

Metabolic reorganization and signal transduction during estivation in the spadefoot toad

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Received: October 22 1999 / Accepted: December 28 1999 / Published: January 18 2000

Abstract. The maximal activities of 28 enzymes, representing multiple pathways of intermediary metabolism, were quantified in the brain, liver and skeletal muscle of spadefoot toads *Scaphiopus couchii*, comparing control toads with animals that had estivated for 2 months. Estivation-induced changes in brain enzyme activities were consistent with suppressed glycolysis and increased ketone body and amino acid catabolism. In liver, estivation resulted in reduced activities of eight enzymes representing carbohydrate, amino acid, ketone body and phosphagen metabolism, but the maximal activity of malic enzyme increased by 2.4-fold. Estivation led to a large-scale reorganization of skeletal muscle affecting most of the enzymes analyzed. Activities of enzymes of carbohydrate catabolism were generally elevated except for glycogen phosphorylase and hexokinase, whereas those of enzymes of fatty acid synthesis and ketone body metabolism were reduced. Increased glutamate dehydrogenase activities in both brain and muscle, as well as activities of other amino-acid-catabolizing enzymes in muscle, correlated with specific changes in the free amino acids pools in those tissues (reduced glutamine activity, increased glutamate, alanine and valine activities) that appear to be related to protein catabolism, for the purposes of elevating urea levels. The effects of estivation on signal transduction systems were also assessed. Total activities of protein kinases A and C (PKA and PKC) were largely unaltered in toad tissues during estivation (except for a 57% reduction in liver total PKC), but in seven organs there were strong reductions in the percentage of PKA present as the active catalytic subunit in estivating animals, and three contained a much lower percentage of membrane-bound active PKC during estivation. Activities of protein phosphatase types 1, 2A, 2B, and 2C were also frequently reduced during estivation. Overall, these results suggest that anuran estivation involves metabolic reorganization, in-

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cluding changing the maximal activities of key enzymes of intermediary metabolism as well as depressing the metabolic rate by suppressing signal transducing enzymes.

Key words. Anuran dormancy · Intermediary metabolism
Metabolic rate depression · Protein kinase · Protein phosphatase
Scaphiopus couchii

Introduction

Estivation is a dormancy induced as a response to dry environmental conditions. Seasonal estivation is common among animals that inhabit arid regions of the Earth and has been described for many anuran species (Pinder *et al.*, 1992; Abe, 1995). Frequently, estivating species are active for only 2–3 months during the rainy season when food and water are plentiful, and then they retreat to sheltered sites, often underground, for the remainder of the year. While estivating, the metabolic rate is suppressed so that internal body fuel reserves can be stretched to support survival for the maximum possible time (Hochachka and Guppy, 1987). Many species also express adaptations that defend the body from water loss during dormancy. The spadefoot toad, *Scaphiopus couchii* (Baird), of the American southwest is a well-known estivating species that typically spends about 9–10 months of the year buried under the desert soil (McClanahan, 1967; Jones, 1980). During this time its metabolic rate drops to 20–30% of the resting rate of awake toads (Seymour, 1973), and the animals rely primarily on the oxidization of stored lipid reserves for energy production. As the soil dries out over time, net water loss from the body begins to occur and up to 60% of total body water (47–50% of body mass) may be lost after several months (McClanahan, 1967). Plasma and tissue water is replenished for as long as possible from a large water reserve in the bladder, but as this runs low a metabolic approach is added to retard further water loss by colligative means. To do this toads increase the catabolism of protein reserves and accumulate the by-product, urea, in their plasma and urine, elevating body fluid osmolality by as much as 300 mM (Jones, 1980).

Metabolic rate depression is a critical component of estivation, as it is for animal survival of various other environmental stresses (e.g., low temperature, oxygen deprivation) and occurs widely as part of torpor, hibernation, diapause, anaerobiosis, anhydrobiosis (Hochachka and Guppy, 1987; Storey and Storey, 1990). Part of the reduction of metabolic rate during estivation can be traced to the cessation of digestion and skeletal muscle movements as well as to strong suppressions of heart and breathing rates. However, a coordinated suppression of the rates of all cellular processes, both energy-consuming and energy-producing, is also needed so that a stable long-term dormancy can be achieved. Thus, one of the components of estivation may be a rearrangement of the metabolic potential of cells and organs, altering the activities of enzymes so that those in necessary metabolic pathways are sustained or en-

hanced whereas those in pathways that are little used during dormancy are suppressed. Furthermore, the sensitivity of metabolism to extracellular stimuli might be altered during dormancy by reducing the potential for responses by signal transduction pathways through changes in their enzyme components.

In the present study we assessed the metabolic potential of toad organs during estivation by conducting a broad survey of the maximal activities of numerous enzymes of intermediary metabolism, comparing control (awake, nonestivating) toads with estivating individuals. We also assessed estivation-induced changes in components of signal transduction pathways, cAMP-dependent protein kinase (PKA), calcium- and phospholipid-dependent protein kinase C (PKC), and protein phosphatases, in order to determine whether the metabolic adjustments of the dormant state include alterations in the potential for reversible phosphorylation control over enzymes.

Materials and methods

Chemicals and animals

Biochemicals were purchased from Sigma (St. Louis, Mo., USA) or Boehringer-Mannheim (Montreal, Quebec) and [γ-^{32}P] ATP (111 TBq/mmol or 3000 Ci/mmol) was obtained from New England Nuclear (Montreal, PQ). Distilled, deionized water was used for the preparation of all solutions. Spadefoot toads (*S. couchii*) were obtained from the Tucson, Arizona area in July within $\cong$1 week after their emergence from estivation and were air-freighted to the Carleton laboratory. Upon arrival the animals were washed in a tetracycline bath and then placed in plastic boxes that were held at 22°C. Control animals were sampled 2 days later. Upon dissection of controls it was clear that all animals had eaten recently before capture and their bladders were well filled. Hence, animals to be used for estivation were not fed anything more before experimentation began. To induce estivation, remaining toads were placed in plastic tubs covered by a wire mesh that contained 15–20 cm of damp soil and then tubs were placed at 15±3°C in a Precision Low Temperature Incubator. Animals burrowed into the soil within the first 24 h and did not emerge again until gently dug out of the soil 2 months later. Each animal was carefully removed so as not to disturb other toads. Mean body masses were 27.42±0.72 g (n=40) for control and 19.37±0.97 g (n=35) for estivated toads. Toads were killed by pithing and tissue samples were quickly excised, frozen in liquid nitrogen and transferred to –70°C for storage.

Enzyme analysis

Samples of frozen tissues were quickly weighed and then homogenized 1:5 w/v in 20 mM imidazole, pH 7.0, 15 mM β-mercaptoethanol, 50 mM NaF, 2 mM EDTA, 2 mM EGTA and 20% v/v glycerol with 0.1 mM phenylmethylsulfonyl fluoride (PMSF) added immediately prior to homogenizing; β-mercaptoethanol was omitted from the homogenization buffer for citrate synthase (CS), carnitine palmitoyltransferase (CPT) and carnitine octanoyltransferase (COT) assays that use 5,5'dithiobis-2-nitrobenzoic acid (DTNB) to measure CoASH release. The homogenate was centrifuged at 14,000 *g* for 25 min at 4°C in a Baxter Canlab Biofuge 15 and the supernatant was removed and stored on ice until use.

Glycogen phosphorylase was measured as in Brooks and Storey (1989). Maximal activities of all other metabolic enzymes were assayed at 25°C in a final volume of 250 µl (plus enzyme extract) using a Dynatech MR5000 microplate reader at an absorbance of 340 nm (unless otherwise noted). Assay conditions for the microplate reader were similar to those described by Brooks and Lampi (1995, 1996) and were optimized for all enzymes using extracts of toad liver; blanks (minus the most specific substrate) were subtracted. One unit is defined as the amount of enzyme that uses 1 µmol of substrate per minute at 25°C. Optimal assay conditions were:

Hexokinase (HK; E.C. 2.7.1.1): 100 mM Tris buffer (pH 8.0), 1 mM EDTA, 2 mM $MgCl_2$, 20 mM glucose, 0.2 mM $NADP^+$, 1 mM ATP and 1 unit (U) glucose-6-phosphate dehydrogenase (G6PDH).

