén et al., 1992). Thus, the apparent functional nonequivalence of Antarctic rockcod and Atlantic cod MAPs is an important issue that remains to be resolved.

E. Concluding Remarks

The coastal fishes of the Antarctic diverged from temperate fishes some 40 million years ago as the southern ocean began to cool (DeWitt, 1971). Subjected to selection by an increasingly unfavorable thermal environment, the microtubule proteins of the southern fishes evolved adaptations that preserve efficient polymerization at low temperatures. Our results, *in toto*, argue strongly that tubulins, rather than MAPs, are the major loci of functional adaptation. Furthermore, our structural analyses suggest that these adaptations are likely to include changes both in the primary sequences of the α and β tubulins and in their posttranslational modifications. Although these changes are likely to be small, they should contribute to an understanding of the structural domains and features of the tubulin dimer that control microtubule formation in all eukaryotes.

VII. SUMMARY

Fishes native to the coastal waters of the Antarctic have adapted to habitat and body temperatures in the range -1.8 to +2 °C. Their cytoplasmic microtubules, unlike those of mammals and temperate poikilotherms, have evolved to assemble efficiently at these low temperatures. To learn about the underlying molecular adaptations, my laboratory is studying microtubule proteins (tubulin αβ dimers and microtubule-associated proteins (MAPs)) and tubulin genes from several Antarctic fishes, including the rockcods *Notothenia coriiceps neglecta* and *N. gibberifrons*, and the icefish *Chaenocephalus aceratus*. We find that the assembly-enhancing adaptations of the fish microtubule proteins reside largely, if not exclusively, in the tubulin subunits. Furthermore, microtubule formation

by Antarctic fish tubulins is strongly entropy-driven, due apparently to an increased reliance, relative to tubulins from other species, on hydrophobic interactions at sites of dimer-dimer contact. Based on analyses of tubulin polypeptides and cDNAs, we suggest that the structural adaptations of Antarctic fish tubulins are likely to include both alterations in the primary sequences of tubulin isotypes and reductions in the acidity of their carboxyl termini (probably due to decreased posttranslational polyglutamylation). With respect to neural β tubulins from other vertebrates, for example, the class II β-tubulin isotype of *N. coriiceps neglecta* brain contains seven unique amino acid substitutions and one novel insertion in its 446-residue primary sequence, and many of these changes are located in a structural domain that forms contacts between tubulin dimers during microtubule assembly. We also find that brain and egg tubulins from Antarctic fishes differ strikingly in their polymerization efficiencies, which demonstrates, in agreement with the multitubulin hypothesis, that tissue-specific tubulin isoforms can possess distinct functional properties. Finally, we show that neural MAPs from Antarctic fishes and from mammals function interchangeably in promoting microtubule assembly at low (or high) temperatures. Thus, Antarctic fish MAPs appear not to be loci for cold adaptation of microtubule assembly. We conclude that study of microtubule proteins from organisms, such as the Antarctic fishes, that have adapted to extreme thermal regimes should contribute significantly to an understanding of the quaternary interactions that control microtubule assembly in all eukaryotes.

ACKNOWLEDGMENTS

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NOTES

1. In this review, the term *isoforms* encompasses both the genetic variants (*isotypes*) encoded by the vertebrate multitubulin gene family (Sullivan, 1988) and their posttranslational derivatives.

2. Arabic subscripts appended to tubulin chain types (α or β) denote electrophoretic variants resolved by SDS polyacrylamide gel electrophoresis (see Detrich et al., 1987 for protein nomenclature). Roman subscripts identify distinct genetic isotypes encoded by the vertebrate α- and β-tubulin gene families (Sullivan, 1988). Each electrophoretic variant contains one or more of the genetic isotypes.

3. The large amino-terminal domain may be further divided into two equal subdomains (de la Viña, 1988).

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SURVIVAL OF MAMMALIAN CELLS EXPOSED TO PURE HYPOTHERMIA IN CULTURE

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I. INTRODUCTION

Hypothermia is employed in heart and brain surgery and in the preservation of human organs to be used for transplantation. Hence, most experiments involving hypothermia are either at the whole animal or organ level. In both cases, the hypothermia is usually accompanied by previous ischemia or anoxia. Unfortunately, the complex factors involved with research on whole organs allow little opportunity for separation of variables which influence the success of the preservation. These variables include optimum temperature, hypertonic perfusion, perfusion pressure and flow rate, pH and pO_2 of perfusate, previous episodes of warm ischemia, possible hypoxia during perfusion, heparin, and drugs to combat rejection (if endpoint is survival of transplant). Some of the above variables also change as a function of time. Furthermore, in the transplant field, two vastly different methods of preservation are used: Method A) hypothermic perfusion; and Method B, cold ischemia. The former usually involves an episode of warm ischemia (during removal of organ or death of donor) followed by perfusion during the storage time using an oxygenated medium containing some energy source. The latter involves filling the organ with some solution and storing in the cold *without* perfusion during the storage time, that is, simultaneous hypoxia and hypothermia.

In tissue culture (let's make this Method C for ease of reference), separation of many of the above variables can be done relatively easily and inexpensively using single cells. Our system, which consists of cells in monolayer culture in plastic flasks, simulates a perfusion system during the hypothermic exposure in that oxygen and nutrients are available in excess and the ratio of total cell volume to total medium volume is such that "wastes" are highly diluted. Cell hypoxia does not occur during the hypothermic exposure time in this system. More importantly, the cells have not been exposed to previous hypoxia. Hence, we are *only* observing the effects of pure hypothermia on cells. Furthermore, the assay we use measures reproductive integrity, that is, clonogenic ability,

of the cells. This is usually the most sensitive criterion of any type of damage to the cells.

Many mechanisms for "hypothermic cell killing" have been proposed. These include disruption of calcium homeostasis; activation of proteases, phospholipases, or other calcium dependent enzymes, inhibition of adenosine nucleotide translocase activity; suppression of calcium ATPases; cell swelling; and membrane instability. Some of these have been reviewed by Pegg (1981), Fuller (1987), Fuller et al. (1988), and Southard and Belzer (1993). However, in many, but not all, of these cases the experiments have involved whole organs, tissue slices and other systems where previous or simultaneous ischemia or hypoxia was also involved. Unfortunately, the mechanisms of cell killing by *pure hypothermia* are not fully understood.

There are obvious difficulties with comparing results from hypothermic perfusion (Method A), cold ischemia (Method B), and tissue culture (Method C). Using one or another of these methods, investigators have suppressed cell swelling (Lindell et al., 1989, Method A; Sundberg et al., 1991, Method B); developed, tested, and compared various perfusion and cold ischemic (static) solutions (Busza et al., 1989; De Mel et al., 1990; Jamieson et al., 1988; all Method B); inhibited phospholipases (Cotterill et al., 1990, Method B; Das et al., 1991, Method A); blocked calcium ion entry (Jacobsson et al., 1992; Pickford et al., 1990; both Method B); inhibited lipid peroxidation (Pickford et al., 1990, Method B; Gower et al., 1989, Method B; Umeshita et al., 1988, Methods A and C); inhibited free radical formation (Pickford et al., 1990, Method B; Gower et al., 1989, Method B; Umeshita et al., 1988, Methods A and C; Fuller et al., 1988); used membrane stabilizers (Saunder et al., 1993, Methods B and C; Mangino et al., 1993, Method B; Sundberg et al., 1988, Method A; Lindell et al., 1989, Method A); improved regeneration of ATP (McAnulty et al., 1988, Method A); suppressed glutathione loss (Vreugdenhil et al., 1991, Method B; Umeshita et al., 1988, Methods A and C); added calcium (Lindell et al., 1989, Method A; Marsh et al., 1990, Methods A and C; Umeshita et al., 1988, Methods A and C) and amino acids (Marsh et al., 1990, Methods A and C); and searched for an optimum pH (Fox et al., 1989, Methods B and C). This is by no means an exhaustive list and has been compiled using only two journals.

Various assays have also been used by different researchers to assess organ damage. Some of these have been reviewed by Pegg (1989) and Southard (1989). The most popular assays, in no particular order, are: ATP/ADP levels (Busza et al., 1989, Method B; Toffa et al., 1991,

Method B; McAnulty et al., 1988, Method A), cell swelling or tissue edema (De Mel et al., 1990, Method B; McAnulty et al., 1988, Method A; Sundberg et al., 1991, Method B; Lindell et al., 1989, Method A; Marsh et al., 1990, Methods A and C), enzyme release (e.g., aspartate or alanine aminotransferase, lactate dehydrogenase, creatine kinase; Jamieson et al., 1988, Method B; Saunder et al., 1993, Methods B and C; Sundberg et al., 1988, Method A; Sundberg et al., 1991, Method B; Das et al., 1991, Method A; Fox et al., 1989, Methods B and C; Lindell et al., 1989, Method A; Marsh et al., 1990, Methods A and C), regeneration of ATP (Saunder et al., 1993, Methods B and C), mitochondrial function (Saunder et al., 1993, Methods B and C;, Marsh et al., 1990, Methods A and C), lipid peroxidation (Pickford et al., 1990, Method B; Gower et al., 1989, Method B; Das et al., 1991, Method A; Umeshita et al., 1988, Methods A and C), release of free fatty acids (Cotterill et al., 1990, Method B), lactate content (Busza et al., 1989, Method B; De Mel et al., 1990, Method B), release of long-chain activated fatty acids (Mangino et al., 1993, Method B), gluconeogenesis (De Mel et al., 1990, Method B), arterial blood flow (Toffa et al., 1991, Method B; Jacobsson et al., 1992, Method B; Das et al., 1991, Method A), bile production (liver; Jamieson et al., 1988, Method B; Sundberg et al., 1988, Method A), tubular reabsorption of glucose and sodium (kidney; Toffa et al., 1991, Method B), glomerular filtration (kidney; Toffa et al., 1991, Method B), active uptake of para aminohippurate (kidney; De Mel et al., 1990, Method B), and functional assessment after transplant (Pickford et al., 1990, Method B; Okouchi et al., 1991, Method B).

If the difficulty of comparing results when researchers are using different assays is not hard enough, let us consider more complications.

1. Results suggest that there are specific sites of injury in the kidney (Toffa et al., 1991, Method B).
2. The various assays used for organs aren't always indicative of true damage. For example, in the liver, bile production, rather than release of various enzymes (Sundberg et al., 1988, Method A), is the most sensitive index of damage (Jamieson et al., 1988, Method B; Sundberg et al., 1988, Method A). For the kidney, gluconeogenesis may be the most informative assay (De Mel et al., 1990, Method B). Lipid peroxidation bears little relevance to the functional outcome in lung (Pickford et al., 1990, Method B).
3. Despite the dogma that media with high potassium concentrations are more protective than ordinary media with sodium as the major

cation in preserving tissues, when assessed by liver transplant, no difference is observed.

4. Furthermore, different organs from different animal species are used.
5. Finally, researchers, using either perfusion or cold ischemia preservation, employ a range of temperatures for storage of organs. Hence, researchers have used rat kidneys (De Mel et al., 1990, Method B, 0 °C; Jacobsson et al., 1992, Method B, 4 °C), rabbit kidneys (Cotterill et al., 1990, Method B, 0 °C; Toffa et al., 1991, Method B, 0 °C; Gower et al., 1989, Method B, 0 °C; Vreugdenhil et al., 1991, Method B, 5 °C; Bennet et al., 1987, Method A, 8 °C; Bennet et al., 1987, Method B, 0 °C), dog kidneys (Mangino et al., 1993, Method B, 4 °C; McAnulty et al., 1988, Method A, 6 °C), rat livers (Okouchi et al., 1991, Method B, 4 °C; Busza et al., 1989, Method B, 0 °C; Sundberg et al., 1991, Method B, 0 °C), rabbit livers (Jamieson et al., 1988, Method B, 0 °C, Sundberg et al., 1988, Method A, 5 °C; Vreugdenhil et al., 1991, Method B, 5 °C; Sundberg et al., 1991, Method B, 0 °C; Lindell et al., 1989, Method A, 5 °C), rat lung (Pickford et al., 1990, Method B, 0 °C), rabbit heart (Vreugdenhil et al., 1991, Method B, 5 °C), rabbit leg (Das et al., 1991, Method A, 0 °C), dog renal tubules (Saunder et al., 1993, Methods B and C, 4 °C), and rat hepatocytes (Fox et al., 1989, Methods B and C, 4 °C; Marsh et al., 1990, Methods A and C, 5 °C; Umeshita et al., 1988, Methods A and C, 0 °C).

It is no wonder that the mechanisms of cell killing by *pure hypothermia* are not fully understood.

Hypothermia will first be discussed here in terms applicable to any stress, then will be treated in terms of its specific features. The exposure of a cell to stress and the assessment of its final survival involve several distinct phases. The first of these is the pre-exposure period (Phase I), where *sublethal* damage from a stress (e.g., hypoxia), different from the eventual experimental stress (e.g., hypothermia), could be accumulated. The sublethal damage from the experimental stress during the second event, the exposure period (Phase II), may or may not be additive or interact with the sublethal damage from Phase I depending on the stresses involved. A number of protective agents may minimize the sublethal and lethal damage during Phase II. Furthermore, the possibility of repair of sublethal damage *during* this relatively long phase, in competion with

accumulation of damage, also exists. Some lethal damage may also manifest itself *during* Phase II. Restricting our discussion to hypothermia, a wealth of literature has accumulated dealing with events in this second phase (Karow and Pegg, 1981; Pegg et al., 1982; Belzer and Southard, 1988; Southard et al., 1990). Furthermore, there is also a third phase, the postexposure period (Phase III), where the stress from Phase II is removed. In whole organ systems, Phase III corresponds to the reperfusion phase (whether *in vitro* or *in vivo*).

In general, many cells destined to die due to exposure to some type of stress during Phase II, nevertheless remain intact and seem viable, as assessed by many criteria including some vital stains, for long periods of time *after* the application of the stress has ceased (Elkind and Whitmore, 1967b; Frim et al., 1978; Kruuv et al., 1984), that is, they still seem viable in Phase III. However, in the case of radiation, if the Phase III conditions are changed, cells, which under normal conditions (i.e., pH 7.4, full nutrients, 37 °C) would die, are sometimes able to repair potentially lethal damage (PLD) and, hence, survive (Little, 1971; Hetzel et al., 1976). A series of such events may also occur after hypothermic storage during Phase III.

Let us now restrict our discussion to hypothermic exposures. First, there is the temperature shock in Phase I in going from 37 °C to the storage temperature. Second, a number of events can occur during Phase II. The shape of the survival curves as a function of hypothermic storage time implies that sublethal damage is accumulated until such damage becomes lethal (Nelson et al., 1971; Kruuv et al., 1983). However, it is not known whether this lethal damage manifests itself during Phase II or during or after the eventual return to normothermia (Phase III). It *is* known that a brief period of normothermia between hypothermic exposures will allow the cells to repair sublethal damage and, hence, increase their survival compared with cells which have had the same total time of exposure to hypothermia but with no intervening time at normothermia (Kruuv et al., 1972).

II. CELL SURVIVAL CURVES AND PARAMETERS

The surviving fraction of cells is determined as a function of "cold dose" (or time at a particular temperature) by assessing the colony-forming ability of single cells at the end of the exposure time. Most of the hypothermia data, whether survival or some other functional assay, that

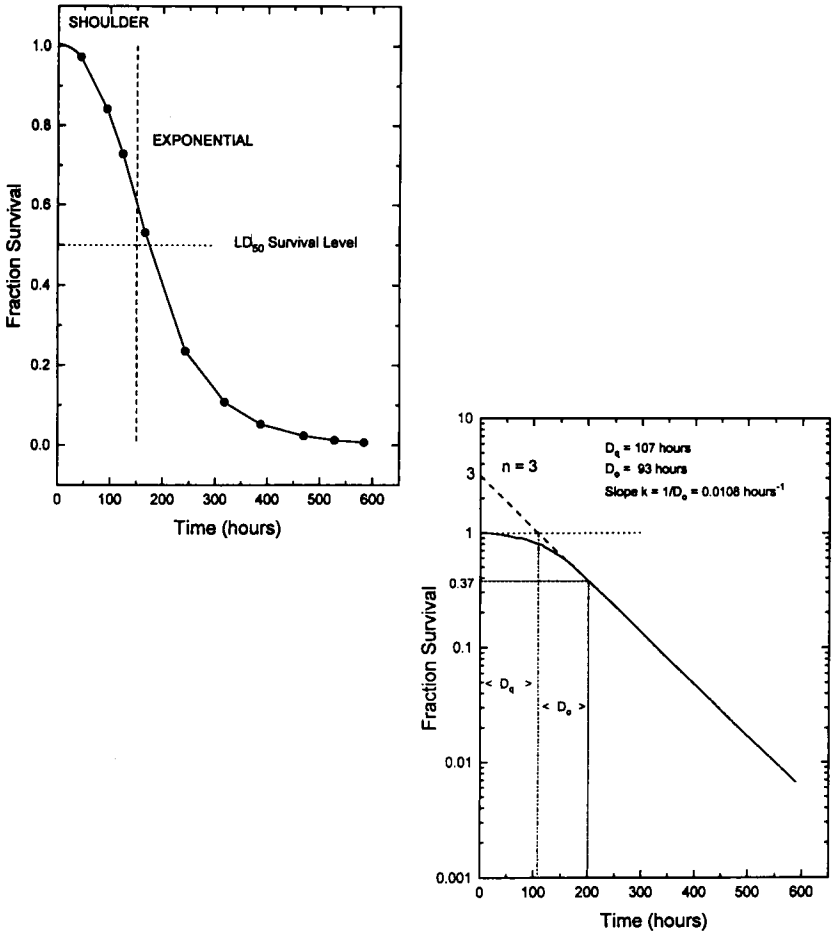

Figure 1. (A) Survival curve for a linear plot as described in the text. The data used is from the 10 °C curve in Figure 2. (B) The survival curve from (A) has been transformed into a semilogarithmic plot. Survival curve parameters are described in the text. The data used is from the 10 °C curve in Figure 2.

we or others have collected has a reverse sigmoid shape when linearly plotted as a function of time (Figure 1a). When plotted semilogarithmically (Figure 1b), the function at longer timès transforms as a straight line with a "shoulder region" at shorter times. The cell survival curve, hence, consists of a semilogarithmic plot of fraction survival versus cold

dose. In accord with standard terminology (Elkind and Whitmore, 1967a), the survival curve can be represented by two numbers, n and D_o. Extrapolation of the linear portion of the curve to the ordinate of a semilogarithmic plot will yield the Y-intercept, which in survival curve terminology is the extrapolation or target number, n. The D_o is defined as the time or cold dose required to reduce the survival by a factor of 1/e on the log-linear portion of the semilog graph. The D_o is inversely related to the cold-sensitivity of the cells. The D_o is also inversely related to the slope, k, of the log-linear portion; k is really the cell inactivation rate and, hence, can be used to generate Arrhenius plots. The ratio of slopes can be used to obtain a protection factor, independent of time of preservation, which can be used to compare results between researchers. If the curve has a shoulder, the size of the shoulder is a quantitative estimate of the amount of sublethal damage a cell can accumulate before this damage becomes lethal. This size is given by the parameter, D_q, the quasithreshold "dose," which can be found by extrapolation of the linear portion of this curve to the point where it meets the horizontal line drawn through 100% survival (i.e., fraction survival = 1). Alternatively, $D_q = D_o \times \ln(n)$. These parameters are shown in graphical form in Figure 1b.

III. PHASE II: THE HYPOTHERMIA EXPOSURE PERIOD

A consideration of events in Phase II illustrates why it is necessary to perform experiments at two different temperatures. We have previously shown (Nelson et al., 1971; Kruuv et al., 1983) that hypothermia survival curves of single cells are classical survival curves having a shoulder region, where sublethal damage is accumulated, followed by an exponential region (Figures 2a and 2b). It can be seen that survival is progressively improved as the storage temperature is lowered between 25 and 10 °C (Figure 2b). However, as the storage temperature is lowered below 10 °C, the survival becomes progressively worse (Figure 2a). The Arrhenius plot of inactivation (killing) rates of V79 Chinese hamster cells exposed to hypothermia (Figure 3) contains a (extrapolated) "break" at approximately 7-8 °C, which should correspond to the minimum inactivation rate (Kruuv et al., 1983). This implies that the mechanisms of hypothermic damage are distinctly different above and below 7 °C. (We will interchangeably use 7 *or* 8 °C for this transition throughout this chapter.) However, the change in survival as a function of tempera-

ture is not equal on both sides of the transition. Cell survival at 10 °C is considerably higher than at 5 °C. While the actual temperature ranges given above may turn out to be slightly different in various tissues and species (see Figures 4a, 4b, and 4c), one would hope that the *mechanisms* of killing will not vary substantially in nucleated mammalian cells. The reasons for this will be discussed in the general conclusions. For the V79 line that we are using, the optimum storage temperature is 10 °C. (Storage at the transition temperature of 7-8 °C does not improve survival (Kruuv et al., 1983) and is not as reproducible as that at 10 °C. This instability is a general feature *right at* membrane lipid phase transitions (discussed below), whether permeability (Rule et al., 1980) or growth rates are measured (Kruuv et al., 1983).

A. Temperature Range I (8-25 °C)

In Range I, the damage mechanism has an inactivation rate which conforms to the Arrhenius equation (k increases exponentially with temperature); the activation energy (E_a) for control cells is about 15 kcal/mole (Kruuv et al., 1983). Presumably this represents the Ea for inactivation of the critical target for hypothermic cell killing. This value is in the range of temperature coefficients of metabolic processes (10-30 kcal/mole) and much less than that for protein denaturation. This suggests that some aspect of metabolism, or unbalance of metabolism (e.g., producing a toxic intermediate which cannot be removed fast enough), is the rate limiting step for hypothermic killing in Range I.

Cell death due to cold exposures in Range I is also proportional to the temperature-time integral of exposure (i.e., total amount of metabolism) suggesting either the accumulation to a critical level of a toxic product or the depletion of a vital and necessary metabolite; cell death below 8 °C does not fit this integral (Nelson et al., 1971) suggesting that a different killing mechanism is involved.

To date, citiolone, a membrane permeable radical scavenger, is the only agent that protects in Range I (Kruuv et al., 1993a). Citiolone, in addition to scavenging of OH° radicals (Aloj Totaro et al., 1985), has the ability to increase superoxide dismutase (SOD) activity (Papaccio et al., 1986). Whether or not this contributes to its protective effect in unknown as we have only added *external* SOD (i.e., to the medium), with no effect (Zieger et al., 1991) in our experiments. When dimethylthiourea (DMTU), another free radical scavenger, is combined with citiolone, a

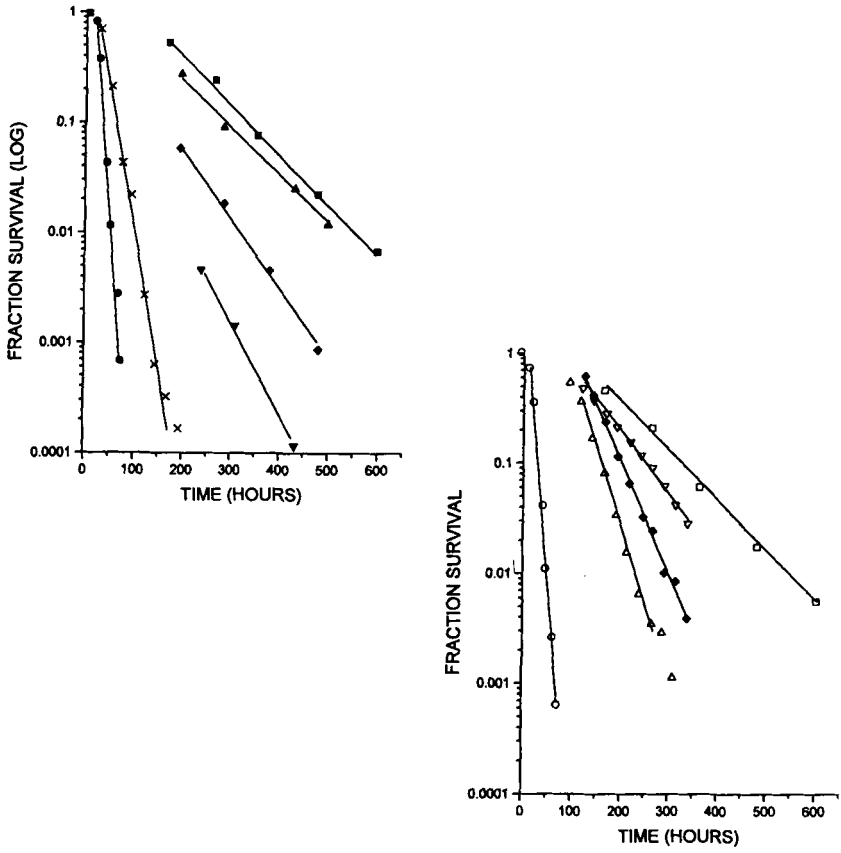

Figure 2. (A) Survival curves of asynchronous attached V79 cells exposed to various temperatures between 0 and 10 °C as a function of time. The standard error bars, which have been left out for clarity, were never any larger than twice the size of the symbol as plotted. The symbols represent data points at 0 °C (circles), 2.5 °C (crosses), 3.5 °C (inverted triangles), 5 °C (diamonds), 7 °C (upright triangles), and 10 °C (squares). (B) Survival curves of asynchronous attached V79 cells exposed to various temperatures between 10 and 25 °C as a function of time. The 0 °C (circles) and 10 °C (squares) curves from Figure 2a are included for comparative purposes. The other symbols represent data points at 15 °C (inverted triangles), 20 °C (diamonds), and 25 °C (upright triangles).

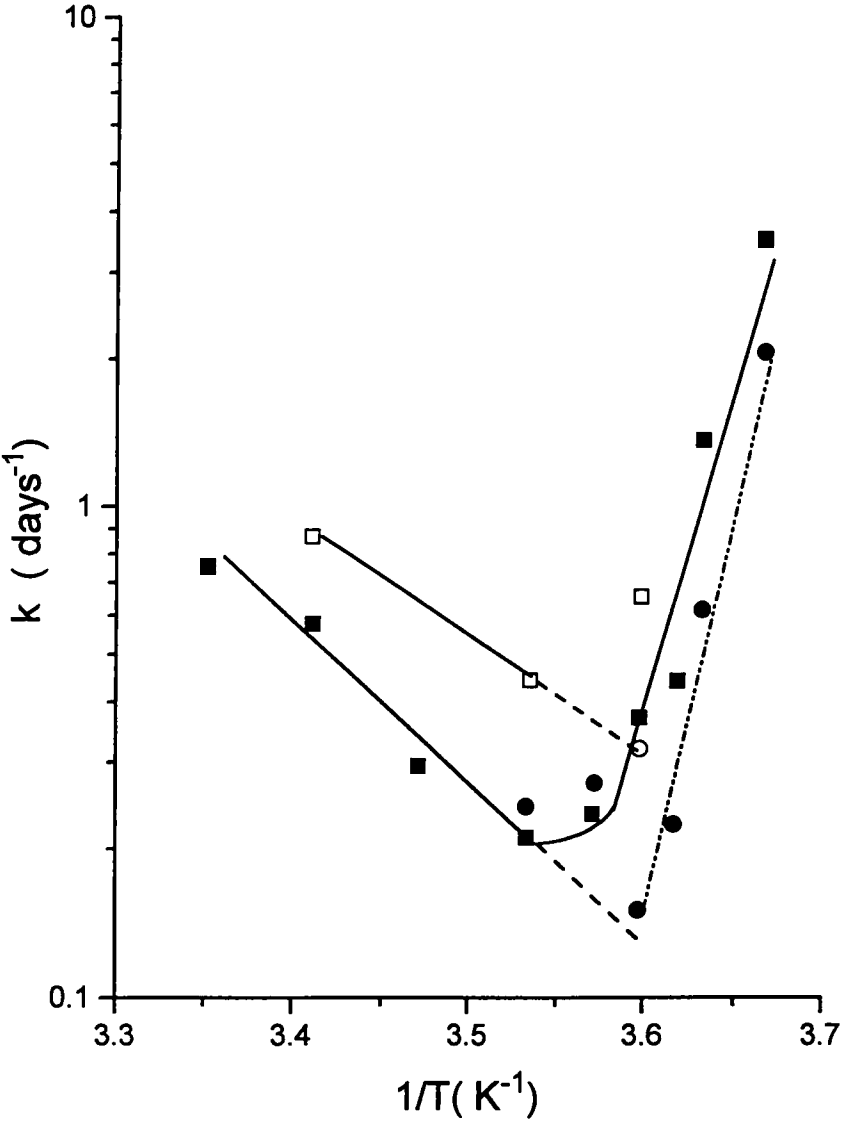

Figure 3. Arrhenius plot of the cell inactivation rate constant for V79 cells that are exposed to various temperatures while attached to plastic plates (closed symbols) or in suspension (open symbols) with (circles) or without (squares) the presence of 0.1 mM BHT. The inactivation rates for the control cells were derived from the final slopes of the curves in Figure 2.

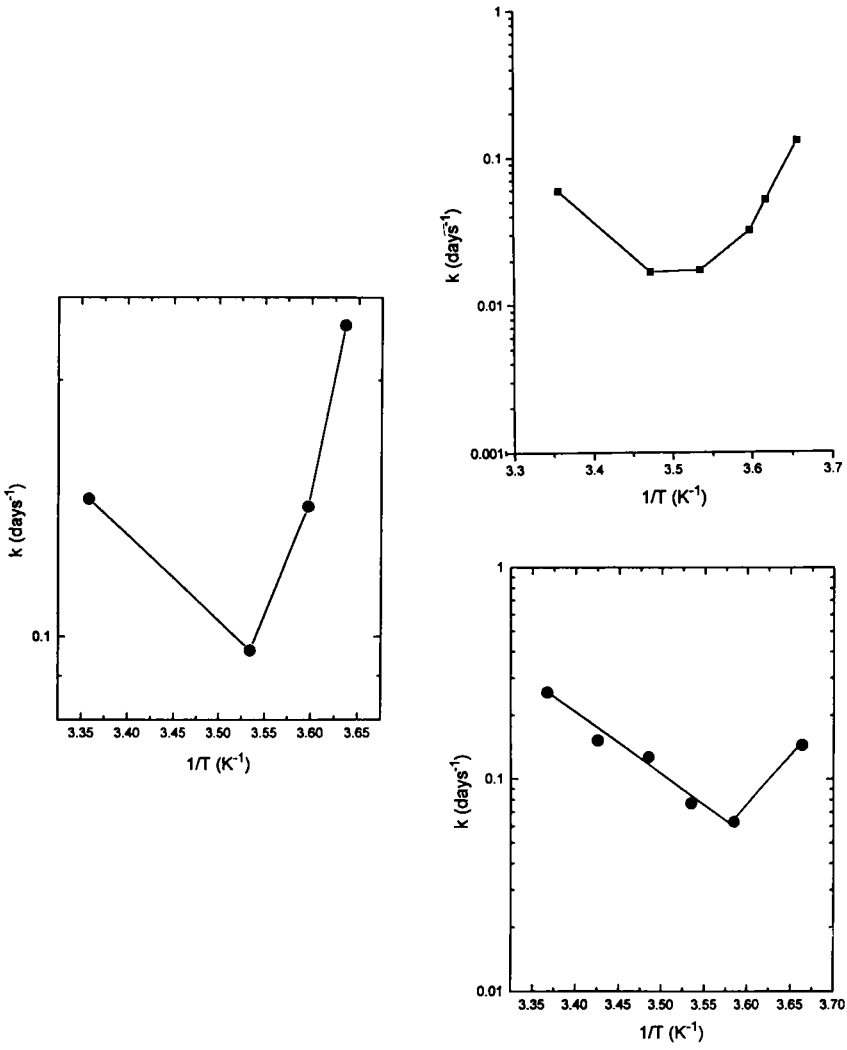

Figure 4. (A) Arrhenius plot of the cell inactivation rate constant for L-929 cells that are exposed to various temperatures while attached to plastic plates (Kruuv et al., 1995). Since there are relatively few data points, the minimum in the curve could still possibly shift to the left, or right. (B) Arrhenius plot of the rate of hemoglobin leakage of human red blood cells that are exposed to various temperatures for up to 48 days (Kruuv et al., 1995). (C) Arrhenius plot of the cell inactivation rate constant for mouse neuroblastoma cells that are exposed to various temperatures while attached. This graph was obtained by plotting the data from van Dongen et al., 1985 in an Arrhenius fashion.

synergistic effect on protection is observed in Range I (Kruuv et al., 1993a). DMTU, by itself, does not protect in Range I.

B. Temperature Range II (0-8 °C)

In Range II, we see the peculiarity of an "apparent negative" activation energy, and progressively greater inactivation rates at lower temperatures. The only way an apparent negative activation energy can be obtained is from a two-step process or mechanism (Muench et al., 1996); an example would be the presence of a transition which changes the nature of the reactants. We have previously given evidence that one of these steps, probably the initial step, involves a membrane lipid phase transition (Kruuv et al., 1983). These phase transitions will be discussed further in General Discussion and Conclusions. (V79 Chinese hamster cells, according to these lipid phase transitions and other data (Young, 1986), behave like mammalian cells from non-hibernators rather than hibernators. Chinese hamsters are not obligatory hibernators.) E_a for control cells is approximately -61 kcal/mole (Kruuv et al., 1983). This is a high value, indicating a strong temperature dependence and implying the involvement of a transition or macromolecular conformation change of some sort. Another possibility besides a lipid transition is that hypothermic killing in Range II is caused by the cold denaturation of a critical protein. Most, if not all, proteins undergo cold denaturation, some above 0 °C (Privalov, 1990). The protection demonstrated by the membrane lipid perturbers, butylated hydroxytoluene (BHT) and adamantanone, in Range II (Rule et al., 1978; Kruuv et al., 1983) suggests if cold denaturation of a protein is the rate-limiting step in Range II, the protein involved is a membrane protein. It should be noted that while BHT has some antioxidant activity, adamantanone does not. Finally, Hansen and Carpenter (1993) have recently shown that hypothermic survival in hemopoietic cell lines correlates with phospholipid membrane phase transitions.

Damage in temperature Range II is associated with formation of gel phase lipid, while damage in Range I is less well understood. For example, BHT protects cells against hypothermic damage in Range II but not in Range (Rule et al., 1978; Kruuv et al., 1983). Also, glycine and DMTU protect in Range II, but not in Range I (Kruuv et al., 1993a).

