HYPOXIC ADAPTATIONS AND CAROTENOIDS OF TWO INTERTIDAL MOLLUSCS

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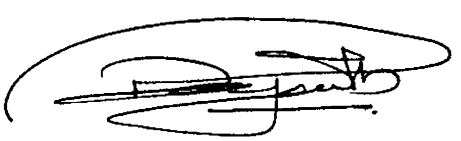
C E R T I F I C A T E

This is to certify that the thesis bound herewith is an authentic record of the research work carried out by Smt. Susan Mathew under my supervision, in partial fulfilment of the requirements for the degree of **Philosophiae Doctor** of the Cochin University of Science and Technology and further that no part thereof has been presented before any other degree.



Cochin - 682 016
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D E C L A R A T I O N

I hereby declare that the thesis entitled, **"HYPOXIC ADAPTATIONS AND CAROTENOIDS OF TWO INTERTIDAL MOLLUSCS"**, is an authentic record of the research work carried out by me under the supervision and guidance of Prof. (Dr.) R. Damodaran in partial fulfilment of the requirements of the Ph.D. degree in the Faculty of Marine Sciences, Cochin University of Science and Technology, and that no part of it has previously formed the basis of the award of any degree, diploma, associateship, fellowship or other similar title of recognition.

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CONTENTS

Chapter 1

INTRODUCTION

Intertidal belt, the narrow strip between the high and low water marks of the spring tides is the haunt of a rich and varied collection of flora and fauna. The conspicuous feature of this habitat is the great variability of environmental conditions prevailing here, which swings from one extreme to the other, twice a day. Biologically this zone is overwhelmingly a marine province.

Alternate submergence and emergence produce an environmental gradient with regard to exposure to air and water resulting in the development of special communities. The intertidal distribution, exposes a marine animal to a wide variety of possible environmental stresses like the abrasive action of waves and ice, discontinuous availability of food, wide variations and extremes of temperature, salinity and desiccation. The habitat also demands the ability, either to withstand periods of anoxia or to gain oxygen from a medium to which the respiratory apparatus is ill-adapted (Bayne *et*

al.,1976b). Added to the above natural variables, stress arising through anthropogenic activities such as release of pollutants, dredging **etc.** also influence the intertidal habitat. Naturally, the organism of such an environment need to be adapted to the periodic excursions to the resistance zone with respect to different environmental parameters. In spite of the harsh nature of the habitat, there is no scarcity of animal or plant life on the sea-shore. Under adverse conditions, intertidal organisms can tolerate and are capable of regulating various physiological activities to a certain extent that allows their successful colonization of the shore.

It is well known that estuaries and adjoining marine realm, in general, are subjected to wide fluctuations in dissolved oxygen and salinity under the impact of seasonal changes. Tidal activity results in the alternate aerial exposure and submergence of the intertidal organisms. Bivalve molluscs response to salinity changes or pollutant stress or even resist desiccation for relatively long periods by closing their shell valves tightly. Valve adduction provides an useful behavioural avoidance mechanism during exposure to adverse environments. Increased production of mucus also helps them in reducing contact with the ambient medium when subjected to unfavourable situations. Shell closure mechanism and mucus secretion cannot contribute to long term survival during periods of stress.During valve closure the animal incurs

penalities related to feeding, reproduction and exchange of gases and metabolites.

Valve closure results in the cessation of aerobic process and the animals switch over to several adaptive mechanisms to sustain the basal metabolic requirements. Such mechanisms include the utilization of anaerobic respiration to sustain basal metabolism, availability of stored food reserves and the ability to tolerate and accommodate levels of excretory products (Akberali and Trueman, 1985).

Molluscs, especially bivalves are often referred to as `facultative anaerobes' (Zwaan, 1977; Zwaan *et al.*,1976 and Hochachka, 1985) on account of their ability to withstand longer periods of valve closure and resultant lack of oxygen (Moon and Pritchard, 1970; Coleman and Trueman, 1971; Akberali and Trueman, 1972; 1985; Widdows *et al.*, 1979). Classical Embden-Meyerhoff pathway for anaerobic glycolysis operating in bivalves, differs from that of the vertebrates (Hochachka and Mustafa, 1972; Zwaan and Zandee, 1972; Zurburg and Kluytmans, 1980; Hochachka, 1985) in that, while vertebrate accumulate lactate, as the main end product of anaerobic catabolism, in molluscs, it is succinate and alanine (Zwaan and Zandee, 1972; Zwaan and Marrewijk, 1973; Zwaan, 1983).

Eventhough, several processes contribute to hypoxic tolerances, metabolic arrest mechanism yields by far the most

effective protection against oxygen lack (Hochachka, 1985). The enhancement of the energetic efficiency of anaerobic metabolism in bivalves in combination with a strong reduction of their energy consumption enable them to withstand long periods of anoxia (Zwaan and Wijsman, 1976). The known anoxic energy producing mechanism such as glycogenolysis or anaerobic glycolysis may contribute energy to a certain extent for the molluscan tolerance to hypoxia/anoxia. The complex biochemical process behind anoxic energy production and reduced metabolic rates are not yet completely understood (Hochachka, 1985). In the intertidal habitat, molluscan populations are often subjected to varying concentrations of oxygen which may fluctuate as a function of time. In addition, some of them regularly experience periods of low pO_2 during shell closure at low tide. Animals respond to varying oxygen tensions by two types of respiratory mechanisms: (1) oxygen dependent or (2) oxygen-independent (Vernberg, 1972), according to the physiological capacity and ecological requirements of the organism.

The resistance of bivalves to low oxygen levels and toxic agents point out that some molecular mechanism must exist that provides energy for cells especially nerve cells in hypoxic conditions. The extreme anoxic tolerance of certain molluscs is found to be connected with the presence of cytosomes in the tissues of these animals (Zs-Nagy, 1967 ; 1971 a,b; Zs-Nagy and Kerpel-Fronius, 1970a,b). Molluscan species

which have no cytosome in the nervous tissues have been reported to display practically no anoxic tolerance (Zs-Nagy, 1971a). The functional activity of carotenoids in animal cells was claimed by Karnaukhov (1969; 1971a,b; 1990) on the discovery of their direct involvement in the oxidative metabolism of the giant neurons of mussels during oxygen deficient conditions.

Regarding the role of cytosomes/carotenoxysomes in the anoxic tolerance of molluscs, two different hypotheses have been put forward independently by Zs-Nagy (1977) and Karnaukhov (1969; 1971a ,b). According to Karnaukhov (1971 a,b; 1990), carotenoids are supposed to take the part of the electron acceptor of the respiratory chain and can be considered as a kind of oxygen depot located in an energy generating intracellular organoid termed carotenoxysome other than mitochondria with peculiar ultra structural organization.

Zs-Nagy (1971a, b; 1974; 1977) and co-workers (Zs-Nagy and Ermini, 1972a, b; Zwaan, 1983) disagreed to the above hypotheses, stating that, a part of the energy production in molluscs during prolonged anoxia is by means of a mechanism called anoxic endogenous oxidation localized in cytosomes. Here the electron acceptor function of molecular oxygen is performed by unsaturated fatty acids,the biodegradation of which represents an oxidative metabolic step. The electron acceptor molecule has a sufficiently high positive redox

potential. Only an initial concentration of this molecule is present in cytosomes, which multiplies in special compartments of cytosomes during anoxic metabolism. It is functionable till the capacity of the electron acceptor is exhausted as in the case of prolonged anoxia. Inspite of their disagreement regarding the functional aspect of carotenoids, both have reached a similar opinion that cytosomes/ carotenoxysomes play a vital role in the anoxic oxidative metabolism of molluscan tissues (Zs-Nagy, 1977; Karnaukhov, 1979).

Ultra structural studies (Karnaukhov *et al.*, 1972; Karnaukhov, 1973b) reflected a close similarity between the chemical composition and the physiological function of the yellow-ageing pigment granules, lipofuscin and carotenoid containing cytosomal granules of molluscan neurons. Lipofuscin accumulation with age (Totaro and Pisanti, 1979) can be considered as cell's adaptation to a decreased rate of oxygen diffusion into tissues, due to the decreased oxygen transferring ability of blood vessels which progresses with age. For the same reason, pigment accumulation is observed in tissues of young animals subjected to hypoxic/anoxic stress (Karnaukhov *et al.*, 1972). Lipofuscin granules, which contain carotenoids, myoglobin and respiratory enzymes like flavoproteins and haemoproteins, have their own terminal oxidation system and oxygen reserve system,providing the energy requirements of cells under conditions of low rate of oxygen diffusion into the tissues (Karnaukhov *et al.*, 1972;

Karnaukhov, 1973b). Thus, concomitant with the rise in the carotenoid concentration during anoxic stress, an increment in the lipofuscin accumulation is also supposed to take place.

The most important climatological aspect of the west coast of India is the occurrence of strong south-west monsoon. It commences in late May or early June, bringing about rapid changes in the hydrographic conditions of the coastal waters off Cochin. A strong upwelling is reported to occur during this season (Banse, 1959; 1968) resulting in a marked reduction in the dissolved oxygen content of the inshore bottom waters. The oxygen content of the upwelling water coming from near the upper intermediate oxygen minimum layer is further reduced by increased consumption on the shelf which is rich in organic matter. In the bottom water below a depth of 4m, the dissolved oxygen becomes very low, even reaching near zero values during July and August (Damodaran, 1973).

A steep decline in the surface salinity is also found to occur during south-west monsoon due to the copious precipitation and flood water influx from backwaters. But the bottom water salinity is hardly affected by monsoon except at shallow water stations. Thereby the organisms of inshore waters off Cochin experience two adverse situations- low oxygen concentration and low salinity in the ambient medium during the monsoon season. Adult species of many of the members of the intertidal fauna were found to survive these

unfavourable conditions. Remarkably none of the species of this habitat is reported to have been completely eliminated by the harsh environment. This clearly points out that organisms of the intertidal habitat have some mechanisms at work which enable them to withstand such near anaerobic or anaerobic conditions. In addition to these natural stress, adverse situations due to the discharge of heavy concentrations of waste materials and pollutants into the inshore waters also confront the organisms forcing them to resort to anaerobic pathway of respiration.

The two bivalves, *Sunetta scripta* and *Perna viridis* selected for the present study are intertidal in their habitat and are often exposed to the above mentioned anaerobic situations. *S.scripta* is an infaunal organism, often found buried in the bottom sand. *P.viridis* being epifaunal, is usually found attached to rocks or hard objects. Both of these species have the capability to withstand such adverse conditions by closing off their shell valves and switching over to anaerobic respiration. Therefore it is worthwhile to study the mechanism by which they are able to thrive well in poorly oxygenated conditions, or condition which demand prolonged valve closure.

Salinity stress is found to enhance the carotenoxysome production in the clam *S.scripta* (Supriya, 1992). The green mussel, *P.viridis* was reported to produce

high carotenoid content in its body under the impact of heavy metal stress (Krishnakumar, 1987; Krishnakumar *et al.*,1987). An excess accumulation of lipofuscin in response to heavy metal exposure have been reported by many workers (George *et al.*,1982; Aloj and Pisanti, 1985; Aloj *et al.*, 1986 a,b; Marzabadi *et al.*,1988 ; Moore,1988; Pisanti *et al.*,1988; Enesco *et al.*, 1989). This may be due to the activation of cytosome/ carotenoxysome of the anaerobic pathway, since metals like copper is supposed to be a respiratory inhibitor even at low concentrations (Scott and Major, 1972; Delhaye and Cornet, 1975), drastically reducing the endogenous respiration (Babu and Rao, 1985). These observations are supported by the general idea that carotenoids do take part in the adaptation of animal cells to low oxygen concentration in environment and mitochondrial inhibitory action (Karnaukhov, 1971 a, b ; 1973a; Schindelmeiser *et al.*,1979).

So far, only a few approaches have been made to study the anaerobic capacity of intertidal molluscs due to environmental/ pollutant stress based on the biochemical and cytological changes. The present investigation is focused on the role of carotenoids in the anaerobic catabolism of intertidal molluscs subjected to anoxic/hypoxic stress. This has been compared with that of the respiratory (mitochondrial) inhibition induced by copper at sublethal levels. Copper has been selected because of its wide recognition as the most common pollutant in the marine environment. It is important

for molluscs, firstly as a part of the respiratory pigment, secondly in the cytochrome system of electron transfer and finally in the enzyme system involved in removing the toxic side products of aerobic metabolism (Simkiss *et al.*, 1982; Simkiss and Mason, 1985). However ,at higher concentrations it has been reported to be hazardous to life (INCRA, 1982).

Considering the functional aspect of carotenoids and lipofuscin granules, the present line of investigation is to have a baseline information on the role of carotenoxy-somes/cytosomes on the anaerobic respiration of the intertidal bivalves. Effect of hypoxic stress and heavy metal stress on the physiological and cytological characteristics have been studied using two indices ——total carotenoid concentration and accumulation of lipofuscin granules.

Chapter 2

MATERIALS AND METHODS

2.1. DESCRIPTION OF THE SPECIES.

2.1.1. *Sunetta scripta*.(Linne').

The clam *Sunetta scripta* is distributed widely along the east and west coasts of India. Important clam bed in Cochin area lies between latitude 9^{o} $28^{'}$ and $10^{o}0^{'}$ N and longitudes $76^{o}13^{'}$ and $76^{o}31^{'}$E on the northern side of the entrance into the Cochin barmouth (Kattikaran, 1988).

Clam beds are largely sublittoral, occurring at depths of 1.5-2.5m. The bottom sediment is composed predominantly of sand with clay and silt forming a small percentage. Salinity of their habitat varies widely ranging from $5X10^{-3}$ (during monsoon) to $36.46X10^{-3}$ (during premonsoon). Eventhough the clam is seen to tolerate lower salinities, the range from $25X10^{-3}$to $35X10^{-3}$ is considered as the zone of tolerance (Thampuran, 1986). Below and beyond this range, they are in their resistance zone.

S.scripta is included in the family Veneridae,

characterized by a shallow borrowing habit and absence of byssus in the adult. It is a sedentary filter feeder and highly tolerant to a wide range of environmental conditions. It is easily available almost through out the year and can be reared with ease in the laboratory. It forms a cheap source of protein food and is economically important, being exploited both for flesh and shell. The shell is used as a raw material for lime and cement industry.

2.1.2. *Perna viridis*.(Linnaeus).

The Indian green mussel *P.viridis*, has a widespread,distribution along the east and west coasts of India (Kuriakose and Nair, 1976; Kuriakose, 1980). It is often found in the mouth of estuaries and rivers (Kuriakose, 1980; Narasimham, 1980; Qasim *et al.*,1980), harbours and in rocky open coast. They occur from intertidal zone to a depth of 15m, attached to rocks and pilings and other hard objects by their byssus thread. Ambient salinity of the mussel is within the range of $30X10^{-3}$ to $37X10^{-3}$,but could tolerate reductions of both the environmental $pO_2$and salinity with no obvious signs of stress (Hawkins *et al.*,1987).

P.viridis, belonging to the family Mytilidae is one of the most exploited bivalve mollusc for human consumption in India and other parts of Indo-Pacific region (Rao *et al.*, 1977) because of its low cost and high proteinaceous meat. It

contributes to substantial sustenance fisheries. In the intertidal habitat, during low tide, the animal is exposed to air for varying periods of time with all the resulting problems of potential desiccation, thermal shock and lack of oxygen.

2.2. LABORATORY ACCLIMATION.

Specimens of *S.scripta* were collected from the clam bed situated on the northern side of Cochin barmouth lying parallel to the sand bar, which is perpendicular to the southern extremity of Vypeen Island. They were brought to the laboratory in plastic buckets containing sea water from the clam bed area and were thoroughly cleaned of the lingering algae, dirt and barnacles attached to their shells. They were acclimated in the laboratory for about 4-5 days in sand filled large plastic basins containing water of the habitat conditions (Salinity: $30\pm2x10^{-3}$, Temperature: $28\pm1^{o}C$, pH :7.8±0.2, Dissolved oxygen: >4ml L^{-1}).

The mussel, *P.viridis* is collected from an unpolluted natural population attached to the sea wall near Narakkal. They were immediately brought to the laboratory in a polythene bag filled with sea water taken from the same site. They were cleaned off the epibiotic growths and acclimated for 4-5 days in the laboratory in tubs containing well aerated unfiltered natural sea water (Salinity :$30\pm2x10^{-3}$, Temperature: $28\pm1^{o}C$, pH:7.8±0.2, Dissolved oxygen : >4ml L^{-1}).

Both the animals were fed on the blue green algae *Synechocystis salina* daily. Feeding was suspended during the experiment. In the case of copper exposed experiments, feeding was stopped two days prior to the commencement of the experiment. The major parameters like salinity, temperature, stocking density, *etc.* were monitored and standardized.

2.3. TEST CONTAINERS.

Bioassays were conducted in specially manufactured colourless polythene tubs of 10L capacity and 14" diameter. The tubs were soaked in Nitric acid (1:1) and Hydrochloric acid (1:1) for 24 h each and washed with copious amounts of deionized water.

2.4. TEST SOLUTIONS.

100 ppm stock solution of copper was prepared by dissolving the analytical grade $CuSO_4 5H_2O$ in glass distilled water. Calculated quantities of the solutions (500ml/animal) were added to the experimental tubs containing a known volume of filtered (42 Whatman) natural sea water (Salinity : $30\pm1X10^{-3}$, Temp : 28 ± 1^{oC}, pH: 7.8±0.2, Dissolved oxygen > 90% saturation) to obtain the required test concentration.

2.5. EXPERIMENTAL PROCEDURES.

Determination of oxygen uptake rate at varying percentage saturation of oxygen:

At the commencement of the experiment, 1L of filtered sea water each with varying percentage saturation of oxygen were run into 4 or 5 beakers. The water in each beaker was covered with a layer of liquid paraffin of nearly 3 cm thick to prevent the diffusion of oxygen from the air. Before the introduction of the animal, samples were drawn from each beaker for determining the initial concentration of dissolved oxygen in the water. In all cases, only those bivalves that were in apparent good health showing activities were used. After the respective intervals of time, samples of water were drawn out. The oxygen content in each sample was determined by the standard Winkler method (Strickland and Parson, 1972). The oxygen consumed at different percentage saturations of oxygen were expressed as the amount of oxygen consumed by a single animal per hour per mg dry wt. This experiment was conducted in three size groups of both *P.viridis* and *S.scripta*.