Phosphofructokinase (PFK; E.C. 2.7.1.11): 20 mM imidazole (pH 7.1), 10 mM $MgCl_2$, 50 mM KCl, 2.5 mM ATP, 0.15 mM NADH, 12.5 mM fructose-6-phosphate (F6P), and 1 U each of aldolase (ALD), triosephosphate isomerase, and glycerol-3-phosphate dehydrogenase.

Aldolase (ALD; E.C. 4.1.2.13): 50 mM imidazole buffer (pH 7.0), 2 mM $MgCl_2$, 0.2 mM fructose-1,6-bisphosphate (FBP), 0.15 mM NADH, 1 U triosephosphate isomerase and 2 U glycerol-3-phosphate dehydrogenase.

Glyceraldehydephosphate dehydrogenase (GAPDH; E.C. 1.2.1.12): 50 mM imidazole (pH 7.2), 20 mM 3-phosphoglycerate, 5 mM $MgSO_4$, 1 mM ATP, 0.15 mM NADH, and 1 U phosphoglycerate kinase.

Pyruvate kinase (PK; E.C. 2.7.1.40): 50 mM imidazole (pH 7.0), 50 mM KCl, 5 mM $MgCl_2$, 0.4 mM phospho*enol*pyruvate (PEP), 2 mM ADP, 0.15 mM NADH, 0.2% v/v rotenone saturated ethanol and 2 U lactate dehydrogenase.

Lactate dehydrogenase (LDH; E.C. 1.1.1.27): 50 mM imidazole buffer (pH 7.0), 2 mM pyruvate, 0.15 mM NADH.

Carnitine octanoyltransferase (COT; E.C. 2.3.1.21): 50 mM imidazole buffer (pH 8.1), 0.2% v/v Triton X-100, 5 mM L-carnitine, 50 µM octanoyl-CoA and 0.1 mM DTNB. Enzyme activity was monitored at 412 nm.

Carnitine palmitoyltransferase (CPT; E.C. 2.3.1.21): 50 mM imidazole buffer (pH 8.1), 0.2% v/v Triton X-100, 5 mM L-carnitine, 50 µM palmitoyl-CoA and 0.1 mM DTNB. Enzyme activity was monitored at 412 nm.

3-Hydroxyacyl-CoA dehydrogenase (HOAD; E.C. 1.1.1.35): 50 mM imidazole buffer (pH 8.0), 0.1 mM acetoacetyl-CoA and 0.15 mM NADH.

β-Hydroxybutyrate dehydrogenase (BDH; E.C. 1.1.1.30): 50 mM imidazole buffer (pH 8.1), 10 mM β-hydroxybutyrate and 2.0 mM NAD^+.

ATP-citrate lyase (CL; E.C. 4.1.3.8): 200 mM Tris-HCl buffer (pH 8.4), 10 mM β-mercaptoethanol, 0.2 mM mM coenzyme A (CoA), 0.15 mM NADH, 10 mM $MgCl_2$, 20 mM potassium citrate, 10 mM ATP, 10 mM $MgCl_2$, and 1 U malate dehydrogenase.

Fatty acid synthetase (FAS): 100 mM potassium phosphate buffer (pH 7.0), 3 mM EDTA, 5 mM dithiothreitol, 0.05 mM malonyl-CoA, 0.032 mM acetyl-CoA, 0.3 mM mM NADPH and 0.2% v/v rotenone saturated ethanol.

Isocitrate dehydrogenase, $NADP^+$-dependent (IDH; E.C. 1.1.1.42): 50 mM imidazole buffer (pH 7.5), 2 mM $MgCl_2$, 6 mM DL-isocitrate and 0.4 mM $NADP^+$.

Glucose-6-phosphate dehydrogenase (G6PDH; E.C. 1.1.1.49): 60 mM Tris-HCl buffer (pH 7.5), 3.3 mM glucose-6-phosphate, 0.4 mM $NADP^+$ and 6 mM $MgCl_2$.

6-Phosphogluconate dehydrogenase (6PGDH; E.C. 1.1.1.43): 50 mM Tris-HCl buffer (pH 7.0), 0.5 mM 6-phosphogluconate, 0.4 mM $NADP^+$.

Malic enzyme (ME; E.C. 1.1.1.40): 75 mM triethanolamine buffer (pH 7.4), 4 mM mM $MnCl_2$, 2.5 mM mM L-malate, 0.2 mM $NADP^+$.

Fructose-1,6-bisphosphatase (FBPase; E.C. 3.1.3.11): 25 mM imidazole buffer (pH 8.0), 5 mM $MgSO_4$, 0.2 mM $NADP^+$, 6 mM FBP and 1 U each of G6PDH and phosphoglucose isomerase.

Phospho*enol*pyruvate carboxykinase (PEPCK; E.C. 4.1.1.32): 100 mM imidazole buffer (pH 6.6), 6 mM β-mercaptoethanol, 50 mM sodium bicarbonate, 1.2 mM PEP, 1.25 mM inosine diphosphate, 1 mM $MnCl_2$, 0.15 mM NADH and 2.5 U malate dehydrogenase. All solutions were degassed prior to assay.

Glutamate dehydrogenase (GDH; E.C. 1.4.1.2): 100 mM triethanolamine buffer (pH 8.0), 5 mM EDTA, 200 mM ammonium carbonate, 7.5 mM 2-oxoglutarate, 0.15 mM NADH, and 1 mM ADP.

Serine dehydratase (SDH; E.C. 4.2.1.13): 200 mM potassium phosphate buffer (pH 8.0), 2 mM EDTA, 100 mM L-serine, 0.11 mM pyridoxal phosphate, 0.15 mM NADH, 0.2% v/v rotenone saturated ethanol, and 1 U lactate dehydrogenase.

Glutamate-oxaloacetate transaminase (GOT; E.C. 2.6.1.1): 500 mM imidazole buffer (pH 7.8), 30 mM β-mercaptoethanol, 15 mM 2-oxoglutarate, 250 mM L-aspartate, 0.11 mM pyridoxal phosphate, 0.15 mM NADH and 1 U malate dehydrogenase (MDH).

Glutamate-pyruvate transaminase (GPT; E.C. 2.6.1.2): 500 mM imidazole buffer (pH 7.3), 30 mM β-mercaptoethanol, 15 mM 2-oxoglutarate, 500 mM L-alanine, 0.11 mM pyridoxal phosphate, 0.15 mM NADH and 1 U lactate dehydrogenase.

5'-Nucleotidase (5NT; E.C. 3.1.3.5): 20 mM imidazole buffer (pH 7.0), 500 mM $MgSO_4$, 12 mM AMP, 0.15 mM NADH, 2.0 mM 2-oxoglutarate and 1 U each of glutamate dehydrogenase and adenosine deaminase.

Adenylate kinase (AK; E.C. 2.7.4.3): 50 mM potassium phosphate buffer (pH 7.5), 5 mM $MgSO_4$, 20 mM KCl, 2 mM AMP, 2 mM ATP, 2 mM PEP, 0.15 mM NADH and 1 U each of LDH and PK.

Creatine kinase (CK; E.C. 2.7.3.2): 50 mM imidazole (pH 7.2), 10 mM $MgCl_2$, 5 mM glucose, 2 mM NAD^+, 1 mM ADP, 100 mM creatine phosphate, 1 U each of G6PDH and hexokinase.

Malate dehydrogenase, NAD-dependent (MDH-1; E.C. 1.1.1.38) or NADP-dependent (MDH-2; E.C. 1.1.1.40): 50 mM imidazole buffer (pH 7.2), 10 mM $MgCl_2$, 10 mM oxaloacetate, and 0.15 mM NADH or 0.15 mM NADPH.

Citrate synthase (CS; E.C. 4.1.3.7): 50 mM imidazole buffer (pH 8.1), 0.2% v/v Triton X-100, 2 mM oxaloacetate, 0.1 mM acetyl-CoA and 0.1 mM DNTB. Enzyme activity was monitored at 412 nm.

