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Determination *In Vivo* of Newly Synthesized Gene Expression in Hamsters During Phases of the Hibernation Cycle

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Abstract: This study measured *in vivo* synthesis of total RNA and protein from cortex, cerebellum and midbrain/brainstem and 6 major organs from Syrian hamsters (*Mesocricetus auratus*) during (a) 33 h of torpor (body temperature 5–6°C); (b) 90 min of the early arousal; (c) 90 min of the middle arousal; (d) 90 min in cold adapted cenothermic (CEN) hamsters of the same circannual period. Appropriate physiological parameters were used to confirm the phase of the hibernation cycle during infusion and incorporation of [^{3}H]-uridine and [^{14}C]-leucine. In torpor, RNA synthesis was 5–25% of CEN levels depending upon tissue. In brain and heart mRNA was not preferentially synthesized. Protein was synthesized at low, tissue specific levels during torpor. Initiation of arousal and the warming of anterior organs via non-shivering thermogenesis during the early arousal occurred without measurable synthesis of RNA or proteins. Tissue specific levels of RNA and protein synthesis occurred later after shivering thermogenesis had been recruited and was strongly influenced by thermal gradients in the body. In the middle arousal phase, protein synthesis is most active in the brain despite modest synthesis of RNA and mRNA. The majority of molecular processing required for the induction and maintenance of torpor and the arousal from torpor up until the onset of shivering thermogenesis occurs during the cenothermic period before the hamster initiates the hibernation cycle. [The Japanese Journal of Physiology 54: 295–305, 2004]

Key words: transcription, protein synthesis, mRNA, torpor.

Hibernation is a circannual adaptation and within the hibernation season, the hibernation cycle of a 'classic' hibernator, such as the Syrian hamster, can be divided into six distinct physiological phases. An entrance phase, when metabolism is inhibited and body temperature decreases [1]. A long period of torpor when metabolic rate (MR) is approximately 2.5% of resting metabolic rate (RMR) [2, 3]. This is followed by an arousal period of three phases: an early arousal phase during which the animal warms anterior crucial organs by non-shivering thermogenesis; a middle arousal phase when shivering thermogenesis is recruited to heat the anterior body to cenothermia (CEN) and a late arousal phase when posterior organs attain CEN and the animal rests or sleeps. An inter-bout arousal phase, of hours to days, during which time the hibernator maintains CEN body temperature before the initiation of another hibernation cycle. At present the onset of the hibernation cycle cannot be predicted. (Cenothermia is the IUPS term replacing euthermia [4]).

Molecularly orientated studies have searched for up-or-down regulation of gene expression in a small number of organs from torpid animals in an attempt to demonstrate molecular changes that are consistent with the enormous physiological changes occurring across the hibernation cycle [5]. Early investigations used radioactively labeled precursors to measure *in vivo* transcription in brains [6, 7], translation in liver [8, 9], and more recently translation in brain and heart [10],

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indicated that during torpor transcription was greatly reduced and translation largely inhibited. The latter as a result of thermodynamic effects coincident with phosphorylation of initiation factor 2a [10, 11]. *Ex vivo* polysomal run-on analysis demonstrated that, in liver, elongation of pre-initiated peptides continues slowly during torpor although initiation of protein synthesis is inhibited [12]. It was also demonstrated that CEN polysomal profiles are re-established when a temperature of 18°C was surpassed [12]. A recent report [13] demonstrates *in vitro* protein synthesis (PS) in brown adipose tissue (BAT) from torpid squirrels. This suggests that inhibition of PS during torpor is not universally associated with low ambient temperature or phosphorylation of initiation factor 2a and raises the possibility of PS *in vivo* in other essential but as yet unexamined organs.

The control of transcription across the hibernation cycle is not well understood. An increasing number of studies have demonstrated decreased [14] or increased [13, 15–21] differential expression of cDNA probed levels of mRNA for a variety of genes, associated with biochemical adaptations considered relevant to hibernation, from tissues extracted at different phases of hibernation cycle. Although increased levels of mRNA in tissues from torpid animals appears to be consistent with earlier *in vivo* studies that demonstrate RNA transcription during torpor, the two procedures are not temporally complimentary.

In heart, brain and liver sufficient evidence is now available to indicate that in torpor, levels of total tissue RNA (as estimated from levels of ubiquitous RNA or mRNA) and protein, in the majority but not all organs examined [14], are not different from levels in CEN [10, 11, 13, 14, 19, 22, 23] while mRNA [24] and probably protein [25] are stabilized in torpor. The preservative character of torpor on the levels of genomic components determines that protein or mRNA levels in tissues harvested from animals in torpor will reflect both the molecular events active during torpor, and also a component residual to changes of synthesis or degradation that occurred prior to the initiation of torpor. Quantification of RNA and protein synthesized *in vivo* during specific phases of the hibernation cycle from labeled precursors provides a means to distinguishing between active gene expression and residual gene expression.

In an investigation of molecular processes that are active during different phases of the hibernation cycle, it is critical to monitor physiological parameters such as BAT temperature (T_{BAT}), heart rate (HR) and respiratory rate (RespR) which when combined are reliable indicators of the phases of the hibernation cycle [26]. Ambiguities arise when assigning the results of previous molecular studies to a specific phase of the hibernation cycle because the majority of researchers have favored the sole use of rectal or abdominal temperature (T_{RECTUM}) to indicate that animals are in torpor. The rectal and abdominal regions are amongst the last tissues to be re-warmed during arousal [26] and as such T_{RECTUM} is a good indicator of the middle arousal phase but unsuitable as an accurate bio-indicator of torpor.

