Trends in Chromatography
Vol. 6, 2010

Review

Effects of estivation on selected metabolites in pulmonate snails as determined by chromatography

James D. Vasta[1], Bernard Fried[2,*], and Joseph Sherma[1]

[1]Department of Chemistry, [2]Department of Biology, Lafayette College, Easton, PA 18042, USA

ABSTRACT

Estivation (also referred to as aestivation), a hypometabolic state used by a variety of organisms to survive harsh environmental conditions, is ubiquitous among the pulmonate snails. While estivation in pulmonates has been well studied at the biochemical and behavioral levels, recent advances in analytical methodology, particularly in the chromatographic methods, have been used successfully to study the effects of estivation on various metabolites in snail tissue and hemolymph. This review discusses the application of various chromatographic methods, including thin-layer chromatography, high performance thin-layer chromatography, high performance liquid chromatography, and gas chromatography, to the determination of selected metabolite classes such as neutral and polar lipids, carbohydrates, carboxylic acids, amino acids, lipophilic pigments, and purine bases in estivating pulmonate snails. Findings generally provide support for the biochemical and physiological processes that occur during estivation, such as metabolic rate reduction, the use of carbohydrates and lipids as the primary nutrients for energy production, and the possible recruitment of anaerobic pathways to supplement a reduced oxidative metabolism.

KEYWORDS: chromatography, estivation, snails, pulmonates, metabolites, hypometabolism, TLC, HPTLC, HPLC, GC

*Corresponding author
friedb@lafayette.edu

INTRODUCTION

Estivation (also referred to as aestivation in some papers) is a biologically important hypometabolic state used by a variety of organisms to minimize the effects of certain adverse environmental conditions, including desiccation and limited food availability. This phenomenon is ubiquitous among the pulmonate snails, a group composed primarily of terrestrial and freshwater gastropods that possess a lung and are capable of breathing air. The ubiquity of estivation among the pulmonates and the ease with which estivation is induced and maintained makes the pulmonate snails ideal models for the study of estivation in invertebrates.

Although the pulmonates are a diverse group, they may be divided into two large subgroups: the aquatic pulmonate snails (mainly planorbids) and the terrestrial pulmonate snails. Model snails from each of these subgroups are discussed. Our discussion of the terrestrial pulmonates is limited to the snails *Cepaea nemoralis* (the grove snail), *Oreohelix strigosa* and *O. subrudis* (mountain snails of the western United States), and *Otala lactea* (the milk snail or Spanish snail). Our discussion of the planorbids will be limited to the snails *Biomphalaria glabrata* [primarily the albino strain of Puerto Rico (PR) and the NMRI strain] and *Helisoma trivolvis* [both the Colorado and Pennsylvania strains].

The behavioral and biochemical effects of estivation in pulmonates have been well characterized and reviewed previously by Storey [1]. Briefly, estivation in pulmonates is an

aerobic state of hypometabolism characterized by apnoic breathing patterns, evaporative water loss, and a decrease in oxygen consumption. To minimize water loss, snails generally retract into their shells, while some may cover the aperture of the shell using a mucus epiphragm or burrow into the ground. During this state, most snails appear to maintain an aerobic metabolism, using the catabolism of endogenous energy reserves including carbohydrates, lipids, and proteins to sustain life. The hypometabolic state appears to be maintained in part by glycolytic rate depression via reversible phosphorylation of key enzymes including pyruvate dehydrogenase, 6-phosphofructo-1-kinase, and pyruvate kinase, among others.

Until recently, comparatively few studies have investigated the effects of estivation on the levels of certain snail metabolites. Perhaps some of the best information on this topic comes from the work of von Brand *et al.* [2], who investigated the effects of estivation on lactic acid and certain volatile acids in the aquatic pulmonate snail *B. glabrata* (formerly *Australorbis glabratus*), and the work of Churchill *et al.* [3], who investigated the intermediary energy metabolites in the land pulmonate *O. lactea*. However, recent advances in analytical methodology, particularly in the chromatographic methods, have allowed for the determination of a variety of additional metabolite classes in various snail tissues during estivation. Here we discuss the use of thin-layer chromatography (TLC), high performance TLC (HPTLC), high performance liquid chromatography (HPLC), and gas chromatography (GC) for the determination of neutral and polar lipids, carbohydrates, carboxylic acids, amino acids, lipophilic pigments, and purine bases in estivating pulmonate snails.

