STUDIES ON THE PHYSIOLOGICAL AND BIOCHEMICAL ASPECTS OF OSMOREGULATION IN TWO INTERTIDAL BIVALVE MOLLUSCS

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This is to certify that the thesis entitled *Studies on the physiological and biochemical aspects of osmoregulation in two intertidal bivalve molluscs* is an authentic record of research work carried out by Shri. P. J. George, under my supervision and guidance in the Division of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology, in partial fulfilment of the requirements for the degree of **Doctor of Philosophy** of the Cochin University of Science and Technology and no part thereof has been presented before for the award of any other degree, diploma or associateship in any University.

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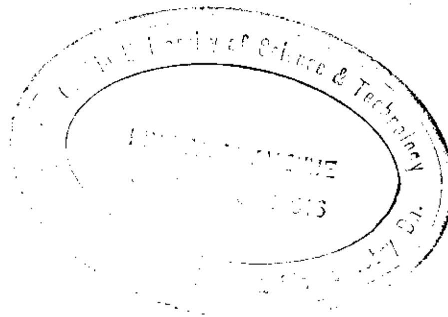
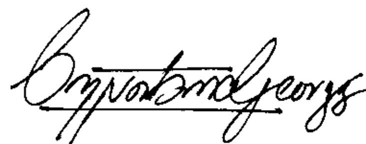


DECLARATION

I, Shri. P. J. George, do hereby declare that this thesis entitled *Studies on the physiological and biochemical aspects of osmoregulation in two intertidal bivalve molluscs* is a genuine record of research work done by me under the supervision and guidance of **Prof. Dr. R. Damodaran**, Division of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology, and has not been previously formed the basis of the award of any degree, diploma or associateship in any University.



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ABBREVIATIONS USED IN THE THESIS

ADP	: Adenosine diphosphate
Ala	: Alanine
ANOVA	: Analysis of variance
Arg	: Arginine
Asp	: Aspartic acid
AST	: Aspartate aminotransferase
^{o}C	: Degree centigrade
Cl^{-}	: Chloride ion (s)
cm	: Centimetre
Conc.	: Concentration
DVB	: Divinylbenzene
E	: East
EC	: Enzyme Commission
EDTA	: Ethylenediaminetetraacetic acid
eq.	: Equivalents
Fig.	: Figure (s)
g	: Gyres
g/g	: Gram by gram
GDH	: Glutamate dehydrogenase
Glu	: Glutamic acid
Gly	: Glycine
h	: Hour (s)
H^{+}	: Hydrogen ion (s)
HCl	: Hydrochloric acid
K^{+}	: Potassium ion (s)
KCl	: Potassium chloride
LDH	: Lactate dehydrogenase
Lys	: Lysine
M	: Molar (Moles per litre)
meq/l	: Milliequivalents per litre
mg	: Milligram
min.	: Minute (s)
Misc.	: Miscellaneous
ml	: Millilitre
mm	: Millimetre
mM	: Millimolar (millimoles per litre)

N	:	Normal
Na^+	:	Sodium ion (s)
NaCl	:	Sodium chloride
NAD^+	:	Nicotinamide adenine dinucleotide
NADH	:	Nicotinamide adenine dinucleotide (reduced)
nm	:	Nanometre
No.	:	Number (s)
NPS	:	Ninhydrin positive substance (s)
Pro	:	Proline
rpm	:	Revolutions per minute
SD	:	Standard deviation
Ser	:	Serine
SH	:	Serine hydrolyase
Tau	:	Taurine
TCA	:	Tricarboxylic acid cycle
Thr	:	Threonine
Val	:	Valine
v/v	:	Volume by volume
$<$	:	Less than
$>$	:	Greater than
%	:	Percentage
Δ	:	Rate of change (delta)

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CHAPTER 1

GENERAL INTRODUCTION

1.1 SCIENTIFIC BACKGROUND

Marine environment is well known for its dynamic and homogeneous nature. A notable feature of this habitat is the stability of environmental conditions over vast areas which allows the biota to enjoy wider distributions. On the contrary, the coastal zones are subjected to continuous biological, chemical, physical, geological and meteorological interactions. The littoral habitat known for its abundance and variety of life is prone to greater interactions of the above environmental parameters and is colonized by species which can tolerate wider fluctuations in ambient environmental conditions. One of the parameters that shows rapid and extensive fluctuations is salinity. Salinity is one of the major environmental variables which affects the performance and ultimately determines the growth, survival and distribution of organisms in the intertidal region (Kinne, 1971).

South west coast of India is subjected to annual impact of monsoon which makes salinity the most critical environmental factor in the intertidal region due to land run off and dilution of the surface waters. Therefore, inhabitants of this region must have a wider salinity tolerance to maintain themselves in the community. The principal problem of life in the marine environment with different salinity regimes is the maintenance of optimum concentration of water and ions in the body fluids through the processes of ion and osmoregulation (Gilles, 1975).

The members of phylum Mollusca have a wide distribution and are represented in almost all habitats of the biosphere. They

certainly constitute one of the most representative phyla of littoral area and have often been chosen as landmarks in the definition of shore levels. Most adult bivalves are relatively immobile and environmental changes of transient, recurrent or permanent nature have to be accommodated by the animal (Akberali and Trueman, 1985).

Molluscs subsist throughout the aquatic environment from fresh to sea water and the regulatory mechanisms involved are closely related to the salinity regime of their natural habitats (Gainey and Greenberg, 1977). Osmoregulation in molluscs has been the subject of various investigations (Schoffeniels and Gilles, 1972; Gilles, 1975, 1982; Henry *et al.*, 1980; Otto and Pierce, 1981; Somero and Bowlus, 1983; Burton, 1983; Zatta and Cervellin, 1987; Matsushima *et al.*, 1987, 1989; Deaton, 1987, 1990, 1992 and Deaton *et al.*, 1989).

Anisosmotic extracellular and isosmotic intracellular regulations are the two major physiological tactics for adaptation of aquatic animals to fluctuating salinity (Matsushima *et al.*, 1987). Marine molluscs are considered as poikilosmotic having very limited power of extracellular regulation (Henry and Mangum, 1980a). Regulation of intracellular osmolytes is the basis of cellular water control and therefore the mechanism that underlies their salinity tolerance (Pierce *et al.*, 1992).

The extracellular-fluid anisosmotic regulation (Florkin, 1962) deals with the processes involved in the control of blood osmotic pressure. The limited capacity of blood osmotic pressure regulation in marine bivalves depends on two major processes

namely, valve closure and ionic regulation (Gilles, 1975; Gainey, 1978a; Akberali and Trueman, 1985; Jeuniaux, 1988). The valve closure is the behavioural response by which the animal can isolate itself temporarily from the external medium (Akberali and Trueman, 1985). Ionic regulation is the maintenance of ionic concentrations in the body fluids which are different from the concentrations to be expected, should passive equilibrium occur between the internal and external media (Robertson, 1964; Bayne, 1976; Burton, 1983).

Intracellular-fluid isosmotic regulation (Florkin, 1962) deals with the regulation of osmotic pressure of the cell. In many species cell volume is thought to be regulated by altering the quantity of intracellular osmolytes at a rate appropriate to match the changes of extracellular osmotic concentration, thereby, minimizing osmotic transfer of water across the cell membrane. Free amino acids including taurine are believed to be the principal regulatory osmolytes, although inorganic ions do play a minor role (Lynch and Wood, 1966; Lange, 1970; Gainey, 1978b; Lombardini *et al.*, 1979; Bishop *et al.*, 1981; Taylor and Andrews, 1988). These nitrogenous osmolytes form a significant component of the intracellular milieu and it is possible, therefore, that these solutes are subject to stringent selection to make the intracellular environment compatible for various life supporting molecular interactions (Yancey *et al.*, 1982; Somero and Bowlus, 1983).

The quantitative and qualitative nature and abundance of the osmolytes in a particular cell at any moment during osmotic

adaptations depend upon the changes in membrane related processes, glycolysis, protein turnover, tricarboxylic acid cycle reactions, transaminases and metabolite shuttling between cellular compartments (Pierce and Greenberg, 1973; Baginski, 1978; Stewart *et al.*, 1979; Pierce and Amende, 1981; Karam *et al.*, 1987; Wright *et al.*, 1989). Though the topic has attracted the attention of several workers, further explanation is required to explain the source of the organic osmolytes and the means of their regulation involved in the process.

A complex cellular machinery is at work for the accumulation and elimination of compatible nitrogenous osmolytes during osmotic adaptations in euryhaline bivalves. The concentration of different intracellular osmolytes to a limited extent depends upon the relative ease of availability of that osmolyte in the system. But what is important is the establishment of a hospitable microenvironment for macromolecular structure and function, irrespective of their relative accessibility from metabolic pathways, or their metabolic cost (Somero and Bowlus, 1983).

1.2 EXPERIMENTAL BIVALVES

1.2.1 *Sunetta scripta* (Linne')

The marine mollusc, *S. scripta* belongs to class Bivalvia and family Veneridae. It is an infaunal siphonate clam inhabiting sandy intertidal region. The animal is one of the dominant species of the littoral community along the south west coast of India.

Meat of the species is used for human consumption and also as a poultry feed. Thick shell of the species is used in cement and lime industry.

S. scripta has established comparatively large beds on the northern side of the Cochin barmouth. During monsoon, the salinity over the clam bed shows very low value ($2x10^{-3}$) and in pre-monsoon period the salinity goes up to $35x10^{-3}$. The annual temperature range of the area is $24^{o}C$ to $31^{o}C$ (Katticaran, 1988). Under laboratory conditions the species is found to tolerate a salinity range of 5 to $40x10^{-3}$ (Thampuran *et al.*, 1982).

The species has been subjected to study by different workers: allometric relationship of the shells (Mohan and Damodaran, 1981); reproductive biology (Katticaran, 1988); haematology (Suresh, 1988) and scope for growth at different salinities (Supriya, 1992).

1.2.2 *Perna viridis* (Linnaeus)

The green mussel, *P. viridis* is an epifaunal bivalve mollusc belonging to the family Mytilidae. The species has got a widespread distribution on hard and stable substratum on the east and west coasts of India. On the east coast, it occurs as small beds along Chilka Lake and extends in its distribution to south up to Porto Novo. On the west coast, extensive beds have been reported in all maritime centres from Quilon to the Gulf of Kutch (Kuriakose, 1980). The nutritious meat of this species is widely used as human food in India and in several countries of the

Indo-Pacific region.

P. viridis is found on the rocky open coasts and in harbours. The distribution extends from the intertidal zone to a depth of about 15 metres (Kuriakose, 1980). The species is found to tolerate wide fluctuations in salinity in their ambient environment (Quasim *et al.*, 1977; Narasimham, 1980). Since the animal has got ideal qualities which make it a sentinel organism, it has been used as a biological indicator of marine environmental quality (Krishnakumar and Damodaran, 1986).

Cultural prospects of the species have been studied by Quasim *et al.* (1977), and Kuriakose (1980); systematic position by Kuriakose (1980); biology by Narasimham (1980) and the metabolic responses to some natural variables by Shafee (1976).

