

# Molecular and Metabolic Aspects of Mammalian Hibernation

*Expression of the hibernation phenotype results from the coordinated regulation of multiple physiological and molecular events during preparation for and entry into torpor*

Bert B. Boyer and Brian M. Barnes

**A**s winter approaches and snow fills the air, mammals that hibernate avoid the energetic demands of maintaining high body temperatures by seeking shelter, falling asleep, and becoming deeply hypothermic. Hibernation is best viewed as an adaptation to anticipated famine and not to winter or cold per se. For example, near the beaches of Santa Cruz, adult California ground squirrels hibernate from late May until November, avoiding the hot summer months when grasses are dried and seeds long blown away. Even in northern climes, hibernators often overlap in distribution with species of similar body size that feed and remain active throughout the winter. Throughout montane and boreal forests, red tree squirrels and flying squirrels continue to move about during winter, high above buried hibernating ground squirrels, in their search for treeborne seeds and dormant insects. These animals also make use of cached cones, fungi, and berries. During winter on the tundra, small voles and lemmings live in the subnivalian space between the ground and depth hoar layers of snow. All winter long they continue to clip and feed from grasses and sedges. In contrast, most hibernating species do not climb trees or

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eat seeds from cones and are too large to use the subnivalian space; for these animals, winter can be a long season without foraging opportunities, and they have therefore evolved the ability to pass winter by while in a torpid state of lethargy.

A highly regulated sequence of physiological events beginning months in advance of winter coordinates entrance into the suspended state of animation known as hibernation. However, with the exception of bears, which become only moderately hypothermic, no hibernating mammal remains deeply hypothermic for longer than several weeks. Instead, for reasons that are not yet known, they expend significant amounts of energy to periodically rewarm back to normal body temperatures for less than a day before recooling.

The seasonal changes in body temperature that occur in an arctic ground squirrel (*Spermophilus parryii*) liv-

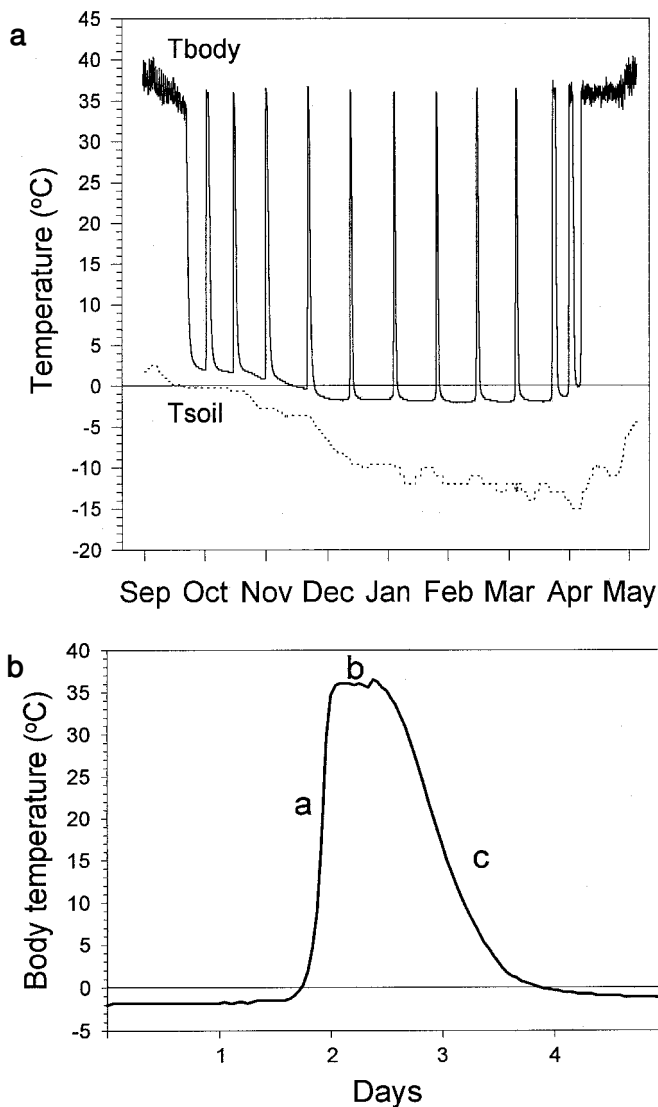
ing near the edge of its northernmost distribution in Alaska are especially profound and provide an extreme example of hibernation physiology (Figure 1). In a male arctic ground squirrel monitored for body temperature throughout hibernation, minimum body temperature decreased in autumn in parallel with the drop in soil temperature, indicating that when ambient temperatures are above freezing there is a passive thermal equilibrium between arctic ground squirrels and their surroundings. In early December, soil temperatures decreased toward  $-15^{\circ}\text{C}$ , but the ground squirrel's body temperature remained a constant  $-2.0^{\circ}\text{C}$  during torpor, indicating that this species actively thermoregulates. Substantial thermogenesis is also required throughout hibernation to fuel arousal episodes that interrupt torpor every 10–21 days.

Although there is a rich literature of physiological research related to the seasonally programmed changes that prepare the body for hibernation and regulate entry into, maintenance of, and recovery from torpor (e.g., Lyman et al. 1982, Carey et al. 1993, Geiser et al. 1996), the molecular and cellular mechanisms of mammalian hibernation have remained largely a mystery. In this article, against a background of the natural history of hibernation, we focus on recent progress defining the molecular and cellular changes that potentially regulate preparation for and entrance into hibernation and that result in differential regulation of gene products during torpor (Fig-

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**Figure 1.** Body temperature during hibernation, which is defined as the period of seasonal heterothermy and consists of substantial hypothermia (low body temperature), or torpor, and recurring arousal episodes. (a) Core body temperature of a free-living male arctic ground squirrel overwintering in its hibernaculum on the North Slope of Alaska ( $T_{\text{body}}$ , solid line) and nearby soil temperature at 1 m below the surface ( $T_{\text{soil}}$ , dotted line). Data are from temperature data loggers implanted in the abdomen of the ground squirrel and buried in the soil. This ground squirrel's hibernation season was composed of 12 torpor bouts of 1–3 weeks in duration separated by regular arousal episodes during which body temperature returned to near normal levels. During hibernation, the minimum body temperature fell to  $-2^{\circ}\text{C}$ , whereas soil temperatures were  $-10$  to  $-15^{\circ}\text{C}$ ; thus, torpid arctic ground squirrels must continually thermoregulate. (b) By graphing a representative arousal episode on an expanded time scale, it is clear that each recurring arousal episode includes three phases: a, rewarming from torpor; b, approximately 24 hours of euthermia (normal high body temperature); and c, slow cooling into torpor. Each tick mark indicates 1 day.



ure 2). We draw together literature from a variety of hibernating species, most frequently highlighting the hibernating arctic ground squirrel, which overwinters at an environmental extreme.

