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Biochemical Adaptation to Extreme Environments

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1. INTRODUCTION

Physiology can be viewed as the collection of mechanisms and processes that allows organisms to deal with challenges from both internal (e.g., exercise, growth, reproduction) and external (e.g., variations in temperature, oxygen and water availability, salinity, pressure, radiation, heavy metals, etc.) sources. In this chapter we focus on solutions to some of the external challenges to life in extreme environments. This subject is a huge one because life on Earth has radiated into every conceivable environment, from the frigid Antarctic to boiling hot springs, from the ocean depths to the tops of mountains, from hypersaline lakes to the driest deserts, and many more. We mainly consider biochemical and molecular solutions by vertebrate animals to environmental challenges of low oxygen and low temperature because these hold lessons that can be applied to the human condition and medical concerns. However, the reader should be aware that the extremes of vertebrate life are bested on every front by the capabilities of invertebrates, plants, bacteria, and archaea and many excellent resources explore life at the extremes from different perspectives; selected texts include those by Hochachka and Somero *(1)*, Schmid-Neilsen *(2)*, Ashcroft *(3),* Margesin and Schinner *(4),* Willmer et al. *(5),* Lutz et al. *(6),* and Gerday and Glansdorff *(7).*

In general, adaptive responses to environmental stresses are needed for two main reasons. First, all biological molecules and all biochemical reactions are directly susceptible to perturbation by multiple environmental parameters including temperature, pressure, pH, ionic strength, solute concentrations, water availability, radiation, and attack by free radicals. Second, to sustain life, all cells must maintain adequate energy turnover by maintaining sufficient energy currencies, primarily adenosine triphosphate (ATP), that is used to drive thermodynamically unfavorable reactions, and reduced nicotinamide adenine dinucleotide phosphate (NADPH), that is used for reductive biosynthesis *(1)*. Depending on the circumstances, adaptation can be undertaken at behavioral, physiological, and/or biochemical levels and can address one (or more) of three goals: compensation, conservation, and protection.

Compensatory responses often deal with short-term or relatively mild stresses under which the organism aims to maintain normal functions. An example of a compensatory response is the rapid increase in ventilation rate and the release of stored erythrocytes that occurs in response to hypoxia (low oxygen) challenge. In man, this is well- known as the first response

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to movement from low to high altitude. Compensation can also be employed for long-term adaptation. For example, homeoviscous adaptation is a common response to temperature change in ectotherms that alters the proportions of saturated, mono- and poly-unsaturated fatty acids in cellular membranes to compensate for temperature effects on membrane fluidity. In fish, a switch from warm to cold water triggers a rapid upregulation of desaturase enzymes that raise monounsaturated levels in membranes at the expense of saturated fatty acids *(8)*.

Conservation responses are typically enacted when organisms face a stress that is too strong or too prolonged and, therefore, incompatible with maintaining normal life. Conservation often includes behavioural responses that move the organism to a sheltered site (e.g., desert toads dig underground during the dry season to limit their exposure to desiccating air; many turtles spend the winter under water to avoid freezing temperatures on land) as well as strong metabolic rate depression (MRD). MRD frequently combines adaptations at both physiological (e.g., reduced rates of heart beat and breathing) and biochemical (e.g., selective inhibition of nonessential metabolic functions) levels to reduce basal metabolic rate to as little as 1 to 20 % of the previous resting rate *(9,10)*. MRD is often brought into play when the stress compromises an organism's access to exogenous fuels (either foodstuffs or oxygen) and thereby greatly extends the time that the organism can survive using only its fixed internal fuel reserves.

Protective responses are typically enacted against stresses that challenge the physical integrity of living organisms. For example, desiccation or high salinity dehydrate cells causing metabolic damage from high ionic strength and compression stress on membranes owing to cell-volume collapse. Exposure to subzero temperatures brings the risk of freezing and the massive physical and metabolic destruction that can arise from the propagation of ice crystals through a body. Protective responses often involve the proliferation of low-molecular-weight metabolites (e.g., high plasma urea limit body water loss in desert amphibians; high glycerol acts as an antifreeze for cold-hardy insects) or the synthesis of new proteins (e.g., heat shock or cold shock proteins that stabilize protein conformations, antifreeze proteins that inhibit ice crystal formation) *(4,11,12)*. These address both cell-volume concerns and the physical stability and conformation of macromolecules. Protective responses often go hand- in- hand with conservation responses.

At the biochemical level, virtually all aspects of cell function and metabolism can be the target of adaptive change in response to environmental stress *(13)* and can include elements such as those that follow:

1. Increasing or decreasing the expression of selected genes to produce corresponding changes in the levels of various proteins.
2. Elaborating novel genes and proteins that address stress-specific concerns.
3. Altering the kinetic and regulatory properties of enzymes and functional proteins.
4. Changing enzyme susceptibility to posttranslational modification, in particular the effects of reversible phosphorylation by protein kinases and protein phosphatases.
5. Modifying sensing and signaling mechanisms by changes to cell surface receptors, signal transduction cascades, cross-talk between signaling pathways, and the targets of signals including proteins, transcription factors, and genes.
6. Changing protein–protein binding interactions that alter the composition of enzyme/protein complexes or localize enzyme/protein function via associations with specific binding proteins or subcellular structures.
7. Changing membrane composition to compensate for changes in environmental factors including temperature, pH, and ionic composition.

8. Producing protective molecules that can defend cell volume and/or stabilize protein or membrane structure/function.

We discuss examples of many of these strategies of biochemical adaptation throughout the remainder of this chapter. In doing so, we draw most of our examples from discussions of three animal strategies for dealing with extreme environments: anoxia tolerance, hibernation, and freeze tolerance. Our treatment of each of these topics is in no way comprehensive, for each is a huge field of its own. We begin with a brief overview of each of these strategies, but then focus on two areas of major new research: the molecular mechanisms of MRD, and the use of new technologies in genomics to provide a comprehensive assessment of the full range of metabolic adaptations that underlies animal survival in extreme environments.

2. ANOXIA TOLERANCE

2.1. Low Oxygen Injuries and Strategies for Survival in Oxygen-Sensitive Organisms

Humans lead a highly oxygen-dependent existence—an interruption of oxygen supply to the most oxygen-sensitive human organ, the brain, for more than about 4 to 5 min can cause irreparable damage. Many other organisms similarly rely on the high rates of ATP generation possible from oxidative phosphorylation to fuel energy-expensive lifestyles such as the homeothermy of mammals and birds and the muscle power requirements flying insects or jetting squid. For such organisms, oxygen limitation can have grave consequences. Their response to hypoxia (low oxygen) is an immediate implementation of compensatory mechanisms that both increase oxygen delivery to tissues (e.g., increased ventilation rate, release of erythrocytes from spleen) and elevate ATP output from anaerobic sources (e.g., activate glycolysis, creatine phosphate hydrolysis). Gene expression is also activated under the regulation of the hypoxia-inducible factor 1 (HIF-1) to provide more long-lasting compensatory responses. Genes activated by HIF-1 include those for vascular endothelial growth factor (to stimulate capillary growth), erythropoietin (to stimulate red blood cell synthesis), several glycolytic enzymes (to increase glycolytic capacity), and glucose transporters (to enhance substrate uptake) *(14)*.

These compensatory responses work well for dealing with mild hypoxia or ischemia (reduced blood flow) but fail as responses to severe hypoxia, anoxia (no oxygen) or severe ischemia (blood flow stopped). This is because an elevated glycolytic rate is rarely sufficient to sustain cellular ATP demands for very long. For example, ischemic mouse brain shows an almost instantaneous increase in glycolytic rate of four- to sevenfold but this only partially compensates for the much lower ATP yield from glycolysis compared with the full oxidation of glucose by brain mitochondria (a net of 2 ATP is produced per 1 glucose catabolized to 2 lactate vs 36 ATP if glucose is catabolized to CO_2 and H_2O). As a result, within 5 min of oxygen deprivation, as much as 90% of the ATP is depleted in mammalian brain because the rate of ATP output from glycolysis cannot keep pace with the unaltered rate of ATP consumption by energy-consuming cell functions.

Indeed, this imbalance between ATP-producing and ATP-consuming processes is the root of low oxygen injuries in all oxygen-sensitive systems as well as the primary reason for the implementation of conservation responses by anoxia-tolerant species. Chief among the causes of metabolic failure is the inability of oxygen-limited cells to meet the ATP demands of the ion pumps that are involved in maintaining membrane potential difference and the

many sensing and signaling functions that rely on transmembrane ion gradients *(15,16)*. For example, the sodium-potassium ATPase alone uses 5 to 40% of cellular ATP turnover in mammals, depending on cell type *(17)*. Membrane potential difference is maintained by a balance between the actions of ATP-dependent ion pumps that move ions against their concentration gradients and facilitative ion channels that allow ions to move down their concentration gradients. If ion pumps fail because of ATP limitation, then membrane depolarization quickly occurs. Depolarization results in a rapid uptake of Na^+ and water (and a loss of K^+) from cells and is followed by an influx of Ca^{2+} through voltage-gated Ca^{2+} channels. Transient elevations of cytosolic Ca^{2+} are critical signaling mechanisms for many cell functions but sustained high Ca^{2+} triggers a range of pathological changes including the activation of phospholipases and proteases that lead to damage and death of cells *(18,19)*.

Not only is oxygen deprivation damaging, but another set of injuries arise when ischemic tissues are reperfused with oxygenated blood. Reperfusion injury, such as occurs after heart attack or stroke, is caused by a burst of reactive oxygen species (ROS) generation, chiefly superoxide radicals, from a highly reduced electron transport chain when oxygen is restored. ROS production can overwhelm existing antioxidant defenses of cells and cause oxidative damage to macromolecules including DNA, proteins, and membrane lipids *(20)*. Indirect damage can also arise such as from an inability of sarco- or endoplasmic reticulum membranes that are damaged by peroxidation to properly re-sequester Ca^{2+}, thereby exacerbating the Ca^{2+}-mediated damage that occurred under anoxia/ischemia.

2.2. *Facultative Anaerobiosis*

Unlike the oxygen-sensitive species discussed earlier, many organisms are well equipped to survive the environmental extreme of anoxia. Indeed, some organisms are obligate anaerobes, whereas others can survive equally well in the presence or absence of oxygen. Among vertebrates, the premier facultative anaerobes are various species of freshwater turtles of the *Chrysemys* and *Trachemys* genera. These hibernate underwater to escape freezing temperatures but in doing so cannot breathe with lungs. However, turtles can survive in cold, deoxygenated water for as long as three months using anaerobic glycolysis as their only source of ATP generation *(21)*. Carp and goldfish also use well-developed anoxia tolerance to support winter survival in small ice-locked ponds where oxygen in the water is depleted by the respiration of all organisms present. Many types of invertebrates are also excellent facultative anaerobes, the best-studied of these being molluscs and annelids of the marine intertidal zone; these gill-breathing animals have full access to oxygen when under water but switch to anaerobic metabolism each time the tide recedes *(22)*.