The two previous studies of *in vivo* synthesis of total RNA during the phases of hibernation are physiologically problematic, are limited to analysis of cerebral tissue and do not examine the RNA being transcribed. In the earlier study on bats [7], intracerebroventricular injections of labeled orotoic acid was used as the precursor for de-novo pyrimidine synthesis. Under physiologic conditions, orotoic acid is a less appropriate substrate than uridine for cerebral RNA synthesis (RNAS) [27] since in most organs, except for liver and to some extent kidney, RNAS usually proceeds predominantly via the salvage pathway [28]. In the more recent study using torpid squirrels, it was demonstrated that intracerebroventricular injection of labeled uridine was incorporated into cerebral RNA at reduced rates relative to non-hibernating squirrels [6].

The aim of this study was to examine *in vivo* PS and RNAS, in Syrian hamsters (*Mesocricetus auratus*) in order to determine if the physiological phases of the hibernation cycle were associated with organ specific, distinct patterns of transcription and translation. We measured *in vivo* synthesis of total RNA and protein from the cortex, midbrain/brainstem and cerebellum and 6 major organs. We also measured mRNA synthesis in heart and midbrain/brainstem during torpor, during early arousal when non-shivering thermogenesis was maximal, during middle arousal when shivering thermogenesis had been recruited. These results were compared with the levels of *in vivo* mRNA synthesis in non-hibernating, cold adapted CEN hamsters of the same circannual period.

MATERIALS AND METHODS

Animals and housing. The following experiments conformed to the ethical guidelines of the Japanese Physiological Society and Asahikawa Medical University (ethics approval # 02166). Male hamsters were housed at 22°C with 12:12 h light dark cycle and *ad lib* food and water until 3 months of age or body weight (bwt) was greater than 120 g. The hamsters were then housed individually, in constant darkness at 4°C, with nest materials and *ad lib* access

to chow and water. An infrared motion sensor and copper/constantan thermocouple attached to each nest were used to monitor the duration of each hibernation bout. Hibernation in mature hamsters can be induced at any time of year after reducing ambient temperature to 5–10°C and housing in constant darkness for about 45 days. Once hibernation is initiated, hamsters will continue a regular hibernation cycle for about 10 weeks. Hamsters that had experienced 2–3 hibernation cycles were aroused and the femoral vein was cannulated after they had attained CEN.

Surgery and animal protocol. Using sterile surgical procedure, under Nembutal anesthesia (45 mg/kg bwt, IP, Dainippon seiyaku, Tokyo) supplemented with topical application of 4% Xylocaine (Astra Zeneca, Osaka) to the skin wound, the femoral vein was cannulated so that the silicon tubing tip of a polyethelene cannula (PE-10, Becton Dickinson, Tokyo) was positioned in the inferior vena cava. The PE cannula was exteriorized between the shoulder blades and first filled with sterile glycerol and saline containing 200 IU heparin (ratio 1:1). After surgery, the skin wound was irrigated with Xylocaine and the hamster was given a 5 ml/kg IP injection of sterile saline. Hamsters were returned to their home cage after recovery from anesthesia. Hamsters usually re-commenced hibernation within 7 days after surgery. Thirty to forty h after the hamster had re-commenced hibernation the cannula was flushed and filled with sterile saline containing 5 IU of heparin and aroused from hibernation. Aroused hamsters usually re-hibernated within 2 days. Hamsters that did not re-hibernate within 1 week after surgery or flushing of cannula were allocated to the cold adapted, hibernation season cenothermic control group (CEN).

Phases of the hibernation cycle and *in vivo* infusion protocol for hamsters. The length of the hibernation cycle of hamster is 80–100 h. Torpor lasts for approximately 70 h during which time the whole body MR of the hamster is approximately 2.5% of cenothermic cold adapted RMR [2, 3, 26]. In torpor, small changes in MR can be performed to defend the reduced body temperature against fluctuations in the low ambient temperature [29, 30]. Arousal from hibernation (rectal and brain temperature 4–5°C) to CEN (35–37°C) takes 3–3.5 h at an ambient temperature of 4°C and, on the basis of physiological parameters it can be divided into early, middle and late arousal phases. The early arousal is associated with a MR that increases from 2.5% to approximately 100% of RMR by non-shivering thermogenesis. RespR changes from Cheyne Stoke pattern and increases to the same rate as at resting CEN levels [2, 3, 26]. The middle arousal is associated with an increasing MR that peaks at 250–300% of RMR after the recruitment of shivering thermogenesis [2, 3, 26]. At this time, BAT, cheek pouch and brain temperatures are near cenothermic. However, rectal and hindlimb temperatures remain low [3, 26, 31] and the animal is incapable of coordinated walking. Late arousal is associated with warming of rectum to CEN, MR and RespR decrease to resting CEN levels [2, 3, 26] and the animal rests or sleeps [32]. These phases are exemplified in Fig. 1 which is a composite of the physiological data from all animals used in this study.

In CEN animals, the chemical pathways for *in vivo* incorporation of uridine [27, 28, 33] and leucine [34] in particular have been well characterized. Previous *in vivo* studies have demonstrated that at CEN, *in vivo* incubation periods of 30 min to 2 h are of suitable duration for quantifiable incorporation of radioactive precursors into RNA and PS and minimize degradation of newly synthesized molecules [27, 33, 34]. In regard to uridine incorporation, uridine is degraded *in vivo* in the liver but sufficient uridine escapes degradation to be incorporated into ribosomal and mRNA [27, 28, 33]. Unincorporated label and metabolites are removed by washing after extraction and subsequent chromatographic separations.

The length of the *in vivo* incubation periods for hamsters in torpor (31–36 h), early and middle arousal (90 min each) were chosen as the longest practical *in vivo* incubation periods that encompassed the physiology that characterizes each of these phases of hibernation. The duration of the early and middle arousal phases, is approximately 90 min however the duration of torpor is often approximately 60 h or double the *in vivo* incubation period. *In vivo* incubation periods of 60 h during torpor were attempted but proved not practical. Arguably, estimates of whole body oxygen consumption during the different *in vivo* incubation periods, rather than time per se, provides a physiological reference point from which amounts of protein or RNA synthesized may be related to the rate of synthesis at CEN. It should be recognized that the contributions of individual organs to whole body MR during torpor, early and middle arousal will be different than during CEN.

