

# Ascorbate dynamics and oxygen consumption during arousal from hibernation in Arctic ground squirrels

Ø. TØIEN,<sup>1</sup> K. L. DREW,<sup>1</sup> M. L. CHAO,<sup>2</sup> AND M. E. RICE<sup>2</sup>

<sup>1</sup>Institute of Arctic Biology, University of Alaska Fairbanks, Fairbanks, Alaska 99775; and <sup>2</sup>Departments of Physiology and Neuroscience and Neurosurgery, New York University School of Medicine, New York, New York 10016

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**Tøien, Ø., K. L. Drew, M. L. Chao, and M. E. Rice.**

Ascorbate dynamics and oxygen consumption during arousal from hibernation in Arctic ground squirrels. *Am J Physiol Regulatory Integrative Comp Physiol* 281: R572–R583, 2001.—During hibernation in Arctic ground squirrels (*Spermophilus parryii*), O<sub>2</sub> consumption and plasma leukocyte counts decrease by >90%, whereas plasma concentrations of the antioxidant ascorbate increase fourfold. During rewarming, O<sub>2</sub> consumption increases profoundly and plasma ascorbate and leukocyte counts return to normal. Here we investigated the dynamic interrelationships among these changes. Plasma ascorbate and uric acid (urate) concentrations were determined by HPLC from blood samples collected at ~15-min intervals via arterial catheter; leukocyte count and hematocrit were also determined. Body temperature, O<sub>2</sub> consumption, and electromyographic activity were recorded continuously. Ascorbate, urate, and glutathione contents in body and brain samples were determined during hibernation and after arousal. During rewarming, the maximum rate of plasma ascorbate decrease occurred at the time of peak O<sub>2</sub> consumption and peak plasma urate production. The ascorbate decrease did not correlate with mouth or abdominal temperature; uptake into leukocytes could account for only a small percentage. By contrast, liver and spleen ascorbate levels increased significantly after arousal, which could more than account for ascorbate clearance from plasma. Brain ascorbate levels remained constant. These data suggest that elevated concentrations of ascorbate {[Asc]} in plasma {[Asc]<sub>p</sub>} provide an antioxidant source that is redistributed to tissues during the metabolic stress that accompanies arousal.

vitamin C; antioxidants; reperfusion; torpor; metabolic rate

ARCTIC GROUND SQUIRRELS (AGS; *Spermophilus parryii*) are one of the most extreme species of hibernators, with changes in body temperature from –3 to +37°C (3) and correspondingly a large, dynamic range of metabolic rates (6) during the hibernation season. Indeed, O<sub>2</sub> consumption falls to 2% of basal levels during torpor, then surges to 300% of minimum hibernating levels during periodic arousals throughout the course of the hibernation season (5). Adaptations and protective mechanisms necessary for successful hibernation

and periodic arousal from torpor, therefore, are also very well developed in this species.

The ability of AGS to withstand profound decreases in heart rate, blood pressure, and blood flow associated with hibernation suggests that this species may represent a natural genetic model of tolerance to dramatic fluctuations in blood flow and O<sub>2</sub> consumption (12). This tolerance arises from a variety of physiological adaptations (5). One of these adaptations may involve antioxidant protection provided by the water-soluble antioxidant ascorbate (10). Ascorbate is an essential component of the antioxidant network and is synthesized in the liver in mammals and transported in plasma to all other tissues, where it is then concentrated by active uptake (24). Ascorbate is the most important antioxidant in plasma (11) and has been shown to minimize ischemic damage in models of cerebral ischemia/reperfusion, both in vivo (16, 23) and in vitro (22).

We have reported previously that [Asc]<sub>p</sub> increase three- to fourfold during torpor in two species of hibernators: AGS and 13-lined ground squirrels (10). Preliminary findings in AGS suggested that these elevated concentrations returned to euthermic levels within 12 h after arousal was initiated (10). One hypothesis resulting from these observations was that elevated plasma ascorbate levels during hibernation might provide an antioxidant source that could be taken up by tissues to prevent oxidative stress during the increase in O<sub>2</sub> consumption that accompanies arousal. This hypothesis predicted that plasma concentrations would fall in parallel with the increase in oxidative metabolism and consequent generation of reactive oxygen species (ROS). Mitochondria produce ROS at a rate that accounts for ~2% of the total uptake of O<sub>2</sub> (4).

In the present study, we examined the time course of ascorbate clearance from plasma in relation to O<sub>2</sub> consumption and other coordinated physiological events associated with arousal from hibernation. We continuously measured O<sub>2</sub> consumption, shivering, respiratory frequency, and body temperatures in proximal and distal parts of the body during arousal from hibernation.

Address for reprint requests and other correspondence: K. L. Drew, Institute of Arctic Biology, Box 757000, Univ. of Alaska Fairbanks, Fairbanks, AK 99775 (E-mail: fflkd@uaf.edu).

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tion. We also compared the time course of the changes in  $[\text{Asc}]_p$  to the reappearance of white blood cells (WBC), a possible sink for plasma ascorbate, and to plasma levels of uric acid (urate), an antioxidant and a marker of increased ROS production (13, 15). In addition, we determined the distribution of ascorbate as well as urate and the related antioxidant glutathione (GSH) in peripheral tissues before, during, and after hibernation. Ascorbate, GSH, and urate levels were also determined in three brain regions: frontal cortex, hippocampus, and cerebellum. We show that brain levels of ascorbate and GSH were relatively constant, indicating important homeostasis. On the other hand, increases in ascorbate content in peripheral tissues after rewarming suggest that redistribution of ascorbate during peak  $\text{O}_2$  consumption could account for the rapid clearance of ascorbate from plasma during arousal from hibernation.

## MATERIALS AND METHODS

*Animals and experimental design.* AGS were trapped during mid-July in the northern foothills of the Brooks Range, AK, ~20 miles south of the Toolik Field Station of the University of Alaska Fairbanks (68°38'N, 149°38'W; elevation 809 m) and transported to Fairbanks. Ground squirrels were housed individually at 21°C and fed rodent chow, sunflower seeds, and fresh carrots and apples ad libitum until mid-September when they were moved to a cold chamber set to an ambient temperature ( $T_a$ ) of 2°C and 2:18-h light-dark cycle. Actual  $T_a$  varied from 21 to 23°C and 2 to 3°C; for clarity in this report, these are designated as 21°C and 2°C to indicate the nominal temperatures of hibernation or rewarming. Experiments were conducted from January to March. Animals were identified as hibernating if wood shavings placed on their backs ~24 h earlier were not disturbed. In animals with implanted temperature transmitters for arousal experiments, an abdominal temperature close to  $T_a$  was taken as indication of hibernation. All procedures were approved by the University of Alaska Fairbanks' Institutional Animal Care and Use Committee.

Three groups of animals were used: a nonhibernating group, a hibernating group, and an arousal group. The nonhibernating group was cold-adapted (winter) euthermic AGS that had not hibernated since being placed in the cold chamber at the same time as hibernators in this study. The other two groups were animals that hibernated.

In the arousal group, indwelling femoral arterial catheters were implanted for blood sampling, and a temperature transmitter was implanted intraperitoneally. The arousal experiment started after the animal resumed hibernation, at least 2 days into the hibernation bout. After a control blood sample was taken without disturbing the animal, arousal was stimulated by implanting a differential electromyographic (EMG) electrode subcutaneously at the pectoral muscle and inserting thermocouples into the rectum and cheek pouch. In most arousal experiments, AGS were kept at  $T_a$  of 2°C throughout the arousal. In some, however, they were transferred to a  $T_a$  of 21°C to increase the rate of arousal. Blood was withdrawn from the indwelling femoral arterial catheter at ~15-min intervals depending on time required to fill the capillary tubes. Actual start and end times of sampling were recorded, and the average sampling time was corrected for dead volume of the catheter.  $\text{O}_2$  consumption was measured continuously by indirect calorimetry. Measurements and blood

sampling continued through the arousal episode until rectal or abdominal temperature reached 35°C.

Tissue samples were not taken from animals aroused at room temperature; these AGS were returned to the cold room to repeat the experiment at a  $T_a$  of 2°C. At the end of arousals at a  $T_a$  of 2°C, samples of cerebrospinal fluid (CSF) and peripheral and brain tissue were taken after anesthetizing the animal.

Similar tissue samples were taken from the hibernation group of animals 2–4 days into a torpor bout (without arousing the animals first) and from the nonhibernating group of AGS. Samples of CSF were obtained from the nonhibernating group only.

Whole organs from one additional euthermic AGS were removed and weighed. These weights were used to estimate the total amount of ascorbate that each organ could have taken up from plasma during arousal.