Given the multiple forms of metabolic injury that can arise because of oxygen limitation in oxygen-sensitive organisms, it is clear that facultative anaerobes must address a variety of issues in order to survive periods of oxygen deprivation. The overriding strategy for anaerobiosis is not compensation but conservation—organisms use strategies to minimize their ATP use, optimize the time that fixed internal fuel reserves can fuel metabolism, and limit the disruption of cellular homeostasis. The following five main categories of biochemical adaptation have been identified (*9,10,13,16)*:

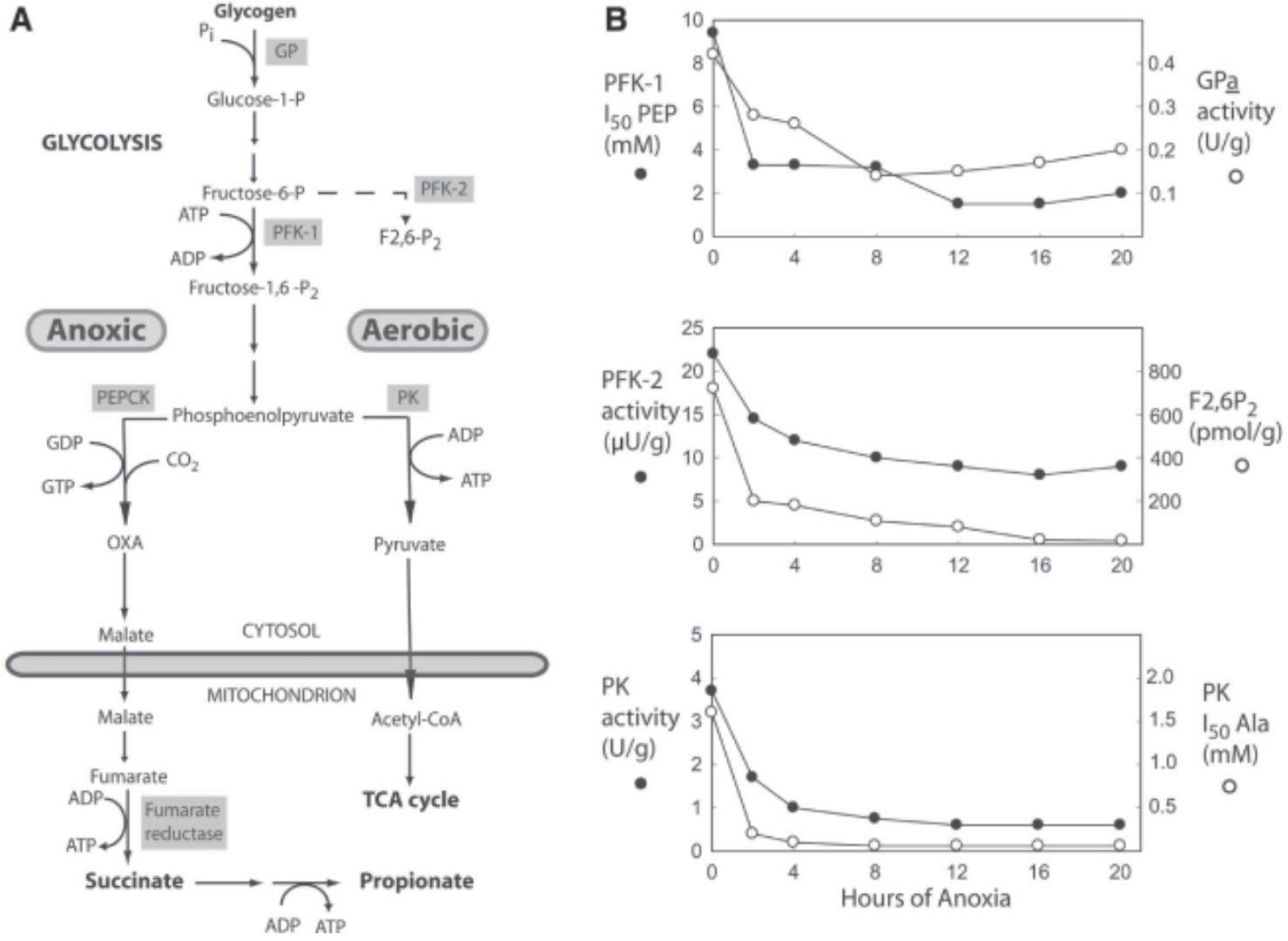
1. *Fuel supply*: Facultative anaerobes maintain large reserves of glycogen in their tissues and marine invertebrates also maintain substantial pools of fermentable amino acids (e.g., aspartate, glutamate).

2. *Enhance ATP yield*: Anaerobic ATP production by the basic glycolytic pathway can be supplemented with other reactions that increase the ATP yield per glucose catabolized. For example, many marine molluscs catabolize glucose to succinate and propionate, with additional substrate-level phosphorylation reactions increasing the yield to 4 or 6 ATP per glucose, compared with 2 ATP per glucose converted to lactate (Fig. 1).
3. *Minimize cytotoxicity*: Cells generating ATP from anaerobic glycolysis ending in lactate production soon undergo significant acidification as well as major end-product accumulation. Solutions include enhanced buffering capacity (e.g., turtles release calcium carbonate from their shell and bone to buffer acid build-up and also move huge amounts of lactate into their shells for storage), making less acidic end-products (e.g., synthesis of succinate or propionate generates a much lower proton load than does lactate output), or making products that can be excreted easily (e.g., carp and goldfish catabolize lactate to ethanol + CO_2 in their skeletal muscles and then excrete both across the gills).
4. *MRD*: A coordinated and strong reduction in the rates of ATP consumption by multiple cell functions reduces ATP demand into line with ATP output from fermentative pathways and greatly extends the time that fixed internal reserves of fermentable fuels can sustain anaerobic survival.
5. *Antioxidant defense*: Well-developed enzymatic and metabolite antioxidant defenses minimize oxidative stress during the transition from anaerobiosis back to aerobic life. For example, anoxia-tolerant freshwater turtles show the highest constitutive antioxidant defenses among ectotherms (closely comparable to mammalian levels of defense) and anoxic or ischemic stresses frequently induce the synthesis of antioxidants in species that encounter low oxygen stress less frequently *(23,24)*.

3. HIBERNATION

A distinguishing characteristic of mammals and birds is endothermy, heating the body from internal biochemical reactions to permit homeothermy, the maintenance of a high and near constant core body temperature (Tb). Endothermy is very costly, the metabolic rate of mammals being four to seven times higher than that of comparably sized reptiles. This must be supported by equally higher rates of fuel consumption, supplied by foraging or, if food supply is limiting, by food caches or body fuel reserves (chiefly adipose). When environmental temperature falls in the autumn and winter, so to does the metabolic rate, Tb and food needs of an ectothermic (cold-blooded) organism. However, the opposite is true of mammals—they lose body heat faster at colder temperatures, thereby necessitating a higher metabolic rate and greater fuel consumption to compensate. Some mammals can meet this challenge and minimize the extent of metabolic compensation needed by strategies including enhanced body insulation, huddling in groups, counter-current heat exchangers in extremities, and so forth *(5)*. However, for others, the combination of cold temperatures and lack of food availability makes winter survival as a homeotherm impossible. The problem is particularly acute for species such as insectivorous bats or grazing herbivores (e.g., ground squirrels, marmots) that have little or no access to edible food in the winter.

For many small mammals, the solution to life in extremely cold environments is hibernation. By abandoning homeothermy and allowing Tb to fall, tracking environmental temperature, they gain tremendous energy savings, sufficient to sustain life until spring. For example, ground squirrels save as much as 88% of the energy that would otherwise be needed to maintain a Tb of 37°C over the winter *(25)*. During hibernation, all aspects of the animal's physiology slow dramatically. Metabolic rate can be as low as 1 to 5% (at Tb = 0–5°C) of the normal resting rate at 37°C. Heart beat in ground squirrels can drop from 200–300 to just 5–


A
Glycogen
Pi
GP
Glucose-1-P
GLYCOLYSIS
Fructose-6-P
PFK-2
ATP
PFK-1
F2,6-P2
ADP
Fructose-1,6 -P2
Anoxic
Aerobic
PEPCK
Phosphoenolpyruvate
PK
GDP
CO2
ADP
GTP
ATP
OXA
Pyruvate
Malate
CYTOSOL
MITOCHONDRION
Malate
Acetyl-CoA
Fumarate
ADP
Fumarate reductase
TCA cycle
ATP
Succinate
Propionate
ADP ATP
B
PFK-1 I50 PEP (mM)
GPa activity (U/g)
PFK-2 activity (μU/g)
F2,6P2 (pmol/g)
PK activity (U/g)
PK I50 Ala (mM)
Hours of Anoxia

10 beats per minute. Breathing rate similarly declines and sometimes includes long periods of apnea (breath-hold). Hibernation is not continuous but consists of a series of torpor bouts that in midwinter typically stretch to 1 to 3 wk and are interspersed with brief periods of arousal, generally lasting 6 to 24 h, when the animal uses nonshivering thermogenesis by brown adipose tissue to return its Tb to 37°C. Arousals are by far the greatest energy expenditures of the winter season although the signals that trigger them and their purpose are still not well understood.

3.1. Hypothermic and Ischemic Injury

From our point of view as nonhibernating mammals, there are multiple risks involved with the hibernation strategy. Hypothermia is a serious problem for most mammals; for example, humans undergo severe, often lethal, metabolic injuries if our core Tb drops below about 25°C. Hypothermic injury arises from two main factors. The first is the differential effects of temperature change on cellular reaction rates that culminate in a mismatch between the net rates of ATP-producing and ATP-utilizing reactions. The result is that energy currencies are depleted and the major manifestation of this energy crisis is membrane depolarization, which sets off a chain of catastrophic events that are much the same as those described earlier for anoxia-induced energy failure *(15).* The second main effect of hypothermia is a decrease in lipid fluidity in both membranes and adipose depots as temperature declines. Membrane lipid fluidity is crucial for allowing protein movements within membranes and the protein conformational changes that are associated with receptor and transporter functions, whereas adipose depots must remain fluid in order for triglycerides to be mobilized as fuels. Normally, the composition of mammalian lipids is optimized for 37°C function and they solidify at about room temperature. Other problems associated with chilling of nonhibernators include the ischemia that develops at extremely low blood-flow rates and a greatly increased risk of blood clotting at low flow rates.

3.2. The Solutions for Hibernators

Summer-active individuals of hibernating species are just as susceptible to hypothermia-induced membrane depolarization as are nonhibernating species. Hence, the preparations for

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Fig. 1. *(continued from facing page)* Control of glycolysis in anoxia tolerant marine molluscs. (A) The glycolytic pathway showing aerobic and anoxic routes of carbohydrate catabolism determined by the fate of phosphoenolpyruvate (PEP) at the PEP branchpoint. The aerobic route feeds PEP via an active pyruvate kinase (PK) into the tricarboxylic acid (TCA) cycle. The anaerobic route, facilitated by phosphorylation-mediated inhibition of PK, feeds PEP via PEP carboxykinase (PEPCK) into reactions of succinate and propionate synthesis that are linked with substrate-level phosphorylation of ADP to increase the ATP yield of anaerobic metabolism. (B) Overall glycolytic rate depression under anoxia is mediated by reversible phosphorylation control at multiple enzyme loci. Data from gill of the whelk, *Busycon canaliculatum*, over the course of 20 hours of anoxia exposure show coordinated reduction in (1) the activity of the active phosphorylated a form of glycogen phosphorylase (GPa), (2) the I_{50} value for PEP of 6-phosphofructo-1-kinase (PFK-1)(one of the enzyme kinetic parameters modified by anoxia-induced phosphorylation of PFK-1), (3) the activity of 6-phosphofructo-2-kinase (PFK-2), (4) levels of the PFK-2 product, fructose-2,6-bisphosphate ($F2,6P_2$), (5) the activity of PK and (6) the I_{50} value for L-alanine of PK. Enzyme activities are in units (or micro-units) per gram wet mass and concentrations are in millimolar. Enzyme data modified from Storey *(45)*.

hibernation must include adaptations that address the variety of potential problems noted previously as well as ensure that adequate fuel reserves, in the form of adipose depots, are accumulated. The main mechanisms known to be involved in mammalian hibernation are discussed here.