Protein kinases and phosphatases

cAMP-dependent protein kinase (PKA)

The soluble activity of PKA was determined by measuring incorporation of radiolabeled [^{32}P]ATP onto the peptide substrate, Kemptide (Jiang and Corbin, 1991). Frozen tissue samples were homogenized 1:10 w/v in 10 mM potassium phosphate, pH 6.8, 15 mM 2-mercaptoethanol, 2 mM EDTA and 20% v/v glycerol with a few crystals of PMSF added immediately prior to disruption with a Polytron PT10 homogenizer. Homogenates were centrifuged for 5 min at 13,000 *g* in a Biofuge 15 microcentrifuge; supernatants were removed and used immediately. Reactions were conducted at 22°C in a 60 µl volume containing 20 mM potassium phosphate (pH 6.8), 75 µM kemptide, 1 mM EDTA, 10 mM magnesium acetate, 0.245 mM ATP, 370 kBq/ml (10 µCi/ml) [^{32}P]ATP (22.2 kBq or 0.6 µCi per assay) and 10 µl of enzyme extract in the absence or presence of 1 µM cAMP for assays of active or total protein kinase activity, respectively. Initial trials optimized time and enzyme amount. Reactions were started by adding [^{32}P]ATP and terminated after 10 min at 22°C by withdrawing a 40-µl aliquot and spotting this onto a 2×2 cm piece of Whatman P81 filter paper. Papers were washed with four changes (50 ml each) of 75 mM H_3PO_4 for 5 min, then rinsed with distilled water and placed in 1.5-ml microcentrifuge tubes held in 20-ml scintillation vials. Radioactivity was counted using Cerenkov methodology on a Packard Model 1900CA scintillation counter. One unit is the amount of enzyme that transfers one micromole of phosphate to Kemptide per minute at 22°C. The percentage of PKA present as the free catalytic subunit was calculated from the activities (mU/g wet weight) in the absence or presence of cAMP.

Protein kinase C

Enzyme activity was assayed essentially as in Mehrani and Storey (1996) with the crude enzyme partially purified by passage through Sephadex DE52; extracts from 0.2 g tissue were loaded onto 0.5-ml columns of DE52, washed with buffer containing 50 mM NaCl, and then activity was eluted with buffer containing 750 mM NaCl. PKC activity was measured with 75 µM FKKSFKL-NH_2 peptide substrate; this substrate represents the target sequence of PKC in MARCKS (myristoylated alanine-rich C kinase substrate) protein, a natural substrate of PKC *in vivo* (Chakravarthy *et al.*, 1991). Assays were incubated for 10 or 15 min at 22°C. Blanks omitted phosphatidylserine and diacylglycerol

and substituted 0.5 mM EGTA for Ca^{2+}. One unit is defined as the amount of enzyme that transfers one micromole of phosphate to peptide substrate per minute at 22°C.

Protein phosphatase type-1 (PP-1)

Activity was measured as the ability to dephosphorylate glycogen phosphorylase *a*. [^{32}P]Phosphorylase *a* was prepared essentially as described by Mehrani and Storey (1995) using a specific radioactivity of [γ-^{32}P]ATP of 1.85 MBq/μmol (50 μCi/μmol, >10^6 cpm/nmol). Aliquots of labeled enzyme were mixed with an equal volume of 100% glycerol and stored at –20°C until use. Samples of frozen tissues were homogenized 1:10 w/v in buffer A (20 mM Tris-HCl pH 7.4, 2 mM EGTA, 15 mM 2-mercaptoethanol) with freshly prepared protease inhibitors: 1 mM PMSF, 1 μg/ml aprotinin, 5 mM benzamidine and 0.1 mM tosylphenylalanylchloromethane. Homogenates were centrifuged at 4°C for 2 min at 13,000 *g*. Supernatants were removed and stored on ice. Dilution buffer (50 mM Tris-HCl pH 7.0, 0.1 mM EGTA, 15 mM β-mercaptoethanol, 2 mg/ml bovine serum albumin) was added to make 1:100 v/v dilutions of enzyme extracts; high dilutions minimize the effects of inhibitor-1 and inhibitor-2, which may be present in crude extracts. A 50-μl aliquot of enzyme extract was added to 2 μl of a 15 mg/ml solution of trypsin and incubated for 10 min at 22°C. Samples of original extract and diluted, trypsin-treated extract were then added to 20 mM Tris-HCl pH 7.0, 0.1 mM EGTA, 10 mM β-mercaptoethanol, 5 mM theophylline, and 2.5 nM okadaic acid in a total volume of 30 μl (2 μl untreated extract, 10 μl of diluted trypsin-treated extract). [^{32}P]Phosphorylase *a* was added (20 μl at 6×10^8 cpm/μmol or 0.3 nmol) and the reaction was incubated for 10 min at 22°C followed by termination with 100 μl of ice-cold 25% w/v trichloroacetic acid/10 mM phosphoric acid. After incubation on ice for 5 min, samples were centrifuged for 5 min at 13,000 *g* at 4°C, and 100 μl was transferred and mixed with 1 ml of dioxane-based scintillation fluid. The % active was determined by dividing the activity (U/mg) of the untreated extracts by that of the trypsin-treated extracts. This ratio reflects the ratio of *in vivo* PP-1 activity to total PP-1 activity, as trypsin has been shown to release the catalytic subunit from its association with proteins (or modulator subunits) that mask catalytic activity (Price *et al.*, 1986). Activity was corrected for time zero and minus enzyme blanks, and the release of ^{32}P was held at <30% of total radiolabeled substrate to ensure linear reaction rates. One unit of PP-1 activity is defined as the amount of enzyme that catalyzes the release of 1.0 μmol phosphate from [^{32}P]phosphorylase *a* per minute at 22°C.

Protein phosphatase types-2A, -2B, and -2C

Tissues were homogenized 1:10 w/v as described for PP-1 (except with 2 mM EDTA added to the buffer) and then centrifuged at 4°C for 15 min at 13,000 *g*. Supernatants were removed and layered onto 5-ml columns of Sephadex G-25 (Helmerhorst and Stokes, 1980) equilibrated in homogenizing buffer at room

temperature and centrifuged in an IEC benchtop centrifuge for 1 min at 2000 rpm; desalted enzyme was stored on ice. Activities were determined essentially as in Ekman and Jaeger (1993). A molybdate/malachite green dye solution was prepared by adding one volume of 10% (w/v) $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ in 4 M HCl to 3 vols of 0.2% (w/v) malachite green in 4 M HCl, followed by gravity filtration. This was then combined with deionized distilled water and a detergent mixture (5% v/v Tween-20, 5% v/v Triton X-100) in a ratio of 60:40:1 to make the final dye reagent. Assays were conducted in the presence of 50 mM imidazole-HCl, pH 7.2, 0.2 mM EGTA, and 15 mM β-mercaptoethanol, along with 150 μM substrate peptide and other additives as indicated. PP-2A activity was detected using the phosphothreonyl hexapeptide substrate, RRApTVA, in the absence or presence of the PP-2A inhibitor okadaic acid (2.5 nM). PP-2B activity was determined in the presence of 10 mM $MgCl_2$, 0.4 mM $CaCl_2$, 50 μg/ml calmodulin and the substrate peptide DLDVPIPGRFDRRVpSVAAE with 1 nM okadaic acid added to inhibit PP-2A. PP-2C activity is absolutely dependent on Mg^{2+} but is unaffected by okadaic acid. Activity was assayed using RRApTVA with 10 mM Mg^{2+} and 1 μM okadaic acid along with two blanks, one in the absence of Mg^{2+} with 1 μM okadaic acid to account for PP-2A activity toward the peptide, and the other in the absence of Mg^{2+} and substrate but with 1 μM okadaic acid to account for nonspecific phosphatase activity. Solutions were pipetted into a 96-well flat-bottom microplate, and reactions were started by adding 5 μl of enzyme extract to each well for a total assay volume of 50 μl. After a 15-min incubation at 22°C, reactions were terminated by adding 50 μl of dye reagent; color was developed for 45 min, followed and then read at 595 nm. Phosphate release was determined by comparison with a standard curve for varying amounts of phosphate. One unit of phosphatase activity is defined as the amount of enzyme that catalyzes the release of 1 μmol of phosphate per minute.

Soluble protein, amino acids and data analysis

Protein was determined by the Coomassie blue G-250 binding method (Bradford, 1976) using the Bio-Rad commercial kit and bovine serum albumin as the standard; a Dynatech MR-5000 microplate reader was used at 595 nm with a total well volume of 310 μl. Enzyme rate data obtained with the microplate reader were interpreted using the MPA program of Brooks (1994). Free amino acids were quantified in sulfosalicylic acid extracts of frozen tissues using a Waters HPLC after precolumn derivatization with *o*-phthalaldehyde (Churchill and Storey, 1992). Statistical analysis used the Student's *t*-test.

