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Effects of Starvation on Haemolymphatic Glucose Levels, Glycogen Contents and Nucleotidase Activities in Different Tissues of *Helix aspersa* (Müller, 1774) (Mollusca, Gastropoda)

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ABSTRACT In the present study, the glucose concentration in the haemolymph and glycogen levels were determined in the various body parts of the *Helix aspersa* snail after feeding lettuce ad libitum and after various periods of starvation. To characterize the effect of starvation on nucleotidase activity, enzyme assays were performed on membranes of the nervous ganglia and digestive gland. Results demonstrated the maintenance of the haemolymph glucose concentration for up to 30 days of starvation, probably due to the consumption of glycogen from the mantle. In the nervous ganglia, depletion of glycogen occurs progressively during the different periods of starvation. No significant changes were observed on ATP and ADP hydrolysis in the membranes of nervous ganglia and no alterations in Ca^{2+}- ATPase and Mg^{2+}-ATPase occurred in the membranes of the digestive gland of *H. aspersa* during the different periods of starvation. Although there were no changes in the enzyme activities during starvation, they could be modulated by effectors in situ with concomitant changes in products/reactants during starvation. *J. Exp. Zool. 301A:891–897, 2004.*

INTRODUCTION

The Mollusca comprise one of the largest phyla within the animal kingdom and has evolved successfully, presenting a widespread distribution and being able to survive in both aquatic and terrestrial environments.

The gastropod class possesses about 35,000 living species and 15,000 fossil records and is the major class of the mollusks described. The successful evolution of mollusks is a consequence of their extraordinary adaptive capacity. Most organisms are exposed to changes in their habitat environment. Such changes include temperature, humidity, photoperiodism and energetic substrate availability. This variable environment can induce metabolic and behavior changes in these organisms. In order to survive, animals can migrate to other locations, change their physical characteristics or enter a hypometabolic condition (Brooks and Storey, '97).

The gastropod mollusks utilize carbohydrate as an essential source of energy (Livingstone and De Zwaan, '83), glycogen and galactogen being the main polysaccharides stored in tissues (Geraerts, '92). Galactogen is restricted to the female albumen gland, whilst glycogen is the most widely distributed polysaccharide and is found in an appreciable amount (Sminia, '72; Goddard and Martin, '96). Present in all cells (Joosse and Geraerts, '83), glycogen is concentrated particularly in specialized ''glycogen cells'' (GC) for storage. These characteristic cells contain a large

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mass of glycogen, occupying almost the entire cell, and may be found in the entire body, although they are particularly abundant in the anterior mantle region, digestive gland and gonad (Sminia, '72).

Haemolymph glucose is the main regulator of glycogen metabolism (synthesis and mobilization) in mollusks (Wijsman and Maaskant, '82; Wijsman et al., '88; Liebsch and Becker, '90; Rossi and Da Silva, '93). In gastropods, and mainly bivalves, glycogen metabolism has been shown to be under the endocrine control of insulin-like substances and hyperglycemic factors (Plisetskaya and Joose, '85; Ebberink and Joosse, '85; Smit et al., '88). The presence of insulin-like substances has been demonstrated by immunocytochemical methods in the central nervous system of the *Helix aspersa* snail (Gomot et al., '92).

Over the last decade, substantial evidence has emerged to show that ATP and other nucleoside di- and tri-phosphates act as extracellular signaling molecules in virtually all tissues (Gordon, '86; Edwards and Gibb, '93; Sévigny et al., 2000). In addition, enzymes located at the cell surface, known as ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases), have been shown to use these molecules as extracellular substrates (Anderson and Parkinson, '97; Lazarowski et al., '97; Zimmerman and Braun, '99). Since the physiological role of extracellular ATP and its metabolic products differ depending on the tissue, these differences also apply to the functional roles of ectonucleotidases, which include neurotransmission, cardiac function, platelet aggregation, muscle contraction, vascular tone and cell adhesion (Edwards and Gibbs, '93; Plesner, '95; Zimmermann, '96; Anderson and Parkinson, '97). ATP has been shown to be stored in nerve terminals and released with several neurotransmitters, including acetylcholine, noradrenaline, serotonin (Di Iorio et al., '88; Rathbone et al., '99) and GABA (Hugel and Schlichter, 2000).