*Surgical procedures.* For the arousal group of animals, hibernating AGS were aroused and fitted with indwelling femoral arterial catheters according to the technique described by Frerichs et al. (12) with slight modifications. Anesthesia was induced with methoxyflurane (Metofane, Schering-Plough Animal Health, Union, NJ) and maintained with halothane (Halocarbon Laboratory, Riveredge, NJ) 1–3% mixed with 100% medical grade  $\text{O}_2$  at a flow rate of 1.5 l/min. During surgery, body temperature was maintained at 35–37°C with a servo-controlled fluid-filled heating pad (Omni medical equipment, Cincinnati, OH). Under strictly aseptic conditions, catheters were inserted into the femoral artery (Tygon tubing; 0.375-mm ID, 0.75-mm OD; Norton, Akron, OH), externalized in the neck area on the back, and sealed. Abdominal temperature transmitters (model WMFH LT 2-cm disc, Mini Mitter, Sunriver, OR) were implanted intraperitoneally via a midline incision through linea alba and sealed with three layers of sutures. Animals were administered 30 mg/kg im chloramphenicol (daily dose) and allowed 1 day postoperative recovery before being returned to the cold chamber to resume hibernation.

*CSF and tissue sampling.* Before initial sampling in euthermic and aroused AGS, animals were lightly anesthetized with methoxyflurane (Pitman-Moore, Mundelan, IL); hibernators were not initially anesthetized. Rectal temperatures were then measured with a thermocouple thermometer, and ketamine (2 mg/kg) and xylazine (2 mg/kg) were administered intracardially. For CSF sampling, the animals were positioned in a stereotaxic frame to stabilize the head and stretch the back of the neck. The atlantooccipital membrane was exposed, and two 10- $\mu\text{l}$  samples of CSF were withdrawn from AGS via cisternal puncture using a 0.75 in.-long, 30-gauge dental needle, a 100- $\mu\text{l}$  gas-tight Hamilton syringe, and Tygon tubing (0.375-mm ID) (34). Because the second sample was often contaminated with blood, only the first sample was used for ascorbate analysis. After decapitation, brains and peripheral tissue samples were immediately removed and dissected. Peripheral tissue samples were taken from spleen, liver, kidney, heart, skeletal muscle, brown fat, white fat, adrenal, and gut. Brain samples were taken from frontal cortex, hippocampus, and cerebellum because these regions were used in our previous study (10). In addition, there are technical advantages to examining these regions. First, they can be dissected rapidly, which is necessary for preserving antioxidant contents. Second, they have higher gray-to-white matter ratios compared with other brain regions, thus decreasing sampling variability [white matter has much lower ascorbate and GSH levels than gray (25)]. Care was also taken to remove white matter from the brain tissue samples. Tissue samples were frozen immediately on

dry ice and stored at  $-80^{\circ}\text{C}$ . Time from decapitation to freezing was  $<10$  min.

**Analyses.** Samples of arterial blood taken from the arterial catheter were collected in capillary tubes, spun in a microcentrifuge, and a 20- $\mu\text{l}$  sample of plasma supernatant was removed and frozen immediately on dry ice. These samples were stored at  $-80^{\circ}\text{C}$  until analysis for ascorbate and urate. On the day of analysis, plasma samples were diluted 1:4 in ice-cold, deoxygenated eluent, spun again at 14,000 rpm for 1 min, and injected directly onto the HPLC (10).

Sample preparation of brain and peripheral tissues for ascorbate, urate, and GSH contents was the same as described elsewhere (10, 25). The tissue samples (50–100 mg) were dissected immediately after decapitation, frozen on dry ice, and stored at  $-80^{\circ}\text{C}$ . On the day of analysis, these were cut into smaller pieces (typically 10 mg), weighed, then sonicated while still frozen in ice-cold deoxygenated eluent. The samples were then centrifuged, and the supernatant was injected directly onto the HPLC system. The HPLC analyses used were based on methods described by Rice et al. (25) using a gold amalgam electrode for ascorbate and GSH and a glassy carbon electrode for urate. Urate and ascorbate both oxidize on glassy carbon electrodes at an applied potential of  $+0.7$  V vs. Ag/AgCl reference; separation of these molecules is achieved using a slight modification of the eluent used for ascorbate and GSH: 16 mM monochloroacetic acid, with 150 mg/l EDTA and 300 mg/l myristyl dimethylbenzylammonium chloride (Myris-100; Jame Fine Chemicals, Bound Brook, NJ), pH 5, with 20% methanol. Retention time for urate is similar to that for GSH under these conditions; however, GSH is not detected on the carbon electrode and thus does not interfere with urate analysis. Standards were prepared in ice-cold deoxygenated eluent at regular intervals throughout the analysis. Tissue contents of ascorbate, urate, and GSH are expressed as micromoles per gram wet weight of tissue. Content per tissue wet weight is most appropriate for these data, because ascorbate, urate, and GSH are water soluble and therefore localized in the fluid compartments of the tissues.

Arterial samples for leukocytes (WBC) counts were collected in separate capillary tubes, 20  $\mu\text{l}$  were diluted 1:100 in 2.86% glacial acetic acid with a Unopette microdilution system and counted manually in a hemacytometer (Bright-line, AO Scientific Instruments, Buffalo, NY). For hematocrit determination, blood samples in capillary tubes were centrifuged for 5 min and then measured; hematocrit was determined in all but the first three experiments.

**$\text{O}_2$  consumption.**  $\text{O}_2$  consumption was recorded by indirect calorimetry in an open-flow system. Air was drawn through the vertical cylindrical-shaped Plexiglas metabolic chamber (diameter 20 cm, height 17.5 cm) at a flow rate of 3 l/min, passed through a drying canister with  $\text{CaCl}_2$ , through a membrane pump, and a mass flowmeter (model AFSC-10K, Teldyne Hastings-Raydist, Hampton, VA) to a distribution tube. A separate pump sampled air from the distribution tube.  $\text{O}_2$  extraction was measured with an  $\text{O}_2$  analyzer (Applied Electrochemistry/Ametek model S-3A, Thermo Instruments Division, Pittsburgh, PA). An infrared  $\text{CO}_2$  analyzer (Beckman model 864, Fullerton, CA) was connected in series with the  $\text{O}_2$  analyzer. The mass flowmeter was calibrated by recording mass loss of a cylinder of compressed air, which was emptied via a demand regulator through the flowmeter over a period of 60 min. All gas volumes are given at  $0^{\circ}\text{C}$  and 760 mmHg dry. The  $\text{O}_2$  analyzer was calibrated with air, and the span was checked by pressure calibration. The  $\text{CO}_2$  analyzer was calibrated with air and a calibration gas (1–2%  $\text{CO}_2$ ). The total system was checked by burning known

amounts of 100% ethanol with a clean burning lamp. The measurements were considered satisfactory if calculated and measured  $\text{O}_2$  consumption agreed within 3%. The ethanol burn also provided a check of the calibration gas for the  $\text{CO}_2$  analyzer because the respiratory quotient (RQ) of burning ethanol is 0.6667. Linearization curves for the  $\text{CO}_2$  analyzer were also determined by burning ethanol at a varying flow rate and calculating  $\text{CO}_2$  concentrations from measured  $\text{O}_2$  concentrations and the known RQ. Linearized  $\text{CO}_2$  values, baseline corrections for  $\text{O}_2$  and  $\text{CO}_2$ , and  $\text{CO}_2$  span corrections were calculated online during the experiments by the data-acquisition software (31).  $\text{O}_2$  consumption corrected for respiratory volume change was calculated according to the principles of equation 3b of Withers (35).

**EMG activity, temperature, and respiratory recording.** EMG was recorded with differential subcutaneous electrodes. The electrodes consisted of two insulated multicord stainless steel wires, each 0.1-mm diameter (0.2 mm including insulation, Cooner Wire, Charlesworth, CA) inserted into position in a Teflon tube. Two 1-mm holes, 4 mm apart, were cut in the Teflon tube and the inner insulation (31). The electrodes were inserted at the beginning of the experiment in hibernating animals with a special tool made from a 17-gauge needle with a machined slit in it. A ground electrode was either placed in a rectal probe or attached to the skin in the anterior back area. The electrodes were connected to EMG amplifiers, and a signal-processing system was connected to the data-acquisition system. The system allowed both recording of the direct electromyogram and the mean rectified value of the EMG signal (31).