Animals were subjected to experimental hypoxic conditions in the following ways:

Clams(30-35mm) were kept out of water for a desired interval of time to provide hypoxic condition. Duration of the

aerial exposure was from 1h to 6h. After the respective intervals of time, 4/5 clams (pooled) were taken out for carotenoid estimation.

Secondly, anoxic environment was created by embedding individual clams (35-45mm) in a ball of modelling clay. 4/5 animals (pooled) were taken for carotenoid extraction after exposure likewise the procedure described above. With another set of clams (35-45mm), exposure time was extended to 48h and pooled samples were taken out at 12h, 24h and 48h intervals of anoxic exposure for carotenoid extraction.

Both *S.scripta* (35-45mm)and *P.viridis* (40-50mm) were subjected to gradually developing hypoxic condition by keeping them in known volume (1000ml/10animal approx.) of natural sea water filtered through millipore filter without aeration. The physico-chemical properties of the experimental sea water were salinity :$30\pm2X10^{-3}$, temp : 28 ± 1^{0C} and pH: 7.8±0.05.

The surface of the water was covered by paraffin oil layer nearly 3 cm in thickness to seal off the water air interphase. At 0h, 24h, 48h of hypoxic exposure and reimmersed after exposure, about 8/10 animals (pooled) were removed for the estimation of both glycogen and total carotenoid content in the body of the molluscs. One set of the sample at the respective intervals of time was take out for histological studies.

Toxicity studies were conducted in both *S. scripta* (35-45mm) and *P.viridis* (55-65mm). *S.scripta* and *P.viridis* were exposed to 2ppm and 7.5ppb of copper in the sea water respectively. Each tub contained 5L of test medium and ten animals. One experimental tub was kept as control without metal solution. Test medium (Salinity: $30\pm2x10^{-3}$,Temp: 28 ± 1^{oC}) was renewed once in 24h to replenish the metal concentration. After 48h of exposure, all the live specimens were removed for carotenoid estimation. From both *S.scripta* (35-45mm) and *P.viridis* (55-65mm), a few samples were withdrawn for lipofuscin detection.

Valve movements of both clams (35-45mm) and mussels (55-65mm) in salinities 30ppt and 33ppt respectively and during aerial exposure followed by reimmersion were observed and recorded using an electronic gadget named Oyster Activity Monitor (Supriya ,1992). A sensor is placed on the valve near the margin at the region of maximum sensitivity. A slight opening of the valve is highly magnified by the recorder and both its frequency and magnitude were calculated.

2.6. BIOCHEMICAL ESTIMATIONS.

2.6.1. Carotenoid extraction and estimation.

The carotenoid extraction and estimation were made

according to the standard procedures given (Karrer and Jucker, 1950; Karnaukhov *et al.*, 1977; Karnaukhov and Fedorov, 1977; Krishnakumar, 1987).

The soft tissues of the animals were dissected out, wiped with filter paper and the wet weight recorded. The weighed tissues were ground with chilled acetone in a glass mortar. The acetone extract was filtered through a sintered glass funnel under reduced pressure. The solid residues were returned to the mortar for further extraction, the process being repeated until the acetone extract become colourless. The volume of the extract was measured and its optical density being recorded using a Hitachi Spectrophotometer (Model 200-20) at 455 nm.

The total carotenoid concentration in mg $100g^{-1}$ of wet tissue weight was calculated from the equation (Karnaukhov *et al.*, 1977),

$$\text{Concentration of carotenoid (mg } 100g^{-1} \text{ of wet wt)} = \frac{0.4DV}{P}$$

Where, D = optical density of the extract measured in the wavelength of the carotenoid absorption maxima (455nm)

V = the total volume of the acetone extract in ml and

P = the total wet weight in grams of the tissue from which the carotenoid was extracted.

2.6.2. Estimation of glycogen.

The modified Pfuger method as given by Hassid and Abraham (1963) was used for the estimation of glycogen. Glycogen is hydrolysed to glucose by refluxing with 0.6NHCl. After neutralization with 0.5N NaOH,glucose is estimated by the procedure given by Heath and Barnes (1970), where 6ml of H_2SO_4 is added after neutralization to develop a pink colour. Glucose (Analar grade)was used as the standard. Concentrations were specrophotometrically determined at 520nm.

2.7. CYTOCHEMISTRY.

2.7.1. Tissue Preparation.

The tissues were prepared for cryostat fixation and sectioning according to the standard procedure (Moore, 1988). Hepatopancreatic tissues were dissected out from both the experimental clams and mussels. Small pieces of the tissue (Ca 5x5x5mm) were placed on aluminium cryostat chucks with 2-3 pieces of tissues in a straight row across the centre. The chuck was then placed for one minute in a small bath of hexane (aromatic hydrocarbon free, boiling range 67-70^{o}c) which had been precooled to -70^{o}C in liquid nitrogen inorder to quench the tissue.

The chuck plus the quenched solidified tissue were then sealed by aluminium foils and stored at -30°C until required for sectioning. Tissue sections (10μm thick) were prepared in a Bright's Cryostat (motorised cutting speed setting 50), with a cabinet temperature of less than -25°C. Sections were transferred to slides at room temperature, which effectively flash dried them (Bitensky *et al.*, 1973). The sections were stored in the cryostat until required, but not longer than four hours.

2.7.2. Determination of Lipofuscin granules.

Lipofuscin granules are often positive to Schmorl ferric chloride-potassium ferricyanide reaction (Hack, 1981).

The granules were detected using the Schmorl technique (Pearse, 1972). Duplicate cryostat sections (10μm) were fixed for 15min. in calcium formol at 4°C. Sections were then rinsed in distilled water and immersed in the reaction medium. This latter contained 1% ferric chloride and 1% potassium ferricyanide in the ratio of 3:1. Sections were stained for 5min. in this solution. Then rinsed in 1% acetic acid for 1min. followed by rinsing in distilled water. They were mounted in aqueous mounting medium. The blue reaction product indicates lipofuscin granules.

The microphotographs were taken with Nikon FX - 35 WA microscope at low magnification (X200).

2.8. QUANTIFICATION OF LIPOFUSCIN GRANULES

To determine quantitatively, whether there is any difference in the number of lipofuscin granules between nonexposed and 48h copper exposed specimens and also between control(0h), 24h hypoxia, 48h hypoxia and reimmersed groups, a comparison was made on the number of granules per unit area by the conventional method (Mohandas *et al.*, 1985).

2.9. STATISTICAL ANALYSES

The regression equation form $Y = a+bx$ is fitted to the data by the method of least squares. The normal equations are:-

$$b\varepsilon x^2 + a\varepsilon x = \varepsilon xy$$

$$b\varepsilon x + ba = \varepsilon y$$

from these two equations,value of a and b are solved and substituting $y = a+bx$, determined the regression of the best fit to the data.

Changes in the total carotenoid concentration at 0h, 24h and 48h of hypoxic exposure and reimmersed were statistically analysed by means of one way ANOVA. The significant difference between control(0h) and reimmersed groups were determined by student's **t** test.

The least significant difference was calculated using the formula:

$$LSD = \sqrt{\frac{2}{r} \times Ve \times t_{0.05}}$$

where, r= number of observations

Ve= the mean of square error

$t_{0.05}$= 5% table value.

The total carotenoid concentration in both *S. scripta* (35-45mm) and *P.viridis* (55-65mm) exposed to sublethal levels of copper were compared statistically using student's *t* test.

Quantification of lipofuscin granules during hypoxic exposure were statistically worked out by means of one way ANOVA. The least significant difference was calculated as mentioned above.

The statistical analyses on the quantification of lipofuscin granules upon exposure to sublethal level of copper were employed using student's *t* test.

Chapter 3

RESPONSES OF INTERTIDAL BIVALVES TO DECLINING OXYGEN TENSION

3.1. INTRODUCTION.

The role of dissolved oxygen as a limiting factor in the respiratory metabolism is of vital importance in the ecology of intertidal animals. Amount of dissolved oxygen may vary markedly in different habitats or in the same habitat at different times. Marine organisms show varying degrees of dependence on dissolved oxygen according to their physiological capacity and ecological requirements. Some require higher concentrations of oxygen in the environment while some others are able to survive temporarily in anaerobic conditions.

Animals, especially those inhabiting the marine intertidal zone are subjected to varying concentrations of oxygen which may fluctuate as a function of time. The tissues of intertidal bivalves regularly experience periods of hypoxia during shell valve closure at low tide. With many species, the rate of oxygen consumption has been found to vary with changes in the environmental variable.

The capacity to regulate the rate of oxygen

consumption during environmental hypoxia varies with the physiological conditions of the animal. Marine bivalve molluscs respond to environmental hypoxia by a variety of physiological compensation. These serve to increase the scope for activity and possibly to maintain the optimum delivery of oxygen to the cells (Bayne *et al.*, 1976b).

Classically aerobic marine organisms are divided into two categories depending upon its response to varying oxygen tension: (1) Oxygen dependent and (2) Oxygen independent (Lockwood, 1967; Vernberg, 1972, Cherian, 1977; Herreid, 1980).

In oxygen dependent category, the oxygen consumption of the animal is directly proportional to the oxygen tension in the medium. Species in which respiration is thus limited by the amount of oxygen present in the surrounding medium are called 'conformers'. In oxygen independent category, the rate of respiration remains unaffected until some critical oxygen tension is reached. Below this critical oxygen tension, the respiratory rate becomes dependent on the availability of oxygen as in conformers. Animals belonging to this category are termed `regulators'.

Metabolic regulation varies not only among species but also between members of the same species. Bayne (1971) has reported that some members of *Mytilus edulis* were found to be regulators, while some others were conformers. The degree of

regulation depends upon the species, the physiological state of the individual and also with temperature and salinity of the environment.

Regulators compensate for a fall of partial pressure by increasing oxygen conductance such as *via* increased ventilation or circulation (Herreid, 1980). Many bivalves control their oxygen uptake rates upto a very low oxygen tension below which it falls to a very low level. Bayne (1973) has observed that *M.edulis* regulated oxygen consumption over a wide range of environmental partial pressures of oxygen, with critical oxygen tension in the range of 40-70mm Hg pO_2.

Studies conducted on the clam *Katelysia opima* by Mane (1975a) has reported that the oxygen consumption rate of the clam appear to be directly related to the oxygen tension of the external medium. The rate of oxygen consumption is found to be considerably retarded at 1.5ml/L oxygen content in the ambient medium..

The oxygen consumption rate of *Perna viridis* studied in relation to body weight and declining oxygen tension (Mohan and Cheriyan, 1980) has revealed that *P.viridis* is a good regulator. In comparison to *Perna indica, P.viridis* is shown to have greater tolerance to reduction of both salinity and environmental oxygen tension (Hawkins *et al.*, 1987).

The present study is undertaken to compare the response of *Perna viridis* and *Sunetta scripta* to declining oxygen tension in the environment in relation to body weight.

3.2.MATERIALS AND METHODS.

The details of the experimental procedure has been dealt with in chapter-2.

3.3. RESULTS.

The 'b' values estimated at various percentage saturation of oxygen for *Sunetta scripta* are given in Table 3.1a. No significant differences in the oxygen consumption has been noticed at 100 and 80 percentage saturation of oxygen. At 70 percentage saturation,a decrease in 'b' value has been observed, which further decreased at 50 percentage saturation (Fig 3.1a).

Table 3.1b represents 'b' values of *Perna viridis* determined at different percentage saturation of O_2. No significant differences in 'b' value has been noticed at 100, 80 and 70 percentage saturation of O_2 (Fig 3.1b). But at 50 percentage saturation,a sharp decline in 'b' value has been observed.

Rate of oxygen consumption obtained for three size

groups (100mg, 200mg and 300mg dry wt) of *S.scripta* at different percentage saturation of oxygen is presented in Table 3.2a. In all the three size groups, rate of oxygen consumption is appreciably decreased at 50 percentage saturation of oxygen, but the reduction is more obvious for 200 mg (dry wt) and 300 mg (dry wt) size groups. At 70, 80 and 100 percentage saturation, all the size groups maintained more or less a high rate of oxygen consumption rate.

Table 3.2b represents the oxygen consumption rate of three size groups (100mg, 250mg and 400 mg dry wt) of *P.viridis* at different percentage saturation of oxygen. Here also, the lowest oxygen consumption rate is observed at 50 percentage saturation in all the size groups. The reduction in oxygen consumption rate at 50 percentage saturation is more pronounced for 250mg and 400mg size groups. At 70, 80 and 100 percentage saturation, all the size groups maintained a high level of oxygen consumption rate. In both *S.scripta* and *P.viridis*, the reduction in oxygen consumption rate at 50 percentage saturation of oxygen is more pronounced in intermediate and larger size groups. In comparison to *S.scripta*, *P.viridis* exhibited a higher oxygen consumption rate per mg dry wt. Oxygen consumption rate of *P.viridis* is found to be almost thrice than that observed in *S.scripta* in the smallest size group. Whereas in other two size groups (intermediate and large), oxygen consumption rate per mg dry weight of *P.viridis* is around 1.4-1.6 times greater than that of *S. scripta*.

3.4. DISCUSSION.

Bertalanffy (1957) has postulated three types of metabolism:

(1) Oxygen uptake rate proportional to surface area (`b' value is 0.67),

(2) Oxygen uptake rate proportional to weight (`b' valve is 1) and

(3) Oxygen uptake rate intermediate between surface area and weight proportionality (`b' value is 0.67 — 1).

The regression coefficient obtained at 100, 80 and 70 percentage saturation of oxygen for *P.viridis* comes in the third type of metabolism proposed by Bertalanffy (1957). The `b' value of *P.viridis* at 100, 80 and 70 percentage saturation of oxygen were found to be comparable with that of the results obtained by other workers on the same species. (Shafee, 1976, Mohan and Cheriyan, 1980). But the `b' value at 50 percentage saturation of oxygen is found to be less than that observed in other percentage saturation of oxygen. In bivalves, the `b' values are reported to vary from 0.24-0.95 under various environmental conditions (Ansell, 1973; Bayne *et al.*, 1973; Ganti *et al.*, 1975; Mane, 1975a; Mangapathi, *et al.*, 1975; Shafee, 1976; Salih, 1978; Mohan and Cheriyan, 1980).

In *S.scripta*, the `b' values at 100, 80 and 70

percentage saturation of oxygen goes well with the third type of metabolism *i.e.* the oxygen uptake rate intermediate between surface area and weight proportionality. At 50 percentage saturation, low 'b' value was obtained as in the case of *P.viridis*.

Both bivalves could control their oxygen consumption rate independent of the variation in oxygen tension till 50 percentage saturation of oxygen in the sea water. For both *S.scripta* and *P.viridis*, 50 percentage saturation of oxygen can be considered as critical oxygen tension because of the sharp decline in the oxygen consumption rate noticed at this level in all the size groups. The reduction is less obvious in the smallest size group. This may be due to their greater ability to maintain a higher ventilation rate in comparison to the other two size groups. The smaller size groups could maintain a high ventilation rate, which has been confirmed from the studies conducted on the copper toxicity of three different size groups of *S.scripta* (Thampuran, 1986),and also on the filtration rate of *Donax cunaetus* (Talikhedkar and Mane, 1977) wherein the highest accumulation of copper and highest filtration rate has been noticed in the smallest size groups of clams. Weight specific filtration rate showed a decreasing trend with increasing body weight in many bivalves (Fox *et al.*, 1937, Nagabhushanam, 1966; Wayne, 1972; Mane, 1975b; Krishnakumar, 1987; Supriya, 1992). Considering the various percentage saturation of oxygen (100, 80, 70), both *S.scripta*

and *P.viridis* are considered as good regulators since their physiological adaptations to decreasing partial pressure exhibits the characteristics of regulators.

Higher oxygen consumption rate (per mg dry wt) exhibited by *P.viridis* points out to a higher energy demand which make them more sensitive to hypoxic/anoxic stress than *S. scripta*. Whereas, *S.scripta* with its low oxygen consumption rate (per mg dry wt) indicates its reduced activity, demanding less energy. Thus, in comparison to mussels, they can be more tolerant to oxygen deficiency in the ambient medium. The differences shown in the oxygen consumption may be due to their dissimilar habitats *i.e. P.viridis* being an epifaunal and *S.scripta* an infaunal species.

It is evident that the metabolic adaptations of these two bivalves to regulate oxygen consumption rate at different oxygen tensions, enable them to carry on metabolic processes at a high rate till a critical level is reached, where they considerably reduce the energy demand, as indicated by the reduction in the oxygen consumption rate noticed at 50 percentage saturation of oxygen.