1.3 SCOPE OF THE STUDY

The present work is focussed on the physiological and biochemical aspects of osmoregulation in *S. scripta* and *P. viridis*. Both the species are euryhaline and are found to tolerate wide ranges of ambient salinity. Though the euryhaline nature of these bivalves has been noticed by various investigators, nobody has so far made any serious contribution to understand the mechanisms involved in their salinity tolerance. This study is an attempt to understand the processes involved in the extracellular and intracellular osmoregulation. Most of the euryhaline bivalves studied show valve closure to tide over short term adverse salinity fluctuations. This behavioural response along with ionic

regulation provide the major means of extracellular-fluid anisosmotic regulation and attempt is made to understand these processes in different size groups of both the bivalves. Studies on various size groups of both the species are conducted on the basis of the view expressed by Kinne (1971) and Skinner and Peretz (1989) that in marine molluscs the degree of osmoregulation is a sensory initiated response which is altered with age. Of the two bivalves studied, the data available on *S. scripta* also indicate that the smaller size groups are more tolerant to variations in salinity than larger ones (Thampuran *et al.*, 1982).

Since the valve closure provides only temporary exemption from unfavourable conditions and the role of ionic regulation is very much limited, the bivalves have to heavily rely on the cellular salinity tolerance mechanism for long term adjustment in salinity variations. Several workers have observed a positive correlation between the external salinity and the concentration of total ninhydrin-positive-substances (NPS) in the intracellular fluid. It has also been noticed that the quality and quantity of the components of the NPS pool is carefully selected in different tissues of bivalves in order to make the intracellular environment suitable for various physico-chemical processes (Somero and Bowlus, 1983). Considering these aspects, the qualitative and quantitative nature of the organic molecules involved in the cellular salinity tolerance is investigated for the first time in different tissues of various size groups of *Sunetta scripta* and *Perna viridis*. The information obtained is expected to fill the lacunae in understanding the physiological and biochemical

adaptations of the species in different levels of salinity.

The cellular machinery involved in the mobilization of the organic osmolytes is still a subject of debate among the scientists. The possible mechanisms suggested include changes in metabolic regulation including protein turnover and membrane transport. Among these mechanisms, the relative importance of one over the other varies with species, environmental conditions and physiological state of the bivalves (Riley, 1980). Hence the concentration of intracellular osmolytes in various tissues of different size groups of both the bivalves and the mechanisms involved in their regulation are relevant themes for study.

CHAPTER 2

EXTRACELLULAR–FLUID ANISOSMOTIC REGULATION

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2.1 ROLE OF VALVE CLOSURE

2.1.1 INTRODUCTION

Bivalve molluscs protect their soft tissues from a variety of adverse environmental conditions by closing their shell valves (Akberali and Trueman, 1985). This behavioural response, to a great extent, helps them to invade the stressful environments like littoral region. The mechanism gains great significance, where a sedentary mode of life restricts the animal to a particular habitat. Valve adduction in bivalves in stressful salinities has been the subject of many studies: Trueman (1983), Akberali and Trueman (1985), Widdows (1985), Genovea *et al.* (1988), Berger (1989) and Supriya (1992).

Amongst bivalves, in general, behavioural responses probably contribute most, to their adaptation to fluctuations in salinity (Bayne, 1976). Valve closure in bivalves prevents drastic changes in osmotic concentration of their body fluids when exposed to short-term fluctuations in salinity (Shumway, 1977). This helps the animal to maintain a microenvironment for its tissues irrespective of the fluctuations in ambient salinity. Milne (1940) have described that the salinity within the mantle cavity of *Mytilus edulis* can be as high as $24x10^{-3}$, when the salinity of the ambient water is only $7x10^{-3}$. Since the animal cannot cut off itself for long periods from the ambient environment, the ambient medium and mantle cavity fluid gain contact and the latter gradually attains the external salinity. During this period of change acclimatization of the internal body fluids to the ambient

salinity does occur (Akberali, 1980). Hence, the tenure of valve adduction is a period of grace which prevents osmotic shock.

In bivalves, isolation of mantle cavity fluid from adverse environmental conditions comprises a three part process (Davenport, 1979). The events start with the closure of exhalant siphon, which ceases the irrigation of mantle cavity. Further change in salinity results in the closure of inhalant siphon. Valve closure occurs in most unfavourable salinities which produces virtually a complete isolation. The sequence of events has been studied for *Katelysia opima* (Mane, 1974), and *Donax dentriculatus* (Genovea *et al.*, 1988) and has found to be the same.

Pierce (1971b) has recorded valve movements by different species of *Modiolus*, during exposure to different levels of salinity. He observed that the animals close their valves on confronting a change in salinity and the period they remain closed could last for some days with short intermittent periods of opening. Studies have shown that during valve closure some exchange or contact with the environment is often maintained (Trueman, 1983; Supriya, 1992). The experiments in *Mytilus californianus* (Moon and Pritchard, 1970); *Mytilus edulis* (Coleman, 1972) and *Scrobicularia plana* (Akberali *et al.*, 1977; Akberali and Trueman, 1979), have indicated that during valve closure the mantle cavity fluid is not totally isolated from the ambient environment and a total isolation is noticed only in forced clamping. It has also been reported that during valve closure the animal intermittently opens the valves to monitor the prevailing environmental conditions (Akberali and Trueman, 1985).

Many bivalves studied so far, utilize valve closure as a protective mechanism to counteract transient or recurrent short-term adverse salinity changes. Such a mechanism is of great biological interest since it provides temporary exemption from the osmotic shock (Hoyaux *et al.*, 1976). Hence, any study on the osmotic and ionic regulations of bivalves necessarily has to consider the changes occurring in the mantle cavity fluid (Leader *et al.*, 1986). The extracellular fluid is always maintained in osmotic equilibrium with mantle cavity fluid, except in extreme conditions (Schoffeniels and Gilles, 1972). Therefore, any study of the extracellular fluid will not attain necessary relevance without considering the mantle cavity fluid.

The valve closing mechanism in maintaining a concentration gradient in the mantle cavity fluid against the ambient environment was monitored along with blood ionic regulation in different size groups of both the species. Sodium and chloride ions, being the dominant ones were selected for the purpose.

2.1.2 MATERIALS AND METHODS

S. scripta were collected from Fort Cochin area (between latitude 9° 28' and 10° 00' and longitude 76° 13' and 76° 11'E). They were brought to the laboratory in plastic bags (containing sea water collected from the same site) unexposed to the sun. In the laboratory, the lingering algae, dirt and the barnacles attached to the shells were cleaned off from the clams. The animals were sorted out into three different size groups as small

(20±2 mm), medium (30±2 mm) and large (40±2 mm).

Acclimation tank consisted of a large fibre glass tank provided with clean sand (natural substratum of the animal) and a biological filter. Well-aerated sea water of $30 \pm 2 \times 10^{-3}$ salinity (optimum salinity for this species; Supriya, 1992) was used for maintaining the animals. The animals were allowed to acclimate for two weeks in the laboratory conditions before being used for experiments. During this period, they were daily fed on blue green algae, *Synechocystis salina* (Wislouch). The sea water in the fibre glass tanks was changed once in two days (salinity = $30 \pm 2 \times 10^{-3}$, temperature = $28 \pm 0.5^{o}C$, pH = 7.85 ± 0.05 and dissolved oxygen >90% saturation).

Experimental tanks were also maintained with clean sand, biological filter and well-aerated sea water of different levels of salinity. All conditions like temperature, pH, dissolved oxygen and feed were the same as that of acclimation tank and only variable was salinity which was adjusted according to the experimental requirement. Salinity adjustment was done using deionized water. Clams acclimated to $30 \pm 2 \times 10^{-3}$ salinity were transferred to four tanks maintained in salinities of 5, 10, 15 and 40×10^{-3} for experiments. Approximately 300 animals were transferred to each tank. Blood and mantle cavity fluid were collected from a pool of six animals before transferring to the experimental salinities and during the period of experiment at regular intervals.

Mantle cavity fluid was collected using a clean dry syringe after blotting off the sea water from the shells. Blood samples

were also taken from the same pool of animals after draining out the remaining mantle cavity fluid and the water adhering to the animal using an absorbent paper. Blood was collected from the adductor sinus with a clean dry syringe. Monitoring the blood and mantle cavity fluid were carried out at regular intervals till the concentration of Na^+ and Cl^- in the mantle cavity fluid attained the ambient concentration.

The pooled samples of blood and mantle cavity fluid were centrifuged at 6,000 rpm for 30 min. to remove suspended particles. The supernatants were diluted with distilled water and were analyzed for sodium by Flame Photometric method of Robinson and Ovenston (1951) using Flame Photometer (Elico Type 22). The chloride content was determined using a Chloride Meter (Elico Model EE 34) which is designed for automatic colorimetric titration.

Perna viridis were collected from an unpolluted natural population attached to the sea wall near Narakkal, Cochin. Mussels were brought to the laboratory in a polythene bag filled with sea water taken from the same site. They were cleaned and divided into two size groups as small (35±2 mm) and large (65±2 mm).

Mussels were acclimated in large fibre glass tank provided with biological filter. Clean granite rock pieces were provided as substratum. The animals were allowed to acclimate to the same environmental and nutritive conditions as mentioned in the case of *S. scripta*.

Considering the salinity tolerance of the animal, the mussels acclimated to $30\pm2\times10^{-3}$ salinity were exposed to 15×10^{-3}

salinity. All conditions except salinity were maintained similar to that of the acclimation tank. Samples of blood and mantle fluid were collected, treated and analyzed as in the case of *S. scripta*.

2.1.3 RESULTS

It is noticed that in large size group of *S. scripta* exposed to $5x10^{-3}$ salinity (Tables 1 and 2), mantle cavity fluid is kept hyperosmotic to the ambient medium for a period of 85-136 h (Fig. 1 and 4). In the case of medium (Fig. 2 and 5) and small (Fig. 3 and 6) size groups, this hyperosmotic state is maintained only for a period of 48-72 h.

When exposed to $10x10^{-3}$ salinity (Tables 3 and 4), ionic equilibrium between mantle cavity fluid and sea water is attained in a period of 48-72 h in large size group (Fig. 7 and 10). But in medium (Fig. 8 and 11) and small (Fig. 9 and 12) size groups, the time taken to attain such a state is 24-30 h.

In $15x10^{-3}$ salinity (Tables 5 and 6), large size group attains ionic equilibrium between, 18-24 h (Fig. 13 and 16) and in medium (Fig. 14 and 17) and small (Fig. 15 and 18) size groups the period is 12-18 h.

In the animals subjected to $40x10^{-3}$ salinity (Tables 7 and 8), increase in ionic concentration of mantle cavity fluid and establishment of an isoionic condition between mantle cavity fluid and ambient sea water is attained in a period of 5-6 h in the case of large animals (Fig. 19 and 22) and of 3-4 h in the case of medium (Fig. 20 and 23) and small (Fig. 21 and 24) size groups.