LIPIDS

The effects of estivation on various types of lipids have been investigated frequently, primarily due to the myriad functions of these molecules in biological systems, as well as the chromatographic methods available for their determination. It has been shown previously that the total lipid content of various planorbid snails is influenced by estivation [2]. However, it was not until recently that researchers began to investigate the roles of specific types of lipids in estivation in pulmonates. We discuss here the use of HPTLC-densitometry (HPTLC-Dens) to investigate the effects of estivation on the neutral lipid and phospholipid content of snails *H. trivolvis* (CO) and *B. glabrata* (NMRI) [4, 5], as well as the use of TLC in conjunction with GC-flame ionization detection (GC-FID) to determine the effects of estivation on mitochondrial phospholipids in the hepatopancreas (digestive gland) of *C. nemoralis* [6, 7].

Neutral lipids

In the case of the neutral lipids, White *et al.* [4] determined free sterols (FSs), free fatty acids (FFAs), triacylglycerols (TGs), and steryl esters (SEs) in *H. trivolvis* (CO) and *B. glabrata* (NMRI) during estivation. The above lipid classes were extracted from snail tissue using the method of Folch [8] in which lipids are separated from aqueous soluble tissue components using a two phase extraction with chloroform-methanol (2:1) and 0.88% KCl at a ratio of 4 parts chloroform-methanol (2:1) to 1 part 0.88% KCl. Neutral lipids isolated using the above method were separated on 10 cm x 20 cm Whatman (Florham Park, NJ, USA) LHPKDF high performance silica gel plates using ascending development with the mobile phase petroleum ether-diethyl ether-glacial acetic acid (80:20:1) for FSs, FFAs, and TGs, or hexane-petroleum ether-diethyl ether-glacial acetic acid (50:25:5:1) for SEs. Following postchromatographic derivatization with phosphormolybdic acid, neutral lipid zones were quantified by slit-scanning densitometry using the absorbance at 610 nm. In all of the analytical TLC and HPTLC methods reported in this review, development of the plate was carried out in a CAMAG (Muttenz, Switzerland) twin trough chamber and densitometry was performed with a CAMAG TLC Scanner II.

Using the above method to study snails estivated for 14-days, it was found that the TG content of *B. glabrata* (NMRI) and *H. trivolvis* (CO) was significantly altered during estivation. TGs were reduced by 97% and 88% in the whole body of *B. glabrata* (NMRI) and *H. trivolvis* (CO) snails,

respectively, and by 84% in the digestive gland-gonad complex (DGG) of *H. trivolvis* (CO). Given that TGs serve a prominent role in energy storage, these results suggest that both *H. trivolvis* (CO) and *B. glabrata* (NMRI) snails derive energy from TGs during estivation.

The SE content of the above snails was also altered during estivation. In general, SEs were found to increase during estivation, with the largest increases occurring in the DGG [214% for *B. glabrata* (NMRI) and 200% for *H. trivolvis* (CO)]. While the role of SEs in snails is not well characterized, it is known that SEs play important roles in lipid storage in the model yeast, *Saccharomyces cerevisiae* [9], and other eukaryotes. Moreover, SEs were also found to increase in the DGG and whole body of *B. glabrata* (NMRI) and *H. trivolvis* (CO) during starvation [4]. Thus, it is likely that the increase in SEs observed during estivation could arise via a stress response during times in which food intake and/or metabolic rate is reduced. In this way, sterols and fatty acids may be stored in lipid droplets as SEs so that these biologically important molecules can be sequestered until the snail returns to a normal metabolic state. This scenario is in agreement with the finding that *S. cerevisiae* also converts FSs to SEs when growth conditions are suboptimal, i.e., during the stationary phase of growth and during nutrient deprivation [10].