Results

The soluble protein content of brain, liver and leg skeletal muscle of spadefoot toads did not change during the 2 months of estivation. Mean values for control and estivated animals combined were 26.5±3.53, 126±16.9, 50.4±6.02 mg soluble protein/gram wet weight (mean ±SEM, n=8) for the three tissues, respectively. This suggests that, despite the loss of 35.7±8.6% of total body water over the 2 months (Grundy and Storey, 1998), the water content of individual organs was not perturbed.

Maximal activities of 28 metabolic enzymes were assessed in three tissues (brain, liver, skeletal muscle) of control and 2-months-estivated *S. couchii*. Activities are presented as milliunits per milligram soluble protein but can be converted to milliunits per gram wet weight using the above values for mg protein/gram wet weight. Table 1 shows enzyme activities in tissues from control animals or, if no effect of estivation occurred, shows combined values for tissues from control and estivated animals. In general, estivation had little effect on activities in the brain: 17 out of 23 enzymes measured were unchanged between control and estivated animals. In liver 16 out of 25 enzymes were unchanged, but activities of most enzymes changed during estivation in hindleg skeletal muscle (only 3 out of 27 were unaffected). Hence, estivation seems to cause specific and widespread reorganization of enzyme activities in skeletal muscle.

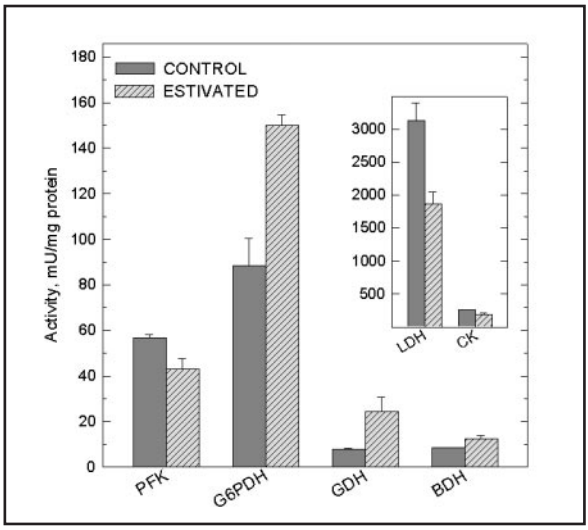


Fig. 1. Maximal activities of selected enzymes in the brains of control and 2-month-estivated spadefoot toads, *S. couchii*. Data are means ±SEM, n=3–4 animals. All values for estivated toads are significantly different from their corresponding control value, P<0.05

Table 1. Maximal activities of enzymes in three tissues of spadefoot toads. Values are mU/mg soluble protein, means ±SEM, with *n* in *parentheses* and represent data for control toads alone or where indicated by ([a]) data for control and estivated toads are combined when no significant difference was found between the two states. Muscle is hindleg skeletal muscle. (*ALD* aldolase, *AK* adenylate kinase, *BDH* β-hydroxybutyrate dehydrogenase, *CK* creatine kinase, *CL* ATP-citrate lyase, *COT* carnitine octanoyltransferase, *CPT* carnitine palmitoyltransferase, *CS* citrate synthase, *FAS* fatty acid synthetase, *FBPase* fructose-1,6-bisphosphatase, *G6PDH* glucose-6-phosphate dehydrogenase, *GAPDH* glyceraldehydephosphate dehydrogenase, *GDH* glutamate dehydrogenase, *GOT* glutamate-oxaloacetate transaminase, *GPT* glutamate-pyruvate transaminase, *HK* hexokinase, *HOAD* 3-hydroxyacyl-CoA dehydrogenase, *IDH* isocitrate dehydrogenase, $NADP^+$-dependent, *LDH* lactate dehydrogenase, *MDH-1* malate dehydrogenase NAD-dependent, *MDH-2* malate dehydrogenase NADP-dependent, *ME* malic enzyme, *n.d.* not determined, generally due to low amounts of tissue available, *5NT* 5'-nucleotidase, *PEPCK* phospho*enol*pyruvate carboxykinase, *PFK* phosphofructokinase, *6PGDH* 6-phosphogluconate dehydrogenase, *PK* pyruvate kinase, *SDH* serine dehydratase)

Enzyme	Brain	Liver U/ mg protein	Musclem
HK	39.8±2.02 (8)[a]	9.94±0.75 (8) [a]	58.3±1.33 (8)
PFK	56.7±1.41 (3)	5.71±0.63 (6) [a]	12.1±0.19 (8)
ALD	437±37.1 (8) [a]	74.6±4.17 (8) [a]	872±79.5 (8)
GAPDH	151±7.68 (6) [a]	123±5.54 (3)	38.7±4.23 (7)
PK	2849±201 (8) [a]	117±10.9 (4)	2374±83.7 (8)
LDH	3134±261 (3)	1943±310 (3)	8121±512 (15) [a]
CS	n.d.	50.0±6.97 (7) [a]	437±21.7 (3)
GDH	7.69±0.57 (4)	314±34.2 (7) [a]	16.7 ±0.59 (7)
GPT	193±10.0 (8) [a]	317±66.0 (3)	27.1 ±1.53 (8)
GOT	n.d.	n.d.	1982±143 (16) [a]
SDH	n.d.	n.d.	0.57 ±0.043 (8)
G6PDH	88.3±12.1 (4)	n.d.	1.05 ±0.13 (6)
6PGDH	37.5±2.62 (8) [a]	17.2±2.05 (8) [a]	11.1±0.86 (14) [a]
FBPASE	7.21±0.67 (8) [a]	13.0±0.46 (4)	4.85±0.40 (8)
PEPCK	19.8±1.58 (7) [a]	38.1±5.11 (7) [a]	n.d.
ME	29.9±0.93 (7) [a]	4.32±0.56 (3)	15.9 ±0.49 (8)
BDH	8.42±0.21 (3)	3.17±0.33 (4)	1.40 ±0.50 (6)
HOAD	45.7±6.74 (7) [a]	21.1±1.84 (7) [a]	74.8 ±8.33 (7)
COT	n.d.	7.11±0.76 (7) [a]	35.7±3.05 (3)
CPT	n.d.	10.5±1.17 (7) [a]	35.7±1.20 (3)
CL	6.47±0.76 (7) [a]	1.28±0.13 (7) [a]	47.79 ±15.87 (6)
FAS	1.51±0.15 (7) [a]	0.61±0.10 (7) [a]	1.22 ±0.12 (6)
MDH-1	349±20.8 (7) [a]	107±12.2 (7) [a]	1173±158 (7)
MDH-2	100±6.97 (7) [a]	19.4±2.49 (7) [a]	102±12.7 (7)
IDH	56.4±7.00 (8) [a]	103±7.39 (3)	106±7.25 (7)
AK	455±38.2 (7) [a]	34.7±2.98 (6) [a]	3438±425 (7)
CK	256±9.32 (4)	8.71±0.90 (3)	41205±3778 (7)
5NT	12.3±1.07 (7) [a]	3.54±0.91 (8) [a]	8.35±0.36 (6)

Brain

Figure 1 shows the effect of estivation on six enzymes in toad brain that were changed significantly during 2 months of dormancy. The activity of a key regulatory enzyme of glycolysis, PFK, decreased by 25% during estivation and that of the terminal enzyme of glycolysis, LDH, decreased by 40%. The maximal activity of CK also decreased by 25%. Activities of three other enzymes increased during estivation. GDH was most strongly affected, rising 3.2-fold whereas G6PDH activity rose by 70%, and BDH by 50%.

Liver

Estivation led to changes in the activities of nine enzymes in the liver (Fig. 2). Of these, only ME increased in activity during estivation, rising by 2.4-fold. Activities of three glycolytic enzymes, GAPDH, PK and LDH, decreased significantly to 75, 78 and 40% of their control values, respectively. Activity of the gluconeogenic enzyme FBPase decreased by 25%, and NADP-linked IDH activity decreased to 50%, GPT to 60%, and BDH to 37.5% of their corresponding controls. Activity of CK also decreased by 50%.