Recently, our laboratory characterized the presence of a Ca^{2+}-ATPase from the nervous ganglia *of Phyllocaulis soleiformis,* a terrestrial snail, which presented a peculiar kinetic behavior when compared to other previously described ATPases (Da Silva et al., 2002). In *Helix aspersa*, we also demonstrated a nucleotidase activity with different kinetic properties in nervous ganglia and the digestive gland (Borges et al., 2004). In order to survive to severe conditions, such as starvation, anoxia, etc., animals may use strategies of metabolic depression (Hochachka and Lutz, 2001; Hochachka et al., '96). In the brain of anoxia-tolerant animals, increased levels of GABA, an inhibitory neurotransmitter, might contribute to metabolic depression by reducing neuronal activity (Nilson and Lutz, '93). In addition, there is some evidence to indicate a contribution of the neuromodulator adenosine (a product of ATP degradation by ectoenzymes) to metabolic depression in vertebrates and invertebrates (Lazou, '89).

Thus, the purpose of the present study was to observe the effect of starvation on glycogen metabolism, haemolymph glucose concentration and on nucleotide hydrolysis observed in the membranes of the nervous ganglia and digestive gland of *H. aspersa.*

MATERIAL AND METHODS

Experimental model

Adult specimens were collected from the metropolitan region of Porto Alegre, RS, Brazil. Animals were maintained in plastic boxes ($68 \times 60 \times 22$ cm) at $25 \pm 5^{\circ}$C with a 12-h light/12-h dark cycle (light on at 07:00 a.m.). All snails used in the experiments were adults and weighted approximately 6 g$\pm$1.5. The animals were fed with lettuce (*Latuca sativa*) at least 7 days before experiments. After this period, the animals were divided into control and starvation groups and maintained in the same room during the experiments. All the snails were maintained in humid conditions in the range of 70–80%.

Isolation of tissues

The animals of the control and starvation (30, 60, 90, and 120 days) groups were cryoanesthetized and the shells removed for collection of the haemolymph. After hemolymph collection, the snails were dissected and the digestive gland and mantle were isolated, weighed and stored at -20 °C. The nervous ring was isolated with a single razor blow in the anterior region of the animal. Under the stereomicroscope, the nervous ring was separated and stored at -20°C. For the experiments, groups consisting of 15 snails each were randomly chosen.

Glycogen amount and glucose determination

The glycogen in different tissues was extracted by the Van Handel ('65) method and the glucose was determined by the enzymatic oxidase method after acid hydrolysis (HCl) and neutralization

(Na_2CO_3), and results were expressed in g%. The glucose concentration in the haemolymph sample (60 μl) was determined by the enzymatic glucose-oxidase method (Kits from Sigma Diagnostic, St. Louis, MO) and results were expressed in mg/dl.

Enzyme assays

The nucleotidase activities were determined in animals feeding lettuce ad libitum (control group) and after 30, 60, 90, and 120 days of starvation. Enzyme activity was assayed in standard reaction medium containing 50 mM Tris-HCl, pH 7.2, 5 mM $CaCl_2$ in a final volume of 200 μl. The membranes of the nervous ganglia and digestive gland of *H. aspersa* (5–10 μg and 2.5–5 μg protein, respectively) were obtained according to Barnes et al. ('93). The membrane fractions were added to the reaction medium and pre-incubated for 10 min at 30°C. The reaction was initiated by the addition of substrate (ATP or other, as indicated) for 20 min at 30°C to a final concentration of 1 mM and stopped by addition of 200 μl 10% TCA (trichloroacetic acid). The samples were chilled on ice for 10 min before inorganic phosphate (Pi) released was measured (Chan et al., '86). Incubation times and protein concentration were chosen in order to ensure the linearity of the reactions. Controls with the addition of the enzyme preparation after mixing with trichloroacetic acid were used to correct non-enzymatic hydrolysis of substrates. Specific activity is expressed as nmol of Pi released.$min.^{-1}.mg^{-1}$of protein. All enzyme assays were run in triplicate. Protein was determined by the Coomassie Blue method, using bovine serum albumin as a standard (Bradford, '76).

Statistical analysis

Statistical analysis was conducted by one-way analysis of variance (ANOVA), followed by multiple range Tukey test, considering a level of significance of 5%.

RESULTS

Effect of starvation on glycogen concentration and haemolymph glucose levels

The results in Table 1 show that the haemolymph glucose concentrations in starved snails differ significantly *($P<0.05$)* at 60 days of starvation when compared to the control animals. Furthermore, a decrease in the glycogen content was observed at 60, 90, and 120 days of starvation in the digestive gland and nervous ganglia of *H. aspersa* (Table 1). The mantle, however, presented lower glycogen contents at 30, 60, 90, and 120 days of starvation, when compared to the control group (Table 1).