Radioactive uridine [5,6-^{3}H] (specific activity: 30 Ci/mmol) and L-leucine [1-^{14}C] (specific activity: 55 mCi/mmol) were purchased from (American Radiolabeled Chemicals Inc. Missouri, USA). Fourteen hamsters were allocated to one of 4 experimental groups. Cold adapted, circannual non-hibernating CEN hamsters (n = 4); hamsters in early arousal (n = 4); hamsters in middle arousal (n = 3); hamsters in the

torpor (n = 3). Eleven hamsters were infused with uridine [5,6-^{3}H] 50 μCi/100 g bwt and L-leucine [1-^{14}C] 10 μCi/100 g bwt dissolved in 0.15 mls of sterile saline. Two CEN and one early arousal hamsters were infused with only uridine [5,6-^{3}H] 50 μCi/100 g bwt dissolved in 0.15 ml of sterile saline. Preliminary experiments were performed in two urethane anesthetized CEN hamsters infused with uridine [5,6-^{3}H] 50 μCi/100 g bwt and L-leucine [1-^{14}C] 10 μCi/100 g bwt.

Experimental procedure and measurement of biophysical parameters. Hamsters in torpor for use in the early or middle arousal groups, were transferred to the dark room maintained at 4°C, 50 to 60 h after commencing a cycle of hibernation. These animals were fitted with pre-calibrated rectal and BAT thermocouples. The skin above the interscapular BAT was anaesthetised with procaine, the interscapular BAT was acutely dissected through a small incision and a sterile, pre-calibrated thermocouple was secured between lobes of BAT with tissue glue. HR was recorded from stainless steel 27G needle electrodes acutely inserted into the skin on either side of the heart. These procedures initiated arousal. Thermocouples were connected to zero temperature reference refrigeration unit (Zero-con, Komatsu Electronics, Tokyo) and a thermocouple interface (IM-7, I-techno, Tokyo). HR was recorded on a biophysical amplifier (AVB-10, NihonKohden, Tokyo) connected to computer interfaces (Mac Lab 2e, ADInstruments, Sydney) and the data was stored electronically. An observer used a video camera coupled to an infrared light source to remotely monitor and count the RespR of hamsters.

Hamsters in the early arousal group were given an infusion of 150 μl of radioactive uridine and leucine at 50 μl/min commencing as soon as the animal was instrumented. Ninety minutes later, before the onset of shivering thermogenesis, the animals were killed with an IV overdose of Nembutal. Hamsters in the middle arousal group were instrumented as above but the infusion of 150 μl of radioactive uridine and leucine at 50 μl/min was delayed until both T_{BAT} reached 11°C and RespR was in excess of 35 b/min. Ninety minutes later, when the RespR was maximal the hamsters were killed with an overdose of Nembutal. The end of the EAP and the beginning of the MAP overlapped by approximately 25 min. Hamsters in the torpor group were transferred to the experimental room and given an infusion of 150 μl of radioactive uridine and leucine at 5 μl/min commencing 18–20 h after the initiation of the torpor. Thirty-one to 36 h later the hamsters were killed with an IV overdose of Nembutal. Freely moving CEN hamsters were transferred to the dark and cold experimental room in their home cage and given an infusion of 150 μL of radioactive uridine and leucine at 50 μL/min. Ninety minutes later the animals were killed with an IV injection of Nembutal.

Tissue preparation and assay. Immediately before death, a blood sample was taken by cardiac puncture from the anesthetized hamster. Heart, liver, BAT, femoris muscle from the hind limb (FeM), rhomboid muscle from under interscapular BAT (RhM), kidney and brain were rapidly excised from dead hamsters. The brain was dissected into 4 parts: cerebellum, cerebral cortex, brainstem/midbrain (excluding olfactory bulb) and remainder including thalamus, septal and hippocampus. All tissues were immediately frozen in liquid nitrogen and then stored at –80°C until assay. Urine in the bladder was collected. Radioactivity in plasma and urine was measured. Tissues were weighted (approx. 140 mg used for each determination) and pulverized frozen, homogenized in Tryzol solution (Life Technologies, USA) and the RNA and protein were separated as per manufacturers procedure. Total RNA was washed extensively in 80% ethanol before non-incorporated radioactive nucleotides and RNA < 20 bases long were removed by size exclusion chromatography (MiniQuick spin RNA column, Roche, USA). In brain and heart, mRNA was separated from approximately 150 μg of total RNA using poly dT affinity chromatography (Oligotex-dt30, Takara, Japan). RNA and mRNA were quantified by measurement of absorbance at 260 nm and purity of sample was estimated by the ratio of absorbance 260/280 nm using Gene Quant II (Pharmacea Biotech, UK). Aqueous solutions of RNA and mRNA were added to a scintillation fluid containing toluene/triton X (ratio 2:1), 10% water and 2,5 Diphenyloxazole (0.5%). Protein extracted from tissue was dissolved in 1% SDS. Total tissue protein concentration was determined using protein assay kit that measured absorbance at 562 nm (Pierce, USA). A known quantity of tissue protein (1500–1800 μg) was precipitated from solution using 7% TCA and applied to 45 μm nitrocellulose filter paper (Millipore, Ireland). The precipitated protein was washed copiously with 5% TCA solution. Filter papers were dried at 70°C for 90 min and applied to an aqueous free scintillation fluid (Atomlight, Perkin Elmer, Tokyo). Since only 2 CEN animals received [^{14}C]-leucine infusions, protein determinations were performed twice from 2 separate tissue extractions from all animals for all tissues except the cerebellum and cerebral cortex. These two tissues were insufficient for repeated extraction. [^{3}H] emissions from RNA solutions and [^{14}C] emissions from protein impregnated filters were measured using scintillation counter