3.2.1. MRD

This is the primary conservation strategy of hibernation. Strong MRD causes the fall in Tb which is unopposed because of an accompanying reduction of the hypothalmic set point for Tb (i.e., the equivalent of lowering a thermostat). Coordination is key to reestablishing balanced rates of ATP production vs ATP use during torpor and selectivity is applied to reorder cellular priorities and shut down various functions that are not needed in the torpid state.

3.2.2. Fuel Accumulation

Although some hibernating species cache food in their burrows and can eat between torpor bouts, most do not. In late summer, animals enter a phase of hyperphagia and lay down huge reserves of lipids in white adipose depots, increasing body mass by 50% or more. Sufficient fuel must be laid down to support winter torpor, periodic arousals, and considerable activity in the spring before eating resumes. Normal hormonal controls on satiety and lipid storage by adipose tissue are overridden during this period to alter the body mass set point; for example, the production of leptin is reduced despite a metabolic situation (rising adiposity) that should elevate levels of this hormone *(26).* Diet selection is also employed to ensure that lipid depots acquire elevated levels of polyunsaturated fatty acids (PUFAs) *(27).* A high PUFA content (particularly linoleic 18:2 and α-linolenic 18:3 fatty acids) is needed to maintain the fluidity of lipid depots down to at least 5°C. However, although the composition of depot lipids is modified before hibernation, homeoviscous adaptation of membranes does not occur and it is not yet clear how functionality of hibernator membranes is maintained in the cold. Indeed, it is possible that impaired membrane function at low Tb values contributes to MRD.

3.2.3. Fuel Metabolism

Both seasonal and hibernation-induced adjustments are made to switch organs over to the use of lipids as the primary fuel supply during the winter with a strong accompanying suppression of carbohydrate use *(28).* For example, entry into a torpor bout triggers the upregulation of fatty acid binding proteins (that provide intracellular transport of fatty acids) and of pyruvate dehydrogenase (PDH) kinase, the enzyme that phosphorylates and inhibits PDH thereby suppressing carbohydrate use during torpor (Fig. 2) *(29,30).* Ketogenesis by liver is also enhanced to supplement fuel supply for the brain and minimize the need for muscle proteolysis to supply amino acids for gluconeogenesis.

3.2.4. Thermogenesis

Arousal from bouts of torpor is dependent on high rates of nonshivering thermogenesis by brown adipose tissue (BAT) that is found in large masses in the interscapular region, the perirenal area, and surrounds the aorta and heart of the hibernator. BAT proliferation and differentiation is responsive to multiple signals including insulin and insulin-dependent growth factor (IGF-I) that are particularly involved in longer term seasonal responses (and are mediated by protein kinase B) and noradrenaline that is responsible for acute activation of nonshivering thermogenesis *(31).* Noradrenaline acts via β3-adrenergic receptors on the BAT plasma membrane to activate protein kinase A which, in turn, triggers lipolysis (activa-

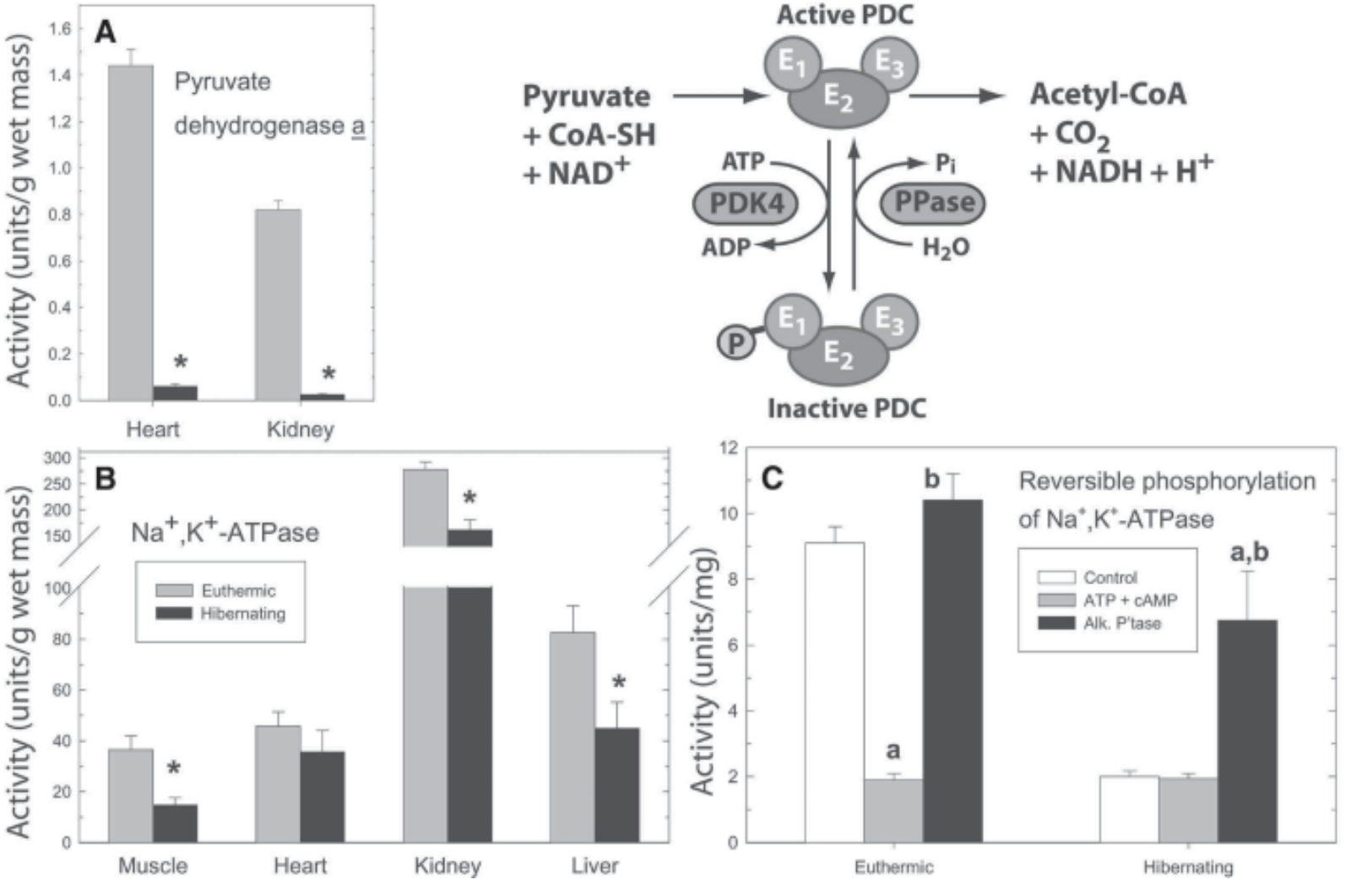
tion of hormone-sensitive lipase) and the upregulation of gene expression, particularly the expression of uncoupling protein 1 (UCP1) that is the key to thermogenesis. The net action of UCP1 is as a protonophore that allows proton re-entry into the mitochondrial matrix without driving ATP synthesis by the F_1F_0-ATP synthase *(32).* Hence, the energy that would normally be trapped in ATP is released as heat. However, recent work ahs shown that UCP1 does not actually carry protons itself. Its physiological substrates are free fatty acid (FFA) anions that it transports out of the mitochondrial matrix. FFAs are protonated in the acidic intermembrane space and then neutral FFA-H diffuse back and dissociate in the more basic pH environment of the matrix. The net effect is that protons re-enter the matrix without driving ATP synthesis.

3.2.5. Differential Temperature Controls on Metabolism

The Tb of hibernators can vary from 37°C in euthermia to near 0°C in torpor but hibernators do not have the option of major metabolic restructuring (e.g., homeoviscous adaptation of membranes) to resculpt metabolism for low temperature function because they must always be prepared for a rapid arousal back to 37°C. However, the effects of temperature change on different enzymes and proteins can be employed to achieve different metabolic outcomes. Several proteins that are key to the hibernation phenotype show temperature-insensitive properties that allow them to function well over the full range of possible Tb values. For example, UCP1 shows temperature-independent properties with regard to both the maximal binding capacity and the dissociation constant (Kd) for guanosine diphosphate, its major allosteric regulator. Hibernator fatty acid binding protein (FABP), that plays a key role in energy metabolism by transporting fatty acid substrates through the cytoplasm to the mitochondria for oxidation, also shows temperature insensitive dissociation constants for both natural and artificial substrates, whereas rat FABP has reduced substrate binding abilities at low temperature *(33).* Various other proteins and enzymes show temperature sensitive properties that can enhance, suppress or radically alter function at low temperature, in some cases contributing to MRD and in others supporting an altered function for the enzyme in the torpid state *(28).* For example, ground squirrel liver glutamate dehydrogenase not only undergoes a stable modification between euthermic and hibernating states but the properties of the hibernating form (particularly sensitivities to ADP and guanosine triphosphate as allosteric effectors) strongly poise the enzyme for a glutamate-utilizing function at low Tb that would aid gluconeogenesis during torpor *(34).*

4. FREEZE TOLERANCE

Hibernating mammals will let their Tb fall to near 0°C, but if ambient temperature in their hibernaculum falls below 0°C they activate a low level of thermogenesis to keep their bodies from freezing. However, ectothermic animals have no such recourse when exposed to subzero temperatures. They have only two choices for enduring this temperature extreme: (a) freeze avoidance—antifreeze mechanisms are used to allow body fluids to remain liquid (supercooled), sometimes to temperatures as low as –40°C, or (b) freeze tolerance—controlled growth of ice in extracellular spaces is allowed coupled with antifreeze protection of the cytosol. Freeze avoidance is used by hundreds of species of terrestrial arthropods (e.g., insects, spiders, ticks, etc.) and other invertebrates and also by coldwater marine teleosts that live in seawater that is colder than the freezing point (FP) of fish blood *(11,35).* Freeze avoidance relies on three main strategies: (a) the proliferation of antifreeze proteins that bind


A
Pyruvate dehydrogenase a
Activity (units/g wet mass)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
Heart
Kidney
Active PDC
E1
E3
E2
Pyruvate
+ CoA-SH
+ NAD+
Acetyl-CoA
+ CO2
+ NADH + H+
ATP
Pi
PDK4
PPase
ADP
H2O
P
Inactive PDC
B
Na+,K+-ATPase
Euthermic
Hibernating
Activity (units/g wet mass)
300
250
200
150
100
80
60
40
20
0
Muscle
Heart
Kidney
Liver
C
Reversible phosphorylation of Na+,K+-ATPase
Control
ATP + cAMP
Alk. P'tase
Activity (units/mg)
12
10
8
6
4
2
0
a
b
a,b
Euthermic
Hibernating

to microscopic ice crystals and keep them from growing, (b) the accumulation of high concentrations (often 2 molar or more) of polyhydric alcohols or sugars that provide colligative suppression of the FP and supercooling point (SCP) of body fluids, and (c) where possible, reducing the probability of ice nucleation by reducing body water content (dehydration), shielding with a cuticle, epiphragm or cocoon that prevents ice contact with body tissues, or clearing potential nucleators from the body (e.g., voiding the gut to get rid of bacteria) *(11)*. Other ectotherms have developed freeze tolerance and in doing so have had to address multiple injurious consequences of ice formation in biological tissues.