Effect of starvation on nucleotidase activities in nervous ganglia and digestive gland

The effect of starvation on nucleotidase activities in membranes from nervous ganglia and digestive gland from *H. aspersa* submitted to starvation is demonstrated in Table 2. There were no significant changes in ATP and ADP hydrolysis

TABLE 1. Haemolymph glucose concentration and glycogen levels in nervous ganglia, the digestive gland and the mantle of Helix aspersa *after 30, 60, 90 and 120 days of starvation*

Groups	Glucose (mg/dl)	Glycogen (g% wet weight)		
		Nervous ganglia	Digestive gland	Mantle
Control	6.63 ± 1.19 (20)	26.76 ± 3.82 (20)	14.72 ± 3.17 (20)	7.90 ± 1.82 (20)
30 days	5.57 ± 0.57 (20)	15.67 ± 4.26 (20)	11.87 ± 3.42 (20)	2.39 ± 0.65[1] (20)
60 days	2.80 ± 0.40[1] (20)	8.78 ± 3.20[1] (20)	1.87 ± 0.55[1] (20)	2.43 ± 0.60[1] (20)
90 days	5.16 ± 0.83 (20)	1.76 ± 0.56[1] (20)	1.92 ± 1.06[1] (20)	2.06 ± 0.38[1] (20)
120 days	3.71 ± 0.26 (20)	1.13 ± 0.09[1] (20)	1.05 ± 0.18[1] (20)	0.96 ± 0.11[1] (20)

Data are given as means ± SEM. Numbers of animals are in parentheses. Data were analyzed statistically by one-way (ANOVA) followed by multiple range Tukey testing.
[1]Values significantly different from control values ($P<0.05$).

TABLE 2. Nucleotidase activities in membranes from nervous ganglia and digestive gland in nervous ganglia, the digestive gland and the mantle of Helix aspersa *after 30, 60, 90 and 120 days of starvation*

	Nervous ganglia		Digestive gland	
Groups	Ca^{2+}-ATPase	Ca^{2+}-ADPase	CA^{2+}-ATPase	Mg^{2+}-ATPase
Control	1124.7 ± 40.4	71.0 ± 4.2	154.0 ± 12.9	232.8 ± 14.7
30 days	1156.6 ± 107.6	87.1 ± 7.4	148.8 ± 15.8	233.7 ± 24.7
60 days	923.2 ± 114.9	59.6 ± 4.2	109.6 ± 12.2	158.6 ± 18.4
90 days	979.6 ± 130.4	68.2 ± 11.3	104.5 ± 13.5	209.0 ± 20.8
120 days	914.2 ± 132.9	80.8 ± 7.2	92.7 ± 21.9	165.9 ± 32.5

Data represent as means ± SEM of five different experiments, each in triplicate. Data were analyzed statistically by one-way (ANOVA) followed by multiple range Tukey testing. All substrates were used at 1.0 mM with 5 mM Ca^{2+} or 5 mM Mg^{2+}. Specific activity is expressed as nmol of Pi released.$min.^{-1}.mg^{-1}$ of protein.

in membranes of the nervous ganglia and digestive gland of *H. aspersa* during the different periods of starvation (30, 60, 90, and 120 days), when compared to the control group (Table 2). Similarly, in the membranes of the digestive gland, Ca^{2+}-ATPase and Mg^{2+}-ATPase activities did not present significant changes after several periods of starvation (Table 2).

DISCUSSION

In the present study, maintenance of the haemolymph glucose concentration was observed for up to 30 days of starvation (Table 1), probably due to the consumption of glycogen from the mantle. A decrease in haemolymph glucose levels at 60 days of starvation may be due insufficient glycogen in the digestive gland for maintenance of the haemolymphatic glucose at this stage (Table 1). The nervous ganglia progressively utilize glycogen during starvation, and the finding that glycogen depletion, in this tissue, occurred from 60 days of starvation suggests that it is utilized by the nervous system during this period (Table 1).

Our findings are similar to those obtained in *Lymnaea stagnalis* (Veldhuijzen, '75a,b; Veldhuijzen and Van Beek, '76; Wijsman and Maaskant, '82; Wijsman et al., '88) and *Aplysia* (Young et al., '91), where the authors suggest that the mantle and hepatopancreas (digestive gland) are the main energetic sources during starvation. It may be postulated that, during a certain period (60 days), glycogen from nervous ganglia is preserved for their own use. Our results demonstrated a decrease in glucose levels in the haemolymph at 60 days of starvation. In addition, despite the significant decrease in the glycogen content in the nervous ganglia at 60 days, this glycogen was preserved more than the glycogen of the digestive gland (Table 1). At 90 days of starvation, an increase in glucose levels in the haemolymph was observed, possibly due to the decrease in glycogen in the nervous ganglia or the occurrence of gluconeogenesis in other tissues. After 90 days of starvation, glucose levels and glycogen contents were observed to stabilize, suggesting that the animals were submitted to metabolic depression with a possible decrease in the glycogen phosphorylase activity. During anoxia in *Megalobulimus oblongus*, a progressive decrease in glycogen phosphorylase activity was demonstrated in nervous ganglia (Zancan et al., '97; De Fraga et al., '98, '99; De Fraga et al., 2000).