(LS6500 Beckman USA). [^{3}H] and [^{14}C] emissions were counted in channels 0–400 and 400–670 respectively. Total radioactivity was counted in channels 0–670. Chromatographic separation of RNA from protein and the high concentration of [^{3}H] administered relative to [^{14}C] determined that the contribution of [^{14}C] to the tritium channels was minimal. In the 2 CEN and 1 early arousal animals tested, tissue levels of incorporation of [^{3}H] in RNA were not different between animals injected only with [5,6-^{3}H] uridine and those injected with both [5,6-^{3}H] uridine and [^{14}C]-leucine. DNA was extracted from cerebral tissue by digestion and purified using affinity chromatography (DNeasy Kit, Qiagen, Japan). The DNA was quantified by fluorometry (excitation 380 nm, emission 460 nm) after reaction of DNA with Hoechst 33258 fluorochrome (BioRad, USA) and comparison against calf thymus DNA.

RESULTS

Figure 1 shows the average T_{BAT} and T_{RECTAL}, RespR profile of hamsters from the 4 *in vivo* incubation periods. Continuous torpor was confirmed by data from a motion sensor and nest thermocouple, and Cheyne Stoke respiratory pattern, HR of 13 ± 1 b/min (n = 3 hamsters) and T_{RECTUM} of 5–6°C immediately before sacrifice after 30–36 h of *in vivo* incubation. On the basis of whole body MR, 60 h of torpor is metabolically equivalent to 90 min of cold adapted CEN, assuming MR during torpor is 2.5% RMR during cold adapted CEN [2, 3, 26]. Thus the metabolic expenditure over 33 h of torpor is approximately 50% of RMR over 90 min at cold adapted CEN.

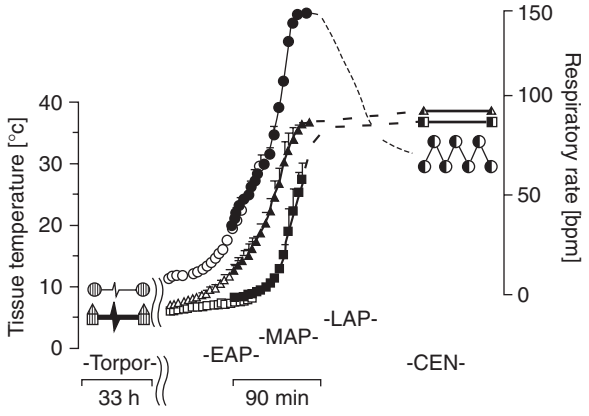

Fig. 1. Averaged physiological profile of all hamsters during *in vivo* incorporation of [^{3}H]-uridine and [^{14}C]-leucine. T_{BAT} °C – triangles, T_{RECTUM} °C – squares, Respiratory rate (breaths/min) – circles (Mean ± SEM). Torpor incubation in 3 hamsters was 33 ± 1 h (striped symbols). Early arousal phase (EAP) incubation in 4 hamsters was 90 min (open symbols). Middle arousal phase (MAP) incubation in 3 hamsters was 90 min (filled symbols). CEN incubation in 4 hamsters was 90 min (half filled symbols). Time course of physiological changes in late arousal phase (LAP, not measured in this experiment) are shown as dotted lines and are estimated from a previous publication by the present authors [26].

In 4 hamsters at the beginning of the early arousal phase, T_{BAT} and T_{RECTAL} were 6.3 ± 0.1 and 5.5 ± 0.1°C respectively. RespR and HR were 2 ± 1 b/min and 18 ± 2 b/min respectively. At the end of the early arousal period T_{BAT} and T_{RECTAL} were 17.6 ± 1.0 and 7.7 ± 0.5°C respectively. RespR and HR were 71 ± 5 b/min and 112 ± 13 b/min, respectively. The oxygen consumption over 90 min of early arousal was estimated to be approximately 40% of oxygen consumption over 90 min at cold adapted CEN. This was estimated by comparing the increase of RespR and T_{BAT} in these experimental hamsters with the increase RespR, T_{BAT} and oxygen consumption in 4 other hamsters arousing at the same ambient temperature over the same time period [26].

Middle arousal phase began when RespR, HR and T_{BAT} were 39 ± 3 b/min and 46 ± 3 b/min and 11°C, respectively. This phase was completed 90 min later, just prior to the animals becoming ambulatory, when RespR was maximal (150 b/min) and T_{BAT} was 36°C. As with animals in early arousal phase, the metabolic expenditure during this 90 min was estimated by matching the increase in RespR and T_{BAT} with previously published measurements of oxygen consumption [26]. The oxygen consumption over 90 min of early arousal phase was estimated to be approximately 150% of RMR over 90 min at cold adapted CEN. CEN animals were not instrumented and slept or displayed low level activity with RespR of 60–70 b/min during the 90 min *in vivo* incubation period.

Table 1 shows that after the *in vivo* incubation periods, plasma levels of [^{14}C]-leucine were greatest in hamsters of the early arousal, intermediate in torpor and CEN and lowest in middle arousal ($P < 0.05$). Plasma levels of [^{3}H]-uridine were lowest in middle arousal and not different in other phases ($P < 0.05$). Plasma [^{14}C]-radioactivity for 4 CEN animals is derived from 2 conscious CEN hamsters and 2 urethane anesthetized CEN hamsters. Total radioactive loss to urine was very low in torpor, early and middle arousal, and greatest during CEN when kidney function was normal. Uptake of radioactive substances by red blood cells during any phase of hibernation was not detectable.

Table 2 shows that total RNA and protein content were different between tissues. The extraction efficiency of RNA or protein from the same organ was

Table 1. Physiological parameters of hamsters during *in vivo* incorporation of [^{3}H]-uridine and [^{14}C]-leucine.