4.1. Freezing Damage

For most organisms on Earth, internal ice formation is highly damaging and frequently lethal. Damage can arise in several ways *(36–38)*. Ice crystals can cause direct physical injury to cells and tissues by shearing and squeezing stresses as ice grows among cells and by ice expansion that can burst delicate capillaries so that on thawing the organism suffers severe internal bleeding. Ice growth inside of cells destroys subcellular architecture and compartmentation and damage is so severe that even freeze-tolerant organisms do not endure intracellular freezing (the only good documented exception to this rule is the Antarctic nematode, *Panagrolaimus davidi [39]*). Ice growth in extracellular spaces also places volume and osmotic stresses on cells. Growing crystals exclude solutes so that remaining extracellular fluid becomes highly concentrated. This sets up a steep osmotic gradient across the plasma membrane that causes water to flow out and cells shrink. Dehydration, elevated ionic strength, and cell-volume reduction all have negative effects on cell morphology and metabolic functions, and this is compounded during thawing when cells can swell so quickly that they can burst. Shrinkage below the critical minimum cell volume also causes irreparable damage to cell membranes owing to compression stress. Finally, freezing disrupts cellular energetics because the delivery of oxygen and fuel supplies is cut off when ice forms in plasma and extracellular spaces. Hence, freezing is an anoxic and ischemic stress and although the low Tb of a frozen animal means that metabolic rate is also very low, the duration of a freezing episode can stretch to weeks or months and strain anaerobic capacities.

Fig. 2. *(continued from opposite page)* Effect of hibernation on the activities of (**A**) the active form of pyruvate dehydrogenase (PDH<u>a</u>) and (**B**) Na^+K^+ATPase in tissues of ground squirrels (*Spermophilus lateralis*). (**C**) Effects of incubation under conditions that stimulate endogenous protein kinase A (10 mM ATP, 10 mM $MgCl_2$, 0.3 mM cAMP) and subsequent alkaline phosphatase treatment (10 units) on Na^+K^+-ATPase activity in skeletal muscle extracts from euthermic and hibernating ground squirrels. Data are means $\pm$ SEM, $n = 4$. Also shown is the reaction of the pyruvate dehydrogenase complex (PDC) and its interconversion between active (dephosphorylated) and inactive (phosphorylated) forms by the actions of PDH kinase versus phosphatase. The PDH complex is composed of three enzymes: E_1 is pyruvate dehydrogenase (phosphorylated by pyruvate dehydrogenase kinase [PDK]), E_2 is dihydrolipoyl transacetylase, and E_3 is dihydrolipoyl dehydrogenase. *, Significantly different from the corresponding euthermic value, $p < 0.05$. a, Significantly different from the untreated control sample; b, significantly different from the protein kinase treated sample, $p < 0.05$. Enzyme data compiled from Brooks and Storey *(48)* and MacDonald and Storey *(49)*.

4.2. Natural Freezing Survival

Natural freeze tolerance is well developed in several species of North American frogs that hibernate on land as well as some reptiles, a variety of intertidal invertebrates, and hundreds of insect species *(36,37).* The main vertebrate model that we use is the wood frog, *Rana sylvatica (38).* Known principles of freeze tolerance include protective strategies that regulate ice growth and preserve cellular macromolecules as well as conservation strategies that ensure metabolic survival during freezing.

4.2.1. Regulation of Ice Growth

Freeze-tolerant animals typically ensure that freezing is initiated close to the equilibrium FP so that the rate of ice formation is slow and there is plenty of time to initiate metabolic adjustments. Freezing may be triggered by contact with environmental ice across a water-permeable skin or via the action of nucleators, most commonly specific ice-nucleating proteins that are added to the blood of the organism or bacteria in skin or gut that have ice-nucleating abilities *(11,38).* By triggering ice growth in extracellular and extra-organ spaces (e.g., the abdominal cavity in frogs), the probability of intracellular ice nucleation is drastically reduced. The plasma of many freeze-tolerant animals also contains proteins with antifreeze actions (when assayed in vitro) but their function in vivo appears to be to limit the size of crystal growth and inhibit recrystallization.

4.2.2. Cell-Volume Regulation

Freeze-tolerant animals frequently endure the conversion of up to 65 to 70% of total body water into extracellular ice and cells/organs undergo substantial dehydration and shrinkage. To prevent cell-volume reduction beyond a critical minimum, high concentrations of low-molecular-weight carbohydrates are typically accumulated to provide colligative limitation of cell water loss. Glycerol and other polyhydric alcohols often do this job but wood frogs use glucose and show extreme freeze-induced hyperglycemia with glucose rising to 150 to 300 m*M* in plasma and organs compared with approx 5 m*M* in unfrozen controls *(36,38).* Other protectants (e.g., trehalose, proline) protect/stabilize cell membranes against compression stress during volume reduction.

4.2.3. Energetics

Extracellular freezing imposes an ischemic state on all cells/organs of frozen animals and therefore freeze-tolerant animals also show well-developed ischemia/anoxia resistance including pathways of fermentative ATP generation, regulated MRD, and antioxidant defenses to provide protection against reperfusion damage during thawing *(38).*

4.2.4. Vital Signs

Freezing halts all vital signs including heart beat, breathing, muscle movement, and nerve transmission. All are sequentially reactivated during/after thawing (heart beat being the first sign of reanimation) but the molecular mechanisms underlying these processes are still largely unknown.

5. METABOLIC RATE DEPRESSION

Although alien to human physiology, the ability to strongly suppress metabolic rate and sink into a hypometabolic state is a life-saving mechanism for many organisms that must endure extreme environmental stress on a periodic or seasonal basis. When faced with envi-

ronmental extremes that threaten normal life, limit food availability or impose severe challenges to their physiology, this conservation strategy allows organisms to endure until conditions are again conducive for active life. Examples of hypometabolism abound as animal responses to high and low temperature, oxygen deprivation, food restriction and water limitation and, coupled with various protective adaptations that apply to specific cases, MRD is the underlying principle of stress survival in phenomena including anaerobiosis, estivation, diapause, torpor, dormancy, hibernation and anhydrobiosis (also called cryptobiosis). Among animals, MRD can range from the nightly torpor (a 20–30% reduction in metabolic rate for a few hours) that eases the energy budget of small birds and mammals in cold environments to many months of seasonal dormancy in response to winter cold (hibernation) or summer drought (estivation), and even to many years in a virtual ametabolic state for cryptobiotes (e.g., many seeds, spores, cysts, eggs; the most frequently studied example of cryptobiosis is the brine shrimp, *Artemia*). The molecular mechanisms of MRD have been the subject of much recent study in our lab and others. By analyzing the mechanisms of MRD in four situations—anaerobiosis, hibernation, estivation, and freeze tolerance—we have documented the principles of metabolic control that extend across phylogeny to regulate MRD in multiple states *(9,10).*

MRD has three main principles: (a) both intrinsic mechanisms within cells and extrinsic mechanisms are involved, (b) the rates of energy-producing and energy-consuming cellular processes are suppressed in a coordinated manner so that a new lower net rate of ATP turnover can be sustained over the long term, and (c) cellular priorities are reorganized to give precedence to key functions (e.g., maintenance of membrane potential difference) and more strongly suppress functions that are less essential (e.g., protein synthesis) under energy-restricted conditions. Extrinsic influences on MRD include a general suppression of physiological functions (heart rate, breathing, digestion, muscle movement) in the hypometabolic state as well as the hypercapnia (due to apnoic breathing patterns) and reduced cytosolic pH that typically accompany entry into hypometabolism and that both suppress metabolic functions. Hypothermia can also be a factor; for instance, many ectotherms voluntarily seek cooler temperatures when challenged by hypoxia and thereby use a decrease in body temperature to help reduce tissue demands for oxygen. An ancient hypoxia–hypothermia interaction may also contribute to the mechanism of MRD that allows small mammals to coordinate the suppression of metabolic rate and Tb as they sink into the hibernating state *(40,41).*

Intrinsic mechanisms of MRD within cells account for at least half of the total MRD. Intrinsic mechanisms lead to both a net decrease in cellular metabolic rate and a reorganization of priorities for ATP use. An example of these principles comes from studies with isolated hepatocytes of anoxia-tolerant turtles *(42).* Five main ATP-consuming processes were identified in hepatocytes and under normoxic conditions the fractional use of cellular ATP turnover was calculated as 28, 36, 17, 3, and 17% for ion pumping by the Na^+K^+-ATPase, protein synthesis, protein degradation, urea synthesis, and gluconeogenesis, respectively. However, when cells were incubated under anoxic conditions, not only did total ATP turnover decrease by 94% but each of the ATP-consuming processes was differently affected with the result that the fractional use of ATP turnover by the five processes in anoxic cells shifted to 62, 21, 9, 8, and 0 %, respectively. In other words, the sodium/potassium pump became the dominant energy sink in the energy-restricted anoxic state. Priorities are also rearranged between organs in hypometabolic states. For example, when the capacity for protein synthesis was analyzed in different organs of hibernating vs euthermic ground squirrels,

the rate of synthesis was unaltered in brown adipose tissue but was reduced by 66% in brain and 85% in kidney (all rates measured in cell extracts in vitro at 37°C) *(43,44)*.

5.1. Regulation by Reversible Protein Phosphorylation

What are the molecular mechanisms of intrinsic MRD? By far the most important mechanism is reversible protein phosphorylation (RPP) carried out by the actions of protein kinases or protein phosphatases on enzymes and functional proteins. Key advantages of RPP as an instrument of MRD include the following:

1. RPP can induce major changes to the activity states of selected regulatory enzymes and functional proteins, often including virtual on–off control.
2. Thousands of proteins in cells are susceptible to RPP so the mechanism provides an excellent way of coordinating the responses by multiple cell functions.
3. Signal transduction cascades involving protein kinases and protein phosphatases are fast and allow a rapid suppression of the activities of multiple ATP-utilizing functions and an equally fast reversal to re-establish normal cell functions during arousal from the hypometabolic state
4. Major changes in the activity states of enzymes and pathways are achieved without the need to change the overall amounts of proteins via synthesis or degradation, another factor that benefits fast recovery from the hypometabolic state.

Recognition of the role of RPP as a mechanism of metabolic suppression arose initially from studies of the control of glycolysis in anoxia-tolerant marine molluscs *(9,22)*. Strong anoxia-induced inhibition of pyruvate kinase (PK) was found to be responsible for rerouting carbohydrate flux at the phosphoenolpyruvate (PEP) branchpoint under anoxia (Fig. 1A). This directs PEP away from the aerobic route of carbohydrate degradation (via PK and into the tricarboxylic acid cycle) and into the PEP carboxykinase reaction and onwards into the reactions of anaerobic succinate synthesis. The suppression of PK activity was traced to anoxia-induced phosphorylation of the enzyme that caused a strong decrease in enzyme activity and major changes in enzyme properties that virtually shut off PK in vivo under anoxic conditions (Fig. 1B) *(45)*. For example, anoxia-induced phosphorylation of whelk muscle PK had the following strong effects: substrate affinity decreased (K_m for PEP rose 12-fold), enzyme sensitivity to the activator, fructose-1,6-bisphosphate decreased (K_a increased 26-fold), and sensitivity to inhibition by L-alanine rose (I_{50} decreased by 490-fold) *(46)*. Inhibitory control is further enhanced by the major accumulation of alanine as an initial product of anaerobic metabolism in marine molluscs.

