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**ANTIOXIDANT DEFENCES DURING ESTIVATION IN THE SPADEFOOT
TOAD *SCAPHIOPUS COUCHI*.**

by

Jean Elizabeth Grundy, B.Sc. (Hons)

A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

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Carleton University
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in partial fulfillment of the requirements for the degree of
Doctor of Philosophy of Science

Chair, Department of Chemistry

Thesis supervisor

Carleton University
1996

ABSTRACT

Antioxidant enzymes and lipid peroxidation damage were analyzed in an estivating species, to determine whether estivation (a dormancy induced by hot, dry environmental conditions) represented an oxidative stress and to determine whether changes in organ antioxidant systems were made in response to estivation. The model animal used was *Scaphiopus couchi*, the spadefoot toad, that estivates for about 10 months per year in the Arizona desert during which it loses about 50 % of its body water and builds up high levels of electrolytes and osmolytes in body fluids. Activities of six antioxidant enzymes (AOE) and xanthine oxidase/xanthine dehydrogenase as well as the small molecular weight free radical scavenger glutathione were measured in liver, lung, heart, kidney, gut and leg muscle of control and dormant toads. Damage to membranes was assessed by measurement of extent of lipid peroxidation by three different methods. The glutathione ratio, [GSSG]/[GSH], rose significantly in several tissues during estivation. In addition, activities of key AOE were reduced in several tissues, perhaps indicating that the metabolic cost of keeping antioxidant defences high during estivation was greater than the cost of maintaining AOE at their previous level. Three antioxidant enzymes were further examined: Catalase (CAT) and glutathione reductase (GR), were purified and characterized from liver and glutathione *S*-transferase (GST) from liver and muscle of awake and estivated toads. CAT activity was lower in liver during estivation but the kinetic properties of catalase were the same in estivated and awake toads. Catalase from toad liver was an active dimer, which has not been demonstrated previously in a eukaryote, to our knowledge. GR and GST of toad liver had altered kinetic properties during estivation, indicating pre-or post-translational modification of the enzyme in the dormant animal. However, GST in muscle was the same for awake and estivated toads. GST is a multiclass family of enzymes which generally render lipid-soluble material soluble in the cytosol, via conjugation to glutathione. Three classes of GST, Mu Pi and Alpha, have different substrate specificities; these parameters were used to classify frog

and toad GSTs. GST, GR and CAT were relatively insensitive to physiological urea concentrations but were strongly modulated by KCl, suggesting that urea buildup during estivation may serve to modulate passive KCl accumulation as a result of dehydration.

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LIST OF ABBREVIATIONS

AI	- 3-amino-1,2,4-triazole
β -MeSH	- β -mercaptoethanol.
BCIP	- bromochloroindolylphenol
BHT	- butylated hydroxytoluene
BSA	- bovine serum albumin
bw	- body water
CAI	- catalase
CB	- Cibacron Blue
CDNB	- 1-chloro-2,4-dinitrobenzene
CHP	- cumene hydroperoxide
DTNB	- 5,5'-dithiobis-2-nitrobenzoic acid
DTT	- dithiothreitol
EDTA	- ethylenediamine tetraacetate
ETC	- electron transfer chain
FOX	- ferrous oxidation/xylene orange complex
G3PDH	- glyceraldehyde-3-phosphate dehydrogenase
G6PDH	- glucose-6-phosphate dehydrogenase
GP _{ox}	- glutathione peroxidase
GR	- glutathione reductase
GR _A	- glutathione reductase from awake toad liver
GR _F	- glutathione reductase from estivated toad liver
GSH	- reduced glutathione
GSSG	- oxidized glutathione
GST	- glutathione <i>S</i> -transferase
H ₂ O ₂	- hydrogen peroxide
HO [•]	- hydroxyl radical

I_{50} - concentration of inhibitor that lowers enzyme velocity by 50%

IgG - immunoglobulin G

IEF - isoelectric focusing

K_m - Michaelis-Menten constant (substrate affinity constant)

KPi - potassium phosphate

MDA - malondialdehyde

NADP⁺ - nicotinamide adenine dinucleotide phosphate, oxidized form

NADPH - nicotinamide adenine dinucleotide phosphate, reduced form

NBT - nitroblue tetrazolium

$O_2^{\cdot-}$ - superoxide

PEP-carboxykinase - phosphoenolpyruvate carboxykinase

Pi - inorganic phosphate

pI - isoelectric point

PMSF - phenylmethylsulfonyl fluoride

RB - Rose Bengal

S-hex GSH - S-hexyl glutathione

SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis

SOD - superoxide dismutase

TBA - thiobarbituric acid

TBA-RS - thiobarbituric acid-reactive substances

TBS - Tris-buffered saline: 150 mM NaCl, 50 mM Tris.Cl, pH 7.0

V_{max} - maximal enzyme velocity

XO/XDH - xanthine oxidase/xanthine dehydrogenase

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CHAPTER 1
GENERAL INTRODUCTION

It was first proposed in 1954 that the known toxic effects of hyperbaric oxygen might involve the actions of oxygen radicals (Gerschmann *et al.*, 1954). The discovery of an enzyme specific for the removal of superoxide ($O_2^{\bullet -}$), superoxide dismutase (SOD), lent further support to this proposal and identified one of the oxygen radicals responsible for O_2 toxicity. It is now known that reactive reduced forms of oxygen or reactive oxygen species (ROS) are continuously produced in all aerobic systems (Sies, 1983; López-Torres *et al.*, 1993). During normal oxidative phosphorylation, oxygen is sequentially reduced with four electrons to form water (tetravalent reduction). However, oxygen may also undergo one-electron reduction *in vivo*, with the formation of a series of ROS (Figure 1.1; Sies, 1993). ROS rapidly react with and damage all cellular components, especially hydroxyl ion ($HO^{\bullet}$) which reacts so rapidly that its half-life is essentially diffusion controlled (10^{-9} to 10^{-10} sec) (Sies, 1983).

Molecular oxygen is the terminal electron acceptor of the respiratory chain in aerobic metabolism. Oxygen is appropriately soluble in water and so is suitable for biological reactions; it also has greater oxidative potential than nitrogen yet its spontaneous oxidation reactions are sluggish (Fridovich, 1976). The slow reaction of molecular oxygen is because the one-electron reduction of molecular O_2 is thermodynamically unfavourable (E° for $O_2 + 1 e^- \rightarrow O_2^{\bullet -} = -0.33V$) (Buettner, 1993). However, thermodynamic barriers may be overcome by tight binding of the intermediates to the enzyme (Naqui *et al.*, 1986). O_2 has an even number of electrons but two unpaired electrons in its outermost molecular orbitals, with the same spin quantum number (thus technically O_2 qualifies as a diradical). In order to oxidize another atom or molecule by accepting a pair of electrons from one of the molecule's orbitals, both the new electrons must be of parallel spin so as to fit into the vacant spaces of the outer orbitals of oxygen, but any pair of electrons sharing an orbital must have opposite spins (Pauli's principle). To overcome this restriction, oxygen can interact with another paramagnetic center to

Fig. 1.1 Reduction of molecular oxygen. Tetravalent and univalent reduction of molecular oxygen. Intermediates in univalent reduction are ROS.

Four-electron

One-electron

participate in exchange coupling, but otherwise must accept electrons one at a time, which slows down the rate at which its oxidation reactions occur. However, one of the two parallel spinning electrons in the outer orbital of O_2 can be turned to produce antiparallel spins for the outer electrons. This produces singlet oxygen (1O_2), which is more easily reduced (E° for $^1O_2 + 1 e^- \rightarrow O_2^{\cdot-} = 0.65V$) because spin restriction has been removed. Singlet oxygen is produced by ultraviolet radiation and also enzymatically by cytochrome P-450 during the metabolism of hydroperoxides (in the absence of electron donors and hydroxylatable substrates) (Naqui *et al.*, 1986).

Formation of ROS

At the end of the electron transport system (ETS), oxygen accepts four electrons, one at a time, from cytochrome oxidase to produce H_2O . Whereas more than 95 % of cellular oxygen is used in this way and is tightly bound, a small proportion of the oxygen is free and may form reactive oxygen species (Reiter, 1995). Often reactions that form ROS occur with other components of the electron transport chain such as complex I (NADH-ubiquinone oxidoreductase) and complex III (ubiquinol-cytochrome *c* oxidoreductase) which are thermodynamically capable of one- or two-electron reductions of O_2 , and which can, therefore, generate $O_2^{\cdot-}$ and H_2O_2 . Indeed, the electron transfer chain in mitochondria is the major source of endogenously produced ROS in cells (Yu, 1994). $O_2^{\cdot-}$ and H_2O_2 are also produced enzymatically by oxidases such as xanthine oxidase, glucose oxidase, and NADPH oxidase, as well as during auto-oxidation of small molecules such as catecholamines and flavins (Yu, 1994; Reiter, 1995). Thus, $O_2^{\cdot-}$ or H_2O_2 are produced naturally from a variety of intracellular reactions. Although they are potentially damaging to some extent by themselves, the most harmful effects of these species arise due to the conversion of H_2O_2 alone, or $O_2^{\cdot-} + H_2O_2$ in combination, to hydroxyl radical ($HO^\cdot$), catalyzed by transition metals such as iron and copper in the Fenton and Haber-Weiss reactions, respectively (Basaga, 1990; Fig. 1.2).

ROS attack on proteins, lipids and DNA can result in irreversible damage to cellular components that, if extensive enough, can be lethal. Once initiated, damage by ROS is greatly exacerbated by free radical propagation or reaction of the ROS with other cell components (Halliwell and Gutteridge, 1990), leaving behind a damaged macromolecule. Damage due to free radical attack has been linked to a variety of pathological conditions including arthritis, arteriosclerosis, diseases affecting the brain (Godin and Garnett, 1992), cancer (Sies, 1993), injuries associated with freezing, amputation, hypoxia and ischemia/reperfusion (Kappus, 1986; Halliwell, 1992; Ames, 1993; Dawson *et al.*, 1993), and the normal degradative processes of aging (Kristal and Yu, 1992).

Various stress situations cause excess ROS generation and oxidative stress *in vivo*. One of the best documented physiological situations occurs during reperfusion with oxygenated blood after an ischemic (interruption of blood flow) episode (Baker *et al.*, 1988; Cao *et al.*, 1988; Granger *et al.*, 1986). Several studies have demonstrated the large-scale proteolytic conversion of xanthine dehydrogenase (XDH) to xanthine oxidase (XO) specifically during ischemic episodes (Mink *et al.*, 1990; Hossain *et al.*, 1995; Korthius and Granger, 1986). At the same time, the energy stress imposed by ischemia leads to a degradation of ATP in several steps to hypoxanthine (Dorion *et al.*, 1993). XDH and XO convert hypoxanthine to xanthine (one-electron reduction) or urate (two-electron reduction), and use oxidizing agents capable of accepting one or two electrons in the process, respectively. But whereas XDH uses either one or two molecules of NAD⁺, XO is a typical oxidase and uses O₂, converting it to O₂^{-•} or H₂O₂. Thus, conversion of XDH to XO is a possible predictor for tissue injury, since XO alone produces ROS. Combined with elevated hypoxanthine due to adenylate degradation and increased oxygen availability upon reperfusion, the mixture is a lethal cocktail for ROS formation. In addition, copper and iron are mobilized following reperfusion (Chevion *et al.*, 1993), increasing the likelihood of HO[•] production. Indeed, ROS formation and damage to

cellular components as the result of $\text{HO}^\bullet$ production have been detected following reperfusion of ischemic tissues (Kadkhodaei *et al.*, 1995).

Protection

To minimize damage due to ROS attack, all cells maintain enzymatic and nonenzymatic antioxidant defenses that continuously scavenge and/or chemically convert free radicals to less toxic species *in vivo*. The situation known as *oxidative stress* arises when the rate of oxygen radical generation overwhelms the natural capacity of cellular antioxidant defences so that net damage accumulates (Sies, 1993). Sophisticated systems exist for the prevention and termination of cellular free radical reactions. For example, in yeast ROS modulate gene expression by acting as second messengers to induce the expression of genes which code for antioxidant enzymes (Fanburg *et al.*, 1992).

Prevention of free radical formation is also accomplished by strict control over the free concentrations and subcellular locations of metal ion catalysts, such as Fe^{++} , via the use of iron binding proteins such as transferrin in blood plasma and desferrioxamine and ferritin in cytoplasm (Gutteridge and Quinlan, 1992; Gutteridge and Halliwell, 1990). Other prevention mechanisms include strict subcellular localization of enzymes of the ETS within the mitochondria as well as the association of specific antioxidant enzymes within mitochondria to degrade ROS before they can leave the organelle.

Termination of ROS occurs via the detoxifying reactions of antioxidant enzymes (AOE) (Fig. 1.2) and by the actions of low molecular weight oxygen radical scavengers. Primary enzymatic ROS detoxifiers include the enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GP_{ox}). Superoxide is converted by SOD to a non-radical species, hydrogen peroxide (H_2O_2). H_2O_2 is removed by CAT or selenium-dependent GP_{ox} and converted to non-radical species. Competing with the enzymatic removal of $\text{O}_2^{\bullet-}$ and H_2O_2 are transition metal-catalyzed reactions (the Haber-Weiss and Fenton reactions) which convert $\text{O}_2^{\bullet-}$ and/or H_2O_2 into $\text{HO}^\bullet$. The reaction rate of $\text{HO}^\bullet$ is

essentially diffusion controlled (Sies, 1986) and it rapidly damages all cellular components. Hydroxyl radical attack on membrane lipids forms lipid hydroperoxides, which rapidly propagate and lead to the destruction of membrane integrity. Lipid hydroperoxides are detoxified by GP_{ox} or by the selenium-independent peroxidative activity of glutathione *S*-transferase (GST). However, the primary function of GST in the cell is the detoxification of electrophilic xenobiotics in the cell, coupling them to reduced glutathione to target them for removal.

A number of low molecular weight species, such as vitamin C, vitamin E, urate and melatonin (in the brain) also scavenge free radicals directly. However, the major ROS scavenger in tissues and blood is glutathione (gamma-glutamyl-cysteinyl glycine), which is one of the most abundant short peptides in eukaryotic cells and the most prevalent intracellular thiol. GSH is also involved in a wide spectrum of other cell functions including protein and DNA synthesis, amino acid transport, prostaglandin synthesis and intermediary metabolism (Redegeld, 1990). Conjugation of GSH to electrophiles occurs both spontaneously and via GST catalysis (Chang, 1992). GSH can be directly oxidized to GSSG by oxygen centered free radicals (Winterbourn and Metodiewa, 1994), or can act as the mandatory substrate for the antioxidant enzymes GP_{ox} and GST (peroxidase activity) whose product is GSSG. GSSG is recycled back to 2 equivalents of GSH by glutathione reductase (GR) in an NADPH-dependent reaction. Glutathione reductase is considered a secondary antioxidant enzyme, as it replenishes an antioxidant but does not itself detoxify free radicals.

Response to oxidative insult

Many environmental conditions or physiological stresses are associated with oxidative stress, and may induce protective responses by the antioxidant systems of organisms. For example, exhaustive exercise increased oxidized glutathione levels and induced antioxidant enzymes in rat muscle (Ji and Fu, 1992), the acquisition of cold-

hardiness is associated with a specific GR expressed in pine needles (Hausladen and Alscher, 1994), exposure to cadmium pollution increases lipid peroxidation in mussels (Ribera *et al.*, 1991), and heat stress depletes blood glutathione and increases lipid peroxidation damage in humans (Ohtsuka *et al.*, 1994).

Desiccation is a stress unique from those listed above in that it results in cell and plasma hyperosmolality. Many animals defend themselves against water loss and its consequences with, for example, highly impermeable integuments and behavioural strategies that make them avoid desiccating conditions. However, amphibians as a group have highly water permeable skins and are the least desiccation resistant of terrestrial vertebrate groups (Shoemaker *et al.*, 1992). As a consequence, they have developed the ability to endure wide variation in their body water content and in the osmolality and ionic strength of their body fluids. For example, amphibians can tolerate 90-200 mM increases in plasma sodium concentrations without severe consequences whereas such a disturbance of ion balance would be lethal in mammals (Hillman, 1988). Despite relatively poor abilities to resist desiccation, amphibians can nonetheless be found in various dry environments. For example, toads of the genus *Scaphiopus* (spadefoot toads) are found throughout the arid regions of the American southwest. They burrow to escape the sun's heat and during the dry seasons of the year retreat underground for months at a time and enter dormancy. Couch's spadefoot toads (*Scaphiopus couchi*) spend about nine months or more of the year buried under the Arizona desert soil. While dormant they tolerate the loss of up to 60 % of their total body water (48% of their body weight) (Hoar, 1966), and dehydration of their tissues, resulting in elevated intracellular potassium and extracellular sodium levels (Flanigan *et al.*, 1993). The toads surface only after summer thunderstorms create sufficient run-off to form temporary pools. They immediately breed at these pools and then over a few short weeks eat sufficient food to sustain them for another year (McClanahan, 1967). When conditions above ground again become too dry, the toads retreat underground and enter a dormant or hypometabolic state that is called

estivation. During estivation large reserves of water in the bladder are gradually resorbed in order to maintain plasma volume and organ hydration for as long as possible. Ultimately, however, as the soil dries out and body water reserves run short, another mechanism is called into play to restrict further evaporative water loss. Toads raise the osmolarity of their plasma and urine by increasing the catabolism of body protein reserves and synthesizing urea which accumulates in high concentrations up to 300 mM. The colligative effects of high urea concentrations retards further water loss.

Entry into estivation involves a coordinated response by all metabolic processes (Pinder *et al.*, 1992). Heart rate and breathing rate slow to one-quarter of normal (Seymour, 1973b). Toads undergo wide variations in oxygen consumption in the transition from awake to estivating states, even though oxygen availability in soil around the toad is no different from that in the air above ground. Some gas exchange also occurs through the skin and toads use apnoic breathing patterns to minimize body water loss during long-term dormancy (Seymour, 1973; Barnhart, 1987; Shoemaker *et al.*, 1992). The activity of enzymes responsible for urea synthesis is increased up to 600 % in response to decreases in the water potential of the soil and dormant toads have higher rates of urea synthesis than awake toads (Shoemaker *et al.*, 1992).

Although most of the toad's metabolic processes are depressed during estivation, its antioxidant defences may not be. In previous studies it has been found that animals in hypometabolic states appear to remain ready for an oxidative stress accompanying an increase in metabolic rate during arousal. Estivation in land snails and freezing in garter snakes and wood frogs all appear to be associated with either high constitutive activities of antioxidant enzymes, or with an increase in antioxidant enzyme activities during dormancy in anticipation of oxidative stress upon arousal or thawing (Hermes-Lima and Storey, 1994; Hermes-Lima and Storey, 1993; Joanisse and Storey, 1996). These species may adopt either strategy to avoid a condition similar to ischemia/reperfusion stress, since arousal from a hypometabolic state involves increased perfusion with oxygenated

blood and increased oxygen consumption. Toads arousing from estivation may also face a similar oxidative challenge and may prepare for emergence from dormancy by sustaining higher activities of AOE during estivation or inducing AOE synthesis upon entry to estivation. If this is in fact the case, modification of AOE activity may be due to quantitative adjustments in the amounts of AOE proteins, or to qualitative changes in antioxidant enzyme expression during estivation that enable toads to deal with specific challenges related to survival in the estivating state.

The objective of the research described in the following chapters of this thesis was to provide a clear analysis of the mechanisms of oxidative damage avoidance that are used during estivation and arousal in spadefoot toads. I hypothesized that adaptation of antioxidant systems could occur at one or more of several levels. Thus, animals might

- a) maintain high antioxidant defences at all times to deal with variable ROS levels,
- b) alter the activities of AOE and/or the concentrations of metabolite antioxidants either to match or in anticipation of the predicted ROS "load" during estivation or arousal,
- c) alter the kinetic properties of AOE to deal with the particular circumstances of the estivating state, or
- d) endure increased levels of ROS formation and the accumulation of products of oxidative damage in cells during dormancy or arousal that could subsequently be cleared.

Studies were designed to analyze all of these possibilities:

(1) To determine whether the antioxidant defenses of toads were adjusted to suit the particular circumstances of estivation/arousal, maximal activities of antioxidant enzymes (AOE) and the concentrations of the reduced and oxidized forms of the major soluble antioxidant, glutathione, were measured in multiple organs of spadefoot toads. AOE activity in liver and muscle were also compared to that of a non-estivator, the leopard frog (*Rana pipiens*). Studies included measurement of the activities of the primary AOE (selenium-dependent and independent GP_{ox}, CAT and SOD) and secondary

AOE (GST and GR) used to defend against ROS toxicity and evaluation of any changes in these that occurred during estivation. Activities of XO and XDH were also measured to determine whether the percentage of the oxidase form changed during dormancy, an occurrence that could elevate ROS formation (Hossain *et al.*, 1995). Measurement of levels of oxidized and reduced glutathione were also made to determine whether GSH was being depleted for specific uses in tissues during estivation, or oxidized to GSSG by ROS. A common measure of oxidative stress is the GSSG/GSH ratio, which typically rises under situations of increased ROS formation (Ambrosio, 1992). Since the concentrations of urea and inorganic ions also build up in toad tissues during estivation, maximal activities of AOE were also analyzed in the presence of physiological levels of urea and/or salt to determine whether natural changes in the levels of these compounds would influence AOE function during estivation.

(2) To determine the effectiveness of AOE in preventing damage to cell components by ROS during estivation, levels of ROS damage products were quantified in tissues. ROS can damage DNA, carbohydrates, proteins and lipids, but ROS attack on lipids (lipid peroxidation) is the most widely studied of the biologically relevant free radical chain reactions (Halliwell and Chirico, 1993), and the accumulation of lipid peroxidation products is frequently cited as evidence for the involvement of ROS in pathological conditions (Aikens and Dix, 1991; Halliwell and Chirico, 1993). Thus, the present studies focused on ROS damage to lipids. The products of free radical attack on polyunsaturated fatty acids (Fig. 1.2) were measured by three methods: 1) quantification of aldehydes, such as malondialdehyde, which are the ultimate products of lipid hydroperoxide breakdown as thiobarbituric acid reactive substances, 2) detection of conjugated dienes, and 3) measurement of the tissue levels of lipid hydroperoxides via a redox-sensitive dye couple. This latter assay, termed the FOX assay was recently adapted for use with animal tissue extracts in our lab (Hermes-Lima *et al.*, 1995).

(3) To determine whether estivation induced alterations in the properties of specific antioxidant enzymes, selected enzymes showing altered maximal activities during estivation were purified and characterized. The kinetic and physical properties of CAT, GR and GST from liver of both awake and estivating toads were examined to determine whether the enzymes showed any unique properties compared with their counterparts from other sources or whether kinetic properties were altered during estivation.

My studies showed that the antioxidant defence response to estivation was tissue-specific and was not generally altered to reflect anticipation of forthcoming oxidative stress. In general, slight oxidative damage was observed during estivation concomitant with diminished antioxidant protection. However unique adaptive changes were made to GST, GR and CAT activity, indicating more complex regulation of AOE activity than simple suppression during estivation.

Fig. 1.2 Reactive oxygen species formation, detoxification and attack on polyunsaturated fatty acids. Endogenously produced superoxide is catalytically converted to hydrogen peroxide, which is itself detoxified, to prevent formation of hydroxyl radical. Abbreviations are: GST, glutathione *S*-transferase; GR, glutathione reductase; CAT, catalase; GP_{ox}, glutathione peroxidase; EI, aromatic hydrocarbon/electrophile; GSH/GSSG, reduced/oxidized glutathione; O₂^{•-}, superoxide; H₂O₂, hydrogen peroxide; HO•, hydroxyl radical. GR is technically an equilibrium enzyme, but in this diagram functions in one direction to replace GSH as a reducing equivalent. Hydroxyl radicals formed by the competing Haber-Weiss and Fenton reactions go on to attack polyunsaturated fatty acids and form conjugated dienes and lipid hydroperoxides.

CHAPTER 2
ANTIOXIDANT RESPONSE AND LIPID DAMAGE IN *SCAPHIOPUS COUCHI*
DURING ESTIVATION

2.1 ENZYMATIC ANTIOXIDANTS AND GLUTATHIONE STATUS IN ESTIVATING *S. COUCHI*

INTRODUCTION

The response to an oxidative insult often includes the increased synthesis of antioxidant enzymes (AOE), the synthesis of stress-specific AOE, and the induction of GSH synthesis or increased recycling of GSSG to GSH, sometimes mediated by ROS themselves. Maintenance of AOE and GSH levels are necessary for cellular homeostasis, since ROS rapidly and irreversibly damage most cell components. Studies to date with invertebrates and lower vertebrates suggest that the response of organisms to oxidative stress induced by dryness or hyperosmolality can be either constitutive or inductive. An example of constitutive adaptation of antioxidant systems to defend against a similar oxidative insult was documented in the wood frog, *Rana sylvatica*. This frog endures the freezing of its extracellular body fluids, a condition in which circulation is halted (imposing ischemia) by the formation of ice in plasma. Thus, thawing and reperfusion of tissues with oxygenated blood is analogous to the reperfusion event that follows an ischemic episode (Hermes-Lima and Storey, 1995b). Oxidative damage is one of the most highly documented sequels to this stress (Downey, 1990; Floyd, 1990; Traystman *et al.*, 1991; Dorion *et al.*, 1993). Studies on frogs prior to, during and following freezing episodes showed that AOE activities in tissues of this species were maintained at levels several-fold higher than in several other freeze intolerant amphibians, suggesting that high constitutive AOE activities aided endurance of oxidative stress events accompanying freeze-thaw episodes (Joannis and Storey, 1996).

One example of inductive adaptation is the response of land snails (*Otala lactea*) to one month without food or water, in dry surroundings. The snails entered estivation and after one month, activity of the primary AOE (SOD, GP_{ox}, CAT) had risen significantly (Hermes-Lima and Storey, 1994). Within minutes after arousal of the snails

was stimulated, accumulation of the products of oxidative damage to lipids was documented indicating that arousal from estivation was an oxidative stress (Hermes-Lima and Storey, 1993). The elevation of antioxidant enzyme activities during dormancy appeared to be an anticipatory adaptation for the burst of oxygen utilization that occurs immediately when arousal is initiated.

The inductive response is more widely seen than the constitutive response, and has been demonstrated for stress situations in other amphibians and reptiles. For instance, *Discoglossus pictus*, a toad, responded to normobaric hyperoxia (which leads to increased cellular generation of O_2^- and H_2O_2) with an increase in the activity of hydroperoxide-detoxifying AOE (Barga de Quiroga *et al.*, 1988). Antioxidant enzyme expression in frogs (*Rana perezi*) was also altered to compensate for CAT inactivation (López-Torres *et al.*, 1993). During both anoxia (zero oxygen) and freezing exposure, garter snake tissues showed increased antioxidant enzyme activity (increased by 50 % or more over pre-stressed animals) (Hermes-Lima and Storey, 1993), indicating an inductive response to these stresses. Obviously, modulation of antioxidant defence capability is an important part of the biochemical mechanisms which support tolerance of varying oxygen tensions in these animals.

This chapter answers the question: is modulation of antioxidant defence mechanisms part of the metabolic adjustment necessary to support estivation in a desert toad? Spadefoot toads, *Scaphiopus couchi*, tolerate months in a hypometabolic state (estivation), buried underground in desert soil without food or water. During estivation, oxygen consumption is low in the organs of the toad, and arousal from estivation, with increased oxygen uptake, could pose an oxidative stress to the animal in a similar manner to arousal from estivation in the snail *O. lactea*. Toads may employ the same strategy as freeze tolerant frogs or estivating snails by maintaining high AOE activity (constitutive adaptation) or may specifically increase AOE activities during estivation (inductive adaptation). Heightened soluble and enzymatic antioxidant defences may be required to

deal with oxidative stress either during or upon arousal from estivation. The studies described in this chapter determine whether specific antioxidant defence mechanisms are altered to support estivation in this species.

To do this, glutathione levels and the maximal activities of five antioxidant enzymes were quantified in six tissues of awake and estivating toads. These tissues were selected for specific reasons. The lung is normally continually exposed to a high partial pressure of oxygen and the liver, due to its central role in metabolism and detoxification, has the predominant rate of generation of H_2O_2 in most animals. Heart and kidney are both well-perfused aerobic organs whereas leg muscle has a relatively high anaerobic capacity (Barga de Quiroga *et al.*, 1988). Stomach and intestine are interesting in that they are in use when the toad is awake, and unused when the toad is dormant.

Comparisons of antioxidant enzyme activities in toads were also made with tissues (liver and leg muscle) of the leopard frog, *Rana pipiens*, a semi-aquatic species that does not estivate.

Glutathione is the major water-soluble, non-enzymatic antioxidant present in cells, and is also a substrate for ROS-detoxifying enzymes. Glutathione is present in reduced (GSH) and oxidized (GSSG) forms in cells and the amount of GSSG is normally maintained at low levels relative to GSH (1/10 or lower) by the NADPH-linked glutathione reductase (GR) catalyzed recycle pathway (Chang, 1992). GSSG is produced by direct attack of ROS on GSH, or as a product of enzymatic detoxification of ROS using GSH as a substrate. Hence, a rise in GSSG content and/or an increase in the ratio of GSSG to GSH is frequently considered to be a good indicator of cellular oxidative stress (Ambrosio, 1992). A decrease in cellular [GSH] also indicates reduced defence against ROS, since GSH is a free radical scavenger by itself as well as a mandatory substrate for AOE. High GSSG levels allow the formation of disulfide bonds in or between proteins, or between proteins and glutathione (mixed disulfides), which can lead to loss of protein function. Disruption of protein function as a result of an altered GSSG/GSH ratio is

twofold: GSSG directly induces protein inactivation, but lower levels of GSH, as the pool is depleted also provide reduced protection. The investigation described in this chapter examined levels of reduced and oxidized glutathione and changes in the redox state of the cell (the GSSG/GSH ratio) as a result of recovery from estivation.

Maximal activities of five antioxidant enzymes were measured in toad tissues. SOD, GP_{ox} and CAT are primary antioxidant enzymes that directly detoxify ROS, converting them to less active species. SOD detoxifies superoxide, the first oxygen radical formed by direct leakage of electrons from the ETC onto molecular oxygen. Three forms of SOD exist; one is localized in mitochondria and contains Mn whereas the Cu- and Zn-containing forms are localized in the cytoplasm. The assay used measured the total activity of all three forms. Se-dependent GP_{ox} detoxifies both hydrogen peroxide and alkyl hydroperoxides, using two equivalents of glutathione. CAT also detoxifies hydrogen peroxide, without a requirement for GSH. Hydroperoxides formed by oxygen radical attack on cellular components are detoxified by both the selenium-dependent and selenium-independent forms of GP_{ox}. Se-independent GP_{ox} activity toward alkyl hydroperoxides is one of the several catalytic functions of GST, which conjugates GSH to a variety of compounds. GST is also involved in the detoxification of xenobiotics and the synthesis of prostaglandins. Oxidized glutathione formed as a product of GP_{ox} activity is regenerated to GSH by GR. In the second half of this chapter (Chapter 2.2) lipid peroxidation damage was measured to evaluate the effectiveness of AOE and GSH defences in estivating *S. couchi*.

As a means of determining the rate of generation of some ROS, the xanthine oxidoreductases were also analyzed in toad tissues. Xanthine oxidase (XO) and xanthine dehydrogenase (XDH) are similar in size and composition of redox centers, as well as acceptance of a variety of substrates. This and other evidence has indicated that they are two forms of the same gene product. These enzymes oxidize hypoxanthine and xanthine, breakdown products of ATP, to xanthine and to urate by either two one-electron

oxidations or one two-electron oxidation. As an electron acceptor, however, XO preferentially utilizes molecular oxygen whereas XDH uses NAD^+ . During the process, XO reduces 2O_2 to $2 \text{O}_2^{\cdot-}$ or 1O_2 to H_2O_2 , respectively. XO is a significant source of ROS and $\text{O}_2^{\cdot-}$. generated by XO has been shown to damage enzymes in situations of ischemia-reperfusion in mammals (Palmer, 1987). Thus, it is important to determine whether XO activity is altered during estivation in toads. Some forms of XO/XDH exist in reversibly interconvertible forms, but an irreversible conversion from the dehydrogenase to the oxidase form can also occur by limited Ca^{++} dependent proteolysis (Hille and Nishino, 1995) initiated during ischemia (Gutteridge and Halliwell, 1990). The relative amount of XO activity compared to total XO + XDH was significantly higher in estivating *O. lactea* compared with control snails (Hermes-Lima and Storey, 1995), indicating increased potential for ROS generation in the estivated state. Thus, a change in the relative activity of XO in toad tissues during estivation would also indicate a conversion of the dehydrogenase to the oxidase form, which could lead to increased generation of ROS in toad tissues.

The activities of many enzymes are perturbed by even moderate salt concentrations such as occur in toad body fluids during estivation. Toads tolerate the loss of up to 50% of their total body water during the time they stay buried. To help retard evaporative water loss across their skin, they actively raise the osmolality of their body fluids by synthesizing high concentrations of urea. The salt concentration of their body fluids also rises passively as the animal dehydrates and NaCl in plasma can reach 300 mM (from a normal concentration of less than 50 mM). The colligative effects of these high solute levels slow water loss, but they could also affect the function of enzymes in the estivating animal (Hochachka and Somero, 1982, Grundy and Storey, 1994). Hence, the effects of physiological levels of these compounds on antioxidant enzyme activities were also tested.

MATERIALS AND METHODS

Chemicals and animals

All chemicals were obtained from Sigma Chemical Co., St. Louis, MO and were of the highest purity available. Leopard frogs (*Rana pipiens*) were obtained locally in the fall and were held at 21°C in a large tank with terrestrial and aquatic areas, and were fed *ad libitum* until use within a few days. Spadefoot toads (*S. couchi*) were captured in the Tucson, Arizona area and were air freighted to Carleton University where animals were washed with deionized, distilled water, treated with a mild solution of tetracycline and rinsed, then placed in plastic basins containing damp soil. Animals burrowed into the soil and entered estivation and were maintained in a dormant state for 2 months at 21°C. Soil humidity was 10-12% at the end of the two months. At this point, some of the toads were sampled from the dormant state whereas twenty were aroused from dormancy. The aroused animals were given free access to water (changed every day) and crickets to eat and were then sampled 10 days later. Toads were killed by pithing and liver (without gallbladder), heart, lung, gut (stomach and intestine combined), hind leg muscle, and kidney were rapidly excised and frozen in liquid nitrogen.

Measurement of total and oxidized glutathione

GSH and GSSG determinations were conducted by the method of Tietze (1969) as modified by Griffith (1980). Frozen tissue samples were quickly weighed and then rapidly homogenized (with sonication) 1:5 w/v under nitrogen gas in ice-cold 5 % w/v sulfosalicylic acid (previously bubbled with nitrogen gas for at least 10 minutes). The homogenate was then centrifuged in an Eppendorf microcentrifuge at 4 °C for 15 minutes at 13,000 x g. The supernatant was removed and divided. Standard 1 mM GSH and GSSG were made up in water, kept on ice and used within a few hours. One portion of supernatant was used immediately for measurement of total glutathione equivalents ($\text{GSH}_{\text{eq}} = \text{GSH} + 2 \text{ GSSG}$). GSH equivalents were measured by following the rate of

reduction of DTNB by GSH at 412 nm. The assay mixture (in a spectrophotometer cuvette at room temperature) contained 0.2 mM NADPH, 0.6 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), 5 mM EDTA, 0.5 U GR and sample/water to final volume of 1 mL. After checking that background rate was zero, the reaction was started by addition of GR and monitored at 412 nm. The rate of the reaction was proportional to the concentration of GSH_{eq} and was compared with a standard curve made with 0-6 μM GSH. GSH equivalents in the second portion of the supernatant were measured after first derivatizing the GSH in the sample with 2-vinylpyridine. The aliquot was treated with 0.5 M 2-vinylpyridine (Aldrich, 20 % v/v in ethanol) and adjusted to between pH 5.5 and 7.5 with 0.5 M KPi buffer, pH 7.0. This preparation was left for one hour at 20 °C to derivatize GSH. This reagent formed a derivatized product with GSH so that it could not be measured in the assay. GSSG was unaffected and reacted normally, being measured as 2 GSH equivalents. These remaining GSH equivalents were thus subtracted from the total glutathione measurement to yield GSH present in the sample: (total GSH - GSH equivalents as GSSG) = GSH in the extract. GSH equivalents in the derivatized sample were divided by two to yield the concentration of GSSG.