Phospholipids

It is well known that estivation results in a coordinated reduction in metabolic rate, and that this reduction is induced, in part, by reversible regulation of key enzymes in cellular respiration. For example, glycolytic rate suppression is mediated by reversible phosphorylation of enzymes such as glycogen phosphorylase, 6-phosphofructo-1-kinase, pyruvate kinase, and pyruvate dehydrogenase [1]. Much less is known about the effects of estivation on mitochondrial metabolism and how those effects contribute to overall metabolic rate reduction. Recently, however, Stuart *et al.* [6, 7] showed that estivation induces significant alterations to the mitochondrial membranes of the hepatopancreas of *C. nemoralis* snails.

Preparative TLC was first used to separate individual phospholipids from neutral lipids in lipid extracts from either whole hepatopancreas or mitochondrial fractions according to the method of Holub and Skeaff [11]. Following separation, fatty acids from individual phospholipids were methylated, redissolved in CS_2, and determined by GC-FID using a Hewlett-Packard (Palo Alto, CA, USA) HP5890 series II gas chromatograph equipped with a DB 225 megabore fused silica column (Chromatographic Specialties, Brockville, ON, Canada). The analysis temperature was 210°C for 30 min, which included an initial ramping from 150 to 210°C during the first min. Using GC-FID, it was possible to determine not only the relative proportions of major phospholipids including cardiolipin (CL), phosphatidyl-choline (PC), Phosphatidyl-ethanolamine (PE), phosphatidyl-inositol (PI), and phosphatidylserine (PS), but also the fatty acid composition of those lipids.

Using the above GC-FID method, Stuart *et al.* [6] reported marked changes in the mitochondrial membranes including an 82.7% reduction in CL and a 71.7% reduction in total mitochondrial phospholipids. Moreover, alterations in the relative proportions of the individual phospholipids included 35% and 14% reductions in CL and PE, respectively, and an 89% increase in PI [6].

In addition to the above changes, it was also found that the fatty acid composition of these phospholipids was altered markedly in estivating snails. Generally, the levels of polyunsaturated fatty acids (especially 18:2n-6 and 18:3n-3) were significantly reduced, while the proportions of monoenes and saturated fatty acids were markedly increased [7]. Such changes are correlated with a more stable lipid bilayer and decreased activity of membrane proteins.

Perhaps the most significant changes occurred in CL, where 18:2n-6 and 18:3n-3 were reduced by 50% and 57%, respectively, and saturated fatty acids and monoenes were increased 13-fold and 9-fold, respectively [7]. CL containing high proportions of 18:2n-6 is known to be required for activity of the respiratory chain enzyme cytochrome c oxidase (CCO) [12], and the

activity of CCO is reduced by 83.9% during estivation in *C. nemoralis* [6]. Thus, stabilizing the lipid bilayer in conjunction with altering the proportions and fatty acid compositions of the phospholipid constituents of the mitochondrial membrane could be a mechanism that contributes to overall metabolic rate reduction during estivation.

Interestingly, while marked changes to the mitochondrial membrane lipids were observed in estivating *C. nemoralis*, the researchers found that the total phospholipid content of the DGG of these snails was unchanged [6]. Such a result might suggest that the remodeling of the mitochondrial membrane during estivation does not involve any *de novo* phospholipid biosynthesis or degradation, but rather a reorganization of existing phospholipids that are sequestered within the cell. Mechanistically, such a phenomenon could operate in a similar manner to regulation of Na^+ leak in the plasma membrane, which is thought to occur by a removal of channel containing membrane fragments that are stored elsewhere in the cell [13, 14]. In the case of estivation, the mitochondrial membrane may be altered by removing certain membrane fragments containing phospholipids that increase the activity of membrane proteins, thus contributing to metabolic downregulation. Moreover, such a mechanism would be quickly reversible, allowing for the rapid recoveries that are observed for all pulmonates during the transition from a hypometabolic to an active state [1].