DMTU, which is highly permeable to cell membranes (Fox, 1984), may have functions other than OH° scavenging (Collins et al., 1979; Wasil et al., 1987) that contribute to its protective effect at 5 °C and synergistic effect with citiolone at 10 °C (Range I). There is no synergism

between DMTU and citiolone at 5 °C. DMTU scavenges HOCl, a powerful oxidant produced by biological systems (Wasil et al., 1987). Citiolone, by itself, does not protect in Range II.

Glycine protected isolated hepatocytes exposed to cold (5 °C) *ischemia* (Marsh et al., 1991); the mechanism of action is unknown. In our case, with pure hypothermia, some interaction of glycine with membranes is implied in Range II, since glycine is ineffective in Range I.

It is significant that our survival experiments on the mouse L-929 cell line (Kruuv et al., 1995) also indicate that there is a break in the Arrhenius plot of cell inactivation (Figure 4a). This is also true for leakage of hemoglobin from human red blood cells (Figure 4b) when plotted in Arrhenius fashion and survival of human lung cells (Kruuv et al., 1995). Furthermore, replotting the cell survival data of van Dongen et al. (1985) for mouse neuroblastoma cells in an Arrhenius graph (Figure 4c) also results in a break at around 6 °C. Hence, a break in the Arrhenius plot of cell inactivation due to hypothermia is not unique to Chinese hamster V79 cells. However, since the break in the above four Arrhenius plots occurred at slightly different temperatures, one should expect some tissue-and cell-specific differences. Also, these Ranges may be different, even in the same cell line, when comparing pure hypothermia with combined hypothermia plus hypoxia, that is, cold ischemia. Other events occurring in Phase II, such as cell swelling and lysing, will be discussed later in the chapter.

C. Summary

The above also further confirms that the hypothermic damage is different in Ranges I and II. Glycine, DMTU, and BHT all protect in Range II, but not in Range I (Kruuv et al., 1983, 1993a). Citiolone protects in Range I but not Range II (Kruuv et al., 1993a). There is synergism in protection between citiolone and DMTU in Range I, but not Range II. Furthermore, cells swell at 5 °C but not at 10 °C (Kruuv et al., 1984a).

The mechanisms of cell killing by *pure hypothermia* are not fully understood but could involve some damage by free radicals, since citiolone and DMTU both protected against hypothermic killing. However, we can not rule out that the agents that were protective in our studies affected some other mechanisms, that is, the cytoprotective effects observed could be due to events unrelated to radical formation. For example, we will see in a later section that the OH° radical scavenger, dimethyl sulfoxide (DMSO), was ineffective as a protector.

IV. PHASE III: (THE POSTHYPOTHERMIA EXPOSURE PERIOD)

One of the best ways of measuring the cells' ultimate reproductive integrity after exposure to some stress, such as radiation or cold, is to use the colony formation assay developed by Puck and Marcus (1955). However, by the time cells form colonies they will have repaired much of the potentially lethal damage present as well as most of the sublethal damage. In other situations, it is important to know when such damage has occurred or when cells have ceased to have the ability to survive. Visual observations usually do not suffice since eventual nonsurvivors of many stresses remain unchanged in appearance for long periods of time.

One example of the above is survival after freeze-thaw damage. Does the lethal damage occur during freezing or thawing? In our case, does lethal damage occur during hypothermic storage, that is, during Phase II or during the return to normothermia (Phase III)? We attempted to answer this question by monitoring the hypothermic damage by analyzing cell and microsome sizing distributions and comparing them with earlier studies on cell survival as measured by the colony formation assay (Kruuv et al., 1984b). The results help to answer some of the questions raised above. The initial temperature shock in shifting from hypo- to normothermia, even with the rather gentle temperature-time profile used, caused some cell lysis (Kruuv et al., 1984b).

Storage at 10 °C is the easiest to analyze since the most data exists for this case. Addition of BHT to cells in suspension in spinner flasks not only helps prevent lysis due to the initial temperature shock in going from normo- to hypothermia, but also minimizes lysing during the subsequent 10 °C (Phase II) storage (Kruuv et al., 1984b). However, since the final survival as assessed by the colony formation technique is the same for 10 °C exposure whether BHT is present or not (Kruuv et al., 1983), it implies that the lysing of cells containing BHT is greater after return to normothermia (Phase III) than in cells without BHT. The latter may not be surprising since BHT greatly increases the membrane fluidity of these (Law et al., 1986) and other (Rule et al., 1979) cells at 37 °C. Nevertheless, for cells without BHT in the 10 °C case, the majority of the lysing still takes place after the cells are returned to normothermia (Phase III). Whether these results apply to attached cells or cells in organs is not known. There is no doubt, however, that the mechanical forces on the cells in spinner flasks are bound to influence survival since it is well

established that attached cells survive hypothermic storage much better than cells in suspension in spinner flasks at any given temperature (Hetzel et al., 1973; Rule et al., 1978).

In the case of 1.5-2.5 and 5 °C preservation, the addition of BHT prior to hypothermic exposure has little or no effect on lysing as a function of hypothermic storage time. However, it is known that the fraction of surviving colonies is greatly enhanced by 0.1 mM BHT at these temperatures (Rule et al., 1978; Kruuv et al., 1983). Hence, considerably more cell lysing must be taking place after the return to normothermia (Phase III) in the case of cells without than with BHT in these experiments. It is of interest that glycine (Saunder et al., 1993), after cold ischemic storage of organs, reduces enzyme leakage in Phase III, but has no effect on ATP or mitochondrial function.

Using the colony formation assay, the survival of cells without BHT is such that, in the temperature range used in these experiments, 10 °C gives the optimum survival followed, in turn, by 5 and 1.5-2.5 °C (Kruuv et al., 1983). Since lysing as a function of (Phase II) storage time increases as the temperature is changed from 5 to 10 to 1.5-2.5 °C, but 10 °C storage results in higher ultimate survival (colony formation) than 5 °C storage, the results suggest that Phase III lysing is greater in the 5 °C case than in the 10 °C experiments. This finding may have clinical implications as some types of post-hypothermic damage to organs may be difficult to manage and/or monitor in patients.

Summary of Phase III: Chinese hamster V79 cells exposed to hypothermia as a function of (Phase II) time lysed at at a higher rate at 5 than at 10 °C. Addition of the membrane lipid perturber BHT changed the lysing rate during 10°C (Phase II) storage. The results imply that posthypothermic (Phase III) lysing at 37 °C is greater after 5 than after 10 °C storage and that a substantial fraction of cell lysing takes place during the Phase III period, sometimes larger than during the hypothermic storage period itself.

V. PHASE I: THE PREHYPOTHERMIA EXPOSURE PERIOD

First, there is the temperature shock in the preexposure period in going from 37 °C to the storage temperature. If the temperature-time profile of this step is steep enough, this event in itself can contribute to some cell lysis and death even when very brief hypothermic exposures and isosmotic solutions are used (Raaphorst et al., 1981; Kruuv et al., 1984b). In

most mammalian spermatozoa, this coldshock causes irreversible damage and may be due to lipid phase transitions in membranes (Drobnis et al., 1993). However, this can be prevented by hydrophobic aromatic compounds such as BHT (Bamba and Miyagawa, 1992; Graham and Hammerstedt, 1992). Second , this period may include warm ischemia damage in the case of procuring organs for transplant. This damage, as in the case of temperature shock, may be lethal or sublethal. Third, cellular resistance or sensitivity to stress in Phase II may be induced by certain procedures or sublethal stresses in Phase I. Pretreatment by hypothermic (25 °C) cycling (PHC) of attached exponential-phase V79 Chinese hamster cells by Method 4 (24 hours at 25 °C + 1.5 hours at 37 °C + 24 hours at 25 °C + trypsin + 3 hours at 37 °C) or Method 3 (48 hours at 25 °C + trypsin + 3 hours at 37 °C) make mammalian V79 cells significantly more resistant to 43 °C hyperthermia (Glofcheski et al., 1993). There is no significant difference in the 43 °C survival curves whether Method 3 or 4 is used for pre-exposure; this implies that the total time at 25 °C is the important factor in inducing resistance rather than the 1.5 hour interval at 37 °C between 24 hour exposures to 25 °C. However, if pre-exposure is at 15 or 10 °C, the resistance to hyperthermia is reduced. PHC by Method 4 increases survival of cells exposed to 5 °C (Range II) and, to a lesser extent, to 10 °C (Range I; Glofcheski et al., 1993). Cell survival is a function of position in the cell cycle (discussed later), however, the increase in hyper- and hypothermic survival following PHC can not be accounted for by changes in cell cycle distribution. Heat shock protein synthesis is not induced by PHC. When cells are made tolerant to hyperthermia by a pretreatment in 2% DMSO for 24 hours at 37 °C, the cells are *not* more resistant to subsequent exposures to hypothermia, either at 5 or 10 °C. The results imply that there may be two mechanisms of inducing resistance to hyperthermia, only one of which also confers resistance to hypothermia (Glofcheski et al., 1993).

As discussed above, not only is the resistance induced by PHC temperature dependent (Glofcheski et al., 1993), but it also may be time dependent in that shorter times (e.g., 16 hours PHC at 25 °C) may produce sensitization instead of protection (Glofcheski and Kruuv, unpublished observations). Furthermore, both dependencies may be different in other cell lines.

It is of interest that hibernating animals cycle their body temperature during hibernation, that is, cycles at 5 °C interrupted by periods at 37 °C in some mammalian species. Prior to hibernation, there are cycles at approximately 15 °C interrupted by 37 °C intervals (Wang 1989). The function of the PHC in animals is not known, but may be required to

preserve the animal during hibernation. It is likely that the sublethal damage accumulated at 5 and 15 °C is repaired during brief exposure to 37 °C and/or, perhaps, the cells develop protection against damage that will be induced at the lower temperatures (Willis et al., 1975).

VI. OTHER FACTORS

A. Cell Cycle and Stationary (G_0) Phases

The variation of hypothermic sensitivity within the cell cycle is known for Chinese hamster cells, viz., S is sensitive, G_2 and M phases are intermediate in sensitivity, and G_1 is least sensitive (Nelson and Kruuv, 1972). This is also true for hypothermic sensitivity of mouse neuroblastoma cell (van Dongen et al., 1985). This rank order of sensitivities is similar to the cell cycle age response curve to hyperthermia for Chinese hamster cells (Westra and Dewey, 1971; Raaphorst et al., 1985) but complementary (i.e., reversed) to that for gamma irradiation (Sinclair and Morton, 1966) and for freeze-thaw damage (McGann et al., 1972) for these same cells. Hence, the arguments for effects of cell cycle redistribution on asynchronous cell responses will be the same for hyper- and hypothermia. The survival response of asynchronous cells to any stress can be calculated if the cell cycle distribution and cell cycle responses are known (Sinclair and Morton, 1965). Any procedure which changes the cell cycle distribution will change the asynchronous survival response.

There have been relatively few survival curves published for mammalian cells in tissue culture exposed to hypothermia in the range of 0 to 15 °C (Nelson et al., 1971; Matsumura et al., 1973; Kruuv et al., 1983; van Dongen et al., 1985). All of the studies have used exponential-phase cells. However, most of the cells in the body or in an organ are in a stationary (G_0) phase. In tissue culture, this phase can, at least partially, be simulated by using plateau-phase cells. It would be of practical interest to know how G_0 cells behave in order to better compare results of cells in tissue culture with those of whole organs.

A shoulder in a cell survival curve indicates that the cells have the ability to accumulate sublethal damage before this damage becomes lethal. That is, since survival is still close to 100% after a certain amount of stress (dose) has been applied, the damage accumulated up to that point must be sublethal. (This type of curve is seen for most mammalian

cells whether the stress is radiation, cold, heat or toxic drugs.) When the cell is saturated with sublethal damage, the lesions result in lethality. The D_q can be used as a measure of this saturation point. Radiation biologists have treated this type of curve from a statistical viewpoint (Elkind and Whitmore, 1967a). From a biochemical viewpoint, the size of the shoulder, when cold is the stress, may possibly be related to the size of a chemical pool (either energetic or otherwise vital, protective, or toxic), which is exhausted or accumulated, following which additional damage becomes lethal to the cell.

Plateau-phase cells are able to accumulate about 3.6 times more cold-induced sublethal damage than exponential-phase cells (Figure 5), that is, the D_q is 3.6 times larger in the former case (Kruuv et al., 1985a). It is not known whether this damage is repairable in plateau-phase cells. However, once the latter cells are saturated with sublethal damage, their rate of inactivation is 3.2 times faster than that for exponential-phase cells (Kruuv et al., 1985a).

At this point it is difficult to assess which cell population (exponential or plateau phase) is the better model for simulating organs being preserved by hypothermia, not that this is the only reason for doing hypothermia research on cells in tissue culture. However, the large shoulder on the survival curve followed by a rapid rate of cell inactivation, as in plateau-phase cells, is somewhat reminiscent of the clinical situation where three-day hypothermically perfused kidneys are viable for transplantation purposes but five-day kidneys are not.

B. Repair of Sublethal Hypothermia Damage

A shoulder in a cell survival curve indicates that the cells have the ability to accumulate sublethal damage before this damage becomes lethal. Whether this sublethal damage is repairable or not can not be determined unequivocally by looking at a survival curve of this type, but must be determined by a split-dose experiment. Split dose refers to splitting the cold exposure into two or more fractions (e.g., three or five days per fraction), each of which is separated by 1.5 hours at a higher temperature (usually 37 °C). Split-dose experiments have shown that exponential-phase cells can, at 37 °C, repair cold-induced sublethal damage accumulated at 5, 10, 15, 20, and 25 °C (Kruuv et al., 1972), that is, sublethal hypothermia damage from five days of storage at temperatures from 5 to 25 °C can be repaired at 37 °C in about 1.5 to 2 hours. Additionally, even a single period at 37 °C in a middle of a 10-day

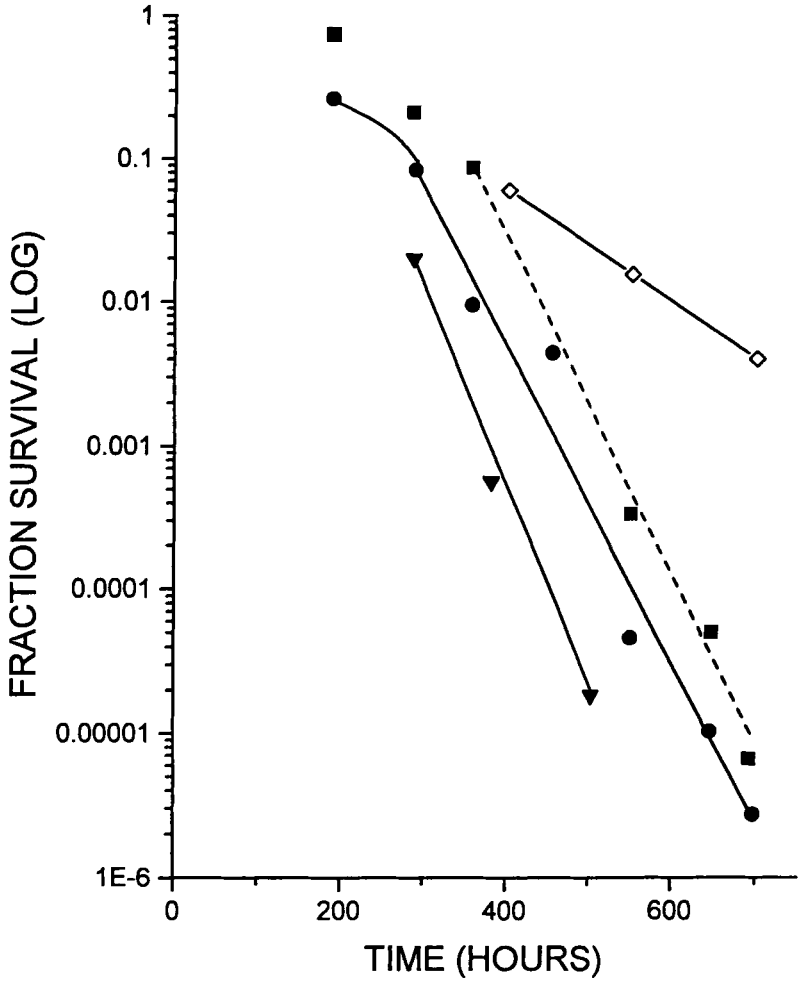

Figure 5. Survival curves of attached plateau-phase cells exposed to high mannitol (circles) or potassium (triangles) supplemented (400 mOsm) medium or to isosmotic medium (squares) and attached exponential-phase cells exposed to isosmotic medium (diamonds) as a function of time at 10 °C.

hypothermic exposure substantially improves survival (Kruuv et al., 1972). We have previously shown that there is no significant cell division during this repair period (Kruuv et al., 1972). However, while we have assumed that the increase in survival is due to repair of sublethal damage in the 37 °C interval, we cannot entirely rule out at this time that the temperature cycling may induce resistance to subsequent hypothermia exposures via a general stress mechanism (Glofcheski et al., 1993). (The

latter explanation seems unlikely in that repair of sublethal damage involved raising the temperature to 37 °C for 1.5 hours, while induction of resistance required preexposure at 25 °C for 24 hours, generally followed by 3 hours at 37 °C.)

Citiolone shows no significant protection for Range II (5 °C) damage (Kruuv et al., 1993a). On the other hand, it does not inhibit split-dose repair of Range II type of damage (Kruuv and Glofcheski, 1993b). Similarly, glycine protects at 5 °C (Kruuv et al., 1993a), but may slightly inhibit repair of Range II damage (Kruuv and Glofcheski, 1993b). At 10 °C, citiolone protects (Kruuv et al., 1993a) but may slightly inhibit repair of this type (Range I) of damage (Kruuv and Glofcheski 1993b). Glycine does not protect and may also slightly inhibit repair (Kruuv and Glofcheski, 1993b) in Range I. DMTU, by itself, does not protect in Range I, but may enhance repair (Kruuv and Glofcheski, 1993b). This may explain our previous work, which showed that there was a significant synergism in 10 °C protection when citiolone and DMTU were combined at 10 °C (Kruuv et al., 1993a). It should also be noted that since the hypothermic damage mechanisms in Ranges I and II are different, the repair mechanisms for damage may also be dissimilar. Repair of 10 °C (Range I) hypothermic damage is more efficient at 37 °C than repair of 5 °C (Range II) damage (Kruuv and Glofcheski, 1993b). Repair of 5 °C hypothermia damage is not very temperature dependent, while repair of 10 °C hypothermia damage shows some temperature dependence (Kruuv and Glofcheski, 1993b). This may imply that lipids are involved in Range II damage, that is, the damage is reversible if not allowed to accumulate too long. Range I repair implies a need for metabolism, which in turn may implicate the need for macromolecular synthesis.

Since repair of Range II damage is not very temperature dependent, it may not be necessary to raise the temperature to 37 °C for the repair period. This would be of practical importance when cycling the temperature in an organ. Finally, we have also shown that split-dose methods significantly improve long-term survival of cells stored at 5 or 10 °C, especially if repeated splits (e.g., 3-day splits for a total of 12 days of storage) are used (Kruuv and Glofcheski, 1993b).

C. Effects of Hypertonic Medium

Hypertonic flush and perfusion solutions are commonly used when organs are stored at hypothermic temperatures prior to transplantation

(Collins et al., 1979), mainly to counteract the generalized tissue edema (Downes et al., 1973) which would otherwise cause blockage of the microcirculation. K+ and mannitol have been popular additives to partially achieve this goal. We decided to investigate if hypertonic solutions of approximately the same osmolality as those used clinically (400 mOsm) could decrease survival in single cells exposed to pure hypothermia in tissue culture.

Survival of *exponential phase* Chinese hamster lung (V79) cells, exposed as a function of time to hypothermia in tissue culture, in isosmotic and various hypertonic media was measured. Addition of NaCl or mannitol to regular growth medium to increase the tonicity from 325 to 400 Osm greatly decreased the survival at 10 °C (Range I), while addition of KCl to regular growth medium had no significant effect. When these experiments were repeated at 5 °C (Range II), addition of the same amounts of either NaCl, KCl, or mannitol to regular growth medium, as in the 10 °C experiments, was detrimental to long-term cell survival. Furthermore, addition of mannitol to the medium did not improve survival when cells were stored at 7 °C. Addition of KCl at 5 or 10 °C or NaCl at 5 °C only decreased the cells' ability to accumulate sublethal damage (i.e., decreased D_q), while addition of mannitol at 5 or 10 °C decreased both the D_q and the cold-sensitivity (D_o) of the cells. Addition of NaCl at 10 °C only decreased the D_o (Kruuv et al., 1985b). The fact that high K^+ (or mannitol) medium affected the survival curve parameters in a qualitatively similar fashion above and below 7 °C may simply mean that the different cold-injury mechanisms are both sensitive to high K^+ effects.Alternatively, there are several injury mechanisms involved in *each* of these two temperature ranges with *some* mechanisms common to both ranges. These experiments suggest that prevention of cell swelling by these conditions, while possibly necessary during clinical hypothermic organ storage, is detrimental to single-cell survival at these temperatures. However, in general, use of hypertonic solutions during hypothermic (Phase II) storage is less harmful in Range I than II (Kruuv et al., 1985b).

Willis and Holeckova (1977) have demonstrated a positive relationship for cold-adapted mouse L-cells between retention of K^+ and ability to survive at 5 °C. If loss of cell viability is also partially related to the inability to reaccumulate K^+ (Southard et al., 1984), then hypothermic preservation in K^+-rich medium may have some benefit as advocated by many groups over the years. Our results show that high K^+ medium is the least harmful of the hypertonic solutions tested for hypothermic preservation in Range I.

While we demonstrated that storage in hypertonic medium at 5 °C (Range II) is detrimental to cell survival, the inference of possible mechanisms from these cell survival curves is not clear-cut. Since extrapolation of these curves gives a negative D_q, we are probably dealing with multi-component (i.e., "resistant tail") curves. This situation could arise from an additive cell cycle killing effect where the tail is due to the population most resistant to the two different stresses, that is, hypertonicity and 5 °C hypothermia. In fact, the survival response curve for the cell cycle for these two stresses is complimentary (McGann et al., 1972; Nelson and Kruuv, 1972); the "resistant tail" is probably due to an early G_1 and/or late S-phase population. One of the common features of these two stresses is an increase in cytoplasmic viscosity (Lepock et al., 1983), the additive effects of which would certainly influence the rate of metabolism within the cytoplasm.

It has previously been shown that V79 cells shrink at 10 °C in isosmotic medium, whereas they swell at 5 °C (Kruuv et al., 1984a). If cell shrinking at the single cell level, as in our experiments, is important for survival, then storage at 10 °C in hypertonic medium would not be expected to increase survival as cells are already shrunken in the 10 °C control. Our results confirmed these predictions. On the other hand, storage of cells in hypertonic medium at 5 °C should prevent cell swelling. Unfortunately, this procedure is detrimental to cell survival. Hence, cell swelling is probably unrelated to the mechanism of cell death due to hypothermia, at least at the single cell level. This is in agreement with data which shows that BHT greatly improves survival of cells exposed to 5 °C (Kruuv et al., 1983) while not decreasing the rate or amount of cell swelling at this temperature (Kruuv et al., 1984a).

Even though the 5 °C results with mannitol were relatively unpredictable, in view of the 7 and 10 °C experiments and the four 5 °C experiments, it seems safe to say that high mannitol medium at 5 °C does not improve cell survival above the isosmotic control for long-term preservation (i.e., longer than 10 days). The variability may be related to the fact that mannitol slowly leaks into cells (Pegg, 1981b) and that leakage into and out of cells at low temperatures is rather inconsistent especially if some of the plasmalemma lipids have undergone a phase transition, which is the case for these cells at temperatures below 8 °C (Kruuv et al., 1983; Lepock et al., 1987).

Since the hypertonic solutions tested did not improve cell survival at 5 or 10 °C, and since no cell swelling is observed at 10 °C (Kruuv et al., 1984a), which is the optimum storage temperature in this cell line (Kruuv

et al., 1983), it is suggested that organ storage at 10 °C (or the low temperature end of Range I) in isosmotic solutions be considered more widely in order to increase the length of time an organ remains viable for clinical transplantation purposes. Furthermore, since post-hypothermic (Phase III) cell lysing at 37 °C is greater after 5 than after 10 °C storage (Kruuv et al., 1984b), there may be fewer complications in transplant patients after organ storage at the latter temperature.

Cells in culture is not a completely irrelevant experimental model for clinical organ storage. Successful kidney preservation is limited to 72 hours of continuous hypothermic (5-10 °C) perfusion while 120 hours usually results in nonviable kidneys. Our tissue culture 5, 7, and 10 °C control curves generally all have shoulders. The average D_q values for this cell line are 53, 51, and 80 hours at 5, 7, and 10 °C, respectively (Kruuv et al., 1983). Examination of survival curves, where the shoulder region has been carefully investigated (Nelson et al., 1971; Kruuv et al., 1972), reveals that cell survival is very high (85-100%) at these temperatures for at least 72 hours. On the other hand, the fact that 72 hours of hypothermic perfusion yields a functioning kidney does not ecessarily mean that 100% of the kidney cells will turn out to be survivors when the kidney is transplanted. Hence, despite the differences from the clinical situation, the study of the effects of hypothermia on mammalian cells in tissue culture can not only provide some insight into mechanisms of cold damage at the cellular level but may also prove to be of value to those using hypothermia in the clinical setting.

Exposure of *plateau-phase* cells, on the other hand, to high mannitol or K^+ medium (400 mOsm) at 10 °C decreases the cells' ability to accumulate sublethal damage (Kruuv et al., 1985a) but does not change the rate of cell inactivation (D_o). This differs from results with exponential-phase cells exposed to high mannitol medium at 10 °C, in which case both D_o and D_q are reduced, compared to controls (Kruuv et al., 1985b). The effect of hypertonic K^+ medium at 10 °C is also dissimilar in that it caused a small reduction in D_q in the case of exponential-phase cells (Kruuv et al., 1985b) but a large reduction in the same parameter in the case of plateau-phase cells (Kruuv et al., 1985a). Furthermore, high K^+ was superior to high mannitol medium at 10 °C for the former cells while the situation was reversed for the latter cells. That is, the exponential-phase cells, unlike plateau-phase cells, are relatively unaffected by exposure at 10 °C to medium made hypertonic by KCl, which parallels the clinical situation where kidneys are perfused with hypertonic, high K^+ Collins' medium (Collins et al., 1979).

D. Effects of Iron and Iron Chelators

The effects of cooling on complex integrated cell metabolism may include the uncoupling of reaction pathways with unpredictable consequences (Pegg, 1981a). The aftermath of some of these consequences is a decrease in mammalian cell survival as a function of time of exposure to hypothermia (0 to 25 °C), even when the cells are not subjected to hypoxia before or during the hypothermic exposure. While evidence has accumulated that free radical damage is involved in cold ischemia stored organs (Fuller et al., 1988), either during organ storage or reperfusion, the mechanisms of cell killing by hypothermia are not fully understood but *could* involve damage by free radicals.

Free radicals are naturally produced as a consequence of oxygen metabolism, and are considered universal mediators of cell and tissue damage, since their reactions are believed to play a part in a great variety of physiological and pathological processes, including ischemia/reperfusion damage to transplant organs (Halliwell and Gutteridge 1985; Feher et al., 1987). Iron has been shown *in vitro* and *in vivo* to catalyze powerful and biologically destructive oxidants (Halliwell and Gutteridge, 1984, and 1986; Aust et al., 1985; Weiss, 1986; Dunford, 1987) and since iron is present intracellularly in a variety of forms and may also be inadvertantly present in the extracellular environment (i.e., capillary bed) via the perfusate, its role as a potential mediator of damage to transplant organs or cells may be significant (Fuller et al., 1986; Green et al., 1986a, 1986b; Myers et al., 1986).

Survival of V79 Chinese hamster cells was assessed by colony growth assay after hypothermic exposure in the presence of iron chelators (Zieger et al., 1990). At 5 °C (Range II), maximum protection from hypothermic damage was achieved with 50 μM of the *intracellular* ferric iron chelator Desferal. A three hour prehypothermic incubation with 50 μM Desferal followed by replacement with chelator-free medium at 5 °C also provided some protection. This was not observed when the *extracellular* chelator DETAPAC (50 μM) was used prior to cold storage.

Treating 5 °C stored cells with Desferal just prior to rewarming was ineffective, but treating cells with Desferal *during* (Phase II) hypothermia exposure after a significant period of unprotected cold exposure ultimately increased the surviving fraction. Submaximal protection *during* hypothermia was achieved to various degrees with extracellular chelators at 5 °C, including 50 μM DETAPAC and 110 μM EDTA. EGTA at a 110 μM concentration had little effect. The sensitization of cells at

5 °C with 200 μM FeCl3 could be reduced or eliminated with Desferal in accordance with a 1:1 binding ratio. At 10 °C (Range I), 50 μM Desferal, 50 μM DETAPAC, and 110 μM EDTA were as or less effective in protecting cells than at 5 °C. An Arrhenius plot of cell inactivation rates shows a break at 7-8 °C, corresponding to maximum survival for control cells and cells in 50 μM Desferal, however, the amount of protection offered by the chelator increases with decreasing temperature below about 19 °C, and sensitization increases above that point (Zieger et al., 1990). It has not previously been shown that iron chelators protect against cellular hypothermia damage which is uncomplicated by previous or simultaneous ischemia. This may be relevant to the low temperature storage of transplant organs, in which iron of intracellular origin and in the perfusate may be active and damaging.

The intracellular iron chelating agent, Desferal, at a concentration of 50 μM increases the activation energy (E_a) to 26.2 kcal/mole in Range I, resulting in progressively less protection with increasing temperature, and sensitization above about 19 °C. This value is still in the range of temperature coefficients of metabolic processes and much less than that for protein denaturation. The effect of Desferal on the reaction rates associated with the mechanism of cell killing below 7 °C (Range II) is to decrease E_a to -44.2 kcal/mole.

Numerous cellular events at hypothermic temperatures have been reported. For example, sharp increases in the rates of DNA and protein synthesis occur in V79 cells between 18 and 21.6 °C (Nelson et al., 1971), and are unaccompanied by cell division, possibly accounting for the increased sensitivity at higher temperatures, that is, 25 °C (Nelson et al., 1971). Electron spin resonance and fluorescence studies of V79 cell membranes show a lipid phase transition centered at approximately 20 °C (Kruuv et al., 1983), and refluidizing the membrane with BHT at or below this temperature facilitates the repair of sublethal radiation damage (Nolan et al., 1981). Ward et al. (1987) have shown that single-strand breaks in DNA and consequential killing of V79 cells by superphysiological amounts of H2O2 is significantly more damaging at 37 than at 0 °C; they hypothesized that site specific .OH damage induced at 37 °C was accompanied by misrepair, while the repair mechanism was not active during the 0 °C exposure. We now have evidence that there is an iron related damage mechanism at low temperature. Thus, hypothermic damage to V-79 cells is probably the combined result of multiple simultaneous mechanisms (perhaps meta-

bolic and free radical) and endogenous repair mechanisms. The process of hypothermic protection by Desferal is likely complicated as well; the iron chelator probably interacts with regular metabolism (Barankiewicz and Cohen, 1987), as well as repair processes and pathological (free radical) reactions. The result is a varied protective response to hypothermic cell damage as a function of temperature (Figure 6). However, since we observe linearity in the log plot of $k_{control}/k_{desferal}$ versus reciprocal temperature (Figure 7), the mechanism of action of Desferal must have a single temperature coefficient throughout the temperature range of 0 to 25 °C. From Figure 6, it is also apparent that Desferal alters the temperature coefficient for hypothermic cell killing (otherwise the line in Figure 7, while linear, would have zero slope). Hence, the decrease in protection as the temperature is raised, eventually resulting in sensitization above 19 °C, is due to the difference in the temperature coefficients for hypothermic cell killing in the presence and absence of Desferal.

The physiological success of iron chelation is dependent on many factors, including the optimal coordination of iron by the chelator, an effective binding affinity for iron, especially in the presence of competing or interfering ions, the bulk of the chelator and its possible effect on the kinetics of iron mobilization, and the environment of iron chelation (Pitt and Martell, 1980). Both Desferal and DETAPAC have very high affinities for ferric iron (standard and effective stability constants are given by Pitt and Martell, 1980), and in view of the excess chelator concentrations used, the difference in hypothermic protection offered probably relates to the environment of iron chelation. Regular medium supplemented with 50 μM of the intracellular agent, Desferal, protects maximally against hypothermic damage. The extracellular agent, DETAPAC, at a concentration of 50 μM, also protects at 5 °C, but less effectively than Desferal. This extra level of protection by Desferal may be due to the inhibition of two distinct damage mechanisms: one external and one internal. It has been speculated that metals in the organ preservation perfusate may be a source of free radicals that are damaging to the vascular endothelium even before reperfusion-generated free radicals appear (Bennet et al., 1987). Such primary lesions may be amplified during reperfusion by the infiltration of neutrophils attracted to the site of injury where they generate superoxide and injure tissue (Bulkley, 1987). We have now shown evidence of an iron promoted extracellular damage mechanism in cultured cells exposed to hypothermia under nonischemic conditions. This low temperature damage mechanism may be free radical mediated, and may contribute to the much-cited is-

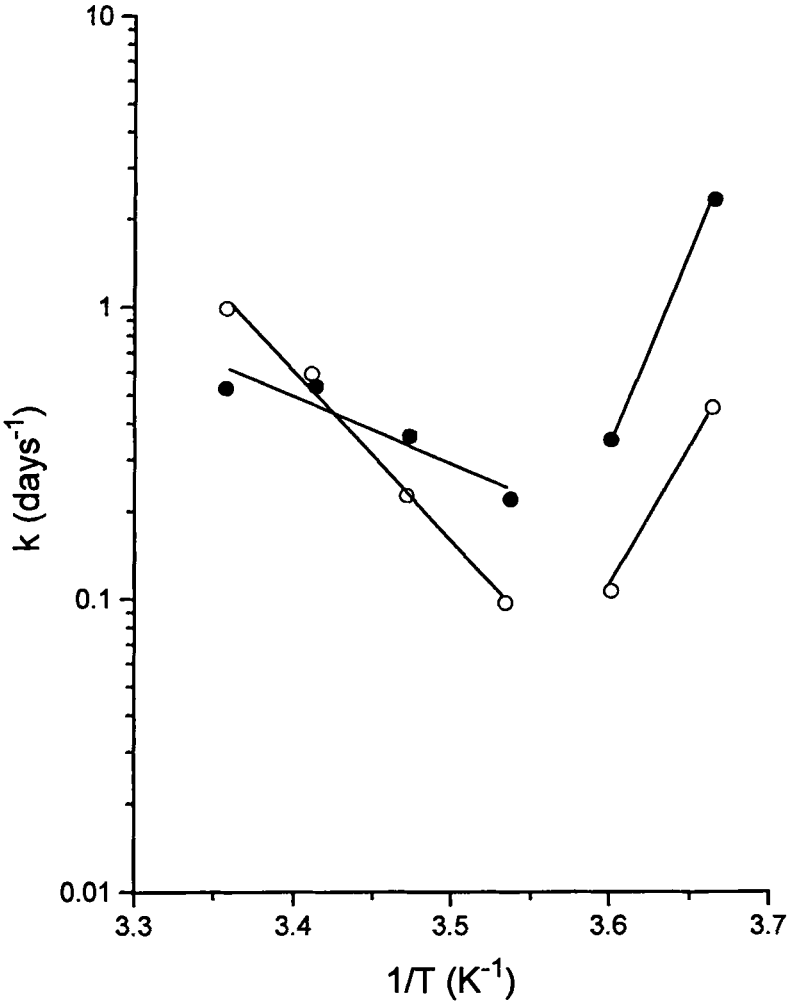

Figure 6. Arrhenius plot of the cell inactivation rate constant for V79 cells that are exposed to various temperatures while attached to plastic plates with (open circles) or without (closed circles) the presence of the intracellular iron chelator Desferal.

chemia/reperfusion injury to transplant organs. Our results reaffirm the strict requirement for ultraclean perfusates in a transplant organ storage regime.