Abdominal temperature ( $T_{\text{abd}}$ ) was recorded with implanted transmitters (see *Surgical procedures*) whose signal was received with RA1000 receivers and BCM100 units connected to a Dataquest III data-acquisition system (Mini Mitter, Sunriver, OR). Rectal ( $T_{\text{rec}}$ ) and mouth ( $T_{\text{mouth}}$ ) temperatures were recorded with copper-constantan thermocouples connected to thermocouple amplifiers (AD595, Analog Devices, Norwood, MA) that interfaced with the data-acquisition system.  $T_{\text{mouth}}$  was used as a measure of the temperature of the frontal part of the body, which is known to warm before the rear part in many hibernators. Respiratory frequency was recorded barometrically with one side of a differential pressure transducer connected to the metabolic chamber. The transducer was connected to a strain-gauge amplifier and triggering system interfaced to counters on the data-acquisition board.

**Statistics.** Data are expressed as means  $\pm$  SE. For arousal experiments,  $n$  is equal to the number of experiments. For tissue samples,  $n$  is equal to the number of animals sampled. To average time-series data from arousal experiments, data were synchronized around a single time point, which was chosen to be the time of peak  $\text{O}_2$  consumption. However, because plasma sampling time varied due to differences in time required to fill capillary tubes,  $[\text{Asc}]_p$  and plasma concentration of urate  $[\text{urate}]_p$  were calculated from curves linearly interpolated from  $\sim 15$ -min sampling intervals to 1-min intervals. Standard error was calculated at 1-min intervals but was only shown at 15-min intervals shifted relative to each other to prevent the error bars from overlapping.

Comparisons between two groups were made with a  $t$ -test. For peripheral tissues, comparisons of ascorbate, urate, or GSH content among states (hibernating, nonhibernating, and aroused) were made using a one-way ANOVA. For brain tissues, comparisons were made using two-way repeated-measures ANOVA, with brain region treated as the within-subjects variable. One-way ANOVAs and Tukey-Kramer post

hoc comparisons followed if significant interactions between tissue or brain region and state were found. Due to small sample size for brain urate data and lack of normality and equal variance, a Kruskal-Wallis one-way ANOVA on ranks was used to test for differences with respect to state followed by a nonparametric post hoc test (Dunn's method). The level of significance was set at  $P < 0.05$ .

## RESULTS

**Arousal at 2°C.**  $[\text{Asc}]_p$  from hibernating AGS before stimulation of arousal was  $183 \pm 20 \mu\text{M}$ . This fell by the end of the arousal experiment to  $40 \pm 6 \mu\text{M}$  (12 experiments in 8 animals,  $P < 0.001$ );  $[\text{Asc}]_p$  was similar whether arousal was at 2 or 21°C so that data from both conditions are included in these averages. Hematocrit decreased from  $51.8 \pm 1.1\%$  to  $45.3 \pm 1.0\%$  (9 experiments in 5 animals,  $P < 0.001$ ).

A representative example of data from one arousal experiment at  $T_a$  of 2°C is shown in Fig. 1. Because of the time to prepare an animal for recording,  $\text{O}_2$  consumption had already increased to levels above those expected in a hibernating AGS by the time the metabolic chamber was closed and the recording started. During the first hour of arousal, slight EMG activity was present in the pectoral muscle, the animal began to breathe more rapidly and deeply (judged visually) than before initiation of arousal, and  $\text{O}_2$  consumption began to increase steadily. In this example (Fig. 1), during the second hour of arousal, the high initial  $[\text{Asc}]_p$  started to decrease shortly before the time  $\text{O}_2$  consumption peaked. During this time,  $T_{\text{mouth}}$  began to increase rapidly, while  $T_{\text{rec}}$  and  $T_{\text{abd}}$  increased only slowly. In this experiment, WBC count began to increase only after 2 h, which was 45 min after  $[\text{Asc}]_p$  began to fall. Muscle shivering (EMG activity) declined before the decline in  $\text{O}_2$  consumption. Respiratory frequency paralleled both  $\text{O}_2$  consumption and shivering. Importantly, peak  $[\text{urate}]_p$  occurred at the same time as the peak in  $\text{O}_2$  consumption.

The mean of all individual responses during rewarming at  $T_a$  of 2°C, synchronized to the time of peak  $\text{O}_2$  consumption, is shown in Fig. 2. The averaged data are largely consistent with the representative sample shown in Fig. 1. Because of averaging and slight differences in timing between experiments, however, the mean responses appear to be somewhat slower and more blunted than the data from an individual experiment. For example, the peak in  $[\text{urate}]_p$  widened to form a plateau, which reflected a shift in maximal urate production toward the time of the maximal shivering in two of eight experiments, in which the shivering response was particularly strong. EMG activity was omitted from these average plots because of its high degree of variability. Despite variability in the timing of the increase in WBC in individual experiments, the time course of the average response did appear to have an inverse correlation with the fall in  $[\text{Asc}]_p$ .

**Arousal at 21°C.** To alter the time course of arousal and thus examine which parameters remained temporally linked, arousal was also initiated at  $T_a$  of ~21°C.

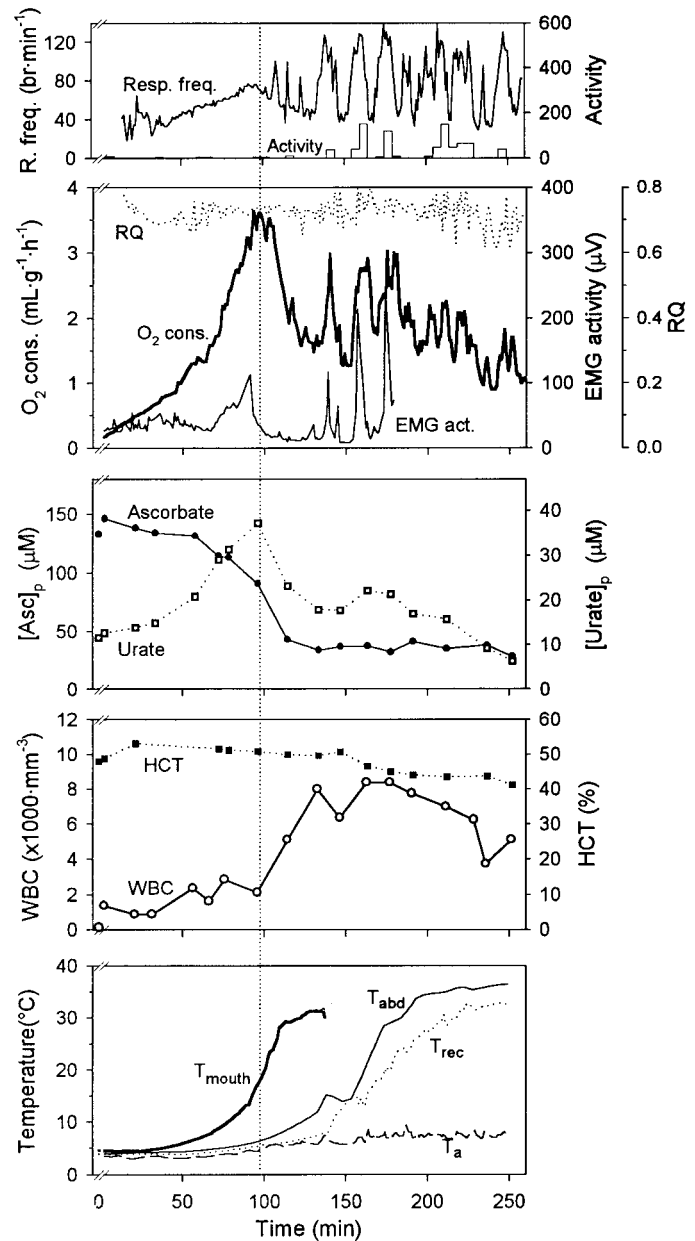


Fig. 1. Individual, stimulated arousal experiment in an Arctic ground squirrel at an ambient temperature ( $T_a$ ) of 2°C. Respiratory frequency (Resp. freq.) and activity,  $\text{O}_2$  consumption (thick continuous line), respiratory quotient (RQ; dotted line), electromyographic activity (EMG act.; thin continuous line), plasma ascorbate concentration ( $[\text{Asc}]_p$ ; circles with continuous line), plasma urate ( $[\text{urate}]_p$ ; square with dotted lines), white blood cell (WBC; open circles with continuous line) count, hematocrit (HCT; closed squares with dotted lines), temperature of cheek pouch ( $T_{\text{mouth}}$ ; thick continuous line), abdominal temperature ( $T_{\text{abd}}$ ; thin continuous line), rectal temperature ( $T_{\text{rec}}$ ; dotted line),  $T_a$  (stippled line). For reference, the thin vertical dotted line shows the time at which  $\text{O}_2$  consumption generally peaked. Data points before the axis break represent plasma samples before disturbance of the animal.

