Calcium, Copper Protein And Oxygen Affinity In Haemocyanins Of Aestivating And Nonaestivating Snails (*Achatina achatina)*

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Abstract

Under dry conditions, land snails withdraw into their shells closing the entrance with a calcified mucous membrane called epiphragm and become dormant (aestivate). In this state, the partial pressure of oxygen gradually falls to low levels whereas the partial pressure of carbon dioxide rises. We have investigated the effect of aestivation on the haemolymph inorganic ions, the oxygen affinity of haemocyanin in the giant African snail, *Achatina achatina*. After four months of aestivation, they were 46.35%, 4.19%, 44.60% decreases in haemolymph protein, pH and body weight respectively and significant increases in haemolymph Cu^{2+} and Ca^{2+}, i.e is 161.16% and 75.09% respectively. The increases in divalent ions which may have resulted from the need to buffer the decrease in extracellular pH during aestivation is likely responsible for the high oxygen affinity of haemocyanin (43.0% increase) from aestivating snails through co-operative oxygen binding.

Key words: Aestivation, snail, *Achatina achatina*, inorganic ions, haemocyanin, absorption spectra, oxygen affinity.

Introduction

Under adverse environment conditions many animals enter a state of dormancy known as aestivation or hibernation which is characterised by a drop in body temperature (Hoffman, 1964, Riddle, 1971,Precht *et al.*,1973,).

Pulmonate land snails, which the giant African snail (*A. achatina*) is an example of, are capable of prolonged aestivation in the dry season (Hodasi, 1979, Umezuruike et al.,1983). Aestivation is the means through which these snails survive adverse life conditions stimulated by reduced environmental humidity (Bailey, 1975,1981), extreme temperatures (Bailey 1981, Barnhart *et al.*, 1987) and limited food supply (Little, 1983, Riddle 1983.). During aestivation, land snails withdraw into their shells, closing the entrance with a calcified mucous membrane (epiphragm) and may remain dormant for many months or even years. Other behavioural, physiological and biochemical adaptations are also put in place to support long-term aestivation in the snail (Guppy, *et al.,* 1994; Books, *et al.,* 1997).

A reasonable number of proteins require a metal ion for their structural stability or their biological function. Furthermore enzymes and other non-enzymic proteins bind metal ions at sites other than structural or functional sites. In fact the elucidation of the three-dimensional structures by x-ray diffraction is dependent upon these latter metal sites (Lipscomb, 1980). In haemocyanins (Hcs) for example, the multisubunit proteins that function as reversible oxygen carriers in arthropods and molluscs (Van

Holde, *et al.,* 1982; Ellerton, *et al.,* 1983; Van Holde, *et al.,* 1995;) interact *in vivo* as well as *in vitro* with a number of metal ions. Their active site consists of a binuclear copper centre and binding of oxygen occurs via a reversible one-electron transfer from each copper ion to the ligand thus the resulting complex is depicted as a [Cu (II) O_2^{2-} Cu (II)] complex, where the peroxide dianion is bound in a $\mu: \eta^2 - \eta^2$ bridging of mode (Ling, *et al.,* 1994)

Although the chemical structure of the active site of Hcs from arthropods and molluscs Hcs seem quite similar, their molecular architecture is entirely different and has been described in reviews (Ellerton *et al., 1983,* Herskovits, 1988; *Herskovits,* et al., 1991, Van Holde, *et al.,* 1982; Van Holde, *et al.,* 1992; Van Holde *et al.,* 1995). Molluscan Hcs are enormous having molecular weights comparable to those of viral proteins and occur as complex structures called decamers or multi-decamers (Ellerton et al., 1983; Herskovits, 1988; Van Holde, *et al.,*1992). The molecular weight of these decamers ranges from $3.5 - 9 \times 10^6$ Da. Monomer units of decamers with molecular weights of 350 – 440 k Da contain seven or eight domains depending on the species (Markl, 1986; Burggren *et al.,* 1991, Van Holde *et al.,* 2001).

In addition to copper, a divalent cation that plays an important role in controlling Hc structure and function is Ca^{2+}. It has been extensively demonstrated that Ca^{2+} (or Mg^{2+}) is required for maintaining the structural stability of many arthropodan and molluscan Hcs (Brouwer *et al.,* 1983; Ellerton *et al.,* 1983, Herskovits, 1988; Herskovits *et al.,* 1991). Removal of Ca^{2+} from these proteins at alkaline pH results in their dissociation into subunits (Herskovits, 1988; Van Holde *et al.,* 1982, 1995). In addition to its structural role, Ca^{2+} also acts as a modulator of Hc function. It has been suggested that increase oxygen affinity through co-operative oxygen binding in arthropodan and molluscan Hcs is conditional upon the presence of Ca^{2+} (Markl 1986; Herskovits, 1988; Morris *et al.,* 1987).