### Preparation for hibernation

For seasonal hibernators (i.e., animals that predictably enter dormancy periods lasting several months each year), entrance into hibernation is anticipated several weeks or more in advance by changes in behavior and physiology that lead to accumulation of energy stores.

**Fuel storage.** Hibernating species prepare for winter by storing food or becoming obese, and sometimes by doing both. Chipmunks, pocket mice, and hamsters do not fatten appreciably but rather store food, principally seeds, in their hibernacula. They then feed from these food caches during their arousal episodes, which occur more frequently than in hibernators that fatten. Males of several species of ground squirrels both fatten and store food. These caches are probably crucial for use during spring, when males end hibernation early to return to high body temperatures during their sexual maturation,

which is prolonged compared to sexual maturation in females (Barnes 1996).

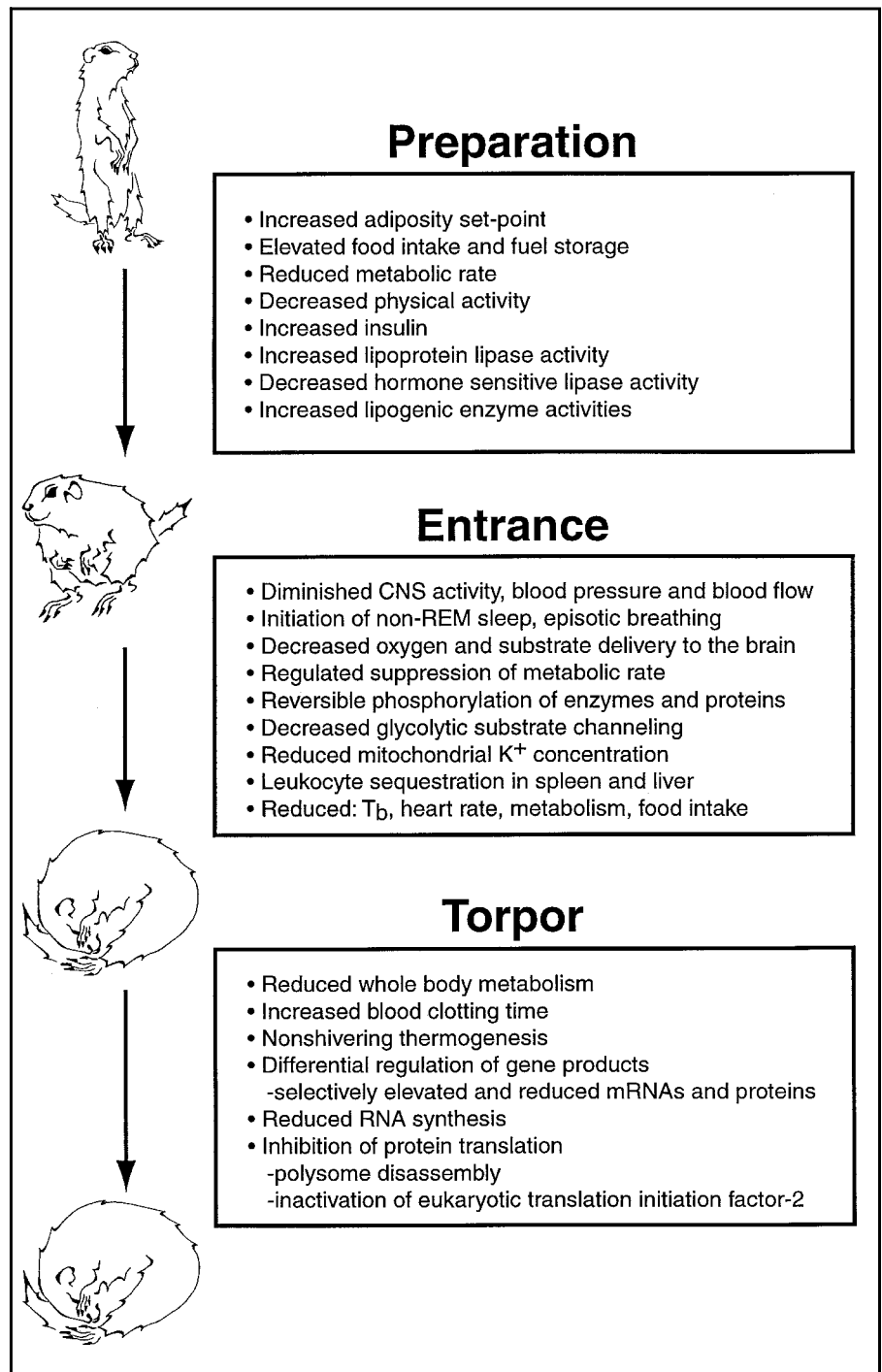
Food intake and body mass of species that fatten, such as ground squirrels and marmots, nearly double over the summer, and 60–80% of this increase can be accounted for as white adipose tissue, which triples in mass (Galster and Morrison 1976, Florant et al. 1990). Adipose tissue represents the most efficient fuel storage form for overwintering animals because, unlike carbohydrates, which are stored as hydrated glycogen, triacylglycerides (glycerol and fatty acids) are hydrophobic and stored without water. Furthermore, fatty acid oxidation provides more than twice as much energy on a per gram basis than carbohydrate oxidation. Although white adipose tissue provides most of the energy used during hibernation, other body reserves are also used and may be essential. For example, as much as 43% of protein (fat-free mass) is lost in arctic ground squirrels during the hibernation season (Galster and Morrison 1976, Buck and Barnes 1999). Protein can serve as a carbon source for carbohydrate synthesis through gluconeogenesis during prolonged fasts, when triacylglyceride turnover and release of glycerol from fat metabolism are low. Hibernating bears use protein as well, but they have solved the problem of protein wastage by recycling urea released by the breakdown of protein back into amino acids (Nelson 1980, Barboza et al. 1997).

Lipid accumulation in adipose tissue results from white adipose cell enlargement rather than increased numbers of adipocytes (Mrosovsky and Faust 1985). Fattening is stimulated by increased plasma insulin in the presence of unchanging glucagon levels (Florant et al. 1985, Tokuyama et al. 1991). Insulin stimulates the synthesis and inhibits the breakdown of glycogen, triacylglycerides, and protein. Insulin levels are high during the late summer peak in body mass, further contributing to fattening by increasing both the synthesis and activity of lipoprotein lipase, which promotes deposition of dietary lipid into fat stores (Wilson et al. 1992). High insulin levels also decrease the activity, and possibly the synthesis, of hormone-

sensitive lipase, the major lipolytic enzyme that releases free fatty acids from triglyceride stores (Wilson et al. 1992). Additional lipogenic enzyme activities increase during fattening, including fatty acid synthase, the multienzyme complex responsible for fatty acyl chain lengthening and lipid synthesis; fatty acid-CoA ligase, the enzyme required to activate fatty acids for glycerolipid synthesis; and diacylglycerol acyl transferase, which catalyzes the acylation of diacylglycerol in triglyceride synthesis (Mostafa et al. 1993).