Preparation of tissue extracts and enzyme assay

Frozen tissues samples were rapidly weighed and homogenized 1:5 w/v in ice-cold 10 mM KPi, pH 7.0 containing 1 mM EDTA using a Polytron PT-10, with a few crystals of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) added. Homogenates were centrifuged at 13,000 $\times$ g in an Eppendorf centrifuge for 15 minutes at 4°C and then the supernatant was removed and stored on ice. Initial trials tested the usefulness of removing low molecular weight species by passing supernatants through small columns of G-25 Sephadex; this had no effect on the activities of GST, GR, or GP_{ox} but reduced SOD and catalase activities and, hence, was not routinely used. Enzyme assays were conducted at room temperature (21°C) using a Gilford recording

spectrophotometer. Substrate concentrations were optimized for all enzymes in each organ type using tissue extracts from control animals. Maximal activities were measured in the presence versus absence of 300 mM urea, 200 mM KCl or both urea and KCl. These two substances were added to simulate cytosolic conditions in estivated toads (Shoemaker 1967; Flanigan *et al.*, 1993) and to examine the effect of increased ionic strength and/or high urea on toad enzymes. Final assay volume was 1 mL in all cases.

A). *Glutathione S-transferase* (E.C. 2.5.1.18)

GST was assayed using the method of Habig and Jakoby (1981). The assay measures the formation of a conjugate between GSH and 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm (extinction coefficient $\epsilon = 9.6 \text{ mM}^{-1}$) in 50 mM KPi buffer, pH 6.5 with 1 mM EDTA; the reaction was started by addition of homogenate. Optimal concentrations of GSH were 5 mM for toad liver, lung, gut and kidney, and 2 mM for toad heart, and frog liver and muscle. Optimal CDNB concentration was 2 mM for toad liver and frog muscle, and 1 mM for all other tissues tested. Two blanks were used to control for nonspecific activity: a minus GSH blank (which was always zero) and a minus homogenate blank (which measured spontaneous reaction between GSH and CDNB). One unit of enzyme activity is defined as the amount that catalyzes the production of one μmol of the conjugate (*S*-2,4-dinitrophenylglutathione) per minute. For liver, 2-5 μL of a 1/100 dilution of supernatant was used for assay; for other tissues, 2-10 μL of a 1/10 dilution of supernatant was used.

B). *Glutathione reductase* (E.C. 1.6.4.2)

GR was assayed by the method of Di Ilio (1983). The standard assay contained 50 mM potassium phosphate buffer (KPi) pH 7.4, with 1 mM EDTA and 0.2 mM NADPH. Optimal assay conditions were 2 mM GSSG for toad liver and heart, and frog liver, 0.5 mM for toad kidney and 1 mM GSSG for all other tissues tested. The reaction was started

with addition of enzyme, and consumption of NADPH was monitored at 340 nm ($\epsilon=6.22 \text{ cm}^{-1} \text{ mM}^{-1}$); 10 μL of supernatant was required for an observable rate. Two blanks were used and subtracted: a minus GSSG blank and a blank activity measured prior to addition of enzyme extract. One unit of GR was defined as the amount of enzyme that oxidizes 1 μmol NADPH per minute. This enzyme is normally present in low amounts, and activity is sometimes represented in mU.

C.) *Glutathione peroxidase* (E.C. 1.11.1.9)

Selenium-dependent glutathione peroxidase was assayed as described by Ahmad and Pardini (1988). The reaction mixture contained 50 mM KPi buffer pH 7.0, 1 mM EDTA, 2 mM NaN_3 , 0.2 mM NADPH, 5 U GR (Sigma) and optimal GSH concentration, and was initiated by the addition of H_2O_2 (0.25 mM for kidney and heart, 0.5 mM for other tissues). H_2O_2 was made fresh in H_2O just before assay. Optimal GSH concentration was 1 mM for toad kidney, 10 mM for toad liver, 2 mM for frog liver and muscle and toad heart, and 5 mM for all other tissues. One unit of activity was defined as the amount that catalyzes the reduction of 1 nmol NADPH per minute.

Total glutathione peroxidase, both selenium dependent and independent (Se-indep GPox; α class GST), was assayed under the same conditions as above but using cumene hydroperoxide (1.2 mM was optimal for all tissues) as the substrate instead of H_2O_2 . Optimal GSH concentration was 1 mM for toad kidney and frog liver and muscle and 5 mM for all other tissues. One unit was defined as the amount that catalyzes the reduction of 1 nmol NADPH per minute. The rate of consumption of NADPH in the absence of GSH, supernatant and peroxide solution were subtracted from the initial rate.

D.) *Catalase* (E.C. 1.11.1.6)

CAT was assayed according to Aebi (1984). The reaction mixture contained 50 mM KPi buffer, pH 7.0 and 10 mM H_2O_2 and decay of peroxide was followed at 240 nm

in a quartz cuvette ($\epsilon=0.0394 \text{ mM}^{-1}$). A low scale expansion was used and the enzyme was diluted until a linear rate could be observed: the reaction was started by addition of 2 to 5 μl of a 1/100 (kidney and liver) or 1/10 dilution (all other tissues) of the supernatant in homogenization buffer. One unit of enzyme activity was the amount that reduces one μmol of H_2O_2 per minute.

E). *Superoxide dismutase* (E.C. 1.15.1.1.)

SOD was measured according to Paoletti (1986). The assay contained 50 mM KPi buffer, pH 7.4, 5 mM EDTA, 2.5 mM MgCl_2 , 0.27 mM NADH and supernatant (this mixture gave no blank reaction). The reaction was started by the addition of 0.39 mM β -mercaptoethanol (made fresh daily and kept on ice). This mixture generates $\text{O}_2^{\cdot -}$ at a constant rate, which in turn oxidizes NADPH, and the oxidation of NADPH is inhibited by SOD. Several cuvettes are run, each with different amounts of supernatant, and results of these are used to generate an inhibition curve (rate of oxidation vs. volume of supernatant). The activity of SOD is expressed as an I_{50} value, one unit being defined as the amount of enzyme that inhibits the oxidation of NADPH by 50%.

F). *Xanthine oxidase/Xanthine dehydrogenase* (EC 1.2.3.2)

XO/XDH was measured as described by Stirpe and Della Corte (1969). XDH and XO were both measured when 0.6 mM NAD^+ was included in the assay whereas XO alone was detected if NAD^+ was omitted. Formation of urate from xanthine was followed at 295 nm in a quartz cuvette ($\epsilon=12.59 \text{ mM}^{-1}$) with an assay system containing 50 mM KPi buffer, pH 7.8 and 0.1-1 mM xanthine. Optimal concentrations of xanthine were 0.5 mM for frog and toad liver. Activity in other tissues was immeasurably low. One unit was defined as the amount of enzyme that produces 1 μmol of urate per minute.

XO and XDH activities were also measured using the assay of Beckman *et al.* (1989). The fluorescence of isoxanthopterin was measured using a Perkin-Elmer

Luminescence spectrometer attached to an IBM 386/33 using Perkin-Elmer FLDM software. XO activity was measured by monitoring isoxanthopterin production (345 nm excitation, 390 nm emission) in an assay containing 50 mM KPi buffer, pH 7.5, and supernatant, and started by the addition of 0.02 mM pterin. XO + XDH activity was followed after the further addition of 0.01 mM methylene blue. Allopurinol (2 mM) was then added to completely inhibit enzymatic activity and the fluorescence intensity of known concentrations of added isoxanthopterin was then quantified as an internal standard for each cuvette. One unit of activity is defined as the amount that produces one nmol isoxanthopterin per minute.

Protein determination

Protein concentration was determined using the Bio-Rad assay kit based on the Coomassie Blue G-250 dye-binding method, with bovine serum albumin as the standard (Bradford, 1976).

RESULTS

Glutathione status

Total, reduced and oxidized glutathione contents (nmol/gram fresh weight), as well as the ratio of oxidized to reduced glutathione in tissues of estivated and awake toads are shown in Table 2.1.1. Total glutathione equivalents were 84 % higher in muscle and lung, and 50 % higher in hearts of estivated toads, compared to the corresponding values from awake toads. In contrast, liver had the opposite distribution: total glutathione content of liver from estivated toads was lower, only 86 % of the level in awake liver. Comparing the distribution of total glutathione between tissues of estivated toads, the distribution was 91:75:47:95:35:100 for liver, heart, kidney, muscle, gut and lung, respectively. This distribution was different in awake animals: 100:48:38:49:32:50 for the tissues in the same order as above. Thus lung had the highest total glutathione in estivated

animals, whereas liver had the highest total GSH content in awake animals, twice the amount in other tissues.

Levels of oxidized glutathione in tissues from estivated toads were significantly higher than levels in the awake animals' kidney, gut, leg muscle, lung and heart—from 50 % higher in kidney, up to 350 % of values in heart from awake animals. Oxidized glutathione was 2 to 10 % of the total pool in most tissues but much higher (18 to 24 %) in lung. GSH levels were raised following estivation in liver, and were lower in lung, muscle and heart. Distribution of GSH in the tissues (in order, down table 2.1.1) was: 94:76:44:100:34:61 in estivated tissues and 100:51:36:49:33:36 in tissues from awake animals. The GSSG/GSH ratio, an index of oxidative stress, was lower following estivation in liver, heart, kidney, gut and lung. The GSSG/GSH ratio in lung was three or more times higher than in any other tissue in both estivated and awake animals.

Tissue soluble protein

Enzyme activities were expressed relative to tissue soluble protein in order to factor out any effect of tissue water loss which could result in large apparent increases in enzyme activities during estivation if activities were reported per gram wet weight. Fig. 2.1.2 shows that soluble protein content was significantly higher in all tissues from estivated animals except gut, when compared with corresponding levels in awake toads. Protein contents of liver and muscle of awake toads were not significantly different from those of frog (*Rana pipiens*) tissues.

Two different assay methods were employed to assess xanthine oxidase and xanthine dehydrogenase activities in toad tissues. Using the spectrophotometric assay, XO activity was 0.255 ± 0.054 μmol urate formed/min/mg protein ($n = 4$) in liver of awake toads, but the enzyme was undetectable in liver of estivated toads. Using the fluorometric assay of Beckman *et al.* (1989), however, XO activity was determined to be 0.306 ± 0.037 ($n = 5$) and 0.644 ± 0.083 ($n = 11$) nmol isoxanthopterin/min/mg protein

for estivated and awake liver, respectively (the value for liver of estivated toads was significantly lower, $P < 0.025$). Total XO and XDH activity was 7.01 ± 0.74 ($n = 5$) and 10.28 ± 0.70 ($n = 11$) nmol isoxanthopterin/min/mg for estivated and awake liver, respectively, again significantly lower in the estivated case ($P < 0.025$). XO alone represented 4 % of the total activity in estivated liver, and 6 % of the total activity in liver of awake animals. Neither XO nor (XO + XDH) activity was detectable in other tissues using either method ($n = 3$ for the spectrophotometric method, $n=1$ for the fluorometric method).

Antioxidant enzyme activity

Table 2.1.2 shows maximal activities of antioxidant enzymes in liver, heart and kidney of estivated and awake toads, expressed per mg soluble protein in the tissue. These three tissues showed major changes in AOE activities between estivated and awake states. They also had higher activities of GST, GR, SOD and CAT than leg muscle, gut and lung.

With the exception of SOD, liver from awake toads exhibited higher activities of all AOE than the corresponding values for estivated toads; values were 170 to 300 % of the values recorded for tissues of estivated animals (Table 2.1.2). SOD activity, however, was 2-fold higher in liver from estivated, compared with awake, toads. Toad liver had the highest activities of GST, SOD and CAT of all the tissues studied, in accordance with the liver's primary function of detoxification (GST activity) and generation of the most ROS of any tissue (SOD and CAT activity). AOE activities in toad liver were also compared with those in liver of a semi-aquatic, non-estivating frog, *R. pipiens*. Liver from awake toads had similar activities of GST, Se-dep GP_{ox}, two-thirds the GR and total GP_{ox}, and twice the CAT activity of *R. pipiens* liver, however SOD activity in awake *S. couchi* liver was 400 % higher than in frog liver.

Heart (Table 2.1.2) also showed higher maximal AOE activities following estivation; AOE activity in awake toads ranged from 190 to 340 % of the corresponding values for CAT, GR, total GP_{ox}, GST and SOD from tissues of estivating toads. Only Se-dependent GP_{ox} activity was unchanged by the experimental state of the animal.

By contrast with the generally higher activities of AOE in liver and heart of awake toads, compared with estivated animals, results for kidney were more variable. GST and GR activity in kidney from estivated animals was 1.5-fold higher and SOD activity was 2-fold higher than the corresponding values for awake animals, but CAT and total GP_{ox} activity were 62 % and 50 %, respectively, of the activity in kidney of awake toads; Se-dep GP_{ox} activity was not different between the two states. Kidney had GST activity 200 % to 1100 % higher and CAT activity at least 1000 % higher than gut, heart, lung or leg muscle, and also the highest GR activity of any tissue.

Table 2.1.3 shows antioxidant enzyme activities in leg muscle, gut and lung of estivated and awake toads. These tissues exhibited generally lower AOE activity during both estivated and awake states, than liver, heart and kidney, except for GP_{ox} activity (Se-dependent and total) which was highest in gut and lung. Muscle and gut may have generally lower antioxidant needs than other tissues. Lung is the tissue that always sees the highest oxygen tensions, and maintains specific defences against these, but gular movements indicating breathing are greatly reduced in estivating toads (Seymour, 1982). With few exceptions AOE activities were generally unchanged between awake and estivated states.

SOD and total GP_{ox} activities changed from estivation to arousal in toad hind leg muscle; activity of both enzymes in awake toads was 70 % and 62 % of the value in estivated toads, respectively. No other antioxidant enzyme activity changed between estivated and aroused states in toads. Leg muscle had the lowest activities of GST, GR, GP_{ox} and CAT, relative to other toad tissues, and was the only tissue which did not show reduced GST activity during estivation. However, except for GR, AOE activities in toad

leg muscle were consistently 3- to 24-fold higher than in leg muscle of *R. pipiens*, indicating a significantly higher antioxidant capacity of the skeletal muscle of the estivating species.

Selected effects of estivation were seen in lung. Only GST and total GP_{ox} increased significantly following estivation by 0.5 -fold and 1.3-fold over activity in estivated toads. Lung had low activities of GST, GR and CAT (relative to other tissues), and characteristically low amounts of SOD, as previously reported for amphibians (Perez-Campo, 1993), that were at most one tenth the activity in liver, heart, kidney and leg muscle.

Gut from toads (combining stomach and intestine) had 100 % higher GST and Se-dep GP_{ox} activity following estivation, but activities of other enzymes were unchanged by experimental state. Total GP_{ox} activity was not significantly different despite the decrease in Se-dep GP_{ox} activity.

Effect of urea and/or KCl on AOE activities

As a result of dehydration during estivation, concentrations of all ions increase in toad body fluids (major ions like K and Na can reach about 200 mM in intra- versus extra-cellular fluids, respectively). In addition, the animals specifically synthesize urea to help retard water loss, accumulating up to 300 mM urea in both extracellular and intracellular spaces. Hence, both the ionic strength and osmolality of toad body fluids is greatly increased during estivation (Flanigan *et al.*, 1993). These parameters could potentially have significant effects on the function of AOE since the activities of many enzymes are sensitive to elevated ion concentrations and urea is commonly used in vitro as a protein denaturant. To determine whether AOE from awake or estivated toads were differentially sensitive to the levels of urea and salt that would be present in the estivated state, selected enzymes (GST, CAT, GR) were assayed under optimal substrate conditions but with the addition to the assay of 200 mM KCl, 300 mM urea, and both

solutes mixed. These assays were conducted concurrently with the assays reported in Tables 2.1.2 and 2.1.3, and hence, the data in these tables can be regarded as control conditions for these studies of salt and urea effects. With three exceptions, the addition of physiological levels of urea (300 mM) to the $V_{\max}$ activity assays had no effect ($n = 3$ to 6) on the measured maximal activities of CAT, GST or GR in tissue from either awake or estivated toads. However, addition of urea inhibited GR activity in kidney of awake toads, reducing it to 26.1 ± 1.24 mU/mg protein (a 20 % reduction), and also lowered lung GST activity to 0.15 ± 0.03 U/mg protein (a 19 % inhibition). Urea did not have inhibitory effects on these same enzymes from tissues of estivated animals. The presence of urea also reduced CAT activity in heart which was 37.9 ± 3.95 U/mg protein (a 25% inhibition) in the presence of 300 mM urea; however, urea did not affect CAT from heart of awake toads.

$V_{\max}$ values for GST and CAT assayed in the presence of 200 mM KCl are shown in Table 2.1.4. Except for gut GST, activity of all enzymes was significantly reduced in the presence of salt, as compared with $V_{\max}$ values for assays without KCl shown in Table 2.1.2 and 2.1.3. In the presence of KCl, maximal GST activity was reduced by 55 ± 5 % in five tissues. Maximal CAT activity was reduced by 51 ± 2 % in the presence of KCl in six tissues. Virtually equivalent values for percent inhibition by KCl were observed in corresponding assays conducted with tissue extracts from estivated toads; the mean percentage inhibition was 54 ± 4 % and 52 ± 4 % for GST and CAT, respectively (data not shown). GR was inhibited by KCl only in liver: the $V_{\max}$ of the enzyme from awake toads was reduced to 6.04 ± 1.25 mU/mg protein (a 40 % inhibition) whereas enzyme activity in extracts from estivated toads was lowered to 2.42 ± 0.23 mU/mg protein (a 25 % inhibition).

When both 300 mM urea and 200 mM KCl were combined as additives in the assay for $V_{\max}$ the effects of the two compounds were found to be additive in cases where both compounds were inhibitory. Where no effect of urea alone had been found, enzyme

activities with 300 mM urea and 200 mM KCl were the same as those observed in the presence of 200 mM KCl alone.

Table 2.1.1. Glutathione status in *Scaphiopus couchi* tissues after 2 months

estivation or following 10 days arousal after estivation. Values are nmol/gram fresh weight, means $\pm$ S.E.M. for $n = 3$ (heart), $n = 4$ (kidney, lung) and $n = 6$ (gut, muscle, liver) samples. ^a, Significantly different from the corresponding value for estivated toads by the Student's t-test, $P < 0.025$; ^b, $P < 0.005$.

	Estivated	Awake
Liver		
GSH-eq	1483 $\pm$ 81	1710 $\pm$ 39 ^a
GSH	1311 $\pm$ 66	1546 $\pm$ 40 ^a
GSSG	86.0 $\pm$ 7.8	81.8 $\pm$ 3.1
GSSG/GSH	0.065 $\pm$ 0.003	0.053 $\pm$ 0.003 ^a
Heart		
GSH-eq	1213 $\pm$ 51	826 $\pm$ 41 ^b
GSH	1063 $\pm$ 60	792 $\pm$ 40 ^a
GSSG	74.9 $\pm$ 6.6	16.9 $\pm$ 1.2 ^b
GSSG/GSH	0.073 $\pm$ 0.01	0.021 $\pm$ 0.001 ^a
Kidney		
GSH-eq	756 $\pm$ 89	653 $\pm$ 32
GSH	615 $\pm$ 76	564 $\pm$ 31
GSSG	70.2 $\pm$ 7.9	44.3 $\pm$ 2.3 ^a
GSSG/GSH	0.116 $\pm$ 0.009	0.079 $\pm$ 0.005 ^a
Leg muscle		
GSH-eq	1543 $\pm$ 32	838 $\pm$ 27 ^b
GSH	1394 $\pm$ 37	765 $\pm$ 27 ^b
GSSG	74.3 $\pm$ 6.1	36.6 $\pm$ 2.5 ^b
GSSG/GSH	0.054 $\pm$ 0.005	0.048 $\pm$ 0.004
Gut		
GSH-eq	572 $\pm$ 34	557 $\pm$ 55
GSH	473 $\pm$ 32	504 $\pm$ 48
GSSG	49.5 $\pm$ 4.1	26.5 $\pm$ 3.7 ^b
GSSG/GSH	0.108 $\pm$ 0.01	0.052 $\pm$ 0.004 ^b
Lung		
GSH-eq	1613 $\pm$ 59	870 $\pm$ 42 ^b
GSH	852 $\pm$ 30	563 $\pm$ 41 ^b
GSSG	381 $\pm$ 20.8	153 $\pm$ 10.0 ^b
GSSG/GSH	0.45 $\pm$ 0.03	0.28 $\pm$ 0.03 ^b

Table 2.1.2. Antioxidant enzyme activities in liver, heart and kidney of *S. couchi*, and liver of *R. pipiens*. Values are means $\pm$ S.E.M., n = 4-9 for liver, or n=3 for heart and kidney. ^a = Significantly different from the corresponding value for estivated loads by the Student's t-test, $P < 0.05$ ^b = $P < 0.025$.

Tissue		GST	GR	GPox (Se-dep)	GPox (total)	SOD	CAT
<i>Scaphiopus couchi</i>							
liver	estivated	0.78 $\pm$ 0.08	3.24 $\pm$ 0.33	31.9 $\pm$ 8.36	35.4 $\pm$ 3.34	104 $\pm$ 10.3	670 $\pm$ 40
	awake	1.61 $\pm$ 0.09 ^b	10.1 $\pm$ 1.79 ^b	75.4 $\pm$ 13.4 ^a	59.8 $\pm$ 4.57 ^a	49.6 $\pm$ 8.27 ^a	1270 $\pm$ 140 ^b
heart	estivated	0.14 $\pm$ 0.01	18.2 $\pm$ 1.6	28.5 $\pm$ 1.9	30.7 $\pm$ 2.5	31.9 $\pm$ 6.3	51.5 $\pm$ 3.4
	awake	0.29 $\pm$ 0.06 ^a	27.1 $\pm$ 1.3 ^a	29.4 $\pm$ 5.7	53.1 $\pm$ 7.5 ^a	67.7 $\pm$ 9.2 ^a	73.3 $\pm$ 11.6 ^a
kidney	estivated	0.97 $\pm$ 0.09	20.0 $\pm$ 2.1	59.0 $\pm$ 12.5	34.1 $\pm$ 5.8	33.8 $\pm$ 3.7	1910 $\pm$ 260
	awake	1.56 $\pm$ 0.18 ^b	32.9 $\pm$ 1.7 ^a	29.6 $\pm$ 4.3 ^a	39.1 $\pm$ 12.4	49.7 $\pm$ 3.6 ^a	1160 $\pm$ 60 ^b
<i>Rana pipiens</i>							
liver		1.15 $\pm$ 0.05	15.48 $\pm$ 1.41	70.0 $\pm$ 10.1	91.7 $\pm$ 24.1	11.14 $\pm$ 0.85	0.488 $\pm$ 0.060

Table 2.1.3. Antioxidant enzyme activities in leg muscle, lung and gut of *S. couchi* and leg muscle of *R. pipiens*. Values are means $\pm$ SEM. n = 4 to 6 for muscle, 4 for lung, and 6 for gut. Other information as in Table 2.1.2.

Tissue		GST	GR	GPox (Se-dep)	GPox (total)	SOD	CAT
<i>Scaphiopus couchi</i>							
leg muscle	estivated	0.11 $\pm$ 0.01	2.77 $\pm$ 0.22	11.5 $\pm$ 1.50	12.1 $\pm$ 0.8	44.9 $\pm$ 4.0	38.3 $\pm$ 4.7
	awake	0.13 $\pm$ 0.02	2.98 $\pm$ 0.41	8.30 $\pm$ 1.81	7.47 $\pm$ 1.77 ^a	31.6 $\pm$ 3.0 ^a	34.9 $\pm$ 3.3
lung	estivated	0.12 $\pm$ 0.01	8.42 $\pm$ 1.3	79.5 $\pm$ 13.1	26.5 $\pm$ 6.5	2.29 $\pm$ 0.09	99.2 $\pm$ 7.1
	awake	0.18 $\pm$ 0.02 ^a	10.7 $\pm$ 0.9	84.4 $\pm$ 4.7	62.0 $\pm$ 7.5 ^a	3.57 $\pm$ 0.79	115 $\pm$ 18
gut	estivated	0.23 $\pm$ 0.02	17.3 $\pm$ 0.87	44.8 $\pm$ 4.8	42.6 $\pm$ 3.52	13.1 $\pm$ 1.43	50.1 $\pm$ 5.29
	awake	0.49 $\pm$ 0.08 ^a	19.3 $\pm$ 2.34	90.5 $\pm$ 10.2 ^b	54.8 $\pm$ 7.7	16.4 $\pm$ 1.5	58.2 $\pm$ 8.96
<i>Rana pipiens</i>							
leg muscle		0.051 $\pm$ 0.003	4.56 $\pm$ 0.81	1.12 $\pm$ 0.09	0.72 $\pm$ 0.12	1.27 $\pm$ 0.07	2.47 $\pm$ 0.56

Table 2.1.4. Activities of GST and CAT in various tissues, with 200 mM KCl added to the assay. Assay conditions were as described in Materials and Methods. KCl was added prior to measurement to a final concentration of 200 mM. Values presented are means $\pm$ SEM, $n = 3$ for heart and kidney, $n = 4$ to 9 for liver, lung and muscle, and $n = 6$ for gut (stomach and intestine combined). ^a = Significantly different from the corresponding value for awake toads with 0 M KCl by the Student-Neuman-Keuls test, $P < 0.05$; ^b, $P < 0.005$. Similar results for both enzymes were obtained with tissues from estivated toads.

Tissue (awake toads)	GST (U/mg)	CAT (U/mg $\times 10^{-3}$)
liver	1.01 ± 0.11^b	0.49 ± 0.08^b
heart	0.12 ± 0.03^b	0.024 ± 0.004^b
kidney	0.83 ± 0.08^b	0.356 ± 0.047^b
leg muscle	0.040 ± 0.01^b	0.018 ± 0.002^a
lung	0.066 ± 0.004^b	0.042 ± 0.005^b
gut	0.35 ± 0.07	0.021 ± 0.003^b

Fig. 2.1.1 Tissue protein levels in mg protein/g fresh weight in estivated (■) and awake (□) toads. L = liver; M = muscle; H = heart; K = kidney; Lng = lung; G = gut. ^b. significantly different from corresponding value from estivated tissue as assessed by the Student's t test, $P \leq 0.01$.

DISCUSSION

Toads enter dormancy with a large reserve of body water (chiefly in the bladder) but as the soil dries out over several months the animals begin to desiccate. When dehydrated very slowly, *S. couchi* can tolerate the loss of 47-50% of total body water (McClanahan, 1967). Metabolism during estivation is primarily fueled by lipid oxidation but when body water loss becomes substantial, toads also increase protein catabolism and accumulate the nitrogenous end product, urea, in body fluids. Urea concentrations of up to 300 mM raise the osmolality of body fluids to help retard further water loss. In *S. couchi*, much of the water lost during dehydration can be accounted for by water loss from extracellular and extra-organ fluid compartments (including fluids in the abdominal cavity and bladder); this is also illustrated by the decreased blood pressure and increased viscosity of the blood at high levels of dehydration (McClanahan, 1967). Studies with other species have shown that water loss from tissues is strongly defended during dehydration and changes in organ water contents are often not apparent until about 25 % of total body water has been lost (Churchill and Storey, 1993, 1995). Nonetheless with extensive dehydration, organ water contents also fall (Flanigan *et al.*, 1993; Churchill and Storey, 1993, 1994, 1995). As the consequence of tissue dehydration, tissue protein concentration would rise and this is undoubtedly the reason for the higher protein concentrations measured in tissues from estivated toads, as compared with awake animals. Altered enzyme activity during estivation could be an artifact of changing tissue water content, so AOE activity was expressed per mg of protein to highlight estivation-specific changes.

Glutathione status

Glutathione is synthesized primarily in liver (Meister, 1983) and is exported in the blood to other tissues (Sies *et al.*, 1983). Liver is a frequently studied organ with regard to antioxidant protection since it generates the most free radicals of any organ in the body.

and thus is both the source of antioxidant equivalents (glutathione) and the primary source of the ROS that glutathione detoxifies. Soluble antioxidants such as GSH comprise an essential part of the antioxidant defences of the liver, and GSH levels in liver are generally the highest of all organs. The GSH levels reported in Table 2.1.1 for liver of estivated and awake *S. couchi* agree well with previous reports for liver of other anurans: *Xenopus laevis* (1000 nmol/gfw), *Rana sylvatica* and *R. pipiens* (1332 and 969 nmol/gfw). However, they were lower than levels in *R. perezi* liver (2250 nmol/gfw) (López-Torres *et al.*, 1993; Joannis and Storey, 1996). Compared to GSH in mammalian tissues, reduced glutathione levels in amphibian liver were low, being only about 1/5 of the levels observed in rat or guinea pig (López-Torres *et al.*, 1993). Hepatic GSSG levels of anurans appear to be more variable; the values of 82-86 nmol/gfw in *S. couchi* were similar to values of 85-100 nmol/gfw reported for *X. laevis* and *R. perezi* (López-Torres *et al.*, 1993) but were much higher than GSSG levels in *R. sylvatica* and *R. pipiens* (1-12 nmol/gfw) (Joannis and Storey, 1996).

GSH and GSSG content of heart from awake *S. couchi* and from *R. sylvatica* were very similar. However, with the exception of *R. sylvatica* kidney, which had 200 % higher GSH than *S. couchi* kidney, GSH and GSSG levels in *S. couchi* tissues were the same as, or higher than levels in other amphibians. For instance, GSH content of lung of aroused *S. couchi* was in agreement with values for *R. perezi* (López-Torres *et al.*, 1993) but GSH content of leg muscle was 400 % and 1000 % higher in *S. couchi* than in muscle from *R. sylvatica* or *R. pipiens* (Joannis and Storey, 1996). In toads, leg muscle GSH content was similar to other tissues, but in *R. sylvatica* and *R. pipiens*, relative levels of glutathione in skeletal muscle were 1/5 of the values in other tissues. *S. couchi* kidney and leg muscle had up to twenty times higher GSSG levels than in *R. sylvatica* or *R. pipiens* (Joannis and Storey, 1996). GSSG content in estivated and aroused *S. couchi* lung was 250 % and almost 800 % higher than levels in *R. perezi* and was closer to levels reported in guinea pig or canary (López-Torres *et al.*, 1993). Higher GSSG content in *S.*

couchi tissues, compared to other sources, indicates perhaps poor conversion of GSSG to GSH in toads, or, conversely, a high tolerance or requirement for oxidized glutathione in toad tissues, relative to tissues from other amphibians.

Oxidized glutathione is primarily formed by detoxification of H_2O_2 by GP_{ox}. As such, an increase the content of oxidized glutathione in a tissue is an indication that more H_2O_2 has been detoxified. However, oxidized glutathione itself can react to form protein-SS-glutathione mixed disulfides or protein-SS-protein disulfides (Chung *et al.*, 1991). While some studies show that the spontaneous oxidation of protein thiol groups in the presence of high concentrations of GSSG may constitute a last defence against oxidative stress (Del Corso *et al.*, 1994), most studies confirm that formation of disulfide bridges or mixed disulfides may inactivate proteins and that formation of disulfide bonds targets proteins for breakdown by proteases such as the multicatalytic proteinase complex (Dean *et al.*, 1993). Formation of disulfide bonds and mixed disulfides can adversely affect the function of numerous cellular proteins, for example coenzyme A (Mannervik and Larson, 1981), pyruvate kinase, phosphofructokinase, PEP-carboxykinase, and glycerol-3-phosphate dehydrogenase, as well as the NADPH-requiring enzymes hydroxymethyl glutaryl CoA reductase, fatty acid synthase and aldose reductase (Del Corso *et al.*, 1994). In addition, oxidation by GSSG may disrupt the ionic homeostasis of cells (via conformational change of membrane transport proteins). This leads to extensive membrane and cell damage and gross exacerbation of the initial injury (Geiger, 1993; Ji, 1992). To prevent this happening, GSSG is quickly reduced by GR back to 2 GSH. In situations of oxidative stress, GSSG accumulation is also prevented by direct export of GSSG from the liver into the bile (Meister, 1982). This may explain why liver was the only tissue that did not have a higher GSSG level during estivation than when the toad was awake. During estivation, GSSG was up to 350 % higher than the corresponding awake values in lung, heart, kidney, leg muscle and gut of toads. The presence of higher tissue GSSG content during estivation was similar to results observed in hepatopancreas

(an organ functionally analogous to liver) and foot muscle of estivating land snails *Otala lactea* (Hermes-Lima and Storey, 1995b).

Higher cellular levels of GSSG in estivating toads, compared to awake toads, indicates that the estivated toad was more vulnerable to disulfide-induced modulation of the activity of many cellular proteins, including key enzymes of the glycolytic cycle. Why, then, does the toad allow accumulation of GSSG? One explanation could be that there might be an adaptive advantage to promoting protein modification by disulfide bond formation during estivation, to achieve altered protein function or regulation. Since the formation of disulfide bonds is reversible (cleavage is catalyzed by a disulfide reductase) conjugation of GSSG to cellular proteins could allow a readily reversible modulation of toad enzymes during estivation. This mechanism also might serve to reduce GSSG content *in vivo*. The possibility of regulation of specific enzyme pathways by reversible modification by thiol compounds has been discussed by Ziegler (1985) and by Del Corso *et al.* (1994). Specific impairment of glycogen phosphorylase phosphatase (Usami *et al.*, 1980), glycogen synthase (Lau and Thomas, 1983) and other enzymes of the glycolytic pathway by GSSG, as well as O₂-dependent glutathione activation of the pentose phosphate pathway in several tissues (Del Corso *et al.*, 1994) implies a role for oxidized glutathione in the activation of cellular processes which produce reducing equivalents in situations with high GSSG (i.e. oxidative stress). Control of cellular processes by thiol oxidation-reduction reactions has not been tested, however, and remains an interesting theory, which, if proven, might account for the presence of high GSSG levels in estivated toad tissues.

Measured changes in the glutathione concentrations of some tissues in the transition from the estivated to the rehydrated, awake toads may have also been partially due to tissue dehydration during estivation. However, cellular dehydration would not alter GSSG/GSH ratio. An increased GSSG/GSH ratio indicates increased susceptibility to oxidative damage, either from a diminution of the scavenging properties of GSH

(Palamanda, 1992) or an increased risk of oxidation by GSSG (Del Corso *et al.*, 1992). In heart, kidney, gut and lung, increased cellular GSSG was at least partially responsible for an increase in the GSSG/GSH ratio (Fig. 2.1.2). GSSG/GSH was 21 % (liver), 50 % (kidney), 60 % (lung), 108 % (gut), and 240 % (heart) higher in tissues of estivating toads, compared to the corresponding tissue from awake animals. The GSSG/GSH ratio in liver of awake *S. couchi* was similar to that calculated for *R. perezii* but lower than the ratio in *X. laevis* (López-Torres *et al.*, 1993). GSSG/GSH was four-fold higher than in liver of *R. sylvatica* and tenfold higher than in liver of *R. pipiens*, mainly due to much lower hepatic GSSG levels in these animals (Joanisse and Storey, 1996). Hepatic GSSG/GSH ratio for *R. sylvatica* and *R. pipiens* were more similar to the GSSG/GSH ratios of mammalian liver (0.003-0.0025) (Chung *et al.*, 1991). The GSSG/GSH ratio was also higher for kidney and leg muscle from aroused and estivated *S. couchi*, than the corresponding tissues from *R. sylvatica* and *R. pipiens*, also due to low GSSG levels in *R. sylvatica* and *R. pipiens*. However, GSSG/GSH for heart from awake *S. couchi* was similar to *R. sylvatica* (Joanisse and Storey, 1996).

The lung is continuously exposed to high oxygen tension and has a high rate of generation of ROS (Barga de Quiroga, 1988). In toads, GSSG in lung was higher in estivated and awake animals than in any other tissue. Lung GSSG/GSH was 400 % higher than GSSG/GSH in liver of aroused toads. By comparison, GSSG/GSH ratio of lung compared to liver was 300 % higher for guinea pig, 700 % higher for pigeon, 75 % higher for the frog *R. perezii*, and slightly lower than GSSG/GSH for the toad *X. laevis* (López-Torres *et al.*, 1993). The glutathione ratio for lung was higher in *S. couchi* than in *X. laevis* or *R. perezii* but was approximately equivalent to ratios in guinea pig, rat, canary and pigeon (López-Torres *et al.*, 1993). Thus, a wide variation exists for glutathione status for lung among vertebrate species, and apparently the maintenance of the GSSG/GSH ratio at a low level is not as high a priority as it is in liver.