Another ion found in the haemolymph of snails is H^+. It has been proposed that the most probable trigger of the molecular events associated with aestivation - induced metabolic depression in snails appear to be the decrease in intra or extra-cellular pH caused by elevations in the partial pressure of carbon dioxide (Busa, *et al.,* 1984; Burton *et al.,* 1987; Guppy, 1994; Scholnick, *et al.,* 1994.). In order to compensate for this haemolymph acidosis, snails mobilize inorganic (mineral) salt to increase both the levels of circulating divalent cations.

Ca^{2+} and other divalent ion salts are among the mineral salts mobilize during aestivation. In view of the important role that divalent cations play in controlling Hc's structure and function which in most cases is by far the most important if not the only protein present in the haemolymph of snails (Ghiretti, 1966), it is surprising that literature available to date shows no attempts made to study the physical properties of Hc from *A. achatina* during aestivation. At present, work has been done on Hc from other animals by investigating the dissociation- association of Hc, oxygen affinity and Ca^{2+} binding by Hc from these active animals (Brix, 1982; Brouwer, *e al,* 1983; Morris, 1986; Morris *et al.,* 1987 Herskovits, 1988).

We have studied variations in haemolymph inorganic ions and changes in some physical properties (absorption spectra, and oxygen affinity) of Hc from active and aestivating *A. achatina*.

Materials and Methods

Tris [tris (hydroxymethyl) amino methane] was purchased from B.D. H Chemical Ltd. Poole, England. All other chemicals and reagents used were of analytical grade.

Adult land snails (*A. achatina*) were bought from the local market and kept inside a cage covered with wire mesh at an appropriate humidity (80% relative humidity and temperature of 25 – 27°C). The snails were fed for a week with fresh leaves of *Telferia occidentalis* after which they were randomly assigned to one of two groups (50 snails each). One group continued to receive adequate supply of fresh leaves and water and the other group of snails were induced to aestivate by being transferred to a dry cage without food and water. The snails were allowed to aestivate for 4 months and before the starvation (aestivation) period, each snail was weighed to determine fed body weight.

Isolation of *A. achatina* Hc: Snails were washed de-shelled and their individual haemolymph collected by punctuating the pervisceral haemoceal. To facilitate collection, the snails were placed on small funnels containing filter papers. The crude haemolymph was centrifuged for 3 min in a cooling box (4 °C) at a speed of 1000g so as to precipitate solid particles.

Measurement of inorganic ions in the haemolymph: Sub-samples of haemolymph taken from individual *A. achatina* were analysed first for pH using a pH meter, and then for Ca^{2+} and Cu^{2+} using an atomic absorption spectrophotometer (Buck Scientific 200 – A) at 422.6 nm and 465 nm respectively. The concentration of Hc was determined by a modified Lowry method as described by Schacterle and Pallack, (1972). When necessary the haemolymph collected from snails was diluted 10 times with 0.05 Tris HCl buffer.