In addition to the GC-FID studies by Stuart *et al* [6, 7], White *et al.* [4, 5] used HPTLC-Dens to investigate the effects of estivation on phospholipids in *B. glabrata* (NMRI) and *H. trivolvis* (CO) snails. In the first study [4], phospholipids were separated on 10 cm x 20 cm Whatman LHPKDF high performance silica gel plates using ascending development with chloroform-methanol-water (65:25:4) as the mobile phase. Lipids were detected by postchromatographic derivatization with 10% $CuSO_4$ in 8% H_3PO_4 and quantified by slit-scanning densitometry using the absorbance at 370 nm. In the second study [5], phospholipids were determined using the HPTLC method described above except that 10 x 20 cm Analtech (Newark, DE, USA) HPTLC-HLF silica gel plates were used instead of the Whatman HPTLC plates. Using the above method, the researchers were able to detect and quantify both PC and PE in the whole body and DGG of *B. glabrata* (NMRI) and *H. trivolvis* (CO) snails subject to a variety of experimental conditions. For *H. trivolvis* (CO), the conditions under study included estivation and starvation [4]. For *B. glabrata* (NMRI), the effects of 14 days of estivation and starvation were investigated [4]. However, *B. glabrata* is the primary intermediate host of the blood fluke *Schistosoma mansoni*, and the researchers were also interested in how the *S. mansoni* infection influences metabolism of the snail. Thus, two additional experimental groups were included in which snails infected with the parasite were either estivated or starved for 7 days [5].

For both *B. glabrata* (NMRI) and *H. trivolvis* (CO), no significant difference in the PE or PC content of the whole body or DGG of estivating or starved snails was observed after 14-days of treatment [4]. This finding is in agreement with those of Stuart *et al.* [6] who reported that the total phospholipid content of the DGG of *C. nemoralis* was unchanged by estivation, despite the marked changes that occurred in the mitochondrial membrane lipids.

Interestingly, *S. mansoni* infected *B. glabrata* (NMRI) snails estivated for 7 days did show a significant decrease in PC, while snails that were infected and starved showed no such decrease. However, no clear explanation for these findings exists at this time.

CARBOHYDRATES

While neutral lipids are an important energy reserve for pulmonates during estivation, carbohydrates also serve a similar function and indeed may be the primary fuel source in some species [1]. Recently, two quantitative TLC studies [15, 16] on the carbohydrate profiles of the DGG and hemolymph of estivating *B. glabrata* (NMRI) and two strains of *H. trivolvis* (CO and PA) also provided evidence that the catabolism of carbohydrates, especially glucose and maltose, can provide a significant source of energy for pulmonates during estivation.

In both investigations, sugars extracted in 70% ethanol from the DGG or hemolymph of the above snails were separated on Whatman 20 x 20 cm

LK5DF silica gel TLC plates by ascending development with a mobile phase of ethyl acetate-glacial acetic acid-methanol-water (60:15:15:10). After development, sugars were derivatized using α-napthol-sulfuric acid reagent [17], after which the dark brown spots corresponding to each sugar were quantified by densitometry using the absorbance at 515 nm.

Investigations on both snail species showed that glucose and maltose were the major sugars in the DGG and hemolymph [15, 16]. These results agree with the findings of Cline *et al.* [17], who used GC-mass spectrometry (GC-MS) to confirm that glucose and maltose were the most abundant sugars in the DGG and hemolymph of *B. glabrata* (NMRI).

In *B. glabrata* (NMRI), maltose and glucose were present at similar levels in the DGG. After a 14 day estivation period, glucose and maltose levels were reduced by 45.9% and 67.4%, respectively, suggesting that these sugars were used as a source of energy during estivation [15]. However, no time series analysis was done for *B. glabrata* (NMRI), so it is difficult to interpret the rate of usage of these carbohydrates in comparison to other energy sources.