Cells obtain iron from extracellular transferrin, and much of this iron is used in the mitochondria to synthesize heme for mitochondrial en-

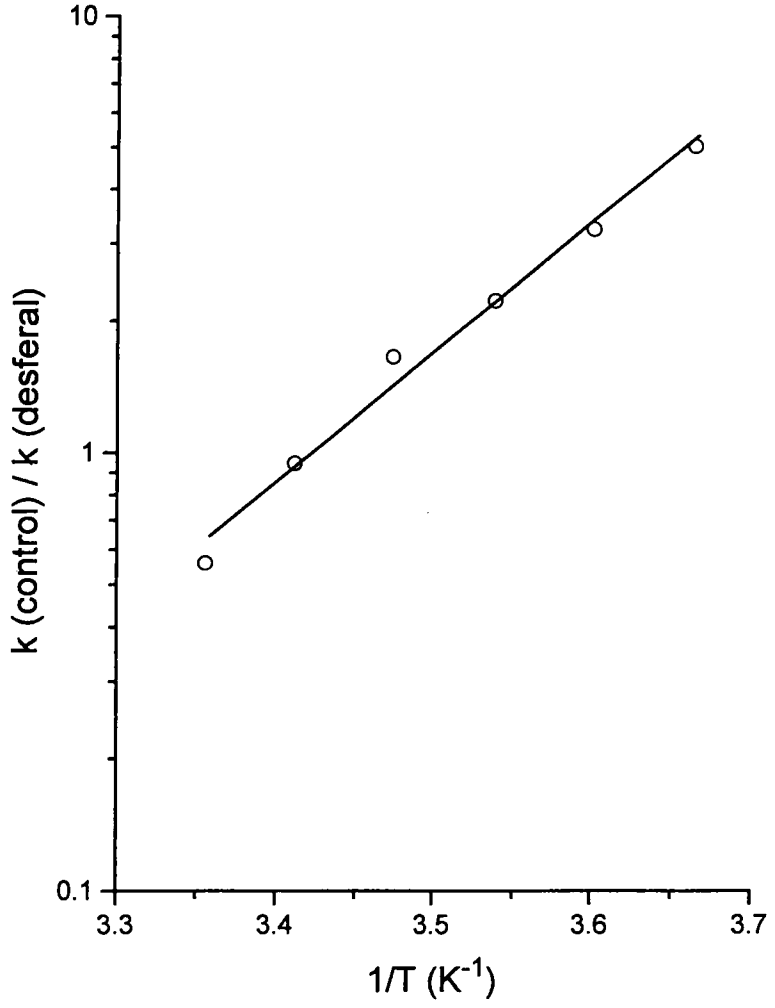

Figure 7. Logarithmic plot of the ratios of cell inactivation rate constants ($k_{control}/k_{Desferal}$ from Figure 5) as a function of reciprocal temperature.

zymes, for widely distributed enzymes like cytochrome P450 or for specialized proteins such as hemoglobin or myoglobin (Jacobs, 1977). Iron which is in excess of metabolic requirements accumulates in the transient storage protein, ferritin (Jacobs, 1977). Jacobs has described an intracellular transit iron pool consisting of low molecular weight iron chelates which maintain an equilibrium between iron uptake, iron stor-

age, and iron utilization within the cell (Jacobs, 1977). This pool may be the target of intracellular chelation by Desferal (White et al., 1976; Bridges and Cudkowicz 1984; Bottomley et al., 1985; Rogers and Munro 1987). Alternatively, iron binding by Desferal may occur in lysosomes where it complexes iron released from ferritin under the conjugate actions of acidic pH and lysosomal enzymes (Laub et al., 1985). Cytoplasmic ferritin is deemed an unlikely source of chelatable iron as the channels in this protein limit free access of the relatively large chelator (Desferal) to the iron core (Kontoghiorghes et al., 1987).

Some intracellular forms of low molecular weight iron chelates can support free radical production *in vitro* (Flitter et al., 1983; Floyd and Lewis, 1983; Graf et al., 1984; Sutton, 1985) and lipid peroxidation *in vivo* (Anderson and Means, 1985; Nayini et al., 1985). Also, it has been proposed that the lysosomal pool is the source of ferric iron necessary for the killing of hepatocytes by H_2O_2 via the Haber-Weiss reaction (Mello Filho et al., 1984; Starke et al., 1985). Free radicals are produced in the cell by many metabolic reactions, and natural scavenging mechanisms exist to keep these metabolites in check (Feher et al., 1987). It is possible that hypothermic stress alone can create a metabolic imbalance by impairing endogenous scavenging mechanisms or by overloading the cell with dangerous free radicals. Such an overload may be the result of iron being released from a safe association or storage site to a more active chelate, capable of catalyzing the production of ·OH or similarly reactive free radicals, and promoting lipid peroxidation.

Desferal was shown to reduce lipid peroxidation in cold (0 °C) ischemic rabbit kidneys when added to the initial flush and cold storage solutions (Green et al., 1986b). Signs of lipid peroxidation predominated when cold storage was followed by *in vivo* reperfusion (Green et al., 1986a); treatment of the tissue homogenates of cold stored porcine kidneys with Desferal just prior to *in vitro* incubation reduced this damage (Fuller et al., 1986). The authors hypothesized that the observed lipid peroxidation products resulted from the iron catalyzed breakdown of lipid hydroperoxides produced in the cold *ischemic* preservation period by the reaction of low density lipoproteins with free radicals (Fuller et al., 1986). However, their experimental methods did not allow them to determine the exact site of damage (intracellular or extracellular), nor could they identify the specific stress responsible.

In our experiments, treating cold stored aerobic cells (5 °C) with Desferal just prior to rewarming was an ineffective means of protection.

This failure of the chelator to act as a rescue agent and the prevalence of fully oxygenated conditions (Zieger 1988) precludes anischemia/reperfusion damage mechanism. Fifty μM Desferal added on the second or sixth day of the cold exposure ultimately salvages survival for some of the cells not yet killed. We observed that by day six, and possibly even by day two, the surviving cells in HEPES control medium have accumulated significant damage (survivals lie on the exponential part of the curve). Without the intervention of the iron chelator, subsequent survivals would lie further along this part of the curve. However, within two days the addition of extra medium, we observe that Desferal minimizes the sensitization experienced by control cells, and within three to six days, Desferal facilitates a decrease in the rate of killing as witnessed by the decrease in the survival curve slope (Zieger et al., 1990). The events corresponding to these curve features may be immediate protection from extracellular mediated damage, with intracellular protection taking effect only after enough chelator has diffused through a solidified plasma membrane.

Thus, we have identified a distinct hypothermic damage mechanism(s) using a *non-ischemic* tissue culture system. This unique form of injury is iron mediated, and our evidence shows that it occurs in both the intracellular and extracellular environments. The precise physiological and biochemical mechanisms are yet to be identified, but may be elucidated with further investigations of the protective actions of iron chelators, and the kinetics of iron mobilization from the cell.

E. Effects of Hepes Buffer, Free Radicals, and H_2O_2

Buffers are essential components of cell culture media and cold storage solutions or perfusates used in the storage of transplant organs. In cell culture, buffers must prevent pH changes that occur during production of cellular metabolites; in organ perfusates, buffers must maintain a physiological or favorable pH despite the metabolic changes instigated by ischemic and hypothermic conditions. In perfusion media, the buffers of choice are often the substituted sulfonic acids known as Good buffers, of which HEPES is the most suitable (Pegg, 1981b). HEPES (N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid) is a zwitterionic buffer commonly used in cell culture because it offers numerous advantages over other buffers and, in hypothermia experiments, it eliminates the need for a 5% CO_2 atmosphere to maintain physiological pH, unlike sodium bicarbonate buffer (Zieger, 1988).

However, cytotoxicity results from the interaction of fluorescent light from a flow hood with HEPES buffered cell culture medium at room temperature (Zieger et al., 1991). Toxicity can be prevented by keeping both cells (V79 Chinese hamster) and medium shielded from direct fluorescent light (dark conditions), or by supplementing the medium with 10 μg/ml catalase; this suggests that extracellular hydrogen peroxide is a major cause of the lethal effect under lighted conditions. No sensitization resulted from the exposure of cells in a sodium bicarbonate (SBC) buffered medium to fluorescent light, nor in a catalase supplemented SBC buffered medium. The HEPES/light reaction during routine cell manipulations (Phase I) presensitized cells to hypothermia (Phase II) damage in the dark with the presensitization being more severe for 5 than for 10 °C hypothermic exposure. Presensitization was prevented by performing the complete experiment under dark conditions or by supplementing the medium with 10 μg/ml catalase. However, catalase did not improve the hypothermic survival when experiments were performed under dark conditions. Hence, 10 μg/ml catalase does not protect cells from hypothermic (5 and 10 °C Phase II damage per se, but rather from HEPES/light *sublethal* (Phase I) damage which interacts with hypothermic sublethal (Phase II) damage to result in lethal lesions.

While dark conditions greatly improve Phase II cell survival at both 5 and 10°C, survival is not further enhanced under dark conditions by supplementing the medium at 5 or 10 °C with either (a) 10 μg/ml superoxide dismutase (SOD), (b) 5 μg/ml SOD plus 5 μg/ml catalase, or (c) 0.1 mM allopurinol. This suggests that extra-cellular superoxide anion, H_2O_2 and internal xanthine oxidase generated free radicals (O_2^-) are not implicated in cell killing during hypothermia 5 or 10 °C Phase II storage uncomplicated by previous ischemia or hypoxia. Furthermore, under dark conditions at 5 °C (Range II), hydroxyl radical scavenging does not improve the survival as neither DMSO nor mannitol improve the survival (Zieger et al., 1991).

The implications of these results to the storage of transplant organs are important for two reasons. First, they reaffirm that different damage mechanisms may interact in a complex manner, if not directly, then by inhibiting repair or protection mechanisms of other types of damage (i.e., hypothermia prevents repair of light/HEPES induced sublethal damage). The corollary is that attempts to protect from one damage mechanism may influence other damage mechanisms in a negative manner (i.e., reducing ischemia injury with low temperature promotes hypothermic damage mechanisms and inhibits repair processes). Second, unac-

counted for stresses may lead to misinterpretation of experimental results; the erroneous conclusion that catalase protects cells in culture from hypothermic damage is an example. Interpretations of successful protection of perfused organs may in fact be manifestations of unaccounted for stresses inflicted by the experimental or storage process. An example is the intensification of ischemia/reperfusion injury by trace iron contaminants in organ perfusates (Wicomb et al., 1986, 1987; Bennet et al., 1987). Similarly, light effects may promote injury to preserved organs and create apparent mechanisms of protection. These and other potential unphysiological stresses must be considered when conducting experiments or devising an organ preservation regime.

F. Cell Swelling

One of the problems of storing organs at low temperatures (0-5 °C) for two days before transplantation concerns cell swelling (Downes et al., 1973). This swelling can cause two problems: (a) the mechanical pressure increase in the tissue may directly lead to necrosis of the cells and/or (b) the swelling will block small blood vessels which in turn will limit the amount of oxygenated fluid that can be pumped through the organ during preservation by perfusion. Hence, the organ perfusion solutions commonly used are hypertonic (Collins et al., 1979). It has been hypothesized that hypothermic preservation inactivates or at least slows down the sodium pump in the plasma membrane causing extracellular sodium to leak in. The cell will then absorb water to try to minimize the osmotic gradient and, therefore, swell. It is not certain at which temperature cells begin to swell, if all the variables involved in transplant research were removed, and what the time-course of this swelling is during the hypothermia (Phase II) exposure.

Therefore, we decided to investigate cell size as a function of temperature in a cell line where cell survival is known as a function of temperature and time (Kruuv et al., 1983) and where membrane fluidity for the major types of cell membranes has also been measured as a function of temperature. Additionally, since BHT affects both membrane fluidity (Rule et al., 1979) and hypothermic survival parameters (Kruuv et al., 1983), its influence on cell size at these temperatures was investigated to discern any relation between cell size and survival at hypothermic temperatures.

Chinese hamster V79 cells exposed to various hypothermic temperatures as a function of time showed that cells exposed to 5 or 1.5-2.5 °C

(Range II) increased in size somewhere *after* four hours of exposure with maximum size reached between 12 and 20 hours. Exposures of a further 13-49 hours resulted in no significant additional swelling. Exposures to 10 °C (Range I) of these cells resulted in cell shrinking for up to six hours in the growth medium and no significant change thereafter for the next 39 hours. Addition of 0.1 mM BHT to the cells changed the kinetics and the magnitude of cell volume changes at some temperatures (Kruuv et al., 1984a).

There seems to be no temporal relationship between cell swelling and survival as single cells in suspension, exposed to 5 °C, have reached their maximum size after 20 hours of exposure whether BHT is present or not. Cell survival, on the other hand, measured by the colony formation assay at this time in the same cell line, is 96% in the presence of BHT and 56% in its absence (Rule et al., 1980). Subsequent exposure to 5 °C results in no further cell swelling but a steady decrease in cell survival. For example, at 69 hours, where we saw no additional increase in cell size at 5 °C, the survival has decreased to 14% for cells in suspension (Rule et al., 1980). Likewise, 0.1 mM BHT greatly increased the survival of cells at 2.5 °C (Kruuv et al., 1983) but not the cell swelling. The situation is similar for cells in suspension exposed to 10 °C. While cell size does not vary between six and 45 hours of exposure, survival in this cell line changes from 88% to 47% in this time interval (Hetzel et al., 1973). The presence of 0.1 mM BHT has little effect on cell survival (Kruuv et al., 1983) or final cell volume at 10 °C.

The fact that no temporal correlation exists between cell swelling and survival should not be interpreted to say that the two phenomena cannot be related. For example, even in single cells, it is possible that the shrinkage may somehow prevent lethal damage by increasing cytoplasmic viscosity (Lepock et al., 1983) and, hence, slow down metabolic activity without necessitating a further decrease in temperature. The fact that in this cell line there is a lipid phase change centered around 8 °C in mitochondrial membranes (Kruuv et al., 1983) may account for the cell swelling observed below this temperature. The mechanism for shrinkage at 10 °C is presently being explored.

Since all hyperosmotic solutions (high K^+, Na^+, or mannitol) tested decreased single cell survival as a function of time at either 5 or 10 °C (Kruuv et al., 1985b) compared to an isosmotic control, hyperosmoticity per se is not the ultimate answer for hypothermic cell or organ preservation. Hyperosmotic solutions have been used in organ preservation to prevent the generalized tissue edema that is seen when organs are stored

or perfused at temperatures between 0 and 5 °C for periods of 24 hours or more. Since 10 °C is the optimum hypothermic storage temperature in this cell line (Kruuv et al., 1983) and in human red blood cells (Lepock et al., 1979), and because no cell swelling is observed when single cells are stored as a function of time at 10 °C, it is tempting to extrapolate that organ (Phase II) storage in isosmotic solutions at 10 °C (Range I) may increase the length of time an organ remains viable for clinical transplantation purposes.

G. Oxygen Consumption and Na-K ATPase Activity

Cell hypoxia does not occur during the hypothermia exposure time in survival experiments in the closed flasks in our tissue culture system. At 5 °C, the rate of oxygen consumption in this cell line is 0.062×10^{-17} moles/cell/sec (Lepock et al., 1987). There are 4 mls of medium plus 59 mls of air in these tissue culture flasks. Applying vapor pressure and salt corrections, the amount of oxygen in the gas phase is more than 500 times that in the 4 mls of liquid when the system is equilibrated (gas/liquid) at 22 °C (the starting point of the medium before the hypothermia exposure). For a survival fraction of 10^{-4}, 10^6 cells are plated to result in 100 survivors in 19 days. Making the extreme assumption that *all* 10^6 cells respire at the rate of undamaged cells for the entire 19 days, the cells still would only consume 0.19% of the oxygen in the system. At 10 °C, for a fraction survival of 10^{-5}, 10^7 cells are plated for 25 days of exposure. The rate of oxygen consumption is 0.117×10^{-17} moles/cell/sec at 10 °C (Lepock et al., 1987). Making the same assumptions, the cells would consume 4.73% of the total oxygen available. While we have not measured the oxygen consumption in these cells in the presence of the various drugs used, the rate of oxygen consumption in *all* the 10^5 or 10^7 cells would have to increase by factors of 500 and 20 for the duration of the hypothermia exposure at 5 and 10 °C, respectively, to have any effect on the amount of oxygen available to the cells. There are no drugs known that *increase* the rate of oxygen consumption by factors as large as the above.

In this cell line, there are lipid transitions in both the plasmalemma (Kruuv et al., 1983) and mitochondrial membranes (Kruuv et al., 1983; Lepock et al., 1987) at approximately 8 °C, correlating with the abrupt change in the cell killing rate. Since there is also cell swelling at 5 °C but not at 10 °C in this cell line (Kruuv et al., 1984a), the activity of the $(Na^+\text{-}K^+)$-ATPase in the plasmalemma in the presence of *excess* ATP was

measured in cell homogenates as a function of temperature to determine if inactivation of this pump could explain the above cell sizing or survival observations. The ATPase measurements indicated a "break" in the Arrhenius plot at 20 °C, but not at 8 °C (Figure 8; Young, 1986). Although it is known that broken membrane ATPase is more sensitive to cooling than the intact pump (Ellory and Willis, 1976; Willis et al., 1978), the effect of temperature on affinity for ATP is very different in intact and broken membrane preparations (Marjanovic and Willis, 1992), and absolute ATPase activity depends on the size of the membrane vesicles (Young, 1986), we were interested in finding breaks in the Arrhenius plot of enzyme activity, which may coincide with the abrupt change in survival or the lipid transitions of these cells, rather than the absolute activity of the ATPase.

To determine if the mitochondrial respiration was limiting ATP production, the rate of oxygen consumption in whole cells was measured as a function of temperature. There were no breaks in the Arrhenius plot (Figure 9) suggesting that if mitochondrial ATP production were limiting the activity of the Na-K ATPase in whole cells, then there may be uncoupling of oxygen consumption and ATP production in mitochondria between 5 and 10 °C (although we have not proved this) since the cells swell at 5 °C. (The oxygen consumption measurements in Figure 9 were further extentions, to 0 °C, of those described earlier in the 10 to 45 °C range (Lepock et al., 1987).)

The cell swelling, at 5 °C, seen in this cell line may be due to uncoupling of oxygen consumption and ATP production in mitochondria below 8 °C, as this is the lower temperature limit of a broad membrane lipid transition in these mitochondria (Lepock et al., 1987). The ATPase activity of the Na-K pump follows Arrhenius kinetics from 20 to 2 °C in the presence of *excess* ATP although uncoupling of ion pumping from ATPase activity at some intermediate temperature cannot be ruled out at this time. However, many other factors could influence the cell swelling at 5 °C, as swelling is the balance between leaks and pump activity. For example, use of impermeant anion instead of chloride in preservation solutions has minimized swelling (Southard and Belzer, 1993). Also, Willis and his colleagues have shown that the failure of cold-sensitive mammalian cells to maintain a Na^+ gradient at low temperatures is due to their inability to sufficiently reduce their passive permeability (Willis, 1987; Willis et al., 1989; Zhao and Willis, 1989; Zhou and Willis, 1989). They investigated amiloride-sensitive (AS) Na^+ *influx* in guinea pig red cells and found that the AS Na^+ influx

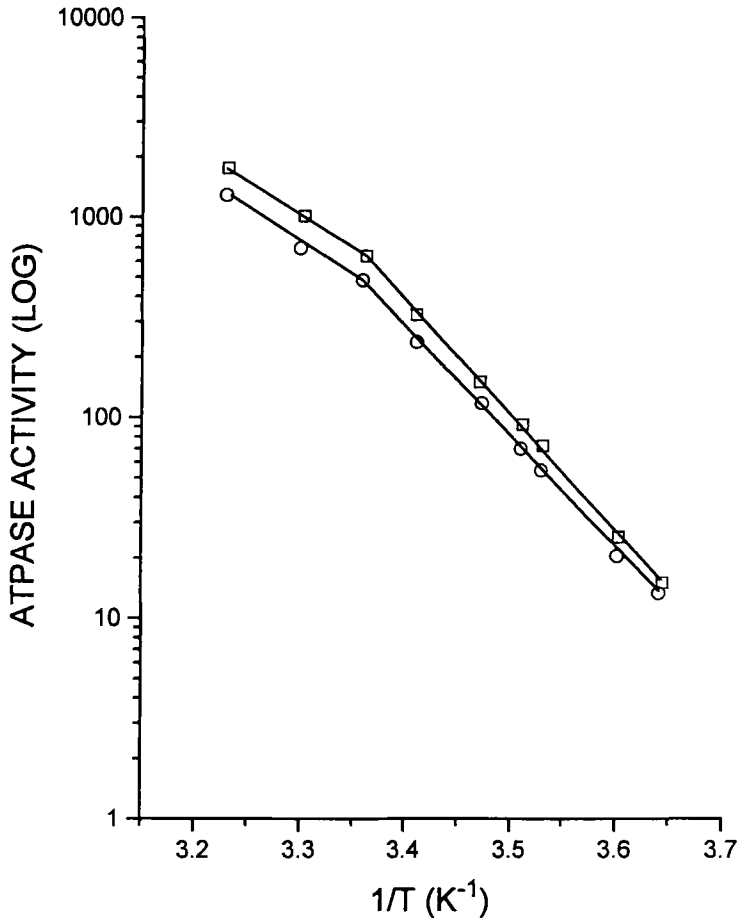

Figure 8. Arrhenius plot of the ouabain-inhibited (circles) and uninhibited (squares) ATPase activities ($\mu g\ PO_4$/mg protein/hr) of V79 cell homogenates. The plasmalemma Na^+-K^+ ATPase activity is calculated by substracting the activities of the curves shown. Obviously, the break in the Arrhenius plot in the activity of the plasmalemma Na^+-K^+ ATPase will occur at the same temperature as in the curves shown.

significantly increased with cooling to 20 °C (Zhao and Willis, 1993).

It should be noted that while it has been shown that there is respectable Na^+-K^+ pump activity even at 5 °C in some cells (Marjanovic and Willis, 1992), those measurements were only carried on for a little more than

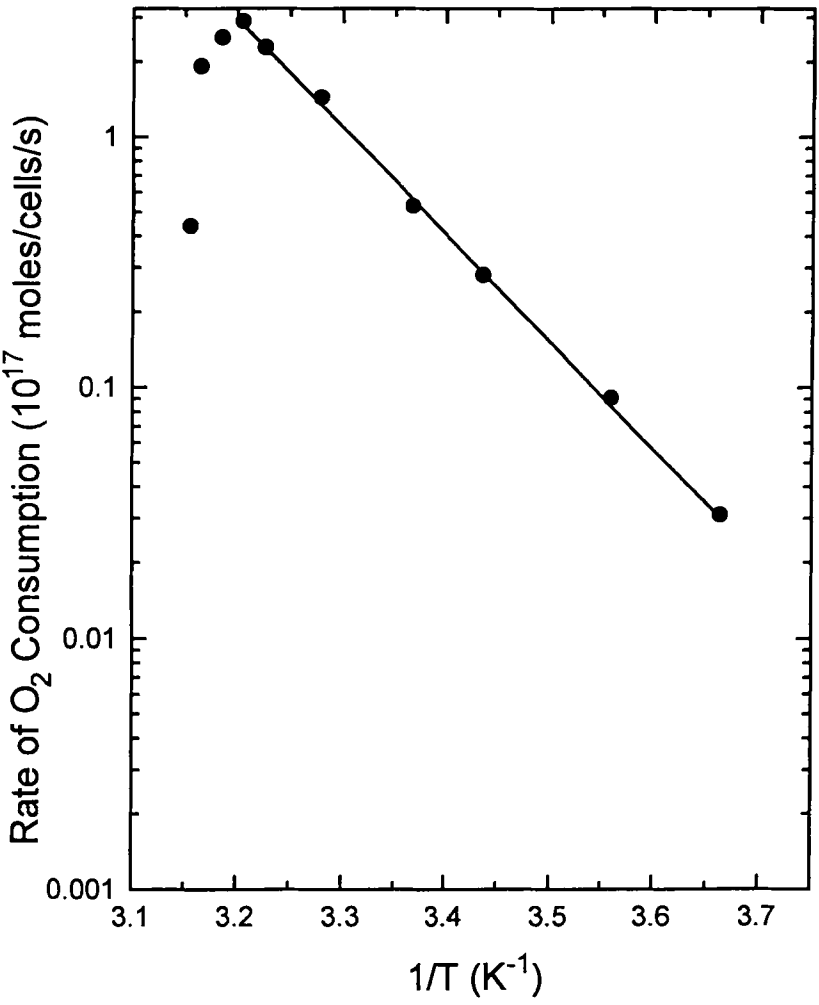

Figure 9. Arrhenius plot of the rate of oxygen consumption of V79 cells.

an hour. The V79 cells show no increase in size at 2 or 5 °C until after four hours of exposure to those temperatures. Detailed measurements of pump activity as a function of time and temperature have yet to be done. In retrospect, since there is no temporal correlation between cell swelling and survival in cultured cells (Kruuv et al., 1984a), activity of the Na^+-K^+ pump is probably not the determining factor in hypothermic cell killing in Range II.

VII. GENERAL DISCUSSION AND CONCLUSIONS

Very few *survival* studies using the colony-formation assay as a function of time and temperature in hypothermic conditions have been done on mammalian cells. Assays that measure membrane permeability do not always correlate with survival as measured by colony formation; usually the former overestimate survival. However, we have now demonstrated that in four different cell lines the Arrhenius plot of cell inactivation (killing) shows a break at temperatures well above zero (Figures 3, 4a, and 4c) as well as a similar plot for leakage of hemoglobin out of human red cells (Figure 4b). Hence, this survival behavior is not confined to cells of hibernators. The question is: what are the events that cause this break?

There is no doubt that membrane-bound enzymes and membrane transport systems cease to function as the membranes undergo the liquid crystalline-to-gel lipid phase transition. Even though we observe weak breaks in the Arrhenius plots of fluidity in isolated plasmalemma of V79 cells at the critical temperatures of 8 and 30 °C, because of the amount of the membrane perturber cholesterol in plasmalemma, it probably doesn't completely solidify at physiological temperatures although the viscosity changes with temperature may reach a critical point where the Na-K pump may cease to transport ions in some cells even if adequate ATP were available. However, this was not true in the case of V79 cells. The more likely target is the mitochondrial membrane, which also showed breaks in the Arrhenius plot of fluidity at the critical temperatures in V79 cells. The inner mitochondrial membrane, which has much more surface area than the outer membrane, contains 70% protein and 30% lipid. The three large respiratory enzyme complexes (NADH dehydrogenase complex, b-c_1 complex, and cytochrome oxidase complex) are embedded in the inner membrane and pump protons out of the mitochondrial matrix space creating the electrochemical proton gradient. A number of active transmembrane transport processes are driven by this proton gradient, for example, the H^+-pyruvate symporter, the H^+-phosphate symporter, the ADP-ATP anti-porter, the import of Ca^{++}(Alberts et al., 1989). Furthermore, the transmembrane ATP synthetase complex, which contains a transmembrane proton carrier, is powered by this electrochemical proton gradient. Obviously, if this membrane were in the gel phase it is not likely that the above proteins would function since they would not be able to change their configuration. Furthermore, a number of shuttles (e.g., the malate-aspartate shuttle) all would be shut down, due to the lipid phase transitions alone, even if ATP *were* available.

Finally, the small, hydrophobic mobile electron carriers, ubiquinone and cytochrome c, are required to diffuse rapidly in the plane of the inner mitochondrial membrane to collide with the three major respiratory enzyme complexes to transfer electrons as part of the electron transfer chain (Alberts et al., 1989). This activity would also stop in the gel phase. The question is: which is the critical temperature for these events if membrane fluidity is the culprit or does one of these proteins cold denature? Since membranes are composed of many different kinds of lipids, the phase transitions, as observed by DSC, are broad (Lepock et al., 1987). For V79 cell mitochondria, this broad transition, as seen by DSC, begins around 8 °C and ends around 30 °C (Lepock et al., 1987). Transitions at these temperatures in V79 cell mitochondria and plasmalemma are also seen by fluorescence and electron spin resonance techniques (Kruuv et al., 1983). Under our own classification system, all membrane lipids, except possibly the "boundary lipids," are in the gel phase in Range II, while in Range I there will be a mixture of lipids in both the gel and liquid crystalline phases. (For consistency, we should have a Range 0, where all membrane lipids are in the liquid crystalline state; for V79 cells, this range would start at 30 °C.) Furthermore, even in the same cells, the transitions of the inner mitochondrial membrane may be different from that of the outer mitochondrial membrane and from the plasmalemma (Hackenbrock et al., 1976). Hence, Arrhenius plots of the activity of a particular membrane-bound *enzyme* may show a break somewhere in the middle of the broad lipid phase transition if particular boundary lipids are involved with this particular enzyme. Therefore, enzyme studies do not tell us the *full* extent of the lipid transition. On the other hand, unless we obtain Arrhenius plots of *cell inactivation rates* of more cell types, we cannot be sure that the break in *survival* corresponds to the lower end of the liquid crystalline-to-gel mitochondrial membrane lipid phase transition, as observed by DSC, in every case.

The lipids of beef heart mitochondria have been shown by X-ray diffraction to undergo a broad transition between -10 and +10 °C (Gulik-Krzywicki et al., 1967). Similar broad reversible thermotropic phase transitions, centered at 0 °C, have been detected in rat liver mitochondria and microsomes by differential scanning calorimetry (Blazyk and Stein, 1972). The transition temperature of the isolated lipids is slightly lower than that of the membranes in both mitochondria and microsomes. In the membranes, no change in the transition occurs following irreversible protein denaturation (Blazyk and Stein, 1972).

However, a number of toxic compounds may induce gel phases in mammalian cell membranes (Packham et al., 1982).

It is possible that the composition of the membrane lipids, especially those of the mitochondria, are different in our cultured cells than in cells of intact animals as diet influences this lipid composition (Daum, 1985). The only lipid supplementation for our cells in culture is from components in the fetal calf serum, with which the medium is enriched. However, a liquid crystalline-to-gel membrane lipid phase transition has to occur in all cell membranes in all species if the temperature is lowered enough. Hence, a Range I and II is present in all cells, the boundary of which, in V79 cells, we correlate with the above transition, although the temperatures are likely to be different for each cell type.

Since detailed *survival* studies as a function of temperature have not been done with other cells, tissues, or organs, it is not known what actual temperatures Ranges I and II encompass in other cells. It is suggested that future investigations be interpreted in terms of Range I and II rather than in absolute temperatures. It is possible that in many clinically important tissues Range II starts at temperatures where water has already undergone a phase change to ice. (This may happen in some hibernators; Pehowich et al., 1988.) In this case, the researchers are always working in Range I. However, there are bound to be tissues where this is not the case and, hence, procedures which were successful in Range I may be ineffective or even detrimental when researchers are actually using tissues in temperature Range II. On the other hand, direct extrapolation of the cell studies to organ preservation can not always be made since experiments with kidneys suggest that there may be specific sites of damage on reperfusion following prolonged cold ischemia storage (Toffa et al., 1991).

VIII. SUMMARY

Classical hypothermia survival curves of single cells, on a semi-logarithmic plot, consist of a shoulder region followed by a straight line. The slope of the line is an inactivation rate and can be used in an Arrhenius plot. The Arrhenius plot of inactivation (killing) rates of V79 Chinese hamster cells exposed to hypothermia changes slope at approximately 7 to 8 °C, which corresponds to the minimum inactivation rate. This implies that there are distinct hypothermic damage mechanisms above (Range I = 8 to 25 °C) and below (Range II = 0 to 8 °C) this

temperature. The actual temperature ranges given above may turn out to be slightly different in various tissues and species. Survival experiments on the mouse fibroblast L-929 and human lung firbroblast cell line also indicate that there is a break in the Arrhenius plot of cell inactivation. This is also true for rate of leakage of hemoglobin from human red blood cells when plotted in Arrhenius fashion. Furthermore, replotting the data of van Dongen et al. for mouse neuroblastoma cells in an Arrhenius graph also results in a break at around 6 °C. Hence, a break in the Arrhenius plot of cell inactivation due to hypothermia is not unique to Chinese hamster V79 cells. Above 8 °C (Range I), the activation energy from the Arrhenius plot for control cells is about 15 kcal/mole. This value is in the range of temperature coefficients of metabolic processes (10-30 kcal/mole) and much less than that for protein denaturation. This suggest that probably unbalanced metabolism is the rate limiting step for hypothermic killing in Range I. Below 8 °C (Range II), the magnitude of the apparent activation energy is large (-61 kcal/mole), indicating a strong temperature dependence and implying the involvement of a membrane lipid phase transition or the cold denaturation of a critical protein.