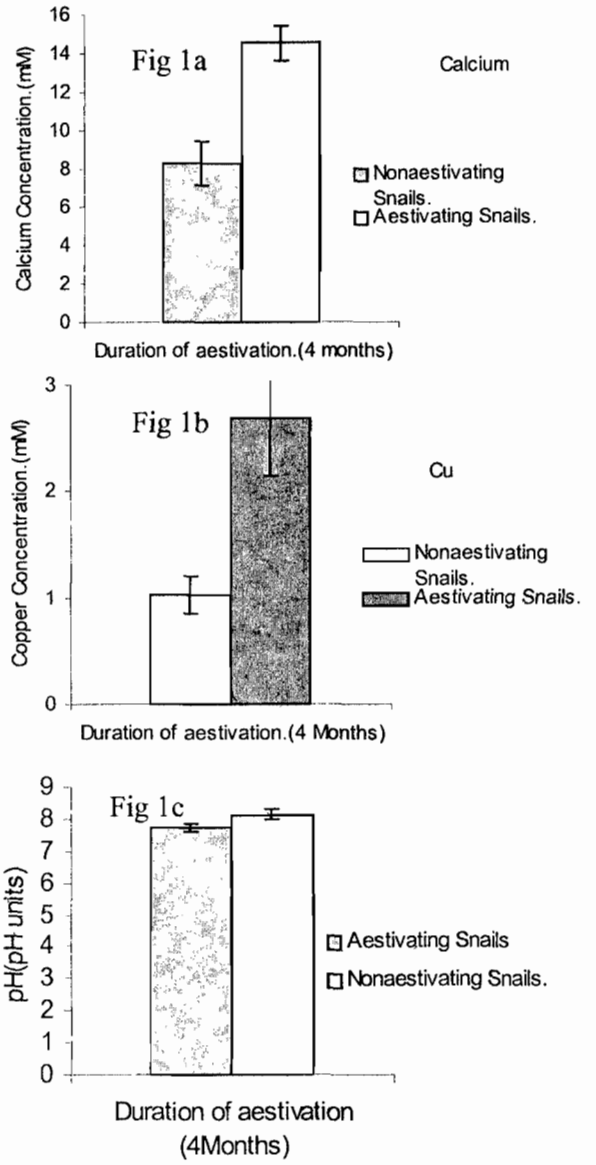
Results

Absorption spectroscopy: All absorption spectra were recorded with 1 cm cells on a Pye Unicam. spectrophotometer S.P. 8 – 100. Samples were diluted with 0.05 Tris–HCl containing 20mM Ca^{2+}. The pH of the buffer was that of the haemolymph in its natural condition i.e. pH 7.5 for Hc from aestivating snails and pH 8.0 for Hc from nonaestivating snails.

Ionic concentration: The measured concentrations of Ca^{2+} and Cu^{2+} in the haemolymph varied significantly ($p < 0.05$) during aestivation. The mean values for $[Ca^{2+}]$ and $[Cu^{2+}]$ in the haemolymph of active *A. achatina* where 8.31 ± 1.15mM and 1.03 ± 0.17mM respectively (Fig. 1 a – c). After 4 months of aestivation, the concentrations of these ions had increased significantly to: Ca^{2+} 14.55 ± 0.89mM and Cu^{2+} 2.69 ± 0.55mM. This gave percentage increases of 75.09% and 161.16% respectively.

The concentration of haemolymph H^+ (pH) did not vary much when compared to the other ions. Haemolymph pH was 8.15 ±0.16pH units for nonaestivating and 7.75 ± 0.14pH units for aestivating snails. This gave a marked drop of 4.19%.

Effects of aestivation on the body weight and haemolymph protein: Body weight and haemolymph proteins are one of the parameters that are most clearly affected by aestivation (Fig. 2a and b). The mean body weight and haemolymph protein



Figs. 1a – c summarises the changes in haemolymph Ca^{2+}, Cu^{2+} and H^{+} respectively when snails aestivate for 4 months

concentration of active *A. achatina* were 109.25 ± 7.39g and 13.96 ± 0.83mg/ml respectively. After aestivation, these valves dropped to 60.55 ± 12.06g and 7.49 ± 0.82mg/ml respectively. This corresponds to 44.60% and 46.35% drop in body weight and haemolymph protein concentration respectively.

Absorption spectra: The absorption spectra of oxyhaemocyanin from nonaestivating and aestivating snails are shown in Fig. 3a and b respectively. The spectra from both Hc show bands at wavelengths that are typical of molluscan Hcs. The normal protein band that occurs around 280nm is at 290nm for nonaestivating and at 279nm for aestivating snails. The copper bands that usually centered at 340nm and 570nm occur at 332nm and 556nm for aestivating and at 338nm and 556nm for nonaestivating snails.

The spectroscopic ratio 338: 290nm for nonaestivating and 332: 278nm for aestivating snails' Hc are informative of the oxygen saturation of the Hc molecules (Gielens *et al.*, 1975) and can serve as a measure for the oxygen binding at copper sites. The values calculated, 0.35, for nonaestivating and 0.50 for aestivating snails (fig. 3c) show that Hc from aestivating snails has a higher affinity for oxygen than Hc form nonaestivating snails.