It was next determined that RPP provided not just PK control in marine molluscs under anoxia but also coordinated an overall suppression of glycolysis (as part of the overall MRD) by targeting enzymes including glycogen phosphorylase, 6-phosphofructo-1-kinase (PFK-1), and 6-phosphofructo-2-kinase (PFK-2; that produces the potent activator of PFK-1, fructose-2,6-bisphosphate) *(45)*. Figure 1B shows these coordinated responses to anoxia by enzymes in gill of the whelk, *Busycon canaliculatum*. The realization that RPP had an even broader role in orchestrating MRD in multiple situations came when the same coordinated phosphorylation of glycolytic enzymes was found to regulate the suppression of carbohydrate catabolism during anoxia exposure in vertebrates (turtles and goldfish) and during estivation, an aerobic dormancy, in land snails and desert toads *(10,12,45,47)*.

Studies with hibernating mammals and estivating snails next confirmed that RPP also controlled enzymes, not just of glycolysis, but also of aerobic substrate catabolism. Strong inhibition of PDH, the enzyme that gates carbohydrate entry into the tricarboxylic acid cycle

was documented in both situations *(12,28).* In hibernators, for example, the amount of PDH present in the dephosphorylated active form dropped from 60 to 80% in euthermia to less than 5% in hibernation (Fig. 2A) *(48).* This supports carbohydrate sparing during torpor and the shift to a primary reliance on lipid oxidation for energy generation. Furthermore, gene-screening studies found a strong hibernation-induced upregulation of PDH kinase, the enzyme that phosphorylates and turns off PDH *(29).*

5.2. Ion-Motive ATPases and Channel Arrest

The examples presented here deal with catabolic enzymes involved in ATP production but hypometabolism requires a balanced suppression of the rates of both ATP-producing and ATP-utilizing pathways so that a new lower rate of ATP turnover is established. Studies with hibernators and anoxia-tolerant turtles have led the way in analyzing the controls on ATP-utilizing reactions in hypometabolic states. As noted earlier, ion-motive ATPases are huge consumers of cellular energy and, therefore, key potential targets for achieving metabolic suppression. Indeed, analysis of Na/K-ATPase in ground squirrel tissues showed that activity of the enzyme was strongly suppressed during hibernation. Activities were just 40 to -60% of the corresponding euthermic values when quantified at the same temperature (25°C) *(49).* Again, the mechanism involved is RPP. Figure 2B shows the results of incubation studies with extracts of ground squirrel tissues. Conditions that promoted the activity of protein kinases suppressed Na/K-ATPase activity in extracts from euthermic tissues, whereas treatment with alkaline phosphatase restored activity and also elevated activity in tissue extracts from hibernating animals. Similar results were found for Ca-ATPase of skeletal muscle sarcoplasmic reticulum (SR); maximal activity was reduced by 50% during hibernation along with comparable reductions in other proteins involved in SR calcium signaling including the SR calcium-release channel (ryanodine receptor) and SR calcium-binding proteins (e.g., sarcalumenin, calsequestrin) *(50).*

Suppression of the activities of ion-motive ATPases must be balanced by concomitant suppression of the ion movements through oppositely directed ion channels in order to maintain membrane potential difference in a hypometabolic state. Channel arrest has received the most attention in studies of vertebrate brain hypoxia/anoxia tolerance, specifically in studies with anoxia tolerant turtles *(51).* Oxygen-sensitive potassium, sodium, and calcium channels have been identified in neuronal plasma membranes controlled by mechanisms including RPP, redox regulation, Ca^{2+}-dependent regulation, and regulation by neuromodulators acting via G protein-coupled receptors. Adenosine is a particularly important neuromodulator in this regard. It accumulates quickly in turtle brain during anoxia exposure and acts via adenosine A1 receptors to suppress excitatory neurotransmission that is mediated largely by Ca^{2+} entry through *N*-methyl-D-aspartate (NMDA) receptors *(52).* Patch-clamp studies using cells from normoxic brain showed that both anoxia exposure and adenosine perfusion reduced the NMDA receptor open probability by 65%, an effect that was antagonized by an A1 receptor blocker. Hence, the activities of both ion channels and ion motive ATPases are suppressed during anoxia in anoxia-tolerant brains, contributing to the overall MRD. Overexpression of adenosine A1 receptors is also known to increase myocardial tolerance of ischemia in transgenic mice *(53)*, suggesting a wide role for adenosine signaling in channel arrest in hypoxia or ischemia. In addition, we have recently documented putative upregulation of the genes for adenosine A1 receptors and 5'-nucleotidase (that synthesizes adenosine from adenosine monophosphate) as a response to freezing in freeze-tolerant wood

frogs, which suggests that adenosine-mediated responses may also aid ischemia resistance in frozen tissues *(54)*. Furthermore, a universal role for adenosine signaling in both aerobic and anaerobic forms of MRD might now be suggested based on recent studies with hibernating hamsters. Shiomi and Tamura *(55)* found that intracerebroventricular injections of adenosine or an adenosine A1 receptor agonist produced profound hypothermia in hamsters on a time course coincident with the normal descent of Tb during entry into natural hibernation whereas an adenosine A1 receptor antagonist could elevate Tb and interrupt hibernation.

5.3. Regulation of Protein Biosynthesis in Arrested States

Another major energy expenditure in cells is protein synthesis, requiring approx 5 ATP equivalents per peptide bond formed and consuming as much as 40% of total ATP turnover in some organs such as liver. Suppression of protein biosynthesis has been widely documented in hypometabolic states, occurring in an organ-specific manner in hibernating ground squirrels (discussed earlier) with a major reduction in the fractional use of cellular ATP by protein synthesis in hypometabolic states (as discussed earlier for turtle anoxia). Suppression can occur rapidly and before cells experience energy stress; for example, the rate of ^{3}H-leucine incorporation into protein in hepatopancreas extracts from marine periwinkle snails (*Littorina littorea*) decreased by 50% within just 30 min when snails were transferred from aerobic to anoxic conditions *(56)*. This indicates that protein synthesis inhibition is a proactive response by cells that is an integral part of metabolic arrest rather than a reactive response to ATP limitation. Indeed, ATP content and energy charge did not change significantly over the course of 72 h of anoxia exposure in this facultative anaerobe species.

Two factors could contribute to protein synthesis inhibition during hypometabolism: (a) mRNA substrate availability, and (b) specific inhibition of the ribosomal translational machinery. The former possibility has been largely discounted by multiple studies that found no change in either global mRNA content or the transcript levels of various constitutively active genes during transitions to or from hypometabolic states *(10)*. This makes intuitive sense because it preserves the cellular pool of mRNA transcripts so that messages are immediately available for translation when organisms arouse from hypometabolism.

The primary mechanism of translational control in hypometabolism is again RPP, this time regulating the activities of ribosomal initiation and elongation factors. The mechanisms identified to date are the same as the RPP controls on translation that have been described for the regulation of mammalian protein synthesis in response to various stresses (e.g., amino acid limitation, hypoxia/ischemia, heat shock, infection) *(57)*. Because most hypometabolic states are also typically states of prolonged starvation, it is perhaps not unexpected that mechanisms for controlling protein synthesis with respect to nutritional status (starved vs fed) are also employed to achieve long-term suppression of biosynthesis during hypometabolism.

Recent studies have documented consistent inhibition of the eukaryotic initiation factor-2 (eIF2) in hypometabolic states. This factor introduces initiator methionyl-tRNA into the 40S ribosomal subunit and phosphorylation of the α-subunit inhibits this function (Fig. 3). The content of phosphorylated, inactive eIF2α increases strongly during hibernation; for example, the figure shows Western blot assessments of total eIF2α protein (no change) vs eIF2α phosphopeptide content (a strong increase in hibernation) in ground squirrel kidney *(44)*. Similarly, in ground squirrel brain, phospho-eIF2α content was less than 2% of the total in euthermia but rose to 13% in hibernation *(43)*. Anoxia exposure had the same effect

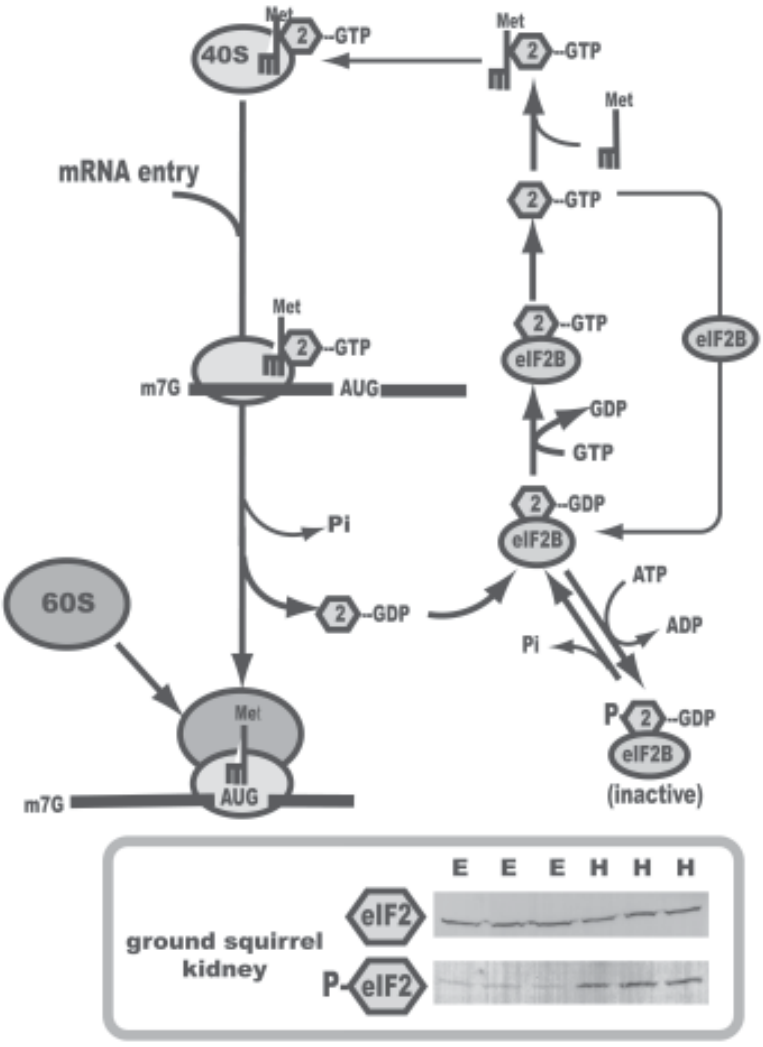


Fig. 3. Reversible phosphorylation control of the eukaryotic initiation factor 2 (eIF2) regulates translation initiation by restricting the availability of methionine tRNA to the 40S ribosomal subunit. Phosphorylation of the α-subunit of eIF2 keeps the protein bound in an inactive complex with the guanine nucleotide exchange factor, eIF2B, and prevents eIF2α recycling between successive rounds of peptide synthesis. The inset shows Western blots of ground squirrel kidney extracts crossreacted with antibodies that recognize total eIF2α versus the phosphopeptide segment of eIF2α. Total eIF2α protein content did not change during hibernation (H) compared with euthermia (E) but the amount of phosphorylated eIF2α rose sharply. Western blot data from Hittel and Storey *(44)*.

on eIF2α in the marine snail, *L. littorea*; total eIF-2α content in hepatopancreas was unaffected over a cycle of anoxia and aerobic recovery but the amount of phospho-eIF2α rose approx 15-fold in anoxic animals, compared with aerobic controls *(56)*. However, when oxygen was reintroduced, phospho-eIF2α fell to control levels or below within 1 h. Inhibitory control of protein synthesis also occurs at other loci. Phosphorylation-mediated inhibition of the eukaryotic elongation factor-2 (eEF2) raised mean transit times for polypeptide

elongation by ribosomes by threefold in extracts of hibernator brain as compared with euthermic ground squirrels *(43).* Regulation was traced to both a 50% higher activity of eEF2 kinase in hibernator tissues and a 20 to 30% decrease in protein phosphatase-2A activity (that opposes eEF2 kinase) *(58).* EF2 kinase is, in turn, subject to phosphorylation and activation by several of the major cellular protein kinases including PKA, MAPKs, $p90^{RSK1}$, and p70 S6 kinase, one or more of which may mediate the hibernation response.