Similar investigations using the identical TLC method described above [15] to study two strains of *H. trivolvis* yielded comparable results to those obtained for *B. glabrata* (NMRI) [16]. In unestivated DGG and hemolymph of both strains, glucose was present at slightly higher levels than maltose, although both sugars were still the most abundant monosaccharides detected. For *H. trivolvis* (CO), a 7-day estivation period was found to reduce glucose by 30.0% and 53.2% in the DGG and hemolymph, respectively, and maltose by 18.9% and 87.0% in the DGG and hemolymph, respectively. Similarly to the investigations of *B. glabrata* (NMRI) discussed above, no time series analysis was performed for this strain.

However, a time series analysis was performed for *H. trivolvis* (PA) that clearly demonstrated reductions and/or depletions of both glucose and maltose in the DGG and hemolymph [16]. With respect to glucose, a 3-day estivation period reduced glucose levels by 99% and 67% in the DGG and hemolymph, respectively. After 5 days, a 90% reduction in glucose was observed in the hemolymph, and glucose was no longer present at detectable levels in the DGG. Maltose also showed similar reductions of 99% and 37% after 3 days of estivation in the DGG and hemolymph, respectively, with the level of maltose in both tissues dropping below the limit of detection after 5 days of estivation.

There is no clear explanation as to why the two strains of *H. trivolvis* show markedly different patterns of sugar usage during estivation. It is possible that each strain has a different level of tolerance to estivation, and indeed, mortality results for both strains suggest that *H. trivolvis* (CO) is more tolerant of estivation than is *H. trivolvis* (PA) [16]. Such findings could suggest that *H. trivolvis* (CO) may have evolved more efficient mechanisms of estivation than *H. trivolvis* (PA), ultimately allowing for increased survival during longer periods of estivation. Further investigation is required to explain the apparent strain differences.

Collectively, the findings of reductions in glucose and maltose in the DGG and hemolymph of estivated snails from both *B. glabrata* (NMRI) and *H. trivolvis* (CO and PA) suggest that both of these sugars may be used to sustain estivating pulmonates. Similar studies in other pulmonates also showed that carbohydrates are important energy reserves for estivating snails. For example, investigations on the mountain snails *O. strigosa* and *O. subrudis* showed that polysaccharides were the most abundant nutrient reserves in these snails prior to estivation and also the first to be catabolized by the snail [18]. Moreover, net protein catabolism did not begin until most of the polysaccharide reserves were depleted, and lipid catabolism occurred at a constant, slow rate throughout estivation [18]. Further investigations using the planorbid snails *B. glabrata* and *H. trivolvis* should be done to investigate the time scale over which different energy reserves are utilized during estivation so as to determine whether planorbids also display energy utilization patterns similar to land pulmonates during estivation.

CARBOXYLIC ACIDS

Carboxylic acids, particularly the tricarboxylic acid (TCA) cycle intermediates, serve a variety of biologically important functions from energy metabolism to serving as substrates in numerous biosynthetic reactions. Previously, the effects of estivation on these important metabolites had not been extensively characterized in pulmonate snails. However, the use of HPLC methods for the determination of carboxylic acids has allowed for more thorough investigations of the role of these molecules in estivating pulmonates.

Recently, HPLC was used to investigate the effects of estivation on the profile of organic acids in the DGG and hemolymph of *B. glabrata* (PR) [19]. In this study, HPLC was performed at room temperature using a Bio-Rad Laboratories (Hercules, CA, USA) Aminex ion exclusion HPX-87H column (300 x 7.8 mm). The mobile phase was 0.5 mM sulfuric acid and the column was eluted isochratically at a flow rate of 0.8 mL/min. Carboxylic acids were detected using the ultraviolet absorbance at 210 nm.

After 7 days of estivation, analyses of the DGG showed that the concentrations of lactate, acetate, succinate, and malate were significantly increased, while pyruvate was significantly decreased. In the hemolymph, the pyruvate and acetate concentrations increased significantly, while lactate, propionate, β-hydroxybutyrate, and fumarate appeared to decrease significantly. After 14-days of estivation, lactate and acetate continued to accumulate in the DGG, while lactate further decreased and acetate further increased in the hemolymph. Moreover, the levels of propionate and β-hydroxybutyrate in the hemolymph remained at levels similar to that of snails estivated for 7 days, while the concentration of acetoacetate was decreased significantly in both the DGG and hemolymph. Interestingly, the levels of pyruvate, succinate, and malate in the DGG after 14-days returned to levels similar to that of unestivated controls, while the levels of succinate, acetoacetate, and malate in the hemolymph decreased significantly relative to both the unestivated controls and snails estivated for 7 days. Lastly, the level of pyruvate in the hemolymph decreased relative to snails estivated for 7 days, although the level remained significantly higher than that of unestivated snails.