Variation of `b' values at various percentage saturation of oxygen

Table 3.1a *Sunetta scripta*

Percentage saturation of oxygen	b
100	0.9133
80	0.8948
70	0.7015
50	0.6652

Table 3.1b *Perna viridis*

Percentage saturation of oxygen	b
100	0.7814
80	0.6940
70	0.7589
50	0.4252

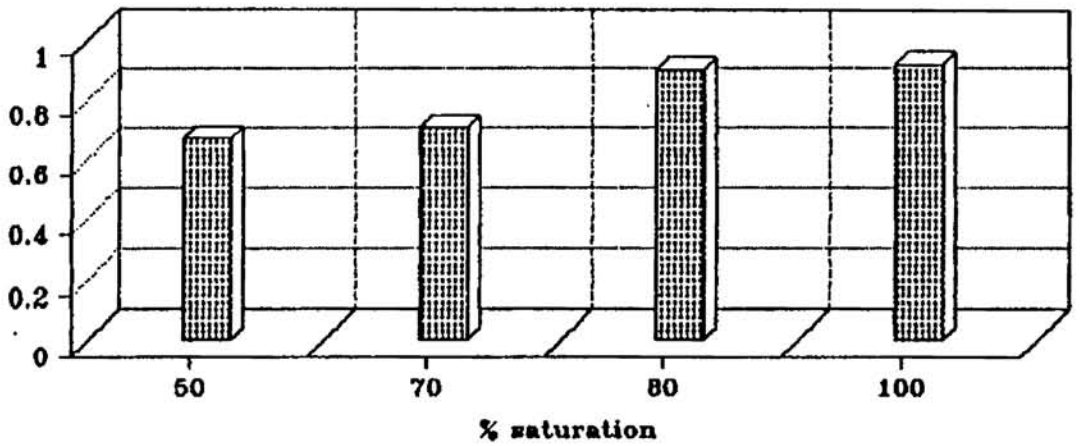

Fig. 3.1a *Sunetta scripta*

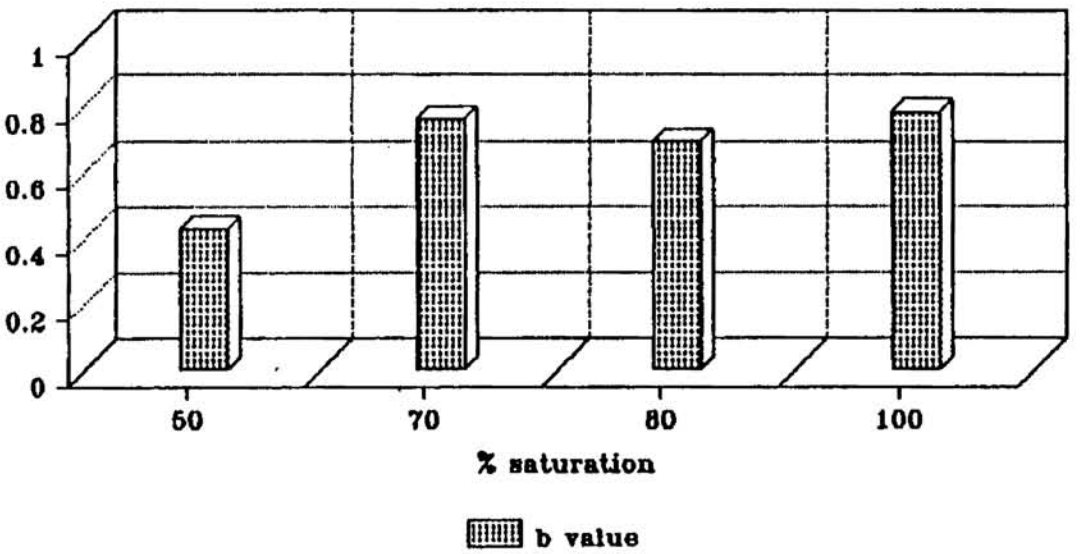

Fig. 3.1b *Perna viridis*

Variation in the O_2 consumption rate (ml $l^{-1}mg^{-1}$dry wt h^{-1}) at various percentage saturation of oxygen

Table 3.2a *Sunetta scripta* (100, 200 and 300 (mg dry wt))

Percentage saturation of oxygen	O_2 consumption rate		
	100mg	200mg	300mg
100	1.450	1.668	1.933
80	1.160	1.740	1.642
70	1.735	2.110	1.803
50	1.045	0.870	0.823

Table 3.2b *Perna viridis* (100, 250 and 400 (mg drywt))

Percentage saturation of oxygen	O_2 consumption rate		
	100mg	250mg	400mg
100	3.190	2.320	2.776
80	2.465	2.028	2.464
70	2.895	2.608	2.741
50	2.030	1.218	1.013

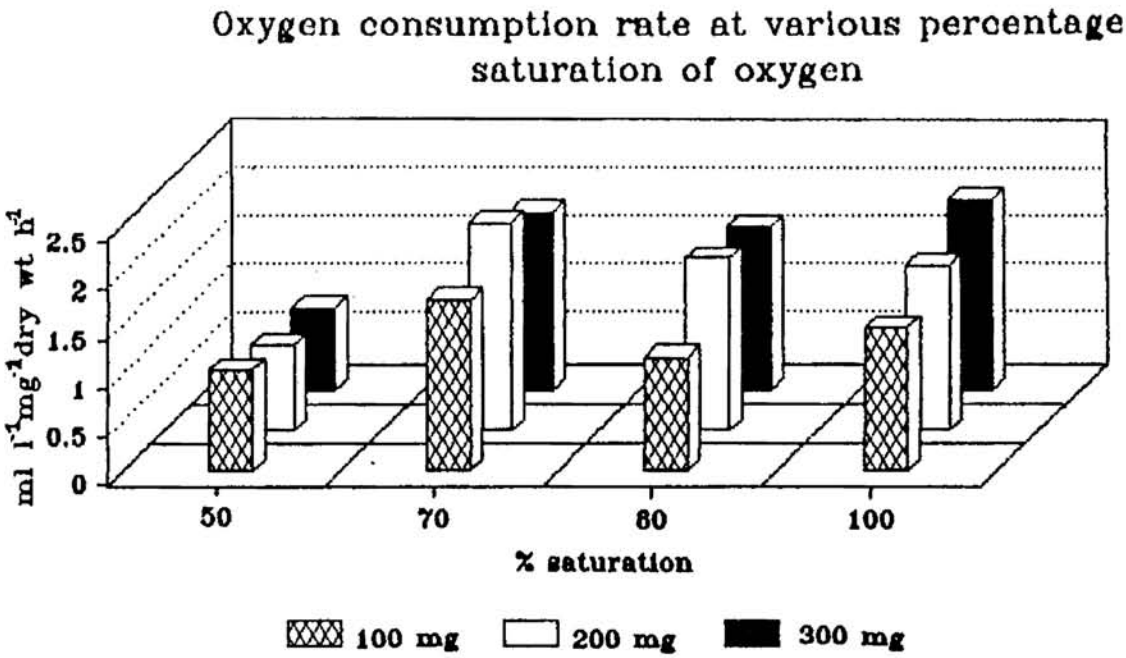

Fig. 3.2a *Sunetta scripta*

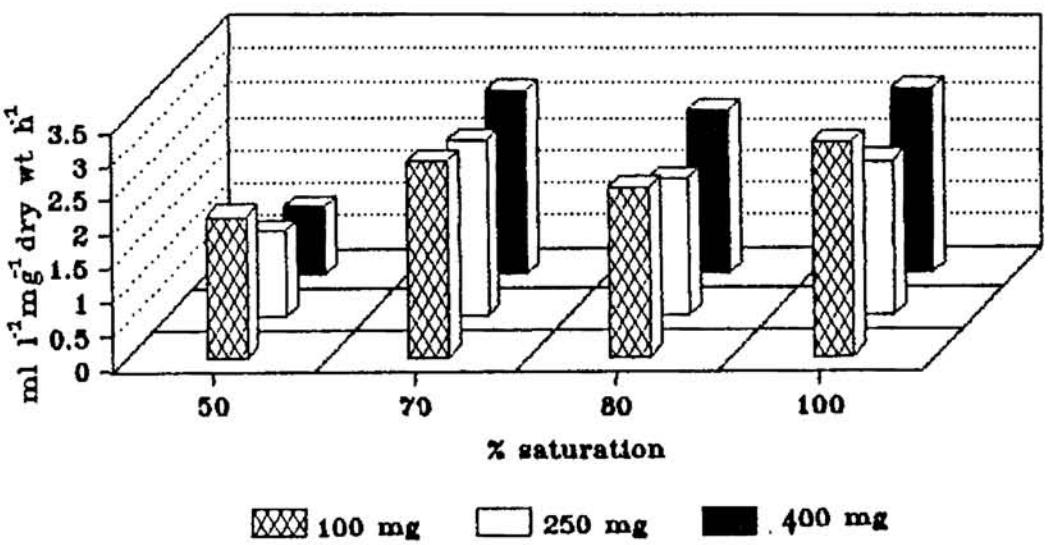

Fig. 3.2b *Perna viridis*

Chapter 4

CAROTENOIDS AND ANOXIC/HYPOXIC STRESS

4.1. INTRODUCTION.

Carotenoids are the most numerous widespread conspicuous biochromes of the terpene group to occur in nature. They are naturally occurring yellow, orange or red pigments of nitrogen-free fat soluble class, composed of carbon and hydrogen with minor proportions of oxygen. The carotenoids may be grouped into hydrocarbons *i.e.* the carotenes proper and their oxygen derivatives — alcohols, aldehydes, carboxylic acids and epoxides.

These pigments are biosynthesised *de novo* by all green plants and also by many achlorophyllous plants (Goodwin, 1952; Fox, 1983). Animals in general do not synthesize them *de novo* and those found in their bodies are either directly accumulated from food or partly modified through metabolic cycles. They are known to be indispensable cellular components in micro-organisms, algae, higher plants, animals and man (Goodwin, 1952; 1984; Karnaukhov, 1973a; Jensen, 1978; Karnaukhov, 1990).

In molluscs (bivalves and gastropods), the major carotenoid present is the hydrocarbon derivative, β carotene as well as lutein or a close analog, with lesser incidence of other carotenoids, characteristic of each species (Goodwin, 1952; 1984). Cephalopodan species have been found to yield no significant quantities of carotenoids from any of their tissues. In bivalves and gastropods, they are encountered mainly in nervous tissue and hepatopancreatic tissues, but they are also found in other organs such as gonads, integument, ripe ova, gills, mantle etc. It is xanthophyll, the oxidative derivative of carotene which is prominently represented in molluscs (Florkin and Scheer, 1972; Goodwin, 1984).

Investigation of the functional aspect of carotenoids in animal cells seemed to be very difficult. Not withstanding the rapid progress in chemistry the biological role of carotenoids in both animals and man is yet to be elucidated. The biological polyfunctionality of carotenoids and their ability to take part in the formation of various intracellular functional mechanism constitute a reason for the slow progress in understanding their role in the physiological activity of bacterial, plant and animal cells. The precise structural formulae of many of the carotenoids are not available nor the principal pathways of their metabolism in the body known.

It is generally accepted that all carotenoids have one trait in common *i.e.* the main portion of the carotenoid

molecules is formed by a chain of conjugated unsaturated bonds. It is this unique structure that accounts for their physico-chemical properties as electron acceptors or donors (Pullman and Pullman, 1963).

The interrelation between exergonic metabolic oxidation-reduction reactions and endergonic functional mechanisms of the single living cell is one of the most real and difficult problems. From the physiological mechanisms of the resistance of animal to low oxygen levels and toxic agents, it is evident that some molecular mechanisms do exist that provide energy to cells, mainly the nerve cells in hypoxic/ anoxic conditions. In fact, carotenoids can be considered to be a part of such a molecular mechanism (Karnaukhov, 1973a,b; 1990).

Many aquatic invertebrates, especially bivalves and snails are able to withstand periods of low oxygen tensions in their habitat (von Brand *et al.*,1950; Karnaukhov 1971b; Zs-Nagy 1971b; Hochachka and Mustafa, 1972; Zs-Nagy and Ermini, 1972 a,b; Hochachka and Somero, 1973; Hochachka, 1985; Gaede, 1987). Environmental hypoxia induces a variety of physiological compensations that constitute systemic adaptation, which rely critically on metabolic strategies directed towards sustained anaerobic function. This serves to increase the scope of activity and possibly to maintain the optimum delivery of oxygen to the cells.

Studies conducted by Karnaukhov, Zs-Nagy and their co-workers have affirmed that the carotenoids participate in the oxidative metabolism of animal cells, when the normal mitochondrial activity is inhibited (Karnaukhov, 1969; 1970; 1971a,b; 1979; 1990; Karnaukhov and Fedorov, 1977; Karnaukhov *et al.*, 1977; Zs-Nagy, 1971a, b; 1973; 1974; 1977). A higher carotenoid concentration is a characteristic of nervous tissue of molluscs (Karnaukhov *et al.*, 1977). Apparently, the carotenoid system of the intracellular accumulation of oxygen (or its electron acceptor equivalent) during anoxic stress condition is a privilege of tissues and cells which are more significant for the animal's survival.

Carotenoids play an important role in molluscan nerve cells in the mechanism of the animal's resistance to environmental pollution and reduced oxygen content in water (Karnaukhov *et al.*, 1977; Karnaukhov, 1979; 1990; Krishnakumar, 1987). It is shown that those molluscs having high carotenoid content in their tissues showed high resistance and survival capacity to environmental pollution than those having low carotenoid content (Karnaukhov *et al.*, 1977). As the pollution increases the carotenoid concentration in the tissues is found to be enhanced. Karnaukhov (1971 a,b; 1979) suggested the term carotenoxysome for the molluscan granules rich in carotenoids to emphasise its universal function in intracellular oxidative metabolism under hypoxic /anoxic condition (Karnaukhov, 1990).

They are considered as universal energy producing structures of cells, phylogenetically older than mitochondria.

A definite correlation between the degree of pigmentation in molluscan ganglia and anoxic tolerance reveals the endogenous anoxic oxidation mechanisms by cytosomes (Zs-Nagy, 1977). Pigmented cell inclusions of the central nervous system of *Mytilus edulis* and *Crepidula fornicata,* contain the different oxidised products of beta carotene serving as an oxygen reserve during anoxic condition. (Damerval, 1985). Species with less pigmented ganglia exhibit shorter anoxic survival, whereas those with unpigmented central nervous systems as in *Pecten jacobaeus* and *Octopus vulgaris* showed no tolerance at all (Zs-Nagy,1971a).

Carotenoxysomes/cytosomes have been reported to occur in organisms other than the molluscs. They exist in higher concentrations in the coelomic cells of many annelids under the name chloragosomes or as cytosomes in the nerve cells of leeches (Moment, 1974; Fischer, 1975). The obvious resistance of these organisms to heavy environmental pollution and pronounced oxygen deficiency is very much likely to be due to the presence of carotenoids in their cells (Karnaukhov, 1990). Fishes having maximum carotenoid concentration in their brain like *Cyprinus carpio* and *Misgurnus fossilis*, have been found to have the highest resistance to oxygen deficiency (Czeczuga, 1977; Karnaukhov, 1990).

A decrease in vitamin A reserve in the liver with corresponding increase of carotenoids in the brain has been noticed in animals during their adaptation to low oxygen deficiency in high altitude (Karnaukhov and Fedorov, 1976; 1977). This reveals the involvement of carotenoid in the adaptation of the animal cells to high altitude hypoxia. Lipofuscin granules which contain carotenoids are found to accumulate in the older cells. The carotenoids together with myoglobin can function as intracellular oxygen stock in the gradually developing tissue hypoxia which progresses with age (Karnaukhov, 1973b; 1990; Karnaukhov *et al.*, 1972).

The recent findings and the speculations regarding the functional aspect of carotenoxysomes or cytosomes paved the way for the main target of the present study *viz.* to understand the role of carotenoids in the anaerobic metabolism of some intertidal bivalves.

4.2. MATERIALS AND METHODS.

The details of the materials and methods are described in Chapter-2.

4.3. RESULTS .

Specimens of *S.scripta* (30-35mm) were exposed to air

for an interval of 1h to 6h (considering the tidal period) and the total carotenoid content was estimated at the respective interval of time (Table 4.1,Fig.4.1). It was noticed that the carotenoid concentration increased initially and subsequently the values levelled off to the control.

The valve movements were monitored in *S.scripta* during aerial exposure, at 30ppt salinity and also on reimmersion after the exposure. It has been observed that during aerial exposure, only a partial valve closure (Fig. 4.5a) is taking place in the species. At 30ppt salinity, an increased magnitude of the valve gape with wider periods of valve addductions have been observed (Fig. 4.5c, Table 4.5). During aerial exposure (Fig. 4.5a), the magnitude of the valve gape was found to be less, with the valve adductions of narrow periods than observed at 30ppt salinity (Table 4.5). Upon reimmmersion (Fig.4.5b) after aerial exposure, the magnitude of the valve gape increased with wider periods of valve adductions.

The valve movement was also studied in *P.viridis*. Here also, during aerial exposure (Fig.4.6a) less valve gaping with narrow periods of valve adductions has been observed. But in *P.viridis* (Table 4.6), the magnitude of the valve gape as well as the frequency of the valve adductions were more with wider periods than observed in *S.scripta*. At 33ppt salinity (Fig.4.6c), an increased valve gaping as well as frequency of

valve adductions have been noticed. Upon reimmmersion (Fig.4.6b), both the frequency of valve adductions as well as the magnitude of the gape increased, the pattern of behaviour being more or less similar to that at 33ppt salinity (Table 4.6).

When the clams (35-45mm) were subjected to total lack of atmospheric air by embedding them in modelling clay (Table 4.2, Fig.4.2),the total carotenoid concentration showed an increase only after a lapse of 4h. When these animals were further subjected to prolonged anoxic condition (Table 4.3,Fig.4.3), the total carotenoid concentration was found to be less than that of the control upto a period of 48h.

In both *S.scripta* (35-45mm) and *P.viridis* (40-50mm), the total glycogen and the total carotenoid concentration were monitored in decreasing partial pressures of oxygen for a period of 48h. It was noticed that there was an increase in the total carotenoid concentration along with a decline in the glycogen content of the body in both *S.scripta* (Table 4.4a, Fig. 4.4a) and *P.viridis* (Table 4.4b, Fig. 4.4b). There was only a limited change in the total carotenoid concentration at 48h than at 24h of exposure in both the species. In *P.viridis*, the glycogen content at 48h remained the same as that at 24h of exposure. When these animals were reimmersed in well aerated sea water for 24h after a period of experimental 48h, the total carotenoid concentration decreased and the glycogen values showed an increment.

4.4. DISCUSSION.

During aerial exposure, a sudden elevation in the total carotenoid concentration was observed after 1h. This increase can be attributed to the operation of the carotenoxysome pathway in response to the sudden anoxia created, which may not have sustained, as emphasised from the fall in the carotenoid concentration down to the control level after a lapse of time.

The recordings of the valve movements of both *S.scripta* and *P.viridis* during aerial exposure have shown that the animals do not close their valves completely during aerial exposure, but maintained a small gape in their mantle margins. The slight gape maintained during aerial exposure may aid in the permeation of oxygen into the mantle water which may be at a low oxygen tension. Valve adductions to utilize atmospheric oxygen during exposure to low tides have been reported in bivalves like *Mytilus edulis, M.demissus, M.californianus, M.galloprovincialis,* and *Cardium edule* (Moon and Pritchard, 1970; Coleman and Trueman, 1971; Coleman, 1973; Bayne *et al.*, 1976b; Bayne and Livingstone, 1977; Widdows *et al.*, 1979).

To cut off totally from any contact with oxygen, *S.scripta* were individually covered in clay balls and subjected to short term anoxic condition. No carotenoid increase has

been noticed except a small increment after 4h. In order to find out whether any carotenoid increase was noticed during long term anoxia, the experiment was extended to 48h under similar experimental anoxic condition. Here also, hardly any increase in the carotenoid concentration has been noticed. Since the carotenoid increment did not persist on after the peak at 4h, it can be assumed that the carotenoxysome pathway is not in function in *S.scripta* when they are totally cut off from the external environment. In all these cases it is reasonable to believe that the carotenoxysome pathway does not function as a routine in the anaerobic respiration of intertidal clams.