During arousal at this temperature, body temperatures tended to increase more rapidly, possibly due to passive warming (see representative example in Fig. 3). Importantly, the timing of the decrease in  $[\text{Asc}]_p$  level relative to the peak in  $\text{O}_2$  consumption remained

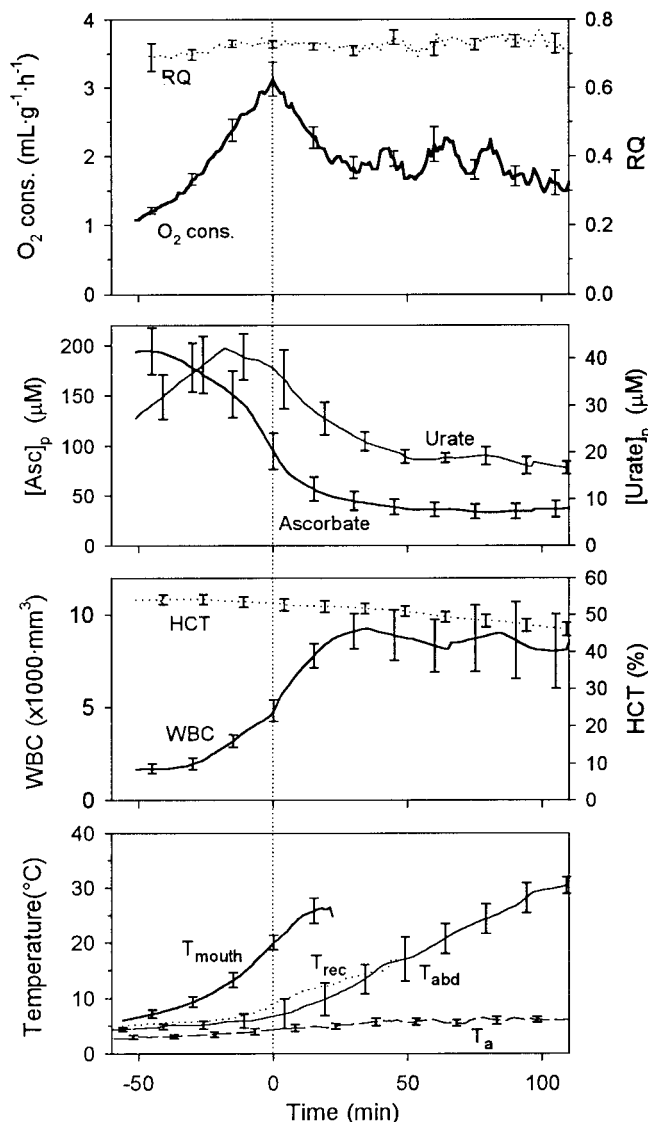


Fig. 2. Mean responses for stimulated arousal experiments in Arctic ground squirrels at a  $T_a$  of  $\sim -2^\circ\text{C}$  (8 experiments in 7 animals). Recordings were synchronized at the time of peak  $\text{O}_2$  consumption. Plasma values were calculated from curves interpolated at 1-min intervals. Standard errors were calculated at 1-min intervals but are only shown at 15-min intervals and shifted relative to each other for clarity. The times at which standard errors are shown are unrelated to actual blood sampling times.

unchanged, and  $[\text{urate}]_p$  again peaked at the same time as  $\text{O}_2$  consumption. In this experiment, WBC count increased gradually and not in concert with the rapid fall in  $[\text{Asc}]_p$ . In the four experiments conducted at  $21^\circ\text{C}$ , the WBC count response varied from a rapid increase at the time of the  $\text{O}_2$  consumption peak to a gradual increase lasting through a major part of the experiment. On average, however, the time course of the increase in WBC count appeared to be inversely related to the decrease in  $[\text{Asc}]_p$  (not illustrated).

**Relationships among  $[\text{Asc}]_p$ ,  $[\text{urate}]_p$ ,  $\text{O}_2$  consumption, and temperature.** The timing of the maximum decrease in  $[\text{Asc}]_p$  relative to the peak in  $\text{O}_2$  consumption can be better shown as the derivative with respect

to time ( $\Delta[\text{Asc}]_p/\Delta t$ ; Fig. 4). In the illustrated experiment at  $2^\circ\text{C}$ , the time at which  $\Delta[\text{Asc}]_p/\Delta t$  reached its most negative value coincided exactly in time with the peak in  $\text{O}_2$  consumption. When data from all arousal experiments at both  $T_a$  were pooled (Fig. 5A), a highly significant correlation was found between the time of maximum decrease in  $[\text{Asc}]_p$  (most negative  $\Delta[\text{Asc}]_p/\Delta t$ ) and the time of the peak in  $\text{O}_2$  consumption ( $y = -9.458 + 1.060x$ ,  $R^2 = 0.936$ ,  $P < 0.001$ ,  $n = 12$ ). A line with a slope of 1 and intercept of 0 falls within 95% confidence interval of the regression line. Importantly, whereas the timing of the decrease in

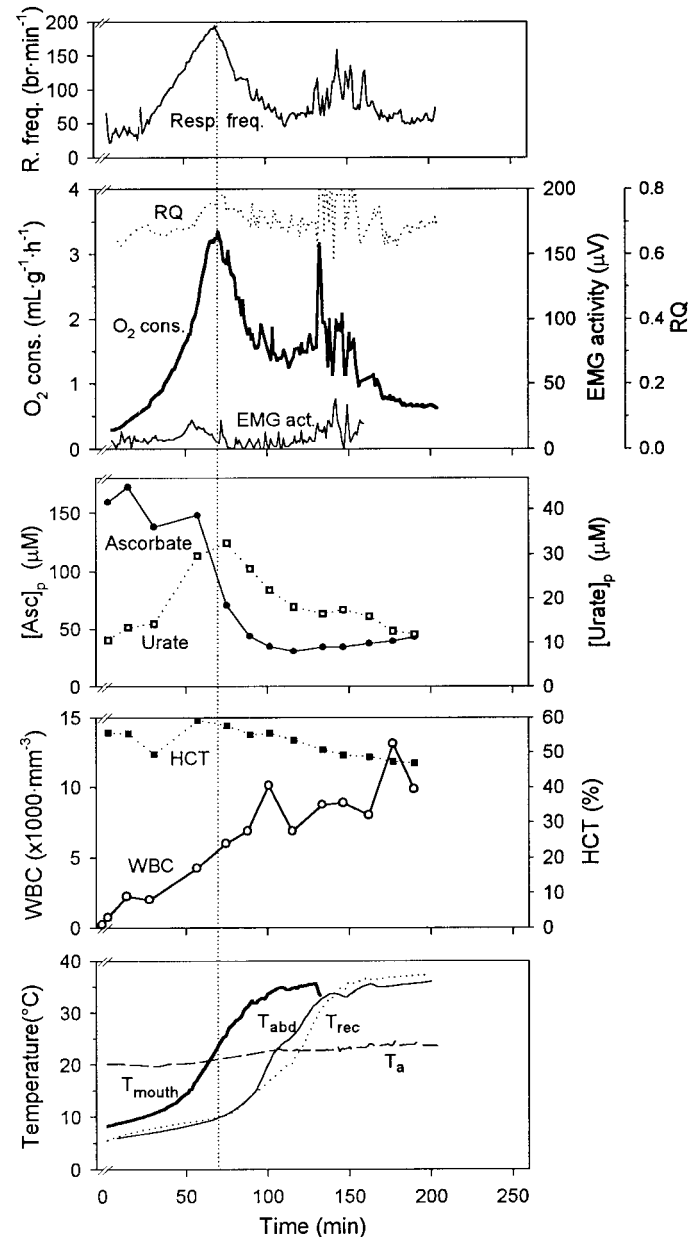


Fig. 3. Stimulated arousal experiment in an Arctic ground squirrel at room temperature. The animal, hibernating at  $\sim -2^\circ\text{C}$ , was moved to a warmer environment ( $\sim 21^\circ\text{C}$ ) during the arousal. Note the more rapid time courses of the physiological changes during arousal than those in Figs. 1 and 2. Abbreviations, symbols, and lines are as in Fig. 1.