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ON THERMAL STABILITY OF CATION GRADIENTS IN MAMMALIAN CELLS

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I. INTRODUCTION

This chapter is an inquiry—rather than a review—into a rarely asked question: Do mammalian cells regulate their cationic composition in the face of altered temperature? This question leads immediately to several others: Do mammalian cells regulate their cationic composition under any circumstances? Why should changing temperature constitute a challenge to cation gradients? What known mechanisms are available for compensation of ion balance with changing temperature?

The central paradigm of regulation of the gradients for Na^+ and K^+ in animal cells (and with them, Cl^- and cell volume) has been the pump-leak model, which postulates that high cell K^+ and low cell Na^+ are maintained by the activity of the Na-K pump offsetting the downhill movements of Na^+ into and K^+ out of the cell. This hypothesis had its inception in the 1940s, was developed further in the 1950s and reached its apotheosis with the classic paper of Tosteson and Hoffman (1960) that compared steady-state of "low-K" and "high-K" sheep red cells. Quantitative amplifications have occurred (Jakobsson, 1980; Stein, 1990) and various aspects of the history of this subject have been reviewed recently (Skou, 1992; Glynn, 1993; Hallows and Knauf, 1994). Over the past 20 years the pathways (channels and carriers) comprising the "leak" have been greatly elucidated and new pathways are being discovered apace (Figure 1). Many of these pathways are involved with rapid responses of cells to swelling or shrinkage, and this fact has modified the original, simple pump-leak concept without, however, eliminating it as the long-term governor of ultimate steady-state.

While the main focus of this discussion is Na^+ and K^+, similar considerations apply to—and indeed are difficult to separate from—the cellular management of H^+, Ca^{2+}, and Mg^{2+}. The regulation of these three cations is more complex because in part they all involve buffering by cytoplasmic components and, in the case of Ca^{2+} and H^+, sequestration within vesicular compartments. Membrane handling of H^+ is very much tied to regulation of Na^+ and Cl^- because the plasma membrane has a low permeability for H^+ and equilibrates excess H^+ produced by metabolism. This equilibration occurs mainly through an exchange with extracellular Na^+ and/or exchange of HCO_3^- with Cl^- through specific carriers. For Ca^{2+} there are both an ATP-dependent Ca^{2+} pump and Ca^{2+} channels, and to this extent its regulation resembles that for Na^+. However, in many cells (notably cardiac and skeletal muscle, nerve endings, liver) there is

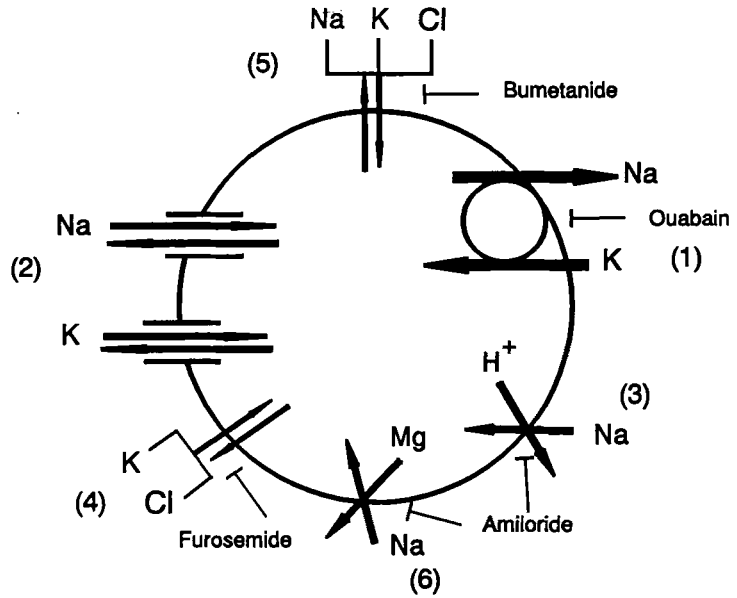


Figure 1. A selection of pathways for permeation of Na^+ and K^+ discussed in this chapter and found in rodent red cells. (1) The ATP-dependent *pumping of* Na^+ *out of and* K^+ *into* the cell (in a stoichiometry of 3:2, at least in red cells) offsets the passive loss of K^+ and gain of Na^+ through pathways of passive permeation (2-6). The Na-K pump is inhibited by ouabain and its activity in intact cells can be conveniently estimated as ouabain-sensitive K^+ influx. (2) The fundamental leak of ions is through channels, which in red cells are ill-defined. In untreated red cells it is not clear whether there are differentiated Na^+ and K^+ selective channels (as suggested by the figure) or undifferentiated "leak" pathways. In cells loaded with Ca^{2+}, a K-selective channel opens. Magnitude of basic leak path for K^+ (also called "residual leak") has been estimated as K^+ influx in the presence of ouabain and bumetanide (however, see Figure 3). (3) Na-H exchanger in some cells can be estimated as amiloride-sensitive Na^+ influx. It is normally quiet at 37 °C in isotonic conditions and unacidified cells. It is activated by cell acidfication and powerfully activated by cell shrinkage. (4) K-Cl cotransport operates to increase K^+ and Cl^- efflux under conditions of swelling in red blood cells. (In other cells, swelling activated K^+ and Cl^- channels achieve this effect.) The K-Cl cotransporter is relatively insensitive to concentrations of bumetanide that inhibit the Na-K-Cl cotransporter (5), but is inhibited by higher concentrations of loop diuretics such as furosemide. (5) The Na-K-2Cl contransporter is present in many cell types. In some cells it is activated by shrinkage and operates to carry solute into the cell. (6) The Na-Mg exchanger is less well-studied and understood than the other pathways shown here; it has properties of both an ATP-dependent pump and an exchange carrier. (There may be more than one pathway present confounding interpretation of results.) It is slightly activated by cell swelling (see Xu and Willis, 1994), and it is inhibited by amiloride.

also an Na-Ca exchange carrier, so that in these cases the regulation of the two ions is connected. Mechanisms of transport of Mg^{2+} are comparatively poorly understood, but the steady-state levels of this important metal ion is maintained below equilibrium, at least in part, by a Na-dependent carrier system that requires ATP, but may or may not hydrolyze it (Flatman, 1991; Xu and Willis, 1994).

II. CATION REGULATION IN THE FACE OF NONTHERMAL CHALLENGE

In its simplest form, the balance of pumps and leaks is a rather static concept; the question raised here is what provision the cell may have for compensating for challenges to the steady state: are cytoplasmic concentrations of Na^+ and K^+ truly regulated?[1] The first distinction that one ought to make here is between mechanisms that serve the whole organism by controlling cell activity and those that are concerned intrinsically with the housekeeping of the cell itself. For example, while thyroid hormone may increase Na-K pump activity and parallel Na-H exchange, thus increasing cell Na^+ turnover without impacting cell $[Na^+]$, this is clearly not a case of *cellular* regulation. Similarly, apparent examples of modification of membrane transport activity in response to challenges of the whole organism (e.g., K^+ depletion, Chan and Sanslone, 1969; starvation, Zhao and Willis, 1988; seasonal cold adaptation, Willis and Zhao, 1991) presumably represent tuning of cells by organismic regulatory systems.

Considerable evidence shows that cells do possess systems for monitoring ionic composition and entraining effector mechanisms that respond to long-term challenge to maintenance of Na-K gradients. In media with low $[K^+]$ or in conditions that artificially elevate cytoplasmic $[Na^+]$ with ionophores, mammalian cells in culture will respond by synthesizing increased Na-K ATPase molecules (Vaughan and Cook, 1972; Lechene, 1988; Unkles et al., 1988; Lyoussi and Crabbé, 1992).

While such findings indicate that there are mechanisms that monitor and regulate cytoplasmic ion concentration, the issue being raised here, impact of changing temperature, is one that generally requires a faster response than could probably be met by protein synthesis or alteration in rate of protein turnover. Analogous challenges that may be used for comparison are work load, metabolic (or pump) inhibition, and anisosmotic exposure.

A. Work Load

Challenge of ion balance by work load—or intensity of activity of the cell—occurs especially in excitable cells (nerve, muscle) and in cells carrying out transepithelial transport involving one of the regulated ions (absorbing or secreting epithelia).

In transporting epithelia, rate of Na-K pump activity at the basal surface often matches Na^+ entry at the apical membrane (through regulated conductive channels or cotransport carrier pathways) without large deviations in cell [Na^+], and increased K^+ channel activity at the basolateral surface may also defend against increased cell [K^+] concentration due to the greater pump activity (Schultz, 1989).

In working muscle, Na-K pump activity certainly increases in response to the elevation of cell [Na^+] caused by increased opening of voltage-activated Na^+ channels during excitation, but the form of the response appears to be no different from that of red blood cells (Sejersted, 1988). Catecholamines may enhance pump activity in muscle; it is not clear whether they affect the activation kinetics by Na^+ and K^+ or only the maximal capacity (Clausen and Flatman, 1977). More to the point of this inquiry, however, is the finding of Everts and Clausen (1994) that Na-K pump rate in *in vitro* preparations of directly stimulated rat muscle exhibit faster pump rate than unstimulated muscle, even in the absence of any measurable rise in cytoplasmic [Na^+]. The mechanism of this effect is not clear but could be as simple as rise in [Na^+] in the immediate local vicinity of the pump sites. Even given elevated pump rates, however, the kinetic compensation by the pump is insufficient to match increased Na^+ influx at high but still physiological rates of stimulation *in vitro* (Clausen and Everts, 1988) and *in vivo* (Sjøgaard et al., 1985). Failure to maintain the Na-K gradients has been suggested as the likely limiting factor causing exhaustion of muscle (Sjøgaard et al., 1985).

B. Slowing the Na Pump by Direct and by Metabolic Inhibition

Metabolic inhibition, according to the original pump-leak model, would affect ion balance and cell volume merely by the deprivation of ATP as the energy source for the Na-K pump (or Ca pump). Indeed, this presumption dominated thinking for decades regarding the deleterious effects of hypothermia and hypoxia. However, ATP was never shown to be a limiting factor in maintenance of cation gradients in hypothermic

mammalian cells, and several studies have demonstrated that ATP concentrations are not limiting in heart (Burlington et al., 1976), brain (Mendler et al., 1972), and red cells (Marjanovic and Willis, 1993; Marjanovic et al., 1993) at low temperature. In human and guinea pig red cells it has been shown that it is a decrease in *sensitivity* of the Na-K pump to ATP, not a decrease in ATP concentration, that contributes to excessive slowing of the pump with cooling. In guinea pig red cells, entry of Na^+ with cooling is less reduced than Na-K pump activity because of uncoupled influx through the Na-H exchanger. K^+ loss from guinea pig red cells at low temperature is aggravated by opening of the Ca-activated K^+ channel, apparently as a secondary result of loss of Ca^{2+} regulation (Hall and Willis, 1984).

With regard to hypoxia, Anderson et al. (1990) proposed that in hypoxic heart, damage occurs not primarily because of diminished ATP but rather as a result of the accumulation of H^+ due to increased glycolysis. Thus, lowered cytoplasmic pH stimulates uptake of Na^+ via the Na-H exchange carrier, which is inhibitable by amiloride and its more specific analogues. Accumulation of cytoplasmic Na^+ results in activation of Na-Ca exchange, and it is the consequent accumulation of cytoplasmic Ca^{2+} that causes the cell damage, especially during reperfusion when extracellular acidosis is relieved. Amiloride analogues protect against this reperfusion injury. (Lemasters and his coworkers, on the other hand, have found that acid perfusion conditions are also protective, a phenomenon dubbed the "pH paradox," not attributable to prevention of Ca^{2+} overload (Bond et al., 1993).)

While none of these observations speak for regulation of ion balance in the face of metabolic inhibition, they do show (a) that the energy limitation of the pump-leak model is not sufficient to account for failure in these physiologically relevant cases, and (b) that it is the linkage between—and possibly regulatory, albeit inappropriate, responses of—ion transport pathways that account for failure. Regulation in the face of temperature change is discussed further below.

Cells of mammals resistant to hypoxia have not so far been investigated with regard to maintenance of ion gradients. Hochachka (1986) has championed a hypothesis that in the face of hypoxia, adapted animals such as freshwater turtles, may reduce Na permeation pathways to reduce the load on the Na-K pump and conserve energy. Like the the freshwater turtle, evidence for this hypothesis has been slow to emerge. Lutz and his collaborators have demonstrated that preparations of hypoxic turtle brain do not exhibit extracellular accumulation of K^+ as do comparable

rat brain preparations and that there is a decrease in voltage-activated Na^+ channel activity in turtle brain cells (see Lutz, 1992). Buck and Hochachka (1993) have observed that ouabain-sensitive Rb^+ influx into cultured turtle hepatocytes is reduced by 70% under anoxic conditions even though ATP concentration and membrane potential are maintained. They have not so far offered data on cytoplasmic Na^+ concentration or unidirectional Na^+ fluxes, let alone specific Na^+ pathways.

One recent study suggests the kind of compensatory interaction between pathways of which mammalian cells may be capable (Doug et al., 1994). When the Na-K pump of a cell culture derived from hair cells is blocked with ouabain, the Na-K-Cl cotransport pathway is activated, and the increase in this avenue of K^+ influx offsets the blocked entry of K^+ through the Na-K pump. The activation of the cotransporter appears to be linked to the opening of K^+ channels, inhibitable by quinidine and apamine. The authors could not determine the linkage between pump inhibition and opening of the K^+ channels (Ca^{2+} activated K^+ channel perhaps?), but the net effect would have been to maintain membrane potential, minimize loss of K^+, and retard cell swelling.

Some such form of compensation may occur in guinea pig red cells when incubated with ouabain. Here, a large decrement in K^+ influx is found when ouabain is present, and from this, presumably pump-related, influx one can compute the rate of Na^+ influx which the pump balances (about 5-6 mmoles/l cells/h). Direct measurement of Na^+ influx gives a value in agreement. Yet the rise in cell [Na^+] concentration is much slower than these measurements of fluxes would indicate (Table 1). The explanation of this disparity between unidirectional fluxes and net concentration change is not clear, but since isotopic unidirectional fluxes are determined quickly (i.e., in less than half an hour), compensatory changes may occur over longer intervals.

C. Cell Volume

Regulation of cell volume in response to anisosmotic challenge (i.e., hypotonic swelling, hypertonic shrinkage) is currently one of the most active areas of investigation in cell physiology. During the 1970s and 1980s numerous pathways were discovered, usually initially in red blood cells (depicted in Figure 1), which (or homologues of which) have subsequently been tied to responses either to shrinkage (Na-H cotransporter) or swelling (K-Cl cotransporter) in other cells, notably transporting epithelia, lymphocytes, and ascites tumor cells. In addition, various

Table 1. Disparity Between Net Gain of Na^+ and Inhibition Of Na-K Pump in Guinea Pig Red Cells Incubated at 37 °C.

Duration of Incubation (h)	*(mmole/l cells/h)* Na-K Pump K^+ *Influx*	Na-K Pump Na^+ *Efflux*	Na^+ *Influx*	*(mM)* [Na+]*cell* *Observed*	*Expected*
0	2.5 ± 0.3	3.8	7.7 ± 0.4	7.7 ± 1.3	—
1				7.7 ± 0.3	11.4
2				8.3 ± 0.5	15.0
3				9.3 ± 0.4	18.5

Notes: Cells were incubated in medium with 150 mM NaCl, 5 mM KCl, 10 mM glucose, 5 mM adenosine, 10 mM MOPS buffer (pH 7.4). K^+ influx determined with ^{86}Rb serving as a congener of K was measured over 20 minutes in the first hour of incubation. Pump K^+ influx was taken as the difference between influx with and without 100 μM ouabain in the medium. Pump Na^+ efflux was taken as 1.5 times ouabain-sensitive K+ influx. Na+ influx was determined over the first 25 minutes of the first hour of incubation using 22Na. Uptake of isotope in the first five minutes was subtracted from uptake at 25 minutes to eliminate an early fast component. Observed cell Na^+ concentrations ($[Na^+]$cell) were determined by flame emission photometry. Expected $[Na^+]$ was computed from the measured Na+ influx and decremented in each hour in proportion to the computed decrease in gradient. The difference between observed and expected $[Na^+]$ are all highly significant, statistically ($p < 0.001$. K+ influx, Na^+ influx, and cell concentrations were obtained in separate sets of experiments. All mean values shown with S.E. represent averages of six or more experiments on cells of different individual guinea pigs.

stretch-activated K^+ and Cl^- channels have also been identified in several kinds of cells (Christensen and Hoffmann, 1992; Sackin, 1994). Finally, it is clear that osmotic balance is achieved in some cells by controlling their cytoplasmic concentration of osmotic ballast molecules such as taurine and other amino acids and polyols, such as sorbitol, by alteration either of their transport or of their synthesis and degradation (Burg, 1994; Yancey, 1994).

Many different molecular regulators of these pathways have been identified: phosphorylation by kinases, modulation by pH or Mg^{2+}, association with the cytoskeletal matrix. Hypotheses of the primary signal for activation range from simple stretch of the membrane to macromolecular crowding or dilution. These matters lie mostly outside the scope of this inquiry and in any case have been both extensively and intensively reviewed (Parker, 1993; Garner and Burg, 1994; Strange, 1994). Their existence serves here to make a point and to raise a question. The point to be made is that, collectively they represent the most persuasive evidence for the existence of specific and elaborate mechanisms for regulation of cell ionic composition or cell volume. In the case of shrinkage-activated Na-H exchange and swelling-activated K-Cl cotransport in dog red cells, there is persuasive evidence that their counter-

poised regulation operates through a common pathway (Parker, 1994). Another example of both the complementary balance between these mechanisms and their response to a natural, physiologically induced, isosmotic challenge is provided by rat salivary acinar cells (Foskett et al., 1994). In these cells cholinerigic stimulation causes a Ca^{2+}-mediated opening of K^+ and Cl^- channels and loss of K^+, Cl^-, and loss of up to 30% of cell water. These events are followed by a rise in cell [Na+] from 7 mM to 30-100 mM within a few minutes as a result of activation of the Na-H exchanger and the Na-K-Cl cotransporter pathways. Recent evidence suggests that the activity of the Na-K pump itself may also be governed by these mechanisms (activity and affinity for cytoplasmic Na^+ increasing with swelling, decreasing with shrinkage; Whalley et al., 1993).

On the other hand, the specific pathways employed are quite diverse among cell types, even though there seems to be a general pattern for cytoplasmic K^+ and Cl^- to be dumped with swelling and for Na^+ and Cl^- to be accumulated during shrinkage. The non-neoplastic models investigated have tended to be either epithelial transporting cells that lie between the isosmotic milieu interieur and highly variable "external" compartments (intestinal lumen, urine, body surface) or circulating cells (red cells, lymphocytes). So, the question arises of whether the mechanisms are general or whether they are specialized features of differentiated cells and if general, whether their role is limited to cell volume regulation.

In this regard it is of interest that Bedford and Leader (1993) found no osmotic accommodation in *in situ* diaphragm skeletal muscle, liver, or renal cortex in rats perfused with various hypertonic or hypotonic solutions and the accommodation that was observed in brain and cardiac muscle was far slower (hours to days) than in isolated cell preparations (minutes). Muscle, liver and renal cortex all exhibit apparent volume regulatory mechanisms when tested *in vitro*.

Macknight (1994) has pointed out that the anisosmotic challenges used in *in vitro* experiments are both more sudden and more extreme than would be met in usual physiological circumstances. He has suggested that the membrane transport pathways identified in drastic cell volume responses normally are part of a network that operates to maintain relative constancy of cytoplasmic ion composition. Addressing this same point, Hallows and Knauf (1994) have stated,

> ...since body fluid homeostatic mechanisms normally regulate the osmotic strength within narrow limits in most mammalian cellular environments, acute

> cell volume regulatory responses *in vitro* would be of questionable physiological significance. In fact, one might speculate that for many cells the capacity to regulate cell volume under anisotonic conditions may be ... [as one possibility] ... a fortuitous activation of transport pathways normally involved in other cell functions, such as acid-base balance or control of the membrane potential.

Credence is added to this interpretation by the fact that most of the carrier pathways implicated in mammalian cell volume regulation are also subject to activation or inhibition by cytoplasmic variables such as pH (Na-H exchanger) and oxidative state (K-Cl cotransporter), as well as cytoplasmic [Mg^{2+}], ATP or kinases, which could tie them to other cellular activities. Cala and Maldonado (1994) have recently confirmed that one membrane transport pathway (Na-H exchanger in *Amphiuma* red cells) can serve as the effector for two different regulatory processes (volume regulatory increase and realkalinization following acidfication) and have shown that priority is established on a first-come-first-served basis (i.e., shrinkage activated cells extrude H^+ regardless of cytoplasmic pH, acidified cells extrude H^+ regardless of cell volume).

Hallows and Knauf (1994) go on to say,

> ... some of the leak flux pathways involved in acute volume regulation (e.g., K-Cl cotransporter) may have tonic activity under steady-state conditions, serving to buffer a cell's volume against the normal slight shifts in ambient osmolality. Such mechanisms could also compensate for occasional imbalance between pump and leak fluxes due, for example to changes in the rate of Na-dependent nutrient uptake.

Thus, these pathways become prime candidates for consideration in regard to effects of temperature on ion balance and in regard to possible regulatory responses to altered temperature.

III. THERMAL CHALLENGE TO CATION BALANCE IN MAMMALIAN CELLS

Why should changing temperature be a problem to mammalian cells? To consider this question, we must first ask what is the extent to which mammalian cells face alterations in temperature, and then we must ask what the expected result would be of such change in the absence of compensation.

A. Thermal Experience of Mammalian Cell Membranes

Even though most cells of most mammals are maintained in relatively constant thermal conditions most of the time, there are numerous departures from constancy. Thus, cells in the periphery are often exposed to reduced temperatures; body temperature may drop several degrees in sleep and several tens of degrees in voluntary hypothermia of hibernation or nightly torpor. Cells of intact mammals may also experience hyperthermia either as the result of fever or as the consequence of sustained exertion in a warm environment. Temperature is therefore a variable in the life even of mammalian cells.

Deviations toward elevated temperatures have been speculatively connected with membrane activity in diverse and sometimes conflicting ways. For example, Kozak (1993) suggested that, since cytokines released in response to infection have a stabilizing effect on membranes (i.e., decrease fluidity), the functional significance of fever is that it allows cells to operate with normal membrane microviscosity. On the other hand, it has been proposed that during exercise-induced hyperthermia cells become leakier to Na^+, which would tend to elevate cell $[Na^+]$ and in turn drive the Na-K pump faster. Greater pump activity would have a feedback effect on cell metabolism (Whittam and Willis, 1963; Soltoff, 1986), leading in turn to increased heat production and the possibility of runaway increase in body temperature and heat stroke (Hubbard et al., 1987).

B. Effects of Altered Temperature on Cation Regulation

Most investigations of the effects of high or low temperature on membrane transport have focused on the failure of these systems at the thermal limits of normal function. Such studies may be broadly categorized between searches for a global cause of failure and studies of specific transport systems or functions. The global hypotheses may in turn be subdivided between the lipid fluidity hypothesis (see Hazel, Chapter 3) and membrane protein deactivation (for high temperature failure see Lepock, Chapter 7; Lepock, 1987).

At limiting high temperatures, most studies of membrane transport and cellular ion balance have been concerned with cell death in various lines of cultured mammalian cells. Burdon and Cutmore (1982), for example, found that in HeLa cells five minutes exposure to 45 °C resulted in 50% decrease in extractable Na-K ATPase, and Vidair and Dewey

(1986) found that 30 minutes exposure to 45 °C (sufficient to cause 98% subsequent cell death) caused a significant rise in cell $[Ca^{2+}]$.

The subject of this chapter, however, is the maintenance of ion balance at moderate and physiologically relevant temperatures. What then should be the expectation over such a moderate range? According to the classic pump-leak model, ion composition would remain unchanged only if the effect of temperature on leak was identical to its effect on Na-K pump rate. In particular, if decreasing temperature were to cause a slower decline in leak than in pump, then the pump would be challenged to catch up, and, similarly, if at higher temperatures leak pathways were to open up more readily than the pump, then again, in the absence of any compensation an imbalance would develop.

What is the likelihood that the intrinsic change in leaks with decreasing temperature will exactly match that of Na-K pumping? The foregoing discussion has illustrated that "leak" is now known to be a multiplex phenomenon, consisting of a variety of parallel carrier and channel pathways, the specific complement of which is rather cell-specific. Furthermore, apart from simple unregulated channels (if indeed such truly exist), most pathways of passive permeation are characterized by having regulatory cofactors (indeed that is how many of them were discovered). Consequently, temperature could well affect not only the rate process of permeation per se, but also the binding affinities of various ligands.

Its effect on the rate process of transport is itself not straightforward. In carrier-mediated systems, for example, the effect of temperature would be influenced by the extent to which the system allowed "slippage" (return of unloaded carrier from one face to the other), by the affinity and availability of acceptable ligands on the two faces, and by the ease of transit of the loaded carrier. Even for a supposedly simple channel, where one might naively expect a Q_{10} similar to that for free diffusion of water, energy barriers could exist in terms of interaction with fixed charges in the channels, and the kinetics of the opening and closing of the channel. Given these considerations, it seems unlikely that the sum of all K^+ and Na^+ passive permeation processes would vary with temperature in tandem with variation in rate of the Na-K pump.

What evidence is there regarding the effects of temperature on these several pathways? Hall and Willis (1986) found that for red cells of several mammalian species ouabain-and-bumetanide insensitive K^+ in-

flux ("residual leak" or passive K^+ permeability) decreased steeply between 37 °C and about 18 °C, but only very gradually with temperature at lower temperatures. In red cells of man (Stewart et al., 1980), guinea pig (Hall and Willis, 1984), dog (Elford and Solomon, 1974), and rat (Friedman et al., 1977; Harris et al., 1984) ouabain-insensitive K^+ fluxes actually tend to rise at temperatures below 12 °C. In red cells of most of these species this rise in K^+ influx at low temperature is attributable to opening of Ca^{2+}-activated K^+ channels (Hall and Willis, 1984, 1986), but this does not account for the rise in primate red cells.

In human red cells bumetanide-sensitive K^+ influx (presumably representing Na-K-2Cl cotransport) falls less steeply with cooling than does Na-K pump activity at temperatures between 37 °C and 18 °C and then parallels the fall in pump activity (Stewart et al., 1980). At temperatures above 37 °C the residual K^+ influx in the presence of ouabain and bumetanide rises steeply in guinea pig red cells, almost doubling between 37 °C and 41 °C (Figure 2). The steepness of this rise is even greater than that observed by Hall and Willis between 18 °C and 37 °C. The bumetanide-sensitive component (presumably Na-K-Cl cotransport) decreases with warming between 37 °C and 41 °C. Although the ouabain-and-bumetanide insensitive component is usually attributable to residual, or basic electrodiffussive, K^+ "leak" (representing passive or minimal P_K) under isotonic conditions, preliminary data indicate that at elevated temperatures swelling-activated K^+ influx is greatly enhanced and ouabain-bumetanide-insensitive K^+ influx does not increase with warming in hypertonic media (Figure 3), suggesting that even under isotonic conditions the K-Cl cotransporter may be turned on at temperatures above 37 °C.

Kolb and Adam (1976) found that in liver cells the temperature-sensitivity of potassium permeability was strongly dependent upon extracellular Ca^{2+} concentration. Thus, with 1 mM Ca^{2+} in the medium P_K was only 50% greater at 39.5 °C than at 25 °C, but with less than 0.1 mM Ca^{2+} in the medium P_K was threefold greater at 39.5 °C than at 25 °C. (Extracellular Ca^{2+} had little effect on the difference in P_K between 25 °C and 0 °C.)

In red cells of ordinarily cold-sensitive mammals, Na^+ influx falls gradually at temperatures below 37 °C (Kimzey and Willis, 1971b; Zhou and Willis, 1989), and the rise with cooling between 18 °C and 0 °C found in human red cells for K^+ influx also occurs for Na^+ influx in human cells. The decline with cooling is steeper in red cells of rodents adapted

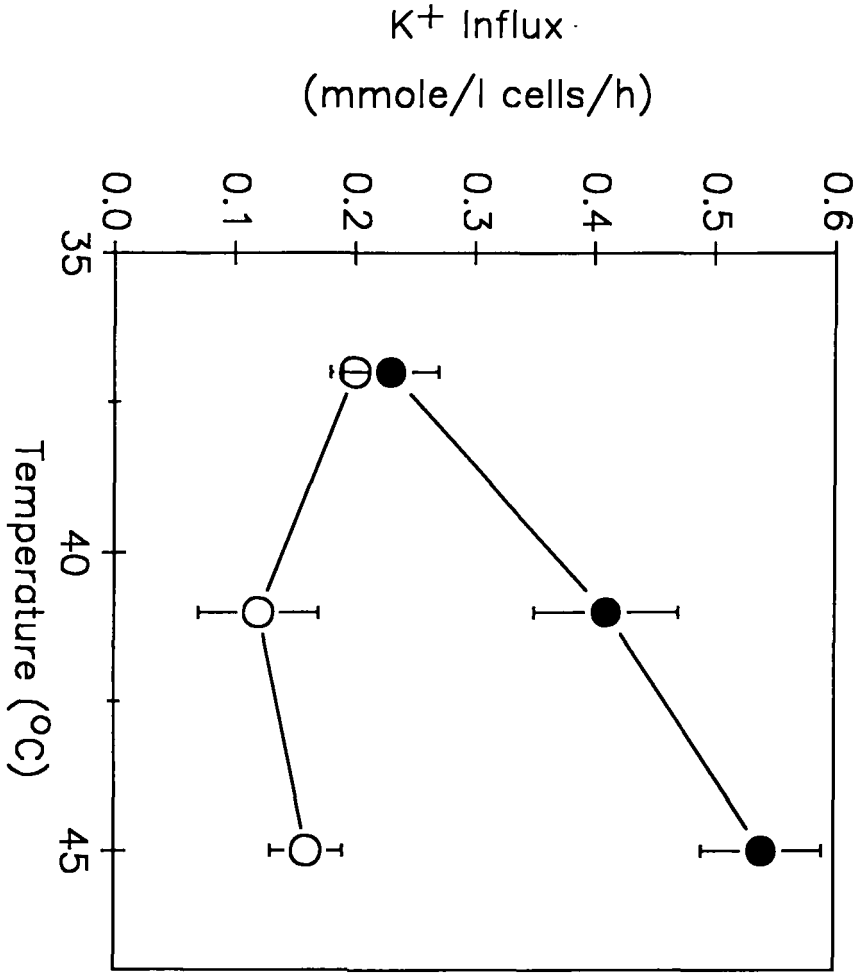


Figure 2. Differential effect of heating on two components of K^+ influx in guinea pig red blood cells. Cells were incubated as described in Table 1. Filled circles, ouabain-bumetanide-insensitive K^+ influx (so-called "residual leak") was measured as K^+ influx (see Table 1) in the presence of 0.1 mM ouabain and 0.4 mM bumetanide. Open circles, Na-K-Cl cotransport was measured as the difference between K^+ influx in the presence of ouabain and K^+ influx in the presence of ouabain and bumetanide. Means ± S.E. of seven experiments are shown. It appears that warming steeply increases residual K^+ influx, but see Figure 3.

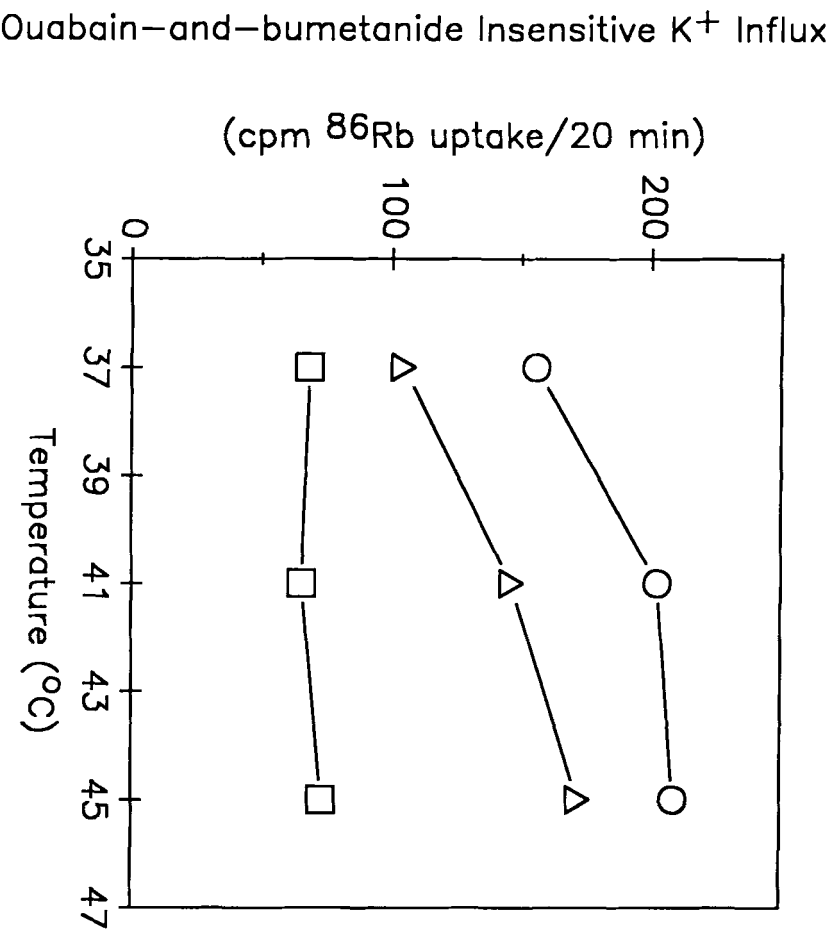


Figure 3. Effect of warming and anisosmotic incubation on ouabain- and bumetanide-insensitive K^+ influx. Cells were incubated at three temperatures in media with three widely different osmotic concentrations. Circles, hypoosmotic medium (200 mOsM); triangles, isotonic medium (300 mOsM); squares, hypertonic medium (510 mOsM). Hypotonicity was achieved by mixing isotonic medium with NaCl-free medium. Hypertonicty was achieved by adding sucrose to isotonic medium. All media contained 0.1 mM ouabain and 0.2 mM bumetanide. Note that in hypertonic medium there is no rise in K^+ influx with warming, whereas there are large increases in K^+ influx in hypotonic and isotonic media with warming. To state the matter differently, there is an osmotically responsive component in "residual influx" even in isosmotic conditions and this becomes larger at elevated temperatures. Results shown are for triplicate determinations of red cells from a single animal and are representative of two similar experiments.