**Set-point hypothesis.** Almost 30 years ago, a “sliding set-point” hypothesis was proposed to explain the seasonal cycle of changing body mass in mammals that hibernate (Mrosovsky and Fisher 1970). According to this model, adipose tissue mass is controlled by a hypothalamic “lipostat” that senses body lipid content and initiates compensatory changes in appetite and energy expenditure to maintain a seasonally appropriate level of adiposity, leading to fat gain during late summer and autumn and loss during winter. Experimental support for an adipose-sensing lipostat in animals that fatten in preparation for hibernation comes from manipulations of adipocyte size and number. Both surgical removal of adipose tissue, which reduces adipocyte number without affecting the size of the cells left behind, and food restriction, which reduces adipocyte size without affecting cell number, trigger compensatory increases in food consumption and metabolism to replace lost adipose mass to seasonally appropriate levels (Heller and Poulson 1970, Dark et al. 1985).

**Leptin.** Molecular support for a lipostat has come from the recent cloning of the *leptin* (*lep*) gene, which was first identified as the gene that is defective in obese *ob/ob* mice. These mice overeat to such an extent that they become as much as four to five times as fat as their lean littermates (Zhang et al. 1994). The *lep* gene, which is expressed in white and brown adipose tissue, codes for a 16 kD protein termed leptin (Greek root, *leptos*, meaning thin). In humans, mice, and rats, blood concentrations of leptin are proportional to total



**Figure 2.** Some of the major physiological events that characterize the hibernation cycle of ground squirrels. See text for details.

body fat. Leptin production is hypothesized to function as a peripheral signaling component of the lipostat, with high levels causing decreased food intake and increased energy expenditure and low levels resulting in greater hunger and energy conservation (Stephens and Caro 1998). Leptin action is mediated through binding to central and pe-

ripheral leptin receptors that belong to the cytokine receptor superfamily.

In arctic ground squirrels, circulating levels of leptin are also positively correlated with adipose tissue mass, although the range of plasma leptin levels, as detected by mouse leptin antibodies, is small (1–4 ng/ml; Bert B. Boyer and Olav Ormseth, unpublished observations) compared

to that in humans (1–100 ng/ml; Considine et al. 1996) and in mice or rats (1–60 ng/ml; Ahren et al. 1997). Leptin in arctic ground squirrels has a protein structure that is 86% identical to the mouse amino acid sequence (Ruth Stafford and Bert B. Boyer, unpublished data). This highly conserved protein structure suggests that leptin may participate with a lipostat in the regulation of seasonally changing levels of white adipose tissue mass in hibernating mammals.

Arctic ground squirrels are capable of responding to leptin in a similar way as mice and rats. For example, administration of recombinant leptin to leptin-deficient *ob/ob* mice normalizes their food intake, body mass, and energy expenditure to that of lean littermate control mice (Halaas et al. 1995). Continuous delivery of mouse recombinant leptin to arctic ground squirrels during prehibernation fattening also inhibits food intake and reduces body mass (Ormseth et al. 1996, Boyer et al. 1997). However, additional studies on the seasonal regulation of leptin production, leptin receptor sensitivity, and post-receptor signaling processes are necessary to determine whether leptin plays a physiological role in the regulation of adipose tissue mass in hibernators.

## Entrance into hibernation

Suitably fat and with sufficient stores of food secured in its burrow, a male arctic ground squirrel finally retreats into its hibernaculum, where it will remain until spring. Among arctic ground squirrels, adult and juvenile males are the last to enter hibernation, waiting until late September; adult females begin hibernating in early August. Emergence from hibernation occurs in the opposite order, males before females, with the first hardy adult male digging up through snow the second week of April and females emerging 1–2 weeks later, ending a 6–8-month hibernation season (Buck and Barnes 1999). Several of the physiological events that characterize entrance into hibernation have been well described.

**Central nervous system.** Electroencephalography (EEG) measurements demonstrate that hibernators enter

torpor via slow-wave sleep (Walker et al. 1977, Kilduff et al. 1993), which in mammals is associated with a small decrease in body temperature and metabolic rate (Glotzbach and Heller 1976, McGinty and Szymusiak 1990). In nonhibernators, body temperature and metabolic rate rise briefly during subsequent episodes of rapid eye movement (REM) sleep and again before waking. In contrast, hibernators suppress REM sleep and instead passively cool to low body temperatures. As the brain cools, the amplitude of the EEG signal diminishes, until at body temperatures below 20 °C it essentially becomes a flat line. Electrophysiological recordings of individual neurons demonstrate the absence of action potentials at brain tissue temperatures of below 15 °C when measured in vitro in the suprachiasmatic nucleus of hypothalamic slices (Miller et al. 1994) or in vivo by single-cell recordings in the posterior thalamus (Krilowicz et al. 1988).

However, central nervous system function persists in this apparently brain-dead state. Some behaviors continue in deep hibernation, such as slow movements to adjust posture and even vocalizations (Strumwasser 1959) and response and habituation to disturbance (Pengelley and Fisher 1968). Brainstem and hypothalamic control over respiration and thermoregulatory responses also continue. Regulation of these and other integrated functions, such as keeping track of time for circadian rhythms (Grahn et al. 1994), must be retained in the absence of action potentials or by the activity of as yet undetected small populations of neurons.

**Cardiorespiratory system.** The arctic ground squirrel's heart, which during euthermia beats 200–300 times per minute, slows gradually during entry into torpor, eventually reaching rates as low as 3–4 beats per minute. Breathing can become episodic, consisting of 5–10 successive breaths followed by periods of apnea that last from several minutes to an hour or more. The slowed heart rate and reduced blood pressure during torpor decrease cerebral blood flow by 80–90% compared to levels during euthermia. In nonhibernating mammals, such as rats or

humans, equivalent reductions in the flow of blood to the brain cause damage and neuronal death within minutes. That hibernators are not subject to such damage is probably due in part to an accompanying 98–99% drop in cerebral metabolic rate and, thus, in the demand for oxygen (Frerichs et al. 1995). However, additional protective factors may be necessary to prevent inflammation and damage from free radicals during rewarming, when high pressures of oxygenated blood suddenly reperfuse the brain.