The presence of a relative increase in oxidizing potential (as GSSG) in five out of six tissues of estivating animals is an indirect indication that greater oxidative stress occurred in this state (Ji and Fu, 1992), as compared with the awake state assessed ten days after arousing the toads and giving them food and water. This is the opposite to the effect observed in tissues of animals subjected to restricted oxygen availability. In situations where oxygen availability is limited or cut off entirely (e.g. ischemia) the GSSG/GSH ratio is lower during stress (Ambrosio *et al.*, 1992) and rises as a consequence of recovery. But for the toad, dormancy is not associated with decreased oxygen availability, but just by reduced oxygen consumption brought on by a concerted suppression of all metabolic processes. It is also worth noting that several studies originating from this lab have indicated either increased or unchanged GSSG/GSH ratio upon exposure to stress, and lower or again unchanged GSSG/GSH ratio upon recovery, in snails during estivation, garter snakes during freezing and anoxia, frogs during bouts of freezing and turtles enduring and recovering from anoxia (Hermes-Lima and Storey, 1993, Hermes-Lima and Storey, 1995b, Joannis and Storey, 1996, Willmore and Storey, in press)

Antioxidant enzymes

Since the ETC is the primary site for leakage of electrons onto O₂, the rate of endogenous production of ROS is directly related to oxygen consumption. Thus, reduced oxygen consumption during estivation would presumably lead to a lower rate of formation of ROS. ROS themselves induce gene expression of AOE (Cowan *et al.*, 1992) via redox-sensitive protein second messengers (Kapoor *et al.*, 1990). Maximal activities of AOE in toad tissues were generally lower in estivated toads than in awake toads, perhaps as a result of fewer ROS present in the estivated state. The reduced state of enzymatic antioxidant defences in the estivating toad, relative to awake toads, contrasts with antioxidant preparedness in the freeze-tolerant frog *R. sylvatica* (Joannis and

Storey, 1996). This frog maintains high activities of AOE, particularly GP_{ox}, in preparation for oxidative stress incurred during thawing: repeated cycles of freeze/thaw may occur during the winter. The toad's antioxidant preparedness in estivation mimics the response to reduced oxygen tension seen in mammalian systems under situations of reduced oxygen availability, namely reduced activities of key antioxidant enzymes such as CAT and GP_{ox}, and for some tissues, SOD as well (Cowan *et al.*, 1992). This response of mammalian systems has been closely linked to oxidative damage during reperfusion following ischemia or reduced oxygen availability (Cowan *et al.*, 1992). Chapter 2.2 of this thesis explores whether decreased AOE protection during estivation results in oxidative damage.

AOE activities in *S. couchi* were highest in liver, heart and kidney, and low in muscle, gut and lung, except for GP_{ox} activity which was roughly the same in 5 tissues and low in skeletal muscle. This distribution was similar to that observed in corresponding tissues of *R. perezi*, *R. pipiens* and *R. sylvatica* (Perez-Campo *et al.*, 1993; Tables 2.1.2 and 2.1.3; Joannis and Storey, 1996). The distribution of AOE in *S. couchi* was also similar to the distribution in mammals as well, indicating that tissue-specific distributions are maintained between species (Perez-Campo *et al.*, 1993; Tappel *et al.*, 1982).

AOE activities of awake *S. couchi* were generally similar to or higher than AOE from other anuran amphibians. Liver from these terrestrial toads had similar AOE activities to liver from the semi-aquatic *R. pipiens* or *R. sylvatica* (GST, GR, SOD) (Table 2.1.2; Joannis and Storey, 1995) and *R. perezi* (SOD, GP_{ox} and CAT), but higher activities than in the aquatic species *X. laevis* (Perez-Campo *et al.*, 1993). Lung AOE activities were also similar between *S. couchi* and *R. perezi* (Perez-Campo *et al.*, 1993). *S. couchi* leg muscle had higher AOE activities than *R. pipiens* leg muscle, and roughly equivalent activities to *R. sylvatica* leg muscle (Table 2.1.2; Joannis and Storey,

1996). Heart and kidney from awake *S. couchi* also had similar GST, GR and SOD activity to the corresponding tissues of *R. sylvatica* (Joannis and Storey, 1996).

Most AOE activities were similar between *R. sylvatica* and *S. couchi*, however the relative activities of H_2O_2 -detoxifying enzymes were different. CAT in spadefoot toad liver, kidney and heart was 3- to 10-fold higher than in *R. sylvatica*, but Se-dep. GP_{ox} activity in the same tissues was one-third to one-fifth of activity in the wood frog (80 to 220 U/mg protein; Joannis and Storey, 1996). Total GP_{ox} activity was also lower in spadefoot toads than in tissues of freeze-tolerant frogs (100 to 300 U/mg protein; Joannis and Storey, 1996) and 2/3 of the activity in *R. pipiens* liver (Table 2.1.2) but tenfold higher in leg muscle (Table 2.1.3). GP_{ox} activity is generally much lower than CAT activity but is considered more important for H_2O_2 detoxification since the enzyme catalyzes the breakdown of both hydrogen peroxide and lipid hydroperoxides (Flohé, 1982) and has a lower K_m for H_2O_2 than catalase (Pérez-Campo *et al.*, 1993). In addition, CAT is mainly localized in peroxisomes (Michiels *et al.*, 1994, Aebi, 1974), although this is not true for all animals (Himeno *et al.*, 1993), whereas GP_{ox} distribution is mainly in the cytosol. Thus, GP_{ox} is preferentially available to detoxify endogenously produced H_2O_2 , whereas CAT activity is compartmentalized and specific to high concentrations of H_2O_2 . However, catalase activity is not dependent on GSH, so in situations where lowered GSH availability could limit GP_{ox} activity, catalase removal of H_2O_2 could become much more important. It is important to note that freeze-tolerant frogs have higher GP_{ox} activity than many other amphibians, whereas GP_{ox} activity in spadefoot toads is not lower than average levels for other frogs. Thus, GP_{ox} in toads is not 'low' *per se*, only lower than *R. sylvatica* GP_{ox} activity.

Removal of one subset of antioxidants, for instance by glutathione depletion or catalase inactivation results in the concomitant activation of other ROS-detoxifying systems (López-Torres *et al.*, 1991, Dhaunsi *et al.*, 1993). The guinea pig has markedly low GP_{ox} activity in all its tissues, compared with other mammals. However, catalase

activity in this species was 2-3 times the activity of this enzyme in tissues of mice or rats (Himeno *et al.*, 1993). A high proportion of CAT activity was found in the cytosol of guinea pig tissues, instead of compartmentalized in peroxisomes, suggesting a compensatory role of this enzyme in detoxification of H_2O_2 (Himeno *et al.*, 1993). Possibly CAT activity in toads is not limited to peroxisomes and CAT activity complements GP_{ox} activity in the cytosol.

S. couchi leg muscle, which has much higher concentrations of both Se-dependent and total GP_{ox} than *R. pipiens* leg muscle, also has tenfold higher activity of CAT.

Glutathione enzymes - GR and GST

While not actively removing radical species, GR activity impacts on the function of other antioxidant enzymes, by furnishing GSH to serve as a substrate for the glutathione-consuming enzymes. Lower GR activity in tissues from estivated, versus awake, toads was correlated with a higher GSSG/GSH ratio for liver, heart and kidney, compared to tissues of awake toads. GR in tissues from estivated animals did not maintain a constant GSSG/GSH ratio between estivation and arousal, rather this ratio was reduced in awake toads. During estivation, GR did not permit the accumulation of more than 86 nmol/g fresh weight of GSSG in any tissue (with the notable exception of lung). The function of GR in tissues of estivated animals may be to prevent GSSG accumulation beyond a minimum level, but not necessarily maintain GSSG/GSH ratio, because of the metabolic cost involved as NADPH is used.

GR is redox sensitive; the enzyme is noncompetitively inhibited by physiological concentrations of GSH (Mutus *et al.*, 1992) and activated by GSSG (Chung *et al.*, 1991). Thus although GR is an equilibrium enzyme, backwards flow converting GSH and $NADP^+$ to GSSG and NADPH would probably not occur. The relatively higher concentration of GSSG in tissues of the estivating toad would not inactivate but protect

GR, yet activity in estivation was markedly lower than in awake animals, which may indicate regulation of GR activity by other methods.

The activity of another GSH-utilizing enzyme, GST, was substantially lower in tissues of estivated toads - about 50 % lower in five tissues compared to awake toads (Tables 2.1.2 and 2.1.3). GST is a multiclass family of enzymes whose primary functions are detoxification of xenobiotics (primarily non-endogenous pollutants such as chlorobenzene, which are not soluble in the aqueous phase) and the production of some prostaglandins. By conjugation of GSH to a xenobiotic, its water solubility is enhanced, aiding export from the cell. One class of GST (α) has peroxidase activity in addition to other activities and is measured together with Se-dep. GP_{ox} in the assay with cumene hydroperoxide. The detoxification activity of GST may be lowered during estivation (likely gene expression is down-regulated) because the toad is a closed system during estivation, except for gas exchange. Since the animals are not feeding, there are no ingested xenobiotics to be excreted (Shoemaker, 1969) and the requirement for GST would be reduced. GST is also inhibited by high concentrations of GSSG and thus may be further inhibited *in vivo* during estivation by metabolite regulation.

It might be expected that a shift to antioxidant systems not dependent on reduced glutathione could occur upon an increase of the GSSG/GSH ratio in most tissues. Compared to awake toads, all tissues from estivated toads generally exhibited either lower GSH-utilizing AOE activity (GST, GP_{ox}) or higher non-GSH-utilizing AOE activity (CAT, SOD). Liver, muscle and kidney in estivated toads had higher activities of non-GSH-utilizing AOE; liver and muscle had higher SOD activity, and kidney had higher CAT activity than was measured in tissues of awake toads. These changes indicate tissue-specific inductive adaptations to estivation, which are altered upon arousal, changing the relative activity patterns of AOE, and possibly decreased dependence on reduced glutathione as an antioxidant. Apart from the lower GP_{ox} activity compared to *R. sylvatica* discussed above, spadefoot toad tissues in general also had higher or equal AOE

activities to those seen in other amphibian species (such as higher CAT activity), which might be a constitutive adaptation which supports estivation.

Overall, activity of several AOE was elevated following estivation, implying that maintenance or induction of the activity of these enzymes is not a mechanism used by the toad during estivation to prevent oxidative damage upon recovery.

Xanthine oxidase and xanthine dehydrogenase

XO generates $O_2^{\cdot-}$ and H_2O_2 by reduction of hypoxanthine to xanthine or urate. Ca^{++} -dependent proteolysis of XDH to XO (as occurs during ischemia) results in a higher percentage of the total (XO + XDH) activity present as XO (Palmer, 1987). If this occurs, the greater quantity of $O_2^{\cdot-}$ and H_2O_2 produced could rapidly attack cell components. These would be detoxified by GSH directly or indirectly using GP_{ox} . Both pathways form GSSG, and cellular GSSG levels would be elevated, indicating oxidative stress had occurred. Increased XO activity during estivation, then, could be a convenient explanation for the high GSSG levels which were observed in several tissues of estivating toads. However, XO and XDH activities were so low as to be undetectable in toad tissues except liver. Furthermore, XO activity was the same relative to total (XO + XDH) activity in both estivated and awake toads, which shows no evidence for proteolytic conversion. Activity of both enzymes was higher in toads which had recovered from estivation, indicating that ROS production by XO would be more of a factor in awake toads than in estivated toads. Activities of (XO + XDH) and of XO alone in *S. couchi* liver were approximately ten times higher than activities in *O. lactea* hepatopancreas (Hermes-Lima and Storey, 1995), but the percentage of total activity as XO was similar for the two species, 4-6 % for the toad and 7-10 % for the snail. However, in estivating *O. lactea*, both XO and (XO + XDH) were higher in dormant animals compared with active animals, and the percentage as XO was significantly higher in dormant animals (Hermes-Lima and Storey, 1995). This indicated a potential induction of oxidative stress in *Itala*

lactea; however the authors concluded that the potential of XO to initiate ROS formation in estivation was extremely limited. Since XO and (XO + XDH) were actually lower in estivating *S. couchi*, compared to awake toads, it is even more unlikely that endogenous generation of ROS by XO could cause oxidative stress in estivating animals. The rate of these enzymes is controlled by the availability of their substrates, which are the breakdown products of ATP. While ATP utilization is no doubt decreased during estivation, there is no evidence for large-scale depletion of adenylate pools during estivation which would result in accumulated hypoxanthine and increased ROS production.

Effect of elevated physiological concentrations of urea and salt on antioxidant enzyme activities

The response of toads to dry conditions is similar to the response of several amphibians to high salinity. Elevated ionic strength of surrounding media causes activation of the urea cycle enzymes and concomitant production of urea (Shoemaker *et al.*, 1992). Concentrations of urea in the range of 300-600 mM are known to denature proteins (Hochachka and Somero, 1984) and several authors have considered the potential problem for enzyme function of accumulating high levels of urea in living systems. Sharks maintain high urea (about 1 M) in their body fluids continuously as a mechanism of maintaining osmotic balance with seawater. Several studies of the effects of urea on organisms which accumulate this solute (such as elasmobranches) have indicated either a requirement for urea for normal protein functioning or perturbing effects of urea on the activity and kinetic properties of proteins (Hochachka and Somero, 1984). These effects appeared to be fully countered by the opposing actions of another class of molecules, trimethylamines (termed “counteracting solutes”) when trimethylamines were present at one-half the concentration of urea (Hochachka and Somero, 1984). Thus, during estivation, toads accumulate levels of urea in body fluids

that could potentially affect the functioning of enzymes (Hand and Somero, 1982). To determine whether physiological levels of urea affected the activities of toad antioxidant enzymes, the maximal activities of these were measured with and without the addition of 300 mM urea. In general, however, physiological concentrations of urea had no effect on AOE activities, indicating that low AOE activity during estivation would not be further reduced *in vivo* due to effects of urea on the enzymes. These data, when combined with a previous study that found virtually no effect of urea on the glycolytic regulatory enzymes pyruvate kinase and phosphofructokinase among others (Grundy and Storey, 1994) indicate that urea should be considered as a non-perturbing solute for amphibian cells.

Dehydration during estivation causes passive elevation both intra- and extra-cellular ion concentrations; NaCl may reach 300 mM in plasma and urine of dehydrated amphibians (Hillman, 1978, 1987). Elevated intracellular ionic strength may logically be assumed to follow suit. Estivating Australian frogs (*Neobatrachus wilsmorei*) have elevated plasma sodium levels (150% of control), and elevated intracellular potassium (165 mM from 102 mM in control) (Flanigan *et al.* 1993). The activities of most enzymes are sensitive to ionic strength and many enzymes are sensitive to one or more specific ions. Hence, the elevation of intracellular ion concentrations that results from dehydration during estivation could affect the activity or kinetic properties of various cellular enzymes. Indeed, a previous study on *S. couchii* showed that 200 mM KCl significantly altered the kinetic parameters and maximal velocity of pyruvate kinase and phosphofructokinase (Grundy and Storey, 1994). The present results show that some AOE in toad organs are quite uniformly sensitive to physiological KCl concentrations, which reduce maximal activity by 30 to 50 % in most cases. The same degree of inhibition may not occur *in vivo* under the high protein concentration in cells, but an effect of high salt *in vivo* could be significant in suppressing of AOE activities in tissues during estivation. Addition of 0.2 M KCl to the assay was found to significantly modulate the maximal activity of all antioxidant enzymes, from tissues of both awake and estivated

toads, except for GR and in some cases GP_{ox}. Interestingly, the AOE which appeared insensitive to modulation with increased ionic strength were the enzymes with the lowest activities, which might be considered "bottlenecks" and for which loss of activity would lead to immediate deleterious consequences. Enzymes such as CAT and GST, which have high activities relative to GR and GP_{ox}, may be less resistant to increased ionic strength because significant loss of activity might not result in loss of protective effect.

It has been proposed that one of the reasons for accumulation of various non-perturbing solutes by animal systems under water stress is that these could alleviate or reverse the effects of perturbing salts (Hochachka and Somero, 1982). Urea was generally non-perturbing of toad AOE but did not reverse salt effects on enzymes. Furthermore, in the few cases where AOE activities were modulated by urea, urea effects were additive with KCl. However, the colligative effects of urea concentrations help to retain intracellular water and thereby, prevent the build-up of overly high KCl levels. Thus, high urea *in vivo* would minimize KCl effects on enzymes during estivation.

**2.2 LIPID PEROXIDATION DAMAGE IN TISSUES OF THE ESTIVATING
TOAD, *SCAPHIOPUS COUCHI*.**

INTRODUCTION

Reactive oxygen species have severe deleterious effects on most cell components, damaging DNA, proteins, carbohydrates and lipids. Lipid oxidation results in rancidity and is a problem in the storage of fats and oils; polyunsaturated fatty acids are especially susceptible to attack by reactive oxygen species, and once initiated, the reaction proceeds as a free radical chain reaction (Gutteridge and Halliwell, 1990). Selenium-dependent GP_{ox} activity and the Se-independent GP_{ox} activity of GST are the antioxidant enzyme defences which detoxify carbon-centered hydroperoxides; these constitute total GP_{ox} activity. Reduction in the activities of these enzymes could, therefore, have deleterious effects on a tissue by allowing lipid peroxidative damage to occur.

Studies detailed in Chapter 2.1 examined antioxidant enzyme activities in *Scaphiopus couchi* tissues following 2-months of estivation and subsequent recovery. Differences in the pattern of antioxidant enzyme induction during the stress were evident in toads, as compared with that seen in other stressed systems. For example, freeze-tolerant frogs maintained high enzymatic antioxidant defences during freeze/thaw episodes whereas estivating snails induced AOE activities while stressed (Hermes-Lima and Storey, 1993; Joanisse and Storey, 1996). Only negligible levels of lipid peroxidation damage were observed in these systems, indicating that the antioxidant defences that were put in place adequately prevented large-scale tissue damage during estivation and freezing (Hermes-Lima and Storey, 1993; Joanisse and Storey, 1996). However, spadefoot toads exhibited a change in their AOE activities from estivated to awake states, and increased AOE activity following estivation. In particular, total GP_{ox} activity in liver, heart and lung of estivated toads was higher following estivation. This represents a situation which might result in decreased protection from ROS during estivation, such that damage to cell components could result. We hypothesized that the reduced AOE activity observed in estivated toads could result in oxidative damage to cell lipids during estivation, in amounts significantly greater than would be present in the tissues of awake

toads. To determine whether toads experienced changes in their level of oxidative stress during estivation, the levels of lipid peroxidation products were assessed in toad tissues by several different methods.

The events of hydroxyl radical attack on lipids can be summarized as follows. Hydroxyl radical attack on polyunsaturated fatty acids results in a carbon-centered free radical (reaction 1). Rearrangement of double bonds then leads to the formation of a conjugated diene moiety, which absorbs specifically at 230-235 nm (Fig. 1.2; Halliwell and Gutteridge, 1990). These molecular species can be measured with greater specificity when second-derivative spectra are constructed (Corongiu and Milia, 1983). The carbon-centered radical reacts with O_2 to form a lipid peroxyl radical (reaction 2). The lipid peroxyl radical can attack membrane proteins, react with another lipid peroxyl radical, forming singlet oxygen, or abstract H from an adjacent membrane lipid (reaction 3), forming a lipid hydroperoxide (LOOH). Thus, one initial oxidant is amplified to result in the oxidation of many fatty acid residues.

Accumulation of LOOH residues in membranes allows leakage of Ca^{++} and Na^+ into the cell. LOOH are polar and repartition to the aqueous interface of the lipid bilayer. The resulting bent S-shaped lipid does not maintain bilayer integrity. Leakage of Ca^{++} from outside the cell and from intracellular stores activate Ca^{++} -dependent proteases and ultimately results in the loss of cellular homeostasis. ROS attack on the lipids in organelle membranes also permits leakage of iron and other catalyzing transition metals from intracellular stores, as well as destruction of membrane proteins, which can also liberate transition metals. These metal ions stimulate further peroxidation by decomposing hydroperoxides to radicals that are capable of abstracting hydrogen and continuing the chain reaction (Halliwell and Gutteridge, 1990). In addition, the conjugate acid of

superoxide, peroxy radical ($\text{HOO}^\bullet$), rapidly reacts with LOOH to form $\text{LOO}^\bullet$ and H_2O_2 (Aikens and Dix, 1991). Thus, lipid hydroperoxides are not necessarily an endproduct of the attack but may themselves initiate a free radical chain reaction (reaction 4) (Halliwell and Gutteridge, 1990; Aikens and Dix, 1991):

(4) LOOH (in the presence of Fe^{+2} , Cu^+ , $\text{HOO}^\bullet$) $\rightarrow \text{LO}^\bullet$ or $\text{LOO}^\bullet \rightarrow$ repeat of reaction (3).

Although specific oxygen radicals can be measured directly *in vivo*, quantification of the products of oxidative damage is the more commonly employed means of determining whether ROS are causing damage in a system. In the present study, measurement of the products of lipid peroxidation was used as an indicator of free radical attack on membranes. Three different methods were employed to evaluate the extent of oxidative damage sustained by lipids.

The first method, quantification of thiobarbituric acid-reactive substances (the TBA test), measured the accumulated end products of lipid oxidation. Breakdown products of lipid hydroperoxides are often aldehydes, such as malondialdehyde. These react readily at low pH and high temperature with thiobarbituric acid (TBA), to form a coloured product which can be measured spectrophotometrically. The TBA test is the most widely used method for measuring lipid peroxidation but may also be the most misleading (Halliwell and Gutteridge, 1990), since TBA can react with various other substances, including bile pigments, amino acids and carbohydrates (Gutteridge and Quinlan, 1983). Consequently, to be used as an indicator of lipid peroxidation damage, the TBA test must be accompanied by correlative data from other sources to describe fatty peroxide formation and decomposition (Janero, 1990). The second method employed to measure ROS damage to lipids involved measurement of the concentration of lipid-soluble material having a conjugated diene moiety, which is one of the first configurational changes to take place upon ROS attack on a polyunsaturated fatty acid (Fig. 1.1). Lipid-soluble materials having this functional group may be specifically

quantified by second-derivative spectroscopy at 230-235 nm (Corongiu and Milia, 1983). This technique measures a direct, specific product of lipid oxidation and the use of second-derivative spectroscopy eliminates possible interferences from other compounds present in tissue extracts (Halliwell and Gutteridge, 1990; Corongiu and Milia, 1983).

The third method employed in the present study was direct quantification of lipid hydroperoxides (LOOH) using the recently-developed xylenol orange assay (Jiang *et al.*, 1992) which was adapted for use with heterogeneous tissue extracts (Hermes-Lima *et al.*, 1995c). This assay is based on the oxidation of Fe^{++} by LOOH at acid pH in the presence of the Fe^{+++} -complexing dye, xylenol orange. The Fe^{+++} -xylenol orange complex formed is coloured and absorbs at 560-580 nm; a corresponding decrease in the absorbance peak of xylenol orange at 440 nm is also noted. Cumene hydroperoxide is added to each assay as an internal standard of hydroperoxide equivalents and the levels of LOOH are thus expressed in CHP equivalents. The present study thus assessed both indices of oxidative damage (lipid hydroperoxides) and their products.

METHODS AND MATERIALS

Materials and animals

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) or Boehringer Mannheim Corporation (Montreal, PQ) unless otherwise specified. Spadefoot toads (*Scaphiopus couchi*), males and females of mean body weight 27.42 ± 0.72 g were captured in the Tucson, Arizona area several days after emergence in July and were air freighted to Carleton University where they were kept at room temperature (21°C). Upon arrival, toads were washed in distilled water and then in a mild solution of tetracycline. Ninety toads were immediately killed and used as controls; food in their gastrointestinal tracts revealed that the toads had been feeding prior to capture. Thirty-five toads were weighed and then placed in wire grille-covered basins filled with sterile soil at about 20%

humidity, without access to food or water. They burrowed into the soil and were allowed to estivate for two months at $15 \pm 3^\circ\text{C}$ in a Precision Low Temperature Incubator. Estivation time was chosen to reflect a stable dormant state; previous studies have shown that oxygen consumption by dormant toads stabilizes at its minimum after 2-3 weeks (Seymour, 1973). Soil humidity was 10-12% at the end of the two months. Toads were then rapidly removed, killed by pithing and weighed. A blood sample was quickly taken by aortal puncture (using a micropipette) and a syringe was used to remove a sample of urine from the bladder. Liver (without gallbladder), heart, hind leg muscle, kidney, gut (stomach and intestines combined) and lungs were then quickly excised and frozen in liquid nitrogen. Tissues and tubes containing whole blood and urine samples were kept frozen at -70°C until use. Lipid peroxidation assays were done shortly after collection of tissues to prevent inaccurate results due to long-term freezing storage (Whiteley *et al.*, 1992).

The percentage decrease in total body water during estivation was calculated as described by Churchill and Storey (1994) where

$$\% \text{ change} = ([W_i - W_d] / [W_i * BWC_i]) * 100\%.$$

W_i = initial body weight, W_d = weight after 2 months estivation in laboratory, and BWC_i = initial body water content of toads prior to dehydration in $\text{g H}_2\text{O/g body weight}^{-1}$. Total body water content of control toads was obtained from initial and final weights of toads that were killed and then dried to constant weight for 72 h at 80°C . Toads were not killed and dried to constant weight from the population that was estivated; the decrease in standard body weight during estivation is primarily due to water loss.

Measurement of urea in toad urine and plasma.

Urea in whole blood and urine was determined by measuring NADH decrease in a standard urease assay. Briefly, aliquots of whole blood or urine were incubated in a system containing 150 mM KPi buffer, pH 7.5, 1 mM EDTA, 25 mM α -ketoglutarate,

0.2 mM NADH, and 24 U glutamate dehydrogenase. After a stable initial absorbance was measured at 340 nm (i.e. free ammonium used up), 5 U urease was added; final absorbance was measured after a 30 minute incubation at 20°C (or when final absorbance became constant). Urea concentration in samples was calculated from a standard curve constructed using 10-100 nM urea.

Quantification of lipid peroxidation

Lipid peroxidation due to reaction with reactive oxygen species was assessed by two methods, as thiobarbituric acid-reactive substances (TBA-RS) and by measurement of conjugated diene content of extracted lipids of the tissue.

Thiobarbituric acid-reactive substances (TBA-RS) were measured as per Uchiyama and Mihara (1978), with modifications. Frozen tissue samples were homogenized 1:5 w/v in phosphoric acid (final concentration = 1.1 % phosphoric acid). Well-mixed homogenate representing total tissue protein was divided into two 0.4 mL aliquots in new glass tubes; one aliquot was used as a blank. To the sample tube, 0.4 mL of 1 % w/v thiobarbituric acid (TBA) + 0.1 mM butylated hydroxytoluene (BHT) in 50 mM NaOH was added. To the blank, 0.4 mL of 3 mM HCl was added. A 0.2 mL aliquot of 7 % phosphoric acid was then added to both and pH was measured and adjusted to approximately 1.6, if necessary. Tubes were immersed in a boiling water bath for 15 minutes and then the resulting pinkish solution was extracted into spectrometry grade butanol. Absorbance of the sample was measured at both 532 and 600 nm, using the blank preparation as a reference cuvette, and subtracting the absorbance at 532 nm from that at 600 nm (considered as baseline). TBA-RS absorb light at 532 nm with an extinction coefficient of $156 \text{ mM}^{-1} \text{ cm}^{-1}$. TBA-RS were reported per gram fresh weight of tissue.

Conjugated dienes were measured using the method of Corongiu and Milia (1983). Tissue samples from control and estivated toads (which had both been frozen for

the same length of time, 2 months) were homogenized 1:20 w/v in ice cold 2:1 v/v chloroform:methanol (spectrophotometric grade), using a glass/glass homogenizer. Samples were centrifuged at 2000 $\times g$ in foil covered glass tubes. The organic supernatant was removed and mixed with 0.5 volume 3 mM HCl in fresh glass tubes and again centrifuged. The organic layer was then transferred to a fresh pre-weighed glass tube and dried on ice under a stream of nitrogen. After a second weighing of the tube to determine the weight of extracted lipids, the lipids were redissolved in spectroscopy grade hexane at a final concentration of exactly 2 mg/mL. Second derivative spectra (220-260 nm) of the samples were obtained in quartz cuvettes using a Perkin-Elmer Lambda 4B spectrophotometer (Norwalk, Connecticut) with pure hexane in a reference cell. The absorbance due to conjugated dienes was measured as the change in second derivative absorbance units at the minimum peak between 233 nm and 240 nm. Values were reported as second derivative absorbance units per mg lipid.

Xylenol orange assay

Xylenol orange forms a complex with Fe^{+++} at acidic pH values in the presence of lipid hydroperoxides, in the FOX assay (Jiang *et al.*, 1992). Reaction is rapid and quantitative, resulting in a coloured product which may be measured spectrophotometrically. Lipid hydroperoxides in tissue extracts can be measured by a modification of the above procedure (Hermes-Lima *et al.*, 1995c). Frozen tissues were homogenized rapidly on ice 1:5 w/v in HPLC grade methanol and spun down in a benchtop centrifuge. An aliquot (2-10 μL) of methanolic supernatant was added to a new plastic cuvette containing freshly prepared 0.25 mM $FeSO_4$, 25 mM H_2SO_4 and 0.1 mM xylenol orange, at room temperature. Samples and blanks (without supernatant) were covered and incubated at room temperature for up to 24 hours, monitoring the colour change periodically with a Gilford model 250 spectrophotometer until the absorbance at 580 nm was maximal. Final absorbance was measured at 580 nm against water. Then, the

absorbance increase due to organic hydroperoxides was calibrated for every cuvette by the addition of 5 μ L of a 1 mM cumene hydroperoxide (CHP) solution (5 nmol) and absorbance at 580 nm was re-measured after 40 minutes. Response of the xylenol orange- Fe^{+++} mixture to the internal CHP standard was used to quantify the amount of lipid hydroperoxides originally present in the extract, using the following formula:

$$\text{CHPE/gfw} = (\text{A}_{580 \text{ nm sample}} / \text{A}_{580 \text{ nm 5 nmol CHP}}) \times 5 \text{ nmol CHP} \times 1000/\text{V}_i \times 6$$

where CHPE = CHP equivalents; V_i = volume of extract used; the factor "6" presumes a 1:5 w/v methanolic extract; and fw = gram fresh weight of tissue. Thus, LOOH in the extracts are expressed as CHPE per gram fw.

RESULTS

Conditions of estivation

Toads buried themselves within 24 hours and did not emerge from the soil for the duration of the time of estivation. Average toad weight dropped from 27.42 ± 0.72 g (n = 35) prior to estivation to 19.37 ± 0.97 g (n = 35) at the end of two months of estivation. The dry carcass weight of oven-dried undissected toads was 4.90 ± 0.33 g (n = 7). Hence, the mean percent body water was 82.13 ± 7.68 % for control toads. Estivated toads lost roughly 36 % of their total body water during estivation. Urea levels in blood and urine were measured and are reported in Table 2.2.1. Urea content of blood of awake toads was 14 mM, less than 10 % of the value in blood of estivating toads. Urea content of urine of awake toads was typically higher than in blood (Hillman, 1978) and was 15 % of the level reported in estivated toads.

Lipid peroxidation

Levels of lipid peroxidation damage products, as estimated by two different methods, and as well as direct estimates of the amount (levels) of lipid hydroperoxides,

are shown in Table 2.2.2 for six tissues of control and estivated toads. The TBA test, which measures the reaction of TBA with malondialdehyde (MDA) produced as a result of decomposition of oxidized lipids, generates results expressed in nmol TBA-reactive substances (TBA-RS) per gram fresh weight of the tissue. TBA-RS levels were significantly higher in liver, lung and gut of estivated animals, by 2.54, 2.02 and 2.04 fold over the corresponding tissues from awake animals. Relative amounts of TBA-RS in liver, heart, kidney, muscle, lung and gut changed from 100: 45: 97: 59: 52: 54 in awake toads to 100: 29: 39: 20: 41: 44 in the corresponding tissues of estivated toads. Thus, liver had the consistently highest TBA-RS of any tissue and TBA-RS levels in estivated liver were generally (except for control kidney) at least 2-fold higher than in any other tissue.

The second test measured the relative level of lipids having a conjugated diene moiety, which is formed as a result of hydrogen abstraction from the double bond of a polyunsaturated fatty acid. Conjugated diene levels, expressed per mg lipid isolated from the tissue, increased significantly by 1.3 to 2.6-fold in all tissues from estivating animals except for lung, where a 40 % decrease was noted. The ratio of the amount of conjugated diene groups detected in different tissues was 27: 60: 34: 8.2: 100: 20 for liver, heart, kidney, muscle, lung and gut of awake animals, respectively, and 44: 95: 100: 28: 76: 35 for the corresponding tissues from estivated animals. Thus, lung had the highest conjugated diene levels of the tissues studied in awake animals, and kidney the highest in estivated animals.

Actual LOOH, measured by the FOX assay, were significantly higher, by 13 % and 2.5-fold, respectively, in liver and muscle of estivated animals (Table 2.2.2) compared with control values. However, lipid hydroperoxides were lower in lung and heart of estivated animals, falling to 20 % and 64 % of the values in tissues of awake toads. Lipid hydroperoxides were unchanged during estivation in gut and kidney. The ratio of LOOH contents among the different tissues was 100: 90: 72: 37: 4: 69 for liver, heart, kidney, muscle, lung and gut, respectively, of awake toads. The ratio for the

corresponding tissues of estivated toads was 100: 51: 74: 83: 0.7: 66. Thus, the distribution of LOOH was highest in liver, and remained constant relative to liver for kidney and gut. The ratio relative to liver rose for leg muscle and fell for heart and lung during estivation.

The three methods were checked for agreement as to whether the measured product of lipid peroxidation was higher, the same or lower in estivated toads, compared to their awake counterparts. The results for liver showed agreement between all three methods with significantly higher levels of TBA-RS, conjugated dienes and LOOH (FOX assay) in liver of estivated, compared with awake, toads. There was also agreement between the conjugated diene assay and the FOX assay for leg muscle and lung, both rising in leg muscle and decreasing in lung of estivated toads. Results for the TBA-RS and FOX assays agreed in kidney (unchanged levels), whereas both TBA-RS and conjugated dienes increased in gut during estivation. However, no correlation between any of the methods was observed for heart which showed unchanged TBA-RS, increased conjugated dienes and reduced LOOH in estivated, versus awake, animals.

Table 2.2.1. Urea levels in blood and urine of awake and estivating spadefoot toads

Urea is reported in millimoles urea per litre of blood or plasma (mM). Data are means $\pm$ S.E.M. of values for blood and urine from three separate toads. ^a, significantly different from the corresponding value in awake toads, $P < 0.01$, 2-tailed Student's t-test

	Awake	Estivated
Blood	14.5 $\pm$ 5.2	206.5 $\pm$ 4.8 ^a
Urine	35.5 $\pm$ 4.0	224.0 $\pm$ 0.7 ^a

Table 2.2.2 Lipid peroxidation in tissues of awake and estivated *S. couchi* measured by three methods.

Data are means $\pm$ S.E.M., $n = 4$. ^a - significantly different from the corresponding value for awake toads as determined by the Student's *t*-test. $P < 0.05$; ^b - $P < 0.005$. Data for the TBA test are expressed as nmol thiobarbituric acid-reactive substances per gram fresh weight (gfw) of tissue; conjugated dienes are presented as the 2nd derivative absorbance unit of a fixed concentration of lipid, and units for the FOX assay are in $\mu\text{mol CHPE}$ per gram fresh weight of tissue.

Tissue	TBA-RS		Conjugated dienes		Xylenol orange	
	Awake	Estivated	Awake	Estivated	Awake	Estivated
Liver	22.6 $\pm$ 2.4	57.3 $\pm$ 7.7 ^a	1.20 $\pm$ 0.17	1.58 $\pm$ 0.25 ^a	7.87 $\pm$ 0.26	8.88 $\pm$ 0.20 ^b
Heart	10.2 $\pm$ 0.7	16.8 $\pm$ 4.3	2.68 $\pm$ 0.47	3.40 $\pm$ 0.43 ^a	7.10 $\pm$ 0.55	4.57 $\pm$ 0.28 ^a
Kidney	21.9 $\pm$ 2.9	22.3 $\pm$ 3.1	1.53 $\pm$ 0.31	3.59 $\pm$ 0.45 ^a	5.66 $\pm$ 0.48	6.61 $\pm$ 0.31
Muscle	13.3 $\pm$ 1.7	11.9 $\pm$ 0.6	0.37 $\pm$ 0.05	0.99 $\pm$ 0.23 ^a	2.88 $\pm$ 0.28	7.33 $\pm$ 0.74 ^b
Lung	11.7 $\pm$ 0.95	23.6 $\pm$ 2.4 ^a	4.50 $\pm$ 0.93	2.72 $\pm$ 0.76 ^a	0.32 $\pm$ 0.05	0.06 $\pm$ 0.01 ^a
Gut	12.3 $\pm$ 2.3	25.1 $\pm$ 0.96 ^a	0.89 $\pm$ 0.06	1.24 $\pm$ 0.29 ^a	5.40 $\pm$ 0.54	5.90 $\pm$ 0.63

DISCUSSION

Estivated animals

Hydrated weight, dry carcass weight and total body water were in good agreement with previous measurements in *Scaphiopus couchi* (McClanahan, 1967). Urea levels were measured in plasma and urine of estivated toads (Table 2.2.1) and were also identical to previously reported levels (McClanahan, 1967). Therefore, the toads used in this study showed physiological changes consistent with estivation suggesting that our laboratory holding conditions sufficiently mimicked natural conditions to allow normal estivation to occur, and that toads held in the laboratory accumulated the known products of estivation in the same way as toads do in the wild.

Lipid peroxidation damage during estivation

Antioxidant enzyme activity was reduced during estivation and we predicted that this would result in decreased protection from free radical species. Oxidative damage to cellular lipids might then increase in cells, and damage products could accumulate during estivation.