Discussion

The 44.60 % drop in body weight was smaller than the ones observed in other pulmonate gastropods after 132 days of starvation by Russel-Hunter and Eversole (1976) who reported weight reduction of about 50%. Emerson and Duerr (1967) also reported weight reduction 62% in their studies. The greater weight losses in these studies may have been due to experimental conditions since high air temperatures and low humidity increase water loss through transpiration and thus increase weight loss. Weight loss in *A. achatina* during 4 months of aestivation was also probably due to the use of a large amount of the energy reserves stored in the digestive glands. However,

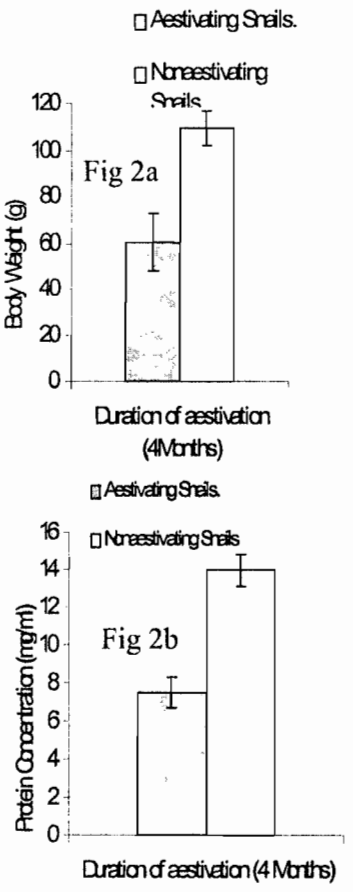


Fig. 2a and b: Effects of Aestivation on body weight and protein concentration respectively

Williams (1970) reported that weight loss during longer periods of aestivation is due to autophagy, which involves the use of animals' own tissues as a source of energy.

Hc makes up 90 – 95% of haemolymph protein in snails (Ghiretti, 1966) and the 46.35% drop in haemolymph protein in *A. achatina* during aestivation is most likely due to decrease Hc concentration. The reduction in protein levels during aestivation may be attributed to its degradation to amino acids and finally keto acids and ammonium ion, by the process of transamination and oxidative deamination. The keto acids if produced may enter the gluconeogenic pathway to produce glucose. The excess amino acid nitrogen from ammonium ions may be utilized in the biosynthesis of purines and other nitrogenous wastes, which are excreted during arousal. The amino acids may also be used for other processes like sexual maturation (Williams, 1970).

The haemolymph pH of 8.15 ± 0.16 pH units obtained for nonaestivating *A. achatina* is comparable to 8.5 ± 0.1 pH units obtained for nonaestivating *A. fulica* (Akintola *et al.*, 1971)) and 8.02 pH units obtained for nonaestivating *Lymnaea truncatula* (Pullin 1971). The decrease in haemolymph pH of aestivating *A. achatina* is also comparable to the 3.83 % drop obtained for aestivating *Helix aspersa* (Scholnick, *et al.*, 1994).

The decrease in haemolymph pH is probably caused by an increase in the partial pressure of carbon dioxide in the haemolymph of snails as a result of epiphragm formation during aestivation (Barnhart, *et al.*, 1987; 1988). In order to prevent the resulting respiratory and metabolic acidosis during aestivation, the snails raise the concentrations of haemolymph, Ca^{2+} in the haemolymph (Busa, *et al.*, 1984; Burton, *et al.*, 1987). Thus the concentration of haemolymph Ca^{2+} is apparently always high in the snails with the main physiological role being to compensate acid-base disruption during aestivation.

The concentration of haemolymph Ca^{2+} increased by 75.09% as the snails become dormant. This increase is similar to the results of Porcol *et al.*, (1996) who observed similar changes in the Mg^{2+}/ Ca^{2+} ratio during 6 months of aestivation in *Helix aspera*.

Cu^{2+} was the most extensively increased ion in the haemolymph of snails during aestivation. Cu^{2+} in the haemolymph of snails exist in 2 forms;