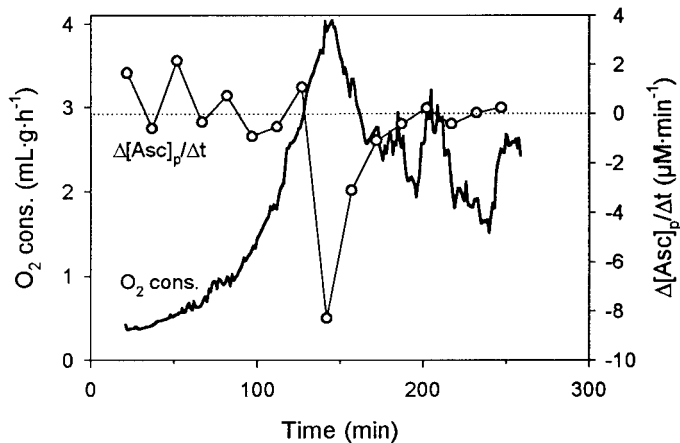


Fig. 4.  $O_2$  consumption ( $O_2$  cons.; thick line) and the rate of change in plasma ascorbate concentration ( $\Delta[Asc]_p/\Delta t$ ; open circles and thin line) vs. time during a stimulated arousal at a  $T_a$  of  $2^\circ\text{C}$ .

$[Asc]_p$  and the peak in  $O_2$  consumption were significantly correlated, the maximal rate of  $[Asc]_p$  decline did not correlate with the magnitude of the peak in  $O_2$  consumption ( $y = 0.220 - 1.765x$ ,  $R^2 = 0.146$ ,  $n = 12$ ,  $P > 0.2$ ).

A highly significant correlation was also found between the time of the peak in  $[urate]_p$  and the time of the peak in  $O_2$  consumption (Fig. 5B) ( $y = 4.118 + 0.858x$ ,  $R^2 = 0.823$ ,  $n = 12$ ,  $P < 0.001$ ). As with  $[Asc]_p$ , the magnitudes of the peak  $[urate]_p$  and peak  $O_2$  consumption were not significantly correlated ( $y = 3.505 + 14.538x$ ,  $n = 12$ ,  $R^2 = 0.203$ ,  $P > 0.1$ ).

$T_{\text{mouth}}$  was the only temperature measurement that increased substantially before the fall in  $[Asc]_p$ . Importantly, however, the time of maximal rate of fall in  $[Asc]_p$  occurred over a wide range of  $T_{\text{mouth}}$  (mean  $18.3^\circ\text{C}$ , range  $9.6$ – $24.7^\circ\text{C}$ ). This maximal rate of  $[Asc]_p$  decrease occurred at similarly wide ranges of  $T_{\text{abd}}$  (mean  $8.4^\circ\text{C}$ , range  $5.2$ – $11.8^\circ\text{C}$ ,  $n = 10$ ) and  $T_{\text{rec}}$  (mean  $8.8^\circ\text{C}$ , range  $4.3$ – $18.2^\circ\text{C}$ ,  $n = 12$ ). Indeed, the maximal rate of fall in  $[Asc]_p$  was not correlated to either  $T_{\text{mouth}}$  ( $R^2 = 0.098$ ,  $n = 12$ ),  $T_{\text{abd}}$  ( $R^2 = 0.023$ ,  $n = 10$ ), or  $T_{\text{rec}}$  ( $R^2 = 0.023$ ,  $n = 12$ ).

*Peripheral tissue contents of ascorbate, urate, and GSH.* Ascorbate content tended to be lowest in heart, skeletal muscle, brown fat, and white fat (Fig. 6). Individual tissues showed significant differences in ascorbate content among the three different states, although state-dependent differences were less than those observed in plasma. There was a significant effect of state of animal on ascorbate content in spleen ( $P < 0.01$ ), liver ( $P < 0.001$ ), and brown fat ( $P < 0.05$ ). Ascorbate content increased significantly in spleen and liver in aroused compared with hibernating AGS, with significantly higher levels in liver after arousal compared with nonhibernators. In brown adipose tissue, ascorbate content was significantly lower in hibernating animals compared with nonhibernating animals.

Interestingly, during hibernation, peripheral tissue levels of urate were significantly lower during hibernation than during euthermia or immediately after

arousal in all tissues except white fat (Fig. 7). GSH levels differed significantly with respect to state only in the spleen where it was decreased in hibernating animals compared with nonhibernating and aroused animals (Fig. 8;  $P < 0.001$ ).

*Antioxidant levels in CSF and brain tissue.* The concentration of ascorbate in CSF ( $[Asc]_{\text{CSF}}$ ) did not differ significantly between nonhibernating ( $170 \pm 45 \mu\text{M}$ ,  $n = 3$ ) and aroused animals ( $173 \pm 49 \mu\text{M}$ ,  $n = 3$ ). CSF was not obtained from hibernating animals in this study.

Tissue contents of ascorbate in frontal cortex, hippocampus, and cerebellum (Fig. 9A) were similar to the highest levels seen in peripheral tissues, exclud-

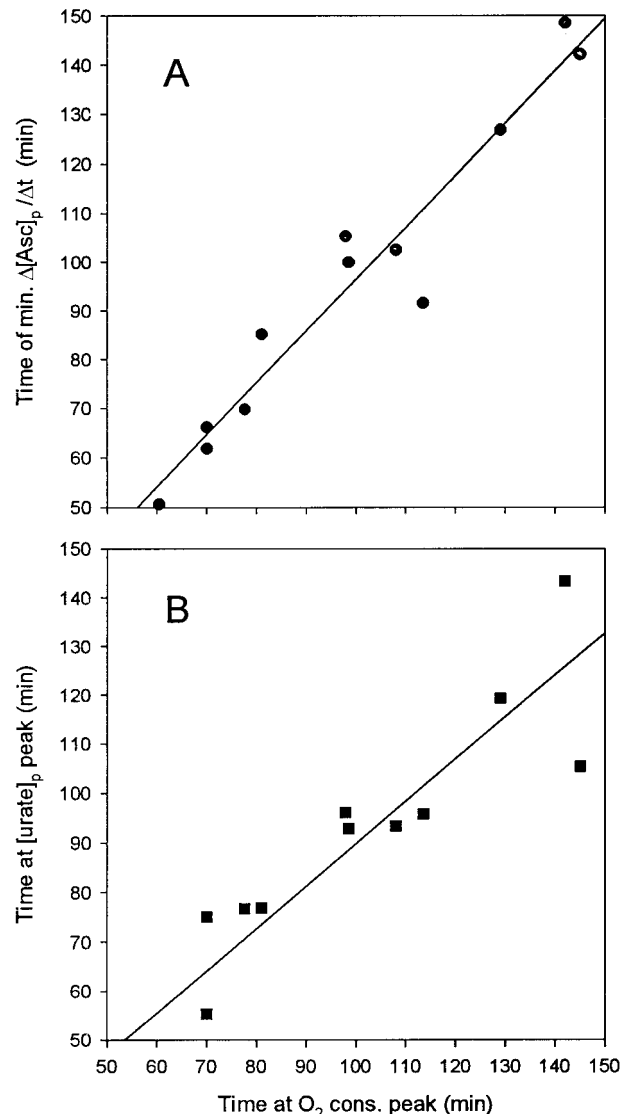
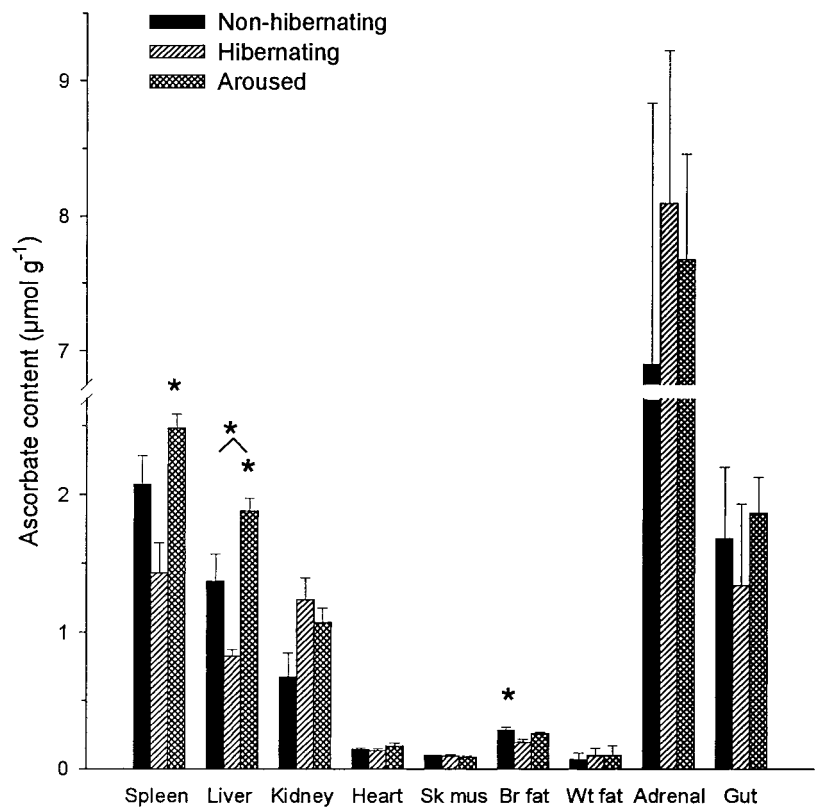


Fig. 5. A: relationship between time of the most rapid decrease in plasma ascorbate concentrations and the time at which  $O_2$  cons. peaked (regression line:  $y = -9.458 + 1.060x$ ,  $R^2 = 0.936$ ,  $P < 0.001$ ,  $n = 12$ ). Line falls within 95% confidence interval of  $y = x$ . B: time of peak in plasma urate concentration ( $[urate]_p$ ) vs. time of peak in  $O_2$  cons. during stimulated arousals in Arctic ground squirrels (regression line  $y = 4.118 + 0.858x$ ,  $R^2 = 0.823$ ,  $P < 0.001$ ,  $n = 12$ ). Data from arousal at both  $2$  and  $21^\circ\text{C}$  are pooled.