Hibernation phase	BWT [g]	BAT Temp [°C]	RespR [b/min]	Urine volume [ml]	Urine activity [cts/min/60 µl]	[^{14}C]-Plasma activity [cts/min/60 µl]	[^{3}H]-Plasma activity [cts/min/60 µl]
CEN n = 4*	127±5^{b,c}	36–37	60–70	0.65±0.2^{b,c}	306976±71510^{b,c}	7832±1432*^{b}	8724±1068
Middle arouse n = 3	101±4^{a}	11–36	39–153	0.2±0.1^{a}	9039±2290^{a}	5274±958^{b}	3911±1674
Early arouse n = 4	108±5^{a}	5–18	2–71	0.1±0.05^{a}	4315±2150^{a}	18117±1330^{a,c}	5927±404
Torpor n = 3	106±8^{a}	5–6	0–1	0.12±0.05^{a}	2257±1143^{a}	10935±1411^{b}	10780±1396^{c}

[^{3}H]-plasma counts – $F(3,13) = 6.5$, $P = 0.01$; [^{14}C]-plasma counts – $F(3,12) = 16.2$, $P = 0.0006$; Urine total counts – $F(3,13) = 12.3$, $P = 0.0015$; Urine volume – $F(3,13) = 4.6$, $P = 0.03$; BWT – $F(3,13) = 4.9$, $P = 0.02$. a – diff from CEN $p < 0.05$; b – diff from early arousal $p < 0.05$; c – diff from middle arousal hamsters. * [^{14}C]-Plasma activity includes [^{14}C]-plasma activity from 2 urethane anesthetised CEN animals. Mean ± SEM or range of the values are shown in this and following tables.

not different between phases of hibernation. For every RNA extraction 260/280 nm absorbance ratio was >1.74. In all tissues, incorporation of [^{3}H]-uridine into total RNA was greatest in CEN, invariably below the detection limit in early arousal and intermediate in torpor. Incorporation during middle arousal phase was consistent within animals but extremely variable as a group, with one animal having levels greater than CEN. In FeM, [^{3}H]-uridine and [^{14}C]-leucine incorporation remained undetectable in middle arousal. Incorporation of [^{14}C]-leucine into protein was below the detection limit in early arousal and low during torpor. The amount of [^{14}C]-leucine incorporation during middle arousal was tissue specific ranging from no incorporation in FeM while in cerebellum and brain stem/mid brain incorporation was not different from CEN.

Comparison of total RNA content of brain (pooling all brain areas) or peripheral tissues (pooling all tissues) from CEN hamsters and hamsters in torpor, as determined from RNA extracted per mg of wet tissue, indicates that brain tissue from hibernating animals has more RNA/mg tissue than CEN hamsters (Torpor – 0.91 ± 0.041 µg RNA/mg tissue (n = 12); CEN – 0.79 ± 0.035 µg RNA/mg tissue (n = 16), Mann Whitney $U = 47$, $P > 0.05$). If hamsters in early arousal were included with torpid hamsters this significant difference was retained (0.89 ± 0.033 µg RNA/mg cerebral tissue (n = 21). The DNA content of brain tissue was not different between torpor (0.81 ± 0.08 µg/mg, n = 11) and CEN hamsters (0.77 ± 0.08 µg/mg, n = 15). There was no difference in the amount of RNA/mg tissue in peripheral organs between torpid (1.44 ± 0.16 µg/mg, n = 39) and CEN hamsters (1.46 ± 0.2 µg/mg, n = 22).

Figure 2 shows a linear relationship ($y = 0.036x$ – 0.064. $r^2 = 0.937$) in the amount of [^{14}C]-leucine incorporated into protein in the 9 tissues between CEN and torpor. Incorporation of [^{14}C]-leucine into FeM during torpor was not detectable. The relationship in the amount of [^{3}H]-uridine incorporated into total RNA in tissues between CEN and torpor is not significant if all tissues were included in a linear analysis. However a significant relationship ($y = 0.041x + 0.469$. r^2 = 0.98) was found when heart and kidney tissue were excluded from the analysis.

Table 3 shows the incorporation of [^{3}H]-uridine into RNA and mRNA from heart and mid brain/brain stem during CEN, middle arousal and torpor. Total RNA for mRNA purification is derived from a separate tissue extraction from data present in Table 2. Total RNA and mRNA contents were different between tissues, however the extraction efficiency of total RNA or mRNA was not different between phases within the same tissue. Incorporation of [^{3}H]-uridine into total RNA was consistent with extractions in Table 2. [^{3}H]-uridine was preferentially incorporated into mRNA in CEN. Incorporation of [^{3}H]-uridine into mRNA is greatest in CEN and middle arousal. Incorporation during middle arousal was variable between hamsters. In torpor, incorporation of [^{3}H]-uridine into mRNA was low in both tissues. According to manufacturer's specifications, retention of [^{3}H]-uridine labeled non-mRNA containing a high concentration of adenosine nucleotides during purification chromatography probably contributes to 10% of [^{3}H]-uridine signal attributed to mRNA. This contribution to [^{3}H] – mRNA signal is small for CEN and large for torpor.

Analysis and presentation of data. Raw physiological data were analyzed by one way ANOVA with Tukey correction for multiple comparisons. In com-

Table 2. Incorporation of [^{3}H]-uridine and [^{14}C]-leucine into RNA and protein in organs during phases of the hibernation cycle.