In Chapter 2.1, measurement of AOE activities in tissues of estivated toads showed that the estivating spadefoot did not anticipate antioxidant stress upon arousal by increasing AOE, nor did it maintain constantly high AOE activities (although activities for some AOE, especially for awake toads, were much higher than in the leopard frog). From these data, two possible results could be predicted for the effect of estivation and lower AOE activities, compared to awake animals, on the levels of lipid peroxidation damage in toad tissues. The first was that higher levels of lipid peroxidation damage might occur during estivation, indicating reduced antioxidant protection. Higher damage could mean that antioxidant protection was inadequate to defend the toad's tissues during estivation and/or that damage products were allowed to accumulate while the toads were dormant. Since the toad suppresses most of its metabolic functions during estivation, it

may be that antioxidant enzyme activity is merely one of the many processes which have a metabolic cost and which are suppressed to conserve energy. The second possibility was that toads would exhibit little or no change in the amount of detectable lipid peroxidation damage and this would imply that antioxidant defences (although generally lowered in estivation) were still sufficient to prevent oxidative damage in the dormant state, or that damage products were removed efficiently during estivation.

Accumulation of free radical damage products has been correlated with metabolic rate (oxygen consumption) in various species (Adelman, 1988). In situations where ROS are produced in greater amounts so as to cause oxidative stress, expression of genes coding for antioxidant enzymes is triggered (Fanburg *et al.*, 1992). Thus during estivation, the toad consumes less oxygen (Seymour, 1973) and also had lower activity of several of its key antioxidant enzymes (Chapter 2.1), a nice correlation potentially explaining the observed rise in AOE activity following estivation. However, if reduced AOE activity during estivation were due solely to reduced ROS production due to low oxygen consumption, the amount of lipid damage would presumably not change; expression of AOE compensation for any new level of ROS generated, either higher or lower. Lipid damage as measured by conjugated diene amounts was higher in estivated toads, indicating that AOE activity in estivated toads was so low as to allow oxidative damage to lipids.

Comparison of the methods

Three methods were used to assess lipid peroxidation. Two (TBA-RS and the test for conjugated dienes) measured the products of lipid peroxidation and are established methods. However, both tests suffer from a lack of specificity (Halliwell and Gutteridge, 1990). The TBA test is the most widely used assay for lipid peroxidation (Halliwell and Gutteridge, 1990; Janero, 1991). The product of LOOH breakdown, malondialdehyde (MDA) is accumulated in tissues and reacts with TBA to form a coloured product.

However the test has fallen under heavy criticism in that it detects not only MDA, which is present in cells as a result of ROS damage to lipids, but also damage products of ROS attack on non-lipid compounds commonly present in biological samples, including carbohydrates, pyrimidines, and hemoglobin (Halliwell and Gutteridge, 1990; Gutteridge and Quinlan, 1983). Decomposition of LOOH may also take place by routes which do not result in formation of MDA and/or result in formation of non-MDA products which react in the TBA test (Janero, 1990). MDA itself is both cytotoxic and formed by other pathways besides the degradation of peroxidized lipids (Janero, 1990). The lipid extract used in this experiment eliminates water-soluble interferences (Wade, 1989), and prevents *in vitro* peroxidation (resulting in grossly exaggerated values) by the inclusion of BHT (Pikul *et al.*, 1983). However, it does not account for MDA formed by other sources such as oxidation of 2-oxydealdose, γ -irradiation of amino acids, or glucose breakdown by ionizing radiation. Arguably, MDA formed by these sources is still a damage product, although not necessarily of oxygen-centered free radical attack.

The nonspecificity of the TBA test may account for the lack of agreement observed between this test and the test for conjugated dienes shown in Table 2.2.2. Moreover, increased production of oxygen centered free radicals may not necessarily lead to increases in TBA-RS: for example, hyperoxic acclimation (a stress known to increase oxygen-centered free radicals) did not increase lipid peroxidation estimated by the TBA test in liver, kidney, heart or skin of *D. pictus* (Barga de Quiroga *et al.*, 1988). TBA-RS were also similarly unaffected in *R. sylvatica* liver and muscle during episodes of freezing and thawing (Joannis and Storey, 1996). Hermes-Lima *et al.* (1995c) reported a correlation between the FOX assay and TBA-RS in several tissues from different animals. However such a correlation was not observed in toad tissues. Thus, the lack of definite agreement between this and other methods of determination of lipid peroxidation, with no obvious reason why this is so, cast the results of the TBA-test into doubt as a tool for assessment of lipid peroxidation in toad tissues. An extensive literature review by

Janero (1990) led to the conclusion that "neither MDA determination nor TBA test response can generally be regarded as a diagnostic index of the occurrence extent of lipid peroxidation, fatty hydroperoxide formation, or oxidative injury to tissue lipid without independent chemical evidence of the analyte being measured and its source" (Janero, 1990, p. 515). Consequently two other tests for lipid peroxidation were done; one measured conjugated dienes and the other measured lipid hydroperoxides (the FOX assay).

In contrast to the variable nature of MDA formation, the long-lived conjugated diene moiety is always formed upon abstraction of a hydrogen from one double bond of a polyunsaturated fatty acid, is not toxic (merely not useful, since it decreases membrane fluidity), and may be recycled to its original conformation by a series of repair enzymes. It may follow, then, that conjugated diene-containing lipids in a tissue would be allowed to accumulate until such time as they pose a threat to the animal or until it is metabolically realistic to expend the energy to recycle them. During estivation this may be a longer interval than in awake toads, and hence accumulation of conjugated diene-containing lipids may be the more likely result of decreased repair (with an associated metabolic cost) during estivation rather than an increased rate of damage accumulation.

The assay of conjugated dienes may be more representative of actual damage incurred. It measures the amounts of lipid-soluble material bearing the functional group of conjugated dienes. Lipids and fatty acids containing this group are a direct product of membrane peroxidation. A problem with the assay is that in some samples octadeca-9,11-dienoic acid, an isomer of linoleic acid, is a contaminating influence, absorbing in the same UV range as conjugated diene products of polyunsaturated fatty acid oxidation (Halliwell and Chirico, 1993). Octadeca-9,11-dienoic acid is not a product of oxidative damage, but of bacterial fatty acid metabolism. However, comparative studies such as the one described here, may still show a relative trend of accumulation or clearance of the products of oxidative damage to fats and lipids.

The FOX assay was recently adapted in this lab for use with tissue extracts (Hermes-Lima *et al* , 1995c), detects lipid hydroperoxides and is standardized by reaction with a known quantity of cumene hydroperoxide. Thus, LOOH in tissues were expressed as $\mu\text{mol CHP/gfw}$. LOOH is a dangerous species, capable of re-initiating lipid peroxidation in reactions catalyzed by reduced iron or copper, or by reaction with the conjugate acid of superoxide (Halliwell and Gutteridge, 1990; Aikens and Dix, 1991). The FOX assay measured this transient species using the formation of a dye complex, with an internal standard added to accurately determine the exact absorbance change due to a known amount of hydroperoxide. LOOH would rapidly react or be cleared by specific AOE (GST, GP_{ox}) *in vivo*. Each sample in the assay was checked following measurement of the colour change with an internal standard (CHP). Problems with the assay include oxidation of extract components by ambient oxygen during colour development (which does not take place rapidly) and the assumption that CHP is useful as an accurate standard for LOOH, i.e. has the same reactivity as a lipid hydroperoxide. If it does not, then the quantity of lipid hydroperoxides would be miscalculated by this method, but as for the other methods, relative changes would still be accurately observed.

The accumulated evidence indicates that none of these methods is a completely accurate depiction of lipid peroxidation in toad tissues, but when taken together, they may provide a more complete picture of the process.

Lipid peroxidation during estivation

The questionableness of the TBA-RS assay might also be seen from the present results (Table 2.2.2) in that in only 3 out of 6 tissues did the results for TBA-RS (up, down, or no change) agree in direction of change with the results of at least 1 of the other tests (agreement in liver, kidney, and gut). The test for conjugated dienes agreed in direction of change with the results for at least 1 other test in 4 tissues (liver, muscle, lung

and gut) whereas FOX agreed with other test results in 4 cases too (liver, kidney, muscle and lung).

In all estivated tissues studied, levels of TBA-RS were unchanged or higher than in tissues of awake toads. Malondialdehyde accumulated to the greatest extent in liver and also rose in lung and gut. The toad reduces the use of its lungs during estivation (this is discussed further below) and empties its gastrointestinal tract prior to burrowing. It is thus interesting that malondialdehyde, which is toxic, is accumulated in the organ most capable of dealing with its toxic effects, and in two organs whose use is reduced during estivation. The results for the assay of conjugated dienes showed that according to this test, oxidative damage to lipids had occurred to a greater extent during estivation in all tissues except lung. Increased levels of conjugated diene moieties in liver, heart, kidney, muscle and gut showed that LOOH attack occurred in these tissues, yet only for liver and gut was there agreement between the test for conjugated dienes and TBA-RS in terms of increased product during estivation. This suggests that malondialdehyde was either stored in less-used tissues or at the very least, cleared from heart, kidney and muscle.

LOOH levels in tissues of spadefoot toads rose during estivation in liver and muscle, and fell in heart, lung and kidney. Lipid hydroperoxides are a product of ROS attack on polyunsaturated fatty acids, but they are not an endproduct. LOOH may react with transition metals (Fe^{+2} , Cu^{+}) and form peroxy radical ($\text{LOO}^{\bullet}$), which may then abstract a hydrogen from a polyunsaturated fatty acid and start another radical chain reaction (Fig. 1.2; Halliwell and Gutteridge, 1990; Halliwell and Chirico, 1993). Thus, one lipid hydroperoxide, if it is not removed, may create many products with conjugated diene moieties (Fig. 1.2). The lipid hydroperoxide moiety may be rapidly converted to a less reactive functional group, however the conjugated diene portion remains in the more stable products (e.g. alcohols resulting from enzymatic detoxification by total GP_{ox}) (Rice-Evans and Diplock, 1991) until it is changed into the cis-conformation during

repair. This may explain why in heart, kidney and gut of estivated toads, levels of conjugated dienes were increased without a corresponding increase in the quantity of lipid hydroperoxides. There was a correlation between measured increases in the levels of conjugated dienes and LOOH in liver, lung and muscle but not in other tissues.

The use of the FOX assay allowed a comparison of actual ROS present (lipid hydroperoxides) to the products of damage which were formed. Liver and muscle had similar results for accumulation of conjugated dienes and actual lipid hydroperoxides present in estivation. Increased conjugated diene levels in liver (Table 2.2.2) could be correlated to increased levels of lipid hydroperoxides. These were not quenched by total GP_{ox} since activity of this enzyme was lower in liver of estivated toads than from awake toads (Chapter 2.1). Muscle from estivated toads showed a twofold increase in conjugated dienes and LOOH, despite higher total GP_{ox} activity. Clearly, muscle experienced oxidative stress during estivation. The possible cause of the increased generation of lipid hydroperoxides in liver or muscle is unknown, since oxygen radical generation should be lower owing to lower oxygen consumption of these tissues during estivation. However, despite levels of lipid hydroperoxides that were similar to most other tissues, muscle showed less than half of the level of conjugated dienes compared with other tissues. This may indicate that propagation of the free radical reaction was slowed in muscle, or that repair enzymes in this tissue rapidly clear the conjugated diene moiety.

Heart, kidney and gut had elevated levels of conjugated dienes, despite lower or unchanged levels of LOOH. Fewer oxygen radicals may be produced as a result of depressed metabolic rate in these tissues. Heart rate, for example, is reduced during dormancy and oxygen utilization decreases for all tissues (Seymour, 1973). The accumulation of lipid damage products may be the result of lower activity of lipid-hydroperoxide-removing enzymes. Indeed, the capacity of the heart and gut to remove lipid hydroperoxides was markedly reduced (GP_{ox} activity was lower) (Chapter 2.1). All three tissues showed significantly elevated GSSG/GSH ratios (Chapter 2.1), which also

indicates decreased protection from lipid peroxidation (Palamanda *et al* , 1992). Total GP_{ox} in heart was higher during estivation and this may account for the lower concentrations of lipid hydroperoxides, compared to heart of an awake animal (Table 2.2.2).

All three tests indicated that spadefoot toads suffered damage to the lipid fraction of their cells during estivation, since TBA-RS, conjugated dienes and FOX units for each tissue were in most cases increased or unchanged but not lowered during estivation.

Differences in lung

Lung was the only tissue to show decreased lipid peroxidation during estivation, by both the conjugated diene assay and the FOX assay. High GSSG levels and GSSG/GSH ratio in lung (Chapter 2.1), as well as the characteristically lower amount of protection from SOD (Perez-Campo *et al.*, 1993; Chapter 2.1), indicate high susceptibility of this organ to free radical damage. Total GP_{ox} activity in lung of estivated toads was less than half the activity in awake toads (Chapter 2.1). Thus, conjugated diene levels in lung far exceeded those of any other tissue in the awake toad, indicating that lipid peroxidation in this tissue was more extensive than in other tissues. However, these levels fell by 40 % (to below the amounts in heart and kidney) in estivated toads. LOOH in toad lung also dropped to 18 % of the value in awake toads. Why was lung the only tissue to show reduced levels of conjugated dienes and LOOH during estivation? While burrowed, toads breathe using an apnoic pattern (i.e. long periods of breath-hold) and also respire through their skin (Seymour, 1973). The lungs in an estivating toad experience long periods of low, declining oxygen tension rather than the continuously high pO₂ of a continuously breathing animal. Hence, the reduced oxygen tension reduces the possibility of formation of oxygen-centered free radicals (Turrens *et al* , 1985, Frank and Sosenko, 1987). Thus, the switch during estivation to an intermittent breathing pattern plus respiration through the skin reduced ROS formation and lipid peroxidation in toad lung.

Interspecies comparisons

The TBA-RS test is the most widely used of all three tests described herein. Thus results for tissues of other frogs and toads were mainly comparable to *S. couchi* only via the results of this test; other tests for lipid peroxidation have not been done on the same animals. TBA-RS accumulated in liver, lung and gut of spadefoot toads during estivation. Levels in liver of awake and estivated *S. couchi* (22.6 and 57.3 nmol/gfw respectively; Table 2.2.2) were lower than in liver of *Rana perezi* (81.7 nmol/gfw; Lopez-Torrez *et al.*, 1991) or *Discoglossus pictus* (118 nmol/gfw; Barga de Quiroga *et al.*, 1988). TBA-RS in heart, kidney, lung and muscle of *D. pictus* (Barga de Quiroga *et al.*, 1988) were generally four-fold higher than in the corresponding tissues of awake *S. couchi*. However, TBA-RS in liver and muscle of *Rana sylvatica* were lower, only one-third of the corresponding values in *S. couchi* (Joannis and Storey, 1996). Thus, TBA-RS levels in amphibians vary widely, with values for *S. couchi* being in the range of those measured for other animals.

For the FOX assay, results for liver of awake toads (7.9 $\mu\text{mol/gfw}$) were roughly half the level in liver of *R. sylvatica* (14.1 $\mu\text{mol/gfw}$; Joannis and Storey, 1996). Lipid hydroperoxides in kidney and muscle of awake *S. couchi* were 5.7 and 2.9 $\mu\text{mol/gfw}$, respectively, compared to 8.1 and 5.3 $\mu\text{mol/gfw}$ for the same tissues from control *R. sylvatica* (Joannis and Storey, 1996). *R. sylvatica* maintains high antioxidant defences by having GP_{ox} activity higher than in most amphibians, including *S. couchi* (Joannis and Storey, 1996; Chapter 2.1). Thus it was interesting to observe that awake toads had similar LOOH levels, despite lower activity of GP_{ox} in toad tissues compared to wood frog tissues.

Reduced AOE activity allows lipid peroxidation during estivation

AOE induction in response to oxidative insult and stress has been well documented. In studies done in this lab, animals such as the freeze-tolerant frog, the garter snake and the estivating land snail tolerate freezing or dry conditions using biochemical adaptations which support cell function under xeric conditions. These alterations are accompanied by specific antioxidant responses which counter the oxidative stress imposed by the stressed state itself, or imposed during recovery from the stressed state (Hermes-Lima and Storey, 1993; Hermes-Lima and Storey, 1995b; Joannis and Storey, 1996). Such responses usually involve higher overall antioxidant defences (AOE and glutathione), or increased defences during the stress. Spadefoot toads do exhibit relatively high AOE activity (Chapter 2.1) but in contrast to the species studied previously, the activity of these enzymes was generally lower in estivated toads than in awake toads. We hypothesized that the difference between the antioxidant response of toads versus other species that tolerate similar stresses would result in net oxidative damage to cellular components, resulting in accumulation of the products of damage. This hypothesis was supported by the increased levels of TBA-RS in three tissues of estivated toads, and the increased conjugated diene content of extractable lipids in five tissues of estivated toads. It should be noted that LOOH as measured by the FOX assay were reduced in both heart and lung, yet the products of oxidative damage were higher. In general, LOOH levels did not correlate with TBA-test results, and agreed with results for conjugated dienes for three tissues only. In heart, kidney and gut, LOOH were lower or unchanged but conjugated diene levels were higher. The lack of agreement may be due to reduced activity of glutathione peroxidase in several tissues, and to elevated GSSG/GSH levels in several tissues, which may have allowed free radical propagation to continue apace in lipids. Another possibility may be the slower clearing of conjugated diene moieties, compared to lipid hydroperoxides. Thus, it appears that reduced antioxidant

enzyme defences allow some peroxidative damage to lipid-soluble components of the cell in several tissues of the spadefoot toad during estivation.

We proposed as well that reduced antioxidant enzyme activity in toad tissues during estivation could be due to decreased expression of genes coding for AOE. However, in this circumstance, no net damage should occur to toad tissues, since expression of AOE should finely match ROS formation. Since net damage occurs, as shown by the results for conjugated dienes accumulation, it would appear that the reduction of AOE activity estivated toads is, at least partially, independent of the rate of ROS formation. Hence, reduced AOE activities may be one of the inescapable metabolic consequences associated with the overall metabolic suppression during dormancy.

CHAPTER 3
PURIFICATION AND CHARACTERIZATION OF A NOVEL CATALASE
FROM
THE ESTIVATING SPADEFOOT TOAD, *SCAPHIOPUS COUCHI*

INTRODUCTION

The products of normal oxidative metabolism (such as the XO/XDH system) as well as of oxidative stress induce the damaging free radical species superoxide ($O_2^{\cdot-}$) (Halliwell and Gutteridge, 1985; Fig. 1.2). This can further react with hydrogen peroxide, via the Haber-Weiss reaction, to form the highly damaging species hydroxyl radical ($HO^{\cdot}$). Hydroxyl radicals can also be formed from hydrogen peroxide via the Fenton reaction (Chapter 1; Fig. 1.2). To minimize damage due to hydroxyl radicals, superoxide that is generated *in vivo* is rapidly detoxified by the enzyme superoxide dismutase (SOD) which converts $O_2^{\cdot-}$ to H_2O_2 and H_2O . Catalase and peroxidases then remove H_2O_2 : catalases catabolize the hydrogen peroxide to oxygen and water; peroxidases (e.g. glutathione peroxidase, GP_{ox}) catalyze the oxidation of various organic compounds by H_2O_2 using a series of one-electron transfers. Besides SOD, various other enzymes also produce H_2O_2 directly, including urate oxidase, the xanthine oxidase/xanthine system (which produces either $O_2^{\cdot-}$ or H_2O_2), D-amino oxidase, glucose oxidase, L-amino acid oxidase, glycolate oxidase and monoamine oxidase (Chance, 1979).

Catalase (CAT) (hydrogen peroxide oxidoreductase, E.C. 1.11.1.6) is an essential antioxidant enzyme, ubiquitous in both aerobic and anaerobic organisms (Michiels *et al.*, 1994). Two types of catalase are known: Mn-catalases, which are present only in prokaryotes and are non-heme hexameric enzymes with an all-alpha fold, and heme catalases, which are more widespread in nature and have an alpha and a beta fold and one heme molecule per subunit (Gouet *et al.*, 1995). Heme catalases are the ones considered in this study. Heme catalases share a high degree of sequence similarity, suggesting a common origin for the enzyme from prokaryotes, fungi, animals and plants (von Ossowski *et al.*, 1993). In most organisms the enzyme is a homotetramer of molecular weight 225-270 kDa (Hochman, 1987, von Ossowski *et al.*, 1993). Each 60 kDa subunit contains a heme (ferric heme IX) group responsible for catalytic activity. Peroxidases are also heme-containing proteins, but they are monomers with a wide variety in their

molecular weight and a broad range of potential substrates (Brown-Peterson and Salin, 1995). Catalase in eukaryotes is located in the mitochondria, glyoxysomes and peroxisomes of most tissues, but in the cytoplasm of erythrocytes (Michiels *et al.*, 1994, Aebi, 1974). Most aerobic organisms have a very high constitutive activity of the enzyme, but the enzyme can also be induced by exposure of cells or organisms to oxidative stresses (Michiels *et al.*, 1994).

Catalase activity can be described by the following two equations, where CAT is the native enzyme, and a two-electron oxidized intermediate of catalase is formed, termed *Compound I*:

Two types of heme catalase have been identified. One form uses H_2O_2 exclusively as the hydrogen donor in reaction (2). The other has peroxidase activity (termed a catalase-peroxidase). Compound I can exist without being immediately decomposed and with certain other substrates (e.g. ascorbate or $\text{O}_2^{\cdot-}$) is converted into *Compound II*, a one-electron reduced form of Compound I:

Compound II is distinct from native CAT and from Compound I, and is catalytically inactive, but is decomposed back to the native enzyme by reaction with ethanol (Lardinois, 1995).

Catalase-peroxidases are a subset of catalases which have only been demonstrated in bacteria. They are characterized by a sharp pH optimum, insensitivity to inhibition with 3-amino-1,2,4-triazole (AT), which binds to Compound I and irreversibly inactivates it, and the ability to accept either H_2O_2 or some low molecular weight species (including ethanol, formate, nitrous acid, thiol compounds, guaiacol, *o*-dianisidine and pyrogallol) as the secondary hydrogen donor (Aebi, 1974, Lardinois, 1995, Margoliash *et al.*, 1960) for

reaction (2) during normal catalase activity. The enzymes have similar structure (native molecular weight and subunit molecular weight) to catalases, although some are dimers instead of tetramers (Nadler *et al.*, 1986).

The extended dormancy of estivating toads appears to be associated with oxidative stress, as a significant elevation of the GSSG/GSH ratio and lipid peroxidative damage in estivating tissues has been demonstrated (Chapters 2.1 and 2.2). Estivating toads also showed widespread changes in tissue antioxidant enzyme activities, including a 100 % increase in catalase activity in liver of toads aroused from estivation (Chapter 2.1). Significant modulation of CAT activity was also observed in the presence of physiological concentrations of KCl which may exist in cells of the toad as a result of dehydration. Changes in enzyme activity during estivation could be due to changes in the type of enzyme expressed (different isozyme), to changes in the amount of enzyme expressed, or to post-translational modifications such as protein phosphorylation (Storey, 1992) or formation of disulfide bonds (del Corso *et al.*, 1995). Multiple forms of catalase have been demonstrated in bacteria (Nadler *et al.*, 1986), plants (Holtmann *et al.*, 1993) and animals (Woodbury *et al.*, 1971). Catalase may also be induced in response to stress (Barga de Quiroga *et al.*, 1988; Brown-Peterson and Salin, 1995). I hypothesized that an isozyme of catalase was expressed in estivating toads which had properties which enabled the toad to better endure the stress of dehydration during estivation. The isozyme may also be able to better withstand hypertonic environments, such as are present in dehydrated toads. This chapter reports on the purification and characterization of catalase from liver of awake and estivating toads. The enzyme is described as "novel" because, unlike all vertebrate catalases studied up to this time, it was found to have a dimeric structure.

MATERIALS AND METHODS

Materials and animals

All chemicals and bovine catalase were obtained from Sigma Chemical Co. (St. Louis, MO) and were of the highest purity available, unless otherwise stated. Spadefoot toads were obtained and treated as described in Chapter 2.2. Briefly, some toads were killed on arrival as awake specimens, and some were reserved and estivated for two months, then sampled.

Catalase and peroxidase activity

Catalase activity was assayed as described in Chapter 2.1. The standard assay contained 50 mM potassium phosphate buffer (KPi) pH 7.0 with 7.5 mM H_2O_2 . Peroxidase activity of the enzyme was also assessed as per Hochman and Shemesh (1987). This assay consisted of 0.016 mM *o*-dianisidine with 0.8 mM H_2O_2 in 0.1M KPi, pH 6.0. The reaction was started by the addition of enzyme and product formation was monitored at 460 nm. One unit of peroxidase activity is defined as the amount of enzyme that caused the disappearance of 1 μmol of *o*-dianisidine min^{-1} ($\epsilon_{460} = 11,300 \text{ M}^{-1} \text{ cm}^{-1}$) (Claiborne and Fridovich, 1979). The preparation was checked for Se-dependent and total GP_{ox} activity, using the assay of Ahmad and Pardoll (1988). No GP_{ox} activity was detected in the purified catalase preparation.

The concentration of H_2O_2 needed to give an apparent half-maximal rate was determined ($K_{m,\text{app}}$) for both estivated and awake toad catalase. Since the concentration of H_2O_2 used in the standard assay was already sub-maximal, the concentrations of KCl, urea, NaN_3 and AT which inhibited the enzyme by 50% (i_{50}), were also tested at 7.5 mM H_2O_2 . Kinetic constants were determined using a computer program (Brooks, 1992).

2

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PRECISIONSM RESOLUTION TARGETS

Purification

All steps of catalase purification were carried out at 4 °C unless otherwise indicated. Frozen toad liver was rapidly weighed and homogenized 1.5 % v/v in 20 mM imidazole pH 7.0, containing 10 % v/v glycerol (Buffer A), using a Tissumizer (Tekmar) homogenizer with a few crystals of PMSF added. The homogenate was centrifuged at 28,600 x g in a Sorvali refrigerated centrifuge for 20 minutes and then the supernatant was decanted into a tube on ice. Samples were then subjected to isoelectric focusing (Vesterberg, 1971) using an LKB 8101 column (110 mL) in a sucrose gradient containing ampholines of the pH range 3.5-10. The column was run for 14-18 h at 450 V constant voltage. After focusing, the column was drained into 1.87 mL fractions and the elution profile of enzyme activity and the pH gradient were measured. Active fractions were pooled and then loaded onto a Cibacron blue agarose column equilibrated in Buffer A at room temperature (21 °C). The column was washed with Buffer A until A_{280} returned to baseline and then catalase was eluted from the column with a 0-1 M KCl gradient in Buffer A. Active fractions were pooled and concentrated to a volume less than 1 mL using Centricon filters in a Hermle refrigerated centrifuge at 5000 rpm for 2-3 hours. The concentrated enzyme was then loaded onto an Sephacryl S-300 size exclusion column equilibrated in 10 mM KPi, pH 7.5 (Buffer B) at room temperature. The column was eluted with the same buffer and fractions containing catalase activity were pooled and concentrated using Centricon filters. In the final preparation of the purified enzyme, samples were adjusted to 40 % v/v glycerol and stored at 4 °C in the short term or at -20 °C for long term storage; neither storage condition altered enzyme activity or kinetic properties. To test whether glycerol was an appropriate stabilizer, a solution of catalase in 40 % v/v glycerol was left at room temperature for two days. Activity remained at 100% (results not shown). Hence, preparations of catalase for storage were kept in 40 % v/v glycerol. Glycerol was removed by centrifugation of the enzyme through a small column of Sephadex G-25 prior to use with no initial loss of enzyme activity. To determine

protein purity, aliquots from each step of the purification procedure were reserved (frozen at -80°C) for subsequent determination of protein content as described in Chapter 2.1.

Molecular weight

For native molecular weight determination, the same Sephacryl S-300 column used for purification was calibrated using blue dextran (void volume), phosphofructokinase (360 kDa), pyruvate kinase (238 kDa), aldolase (150 kDa), hexokinase (100 kDa) and hemoglobin (64 kDa). Elution of protein standards was followed using A_{280} . Bovine catalase was also run separately for comparison to toad catalase and activity was measured using the standard assay. For subunit molecular weight determination, the purified enzyme was subjected to discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Purified catalase was mixed 1:1 v/v with SDS-PAGE loading buffer (50 mM Tris-HCl, 2 % w/v SDS, 10 % v/v glycerol, 0.1 % w/v bromophenol blue, 5 % v/v β -MeSH) and boiled for 5 minutes. The enzyme preparation was then loaded on the gel and run adjacent to molecular weight standards (Sigma). The stacking gel was 5 % w/v acrylamide (30:0.8 w/w acrylamide: bisacrylamide) and the separating or resolving gel was 10 % w/v acrylamide. The gel was run at 200 V for 1 hour and then removed to a fixing solution of 30 % v/v methanol, 10 % v/v acetic acid. The gel was fixed for one hour at room temperature on a rotary shaker, and then washed by shaking in three changes of distilled, deionized water for 15 minutes each. Protein was detected by staining using silver nitrate (Nielsen and Brown, 1984). Presence of a single band was taken to determine high purity. Protein standards used were: myosin (200,000 Da), β -galactosidase (116,250 Da), phosphorylase B (97,400 Da), serum albumin (66,200 Da), ovalbumin (45,000 Da), carbonic anhydrase (31,000 Da), trypsin inhibitor (21,500 Da), lysozyme (14,400 Da) and aprotinin (6,500 Da). The log molecular weight of standards was plotted against their relative migration (R_f) in the gel to create the standard curve. The R_f of the single band of toad catalase was

compared to the standard curve to determine the molecular weight of the subunit monomer.

Peroxidase activity

For a positive control of catalase-peroxidase activity, *Escherichia coli* were obtained from an in-lab culture at the end of the logarithmic phase of growth, and a crude cell extract was made using a modification of the procedure of Nadler *et al* (1986). A 20 mL aliquot of approximately 37.5×10^8 cells/mL (determined by A_{600} absorbance of the cell suspension) was harvested by centrifugation and washed twice with 100 mM NaPi pH 6.8, 1 mM EDTA. Cells were frozen and thawed twice, then resuspended in 1 mL of 50 mM NaPi, 10 mM EDTA, pH 6.8. The extract was sonicated on ice using a Kontes Micro-Ultrasonic Cell Disrupter with three 10 second bursts and was then centrifuged at 4°C for 40 min at $19,200 \times g$ to precipitate debris. The resulting supernatant was kept on ice and immediately used to assay catalase-peroxidase activity using *o*-dianisidine as previously described.

pH profile

Purified catalase from toad liver (n=3) and from bovine liver (n=1) was assayed between pH 3 and 9.5 in 50 mM phosphate buffer to determine the optimum assay pH of each enzyme. Where phosphate did not buffer the assay appropriately (below pH 5 and above pH 8.5) pH was adjusted with HCl or KOH. Assay pH was measured immediately prior to and following the assay. Assays were done at room temperature with 7.5 mM H_2O_2 as the substrate.

Arrhenius plots

An Arrhenius plot was generated by monitoring the activity of the purified enzyme at different assay temperature. Assay temperature was adjusted up or down using

a Lauda circulating bath attached to the four-cuvette holder in the Gilford spectrophotometer. Cuvettes were allowed to equilibrate to the desired temperature, then temperature of each cuvette was checked before and after the assay with a small thermocouple (Yellow Springs Instruments). The logarithm of enzyme velocity was plotted against the reciprocal of the assay temperature (in °K) using a program provided by Dr. S.P.J. Brooks (Brooks, 1992).

Temperature incubation studies and inhibition with salts

Purified catalase from awake toads was incubated in glass tubes at various temperatures with either no effector, 300 mM urea, 200 mM KCl, or 150 mM TMAO ($n = 1$ for all assays). Final protein concentration was the same for all incubations. Activity was followed over time using the standard assay (50 mM KPi, pH 7.0, containing 7.5 mM H_2O_2 at room temperature).

The activity of purified catalase with the addition of various salts to the assay was also tested using the standard assay containing 0.2 M of either: NH_4Cl , KCl, NaCl, CsCl, LiCl, $MgCl_2$, NaF, $(NH_4)_2SO_4$, K_2SO_4 , Na_2SO_4 , or $MgSO_4$. Activities in the presence of 50 mM glycerol or 50 mM glucose were also tested. Activity in the presence of these chemicals was compared to the activity with no added effector, using the Student's *t*-test, two-tailed.

Fluorescence of the purified enzyme

Fluorescence spectra of the purified enzyme were analyzed at room temperature on a Perkin-Elmer LS-50 fluorescence spectrophotometer. Purified catalase (80 μg) from awake toads was used and placed in 10 mM KPi, pH 7.5. Excitation was at 280 nm with monitoring of the fluorescence spectrum between 300-500 nm. An equal amount of enzyme was placed in 5 M urea and denatured for 12 hours at room temperature before fluorescence monitoring. In all cases, the baseline was subtracted; this was the spectrum

of buffer B alone or in the case of 5 M added urea, buffer B with 5 M urea. The spectrum after 12 h incubation in 5 M urea was compared with the spectrum of the native enzyme

Low molecular weight peroxidase activity

Peroxidase activity with respect to ethanol methanol was tested qualitatively using the method of Mitchell *et al* (1991). Purified enzyme (30 μ L) in 10 mM KPi, pH 7.5 was added to 4 sets of Eppendorf tubes (9 tubes each), followed by 20 μ L of water (set 1) or 5 mM H₂O₂ (set 2-4). The mixture was then incubated for 2 min followed by the addition of 5 % v/v ethanol to set 3 and 5 % v/v methanol to set 4. An aliquot (50 μ L) from different stock AF solutions was then added immediately to give final AF concentrations of 0, 2, 5, 10, 20, 40, 80, 100 or 150 mM. Tubes were incubated for two hours at room temperature and then enzyme activity was measured.

RESULTS

Catalase activity

Catalase activity in extracts of liver from awake toads (1270 U/mg soluble protein) was similar to that reported for frog (*Rana perezi*) liver (Perez-Campo *et al* , 1993) and rat liver (Ji and Fu, 1992). In extracts from estivated toads, however, liver catalase activity dropped to 793 U/mg soluble protein, a decrease of over 38 % during two months of estivation. The values obtained in this study agree closely with those reported in Chapter 2.1 for a different sample of toads which were estivated, then awoken. This change in liver catalase activity during estivation led to the question of how catalase was modified during estivation. Several possibilities exist including a simple change in the amount of enzyme protein in liver, a stable modification of the enzyme and its properties in liver of estivating animals. To investigate the reason behind the reduced CAT activity in liver of estivated toads, studies began with the purification and characterization of the enzyme from liver of awake versus estivated toads.

Purification

CAT activity upon elution from the IEF column was associated with red coloured bands (but no precipitate) and eluted from the column in one peak for both awake and estivated toad liver, virtually identically for both (Fig. 3.1, A and B). Yield was between 20 and 50 % of the initial activity (Table 3.1). Peak fractions were pooled and applied to a Cibacron Blue agarose column, and then eluted with a salt gradient (Fig. 3.2). The red colour came off in the wash and from this point on, the preparation was colourless. The elution profile was again identical for purifications from liver of awake and estivated toads. This step represented a 5- to 7-fold purification over the original supernatant; yield was between 6 and 21 % of initial activity (Table 3.1). Finally peak fractions were pooled and applied to a Sephadex S-300 column in dilute buffer. Void volume for the column was 27 mL (Fig. 3.3) and catalase from both awake and estivated toads eluted at 45 and 46 mL in an identical manner. In a separate experiment, commercial bovine catalase (Sigma) was applied to the S-300 column and eluted at 37 - 38 mL. Final specific activity of catalase from liver of awake and estivated toads was roughly 30,000 U/mg protein, essentially the same for both. The fold purification was 30-40 but the final % yield was only 3 % (Table 3.1).

Characterization of the purified enzyme

The pH of fractions eluted from the IEF column allowed the determination of the pI of toad catalase, and revealed that there was no significant difference between the pI values for the enzyme from liver of awake versus dormant toads (Fig. 3.1). Mean pI values were 7.40 ± 0.17 ($n = 4$) and 7.55 ± 0.15 ($n = 5$), respectively.

Sephacryl S-300 is a size-exclusion column which separates proteins on the basis of their size, which is directly related to their molecular weight. Heavy proteins elute first, lighter proteins later. The void volume of the column was checked with Blue Dextran, a

dye of approximate molecular weight 2×10^6 g mole. Several proteins of known molecular weight were then run on the column, and a standard curve was generated (Fig. 3.4). Native molecular weight of toad catalase was determined by comparison of the molecular weight for toad catalase to the standard curve. The elution volume for toad liver catalase on Sephacryl S-300 (45-46 mL) was very different from that of bovine liver catalase, which eluted at 37-38 mL (Fig. 3.3) indicating that the toad enzyme had a different native molecular weight than the mammalian enzyme. Toad catalase was a lighter protein, with an apparent molecular weight of 120 ± 4 kDa ($n = 3$ catalase from awake toads, $n = 1$ estivated toad catalase). Catalase from bovine liver (Sigma), eluted from the same column, had a molecular weight of 240 kDa, similar to the previously reported molecular weight of about 245 kDa for the bovine enzyme (Fig. 3.3, Chance, 1948).