The results above for *B. glabrata* (PR) differ from those reported by Brown *et al.* [20] for *H. trivolvis* (CO and PA). The HPLC method used in this investigation was identical to that used by Bezerra *et al.* above [19], except that HPLC was performed using an Agilent Technologies (Wilmington, DE, USA) 1100 Series HPLC instrument. For *H. trivolvis* (CO), it was found that the level of succinate in the DGG increased by 225% after 7 days of estivation. For *H. trivolvis* (PA) estivated for up to 7 days, it was found that by day 3, the levels of malate, pyruvate, and succinate in the DGG dropped to levels that were no longer detectable by the above HPLC method, indicating that these acids were essentially depleted in the DGG of estivating *H. trivolvis* (PA) snails.

Considering the markedly different results above between each of these three planorbids, it appears that that the effects of estivation on carboxylic acid metabolism are not unified between the three snails. In the case of *B. glabrata* (NMRI), increased levels of lactate, acetate, and succinate in the DGG could suggest that anaerobic pathways contribute to overall metabolism during estivation. However, such accumulations could result from a variety of circumstances involving a decrease in the flux through the TCA cycle. For example, in the pulmonate land snail *Otala lactea*, pyruvate dehydrogenase (PDH) is known to be downregulated by approximately 30% during estivation, thus decreasing the flux of pyruvate into the TCA cycle. If such a mechanism exists in *B. glabrata*, downregulation of PDH could result in an increased flux through the fermentative pathways, as evidenced by the accumulation of lactate and acetate, two known fermentative end products produced by *B. glabrata* [21]. However, the observation that other components of the TCA cycle including malate and fumarate remain at levels comparable to active snails is an interesting finding and in agreement with the previous findings that oxygen uptake (and thus aerobic metabolism) by *B. glabrata* is reduced but not completely halted during estivation [22, 23]. Thus, although it is not completely clear which metabolic pathways remain operative during estivation in *B. glabrata*, it is possible that both aerobic and anaerobic pathways may contribute to overall metabolism in this state.

Comparatively less is known about overall metabolism in *H. trivolvis* during estivation. Thus, it is difficult to put the findings above for carboxylic acids into context. However, in the case of *H. trivolvis* (CO), an accumulation of succinate during estivation could also suggest the use of fermentative pathways to provide energy, as succinate is known to be a fermentative end product in numerous bivalves and gastropods [24]. In the case of *H. trivolvis* (PA), there is no clear explanation for the depletion of malate, pyruvate, and succinate in the DGG during estivation. Given that *H. trivolvis* snails appear to use a large proportion of their energy reserves (lipids and carbohydrates) during the first few days of estivation [4, 16], and that these snails have a low rate of survival during estivation [16], it is possible that the depletions of pyruvate, malate, and succinate may result from the inability to replenish these intermediates. Such a result could also indicate that the mechanisms responsible for metabolic down regulation during estivation in *H. trivolvis* (PA) are inefficient compared to other planorbid snails that display a much higher tolerance to estivation.

AMINO ACIDS

While the effects of purely anaerobic metabolism on free amino acids have been studied extensively in gastropods, the effects of estivation on these metabolites has only recently begun to be investigated [1, 25]. Representative of the land dwelling pulmonates, the amino acid response to estivation and anoxia in various tissues of the snail *Otala lactea* was investigated by Storey [1] using HPLC. Free amino acids in 0.5% sulfosalicylic acid extracts of the hepatopancreas, foot, or mantle of the snail were derivatized prechromatographically using *o*-phthalaldehyde and determined using a Waters (Milford, MA, USA) high performance liquid chromatograph.