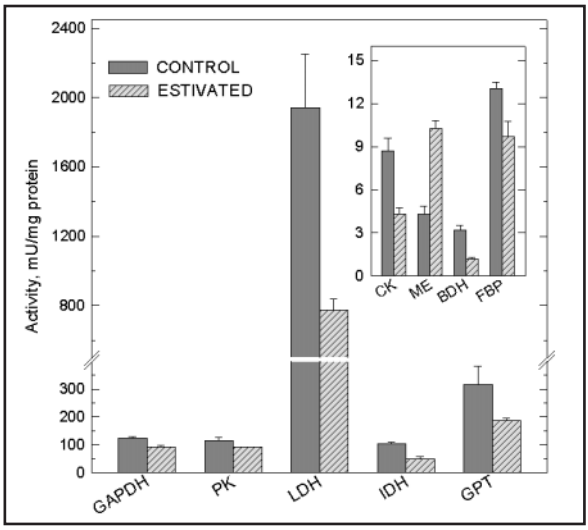
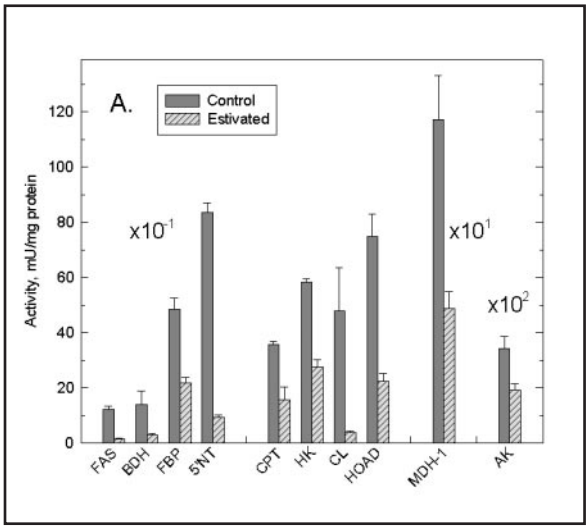


Fig. 2. Comparison of the maximal activities of selected enzymes in the livers of control and 2-month-estivated *S. couchii*. (*FBP* Fructose-1,6-bisphosphatase)



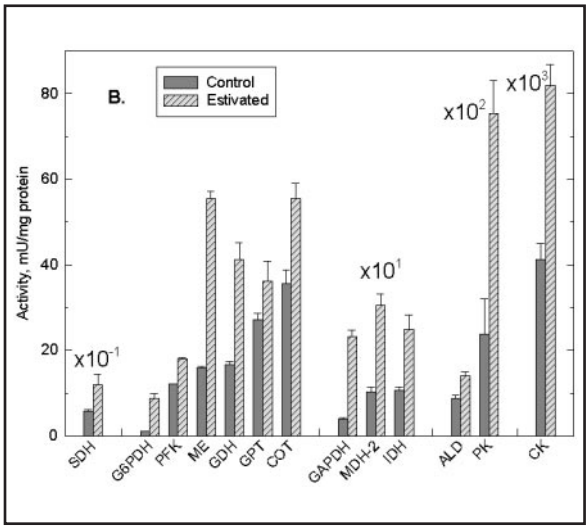


Fig. 3A,B. Comparison of the maximal activities of selected enzymes in skeletal muscles of control and 2-month-estivated *S. couchii*. **A** Enzymes whose activity decreased significantly during estivation. **B** Enzyme activities that increased significantly during estivation. Activities of different enzymes differed by over 1000-fold in some cases and are therefore grouped in horizontal blocks that must be multiplied by the associated power of magnitude to give the absolute activity *in vivo*. Other information as in Fig. 1

Skeletal muscle

Enzymes in skeletal muscle were broadly affected during estivation. Activities of 14 enzymes rose significantly whereas those of 10 others decreased (Fig. 3). Pronounced increases were seen for G6PDH (8.4-fold), GAPDH (6-fold), and CS (5.5-fold) with intermediate increases in PK (3.2-fold), MDH-2 (3-fold), IDH (2.4-fold), ME (3.5-fold), GDH (2.5-fold), SDH (2.1-fold) and CK (2-fold). Other enzymes rose by lesser amounts: GPT by 33%, PFK by 50%, COT by 56%, and ALD by 60%. Strong reductions in enzyme activities during estivation were seen for 5NT, CL, FAS, BDH, and HOAD which were 8, 11, 13, 21 and 30% of their corresponding controls, respectively. Five others fell by 40–60% (HK, FBPase, CPT, AK, MDH-1).

Glycogen phosphorylase

Maximal activities of glycogen phosphorylase and the percentage of enzyme in the active *a* form were measured in five toad organs. Total activities were unaffected by estivation and overall mean activities were 5.64±0.42, 4.24±0.41, 2.84±0.17, 0.90±0.14, 11.3±0.86 μmol glucose-1-P produced per minute per gram wet weight (n=24) in liver, heart, brain, kidney and skeletal muscle, respectively. The percentage of the active *a* form was also unchanged in four organs, being 6.95±1.17, 29.4±2.47, 21.0±2.30, 2.75±0.30% in heart, brain, kidney and muscle, respectively. However, the percentage of phosphorylase *a* decreased significantly in the liver of 2-month-estivated toads, from 27.6±3.33% in controls to 16.8±1.97% in estivated animals.

Protein kinases and phosphatases

Table 2 shows the effect of estivation on PKA activity in seven organs of *S. couchii*. Total PKA activity varied widely between tissues, being greatest in heart and brain (25–30 mU/gram wet weight), intermediate in skeletal muscle, liver and lung (6–10 mU/g wet weight) and lowest in gut and kidney (1.5–3.7 mU/g wet weight). Total PKA activity did not change during estivation in any tissue but the amount of enzyme present as the free catalytic subunit (PKAc) decreased significantly. The %PKAc dropped by one-third to one-half in all cases; for example, PKAc content in brain dropped from 57% in control to 25% in estivated toads whereas in liver PKAc fell from 97% to 62%.

Table 3 shows responses of PKC to estivation. Total PKC activity varied widely between organs, ranging in controls from 48.5 mU/g wet weight in liver to 0.31 mU/g wet weight in skeletal muscle. Total activity changed significantly during estivation in two organs, decreasing by 57% in liver but rising by

24% in the kidney. In cells, PKC is distributed between an inactive cytosolic pool and active enzyme bound to the plasma membrane. Estivation did not change PKC distribution in heart or skeletal muscle, but in three organs the percentage of membrane-bound enzyme was strongly reduced during estivation. Membrane-bound PKC in brain, liver and kidney was lowered from 46–71% bound in controls to 16–22% bound in estivated toads.

Table 4 shows the effect of estivation on PP-1 in toad organs. A key target of this phosphatase *in vivo* is glycogen phosphorylase *a*, and PP-1 activity is typically measured by its ability to dephosphorylate this enzyme. Assays were conducted in the presence of high okadaic acid (2.5 nM) concentrations, which inhibit PP-2A, the only other phosphatase that can dephosphorylate phosphorylase *a*. Mammalian PP-1 can be complexed with other proteins that mask its activity in crude extracts (Ballou and Fischer, 1986; Cohen, 1989), but this association is released by partial digestion with trypsin, transforming the catalytic subunit into a truncated form dependent on Mn^{2+} (Price *et al.*, 1986). As Table 4 shows, PP-1 activity increased following trypsin treatment in all toad tissues, which suggests that the toad enzyme follows the mammalian model of control. Total activity of PP-1 (in trypsin-treated tissue extracts) changed during estivation in four tissues. In liver and gut, total PP-1 decreased significantly; in particular, in the gut of estivating toads PP-1 was only one-fifth of the control value. By contrast, total PP-1 activity rose by 60% in the kidney and heart of estivating animals. Assays of extracts that were not trypsin-treated showed that the amount of free, active enzyme *in vivo* was much lower, with the % active ranging from a low value of 8% of the total in gut to 39% in the kidney of control toads. The % active was influenced by estivation in four tissues; in kidney and heart the % active decreased significantly during estivation whereas in liver and gut it increased.

The effects of estivation on the activities of PP-2A, PP-2B and PP-2C were also assessed. PP-2B could be detected only in the brain. Activity was 2.96±0.49 and 3.67±0.22 nmol phosphate released per minute per milligram protein (n=4) in the brain of control and estivated toads, respectively; the values were not significantly different. Activities of PP-2A and PP-2C are shown in Table 5. PP-2A activity decreased significantly in three tissues during estivation, falling to 71, 62, and 57% of the control values in liver, heart, and gut, respectively. PP-2C activity changed significantly during estivation in four tissues. Activity decreased by 50% in liver but rose by the same amount in skeletal muscle. Brain and gut showed greater increases during estivation, PP-2C activity rising by 2.25-fold in both cases.