It has been reported by the previous workers (Zs-Nagy, 1977; Zwaan, 1983) that the carotenoxysome pathway does not function during short term anoxia, but is functionable only in gradually developing anoxic condition. So both *S.scripta* and *P.viridis* were subjected to gradually developing hypoxic stress and the glycogen and total carotenoid were monitored for a period of 48h. After 48h of exposure, the animals were reimmersed in well aerated sea water for 24h and again both glycogen and total carotenoid concentration were estimated.

In both *S.scripta* and *P.viridis*,an increase in the total carotenoid concentration has been noticed during gradually developing hypoxic condition. The carotenoid increase is found to be more in *P.viridis* (87% increase than

that of the control) in comparison to *S.scripta* (40% increase than that of the control). It was noticed that the oxygen consumption rate (per mg dry wt) is found to be higher in *P. viridis* when compared to *S. scripta* (Chapter-3, Fig. 3.2a and Fig. 3.2b). Thus, it is likely that the mussels, being more sensitive to oxygen deficiency than clams, depend much on the internal oxygen stock resulting in a higher carotenoid accumulation noticed in mussels during hypoxic stress.

Glycogen depletion along with the carotenoid elevation in both *P.viridis* and *S.scripta* is in accordance with that observed in *Villorita* sp. exposed to heavy metals (Sathyanathan *et al.*, 1988). The results indicate that the animals are depending on anaerobic glycolysis as well as carotenoxysome/cytosome endogenous oxidation pathway. These pathways could very well afford the animals to survive anoxic conditions since the bivalves could reduce their basal metabolic rate very much (as much as 5% of the aerobic level) (Zwaan, 1983). During stress, the bivalves close their valves tightly, switching over to anaerobic pathway of respiration. Utilisation of glycogen during aerial exposure and anaerobic conditions has been reported in many instances (Zwaan and Zandee, 1972; Gabbott and Bayne, 1973; Zwaan, 1983; Gaede, 1983; 1987; Akberali and Trueman, 1985; Lakshmanan and Nambisan, 1985; Oeschger, 1990). The glycogen content at 48h of exposure remained the same as that at 24h of hypoxia in *P.viridis*. Substantially a very little decrease in the glycogen content

has been noticed in *S.scripta* at 48h than at 24h of hypoxic exposure.

In both the species,the total carotenoid concentration showed a decrease after reimmersion. This may be due to the oxygenation of the unsaturated double bonds of carotenoids. This increased oxygen consumption upon reimmersion is well supported by the increased rate of valve adductions and valve gape observed in both the species upon reimmersion. Increased glycogen noticed after reimmersion can be due to the activity of reverse glycolytic pathway resulting in its re-establishment to the control values when animals were returned to aerobic condition. This re-establishment in the glycogen content is reported in *Patella caerulea* on return to aerobic condition after prolonged anoxia (Lazou *et al.*, 1989).

The monitoring of the carotenoid increment and the glycogen content decrease under gradually developing hypoxic condition indicated that both anaerobic glycolysis and carotenoxysomal pathways may be in operation simultaneously in *S.scripta* and *P.viridis* under similar natural situations.

Table 4.1 Total carotenoid : Hypoxic condition developed by keeping clams (*Sunetta scripta* 30-35mm) out of water for an interval of 1h to 6h

Exposure time (h)	Total carotenoid mg $100g^{-1}$ wet wt
0	0.3203 ± 0.018
1	0.9929 ± 0.241
2	0.7086 ± 0.051
3	0.2906 ± 0.022
4	0.2881 ± 0.046
5	0.4093 ± 0.067
6	0.4330 ± 0.038

Table 4.2 Total carotenoid : Anoxic condition developed by embedding individual clams (*Sunetta scripta* 35-45mm) in separate balls of modelling clay for an interval of 1h to 6h

Exposure time (h)	Total carotenoid mg $100g^{-1}$ wet wt
0	0.0759 ± 0.009
1	0.0861 ± 0.004
2	0.0721 ± 0.011
3	0. 1015 ± 0.001
4	0.0841 ± 0.009
5	0.1885 ± 0.006
6	0.1339 ± 0.015

Table 4.3 Total carotenoid during long term anoxia when the clams (*Sunetta scripta* 35-45mm) were kept outside water embedded individually in separate clay balls

Exposure time (h)	Total carotenoid mg $100g^{-1}$ wet wt
0	0.1723 ± 0.066
12	0.1143 ± 0.024
24	0.1044 ± 0.015
48	0.1389 ± 0.046

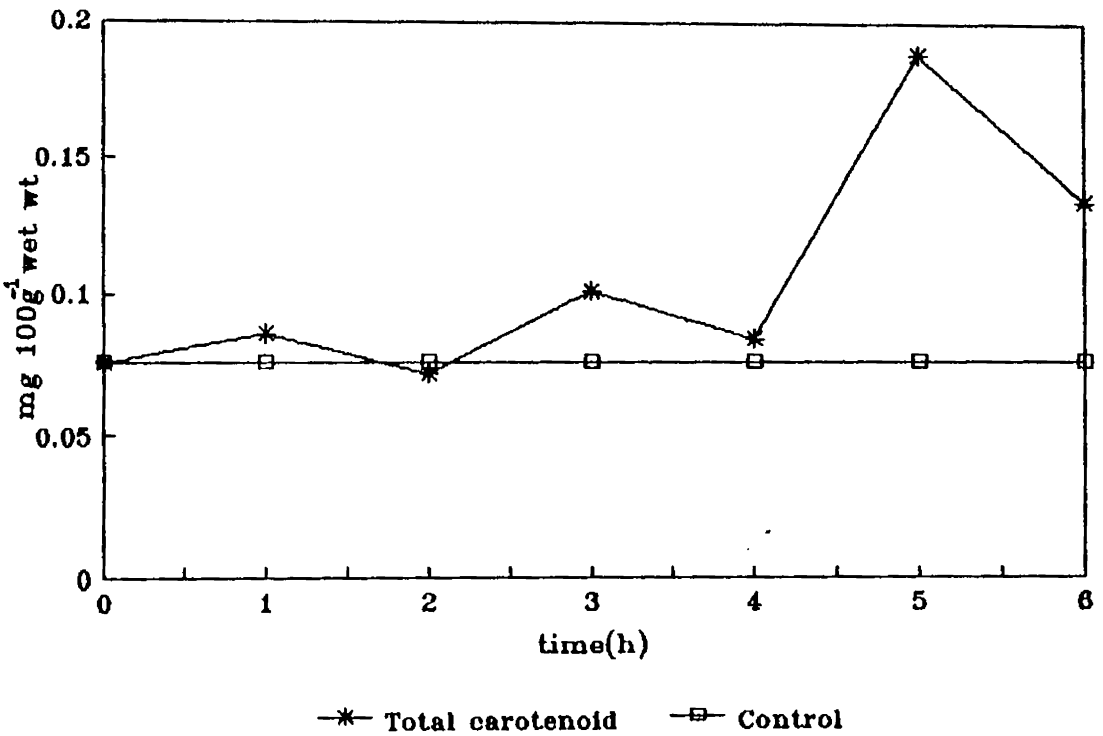

Fig. 4.2 Total carotenoid: Anoxic condition developed by embedding individual clams (*Sunetta scripta* 35–45mm) in separate balls of modelling clay for an interval of 1h to 6h

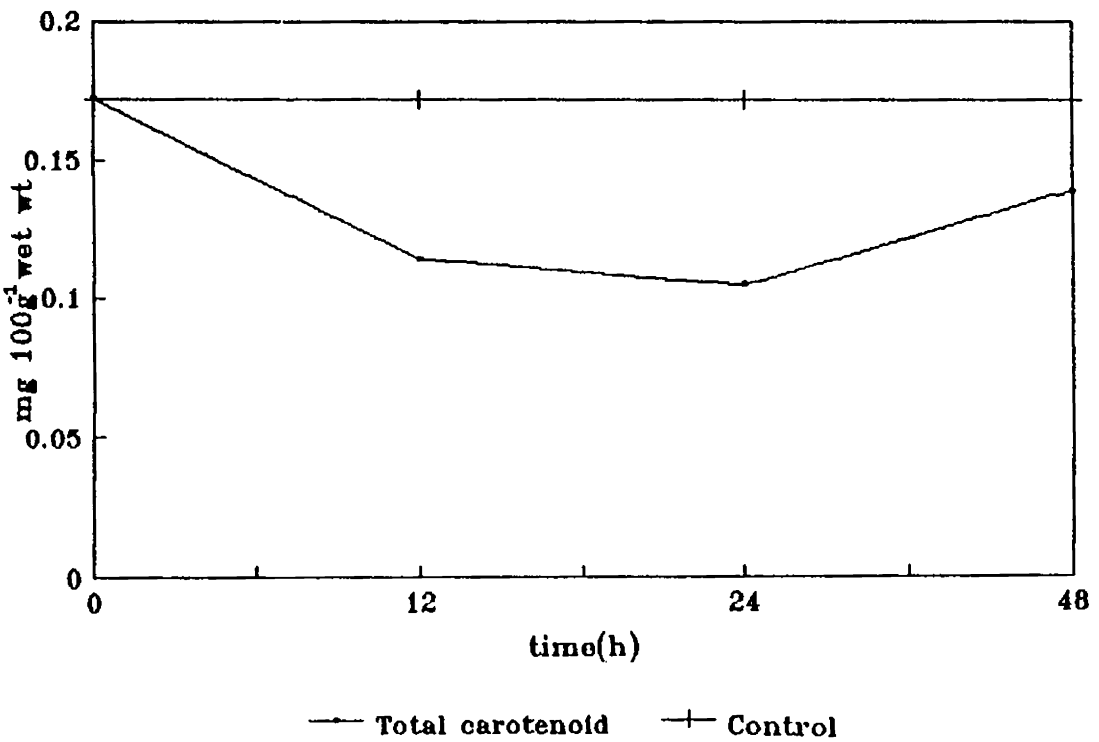

Fig. 4.3 Total carotenoid during long term anoxia when *Sunetta scripta* (35–45mm) were kept outside water embedded individually in separate clay balls

Total carotenoid and Glycogen content during hypoxic condition

Table 4.4a

Sunetta scripta (35-45mm)		
Exposure time (h)	Total carotenoid mg $100g^{-1}$ wet wt	Glycogen mg g^{-1}
0	0.0559 ± 0.005	35.32 ± 1.750
24	0.0862 ± 0.006	26.49 ± 5.150
48	0.0788 ± 0.004	16.11 ± 1.084
after reimmersion	0.0549 ± 0.003	34.08 ± 5.840

Table 4.4b

Perna viridis (40-50mm)		
Exposure time (h)	Total carotenoid mg $100g^{-1}$ wet wt	Glycogen mg g^{-1}
0	0.1019 ± 0.002	189.59 ± 33.63
24	0.1863 ± 0.001	98.37 ± 20.10
48	0.1903 ± 0.008	98.27 ± 9.49
after reimmersion	0.1488 ± 0.001	197.39 ± 7.27

Total carotenoid and Glycogen concentration

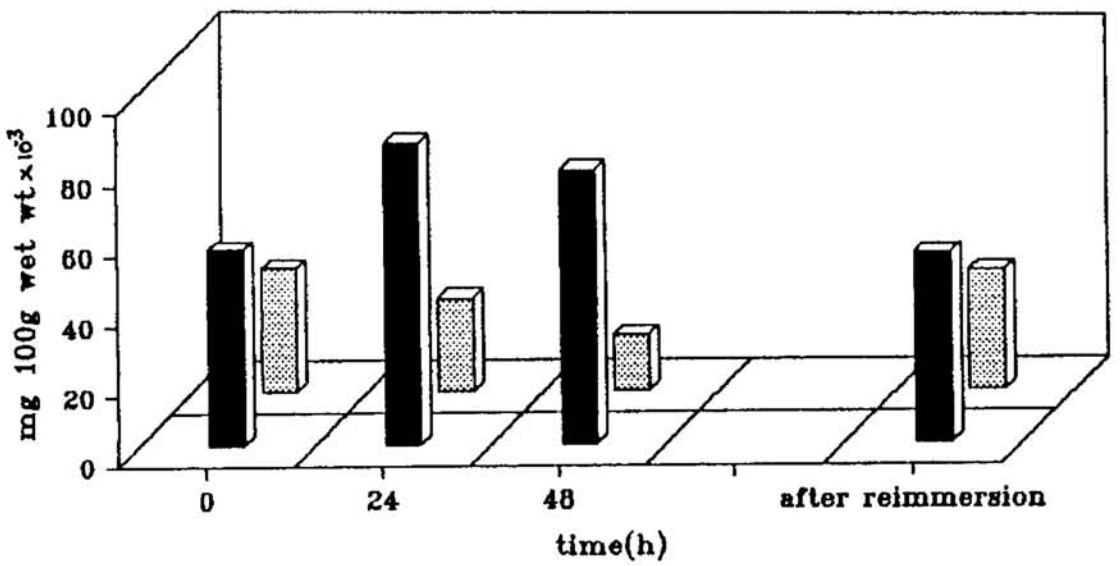

Fig.4.4a *Sunetta scripta* (35–45mm)

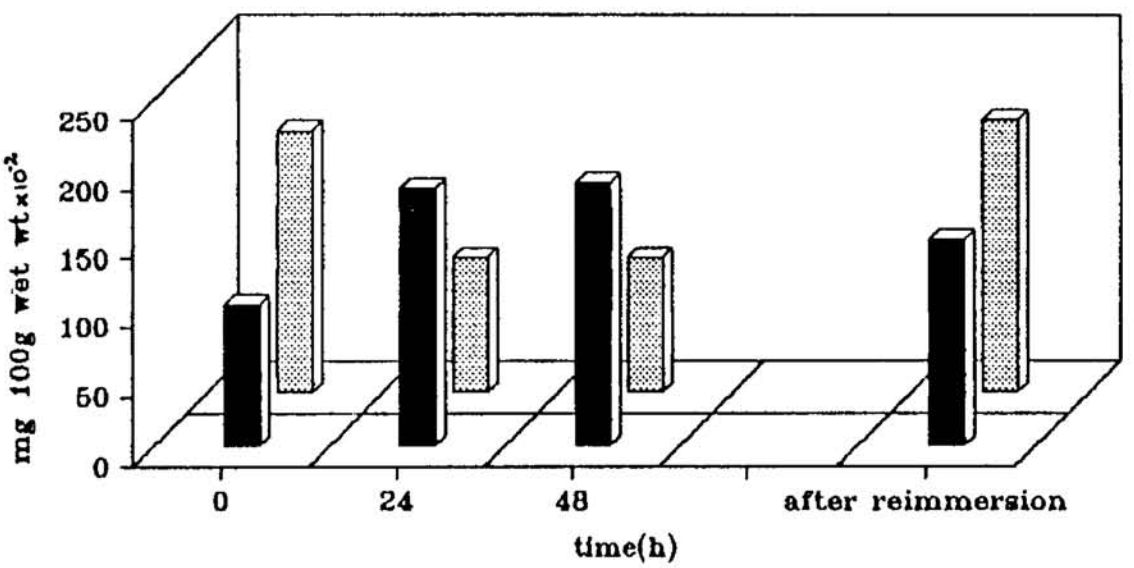

Fig.4.4b *Perna viridis* (40–50mm)

Valve Movements

Table 4.5

Sunetta scripta (35-45mm)		
Type of exposure	Frequency approx. (moments/minute)	Magnitude of the valve gape approx. (mm)
Aerial	4.67 ± 0.58	0.64 ± 0.04
After reimmersion	4.33 ± 0.29	1.33 ± 0.32
30ppt Salinity	5.67 ± 0.29	1.44 ± 0.31

Table 4.6

Perna viridis (55-65mm)		
Type of exposure	Frequency approx. (moments/minute)	Magnitude of the valve gape approx. (mm)
Aerial	4.50 ± 0.50	0.75 ± 0.10
After reimmersion	4.83 ± 0.29	2.50 ± 0.07
33ppt Salinity	2.63 ± 0.25	2.31 ± 0.09

Shell valve movements of *Sunetta scripta* (35–45mm)

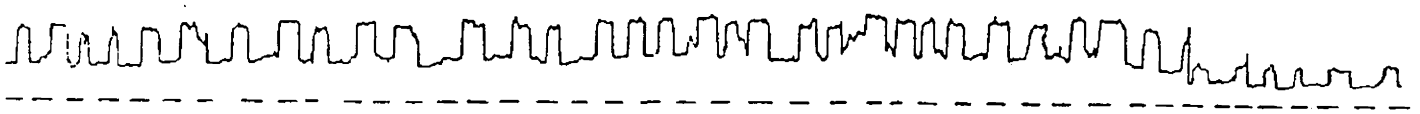

Fig.4.5a Aerial exposure

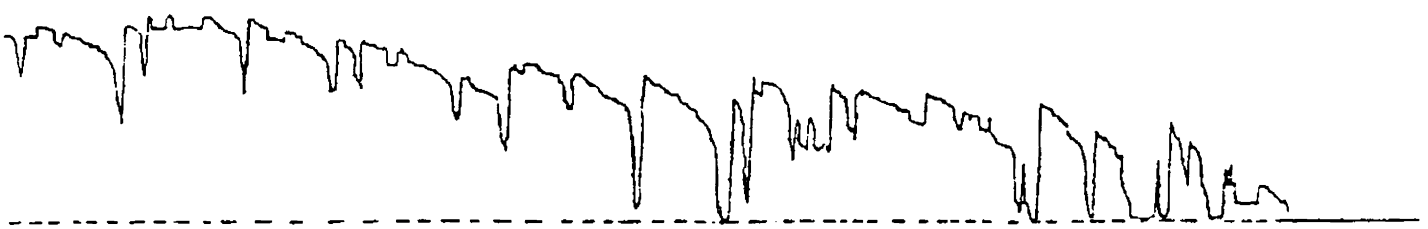

Fig. 4.5b Reimmersion after exposure

Fig.4.5c 30ppt Salinity

Shell valve movements of *Perna viridis* (55–65mm)

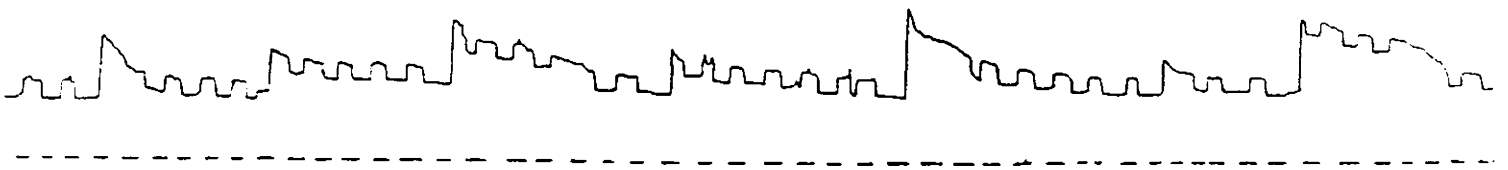

Fig. 4.6 a Aerial exposure

Fig.4.6 b Reimmersion after exposure

Fig. 4.6 c 33ppt Salinity

Chapter 5

EFFECT OF AMBIENT OXYGEN CONCENTRATION ON LIPOFUSCIN ACCUMULATION

5.1. INTRODUCTION .

Most of the cell types in the body of the multicellular organism exhibited an age-associated increase in the content of a special organelle, which has a variety of synonyms including age pigment, chromolipid, lipopigment or lipofuscin. Their abundance are linked to the organism's age or to the senescence of a cell. Not only normal ageing, but a variety of dietary and toxic factors or other miscellaneous environmental stresses can induce an excess accumulation of lipofuscin granules (Aloj and Pisanti, 1985; Aloj *et al.*, 1986a,b; Sohal and Wolfe,1986).