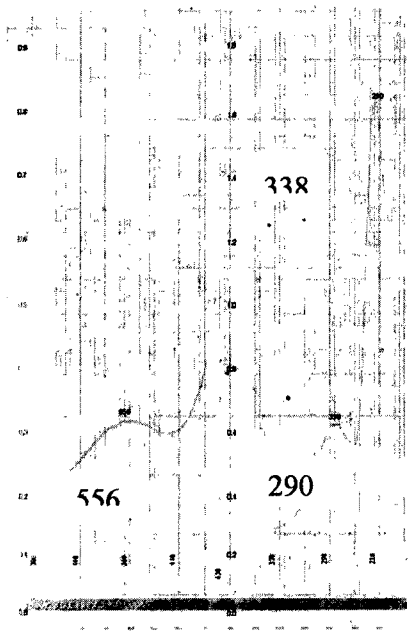


Fig: 3a. Nonaestivating

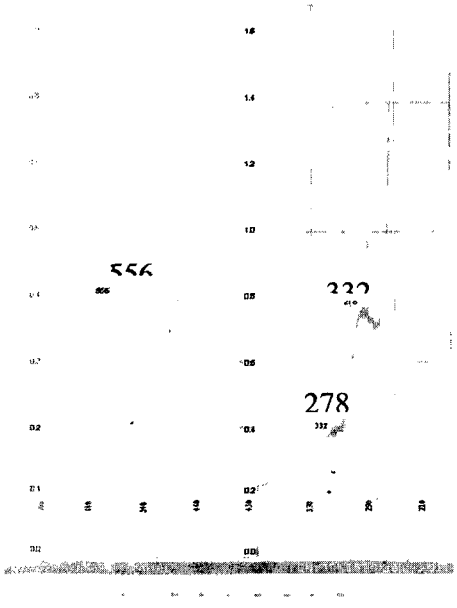


Fig: 3b. (Aestivating snails)

Fig: 3a & b: Absorption spectra of oxygenated haemocyanin from snails. Protein concentration, 0.16mg/ml U.V region & 5.27mg/ml visible region in pH 7.5 (aestivating snails), 8.0 (non aestivating snails) Tris. buffer

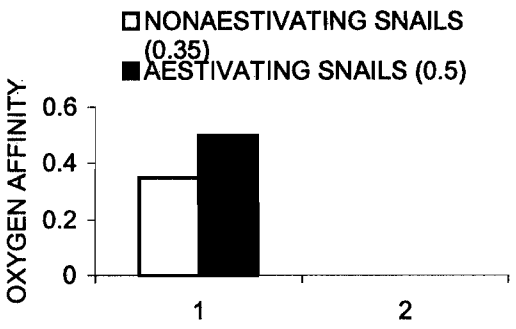


Fig: 3c. Histogram of changes in oxygen affinity of haemocyanin as snails aestivate for 4 months

bound Cu^{2+} at the active site of Hc and free Cu^{2+} dissolved in the haemolymph (Brouwer *et al.,* 1983).From our results, the large increase in the $[Cu^{2+}]$ is due to increases in free Cu^{2+} resulting from the break down of Hc into its constituent amino acids. The free Cu^{2+} together with Ca^{2+} may help to offset metabolic and respiratory acidosis by buffering the extracellular fluid and haemolymph (Busa, *et al.,* 1984; Burton, *et al.,* 1987).

Ca^{2+} ions are usually released from reserves located in the shell as calcium and magnesium carbonates. Shell thinning during prolonged starvation results from the release of mineral salts (mostly Mg and Ca) from the ostracum and hypostacum (Williams, 1970; Burton, 1972,) where they are transported to the haemolymph and other cells.

From fig 3a and 3b, the absorption spectra of Hc from nonaestivating and aestivating snails differ only in the uv region. The bands of 290nm and 278nm for nonaestivating and aestivating snails respectively correspond to the transfer of charges in aromatic amino acids found in the Hc molecules (Wetlaufer, 1962). The bands at 388nm and 332 nm for nonaestivating and aestivating snails

respectively correspond to the transfer of charges from histidine residues found in the Hc molecules to bound oxygen molecules while the bands at 556nm correspond to the transfer of charges from bound oxygen molecules to Cu^{2+} in the Hc molecules (Ling, *et al*1994). These differences demonstrate that during aestivation there are electronic changes in apohaemocyanin that affect the transfer of charges within apohaemocyanin and between apohaemocyanin and its ligands (copper, calcium and oxygen). These changes may be caused by the oligomerization of Hc during aestivation or by the drop in haemolymph pH. That is, oligomerization leads to the disturbance (masking) of tryptophans and tyrosine residues in Hc. While an increase in Ca^{2+} binding to Hc (due to pH drops) also leads to an increase in oxygen binding. These changes therefore affect the transfer of electrons within the Hc molecule.

In relation to oxygen affinity it has been known for years that Ca^{2+} and other divalent ions affect the structural and functional properties of Hc (Herskovits, 1988; Van Holde *et al.,* 1995). The presence for Ca^{2+} in the haemolymph stimulates the oligomerization of Hc and the cooperative binding of oxygen molecules to Hc. Therefore the increase oxygen affinity in Hc from aestivating snails probably results from a higher cooperativity of oxygen binding as a result of increased haemolymph Ca^{2+} concentrations (Morris, 1986; Morris *et al., 1987*).

In conclusion, our findings in *A. achatina* after 4 months of aestivation show that during aestivation haemolymph $[Ca^{2+}]$ and $[Cu^{2+}]$ are raised so as to buffer drops in haemolymph pH. The increase $[Ca^{2+}]$ and $[Cu^{2+}]$ helps to stabilize the functional properties of Hc by increasing its affinity for oxygen through co-operative oxygen binding.

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