Other ribosomal initiation factors are also involved in the suppression of translation under situations such as starvation in mammals and, although they have not yet been investigated as elements of hypometabolism, it is likely that they will prove to be involved. For example, the eukaryotic initiation factor-5 (eIF5), a GTPase-activating protein that promotes GTP hydrolysis within the 40S initiation complex, is also regulated by RPP. Similarly, the eukaryotic initiation factor-4E binding protein (4E-BP1) is subject to RPP and when dephosphorylated it binds to and inhibits eIF4E. Another mechanism, proteolytic fragmentation, controls subunit G of eIF4. Fragmentation occurs under stress (e.g., ischemia) and changes the type of mRNA that can be translated because intact eIF4G is needed to allow eIF4E-bound m^7G-capped mRNAs (the vast majority of cellular mRNAs) to bind to the 40S ribosomal subunit (Fig. 3) *(57).* Without intact eIF4G, message selection favors only those messages that contain an internal ribosome entry site (IRES) *(59).* Such messages often code for proteins involved in apoptosis but the mRNA transcripts of several stress-responsive proteins also contain IRES elements so that these can be translated under stress conditions (e.g., hypoxia, amino acid limitation) that normally inhibit protein synthesis. For example, the mRNA for HIF-1 α-subunit contains an IRES that allows enhanced synthesis of HIF-1α to occur under hypoxic conditions when overall protein synthesis is suppressed *(60).* In turn, enhanced levels of the α-subunit, when combined with the more stable ß-subunit, can elevate overall HIF levels and stimulate the expression of HIF-1 regulated genes whose protein products function to alleviate hypoxia stress. The presence of an IRES may also be key to the translation of the protein products of the selected few genes that are upregulated during entry into hypometabolic states including hibernation, anaerobiosis and freezing (see section on gene expression).

5.4. Regulation of Protein Synthesis by Changes in Ribosome Assembly

The activity state of protein synthesis in a cell can generally be inferred from the state of ribosome assembly—active translation occurs on polysomes (aggregates of ribosomes moving along a strand of mRNA), whereas monosomes are translationally silent. Polysome dissociation is a recognized cellular response to stress. For example, stresses such as hypoxia, starvation, and diabetes all trigger polysome dissociation in rat tissues. Recent work has also shown that polysome dissociation is a mechanism of MRD in stress-tolerant organisms.

The situation is well- illustrated by recent studies with hibernators. To assess the state of ribosomal assembly, tissue extracts are separated on a sucrose gradient and fractions collected. Polysomes migrate into denser fractions whereas monosomes are found in lighter fractions; ribosomal RNA presence is detected by absorbance at 254 nm, ethidium bromide staining, or, as shown in Fig. 4, by Northern blotting for 18 S rRNA. When tissue extracts from euthermic ground squirrels (Tb = 37°C) were assessed, most ribosomes were present in the higher density polysome fraction as was a high proportion of the mRNA for constitutively active genes as illustrated by the distribution of cytochrome c oxidase subunit 4 (*Cox4*) mRNA in the figure, as detected by Northern blotting *(44).* When animals entered hiberna-

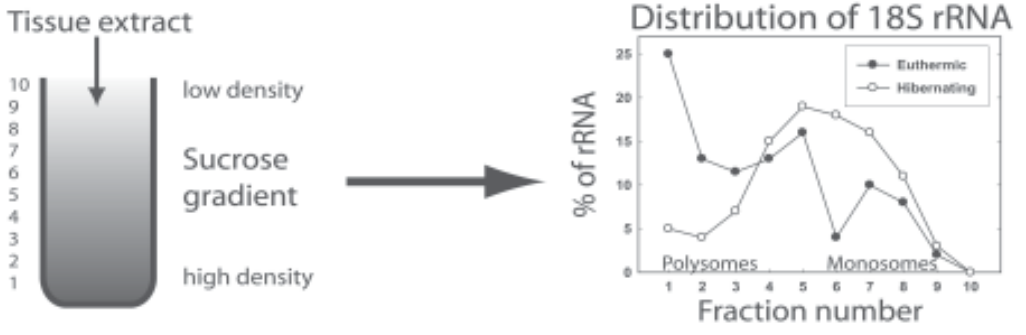
tion, however, tissues showed consistent polysome disassembly with movement of a high percentage of 18S rRNA and *Cox4* mRNA into the monosome fractions. Hence, the principle here is that an overall suppression of protein synthesis during hibernation is achieved by the dissociation of active polysomes and the storage of mRNA transcripts in the translationally silent monosome fraction. During arousal the reverse transition occurs and allows protein synthesis to be rapidly reinitiated without a need for *de novo* gene transcription. Note also that by this mechanism an effective "life extension" of mRNA transcripts is achieved.

However, transcripts of genes that are specifically upregulated during entry into hibernation behaved differently. Transcript levels of fatty acid binding protein (*fabp*) increase several-fold in most ground squirrel tissues during hibernation *(30)* and, in addition, they remain associated with polysomes, ensuring their active translation and leading to a strong increase in FABP protein content in hibernation versus euthermia as illustrated by the Western blots in Fig. 4. By contrast, COX4 protein levels were unchanged or declined slightly during hibernation. This illustrates another principle of metabolic control—the rate of translation of individual mRNA species can be altered by differential distribution of transcripts between translationally active and inactive ribosomes. The polysomes remaining in hibernator tissues contain disproportionately higher numbers of those mRNAs (such as *fabp*) that are crucial to the hibernation phenotype, whereas mRNA species that are not needed during hibernation are relegated into the translationally silent monosome fractions.

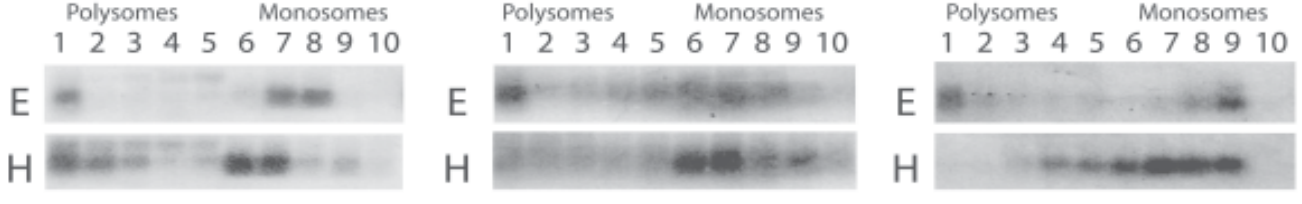
The same mechanisms characterize the response to a very different stress (anoxia) in a very different species (the marine snail, *L. littorea*) and, together with a number of other studies, show that polysome disassembly is a common feature of MRD across phylogeny. Anoxia-induced MRD in *L. littorea* was also accompanied by a movement of rRNA and the mRNA for constitutive genes (α-tubulin in this case) into the monosome fractions, whereas mRNA for genes that were upregulated in anoxia (e.g., ferritin) stayed in the high-density fractions that contained the few remaining polysomes *(56,61)*. The result was a twofold rise in ferritin protein levels in anoxic snails; by increasing iron storage, elevated ferritin is believed to contribute to minimizing oxidative stress during the transition back from anoxic to aerobic life. However, when snails were returned to aerobic conditions, the control situation was reestablished within 6 h with a return to a high polysome content and a high percentage of all mRNA localized with the polysomes.

The trigger for polysome disaggregation is not known with certainty but in hibernators there is evidence that temperature is a factor. When the distribution of rRNA (monitoring ribosomes) and actin mRNA (monitoring constitutive transcripts) was assessed in liver samples taken at different Tb values a distinct shift occurred when core Tb reached 18°C *(62)*. In euthermia, actin mRNA was localized mainly in the polysomes and remained there during entry into torpor until animals cooled to 18°C. Below 18°C a large portion of the transcripts, as well as rRNA, suddenly shifted to the monosome fraction and remained there throughout torpor. Conversely, during arousal, polysome reassembly was first evident when Tb rose to 18°C. Whether this temperature effect derives from a passive influence of temperature on polysome assembly or is due to temperature-stimulated regulation of one or more ribosomal proteins is not yet known.

Another variation on translational control has also been illustrated from our studies with hibernators. A prominently upregulated gene in kidney of hibernating ground squirrels is the organic cation transporter type 2 (*Oct2*); *Oct2* transcript levels were two to threefold higher



Northern blots of transcript distribution on gradient



Western blots of protein levels

FABP

Hibernation-induced
- transcripts up
- mRNA in polysomes
- protein up

COX4

Constitutive
- transcripts constant
- mRNA in monosomes
- protein constant

OCT2

Anticipatory
- transcripts up
-mRNA in monosomes
-protein down

in torpid animals compared with euthermic controls *(44)*. However, despite this, OCT2 protein content actually decreased during hibernation. Why would this be? The answer came from an analysis of *Oct2* transcript distribution on polysome profiles. Although transcript levels were much higher in hibernation, they were largely sequestered into the translationally silent monosome fraction during hibernation (Fig. 4). We suggested that the reason for this is to allow OCT2 protein to be produced very rapidly from existing *Oct2* transcripts as soon as torpor is broken. Kidney function is virtually shut down during hibernation and it is possible that this includes an actual degradation of OCT2 protein (rather than a reversible inactivation) since immunoblotting showed much lower levels of OCT2 in hibernator kidney, compared with euthermia (Fig. 4). In such a situation, resumption of transporter action to support renewed kidney function during interbout arousals would require the rapid synthesis of OCT2, which could be potentiated using the high levels of *Oct2* transcripts already present. Hence, this suggests another possible principle of translational control—anticipatory upregulation of selected transcripts during hibernation can support a rapid activation of protein synthesis during arousal that does not depend on enhanced gene expression.

Hence, multiple mechanisms of translational control are available for use in association with MRD and these can accomplish a variety of specific goals including a general suppression of protein translation (via inhibition of ribosomal factors and mRNA sequestering into the monosome fraction), the specific upregulation of selected transcripts (e.g., IRES-mediated translation, preferential transcript presence in polysomes), and anticipatory upregulation with delayed translation. In combination, all of these mechanisms (and probably others yet to be uncovered) contribute to the overall suppression of protein synthesis as part of the general conservation strategy of MRD while still providing the means to regulate the stress-induced production of selected proteins that are needed for the long-term survival of the organism in the hypometabolic state.