Free amino acids

Free amino acid levels were quantified in brain, liver and skeletal muscle of control and 2-month-estivated toads (Table 6). The total free amino acid content of each tissue rose by 4.8, 2.7, and 3.7 μmol/g wet weight, respectively, but significant changes in amino acid levels during estivation were restricted to

Table 2. Effect of estivation on cAMP-dependent protein kinase A (*PKA*) in spadefoot toad tissues. Activities are mU (nmol phosphate transferred/min) per gram wet weight at 22°C, means ±SEM; *n*=8 different animals for total activity and *n*=4 for other values with assays on each extract done in quadruplicate. Muscle is hindleg skeletal muscle; gut combines stomach and intestine. (*PKAc* The free catalytic subunit of PKA)

Tissue	Total PKA activity (mU/g wetweight)	PKAc Activity (mU/g wet weight)		% PKAc	
		Control	Estivated	**Control**	**Estivated**
Brain	30.2±4.53	17.2±2.50	7.55±1.02[a]	57.0±8.0	25.0±3.0[a]
Liver	9.56 ±3.14	9.27±0.42	5.93±0.66[a]	97.0±5.0	62.0±8.0[a]
Muscle	6.27±0.38	5.02±0.35	2.99±0.12[b]	80.0±6.0	47.7±1.0[b]
Heart	25.2±3.02	9.58±0.96	5.04±0.93[a]	38.0±3.0	20.0±5.0[a]
Lung	10.3±1.95	9.99±0.15	6.49±0.01[b]	97.0±2.0	63.0±0.1[b]
Kidney	1.52±0.61	1.25±0.03	0.94±0.03[b]	82.0±2.0	62.0±4.0[b]
Gut	3.69±0.48	1.48±0.21	0.77±0.12[a]	40.0±6.0	21.0±4.0[a]

[a]*Significantly different from the corresponding control values, P<0.05;* [b]*P<0.005*

Table 3. Protein kinase C in tissues from control and estivating spadefoot toads. Activities are mU (nmol phosphate transferred/min) per gram wet weight at 22°C, means ±SEM, *n*=3–5

		Cytosolic (mU/g wet weight)	Membrane (mU/g wet weight)	Total activity (mU/g wet weight)	% Membrane bound
Brain	Control	21.7±0.57	18.4±1.16	40.0±1.37	45.8±1.6
	Estivated	23.4±2.87	8.84±1.38[a]	32.7±2.67	21.5±3.2[a]
Liver	Control	18.4±1.14	16.7±3.54	48.5±6.05	46.6±5.7
	Estivated	11.8±1.56[a]	2.23±0.15[a]	20.8±4.00[a]	16.4±2.4[a]
Muscle	Control	0.22±0.07	0.05±0.004	0.31±0.10	29.6±5.6
	Estivated	0.13±0.03	0.04±0.008	0.17±0.02	29.8±3.9
Heart	Control	0.72±0.04	1.03±0.19	1.89±0.21	62.2±1.4
	Estivated	1.18±0.18	1.31±0.27	2.45±0.63	65.6±1.1
Kidney	Control	7.56±0.81	18.3±2.28	20.7±1.81	70.8±12.0
	Estivated	21.1±3.28	4.58±0.74[a]	25.7±2.86[a]	18.7±4.7[a]

[a]*Significantly different from the corresponding control value, P<0.05*

Table 4. Protein phosphatase type-1 (*PP-1*) activities in tissues from control and estivating spadefoot toads. Activities are mU (nmol phosphate released per minute) per milligram soluble protein at 22°C, means ±SEM, *n*=4. The percentage of active PP-1 was determined from the ratio of activity (mU/mg) in untreated vs. trypsin-treated (total) extracts

Tissue	Control			Estivated		
	Total activity (mU/mg)	Active enzyme (mU/mg)	% Active	Total activity (mU/mg)	Active enzyme (mU/mg)	% Active
Brain	3.90±0.77	0.81±0.07	21±3	3.99±0.03	0.62±0.09	16±1
Liver	2.78±0.13	0.43±0.01	15±0	2.24±0.08 [a]	0.59±0.07	26±3 [a]
Muscle	2.33±0.26	0.46±0.10	20±3	2.80±0.77	0.36±0.05	13±3
Heart	2.57±0.52	0.97±0.09	29±3	4.10±0.26 [a]	0.66±0.09	16±2 [a]
Lung	3.28±0.22	0.60±0.07	18±2	3.06±0.67	0.49±0.11	16±4
Kidney	2.98±0.06	1.15±0.25	39±5	4.78±0.42 [a]	0.33±0.09	7±2 [a]
Gut	9.64±0.95	0.70±0.06	8±1	2.05±0.37 [a]	0.60±0.01	25±4 [a]

[a] *Significantly different from the corresponding control, P<0.05*

Table 5. Protein phosphatase 2A (*PP-2A*) and 2C (*PP-2C*) activities in control and estivated spadefoot toads. Data are in mU (nmol phosphate released per minute) per milligram soluble protein at 22°C, means ±SEM, *n*=4

	PP-2A		PP-2C	
	Control	Estivated	Control	Estivated
Brain	3.66±0.48	2.48±0.17	1.56±0.20	3.50±0.25 [a]
Liver	2.62±0.24	1.85±0.12 [a]	3.06±0.43	2.86±0.19
Muscle	4.08±0.90	3.08±0.33	2.75±0.19	4.23±0.45 [a]
Heart	3.23±0.17	2.00±0.09 [a]	3.11±0.17	1.61±0.07 [a]
Lung	2.68±0.13	2.05±0.33	1.17±0.06	1.71±0.27
Kidney	3.77±0.49	3.01±0.03	6.43±0.86	3.89±0.62
Gut	4.00±0.21	2.29±0.25 [a]	0.89±0.05	2.02±0.22 [a]

[a] *Significantly different from the corresponding control by the Student's t-test, P<0.05*

Table 6. Free amino acids in three organs of control versus 2-month-estivated spadefoot toads. Data are μmol/g wet weight, means ±SEM, *n*=3–4. Other amino acids did not change significantly during estivation. Mean levels of most other free amino acids were <0.5 μmol/g wet weight except for lysine (≅1 μmol/g wet weight in all tissues), glycine which averaged 1.5 mol/g wet weight in brain and 3.8 μmol/g wet weight in muscle, and arginine in muscle (≅1 μmo l/g wet weight)

Amino acid	Brain		Liver		Muscle	
	Control	Estivated	Control	Estivated	Control	Estivated
Aspartate	0.88±0.10	1.47±0.2[a]	0.93±0.15	0.42±0.09[a]	0.51±0.09	0.58±0.18
Glutamate	5.65±0.33	9.72±0.90[a]	2.58±0.36	3.76±0.39[a]	3.93±0.67	6.29±0.53[a]
Glutamine	7.93±0.49	4.95±0.51[a]	0.44±0.11	1.56±0.35[a]	4.46±0.44	2.79±0.40[a]
Alanine	0.98±0.15	1.73±0.22[a]	2.64±0.20	3.43±1.14[a]	4.64±0.34	7.04±0.55[a]
Valine	0.77±0.11	1.66±0.28[a]	1.12±0.06	1.72±0.33	3.83±0.28	6.28±0.35[a]
Total	22.5±0.77	27.0±0.86[a]	11.3±0.71	14.0±0.52[a]	26.7±0.83	30.4±0.89[a]

[a]*Significantly different from the corresponding control, P<0.05*

five amino acids. Glutamate and alanine levels rose in all three tissues whereas valine increased in the brain and muscle, and aspartate also increased in the brain. In contrast, glutamine content decreased by 4 μmol/g wet weight in brain and by 1.7 μmol/g wet weight in muscle but increased by 1.1 μmol/g wet weight in liver.