		Heart	Liver	BAT	FeM	RhM	Kidney	Cereb-ellum*	Cortex*	Brain stem
RNA extract	µg/mg (*n*)	0.96± 0.03 (13)	3.55±0.11 (13)	1.3±0.06 (13)	0.56±0.03 (13)	0.66± 0.03 (13)	2.15± 0.06 (12)	1.03± 0.03 (13)	0.92 ± 0.03 (13)	0.75 ± 0.03 (13)
Protein extract	µg/mg (*n*)	28±2 (22)	52±2 (22)	44±4 (22)	14±1 (22)	17±1 (22)	33±2 (22)	24±1 (22)	17±1 (22)	22±2 (22)
CEN	**RNA ^{3}H cts/µg**	**19.8±3.7 (4)**	**2.6±0.6 (4)**	**33.8±7.7 (4)**	**10.8±3.4 (4)**	**11.1±3.5 (4)**	**21.1±4.7 (4)**	**4.0±1.2 (4)**	**2.4±0.7 (4)**	**2.2±0.6 (4)**
	% of CEN	**[100]**	**[100]**	**[100]**	**[100]**	**[100]**	**[100]**	**[100]**	**[100]**	**[100]**
	Protein ^{14}C cts/µg	0.39± 0.03 (4)	0.97±0.04 (4)	0.41± 0.01 (4)	0.11± 0.01 (4)	0.22± 0.02 (4)	1.16± 0.07 (4)	0.31 (2)	0.45 (2)	0.34± 0.01 (4)
	% of CEN	[100]	[100]	[100]	[100]	[100]	[100]	[100]	[100]	[100]
Middle arousal	**RNA (*n*)**	**27.2±18.9 (3)**	**0.2±0.1 (3)[a]**	**7.1±4.9 (3)**	**0.2±0 (3)[a]**	**8.1±5.8 (3)**	**2.5±1.9 (3)**	**1.3±0.9 (3)**	**1.1±0.8 (3)**	**1.2±0.7 (3)**
	% of CEN	**[138]**	**[7]**	**[21]**	**[1]**	**[72]**	**[12]**	**[31]**	**[45]**	**[53]**
	Protein (*n*)	0.22 ± 0.03 (6)[a]	0.32± 0.08 (6)[a]	0.19± 0.01 (6)[a]	0.00±0 (6) [a]	0.13± 0.02 (6)[a]	0.26± 0.02 (6)[a]	0.32± 0.03 (3)	0.38± 0.03 (3)	0.36± 0.02 (6)
	% of CEN	[55]	[33]	[47]	[0]	[59]	[22]	[105]	[83]	[106]
Early arousal	**RNA (*n*)**	**0.4±0 (4)[a]**	**0±0 (4)[a]**	**0.2±0 (4)[a]**	**0.1±0 (4)[a]**	**0±0 (4)[a]**	**0.1±0 (4)[a]**	**0.1±0 (4)[a]**	**0±0 (4)[a]**	**0.1±0 (4)[a]**
	% of CEN	**[2]**	**[2]**	**[1]**	**[1]**	**[0]**	**[0]**	**[2]**	**[0]**	**[2]**
	Protein (*n*)	0±0 (6)[a]	0±0 (6)[a]	0±0 (6)[a]	0±0 (6)[a]	0±0 (6)[a]	0±0 (6)[a]	0.01±0 (3)[a]	0.01±0 (3)[a]	0±0 (6)[a]
	% of CEN	[1]	[0]	[1]	[0]	[0]	[0]	[2]	[1]	[1]
Torpor	**RNA (*n*)**	**4.1±0.2 (3)**	**0.7±0.1 (3)**	**1.9±0.3 (3)**	**0.9±0.1 (3)**	**0.9±0.2 (3)**	**3.8±1.3 (3)**	**0.6±0.1 (3)**	**0.6±0.0 (3)**	**0.6±0.1 (3)**
	% of CEN	**[20]**	**[25]**	**[6]**	**[8]**	**[8]**	**[19]**	**[14]**	**[26]**	**[26]**
	Protein (*n*)	0.01±0 (6)[a]	0.04±0.0 (6)[a]	0.02±0 (6)[a]	0±0 (6)[a]	0.00±0 (6)[a]	0.03±0.01 (6)[a]	0.01±0 (3)[a]	0.02±0 (3)[a]	0.01 ±0 (6)[a]
	% of CEN	[3]	[4]	[5]	[0]	[1]	[3]	[2]	[4]	[2]

RNA in bold, expressed as [^{3}H] counts/µg RNA (number of determinations). Labeled RNA from each tissue significantly different between phases of hibernation – KW > 8.7; $P > 0.01$ for all. Within same tissue early arousal phase different from others $p > 0.05$, except FeM during middle arousal phase. Protein in plain font, expressed as [^{14}C] cts/µg protein (number of determinations). Labeled protein from each tissue significantly different between phases of hibernation – KW > 16.7; $P > 0.001$ for all. Within same tissue a – different from CEN $p > 0.05$.% of CEN – mean incorporation into tissue during phase of hibernation expressed as % of mean incorporation of same tissue during CEN. * tissue assayed for protein incorporation only once. Other tissues assayed twice from separate extractions.

parison of RNAS and PS in phases of hibernation, data were analyzed by either Mann Whitney test for 2 groups or Kruskal-Wallis nonparametric ANOVA with Dunn's correction for multiple comparisons. Means ± SEM. (*n*). $P < 0.05$ two tailed significance.

DISCUSSION

In CEN hamsters, the relative amount of PS and RNAS expectedly varied between organs while the low rate of RNAS synthesis in the liver is consistent with the dominance of de-novo synthesis in this organ [28]. In regard to *in vivo* PS and RNAS during torpor, the results of the present study are in good agreement with previous *in vivo* PS [8–10] and *in vivo* RNAS studies [6, 7]. The additional organs assayed in this study provide a more complete picture of PS and RNAS during torpor and the other phases of the hibernation cycle. Labeled leucine was transported from the blood and translation occurred at tissue specific, reduced