SDS-PAGE of purified liver catalase from either awake or estivated toads showed a single band, which indicated that the enzyme was homogeneous (Fig. 3.4). The estimated subunit molecular weight was determined to be $65,900 \pm 1,600$ kDa ($n = 3$ catalase from awake toads, $n = 1$ estivated toad catalase) (Fig. 3.3). When combined with the measured native molecular weight of toad catalase, the data indicated that the toad enzyme was a dimer. The concentration of catalase protein in toad liver extract was approximately 1 mg/30 mg of total protein, but I was unable to identify a strong band in the lane for liver extract which corresponded to that of catalase. The reason for this is unknown.

Enzyme activity and kinetics

For standard assays, catalase activity was quantified using 7.5 mM H_2O_2 (Aebi, 1984); activity continued to increase at higher peroxide concentrations with an apparent V_{max} reached at over 80 mM. However, at such high peroxide levels the A_{240} of the reaction is off-scale and so catalase activity is routinely reported for the suboptimal

peroxide concentration which gave a velocity about 20 % of the calculated apparent $V_{\max}$. For the assay, the enzyme was diluted before each measurement until a linear rate could be observed for the activity, and then the initial rate of the reaction was quickly taken, since catalase can be rapidly inactivated by hydrogen peroxide.

Toad catalase was tested for catalase-peroxidase activity. Assays conducted with the typical substrate used to measure catalase-peroxidase activity, *o*-dianisidine (Margoliash *et al.*, 1960), showed no activity of purified toad liver catalase with this substrate. Furthermore, crude extracts of toad liver also showed no activity with this compound, indicating that no other proteins in the soluble protein component of the cell act as peroxidases capable of using *o*-dianisidine in toad liver. As a positive control to ensure that the assay could actually detect catalase-peroxidase activity, a cell-free extract of *E. coli* was prepared as described in Materials and Methods and assayed. This exhibited typical catalase-peroxidase activity, accepting *o*-dianisidine as a hydrogen donor for reaction (2). In addition, purified catalase from toad liver did not show any activity with cumene hydroperoxide, such as is exhibited by Se-dependent and Se-independent glutathione peroxidases, and alkyl hydroperoxide reductase, again confirming that it was a true catalase.

Some kinetic properties of toad liver catalase were assessed. The apparent K_m value for H_2O_2 was 32.92 ± 3.02 mM and 28.10 ± 0.39 mM for the enzyme from awake and estivated toads, respectively ($n = 3$ for both; not significantly different from each other). Based on similarities in *pI*, affinity for chromatographic media, native and subunit molecular weights and apparent K_m values, purified catalases from liver of awake and estivated toads were concluded to be identical and the enzymes from both sources were pooled for use in other tests.

Catalases are heme proteins and are inactivated by sodium azide. CAT was fully inhibited by very low concentrations of NaN_3 ($I_{50} = 0.78 \pm 0.02$ μ M) ($n = 4$). Inhibition with 3-amino-1,2,4-triazole (AT), a specific inhibitor of typical catalases (Margoliash *et*

al., 1960) was also tested. The enzyme showed inhibition with AF ($I_{50} = 22.6 \pm 3.73$ mM) ($n = 3$).

Toad liver catalase showed moderate activity ($\sim 75\%$ of optimal) over a pH range from pH 5.5 to 8 with an optimal assay pH of 6 (Fig. 3.5). The pH optimum for bovine catalase was pH 5, but the enzyme showed $\sim 75\%$ of this activity between pH 5 to 7.3. Toad catalase activity fell sharply below pH 6, and at pH 5 was less than 40% of the maximal activity.

Fig. 3.6 shows a typical Arrhenius plot ($\log V$ vs. $1/^\circ\text{K}$) of the effect of assay temperature on reaction velocity. The relationship was linear over the full temperature range (2.5 to 40 $^\circ\text{C}$) tested. The slope of an Arrhenius plot is equivalent to $-E_a/RT$, where E_a is the activation energy of the enzyme. Activation energy was determined for a preparation of catalase purified from liver of awake toads. E_a was measured from three separate Arrhenius plots and the results averaged: E_a was 20.6 ± 0.62 kJ/mol.

Urea and salt effects

Dehydration during estivation results in elevated ion concentrations in body fluids and stimulates the production of up to 300 mM urea, which exerts a colligative effect to restrict further water loss. Hence, the effects of high salt and urea on enzyme function were tested.

Catalase activity in awake and estivated toad liver supernatant (i.e. crude extracts) was not inhibited by the addition of physiological levels of urea to the assay (Chapter 2.1). Urea was only a weak inhibitor of toad liver catalase. The calculated I_{50} value at the set substrate concentration of 7.5 mM H_2O_2 was 1.85 ± 0.36 M, well above measured urea levels in body fluids of estivated toads (about 200 to 300 mM; Chapter 2.2; Shoemaker, 1967). The enzyme was considerably more sensitive, however, to KCl and showed an I_{50} for the salt of 0.26 ± 0.024 M.

Studies were done to determine whether different solutes resulted in accelerated denaturation or conferred thermal stability. The activity of purified toad CAT, at various temperatures, was measured with enzyme alone (shown in Fig. 3.7), or in the presence of a destabilizing or "perturbing" solute, urea (Hochachka and Somero, 1984), a stabilizing or "compatible" solute, trimethylamine oxide (TMAO) (Hochachka and Somero, 1984), or KCl, both K^+ and Cl^- ions being concentrated in cells as a result of dehydration (Flanigan *et al.*, 1994). Preparations of purified enzyme with the addition of the various effectors (at the same final concentration of enzyme) were incubated over time at various temperatures. Catalase activity was then assayed at room temperature using standard assay conditions. The volume of incubated used for each activity assay was 5 μ L (in a final volume of 1 mL); hence, the added solutes (urea, TMAO or KCl) were diluted out to very low concentrations in the final assay. The enzyme showed appreciable loss of activity at every temperature studied and was fully inactivated at 55 °C within just a few minutes. There were no significant differences in the pattern of activity loss over time at any temperature when incubations were conducted instead in the presence of added 300 mM urea, 150 mM TMAO or 200 mM KCl.

Activity of pure toad CAT was not altered by inclusion of 300 mM urea in the assay (results not shown). Figure 3.8 shows comparisons of CAT activity when different salts were included in the assay. Results are means of $n = 3$ trials and were analyzed by the Student-Neuman-Keuls test for multiple pairs of comparisons (administered through a computer program provided by Dr. S.P.J. Brooks (Brooks, 1992)). Significance is reported relative to no salt added, and also in comparison to KCl addition as this represents the intracellular ions that would accumulate in highest levels during dehydration and would thus presumably be most likely to exert an effect on catalase. CAT activity in the presence of 0.2 M salts where the anion was Cl^- was generally only about 50 % of control (0 salt added); and activity in the presence of 0.2 M $MgCl_2$ was only 14 % of control. By contrast, F^- reduced enzyme activity by only 16 %, and SO_4^{2-}

also had only a slight effect, reducing enzyme activity by no more than 20%. Of the cations tested, salts of NH_4^+ were the least inhibitory, and K^+ , Na^+ , Cs^+ , Li^+ and Mg^{++} salts all inhibited CAT activity very slightly, so as to be almost negligible. For example, inhibition with KCl, NaCl, CsCl and LiCl was not significantly different. Hence, Cl^- is inhibitory, with the cation making little or no difference. Also the activity of catalase in the presence of 0.2 M NaF or Na_2SO_4 was not significantly different, despite the facts that with 0.2 M Na_2SO_4 the concentration of Na^+ was 0.4 M, compared to 0.2 M for NaF.

Glucose and glycerol were also tested (Fig. 3.8) to see if low molecular weight sugars or alcohols affected enzyme activity, as these compounds could potentially act as a secondary hydrogen donor to the intermediate Compound I of catalase. Glycerol (0.5 M) reduced enzyme activity slightly (by 12 %) but glucose had no effect.

The results of the test to measure whether CAT from toads can accept low molecular weight alcohols as hydrogen donors are shown in Fig. 3.9. Without any added substrate, or with hydrogen peroxide (circles and squares), AT rapidly and irreversibly inhibited the enzyme, indicating that a significant portion of CAT was Compound I. When the enzyme was briefly incubated with first hydrogen peroxide and then methanol or ethanol, increasing concentrations of AT only slightly inhibited enzyme activity (triangles). This indicated that Compound I was removed from the solution by rapid reaction with methanol and ethanol, and that toad CAT can accept these molecules as hydrogen donors for reaction (2). Thus, toad CAT is apparently a catalase-peroxidase, but only with low molecular weight hydrogen donors.

Fluorometric data showed an emission λ_{max} typical of catalases at 339 nm and an increase of λ_{max} to 347 in the enzyme denatured by incubation with 5 M urea. Both results are consistent with the spectrum of porcine catalase and the known change in its fluorescence spectrum in response to denaturation (Takeda *et al.*, 1983).

Spectrophotometry of 80 μg of purified catalase showed a Soret band typical of heme catalases at 407 nm (Fig. 3.11).

Table 3.1. Purification of catalase from liver of awake (A) and estivated (E) toads.

Results are each from one purification: essentially equivalent results were obtained with subsequent preparations of purified enzyme.

	Total protein (mg)	Total activity (U)	Fold purification	% yield	Specific activity (U/mg protein)
<u>Awake</u>					
supernatant	30.1	38221	1	100	1270
IEF	4.09	6515	1.25	17.05	1593
Cibacron blue agarose	0.904	6281	5.47	16.43	6944
S-300	0.031	1177	29.6	3.07	37640
<u>Estivated</u>					
Supernatant	35.1	27838	1	100	793
IEF	3.67	14316	4.9	51.42	3900
Cibacron blue agarose	1.19	6893	7.32	24.76	5807
S-300	0.029	977	42.5	3.51	33695

Figure 3.1 Typical elution profile of catalase activity from an isoelectric focusing column. Catalase from liver of control (A) and estivated (E) toads was isoelectrofocussed in a sucrose gradient at 450 V at 4°C for 14-18 h in a column containing pH 3.5-10 ampholines. Open circles show enzyme activity; dotted line shows the measured pH gradient.

Figure 3.2 Chromatography of catalase from liver of awake toads on Cibacron blue agarose. The elution profile was identical for catalase from estivated toad liver. Catalase was eluted using a linear gradient of 0-1M KCl in 20 mM imidazole, 10% v/v glycerol, pH 7.0. Fractions with over 10% of the peak activity were pooled. Circles show enzyme activity; dotted line shows KCl gradient.

Figure 3.3 (A) Native molecular weight of toad liver catalase determined by Sephacryl S-300 gel filtration chromatography. Standard curve shows log molecular weight versus elution volume for protein standards: phosphofructokinase (360 kDa), pyruvate kinase (238 kDa), aldolase (160 kDa), hexokinase (100 kDa) and hemoglobin (64.5 kDa). Arrows indicate elution volumes of bovine and toad liver catalase.

(B) Subunit molecular weight of toad liver catalase as determined by SDS-PAGE. Standard curve shows log molecular weight versus relative migration of protein standards. Standards were: myosin (200 kDa), β -galactosidase (116 kDa), phosphorylase b (97.4 kDa), serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa) and aprotinin (6.5 kDa). Arrow indicates R_f of toad liver catalase.

A

B

Figure 3.4 SDS-PAGE (10% w/v acrylamide gel) of purified catalase from liver of awake toads, stained with silver. Lane 1, supernatant from awake toad liver. Lane 2, catalase pool from IEF. Lane 3, catalase pool from Cibacron blue agarose. Lane 4, catalase pool (concentrated in sucrose) from S-300. Lane 5, molecular weight standards.

Figure 3.5 pH profile of purified toad liver catalase activity in 50 mM potassium phosphate buffer. At lower and higher ends of this pH range, phosphate does not buffer effectively; hence, at pH 3, 4.5, 5, 8.5, 9 and 10, pH of a 50 mM potassium phosphate solution was adjusted using HCl or KOH to the desired pH. pH of the assay cuvettes were measured immediately prior to addition of enzyme, and assay pH was confirmed immediately following measurement of activity. Filled circles show toad liver catalase (mean $\pm$ S.E.M., n=4); open circles show commercial bovine liver catalase (n=1) for comparison.

Figure 3.6 Representative Arrhenius plot showing the effect of assay temperature on the activity of purified toad liver catalase. Activity was measured over the range 2.5 °C to 40 °C. Slope of the plot = $-E_a/RT$ where E_a is the activation energy of catalase.

Figure 3.7 Stability of toad catalase at different incubation temperatures. Purified toad catalase was incubated at 6, 12, 19, 27, 37, 45 or 55 °C in 5 mM KPi, pH 7.0, 10 %
v/v, glycerol. Data are n = 1 for all determinations. At timed intervals, aliquots were removed for activity assays, all conducted at room temperature.

Figure 3.8 Activity of purified toad liver catalase in the presence of various additions to the cuvette. Catalase activity was monitored in the standard assay in the presence of 0.2 M salts or 0.5 M glucose or glycerol. Data are means $\pm$ S.E.M., $n = 4-6$. Significantly different from the control (no additions) value by the Student-Newman-Keuls test, $P < 0.05$; ^a - significantly different from the activity in the presence of KCl

Figure 3.9 Inhibition of purified toad liver catalase by 3-amino-1,2,4-triazole (AT).

Open circles, pure catalase with no pretreatment; filled squares, catalase with prior incubation with 5 mM H_2O_2 ; filled triangles, catalase briefly incubated with 5 mM H_2O_2 followed by addition of 5% v/v ethanol; open triangles, addition of 5% v/v methanol instead. Data are $n = 1$.

Figure 3.10 Fluorometric analysis of urea denaturation of catalase. Catalase was treated as described in Materials and Methods with 5 M urea, and the fluorescence spectrum obtained after overnight denaturation with urea was compared to the spectrum of the native catalase. Protein concentration was 80 μg for each determination. Fluorescence spectra were gathered on a Perkin-Elmer LS-50 Fluorescence spectrophotometer.

Figure 3.11 Absorbance spectrum of 80 µg native catalase in 10 mM KPi, pH 7.5.
Spectrum was monitored at room temperature on a Varian Cary 3 UV-Vis spectrophotometer.

DISCUSSION

Induction of CAT activity in response to oxidative stress and ROS production has been documented in toads (*Bufo bufo*; Barga de Quiroga *et al.*, 1988), and in response to hypotonic stress in the halophilic bacterium *Halobacterium halobium* (Brown-Peterson and Salin, 1995). In spadefoot toads, catalase activity rose following estivation (Chapter 2.1). Catalase was purified and characterized from spadefoot toads in order to determine whether the form of CAT expressed during estivation differed from that in the awake state. My hypothesis was that although catalase is a remarkably conserved enzyme (von Ossinger *et al.*, 1995), an isozyme was expressed in estivating toads which better enabled the toad to detoxify H_2O_2 during estivation.

Catalase purification is traditionally accomplished with an initial step using a denaturing chemical: for example, either chloroform-ethanol denaturation of other proteins followed by ethanol precipitation (Deisseroth and Dounce, 1970), acetone fractionation (Chatterjee and Sanwal, 1993) or ammonium sulphate fractionation (Miyahara *et al.*, 1978). This is typically followed by chromatography on phenyl Sepharose, DEAE, and/or Sepharose 6B (Hochman and Shemesh, 1987; Miyahara *et al.*, 1978; Chatterjee and Sanwal, 1993). The initial step used in the present purification of toad liver catalase, namely separation on the basis of isoelectric point, is apparently novel for catalase. The steps which followed were similar to those used by Chatterjee and Sanwal (1993) for goat lung catalase, namely chromatography on a Cibacron Blue-conjugated bead. These authors used Blue Sepharose Cl-6B chromatography, whereas the purification described here used Cibacron Blue agarose. During optimization of the purification protocol, DEAE-Cellulose and DE-52 Sephadex were also tested as possible purification steps but the enzyme was not retained by these, unlike catalases from other sources (Hochman and Shemesh, 1987; Miyahara *et al.*, 1978; Deisseroth and Dounce, 1970). The final step of the purification (use of Sephadex G-200 or G-300) is one which is commonly used to determine catalase molecular weight and (in this case) was expected

to separate out the majority of proteinaceous contaminants, because heme catalase is a relatively heavy protein (native molecular weight 240-320 kDa) (Gouet *et al.*, 1995). Specific activity of the purified toad liver catalase (U/mg protein) was similar for the enzyme from awake and estivated toads (Fig. 3.1), and was also similar to catalase from goat lung (Chatterjee and Sanwal, 1993).

Catalase is essentially a zero order enzyme for concentrations of H_2O_2 which would occur *in vivo*. Cellular $\text{O}_2^{\cdot-}$ levels are 10^{-11} M; levels of H_2O_2 are three to five orders of magnitude higher, at 10^{-7} to 10^{-9} M (Cadenas, 1989). There is disagreement in the literature as to whether the K_m for catalase can be measured. Increasing concentrations of H_2O_2 rapidly inactivate catalase (Nies and Schlegel, 1982). Very rapid measurement of catalase activity (the rapid-flow two-mixer method; Ogura, 1955) showed that the enzyme exhibits zero-order kinetics (rate increasing linearly with substrate) up to 2.3 M. Eventually, however, the enzyme exhibits Michaelis-Menten kinetics at very high concentrations of H_2O_2 and has a K_m for H_2O_2 of 1.1 M (Ogura, 1955). Some authors have just measured catalase activity with respect to increasing H_2O_2 , and have reported much lower K_m values (70 mM), describing typical Michaelis-Menten behavior with respect to H_2O_2 (Chatterjee and Sanwal, 1993). Other authors claim that the $V_{\max}$ for CAT can never be reached and that the definition of K_m for the enzyme that has been used in the literature should be avoided (Aebi, 1974; Hochman and Shemesh, 1987).

Even so, high concentrations of H_2O_2 in the standard assay rapidly inactivate the enzyme in such a manner that an apparent K_m (abbreviated $K_{m\text{app}}$) can be measured, and this value has been reported (knowingly and unknowingly) by several authors. It should be remembered that this number is **not a real K_m** , merely an indication of how easily the enzyme is inactivated by H_2O_2 . The $K_{m\text{app}}$ for toad liver catalase (28-32 mM H_2O_2) was lower than the typical values reported in the literature. The $K_{m\text{app}}$ of catalase activity occurs at 250 to 1390 mM H_2O_2 for mammalian and some bacterial catalases (Hochman and Shemesh, 1987), although $K_{m\text{app}}$ for catalase of goat lung was 70 mM (Chatterjee

and Sanwal, 1993), *Klebsiella pneumoniae* had a K_{mapp} of 30 mM (Goldberg and Hochman, 1989) and catalase from insects (cabbage loopers) had a K_{mapp} of 16 mM (Mitchell *et al.*, 1991). The K_{mapp} for H_2O_2 for catalase-peroxidases is typically lower; at 4.2 mM for *Rhodopseudomonas capsulata*, 3.9 mM for *E. coli* and 1.9 mM for *Comamonas compransoris* (Hochman and Shemesh, 1987). Since toad catalase was more sensitive to inhibition by H_2O_2 (had a lower K_{mapp} than was seen with other catalases), I wondered whether toad catalase was a catalase-peroxidase.

To test my hypothesis that catalase was expressed as an isozyme in estivated toads, properties of the enzyme from awake versus estivated toads were compared. Catalase from liver of control and estivated toads showed identical elution profiles in the pH gradient created in the IEF column (Fig. 3.1), had the same pI, and showed the same elution profiles from Cibacron blue agarose (Fig. 3.2) and Sephacryl S-300 (Fig. 3.3). Based on the observed similarities between catalase from liver of estivated and awake toads, such as elution profiles, specific activity, pI and apparent K_m for H_2O_2 , the catalases purified from awake and estivated toad liver were concluded to be identical and further tests were done on pooled samples, or on later purifications which were done from liver of awake toads, since it had higher activity per gram fresh weight. Thus catalase from estivated animals was similar to the enzyme from awake animals. Most of the parameters listed (like apparent K_m , elution profiles on ion exchange, pI) help to rule out post-translational modification as the basis of differences between forms which may be present in the estivated or awake animal, as a modified protein should behave differently in these tests. Hence, the total amount of active CAT protein appears to be changed in estivated liver – either by altered synthesis and/or degradation rates.

Sodium azide binds irreversibly to heme and prevents catalytic activity of heme-containing enzymes. Catalase from toads was strongly inhibited by sodium azide (I_{50NaN_3} of $0.78 \pm 0.2 \mu M$). By comparison, I_{50NaN_3} values of 150 μM , 20 μM and 1.7 μM were reported for catalase-peroxidase from *Rhodopseudomonas capsulata* (Hochman and

Shemesh, 1987), catalase from spinach leaf (Galston, 1955) and goat lung catalase (Chatterjee and Sanwal, 1993). This indicated that toad catalase had an $I_{50\text{NaN}_3}$ closer to that reported for two "typical" catalases, and that it also had a heme group, similar to other catalases. The presence of a heme group was confirmed by the Soret absorbance at 407 nm of the purified enzyme (Fig. 3.11) (Eaton and Hofrichter, 1981).

Unlike other H_2O_2 -detoxifying enzymes, for which there is considerable diversity in substrate identity, catalases display little or no functional diversity in their active-site environment, accepting only H_2O_2 as a substrate for reaction (1) (Andersson, 1995), and H_2O_2 , ethanol, methanol or formate as substrates for reaction (2). Catalase-peroxidases (which have only been identified in bacteria to date) can use *o*-dianisidine, guaiacol and other alcohols of higher molecular weight than ethanol or methanol, as the second hydrogen donor for reaction (2) (see Introduction). Toad catalase was tested for catalase-peroxidase activity, since most dimeric catalases identified to date have been catalase-peroxidases (Nadler, 1986, Brown-Peterson and Salin, 1993). Toad liver catalase was not active with *o*-dianisidine, and its activity was also inhibited by AT, indicating that it was not a catalase-peroxidase.

"Typical" catalases, which cannot accept high molecular weight alcohols, such as *o*-dianisidine or guaiacol, as substrates for reaction (2) can accept lower molecular weight alcohols (Mitchell *et al.*, 1991). The ability of toad catalase to accept low molecular weight alcohols as substrates was tested qualitatively using an indirect method (Mitchell *et al.*, 1991). Compound I of all catalases is irreversibly inhibited by AT (Margoliash *et al.*, 1960). During continuous H_2O_2 generation, studies have shown that a steady-state in the concentrations of both CAT and Compound I is obtained (Chance, 1950, Kirkman *et al.*, 1987). Compound I concentration ranges from 30 to 50 % of the total catalase concentration for mammalian catalases during normal H_2O_2 catalysis (Lardinois, 1995). But in a catalase-peroxidase, Compound I is very rapidly broken down by a suitable low molecular weight hydrogen donor (Ji and Fu, 1992). The decrease in the concentration of

Compound I due to the addition of an alcoholic substrate for reaction (2) shifts the equilibrium toward a higher level of native enzyme (Lardinois, 1995) and thus the concentration of Compound I is lower in catalase-peroxidases, relative to total catalase concentration. The results of AT inhibition tests (Fig. 3.9) show that toad catalase in the presence of ethanol or methanol was less inhibited by AT, than catalase without these alcohols. Thus, toad catalase was capable of accepting these molecules as hydrogen donors for reaction (2). Ethanol and methanol were used in the study since toad catalase did not exhibit detectable activity toward *o*-dianisidine. This is similar to catalase from *Trichoplusia ni* reported by Mitchell *et al.* (1991) which cannot accept guaiacol but can accept ethanol as the hydrogen donor, as shown by the minimal inhibition of *T. ni* catalase by 3-amino-1,2,4-triazole (AT) in preparations pre-incubated with hydrogen peroxide and ethanol (Mitchell *et al.*, 1991).

Thus, very small molecular weight alcohols can serve as substrates for toad liver catalase, and may be used in reaction (2) as the hydrogen donor. The slight inhibition of H_2O_2 consumption by catalase observed with 50 mM glycerol (Fig. 3.8) but not with glucose may further indicate that toad catalase is also capable of accepting the polyhydric alcohol, glycerol, as a hydrogen donor (thus, reducing its consumption) but is incapable of accepting higher molecular weight aldoses, such as glucose and other sugars, as hydrogen donors for reaction (2).

Toad liver catalase is not similar to catalase-peroxidases measured previously (Brown-Peterson and Salin, 1993) for it could not use *o*-dianisidine as a substrate, had a broad pH optimum, and was inhibited by AT (Figs. 3.5, 3.9). However, most catalases do have catalase-peroxidase activity toward ethanol and methanol (Aebi, 1974), and toad catalase appears to share this characteristic.

Toad liver catalase showed one highly novel property - its dimeric nature. The molecular weight of the subunit monomer of toad catalase, at 65.9 kDa, was similar to the range of 55-62 kDa reported for catalase from other sources (Deisseroth and Dounce,

1970; Baird *et al.*, 1977; Brown-Peterson and Salin, 1995; Miyahara *et al.*, 1978; Holtmann *et al.*, 1993). However, the native molecular weight, determined from Sephacryl S-300 chromatography, was just 120 kD, which indicated that the native enzyme was a dimer. This contrasts with the heme-containing catalases from most bacteria, plants, animals or fungi, which are tetramers of 200 to 330 kD (Gouet *et al.*, 1995; Deisseroth and Dounce, 1970). One alternative grouping of 60 kDa subunits has been previously shown in vertebrates: hexameric catalase was reported together with tetrameric catalase in mouse liver, both forms being apparently composed of the same 60 kD subunits (Baird *et al.*, 1977).

A dimeric catalase has been reported in one instance in the bacterium, *Klebsia pneumoniae* (Goldberg and Hochman, 1989), but dimeric catalase-peroxidases are more common, such as in *Streptomyces* spp. (Youn *et al.*, 1995), *C. communis* and *M. tuberculosis* (Hochman and Shemesh, 1987). Catalases from *K. pneumoniae* and *Streptomyces* spp. have native molecular weights of 152 to 160 kD and subunit molecular weights of 78-80 kD (Goldberg and Hochman, 1989; Youn *et al.*, 1995). Toad catalase, by contrast, had a subunit molecular weight that was considerably lower than that of the bacterial dimeric catalases and more similar to catalase from mammals and plants. This indicated that toad catalase probably had a similar subunit structure to catalase from mammals and plants.

The pI value of catalase from toad liver (7.4 to 7.5) also differed substantially from the values for catalases and catalase-peroxidases from bacteria which are acidic proteins with isoelectric points of 4.5 (Hochman and Shemesh, 1987), or from eukaryotes in general, with pI of 5.5 (Brown-Peterson and Salin, 1995). Exceptions among eukaryotes are the isoelectric points of catalase from goat liver (5.8 to 6.1) (Miyahara *et al.*, 1978) and barley (pI 6.0) (Holtmann *et al.*, 1993), which are closer but still significantly lower than the pI of toad liver catalase.

pH dependence of catalase from toad liver was fairly broad, but the enzyme had a definite pH optimum at about pH 6. Below pH 6, activity rapidly decreased, similar to catalase from seaweed (Nakano *et al.*, 1995). Catalases typically have high activity over a broad pH range (ca. pH 5 - 9) (Chatterjee and Sanwal, 1993; Hochman and Shemesh, 1987), whereas catalase-peroxidases exhibiting dual activity display a very narrow pH optimum of about pH 7 to 7.5, with no activity at pHs above or below the pH optimum (Nadler, 1986).

When the enzyme was incubated for varying times at temperatures ranging from 6 °C to 55 °C, the activity declined proportionally with temperature (Fig. 3.7). The enzyme was completely inactivated within half an hour at 55 °C. This is in contrast to the results of Brown-Peterson and Salin (1995) who claim that catalases are "typically resistant to thermal denaturation". For example, seaweed catalase showed no change in activity over time (with no added stabilizer) with respect to temperature, until an abrupt decrease in activity over 40 °C (Nakano *et al.*, 1995). However, observation of a decrease in enzyme activity even upon standing at 0- 4 °C has been noted for catalase previously, as well as considerable variability in the specific activity of the purified enzyme, even for preparations isolated from the same animal (Deisseroth and Dounce, 1970). It is apparent from these results that toad catalase activity is not stable in dilute buffers without glycerol at any temperature; however, *in vivo* the enzyme is probably strongly stabilized by high levels of surrounding cellular protein.

It is possible that the identification of a dimeric catalase in toad liver could have been the result of denaturation of an unstable native tetramer, the dimers retaining catalytic activity. However, other catalases, when denatured with acid, 6-9 M urea, lyophilization, 10 M formamide, or weak succinylation, produced dimers or monomers with no catalytic activity (Deisseroth and Dounce, 1970, Samejima *et al.*, 1968). The aforementioned methods are also somewhat rough on the enzyme compared to the steps in the purification procedure, which were mild. Bovine liver catalase only dissociates into

two dimers at $\text{pH} < 3$, or four subunits at $\text{pH} 12$, and in neither case show activity, but upon dilution with buffer back to $\text{pH} 7$ the enzyme reassociates into a tetramer and recovers full activity (Samejima *et al.*, 1968). However, upon complete denaturation in urea and unfolding of subunits, followed by removal of urea, porcine catalase refolded (and reassociated into a tetramer with similar fluorescence spectra) but had no catalytic activity (Takeda *et al.*, 1983). Thus, it appears unlikely that the dimeric toad catalase found here represented a dissociated native tetramer, since the conditions used in the experiment were insufficient to denature most catalases. Also supporting this argument were the kinetic properties of the isolated toad dimeric catalase, the $K_m \text{H}_2\text{O}_2$ and $I_{50} \text{NaN}_3$ being similar to dimeric catalase from *Klebsiella pneumoniae* and other catalases, and the specific activity of the enzyme which was as high as some preparations of mammalian catalase (Chattergee and Sanwal, 1993). Thus, whereas the possibility of dissociation of a native tetramer cannot be completely ruled out, it is still significant that toad catalase is a fully functional, seemingly-normal catalase, although dimeric in nature. An active dimeric catalase from a eukaryotic organism has not been previously identified in the literature, to my knowledge.

Temperature studies such as the Arrhenius plot (Fig. 3.6) showed that catalase activity is not affected by changes in enzyme conformation or changes in solvent interactions around the active site in response to varying assay temperature. This stability may indicate that catalase is initially stable at several temperatures, losing activity upon longer incubations, or that the enzyme resists denaturation in the presence of its substrate, which might prevent unfolding. Since compound I is present in 30 to 50% of the enzyme concentration during steady-state catalysis (Lardinois, 1995), this might be the case if compound I was more resistant to denaturation than native CAT. No studies have been done which might show this result, however. The activation energy of 20.62 ± 0.62 kJ/mol is different from the previously reported values of 6.81 for *Halobacterium halobium* catalase (Brown-Peterson and Salin, 1995).

Incubation of toad catalase at varying temperatures in the absence versus presence of urea, KCl or TMAO (Fig. 3.7) (with subsequent activity assays at room temperature) showed that catalase without stabilizing glycerol rapidly lost activity at most temperatures and that urea, KCl and TMAO did not significantly affect the activity loss. Previous studies of the effect of 400 mM urea on thermal denaturation of RNase have shown that urea lowers the temperature at which RNase inactivation occurs, and hence can be termed a "perturbing solute" (Yancey and Somero, 1979). So-called counteracting solutes such as TMAO reverse the effects of urea at concentrations approximately equal to one-half of urea concentrations (Hochachka and Somero, 1984). These results indicate that the function of catalase *in vivo* would probably not be affected by urea accumulated during estivation.

Salt inhibition studies showed that cations generally had little effect on catalase activity; there was no significant difference in the extent of inhibition of enzyme activity with either the four salts (Na^+ , K^+ , Li^+ , NH_4^+) of chloride or of sulfate. The anion was, instead, the critical factor: Whereas Cl^- inhibited CAT activity, F^- and SO_4^{2-} had only mild inhibitory effects. Chloride is the major intracellular and extracellular anion. When toads are dehydrated to 40 % of total body water lost, extracellular chloride concentrations increase 1.5- to 2-fold over levels in fully hydrated animals (Hillman, 1978; McClanahan, 1967; Flanigan *et al.*, 1994). Thus, since the major effectors of CAT activity were anions, elevated Cl^- concentrations *in vivo* during estivation could strongly modulate enzyme activity.

Urea is a reversible denaturant which has previously been shown to affect catalase (Takeda *et al.*, 1983). Fluorescence intensity rose following denaturation with urea both in the previous reference (porcine liver catalase) and toad catalase (Fig. 3.10). Fluorescence intensity of the toad catalase peak did not increase as much as in the previous reference (a threefold increase in fluorescence intensity).

I hypothesized that catalase expression was reduced during estivation and a different catalase was expressed, since catalase isozymes have been demonstrated in bacteria, plants and animals. This hypothesis was not supported: catalase from liver of estivated toads appears to be kinetically and structurally identical to catalase from liver of awake toads. Incubation studies indicated that neither KCl nor urea contribute to irreversible catalase inactivation, at least at physiological levels of these compounds. Catalase serves many functions, including being part of the heat shock response observed in hyperthermia, and myocardial protection against hydrogen peroxide produced in ischemia-reperfusion (Wall *et al.*, 1993). Expression of catalase isozymes is controlled in bacteria by genes which regulate antioxidant response via direct interaction with ROS (Harris, 1992). Catalase is expressed in response to increased oxygen, as demonstrated for *Bufo bufo* (Barga de Quiroga *et al.*, 1988). Lower ROS production during periods of low oxygen consumption in toads may suppress expression of catalase genes and may represent an inductive response during estivation. Reduced catalase activity *in vivo* is probably due to reduced transcription of the gene coding for catalase.

CHAPTER 4
KINETIC PROPERTIES OF GLUTATHIONE REDUCTASE FROM AN
ESTIVATING TOAD, *SCAPHIOPUS COUCHI*.

INTRODUCTION

Glutathione reductase (GR) (E.C. 1.6.4.2) is a ubiquitous flavoenzyme which catalyzes the reduction of oxidized glutathione by NADPH (Williams, 1976), according to the following reaction:

The reduced glutathione supplied by this reaction has several fates including maintaining the thiol groups of soluble and structural proteins in their proper redox state, directly scavenging oxygen radicals, and acting as a substrate for antioxidant enzymes such as GP_{ox} and GST (López-Barea and Lee, 1979). Maintenance of a low ratio of [GSSG]/[GSH] is important for cellular homeostasis; a rise in this ratio is a hallmark of oxidative stress. The key role of glutathione in cellular metabolism has prompted the study of GR from a variety of sources, and the enzyme has been purified from micro-organisms, plants, mollusks, fish and various mammals (Schirmer *et al.*, 1989). A GR isoform expressed in response to cold stress has been identified and characterized from pine needles (Hausladen and Alscher, 1994) and other stress situations have been correlated with *de novo* expression of GR isoforms (Tanaka *et al.*, 1994). GR activity is linked to the NADPH-regeneration systems. GR is an equilibrium enzyme but it is inactivated by moderate to high NADPH, GSH and reducing conditions in general (Garcia-Alfonso *et al.*, 1993). The loss of activity is due to covalent oxidative modification of the reduced enzyme, which occurs at physiological concentrations of GSH and NADPH and is not alleviated by removal of the reducing agent. However, the inactivation is rapidly reversed by GSSG and to a lesser extent by NADP⁺ (López-Barea and Bárcena, 1988).

Glutathione status and GR were previously measured in liver of spadefoot toads (*Scaphiopus couchi*) as part of a larger survey of antioxidant defences during estivation

(Chapter 2.1). The [GSSG]/[GSH] ratio rose in liver during estivation, solely due to a significant decrease in [GSH]. As well, GR activity in toad liver during dormancy was less than 50 % of the activity in liver of awake animals (Chapter 2.1). Since GR isozymes have previously been shown to be expressed in response to stress (Hausladen and Alscher, 1994; Tanaka *et al.*, 1994), one of the possible mechanisms that could underlie the decrease in GR activity in estivated toad liver may be a change in the form of the enzyme in estivation.

I hypothesized that a different form of GR was present in estivated toads, expressed or subject to post-transcriptional modification as a result of estivation. To study this, GR was isolated from liver of awake and 2-month-estivated spadefoot toads and its kinetics were examined with respect to its substrates, GSSG and NADPH, as well as the influence of physiological levels of urea, KCl, NaCl and GSH on enzyme activity.

MATERIALS AND METHODS

Chemicals and animals

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO), and were of the highest purity available, unless otherwise stated. Spadefoot toads were obtained and treated as described in Chapter 2.2.

Glutathione reductase activity and kinetics

GR activity was measured as described in Chapter 2.1. K_m for GSSG and NADPH were measured at cosubstrate concentrations of 0.2 mM NADPH and 2 mM GSSG, respectively. Inhibition to 50% of V_{max} (I_{50} values) was measured for various salts and for reduced glutathione, using sub- V_{max} concentrations of GSSG: 1 mM for GR from awake toads or 0.5 mM GSSG for essentially purified GR from estivated animals, and V_{max}

concentrations of NADPH (0.2 mM). Kinetic parameters and statistical significance were determined using computer programs provided by Dr. S.P.J. Brooks (Brooks, 1992).