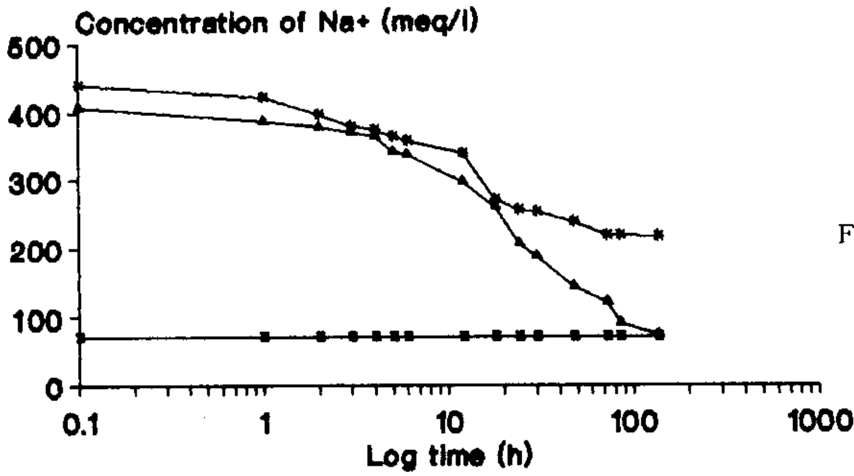

Fig. 1. Large

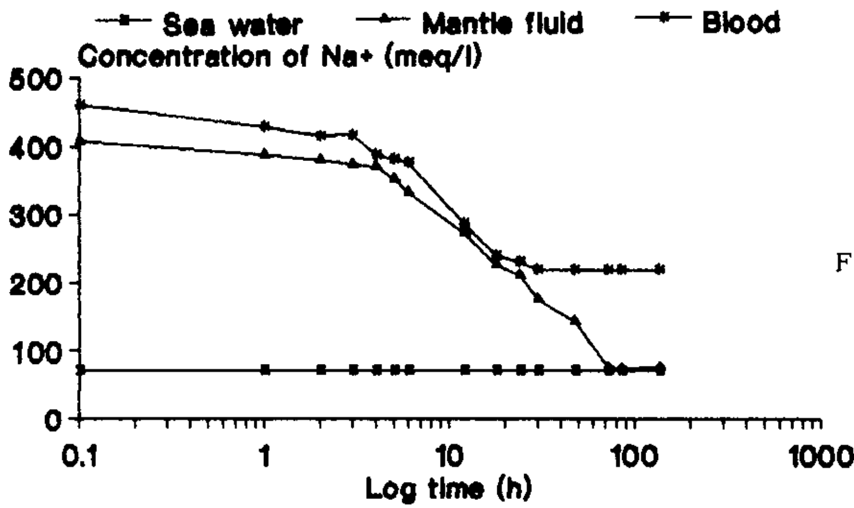

Fig. 2. Medium

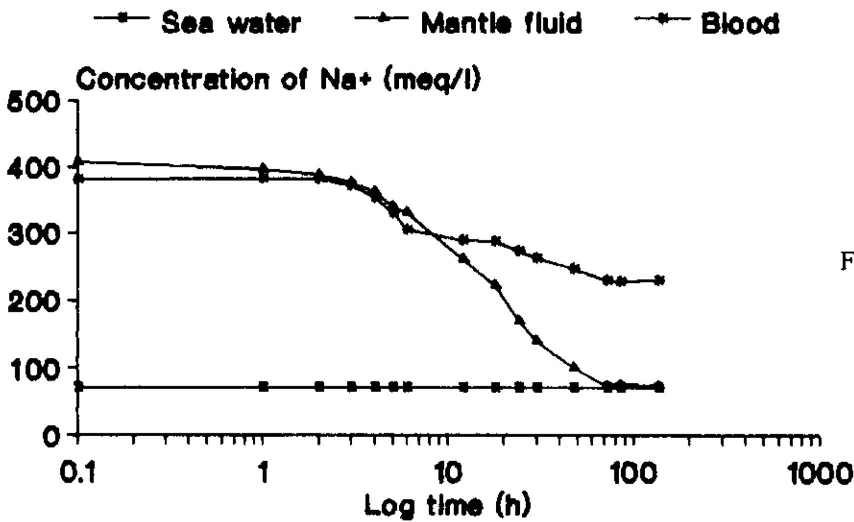

Fig. 3. Small

Concentration of Na^+ in the hemolymph and mantle cavity fluid of *Sunetta scripta* transferred to $5x10^{-3}$ salinity.

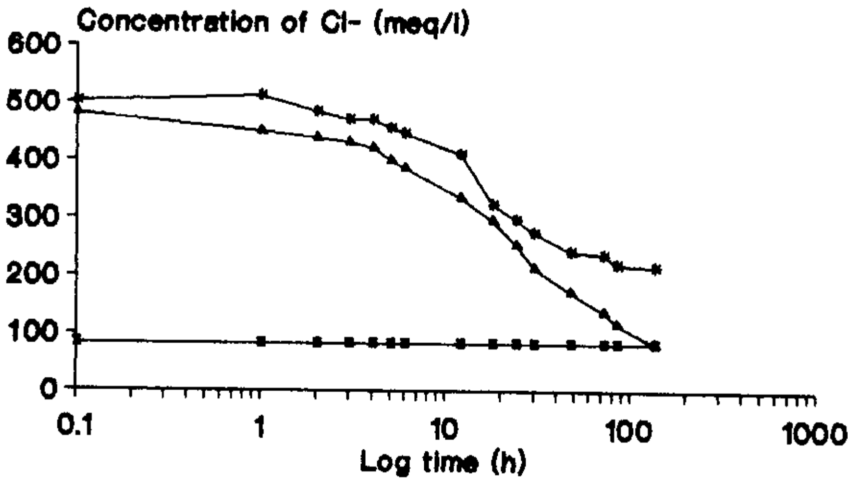

Fig. 4. Large

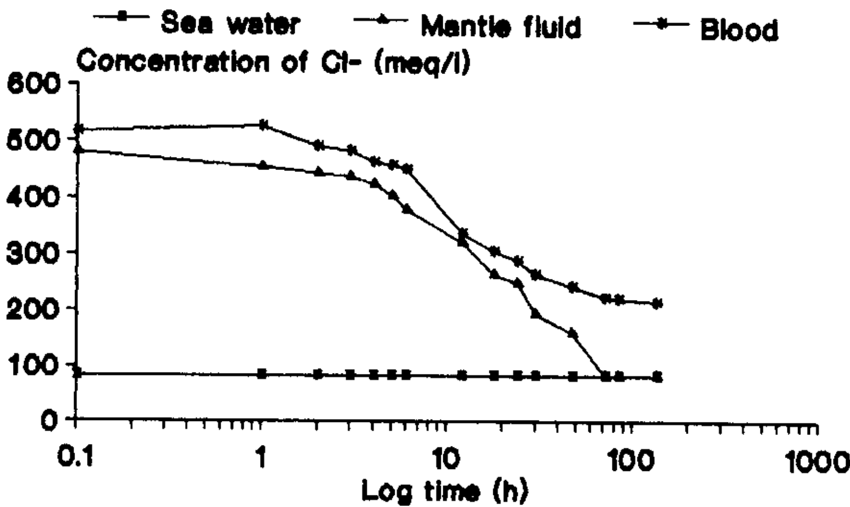

Fig. 5. Medium

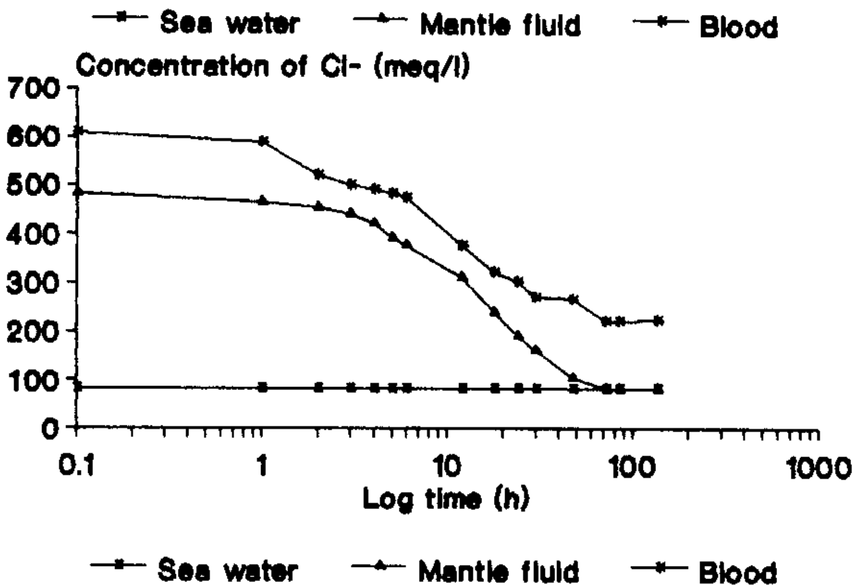

Fig. 6. Small

Concentration of Cl^- in the hemolymph and mantle cavity fluid of Sunetta scripta transferred to $5x10^{-3}$ salinity.

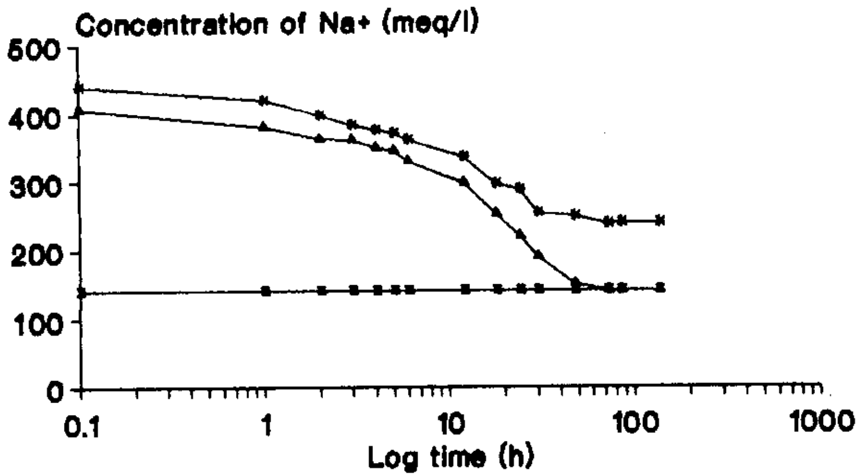

Fig. 7. Large

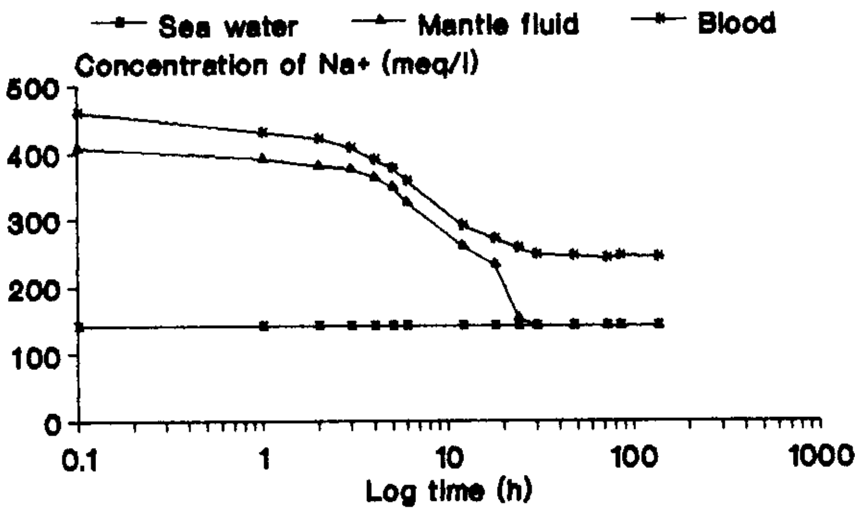

Fig. 8. Medium

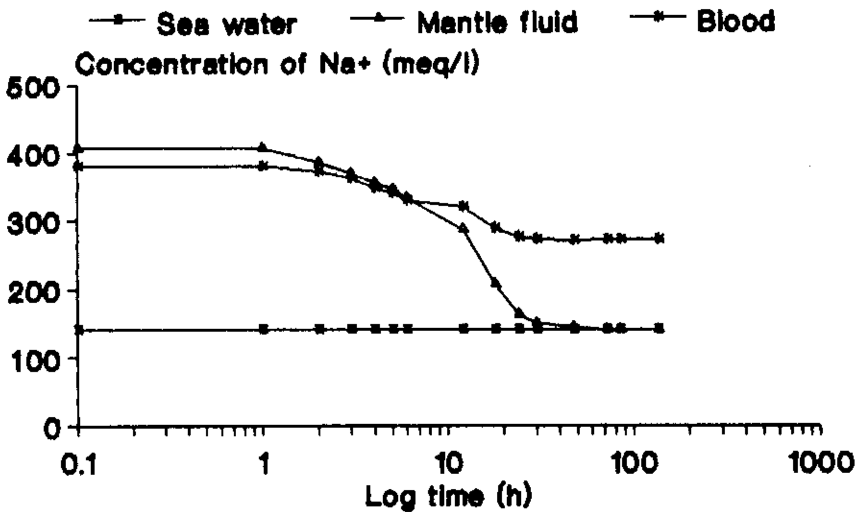

Fig. 9. Small

Concentration of Na^{+} in the hemolymph and mantle cavity fluid of *Sunetta Scripta* transferred to $10x10^{-3}$ salinity.