Using the above method, aspartate, asparagine, glutamate, glutamine, arginine, alanine, and valine were detected and quantified. In control snails, glutamine was found to be the most abundant amino acid in the foot and hepatopancreas, while glutamate was the most abundant in the mantle. In snails estivated for 22 days at 21°C, glutamine levels dropped by 86% and 68% in the foot and hepatopancreas, respectively, and these decreases were correlated with 3.6 and 3.7 fold increases in glutamate in the foot and hepatopancreas, respectively. Glutamine is known to be involved in pH and ammonium balance [26], and it is likely that conversion of glutamine to glutamate, which releases in ammonium ion, could play a role in providing ammonium for urea biosynthesis. Urea is known to accumulate in pulmonates during estivation [18] as a result of protein catabolism. Interestingly, asparagine was also significantly decreased in all tissues of estivated snails, which likely reflects a breakdown of asparagine to aspartate and ammonium, both of which are used as substrates the urea cycle. Aspartate was also decreased in the hepatopancreas, which provides further support that urea biosynthesis is likely used by estivating *O. lactea* to ameliorate the effects of ammonia accumulation resulting from protein catabolism. In addition to the disturbances in glutamine/glutamate and asparagine/aspartate metabolism discussed above, arginine increases significantly in the mantle during estivation, valine decreases significantly in the foot and hepatopancreas, and total amino acids increase by about 1.7 fold in the mantle.

The more important insight gained from these experiments is that amino acid response to estivation in *O. lactea* appears slightly different than the response during anoxic conditions. While the levels of glutamine, glutamine, asparagine, and valine do not appear to differ much qualitatively, alterations in arginine, alanine, and aspartate appear markedly different between anoxia and estivation. For example, arginine levels are significantly increased during anoxia compared to estivation in both the foot and mantle. While the source of the increase in arginine during anoxia has yet to be completely characterized, such an increase may result from an increased use of the phosphagen arginine phosphate, which is a known energy source used by some crustaceans during anoxia [27]. In the case of aspartate and alanine, aspartate was greatly reduced in the hepatopancreas during anoxia, while alanine accumulated in both the foot and mantle. In contrast, aspartate was only mildly reduced in the hepatopancreas during estivation, while alanine levels during estivation remained equivalent to that of unestivated controls. Aspartate is a known substrate in fermentative

metabolism in most gastropods, while alanine is a known fermentative end product [24]. Thus, the changes observed in alanine and aspartate levels during anoxia in *O. lactea* are not unexpected. The lack of such changes in alanine and aspartate levels during estivation, as well as the lack of accumulation of arginine suggests that free amino acids may play different roles in these two metabolic states.

In contrast to the land pulmonate *O. lactea*, alterations in amino acid levels in the planorbid *B. glabrata* (NMRI) may indicate that fermentative pathways do play a role in estivation. In this study by Vasta *et al.* [25], free amino acids were determined by HPTLC-Dens in the DGG of *B. glabrata* (NMRI) snails estivated for 7 days. For identification, amino acids were separated on either 20 x 10 cm HPTLC silica gel plates (EMD Chemicals, Inc., Gibbstown, NJ, USA, an affiliate of Merck KGaA, Darmstadt, Germany) or 20 x 10 cm HPTLC cellulose F plates (EMD Chemicals, Inc.) by ascending development with a mobile phase of either 2-butanol-pyridine-glacial acetic acid-water (39:34:10:26), or 2-butanol-pyridine-25% ammonia-water (39:34:10:26). Amino acids were detected by post-chromatographic derivatization with ninhydrin, and quantified by slit-scanning densitometry using the absorbance at 610 nm.

The amino acids alanine, arginine, lysine, serine, valine, and leucine/isoleucine were unequivocally identified and quantified in both estivated and unestivated *B. glabrata* snails. Alanine was found to be increased by two fold in the DGG of estivated snails vs. that of unestivated controls, while the levels of the other amino acids remained unchanged. As discussed above, alanine is a known fermentative end product in a variety of gastropods, and its accumulation may support the hypothesis that fermentative pathways could play a role in energy production during estivation in *B. glabrata*. This hypothesis is further supported by the finding (discussed above) that lactate and acetate also accumulate in the DGG of *B. glabrata* (PR) during estivation [19].