**Leukocyte sequestration.** During entrance into torpor or during torpor itself, several changes in blood composition take place that may contribute to the ability of torpid animals to survive reduced oxygen and substrate delivery to the brain. Hibernation is associated with increased blood-clotting time, reduced blood volume, and decreased plasma erythrocyte and leukocyte concentrations. Concentrations of leukocytes (white blood cells) are reduced to less than 10% of their original levels during torpor, which may partially explain the ability of hibernating ground squirrels to survive profoundly reduced oxygen and substrate delivery to the brain (Yasuma et al. 1997). If the concentrations of circulating leukocytes were not reduced, their presence could lead to an inflammatory cascade resulting in cerebral ischemia, such as that observed following stroke or traumatic head injury.

Prevention of cerebral ischemia in hibernating ground squirrels is thought to occur by sequestration of circulating leukocytes into the spleen and liver. The proposed mechanism for leukocyte removal involves the up-regulation of intracellular adhesion molecules (ICAM-1) that participate in the migration of circulating leukocytes across the endothelial cell membrane (Yasuma et al. 1997).

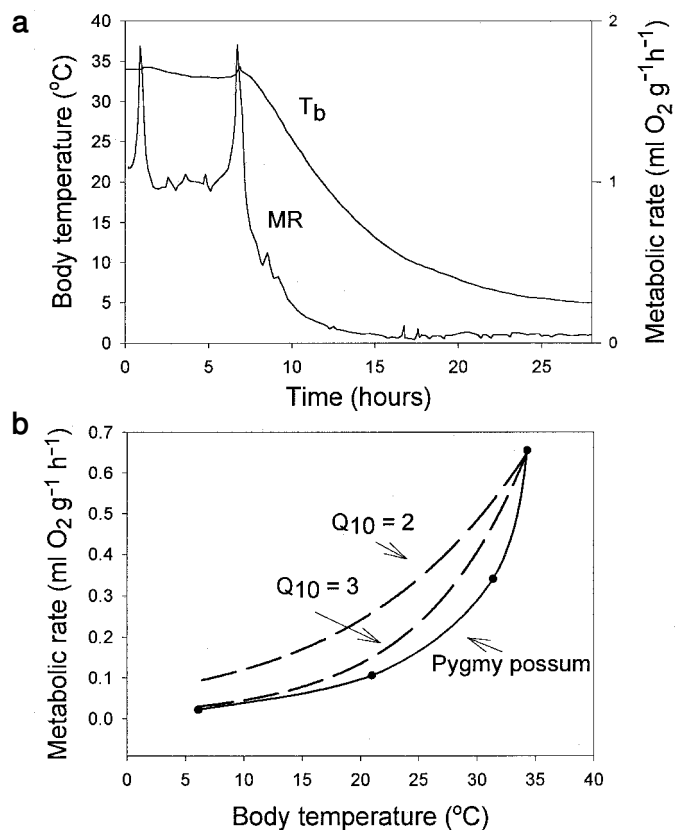
**Body temperature regulation.** Most temperate-zone hibernators protected within a warm nest and buried beneath insulating layers of earth and snow rarely experience subfreezing temperatures. Their bodies passively cool to near ambient temperatures, typically leveling off at 0.2–1 °C

higher than surrounding soil temperatures. Arctic hibernators face far more severe conditions. In their hibernacula, trapped between concrete-like permafrost below and extreme arctic winter above, hibernating arctic ground squirrels endure burrow temperatures that can reach  $-18^{\circ}\text{C}$  or lower (Barnes 1989). Under these conditions, their core body temperature falls to as low as  $-2.9^{\circ}\text{C}$ , or  $2.3^{\circ}\text{C}$  below the equilibrium freezing point of their body fluids. At these body temperatures, arctic ground squirrels avoid freezing by supercooling, which refers to the metastable state that fluids enter when cooled below their crystallization temperature in the absence of catalysts of freezing, or nucleators.

Arctic ground squirrels are the largest animal to use the precarious strategy of supercooling to resist freezing (Lee and Costanzo 1998), and in so doing they adopt the lowest body temperature measured in any mammal. This strategy is dangerous because, in the supercooled condition, arctic ground squirrels are vulnerable to both exogenous and endogenous nucleation of freezing. Exogenous nucleation can occur when supercooled arctic ground squirrels are penetrated by an ice shard. We have demonstrated that freezing can occur through inoculative nucleation, in one scenario by ice simply touching a toe (Brian M. Barnes, unpublished data). Endogenous nucleation occurs when some molecular substance within tissues or blood acts to nucleate freezing from within. Endogenous nucleation can also occur if ambient temperatures drop too fast and torpid arctic ground squirrels, unable to rapidly increase rates of heat generation, cool below their supercooling points (Brian M. Barnes, unpublished observations). It is not yet known how common freezing is in the field.

**Metabolic rate.** During steady-state torpor, and at ambient temperatures greater than  $0^{\circ}\text{C}$ , whole-body metabolism is profoundly slowed. Heat production is almost completely absent, and rates of oxygen consumption in arctic ground squirrel decrease to as low as  $0.01\text{ mL}\cdot\text{g}^{-1}\cdot\text{hr}^{-1}$ , corresponding to only 1% of the basal metabolic rate (BMR) during eu-

**Figure 3.** The decline in metabolic rate of hibernating mammals. This decline precedes and is greater than what can be attributed to the decline in body temperature. (a) Metabolic rate (MR, given as rate of oxygen consumption) and body temperature ( $T_b$ , abdominal temperature) change, as a golden-mantled ground squirrel (*Spermophilus lateralis*) enters torpor. Ninety percent of the decrease in MR occurs before body temperature declines 20%. (b) Metabolic rate of a hibernating animal would be predicted to decrease with decreasing body temperature if inhibition were due solely to temperature effects, with a  $Q_{10}$  of 2 or 3 (dashed lines;  $Q_{10}$  is the factorial difference between two rates  $10^{\circ}\text{C}$  apart). However, in the case of the pygmy possum (*Cercartetus nanus*), the actual rate of decline in metabolic rate (solid line) has a  $Q_{10}$  of greater than 3, which suggests that metabolic rate is being actively suppressed. Data are from Heldmaier et al. (1993) and Song et al. (1997).



thermia (Geiser and Ruf 1995). How this reduction in metabolic rate is achieved is controversial. The reduction in body temperature is generally considered to lead to a slowing of cellular processes, in accordance with the van't Hoff generalization, which states that rates of biochemical reactions typically halve with each  $10^{\circ}\text{C}$  decrease in temperature. For rates of physiological processes (e.g., metabolism, heartbeats), this temperature-sensitivity factor, or  $Q_{10}$  (the factor of difference between two rates  $10^{\circ}\text{C}$  apart), is typically 2–3. Thus, for an arctic ground squirrel passively cooling a maximum of  $40^{\circ}\text{C}$  from a euthermic body temperature of  $38^{\circ}\text{C}$  to  $-2^{\circ}\text{C}$ , metabolic rate during torpor would be expected to be reduced by a factor of 16, to 6.25% of BMR, for a  $Q_{10}$  of 2, and by a factor of 81, to 1.2% of BMR, for a  $Q_{10}$  of 3.