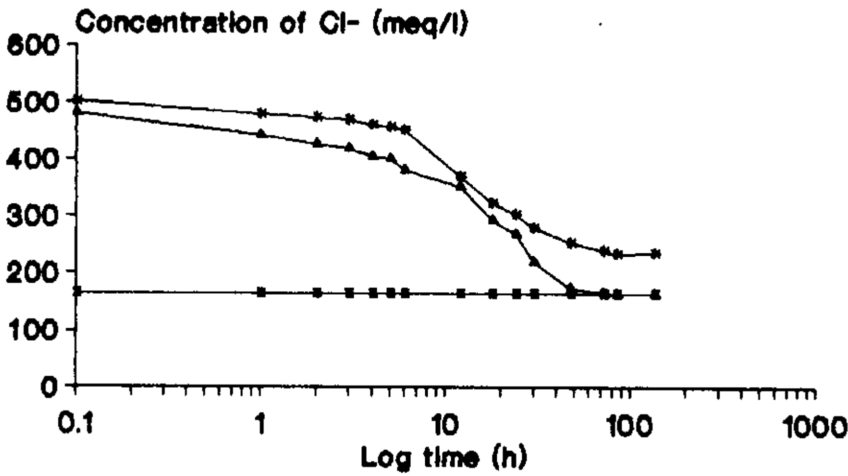

Fig. 10. Large

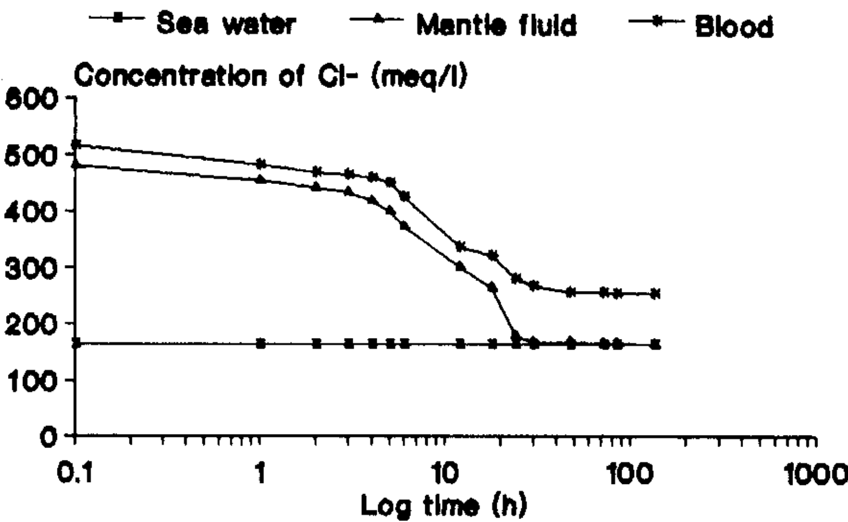

Fig. 11. Medium

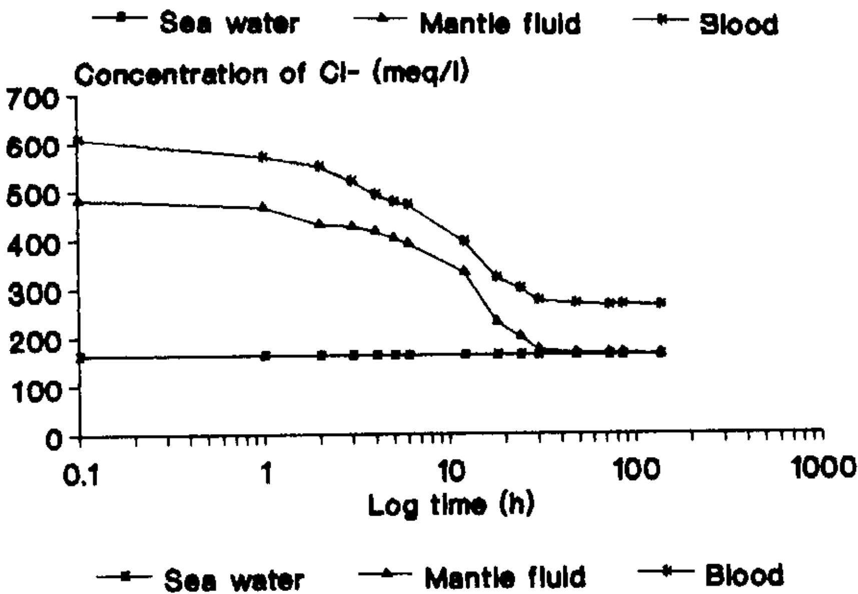

Fig. 12. Small

Concentration of Cl^- in the hemolymph and mantle cavity fluid of *Sunetta scripta* transferred to 10×10^{-3} salinity.

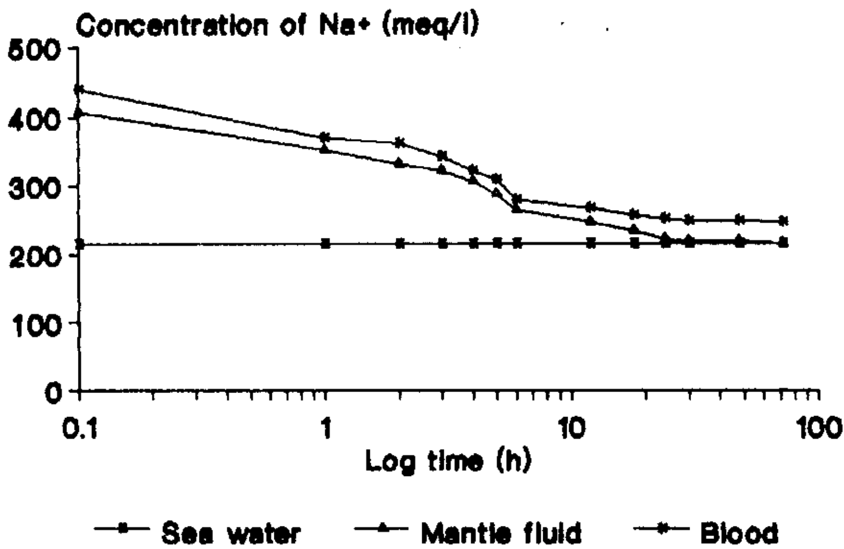

Fig. 13. Large

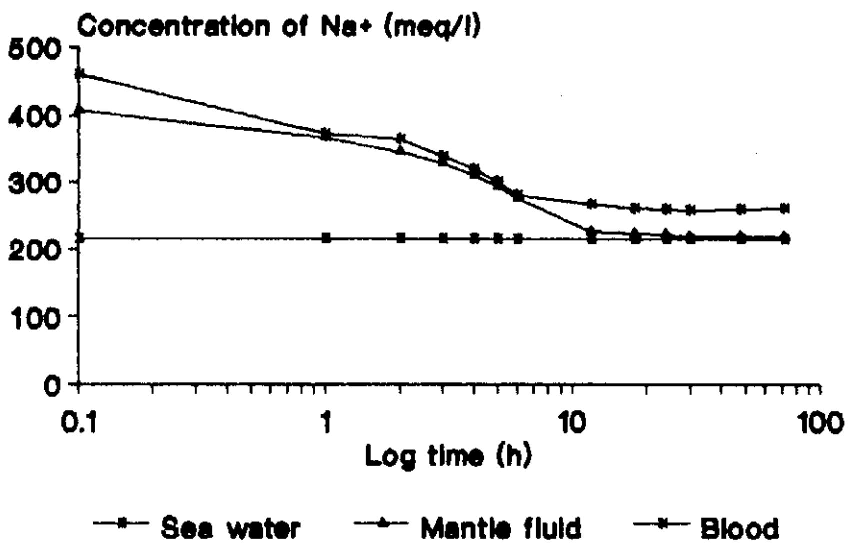

Fig. 14. Medium

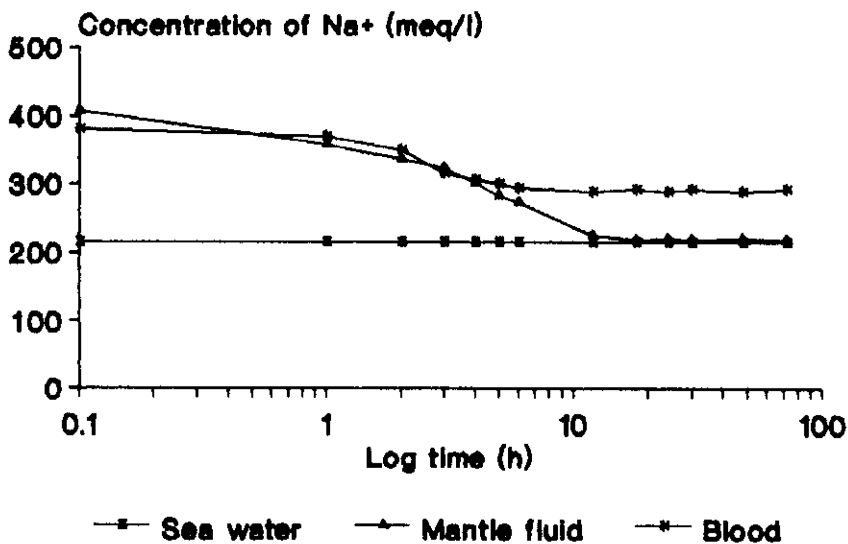

Fig. 15. Small

Concentration of Na^+ in the hemolymph and mantle cavity fluid of *Sunetta scripta* transferred to 15×10^{-3} salinity.