Enzyme purification

All purification steps were conducted at 4°C, unless otherwise indicated. A sample of frozen toad liver was quickly weighed and homogenized 1:5 w/v in 20 mM imidazole, pH 7.0 containing 10 % v/v glycerol (Buffer B) with a few crystals of PMSF added. The homogenate was centrifuged at 28,600 x g at 4°C in a Sorvall refrigerated centrifuge for 20 minutes and then the supernatant was decanted into a tube on ice. GR activity was measured and supernatant was then subjected to isoelectric focusing (Vesterberg, 1971) in an LKB 8101 column (110 mL) as described in Chapter 3. Fractions (1.87 mL) with the highest GR activity were pooled and loaded onto a 3 mL 3',5' ADP-agarose column equilibrated in Buffer B at 21°C. The column was washed with Buffer B until A₂₈₀ of the eluant fell to zero and then enzyme activity was eluted with a linear gradient of 0-1 M KCl in Buffer B. Active fractions were pooled and concentrated with Centricon filters (Diamed) in a Hermle refrigerated centrifuge. The putatively pure enzyme was stored at -20 °C in 40 % v/v glycerol and was stable for several weeks. Before assay, glycerol was removed by centrifuging the enzyme through a small column of Sephadex G-25. Aliquots of peak activity from different steps of the purification were reserved at -20 °C and protein was determined as described in Chapter 2.1.

Subunit molecular weight

Sample preparation and running of SDS-PAGE was performed essentially as described in Chapter 3. The separating gel was 10 % w/v acrylamide. The gel was stained

with silver nitrate as described in Chapter 3. The protein standards used were Bio-Rad broad range molecular weight standards.

Arrhenius plots

The effect of assay temperature on activity was determined in 50 mM KPi, pH 7.4 with substrate concentrations of 2 mM GSSG and 2 mM NADPH over a range of temperatures between 2 °C and 40 °C. Log of enzyme velocity versus 1/Temperature (°K) was plotted and the activation energy of the enzyme (E_a) was determined from the slope of the best-fit line using a program provided by Dr. S. P. J. Brooks (Brooks, 1992). Arrhenius plots were done in triplicate for essentially purified GR from both awake and estivated toads as well as the commercial bovine intestinal mucosa enzyme (Sigma). Arrhenius plots were also done in triplicate for the three enzymes assayed in the presence of 300 mM urea in the assay buffer. Comparisons were made against GR_A with 0 urea using the Student's *t* test, 2-tailed.

RESULTS

GR was partially purified from liver of both awake toads (GR_A) and estivated animals (GR_E). The measured maximal activity of GR_A and GR_E in the crude supernatant from spadefoot toad liver supernatant agreed with values obtained previously (Chapter 2.1) and confirmed that maximal activity of liver GR was significantly reduced during estivation, compared to when toads were awake (Table 4.1). Isoelectric focusing purified the enzyme 60-fold from liver of awake toads, and 8-fold from estivated toads with a high yield (95-98 %). Chromatography on 3'-5'-ADP-agarose purified the enzyme further, 270-fold and 15-fold for GR_A and GR_E, respectively. The enzyme from liver of both awake and estivated toads was obtained with similar final yields of activity: GR_A at 31 % yield,

GR_I at 29 % yield (Table 4.1). However, the specific activities of the final preparations of toad GR varied widely, from 3.67 U/mg for GR_A to 50-fold lower at 0.07 U/mg for GR_E, indicating that even when essentially pure, the enzymes had obvious differences in GR activity.

The isoelectric point (pI) of GR_A, obtained from isoelectric focusing as a purification step, was 5.89 ± 0.19 ($n = 4$) (Fig. 4.1). The value for GR_E was 5.47 ± 0.16 ($n = 4$), not significantly different from GR_A. SDS-PAGE revealed that GR_A and GR_E were partially purified from toad liver (SDS-PAGE for GR_A is shown in Fig. 4.2). The gel lane containing the purified enzyme after ADP-agarose chromatography showed two bands, a prominent one and a small second band above it. GR_A and GR_E both showed similar distribution of these bands on SDS-PAGE. Subunit molecular weights of the predominant protein band were determined from the SDS-gels and were 53.2 kDa for GR_A and 54.9 kDa for GR_I (one determination for both).

GR_A and GR_E had significantly different K_m values for both substrates. Substrate affinity constants for toad liver GRs are shown in Table 4.2. Both enzymes showed hyperbolic velocity versus substrate concentration relationships for GSSG and NADPH; Fig. 4.3 shows the relationship for GSSG. The K_{mGSSG} of GR_A was 4.4-fold higher than that of GR_E, and similarly the K_{mNADPH} of GR_A was also 3.6-fold higher than the corresponding value for GR_E.

Table 4.2 also shows inhibition of GR_A and GR_E by various effectors. Both KCl and NaCl inhibited GR, in agreement with the results of García-Alfonso *et al.* (1993). Inhibition by KCl was significantly stronger for GR_E, the I_{50} of the enzyme from estivated animals being 3-fold lower than the value for GR_A. Urea also inhibited both enzymes but only at high concentrations (I_{50} was about 1.5 M). GSH is a product inhibitor of GR (Sexton *et al.*, 1992) but the two toad enzymes were differentially affected. GSH

inhibited GR_A at moderate concentrations (I_{50} was 4.4 mM) but GR_E showed no inhibition by the product even at 10 mM GSH.

Arrhenius plots (log velocity versus $1/^\circ\text{K}$) for toad liver GR and bovine intestinal mucosa GR produced linear relationships over the entire temperature range tested (2 to 40 $^\circ\text{C}$), both with and without 300 mM urea. Calculated activation energies for the two enzymes differed somewhat (Table 4.3), GR_E having an activation energy 9 % higher than GR_A , when assayed without urea. However, in the presence of 300 mM urea, E_a values for the two enzymes were the same. Activation energies for both toad enzymes were higher than values for commercial GR (Sigma) from bovine intestinal mucosa. The presence of added urea did not significantly change the E_a of any of the three enzymes.

Both GR_A and GR_E showed similar pH optima (Fig. 4.4) of roughly 6.75 to 7.5. Activity markedly declined by pH 6 or pH 8.

Table 4.1. Purification of GR from liver of awake and estivated toads. A = awake toads. E = estivated toads. Results are means $\pm$ S.E.M. for four (awake) or three (estivated) similarly sized purification preparations. *. Significantly different from the corresponding values for specific activity of GR from liver of awake toads GR as determined by the Student's t-test. $P < 0.005$. One unit of activity is the amount of enzyme which uses 1 μ mol NADPH/min.

	total protein (mg)	total activity (U)	fold purification	% yield	specific activity (U/mg)
GR_A					
crude supernatant	21.7 $\pm$ 2.47	0.29 $\pm$ 0.06	1	100	0.013 $\pm$ 0.001
IEF	4.12 $\pm$ 1.3	0.23 $\pm$ 0.08	4.29 $\pm$ 0.55	79 $\pm$ 30	0.056 $\pm$ 0.027
3',5' ADP-agarose	0.023 $\pm$ 0.009	0.085 $\pm$ 0.047	274 $\pm$ 99	31.1 $\pm$ 13.3	3.67 $\pm$ 0.56
GR_E					
crude supernatant	31.2 $\pm$ 2.16	0.14 $\pm$ 0.02	1	100	0.0043 $\pm$ 0.0005*
IEF	3.88 $\pm$ 0.53	0.12 $\pm$ 0.03	7.9 $\pm$ 2.98	85 $\pm$ 25	0.031 $\pm$ 0.008*
3',5' ADP-agarose	0.69 $\pm$ 0.22	0.036 $\pm$ 0.011	16.5 $\pm$ 5.6	29.6 $\pm$ 7.6	0.071 $\pm$ 0.031*

Table 4.2. Kinetic properties of partially purified GR from liver of awake and estivated toads. Data are means $\pm$ S.E.M. of three or more determinations. ^a . Significantly different from the corresponding value for the enzyme from awake toads as determined by the Student's t-test (two-tailed). $P < 0.05$.

enzyme	K_m GSSG (μ M)	K_m NADPH (μ M)	I_{50} Urea (M)	I_{50} KCl (M)	I_{50} NaCl (M)	I_{50} GSH (mM)
GR _A	392 $\pm$ 25.3	48.6 $\pm$ 4.85	1.58 $\pm$ 0.19	0.37 $\pm$ 0.03	0.46 $\pm$ 0.07	4.42 $\pm$ 0.20
GR _E	88.1 $\pm$ 26.0 ^a	13.4 $\pm$ 0.38 ^a	1.66 $\pm$ 0.17	0.12 $\pm$ 0.04 ^a	0.34 $\pm$ 0.07	> 10 ^a

Table 4.3. Activation energies (kJ/mol) determined from Arrhenius plots for essentially purified GR_A and GR_F, and commercial bovine intestinal mucosa GR (GR_{BIM}) in the presence and absence of 300 mM urea. Data are means $\pm$ S.E.M, n = 3. * - Significantly different from the activation energy for GR_A without added urea as determined by the Student's t-test, 2-tailed, P < 0.05; ** P < 0.001.

enzyme	no urea	+ 300 mM urea
GR _A	78.7 $\pm$ 1.45	80.1 $\pm$ 2.80
GR _F	85.7 $\pm$ 2.43*	78.2 $\pm$ 2.75
GR _{BIM}	61.1 $\pm$ 1.46**	60.4 $\pm$ 0.39**

Fig. 4.1. IEF elution profiles for liver GR from awake (A) and 2 month estivating (E) toads. Fractions with maximal activity were pooled for further use. Assays were conducted at room temperature at substrate concentrations giving maximal activity, as described in Materials and Methods.

Fig. 4.2. SDS-Polyacrylamide gel electrophoresis of glutathione reductase from liver of awake toads. Lane 1, horse-liver GR (Sigma Chemical Co.). Lane 2, crude supernatant. Lane 3, pooled IEF fractions. Lane 4, pooled fractions from 3', 5'-ADP-Sepharose. Lane 5, Molecular weight standards (Bio-Rad, broad range). Molecular weight of the subunit was calculated by comparison of the R_f to the log of the molecular weight standards. A similar subunit molecular weight was obtained for GR from estivated toad liver (see Results).

Fig. 4.3. Velocity versus GSSG concentration relationship for liver GR from awake (open circles) and estivated (filled circles) toads.

Fig. 4.4. pH dependence of glutathione reductase. Open circles, awake toad GR; filled circles, GR from estivated toads. Data are means $\pm$ S.E.M., n = 4. Where error bars are not shown they are contained within the symbol.

DISCUSSION

Attainment of a stable hypometabolic state (such as estivation) involves coordinated suppression of both energy-requiring and passive cellular processes. This goal is achieved through the use of various mechanisms that modulate enzyme activity or protein function to suit the new state, including post-translational modification, changes in subcellular localization of enzymes, and regulation of the anabolic uses of fuels (Storey and Storey, 1990) as well as differential gene expression upon entry into the hypometabolic state (Hand and Hardewig, 1996). During estivation, accumulation of GSSG or an increase in the GSSG/GSH ratio has been shown in *Otala lactea* and *Scaphiopus couchi* (Hermes-Lima and Storey, 1995; Chapter 2.1). Modification of sulfhydryl-containing enzymes and proteins by GSSG alone forms protein disulfides and mixed disulfides, inactivating or reducing enzyme/protein function (this was further discussed in Chapter 2.1). In normal systems this is one of the symptoms of oxidative stress and may also greatly perturb enzyme function. For this reason the role of GR is essential in antioxidant protection, to replenish GSH and thus maintain the cellular ratio of GSSG/GSH at a low level (Rendón *et al.*, 1995).

Most forms of GR show similar regulation by reduction or oxidation by chemicals which include its substrates and products. Incubation with reduced substrates, such as NADPH, GSH or DTT inactivates GR in a manner which is rapidly reversed by the addition of GSSG (especially) or NADP⁺ or other oxidizing compounds (López-Barea and Lee, 1979; Carlberg and Mannervik, 1975). Reversible post-translational modifications other than oxidative modification have not been demonstrated in the literature. Thus, the redox state of the cell greatly impacts on GR activity, with greater activity being associated with an oxidized state, and availability of GSSG is the main determinant of enzyme rate.

Situations which change the glutathione ratio generally bring about a change in GR activity. For instance, GR activity was over 120 % higher in GSH-depleted mice, and

maintained the GSSG/GSH ratio constant, despite total glutathione pool depletion (Leeuwenburgh and Ji, 1995). In estivated toad liver, however, an increased GSSG/GSH ratio (as a result of GSH depletion) (Chapter 2.1) was present together with reduced GR activity, suggesting that the enzyme in liver of estivating toads was present in a less active form during estivation, one which does not maintain the glutathione ratio at the levels seen in awake liver. Obviously, toad liver GR did not maintain the glutathione ratio during estivation as it did when the toad was awake and activity of the enzyme was not increased as a result of oxidative activation, or an increase in enzyme quantity, as other authors have suggested normally occurs in this situation (Leeuwenburgh and Ji, 1995; López-Barea and Bárcena, 1988).

The sequence of GR is highly conserved; for example, the *E. coli* enzyme shows high homology with the human enzyme (Greer and Perham, 1986). Different GR isozymes have been demonstrated in few cases. Typically, only one form is found in each tissue but tissue-specific isozymes have been found in spinach (Tanaka *et al.*, 1994). Some animals, such as the marine snail, *Littorina littorea*, appear to have the same isozyme in multiple tissues (Pannunzio, 1994). Modification of GR activity or expression of novel isozymes has been correlated in other organisms to tolerance of various environmental stresses, such as heat (Nieto-Sotelo and Ho, 1986), chilling (Schoner and Krause, 1990; Hausladen and Alscher, 1994), hyperoxia (Foster and Hess, 1980), air pollution (Tanaka *et al.*, 1988), heavy metals (Griff *et al.*, 1985), high light intensity and magnesium deficiency (Cakmak and Marschner, 1992) and water deficiency (Smirnoff and Colombe, 1988; Tanaka *et al.*, 1990). This evidence and the fact that GR activity was not increased in response to a change in GSSG/GSH ratio led to my hypothesis that GR was expressed in a different form during estivation, which maintained a different GSSG/GSH ratio from the awake form.

Several different purification techniques were tested on toad liver GR, based on previous purifications reported in the literature. Purification of mammalian liver GR has

previously been accomplished using ammonium sulfate fractionation, ion exchange chromatography (DEAE-cellulose, DE-52-Sephadex, CM-Sephadex, hydroxylapatite), size exclusion chromatography, and affinity chromatography on ADP-linked to agarose or Sepharose (López-Barea and Lee, 1979; García-Alfonso *et al.*, 1993; Carlberg and Mannervik, 1975; Pannunzio, 1994). However, GR from toad liver was not retained on either the positive or negative columns listed above, or hydroxylapatite, and so other techniques were tested. Isoelectric focusing provided a high-yield first step in the purification which purified the enzyme up to 60-fold (Table 4.1; Fig. 4.1). Chromatography on 3',5'-ADP-agarose was then used to provide an essentially pure enzyme.

Enzyme characteristics

Specific activity of essentially purified toad GR_A and GR_E (3.67 and 0.07 U/mg) (Table 4.1) was lower than values reported for the enzyme from other sources (9.5 U/mg for rat liver (Carlberg and Mannervik, 1975), 30 U/mg from maize (Turner and Pollock, 1993) and ca. 150-250 U/mg for pea chloroplast, mouse liver and horse liver) (Connell and Mullett, 1986; López-Barea and Lee, 1979; García-Alfonso *et al.*, 1993). The value from awake toads was closer to specific activity from hepatopancreas and foot of *Littorina littorea* (2.14 and 1.20 U/mg, respectively) (Pannunzio, 1994).

The pI of GR_A and GR_E were slightly lower than pI of mouse liver GR (López-Barea and Lee, 1979), horse liver GR (García-Alfonso *et al.*, 1993a) and GR from calf and rat liver (Carlberg and Mannervik, 1985). In general, GR from these mammals has a pI of slightly above 6.

The K_{mGSSG} of GR_E (88 μM; Table 4.2) was similar to that reported for the enzyme from other sources: 107, 62, 59, 63, and 50 μM for the enzyme from mouse liver, pea chloroplast, horse liver, rat liver and hepatopancreas of the marine snail *Littorina littorea*, respectively (López-Barea and Lee, 1979; Connell and Mullett, 1986; Carlberg

and Mannervik, 1975; García-Alfonso *et al.*, 1993; Pannunzio, 1994). K_{mGSSG} of GR_A was three-fold higher than the value for GR_E , at 392 μ M. K_m values for NADPH of GR_E (13 μ M) also resembled previously reported values which ranged from 4.9 - 8.8 μ M but K_{mNADPH} of GR_A was 5 - 10 fold higher, at 48.6 μ M (Table 4.2). This data constitutes evidence of a new or modified form of GR in estivated toads.

Product inhibition with GSH was only observed for one isozyme, GR_A . The I_{50} was 4.4 mM, however, compared with 1.4 to 1.8 mM as physiological GSH concentrations (Chapter 2.1); hence, importance of inhibitory effects by GSH *in vivo* may be of limited significance to enzyme control. GR_E appeared insensitive to inhibition by high concentrations of GSH (higher than physiological), indicating that GSH accumulation *in vivo* would not inhibit activity of the enzyme in estivated liver.

Toads accumulate up to 300 mM urea in plasma and urine during estivation. The main function of urea in toad fluids is as a cellular osmolyte to limit water loss. Urea is also a reversible protein denaturant and in similar concentrations (400 mM) perturbs the function of some enzymes significantly (Hochachka and Somero, 1982). The I_{50} values for urea of both GR_A and GR_E were higher than the concentrations of urea that occur *in vivo*, even at the end of estivation (Table 4.2) (McClanahan, 1967) and inclusion of 300 mM urea in the normal assay had no effect on GR activity (Chapter 2.1). Overall, then, urea would not affect enzyme activity *in vivo*.

A more likely modulator of enzyme activity is cellular [KCl], or increased ionic strength in tissues of the estivating toad. GR_A and GR_E differed in their responses to inhibition by KCl, the estivated form being more sensitive to inhibition with this salt. By contrast, the I_{50} NaCl was not different for the two isozymes, and was also higher than the I_{50} for KCl, indicating that KCl inhibition of GR may be specific. KCl and NaCl inhibit GR from horse liver at concentrations below 0.1 M (García-Alfonso *et al.*, 1993) and GR from human erythrocytes, yeasts and mussels is similarly sensitive to changes in ionic strength (Icen, 1967; Moroff and Brandt, 1975; Ramos-Martínez and Rodríguez-Torrez,

1985). As well, KCl inhibited final preparations of GR_F at physiological concentrations (Table 4.2), lower than intracellular KCl concentrations reported in dehydrated Australian frogs (300 mM) (Flanigan *et al.*, 1994). Thus, cellular ion levels may exert more of an effect on the activity of GR *in vivo* than urea-induced denaturation.

The pH dependence of GR from toad liver showed that GR_A and GR_F functioned well at neutral pH values but lost activity below pH 6.5 or above pH 7.5. This was consistent with the pH dependence previously reported for GR from rat liver (7.0) and mouse liver (7.25) (Carlberg and Mannervik, 1975; López-Barea and Lee, 1979). There is evidence that dormant toads undergo hypercapnia during estivation, which would lower body pH (Hillman, 1978). A change in intracellular pH would also contribute *in vivo* to modulation of GR activity.

Subunit molecular weight of GR_A and GR_F (approximately 53 kDa) was similar to GR from horse liver (58 kDa) (García-Alfonso *et al.*, 1993). GR is a homodimer of molecular weight 100 kDa and subunit molecular weight approximately 50 kDa, with a similar subunit structure identified in yeast, rats, and human erythrocytes (Carlberg and Mannervik, 1975).

The activation energies of GR_A and GR_F were only slightly different from each other, but were higher than the activation energy for bovine intestinal mucosa GR. For all conditions tested, GR activity was linear with respect to assay temperature and the enzyme remained active even at temperatures close to 40 °C, indicating that the enzyme had some degree of thermal stability at these temperatures. That the Arrhenius plot was straight indicated that a change in the activation energy of the enzyme did not occur at different temperatures as a result of conformational changes in the enzyme itself, or in arrangement of substrate or solvent molecules around the active site. Inclusion of urea in the assay had no effect even on an animal which does not accumulate high concentrations of urea. The "perturbing" effects of urea on enzyme function (Hochachka and Somero, 1982) were thus not seen, even at these various temperatures.

Differences in the kinetic properties of both GR_A and GR_E indicate that GR in estivated toads is different from the enzyme in awake animals. Thus reduced activity of the enzyme in estivated animals may be due to a specific change induced in response to estivation, which modulates the treatment of and response to both oxidized and reduced glutathione.

In summary, both types of toad GR were similar to GR isoforms in other organisms. The two forms of toad liver GR had the same subunit molecular weight and activation energies, and a similar pI as other glutathione reductases. However, the two forms exhibited substantial changes in enzyme kinetic properties. These results suggest that the enzyme in liver of estivated animals was present either by expression of another form of GR, or due to a post-translational modification of the existing enzyme during estivation.

CHAPTER 5
PURIFICATION AND CHARACTERIZATION OF GLUTATHIONE S-
TRANSFERASE FROM LIVER AND MUSCLE OF *SCAPHIOPUS COUCHI* AND
***RANA PIPIENS*.**

INTRODUCTION

Glutathione-S-transferase (GST) is an ubiquitous multigene enzyme superfamily in aerobic organisms; the enzymes are distributed mainly in the cytosol but have some microsomal distribution (Mannervik and Danielson, 1988, Ketterer *et al.*, 1988, Pickett and Lu, 1989, Coles and Ketterer, 1990, Morgenstern and DePierre, 1988, Habig and Jacoby, 1981). GSTs are catalysts of reactions in which reduced glutathione (GSH) acts as a nucleophile (i.e. glutathione thiolate GS^- is a participant). This is shown below with CDNB as the electrophile:

This enzyme operates by binding GSH and the second substrate at different locations (Rushmore and Pickett, 1993); formation of the nucleophilic thiolate anion at the active site is stabilized by Tyr-9 (Atkins *et al.*, 1993). Endogenous second substrates for GST are toxic products generated from tissue damage. These include compounds generated by lipid peroxidation of biological membranes such as reactive alkenes, epoxides, hydroperoxides and aldehydes. Cholesterol α -oxide, arachidonic acid hydroperoxide, 4-hydroxyalkenals and linoleate hydroperoxide are also substrates of GST. The resultant conjugated products are exported via membrane ATP-dependent pump systems (Ishikawa, 1992), degraded by the mercapturate pathway and then eliminated. GSTs also catalyze the formation of prostaglandins and leukotriene C, and remove aromatic xenobiotics by conjugating them to GSH (Simons and Vander Jagt,

1977). GSTs in the central nervous system may participate in: (a) the binding and transport of certain hormones, neurotransmitters, and drugs, (b) protecting the myelin sheath against toxic substances, (c) eicosanoid metabolism, (d) forming a first line of defence against (electrophilic) xenobiotics, and (e) normal functioning of the blood-brain barrier. GST also serves as a carrier protein for organic substances (Mannervik and Danielson, 1988). In most organisms with GST activity, multiple forms of the enzyme have been discovered; some of which are tissue-specific.

Four classes of GST, each containing more than one isozyme, have been identified on the basis of substrate and inhibitor properties, antisera, and N-terminal amino acid sequence; these have been named Alpha, Mu, Pi and Theta (Mannervik and Danielson, 1988; Mannervik *et al.*, 1992). Classes were originally identified for mammals, but non-vertebrate enzymes generally resemble very closely those from mammals (Clark, 1989; Sheehan and Casey, 1993). Cytosolic GST is a homo- or heterodimer of average molecular weight 43 ± 8 kDa (range 36 - 54 kDa for mammals and invertebrates) (Bogaards *et al.*, 1992; Clark, 1989). Subunit molecular weights range from 17 to 28 kDa (Daniel, 1993) with a mean of 24.9 ± 3.1 kDa (Clark, 1989) and heterodimers only form within a class (Dirr *et al.*, 1994).

Most work on the cytosolic enzymes to date has concentrated on the Alpha, Mu and Pi classes of the enzyme, previously known also as the basic, near-neutral and acidic forms. Alpha class isozymes have high levels of Se-independent peroxidase activity as well as detoxification activity whereas other classes have much lower peroxidase activity (Mannervik and Danielson, 1988). This nomenclature system was developed for rat tissue, but similar isozymes have been found in mice, humans, and hamsters (Mannervik and Danielson, 1988), and comparable class homologies have been noted in bacterial and amphibian sources as well (Ketterer *et al.*, 1988).

Most GST activity in organisms is found in liver and kidney, although all tissues show some activity. Some forms of GST are transcriptionally regulated by xenobiotics or

environmental stress (Makar *et al.*, 1994). In spadefoot toads the maximal activity of GST in liver was greatly reduced during estivation (Chapter 2.1). The mechanism of this change could be: 1) expression of a different GST isozyme within the same or a different class during estivation, 2) expression of the same isozyme but in different amounts, and 3) the same GST and the same expression, but with post-translational modification, perhaps by reversible phosphorylation, to convert the enzyme to a less active form. Physiological changes such as maturation from embryo to adult toad (*Bufo bufo*) have been shown to correlate with expression of different GST isozymes (Barga de Quiroga *et al.*, 1988).

My hypothesis in this study was that, as one of the physiological changes occurring as toads enter estivation, spadefoot toads would express distinct form(s) of GST, i.e. mechanism (1) above. This GST would be better suited for the challenges imposed by the estivating state. GSTs from *S. couchi* were purified from liver and muscle of active and 2-month estivated toads by a simple two-step procedure, and compared to GSTs purified from liver and skeletal muscle of a non-estivating frog, *Rana pipiens*. GST from liver of awake toads has similar maximal activity (Chapter 2.1 results) to GST from the leopard frog, and may be similar kinetically as well. Incidental to kinetic characterization of GSTs from frog and toad, classification can be accomplished on the basis of inhibitor and substrate properties (Tahir *et al.*, 1985; Ketterer *et al.*, 1988). In order to determine whether toad GSTs were similar to GSTs from another toad, cross-reactivity of the isolated GST isozymes from spadefoot toads was tested with polyclonal antisera to the major GST from *Bufo bufo*. This toad species expressed several GST isoforms in liver, which were separable on the basis of isoelectric point. Di Ilio *et al.* (1993) raised polyclonal antibodies to the major isoform, which was present at pH 7.64, and this did not cross-react with antisera against human Alpha, Mu and Pi class GST. Essentially pure toad and frog GSTs and several crude homogenates were tested for cross-reactivity against GST-7.64.

GST from spadefoot toads may also be more resistant to denaturation by urea than GST from animals which do not accumulate urea. Stability of the purified toad enzymes in aqueous urea and KCl solutions was compared to mammalian GST to see if toad enzymes were more resistant to these potential denaturants.

MATERIALS AND METHODS

Chemicals and animals

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO), and were of the highest purity available, unless otherwise stated. *Rana pipiens*, males and females, were obtained locally in the autumn (or spring, where noted) and were sampled immediately. *S. couchi* tissue samples were from the animals described in Chapter 2.2.

GST assay

GST activity was assayed as described in Chapter 2.1. Optimum substrate concentrations which gave maximal velocity ($V_{\max}$) were: 5 mM GSH and 2 mM CDNB for toad liver, 2 mM GSH and 2 mM CDNB for frog muscle, and 2 mM GSH and 1 mM CDNB for all other tissues. Enzymatic analyses were conducted at room temperature (21°C). Total (Se-independent and dependent) GP_{ox} activity, of which GST activity is the Se-independent activity was measured with cumene hydroperoxide (CHP) as described in Chapter 2.1; one unit of CHP activity was defined as that amount which causes the net oxidation of 1 μ mole of NADPH per minute in the coupled enzyme assay with GR.

Sample preparation for assay

Samples of frozen liver or muscle from *R. pipiens* or *S. couchi* were rapidly weighed and homogenized 1:5 w/v in 5 mM KPi, pH 6.0 containing 1 mM EDTA (Buffer A) using a Polytron PT-10 sonicating homogenizer. The homogenate was centrifuged at

28,600 g for 20 minutes at 4 °C in a Sorvall RC-5B refrigerated centrifuge and the supernatant was decanted and desalted by elution through a small column containing Sephadex G-25 equilibrated in Buffer A or by dialysis (1:1000 ν / ν) against Buffer A. GST activity was measured and supernatant was then used immediately for other tests.

To test for GST susceptibility to reversible protein phosphorylation, GST from liver of awake toads was subjected to a variety of *in vitro* incubation conditions designed to promote phosphorylation or dephosphorylation of the enzyme (Brooks and Storey, 1990). Briefly, frozen liver from awake toads was homogenized 1:5 ν / ν in 50 mM imidazole, pH 7.0, containing 30 mM β -MeSH, 5 mM EGTA, 5 mM EDTA and 100 mM NaF, with a few crystals of PMSF. The homogenate was centrifuged at 28,600 x g in a Sorvall refrigerated centrifuge at 4 °C for 20 minutes to precipitate debris, and the supernatant was eluted through a column of Sephadex G-25 which had been equilibrated in Incubation buffer (40 mM imidazole, pH 7.0, 10 mM K_2HPO_4 , 10 mM β -MeSH, 0.1 mM EDTA and 20 % ν / ν glycerol). To promote phosphorylation, tubes 1-6 were set up as shown in Table 5.1, with different protein kinase activators, plus phosphatase inhibitor. Tube 7 was set up to promote *in vitro* dephosphorylation by incubating with Mg^{++} in the absence of ATP or NaF. All volumes are in microlitres; final volume was made to 150 μ L with water. Samples were incubated at room temperature and aliquots were removed for assay of GST maximal activity and K_m values for GSH and CDNB after 0, 2 or 20 hours.

Table 5.1 Setup of incubations promoting action of endogenous kinases or phosphatases. Tubes were set up as described in Materials and Methods and K_m of GSH was monitored over time of incubation.

tube	supernatant	Incubation buffer	NaF, 0.5 M	EDTA 0.2 M	MgCl ₂ , 0.5 M	ATP, 0.1 M	Effector
1	40	80	9	15	none	15	none
2	40	80	9	none	5	15	none
3	40	80	9	none	5	15	5 μ L 0.1 M cAMP
4	40	80	9	none	5	15	5 μ L 0.1 M cGMP
5	40	80	9	none	5	15	2 μ L 0.1M CaCl ₂
6	40	80	9	none	5	15	5 μ L 0.1 M cAMP 5 μ L 0.1 M cGMP 2 μ L 0.1 M CaCl ₂
7	40	80	none	none	5	none	none

GST purification

The purification procedure was optimized for *R. pipiens* liver and was then used for subsequent purifications of GST from other tissues. In initial tests, aliquots of *R. pipiens* liver supernatant (prepared as above in Buffer A) were loaded onto DE-52 Sephadex, DEAE-cellulose, phosphocellulose, or carboxymethyl cellulose columns of 1 to 2 mL, washed with Buffer A and eluted using bumps of 0.25, 0.5, 1, 2, and 4 M KCl. Enzyme binding/elution from hydroxylapatite was also tested by similar methods but with 0.25 to 4 M potassium phosphate used for elution. Hydroxylapatite was determined to be the most suitable column material for GST purification and was used in subsequent purification procedures.

An aliquot of desalted supernatant was loaded onto a hydroxylapatite (Bio-Rad) column (3 mL) equilibrated in Buffer A. The column was washed with 50 mL of Buffer A, or until A_{260} of the eluant dropped to zero. GST was then eluted from the column with a gradient of 5-500 mM KPi in buffer A. Activity eluted in two sharp peaks and the most active fractions of each peak were pooled. Pooled fractions were desalted by dialysis (SpectraPor dialysis tubing, 12-14 kDa molecular weight cutoff) or centrifugation through a small column of Sephadex G-25 into Buffer A. Enzyme was then applied to a 1 mL S-hexylglutathione agarose (SH-GSH, Sigma #H 7011) affinity column, previously equilibrated in Buffer A. The column was washed with 15 mL of Buffer A and then 15 mL of 5 mM KPi, 1 mM EDTA, pH 9.0. GST activity was then eluted with three sequential 2 mL washes of 5 mM GSH in the pH 9 buffer. The column was regenerated by washing with a small aliquot of 1 M β -MeSH and rinsing well with buffer A until no odour remained. Two columns were kept at 4°C and used repeatedly for all purifications, with no loss of function over the course of the experiment. Fractions with the highest enzyme activity (always the first two 2 mL elutions) were pooled and eluted through a small column of Sephadex G-25 to remove GSH, then frozen at -80 °C. Portions from each stage of the purification were reserved for protein analysis and were measured as

described in Chapter 2.1. Purified GSTs could be frozen for four months or more at -80°C in dilute buffer containing 1 mM EDTA with no concomitant loss of activity or alteration in kinetic parameters.

Kinetics and temperature dependence of GSTs

Substrate affinity constants (K_m) of GSH and CDNB were determined for all enzymes along with I_{50} values (the concentration of inhibitor that reduces activity by 50 %) for various salts. I_{50} determinations were conducted at optimal CDNB concentrations but with GSH at approximately K_m levels: 0.5 mM GSH for frog liver GST peak A, estivated toad liver GST, frog muscle and awake and estivated toad muscle, and 1 mM GSH for frog liver GST peak B and GST from liver of awake toads. Temperature dependence of GST activity was assessed over the range from 2 - 3 $^{\circ}\text{C}$ up to 23 $^{\circ}\text{C}$ for frog muscle GST and 40 - 45 $^{\circ}\text{C}$ for all other samples. Activity was measured under $V_{\max}$ substrate concentrations; temperature effects were analyzed using an Arrhenius plot of $\log V$ versus $1/^{\circ}\text{K}$. Kinetic constants, E_a from Arrhenius plots, and statistical analyses were done using a program provided by Dr. S.P.J. Brooks (Brooks, 1992).

Native molecular weight of GSTs

Purified GSTs were injected using a 1-mL injection loop, onto a size exclusion HPLC column (Variant TSK 4000 column 0.75 x 60 cm with a guard column) equilibrated and eluted with 10 mM potassium phosphate buffer, pH 7.0, containing 100 mM KCl. The pump attached to elute the column was an Altex Model 100A pump with an Altex Model 153 detector. Protein was detected by monitoring the change in absorbance of the eluting sample at 260 nm. Elution times were detected and compared to the elution time of a sample of known molecular weight (horse liver GST (46 kDa); Sigma).

SDS-PAGE of purified GSTs

SDS-PAGE of purified GSTs was performed essentially as described in Chapter 3, with a separating gel of 12.5 % w/v acrylamide instead of 10 % w/v acrylamide. One lane contained commercial (Sigma) molecular weight standards. Gels were run at 200 V using the discontinuous buffer system of Laemmli (1970) until the bromophenol blue leading edge was eluted from the gel. The gel was then fixed in 10 % : 30 % v/v acetic acid:methanol for one hour, stained with Coomassie brilliant blue stain for 45 minutes, and destained using several successive rinses of fixing solution.

pH dependence

The dependence of GST on assay pH was analyzed in KPi buffer with optimal concentrations of GSH and CDNB. Initially the buffer pH was obtained by mixing appropriate amounts of KH_2PO_4 and K_2HPO_4 . At low and high pH values, however, phosphate is not a suitable buffer, so pH was further adjusted using HCl or KOH. Assay pH in the cuvette was measured both prior to and immediately following the enzyme assay.

Immunoreactivity

Polyclonal antibodies against GST-7.64 from the toad *Bufo bufo* (a gracious gift from Dr. C. Di Ilio; Aceto *et al.*, 1993) were tested for cross-reactivity against spadefoot toad and leopard frog GST isoforms. Lyophilized antisera was reconstituted in distilled water and immediately frozen in aliquots. Samples of purified GSTs were pipetted onto a pre-equilibrated nitrocellulose membrane under vacuum, allowed to dry, and then immediately blocked with 1% BSA w/v in Tris-buffered saline (TBS) containing 0.05 % v/v Tween 20. Membranes were washed three times with TBS/Tween and then left overnight at room temperature (21°C) to incubate with a 1/1000 v/v dilution of the primary antibody in TBS-Tween. The membranes were then gently washed three times at

room temperature by floating in TBS-Tween for 15 minutes per wash. Subsequently, membranes were incubated with secondary antibody, alkaline phosphatase-conjugated goat anti-(rabbit IgG) for one hour. The membranes were again washed and then were developed with bromochloroindolyl phenol (BCIP) with nitroblue tetrazolium (NBT). Blots were washed with distilled water, observed for evidence of cross-reactivity with the supplied primary antibody, and then photographed.

In another test, aliquots of crude supernatant from liver and muscle of various animals (Table 5.5) were separated on SDS-PAGE and electroblotted onto nitrocellulose. Cross-reaction with the antibodies against *B. bufo* GST-7.64 was then performed as above.