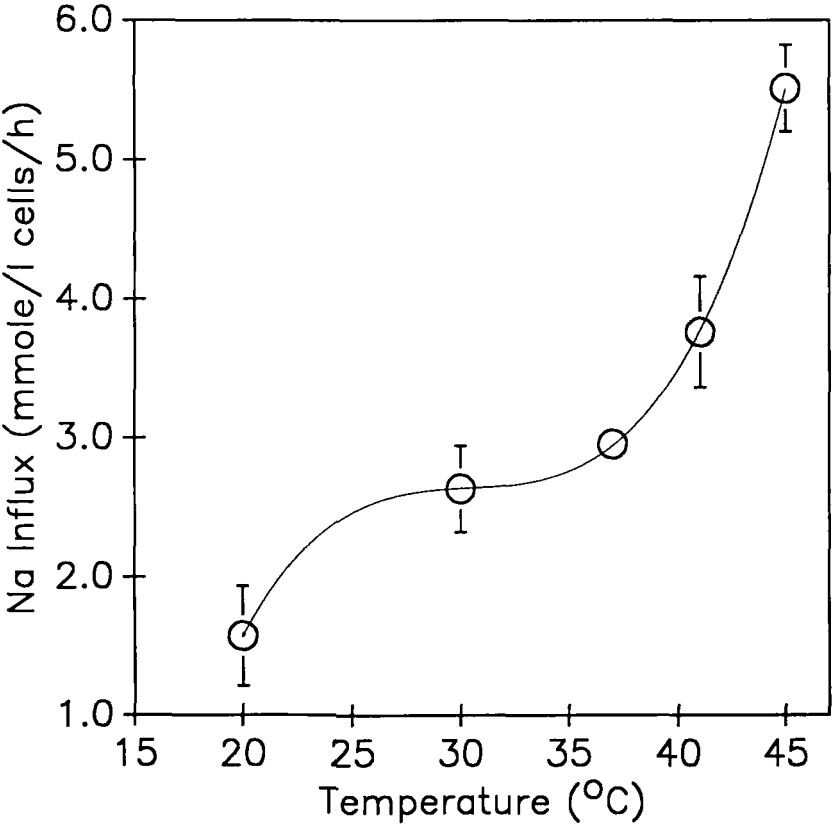
to low body temperature (ground squirrels, hamsters; Willis et al., 1989; Zhou and Willis, 1989).

Part of the difference in decline has been attributed to turning on of uncoupled Na^+ entry through the Na-H exchange pathway in guinea pig red cells (Zhou and Willis, 1989; Willis et al., 1989). This same effect was apparently already observed by Elford in 1975. He found that in isotonically incubated dog red cells, total Na influx increased with cooling from 39.5 to 20 °C, then declined at lower temperatures. When the dog red cells were incubated in hypertonic medium, thereby maximizing Na^+ influx, Na^+ influx declined with cooling from 39.5 °C to 0 °C (Elford, 1975). It is now recognized that the shrinkage-activated pathway in dog cells is the Na-H exchanger. Thus, Elford's results are consistent with the view that cooling activates this component of Na^+ entry. This pathway is not expressed in red cells of species showing steep decline of Na^+ influx with cooling such as ground squirrels, hamsters, and rat (Willis et a., 1992).

At temperatures above 37 °C, Na+ influx rises steeply in guinea pig red cells (Figure 4). There is no amiloride-sensitive Na^+ influx apparent in cells in the absence of any inhibitor, and the sensitivity and magnitude of shrinkage-activated increase of amiloride-sensitive Na^+ influx (Na-H exchange) is decreased with warming between 37 and 45 °C in guinea pig red cells (Figure 5). In rat hepatoma cells Boonstra et al. (1984) found that Na^+ influx followed a single Arrhenius slope between 32 and 44 °C that was parallel to that for ouabain-sensitive K^+ influx.

The effect of temperature on Na-K pump activity has been investigated in several kinds of preparation and in several contexts, yet seldom over a narrow, "functional" temperature range. The few studies in this category have been based upon measurements of Na-K ATPase activity in membrane preparations. These indicate a steep reduction of activity with cooling between 37 and 0 °C, and, when the data are plotted as an Arrhenius curve, there is usually a break in the function and a still steeper decline at temperatures below 13-15 °C (e.g., human red cells, Wood and Beutler, 1967; rat brain synaptosomes, Bowler and Tirri, 1974; for review see Willis et al., 1981). (A point that will be discussed more fully below, however, is that inhibition of Na-K ATPase activity of broken membrane preparations by cooling below 37 °C probably is often much greater than observed in comparable whole cell preparations.)

There have been few studies of the effect of temperatures above 37 °C. Bowler and Tirri (1974) investigated the full range from 55 to 5 °C. In



the range corresponding to temperatures above 30 °C, their Arrhenius plot showed a reduced slope. Between about 37 and 48 °C they observed a doubling of activity. This magnitude of change accords well with those based on ion flux studies in Chinese hamster ovary (CHO) cells (Bates and Mackillop, 1985), but is considerably greater than that observed in guinea pig red cells (Table 2), and rat hepatoma cells (Boonstra et al., 1984). Above 48 °C Na-K ATPase activity in the rat brain synaptosomes declined steeply as did ouabain-sensitive K+ influx in CHO cells above 45 °C, presumably in both cases because of denaturation.

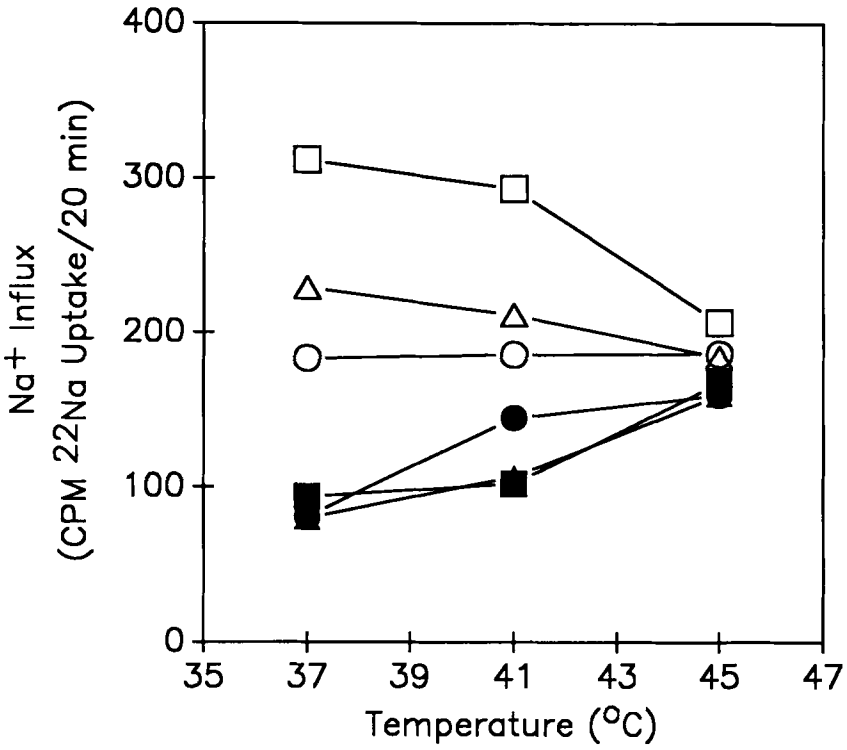


Figure 5. Effect of higher temperature on shrinkage-activated Na^+ influx in guinea pig red cells. Cells were incubated and Na^+ influx determined as described in Table 1 at three temperatures and three hypertonic osmolalities (410 mOsM, circles; 450 mOsM, triangles; and 510 mOsM, squares) and with (closed symbols) or without (open symbols) 1 mM amiloride (see Figure 1). Tonicity was increased by adding sucrose to isotonic medium. The value of 410 mOsM was chosen based on preliminary experiments that showed that this was the lowest osmolality at which an easily observable increase in Na^+ influx could be observed. Amiloride-insensitive Na^+ influx (closed symbols) increases with warming, but shrinkage-activated and amiloride-sensitive Na^+ influx (difference between control and amiloride-exposed cells) decreases with warming. Results represent means of triplicate determinations on red cells of a single animal and are representative of two similar experiments.

Thus, the anticipated problem for ionic balance does appear to manifest itself in some cases: fundamental leaks appear to decline less steeply than pumps with cooling and rise more steeply with warming. However, there are interesting disparities in behaviors of specific pathways, some of which might contribute to thermal compensation.

Table 2. Stability of $[K^+]_{Cell}$ and $[Na^+]_{Cell}$ in Guinea Pig Red Cells at Elevated Temperatures and Balance Between Na-K Pump and Na^+ Influx

Identification and Condition	*37 °C*	*41 °C*	*45 °C*
		(mM)	
$[K^+]_{cell}$, initial	83 ± 3	—	—
$[K^+]_{cell}$, after 2 hours incubation	81 ± 3	80 ± 3	78 ± 3
$[Na^+]_{cell}$, initial	7 ± 1		
$[Na^+]_{cell}$, after 2 hours incubation	7 ± 1	7 ± 1	7 ± 1
		(mmole/l cells/h)	
Measured ouabain-sensitive K^+ influx	2.5 ± 0.3	2.9 ± 0.4	3.0 ± 0.4
Computed Na-K pump efflux of Na^+	3.8	4.4	4.5
Measured Na^+ influx	3.8 ± 0.7	4.3 ± 0.4	5.4 ± 0.5

Notes: Details of incubation and analysis are provided in Table 1. Data for cell concentrations and for ion flux determinations are obtained in separate experiments. Mean values for observations are based upon results from six or more individual guinea pigs.

IV. IS THERE THERMAL COMPENSATION OF ION REGULATION IN MAMMALIAN CELLS?

We return, now, to the original question, whether mammalian cells possess mechanisms that compensate for changes in temperature so as to maintain ion balance and volume regulation. Since this is an issue that does not seem to have been directly addressed in these terms before, the search must be among data that were obtained in pursuit of answers to other questions. The starting point should logically be simply the measurement of ion status as a function of time and temperature over a moderate range. This information should then be supplemented by determinations of whether opposed unidirectional fluxes into and out of the cell are in balance. Following this, a further step should be to consider differential responses of specific pathways to changes in temperature, as a clue to what the participants might be in any game of thermal compensation.

A. Ion Balance

At temperatures below 37 °C, measurements of Na^+ and K^+ content have seldom been carried out over finely graded ranges. Usually the intent has been to determine the effects of profoundly hypothermic temperatures (i.e., near 0 °C). Human red blood cells have been investi-

gated exhaustively in this way for the sake of improved blood banking. Cells of hamsters and ground squirrels held at 5 °C for five days lose only 10-12% of their K^+, corresponding to the loss *in vivo* during a bout of hibernation with a body temperature of 5 °C (Kimzey and Willis, 1971a). In red cells of ground squirrels stored at 5 °C the gain of Na^+ only just matches the loss of K^+ for up to nine days (Zhao and Willis, 1989). The ratio of K^+ influx to K^+ efflux in ground squirrel red cells was found to be 1.0 at 30 °C, to drop steeply at higher temperatures (to 0.3 at 37 °C), and to fall gradually at lower temperatures (to 0.65 at 6 °C). In guinea pig red cells the ratio of K+ influx to K+ efflux was 1.0 at 37 °C and dropped to 0.65 at 20 °C (Kimzey and Willis, 1971b). The ratio of Na+ influx to Na^+ efflux at 37 °C was found in a similar study to range between 0.8 and 1.3 among red cells of several species of mammal: at 5 °C the ratio was 1.2 for hamster red cells; 1.7 for ground squirrel cells; and 3-5 for rat, gray squirrel, human, and guinea pig red cells (Willis et al., 1989).

In cells of primary cultures made from kidney cortex of hamsters and ground squirrels there was less than a 10% decrease in cytoplasmic [K^+] in four hours at 5 °C, and measured unidirectional influx was the same as unidirectional efflux of K^+ over the same period (Zeidler and Willis, 1976). Cultured cells from guinea pig kidney lost 50% of their K^+ in one hour at 5 °C due to a decrease in K^+ influx of about 80% compared with only a 50% reduction in K^+ efflux (Zeidler and Willis, 1976). Although guinea pig kidney cells cannot maintain a K^+ gradient at 5 °C, Mudge (1951) found that rabbit kidney slices maintained a high gradient of K^+ between 12 °C and 38 °C with the optimal temperature being 25 °C. This was confirmed in a later study of K^+ uptake by leached slices of rabbit kidney cortex—a broad peak of favorable temperatures from 15 °C to 38 °C with 25 °C as the optimum; all net uptake was blocked by ouabain (Willis, 1968). (Decline in K^+ content of incubated kidney slices at 38 °C compared with 25 °C was attributed to cell damage due to oxygen limitation in both studies.)

No change in cell [Na^+] or [K^+] was observed during a half hour exposure to 42 °C in rat hepatoma cells (Boonstra et al., 1974) nor during a 15 minute exposure in CHO cells (Stevenson et al., 1983). In the rat hepatoma cells rise in ouabain-sensitive K^+ influx matched the rise in Na^+ influx between 32 °C and 44 °C so that an apparent 3:2 stoichiometry was maintained throughout. Rise in K^+ efflux between 37 °C and 42 °C was twofold, the same as ouabain-sensitive K^+ influx, so that the two remained in balance. In CHO cells, both Stevenson et al. (1983) and

Bates and Mackillop (1985) found no increase in ouabain-insensitive K^+ influx between 37 °C and 42 °C. Similarly, Bates and Mackillop (1985) found no increase in ouabain-insensitive Rb^+ influx between 34 °C and 45 °C, but Rb^+ efflux increased between 31 °C and 40 °C in about the same proportion as the pump rate. (However, between 40 °C and 45 °C pump rate continued to increase whereas Rb^+ efflux declined.) In guinea pig red cells incubated at 37 °C, 41 °C or 45 °C there is no statistically significant change in cell [K^+] or cell [Na^+] within two hours (Table 2). At 41 °C, but not at 45 °C, the rise in rate of ouabain-sensitive K^+ influx from 37 °C just matches the rise in rate of Na^+ influx (Table 2).

Thus, results based on several different cellular models, and on flux ratio as well as on ion content, suggest that the ability to maintain ion balance at lower temperatures is very diverse and the limits poorly defined in most cases except with respect to the extreme cases of cold-tolerant species (i.e., hibernators). At moderately elevated temperatures (i.e., up to 44 °C) mammalian cells appear to maintain ion balance at least for short intervals.

B. Differential Effects of Temperature on Transport Pathways

Pump

Studies of activity of Na-K pump by isotopic fluxes in intact cells indicate a somewhat lower absolute temperature-sensitivity of the Na-K pump than suggested by enzymatic activity of broken membrane preparations. Thus, for example Wood and Beutler (1967) found that Na-K ATPase of activity of broken cells at 4 °C was 0.01% of that at 37 °C, whereas Stewart et al. (1980) found that ouabain-sensitive K^+ influx in intact human cells at 6 °C was 1% of that at 37 °C. A direct comparison between Na-K ATPase of broken cells and active K^+ flux in intact cells in red cells of guinea pig, ground squirrel, and woodchuck (Ellory and Willis, 1976) and cultured kidney cells of guinea pig and ground squirrel (Willis et al., 1980) showed the same sort of disparity.

In the light of the discussion above, it seems worth considering that the reduced sensitivity of the pump to cooling, observed in intact cells relative to that of the cell-free Na-K ATPase, represents a regulation of pump activity in the face of falling temperature. One obvious possibility would be the simple kinetic activation by rising cell [Na^+] combined with increased affinity of the pump for cytoplasmic Na^+ and extracellular K^+ (Ellory and Willis, 1982). This seems to be ruled out by the slowness of

change in [Na^+] in red blood cells and by the observation that resealed ghosts of red cells with pump rate maximized by high cytoplasmic [Na^+] showed the same sensitivity to cooling as intact cells (Willis et al., 1980). Any regulation that would thus anticipate change in cytoplasmic [Na^+] would have to be less simple and less direct.

Passive Permeation

Noted above were several cases of differential effects of temperature on various pathways of passive permeation:

1. Cooling turns on entry of Na^+ through the Na-H exchange pathway in dog and guinea pig red cells. In guinea pig red cells warming suppresses this pathway even in the presence of shrinkage.
2. In guinea pig red cells warming (at least to 41 °C) decreases K^+ influx through the Na-K-2Cl pathway while increasing K^+ influx through the "residual leak" pathway. At reduced temperature in human and ground squirrel cells the relative influx through this pathway is increased.
3. In hypotonically incubated guinea pig red cells, warming activates K^+ entry (and presumably exit) through the K-Cl cotransport pathway.

Finally, the results of Stevenson et al. (1983) and of Bates and Mackillop (1985), taken together, seem to show that in CHO cells warmed to 42-43 °C K^+ efflux increased to match rise in ouabain-sensitive K^+ influx *with no increase in ouabain-insensitive K^+ influx* (i.e., a pathway selectively favoring efflux is turned on as opposed to a simple diffusive pathway). The combination of all these observations offers an intriguing interpretation: cells respond to warming as they do to swelling and they respond to cooling as they do to shrinkage.

Of course, such effects could merely be the by-products of the effect of temperature and part of the pathology of ion imbalance with temperature change. Conceivably, for example, Na-H pathway activation, which occurs because of apparently increased sensitivity of the cytoplasmic regulatory site for H^+, might be a reflection of the increased H^+/OH^- ratio at constant pH with lowered temperature. Similarly, activation of K-Cl cotransport might merely be a reflection of enhancement of oxidative damage at elevated temperatures.

Is there a way that such changes could be viewed as preserving ionic balance or cell volume regulation? None is apparent if one adheres strictly to a simple pump-leak hypothesis. If, however, one also chooses to consider the balance *between* Na^+ and K^+ *leak*, then a plausible explanation may emerge. Let us assume (a) that pump stoichiometry is fixed and (b) that the effect of change of temperature on fundamental Na^+ permeability (P_{Na}) is much steeper than its effect on fundamental K^+ permeability (P_K). (In this context P_K and P_{Na} refer to the diffusive pathway, 2 in Figure 1.) Under these assumptions the cell's problem with cooling is that it will lose K^+ and shrink. With warming, the cell's problem would be that with increased Na-K pump activity it will gain K^+ and swell. Under this scenario the observed differential responses make sense: increased Na^+ uptake with moderate cooling would promote Na-K pump activity and also would preclude shrinkage; with warming increased K-Cl dumping would balance increased Na-K pump activity driven by temperature.

While there is no experimental basis for this scenario, it accords with the patterns described above. Thus, Na^+ influx is steeply temperature dependent in guinea pig red cells when influx through the Na-H pathway is blocked by amiloride (Zhou and Willis, 1989). Furthermore, the large increase in ouabain-insensitive K^+ efflux observed in CHO cultured cells accords with an activation of K-Cl cotransport in guinea pig red cells and suggests that the steep decline in ouabain-bumetanide K^+ influx observed by Hall and Willis (1984, 1986) included a component of K-Cl cotransport down to 18 °C. Consequently, the Q_{10} for P_K in the red cells, like that in the CHO cells at high temperature, would be well below 2.

The stability of ion gradients with significant departures in temperature, combined with the observation of divergent effects of temperature on components of passive permeation, demonstrate that balance of pump and leak with change in temperature is a rich subject for exploration.

V. SUMMARY

The classic pump-leak hypothesis would lay most of the burden of regulatory compensation on the Na-K pump. Pump activity can be rapidly modified in the face of changing cell concentrations, temperature, or volume through a variety of mechanisms including kinetic response to cell $[Na^+]$ (Lechene, 1988), change in affinity for cell Na^+ (Ellory and Willis, 1986; Whalley et al., 1993), or modification of number of pump sites (Clausen and Everts, 1994). Today, however, we

recognize that mammalian cells also possess a rich assortment of regulated passive permeation mechanisms for Na^+ and K^+. Some link the movements of the two ions, some link movement of cations to movement of anions, some link movement of monovalent cations to movement of divalent cations and some link movement of monovalent ions to H^+ or to pH regulation.

Regulatory compensation of the activity of these pathways to preserve steady-state concentration of Na^+ and K^+ or cell volume has been demonstrated or inferred for several kinds of challenge. Because past interest in the effects of temperature has focused on failure of ion regulation at either very low or very high temperature, evidence for compensation in the face of more moderate thermal challenges in mammalian cells has seldom been sought. Nevertheless, the available evidence, though sketchy, suggests that even stenothermic mammalian cells can maintain ion gradients, at least for short intervals, over a range of temperatures possibly as wide as 25 °C. This capacity very likely involves more than just balancing the activity of the Na-K pump against the rate of leakage of Na^+ or K^+; it may also be necessary for cells to compensate for a difference in the effect of temperature change on fundamental P_K and on P_{Na}. This possibility might provide a physiological role for the observations that Na-H exchange pathway is activated by cooling and that the K-Cl cotransport pathway (in red cells, possibly K^+ channels in other cells) are opened with warming. Accordingly, prime candidates as effectors for thermal compensation are these already known, regulatable passive permeation pathways.

NOTE

1. Many authors refer to this as cellular homeostasis. This is a regrettable term for several reasons. Too often it is taken as a given that there is a "homeostasis" of a cellular component—pH, calcium, even membrane lipid composition—without recognition of the need to demonstrate this. The term, homeostasis, as coined by Walter B. Cannon, applied to the internal environment of the organism, a concept which explicitly excludes the cytoplasm. Cells can contribute to the constancy of the internal environment either by controlling input to—and output from—the local external environment or by serving as sinks and sources themselves. Thus, true homeostasis is concerned with feedback mechanisms controlling input and output, not just with buffering. Homeostasis, therefore, does not relate to constancy of the cytoplasm nor even necessarily envision it. Nor does constancy of any constituent in a compartment, even if demonstrated, necessarily imply true homeostasis. To degrade the term to a mere synonym for "regulation" or "control" thus destroys its well-defined and useful meaning. Macknight

(1994) has acknowledged the risk of extending the term to the cellular level, but uses the term to imply the interlinkage of regulation of diverse factors, pH, [Ca2+], and so forth

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PROTEIN DENATURATION DURING HEAT SHOCK

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I. INTRODUCTION

All cells and organisms respond to supraoptimal temperatures (heat shock or hyperthermia) in a number of specific ways depending on the severity of the heat shock. The range of temperatures which can be defined as hyperthermia varies depending on normal growth or body temperature. For mammalian cells, which will be the primary topic of this review, temperatures in excess of 40-41 °C constitute hyperthermia and result in a heat shock. Mild heat shock causes an inhibition of DNA, RNA, and protein synthesis (McCormick and Penman, 1969; Henle and Leeper, 1979). Upon return of mammalian cells to 37 °C, protein synthesis returns to normal levels but the pattern of synthesis is transiently altered. The synthesis of a class of proteins referred to as heat shock proteins (hsps) or stress proteins is greatly enhanced (for reviews see Craig, 1985; Nover, 1991).

The enhanced synthesis of hps has been termed the heat shock response, however, there are numerous other changes in cellular physiology during and following exposure to elevated temperature (for a review see Laszlo, 1992). Both glycolysis and respiration are inhibited by heat shock (Dickson and Calderwood, 1979). There are major alterations in the organization of the cytoskeleton (Laszlo, 1992) with subsequent rounding, blebbing, and other changes in cell morphology (Lin et al., 1973). Alterations in nuclear morphology also occur, primarily in the nucleolus (Simard and Bernhard, 1967). Isolated nuclei and chromatin have a dramatic increase in the protein-to-DNA ratio, indicating an increase in the strength of the interaction of proteins with the nucleus (Tomasovic et al., 1978; Roti Roti et al., 1979). Major alterations in membrane function are also observed (for reviews see Lepock, 1987;

Laszlo, 1992). The general conclusion from these observations is that the damage from heat shock is not localized in a few components, but many cellular components and processes are affected.

In addition to multiple effects on cellular physiology, relatively short exposure to 41-45 °C kills mammalian cells, sensitizes them to ionizing radiation and many chemotherapeutic agents (Hahn, 1982) and induces a state of resistance, termed acquired thermotolerance, to subsequent heat treatment (Gerner and Schneider, 1975). A great amount of information has been acquired over the last 10 to 20 years on the effects of hyperthermia on cells. Extensive protein denaturation has been demonstrated to occur in mammalian cells during exposure to 43-45 °C for moderate periods of time, numerous cellular functions damaged or inactivated have been identified, and much has been learned about how hsps might be involved in acquired thermotolerance. However, at the present time the initial critical targets of heat damage have not been identified. Thus, the specific mechanisms of hyperthermic cytotoxicity are not known, and there is lack of agreement as to the most likely targets.

The general approach that will be employed in this review to try to explain these multiple cellular responses is to postulate that cells contain numerous components sensitive to heat shock, each with a different sensitivity to inactivation by elevated temperature. Thus, each thermolabile component can be thought of as a target whose inactivation results in a detrimental alteration in cellular physiology which is observable as a change in cellular behavior. This approach leads to the prediction that many, if not all, cellular effects of hyperthermia are due to inactivation of thermolabile, subcellular components, either molecular or supramolecular, which can be viewed as critical targets. Thus, identification of mechanisms of thermal cytotoxicity reduces to identification of the critical targets followed by a determination of the secondary responses to the initial inactivation ultimately resulting in a final cellular response.

Hyperthermia may also result in activation. For example, the phosphorylation of hsp27 is activated by heat shock, and the proposal has been made that signal transduction systems may be activated inappropriately during stress (Landry et al., 1992). Activation of lysosomal enzymes has long been considered as a deleterious effect of hyperthermia but this does not appear to be true (Tamulevicius and Streffer, 1983).

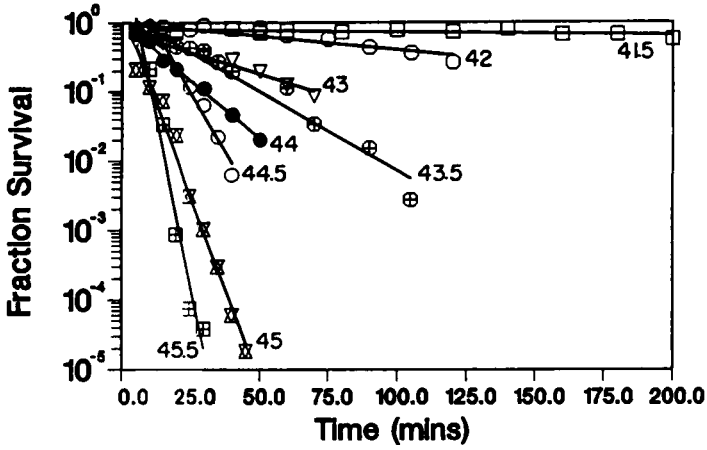
Three terms must be clearly defined at this point: inactivation, denaturation, and irreversibility. Inactivation is defined as the irreversible loss of biological function of any macromolecule. The loss of enzyme catalytic activity has been most extensively studied. There are at least seven

major causes for the inactivation of proteins: (a) aggregation, (b) thiol-disulfide exchange, (c) chemical modification of sensitive groups, (d) cleavage of disulfides, (e) dissociation of prosthetic groups, (f) dissociation of oligomeric proteins, and (g) irreversible conformational changes (Mozhaev and Martinek, 1982; Zale and Klibanov, 1986). These processes are not independent since the first six processes often occur only after the major conformational change of reversible unfolding and consequently lead to irreversibility. By definition inactivation is irreversible. The term denaturation has been used in many ways, which sometimes leads to confusion. A reasonably precise definition has been proposed: "Denaturation is a conformational alteration of a biological macromolecule which entails a reversible or irreversible loss of its ability to perform a certain biological function" (Kushner, 1977). Thus, denaturation and inactivation are not synonymous since denaturation involves a major loss of native structure while inactivation can occur by chemical modification alone. In addition, as will be discussed below in more detail, the conformational change associated with denaturation is a first order, all or none transition from the native to a disordered state which can range in structure from a random coil to the molten globule state. Irreversibility is a relative term meaning that activity or structure is not regained for a long period of time with respect to the time of interest. The reason for irreversibility may be kinetic or thermodynamic.

II. CELL KILLING BY HYPERTHERMIA

A. Clonogenic Survival and Arrhenius Analysis

Considerable information about the response of mammalian cells to hyperthermia has been obtained from measurements of cellular thermal sensitivity, usually by survival assays based upon clonogenic capacity. Survival curves for Chinese hamster lung (CHL) V79 cells from 41.5 to 45.5 °C are shown in Figure 1a. These curves are similar to many others obtained previously for other cell lines (e.g., Westra and Dewey, 1971) and are characterized by a shoulder and an exponential region (see Kruuv, Chapter 5, for a detailed description of survival curves). In addition, below a critical temperature, usually 42-43 °C but dependent upon cell sensitivity, a plateau in survival occurs at longer heating times. This is indicative of the induction of thermotolerance during heating, usually referred to as chronic thermotolerance to distinguish it from acute



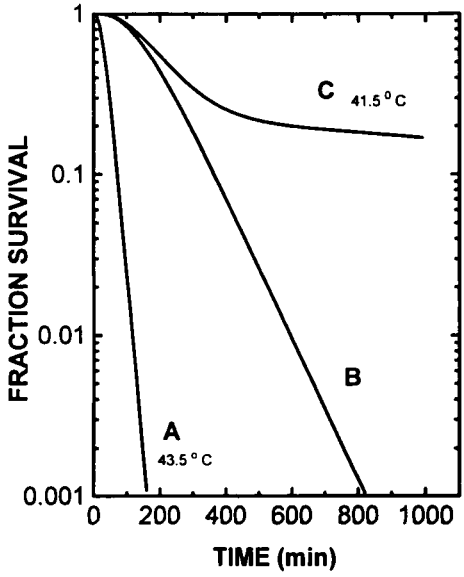


Figure 1. (A) Clonogenic survival curves of CHL V79 cells heated in suspension at temperatures from 41.5 to 45.5 °C (from Lepock et al., 1990a). (B) Model survival curves generated from the equation $S(t) = 1 - [1 - (1 - k)^t]^n$ for $n = 2$ and $k = 0.05$ min^{-1} (curve A), $k = 0.01$ min^{-1} (curve B), and $k = 0.01$ min^{-1} for 80% of the cells and $k = 0.001$ min^{-1} for 20% of the cells (curve C).

thermotolerance (i.e., tolerance that develops at 37 °C following acute heat shock). Chronic thermotolerance is observable for the 41.5 °C curve at exposures greater than 200 minutes (results not shown). In addition, the slope of the exponential region is decreased by chronic thermotolerance below 42-43 °C. There is no convincing evidence that the mechanisms of acute and chronic thermotolerance differ.

Survival in the exponential region can be approximated by

$$S(t) = So\, e^{-kt}$$

where k is the rate of killing which corresponds to an inactivation rate, $S(t)$ the number of surviving cells at time t, and S_o the number of cells at $t = 0$. This suggests that a direct correlation can be made with the inactivation of a molecular target since inactivation of enzyme activity by hyperthermia obeys similar kinetics (Mozhaev and Martinek, 1982).

In the shoulder region survival decreases less than exponentially for short times of exposure. If damage is proportional to dose, which for hyperthermia is dependent on both temperature and time of exposure, the presence of sublethal damage is implied, which can be interpreted in various ways depending on the model one uses. One interpretation is that this implies the existence of multiple targets or multiple copies of a target, all of which must be inactivated before death ensues.

Model survival curves to illustrate these characteristics were generated using the multitarget model of Hahn (1982) and are shown in Figure 1b. The value of the inactivation rate is 0.05 min^{-1} for curve A. This corresponds to the rate of killing of V79 cells at 43.5 °C. The shoulder was generated assuming a target number (n) of 2 which gives a shoulder (referred to as D_q) of 13 minutes. Curve B corresponds to inactivation at 41.5 °C without the induction of thermotolerance. Curve C models a survival curve in which thermotolerance is induced in 20% of the cells yielding a resistant tail composed of cells that are 10-fold more resistant to killing. This closely matches the actual survival curve measured at 41.5 °C for V79 cells.

Arrhenius plots of k (Figure 2) have a sharp bend at 42-43 °C (Leith et al., 1977), due to the induction of chronic thermotolerance during the survival assay, but there is evidence they are linear for temperatures above this critical temperature to at least 57 °C (Borrelli et al., 1990). The linear relationship between ln k and $1/t$ implies that the temperature dependence of the rate constant for killing can be approximated by

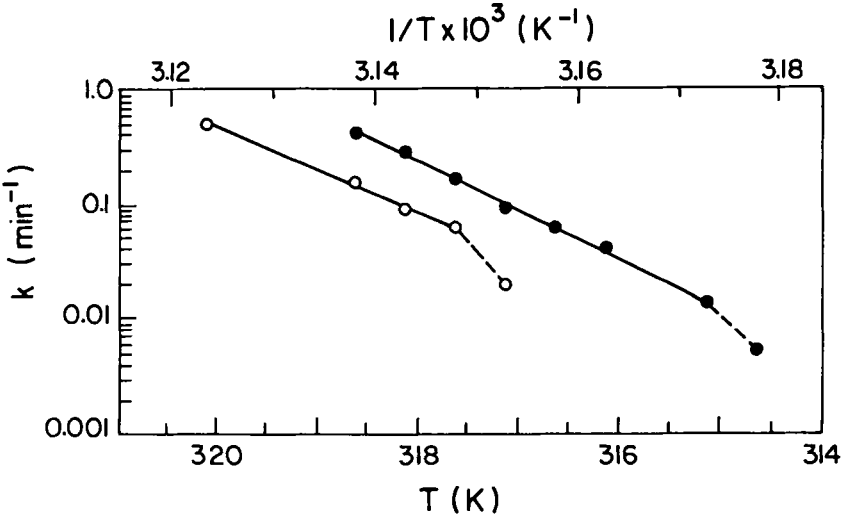


Figure 2. Arrhenius plots of cell killing (ln k versus $1/T$) for control (solid circles) and thermotolerant (open circles) CHL V79 cells. Thermotolerance was induced by a 10 minute exposure to 43 °C followed by two hours at 37 °C. The values of E_a and A obtained from these curves was used to predict the transition profiles of the critical targets shown in Figure 12 (from Lepock et al., 1990a).

$$K(T) = e^{A\text{-}Ea/RT}$$

where E_a is the slope and is referred to as the activation energy. Two interpretations of E_a are possible. The less mechanistic interpretation is that E_a is just a measure of the temperature dependence. A more specific interpretation relates cell killing to the inactivation of a molecular target: E_a represents the energy barrier for formation of the transition state of the critical target during the rate limiting step of inactivation. Westra and Dewey (1971) have suggested that the very high value of E_a (150-200 kcal/mole) for hyperthermic cell killing implies that protein denaturation is the rate limiting step.