6. ADVANCES IN BIOCHEMICAL ADAPTATION THROUGH GENE DISCOVERY

One of the major mechanisms of animal response to extreme environmental stress is the activation of gene expression to synthesize new protein products. Stress responses, particularly in situations where MRD is also employed, are typically selective with a relatively small group of genes upregulated against the background of an overall suppression of protein synthesis in the energy-limited state. For many years, the approach to finding proteins that aided biochemical adaptation worked from physiological phenotype backward; that is, gross differences between stress-tolerant and intolerant species were identified (e.g., a major

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Fig. 4. *(continued from opposite page)* Effect of hibernation on the distribution of ribosomes and mRNA between translationally active polysomes and translationally silent monosomes. Tissue extracts were fractionated on a sucrose gradient. The graph shows 18S ribosomal RNA content in the fractions, documenting the shift in rRNA presence from predominance in the polysome fractions in euthermia (solid circles) to the monosome fractions in hibernation (open circles). Northern blots of RNA extracts of each fraction show changes in abundance and position on the gradient of mRNA for three genes in euthermia (E) versus hibernation (H): fatty acid binding protein (*fabp*) and cytochrome *c* oxidase subunit 4 (*Cox4*) from brown adipose tissue and the organic cation transporter type 2 (*Oct2*) from kidney. Western blots show corresponding changes in protein contents. Data compiled from Hittel and Storey *(44)*.

hysteresis between freezing and melting points of the blood of coldwater marine fish, nonshivering thermogenesis by arousing hibernators) and then the underlying protein adaptations were traced. For instance, in the above examples this led to the discovery of novel antifreeze proteins in marine fish and of UCP1 in brown adipose of hibernating mammals. Such an approach is excellent for exploring adaptations that have an obvious "footprint" but is less good for elucidating more subtle changes in gene/protein expression that may be of equal importance to overall survival of the organism.

New advances in gene-screening technology are revolutionizing the exploration of biochemical adaptation. Broad-based screening techniques can produce an unbiased assessment of the genes that are upregulated in response to stress and are identifying many genes, protein products and cell functions that have never before been linked with the response to a particular environmental stress. For example, in our first use of cDNA library screening to evaluate freeze-induced gene upregulation in wood frog liver, we expected to find increased expression of genes associated with previously identified adaptations for freeze tolerance such as cryoprotectant biosynthesis. What we found instead was strong upregulation of the genes encoding fibrinogen subunits and the mitochondrial ADP–ATP translocase (AAT) *(63,64)* and this led us to two previously unrecognized facets of freezing survival. One is the need for improved plasma-clotting capacity, provided by the synthesis and export of fibrinogen (and perhaps other clotting factors as well), as a mechanism of dealing with any physical injuries caused by ice expansion within the delicate microvasculature of organs. Indeed, such damage, which causes a loss of vascular integrity upon thawing, is a major reason for the current failure of solid organ cryopreservation in medicine. The rationale for AAT upregulation is still not confirmed but interestingly, the stress-induced upregulation of membrane transporters is proving to be a common theme in organs of freeze tolerant frogs with two more examples recently documented: the mitochondrial inorganic phosphate transporter and the monocarboxylic acid transporter *(38,65).* Similarly, another recurring theme that has been highlighted from gene screening, but never previously considered, is the stress-induced upregulation of mitochondrially encoded subunits of respiratory chain proteins that we have now identified in anoxia-tolerant, freeze-tolerant, and hibernating animals. This includes subunits 2, 4, and 5 of NADH ubiquinone-oxidoreductase (complex I), cytochrome b (from complex III), subunit 1 of cytochrome c oxidase (COX, complex IV) and ATPase subunits 6 and 8 (from the F_1F_0ATPase, complex V) *(54,66–69).* The rationale for upregulation of these mitochondrial-encoded proteins has not yet been adequately explained although, clearly, this is a common theme among stress-tolerant animals that potentially represents a new principle of MRD. All of the mitochondrial respiratory chain complexes are large multisubunit enzymes with only a few subunits encoded on the mitochondrial genome and, interestingly, when we assessed the responses of nuclear-encoded genes of the same proteins these were not stress-induced (e.g., no changes were seen in transcript levels of subunit 4 of COX and ATPα, a nuclear encoded subunit complex V) *(69).* Hence, the phenomenon is peculiar to the mitochondrial genome and our working hypothesis is that selective changes to protein components of the respiratory complexes help to stabilize mitochondrial energetics and preserve viable organelles in stress situations.

Gene-screening techniques have also made major inroads in identifying many more adaptations that support mammalian hibernation. Studies by my lab and others have identified hibernation-responsive upregulation a variety of genes including of α_2-macroglobulin in liver, moesin in intestine, isozyme 4 of pyruvate dehydrogenase kinase (PDK4) and pancreatic lipase in heart, isoforms of UCP and FABP in multiple tissues, the ventricular isoform of

myosin light-chain 1 ($MLC1_v$) in heart and skeletal muscle, OCT2 in kidney, the melatonin receptor, and four genes on the mitochondrial genome *(10,41).* Although the genes identified to date are a disparate group, once again principles of adaptation are emerging that are providing new directions for study. For example, the upregulation of $MLC1_v$ in ground squirrel heart *(67),* when combined with studies of hamster heart that show changes in the proportions of myosin heavy-chain isoforms during hibernation *(70),* suggests that myosin restructuring occurs during hibernation. This would presumably provide an optimal mix of myosin isoforms to adapt the contractile apparatus of the heart to the new workload and thermal conditions of the torpid state. Other studies suggest that adjustments are made to minimize the risk of thrombosis in the microvasculature under the very low blood flow (ischemic) conditions during torpor. Up-regulation and export of α_2-macroglobulin (which inhibits proteases of the clotting cascade) from the liver, reduced platelet numbers (sequestered into the spleen) and reduced levels of several clotting factors all support a decreased clotting capacity during torpor *(71).* Indeed, these results illustrate the natural solution to the clotting problems that are typically noted during organ ischemia caused by heart attack or stroke.

6.1. Gene Discovery From cDNA Library Screening

cDNA library screening is one of two major methods of gene discovery that have been used to make major advances in our understanding of biochemical adaptation to extreme environments. The other is cDNA array screening (discussed later). The construction and screening of cDNA libraries is often difficult and time-consuming and favors the detection of genes that have abundant transcripts. However, because the library is made from the organism under study, it has one key advantage and that is its ability to detect the presence of novel species-specific genes. For example, we have discovered two novel genes that are upregulated in response to anoxia exposure in the marine snail, *L. littorea*, and three novel genes in freeze-tolerant frogs *(72–76)*; none of these show similarity to any gene/protein sequences present in international databases. Each of the protein products encoded by these genes may have a key role to play in stress tolerance and a wide range of technologies can be applied to elucidate their functions. Among others, we have used (a) proteomics programs to identify regionalities, functionalities and structural elements within the proteins; (b) Northern blotting or polymerase chain reaction (PCR) techniques to assess time-, organ- and stress-specific changes in mRNA transcript levels; (c) Western blotting using peptide antibodies raised against segments of the putative amino acid sequence to assess comparable time-, organ- and stress-specific changes in protein levels as well as subcellular location of the protein; (d) in vitro tissue incubations to test the influences of external stresses, hormones, and second messengers; and (e) techniques of transgenics and cloning to insert the novel gene into cell lines for high yield manufacture of the gene product for use in protein chemistry or analysis of the effects of the protein on stress survival of the transgenic cell. For example, such techniques when applied to analyzing the snail anoxia-responsive protein (SARP)-19, in *L. littorea* correlated the presence of two EF-hand Ca^{2+}-binding domains in the protein sequence with the upregulation of *sarp* transcript levels in hepatopancreas explants incubated with calcium ionophore A23187 or with phorbol 12-myristate 13-acetate, a stimulator of the Ca^{2+} and phospholipid dependent protein kinase C (PKC) *(73).* With the further information from Northern blots that showed a progressive rise in *sarp* transcripts over several days of anoxia exposure but a rapid reversal within 1 h of aerobic recovery, we proposed an important function for SARP-19 in Ca^{2+} signaling under anaerobic conditions.

Our investigations of three novel freeze-responsive genes identified by cDNA library screening of wood frog liver are also providing fascinating results. FR10, Li16, and FR47 encode proteins of 10, 13, and 47 kD, respectively *(74–76)*. They share no structural features in common other than the presence of a hydrophobic region of 21 amino acids in length in each; in FR10 and Li16 this region is N-terminal but it is near the C-terminus in FR 47. Hydrophobic regions often represent transmembrane segments and this suggests that all three proteins may associate with membranes. Transcripts of all three are elevated by three to five-fold in liver after 24 h of freezing at –2.5°C and Western blotting showed a comparable rise in Li16 and FR47 protein in frozen frogs (FR10 has not been tested) reaching a maximum of 8.4- and 3.5-fold higher than control values after 2 h of thawing at 5°C (before subsequently declining) (*see* Fig. 5 for *li16*). The occurrence of maximum protein levels in 2 h thawed frogs (that still have substantial internal ice, no heart beat and visibly shrunken organs) with a strong reduction by 8 h thawed (when heart beat and breathing have resumed and liver appears visibly restored to normal size) suggests that both proteins play roles in dealing with the ischemic or cell volume stresses associated with freezing and are no longer needed once recovery is well advanced. However, despite similar patterns of transcript and protein changes during freeze–thaw, the three proteins show very different organ distributions and responses to other stimuli, which argues for very different functions for each. For example, transcripts of *fr47* are found only in liver, *li16* occurs in liver, heart, and gut, whereas *fr10* was found in all organs that we tested. Furthermore, FR47 protein was detected in liver of two other freeze-tolerant frog species *(76)* but not in intolerant species, which is a strong indication of a freeze-specific function.

We gained further information about these three proteins by testing their responses to stimuli both in vivo and in vitro. Two main components of freezing stress are (a) ischemia that results from plasma freezing, and (b) cellular dehydration that results from water outflow into extracellular ice masses. In early studies of the control of cryoprotectant (glucose) synthesis in wood frogs we found that dehydration of frogs was just as effective as freezing in stimulating the hyperglycemic response whereas anoxia exposure (mimicking ischemia) had no effect on plasma glucose levels *(37)*. This suggested that glucose synthesis as a colligative cryoprotectant is triggered or regulated by changes in cell volume. We used the same strategy to assess the expression of the three novel proteins and found that transcripts of *fr10* were strongly upregulated by dehydration (suggesting a role for FR10 in cell volume regulation), whereas both *li16* and *fr47* transcripts responded strongly to anoxia exposure (suggesting links to ischemia resistance) *(74–76)*. Li16 protein levels increased strongly under anoxia and also increased somewhat in 40% dehydrated frogs (Fig. 5); note, however, that high dehydration values also impose a hypoxic stress owing to high blood viscosity and low blood volume that impairs tissue oxygenation. Other studies used in vitro incubation of liver slices with different second messenger molecules to derive information about the signal transduction pathways that regulate the *li16* and *fr47* genes. The cryoprotectant response in frogs is regulated by β-adrenergic receptors and a cyclic AMP (cAMP)-dependent activation of liver glycogenolysis *(37,38)* but neither *li16* nor *fr47* responded to tissue incubations with dibutyryl cAMP. However, *li16* transcript levels were stimulated about twofold by dibutyryl cyclic GMP, whereas *fr47* responded to phorbol 12-myristate 13-acetate, indicating PKC involvement *(75,76)*. The response of *li16* to both anoxia and cGMP is very interesting because adenosine receptor signaling is mediated intracellularly by cGMP and the