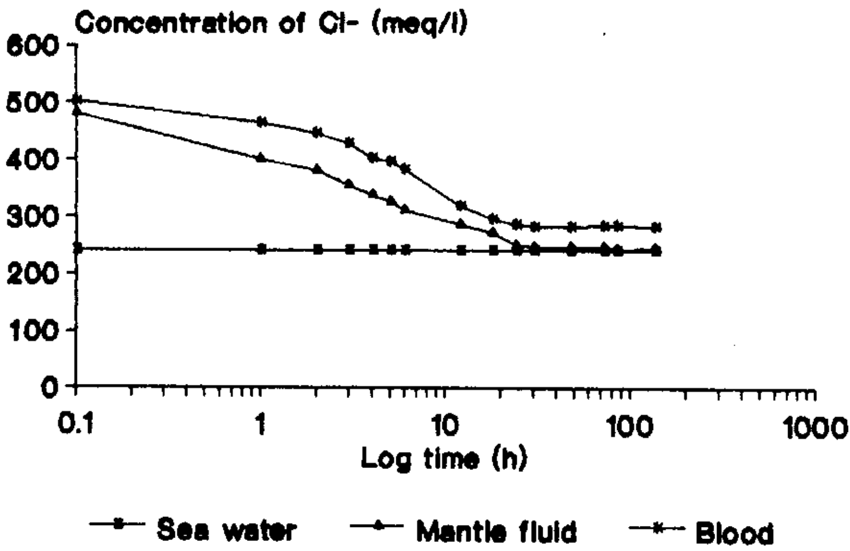

Fig. 16. Large

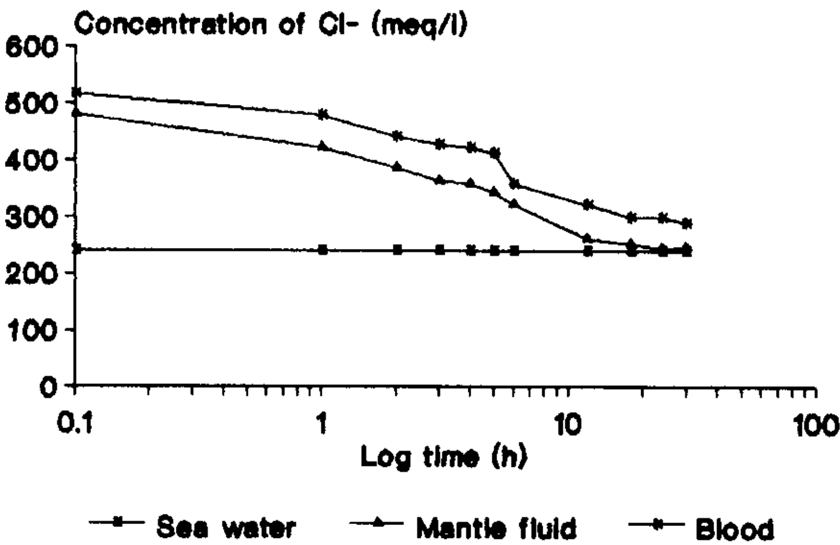

Fig. 17. Medium

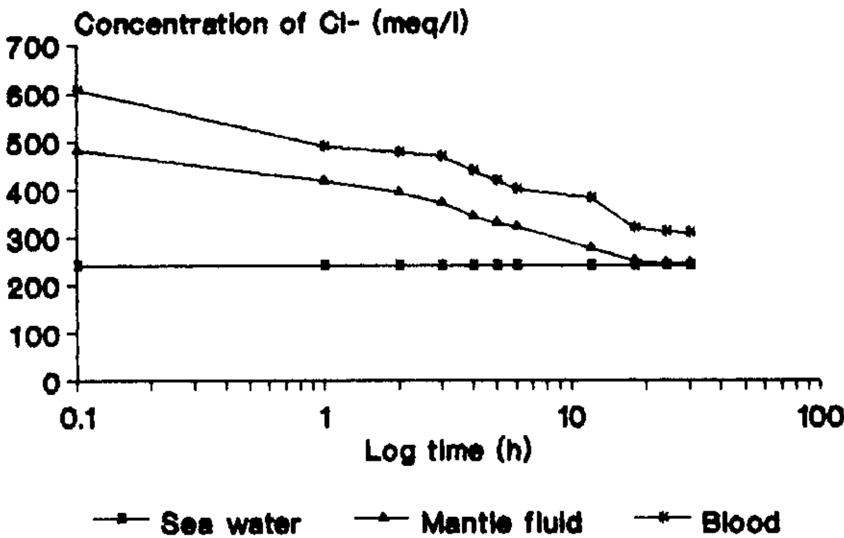

Fig. 18. Small

Concentration of Cl^- in the hemolymph and mantle cavity fluid of <u>Sunetta scripta</u> transferred to $15x10^{-3}$ salinity.

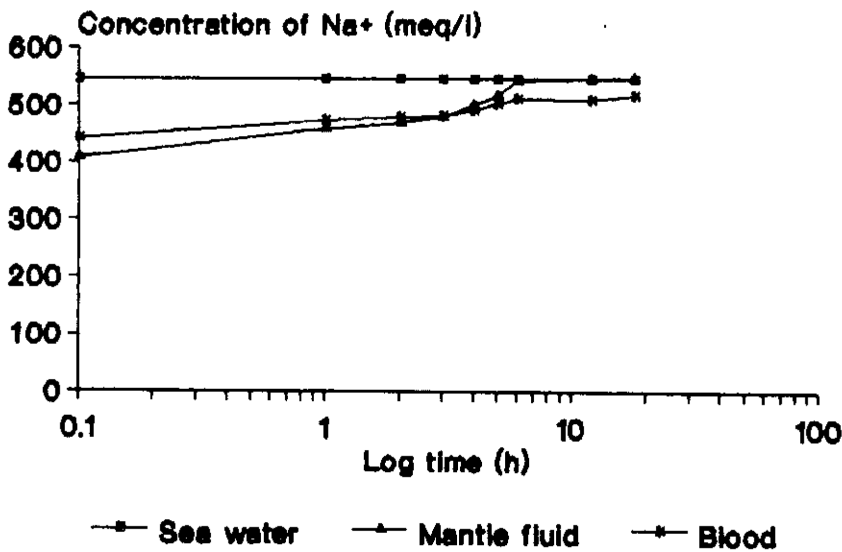

Fig. 19. Large

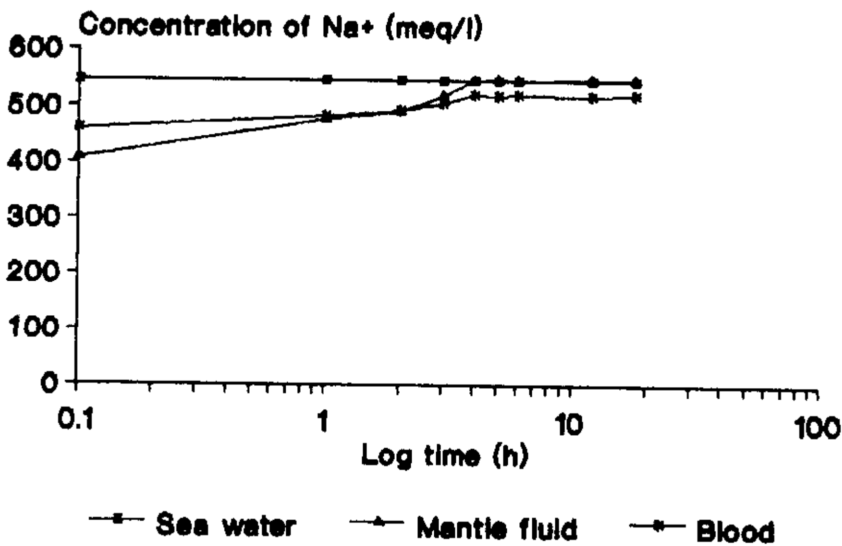

Fig. 20. Medium

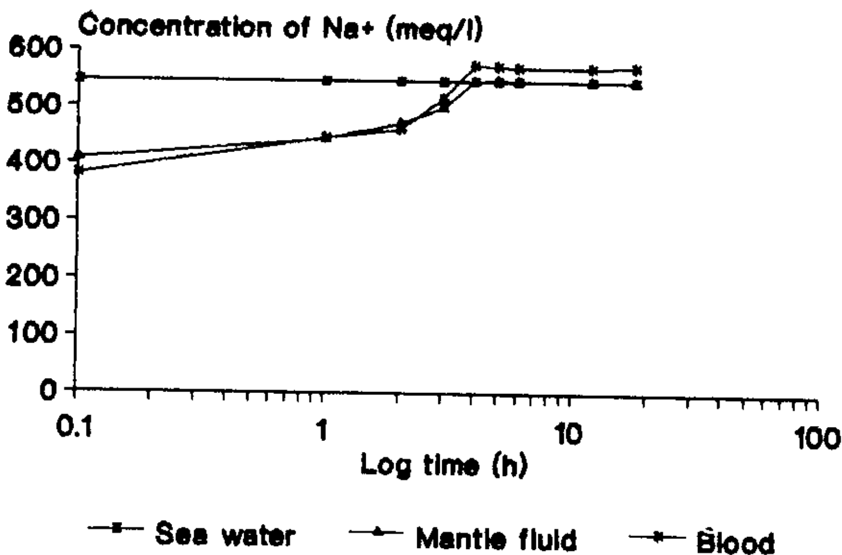

Fig. 21. Small

Concentration of Na^+ in the hemolymph and mantle cavity fluid of *Sunetta scripta* transferred to 40×10^{-3} salinity.

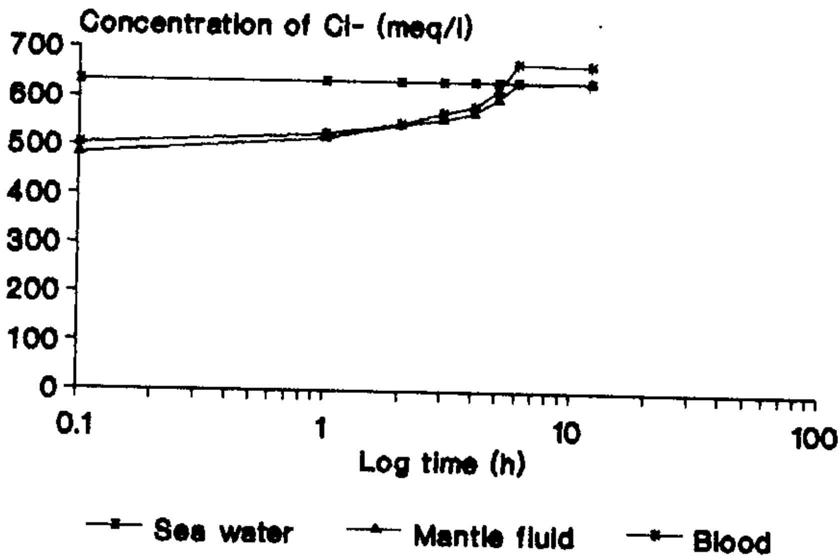

Fig. 22. Large

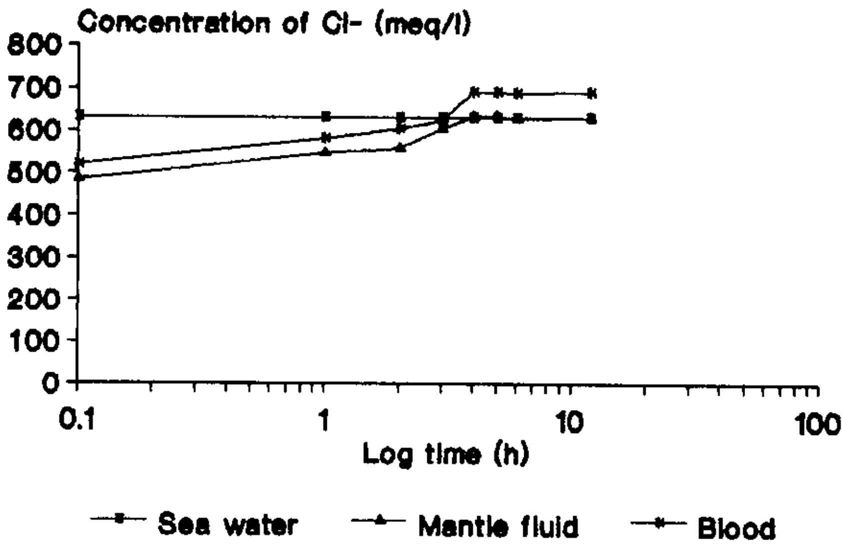

Fig. 23. Medium

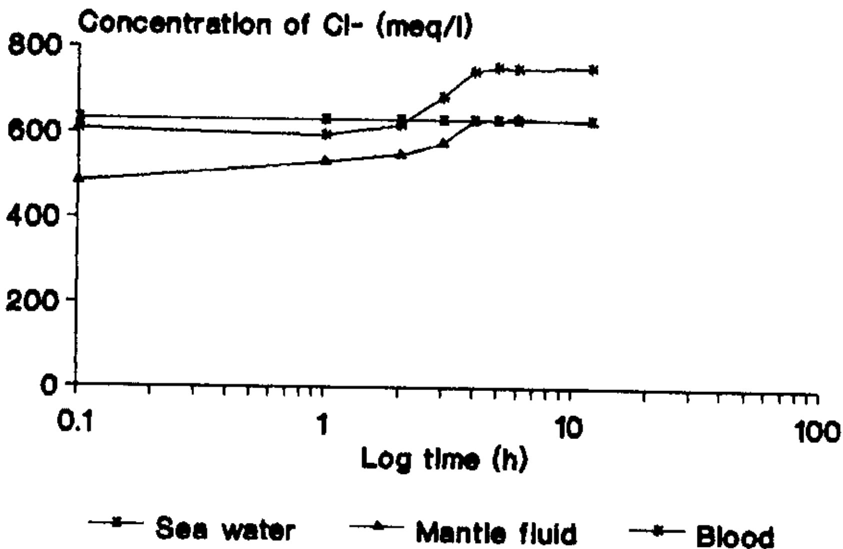

Fig. 24. Small

Concentration of Cl^- in the hemolymph and mantle cavity fluid of *Sunetta scripta* transferred to $40x10^{-3}$ salinity.