Lipofuscin granules are recognized by their specific distribution,in the cytoplasm of certain cells, notably non-mitotic, non-renewable tissues like muscle or nervous tissue (Lamb, 1977). They are also present in other cells such as spleen, pancreas, thymus, gonads, hepatocytes and kidneys.

In the interrenal cells of fishes, lipofuscin pigment granules were found to reveal conspicuous accumulations with advanced age (Mikio , 1986). Even in fungi, both marine and

terrestrial, lipofuscin granules are considered as a characteristic ageing product. In fact the increase of the pigment in the cell is proportional to the development of fungus (Aloj *et al.*, 1986a).

On the basis of current information, lipofuscin granule can be defined as a membrane bound lysosomal organelle which contains lipochrome moieties, exhibit yellow to brown coloration, emits yellow greenish autofluorescence under ultra-violet and accumulation in the cytoplasm progressively with age under normal physiological conditions (Sohal and Wolfe, 1986).

This yellow pigment granules functionally represents the changing modes of cells in response to changing biological requirements as they mature, with no serious adverse effects to the life of the cells (Hack, 1981). Lipofuscinogenesis is a dynamic process, during which morphology and chemical composition of the organelle undergoes progressive modification. Furthermore, the composition of the granule may vary in different cell types and under different dietary and physiological conditions (Sohal and Wolfe, 1986).

The progressive accumulation of lipofuscin granules with age is considered to be an indication of the degree of cellular senescence. It also reflects a state of cellular traumatisation, resulting from an elevated oxidative level

related to the presence of free radicals, particularly superoxide radical (Glees and Hasan, 1976; Sohal and Wolfe, 1986) generated during univalent reduction of oxygen.

Thus lipofuscin accumulation could have ultimately been caused by the inefficiency of certain biochemical mechanisms which manifest gradually during the ageing process. Biochemical studies suggest that the granular concentration is the end result of the peroxidation of polyunsaturated membrane lipids by free radicals derived from partially degraded mitochondria and other organelles present in secondary lysosomes (Chio and Tappel, 1969).

Rate of lipofuscin formation have been shown to respond to certain pathological and physiological conditions. Three different factors seem to affect the rate of its formation:

1. an increase in functional activity,
2. oxidative stress and
3. a disturbance or inadequacy of lysosomal function (Sohal and Wolfe, 1986).

Metabolic rate, lipofuscin formation and ageing are linked together probably by oxygen free radicals (Sohal and Wolfe, 1986). As a result, the granular accumulation can be considered as a function of physiological age (Sohal, 1981) or as indicators of the results of cell damage by anoxia (Lamb,

1977) rather than strict chronological age. Factors affecting the metabolic rate such as temperature, calorific intake (starvation) and activity level (hypoxia) were shown to induce lipofuscinogenesis (Sohal, 1981).

Accumulation of lipofuscin granules in relation to the activity level of cell is evident by the increase in their frequency and size in cultured rat cardiac myocytes in direct relation to ambient oxygen concentration and age of the culture (Sohal *et al.*, 1989). Studies conducted on the nervous ganglia of the mussel, *Mytilus edulis* subjected to experimental anoxia also revealed the cytochemical changes in the pigment inclusions, which become lamellar bodies after 24h of hypoxia (Damerval, 1987; Damerval and Prunus, 1989). Vitamin E deficiency (antioxidant deficiency) seems to increase the rate of lipofuscin accumulation and increased free radical action on unsaturated lipids (Lamb, 1977).

Studies by Karnaukhov *et al.* (1972) have confirmed that carotenoids are a component of lipofuscin granules. Their accumulation with age is the result of cells's adjustment to the deficiency of tissue oxygen caused by impaired permeability of blood vessels for oxygen which progressively increases with age (Karnaukhov, 1969; 1972; 1973b; 1990; Karnaukhov *et al.*, 1972).

Presence of carotenoids, myoglobin and oxidative

enzymes in the lipofuscin granule suggest that they can provide energy requirements for the cells under conditions of low rate of oxygen penetration into tissues (Karnaukhov *et al.*, 1972). The carotenoids together with myoglobin appear to function as intracellular oxygen stock, whereas the oxidative enzymes seem to be components of a specific system for terminal oxidation (Karnaukhov,1973b). This specific system for terminal oxidation functions only when the normal mitochondrial system is inhibited (Karnaukhov, 1973a).

The terminal electron acceptor for this system is either oxygenated carotenoid or oxygen from the intracellular stock. Carotenoids being good electron acceptor and electron donor can provide such a possibility (Pullman and Pullman, 1963 ; Dingle and Lucy, 1965). It can serve as an intrinsic cysotosmal electron acceptor substances (which is activated during conversion process) substituting the electron acceptor function of molecular oxygen for a considerable time. This would maintain the ATP regenerating process even in anoxia (Zs-Nagy, 1971a; Zs - Nagy and Ermini, 1972a,b).

The energy providing mechanism of lipofuscin granule during hypoxic condition is supported by the observation of the increase in strontium ion accumulation in cytosomes and decrease activity in mitochondria of molluscoid neurons during anaerobiosis (Zs-Nagy, 1971a). In aerobic condition, the situation is reversed —— the mitochondrial energy production

being predominant in neurons,while cytosomes showed only little activity.

Based on the background literature, it is reasonable to assume that along with the carotenoid increase, an enhancement of lipofuscin accumulation takes place during hypoxic exposure. Thus the aim of the present study being : (i) to assess the impact of hypoxia at the cellular level in intertidal molluscs, using lipofuscin granules as the physiological index of cellular oxygen stress and (ii) to set up a relation between lipofuscin granules and carotenoid concentration thereby taking these parameters as indices of oxygen stress condition.

5.2.MATERIALS AND METHODS.

Materials and methods are described in Chapter-2.

5.3. RESULTS .

When *S.scripta* is exposed to hypoxic stress, a significant difference ($P<0.01$) in the total carotenoid concentration between control (0h) and 24h hypoxia has been noticed statistically. The mean carotenoid concentrations of 24h and 48h hypoxic exposed animals were significantly higher than that of the control(0h) animals. Between 24h hypoxia and 48h hypoxia, substantially very little difference in the total

carotenoid concentration has been noticed. The differences in the total carotenoid concentration between control (0h), 24h and 48h hypoxic exposed groups of *S.scripta* were compared and the results have been presented in Table 5.1a.

P.viridis, when subjected to hypoxic stress condition, showed an increased total carotenoid concentration at 24h hypoxia. A significant difference ($P<0.01$) in the total carotenoid concentration has been noticed between control(0h) and 24h and 48h hypoxia. There is hardly any significant difference in the total carotenoid concentration between 24h and 48h hypoxic exposed animals. The results have been presented in Table 5.2a.

When both *S.scripta* (Table 5.1b) and *P.viridis* (Table 5.2b) were reimmmersed for 24h in well aerated sea water after 48h of hypoxic exposure, the total carotenoid concentration was found to decrease markedly ($P<0.01$) from that of 24h and 48h hypoxic values. The significant difference in the total carotenoid concentration between the control (0h) and the reimmersed animals was tested statistically by employing student's t test. In *S. scripta*, the t value calculated was not significant at 5% level, indicating that upon reimmersion, the total carotenoid concentration decreased to that of the control (0h) values. But in *P.viridis*, eventhough a reduction in the total carotenoid concentration occurs upon reimmersion, the value was found to be significantly higher ($P<0.05$) than that

of the control (0h) value. The mean carotenoid concentration of the control (0h), 24h hypoxia, 48h hypoxia and reimmersed groups of *S.scripta* and *P.viridis* were graphically represented in Fig 5.1a and Fig 5.1b respectively.

The morphological study of the lipofuscin granules present in the hepatopancreatic cells of the animals subjected to experimental hypoxic condition were carried out in both *P. viridis* (40-50mm)(Fig 5.3a-5.3d) and *S.scripta* (35-45mm)(Fig 5.2a-5.2d) The characteristic parameters of lipofuscin granules of the control(0h), 24h hypoxia, 48h hypoxia and reimmersed groups(after 48h hypoxic exposure) have been presented in Table 5.3a and 5.3b for *S.scripta* and *P.viridis* respectively.

In both the control (Fig 5.2a and 5.3a) as well as in reimmersed animals (Fig 5.2d and 5.3d), the lipofuscin granules are found to be scattered in the cytoplasm. Whereas, in both species, at 24h (Fig 5.2b and 5.3b) and 48h (Fig 5.2c and 5.3c) of hypoxia, the pigment is found to be collected in cytoplasmic aggregates. In *S. scripta* an increase in the granular size has been observed with the advancement of hypoxic exposure time, the size being largest at 48h of hypoxic exposure (Fig 5.2c). After reimmersion (Fig 5.2d),granular size became smaller than that of the control (0h). In *S.scripta*, eventhough a slight difference in the granular number between periods have been noticed, statistically there is hardly any significant

difference between control (0h), 24h hypoxia, 48h hypoxia and reimmersed groups (Table 5.4b). Table 5.4a represents the mean differences in the number of lipofuscin granules /unit area of the control (0h), 24h hypoxia, 48h hypoxia and reimmersed group.

In the case of *P. viridis* (Fig 5.3a - 5.3d), the granular size remains same for the control (0h), 24h hypoxia, 48h hypoxia and reimmersed group. With the progress of hypoxic exposure time the number of granules as well as its clustering increases. At reimmersion,the number as well as the aggregative characteristics decreases (Fig 5.3d). *P.viridis*, in contrast to *S. scripta*, showed significant differences in the granular number between periods at 1% level (Table 5.5b). The mean lipofuscin granules/unit area of the control (0h), 24h hypoxia, 48h hypoxia and reimmersed groups were 1.916, 10, 6.625 and 1.625 respectively. The least significant difference between periods at 5% is 4.11. No significant difference in the granular number has been noticed between the control (0h) and reimmersed groups and also between 24h and 48h of hypoxic exposure. The mean differences in the number of lipofuscin granules/unit area of the control (0h), 24h hypoxia, 48h hypoxia and reimmersed group of *P.viridis* have been statistically presented in Table 5.5a.

5.4.DISCUSSION.

Experimental data showed a definite correlation between the degree of pigmentation and hypoxic exposure time in both *S.scripta* and *P.viridis*. It is evident from the microphotographs that, as the time of hypoxic exposure advances, an increase in the lipofuscin accumulation in the hepatopancreatic tissues has been observed. The carotenoid concentration estimated in the whole tissue was also found to be increasing with the advancement of hypoxic exposure time, but is more prominent in *P.viridis* rather than *S.scripta*. In both species, the increase in the lipofuscin accumulation and carotenoid concentration were noticed only at 24h hypoxia with no substantial change in experiments extended up to 48h.

It has been reported that prolonged hypoxia/anoxia induces structural changes in the cytosomes of *Anodonta cygnea* (Zs-Nagy, 1977) and cytological changes in the pigmented inclusions in the nervous ganglia of mussels (Damerval, 1987; Damerval and Prunus, 1989). Histochemical studies of the pigmented inclusions show that they are protein bound carotenoids, most of them are different oxidised products of β carotene and may serve as an oxygen reserve during hypoxia (Damerval, 1985).

In both the animals, marked morphological difference from that of the control (0h) has been noticed in lipofuscin

granules at 24h hypoxia and 48h hypoxia. Conspicuous large heterogenous granulation has been observed only in *S.scripta* at 48h of hypoxic exposure. This may be due to the high clustering activity of the granules. A similar transformation in cytosomal structure during prolonged anoxia has been reported (Zs-Nagy and Borovyagin, 1972; Zs-Nagy, 1977). In *Mytilus edulis*, after 24h of anoxia, mitochondrial degradation has been reported and the pigmented inclusions becomes lamellar bodies (Damerval and Prunus, 1989). Prolonged exposure to anoxic conditions also increases the frequency of lamellar type cytosomes (Paparo, 1985).

After reimmersion, *P.viridis* showed a marked decrease in the number of granules in the hepatopancreatic tissues, whereas in *S.scripta*,the granular size become reduced. An increased frequency of "transforming" mitochondria has been observed in *Anodonta cygnea* during reoxygenation after prolonged anoxia (Zs-Nagy, 1977). This phenomenon exhibited in the present study could be due to a sort of regeneration taking place leading to the formation of precytosomes/ precarotenoxysomes as reported in *A.cygnea* (Zs-Nagy, 1977). It has been suggested that the reorganization of cytosomes/carotenoxysomes probably takes place when oxygen supply is sufficient thereby acting as a reserve, until it is used up during subsequent oxygen shortage (Karnaukhov, 1971a, b; 1990; Karnaukhov *et al.*, 1972). This proposal goes well with the present data and observations.

In *S.scripta*, the control(0h) total carotenoid concentration coincides well with that of the reimmersed value. But in *P.viridis*, even though a marked decrease in carotenoid content has been noticed after reimmersion, it is not coming upto the level of the control (0h) even after 24h of well aeration. This may be due to the high carotenoid build up noticed in *P.viridis*, when compared to *S. scripta* during hypoxic exposure. *P.viridis* also differs from *S.scripta*, with regard to the steady size of the lipofuscin granules of non-exposed, exposed and reimmersed groups, as well as with high carotenoid concentration than the control(0h), prevailing even after reimmersion for 24 h. One of the reasons for this may be due to the differences in their mode of habitat. *S.scripta*, being infaunal is living in a habitat where hypoxic/anoxic environment often occurs enabling them to have a high anaerobic tolerance capacity. The oxygen consumption rate (per mg dry wt of tissue) has shown that, it is higher in *P.viridis* than in *S.scripta*. (Chapter-3). Thus, *P.viridis*, because of its less anaerobic tolerance capacity and high oxygen consumption rate relies more on the intracellular oxygen depot of carotenoxysomes, resulting in enhanced carotenoid build up than *S.scripta*. This may be the reason for the prolonged time needed for *P.viridis* to resume to the control (0h) carotenoid concentration level in comparison to *S.scripta*.

It has been shown by Karnaukhov and co-workers that

carotenoids participate in the formation of lipofuscin granules (Karnaukhov, 1972; 1973b; 1990; Karnaukhov *et al.*, 1972). As a result, an increase in the pigment granules would be synchronous with that of the increment of carotenoid concentration as observed in the present study.

In conclusion it can be suggested that the lipofuscin accumulation can be considered as a physiological index of oxygen stress at the cellular level. This has been supported by the study on lipofuscin accumulation in cultured rat heart myocytes due to oxygen stress (Sohal *et al.*, 1989).

Hypoxia induced variations in total carotenoid concentration

able 5.1a *Sunetta scripta* (35-45mm)

Exposure time (h)	Mean value	Source of variation	Sum of squares	Degrees of freedom	Estimated variance	Variance ratio=F
0	0.0559	Total	22.21	11		
24	0.0862	Periods	20.05	2	10.025	**
48	0.0788	Error	2.16	9	0.24	41.77

** - P<0.01

able 5.1b *Sunetta scripta* (35-45mm)

Exposure time (h)	Mean value	Source of variation	Sum of squares	Degrees of freedom	Estimated variance	Variance ratio=F
24	0.0862	Total	23.13	11		
48	0.0788	Periods	21.35	2	10.675	**
Reimmersed after exposure	0.0549	Error	1.78	9	0.198	53.91

** - P<0.01

t value between control and reimmersed = 0.3435

Hypoxia induced variations in total carotenoid concentration

able 5.2a *Perna viridis* (40-50mm)

Exposure time (h)	Mean value	Source of variation	Sum of squares	Degrees of freedom	Estimated variance	Variance ratio=F
0	0.1019	Total	10.10	5		
24	0.1863	Periods	9.95	2	4.975	**
48	0.1903	Error	0.15	3	0.05	99.5

** - P<0.01

able 5.2b *Perna viridis* (40-50mm)

Exposure time (h)	Mean value	Source of variation	Sum of squares	Degrees of freedom	Estimated variance	Variance ratio=F
24	0.1863	Total	22.00	5		
48	0.1903	Periods	21.25	2	10.625	**
Reimmersed after exposure	0.1488	Error	0.75	3	0.25	42.5

** - P<0.01

t value between control and reimmersed = 133.86

Total carotenoid concentration during hypoxic exposure and after reimmersion

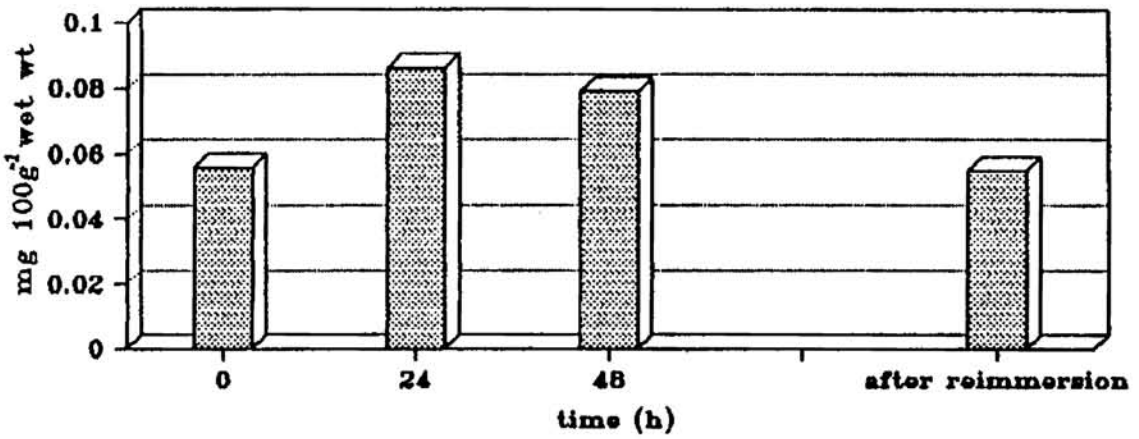

Fig. 5.1a *Sunetta scripta* (35–45mm)

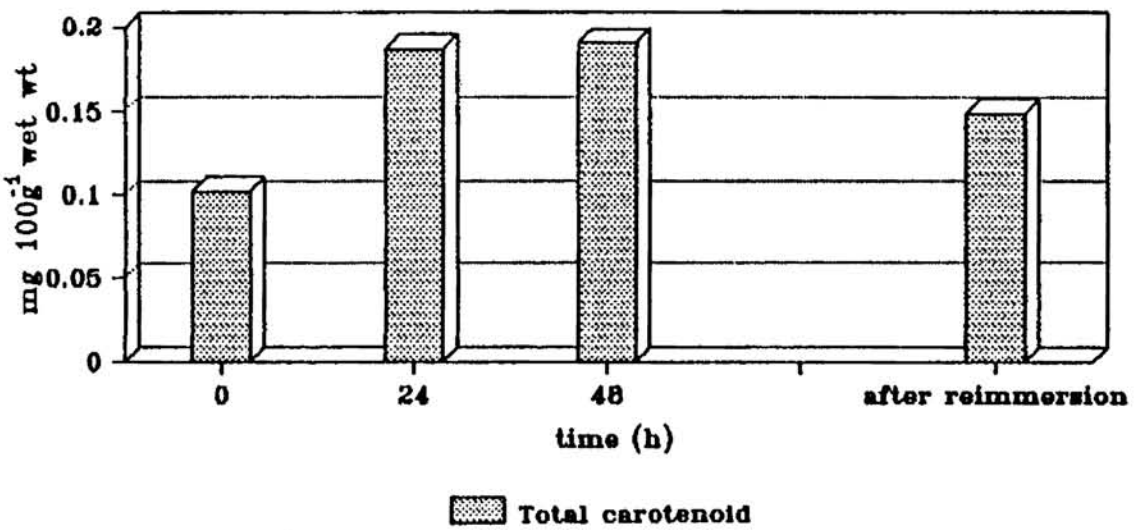

Fig. 5.1b *Perna viridis* (40–50mm)

Characteristics of lipofuscin granules in control and hypoxic exposed animals.