Fig. 6. Ascorbate content (means + SE) of peripheral tissues in nonhibernating, hibernating, and aroused Arctic ground squirrels. Sk Mus, skeletal muscle; Br fat, brown fat; Wt fat, white fat; \* $P < 0.05$  vs. hibernating state. \* and ^ indicate  $P < 0.05$  aroused vs. nonhibernating state ( $n = 3-6$ ).

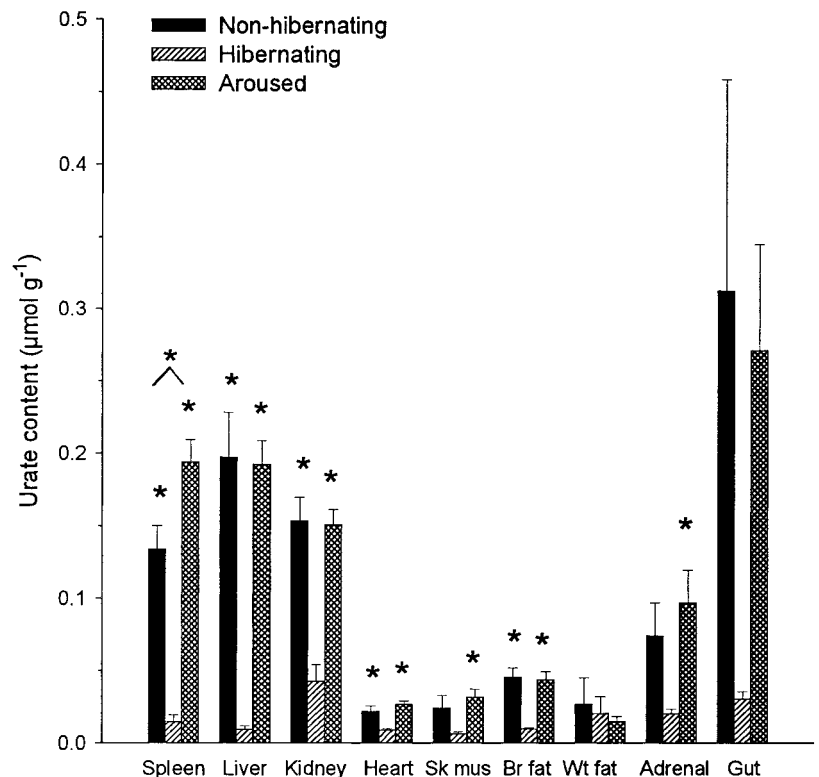


ing the adrenal gland (Fig. 8). Ascorbate content in these brain regions showed no significant differences with state of the animal ( $P > 0.5$ ). Although significant regional differences in content were apparent,

there was no interaction between hibernation state and region (region  $P < 0.01$ ; state  $\times$  region  $P > 0.05$ ).

The urate content in each brain region examined was very low compared with peripheral tissues, regardless

Fig. 7. Urate content (means + SE) of peripheral tissues in nonhibernating, hibernating, and aroused Arctic ground squirrels. Abbreviations are as in Fig. 6; \* $P < 0.05$  vs. hibernating state; \* and ^ indicate  $P < 0.05$  aroused vs. nonhibernating state ( $n = 3-6$ ).



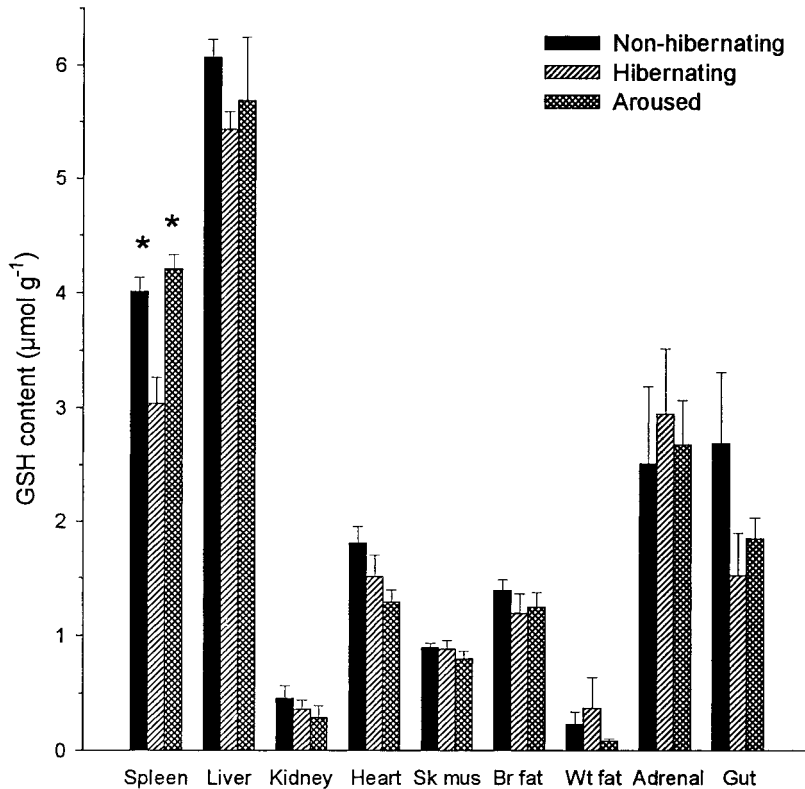


Fig. 8. Glutathione (GSH) content (means + SE) of peripheral tissues in nonhibernating, hibernating, and aroused Arctic ground squirrels. Abbreviations are as in Fig. 6; \* $P < 0.05$  vs. hibernating state ( $n = 3-6$ ).

of hibernation state; indeed, brain levels were similar to or lower than those in the periphery during hibernation. In further contrast to peripheral tissues, brain urate content decreased significantly in aroused compared with hibernating animals (Kruskal-Wallis 1-way ANOVA on ranks,  $P < 0.05$ ; Fig. 9B).

Brain GSH was not altered by hibernation or reawakening (effect of state  $P > 0.5$ ; Fig. 9C) and was generally similar to the GSH content of peripheral tissues ( $\sim 2 \mu\text{mol/g}$ ).

## DISCUSSION

The present results are the first to describe, with high time resolution, changes in  $[\text{Asc}]_p$ ,  $[\text{urate}]_p$ , and WBC parallel with continuous monitoring of  $\text{O}_2$  consumption and body temperature during arousal from hibernation. These results provide a foundation to understand the interrelationships and regulatory mechanisms governing ascorbate dynamics during arousal from hibernation. Importantly, these data indicated that the underlying signal for ascorbate clearance was not simply temperature but rather was associated with peak  $\text{O}_2$  consumption. In addition, two possible sinks for plasma ascorbate were identified, which were WBCs and peripheral organs. Lastly, the data indicate the overall stability of brain antioxidant regulation during the metabolic challenges that accompany hibernation and reawakening.

*Ascorbate clearance from plasma is associated with  $\text{O}_2$  metabolism.* The highly significant correspondence between the time of peak  $\text{O}_2$  consumption and the time of maximal rate of fall in  $[\text{Asc}]_p$  (Fig. 5A) is consistent

with the hypothesis that clearance of plasma ascorbate is associated with oxidative metabolism and that ascorbate may function as an antioxidant during arousal. The time of peak  $\text{O}_2$  consumption presumably coincides with reperfusion of central organs, including the central nervous system, heart, lungs, and liver that are located in relatively close proximity to brown adipose tissue. In many experiments, the drop in  $[\text{Asc}]_p$  started abruptly, near the peak in  $\text{O}_2$  consumption, and not parallel with the gradual increase in  $\text{O}_2$  consumption (Fig. 3). This suggests that the process of reperfusion and subsequent redistribution of ascorbate into tissues is initiated rapidly.