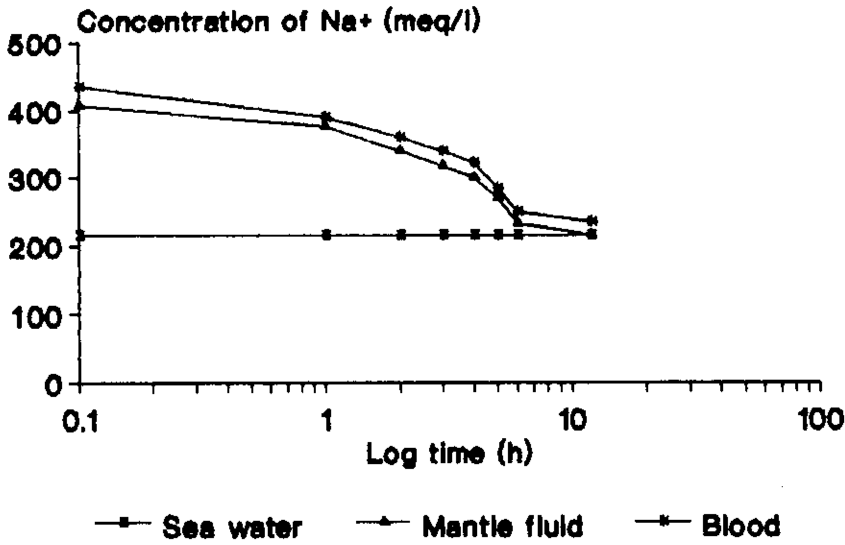

Fig. 25. Large

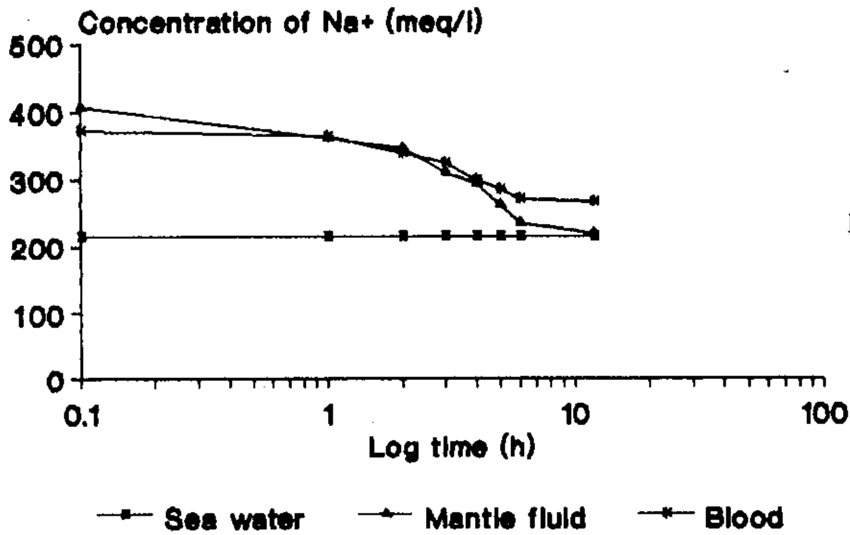

Fig. 26. Small

Concentration of Na^+ in the hemolymph and mantle cavity fluid of Perna viridis transferred to $15x10^{-3}$ salinity.

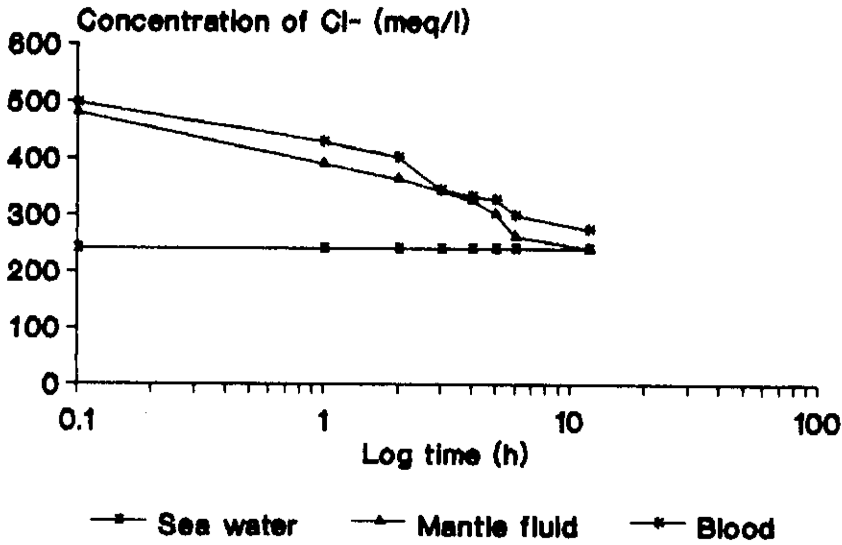

Fig. 27. Large

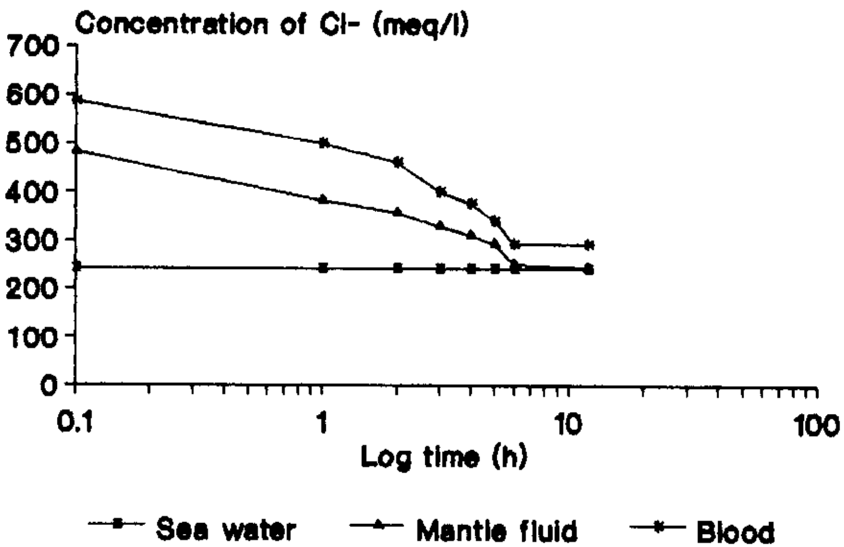

Fig. 28. Small

Concentration of Cl^- in the hemolymph and mantle cavity fluid of <u>Perna viridis</u> transferred to 15×10^{-3} salinity.

The two size groups of *P. viridis*, from the same population were transferred from acclimation tank to $15x10^{-3}$salinity. Other salinities experimented in the case of *S. scripta* were not used considering the lower range of tolerance of this species. The ionic variations in blood and mantle cavity fluid are given in Tables 9 and 10. It is found that in both the size groups the ionic equilibrium between mantle cavity fluid and ambient sea water is attained in a period of 6-12 h (Fig. 25, 26, 27 and 28).

In both *S. scripta* and *P. viridis* decrease in the concentration of $Na^+$and Cl^- in the mantle cavity fluid is followed by a decrease in the concentration of these ions in the blood. It is also noticed that the blood always maintains a slightly higher concentration of these ions than that in the mantle cavity fluid.

2.1.4 DISCUSSION

Any study on the physiological mechanisms of bivalves in response to changes in salinity must consider the possible role of the behavioural response (Burton, 1983; Akberali and Trueman, 1985). The prominent behavioural mechanism by which bivalves respond to changes in salinity is valve closure. It eliminates sudden contact with the ambient environment and ceases irrigation of the mantle cavity. The duration and degree of valve closure in any salinity depend upon the extent of unfavourableness of that ambient medium. But this behavioural avoidance is only a period of temporary exemption under long-term salinity variations and acts as an interim measure to make cellular adjustments. In addition,

the grace due to valve closure provides an absolute means to tide over short-term salinity fluctuations of their ambient medium. Studies on the ionic concentration of mantle cavity fluid along with ambient environment clearly indicate these points.

In the case of *S. scripta*, the mantle cavity fluid is maintained at a higher concentration when transferred to lower salinities and at a lower concentration when exposed to 40×10^{-3} salinity for a considerably long period, but the homogeneity of the mantle cavity fluid with the ambient environment is attained faster in salinities closer to the optimum, whereas, in salinities which considerably deviate from the optimum, the time taken to attain this condition is longer. It has already been noted by several workers that the tenure of temporary exemption from the ambient environment increases towards the extremes of salinity (Schoffeniels and Gilles, 1972; Akberali, 1978; Leader *et al*., 1986). In the case of *S. scripta* maximum time is taken in 5×10^{-3} which has already been reported as the most unfavourable salinity for this species (Supriya, 1992).

When different size groups are considered, the medium and the small size groups attain homogeneity of the mantle cavity fluid with the ambient environment at a faster rate than the large size group. Previous studies have indicated that the larger size groups of molluscs are less equipped to withstand extremes of salinity than the smaller size groups (Kinne, 1971; Skinner and Peretz, 1989). Thampuran *et al*. (1982) have made similar observation in *S. scripta* also. It is possible that cellular adjustments in response to changes in salinity may be slower in

large size group than medium and small sized animals. This may be the reason for their greater dependence on valve closure mechanism. Most probably, the higher metabolic rate of smaller animals may be helping them to evoke intracellular adjustment faster than large size group. The valve closure mechanism also helps to prevent sudden exposure of the tissue to osmotic shock and offers time for the cell to gradually adjust to the altered environment.

In the case of *P. viridis*, the changes in the ionic concentration of mantle cavity fluid indicate a similar pattern as in the case of *S. scripta*. But this species is depending on the mechanism for a much shorter time than *S. scripta*.