B. Models of Cell Killing by Hyperthermia

Only a few models of cell killing suitable for fitting survival curves have been developed for hyperthermia. Some information about poten-

tial mechanisms of killing can be obtained from models. However, the assumptions inherent to the model are very important and must be consistent with experimental data. Hahn (1982) presented a multitarget model similar to those used for killing by ionizing radiation based on the assumption of n targets per cell, each requiring a critical kinetic energy (E_c) for inactivation. What constitutes a target is not defined. If the kinetic energy is distributed among the targets according to the Boltzman distribution, then the rate of inactivation of any target is given by

$$k\,(T) = e^{-Ec/RT}$$

where E_c is the critical kinetic energy for that target. The probability (P_c) of a cell surviving an exposure for time t to a temperature T is

$$P_c\,(S) = 1 - [1-(1-k)^t]^n$$

This model can fit the usual hyperthermic survival curves with a shoulder and an exponential region and gives values of n between 1 and 30. Since the molecular definition of a target is unclear, the exact meaning of n is unclear. If one accepts the simplest definition, that a target represents a single protein, then this model suggest that it is unlikely that the critical target is a simple protein, which would be present in more than the few copies per cell implied by n. Thus, it is more likely that the target is a complex structure.

Jung (1986) has developed the lesion conversion model based on the assumptions that nonlethal lesions are produced at a rate p and are then converted to lethal lesions at a rate c with further heating. One lethal lesion is sufficient to kill a cell. The nature of the lesions is not specified; thus, it may be possible to apply it to many forms of molecular damage. This is a two-step model, which may make it especially applicable to the two-step model of irreversible protein denaturation: unfolding followed by aggregation (discussed below).

Mackey and Roti Roti (1992) have approached this problem with a somewhat different model. The basic assumptions are that there is a distribution of some parameter $\in$ in a cell population, and that the value of $\in$ decreases with time of heating. The reduction in $\in$ below a critical, minimum value ($\in_{min}$) in a cell causes that cell to die. The distribution of $\in$ and the way in which $\in$ decreases with time determines the shape of the survival curve. This model is not based on the inactivation of specific targets as the other two models are, but it still involves the

inactivation (i.e., reduction in value) of a cellular function represented by the parameter ϵ. The reduction in the value of ϵ may be related to the inactivation of unknown cellular targets.

The premise behind this review is that hyperthermic killing of cells is due to the inactivation of a target or a group of targets with similar inactivation kinetics. A modification of the target model of Hahn will be used to predict the inactivation temperature of the critical targets responsible for killing (see below).

C. Modifiers of Cellular Response

Numerous compounds sensitize cells to hyperthermia, and a few protect, when present during heating. Table 1 is a partial listing of hyperthermic sensitizers and protectors. At 37 °C, nearly all the sensitizers have been shown to induce thermotolerance and hsp synthesis, while the protectors inhibit hsp synthesis and reduce the level of thermotolerance when present during heating (Hahn and Li, 1990). Information about the identity of the molecular target can be gained from the use of modifiers of cellular sensitivity if these agents have a specific mechanism of action and interact with a specific cellular component. The compounds listed in Table 1 have little in common except that most, and possibly all, of the sensitizers are known to denature proteins or perturb protein structure. Both protectors, glycerol and D_2O, are well-established protein stabilizers, and the other two resistant conditions, acquired thermotolerance and the tolerance induced by cyclohexamide, are associated with protein stabilization in cells (Lepock et al., 1990a; Borrelli et al., 1991).

The fact that numerous agents and conditions that sensitize cells to hyperthermia also induce thermotolerance and HSP synthesis suggests

Table 1. Hyperthermic Sensitizers and Protectors

Sensitizers	*Protectors*
Alcohols (methanol to octanol)	Polyhydroxyl alcohols (e.g., glycerol)
H^+ (low pH)	D_2O
Solvents (e.g., DMSO)	[Thermotolerance]
Sodium arsenite	[Cyclohexamide]
Sulfhydryl reagents (e.g., diamide)	
Amino acid analogs	
Local anesthetics	
Amphotericin B	
Alkylating agents	

that the mechanisms by which the effects of elevated temperature yield those responses are similar. Furthermore, the finding of Hahn and Li (1990) that the Arrhenius plots above 43 °C for cell killing are indistinguishable from those for induction of thermotolerance and for induction of hsps also supports the hypothesis of a common mechanism for each response. Hightower first proposed that the induction of hsp synthesis is due to damaged or denatured protein (Hightower and White, 1981; Hightower et al., 1985), and since then it has been demonstrated that microinjection of denatured protein into cells is sufficient to induce hsp synthesis (Ananthan et al., 1986; Mifflin and Cohen, 1994), suggesting that protein denaturation also plays a role in cell killing and induction of thermotolerance.

III. DIRECT EFFECTS OF ELEVATED TEMPERATURE

A. Increased Kinetic Energy

When temperature is increased there is an increase in the rotational, vibrational, and translational kinetic energy of all cellular molecules. All effects of hyperthermia must follow from the increased kinetic energy which is just a consequence of increased temperature. Other than for increasing temperature, heat flow into cells and organisms is unimportant, except possibly for the small heat flow into macromolecules and cellular structures during transitions. This is important since it means that any measure of heat dose must be based on temperature, not the heat flow that increases temperature. There are three direct consequences of increased kinetic energy: (a) increased motion, (b) increased metabolism (i.e., biochemical reactions), and (c) transitions (e.g., protein denaturation, lipid phase changes, DNA melting, etc.).

One example of increased motion that has been proposed to play an important role in hyperthermic cell killing is increased membrane fluidity (Dennis and Yatvin, 1981). The general proposal is that the physical properties of the lipid bilayer, characterized by fluidity, change as temperature is increased from 37 °C to within the lethal hyperthermic region. A disruption of membrane structure or function occurs when fluidity exceeds a critical, maximum value. There is some experimental evidence for this proposal; however, a number of studies indicate that membrane fluidity is not involved in hyperthermic killing at moderate temperatures or is at best no more than a modulating factor in determining cellular sensitivity rather than the critical event. This topic has been

discussed in detail in a number of reviews (Lepock, 1982, 1987; Anghileri, 1986). The apparent minimal involvement of membrane fluidity however does not eliminate membranes, or even lipids, as critical targets in other ways (e.g., denaturation of membrane proteins, disruption of lipid asymmetry, reorganization of membrane domains, etc.).

Increased metabolism at hyperthermic temperatures, resulting in unbalanced metabolism due to different temperature coefficients for different reactions, was proposed long ago as a cause of lethality. This concept can be generalized to include any reaction, second messenger pathway, or ion flow directly stimulated by elevated temperature without the need for inactivation. Information about the importance of unbalanced metabolism and altered reactions rates in general can be obtained from Arrhenius plots of killing. The activation energies associated with responses limited by enhanced reactions should be in the range of activation energies for chemical reactions (10-30 kcal/mole).

As shown in Figure 3, growth increases uniformly from 30 to 40 °C for V79 cells with an activation energy of 18 kcal/mole, implying that the rate of growth and division is limited by metabolic processes (Kruuv et al., 1983). There is a sharp transition at 30 °C from net growth to killing. Hypothermic survival curves are similar to hyperthermic survival curves such as those shown in Figure 1, with a shoulder and an exponential region, except that killing is two to three orders of magnitude slower (see Kruuv, Chapter 5). The rate of killing decreases from 25 to 8 °C with an activation energy of 15 kcal/mole, suggesting that metabolism, probably unbalanced metabolism, is the rate limiting step for hypothermic killing in this temperature range.

Below approximately 8 °C there appears to be a dramatic shift in the mechanism of killing of V79 cells. The slope of the Arrhenius plot changes sign resulting in an apparent "negative activation energy" in the temperature range of 8 to 0 °C (see Kruuv, Chapter 5, for a detailed discussion of this phenomenon). The apparent "negative activation energy," which can be explained by a two step process (Muench et al., 1996), is very high (-83 kcal/mole), which is a strong temperature dependence and implies the involvement of a transition or macromolecular conformational change of some sort. Two possible transitions are a membrane lipid transition or the cold denaturation of a critical protein. Most, if not all, proteins undergo cold denaturation, some above 0 °C (Privalov, 1990).

The Arrhenius plot in the hyperthermic region illustrates the normal properties of hyperthermic killing. Killing is first detectable at 40.5-41 °C, there is a bend in the curve at 42 °C, and the activation energy is very high

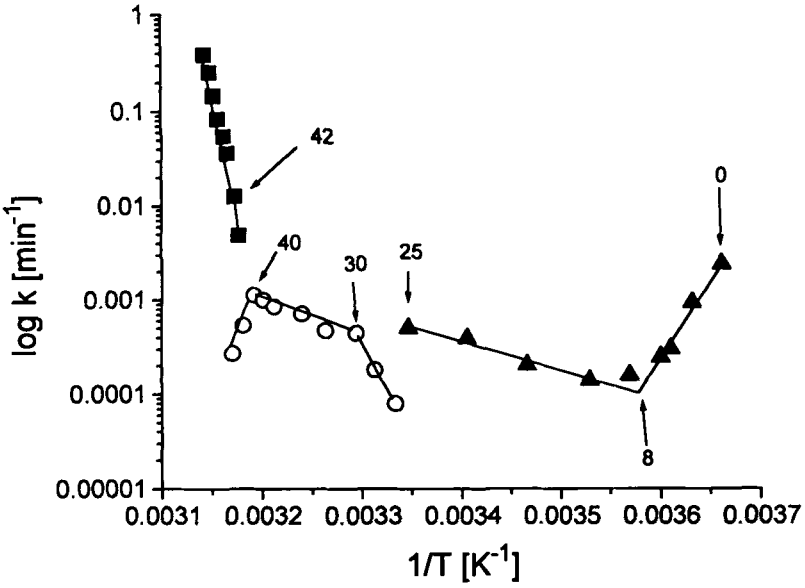


Figure 3. Arrhenius plots of the rates of growth (circles), hypothermic killing (triangles), and hyperthermic killing (circles) from 0 to 45.5 °C for CHL V79 cells.

above this break (greater than 150 kcal/mole). The high activation energy for killing is strong evidence that the rate limiting step for hyperthermic killing is a transition in a critical target. Thus, there are several critical temperatures for growth and killing of V79 cells. These occur at 8, 25-30, 40-41, and 42-43 °C, and there is reason to believe that each involves a transition in some cellular component. Only growth and hypothermic killing from 8 to 25 °C appear to be limited directly by metabolic events. Hypothermic killing below 8 °C and hyperthermic killing above 41 °C are caused by transitions, both possibly involving protein conformational changes.

B. Transitions

Transitions occur in numerous cellular components. The question that must be answered is what is the transition that causes hyperthermic killing above 40-41 °C in mammalian cells? There are many potential targets. Transitions in the membrane lipid bilayer have been intensely investigated. They have a strong temperature dependence that is consis-

tent with the high activation energy for killing. The most obvious lipid transition is the gel-to-liquid crystalline (melting) transition. The hypothesis has been presented that hyperthermic damage would result from the melting of normally solid domains present at 37 °C when the temperature is increased. This is fundamentally different than the critical fluidity hypothesis which postulates that death is caused merely by exceeding a maximum tolerable fluidity in the fluid, liquid crystalline regions of the membrane. There is no strong evidence for either gel lipid at 37 °C or for a gel-to-liquid crystalline lipid transition above 37 °C in mammalian cells, both of which are necessary conditions for death to be caused by a gel-to-liquid crystalline transition (Lepock, 1987). The lamellar-to-hexagonal transition often occurs at high temperatures, which makes it attractive as a possible rate limiting transition, but there is no evidence that it is important in hyperthermic killing, although it has not been investigated in detail.

Order-disorder transitions also occur in DNA and RNA. The main transitions in DNA are unlikely to play a role in killing since they are normally reversible and occur at high temperatures (in the range of 85-90 °C). However, localized melting of destabilized regions could be important. Structured RNA, such as double stranded regions, undergoes a melting transition similar to DNA. Likely RNA targets are tRNA and other forms of compact RNA such as rRNA and the small, nuclear ribonucleoprotein (snRNP) complexes. Very little is known about the stability of RNA, but isolated yeast $tRNA^{phe}$ melts through four transitions over the temperature range of 30-90 °C (Biltonen and Freire, 1978). With the recent discoveries of the large number of enzymatic functions performed by RNA and its likely structural role in many protein-RNA complexes, it must be considered as a potential target much like protein.

The most likely target for heat shock is protein. The denaturation or partial unfolding of proteins during heat exposure, resulting in damage due to the inactivation of crucial cellular functions or the disruption of cellular structures, is most consistent with the large body of experimental observations. The process of protein denaturation and its detection in cells is discussed below.

IV. PROTEIN DENATURATION

A. Molecular Characteristics of Protein Denaturation

Proteins undergo an order-disorder transition from the native to the unfolded or denatured state referred to as protein denaturation. This

process is first order (sometimes referred to as all-or-none), but it is still not well-understood because the nature or structure of the thermally denatured state is not completely described and the steps of unfolding (i.e., the structures of all intermediate states) have not been determined. In spite of this lack of specific structural information regarding the process of unfolding, the general concepts are clear.

The process of denaturation induced by strong chemical denaturants such as urea or guanidinium chloride (GdmCl) is better understood than thermal denaturation. Tanford (1968) concluded that high concentrations of such chemical denaturants completely unfold most proteins to a state approximated by a random coil. However, small regions of stable structure may still exist. Unfolding is less complete at lower concentrations. Measurements of unfolding as a function of denaturant concentration demonstrates the existence of stable unfolding intermediates for most proteins at low denaturant concentration (Ohgushi and Wada, 1983). These equilibrium intermediates have been given the name molten globule.

The molten globule is nearly as compact as the native state with about a 50% increase in volume; however, there is evidence for a relatively dense core (for a review see Ptitsyn, 1992). Thus, most of the volume increase may be due to an unfolding of loops and turns. Considerable secondary structure is retained in the molten globule, and for some proteins at least some of the secondary structure is native. For example, three native α-helices (A, G, and H) are present in the apomyoglobin molten globule (Hughson et al., 1990). Thus, the molten globule is a partially unfolded state in which some secondary structure is present but tertiary structure appears to be lost. It is frequently observed at low pH, high salt, and in low concentrations of urea and GdmCl. Higher denaturant concentrations cause complete unfolding.

These two fundamental observations, that protein denaturation is all-or-none and that the denatured state can be similar to a molten globule (e.g., at high temperature or low pH) or a random coil (e.g., at high GdmCl concentration), must be explained in any theory of denaturation. Shakhnovich and Finkelstein (1989) proposed that protein denaturation is due to a destruction of tight packing and that the denatured state is stabilized by the resulting increase in entropy. The structure of the denatured state that results, molten globule or random coil, depends upon temperature and solvent characteristics.

The thermally denatured state of a protein has two important characteristics: inactivation (e.g., loss of enzyme activity) and aggregation (due to exposure of hydrophobic residues). Both of these characteristics are of relevance to heat shock damage.

B. A Kinetic Model of Protein Denaturation

The increased kinetic energy associated with increased temperature is deposited homogeneously on a gross level. However, it is discrete on a molecular level. Because of the distribution of kinetic energy among all cellular molecules, the probability that a macromolecule or supramolecular structure will have sufficient energy to undergo an endothermic transition is described by the Boltzman distribution and is random. Thus, protein denaturation is stochastic and can be treated using a simple model such as

$$N \underset{k_1}{\overset{k_1}{\Leftrightarrow}} U \tag{1}$$

where N represents the native state, U the unfolded, denatured state (either molten globule or random coil), and k_1 and k_2 are the forward and reverse rate constants, respectively. This one step model assumes that intermediate states of unfolding are not populated to a significant extent and can be neglected. Thus, unfolding is first order.

If denaturation is completely reversible, then all proteins unfolded during hyperthermia would refold when the cell is returned to 37 °C. Irreversibility appears to be due to a second step following unfolding. This can be modeled by a two step kinetic scheme of the form

$$N \underset{k_2}{\overset{k_1}{\Leftrightarrow}} U \overset{k_3}{\rightarrow} D \tag{2}$$

where k_3 is the rate constant for formation of irreversibly denatured protein (D) from unfolded protein.

A number of processes have been shown to contribute to the irreversible inactivation of lysozyme and ribonuclease at 90-100 °C, with the formation of incorrect, non-native structures predominating at neutral pH (Zale and Klibanov, 1986). The native state of most, if not all, proteins is also the state of lowest energy at the normal growth temperature. Aggregation, due to either mixed disulfide formation or hydrophobic interactions, is an important process locking proteins in non-native structures.

If the irreversible step is fast (i.e., $k_3 \geq k_1$), then the two-step model can be simplified to an irreversible one-step model of the form

$$N \overset{k_{app}}{\rightarrow} D \tag{3}$$

where k_{app} is dependent on k_1, k_2, and k_3. The last equation is useful since a similar equation can be written for cell killing, and k_{app} as a function of temperature can be obtained from survival curves assuming that cell killing is proportional to the fraction of the critical, rate limiting target inactivated. This has been used to predict the denaturation profile of the critical target for the hyperthermic killing of V79 cells which gives the transition temperature (T_m) of the macromolecules constituting this target (Lepock et al., 1988, 1990a).

Many studies have been conducted, some nearly 20 years ago, searching for transitions, particularly membrane transitions, that can be correlated with hyperthermic cell killing (Verma et al., 1977; Lepock et al., 1983, 1987; Burgman and Konings, 1992). These studies have demonstrated irreversible protein conformational changes commencing at temperatures near 40 °C in plasma and other membranes isolated from mammalian cells. However, transition temperatures for irreversible processes such as protein denaturation must be dependent on both temperature and time of exposure. Thus, T_ms obtained by the usual method of measuring a parameter sensitive to protein conformation must depend on the rate that temperature is increased during the measurements since this influences the total exposure time.

This also holds true for the T_m for the inactivation of the critical target which is 46 °C when temperature is increased at a rate of 1 °C/min as predicted from survival curves of V79 cells (Lepock et al., 1988). The T_m for the critical target is not the same as the critical temperature for the onset of hyperthermic killing, 40-41 °C, since the latter is obtained from measurements as a function of time at constant temperature. Thus, hours are available for damage to accumulate at 41-42 °C during survival assays, while only minutes are available at a scan rate of 1 °C/min during assays for denaturation, effectively shifting the measured T_m several degrees higher. This analysis give a specific temperature (46 °C) at which to expect the critical transition resulting in hyperthermic cytotoxicity.

C. Approach to Relate Hyperthermic Damage to Protein Denaturation

Is the denaturation of a thermolabile protein(s), defined as the critical target, the initial, rate-limiting event occurring during exposure to hyperthermia? This is the fundamental question concerning hyperthermic

killing and protein denaturation. An approach to answering this question is to proceed through four steps.

1. Does protein denaturation actually occur at the relatively moderate temperatures experienced by cells during hyperthermia? Specifically, do proteins of mammalian cells denature with a transition temperature of 46 °C, the predicted T_m of the critical target?
2. What is the identity of the thermolabile proteins denaturing during hyperthermia?
3. Which specific protein(s) denaturing with $T_m = 46$ °C is the critical target?
4. What are the secondary effects of the denaturation of these thermolabile proteins?

Question 3 cannot be answered independently of question 4 since it does not appear possible to identify a specific critical target without knowing how damage to this target leads to death.

D. Detection of Protein Denaturation

Numerous techniques exist for measuring protein denaturation. Any parameter sensitive to protein conformation can be used. These include fluorescence spectroscopy, electron paramagnetic resonance (epr) spin labeling, circular dichroism, nuclear magnetic resonance (nmr), and Raman spectroscopy. The general requirement is that the parameter measured have a different value in the denatured, unfolded state (Y_D) than in the native, folded state (Y_N). Then $Y_{DN} = Y_D - Y_N$ gives the extent of denaturation.

An alternate approach is to measure a property of the transition itself. Temperature induced transitions such as protein denaturation are endothermic. Thus, heat is absorbed during the transition. Measurement of the extent of heat absorbed, the calorimetric enthalpy (H_{cal}), is a measure of the extent of the transition. Differential scanning calorimetry (DSC) is based on this principle. The excess specific heat (c_p) of a sample is measured as temperature is increased at a uniform rate. The profile of c_p versus temperature for a pure protein is a peak whose shape is dependent upon the particular steps occurring during denaturation. H_{cal} is the integral of excess c_p versus temperature, and the transition temperature (T_m) is defined as the temperature of half completion of the transition (i.e., where H_{cal} is equal to half the maximum value of H_{cal}).

V. CELLULAR PROTEIN DENATURATION DURING HYPERTHERMIA

A. Erythrocytes

DSC is a powerful technique when applied to the study of protein denaturation in whole cells since every temperature induced transition is potentially detectable, and many possible artifacts associated with spectroscopic techniques, particularly those using probes, are not present. One would expect the DSC profile of cells to consist of the sum of the individual transitions of all cellular components, each occurring at a T_m determined by its stability. Human erythrocytes were used as a model cellular system to determine if it is possible to detect cellular protein denaturation by DSC and to identify the T_ms of the major protein species (Lepock et al., 1989).

Figure 4 shows a DSC scan (C_p vs. temperature) for human erythrocytes, scanned at 1 °C/min, and the rescan. Each endothermic peak represents a single transition or a group of transitions with similar

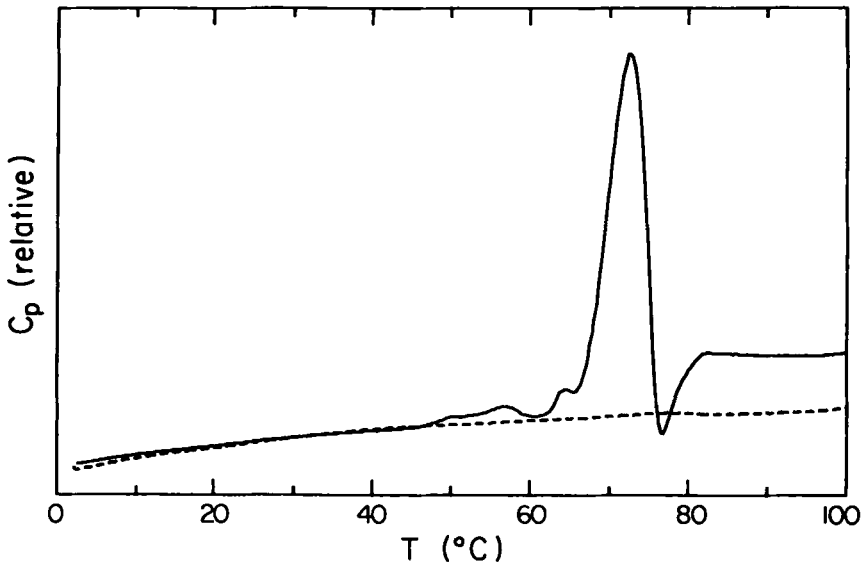


Figure 4. DSC scans (1 °C/min) of human erythrocytes in phosphate buffered saline (solid line) and a rescan (broken line) of the same cells after heating to 100 °C (from Lepock et al., 1989).

transition temperatures (T_m). The evidence indicates that this profile is due almost exclusively to protein denaturation. The large endothermic peak at 72 °C and the exotherm at 78 °C are due to the denaturation of hemoglobin and its subsequent aggregation, respectively. The rescan is flat, demonstrating that denaturation is irreversible. The shift in baseline above 85 °C between the scan and rescan (ΔC_p) is believed to be due to the exposure of hydrophobic amino acid residues upon unfolding.

The scan corrected for the shift in baseline (ΔCp) is shown in Figure 5. This correction was applied to all other scans shown. Deviation of Cp from the baseline is first detectable at about 45 °C, implying that significant protein denaturation commences at this temperature in erythrocytes. A best fit curve for the hemoglobin peak was generated assuming that hemoglobin denaturation obeys a two state, irreversible model of the form $_{N\rightarrow Do}^{k}$. The region of the hemoglobin peak perturbed by the exotherm at 78 °C was not used for fitting.

The best fit hemoglobin curve was subtracted to obtain the profile for the low temperature transitions A, B, and C (Figure 6). Membranes (ghosts) were isolated and the scan is also shown in Figure 6. The peaks

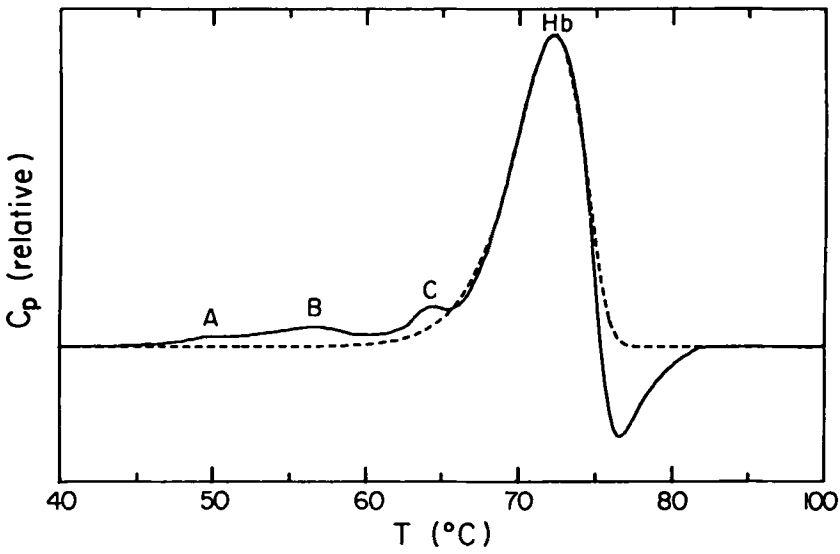


Figure 5 DSC scan of human erythrocytes corrected for the baseline (solid line) and the best fit curve of the hemoglobin transition (broken line) (from Lepock et al., 1989).

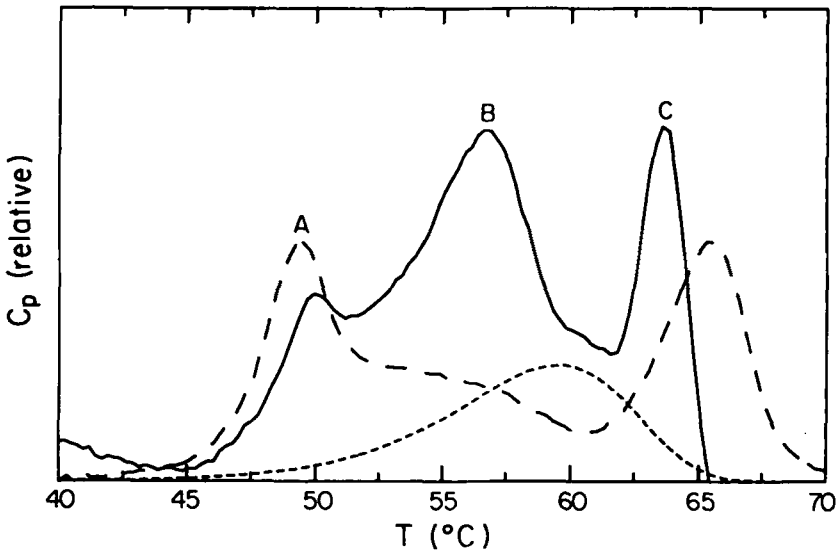


Figure 6. DSC scans of human erythrocytes with the hemoglobin peak subtracted (solid line), erythrocyte ghosts (dashed line), and the predicted transition of the critical target of the rate-limiting step for hemoglobin leakage during hyperthermia (dotted line; from Lepock et al., 1989).

labeled A, B, and C match the peaks in isolated membranes (Brandts et al., 1977) and have been identified as representing the denaturation of spectrin (A), several components of the membrane skeleton (B), and the transmembrane portion of the band 3 protein (C), respectively (Lysko et al., 1981). Thus, the membrane skeleton is the most thermolabile major component of the erythrocyte. The goal of using DSC is to obtain denaturation profiles and to identify the thermolabile proteins of mammalian cells in a similar fashion.

Hemoglobin leakage as a function of time was measured at hyperthermic temperatures from 47-54 °C (results not shown). These are similar in shape to the survival curves shown in Figure 1. The rates of leakage were used to predict the transition profile of the critical target, the denaturation or inactivation of which is rate limiting for hemoglobin leakage during hyperthermia. This profile with a T_m of 60 °C is also shown in Figure 6. The T_m for the critical target is much higher than that of spectrin but corresponds to the denaturation of the other components of the membrane skeleton. Thus, we infer from these results that the rate

of heat-induced hemoglobin leakage is limited by the denaturation of a protein of the membrane skeleton but not by the denaturation of spectrin.

B. CHL V79 Cells

The same approach used for erythrocytes was applied to CHL V79 cells, a more complex, mammalian line of dividing cells. The DSC profile of V79 cells is shown in Figure 7. The curve of c_p versus temperature is similar to that of erythrocytes in that it consists of a number of peaks but is more complex in that the peaks are broad and overlapping. (For a more detailed account of protein denaturation in mammalian cells see Lepock et al. (1988, 1990a), Borrelli et al. (1991), and Lepock et al. (1993.)) It consists of five broad peaks labeled A-E, some of which can be resolved into subcomponents, with T_ms of 48 to 98 °C. Deviation from the baseline is detectable at 37-38 °C with significant deviation at 40-41 °C, indicating the onset of a transition. Similarly shaped profiles have been obtained for Chinese hamster ovary (CHO; Borrelli et al, 1991), radiation-induced fibrosarcoma (RIF), mouse L929, and 3T3 cells (data not shown). Thus, this five peak profile appears to be characteristic of cultured mammalian cells.

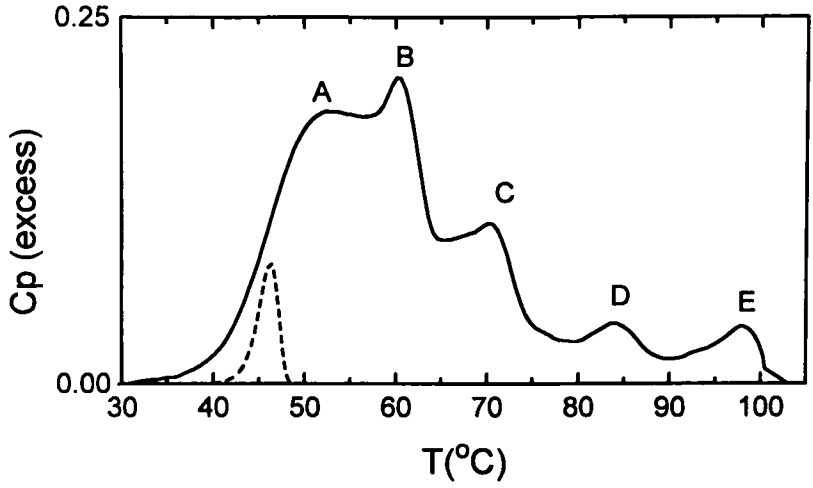


Figure 7. DSC profile of CHL V79 cells (solid line) with the baseline corrected as described for erythrocytes and the predicted transition profile (broken line) for the rate limiting, critical target for killing.

Also shown in Figure 7 is the predicted DSC profile for the critical target. The T_m of the critical target of V79 cells, calculated as described above from the rate of killing obtained from survival curves (Figure 1), is 46 °C. The amplitude is arbitrary since it is not possible to determine the amount of critical target present. Some transitions occur with a $T_m <$ 46 °C and, thus, are predicted to be non-lethal. This was proven by scanning to 45 °C, immediately cooling, and then obtaining a complete scan to 100 °C. This procedure denatures about 5% of total cellular protein. Only 15% of the cells are killed by this procedure, but the beginning of the scan is shifted up to match the predicted profile of the critical target. Thus, the irreversible transitions below 46 °C are not lethal and, hence, cannot occur in the critical target.

These DSC profiles demonstrate that endothermic processes that appear to be transitions occur in mammalian cells above 40-41 °C, the maximum growth temperature and the onset temperature for hyperthermic killing. In addition, there are transitions in the region of Tm = 46 °C, the predicted Tm of the critical target. The first question that must be addressed is do these transitions represent protein denaturation, especially the transitions in the region of 40-50 °C, or are they due to other mechanisms?

It is very difficult to prove unequivocally that all the excess c_p is due to protein denaturation in the complex mixture of proteins, other macromolecules, and supramolecular structures that comprise the cell. Other macromolecular transitions are known to contribute to the profile. At least part of peaks D and E at 85 °C and 98 °C, respectively, are due to the unfolding of DNA, and it would be surprising if the unfolding of compact RNA does not contribute to at least some of the profile. The evidence that the vast majority of the profile is due to protein denaturation can be summarized as follows.

1. The value of H_{cal} = 9.74 cal/g cell protein (Lepock et al., 1990a) obtained from DSC scans such as that shown in Figure 7 is similar to the average value of 6.7 ± 1.2 cal/g for the proteins analyzed by Privalov and Khechinashvili (1974).
2. Proteins are present in much greater quantity than any other cellular macromolecule that can undergo a transition. Hence, their denaturation must dominate the profile.
3. There is lack of evidence for a strong lipid transition in the hyperthermic region that could contribute significantly to the profile (Lepock et al., 1983, 1987; Lepock, 1987).
4. The calorimetric enthalpy of other order-disorder transitions in supramolecular structures, such as depolymerization, is on the

order of 100 times smaller than the calorimetric enthalpy of denaturation.

5. The transitions observable in the hyperthermic region for isolated organelles are more likely due to protein denaturation (see below). Thus, denaturation must occur in the same organelles in intact cells.

In addition to the evidence from DSC, there is indirect evidence that protein denaturation occurs during hyperthermia. Increased proteolytic degradation during hyperthermia suggests the formation of rapidly degradable denatured protein (Parag et al., 1987; Heynen et al., 1989). Induction of the heat shock response by injection of denatured protein suggests that protein denatured during heat shock is the inducing signal (Ananthan et al., 1986). Further evidence comes from the formation of protein aggregates during heating, presumably due to protein denaturation (Hahn et al., 1992). There is strong evidence that protein denaturation occurs in isolated erythrocyte membranes (Brandts et al., 1977; Lysko et al., 1981) and HeLa cell membranes (Burgman et al., 1992). The general similarity of killing due to hyperthermia and the induction of the heat shock response is consistent with the involvement of protein denaturation in each. Dewey (1989) has reviewed other evidence supporting the involvement of protein denaturation in hyperthermic killing.