Discussion

The spadefoot toads used in the present study all survived 2 months of estivation. During that time body mass declined by 29.4% and, although an overestimate, if all of this weight loss was water, it would represent a loss of 35.7% of total body water (Grundy and Storey, 1998). Toads accumulated urea during estivation with mean blood levels rising from 14.5 μmol/ml in controls to 207 μmol/ml after 2 months (Grundy and Storey, 1998), values similar to previous reports for this species (Jones, 1980). Urea accumulation indicates that the toads were experiencing some water stress, but the maintenance of constant soluble protein levels in toad organs suggests that dehydration had not yet affected organ water content. This is not unexpected since toads typically enter estivation with a huge reserve of water stored in the bladder which is slowly used up to defend organ water contents against desiccation as the surrounding soil dries out (McClanahan, 1967).

After 2 months of estivation, the metabolic make up of spadefoot toad organs had changed considerably. Organ-specific patterns were apparent in the responses of both metabolic enzymes (Figs. 1, 2, 3) and signal transduction systems (Tables 2, 3, 4, 5). In brain, only 25% of the metabolic enzymes assayed

showed changes during estivation, suggesting that relatively little metabolic reorganization is needed in the brain during dormancy. Estivation affected more enzymes (38%) in the liver, whereas skeletal muscle appeared to undergo a profound metabolic reorganization that affected 86% of the metabolic enzymes assayed. Estivation is a complex phenomenon and there are several influences at work that differentially affect the metabolic make up of different organs. Starvation is one element that will change the pattern of use of stored fuels and the activities of the catabolic enzymes involved. In general, the first priority in short-term starvation is to maintain the glucose supply to those organs that are highly dependent on it (e.g., brain, red blood cells) and this is done by depleting glycogen reserves, switching most organs to a primary dependence on lipid oxidation, and increasing gluconeogenesis from glycerol or from amino acids supplied by the breakdown of muscle protein. When starvation is prolonged, however, protein catabolism could place too much of a drain on skeletal muscle mass and so the liver begins to synthesize and export ketone bodies (β-hydroxybutyrate, acetoacetate), which are products of lipid oxidation, as an alternative fuel to replace glucose. Thus, for estivating toads the general expectations associated with the starved state would be suppression of glycolysis and fatty acid synthesis and increased protein catabolism, gluconeogenesis, ketone body metabolism, and lipid oxidation. Another influence on metabolic reorganization during estivation is metabolic arrest. An overall suppression of metabolic rate by about 80% in estivating animals (Storey and Storey, 1990; Land and Bernier, 1995) may require changes in the activities of selected enzymes to suppress nonessential metabolic functions during long-term dormancy. Furthermore, defense against dehydration requires increased activities of urea cycle enzymes and enhanced protein catabolism to supply the necessary nitrogen (Balinsky, 1981). Reproductive tissues also undergo change (e.g., egg maturation) during estivation, so that animals are ready to breed immediately when aroused by summer rain (Seymour, 1973). All of these factors can impact on the metabolic make up of toad organs to alter enzymatic pathways to meet the challenges of long-term dormancy.

During estivation the activities of only six metabolic enzymes changed significantly in the brain. Activities of two enzymes of glycolysis, PFK and LDH, were reduced; notably, PFK is an important regulatory enzyme of the pathway, suggesting that the glycolytic rate was suppressed. BDH activity rose, which suggests the possible enhanced use of ketone bodies as an aerobic fuel for the brain. It is also interesting to note that the toad brain contained substantial activities of FBPase, PEPCK and ME, enzymes that all have roles to play in gluconeogenesis. Mammalian brain has only a low gluconeogenic capacity, primarily associated with astrocytes (Schmoll *et al.*, 1995). Significant amounts of PEPCK activity are restricted to neonatal life (Cruz *et al.*, 1998) and PEPCK is very low or undetectable in the brain of adult mammals or birds (Wiese *et al.*, 1991). However, along with the current data for toad brain, we have also detected substantial activities of FBPase, PEPCK and ME in frog (*Rana sylvatica*) and turtle (*Trachemys scripta*) brain (K.J. Cowan and K.B. Storey, unpublished) so that gluconeogenesis may have a much larger role to play in the brain metabolism of ectothermic vertebrates than in mammals and birds.

GDH activity was also enhanced in the brain (by 3.2-fold). This enzyme is a key link between carbohydrate and amino acid metabolism, and previous studies of hypersalinity-induced urea biosynthesis by euryhaline anurans have correlated increased tissue GDH activities with the elevation of urea cycle enzymes. This suggests that GDH probably plays a role in supplying the NH_4^+ needed by the urea cycle (Lee *et al.*, 1982). The near quantitative correlation between the glutamine decrease and glutamate increase in toad brain (Table 6) also implies enhanced NH_4^+ production by the glutaminase reaction during estivation. Elevated aspartate and alanine pools also represent amino acids that can readily donate their amino groups to glutamate via transamination reactions. Thus, although no significant changes in the soluble protein levels in the brain or other organs were detected during estivation, the 200 mM rise in urea (Grundy and Storey, 1998) and the elevated levels of selected amino acids that are readily interconvertible with glutamate indicate that some protein catabolism must have taken place. Elevated levels of amino acids in the brain and other organs also provide a small increment to tissue osmolality themselves, with total free amino acids 3–5 mM higher in organs of estivated compared to control toads (Table 6).

In liver, changes in the activities of metabolic enzymes were consistent with changes in fuel use patterns and with a starved state while estivating. The strong trend among those enzymes that changed was for reduced activities (8 out of 9 cases) (Fig. 2 and glycogen phosphorylase). Glycogen phosphorylase and three glycolytic enzymes were reduced in the liver of estivating toads, which is consistent with carbohydrate sparing. PK activity is also typically reduced or inhibited when gluconeogenesis is promoted, although FBPase activity was also lowered somewhat during estivation. However, the relative balance between glycolytic and gluconeogenic activities in an organ is often reflected in the relative activities of PFK (5.7 mU/mg) and FBPase (13 mU/mg control, 10 mU/mg estivated), which gate irreversible steps in the two pathways, respectively. In toad liver, the relative activities clearly favor gluconeogenesis. The reduced percentage of glycogen phosphorylase present in the active a form (27.6% in controls versus 16.8% in estivation) is also consistent with the reduced amount of PKA active catalytic subunit (Table 2), PKA being the primary protein kinase responsible for phosphorylase activation (via stimulation of phosphorylase kinase). Malic enzyme has an anaplerotic role in converting providing four-carbon units (oxaloacetate, derived from malate) to the tricarboxylic acid cycle for condensation with acetyl-CoA. During lipid oxidation (providing two-carbon acetyl-CoA inputs only), the supply of oxaloacetate can be rate-limiting and malate synthesis from an accompanying low rate of carbohydrate catabolism (providing pyruvate) keeps the TCA cycle primed with sufficient four-carbon units. Hence, a 2.4-fold increase in ME activity during estivation would increase the potential for malate synthesis from pyruvate when the organ switches to fatty acids as a primary fuel during estivation. Reduced BDH activity in the liver during estivation seems surprising, since the liver is the primary source for ketone body synthesis in vertebrates. However, reduced activities of NADP-dependent IDH, which is typically

viewed as one of the sources of NADPH for lipid biosynthesis, may be linked with low rates of fatty acid synthesis in the estivating state.

The effect of estivation on skeletal muscle enzymes suggests large-scale changes in metabolic potential in different pathways of intermediary metabolism. Contrary to the results for the two other organs, activities of glycolytic enzymes (PFK, ALD, GAPDH, PK) increased during estivation, although reduced HK and no change in glycogen phosphorylase argue against increased carbohydrate utilization. The purpose of these changes is not clear. One possibility is that enhanced glycolytic capacity, coupled with elevated CK (producing quick ATP from creatine phosphate) and CS (increasing the capacity for carbohydrate entry into the Krebs cycle), could develop during estivation in anticipation of the high levels of muscular work required upon emergence from estivation to support the frenzied breeding and feeding that is characteristic of this species. Such a proposition, however, will require more detailed measurements of muscle enzymatic make up, both seasonally and over estivation/arousal cycles. The PFK:FBPase ratio also changed substantially during estivation from 2.5:1 in controls to 8.3:1 in estivating toads, again suggesting that estivator muscle has an increased glycolytic potential. Amino acid oxidation also appears to be facilitated in the muscle of estivating toads, with elevated levels of SDH, GPT, and GDH. Elevated GDH, as in the brain, also suggests increased capacity for NH_4^+ production in support of urea synthesis by the liver. GDH activities in toad liver did not change during estivation but, notably, they were very high to start with, 18-fold higher than in muscle. Again in muscle, a common suite of changes in free amino acid levels was seen: glutamate, alanine and valine were elevated and glutamine was reduced. Except for glutamine, the same pattern was also seen in the liver so it appears that these changes may be characteristic of some anuran tissues when poised for urea synthesis from protein catabolism. BDH activity was reduced in the muscle of estivating toads and this may suggest that ketones are preferentially oxidized by other tissues, such as the brain. However, reduced activity of HOAD, an enzyme of β-oxidation, seems contrary to expectations in a tissue that should be primarily oxidizing lipid during estivation, but increased ME could, as in the liver, improve the supply of four-carbon inputs to the TCA cycle to aid acetyl-CoA oxidation. Directly contrasting changes in COT and CPT may reflect the predominant type of fatty acid being released from lipid depots for oxidization during estivation. Suppressed activities of enzymes involved in fatty acid synthesis (FAS, CL) are consistent with the starved state during estivation.