On average, the increase in  $T_{\text{mouth}}$ , which probably reflects temperature of anterior organs such as heart, brain, liver, and brown adipose tissue located under the arm pits, around the heart, and along the spinal cord, occurred parallel with the initial increase in  $\text{O}_2$  consumption (Figs. 1–3). By the time of peak  $\text{O}_2$  consumption,  $T_{\text{mouth}}$  increased from  $3^\circ\text{C}$  to  $\sim 18^\circ\text{C}$ , but  $T_{\text{rec}}$  and  $T_{\text{abd}}$  were still on average  $8-9^\circ\text{C}$ . This suggests that reperfusion of central organs plays a greater role in the peak in  $\text{O}_2$  consumption than reperfusion of distal tissues. Furthermore, the peak in  $\text{O}_2$  consumption was dissociated from the peak in EMG activity (31), indicating that a major part of  $\text{O}_2$  consumption was due to nonshivering heat production by brown adipose tissue. The observation that time of peak  $\text{O}_2$  consumption occurred after the peak in shivering suggests that metabolic demand of skeletal muscle contributed less to peak  $\text{O}_2$  consumption than other thermogenic tissues.

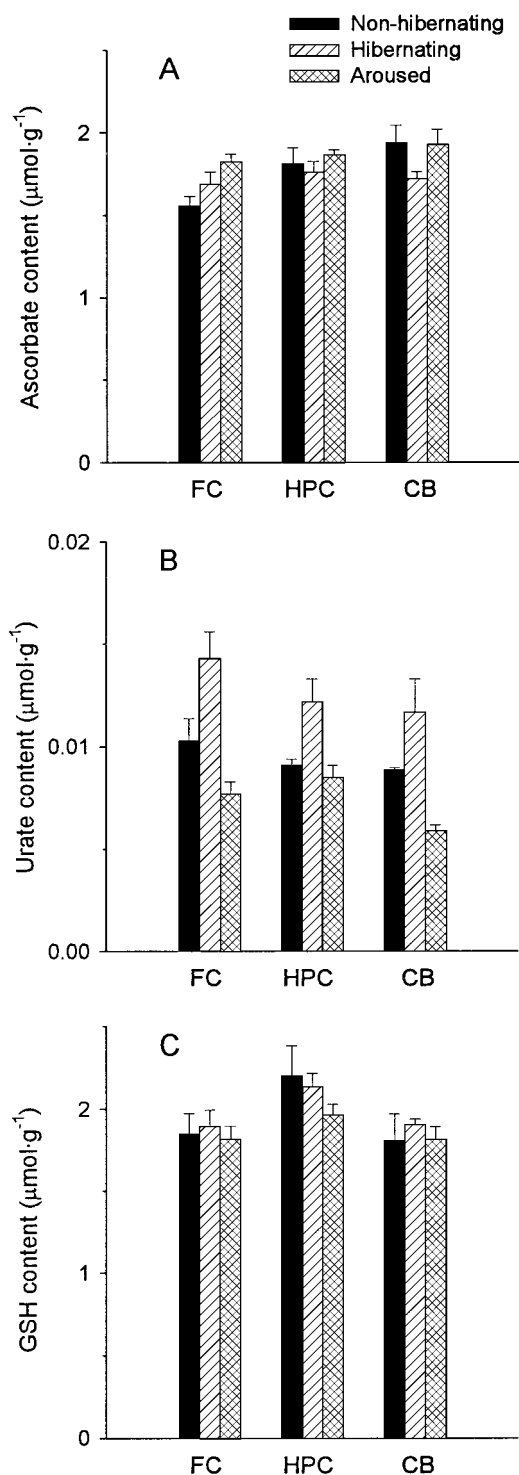


Fig. 9. Content of ascorbate (A), urate (B), and GSH (C) in different brain regions of nonhibernating, hibernating, and aroused Arctic ground squirrels. FC, frontal cortex; HPC, hippocampus; CB cerebellum; \* $P < 0.05$  vs. hibernating state. (ascorbate and GSH  $n = 4-6$ , urate, nonhibernating  $n = 2$ , hibernating and aroused  $n = 3$  or 4). Data are means  $\pm$  SE.

*Evidence for increased ROS production during peak  $O_2$  consumption.* Redistribution of ascorbate at the time of peak  $O_2$  consumption might place this water-soluble molecule in tissues with high thermoregulatory

heat production and  $O_2$  demand, where it could act as an antioxidant and free-radical scavenger. The fact that a peak in  $[urate]_p$  occurred at the same time as the peak in  $O_2$  consumption is consistent with increased ROS production during this phase of arousal and thus a need for increased antioxidant availability to maintain redox balance. Urate is a product of purine metabolism catalyzed by xanthine oxidase. Xanthine dehydrogenase (XDH) and xanthine oxidase (XO) are found in highest concentrations in hepatocytes and vascular endothelium and are released during hypoxia (21). In vivo, the enzyme exists primarily as XDH. However, conversion to XO occurs rapidly during hypoxia and oxidative stress. XO transfers electrons directly to  $O_2$ , producing superoxide anion,  $H_2O_2$ , and urate in the process (13, 15, 21). The XDH-to-XO conversion, via sulfhydryl oxidation, may be extremely fast, requiring less than 1 min (36). Increased  $[urate]_p$  is therefore consistent with increased XO activity and is indicative of ROS generation in vivo at the time of peak  $O_2$  consumption. The production of urate during conditions of oxidative stress has also been proposed to provide additional antioxidant protection (1).

*Lack of temperature dependence of  $[Asc]_p$  clearance during rewarming.* The observation that the maximal rate of  $[Asc]_p$  clearance occurred over a wide range of body temperatures argues against a limiting role for temperature-dependent ascorbate uptake in this process. Ascorbate is taken up into tissues via active,  $Na^+$ -dependent transporters (24, 32). If temperature alone regulated this process, the maximal rate of ascorbate clearance from plasma would be expected to occur within a narrow range of temperature, corresponding to the optimal temperature for ascorbate uptake. However, the point at which greatest change in  $\Delta[Asc]_p/\Delta t$  occurred was not correlated with the  $T_{mouth}$ ,  $T_{abd}$ , or  $T_{rec}$  recorded at that time. This indicated that the disappearance of ascorbate from plasma was not predominantly governed by temperature-dependent transport.

*Uptake of ascorbate into WBC during recovery from leukocytopenia.* When experiments at  $T_a$  of 2°C were averaged (Fig. 2), the apparent time course of the increase in WBC count during rewarming coincided with the decrease in  $[Asc]_p$ , although the WBC increase lagged behind the drop in  $[Asc]_p$  in a few individual experiments (e.g., Fig. 1). This release of ascorbate-deficient leukocytes from marginated pools in AGS during arousal could therefore provide a sink for  $[Asc]_p$ . It has been shown previously that freshly isolated human neutrophils contain 1.0–1.4 mM ascorbate, which can increase to a maximum of 2.6 mM after addition of external ascorbate to the medium (33). Assuming a maximal increase in intracellular WBC ascorbate content from 0 to 2.5 mM, a fall in  $[Asc]_p$  of only 5.8  $\mu M$  would be predicted based on the further assumptions that the cell volume for neutrophils [ $0.31 \mu l/10^6$  cells (33)] applies to all WBC, that WBC count increases to 15,000/mm<sup>3</sup> (about the maximum observed in our experiments), and that the fall of 143  $\mu M$   $[Asc]_p$  occurs in a plasma volume of roughly 40 ml

(assuming 5% of the body wt is plasma in an 800-g animal). Maximal ascorbate uptake into WBC could thus account for only a minor part of the changes in plasma ascorbate that occur during arousal.

*Ascorbate accumulation by liver, spleen, and other tissues.* Similar calculations can be made to estimate contribution of uptake into other tissues during redistribution of plasma ascorbate. In general, the organ-specific levels of ascorbate found in peripheral tissues of AGS (Fig. 6) were similar to those previously reported for other mammals, including humans (17). The high content of ascorbate in the adrenal is consistent with its role as coenzyme for dopamine- $\beta$ -hydroxylase in catecholamine biosynthesis (9). Although there was a tendency for ascorbate content to fall during hibernation and increase again during arousal, spleen and liver were the only peripheral tissues that showed a significant increase in ascorbate from hibernating to aroused state. The increase in ascorbate levels in kidney during hibernation was consistent with the role of this organ in excretion of ascorbate.