Because this range well represents measured values of how far metabo-

lism is decreased overall in the arctic ground squirrel, from a BMR of approximately  $0.6\text{ mL O}_2\cdot\text{g}^{-1}\cdot\text{hr}^{-1}$  to a minimum BMR during torpor of  $0.01\text{ mL O}_2\cdot\text{g}^{-1}\cdot\text{hr}^{-1}$ , our data are consistent with the van't Hoff generalization (Barnes et al. 1998). However, for other hibernating species, particularly smaller rodents and marsupials, calculated  $Q_{10}$  values for comparing metabolic rate during euthermia and torpor can exceed expected values, reaching 4 or more. Furthermore, close inspection of the time course of decreases in body temperature and metabolic rate of these mammals, such as the pygmy possum (*Cercartetus nanus*), during entry into torpor reveals that substantial reductions in metabolism often precede significant decreases in body temperature (Figure 3). These observations suggest that entry into torpor can result from a regulated suppression of metabolic rate and that body temperature then drops as a

consequence of the lowered rates of heat production, rather than the lowered body temperature causing the lowered rates of metabolism. This mode of entry into torpor has been referred to as a temperature-independent, or regulated, suppression of metabolism.

**Regulation of enzyme activity.** Several cellular mechanisms may contribute to the regulated suppression of metabolism and thermogenesis during entrance into torpor. Reversible phosphorylation of enzymes and proteins, as well as reductions in the levels of allosteric activators and substrates, limit rates of flux through metabolic pathways (Storey 1997). Reductions in glycolytic enzyme binding to the cytoskeleton and mitochondria and diminished maximal enzyme activity also decrease glucose breakdown during entrance into torpor by altering enzyme conformation and reducing substrate and product channeling between sequential membrane-bound glycolytic enzymes (Nestler et al. 1997). Inhibition of potassium transport and the subsequent reductions in mitochondrial potassium concentrations may further suppress metabolic rate during entrance into torpor by reducing the influx into, and oxidation of, respiratory fuels in mitochondria (Fedotcheva et al. 1985).

**Thermogenesis.** Brown adipose tissue is one of the major thermogenic tissues in hibernating rodents, providing the metabolic heat necessary for arousals from hibernation. Like white adipose tissue, brown adipose tissue undergoes a two- to threefold mass increase in arctic ground squirrels during preparation for hibernation (Feist et al. 1986), resulting in brown adipocyte enlargement due to lipid accumulation (Boyer et al. 1993). Brown fat grows in large masses alongside the shoulder blades of hibernating ground squirrels and wraps along the aorta and heart like heat tape.

As body temperature decreases during entry into torpor, shivering and other heat-generating mechanisms for thermoregulation are not activated because the hypothalamic set-point for body temperature control is apparently reprogrammed. Results of experimentally cooling and heating

the hypothalamus of hibernating ground squirrels and marmots while simultaneously measuring thermogenic responses suggest that this thermostat is gradually lowered during entry into torpor, from euthermic levels of 37–38 °C to a new level, often between 0 °C and 5 °C (Heller et al. 1977). This temperature represents the minimum level to which a hibernator will allow its body temperature to cool before either arousing back to euthermic levels or actively thermoregulating to maintain a constant temperature.

### Differential regulation of gene products during torpor

The evolutionary origin of hibernation is not known, with both ancestral and recent derivations hypothesized (Malan 1996, Geiser 1998). Species capable of entering profound and regulated hypothermia and hypometabolism have been found in diverse families among seven orders of mammals: Monotremata, Marsupialia, Primates, Insectivora, Chiroptera, Carnivora, and Rodentia. The interspersed phylogenetic distribution of hibernating and nonhibernating species has led to the hypothesis that, rather than requiring the creation of novel gene products, the hibernation phenotype results from the differential expression of existing genes during torpor (Srere et al. 1992).

Simple thermodynamic considerations suggest that low body temperature globally reduces rates of macromolecular synthesis and degradation, thereby maintaining constant RNA and protein concentrations during torpor (Figure 4). According to this model, the net result of low body temperature would be a reduction in the activity of all enzymes related to protein homeostasis, such that the rates of transcription, translation, mRNA degradation, and protein degradation should all decrease proportionately as a function of reduced body temperature. However, the identification of gene products (mRNAs and proteins) that accumulate or are reduced relative to others in torpid hibernators suggests that specific mRNAs and proteins are preferentially synthesized, stabilized, or de-

graded during hibernation. These results are consistent with the hypothesis that differential gene regulation is necessary to hibernate successfully. The molecular mechanisms underlying the differential gene regulation associated with hibernation in mammals are just beginning to be unraveled.

**Gene expression in the liver.** The first reports of differentially regulated gene products in hibernators were identified from plasma of Asian chipmunks (*Tamias asiaticus*; Kondo and Kondo 1992). Three of these differentially regulated proteins (HP-20, HP-25, and HP-27), each of which has a collagenlike amino acid domain, exist in blood of summer animals (hibernators that are not in torpor) as a 140 kD protein complex (Kondo and Kondo 1992). Although the function of the three proteins of the complex is not known, they are reduced in concentration before hibernation, disappear from the blood during hibernation, and do not reappear until hibernation has ended. These three proteins are not found in tree squirrels or rats, suggesting that they may be specific to hibernators. Cloning of the genes coding for these proteins revealed that their DNA sequences are similar, their mRNAs are restricted to the liver, and changes in their mRNA levels reflect observed changes in plasma concentrations of the proteins (Takamatsu et al. 1993).