The generalized picture that is coming out of these experiments is the maintenance of hyperosmotic mantle cavity fluid in animals transferred to hypoosmotic sea water. The exposure of *S. scripta* to hyperosmotic sea water exhibits a maintenance of hypoosmotic mantle cavity fluid. In all experiments, the mantle cavity fluid attains isosmoticity with the ambient medium after a lag of time. The change in ionic content in the mantle cavity fluid is reflected without much delay in their concentrations in the hemolymph. This may be taken as an expression of lack of anisosmotic extracellular regulation in both the bivalves. The transitory hyper or hypoosmotic state observed in the blood of the bivalves is a reflection of a similar situation in the mantle cavity fluid. It seems that the main purpose of valve closure is to reduce the impact of osmotic shock and is of immense significance in relation to their intertidal habitat.

TABLE NO : 1

Concentration of Na^+ in the hemolymph and mantle cavity fluid of *S. scripta* transferred to a salinity of $5x10^{-3}$.

Concentration of Na^+ is expressed in meq/litre.

TIME (h)	SIZE GROUPS					
	LARGE (40±2mm)		MEDIUM (30±2mm)		SMALL (20±2mm)	
	MANTLE $[Na^+]$	BLOOD $[Na^+]$	MANTLE $[Na^+]$	BLOOD $[Na^+]$	MANTLE $[Na^+]$	BLOOD $[Na^+]$
0	408 ± 03	441 ± 05	408 ± 03	461 ± 09	408 ± 03	381 ± 06
1	387 ± 12	422 ± 08	389 ± 12	430 ± 09	397 ± 13	382 ± 11
2	378 ± 11	397 ± 13	380 ± 11	415 ± 10	389 ± 09	382 ± 14
3	369 ± 19	379 ± 10	374 ± 13	417 ± 09	376 ± 09	371 ± 08
4	363 ± 07	372 ± 09	371 ± 11	389 ± 10	362 ± 10	354 ± 12
5	342 ± 07	364 ± 12	354 ± 12	382 ± 08	339 ± 10	330 ± 09
6	336 ± 07	357 ± 08	333 ± 10	377 ± 09	331 ± 09	305 ± 09
12	296 ± 13	338 ± 11	273 ± 09	287 ± 12	261 ± 11	289 ± 09
18	259 ± 06	270 ± 10	226 ± 08	241 ± 07	222 ± 08	287 ± 08
24	206 ± 08	254 ± 10	211 ± 09	231 ± 09	170 ± 11	274 ± 08
30	187 ± 09	252 ± 09	176 ± 11	218 ± 06	139 ± 08	263 ± 06
48	143 ± 13	238 ± 12	143 ± 10	219 ± 06	100 ± 09	246 ± 09
72	121 ± 07	217 ± 10	075 ± 03	218 ± 05	073 ± 02	229 ± 06
85	091 ± 09	217 ± 06	074 ± 03	219 ± 06	075 ± 03	228 ± 05
136	073 ± 02	215 ± 04	075 ± 03	218 ± 05	074 ± 03	229 ± 05

Values are the mean of six different observations ± SD.

Each observation is from a sample of six animals pooled together.

TABLE NO : 2

Concentration of Cl^- in the hemolymph and mantle cavity fluid of *S. scripta* transferred to a salinity of $5x10^{-3}$.
Concentration of Cl^- is expressed in meq/litre.

TIME (h)	SIZE GROUPS					
	LARGE (40±2mm)		MEDIUM (30±2mm)		SMALL (20±2mm)	
	MANTLE $[Cl^-]$	BLOOD $[Cl^-]$	MANTLE $[Cl^-]$	BLOOD $[Cl^-]$	MANTLE $[Cl^-]$	BLOOD $[Cl^-]$
0	482 ± 02	502 ± 11	482 ± 02	518 ± 07	482 ± 02	607 ± 07
1	449 ± 10	511 ± 07	455 ± 08	527 ± 06	465 ± 07	588 ± 09
2	438 ± 05	484 ± 08	443 ± 06	491 ± 08	453 ± 08	520 ± 07
3	431 ± 07	470 ± 06	437 ± 07	483 ± 06	440 ± 09	500 ± 08
4	421 ± 09	470 ± 08	424 ± 10	465 ± 09	421 ± 08	491 ± 07
5	398 ± 06	455 ± 09	404 ± 05	458 ± 06	392 ± 07	481 ± 05
6	384 ± 08	446 ± 10	379 ± 11	451 ± 05	377 ± 08	474 ± 09
12	333 ± 07	409 ± 09	321 ± 08	338 ± 08	311 ± 06	376 ± 07
18	295 ± 09	323 ± 11	264 ± 06	306 ± 10	240 ± 05	322 ± 06
24	253 ± 06	297 ± 06	248 ± 09	289 ± 06	190 ± 10	302 ± 08
30	213 ± 09	274 ± 08	194 ± 08	264 ± 04	163 ± 08	271 ± 09
48	171 ± 06	241 ± 06	160 ± 06	243 ± 09	106 ± 07	266 ± 10
72	138 ± 09	235 ± 09	083 ± 08	223 ± 07	083 ± 09	223 ± 07
85	116 ± 10	218 ± 12	082 ± 09	220 ± 06	082 ± 08	223 ± 05
136	083 ± 07	215 ± 08	082 ± 06	215 ± 08	083 ± 05	224 ± 04

Values are the mean of six different observations ± SD.
Each observation is from a sample of six animals pooled together.

TABLE NO : 3

Concentration of Na^+ in the hemolymph and mantle cavity fluid of *S. scripta* transferred to a salinity of $10x10^{-3}$.

Concentration of Na^+ is expressed in meq/litre.

TIME (h)	SIZE GROUPS					
	LARGE (40±2mm)		MEDIUM (30±2mm)		SMALL (20±2mm)	
	MANTLE $[Na^+]$	BLOOD $[Na^+]$	MANTLE $[Na^+]$	BLOOD $[Na^+]$	MANTLE $[Na^+]$	BLOOD $[Na^+]$
0	408 ± 03	441 ± 05	408 ± 03	461 ± 09	408 ± 03	381 ± 06
1	380 ± 08	420 ± 10	392 ± 08	431 ± 09	407 ± 09	380 ± 07
2	364 ± 08	398 ± 10	380 ± 07	421 ± 08	387 ± 10	372 ± 06
3	361 ± 09	384 ± 11	376 ± 08	407 ± 10	369 ± 08	364 ± 07
4	351 ± 08	376 ± 06	364 ± 11	390 ± 08	357 ± 08	349 ± 08
5	346 ± 07	371 ± 07	349 ± 10	378 ± 09	347 ± 05	342 ± 06
6	331 ± 08	361 ± 11	325 ± 05	358 ± 08	334 ± 07	331 ± 06
12	298 ± 11	337 ± 07	261 ± 07	291 ± 09	288 ± 06	320 ± 08
18	253 ± 08	297 ± 09	234 ± 08	272 ± 07	208 ± 10	289 ± 07
24	220 ± 09	289 ± 11	152 ± 05	257 ± 12	163 ± 09	276 ± 06
30	190 ± 06	254 ± 06	142 ± 08	249 ± 05	151 ± 08	273 ± 06
48	150 ± 08	250 ± 07	142 ± 00	246 ± 05	145 ± 02	272 ± 06
72	142 ± 00	239 ± 06	142 ± 00	244 ± 05	142 ± 03	273 ± 05
85	142 ± 00	240 ± 06	142 ± 00	246 ± 03	141 ± 02	274 ± 05
136	142 ± 00	241 ± 06	142 ± 00	245 ± 03	142 ± 02	273 ± 04

Values are the mean of six different observations ± SD.

Each observation is from a sample of six animals pooled together.

TABLE NO : 4

Concentration of Cl^- in the hemolymph and mantle cavity fluid of *S. scripta* transferred to a salinity of $10x10^{-3}$.
Concentration of Cl^- is expressed in meq/litre.

TIME (h)	SIZE GROUPS					
	LARGE (40±2mm)		MEDIUM (30±2mm)		SMALL (20±2mm)	
	MANTLE [Cl^-]	BLOOD [Cl^-]	MANTLE [Cl^-]	BLOOD [Cl^-]	MANTLE [Cl^-]	BLOOD [Cl^-]
0	482 ± 02	502 ± 11	482 ± 02	518 ± 07	482 ± 02	607 ± 07
1	442 ± 08	479 ± 09	455 ± 08	484 ± 08	466 ± 09	571 ± 08
2	427 ± 09	473 ± 10	442 ± 09	470 ± 09	432 ± 08	550 ± 06
3	419 ± 11	469 ± 08	434 ± 07	466 ± 10	426 ± 09	520 ± 09
4	404 ± 10	460 ± 07	419 ± 06	461 ± 09	416 ± 08	494 ± 06
5	400 ± 08	456 ± 06	400 ± 08	452 ± 07	402 ± 07	478 ± 07
6	382 ± 07	451 ± 08	373 ± 10	426 ± 08	391 ± 06	471 ± 08
12	351 ± 09	369 ± 08	301 ± 06	338 ± 07	332 ± 08	397 ± 09
18	293 ± 06	322 ± 09	264 ± 06	323 ± 08	230 ± 06	321 ± 06
24	267 ± 09	301 ± 11	178 ± 07	281 ± 07	199 ± 05	300 ± 10
30	219 ± 10	279 ± 08	167 ± 08	267 ± 08	174 ± 08	274 ± 09
48	174 ± 07	252 ± 06	167 ± 05	257 ± 07	167 ± 09	269 ± 06
72	165 ± 06	240 ± 07	165 ± 05	256 ± 05	167 ± 08	264 ± 05
85	164 ± 08	233 ± 06	166 ± 04	255 ± 07	167 ± 06	265 ± 04
136	163 ± 05	236 ± 08	163 ± 05	254 ± 05	165 ± 05	264 ± 03

Values are the mean of six different observations ± SD.
Each observation is from a sample of six animals pooled together.

TABLE NO : 5

Concentration of Na^+ in the hemolymph and mantle cavity fluid of *S. scripta* transferred to a salinity of $15x10^{-3}$.
Concentration of Na^+ is expressed in meq/litre.

TIME (h)	SIZE GROUPS					
	LARGE (40±2mm)		MEDIUM (30±2mm)		SMALL (20±2mm)	
	MANTLE $[Na^+]$	BLOOD $[Na^+]$	MANTLE $[Na^+]$	BLOOD $[Na^+]$	MANTLE $[Na^+]$	BLOOD $[Na^+]$
0	408 ± 03	441 ± 05	408 ± 03	461 ± 09	408 ± 03	381 ± 06
1	352 ± 08	370 ± 09	367 ± 09	373 ± 10	359 ± 08	369 ± 08
2	332 ± 07	362 ± 07	346 ± 06	365 ± 09	336 ± 09	351 ± 07
3	322 ± 07	343 ± 07	328 ± 06	339 ± 08	324 ± 11	316 ± 09
4	306 ± 07	322 ± 11	312 ± 05	320 ± 08	302 ± 07	306 ± 11
5	287 ± 07	310 ± 07	294 ± 07	302 ± 07	283 ± 10	300 ± 05
6	264 ± 07	280 ± 06	277 ± 06	282 ± 07	273 ± 08	294 ± 06
12	246 ± 06	267 ± 05	227 ± 05	267 ± 07	225 ± 09	290 ± 08
18	234 ± 08	256 ± 05	223 ± 05	261 ± 06	219 ± 05	292 ± 06
24	221 ± 05	252 ± 06	221 ± 03	260 ± 05	220 ± 06	290 ± 05
30	218 ± 01	248 ± 05	219 ± 02	258 ± 08	218 ± 05	293 ± 08

TABLE NO : 6

Concentration of Cl^- in the hemolymph and mantle cavity fluid of *S. scripta* transferred to a salinity of $15x10^{-3}$.
Concentration of Cl^- is expressed in meq/litre.