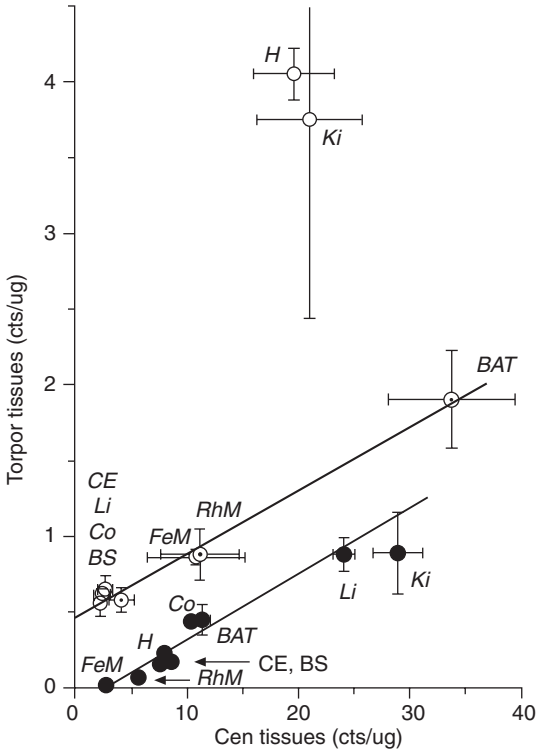

Fig. 2. The relationship between incorporation of [^{14}C]-leucine into protein ([^{14}C]-cts/μg) and [^{3}H]-uridine into total RNA([^{3}H]-cts/μg) in 9 tissues during CEN and torpor. Filled circles – protein, tissue in bold script. Open circles – Total RNA, tissue in italic script. In order to accommodate both data sets on the same graph the data for protein incorporation has been multiplied by 25. Mean ± SEM for each tissue (n = 4 for CEN; n = 3 for torpor). Linear regression for [^{14}C]-leucine incorporation into protein for 9 tissues $y = 0.036x - 0.064$. $R^2 = 0.936$. Linear regression for [^{3}H]-uridine incorporation into RNA for 7 tissues $y = 0.041x - 0.469$. $R^2 = 0.987$. (Heart and kidney excluded from RNA regression). H – heart, Li – liver, Ki – kidney, BAT – brown adipose tissue, FeM – femoris muscle from hindlimb, RhM – Rhomboid muscle from under interscapular BAT, Co – cortex, BS – brain stem/mid brain, Ce – cerebellum.

amounts relative to CEN in all organs assayed during torpor. In each organ assayed, the amount of PS during torpor is greatly reduced but directly related to the amount of PS during CEN. This implies a mechanism of inhibition common to all tissues carried over from CEN and is consistent with continued elongation of pre-initiated polypeptides after PS had been inhibited during torpor, as has been demonstrated *ex vivo* in liver polysomes [12]. In contrast to *in vitro* analysis of ground squirrel BAT [13], PS *in vivo* in hamster BAT was not increased relative to other tissues. In the hamsters, it would appear that none of the organs examined synthesized large quantities of protein at low body temperatures of torpor.

In general, the level of RNAS during torpor when body temperature is 5–6°C, is surprisingly high ranging from 5 to 25% of cenothermic levels depending upon organ (Table 2). It is not known if RNAS occurs at a constant rate for the duration of the torpor. However should this occur, then the amount of RNAS measured during the 33 h of torpor could be doubled to facilitate a comparison between synthesis during torpor and CEN based upon equal whole body metabolic expenditure. The amount of synthesis during torpor then becomes quite remarkable. Irrespective of a metabolically based adjustment of synthesis, the results clearly demonstrate that the processes involved in uridine incorporation into total RNA, such as transport by the energy dependent concentrative and equilibrative nucleoside transporters [35], enzymatic phosphorylation and ultimately incorporation into RNA [28] are only modestly inhibited by thermodynamic factors during torpor.

In each organ, with the exception of heart and kidney tissue, the amount of [^{3}H]-uridine incorporated into total RNA during torpor is proportional to the incorporation during CEN (Fig. 2). As with the inhibition of PS, this suggests the existence of regulatory mechanisms common to all tissues, except kidney and heart, the activity of which is carried over from CEN to torpor. Speculatively, this may involve reduction of the rates of initiation and/or elongation of total RNA, analogous to the mechanisms that depress protein synthesis [12] and reduce rates of initiation of mRNA transcription [36]. Relative to CEN, regional blood flow to the heart is proportionally greater during torpor [37], and thus the higher rates of [^{3}H]-uridine incorporation into heart, and possibly kidney, may be influenced by relative increase in substrate availability.

Although technical differences between *in vivo* studies make comparisons of the magnitude of incorporation of label into RNA difficult in these three families of hibernators, each study clearly demonstrates transcription of total RNA during torpor. Collectively these results imply active synthesis and degradation of total RNA during torpor in light of the finding that total RNA content of peripheral tissues is unchanged or slightly increased in brain tissue during torpor relative to CEN. In contrast, mRNA appears not to be synthesized or degraded during torpor.

Labeled RNA from heart and brain tissues, considered the most atypical and typical tissues respectively, were chosen for purification and analysis of incorporation of [^{3}H]-uridine into mRNA. The profile of radioactive incorporation into pre-mRNA and mature mRNA, as determined by oligo-poly dT chromatographic purification and scintillation counting, appears consistent for both heart and midbrain/brainstem across

Table 3. Incorporation of [^{3}H]-uridine in RNA and mRNA in heart and brain tissue during phases of the hibernation cycle.