A fourth protein depleted from the plasma of hibernating Asian chipmunks (HP-55) interacts weakly with the 140 kD complex and is highly homologous to  $\alpha_1$ -antitrypsin, a member of the serine protease inhibitor (serpin) superfamily and an acute phase reactant protein. Acute phase reactant proteins participate in defense during injury and infection; these proteins include a number of hepatic proteins, such as fibrinogen, complement proteins, and clotting factors. Other members of the serpin superfamily are proteolytic inhibitors that are involved in inflammatory cascades and hormone binding (Takamatsu et al. 1993). Four additional members of the  $\alpha_1$ -antitrypsin-like gene family have now been cloned from Asian chipmunks (Takamatsu et al. 1997), although the functional significance of these

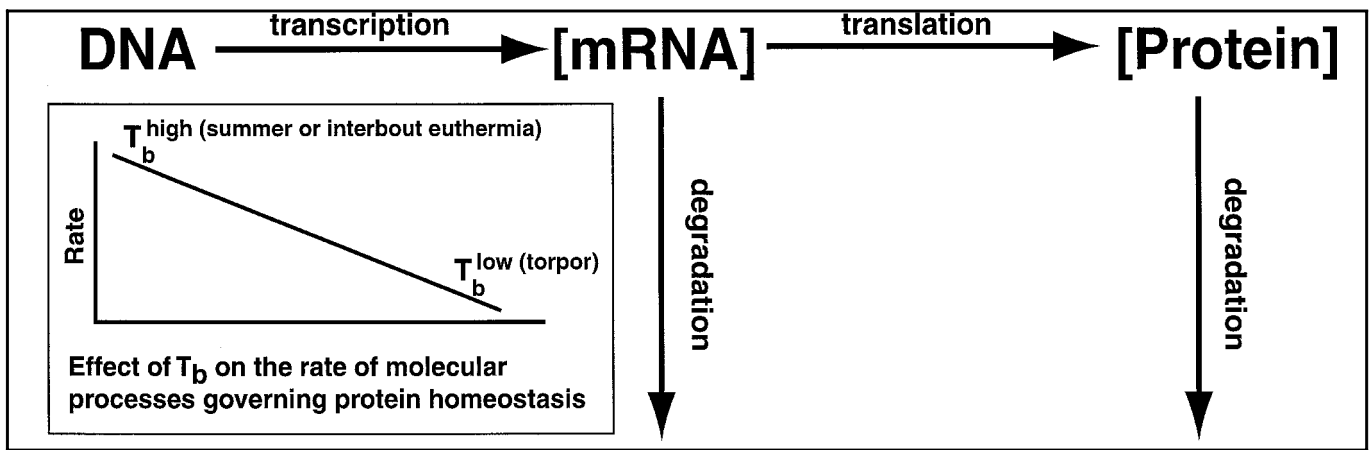


Figure 4. Molecular processes governing protein homeostasis. Inset box shows the variable effect that body temperature ( $T_b$ ) may have on rates of transcription, translation, mRNA degradation, and protein degradation. For example, it is now known that protein synthesis is very low in hibernating ground squirrels (Frerichs et al. 1988).

proteins in hibernation is not known.

**Control of blood clotting.** A different acute phase reactant protein,  $\alpha_2$ -macroglobulin, increases in the plasma of hibernating ground squirrels; these changes in plasma protein levels are accompanied by increases in mRNA for  $\alpha_2$ -macroglobulin in the liver (Srere et al. 1992). Although  $\alpha_2$ -macroglobulin levels are elevated in hibernating mammals, the entire acute phase response is not activated during hibernation (Srere et al. 1995). Rather, the functional role of increased  $\alpha_2$ -macroglobulin levels and activity may be to facilitate microcirculation by inhibiting blood coagulation when blood pressure and temperature are low.

**Gene expression in the brain.** The *c-fos* gene codes for the Fos protein, a transcription factor that can heterodimerize with other transcription factors (e.g., Jun) to activate the transcription of other genes. The abundance of *c-fos* mRNA is slightly elevated in the suprachiasmatic nucleus of the hypothalamus (the location of the mammalian circadian clock) during torpor and is significantly elevated during arousals from hibernation (Bitting et al. 1994). Accumulation of *c-fos* mRNA during arousals may initiate a “warm-up” signal that leads to the transcription of additional genes required during euthermia.

**Metabolic rate depression.** Several enzymes involved in carbohydrate metabolism are inactivated by phos-

phorylation during the transition to torpor, thereby preserving limited carbohydrate stores and permitting selective oxidation of lipid reserves (Storey 1997). Recent evidence suggests that selective fuel use may be controlled in part by differential gene expression. Reduced glyceraldehyde-3-phosphate dehydrogenase mRNA levels in muscle have been observed in hibernating jerboas (*Jaculus orientalis*), a rodent from the Moroccan Highlands (Soukri et al. 1996). Although liver mRNA levels for this enzyme are unchanged during hibernation, enzyme activity is reduced significantly. Thus, both transcriptional regulatory mechanisms and decreases in enzyme activity reduce glycolytic flux and preserve stored carbohydrate during hibernation.

Another enzyme that is phosphorylated and inactivated during torpor is pyruvate dehydrogenase (PDH). This enzyme complex converts pyruvate to acetyl CoA, thereby controlling aerobic oxidation of carbohydrates in the mitochondrial tricarboxylate acid cycle. PDH activity is controlled by several allosteric effectors and by covalently bound kinases and phosphatases that, respectively, phosphorylate (inactivate) and dephosphorylate (reactivate) the PDH complex. Dramatic reductions in aerobic carbohydrate oxidation during torpor have been explained by PDH kinase-mediated phosphorylation of the PDH complex (Storey and Storey 1990). The recent observation that PDH kinase isozyme 4 mRNA is sig-

nificantly up-regulated in the heart and skeletal muscle of thirteen-lined ground squirrels (*Spermophilus tridecemlineatus*) during torpor provides a molecular mechanism that accounts for increased PDH phosphorylation and reduced enzyme activity in these tissues (Andrews et al. 1998).

Employing the same differential gene expression screen that was used to identify up-regulated PDH kinase isozyme 4 mRNA, Andrews et al. (1998) discovered that pancreatic lipase mRNA and enzyme activity are also elevated in the heart of hibernating thirteen-lined ground squirrels. Pancreatic lipase is normally expressed in the pancreas and secreted into the small intestine, where it hydrolyzes dietary triglycerides into free fatty acids. Soluble heart fractions obtained from hibernating ground squirrels not only have higher pancreatic lipase activity than fractions from nonhibernating ground squirrels at the typical assay temperature of 37 °C, but also maintain significant enzyme activity at low assay temperatures (7 °C). Thus, hibernating ground squirrels may contain a cold-tolerant form of the enzyme that would provide a steady supply of free fatty acids for mitochondrial  $\beta$ -oxidation during torpor.

Collectively, these results indicate that genetic controls participate in directing the flux of metabolic fuels by limiting carbohydrate oxidation and increasing free fatty acid availability in hibernating ground squirrels. Moreover, these results further

support the hypothesis that differential regulation of existing genes contributes to the adaptive changes that define the hibernating phenotype (Srere et al. 1992).