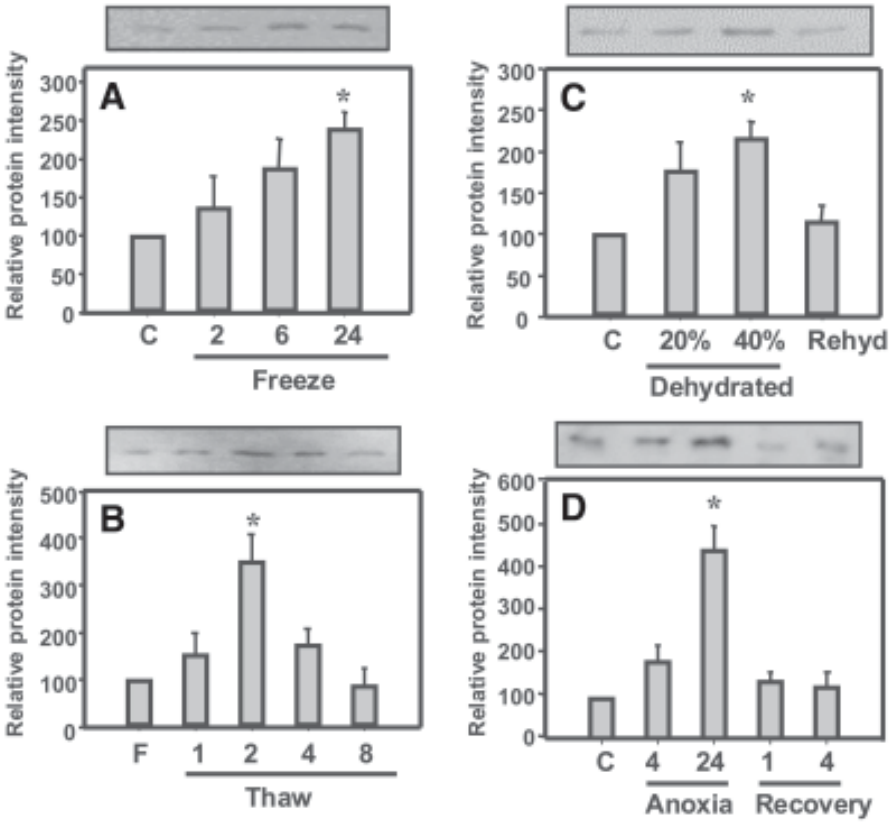

Fig. 5. Changes in the levels of the novel freeze-responsive protein, Li16, in liver of wood frogs, *Rana sylvatica*, under different conditions: (**A**) freezing at –2.5°C for 2, 6, or 24 h, (**B**) thawing at 5°C after 24 h frozen for 1, 2, 4, or 8 h; note that on this graph data are expressed relative to protein levels in 24 h frozen (F) frogs, (**C**) Dehydration at 5°C to 20 or 40% of total body water lost followed by 24 h rehydration, and (**D**) Anoxia exposure under a nitrogen gas atmosphere at 5°C for 4 or 24 h followed by aerobic recovery for 1 or 4 h. Shown are representative Western blots with histograms showing mean values for relative band intensities, $n = 3$ trials. C– control at 5°C. * , significantly different from zero time value, $p < 0.01$. (Data complied from McNally et al. *[75]*).

adenosine A1 receptor is one of freeze-responsive genes that have been identified from cDNA array screening in wood frogs *(54).* This suggests a role for *li16* in ischemia resistance during freezing. FR47, by contrast, seems to be regulated by PKC and in other studies we have shown that levels of the PKC second messenger, inositol 1,4,5-trisphosphate (IP_3), rise over time during freezing or anoxia exposures in wood frog liver *(77).* Overall, then, it appears that the three novel proteins are regulated by different signal transduction pathways and probably serve quite different, although as yet unknown, functions in the cell under freezing stress. Interestingly, one of the key conclusions from these studies is that natural freezing survival involves multiple pro-active responses by cells that include the upregulation of both known and unknown genes and regulation via at least three different signal transduction pathways.

6.2. Gene Discovery From cDNA Array Screening

The use of cDNA arrays to screen for stress-induced gene expression is revolutionizing the study of biochemical adaptation. State-of-the-art glass microarrays now have thousands of nonredundant cDNAs bound to them and offer one-step screening for stress-responsive genes. Critical advantages of array screening over other technologies include (a) a capacity to detect and quantify transcripts of different genes in a single sample that can vary as much as 1000-fold in abundance, (b) most of the genes are identified so screening allows evaluation of a huge number of genes involved in a wide array of cell functions, and (c) the ability to analyze the responses of functional groupings of genes to an imposed stress (e.g., families of transmembrane transporters, enzymes of different signal transduction cascades, enzymes in specific metabolic pathways, transcription factors, etc.). An illustration of this latter point came when we used rat macroarrays (Clontech ATLAS™) to screen for hibernation-responsive genes in ground squirrel skeletal muscle. The results showed that several genes that encode components of the small and large ribosomal subunits were consistently downregulated during hibernation including L19, L21, L36a, S17, S12 and S29 *(78).* This further implicates control of the ribosomes as critical to the inhibition of protein synthesis in hibernation. Array screening of liver and kidney samples from both ground squirrels and bats also showed consistent upregulation of genes associated with antioxidant defense during hibernation. Transcript levels of superoxide dismutase, glutathione peroxidase, and glutathione-*S*-transferase were elevated by twofold or more in hibernator kidney, whereas these genes plus peroxiredoxin and metallothionein were upregulated in liver *(78).* It is well known that hibernators elevate antioxidant defenses in brown adipose tissue as a means of dealing with high rates of oxygen-free radical generation during thermogenesis *(79)* but our screening data now suggests that the improvement of antioxidant defenses is widespread in multiple tissues. This would aid all organs in defense against oxidative damage during arousal when oxygen consumption can rise by 10- to 20-fold within minutes as the animal rewarms to 37°C.

In other studies, we used 19,000 gene human microarrays (Ontario Cancer Institute) to screen for genes that were upregulated in wood frog heart during freezing (comparing 5°C acclimated controls with frogs frozen for 24 h at –3°C) *(54).* More than 200 genes were designated as putatively upregulated during freezing by at least 1.5-fold, some by as much as four to sevenfold. These included a variety of protein kinases and protein phosphatases that further stresses the point made earlier that the cells of freeze-tolerant organisms respond actively to the encroachment of extracellular ice around them with responses by a wide variety of cellular systems, triggered and coordinated by multiple signal transduction cascades. Furthermore, the key advantages of array screening for identifying functionally related groups of genes as well as genes that had not previously been associated with freeze tolerance was again apparent. Putative upregulation of genes involved with multiple metabolic functions was documented including antioxidant defense (e.g., glutathione-*S*-transferase, metallothionein, thioredoxin), transmembrane carriers and ion motive ATPases (monocarboxylic acid transporter, adenine nucleotide translocator, Na^+-K^+ ATPase), signal reception (e.g., adenosine A1 receptor, atrial natriuetic peptide [ANP] receptor), glucose production and transport (e.g., glucose transporter type 4, glucose-6-phosphatase), defense against high glucose damage (e.g., receptor for advanced glycosylation end products [RAGE]), and hypoxia-related proteins (HIF-1 α-subunit, F_o subunit c of the F_oF_1 ATPase complex *(54).* In particular, the putative upregulation of receptors has provided key new clues about freeze tolerance that we are currently pursuing. For example, adenosine A1 re-

ceptor upregulation suggests that mechanisms of hypoxia-ischemia-induced MRD are activated during freezing. Up-regulation of the ANP receptor has implicated ANP, a cardiac hormone that regulates intravascular volume, in regulating the major changes in fluid dynamics of the cardiovascular system that occur during freeze–thaw. Up-regulation of RAGE suggests that advanced glycosylation end-products may accumulate in frogs as a result of nonenzymatic glycation of proteins under the extreme hyperglycemia of the frozen state. How frogs deal with this problem may suggest solutions for human diabetics because nonenzymatic glycation of long-lived proteins is the cause of diabetic vasculopathies and cataract.

The use of cDNA arrays for screening in comparative animal systems raises the critical issue of heterologous probing—the use of arrays containing immobilized cDNAs from one species to screen the mRNA populations of another species. Clearly, gene-sequence differences between species will prevent 100% cross-reaction between sample and array in any heterologous pairing. Predictably, our trials with 19K human cDNA arrays show that cross-hybridization falls off rapidly with phylogenetic distance. After optimization, we achieved cross-hybridization of 85 to 90% for human arrays hybridized with cDNA from hibernating mammals (ground squirrels or bats), 60 to 80% for frogs, and only approx 18% for hepatopancreas of *L. littorea (22,78).* The latter value for snails may seem very low especially when only 10.6% of the genes that hybridized were scored as putatively upregulated this still provided us with more than 300 anoxia-responsive candidate genes for future work. Among the anoxia-responsive genes identified by this screening were protein phosphatases and kinases, mitogen-activated protein kinase interacting factors, translation factors, antioxidant enzymes, and nuclear receptors *(22).* Heterologous screening would clearly have problems if the experimental goal was to evaluate qualitative and quantitative responses to stress by specific genes or groups of genes. However, our use of array screening has been as a general screening tool to find any genes that are stress-responsive and, in this mode, heterologous screening can be highly successful, providing the researcher with tens or hundreds of positive "hits" that provide multiple new directions for future research. Indeed, as noted above for frogs, array screening has provided numerous new directions to follow (e.g., receptors, antioxidants, glucose-related proteins, etc.) in our exploration of vertebrate freeze tolerance.

Another issue that has caused concern with heterologous probing is the possibility of false-positives (sample cDNA binding to cDNA on the array that is not its homologue) but in our work, to date, we have not encountered any case of a false-positive result. We treat all results from heterogeneous array screening with caution until they are verified by other techniques (e.g., RT-PCR or Northern blotting) using homologous probes. Indeed, our current protocol for the follow-up of genes that are highlighted as putatively upregulated from array screening is as follows. We search Genbank to gather sequences for the gene of interest from multiple sources, preferably from species phylogenetically close to the one under study, and then use those to identify a consensus sequence from which we design a PCR probe. The probe is then used to isolate the species-specific PCR product from a total RNA mix. We then sequence the species-specific product, confirm its identity, and use it to assess relative mRNA expression levels under multiple conditions via quantitative PCR. With the further use of peptide antibodies designed from the translated species-specific amino acid sequence we can also assess stress-induced changes in the accompanying protein levels. Thus, when combined with appropriate follow-up techniques, heterologous probing of cDNA arrays can provide an amazingly powerful search tool for gaining insights into the genes/proteins that underlie biochemical adaptation to extreme environmental stress.

In addition to gene-screening technologies, a variety of other new molecular methods are allowing enormous advances to be made in the field of biochemical adaptation. Proteomics approaches are rapidly developing, combining two-dimensional gel electrophoresis for protein isolation with analysis of tryptic peptides via liquid chromatography and mass spectrometry to identify stress upregulated proteins. The development of peptide and phosphopeptide antibodies for dozens of protein kinases, transcription factors and other proteins has greatly improved the ability to trace stress-activated signal transduction pathways to identify the stimulators of gene expression and of enzyme phosphorylation. For example, we used this technology to trace the upregulation of FABP in hibernator organs to stimulation by the γ isoform of the peroxisome proliferator-activated receptor (PPARγ) transcription factor and its co-activator, PGC-1 *(80).* Techniques for evaluating protein–protein interactions and the roles of targeting proteins are allowing researchers to decipher the three-dimensional organization and compartmentation of metabolism *(81).* These technologies and many more are allowing researchers to elucidate the unifying principles of biochemical adaptation that allow organisms to exploit and endure every extreme environment on Earth.

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