Table 5.3a *Sunetta scripta* (35-45mm).

Hypoxic exposure time (h)	Configuration	Distribution
0	Small spherical granules, fewer in number less pigmentation with non-aggregation.	Granules scattered in the cytoplasm.
24	Granular size increases. Heterogenous granulations due to clustering.	Mainly located as groups in the cytoplasm.
48	Intensively pigmented; increased size of the granules due to high heterogenous aggregation.	Large clusters of granules of various sizes were observed.
Reimmersed after 48h hypoxia	Very fine non - aggregative granules smaller than that of the control were noticed	Very fine granules scattered in the cytoplasm.

Contd...

Table 5.3b *Perna viridis* (40-50mm)

Hypoxic exposure time (h)	Configuration	Distribution
0	Small spherical bodies fewer in number with no aggregation	Granules scattered in the cytoplasm
24	Granular size same as that of the control; increased number with more aggregations resulting in heterogenous granulations.	Located mainly as groups in the cytoplasm
48	Granular size remains unaltered; no increase in number but showed heterogenous granulation as in 24h hypoxia.	Located mainly as groups in the cytoplasm
Reimmersed after 48h hypoxia	Granular size same as that of control. Fewer in number with non aggregation	Granules scarcely distributed in the cytoplasm.

Comparison of the number of lipofuscin granules of control and hypoxic exposed animals

Table 5.4a *Sunetta scripta* (35-45mm)

Hypoxic exposure time (h)	No.of lipofuscin granules/cm^2 (± SD[a])
0	3.042 ± 4.25
24	4.042 ± 3.17
48	5.792 ± 4.74
Reimmersed after exposure	6.080 ± 7.97

a = no.of lipofuscin granules/cm^2 was ascertained on micrographs (200X)

Table 5.4b *Sunetta scripta* (35-45mm)

Source of variation	Sum of squares	Degrees of freedom	Estimated variance	Variance ratio = F
Total	1328.995	47		
Periods	75.391	3	25.130	NS
Error	1253.604	44	28.491	0.882

NS - Not significant

Comparison of the number of lipofuscin granules of control and hypoxic exposed animals

Table 5.5a *Perna viridis*(40-50mm)

Hypoxic exposure time (h)	No.of lipofuscin granules/cm^2 (± SD[a])
0	1.916 ± 2.91
24	10.00 ± 6.96
48	6.625 ± 6.38
Reimmersed after exposure	1.625 ± 1.91

a = no.of lipofuscin granules/cm^2 was ascertained on micrographs (200X)

Table 5.5b *Perna viridis*(40-50mm)

Source of variation	Sum of squares	Degrees of freedom	Estimated variance	Variance ratio = F
Total	1696.42	47		
Periods	582.38	3	194.125	**
Error	1114.042	44	25.319	7.67

** - $P<0.01$

Effect of hypoxic exposure and reimmersion on lipofuscin accumulation (X200)

Sunetta scripta **(35–45mm)**

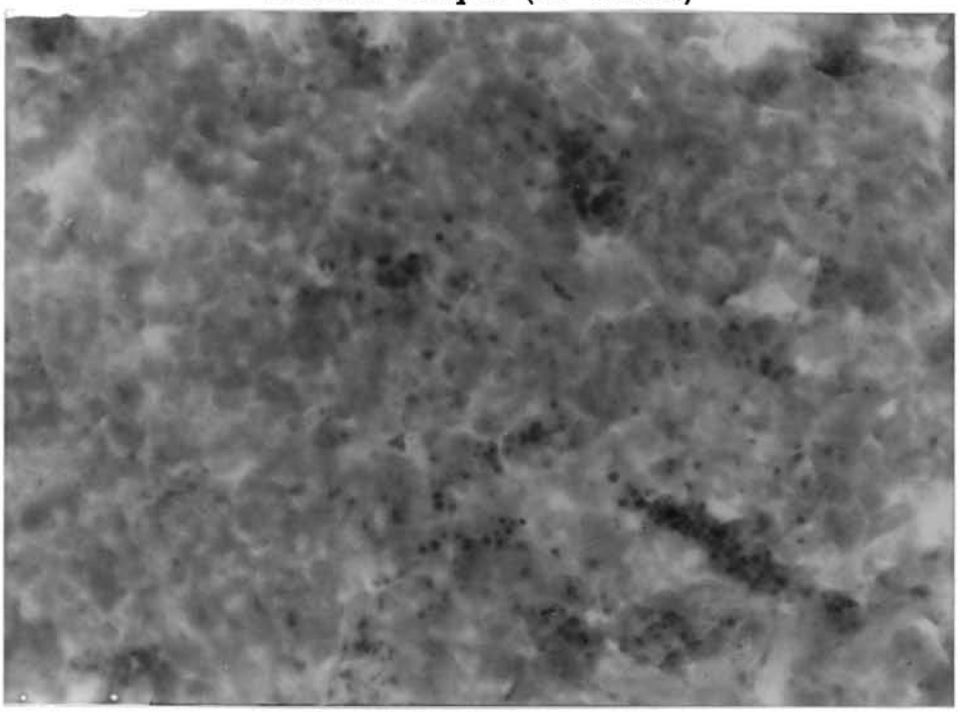

Fig. 5.2a Control (0h)

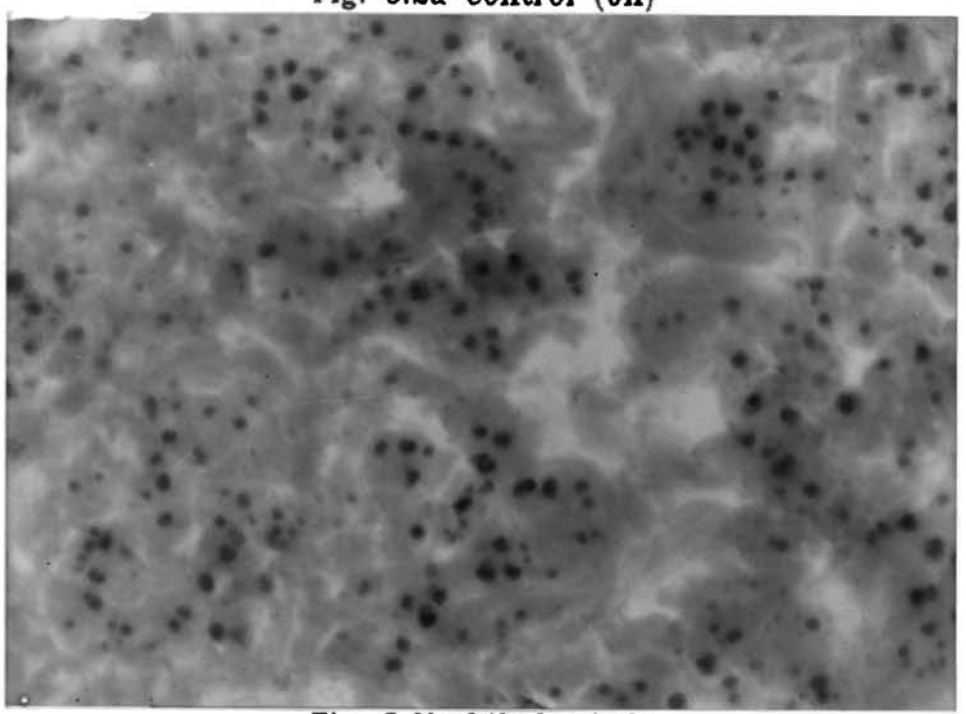

Fig. 5.2b 24h hypoxia

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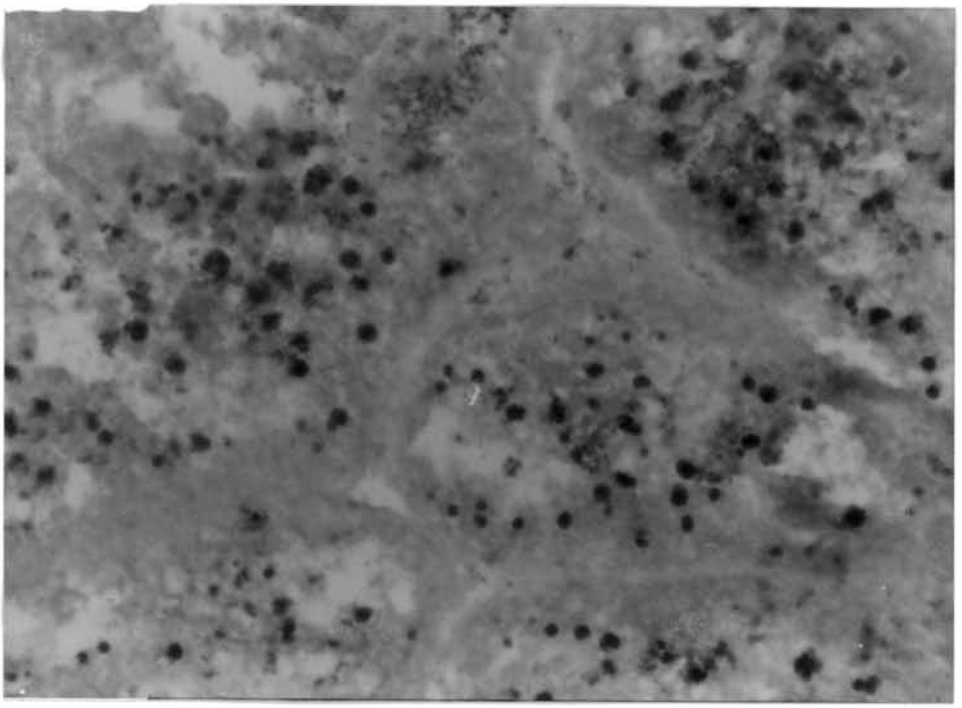

Fig. 5.2c 48h hypoxia

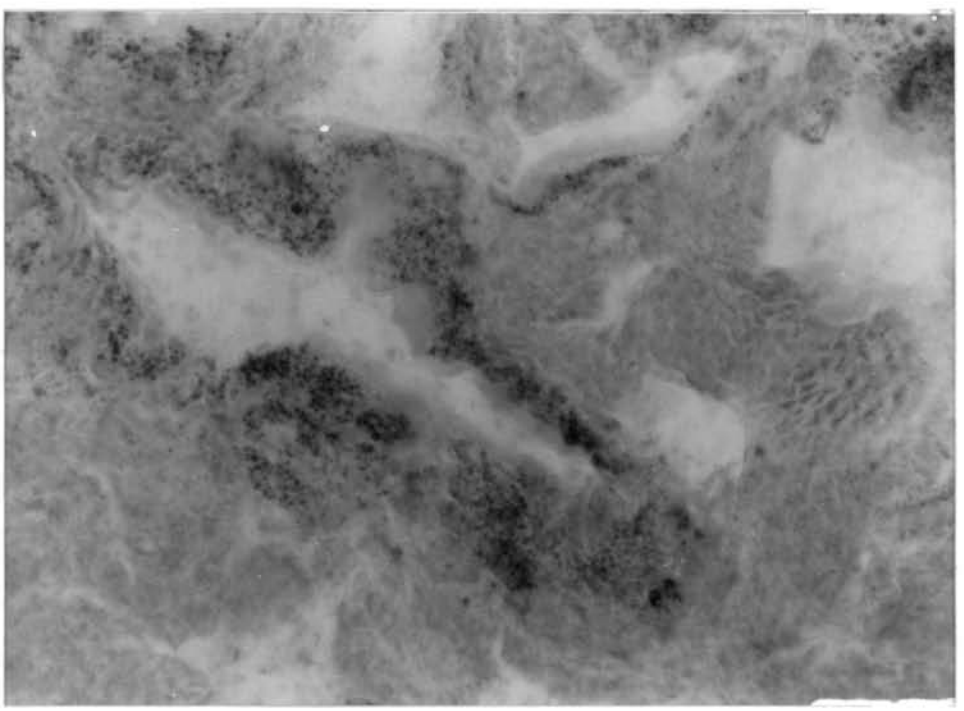

Fig. 5.2d Reimmersed

Effect of hypoxic exposure and reimmersion on lipofuscin accumulation (X200)

Perna viridis (40–50mm)

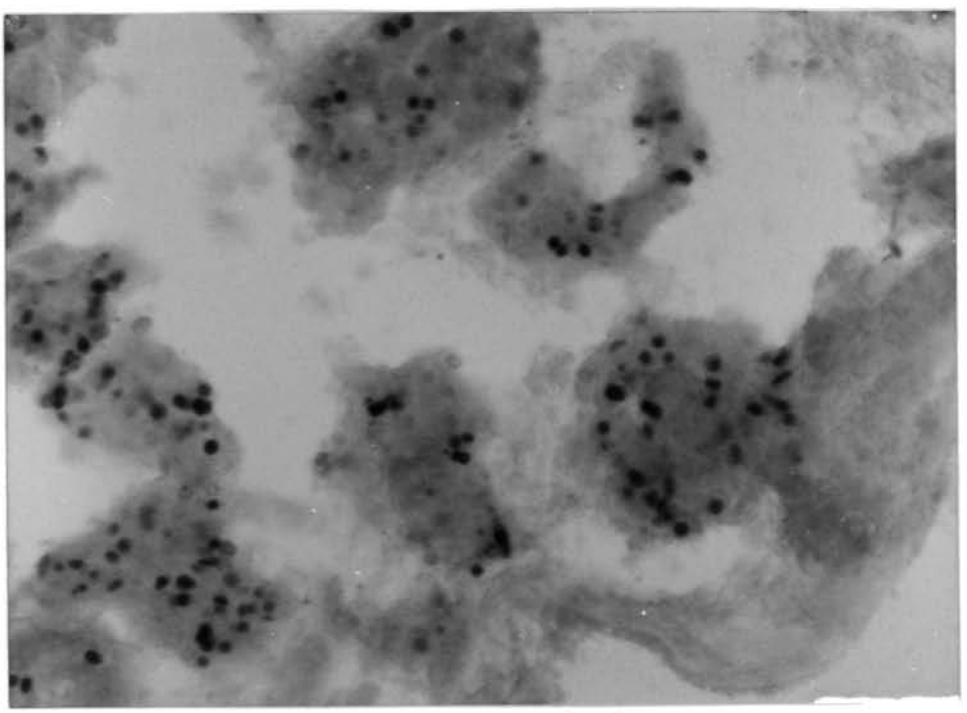

Fig. 5.3a Control (0h)

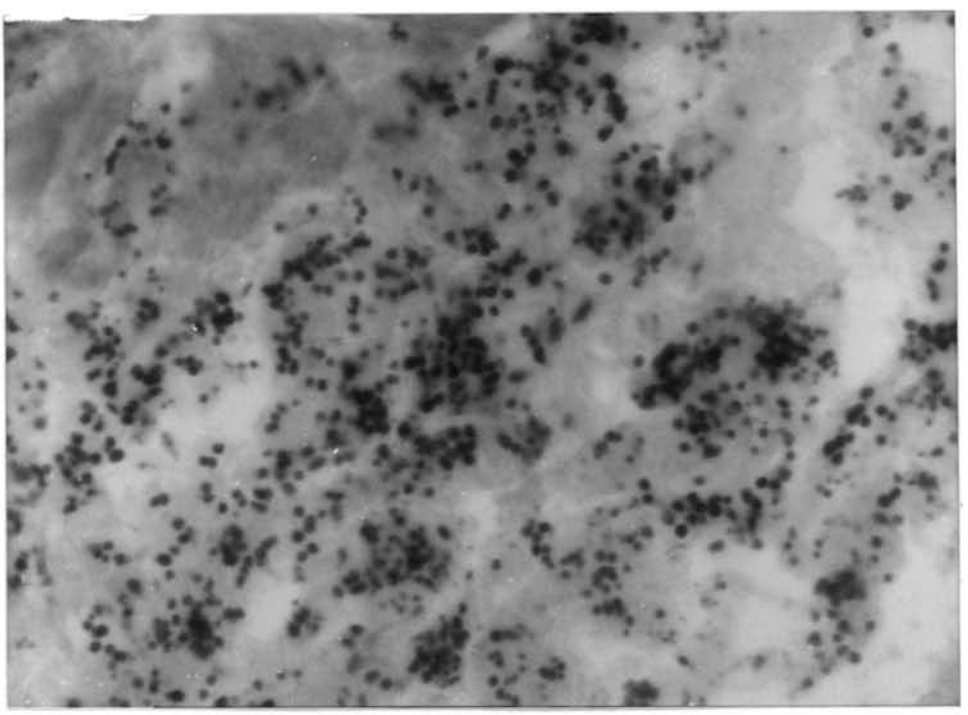

Fig. 5.3b 24h hypoxia

Contd...