Studies have shown that, during longer starvation periods, animals may be submitted to metabolic depression (Hochachka and Somero, '84; Storey and Storey, '90; Guppy et al., '94; Hand and Hardewig, '96, Brooks and Storey, '97). This condition induces a metabolic adaptation, explaining the maintenance of haemolymphatic glucose levels at 90–120 days of starvation, probably by gluconeogenesis (Moon, '88) and/or by a decrease in the use of peripheral glucose (Goudsmit, '72; '73). Furthermore, it may be suggested that lipids may be used as an energetic source. Lipids are usually used in the reproductive phase as substitutes for glucose, rather than as an energetic source. In situations such as starvation, however, reproductive activity is reduced and, consequently, a reduction occurs in the peripheral use of glucose. Studies have demonstrated that, during starvation, the glycogen reserve is depleted, whilst protein and lipid are preserved (Emerson, '67; Veldhuijzen and Van Beek, '76; Schmitz et al., '89). In *Lymnaea stagnalis,* starvation for 52 days did not alter haemolymph glucose levels (Wijsman et al., '82), however, after 15 days of starvation, the polysaccharide reserve decreased significantly,

when compared to the control group (Veldhuijzen and Van Beek, '76). In the pulmonate snail, *Planorbis corneus*, Emerson ('67) observed a significant decrease in the total reserve of polysaccharides after 58 days of starvation, which induced death in the animals, despite the maintenance of lipid and protein reserves. Our study also demonstrates, for the first time, the effect of starvation for 90 and 120 days on these biochemical parameters in mollusks.

Several mechanisms are able to regulate metabolic processes under adverse situations such as starvation. The functional response is intracellular, occurring via stoichiometric and allosteric effects, enzyme phosphorylation-dephosphorylation, and enzyme synthesis and degradation (Lazou, '89; Storey and Storey, '90; Van Den Thillart et al., '92; Brooks and Storey, '94; '95; '97; Storey, 2002). The stimuli that trigger intracellular metabolic alterations are mediated by messengers, such as hormones, neurotransmitters, and local mediators. The presence of a hyperglycemic factor has also been postulated in mollusks, this factor is probably a neurohormone that can inhibit the synthesis and stimulate glycogen degradation in the mantle in *Lymnaea stagnalis* (Hemminga et al., '85a,b). In bivalve *Mytilus edulis,* this factor is able to mobilize glycogen extracted from cerebral ganglia and the haemolymph (Robbins et al., '90; '91). Several studies have suggested that the synthesis and mobilization of glycogen in mollusks is controlled by hormonal factors. Recently, ''light green cells'' (LGCs), localized in the cerebral ganglia of *Lymnaea stagnalis*, were shown to express, produce and release hormones of the insulin superfamily called ''molluscan insulin-related peptides'' (MIPs) (Li and Geraerts, '92; Li et al., '92a,b), which are involved in growth and in the control of carbohydrate metabolism in this gastropod (Geraerts, '76; '92). Studies performed with *Strophocheilus oblongos* demonstrated that the insulin of mammalians is able to decrease haemolymph glucose concentrations and increase the glycogen content in muscle (Marques and Falmer, '76). Hormones, such glucagon, insulin, catecholamines and glucocorticoids modulate, in a distinct manner, gluconeogenesis (Moon, '88).

Recently, we demonstrated a nucleotidase activity with different kinetic properties in the membranes of nervous ganglia and the digestive gland of *Helix aspersa* (Borges et al., 2004). Lazou ('89) demonstrated 5'-nucleotidase, adenosine deaminase, adenosine kinase and AMP deaminase activities in a large number invertebrates, including mollusk gastropods and the genus *Helix*.

ATP, and its metabolic products, obtained by the action of nucleotidases, such as ADP, AMP and adenosine, are signaling molecules located on the cell surface which act via the receptors P2 (P_{2x} and P_{2y}) and P1 (A_1, A_2, A_{2a}, and A_3), respectively. Furthermore, AMP is a positive allosteric modulator of glycogen phosphorylase and phosphofructokinase 1. Despite their important modulation functions, no changes in nucleotidase activities were found under our experimental conditions. These enzyme activities, however, could be modulated by effectors in situ, with concomitant changes in products/reactants (ATP/ADP/AMP) during starvation. Further studies will be required to evaluate the nucleotide levels *in H. aspersa* after long starvation periods.

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