Reversible protein phosphorylation is a widespread and highly effective mechanism for regulating changes in the activities and properties of enzymes and functional proteins. Through the actions of protein kinases or protein phosphatases, enzymes and proteins may be rapidly interconverted between high and low activity states, allowing quick changes in pathway flux without a need to alter the total amount of the protein present. Studies of a variety of animal systems have shown that changes in the phosphorylation state of enzymes and other proteins commonly occur in dormant or arrested states (Storey and Storey, 1990). For example, studies with land snails have documented

estivation-induced covalent modification of enzymes of carbohydrate metabolism (glycogen phosphorylase, PFK, PK, pyruvate dehydrogenase) that result in less active enzyme forms during dormancy (Brooks and Storey, 1997). The present study shows that the amount of phosphorylated, active glycogen phosphorylase was reduced in the liver of estivating *S. couchii* and another study documented changes in the ratio of high to low phosphate forms of PFK and PK in skeletal muscle during estivation (Cowan and Storey, 1999).

Given this background, we predicted that the activities of cellular protein kinases and protein phosphatases in toad organs would change during estivation. Indeed, results for cAMP-dependent protein kinase were highly consistent in showing a strong reduction in the amount of PKA that was present as the active catalytic subunit (PKAc) in all seven tested organs of estivating toads (Table 2). A main role for PKA in many cases is the activation of fuel catabolism, frequently as a response to hormones; for example, both glycogen phosphorylase and hormone-sensitive lipase are activated via PKA-mediated phosphorylation to increase the breakdown of carbohydrate and lipid stores, respectively. A reduced content of PKAc in organs from estivating animals may be consistent, therefore, with lower rates of fuel catabolism in estivating toads. However, total PKA activity was unaltered during estivation and this maintains organ sensitivity to PKA-mediated signals so that fuel catabolism and metabolic rate can be rapidly re-elevated during the arousal process.

Ca^{2+}-activated, phospholipid-dependent PKC mediates the intracellular responses that underlie a variety of events including cell proliferation, differentiation, exocytosis, and neural activity (Kikkawa *et al.*, 1989). PKC is active in its membrane-bound form and, in response to extracellular stimuli that elevate phosphatidylserine and Ca^{2+}, the enzyme is translocated from inactive pools in the cytosol to the plasma membrane where it becomes active. Therefore, the key parameter in evaluating the state of PKC activity in tissues is determining the percentage of the enzyme that is membrane bound. As Table 3 shows, this percentage was strongly reduced during estivation in three toad organs (brain, liver, kidney). Furthermore, total PKC activity also dropped by half in the liver. Overall, then, the activity of membrane-bound PKC in estivating animals was only 50% of the control value in brain and kidney and just 13% of the control in liver. In estivating animals there was no overall change in the PKC distribution in muscle and heart, which have lower activities of PKC overall. As for PKA, an overall reduced content of active PKC in organs is consistent with a lower organ metabolic rate and also, undoubtedly, with specific suppression of selected metabolic processes that are PKC activated. These results for both protein kinases clearly suggest, therefore, that a key factor in inducing and maintaining a suppressed metabolic state during estivation is control over the protein-kinase-mediated signal transduction pathways in organs.

Protein-kinase-mediated phosphorylation of enzymes is reversed by the action of protein phosphatases and the two enzyme types are typically oppositely regulated. PP-1 is the best studied of the phosphatases involved in intracellular signal transduction. It cleaves covalently attached phosphate from a variety of regulatory enzymes (Hubbard and Cohen, 1989), including enzymes

of glycogen metabolism. PP-1 dephosphorylates and activates glycogen synthetase, and dephosphorylates and inactivates glycogen phosphorylase a; this latter capacity is generally used to assay its activity. Type-2 protein phosphatases also have roles in the regulation of intermediary metabolism (Cohen, 1989). PP-2A is related to PP-1 in terms of its structure and inhibition caused by okadaic acid, but is found in much lower abundance. It is involved in glycogen metabolism and muscle contraction, in particular regulating the phosphorylation state of PP-1. PP-2B, also known as calcineurin, is structurally related to PP-2A and PP-1 but is largely restricted to nervous tissue. Indeed, of seven toad tissues tested, PP-2B was detected only in brain. PP-2C is involved in the cell's response to stress. In yeast its activation results in the downregulation of the PBS2/HOG1-MAP kinase pathway, and in mammalian cells it stops cholesterol and fatty acid biosynthesis through elevated AMP-ATP ratios. The present study demonstrates the presence of PP-1, PP-2A and PP-2C activities in all toad organs tested.

Studies with mammalian systems indicate that PP-1 exists in two formats *in vivo*; some portion of the enzyme is present as the free catalytic subunit whereas the remainder is found complexed with other proteins that mask its activity (Ballou and Fischer, 1986; Cohen, 1989). This association can be released by partial digestion with trypsin. The same system of PP-1 control appears to occur in toad tissues, since incubation of enzyme extracts with trypsin increases the measurable activity of PP-1 in all tissues (Table 4). Indeed, the majority of PP-1 activity was masked, with the percentage of free, active PP-1 representing only 8–39% of the total activity in different organs. Estivation altered PP-1 activities in four organs, in all cases resulting in significant changes in both total activity (in trypsin-treated extracts) and the percentage of free, active enzyme (activity in untreated extracts). The changes in total and % active amounts generally occurred in opposite directions, so that the net change in the amount of free, active enzyme in the liver, heart and gut of estivated toads was small. However, kidney PP-1 was strongly reduced during estivation, with activity decreasing from 1.16 U/mg in control toads to 0.33 U/mg in estivated animals. Along with reduced PKA and PKC activities in kidney of estivating toads, this suggests an overall suppression of signal transduction mechanisms in the tissue during estivation.

Unlike the situation with the protein kinases, there were no consistent changes in PP-1 total activities or in the amounts of active PP-1 catalytic subunits in the different tissues of estivating toads. This suggests that control over the responsiveness of signal transduction pathways during estivation probably lies primarily with the modulation of protein kinases, as opposed to the phosphatases. It should be pointed out, however, that in many metabolic situations where protein kinase activities are suppressed to reduce specific metabolic functions, protein phosphatase activities are oppositely enhanced to activate others. This did not happen in estivating toads, suggesting that suppression of both arms of signal transduction systems (kinases and phosphatases) facilitates the overall state of metabolic rate depression.

Results for PP-2 were different. PP-2A activity was reduced to 60–70% of control values in three organs (liver, heart, gut) whereas PP-2C activity in-

creased in three organs (brain, muscle, gut) and decreased by 50% in heart. Estivation only had a consistent effect on the two activities in the heart; reduced activities of both phosphatases, PKA and PKC in the heart may be part of an overall metabolic suppression that manifests itself in a profound bradycardia during estivation. In other organs, the organ-specific changes in the two phosphatases during estivation may suggest differential regulation of the enzymes/proteins that are regulated by PP-2A and PP-2C *in vivo* to achieve specific outcomes during estivation.

Acknowledgements. We thank Mr. C.J. May, Tucson, Arizona for collecting spadefoot toads, J.E. Grundy for conducting animal experiments, S.P.J. Brooks for measuring glycogen phosphorylase and T.A. Churchill for amino acid analysis. Supported by a N.S.E.R.C. research grant to K.B.S. and N.S.E.R.C. postgraduate scholarships to K.J.C and J.A.M.

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