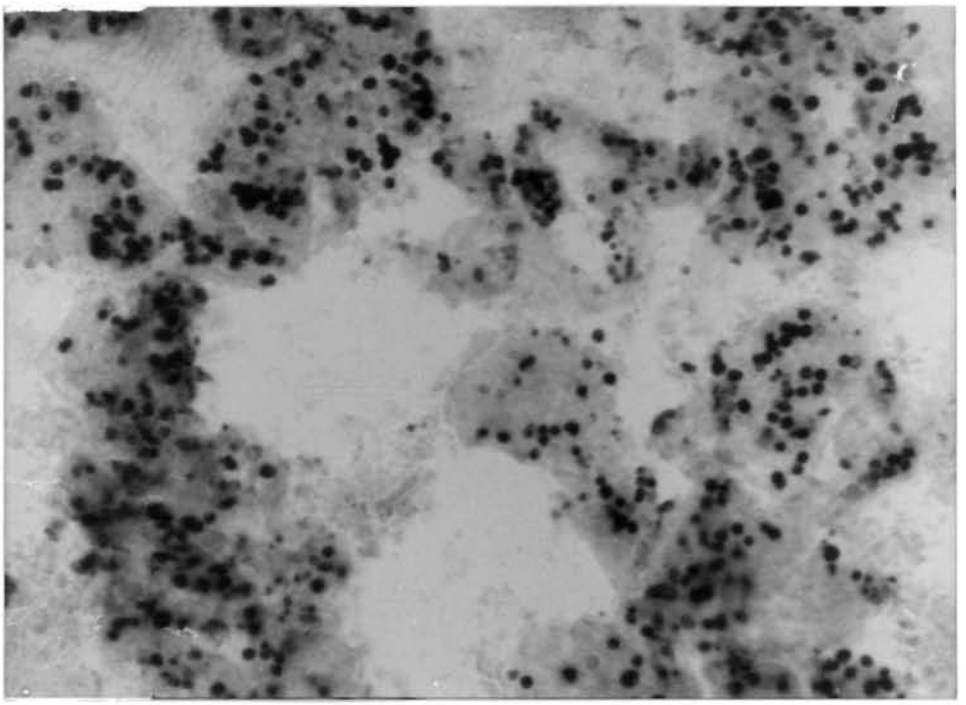

Fig. 5.3c 48h hypoxia

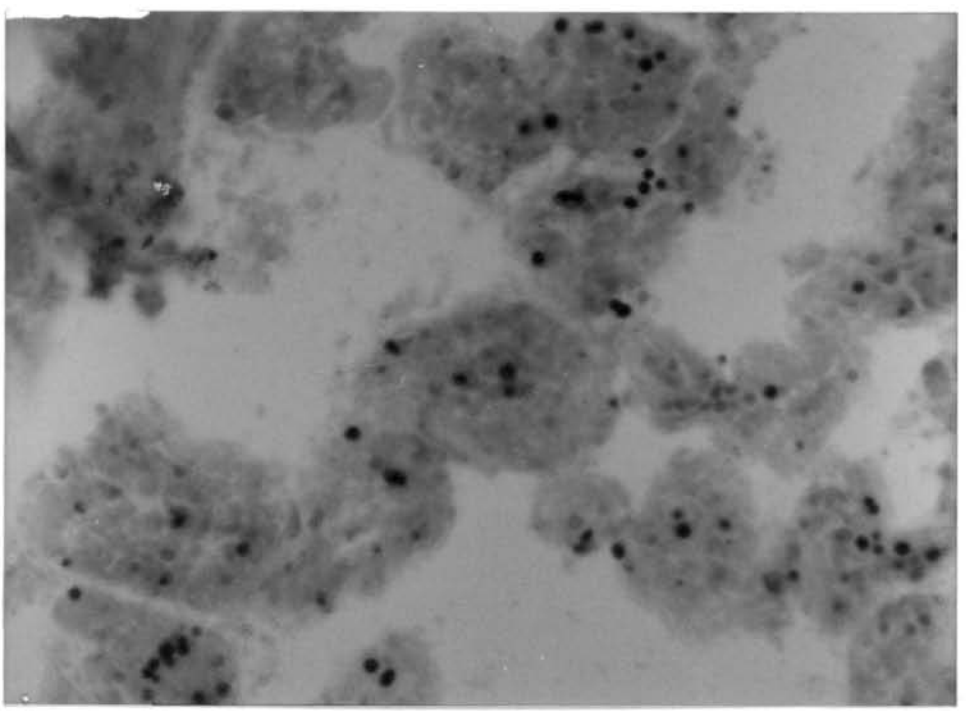

Fig. 5.3d Reimmersed

Chapter 6

LIPOFUSCIN AS PHYSIOLOGICAL INDICATOR OF HEAVY METAL STRESS

6.1. INTRODUCTION.

Heavy metals form a dangerous group of potentially hazardous pollutants, which are released into the marine realms threatening the very existence of aquatic biota(Bryan, 1984). The response of marine animals to a pollutant can be detected at different levels of organization and responses. All species are tolerant to a certain amount of environmental variation . However,beyond the tolerant limits, characteristic biological responses related to the ultimate survival or death of the individual are elicited (Blackstock, 1984; Bayne *et al.*, 1985). The biological responses include physiological, biochemical, morphological, genetic and behavioural responses of organisms to stress (Widdows, 1985).

Most organisms are able to concentrate heavy metals in their body. This holds true for bivalves too. These organisms concentrate heavy metals at very high levels in the different tissues. However, it has been seen that they are able to survive and apparently reproduce normally, which indicates that they have evolved control or tolerance

mechanisms at the cellular level (Akberali and Trueman, 1985).The toxic action of heavy metals is generally attributed to the inhibitory effect on the enzyme system.

In general, high concentration of heavy metals have harmful effects on living organism, although in some cases very low concentration (for copper and mercury) are toxic to some organisms. Since sublethal concentration of metals manifest many physiological changes in the animal, they are considered to be more deleterious and harmful than lethal concentration. It can ultimately affect the population as a whole without the danger being noticed.

Copper is one of the trace elements required by many marine organisms for normal growth and development (Bryan, 1971). Its absorption from food or water, in which the organisms live, can be regulated by different mechanisms in different animals. At lower concentration, copper acts as an essential element (Villarreal *et al* .,1986 ; William *et al*.,1987), whereas at higher concentration, it becomes inhibitory and toxic and can be used as a molluscide (Simkiss and Mason, 1985).

Many pollutants exert their effect on biological system by inducing the production of free radicals (Halliwell, 1981; Halliwell and Gutteridge, 1985). According to Pisanti *et al*. (1988), all transition metals were found to induce

lipofuscinogenesis. The transition metals can be considered as free radicals since they have one or more unpaired electrons (Halliwell and Gutteridge, 1985). Therefore they undergo variations in their oxidative state tending towards the donation or acquisition of electrons inorder to couple the unpaired electrons. Due to this character,transition metals react with oxygen, producing dangerous free radicals such as superoxides and hydroxyl radicals. Thus, copper being a transition metal, may act as a catalyzer of peroxidative reaction. Following are the two possible mechanisms by which copper can induce the formation of free radicals (Sreekumar *et al* .,1978).

$$Cu^{+} + O_2 \longrightarrow Cu^{2} + O_2^{-}$$

$$Cu^{+} + H_2O_2 \longrightarrow Cu^{2+} + OH + OH^{-}$$

The reaction of such radicals with biological macromolecules like lipoproteins causes peroxidative phenomenon leading to lipofuscin production (Sohal, 1981; Aloj and Pisanti, 1985; Aloj *et al*., 1986b; Marzabadi *et al*.,1988; Pisanti *et al*., 1988).

The interference of copper in the histogenesis of lipofuscin granulation may be explained hypothetically in two ways:

(1)a direct induction of peroxidation of the poly-unsaturated fatty acids of membranes (Sreekumar *et al*.,1978).

(2) an indirect action *via* a removal of thio groups which are indispensable for the metabolic role of glutathione dependent enzymes and some metallothioneins which are known to have the capacity for binding the excess heavy metals found in the tissues (Piccinni and Coppellotti, 1982; Viarengo, 1985).

Thus lipofuscin, a granular pigment of wear and tear, is considered to be a marker of cell damage. It is present particularly in post mitotic cells like neurons, which reveal the life history of an individual. They can be considered as an organelle, serving the function of a depot or store house of indigestible, unexcreted cellular wastes, primarily consisting of intracellular membranes (Sohal and Wolfe, 1986).

Massive accumulation of residual bodies in kidney is reported as a metal induced response (George *et al.*, 1982). Copper exposure in the neurons of *Torpedo* sp. has resulted in a significant increase in lipofuscin granules with extensive damage to mitochondria (Aloj and Pisanti, 1985; Aloj *et al.*, 1986b; Enesco *et al.*, 1989). Presence of transitional metals like copper, iron, chromium, vanadium *etc.* in the culture medium induces a rise in the lipofuscin production in marine mycete *Corollospora maritima* (Pisanti *et al.*, 1988). Increased lipofuscin accumulation in the digestive cells have been reported in *M. edulis* and *Littorina littorea* exposed to copper and hydrocarbons (Pipe and Moore, 1986; Moore, 1988) and in

P.viridis exposed to copper and mercury (Krishnakumar *et al.*, 1990).

Heavy metals were reported as the most potent inhibitor of mitochondrial respiration and oxidative phosphorylation (Zaba and Harris, 1976; Akberali and Earnshaw, 1982; Somasundaram *et al.*, 1984; Tort *et al.*, 1984 a, b; Babu and Rao, 1985; Crespo and Sala, 1986; Krishnakumar, 1987). Upon exposure to heavy metals, molluscs tightly close their valves resulting in a decreased rate of respiration. The copious secretion of mucus further reduces the efficiency of gaseous exchange across the gills thereby subjecting the animal to an anaerobic state. This hypoxia leads to the activation of terminal oxidative system of lipofuscin granules (Karnaukhov *et al* ., 1972; Karnaukhov, 1973b). The carotenoids being a component of the lipofuscin granule can provide the cells with energy required under hypoxia (Karnaukhov, 1973b; 1990).

Karnaukhov *et al.* (1977) observed an increase in the population of molluscs having high concentration of carotenoids, in the polluted area of Black Sea. At the same time, population with low concentration of carotenoid content decreases with pollution. A correlation has been observed by Krishnakumar (1987) in *Perna viridis,* wherein the mussels having high carotenoid concentration in their body were found to be able to withstand acute mercury, zinc and copper toxicity. The increase in the carotenoid concentration in the

body of the mussel, *P.viridis* was found to be linear with the increment in the metal concentration in the ambient medium.

The bivalve molluscs, are found to accumulate and concentrate most of the pollutants within their tissues to concentration significantly above the ambient level in the environment, thus facilitating accurate chemical analysis and assessment. The present study aims at elucidating the effect of sublethal exposure of copper on both the carotenoid concentration and the lipofuscin accumulation.

6.2.MATERIALS AND METHODS.

Details regarding the materials and methods have been described in Chapter-2.

6.3.RESULTS.

The sublethal levels of 2ppm and 7.5ppb copper taken for *S.scripta* and *P.viridis* respectively, were based on the previous copper toxicity studies conducted on these two species (Thampuran *et al.*, 1982; Krishnakumar, 1987). The sublethal effect of copper (2ppm) on the carotenoid concentration of *S.scripta* (35-45mm) has been presented in Table 6.1a. From the table, it could be seen that there is significant difference ($P<0.01$) between the time intervals (0h and 48 h). The readings at the 48h exposure were significantly

higher than that of the control (Fig.6.1).

Table 6.1b represents the variations in the carotenoid concentration of *P.viridis* (55-65mm) exposed to sublethal levels of copper (7.5ppb). It has been graphically represented in Fig.6.2. A significant difference ($P<0.01$) in the carotenoid concentration between the control (0h) and 48h of exposure has been noticed.

A morphological examination of lipofuscin granules in the hepatopancreatic tissues of *S.scripta* at 0h and 48h revealed characteristic features which are given in Table 6.2a. In comparison to control (0h), 48h copper exposed animals exhibit more pigmentation with increased granular size and number. At 48h of exposure, heterogenous granulations could be noticed due to high aggregative nature (Fig.6.3a and 6.3b).

Characteristic morphological features of lipofuscin granules of *P.viridis* in the hepatopancreatic tissues have been described in Table 6.2b. Corresponding carotenoid concentration has been illustrated in Fig.6.2 . The main contrast depicted in the lipofuscin granules between *P.viridis* and *S.scripta* is in the size of the granules (Fig.6.4a and 6.4b). In the former species, granular size remains the same at 0h and 48h of exposure, whereas in latter, an increase in granular size has been observed at 48h of exposure, resulting in huge heterogenous granulations due to aggregation. The total

carotenoid concentration and morphological characteristics of lipofuscin granules at 48h of heavy metal exposure is similar to that observed at 48h of hypoxic exposure in both *S.scripta* (Table 5.1a and 5.3a; Table 6.1a and 6.2a) and *P.viridis* (Table 5.2a and 5.3b; Table 6.1b and 6.2b).

Tables 6.3a and 6.3b represents the number of lipofuscin granules/cm^2 in *S.scripta* and *P.viridis* respectively under heavy metal exposure. In both the species, a significant difference ($P<0.5$) in the number of lipofuscin granules has been observed between the control and the copper exposed animals. Exposed animals gave more number of granules /cm^2 in comparison to non-exposed in both the species.

6.4.DISCUSSION.

The data presented here demonstrate that the exposure to sublethal levels of copper produces remarkable biochemical changes at the cellular level which has been indicated by an elevated carotenoid concentration and lipofuscin accumulaton. In both *S.scripta* and *P.viridis*, high carotenoid concentration at 48h of exposure in comparison to control (0h) seems to be consistent with that observed in *P.viridis* and *Villorita cyprinoides* var *cochinensis* exposed to acute mercury, zinc and copper and sublethal levels of mercury and copper respectively

(Krishnakumar, 1987; Krishnakumar *et al.*, 1987; Sathyanathan *et al.*, 1988).

In both *P.viridis* and *S.scripta*, lipofuscin granules displayed marked morphological changes after 48h of heavy metal exposure in comparison to the control. The control (0h) exhibits fewer number of granules, scattered in the cytoplasm with less pigmentation and non aggregative nature in both the species. At 48h of exposure, more pigmentation with increased clustering resulting in heterogenous granulations have been observed. In *P.viridis*, the granular size remains unaltered at 0h and 48h of exposure, whereas in *S.scripta*, an increase in the granular size has been noticed at 48h of exposure.

A similar change in the lipofuscin granule has been observed in both the species at 48h of hypoxic exposure (Chapter-5). In *P.viridis*, as in heavy metal stress, 48h of hypoxia exhibits more pigmentation with increased granular number and aggregation, resulting in heterogenous granulations. The granular size remains the same for 0h and 48h of hypoxic exposure. Regarding *S.scripta*, during hypoxic exposure as well as in heavy metal exposure , more pigmentation with increased size and aggregation have been observed. Hardly any difference in the granular number between 0h and 48h of hypoxia has been noticed on statistical analyses. But during heavy metal exposure, an increase in the granular number has been noticed at 48h of exposure. The increased granular size noticed in

S.scripta may be due to the high clustering or aggregative nature exhibited by the lipofuscin granules. The characteristic differences in the lipofuscin granules of both *S.scripta* and *P.viridis* need further study.

Since carotenoid is a constituent of the lipofuscin granules (Karnaukhov *et al* ., 1972; Karnaukhov,1973b; 1990), at 48h of exposure, concomitant with the increase in lipofuscin accumulation, an enhancement of the total carotenoid concentration has been observed in both the species. In both the stressed condition, a rise in the total carotenoid concentration and lipofuscin accumulation were noticed at 48h of exposure.

Regarding *S.scripta*, the total carotenoid increase during 48h of hypoxic exposure as well as at 48h of heavy metal exposure have been found to be nearly 40% greater than that of the control value. In the case of *P.viridis*, the carotenoid build up during 48h of heavy metal stress is nearly 95% greater than that of the control (0h), whereas at 48h of hypoxic exposure, the increase is found to be nearly 87% higher when compared to the control (0h).

Both carotenoid and lipofuscin accumulation are taken as an index of heavy metal stress in molluscs (Krishnakumar *et al*., 1987; 1990; Moore, 1988). When the animals were exposed to heavy metals, they were found to be subjected to

hypoxic stress. The lowering of the metabolic rate with a reduced oxygen uptake rate had been reported in both *P.viridis* (Krishnakumar, 1987) and *S.scripta* (Thampuran, 1986) during heavy metal exposure. Both partial and complete shell valve closure during heavy metal exposure is known to reduce the heart beat rate in molluscs (Bayne *et al.*,1976a). Reduction in the heart rate will bring about reduction in ventilation within tissues, thus influencing the oxygen uptake. The magnitude of the carotenoid increase during 48h hypoxic exposure is very much similar to that observed at 48h of heavy metal stress. Characteristic features in the accumulation of the lipofuscin granules at 48h of hypoxia were found to be similar to that occurring at 48h of heavy metal exposure. As the quantitative changes in the carotenoid content and lipofuscin accumulation under hypoxia and heavy metal stress are of the same magnitude, it is reasonable to presume that the increase in carotenoid concentration and lipofuscin accumulation expressed by bivalves under heavy metal stress can be due to the indirect effect of hypoxia.