At this time, the conflicting results between the land pulmonate *O. lactea* and the planorbid *B. glabrata* (NMRI) for changes in free amino acid levels during estivation do not allow for any generalizations to pulmonate snails as a whole. Future studies are needed to determine whether there are additional differences in hypometabolism between land pulmonates and planorbids, and how such differences might be reflective of the differing strategies used by each for surviving in an aquatic versus a terrestrial environment.

MISCELLANEOUS METABOLITES

In addition to the primary metabolites discussed above, a few additional metabolites have also been determined by chromatography in estivating pulmonates. These include the lipophilic pigments lutein and β-carotene and the nitrogenous bases uric acid, guanine, and xanthine.

Lipophilic pigments

The lipophilic pigments lutein and β-carotene were determined in *H. trivolvis* (CO) and *B. glabrata* (NMRI) snails estivated for 2 weeks using HPTLC-Dens [28]. Yellow pigment zones were separated on 10 x 20 cm C-18 chemically bonded reversed phase silica gel plates (EMD Chemicals, Inc.) by ascending development with the mobile phase petroleum ether (37.8-53.5°C)-acetonitrile-methanol (1:1:2). Naturally colored pigment zones were quantified by slit-scanning densitometry using the absorbance at 448 nm for lutein and 455 nm for β-carotene.

Results showed that the levels of these pigments in *B. glabrata* (NMRI) snails were unaffected by two weeks of estivation, while lutein was significantly decreased in the DGG of estivating *H. trivolvis* (CO) snails. These planorbids obtain lutein from their diet, which is composed primarily of romaine lettuce under standard laboratory conditions, and indeed, the concentrations of lutein and β-carotene increase markedly in snails maintained on a diet of lettuce vs. snails maintained on a diet of egg yolk [29]. The decrease in lutein in estivated *H. trivolvis* (CO) snails likely reflects an inability to replenish sequestered lutein that is used during metabolism, as these snails are not capable of synthesizing lutein *de novo* and must obtain it through their diet. The function of lutein in *H. trivolvis* (CO) is currently unknown, although it may play a role as an electron acceptor during anoxic conditions [30].

Nitrogenous bases

The nitrogenous bases uric acid, guanine, and xanthine were determined in the land snails *O. strigosa* and *O. subrudis* during estivation using HPLC [18]. Bases were separated using a LDC/Milton Roy (Ivyland, PA, USA) HPLC system equipped with a Waters μBondpak C-18 reversed phase column (30 cm x 3.9 mm i.d.). Bases were eluted isochratically using 4 mM potassium phosphate (pH 3.6) containing 1% (v/v) methanol as the mobile phase. The bases were detected and quantified using the absorbance at 265 nm.

It was found that all three of the above nitrogenous bases accumulated during estivation in both *Oreohelix* species. As with urea, these bases began to accumulate just after the onset of protein catabolism, which began after about 2 months of estivation. Whereas urea accounted for about 50% of the nitrogen derived from protein, the purine bases only accounted for about 10% of protein nitrogen, with uric acid accumulating the most of the three bases. In general, the purine bases represent a low toxicity end product of nitrogen metabolism for the detoxification of ammonia produced from protein catabolism. Urea appears to be the dominant end product, despite its increased toxicity when compared with the purine bases. The biosynthesis of purine bases requires a higher input of energy and carbon sources than urea biosynthesis. Thus, accumulation of urea as the dominant end product of nitrogen metabolism likely represents a compromise between energy expenditure and toxicity, especially in a hypometabolic state such as estivation where nutrient reserves are limiting.

ACKNOWLEDGEMENTS

We would like to thank the Camille and Henry Dreyfus Foundation for continued funding of J. Sherma through its Senior Scientist Mentor Program, which allowed support of the Lafayette College students who performed much of the research presented.

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