C. Distribution of Cellular Transitions

The multicomponent DSC profile shown in Figure 7 indicates the presence of numerous transitions in cells. It is important to understand how the individual transitions produce the complex profile of whole cells. One way to approach this is to simulate the cellular profile by summing the profiles for single transitions with a specific distribution of T_ms. This is shown in Figure 8 (Lepock et al., 1993). The sum of 20 transitions gives a profile of three peaks. Each peak is resolvable because of the superposition of several strong transitions with similar T_ms. This supports the hypothesis that the peaks A-E in the DSC scans of cells are also due to the superposition of many, non-resolvable transitions. One of the non-resolvable transitions comprising peak A is the transition in the critical target for killing at 46 °C.

This simulation illustrates what the DSC profile of a cell represents. It is a distribution of protein stability. Stability is related to T_m, the position of a transition on the temperature axis. Thus, transitions are

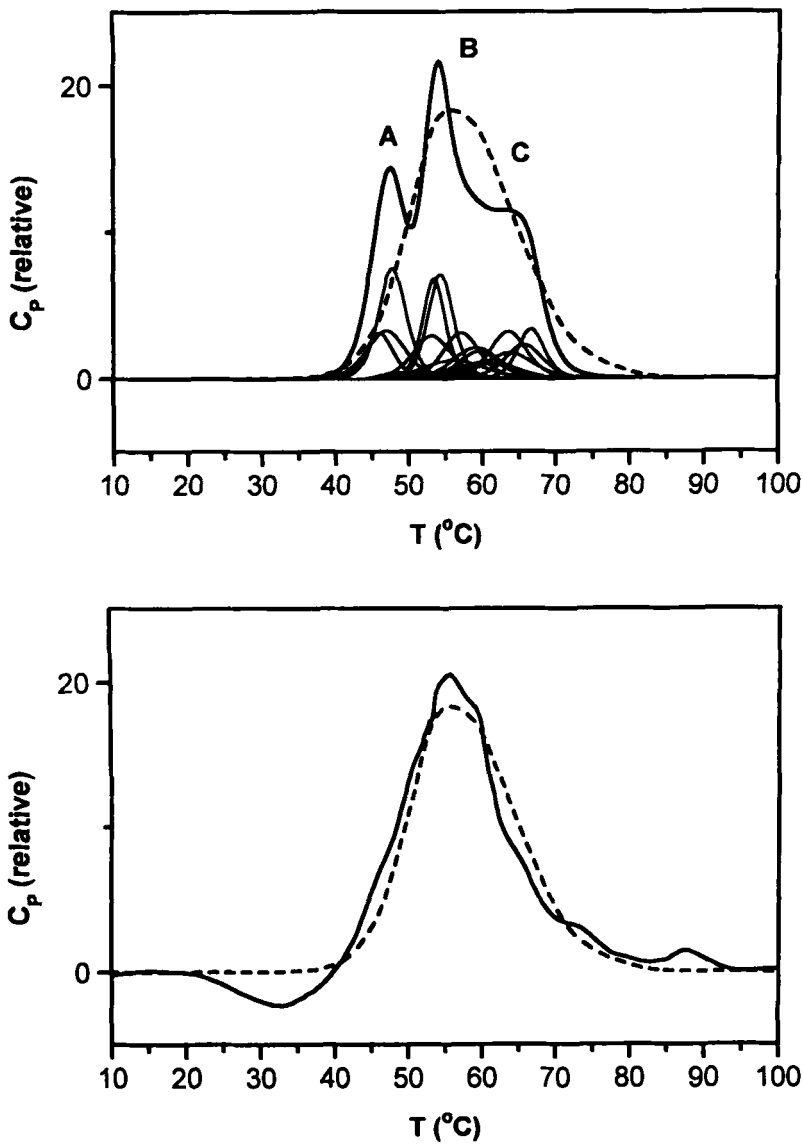


Figure 8. Simulations of the multi-component DSC profile of mammalian cells. (A) Twenty transitions of random amplitude with T_ms selected from a Poisson distribution with an average T_m of 55 °C and a minimum of 40 °C (small peaks), the sum of these 20 transitions (solid line), and the sum of 1,000 transitions (broken line). (B) The DSC profile of isolated hepatocytes (solid line) and the sum of the distribution of 1,000 transitions (broken line; from Lepock et al., 1993).

separated on the basis of stability, and the value of the excess c_p (y-axis) indicates the amount of cellular material undergoing a transition at any temperature. The temperature midpoint of the DSC profile, properly weighted, is a measure of the average stability of all cellular proteins.

An accurate Poisson distribution of T_m can be obtained by using a large number of transitions in the simulation. As shown in Figure 8b, the simulation using 1,000 transitions fits the profile for hepatocytes, which is similar to the profile for V79 cells except that it lacks the high temperature transitions due to the greater cell/nucleus volume ratio of hepatocytes. Thus, the hepatocyte profile is more representative of general protein stability. This distribution of T_ms is the distribution of stability of all cellular proteins. Thus, the average stability is given by a T_m of 55 °C with a minimum stability of T_m = 40-45 °C and a distribution of stabilities skewed toward the high temperature side. This distribution of protein stability is nonrandom, and the relatively low stability of many proteins is probably required for optimum cell function. Most proteins whose denaturation has been investigated in detail are highly stable (T_m = 60-80 °C) and may not be representative of the most thermolabile (T_m = 40-50 °C) proteins in cells.

The hepatocyte profile has a broad exotherm centered at 33-34 °C (Figure 8b) which is due to metabolic heat released during the scan. This exotherm is frequently observed with more metabolically active cells and can be eliminated with potassium cyanide.

D. Cellular Organelles

An important question is does protein denaturation occur uniformly in the cell or are there specific organelles and components that are more thermosensitive and undergo more denaturation during hyperthermia? An answer to this question would aid in the identification of the critical target. This question was approached by obtaining DSC scans from isolated microsomes, mitochondria, nuclei, and a cytosolic fraction from rat liver (Figure 9). Each profile is different with the cytosol contributing primarily to peak A, the membrane fractions to peak B, and nuclei to the high temperature peaks C-E. Thus, the DSC profile of cells (Figure 7) can be approximated as the sum of the organelles and components comprising the cell. Denaturation first begins at approximately 40 °C in each organelle. Thus, all of these organelles consist of some thermolabile proteins, and any organelle or all organelles, could contain the critical target with T_m = 46 °C.

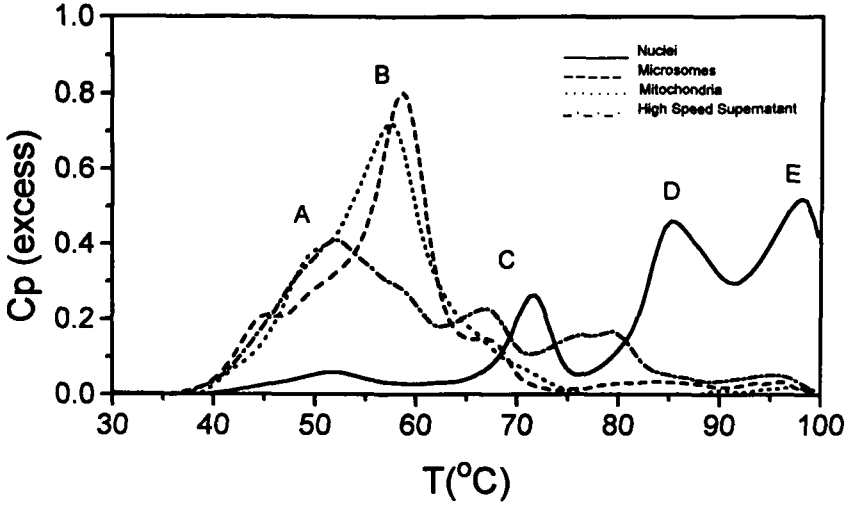


Figure 9. DSC scans of isolated nuclei, microsomes, mitochondria, and a cytosolic fraction (labeled high speed supernatant). The locations of the five peaks in whole cells are labeled A-E.

These scans of isolated organelles support the hypothesis that the DSC profile of whole cells is primarily due to protein denaturation. All of the peaks or transitions of isolated erythrocyte membranes are due to the denaturation of membrane proteins (Lysko et al., 1981). Comparison of the DSC profile of erythrocyte membranes to whole erythrocytes and identification of the major transition of erythrocytes with the denaturation of hemoglobin (Figure 5) indicates that the DSC profile of intact erythrocytes is due primarily, if not exclusively, to protein denaturation. Comparison of the DSC profiles of the isolated organelles, which must be primarily due to protein denaturation, to that of intact cells (Figure 7), also supports the hypothesis that the DSC profile of more complex mammalian cells is due primarily to protein denaturation.

E. Fractional Denaturation

Denaturation is almost completely irreversible in the cell, probably because of the high protein concentration (Lepock et al., 1988; 1993). The area under the curve of c_p versus temperature is a rough estimate of the extent of reversible denaturation occurring on increasing the temperature to any value. Since cellular denaturation appears to be almost

totally irreversible, the area should approximate the extent of irreversible denaturation. (A detailed rationale for this is given in Lepock et al., 1988.) The fractional denaturation of several cells and organelles on heating to 45 °C at 1 °C/min and then immediately cooling is given in Table II. Fractional denaturation is defined as the fractional area under the c_p versus temperature curve on scanning to a given temperature. These estimates are based on several assumptions and the overall accuracy is difficult to determine. Fractional denaturation is probably indicative of the fraction of total cellular proteins undergoing an all-or-none transition rather than a partial unfolding of all proteins. Thus, approximately 5% of all cellular proteins denature and inactivate during this mild heat shock. The level of denaturation is increased by about one-half if the exposure to 45 °C is increased to 10 min.

F. Bacteria

It is important to determine if there is a relationship between protein denaturation and hyperthermic cell killing. This was investigated by altering cellular sensitivity to hyperthermia and determining if there was a corresponding change in protein stability. Altered cellular sensitivity was achieved in several ways. The first was to use a series of species of *Bacillus* with widely different growth ranges (Lepock et al., 1990b). These varied from a maximum growth temperature (T_{max}) of 32.5 °C for the psychrophile *B. psychrophilus* to T_{max} = 69 °C for the thermophile *B. stearothermophilus* (ATCC strain). The DSC scans for the four species are shown in Figure 10, and the values of T_{max} are given in Table 3. The peak with T_m ~ 90 °C in each scan is due to the unfolding of DNA. In addition, *B. stearothermophilus* and *B. megaterium* have reversible lipid transitions centered at 20-30 °C (not shown). Any lipid transition in *B. psychrophilus* occurs below 0 °C.

Table 2. Fractional Denaturation (after scanning to 45 °C at 1 °C/min)

	f_d(%)
V79 cells	5.5
Liver homogenate	5.5
Hepatocytes (rat)	4.5
Microsomes	7.0
Mitochondria	4.3
Cytosol	5.8
Nuclei	0.8

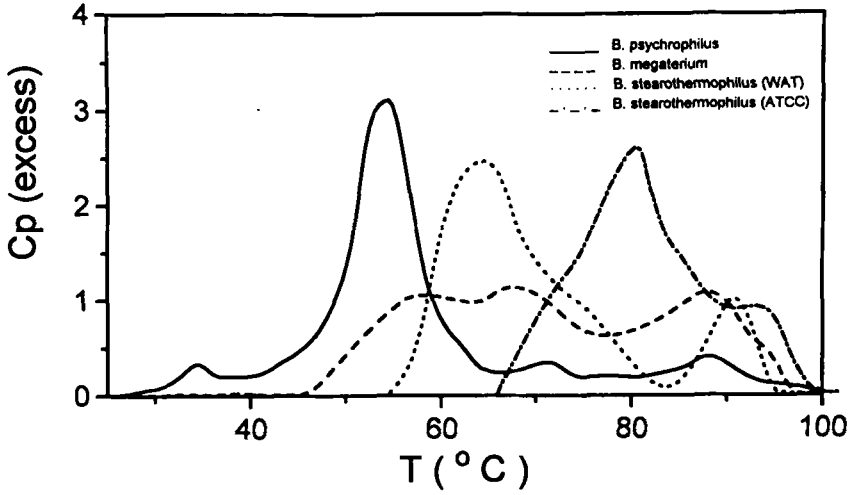


Figure 10. DSC scans of four species of Bacillus. Values of Tmax are given in Table 3.

Table 3. Maximum Growth Temperture (Tmax) Versus Onset of Denaturation(T1) for Various Species of *Bacillus*

	T_{max} (°C)	T_1 (°C)
B. psychrophilus	32.5	30
B. megaterium	48	46
B. stearothermophilus (WAT)	56	55
B. stearothermophilus (ATCC)	69	65

The important parameter for heat damage is the onset temperature for denaturation T_1. This ranged from 30 to 65 °C (Table 3). For each species T_{max} was 1-4 °C higher than T_1, implying that a small amount of denaturation was tolerable before growth was inhibited. This relationship holds over a temperature range of 40 °C, from T_{max} = 32-69 °C).

G. Modifiers of Cellular Sensitivity

The hyperthermic sensitivity of mammalian cells (V79 and CHO) was also varied, but over a much narrower temperature range than for the

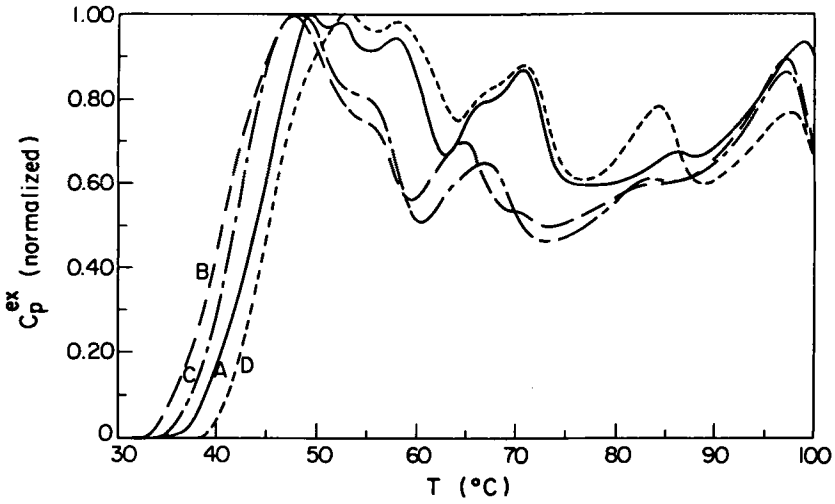


Figure 11. DSC scans (normalized) of A, control V79 cells and cells exposed to B, t-butanol (3%); C, ethanol (3%); and D, glycerol (5%) during scanning. From Lepock et al., 1988.

bacteria, through the use of short-chain alcohols as sensitizers (Lepock et al., 1988) and glycerol and D_2O as protectors (Lepock et al., 1988; Borrelli et al., 1992). The effects of ethanol, t-butanol, and glycerol on the DSC scans of CHL V79 cells are shown in Figure 11. These scans were not corrected for the shift in baseline ΔCp. Thus, Cp is elevated in the high temperature region compared to the V79 scan shown in Figure 7. However, the beginning of the curve is unaltered and the onset of denaturation can be determined accurately.

Ethanol (3%) shifts the beginning of the curve to lower temperatures by 1.8 °C. The shift from t-butanol is larger, about 3.1 °C. Both of these alcohols, with t-butanol being more potent, sensitize cells to heat killing and induce the synthesis of hsps. Glycerol, which protects cells from killing and inhibits the induction of hsps, shifts the profile to higher temperatures. In each case increasing cellular sensitivity to hyperthermia decreases the stability of cellular proteins and increasing cellular resistance increases protein stability as determined by DSC. Thus, these chemical modifiers appear to alter cellular sensitivity to hyperthermia by altering the stability of cellular proteins.

H. Thermotolerance

Cells can be made transiently tolerant to hyperthermia by a prior heat shock followed by a period of recovery (Gerner and Schneider, 1975). This state is usually referred to as acquired thermotolerance to distinguish it from the permanent state of heat resistance that has been observed in certain variant or mutant cell lines (Campbell et al., 1983). The acquisition and decay of acquired thermotolerance correlates well with the synthesis of hsps (Landry et al., 1982; Li and Werb, 1982). However, the fundamental mechanism of thermotolerance is not understood. One possibility is that the most thermolabile proteins are stabilized in thermotolerant cells by hsps or some other factors. In particular those proteins constituting the critical target with T_m = 46.0 °C would be stabilized (i.e., the T_m would be increased to a higher value).

CHL V79 cells were made thermotolerant by an exposure to 43 °C for 10 minutes followed by incubation at 37 °C for 0.25 to 70 hours. The level of thermotolerance was assayed by obtaining survival curves at 45 °C, and DSC scans were obtained at the same times following the primary heat shock (Lepock et al., 1990a). The results after two hours incubation at 37 °C are shown in Figure 12. Critical target analysis using rates of killing predicts that the T_m of the critical target should be shifted from 46.0 to 47.3 °C, an increase of 1.3 °C, if thermotolerance is due to stabilization of the critical target. There is no significant change in the DSC profile immediately after the priming heat dose. However, the beginning of profile is shifted upward by 1.2 °C (ΔT) for cells incubated for two hours at 37 °C. A similar shift and a similar degree of heat protection is obtained with 10% glycerol (Figure 12). From these results we infer that stabilization of thermolabile proteins is sufficient to account for the observed degree of acquired thermotolerance. The mechanism of stabilization is still not understood completely, but it appears that hsps are involved (Martin et al., 1992).

A transient state of thermotolerance can also be induced by a number of inhibitors of protein synthesis, including cyclohexamide (Borrelli et al., 1991). Treatment of CHO cells with cyclohexamide shifts the DSC profile to higher temperatures, as was observed for thermotolerant V79 cells and glycerol treated cells, indicating that cyclohexamide induced tolerance to heat is accompanied by increased protein stability (Borrelli et al., 1991).

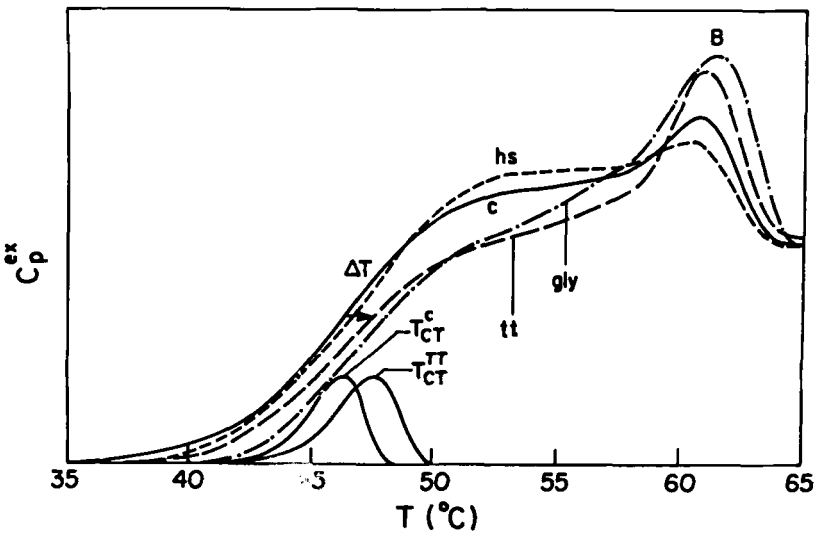


Figure 12. DSC scans (c_p versus temperature) from 35-65 °C of CHL V79 cells. The curves are labeled: c (solid line), control (unheated); hs (dashed line), heat shocked for 10 minutes at 43 °C; tt (long dashed line), thermotolerant (incubated for two hour at 37 °C following heat shock); gly (dot dashed line), unheated cells in 10% glycerol. Also shown are the predicted profiles for control (T_{ct}^{c}) and thermotolerant (T_{CT}^{TT}) cells (from Lepock et al., 1990a).

VI. CONCLUSIONS

There are several reasons to think that the initial, rate-limiting event of hyperthermic killing is a transition causing the irreversible inactivation of a critical target. Protein denaturation is the most likely candidate for this transition. Considerable evidence, both direct from DSC and other physical techniques and indirect, demonstrates that protein denaturation occurs during exposures to temperatures in excess of 40 °C in mammalian cells. A rough estimate is that 5-10% total denaturation occurs during a 15-30 minute exposure to 45 °C. Protein denaturation does not appear to be localized specifically to any organelle or cellular component but occurs throughout the cell. Similar levels of denaturation occur in both membrane and soluble proteins. The critical target has not yet been identified and could be located in any organelle or component of the cell. A number of treatments that either sensitize cells or protect cells from

hyperthermia also sensitize cellular proteins to or protect cellular proteins from thermal denaturation. A better understanding of the biochemical and physiological responses of the cell to protein denaturation is probably necessary to identify which thermolabile proteins constitute the critical target.

Cell death following exposure to hyperthermia takes several hours to several days, depending on the severity of treatment, and heated cells can even divide before finally dying. Thus, there must be many subsequent steps occurring after the initial denaturation before death ensues. These secondary effects ultimately resulting in death must be determined for a complete understanding of thermal cytotoxicity.

There are two general effects of protein denaturation that one would expect to be particularly harmful to cells: direct inactivation of protein function and disruption of complex structures. Inactivation of enzyme activity, membrane receptors, and ion transporters have been shown to occur during hyperthermia (Laszlo, 1992). Death may be caused by damage of this kind; however, a more difficult damage to repair may be structural damage. This can consist of membrane permeability changes (e.g., increased Ca^{2+} flux), depolymerization of complex structures (e.g., disruption of cytoskeletal elements), and aggregation (e.g., aggregation of membrane proteins and binding of proteins to the nuclear matrix). Damage of this type can have pleiotrophic effects on cellular physiology and may ultimately be the cause of death.

VII. SUMMARY

There is circumstantial evidence that protein denaturation occurs in cells during heat shock at hyperthermic temperatures and that death is caused by denatured or damaged protein which is also the primary inducer of the heat shock response. However, until recently there was no direct evidence regarding the extent of denaturation of normal cellular proteins during heat shock. This review describes measurements of protein denaturation in cells and correlates denaturation with cell death due to hyperthermia. DSC is the most direct method of monitoring protein denaturation or unfolding. Due to the fundamental parameter measured, heat flow, DSC can be used to detect and quantitate endothermic transitions in complex structures such as isolated organelles and even intact cells. DSC profiles demonstrating endothermic transitions in the hyperthermic region are given for human erythrocytes, a number of tissue

culture lines, rat hepatocytes, and several species of *Bacillus*. The profiles for the mammalian cells (isolated rat hepatocytes, liver homogenate, and the tissue culture lines) have several common features. Five main transitions (A-E), several of which are resolvable into subcomponents, are observed with transition temperatures (T_m) of 45-98 °C. The onset temperature is approximately 40 °C, but some transitions may extend as low as 37-38 °C. In addition to acting as the primary signal for hsp synthesis, the inactivation of critical proteins may lead to cell death. Critical target analysis implies that the rate limiting step of cell killing for CHL V79 cells is the inactivation of a protein with $T_m = 46$ °C within the A transition. Isolated microsomal membranes, mitochondria, nuclei, and a cytosolic fraction from rat liver have distinct DSC profiles that contribute to different peaks in the profile for intact hepatocytes. Thus, the DSC profiles for intact cells appears to be the sum of the profiles of all subcellular organelles and components. The presence of endothermic transitions in the isolated organelles is strong evidence that they are due to protein denaturation. Each isolated organelle has an onset for denaturation near 40 °C and contains thermolabile proteins denaturing at the predicted T_m (46 °C) for the critical target.

The extent of denaturation at any temperature can be approximated by the fractional calorimetric enthalpy. After scanning to 45 °C at 1 °C/min and immediately cooling, a relatively mild heat shock, an estimated fractional denaturation of 4-7% is found in hepatocytes, V79 cells, and the isolated organelles other than nuclei, which undergo only 1% denaturation because of the high thermostability of chromatin. Thus, thermolabile proteins appear to be present in all cellular organelles and components, and protein denaturation is wide-spread and extensive after even mild heat shock.

A number of conditions which alter cellular sensitivity to hyperthermia also alter the thermostability of cellular proteins. A number of short-chain alcohols sensitize cells to killing by hyperthermia and lower the onset temperature for cellular protein denaturation. Glycerol and D_2O both protect cells and increase the temperature of denaturation. Cellular proteins are more stable in cells made thermotolerant by either a prior heat shock or cyclohexamide. In addition, the onset temperature for protein denaturation correlates very well with the maximum growth temperature (T_{max}) for several species of *Bacillus* with $T_{max} = 32$ to 69 °C. Thus, there is an excellent correlation between protein denaturation and hyperthermic killing.

ACKNOWLEDGMENTS

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THE ROLE OF HEAT SHOCK PROTEINS IN THERMOTOLERANCE

Andre Nussenzweig, Paul Burgman, and Gloria C. Li

I. INTRODUCTION

One of the most interesting aspects of thermal biology in the mammalian system is the response of heated cells to subsequent heat challenges. Mammalian cells, when exposed to a non-lethal heat shock, have the ability to acquire a transient resistance to one or more subsequent exposures at elevated temperatures. This phenomenon has been termed

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thermotolerance (Gerner and Schneider, 1975; Henle and Leeper, 1976; Henle and Dethlefsen, 1978; Gerner, 1983). The molecular mechanism(s) by which cells develop thermotolerance is not well understood, but early experimental evidence suggested that protein synthesis is required for its manifestation. On the molecular level, heat shock activates a specific set of genes, so-called heat shock genes, and results in the preferential synthesis of heat shock proteins (hsps; Lindquist, 1986; Lindquist and Craig, 1988; Morimoto et al., 1990). The heat shock response has been extensively studied in the past decade, and has attracted the attention of a wide spectrum of investigators ranging from molecular and cell biologists to radiation and hyperthermia oncologists. There is much data supporting the hypothesis that hsps play a key role in modulating the cellular response to environmental stress, and are involved in the development of thermotolerance.

We begin this review by characterizing and comparing the phenomena of thermotolerance and permanent heat resistance. We then describe the biochemical and molecular mechanisms for the induction of thermotolerance and the role that hsps play in its development and decay. Finally, we discuss the involvement of hsps in normal cellular metabolism.

II. THERMOTOLERANCE AND HEAT RESISTANCE

Exposure of mammalian cells in culture to temperatures above 40 °C leads to reproductive death. Survival curves, when plotted as a function of duration of heating, resemble X-ray survival curves and are characterized by an initial shoulder region followed by exponential decrease in surviving fractions (Dewey et al., 1980; Lepock, Chapter 7). The survival of cells after hyperthermia depends on both the applied temperature and the duration of exposure (Figure 1). The time required to reduce the survival in the exponential region to 37% of its initial value is defined as D_o. The kinetics of heat killing for a wide variety of mammalian cells has been analyzed in terms of Arrhenius plots, where l/Do is plotted as the inverse of the absolute temperature. For most cell lines studied, the Arrhenius plots of cell inactivation seem to be composed of two segments, with a break at or near 43 °C (Westra and Dewey, 1971; Dewey et al., 1977; Bauer and Henle, 1979; Lepock, Chapter 7). Above that temperature, the activation energy is between 110 and 150 kcal/mol, a value consistent with the view that protein damage is respon-

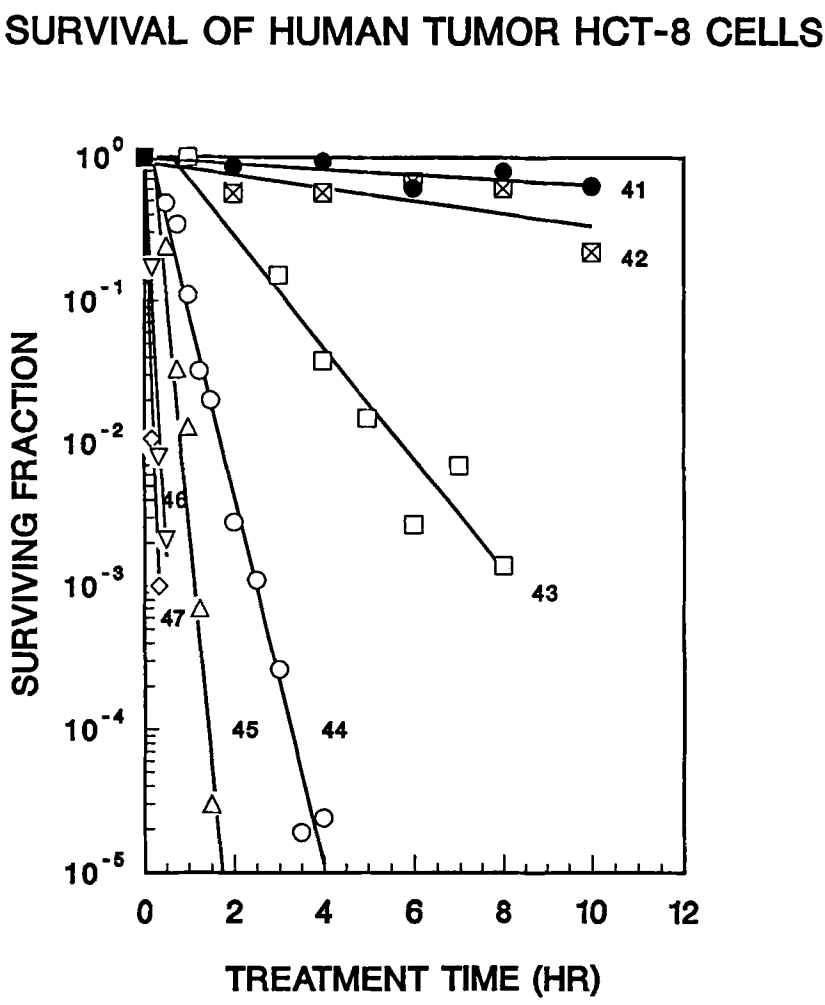

Figure 1. Survival curves at different temperatures (in °C) for human tumor HCT-8 cells. Monolayers of exponentially growing cells were heated at temperatures ranging from 41 to 47 °C for the indicated time periods. The survival fraction was determined by the colony formation assay.

sible for cell death. At lower temperatures, the activation energy is relatively higher, that is, around 300-400 kcal/mol. On the basis of the difference in activation energies, several authors have suggested that there may be different primary modes of heat-induced cell death, one dominant above 43 °C and the other below 43 °C. On the other hand, the change in slope of Arrhenius plots below 43 °C could be a manifestation of the ability of the cells to develop thermotolerance (Sapareto et al., 1978; Li and Hahn, 1980).

Mammalian cells vary appreciably in their intrinsic thermal sensitivity. In a study performed by Raaphorst et al. (1979), it was demonstrated that cells derived from different species vary in their survival response to heat shock as assayed by the colony formation assay. Furthermore, their results show that the normal body temperature of the animal from which the particular cell line was derived correlates well with the observed thermal sensitivity. For example, cells derived from animals whose normal body temperature is high are heat-resistant; conversely, cells derived from animals with low body temperatures are heat-sensitive. Among the most heat-resistant cells are those derived from birds; during flight their normal body temperature may rise to 43 °C. A possible interpretation of these findings is that permanent heat resistance is simply a genetic alteration of constitutive levels of macromolecules transiently induced in thermotolerant cells.

Henle and Leeper (1976) and Gerner and Schneider (1975) first showed that cultured mammalian cells exposed to a nonlethal heat treatment have the ability to develop resistance to subsequent heat challenge. During this tolerant state, cells are much more heat resistant than cells which have never been preexposed to elevated temperatures. Several excellent reviews have discussed this phenomenon in considerable detail (Henle and Dethlefsen, 1978; Field and Anderson, 1982; Gerner, 1983). *In vitro*, thermotolerance can be induced by a short initial heat treatment at temperatures above 43 °C followed by a 37 °C incubation before the second heat challenge. The survival curves after the second heat exposure show an increase in D_o, as well as an increase in the width of the shoulder.

Thermotolerance can also be induced during continuous heating at temperatures below 43 °C (Palzer and Heidelberger, 1973; Gerweck, 1977; Harisladis et al., 1977; Sapareto et al., 1978), and the degree of thermotolerance developed can be dramatic: increases in survival levels by several orders of magnitude are commonplace. However, when Bauer and Henle (1979) compared the responses of tolerant cells and nontol-

erant control cells using Arrhenius analysis, they found that the large increase in survival corresponds to an equivalent decrease in temperature of only 1-2 °C.

The thermal history, heat fractionation interval, and recovery conditions all significantly modify the kinetics of thermotolerance (Henle and Dethlefsen, 1978; Gerner, 1983; Lepock, Chapter 7). The proliferative and nutritional status of mammalian cells also significantly affects their thermal sensitivity (Hahn 1982). Nielsen and Overgaard (1979) and Goldin and Leeper (1981) examined the effect of the extracellular pH on thermotolerance induction during fractionated hyperthermia; their data indicate that reducing the extracellular pH can partially inhibit the induction and expression of split-dose thermotolerance. Gerweck (1977) has shown that the development of thermotolerance during continuous heating can be delayed below physiological pH values.

The effects of varying the temperature and the duration of the first heat treatment on the subsequent expression of thermotolerance in HA-1 cells in culture were studied in detail by Li and coworkers (1982). These sets of experiments (Figure 2) indicated that temperatures of 43 °C or higher did not permit the development of thermotolerance during this first heat exposure. A subsequent incubation at 37 °C was found to be required for its manifestation. In contrast, if the priming dose was at 41 °C, thermotolerance was almost fully expressed by the end of the initial treatment. On the basis of these and other data, Li and Hahn (1980) proposed an operational model of thermotolerance. The authors suggest that thermotolerance can be divided into three complementary and sometimes competing processes: an initial event ("trigger"), the expression of resistance ("development"), and the gradual disappearance of resistance ("decay"). Each of these components may have its own temperature dependence as well as dependence on other factors such as pH and nutrients. Conceptually, the three components of thermotolerance may be considered to be independent processes. However, independent measurements of each component are not always possible.