Sublethal effect of copper on total carotenoid concentration (mg $100g^{-1}$ wet wt)

Table 6.1a *Sunetta scripta* (35-45mm)(2 ppm)

Exposure time (h)	Total carotenoid mg $100g^{-1}$ wet wt	*t* value
0	1.600 ± 0.141	**
48	2.250 ± 0.071	12.748

Table 6.1b *Perna viridis* (55-65mm)(7.5 ppb)

Exposure time (h)	Total carotenoid mg $100g^{-1}$ wet wt	*t* value
0	0.197 ± 0.001	*
48	0.385 ± 0.054	3.487

* - $P<0.1$

** - $P<0.01$

Total carotenoid concentration during sublethal exposure of copper

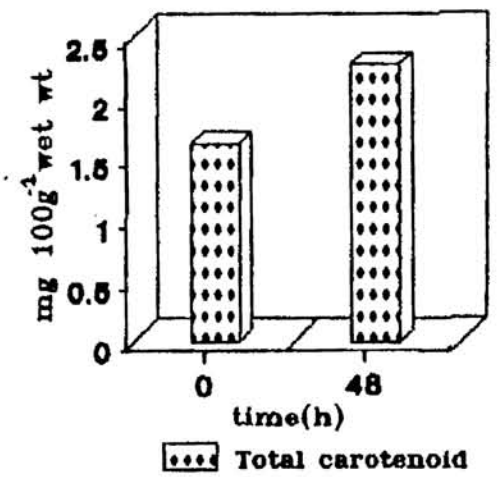

Fig.6.1 *Sunetta scripta* (35–45mm)

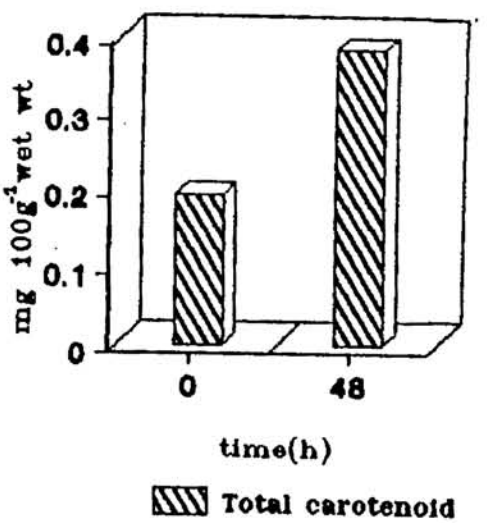

Fig.6.2 *Perna viridis* (55–65mm)

Morphological features of lipofuscin granules observed in control and copper exposed animals

Table 6.2a *Sunetta scripta* (2ppm) (35-45mm)

Exposure time (h)	Characteristics of lipofuscin granules
0	Less pigmentation, small spherical bodies with no aggregation.
48	More pigmentation, high clustering resulting in heterogenous granulations with increase in the granular size.

Table 6.2b *Perna viridis* (7.5ppb) (55-65mm)

Exposure time (h)	Characteristics of lipofuscin granules
0	Mainly scattered, spherical granules fewer in number with less pigmentation and non aggregation.
48	More pigmentation, granular size same as that of control. Increased number with more clustering resulting in heterogenous granulations.

Comparison of the number of lipofuscin granules of control and copper exposed (sublethal) animals

Table 6.3a *Sunetta scripta* (35-45mm)

Exposure time (h)	No:of lipofuscin granules/cm^2(± SD)[a]	*t* value
0	2.208 ± 2.70	***
48	4.083 ± 5.21	1.1042

Table 6.3b *Perna viridis* (55-65mm)

Exposure time (h)	No:of lipofuscin granules/cm^2(± SD)[a]	*t* value
0	2.750 ± 2.49	***
48	3.875 ± 3.79	0.8592

a = no:of lipofuscin granules/cm^2 was ascertained on micrographs (200X)

*** - P<0.5

Effect of sublethal exposure of copper (2ppm) on lipofuscin accumulation (X200)

Sunetta scripta (35–45mm)

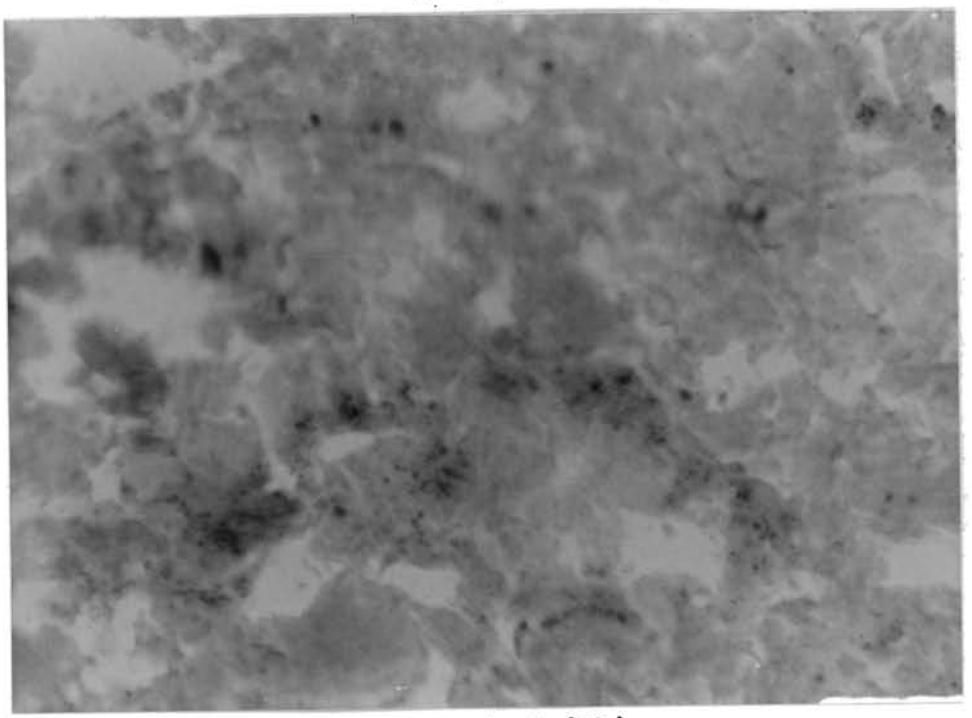

Fig. 6.3a Control (0h)

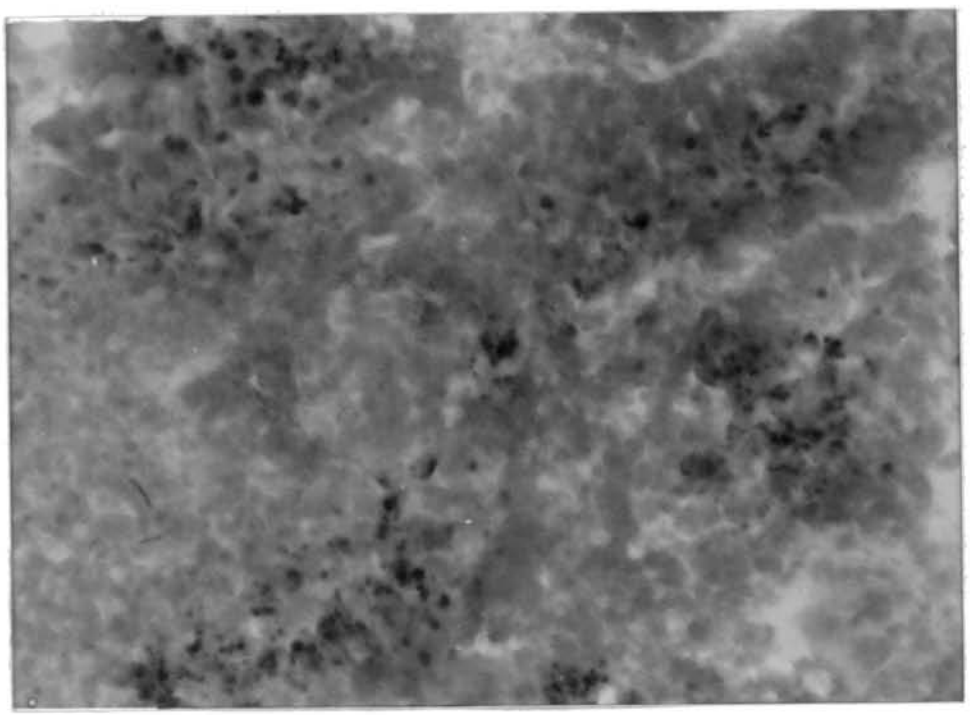

Fig. 6.3b Exposed (48h)

Effect of sublethal exposure of copper (7.5ppb) on lipofuscin accumulation (X200)

Perna viridis (55–65mm)

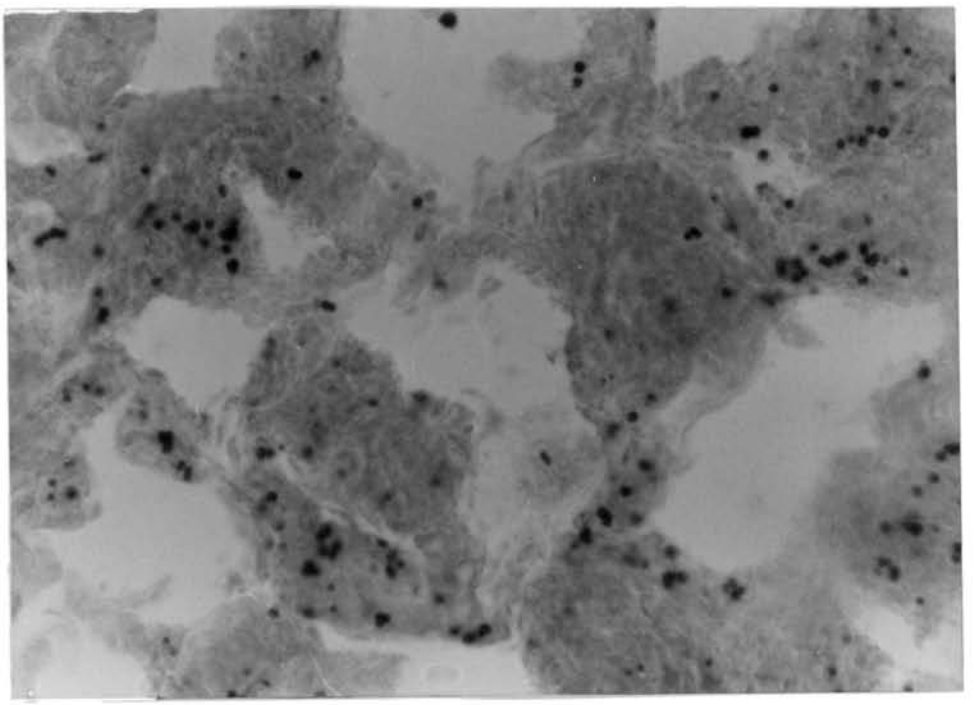

Fig. 6.4a Control (0h)

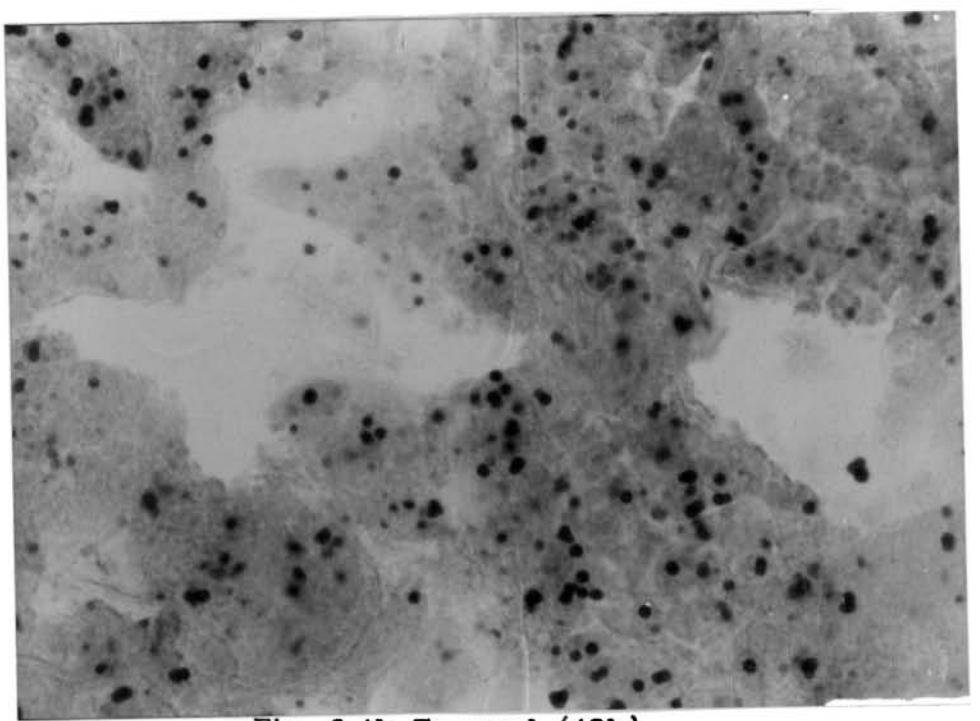

Fig. 6.4b Exposed (48h)

Summary

SUMMARY

Intertidal habitat exposes their inhabitants to various naturally occurring stresses, together with those resulting from the anthropogenic activities. Measurement of physiological and cellular responses of an organism to environmental stress is useful in quantifying the overall fitness of an organism, its performance and the efficiency with which it functions under adverse conditions. The physiological ecology of the animals is of great significance since it reveals the physiological flexibility of an organism in relation to the environmental demand.

The present investigation is to find the hypoxic adaptations and role of carotenoids in the anaerobic catabolism of two intertidal bivalves - *Sunetta scripta* and *Perna viridis*. Physiological and cytological responses during hypoxic stress have been studied and compared to that of sublethal heavy metal (copper) exposure using two indices : total carotenoid concentration and accumulation of lipofuscin granules.

Responses of *S.scripta* and *P.viridis* to declining oxygen tensions in the environment in relation to body weight have revealed that both species are good regulators. They could maintain the oxygen uptake rate independent of the external oxygen tension under various percentage saturation of oxygen (100, 80 and 70). In both the species, a sharp decline in the oxygen uptake rate is noticed only at 50 percentage saturation of oxygen. This decline is less obvious in smaller size groups suggesting their greater ability to maintain a higher ventilation rate at lower oxygen tension than intermediate and larger size groups. Oxygen consumption rate was found to be higher in *P.viridis* than in *S.scripta*. It may be because of their habitat differences. The metabolic adaptations exhibited by these bivalves help them in carrying out metabolic processes at a higher rate till a critical level is reached (50 percent saturation of oxygen), where they reduce their energy demand considerably, as indicated by a low oxygen uptake rate.

Sustained increase in the total carotenoid concentration is not noticed in *S.scripta* during aerial exposure. In both *S.scripta* and *P.viridis*, the recordings of the valve movements during aerial exposure have revealed that they do not close their valves completely, but maintained a small gape in their mantle margins, which may aid in the permeation of oxygen into the mantle water.

Estimation of the total carotenoid concentration in *S.scripta* during short term and long term anoxia indicated that the carotenoxysomal/cytosomal pathway of anaerobic respiration does not trigger on when the animals are totally cut off from the external environment. During gradually developing anoxic condition, an increase in the total carotenoid concentration together with a decrease in the glycogen content of the body have been observed in both *S.scripta* and *P.viridis*. The increment in the total carotenoid concentration is found to be more in *P.viridis* (nearly 87% increase) than in *S.scripta* (nearly 40% increase). Thus it is likely to suggest that mussels due to their more sensitiveness to oxygen stress than clams, depend much on the carotenoxysomal pathway during hypoxic stress. The carotenoid increment with the decrease in the glycogen content during gradually developing hypoxic condition have indicated that both the anaerobic glycolysis and carotenoxysomal pathways are functionable simultaneously in both *S.scripta* and *P.viridis*.

Cellular and biochemical responses of both *S.scripta* (35-45mm)and *P.viridis*(40-50mm) upon exposure to hypoxic stress have been dealt with. It has been inferred from the present study that concomitant with the increase in the lipofuscin accumulation in the hepatopancreatic tissues, an enhancement in

the carotenoid concentration has also been observed. In both the species, the increase in the lipofuscin accumulation and carotenoid concentration were appreciable during 24h hypoxia, with very limited change when the experiments were extended upto 48h of hypoxia.

Marked morphological differences in the lipofuscin granule have been noticed between control (0h) and 24h and 48h hypoxia in both *S.scripta* and *P.viridis*. An increase in the granular size due to aggregation has been observed in *S.scripta* at 24h and 48h of hypoxic exposure. Whereas in *P.viridis*, an increase in the granular number with more heterogenous granulations has been observed with the advancement of hypoxic exposure time. The granular size remains the same for control (0h), hypoxic exposed (24h and 48h),and reimmersed groups in *P.viridis*. At reimmmersion, both the total carotenoid concentration and accumulation of lipofuscin granules decrease to that of the control level. Upon reimmersion, a marked decrease in the lipofuscin granular number has been noticed in *P.viridis*, whereas in *S.scripta*, the granular size becomes reduced.

The increase in the total carotenoid concentration with the advancement of hypoxic exposure time was found to be

more prominent in *P.viridis* than in *S.scripta*. This may be due to the differences in their mode of survival. *S.scripta*, being infaunal, is living in a habitat where it may experience hypoxic/anoxic environment quite regularly enabling them to have a high anaerobic tolerance capacity. Rather *P.viridis*, because of its high oxygen consumption rate, may be relying more on the intracellular oxygen stock of carotenoxysomes during hypoxia. The study indicates that the lipofuscin accumulation can be considered as an important physiological index of hypoxic stress at the cellular level.

Sublethal exposure to copper induces enhanced total carotenoid concentration synchronous with increased lipofuscin accumulation at 48h of exposure in both *S.scripta* and *P.viridis*. A close similarity has been observed between hypoxic exposed and copper (sublethal) exposed animals regarding the total carotenoid concentration and lipofuscin accumulation. In the case of *S.scripta*, the total carotenoid increase at 48h of both hypoxic and heavy metal exposure was found to be nearly 40% greater than that of the control (0h). Whereas in *P.viridis*, the increment in the total carotenoid concentration at 48h of hypoxic exposure and 48h of heavy metal exposure were found to be nearly 87% and 95% higher than that of the control (0h) respectively.

Regarding the lipofuscin accumulation, in both *S.scripta* and *P.viridis*, the characteristic features of the granule at 48h of hypoxia is very much similar to that observed at 48h of heavy metal exposure. Thus, the present study suggests that the increase in carotenoid concentration and lipofuscin accumulation expressed by bivalves under heavy metal stress can be due to the indirect effect of hypoxia.

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