Importantly, the increases in ascorbate content in liver and spleen could more than account for the loss from plasma. The increase in liver ascorbate content after arousal (Fig. 6) would be  $\sim 26 \mu\text{mol}$  (an increase of  $1.06 \mu\text{mol/g}$  tissue wet wt in a liver with a mass of 25 g), which would be over fourfold greater than the net loss of  $5.7 \mu\text{mol}$  of ascorbate from plasma (a decrease of  $183 \mu\text{M}$  in 40 ml). This excess ascorbate content may reflect, in part, increased ascorbate synthesis during arousal, because the liver is the site of ascorbate synthesis in squirrels (8). However, ascorbate is avidly taken up by the liver as well. Within an hour after peripheral injection of radiolabeled ascorbate in vivo, the accumulation of labeled ascorbate is greater in liver than in most other organs (17, 19), with uptake into both hepatocytes (14) and liver microsomes (2). Consistent with these data, the recently discovered ascorbate uptake transporter SVCT1 is strongly expressed in liver (32).

In contrast to ascorbate levels in liver, the increase in spleen ascorbate content after arousal could reflect only increased uptake from plasma (17, 19). With a spleen mass of  $\sim 1.1$  g, the observed increase in spleen ascorbate content (by  $1.06 \mu\text{mol/g}$ , similar to that in liver) during arousal would account for  $\sim 1.2 \mu\text{mol}$  or 20% of the average decrease in  $[\text{Asc}]_p$  during arousal. Interestingly, the low ascorbate content in the spleen of hibernating AGS argues against the hypothesis that the spleen could be a site for storage of WBC enriched with ascorbate, although ascorbate-deficient cells could be sequestered. Although hematocrit showed a slight decrease during the time course of the experiment, this was a gradual response unrelated to the abrupt change in ascorbate content of plasma. Thus enrichment of ascorbate in red blood cells that were possibly sequestered was unlikely to contribute significantly to the more abrupt decrease in plasma ascorbate. Similarly, the modest (8%) change in total plasma electrolyte concentrations reported by Drew et al. (10) could not explain the differences in  $[\text{Asc}]_p$ .

Other tissues in which ascorbate content tended to increase during rewarming, although not significantly, include the digestive system and brown fat. Because of the mass of the digestive system ( $\sim 17$  g), an increase of only  $0.3 \mu\text{mol/g}$  could account for 100% of the change in plasma. Brown fat, with its increase of  $0.06 \mu\text{mol/g}$  and an estimated mass of 10 g, could contribute  $\sim 12\%$  to observed changes in  $[\text{Asc}]_p$ . Thus redistribution of  $[\text{Asc}]_p$  to liver and spleen, as well as possibly to other organs, could readily explain the observed changes in plasma ascorbate.

*Changes in urate content in peripheral tissues in hibernation and arousal.* In addition to elucidating dynamic changes in  $[\text{urate}]_p$  during arousal from hibernation, the present study also showed for the first time that urate content of peripheral tissues is markedly decreased in hibernating animals compared with nonhibernating and recently aroused animals. As discussed above, urate and its oxidation products are markers of oxidant generation in vivo (13, 15). Low tissue urate concentrations during hibernation are consistent with minimal oxidative metabolism during steady-state torpor and concomitant metabolic suppression.

*CSF and brain tissue ascorbate regulation.*  $[\text{Asc}]_{\text{CSF}}$  was the same in recently aroused animals and in animals that were not hibernating. Although CSF was not obtained from hibernating animals in the present study, we previously reported a doubling of the  $[\text{Asc}]_{\text{CSF}}$  during hibernation ( $[\text{Asc}]_{\text{CSF}}$  to  $\sim 350 \mu\text{M}$ ) (10). The present results therefore indicate that  $[\text{Asc}]_{\text{CSF}}$  returns to nonhibernating levels ( $\sim 170 \mu\text{M}$ ) during the process of arousal. Ascorbate is actively transported from plasma into CSF at the choroid plexus and blood-brain barrier (24). Drew et al. (10) suggested that this increase in  $[\text{Asc}]_{\text{CSF}}$  during hibernation might provide a pool of ascorbate that was readily available for uptake into cells, analogous to enhanced  $[\text{Asc}]_p$  for peripheral tissues. The CSF pool would then facilitate homeostatic maintenance of intracellular  $[\text{Asc}]$  during periods of potential increased ROS production during arousal. The decrease in  $[\text{Asc}]_{\text{CSF}}$  by the end of arousal would be consistent with uptake into brain cells. Presumably, this would be primarily into neurons, rather than glia, because neurons appear to express the SCVT2 isoform of the ascorbate transporter, but glia do not (32).

Although the increases in ascorbate content between hibernation and arousal in frontal cortex, hippocampus, and cerebellum did not reach significance, a trend toward an increase was clear. When raw data from the present study and those of Drew et al. (10) were combined, the effect of state on ascorbate content approached significance ( $P = 0.078$ ). Indeed, one can calculate that the absolute increase in tissue content would be roughly the same as that observed (Fig. 9A). If one assumes that uptake into brain cells is from brain extracellular fluid (ECF) (20), that  $[\text{Asc}]$  in ECF is the same as  $[\text{Asc}]_{\text{CSF}}$ , that the volume of the ECF compartment is 20% of total brain volume (20), and that the volume of the intracellular compartment is

60% of brain volume (26), a decrease of  $\sim 180 \mu\text{M}$  in ECF, therefore, would be redistributed to a threefold larger volume to give an increase in tissue content of only  $0.06 \mu\text{mol/g}$ . The actual increases seen were slightly higher ( $\sim 0.1 \mu\text{mol/g}$ ), which presumably also reflected uptake from the additional volume of CSF. Such small differences in tissue content ( $\sim 5\%$ ) would require a much larger sample size to confirm, given the usual animal-to-animal and analytic variability.

The overall stability of brain tissue ascorbate content during hibernation and arousal reflects the profound homeostasis of brain levels. It is unlikely that the decrease in  $[\text{Asc}]_{\text{CSF}}$  during arousal reflects reverse transport back into plasma, given the strong retention of brain ascorbate levels after uptake (17), as well as the tendency toward an increase in brain tissue content after arousal.

**Brain GSH and urate levels.** Brain tissue contents of GSH were relatively stable throughout hibernation. Results reported here confirmed previous findings in hibernating and euthermic animals (10) and extend these observations to include tissue levels after arousal from hibernation. Absolute levels of both ascorbate and GSH in AGS of the present study were similar to those in other small mammals, particularly those in guinea pigs (25, 26). The present results demonstrate that regulation of brain levels of GSH is stable, even during hibernation and arousal.

Brain levels of urate were extremely low compared with peripheral tissues, presumably reflecting low levels of XO in the brain (21). The significance of decreased brain urate content after arousal compared with hibernating levels is unclear, although it is tempting to suggest that this might reflect a consequence of uptake of CSF ascorbate, which might contribute to enhanced antioxidant protection.

In summary, ascorbate is an essential water-soluble antioxidant that has been shown to be an effective scavenger of ROS (24) and is particularly effective as a plasma antioxidant (11). The present study has shown a rapid decrease in plasma ascorbate during arousal in AGS that occurs exactly when  $\text{O}_2$  consumption peaks. Increased ascorbate levels in liver and spleen after arousal suggest that ascorbate is redistributed to metabolically active tissues and thus is poised to protect against oxidative stress at this vulnerable time of high metabolic demand. Further studies would be needed to quantify turnover vs. distribution in different tissues.

### Perspectives

Hibernating species appear to use a variety of adaptive processes that could act in concert to protect vital organs such as the heart and brain during the pronounced fluctuations in blood flow and oxidative metabolism characteristic of hibernation and periodic arousals. A number of parallels exists between hibernation physiology and manipulations found to be neuroprotective in experimental models of stroke and cardiac arrest, including 1) hypothermia (7), 2) suppressed immune function and decreased number of circulating

leukocytes (12, 18, 27, 28), and 3) increased blood clotting time (29, 30), all of which we have observed in AGS (K. L. Drew, unpublished data).

The present data are consistent with our hypothesis (10) that an increase in  $[\text{Asc}]_{\text{p}}$  is another physiologically significant adaptation. Because oxidative stress is understood as an imbalance between oxidant production and antioxidant protection, elevated  $[\text{Asc}]_{\text{p}}$  that is available for uptake into cells during arousal would help maintain redox balance and prevent oxidative damage. The increase in  $[\text{urate}]_{\text{p}}$  during peak  $\text{O}_2$  consumption indicates increased production of ROS during arousal, yet hibernating mammals clearly tolerate periodic rewarming, suggesting that redox balance is indeed maintained. Reestablishment or maintenance of ascorbate levels at nonhibernating levels by the end of arousal may have an important role in this process.

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