**Nonshivering thermogenesis.** As soil temperatures fall below  $-5^{\circ}\text{C}$ , the body temperature of arctic ground squirrels remains constant (Figure 1). To maintain body temperature as the thermal gradient between body temperature and soil temperature expands during winter, arctic ground squirrels must produce heat constantly, most likely through nonshivering thermogenesis. Nonshivering thermogenesis is analogous to heat production provided by an idling engine, in this case fueled by the oxidation of endogenous substrates (fatty acids and glucose) and, ultimately, by proton transport across the mitochondrial membrane. Nonshivering thermogenesis in hibernating mammals involves the activation of a brown adipose tissue-specific mitochondrial uncoupling protein (UCP1), which promotes futile proton transport across the mitochondrial membrane without the production of ATP (Smith and Horwitz 1969). Loss of the proton gradient without the subsequent production of ATP, together with increased oxidation of fuels to reestablish the proton gradient necessary for adequate ATP synthesis, result in metabolic heat production, or nonshivering thermogenesis.

**Uncoupling proteins.** Two new uncoupling protein homologues (UCP2 and UCP3) have recently been identified that may contribute to nonshivering thermogenesis and energy expenditure. In rats and mice, *Ucp2* mRNA is present at high levels in white adipose and spleen and is detectable in several additional tissues; *Ucp3* mRNA is expressed at high levels in muscle and brown adipose tissue (Fleury et al. 1997, Vidal-Puig et al. 1997). To determine the expression pattern of *Ucp* homologues in animals that hibernate, we compared mRNA levels of *Ucp1*, *Ucp2*, and *Ucp3* in brown and white adipose tissue and skeletal muscle of arctic ground squirrels both before and during hibernation (Boyer et al. 1998). *Ucp1* mRNA levels were not

significantly increased following the onset of hibernation, and they remained unchanged during hibernation when ambient temperatures were above  $0^{\circ}\text{C}$ . This result confirms earlier findings that demonstrated that thermogenic capacity in brown adipose tissue increases before hibernation as a result of increases in brown adipose tissue mass, not UCP1 concentration (Milner et al. 1989).

In contrast, *Ucp2* mRNA levels were induced 1.6-fold in white adipose tissue, and *Ucp3* mRNA levels were induced 3-fold in skeletal muscle, of arctic ground squirrels in hibernation compared to ground squirrels that had not yet hibernated. Furthermore, mRNAs for *Ucp1*, *Ucp2*, and *Ucp3* were elevated approximately 2-fold in white adipose tissue, brown adipose tissue, and skeletal muscle of arctic ground squirrels hibernating at ambient temperatures below  $0^{\circ}\text{C}$ , compared to their levels in squirrels hibernating at temperatures above freezing (Boyer et al. 1998).

The differential regulation of *Ucp* gene homologues under differing thermogenic conditions in hibernating arctic ground squirrels suggests that several tissues, including brown adipose tissue, skeletal muscle, and white adipose tissue, may participate in nonshivering thermogenesis in hibernators. Future studies aimed at determining the thermogenic contribution of UCP homologues in multiple tissues of animals that are in hibernation will be necessary to define the physiological relevance of these differentially regulated *Ucp* mRNAs.

**Digestion.** The small intestine atrophies as food intake is significantly reduced or abolished during hibernation, yet intestinal gene expression persists (Carey and Martin 1996). This result is somewhat surprising in light of the necessary biosynthetic costs of gene expression and the observation that intestinal activity is significantly reduced at the low temperatures of deep hibernation. Recently, increased levels of moesin, a cytoskeletal linking protein, have been observed in intestinal brush border membranes of torpid thirteen-lined ground squirrels (Gorham et al. 1998). This observation is intriguing because moesin is not normally expressed in this cell

type in nonhibernating rodents and is not expressed at all in euthermic ground squirrels. Although the functional significance of this protein for hibernation is currently unknown, an intriguing possibility is that it may be involved in stabilizing membrane-cytoskeletal associations during torpor.

Several laboratories are just beginning to identify mRNAs and proteins that appear to be differentially regulated during hibernation; however, much remains to be learned about their physiological role. Future studies aimed at elucidating tissue-specific transcription rates, translation rates, and half-lives of gene products during each stage of hibernation will be necessary to understand the mechanisms responsible for differential gene regulation in hibernating mammals. In addition, such studies should be informative in revealing potentially unique adaptations of biochemical regulation at low body temperatures in mammals, as well as in deciphering the evolutionary origin of hibernation.

## mRNA and protein turnover

The cells of hibernators face dramatic metabolic adjustments and appear to function extremely well over a wide range of temperatures not faced by other mammals. Total RNA and protein concentrations are moderately decreased in liver (Whitten and Klain 1968), muscle (Wickler et al. 1991), and brain (Bocharova et al. 1992a) tissue of hibernating ground squirrels, compared to ground squirrels that are not hibernating. RNA and protein synthesis rates are also reduced during torpor and up-regulated during each arousal (Whitten and Klain 1968, Zhegnuov et al. 1988). Several potential mechanisms could account for the reduced mRNA and protein synthesis during hibernation.

**RNA synthesis during torpor.** Reduced RNA synthesis during torpor may result from reduced RNA polymerase activity at low temperatures or reduced supplies of the nucleotide precursors for RNA polymerases. For example, in one of the few studies to investigate the temperature sensitivity of uptake of labeled precursors

into RNA, DNA, and protein in nonhibernators (cultured chick embryo cells), incorporation rates at 4 °C were less than 1% of those at 37 °C (Scholtissek 1967a). In hibernating arctic ground squirrels, incorporation of labeled uridine injected into the lateral ventricles of the brain is reduced approximately eightfold during torpor compared to after an arousal (Bocharova et al. 1992b). Interestingly, however, rates of nucleotide uptake and phosphorylation are equivalent in torpid and euthermic squirrels (Bocharova et al. 1992b), whereas studies on cultured chick embryo cells indicate that the rate of uridine phosphorylation at 4 °C is 20% of the rate at 37 °C (Scholtissek 1967b). Collectively, these findings suggest that uridine transporters and kinases of ground squirrels operate in a “hypothermia-tolerant” mode, in contrast to those of nonhibernating animal cells, and that the supply of nucleotide precursors is not limiting during torpor. Thus, the reduced RNA concentration in torpid hibernators may result primarily from reductions in RNA polymerase activity.

**Protein synthesis during torpor.** In a landmark paper, Whitten and Klain (1968) demonstrated that the rate of hepatic protein synthesis is substantially decreased during hibernation. They also observed that hepatic polyribosomes disappear during torpor and reassemble during arousal episodes. Surprisingly, however, they found that protein synthesis rates in liver microsomes obtained from hibernating or aroused squirrels and assayed at 37 °C were threefold lower than rates in preparations obtained from euthermic arctic ground squirrels sampled outside the hibernation season. Based on this evidence, Whitten and Klain (1968) suggested that either protein synthesis must be actively inhibited during hibernation by factors in addition to temperature or critical components of the protein synthetic apparatus are missing in hibernating animals.