		Heart	Brain stem/midbrain
	μg RNA/mg tissue (*n*)	0.94 ± 0.03 (10)	0.59 ± 0.06 (10)
	μg mRNA/μg RNA (*n*)	0.022 ± 0.001 (10)	0.048 ± 0.005 (10)
CEN	[^{3}H] cts/μg total RNA	11.8 ± 2.7	2.2 ± 0.6
(*n* = 4)	Percent CEN RNA	[100 ± 23%)]	[100 ± 26%]
	[^{3}H] cts/μg mRNA	**105.9 ± 16**	**12.1 ± 2.0**
	Percent CEN mRNA	**[100 ± 15%]**	**[100 ± 16%]**
Middle	[^{3}H] cts/μg total RNA	16.4 ± 11.8	1.3 ± 0.8
Arousal	Percent CEN RNA	[139 ± 100%]	[58 ± 35%]
Phase	**[^{3}H] cts/μg mRNA**	**132.9 ± 96.6**	**4.2 ± 3.6**
(*n* = 3)	**Percent CEN mRNA**	**[126 ± 91%]**	**[35 ± 29%]**
Torpor	[^{3}H] cts/μg total RNA	2.7 ± 0.2	0.9 ± 0.1
(*n* = 3)	Percent CEN RNA	[23 ± 2%]	[40 ± 5%]
	[^{3}H] cts/μg mRNA	**11.2 ± 3.8**	**1.4 ± 0.1**
	Percent CEN mRNA	**[11 ± 4%]**	**[11 ± 1%]**

Total RNA in plain font, expressed as [^{3}H] counts/μg RNA. mRNA in bold font, expressed as [^{3}H] counts/μg RNA. % of CEN RNA – mean incorporation into tissue during phase of hibernation expressed as [%] of mean incorporation of same tissue during CEN.

the phases of the hibernation cycle. [^{3}H]-uridine is preferentially incorporated into mRNA in CEN and more variably incorporated in the middle arousal phase. In contrast to the significant incorporation into total RNA during torpor, incorporation of labeled uridine into mRNA during torpor was low in both tissues, and close to the limit of purity that can be achieved by the chromatographic procedure. This lack of *in vivo* synthesis of mRNA, is consistent with the near cessation of mRNA synthesis predicted on the basis of reduced mRNA elongation rates from nuclear run on assays performed at the low temperatures approximating torpor in liver tissue [36]. Thus in the brain and heart (this experiment) and liver [36], mRNA appears not to be preferentially synthesized or degraded [24] during torpor.

The oxygen consumption during *in vivo* incubations of torpor and early arousal was estimated to be 50 and 40%, of the CEN incubation period, respectively. However, the characteristics of PS and RNAS in the early arousal phase were distinct from those during torpor. In the early arousal phase, both PS and RNAS were arrested, and below the detectable limit in the majority of organs, despite a substantial increase in MR and temperature via non-shivering thermogenesis. The inhibition of mRNA synthesis *in vivo* during torpor and early arousal is in contrast to a number of previous studies reporting increased levels of cDNA probed mRNA in tissues from animals sampled across the hibernation cycle. As stated previously the preservative character of hibernation determines that this technique is not temporally specific and suggests that some previous studies should be re-assessed with a more exacting emphasis on distinguishing between molecular events preparatory for the onset of torpor and the events occurring during torpor.

Plasma concentrations of labeled precursors indicated that transport of [^{14}C]-leucine, but not [^{3}H] uridine or labeled metabolites, from the circulation appears reduced during the early arousal phase. Intriguingly, leucine exchangers functioned earlier at colder body temperatures during torpor (from 20 h up until 50 h) despite very little PS and also later in the middle arousal phase when tissue temperature and MR further increased and PS was re-initiated. It is not known if the absence of PS during early arousal or some aspect of physiology specific to early arousal, such as an increase in whole body pH via respiratory alkalosis, a putative prerequisite for reversal of metabolic inhibition and initiation of arousal [1], alters the intracellular environment such that an appropriate ligand for the system L amino acid exchangers [38] becomes less available and transport of [^{14}C]-leucine is reduced.

It is clear that the middle arousal is the most energetically demanding phase of the hibernation cycle [3, 26]. Low plasma concentrations of labeled precursors and metabolites indicate a high rate of trans-membrane transport. PS, RNAS and mRNA synthesis during the middle arousal phase were significantly elevated, somewhat heterogeneous between animals in respect to RNA and mRNA synthesis, and strongly

influenced by tissue temperature. FeM tissue and RhB tissue are both skeletal muscles but the latter underlies BAT and is warmed much earlier than the former during arousal. PS and RNAS during middle arousal phase remained undetectable in the colder FeM while synthesis approached half CEN levels in the warmer RhM. These results complement previous polysomal run on analysis in liver [12] and provide *in vivo* evidence to support the finding that the mechanisms depressing both PS and RNAS, in all body tissues, requires a temperature threshold of about 18°C to be surpassed before synthesis can be re-established.

The magnitude of PS during the middle arousal was tissue specific. Relative to CEN levels, PS in brain tissue, particularly midbrain/brainstem and cerebellum during middle arousal, is greater than in other organs assayed despite modest increases in RNAS and lower tissue temperature than during CEN. The high level of PS in these brain structures may be indicative of a need for PS to replace the proteins utilized in the coordination of torpor and the early arousal phase. *In vivo* PS and RNAS and mRNA synthesis during the middle arousal phase is consistent with previous experiments where tissues from animals killed in the middle arousal to late arousal phase showed increased expression of brain tissue levels of transcription factors [16, 19], glucose regulating proteins and messenger proteins JNK, Akt and PKC [39] and UCP mRNA [20] relative to pre aroused animals.

In conclusion, the results of this study in hamsters show that significant gene expression occurs during the middle to late arousal phase of the hibernation cycle in organs that have surpassed a temperature threshold. In contrast, the initiation of arousal from torpor and the warming of anterior organs during early arousal by non-shivering thermogenesis occurred without measurable transcription or translation. During torpor, low levels of PS occurred in all organs. The brains of torpid animals have a slightly higher content of RNA (RNA/wet weight) than cold adapted non-hibernation hamsters. Significant amounts of total RNA are synthesized during torpor at tissue specific levels often related to the level of synthesis during CEN. The function of this RNA is unknown. If brain, heart and liver tissues are indicative of other organs, it is unlikely that functional levels of mRNA are synthesized during torpor. The low level of PS and mRNA synthesis during torpor and early arousal suggest that the majority of molecular processing required for the induction of hibernation and the arousal from hibernation up until the onset of shivering thermogenesis occurs during the cenothermic period before the hamster initiates the hibernation cycle.

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