A simplified schematic diagram of this model is shown in Figure 3. Thermotolerance develops in at least two steps. First, the triggering event converts normal cells to the triggered state with a rate constant k_1. This process very likely involves the activation of the heat shock transcription factor, HSF1 (Lis and Wu, 1993; Morimoto, 1993). Second, these triggered cells are converted to thermotolerant cells with a rate constant

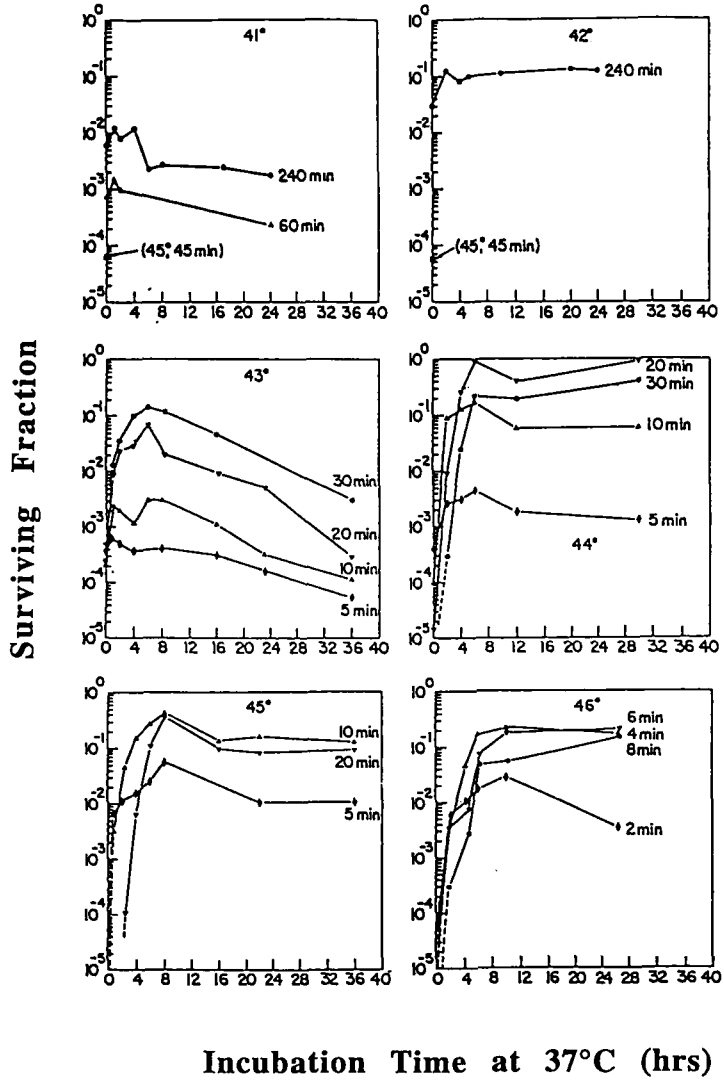

Figure 2. Kinetics of induction of thermotolerance in plateau-phase HA-1 cells. Cells were initially exposed to the designated temperature for the durations noted. In each case the second heat treatment was 45 °C for 45 minutes. The surviving fractions are plotted as a function of the duration of the 37 °C incubations between the first and second heat treatments. Note that survival at 0 hour is at (or near) maximum if the initial treatment was 41 °C. This is in contrast to the survival kinetics following the initial treatments at higher temperatures, where survival at 0 time is minimal; an incubation interval at 37 °C is required before thermotolerance manifests itself.

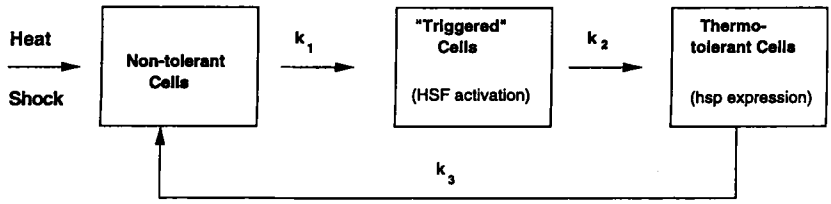

Figure 3. Phenomenological model of thermotolerance development and decay. Thermotolerance develops in two steps. First, a triggering event (e.g., heat shock) converts nontolerant cells to the triggered state. Second, thermotolerance then develops at a rate determined by the highly temperature-sensitive constant, k_2. Finally, thermotolerant cells reconvert to their sensitive state at a slow rate governed by k_3.

k_2. Above 42.5 °C, $k_2 \rightarrow 0$; the triggered cells remain sensitive, and if transferred to 37 °C become converted to thermotolerant cells. This thermotolerant state is manifested by the elevated expression of hsps, enhanced protection, and faster recovery from thermal damage. Finally, thermotolerant cells all reconvert to their sensitive state at a slow rate governed by rate constant k_3. An Arrhenius plot of the induction of thermotolerance as measured by the cell survival assay is shown in Figure 4b. There it is compared to the Arrhenius plot for cell killing (Figure 4a), for the induction of synthesis of one of the hsps (hsp70) (Figure 4c), and for the activation of the heat shock transcription factor (HSF1). The slopes of the straight lines can be used to calculate the activation energy for the rate-limiting reaction involved. The calculated value of activation energy is ~120 kcal/mole, suggesting protein unfolding or protein denaturation. The similarity in the four graphs (at temperatures above 43 °C) strongly suggests that cell killing by heat, the induction of thermotolerance, and the induction of heat shock response both in terms of hsp70 synthesis and HSF1 activation all have similar origins.

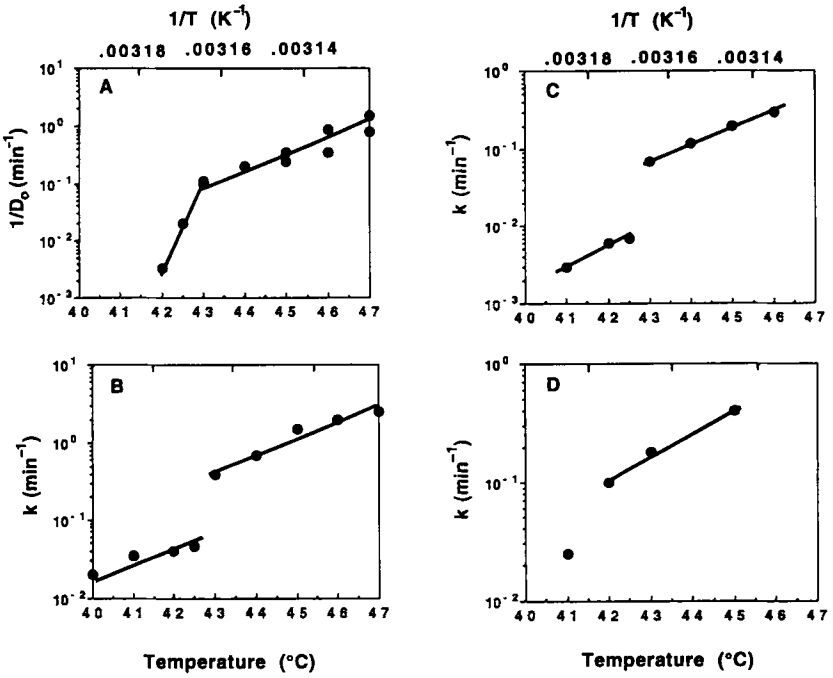

Figure 4. Arrhenius plots for heat-killing, induction of thermotolerance, induction of hsp 70, and heat shock factor (HSF1) activation. (**A**) Plot of the inverse of the log of the slope of the survival curves obtained at various temperatures against the inverse of the absolute temperature. (**B**) Rate at which thermotolerance is induced as a function of the duration of the triggering. This rate is plotted versus the inverse of the absolute temperature of the appropriate treatment. (**C**) Similar to (**B**), except that the time to induce a maximum amount of hsp70 was used to determine the ordinate. (**D**) The rate of maximum HSF1 binding activity to the heat shock element (HSE) of the rat heat shock promoter, determined by quantifying bands of HSE-HSF1 complexes that were obtained using the gel-mobility shift assay. Extracts were prepared immediately after cells were heat shocked for different times at a given temperature.

III. THE ROLE OF HEAT SHOCK PROTEINS IN THERMOTOLERANCE AND HEAT RESISTANCE

The mechanism for thermotolerance is not well understood, although several studies suggest that the hsps may be involved in its development (Landry et al., 1982; Li and Werb, 1982; Subjeck et al., 1982). Qualitative evidence exists for a causal relationship between hsp synthesis and

thermotolerance (Landry et al., 1982; Li and Werb, 1982; Subjeck et al., 1982; Laszlo and Li, 1985; Li, 1985): (a) heat shock induces transiently enhanced synthesis of hsps that correlates temporally with the development of thermotolerance; (b) the persistence of thermotolerance correlates well with the stability of hsps; (c) agents known to induce hsps induce thermotolerance; (d) conversely, agents known to induce thermotolerance induce hsps (see Table 1); and (e) stable heat-resistant variant cells express high levels of hsps constitutively.

One notable exception to the correlation summarized above is that amino acid analogues have been shown to induce hsps but not thermotolerance; HA-1 cells treated with such compounds are more sensitive to elevated temperatures (Li and Laszlo, 1985). This apparent lack of correlation can be attributed, however, to the dysfunction of analogue-substituted hsps: the nonfunctional, analogue-substituted hsps would not

Table 1. Relation Between Induction of Thermotolerance and Induction of hsp Synthesis

Thermotolerance Inducing Treatment	*hsp Synthesis*	*Reference*
Heat	+	Li (1983); Laszlo (1988)
Heavy metals	+	Li and Mivechi (1986)
Ethanol	+	Li and Hahn (1978); Li (1983); Henle et al. (1986); Boon-Niermeijer et al.(1988); Burgman et al. (1993)
Sodium arsenite	+	Crete and Landry (1990); Kampinga et al., (1992); Li, (1983)
Procaine, lidocaine	+	Hahn et al. (1985)
Aliphatic alcohols (C_5-C_8)	+	Hahn et al. (1985)
Dinitrophenol[a]	0	Boon-Niermeijer et al. (1986); Haveman, et al. (1986)
	+[c]	Ritossa (1962, 1963)
CCP[a]	+	Haveman et al. (1986)
Puromycin[b]	+	Lee and Dewey (1987)
Prostaglandin A	+	Amici et al. (1993)

Notes: +, increased; 0, unaffected.

[a] Not observed by Rastogi (Rastogi et al., 1988); this might, however, be due to the long interval between DNP or CCP treatment and test heating, and the low concentrations of CCP and DNP used.

[b] Only at intermediate concentrations of puromycin (3-30 μg/ml) that inhibit protein synthesis by 15-80%; not by higher concentrations.

[c] Induction of new puffs in *Drosophila* salivary gland giant chromosomes was observed, a phenomenon shown to be involved in the induction of synthesis of new proteins (Tissieres et al., 1974).

be expected to protect cells from thermal stress. In support of this notion, Li and Laszlo (Li and Laszlo, 1985; Laszlo and Li, 1993) found that the incorporation of amino acid analogues into cellular proteins inhibits the development of thermotolerance and that thermotolerant cells or permanently heat-resistant cells are more resistant to the thermal-sensitizing action of amino acid analogues.

Quantitatively, of the many hsps preferentially synthesized after heat shock, the concentration of the 70 kDa hsps (hsp70s) appears to correlate best with heat resistance, either permanent or transient (Li and Werb, 1982; Laszlo and Li, 1985; Li, 1985). However, good correlation between a 27 kDa hsp (hsp27) and thermal resistance also has been reported (Landry et al., 1989).

In mammalian cells, three types of experiments were performed before 1990 to vary the intracellular concentration of hsp70 and to correlate this change with thermal-stress response.

1. Microinjection of affinity-purified anti-hsp70 antibodies into rat cells appeared to prevent the nuclear and nucleolar accumulation of hsp70 after a test heat shock and greatly increased the lethality of a 45 °C, 30 minute heat treatment (Riabowol et al., 1988).
2. The 5'-control region of the hsp70 encoding gene was inserted into a plasmid containing the dihydrofolate reductase gene; this recombinant plasmid was then introduced into a Chinese hamster ovary (CHO) cell line, and a 20,000-fold elevation in its copy number was achieved by selection of cells with methotrexate. These copies of the hsp70 regulatory region presumably competed with the endogenous hsp70 encoding gene(s) for factors that activate hsp70 expression to reduce heat-inducible expression from the intact endogenous gene for hsp70 by at least 90%. It was found that cells containing the amplified regulatory sequences display increased thermosensitivity (Johnston and Kacey, 1988).
3. Human hsp70 microinjected directly into CHO cells increased the resistance of cells to 45 °C heating (Li, 1989).

The expression of hsp70 under heterologous promoters has yielded additional insight into its structure and function. Thus, transient expression of *Drosophila* hsp70 in monkey COS cells demonstrated that hsp70 accelerates the recovery of cell nucleoli after heat shock (Munro and Pelham, 1984). Similarly, the domains of human hsp70 responsible for

nucleolar localization and for ATP-binding were dissected (Milarski and Morimoto, 1989). Recently, using retroviral-mediated gene transfer technique, Li et al. (1991) have established rat cell lines stably and constitutively expressing a cloned human hsp70 gene. These cell lines provide a direct means of studying the effects of selected hsp70 expression on cell survival after heat shock.

Survival curves for both pooled populations and individually cloned lines of Rat1 cells constitutively expressing human hsp70 are plotted in Figure 5. Figure 5a shows that MV6-infected Rat-1 cells (vector control) exhibited similar clonogenic survival compared to Rat-1 cells after 45, 60, or 75 minutes of heat shock at 45 °C. By contrast, six independent pools of MVH-infected Rat-1 cells (expressing human hsp70) exhibited approximately 100-fold higher survival after 60 and 75 minutes of 45 °C heat treatment. Similar results were obtained when the 45 °C cell survival experiments were done with individual clones of Rat-1 cells expressing human hsp70 (Figure 5b). To evaluate the protective effect of different levels of human hsp70 expression, Li et al. (1992) measured the level of human hsp70 at 37 °C in individual clones by flow cytometry. In a parallel experiment the clonogenic survival of these cells after 75 minutes at 45 °C heat shock was determined. Figure 5c shows that clones expressing more human hsp70 generally survive thermal stress better than clones expressing lower levels, and there appears to be a good correlation between levels of exogenous human hsp70 expressed and the degree of thermal resistance. Interestingly, increases in human hsp70 expression beyond a certain point do not yield greater thermal protection. These data provide direct evidence for a causal relation between expression of a functional form of mammalian hsp70 and survival of cells at elevated temperatures.

Production of hsp70 is only part of the program of protein biosynthesis initiated after heat shock, and other components of this response might also enhance cell survival. This hypothesis is supported by the observation that Rat-1 cells rendered thermotolerant by a prior exposure to sublethal heat treatment and expressing the full panoply of hsps can better withstand heat shock than pooled cells only expressing human hsp70. However, the survival difference between thermotolerant and heterogeneous populations of recombinant Rat-1 cells may also be from a quantitative difference in the level (or concentration) of rat and human hsp70 expressed in the respective cells before heat shock, because cell clones expressing the highest human hsp70 levels are nearly as resistant to heat shock as the thermotolerant cells.

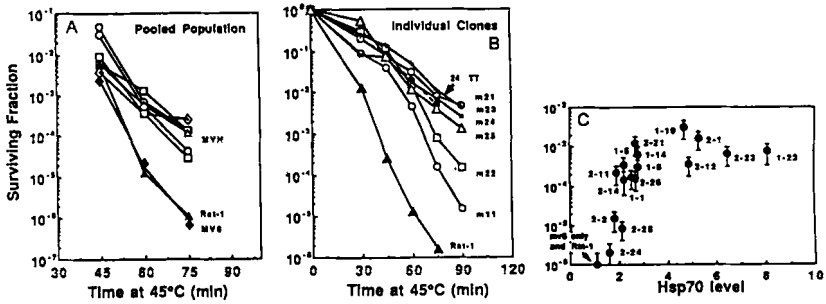

Figure 5. Expression of human hsp70 gene confers thermal resistance to Rat-1 cells. (A) Survival at 45 °C of pooled MVH-infected (expressing human hsp70), MV6-infected (vector control), and uninfected Rat-1 cells. Survivals from six pooled populations of MVH-infected cells independently derived from separate infection experiments are shown (open symbols). Each pool is derived by pooling 200-600 colonies. Survival values after 30 minutes heating at 45 °C are clustered around 20% for all cells and are, therefore, omitted for clarity. (B) Survival at 45 °C of individually cloned lines derived from MVH-infected cells. Each cell line expresses human hsp70 in the following order: m21 (highest) >m23 >m24 >m25 >m22 >m11 (lowest). Survival data for 24 hour thermotolerant Rat-1 cells (24 TT) are shown for comparison. Thermotolerance was induced by a heat treatment at 45 °C for 15 minutes, followed by 24 hour incubation at 37 °C. (C) Monolayers of exponentially growing individual clones of MVH-infected cells were exposed to 45 °C for 75 minutes, and survival was determined. In parallel experiments, relative levels of human hsp70 in these MVH clones were measured by flow cytometry (Becton Dickinson, FACS 440), using mAb C92F3A-5, specifically against human hsp70. Relative levels of hsp70 were estimated by mean FITC flourescence intensity of cell populations. At least 20,000 cells were analyzed for each flow cytometric measurement. Thermal survivals of various infected cell lines are plotted against the relative level of human hsp70.

Recently, we have examined the role played by hsp70 in thermotolerance development (Nussenzweig and Li, 1993) by studying the heat shock response in a cell line that constitutively expresses antisense hsc70 RNA. The antisense RNA, which is complementary to 712 nucleotides of the coding strand of the rat hsc70 gene, was designed to target both hsp70 (p72) and hsc70 (p73), which share greater than 75% identity in nucleotide sequence. As shown in Figure 6a, the expression of hsp70 is significantly reduced and delayed after heat shock in the transfected cells compared with the parental Rat-1 cells. For example, the hsp70 band appears as early as four hours after a 45 °C, 15 minute heat shock in Rat-1, while its appearance is two to four hours delayed in the transfected cells. However, the western blot does not show significant changes in hsc70 concentration in the different cell lines. It is plausible that higher levels of antisense RNA must be present to produce significant inhibition of hsc70, which constitutes approximately 0.1% of total mRNA in Rat-1 cells. This is consistent with previous attempts to regulate gene expression by antisense RNA in which a large excess of antisense over sense transcripts is often necessary to produce significant results. This may explain why hsc70 levels are more difficult to modify than hsp70, which begins to accumulate only after heat shock

Figures 6b and 6c show that the antisense transfected cells are more heat-sensitive than Rat-1, and impaired in thermotolerance development. While greater than 20% of the parental cells survive a 30 minute exposure at 45 °C, only 5% of A12 cells and 0.1% of A13 cells survive such a heat treatment. It is clear from Figures 6a and 6b that the cell line exhibiting the greatest delay in hsp70 synthesis (A13) is also the most heat sensitive. Cells expressing antisense RNA also show a reduced maximum tolerance level, as well as a delay in thermotolerance development (Figure 6c). Clearly, thermotolerance does develop in cells in which hsp70 levels are reduced, but to a much lesser degree than that in the parental cells.

It is well established that heat shock inhibits RNA and protein synthesis. This inhibition is reversible, and the transcriptional and translational activity recovers gradually when heated cells are returned to 37 °C incubation. The role of hsp70 in these processes was examined using Rat-1 fibroblasts expressing a cloned human hsp70 gene, designated M21 (Li et al., 1992). The constitutive expression of the human hsp70 gene in Rat-1 cells confers heat resistance as evidenced by the enhanced survival of heat-treated cells and resistance against heat-induced translational inhibition (termed translational tolerance; Liu, et al., 1992). In addition, after a 45 °C, 25 minute heat treatment, the time

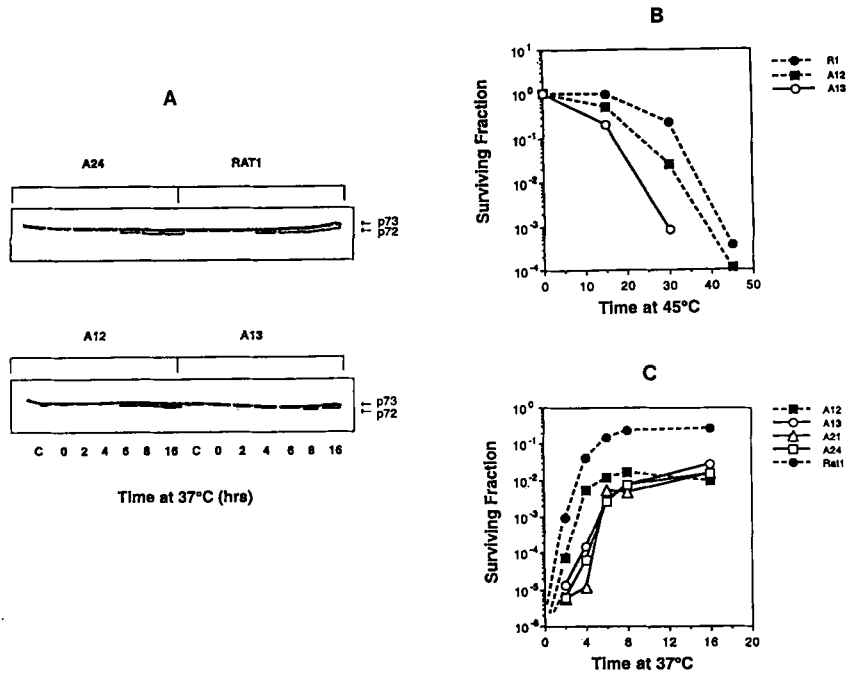

Figure 6. Cells expressing antisense hsc70 (p73) show a reduction in hsp70 (p72) synthesis, increased thermal sensitivity, and a delay in the development of thermotolerance. (A) Kinetics of hsp70 accumulation following heat shock is demonstrated by Western analysis. Antisense transfected cells are designated A11-A13 and A21-A24. Note that the band for p72 appears later in the lines expressing antisense RNA. Equal amounts of protein are separated by one-dimensional polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and probed with a mixture of mAb C92F3A-5 (specific for hsp70 (p72) and N27F3-4 (recognizes both hsc70 (p73) and hsp70 (p72)). (B) Cellular survivals after heat shock at 45 °C, determined by the colony formation assay. (C) Development of thermotolerance. Exponentially growing cultures are first exposed to a 45 °C, 15 minute pretreatment and then incubated at 37 °C for various times. After such incubations cells are challenged with a second treatment at 45 °C for 45 minutes, and survival curves are plotted as a function of the 37 °C incubation time between the two heat treatments. Compared to the wild-type, Rat-1, cells transfected with antisense hsc70 show a greatly reduced maximum tolerance level as well as a delay in thermotolerance development.

required for RNA and protein synthesis to recover was considerably shorter in M21 cells than control Rat-1 cells (see Figure 7). These data demonstrate that the expression of human hsp70 in Rat-1 cells, by itself, not only confers heat resistance and translational tolerance, but also

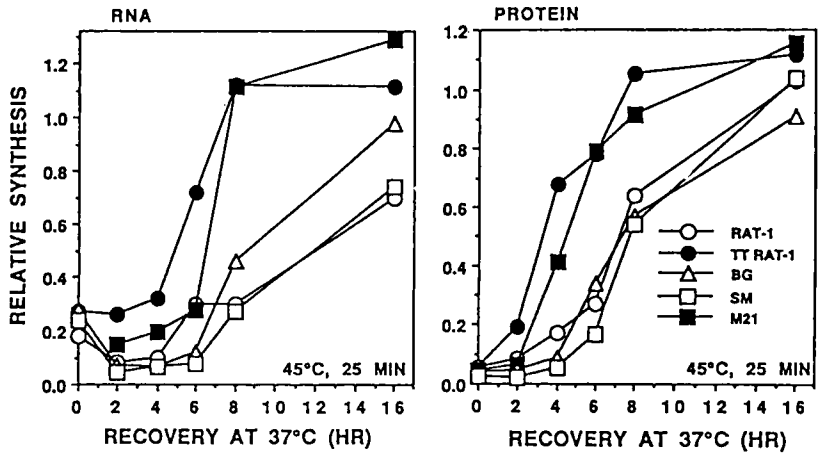

Figure 7. Recovery of RNA and protein synthesis following 45 °C, 25 minute heat treatment in Rat-1 cells and Rat-1 cells expressing intact or mutant human hsp70. Monolayers of cells were heated at 45 °C for 25 minutes and subsequently incubated at 37 °C for various times. At the indicated time after a given heat treatment, cells were labeled for 10 minutes with [^{3}H]-uridine or [^{14}C]-leucine. Radioactivity in the samples was determined by scintillation counting and normalized to the protein content of the sample. Left panel, RNA synthesis; right panel, protein synthesis. Experiments were performed on Rat-1 cells, M21 cells which overexpress human hsp70, MVHΔBg cells expressing mutant hsp70 missing its ATP-binding domain, and MVHΔSm cells expressing mutant hsp70 missing its nucleolar localization domain. Note that the time required for protein or RNA synthesis to recover is considerably shorter for M21 and TT Rat-1 cells (cells expressing high levels of rat hsps) than for Rat-1, MVHΔBg, and MVHΔSm cells.

facilitates the ability of cells to recover from translational and transcriptional inhibition after thermal stress.

In parallel experiments, the effects that mutations of hsp70 have on the cellular transcriptional/translational activity after heat shock were studied. Both a 4-bp out-of-frame deletion (Δ21) and in-frame deletion of the nucleolar localization domain (ΔSm) of human hsp70 had no significant affect on the cell's intrinsic heat sensitivity, heat-induced transcriptional/translational inhibition, or on the subsequent recovery at 37 °C. On the other hand, cells expressing a mutant human hsp70 missing the ATP-binding domain (ΔBg) were found to be heat resistant. However, when the recovery kinetics of protein synthesis were evaluated, no significant differences were observed between Rat-1 cells and cells expressing any of these mutated forms of hsp70 (Figure 7).

These studies provide strong evidence for a direct link between the expression of a functional form of mammalian hsp70 and protection of cells translational machinery at elevated temperature. They suggest that ATP-binding and/or hydrolysis by hsp70 are dispensable in the hsp70-mediated protection against thermal killing and translational inhibition. It is plausible that hsp70 lacking its ATP-binding domain can still bind to cellular proteins (such as RNPs), stabilize them and prevent their aggregation at elevated temperatures. On the other hand, because the mutant hsp70 missing its ATP-binding domain does not facilitate the recovery from translational inhibition, ATP-binding and/or hydrolysis may be important in enabling the dissociation of hsp70 from its substrates, or in facilitating the dissociation of aggregated protein complexes to restore their functional integrity.

The ability of hsps to protect various cellular processes and enzymatic activities during stress has been demonstrated in a series of experiments. *In vitro* studies by Skowyra et al. (1990) have shown that the *Escherichia coli* hsp70 homologue, DnaK, can protect RNA polymerase from inactivation during heat treatment, and that thermally inactivated RNA polymerase can be reactivated by DnaK in a process that requires ATP hydrolysis. Furthermore, the thermal protection efficiency of DnaK is enhanced by the action of its partner proteins DnaJ and GrpE (Hendrick and Hartl, 1993). Similarly, mitochondrial hsp60 has been shown to have thermal protective functions through its ability to prevent the thermal inactivation of dihydrofolate reductase imported into the mitochondria (Martin et al., 1992). The *in vivo* enzymatic activities of luciferase and ß-galactosidase were also demonstrated to be protected in thermotolerant mouse and *Drosophila* cells given a heat challenge (Nguyen et al., 1989).

Heat shock proteins have also been implicated in protecting the splicing machinery from disruption by heat shock (Yost and Lindquist, 1986, 1988, 1991): high temperatures are found to result in the accumulation of mRNA precursors due to the block in splicing. However, in cells made thermotolerant, splicing is no longer disrupted and mature mRNAs accumulate.

In addition to hsp70, the small hsp hsp27 has been shown to have thermal protective functions. Landry and coworkers have overexpressed hsp27 from various species into different cell lines and have observed that resistance to heat shock correlates with levels of hsp27 (Landry et al., 1989; Huot et al., 1991; Lavoie et al., 1993). Also, it appears that phosphorylation of hsp27 may play an important role in its thermal protective function. In support of this, Crete and Landry (1990) observed

that chemical agents such as cycloheximide, A23187, and EGTA, which induce phosphorylation but not the accumulation of hsp27, resulted in a significant degree of thermal protection. Furthermore, phosphorylation mutants of hsp27 failed to protect cells from heat stress (Landry et al., 1993). However, this point remains controversial because a recent report provides evidence that the chaperone properties of the small hsp contributes to the increased cellular thermoresistance in a phosphorylation-independent manner (Knauf et al., 1994). These authors suggest that the phosphorylation-dependent function of hsp27 is distinct from its thermoresistance-mediating functions.

IV. THE ROLE OF HEAT SHOCK PROTEINS IN NORMAL CELLULAR PROCESSES

Although hsps were first noticed because of their dramatically increased synthesis upon heat treatment, most hsps are abundantly expressed under physiological conditions, and many are essential for cell viability (Lindquist and Craig, 1988; Fayet et al., 1989; Georgopoulus et al., 1990). During basic biochemical processes such as polypeptide synthesis, protein assembly into oligomeric structures, and transport into organelles, hsps bind noncovalently and reversibly to a wide variety of protein surfaces that are transiently exposed. The interaction with hsps effectively shields partially unfolded and denatured polypeptides from their tendency to form aggregates. Utilizing energy in the form of ATP hydrolysis, the polypeptides are then released from hsps in a manner that allows for their proper folding. Because of their ability to assist in the folding of a wide variety of proteins, hsps have been referred to as molecular chaperones. In this section we briefly summarize the important chaperone activity of hsps in cell physiology. For extensive reviews see Gething and Sambrook (1992), Georgopoulos and Welch (1993), and Hendrick and Hartl (1993).

Chaperones have been discovered in virtually every organism and throughout all cellular compartments of the eukaryotic cell. Cytosolic hsp70 has been shown to associate transiently with nascent polypeptide chains as they are being synthesized (Beckmann et al., 1990). Similarly, Nelson et al. (1992) showed that the SSB-hsp70 subfamily from *Saccharomyces cerevisiae* is associated with translating ribosomes, and suggested that hsp70 may facilitate the passage of nascent polypeptides

through the ribosome channel. This is analogous to the role played by organellar hsp70 during membrane translocation and folding. For example, mitochondrial hsp70s have been shown to interact with extended polypeptide precusursors as they are being imported into this organelle. This interaction with hsp70 is thought to help "pull" the protein through the channel (Hartl and Neupert, 1990; Kang et al., 1990; Neupert et al., 1990). The extended polypeptide chain then interacts with the mitochondrial hsp60 chaperone which helps it fold to its mature conformation.

In the cytosol, hsp70 members have also been implicated in maintaining polypeptides precursors in a competent state for translocation into organelles (Chirico et al., 1988; Deshaies et al., 1988; Zimmerman et al., 1988), and shown to be essential for transport of some nuclear-targeted proteins (Imamoto et al., 1992; Shi and Thomas, 1992).

In the endoplasmic reticulum (ER), another member of the hsp70 family, named BiP, plays a key role in the folding and oligomerization of newly synthesized proteins, and may also be involved in the translocatian of precursors across the ER membrane (Rothblatt et al., 1989; Vogel et al., 1990). BiP has also been found to bind to Ig heavy chains, and is capable of retaining the incompletely assembled immunoglobulin until the chaperone is displaced by light chains (Hendershot et al., 1987). Similarly, a chaperone bound to ER membranes, referred to as calnexin, has recently been shown to promote retention of incomplete components of class I histocompatibility and T cell receptor molecules in the ER, a chaperone function that may contribute to the efficient assembly of multisubunit complexes (Jackson et al., 1994; Rajagopalan et al., 1994).

Though only briefly alluded to in this review, members of the hsp60 chaperone family play a fundamental role in protein folding (for a recent review see Hendrick and Hartl, 1993). These proteins, referred to as chaperonins, are made up of a 14 subunit oligomer with a double torroid appearance. The ATP-dependent folding reaction is thought to take place in the protected microenvironment of the central cavity. In *E. coli*, the 60 kDa chaperonin, GroEL, interacts functionally with GroES, a 10 kDa polypeptide that forms a single ring of 7 subunits that assists in releasing of bound polypeptides from GroEL. Recently, Hartl and colleagues have described the entire protein folding cycle of GroEL and GroES (Martin et al., 1994). The folding of a number of enzymes by GroEL has also been demonstrated (reviewed in Gething and Sambrook, 1992; Kelley and Georgopoulos, 1992; Hendrick and Hartl, 1993).

Other cellular processes in which chaperones have been implicated include: (a) antigen presentation, in which the peptide binding protein Ppb 72/74 plays a role (VanBuskirk et al., 1991); (b) lysosomal degradation of intracellular proteins, assisted by Prp73, which is biochemically identical to hsc70 (Chiang et al., 1989); (c) cytoskeletion assembly by the cytosolic TCP-1 chaperonin (Gao et al., 1992; Yaffe et al., 1992); (d) regulation by hsp90 of steroid hormone receptor (Picard et al., 1988, 1990; Pratt, 1990), and protein kinase activity (Sefton et al., 1978; Oppermann et al., 1981; Brugge, 1986; Miyata and Yahara, 1992); and (e) the regulation of the heat shock response by hsp70 (Abravaya et al., 1992; Baler et al., 1992).

It is clear from this synopsis that in any process involving polypeptide maturation, intracellular transport, assembly and disassembly, protein degradation, or protection from environmental stress, the ubiquitous chaperone machine is likely to be a key player.

V. SUMMARY

Research from the past few years has made it clear that hsps are required for the development of thermotolerance. The fact that mammalian cells become permanently thermoresistant when transfected with hsp70 and,conversely, that cells become thermal sensitive when hsp70 levels are reduced, directly demonstrates that hsp70 plays a vital role in thermotolerance. hsp70 acts by stabilizing and preventing thermal denaturation of proteins, and by facilitating the dissociation of protein aggregates that are formed during conditions of stress. Other hsps, such as hsp27, may also have thermal protective functions, and there may even be cooperative action among members of the hsp family during thermotolerance development, as has been shown for the hsp70 and hsp60 chaperones during the folding of denatured proteins (reviewed by Hendrick and Hartl, 1993).

Studies both *in vitro* and *in vivo* have shown that chaperones have the ability to recognize structures that are exposed in unfolded proteins, protect polypeptides from aggregation or premature folding, and promote the refolding of proteins after denaturation. These folding and unfolding reactions are critical both during normal cellular biogenesis and during metabolic stress. Under various conditions of stress the increased need to repair denatured proteins and protect vital structures is fulfilled by the enhanced and preferential synthesis of chaperones.

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