Over 30 years later, investigators are just beginning to unravel some of the molecular mechanisms responsible for the inhibition of protein synthesis in hibernating ground squirrels. Using elegant *in vivo* label-

ing techniques, Frerichs et al. (1998) have not only confirmed and quantified the dramatically decreased rate of protein synthesis in the liver of thirteen-lined ground squirrels during hibernation but also shown that protein synthesis is decreased in the brain and heart as well. Use of cell-free translation systems showed that the suppression of protein synthesis in the brains of hibernators continues even at incubation temperatures of 37 °C, demonstrating that, like the decline in overall metabolic rate, inhibition is not due solely to low temperatures but rather results from a regulated shutdown. Frerichs et al. (1998) demonstrated that approximately 13% of the alpha subunit of eukaryotic translation initiation factor-2 (eIF2 $\alpha$ ) in the brains of hibernating ground squirrels is phosphorylated, compared to only 2% in the brains of active squirrels. Phosphorylation of eIF2 $\alpha$  inhibits the initiation of protein synthesis, the primary step for translational control of gene expression. Translation may also be controlled through modifications of the elongation machinery, and indeed, Frerichs et al. (1998) showed that the rate of protein elongation is reduced threefold in extracts from hibernating brains compared to extracts from active brains. Although ADP ribosylation of eukaryotic elongation factor-2 could explain the observed reductions in elongation rates, no ADP ribosylation was detected in extracts derived from active or hibernating squirrels. Frerichs et al. (1998) suggest that these molecular changes, which result in neuroprotection during hibernation, may offer clues for treating or preventing stroke-related brain injury in humans, when blood flow transiently reaches the low levels typical of hibernation.

Although these studies have provided valuable clues to explain the dramatic reductions in protein synthesis (i.e., the decreased initiation and elongation rates) during hibernation, additional investigations are necessary to completely understand the molecular controls governing this suspended state of animation. What is becoming clear, however, is that hibernation results from a highly regulated and coordinated shutdown of cellular activity, stabilizing physi-

ological systems in the face of profound changes in tissue temperature and the availability of energy, substrates, and oxygen.

## Perspectives

Over the last decade, significant progress has been made in defining the molecular architecture of the hibernation phenotype. However, scientists are just beginning to understand some of the molecular physiological controls governing the preparation, entrance, and torpor stages of hibernation, and many classical questions about hibernation are still unanswered.

**Arousals from torpor.** The striking periodicity of arousal episodes constitute one of the most dramatic features of the hibernation cycle (Figure 1). Arousal episodes represent a tremendous shift in physiological and metabolic activity. The frequency of arousal episodes varies seasonally, among species, and among individuals of a species according to sex and age, but frequency is not closely correlated with body size, mass of stored adipose tissue reserves, or availability of stored food (Willis 1982). However, the fact that arousals are energetically expensive, yet shared by all hibernating species, has led to the suggestion that some “inescapable constraint” of low body temperature makes rewarming an absolute necessity (Willis 1982). Recent hypotheses proposed to explain the need for arousal episodes include the necessity to sleep (Daan et al. 1991, Trachsel et al. 1991), to restore “neuronal connectivity” (Strijkstra and Daan 1998), to replace neurochemicals (Wang 1993), and to resynthesize gene products (Martin et al. 1993). Research in these areas will help to define the physiological and molecular mechanisms that allow hibernators to survive extended periods without food or water in extreme climates. Additional overwintering strategies used by hibernating animals to survive famine and hypothermia undoubtedly await discovery.

**Hibernation induction trigger.** Many years ago, Dawe and Spurrier (1969) hypothesized that a “trigger” for mammalian hibernation in the blood

of thirteen-lined ground squirrels in deep hibernation was capable of initiating hibernation when transfused into ground squirrels that were not hibernating. Since then, tissue and body fluid extracts from hibernating animals potentially containing the elusive "hibernation induction trigger molecules" (HIT) have been injected into nonhibernating and hibernating species, with inconsistent results (Wang 1988, Vybiral and Jansky 1997). HIT molecules have never been purified, and their existence remains controversial. Available data in support of the existence of HIT molecules has led investigators to speculate that they are small, thermolabile proteins that are strongly bound to plasma albumin and may act through opiate receptors.

Based on the inconsistent and negative results of investigators attempting to identify HIT molecules (for reviews, see Wang 1988, Vybiral and Jansky 1997) and reproduce the initial results of Dawe and Spurrier (1969), data indicating that circulating HIT molecules lower body temperature and initiate hibernation should be viewed with extreme skepticism. A true hibernation induction substance would have to be multifactorial in its action to sequentially initiate each of the diverse physiological events required for hibernation. Furthermore, this substance would have to produce regulatory modifications at such disparate levels of control as appetite, fattening, thermoregulation, and blood clotting, among others.

Although there has not been much support for a multifactorial hibernation induction substance, research to identify a potential trigger has led to the discovery of an opiatelike compound, DADLE (D-Ala<sup>2</sup>, D-Leu<sup>5</sup> enkephalin), which extends the time that isolated animal organs (e.g., heart, liver, and kidneys) can be kept viable before transplantation (Chien et al. 1994). The mechanisms responsible for the increase in tissue survival time are not known, but a DADLE-induced regulated inhibition of cellular metabolism and protein synthesis, as occurs in the blood-deprived brains of hibernators, should contribute to organ survival in vitro.

Clearly, many questions remain

to be answered about the molecular and physiological mechanisms that are responsible for the hibernation phenotype. Does a single master control gene trigger the diverse cascade of physiological and behavioral events that lead to the suite of adaptations that characterize hibernation? Do low body temperatures indiscriminately drive the thermodynamic suppression of molecular and physiological processes? What accounts for thermoregulation during hibernation, and how are low body temperatures reversed? What cellular adjustments are necessary to allow mammals to survive low body temperatures and associated changes in substrate availability? Is there a gradual loss of homeostasis during torpor that may explain the necessity for periodic arousals to euthermic body temperature? Future research in these areas will undoubtedly solve many of the mysteries surrounding this fascinating physiological adaptation.

## Acknowledgments

Research in the authors' laboratories has been supported by the American Heart Association Alaska Affiliate, a National Institutes of Diabetes Digestive and Kidney Diseases grant (DK-45711), and a National Science Foundation CAREER Award (IBN 9514675) to B. Boyer, as well as National Institute of Child Health and Human Development grants to B. Barnes (HD-23383 and HD-00973). The authors would also like to thank Ms. Claudia Ihl for the arctic ground squirrel drawings.

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