Denaturation with urea or KCl

The effects of *in vitro* incubation with KCl or urea were tested at room temperature on liver GST from commercial sources (horse, rabbit) and from awake versus estivated toads. Aliquots of GST (final concentration 25 µg/mL) were incubated with 0, 1, or 2 M KCl (added from a 4 M stock) or 0, 0.3, 1, 2, 3, 4, or 5 M urea (added from a 10 M stock) in Buffer A. Final pH of samples was checked and was the same for all incubations. After incubation for various times, GST activity was assayed at room temperature with 5 mM GSH and 2 mM CDNB. A blank rate without enzyme representing the spontaneous conjugation of GSH and CDNB was subtracted from the final rate. The amount of incubation mixture added to each 1 mL assay cuvette (5 µL for rabbit liver GST, 2 µL for toad liver GST) diluted effector concentrations to a maximum of 25 mM for urea or 10 mM for KCl; initial assays with non-incubated enzyme showed that these levels of effectors did not alter GST activity in the cuvette.

pl of toad liver GSTs

Toad liver supernatant was subjected to isoelectric focusing (Vesterberg, 1971) as described in Chapter 3. After overnight focusing, the column was drained into 1.87 mL fractions and the elution profile of enzyme activity and the pH gradient were measured.

RESULTS

GST activity

Results of a typical purification of GST from toad (*S. couchi*) liver and muscle are shown in Table 5.2. Results for the corresponding tissues from *R. pipiens* are shown in Table 5.3. The maximal activities of GST in liver of awake and estivated toad and leopard frog were 2.12 ± 0.29 U/mg soluble protein ($n = 5$), 1.45 ± 0.10 ($n = 3$) and 0.97 ± 0.11 U/mg ($n = 3$), respectively. Values were comparable to those from liver of another toad (*Bufo bufo*) (Aceto *et al.*, 1993). Frog liver supernatant had similar maximal GST activity to that reported in Chapter 2.1, however values for awake toads were 43 % higher and for estivated toads were 66 % higher than the corresponding values for toad liver reported in Chapter 2.1 (Table 2.1.2). Maximal activity for toad liver GST was 56 % lower for estivated animals, compared to awake animals (difference of 50 % was reported for these values in Chapter 2.1). CHP-dependent activity of extracts from awake toads ($0.051 \mu\text{mol/mL/mg}$; $n = 2$) was only 2.4 % of the CDNB activity. The enzyme from estivated toads also showed CHP activity ($0.046 \mu\text{mol/mL/mg}$; $n = 2$), roughly 3.6 % of the activity with CDNB. Liver of *Rana pipiens* showed higher activity with CHP, $0.063 \mu\text{mol/mL/mg}$ ($n = 2$), which represented 6.5 % of the activity with CDNB.

Frog and toad muscle had lower GST activity in crude extracts than did liver (also in agreement with results of Chapter 2.1) and lower specific activities for the purified enzyme (Tables 5.2 and 5.3). Activity for muscle of awake toads was 0.30 ± 0.04 U/mg ($n = 4$) and the activity for estivated toads was 0.13 U/mg (average of two values). The

value for estivated toads agrees with the corresponding value in Table 2.1.3 of Chapter 2.1, but the value for awake toads disagreed, being 1.7-fold higher.

The differences between awake and estivated toad GST maximal activities in both liver and muscle likely represented population differences between the sample of toads used in Chapter 2.1 and the sample used for work reported in later chapters. However, the final results were similar in that for both populations, the maximal activities of GSTs were significantly lower for the estivated population than for the awake population.

GST in extracts from liver of awake toads showed no change in K_m for GSTH in response to incubation *in vitro* with second messengers and substrates for endogenous protein kinases or protein phosphatases.

GST Purification

GST in extracts of liver from awake *S. couchi* eluted from hydroxylapatite in one peak, at 175 mM KPi (Fig. 5.1); this peak showed both CDNB and CHP activity, although CHP activity (0.101 U/mg) was only 0.28 % of the activity with CDNB. GST in extracts of estivated liver eluted at 125 mM KPi off hydroxylapatite (Fig. 5.1); activity with both CDNB and CHP eluted in the same peak. Activity with CHP was 0.066 U/mg, representing 0.96 % of the activity with CDNB.

Figure 5.2 shows the elution pattern for GST from *S. couchi* skeletal muscle; only a single peak was detected that eluted at the same salt concentration with extracts from both awake or estivated toads. GP_{ox} activity (CHP as substrate) was so low as to be negligible in muscle of awake and estivated toads.

GST activity from liver of autumn-collected *R. pipiens* eluted from the hydroxylapatite column in two peaks; an approximate 1:2 ratio of activity (Fig. 5.3). CDNB activity showed two distinct peaks; CHP showed one major peak of activity. The pattern of CHP-dependent activity indicated that the two peaks represented separate isozymic forms of the enzyme and so selected fractions from each peak were pooled for

further purification (indicated by asterisks) and named GST A (CDNB and CHP activity) and B (CDNB activity only), respectively (Fig. 5.3). CHP activity of the pooled peaks, termed A and B, was 0.225 U/mg and <0.010 U/mg, respectively. The ratios of CHP activity to CDBN activity were 13.2 % and less than 0.19 %, respectively (n = 2 for GST A; n = 1 for GST B); a second purification of GST B yielded no CHP activity in this fraction).

Leg muscle of autumn-collected *R. pipiens* showed one major and one minor peak of GST activity with CDBN as the substrate but no detectable CHP activity (Fig. 5.4); the major peak was purified for further characterization.

The second step in GST purification was affinity chromatography on S-hexylglutathione agarose. The final percent yield of activity (measured with CDBN as the substrate) after this step was between 22 and 60 % of the starting activity for *S. couchi* enzymes (Table 5.2), and 56 % for *R. pipiens* muscle GST (Table 5.3), but lower for *R. pipiens* liver GST, since the sample was split into two fractions, A and B. CHP activity of the pooled sample in the final step was 0.24 U/mg for GST from liver of awake toads, and 0.39 U/mg from liver of estivated toads (the average from two purifications), representing 0.3 % and 0.6 % of the final activity with CDBN, respectively. For GST from liver of *R. pipiens*, CHP activity was 3.9 U/mg for the final preparation of frog liver GST A (n = 2), representing 12.3 % of the activity with CDBN. CHP activity was undetectable in the final preparation of frog liver GST B. The final purified enzyme was frozen at -80°C in Buffer B and was stable indefinitely. Further concentration of the essentially pure enzyme using Centricon filters (Diamed) or by dialysis against solid sucrose or polyethylene glycol resulted in lower activity, presumably due to the enzyme sticking to the polymer or plastic membrane, or to inhibition of activity by some property of the concentrated enzyme, thus the enzyme was routinely stored in dilute solution. No change in enzyme $V_{\max}$ or K_m for GSH or CDBN was detected over time stored at -80 °C, for the purified enzymes.

Subunit molecular weight

Analysis of the proteins separated on SDS-PAGE and stained with silver showed one or two bands in each preparation, indicating that samples were basically pure as measured by electrophoresis (Fig. 5.5). Since GST can exist as either homo- or heterodimers, with subunit molecular weights of approximately 25 kDa, the presence of two bands in some preparations is consistent with the existence of a heterodimer in that tissue. From a plot of log molecular weight of standards vs. migration on the gel (Fig. 5.7), the subunit molecular weight of the major bands of purified GST were determined to be between 17 and 22 kDa for the *R. pipiens* liver and *S. couchi* liver and muscle enzymes (Table 5.4). Examination of the banding patterns indicated that frog liver GST B and toad muscle were composed mostly of one subunit of molecular weight 20.3 and 20.4 kDa, respectively. Both samples had a small band in addition to the major one, at 19.8 kDa for frog liver B and at 22 kDa for frog muscle. Liver GST from awake and estivated toads was a heterodimer, both with bands of 22.8-22.9 and 21.2 kDa. The distribution of the two bands differed somewhat between the two physiological states; GST from liver of estivated toads had a higher amount of the 21.2 kDa band, whereas GST from liver of awake toads had approximately equal amounts of the two. Frog liver GST A had one different molecular weight subunit than all other GSTs, having a faint band at 21 kDa and a stronger band at 17 kDa. From size-exclusion HPLC, GSTs from toad and frog liver and muscle were compared to GST from equine liver (Sigma) and since elution volume was similar, molecular weight of the purified isozymes was determined to be equivalent for all forms of GST. Thus all GSTs isolated, with the exception of frog muscle, described below, were heterodimers.

Assignment of homo- or heterodimeric structure could not be made for frog muscle GST. There was one major band at 38.5 kDa with minor bands underneath at 35.5 and 22.2 kDa (the results are the average of two separate purifications run on SDS-

PAGE), possibly indicating that the enzyme had only been partially dissociated into subunits by boiling in the SDS-PAGE loading buffer.

Kinetic constants

Toad GSTs. Table 5.5 shows the kinetic properties of purified toad liver and muscle GST. The GST forms from liver of awake versus estivated toads showed distinctly different kinetic properties, differing in their K_m values for both substrates as well as in the I_{50} values for GSSG, urea and several salts. Thus, compared with the enzyme form in awake toads, GST from liver of estivated toads for a lower K_m for GSH (55 % of the awake value) but a higher K_m for CDNB (53 % higher). The estivated enzyme form was also much more strongly inhibited by the product of the reaction, GSSG, with an I_{50} value 5-fold lower than that of the awake isoform. The estivated form was also more sensitive to NaCl inhibition but less sensitive to inhibition by either urea or KCl, compounds that accumulate during estivation.

Purified muscle GST from awake toads had $K_{mCDNB} = 0.72 \pm 0.09$ mM ($n = 6$), which was not significantly different from estivated toad muscle GST ($K_{mCDNB} = 0.86 \pm 0.17$ mM, $n = 5$). K_{mGSH} was also not significantly different for awake and estivated toad muscle GST: 0.29 ± 0.04 mM ($n = 5$) and 0.23 ± 0.04 mM ($n = 6$), respectively. The muscle enzyme also showed no differences in any other kinetic parameters between the awake and estivated animals and, hence, values for muscle GST kinetics shown in Table 5.5 are the means of enzyme from both control and estivated animals. The toad muscle isozyme showed properties that were different from either of the liver forms. It exhibited the lowest K_m for GSH of the three enzymes but was the least sensitive to GSSG and urea inhibition. However, the enzyme was strongly inhibited by KCl and NH_4Cl ; the I_{50} for KCl was only 80 mM, well within the intracellular range.

Frog GSTs - Kinetic properties of purified frog liver GST forms A and B and frog muscle GST are shown in Table 5.6. In addition to the differential use of CHP as a

substrate by the A form but not the B form (noted previously; Fig. 5.1), the two liver enzymes showed distinctly different affinities for both GSH and CDNB as well as differential sensitivities to inhibitors. GST A showed a K_{mGSH} that was 70 % higher than the B form but oppositely, a K_{mCDNB} that was lower (57 % of the value for GST B). The K_{mGSH} of GST A was affected by the addition of salt or urea, and was 1.86- and 2.4-fold higher in the presence of added 0.2 M KCl or 0.3 M urea, respectively. By contrast, the K_m of GST B was not affected by these compounds. The enzymes also differed significantly in their responses to product inhibition by GSSG as well as salts and urea.

Frog muscle GST exhibited kinetics that were quite similar to the liver B form; for instance, K_{mGSH} , K_{mCDNB} , I_{50KCl} and I_{50urea} were similar to those for frog liver GST B and were significantly different from values from frog liver GST A. However, the I_{50GSSG} was 70 % higher than the corresponding value from the B peak of frog liver.

pH and temperature dependence of GSTs

Fig. 5.7 shows the effect of assay pH on the activity of purified GST from frog and toad liver and muscle. The enzyme from all sources showed optimal activity at pH 7. Toad GSTs and frog muscle GST showed a somewhat broader optimal range than did the frog liver enzymes with activity at pH 6.6 virtually equivalent to that at pH 7 but the toad enzymes showed a much sharper reduction in activity at higher pH values than the frog enzymes. Activity of toad liver GST was virtually reduced to zero at pH 7.5, whereas frog liver GSTs retained about 40% of the maximal activity at this pH.

Activation energies were derived from Arrhenius plots as previously described in Chapter 3. The Arrhenius plots were linear over the whole range (2 to 40 °C; 2 to 25 °C for frog muscle) tested. Frog liver peak A had the lowest activation energy (37.2 ± 1.1 kJ/mol) of all GSTs isolated (Table 5.7) whereas the activation energy for frog GST B was very similar to that of the enzyme from toad liver (ranging from 49-55 kJ/mol). GST

from leg muscle of both species also showed similar activation energies that were considerably higher, 67-71 kJ/mol, than the liver values..

Immunoblotting

Immunoblotting against antisera to GST-7.64, raised against the major GST from toad (*Bufo bufo*) liver, was done first against the essentially pure enzymes and next against crude extracts from liver and muscle of various animals (following paragraph). According to the results of Aceto *et al* (1993), *B. bufo* adult liver GST-7.64 was immunologically distinct from mammalian Alpha, Mu and Pi GST. Since this was isolated from liver of another toad, antisera from this isoform was tested also against basically purified GSTs from *S. couchi* and *R. pipiens* to check for homology between amphibia. The "dot blot" technique was used to determine whether purified GSTs from the two species was recognized by a polyclonal antibody to GST-7.64 of *Bufo bufo* (Fig. 5.8). Positive recognition of the antisera was observed for both *S. couchi* liver GSTs as well as *R. pipiens* liver GST A and leg muscle GST, indicating that these enzymes had significant homology to *B. bufo* GST-7.64 to be recognized by its antisera. However, *R. pipiens* liver GST B did not crossreact, and neither did *S. couchi* muscle (both awake and estivated muscle was tested). A commercial preparation of horse liver GST (Sigma) was also recognized by the *B. bufo* GST-7.64 antisera but commercial rabbit liver GST (Sigma) was not recognized (Fig.5.8). These commercial preparations were mixtures of GST classes. Recognition by the antisera was not class-specific, since frog liver GST A (Alpha class) was recognized together with other GSTs which were not Alpha class, but may have been generally tissue-specific to liver GSTs.

To further analyze the species/tissue distribution of GSTs that crossreact with antibodies to *B. bufo* GST-7.64, proteins in crude extracts of liver and muscle from a variety of animals were separated on SDS-PAGE, electroblotted onto nitrocellulose, and then incubated with the *B. bufo* GST-7.64 antisera (Table 5.8). With the exception of

rabbit liver GST, liver GST from a variety of mammalian, reptilian and amphibian sources was recognized by antibodies to *B. bufo* GST-7.64. The antibodies used were polyclonal in origin and thus a small amount of cross-reactivity with other proteins was observed. However, positive immunostaining was observed in the 17-25 kDa range (the molecular weight of GST subunits) for several tissues. The only muscle GST that was recognized was that from leopard frogs, confirming the results of the dot blots (above).

However, GSTs may also be classified on the basis of inhibition by various compounds (Mannervik *et al.*, 1994; Tahir *et al.*, 1985). Table 5.9 shows that *Bufo bufo* GST-7.64 shared no common inhibition characteristics with leopard frog or spadefoot toad GSTs and that it did not fit the classification system based on inhibition profiles that was developed by Tahir *et al.* (1985).

Denaturation of GSTs

Urea is well known as a chemical agent used to denature proteins, and it exerts a perturbing effect on several enzymes (Hochachka and Somero, 1982). Yet urea is accumulated naturally during estivation in spadefoot toads (Chapter 2.2) and acts as an osmolyte to reduce body water loss by colligative means. To determine the effects of urea on the stability of GSTs, samples of rabbit liver GST, purified toad liver GSTs ($n = 3$), and horse liver GST ($n = 1$) were incubated with varying concentrations of urea up to 3 M (toad) and 5 M (rabbit). Activity was tested at intervals over 22 hours, using standard assay conditions (Fig. 5.9).

Results for incubations with urea for horse liver GST ($n = 1$) were the same as those for rabbit liver GST and are not shown. Data in Fig. 5.9 are presented as the average of three separate experiments $\pm$ s.e.m. In the absence of added urea, liver GST from awake toads was completely stable over the 22 h incubation at room temperature whereas the enzyme from estivated animals lost only about 20 % of its original activity. Rabbit liver GST lost about 26 % of its initial activity during incubation. Physiological

concentrations of urea (0.3 M) had only a slight effect on enzyme stability over time. Higher urea concentrations, however, resulted in increased loss of enzyme activity. This was apparently due to irreversible denaturation of the enzyme during incubation and not due to urea effects on the ability to assay GST in the cuvette; initial tests showed that urea concentrations of 25 mM (the maximum that would result from the addition of an aliquot of incubation mixture to an assay cuvette) had no effect on the measured GST activity in the standard assay.

After 21.5 hours of exposure to various concentrations of urea, final activity was assayed. GST from liver of awake toads had remaining activities of 105, 98, 66, 28 and 2.9 % of the initial activity, for enzyme incubated in urea concentrations of 0, 0.3, 1, 2 and 3 M urea, respectively. GST from liver of estivated toads had remaining activities of 80, 70, 50, 24 and 3.6 % of the initial activity, for the same incubation conditions. When incubations were done with 4 and 5 M urea, activity fell to 5 % of initial activity in less than one hour (not shown on the figure). By contrast, rabbit liver GST, after 21.5 hours, had remaining activities of 73, 64, 52, 47, 41, 14 and 10 % of the initial activity, for urea concentrations of 0, 0.3, 1, 2, 3, 4 and 5 M urea, respectively. This indicated that a greater strength of urea was required to denature GST from rabbit liver.

KCl, which also builds up in elevated concentrations in toad cells as the result of cell water loss, was used in a similar experiment as a comparison. GST from awake and estivated toad liver and from horse liver was incubated for 8 hours at room temperature in buffer with salt concentrations ranging from 0-2 M KCl. All preparations (toad and horse GSTs) lost some activity over time but there were no net effects of salt concentration on the final percentage of activity lost in any preparation (overall activity loss in all trials with KCl ranging from 0-2 M was $12\% \pm 3\%$, $n = 9$, data not shown). Thus, concentrated GST is quite stable at room temperature and KCl concentrations up to 2 M have no influence, either positive or negative, on enzyme stability.

pl of toad liver GSTs

GSTs from toad liver were also characterized by isoelectrofocusing. Different isoforms of enzymes typically have different isoelectric points, and the GST classes may be generally grouped by isoelectric point. Fig. 5.10 shows the isofocusing elution profile for toad liver GST from awake and estivated animals. One form of GST was detected in each case but the *pl* values differed significantly between awake and estivated liver. GST from liver of awake toads had a *pl* of 7.12 ± 0.04 ($n = 3$) whereas that for estivated toad liver GST was 6.76 ± 0.07 ($n = 4$) ($P < 0.025$, Student's *t*-test, 2-tailed).

Table 5.9 illustrates how GSTs may be grouped into Alpha, Mu or Pi class according to their behaviour with various inhibitors. Tahir *et al.* (1985) established the system using human GST isozymes, and Cibacron Blue (CB), Rose Bengal (RB) and S-hexylglutathione (S-Hex) were selected and tested with toad enzymes, since these chemicals all inhibit the three GST classes to varying degrees. According to the results of Tahir *et al.* (1985), Alpha class is the least inhibited by CB whereas Mu is strongly inhibited. Pi class has an I_{50} for RB 14 times higher than Mu or Alpha. The I_{50} for S-hexylglutathione is 3 μM for Alpha class; for Mu and Pi the I_{50} s are twofold and sixfold higher, respectively. Frog liver GST A had an I_{50} for CB which was closest to that shown by human Alpha class GST, and one hundred times higher than for the other frog and toad GSTs (Table 5.9). Other GSTs had I_{50} s for CB which most closely resembled that of human Mu class GST. Frog and toad GSTs showed variable I_{50} values for RB of between 0.06 (liver of awake toads) and 1.22 (Frog liver B).

Table 5.2. Purification of GST from *Scaphiopus couchi* liver and muscle (CDNB activity). Results are from one purification: further purifications yielded similar results.

GST	awake liver			estivated liver			awake muscle			estivated muscle		
	U/mg	fold	% purif	U/mg	fold	% purif	U/mg	fold	% purif	U/mg	fold	% purif
crude (U/mg)	2.3	1	100	1.3	1	100	0.30	1	100	0.13	1	100
OH-Ap	35.6	24.19	63	6.8	5.1	51.5	0.95	2.64	48.5	0.30	2.7	58
SH-GSH	81.1	46.7	36	66.1	49.6	22.5	9.79	34.3	49	8.97	80	60

Table S.3 Purification of GST from *Rana pipiens* liver and muscle (CDNB activity). Results are from one purification; further purifications yielded similar results.

	liver peak A			liver peak B			muscle		
	U/mg	fold purif.	% yield	U/mg	fold purif.	% yield	U/mg	fold purif.	% yield
supernatant	0.97	1	100	1.1	1	100	0.069	1	100
OH-Apatite	1.7	1.56	13.8	4.0	3.6	23	0.75	10.9	75
SH-GSH	31.5	29.6	5.6	51.3	47.9	11.5	8.12	117	56

Table 5.4. Elution times for purified GST isozymes off size-exclusion HPLC, and subunit sizes as determined by SDS-PAGE.

GST isozyme injected	Elution volume (mL)	MW of subunit(s) (kDa)
Frog liver A	30.6	21, 17
Frog liver B	30.6	20.3, 19.8
Frog Muscle* note: did not fully dissociate on SDS-PAGE	30.8	39, 36, 22.3
Toad liver, awake	30.8	22.9, 21.2
Toad liver, estivated	30.8	22.8, 21.2
Toad muscle, awake	30.9	22, 20.4
Toad muscle, estivated	30.9	22, 20.4
Horse liver	31.0	23, 21

Table 5.5 Kinetic parameters of GST isozymes from *Scaphiopus couchi* liver and muscle.

Results are means of three or more determinations, $\pm$ S.E.M. ^a, significantly different at $P < 0.01$ from awake *Scaphiopus couchi* liver GST, determined using Student's t test (2-tailed). Awake and estivated muscle were identical kinetically, and the data reported here pool data for both preparations.

	awake liver	estivated liver	muscle
K_m GSH (mM)	0.92 ± 0.03	0.51 ± 0.05 ^a	0.25 ± 0.03 ^a
K_m CDNB (mM)	0.51 ± 0.11	0.78 ± 0.07 ^a	0.77 ± 0.08 ^a
I_{50} GSSG (mM)	1.05 ± 0.11	0.20 ± 0.001 ^a	1.26 ± 0.29
I_{50} urea (M)	0.85 ± 0.06	1.57 ± 0.16 ^a	4.22 ± 0.02 ^a
I_{50} KCl (M)	0.23 ± 0.03	0.49 ± 0.06 ^a	0.08 ± 0.01 ^a
I_{50} NaCl (M)	0.36 ± 0.04	0.17 ± 0.01 ^a	0.41 ± 0.02
I_{50} NH ₄ Cl (M)	0.19 ± 0.01	0.15 ± 0.003	0.073 ± 0.005 ^a
I_{50} Na ₂ SO ₄ (M)	0.15 ± 0.02	nd	nd

Table 5.6 Kinetic parameters of GST isozymes from *Rana pipiens* liver and muscle. Results are means of three or more determinations, $\pm$ S.E.M. ^a, significantly different from the value for *Rana pipiens* liver GST peak A by the Student's t-test (2-tailed), $P < 0.01$. ^b, significantly different from the K_m GSH without modulators, $P \leq 0.05$. When added, KCl was 200 mM and urea was 300 mM. nd = not determined. Citrate and $MgCl_2$ together in equal concentrations is abbreviated C-Mg.

	liver GST A	liver GST B	muscle
K_m GSH (mM)	0.70 ± 0.06	0.41 ± 0.05^a	0.39 ± 0.04^a
K_m CDNB (mM)	0.41 ± 0.08	0.72 ± 0.06^a	0.71 ± 0.05^a
K_m GSH (+KCl)	$1.30 \pm 0.14^{a,b}$	0.39 ± 0.05^a	
K_m GSH (+Urea)	$1.70 \pm 0.30^{a,b}$	0.43 ± 0.03^a	
I_{50} GSSG (mM)	>10 mM	1.00 ± 0.05^a	1.7 ± 0.1^a
I_{50} KCl (M)	0.16 ± 0.01	0.11 ± 0.02	0.15 ± 0.01
I_{50} urea (M)	1.05 ± 0.03	2.04 ± 0.04^a	1.61 ± 0.06^a
I_{50} Na_2SO_4 (M)	0.163 ± 0.045	>0.5 ^a	
I_{50} NH_4Cl (mM)	134 ± 6.0	82.5 ± 4.1^a	
I_{50} C-Mg (mM)	67.6 ± 3.1	245 ± 11.3^a	

Table 5.7. Activation energies (kJ/mol) of GST from toad and frog liver and muscle.

Results are means $\pm$ S.E.M. of three (estivated toad liver, toad muscle, frog muscle) or four (frog liver peak A, frog liver peak B, awake toad liver) separate determinations. Statistical differences between the means were tested using one-way analysis of variance followed by the Student-Newman-Keuls test; values sharing the same letter designation (^a, ^b, or ^c) are not different from each other whereas those with different letter designations are statistically different from each other, $P < 0.05$.

GST	E_a (kJ/mol)
awake toad liver	55.2 ± 5.68^a
estivated toad liver	53.7 ± 0.62^a
awake toad muscle	67.1 ± 1.82^b
frog liver peak A	37.2 ± 1.11^c
frog liver peak B	48.7 ± 2.87^a
frog muscle	71.1 ± 0.10^b

Table 5.8. Crossreactivity of various tissue extracts with antibodies to *Bufo bufo* GST 7.64. Proteins in cell-free extracts were separated by SDS-PAGE (12% acrylamide), blotted onto nitrocellulose, and treated as described in Materials and Methods. +, positive recognition for *B. bufo* GST-7.64 antisera; -, no recognition. Mild cross-reactivity was observed for several proteins in the tissue extracts, since the antibodies provided were polyclonal. Positive cross-reactivity was reported when a strong reaction was observed for one or more protein band of between 20 - 30 kDa molecular weight.

Extract	Reaction
Rabbit liver	-
Rat liver	+
Prairie dog liver	+
Prairie dog muscle	-
Ground squirrel liver	+
Ground squirrel muscle	-
Turtle liver	+
Toad liver (awake toads)	+
Toad liver (estivated)	+
Toad muscle (awake toads)	-
Toad muscle (estivated)	-
Wood frog liver	+
Wood frog muscle	-
Leopard frog liver	+
Leopard frog muscle	+

Table 5.9 Classification of purified GSTs from frog and toad liver and muscle based on their I_{50} values for different inhibitors. Results are mean $\pm$ S.E.M. of three or four determinations. Data for inhibition of human Alpha, Mu and Pi class GSTs was modified from Tabir *et al.* (1985). Data for adult *Bufo bufo* liver GST-7.64 and embryonic *B. bufo* GST-1 was from Aceto *et al.* (1993) (†). †, significantly different from corresponding I_{50} for frog liver GST-A^b, significantly different from corresponding I_{50} for awake toad liver. Significance was assessed using Student's *t* test, 2-tailed, *P* < 0.05

	Ciba Ion Blue (μ M)			Rose Bengal (μ M)			S-Hexylglutathione (μ M)		
Alpha class (human)	5			1			3		
Mu class (human)*	0.05			1			10		
Pi class (human)*	0.5			15			20		
load liver, awake	0.041 $\pm$ 0.003			0.064 $\pm$ 0.006			12.66 $\pm$ 1.41		
load liver, estivated	0.025 $\pm$ 0.002 ^b			0.18 $\pm$ 0.02 ^b			17.31 $\pm$ 1.34		
load muscle (awake + estiv.)	0.009 $\pm$ 0.001 ^b			0.66 $\pm$ 0.02 ^b			12.2 $\pm$ 0.6 ^c		
Frog liver GST-A	3.0 $\pm$ 0.5			1.01 $\pm$ 0.21			11.92 $\pm$ 0.35		
Frog liver GST-B	0.034 $\pm$ 0.006 ^a			1.22 $\pm$ 0.06 ^a			11.12 $\pm$ 0.44		
Frog muscle	0.035 $\pm$ 0.004 ^a			0.97 $\pm$ 0.16 ^a			11.61 $\pm$ 1.54		
Embryonic <i>Bufo bufo</i> GST-†	0.1			150			14		
Adult <i>Bufo bufo</i> GST-7.64†	0.25			15			0.05		

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Figure 5.1. Typical elution profile of GST from liver of awake and estivated *Scaphiopus couchi* from hydroxylapatite. The enzyme was eluted with a linear gradient of KPi (5-500 mM). Activities are expressed as % yield compared with GST activity in the supernatant prior to loading on the column. GST activity in liver extracts from awake and estivated toads each eluted in one peak, but at different KPi concentrations. Hydroperoxide activity was associated exactly with the peak for CDNB activity. Open or closed circles represent GST activity from awake or 2 months estivated toads, respectively.

Figure 5.2. Typical elution profile of GST from skeletal muscle of awake and estivated *Scaphiopus couchi* from hydroxylapatite. The column was eluted and activity was expressed as described in Fig. 5.1. GST activity eluted in one peak with mainly CDNB activity and very low hydroperoxide activity paralleling the CDNB activity peak.

Figure 5.3. Typical elution profile from hydroxylapatite of liver GST from autumn-collected *Rana pipiens*. The column was eluted and activity was expressed as described in Fig. 5.1. The enzyme eluted in two peaks, named A and B. Open circles, peroxidase activity with CHP as the substrate. Filled circles, detoxification activity with CDNB as the substrate. Fractions that were pooled and used for further purification are indicated by asterisks.

Figure 5.4. Typical elution profile from hydroxylapatite of skeletal muscle GST from autumn-collected *Rana pipiens*. The column was eluted and activity was expressed as described in Fig. 1. The enzyme eluted in two peaks, one major and one minor. Hydroperoxide activity (CHP as substrate) was undetectable in any fraction. Peak fractions from the major peak were pooled and used for further purification.

Figure 5.5 SDS-Polyacrylamide gel electrophoresis of purified GST from frog and toad tissues. The gel was 12 % w/v acrylamide (30:0.8 acrylamide: bisacrylamide) and was stained with silver. Lanes 6 and 10 show molecular weight standards (Sigma). Lane 1, Horse liver GST. Lane 2, GST from muscle of estivated toads. Lane 3, GST from muscle of awake toads. Lane 4, GST from liver of estivated toads. Lane 5, GST from liver of awake toads. Lane 7, GST from frog muscle. Lane 8, GST from frog liver, peak B. Lane 9, GST from frog liver, peak A. Both the lanes from GST of awake toad liver (lane 5) and frog liver A (lane 9) were contaminated by neighbouring lanes which contained molecular weight standards, and some of the bands corresponding to these standards appear in these lanes.

1 2 3 4 5 6 7 8 9 10

Figure 5.6. Standard curve used to determine the subunit molecular weight of GSTs: log molecular weight of standard versus relative migration on SDS-PAGE. The standards and their molecular weights (kDa) were: (1) α_2 -macroglobulin, 205; (2) β -galactosidase, 116; (3) phosphorylase b, 97; (4) bovine serum albumin, 67; (5) pig heart fumarase, 48.5; (6) carbonic anhydrase, 29; (7) horse liver GST, 21; (8) β -lactoglobulin, 18.4; α -lactalbumin, 14.2. ($r^2 = 0.97$).

Figure 5.7. pH profiles of purified GSTs. (A) *Rana pipiens* liver GST peak A; (B) *R. pipiens* liver GST peak B; (C) *Scaphiopus couchi* awake liver GST; (D) *R. pipiens* muscle GST; (E) *S. couchi* muscle GST. Values are means of four trials performed on a single enzyme preparation, $\pm$ S.E.M. Where error bars are not visible they are contained within the symbol. Phosphate buffer was used for all assays; the pH was checked immediately prior to and following the assay.

Figure 5.8. Nitrocellulose blot of GSTs probed with antibodies to GST-7.64 (Di Ilio et al., 1993). Purified GST from toad and frog liver and muscle was pipetted onto nitrocellulose equilibrated in TBS-Tween, then incubated as described in Materials and Methods. The coloured reaction was developed with alkaline phosphatase. TLC, GST from liver of awake toads (*Scaphiopus couchi*); TLE, GST from liver of estivated toads; HL, horse liver GST (Sigma); RL, rabbit liver GST (Sigma); TMC, GST from muscle of awake toads; TME, GST from muscle of estivated toads; 1 and 2, GST from liver of *Trachemys scripta elegans*, normoxic and anoxic, respectively. FLA, frog (*Rana pipiens*) liver GST peak A; FLB, frog liver GST peak B; FM, frog muscle GST. Dark spots indicate cross-reactivity; the spot for FLA was very faint.

Figure 5.9. Denaturation of GST from awake and estivated toads and rabbit liver by varying concentrations of urea. (A) awake toad liver GST. (B) estivated toad liver GST. (C) rabbit liver GST. Activity is shown as the percentage of initial activity remaining in the presence of: 0 effector (urea or KCl) at 0 time. Incubations were done in: 0 M urea (●), 0.3 M urea (○), 1 M urea (■), 2 M urea (□), 3 M urea (▲), 4 M urea (Δ), 5 M urea (◆). Data are means $\pm$ S.E.M., $n = 4$. Where error bars are not shown they are contained within the figure.

Figure 5.10. Isoelectric focusing profile of GST in liver extracts from awake and estivated toads. Column isoelectric focusing was performed as described in Materials and Methods. (A) awake toad liver. (E) estivated toad liver. The pI (isoelectric point) of the enzyme was taken as the pH value where the peak of the GST activity intersected the pH curve. Dashed line shows measured pH of fractions.

DISCUSSION

During estivation the maximal activity of glutathione *S*-transferase in liver and muscle of spadefoot toads was reduced substantially to 56 % and 43 %, respectively, of the corresponding activities in tissues of awake toads (Table 5.2). This decrease could result from a number of causes including (a) a reduced amount of enzyme protein present in the tissue (due to either reduced synthesis or increased catabolism of the enzyme during estivation), (b) a stable change in the activity state due to post-translational modification of the enzyme, or (c) the expression of a different GST isozyme during estivation (Storey, 1992). These three possibilities can be differentiated by comparing various properties of GST isolated from awake and estivated toads. The first possibility would be supported by identical kinetic and physical properties of the enzyme in both states indicating that the enzyme varied in amount only between the two states. The second possibility would be indicated by the occurrence of different kinetic and physical properties between the two states but properties that are interconvertible by *in vitro* treatments that promote the phosphorylation or dephosphorylation of the protein. The third possibility would also be indicated by different kinetic and physical properties between the two states but these would not be altered by reversible phosphorylation treatments. Other characteristics that might also help to differentiate between a change in isozyme type and reversible phosphorylation of a single isoform could include differences in immunocrossreactivity of the two forms or very different substrate preferences (e.g. ability to use CHP). Thus, GST from liver and muscle of awake and estivated toads was purified and characterized with these possibilities in mind in order to determine the mechanism of GST modification during estivation. GST was also purified, for comparative purposes, from liver and muscle of the leopard frog (*Rana pipiens*), a semi-aquatic species which does not estivate.

All forms of GST can bind to an affinity column which has GSH attached to the stationary phase (Nishinaka *et al.*, 1992), and this forms the basis for simple purification

of the enzyme. Three forms of GST were purified from *R. pipiens* and four from *S. couchi*. The purification procedure was rapid and straightforward involving only 2 steps, ion exchange chromatography on hydroxylapatite and affinity chromatography on *S*-hexyl-glutathione conjugated agarose. For *S. couchi* tissues, the procedure resulted in 22-60 percent yields and a fold purification of 50 for the liver and 35 to 80 for the muscle enzyme. Yield and fold purification were also good for the *R. pipiens* enzymes; the apparent lower yields for liver GST was due to the separation into A and B forms of the enzyme. Specific activity of amphibian GST liver isozymes (Tables 5.2 and 5.3) was similar to the range reported for GST isozymes from human liver (16-37 U/mg) (Habig and Jakoby, 1981). Final specific activity was also similar to GST 7.64 (54.7 U/mg) and other GST isozymes (23.6 to 48.1 U/mg) from adult toad (*Bufo bufo*) liver (Aceto *et al.*, 1993). Muscle isozymes from toads had nearly equal final specific activities, which at 9 U/mg, were lower than the values for typical liver GSTs reported above, but was similar to the 11.4 U/mg reported for foot muscle of *Littorina littorea* (Pannunzio, 1994).

Toad and frog GSTs showed a variety of properties common to the enzyme from other sources. The enzymes showed the same retention time on size exclusion HPLC as did purified commercial horse GST (Table 5.4) indicating that they had the same native molecular weight as horse GST (44 kDa). Molecular weights of between 37 and 50 kDa have been reported for GSTs from various sources such as flatworms (37 kDa; Clark, 1989), mollusks (40 kDa; Balabaskaran *et al.*, 1986); snail hepatopancreas (44 kDa; Ajele and Afolayan, 1992), toad liver (*Bufo bufo*) (50 kDa; Aceto *et al.*, 1993) and human liver (45 kDa; Comstock *et al.*, 1994). Subunit molecular weight of toad and frog GST enzymes was 17-23 kDa as determined by SDS-PAGE which fits well within the range of subunit molecular weights that have been reported for other animals including 24-26.5 kDa for the bivalve mollusk, *Mytilus edulis* (Fitzpatrick and Sheeham, 1993), and 22-25 kDa for an isozyme expressed in various tissues of the rat (Balabaskaran *et al.*, 1986). Frog muscle was a possible exception to this and with a molecular weight from SDS-

PAGE of 40 kDa might not have dissociated. GST from other sources exists as both homo- and hetero-dimers (Clark, 1989) and the results of SDS-PAGE indicated that this was also true of toad and frog GST. All toad and frog GSTs with the possible exception of frog muscle, proved to be composed of at least two subunits (Figure 5.5), which indicated either a mixture of homodimers or heterodimers. For frog liver GST B, estimated toad liver and toad muscle GST, the amounts of the respective subunits was obviously different, indicating that the samples represented mixtures of GST isozymes with different subunit compositions.

Activation energies for the purified GSTs were distributed in three groups by analysis of the results with the Student-Neuman-Keuls test. Frog liver GST A had the lowest activation energy, with toad liver GSTs and frog GST B having higher E_a , and muscle GSTs having the highest activation energies of all. Activation energy of GST purified from an insect cell line (classified as Mu or Pi based on its inability to accept CHP as a substrate) was 49.1 kJ/mol (Chang *et al.*, 1994), similar to GST B from frogs and GSTs from toad liver.

Three classes of GSTs have been identified in the cytosol, and prior to 1992 the classification of GSTs in the literature was based on their pI values (Mannervik *et al.*, 1992). Acidic, near-neutral and basic forms for GST were distinguished and these are now termed Pi, Mu and Alpha classes. Heterodimers of GST form only within a class (Gilliland, 1993). By this system, the toad liver enzymes, with pI values of 6.8 and 7.1 could be considered to belong the near-neutral or Mu class. However, the present results showing only one peak of GST activity in *S. couchi* liver contrasted with those of Aceto *et al.* (1993) who found five forms of GST (separable on the basis of isoelectric point) in adult *Bufo bufo* liver.

Other characteristics also distinguish the classes. Alpha GSTs, for example, exhibit substantial peroxidase activity which is detected as their ability to use CHP as a substrate in Se-independent GP_{ox} activity (Mannervik and Danielson, 1988). The CHP

activity for this class of isozymes is high; in human liver, hamster tissues (2 isoforms), human lung, and from a nematode CHP activities were 16, 32, 49, 63 and 87 % of the corresponding activity with CDNB, respectively (Mannervik *et al.*, 1983; Bogaards *et al.*, 1992; Singhal *et al.*, 1992; Brophy *et al.*, 1994). Hence frog liver A, showing CHP activity that was 12.3 % of the CDNB activity, probably belongs to this class. The presence of high Se-independent glutathione peroxidase activity in liver extracts of *R. pipiens* (Chapter 2.1, relative to toad liver) is explained by the high activity of this GST isozyme in frog liver, but not toad liver.

Some Mu and Pi class GSTs do accept CHP, but for those that do, the ratio of activity with these substrates is markedly lower. Two of the four human Mu class GSTs do not recognize CHP as a substrate. The other two have low activity with CHP, showing only 1-4% of the activity with CDNB (Comstock *et al.*, 1994). Mu class isozymes from other animals have similar low activity: rat (0.2 %; Ketterer *et al.*, 1985) and hamster (0.5 and 0.03 %; Bogaards *et al.*, 1992). The Pi class of GST from many organisms also shows low activity with CHP: 1.3 % (dog lens, Nishinaka *et al.*, 1992); 0.05 % (rat, Ketterer *et al.*, 1985); 0.07 % (hamster, Bogaards *et al.*, 1992) and 0.03 % (human, Mannervik and Guthenberg, 1981) of the CDNB activity. This was similar to CHP activities reported for GST from toad liver which were 2.0 and 2.3 % in supernatant, and 1 % and 0.6 % for the purified enzymes of the corresponding CDNB activity for awake and estivated toads, respectively. Frog liver GST B, frog muscle and toad muscle GST had negligible activity toward CHP. These results show that toad enzymes, frog liver GST B and frog muscle GST are likely of the Mu or Pi isozyme class. By comparison, the ratio of CHP activity to CDNB activity was 0.04 for *B. bufo* GST-7.64 (Aceto *et al.*, 1993). GST-7.64 was the major isozyme isolated from *Bufo bufo* liver, and could not be classified on the basis of antibody recognition: these results only indicated that the enzyme most probably was not Alpha class.

More recently, GST classification has also been based on enzyme reaction to various inhibitors, different classes having characteristic I_{50} values for Cibacron Blue, rose bengal, and S-hexylglutathione (Tahir *et al.*, 1985), as well as the immunological properties of the enzyme (Aceto *et al.*, 1993). Human near-neutral class (Mu) GST shows the greatest inhibition by CB, acidic class (Pi) is characterized by low inhibition (high I_{50}) by RB, and basic class (Alpha) GST shows the strongest inhibition by SH-GSH as well as low inhibition by CB. As discussed in subsequent paragraphs the frog and toad GSTs can be fitted into this classification system proposed by Tahir *et al.* (1985) (Table 5.9). The classification system also works for *B. bufo* embryo GST, which exhibits a very high I_{50} for RB and is recognized by antibodies to Pi class GST (Di Ilio *et al.*, 1992) but not for adult *B. bufo* GST-7.64 which appears to be distinct from other GSTs.

GST-7.64 was one of five isoforms identified in liver of adult *Bufo bufo* liver (Aceto *et al.*, 1993). The enzyme did not cross-react with antisera to human Alpha, Mu or Pi class GST (Biotrin, Dublin), and antisera to GST-7.64 did not recognize Alpha, Mu or Pi class GST from mammals, and from microbes and plants (Aceto *et al.*, 1993). Thus, it was immunologically distinct from GSTs studied to date. In addition, inhibitor properties indicated that the enzyme did not fit the typical inhibition profiles for the classes proposed by Tahir *et al.* (1985). Cross-reaction had not been demonstrated for this GST with those from any other source, except GST from embryonic *Bufo bufo*. The antibodies provided by Dr. Di Ilio were also the only antibodies to amphibian GSTs available, thus the cross-reactivity of GST-7.64 was studied against toad and frog GSTs.

The dot blot showed recognition of most toad and frog liver GSTs by GST-7.64 antisera, indicating that the antibodies recognized GSTs from some amphibian sources. By contrast, the major GST from toad muscle was not recognized, indicating that the isoform in toad muscle although probably of the same Mu class as toad liver GST, is not homologous to liver isozymes at the amino acid sequence that the antibody recognized. Nitrocellulose binds protein tightly so it is unlikely that protein was lost during the

washing or blocking steps. Frog muscle was the only muscle sample which cross-reacted with the *B. bufo* liver GST-7.64 antisera; frog liver B was not recognized, however frog liver A was. Frog liver A GST was classified as Alpha class, but *B. bufo* GST-7.64 did not fit Alpha class designation, since the pI was neutral and the activity with CHP, relative to CDNB activity, was 4 % (Aceto *et al.*, 1993). These results suggest that *B. bufo* GST-7.64 antibodies can recognize GST from another class. All other GST isozymes isolated from frog and toad were concluded to be Mu class; *B. bufo* GST-7.64 antibodies did not recognize all of these isoforms, differentiating between those which showed substantial kinetic similarity (e.g. frog liver GST B and frog muscle GST). Thus recognition of GSTs by antisera to GST-7.64 from another toad was not class-specific but may be generally tissue-specific.

Recognition of GSTs from other species was then tested using crude tissue extracts, to determine if sequence similarity existed for GSTs from sources other than just amphibians. Liver GSTs in general were recognized by the antisera to *B. bufo* GST-7.64 (Table 5.8), indicating a common amino acid sequence for some mammalian, reptilian and amphibian liver GSTs. Frog muscle GST was again the only muscle sample recognized, confirming the results above and that expression of GST types may be either tissue- and/or species-specific. So despite the fact that the GST 7.64 does not fall into any of the three known classes it still crossreacts with liver GST from many sources, both amphibian and mammalian. This may indicate that: a) most liver GSTs, and some muscle forms, have an isoform with a common sequence which will crossreact immunologically with GST-7.64. Or, b) Liver from most animals, which usually has all three forms of GST (although distribution varies) may have some low amount of a form sufficiently homologous to the GST-7.64 isoform, that can be detected immunologically but (in frogs or toads) doesn't seem to have been detected by other means of separation and characterization, perhaps indicating that it co-purified with one of the other isoforms.

Spadefoot toads showed only one form of GST in liver; both hydroxylapatite chromatography and IEF confirmed this. However, the liver enzyme form changed during estivation and the accumulated evidence indicates that this was due to the expression of a new isozyme type during estivation. Thus, treatments designed to promote *in vitro* phosphorylation or dephosphorylation of liver GST from awake toads had no effect on enzyme K_m values for GST and CDNB indicating the significant changes in these parameters seen in the estivated enzyme form (Table 5.2) were probably not the result of estivation-induced reversible protein phosphorylation of the enzyme. GST is not subject to other forms of covalent modification. The estivated isozyme differed distinctly from the awake form in several ways including binding to hydroxylapatite, isoelectric point, K_m values for substrates, and inhibition by GSSG, urea, salts, Cibacron blue and rose bengal. However, the two also shared characteristics including activation energies (53-55 kJ/mol), molecular weight, and immunocrossreactivity with *B. bufo* GST1-7.64 antisera. Despite significant differences in their I_{50} values for CB and RB, the general response of both enzymes to these inhibitors and to SH-GSH was one that is characteristic of a Mu type enzyme; thus, the enzymes were both highly sensitive to CB inhibition but showed only moderate inhibition by RB or SH-GSH (Table 5.9). Their near-neutral pI values were significantly different, but both also agreed with assignment to the Mu category. Kolesnichenko *et al.* (1991) claim that the Mu family of GST isozymes is regulated by cAMP-dependent protein kinase, and activated by cAMP, but this was not the case for toad liver GST whose properties were not altered by attempts to modify the phosphorylation state of the enzyme *in vitro*.

Unlike the liver enzyme, toad muscle GST was not different in estivated animals. Several comparisons of the enzyme from awake versus estivated toads, including substrate affinity constants, subunit molecular weight and immunocrossreactivity indicated that the same, single enzyme form was found in muscle in both metabolic states (although with a lower maximal activity during estivation). The muscle enzyme differed

from the liver forms in several ways including substrate affinities, sensitivity to urea and KCl inhibition, activation energies, and immunological properties. However, it very closely resembled the frog muscle GST isozyme in its K_m values for GSH and CDNB, I_{50} values for GSSG, urea and KCl and in its responses to CB, RB and SH-GSH. These common properties may indicate that the two muscle isozymes are of the same class, and muscle of anuran species may, therefore, exhibit a common GST isozyme of the Mu class. One difference which should be noted was that frog muscle GST cross-reacted with antisera to *B. hufo* GST-7.64, whereas toad muscle did not, indicating that the two were not equally recognized by the antibody.

I initially hypothesized that liver GST from awake toads would be similar to frog liver GST B, both enzymes coming from animals that were actively feeding and under no dehydration stress. In fact, elution profiles of these enzymes off hydroxylapatite as well as initial measurements of K_m values for GSH and CDNB showed that these were quite similar (although the match between estimated toad liver GST K_m values and frog liver GST B was even closer) (Tables 5.5 and 5.6). Inhibition by GSSG, urea and KCl was also similar between toad liver GSTs and frog liver GST B, and in addition all had more than one subunit identified on SDS-PAGE and very similar activation energies (Table 5.7). However, further studies indicated that the enzymes differed in several other properties including different pH profiles (Fig. 5.7), immunocrossreactivity and a much stronger inhibition by RB of the toad enzyme (Table 5.9). Given the similarities, it is likely that the enzymes belong to the same isozyme class.

Frog liver clearly had two distinct forms of GST that were readily separable by hydroxylapatite chromatography, and these showed numerous properties that were distinct from one another, including hydroperoxide activity (use of CHP as a substrate), substrate affinities for GSH and CDNB, responses to inhibitors, recognition by antibodies to GST-7.64 from *B. hufo* liver, and activation energies. As noted above, frog liver GST A had hydroperoxide activity which indicates that it probably belonged to the Alpha

class. This classification was also supported by its low inhibition by CB (high I_{50}) (Table 5.9) but the enzyme did not display the strong inhibition (low I_{50}) by SH-GSH that characterizes the mammalian Alpha form (Tahir *et al.*, 1985). Liver of most vertebrates has at least one Alpha class enzyme, for example human liver has five basic forms (Alpha class), one near-neutral (Mu) and two acidic forms (Pi class) (Warholm *et al.*, 1985). However, expression of GST isoforms varies not only between tissues but also between species; for instance in guinea pig and human liver, Alpha class enzymes are present in abundance, whereas in hamster Alpha and Pi class enzymes are present at low levels, and a Mu class GST predominates. Thus, it was not unusual that an Alpha class GST was found in frog liver; rather it was strange that one was not detected in toad liver. Alpha class GSTs are directly responsible for detoxification of lipid hydroperoxides. This indicates that spadefoot toads probably rely more heavily on selenium-dependent glutathione peroxidase for this function, whereas frogs have high activities of both enzymes. Differences in GST class distribution between *Rana pipiens* and *Scaphiopus couchii* may be normal differences between the two species or the presence of the Alpha GST in frogs may be a response to an environmental chemical inducer present in the frog's environment but not in the toad's. Expression of GSTs is induced in response to various chemicals such as barbiturates, polyaromatic hydrocarbons, butylated hydroxytoluene, and others (Ketterer *et al.*, 1988). Specific subunits of Alpha and Mu class GSTs are more readily induced than other forms (Ketterer *et al.*, 1988).

Frog liver GST B did not exhibit hydroperoxide activity and was kinetically distinct from GST A. Frog muscle GST also lacked CHP-dependent activity and resembled GST B in various other kinetic and inhibitor properties (Tables 5.5, 5.6 and 5.9). However, the enzymes differed distinctly in their activation energies (Table 5.7), behaviour on SDS-PAGE (Fig. 5.5) and immunocrossreactivity with antibodies to *B. hufscholtzi* GST-7.64 (Fig. 5.8). Both enzymes were strongly inhibited by CB (I_{50} about 0.035 μ M) (Table 5.9) and this indicates that, although distinct isozymes, both belonged to the Mu

class of GSTs. The presence of multiple isozymic forms of GST in animals (at least 3 in *R. pipiens*) is not unusual; for example, eight isozymes of GST, each with unique properties and amino acid compositions, were isolated from hamster liver, kidney and testis (Bogaards *et al.*, 1992), and five forms were separable from liver of *B. bufo* (Aceto *et al.*, 1992), although these have not, as yet, been assigned to classes.

The Alpha class of GST isozymes exhibits higher activity for carbon-centered hydroperoxides, reducing them and producing GSSG as the product (Ketterer *et al.*, 1988). Frog GST A had high Se-independent GP_{ox} activity and, as well, it was not inhibited by levels of GSSG much higher than physiological (Joannis and Storey, 1996). GSSG inhibited other GSTs by 50 % at close to 1 mM GSSG, higher than physiological levels reported in Chapter 2.1. Estivated toad GST had the lowest I₅₀GSSG (0.2 mM; Table 5.5), however this value was still 1.5-fold higher than GSSG levels in estivated liver (Chapter 2.1). Hence *in vivo* levels of GSSG may not modulate GST activity.

The effects of urea on both kinetics and stability of GSTs were examined. GSTs from both species were highly resistant to inhibition by urea as a metabolite effector; I₅₀ values for urea were about 1 M or higher (Tables 5.5 and 5.6) and V_{max} was unaffected (Chapter 2.1) by physiological levels of urea (which rise to about 300 mM *in vivo* during estivation). Of note was the fact that the I₅₀ for urea nearly doubled for the estivated versus awake toad liver enzyme which indicates that one of the reasons for the change in isozyme expression during estivation may be to put an enzyme in place that is highly insensitive to the metabolites and ions that increase in concentration during estivation. Not surprisingly, then, the isozyme from estivated toads also showed a 2-fold higher I₅₀ for KCl, compared with the awake isozyme, so sensitivity to salt inhibition is also reduced during estivation. Indeed, KCl would probably have a greater effect *in vivo* upon the GSTs since it inhibits the enzyme at levels that are closer to the physiological levels observed during estivation (intracellular K⁺ concentrations = 200 mM; Cl⁻ concentrations = 300 mM) (Flanigan *et al.*, 1994), than those of urea. This would be particularly true of

toad muscle GST which showed a low $I_{50\text{KCl}}$ of only 80 mM whereas $I_{50\text{UREA}}$ was over 4 M (Table 5.5). During incubation experiments to determine the stability of toad liver GSTs in urea and contrast this to the response of a mammalian GST, toad liver GST was more susceptible to inhibition with urea at the same protein concentration than was horse or rabbit enzyme, and there was no difference between the estivated and awake enzyme in terms of susceptibility to denaturation by urea. By comparison, frog enzymes exhibited similar $I_{50\text{UREA}}$ to toad enzymes, however the liver isozymes were more sensitive to inhibition by KCl. Salt would not normally accumulate *in vivo* to the same extent as in toads, since the semi-aquatic frog, although tolerant of desiccation (Churchill and Storey, 1995), does not desiccate as the spadefoot toad does, as part of its normal lifestyle.

Whereas urea effects on GST activity are probably of little physiological importance, even under the urea concentrations of estivation, a potentially greater negative impact of urea may derive from its ability to denature enzymes. Urea at physiological levels (0.3 M) had little effect on the stability of purified GST incubated over time but higher levels caused serious, irreversible denaturation of the enzyme (Fig. 5.9). Toad GST was also more sensitive to urea denaturation than were mammalian GSTs; thus, toad liver GST activity (both awake and estivated) was rapidly lost in 3 M urea, whereas similar effects were seen only with 5 M urea with mammalian GST (horse and rabbit). By contrast, high KCl concentrations caused no enzyme denaturation. Glycolytic enzymes from toad liver, in a previous study (Grundy and Storey, 1994), also failed to show changes in kinetic properties in response to elevated urea concentrations, indicating that toad enzymes in general may be insensitive to urea, traditionally considered a perturbing solute (Yancey and Somero, 1979). In the same study, KCl both lowered V_{max} and changed K_m values for substrates (Grundy and Storey, 1994). Thus, the accumulation of urea in high concentrations during estivation may alleviate the accompanying negative effects of high salt on overall enzyme activities during desiccation.

In conclusion, the lower maximal activity of GST in spadefoot toad liver and muscle during estivation was the result of the expression of a different GST isozyme in liver, but of reduced expression of a single GST isozyme in toad muscle. All forms of toad GST that were isolated had characteristics of the Mu class of GST isozymes. Distinct isozymes from liver and muscle of autumn-collected leopard frogs were identified, two probably belonging to the Mu class, and one of the Alpha class. Compared to mammals, toad GSTs were more susceptible rather than more resistant, to urea denaturation, but denaturation and inhibition with urea occurred only at levels that greatly exceeded physiological levels. KCl did not contribute to enzyme denaturation, but modulated GST activity at close to physiological concentrations, and therefore may exert some inhibitory effect on enzyme activity *in vivo* during estivation, as part of the overall metabolic regulation seen during dormancy in these animals.

CHAPTER 6
GENERAL DISCUSSION

Oxygen radicals and ROS are continuously produced *in vivo* by all oxygen-consuming organisms and have been linked to many pathological states. All organisms have built-in defences against attack by these species. Protection against lethal damage by ROS is conferred by prevention and termination of free radical generation and propagation, and by repair of the damage incurred. Termination is accomplished using enzymatic detoxification of ROS and low molecular weight metabolites that are suicide scavengers of the active oxygen species. Certain physiological conditions are particularly associated with oxidative stress including hyperoxia (Barga de Quiroga *et al.*, 1988), hypoxia (Bhat *et al.*, 1992), ingestion of toxic substances (Lee and Berenbaum, 1993), muscle atrophy (Kondo *et al.*, 1994), and exposures to extremes of cold (Barga de Quiroga, 1992), or heat (Ohtsuka *et al.*, 1994), pollution (Livingstone *et al.*, 1990; Ribera *et al.*, 1991), or ultraviolet radiation (Shick *et al.*, 1981). In some cases oxidative stress occurs following physiological stresses, such as may occur upon recovery from ischemia. Endurance of freezing, or entry into hibernation or estivation are all accompanied by depression of metabolic rate and oxygen consumption (Storey and Storey, 1990). Thus, the organism under stress may endure ischemia or hypoxia - either a cutoff of the blood supply (such as occurs during freezing) or, for hibernation or estivation, wide intermittent variations in oxygen levels from full oxygenation to hypoxic states between breaths. Ischemia is a more stressful state since plasma-borne delivery of oxygen and other substances to tissues is cut off and CO₂ and other wastes are not removed. During hibernation and estivation, blood supply is not impaired and tissues remain aerobic although tissue oxygen consumption is decreased and oxygen levels vary over a wide range. Following ischemia, re-introduction of oxygenated blood, rapidly increased metabolic rate and a concomitant burst of oxygen free radical production is coupled with increased levels of iron and copper (Chevion *et al.*, 1993) and hypoxanthine (Rubin *et al.*, 1992) and all these factors combine to lead to increased production of HO[•] and tissue injury. Recovery from hibernation or estivation involves rapidly increased oxygen

consumption and may be functionally similar to reperfusion: damage products of lipid peroxidation have been observed to increase upon arousal in snails (Hermes-Lima and Storey, 1993). Animals which endure freezing or estivation have adapted to easily circumvent the injurious consequences of oxidative stress upon recovery. They do this either by continually maintaining higher tissue levels of antioxidants than are present in comparable species that are intolerant of the stress, or by AOE gene expression during the initial stress, in anticipation of increased generation of reactive oxygen species upon recovery. Examples of these two strategies can be found in tissues of the wood frog, *Rana sylvatica*, which maintains high levels of AOE even while frozen, and the estivating land snail *Otala lactea*, which synthesizes AOE as a defence against the stress that occurs when oxygen consumption is rapidly increased upon arousal (Joanisse and Storey, 1996; Hermes-Lima and Storey, 1994). In both cases these animals experience hypometabolic states that are reversed during thawing or arousal with a rapid increase in oxygen consumption. Indeed, the freeze-thaw transition is functionally analogous to an ischemia/reperfusion event (Joanisse and Storey, 1996), which in mammalian systems is well known to cause substantial free radical damage (Campos *et al.*, 1993; Downey, 1990; Hossain *et al.*, 1995).

The possible association of estivation with oxidative stress had not been considered previously for a vertebrate animal, although other studies in our lab have examined the phenomenon in estivating land snails, *O. lactea* (Hermes-Lima and Storey, 1993). However, the spadefoot toad was considered to be a fitting model of extreme stress due to the degree of tissue desiccation it tolerates during estivation (McClanahan, 1967). Spadefoot toads inhabit one of the most xeric environments on earth. Conditions of extreme heat and dryness, coupled with scarcity of food, force the toad to remain buried for about 10 months of the year, only to emerge when run-off from thunderstorms creates temporary pools where the toads feed and breed (McClanahan, 1967). Dormant toads at 15 °C have a heartbeat of 5 beats per minute, compared to 20-25 beats per minute

in an awake toad at the same temperature (Seymour, 1973), thus reflecting their low metabolic rate during estivation.

Studies were designed to test which mechanisms of oxidative damage avoidance were used in spadefoot toads. The first study quantified antioxidant enzyme activities and soluble antioxidant (glutathione) status in spadefoot toads during estivation. These results presented evidence that toads do not enter estivation with waking up in mind; that is, they did not employ an anticipatory strategy that set them up during estivation with the biochemical adaptations to deal with oxygen radical stress and damage upon arousal. The results clearly showed that the maximal activities of most antioxidant enzymes were unchanged or lowered in tissues of estivating animals. Thus, maintenance or induction of the toad's antioxidant complement of enzymes and soluble glutathione was not essential for survival during estivation. In addition, the activity of XO, which generates reactive oxygen species, was significantly lower in estivated toads than in awake toads, and the ratio of XO to XDH did not change (XO is formed from XDH by proteolytic cleavage following oxidative stress). The conversion of XDH to XO and the subsequent increase in this ratio are predictive criteria for oxidative stress, but toads recovering from estivation showed no such increase and hence probably escaped oxidative stress induced by increased concentrations of XO, upon recovery from estivation.

It is unlikely that increased numbers of oxygen-centered free radicals were formed during estivation since oxygen consumption is much lower in this state than in tissues of the awake toad (Seymour, 1973b). The rate of generation of free radicals is directly proportional to metabolic rate (*i.e.* to oxygen consumption) and should be lower in estivated toads. However, recovery from estivation involves a rapid increase in metabolic rate which should stimulate the production of a burst of oxygen radicals. Indeed, lipid peroxidation increased following arousal from estivation in the snail, *O. lactea* (Hermes-Lima and Storey, 1993). Entry into estivation for this land snail was accompanied by higher antioxidant enzyme activity in anticipation of this event during arousal. This

protective mechanism or one such as the constitutively high AOE activities in tissues of the freezing frog *R. sylvatica* were expected in *S. couchi*.

As a result of lower AOE activities during estivation, it was hypothesized that increased damage to toad tissues would be detected in estivated animals. Analysis of a common marker for oxidative damage - lipid peroxidation - showed that despite lower O_2 consumption and presumably fewer ROS generated during estivation, lipid peroxidation damage was slightly increased in toad tissues. The results of three techniques to quantify lipid peroxidation in toads showed increased levels of TBA-RS in three tissues, increased conjugated diene groups in five tissues, and increased LOOH in three tissues of estivating toads. Since ROS themselves induce expression of several AOE in bacteria and eukaryotes, reduced AOE activity appears to be due to repressive influences other than reduction in the amount of ROS. In other words, influences outside reduced levels of ROS cause the reduction of AOE activity during estivation (otherwise there would not have been a net accumulation of damage).

Estivation for toads was a stress distinct from, but involving some of the hallmarks of an oxidative insult; thus, lipid peroxidation increased during estivation, and the GSSG/GSH ratio rose. This was also shown for the estivating land snail *O. lactea* (Hermes-Lima and Storey, 1993), but snails also showed elevated activities of key antioxidant enzymes, presumably in anticipation of arousal. Three antioxidant enzymes were studied from awake and estivated toad liver to determine whether adjustments in AOE activities in toad liver were due to expression of novel isozymes specific to estivation.

Purified AOE

Liver is the major source of H_2O_2 in the body (Barga de Quiroga *et al.*, 1993). During estivation, liver of spadefoot toads showed reduced activities of CAT, GR and GST, and increased levels of lipid peroxidation and lipid hydroperoxides. These enzymes

that showed reduced activities during estivation were purified from liver to determine whether the estivation-induced changes resulted from a decreased amount of enzyme protein or were due to the presence of modified or novel isoforms of the enzyme.

CAT

Catalase, an essential antioxidant enzyme, was purified to homogeneity from toad liver, with a final specific activity of between 37600 and 33700 $\mu\text{mol H}_2\text{O}_2$ catabolized /min/mg for the enzyme from both awake and estivated toads, with a yield of 3 %. Both specific activity and final % yield of the purification were similar to purifications from other animals (Chatterjee and Sanwal, 1993). The enzyme from tissue of awake toads was not different kinetically from catalase purified from estivated liver. However, toad catalase was significantly different in structure from other plant and animal catalases by virtue of being a dimer. No tetrameric enzyme form was detected in toads, although subunit monomer molecular weight was similar to catalases from other animal and plant sources. It is highly unlikely that toad catalase was denatured from a tetramer to an active dimer by the process of purification, because none of the purification steps were novel (except for IEF) nor particularly harsh and the properties of the dimeric toad catalase were similar to tetrameric catalases from other sources. An active dimeric catalase has not been demonstrated previously from any organism other than bacteria, and may represent a genetic mutation of the enzyme in toads or even within the Amphibia. Unlike the majority of dimeric catalases, toad catalase did not exhibit catalase-peroxidase activity and was similar to catalase from *Klebsiella pneumoniae*. Catalase purification has not been described for other amphibians; until now information on the enzyme has been derived from only a few widely different sources (plants, bacteria, insects and mammals - for reviews see Nadler et al., 1986; von Ossowski et al., 1993; Deisseroth and Dounce, 1970). The toad enzyme was unstable in dilute buffers but denaturation was not affected by urea or KCl. However, KCl exerted a strong modulating effect on activity and this was

determined to be due to the Cl^- anion, which is elevated in concentration in estivated tissues (Flanigan *et al.*, 1995). Thus, modulation of CAT activity in estivated toads may be due to heightened intracellular KCl levels.

GR

Glutathione reductase, responsible for the maintenance of the glutathione status in cells, has been studied from many sources (Tanaka *et al.*, 1994). To date, modification of the enzyme in response to stress has been documented for several organisms (Hausladen *et al.*, 1992; Tanaka *et al.*, 1994). GR was purified with a final specific activity of 3.67 U/mg for the enzyme from awake toads and 0.07 U/mg from estivated toad liver. The results of enzyme purification and characterization showed that GR from estivated toads (termed GR_E) was different from the enzyme from awake toads (GR_A). The kinetic properties of GR_A and GR_E were different from GR from other sources and from each other. Most notably, GR_E did not exhibit product inhibition, in contrast to GR_A , and to previous reports from the literature (Sexton and Mutus, 1992) and was also inhibited by KCl to a greater extent than GR_A . Subunit molecular weight and activation energy were similar for the two enzymes. GR is purportedly activated in response to depleted GSH concentrations, to keep a constant GSSG/GSH ratio (Leeuwenburgh and Ji, 1995). However, in toads, alteration of the GSSG/GSH ratio in estivation did not induce a change in GR activity to compensate; GR from liver of estivated toads had a much lower specific activity than GR from awake toads. This indicated that GR_E does not maintain the same GSSG/GSH ratio as in awake toads, and suggests that the maintenance of this ratio is not essential for the survival of estivation.

GST

Glutathione *S*-transferase is an important multifunctional enzyme involved in a variety of cell processes, one of which is detoxification of alkyl hydroperoxides. GST was

purified from liver and muscle of awake and estivated spadefoot toads using a simple two-step procedure. The enzymes were characterized and compared to each other and to GST purified using the same procedure, from liver and muscle of the leopard frog, *Rana pipiens*. Liver GST from awake toads differed in several respects from estivated toads, and was also different from frog liver GSTs. Leopard frog liver exhibited two forms of GST, termed GST A and GST B, which were separable on hydroxylapatite; toad liver exhibited one form only. By contrast, muscle of both species apparently only had one form of GST that was unchanged in the toad during estivation; kinetic characterization of the enzymes from the two species showed that they were very similar.

GSTs may be grouped into three classes on the basis of their substrate and inhibitor properties. Most of the isozymes purified were identified on this basis as belonging to Mu class, except for one isozyme from frog liver which was identified as Alpha class, primarily due to its high activity with CHP. The presence of this isozyme probably accounted for the higher total GP_{ox} activity observed in frog liver as compared with toad liver (Chapter 2.1). The frog apparently required greater protection by an enzyme which detoxifies lipid hydroperoxides; specific GST classes, including Alpha, are inducible by various external xenobiotics. The frog, in continual contact with an aquatic environment, may absorb greater amounts of inducers for lipid peroxidation and thus express this isozyme in response.

Thus adaptations in toad liver to the challenges of estivation and dehydration showed that antioxidant enzyme response was both qualitative and quantitative. For those enzymes which were different in estivation, the specific activity was generally lower than for the enzyme from awake animals.

Urea effects

The skin of amphibians is freely permeable to water and, as desiccation stress builds, toads catabolize protein reserves to build up urea concentrations to high levels as a

colligative barrier to desiccation. Nevertheless, this strategy is only partially effective and toads lose up to 50% of their total body water while dormant. The consequence of this is that plasma and intracellular ion levels are also elevated. Although many amphibians are much more tolerant of wide variations in the ionic strength and osmolality of their body fluids (Hillman, 1978) than are most other vertebrates, very high ionic strength is still damaging and one of the proposed reasons for accumulating organic osmolytes (such as urea) under desiccating conditions is elevate osmolality without the need to greatly increase ionic strength. These organic osmolytes should, therefore, be non-perturbing solutes that do not interfere with protein function even when occurring at high concentrations (Haüssinger, 1996). Accumulation of urea (Shoemaker *et al.*, 1992) takes place in direct response to osmotic water loss to the soil, and levels in toads reach 300 mM in plasma and urine (Shoemaker *et al.*, 1992; Chapter 2.2). Urea freely permeates cell membranes (Cleworth, 1967) and, thus, reaches similar levels throughout the toad. In other animals and plants volume-regulating osmolytes are polyols, methylamines or certain amino acids (Weretilnyk *et al.*, 1989; Haüssinger, 1996), however in anurans, accumulation of urea is a common volume-regulating response to dryness. Other authors have considered urea to be a perturbing solute and have suggested that physiological concentrations of urea may modulate enzyme function *in vivo* (Hand and Somero, 1982). The present study, however, shows that physiological concentrations of urea had little or no effect on AOE in toad tissues and had virtually no effect on purified CAT and GST. Even the effects of urea on GR from estivated animals, such as a lowered E_{a} , were minimal. This was in agreement with previous work on the effects of urea on glycolytic enzymes of the spadefoot toad (Grundy and Storey, 1992). Thus, the compound clearly appears to be non-perturbing of enzyme function in spadefoot toads, and a suitable osmolyte that may be accumulated in high amounts in cells in response to dry conditions.

Urea may also have another function: hyperosmolarity and the presence of urea are so-called "catabolic signals" which activate certain genes coding for enzymes that

regulate cell volume through activation of ion transport systems across the plasma membrane (Haüssinger, 1996). Regulatory volume increase in this manner does not completely restore the initial cell volume, and the extent of the difference may act as a signal to regulate cell function (Haüssinger, 1996). Urea does not perturb toad enzyme function at physiological levels so it is likely that it is the only osmolyte used by the toad to prevent desiccation, however it may also serve as a volume regulator in a manner unrelated to its osmotic function.

Salt effects

Initially salt effects on enzymes were tested as a comparison for the proposed perturbing effects of urea on enzymes. Both extracellular Na^+ and intracellular K^+ are elevated as anurans dehydrate (Hillman, 1988). However, it soon became apparent that urea was not the perturbing solute; rather the culprit was salt. All the enzymes studied were strongly affected by KCl; activities in both crude cell extracts and in purified form were lowered by up to 50 % in some cases. In addition, each of the three purified enzymes showed I_{50} values for KCl that were close to the known physiological levels of the intracellular ions. This indicates that intracellular ion concentrations *in vivo* may have important effects on the activity of various enzymes, and may modulate the response of these enzymes in tissues of the estivating animals. The actions of high urea may include not just increasing osmolality to minimize water loss due to colligative effects but by building up an organic solute you do not have to endure the very high ionic strength that would otherwise occur.

Concluding Remarks

Entry into estivation for *S. couchi* involves depression of all cellular processes, except urea synthesis (Shoemaker *et al.*, 1992). The present studies show that most antioxidant defences change during estivation, but unlike the case in land snails, the

majority of these changes represented lower antioxidant defences in the estivating state. During estivation the toad generally had lower activity of key antioxidant enzymes (CAT, GR, GST). GR and GST purified from estivated toads had lower specific activity. As a result of this decreased protection, some oxidative damage occurred. Response of antioxidant systems to estivation was tissue-specific and modulation of AOE activity in liver was due to reduced biosynthesis of AOE (for catalase) or (for GST and GR) to expression of a novel form of the AOE or posttranslational modification.

The results of this investigation show that the toad fitted part (d) of the original hypothesis stated in Chapter 1: the spadefoot toad endured oxidative damage during estivation, presumably clearing the products of damage upon arousal. Reduced AOE activity during estivation may be a consequence of overall depression of all enzymes - a new balance between ROS and AOE is apparently formed during estivation.

Future work on antioxidant enzymes of the spadefoot toad should center on activities of superoxide dismutase, catalase and glutathione peroxidase (Se-dep and total), which were elevated in selected tissues of the estivating toad. Further studies should determine whether these represent isoforms specific to estivation, and what signals induce their increased activity.

The third strategy

Both freeze-tolerant frogs and snails which estivate possess strategies which protect them from oxidative damage during recovery. These have been termed "constitutive" and "anticipatory" strategies, respectively, in this thesis. In contrast to these animals, toads enduring similar stresses did not maintain nor increase antioxidant defences to dealing with oxidative stress upon arousal. Antioxidant defences were lowered and glutathione status reflected a more oxidized state of the cell during estivation. These alterations took place at the expense of cell components, as lipids showed higher levels of the products of oxidative damage, such as conjugated dienes.

Closer examination of the AOE involved in the protection of liver cells indicated that lower AOE activity was due in part to the presence of isozymes with lower specific activities, produced in response to a trigger, as yet undefined. Thus, in addition to constitutive and anticipatory strategies for defence against reactive oxygen species during a return to high metabolism and high oxygen consumption, it seems a third strategy for dealing with this type of stress exists: a finely tuned lessening of antioxidant defences, in part quantitative, in part qualitative, which allows some damage but presumably conserves the toad's reserves for long-term survival of estivation. Previous work on the effects of urea and salt on toad enzymes confirmed that toad enzymes are generally not affected by physiological levels of urea, but are significantly affected by high concentrations of the cellular salt, KCl.

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