TIME (h)	SIZE GROUPS					
	LARGE (40±2mm)		MEDIUM (30±2mm)		SMALL (20±2mm)	
	MANTLE $[Cl^-]$	BLOOD $[Cl^-]$	MANTLE $[Cl^-]$	BLOOD $[Cl^-]$	MANTLE $[Cl^-]$	BLOOD $[Cl^-]$
0	482 ± 02	502 ± 11	482 ± 02	518 ± 07	482 ± 02	607 ± 07
1	400 ± 08	464 ± 09	422 ± 08	480 ± 10	418 ± 10	491 ± 08
2	381 ± 07	445 ± 08	387 ± 09	441 ± 09	394 ± 09	477 ± 07
3	354 ± 08	428 ± 07	365 ± 06	428 ± 11	372 ± 08	468 ± 06
4	337 ± 09	401 ± 11	359 ± 07	422 ± 08	344 ± 06	441 ± 09
5	327 ± 08	397 ± 07	343 ± 09	414 ± 06	331 ± 07	419 ± 06
6	311 ± 09	383 ± 08	322 ± 08	359 ± 07	322 ± 08	400 ± 08
12	286 ± 05	318 ± 06	263 ± 10	323 ± 08	276 ± 07	382 ± 07
18	271 ± 09	296 ± 07	252 ± 09	299 ± 07	251 ± 08	318 ± 06
24	251 ± 07	286 ± 08	245 ± 08	299 ± 08	247 ± 06	311 ± 09
30	246 ± 09	282 ± 07	246 ± 06	291 ± 06	246 ± 05	308 ± 05

Values are the mean of six different observations ± SD.
Each observation is from a sample of six animals pooled together.

TABLE NO : 7

Concentration of Na^+ in the hemolymph and mantle cavity fluid of *S. scripta* transferred to a salinity of 40×10^{-3}.

Concentration of Na^+ is expressed in meq/litre.

TIME (h)	SIZE GROUPS					
	LARGE (40±2mm)		MEDIUM (30±2mm)		SMALL (20±2mm)	
	MANTLE $[Na^+]$	BLOOD $[Na^+]$	MANTLE $[Na^+]$	BLOOD $[Na^+]$	MANTLE $[Na^+]$	BLOOD $[Na^+]$
0	408 ± 03	441 ± 05	408 ± 03	461 ± 09	408 ± 03	381 ± 06
1	459 ± 09	474 ± 09	478 ± 11	484 ± 09	444 ± 08	446 ± 11
2	470 ± 11	479 ± 08	490 ± 09	492 ± 11	471 ± 05	460 ± 13
3	480 ± 06	482 ± 10	520 ± 08	505 ± 07	499 ± 09	516 ± 08
4	501 ± 10	490 ± 06	545 ± 10	521 ± 12	546 ± 08	573 ± 07
5	518 ± 08	502 ± 11	547 ± 06	520 ± 06	547 ± 06	572 ± 09
6	541 ± 12	511 ± 09	546 ± 08	521 ± 08	545 ± 09	570 ± 08
12	545 ± 09	509 ± 05	548 ± 09	520 ± 09	546 ± 08	571 ± 09

TABLE NO : 8

Concentration of Cl^- in the hemolymph and mantle cavity fluid of *S. scripta* transferred to a salinity of 40×10^{-3}.

Concentration of Cl^- is expressed in meq/litre.

TIME (h)	SIZE GROUPS					
	LARGE (40±2mm)		MEDIUM (30±2mm)		SMALL (20±2mm)	
	MANTLE $[Cl^-]$	BLOOD $[Cl^-]$	MANTLE $[Cl^-]$	BLOOD $[Cl^-]$	MANTLE $[Cl^-]$	BLOOD $[Cl^-]$
0	482 ± 02	502 ± 11	482 ± 02	518 ± 07	482 ± 02	607 ± 07
1	516 ± 09	523 ± 09	546 ± 12	582 ± 10	530 ± 08	595 ± 07
2	543 ± 11	545 ± 08	559 ± 08	604 ± 06	548 ± 09	620 ± 06
3	555 ± 10	569 ± 07	604 ± 09	624 ± 06	576 ± 07	684 ± 09
4	569 ± 08	582 ± 06	634 ± 08	693 ± 08	628 ± 06	746 ± 10
5	597 ± 12	615 ± 11	634 ± 07	691 ± 07	632 ± 08	756 ± 12
6	629 ± 10	669 ± 06	630 ± 05	689 ± 06	634 ± 05	753 ± 08
12	631 ± 05	668 ± 05	632 ± 04	692 ± 08	630 ± 06	755 ± 07

Values are the mean of six different observations ± SD.

Each observation is from a sample of six animals pooled together.

TABLE NO : 9

Concentration of Na^+ in the hemolymph and mantle cavity fluid of *P. viridis* transferred to a salinity of 15×10^{-3}.
Concentration of Na^+ is expressed in meq/litre.

TIME (h)	SIZE GROUPS			
	LARGE (65±2mm)		SMALL (35±2mm)	
	MANTLE [Na^+]	BLOOD [Na^+]	MANTLE [Na^+]	BLOOD [Na^+]
0	408 ± 03	436 ± 09	408 ± 03	373 ± 09
1	376 ± 09	390 ± 10	362 ± 09	365 ± 12
2	340 ± 12	360 ± 08	348 ± 12	340 ± 08
3	318 ± 10	339 ± 11	312 ± 09	325 ± 09
4	301 ± 11	323 ± 08	294 ± 10	300 ± 08
5	270 ± 13	284 ± 09	262 ± 08	286 ± 07
6	232 ± 09	250 ± 07	236 ± 09	272 ± 05
12	216 ± 06	235 ± 06	218 ± 07	268 ± 07

TABLE NO : 10

Concentration of Cl^- in the hemolymph and mantle cavity fluid of *P. viridis* transferred to a salinity of 15×10^{-3}.
Concentration of Cl^- is expressed in meq/litre.

TIME (h)	SIZE GROUPS			
	LARGE (65±2mm)		SMALL (35±2mm)	
	MANTLE [Cl^-]	BLOOD [Cl^-]	MANTLE [Cl^-]	BLOOD [Cl^-]
0	482 ± 04	498 ± 10	482 ± 04	585 ± 05
1	390 ± 11	430 ± 10	382 ± 09	500 ± 12
2	364 ± 10	402 ± 08	356 ± 10	460 ± 09
3	344 ± 13	345 ± 12	330 ± 08	400 ± 10
4	326 ± 11	334 ± 08	311 ± 11	376 ± 09
5	302 ± 09	328 ± 09	292 ± 09	340 ± 08
6	263 ± 08	300 ± 09	250 ± 08	292 ± 07
12	241 ± 06	276 ± 06	243 ± 07	293 ± 05

Values are the mean of six different observations ± SD.
Each observation is from a sample of six animals pooled together.

2.2 CONTRIBUTION OF IONIC REGULATION

2.2.1 INTRODUCTION

As far as the living cells are concerned the concentration of intracellular and extracellular inorganic ions are of great functional significance and this is especially true in the case of few species of inorganic ions. These ions function as cofactors in many enzyme reactions, provide chemical gradient, act as stores of potential energy and influence the permeability of biological membranes to other solutes (Gilles, 1975; Burton, 1983; Prusch, 1983).

Regulation of inorganic ions occurs essentially at two levels, between cell fluid and body cavity fluids and between body cavity fluids and external medium (Berger *et al.*, 1978). As a matter of fact, in many species, concentration of several inorganic ions in the blood is maintained at a level different from that in the environment through ionic regulation. The primary aim of the mechanism is to provide the necessary microenvironment for the cell to function. However, in some euryhaline species exposed to diluted media, the ionic regulation contributes substantially to the blood osmotic pressure regulation (Bedford, 1972; Stickle and Ahokas, 1975; Deaton, 1981; Deaton *et al.*, 1989).

Molluscs of the aquatic realm vary greatly in their ability to regulate the ions in their internal media. The extent of regulation is high in freshwater species and decreases gradually as the ambient salinity increases and is found to be very low in

marine species (Burton, 1983). In general, molluscs maintain an isosmotic or slightly hyperosmotic body fluid in sea water, and in diluted media this hyperosmotic state becomes more pronounced (Freeman and Rigler, 1957; Todd, 1964; Gilles, 1972a; Bedford, 1972; Rumsey, 1973; Fyhn, 1976; Shumway, 1977; Davenport, 1979; Deaton, 1981; Burton, 1983; Deaton *et al.*, 1989; Salomao and Lunetta, 1989). The hyperosmotic state is achieved mainly by the regulation of sodium, potassium and chloride ions (Schoffeniels and Gilles, 1972; Burton, 1983; Leader *et al.*, 1986; Salomao and Lunetta, 1989; Deaton, 1992).

Both passive mechanisms like Donnan effect and permeability of the body wall and active mechanisms like ion pump are involved in the ionic regulation. While dialyzing body fluid samples against sea water, Robertson (1949, 1953) has found that a small Donnan effect occurs in some molluscs. Pierce (1970) has examined four species of *Modiolus*, among which two were poikilosmotic stenohaline and other two were poikilosmotic euryhaline. In all cases the extracellular fluids were hyperosmotic to the medium. Similar results on dialysis made him to conclude that the hyperosmoticity of body fluids is neither a function of the habitat of the species nor an active process but it is due to passive Gibbs-Donnan effect.

The hemolymph of the marine molluscs constitutes 30-80% of soft body parts, and this dynamic tissue establishes contact with the ambient medium through the body wall and hence the permeability characteristics of the membrane are of crucial importance for survival in low salinities (Potts, 1954; Schoffeniels and Gilles, 1972; Deaton, 1981, 1992). Bivalves with

their large surface area (mantle and convoluted gills) exposed directly to the medium will have higher permeability, which will impose an upper limit on the extent to which their blood can be maintained hyperosmotic to the medium without incurring a large metabolic cost.

Even where the Donnan effect significantly modifies the ionic distribution, it accounts only for a small portion of the ionic regulation and active charge and discharge phenomena of ions appear to be the main system involved in the ionic regulation in marine bivalves (Schoffeniels and Gilles, 1972; Gilles, 1975; Otto and Pierce, 1981). This active process becomes more prominent in diluted media where, in some euryhaline bivalves it has to serve an additional function of blood osmotic pressure regulation (Otto and Pierce, 1981). In many euryhaline bivalves the extracellular concentration of K^+ remained high due to active regulation in all salinities and the concentration of $Na^+$and Cl^- followed the trend of osmotic pressure of the extracellular fluid (Schoffeniels and Gilles, 1972; Veiga *et al.*, 1988; Mahasneh and Pora, 1981; Salomao and Lunetta, 1989).

The blood is almost similar to the ambient medium in ionic composition in species surviving in stable environments. However, such a state can never be maintained in a fluctuating environment without profound ionic regulation. This variation in ionic concentration and the resultant osmotic pressure are believed to be the cause of evocation of cellular salinity tolerance mechanism by intracellular-fluid isosmotic regulation. Therefore, study on the ionic regulation is rewarding on species surviving in unstable environments (Burton, 1983; Treherne, 1980).

The aim of the present study is to understand the extracellular concentration of Na^+, K^+ and Cl^- in different levels of salinity and its role, if any, in the extracellular-fluid anisosmotic regulation in both *Sunetta scripta* and *Perna viridis*. Since these species have been found to show variation in salinity tolerance depending on size, the ionic concentration in different size groups were also studied.

2.2.2 MATERIALS AND METHODS

In these experiments, bivalves acclimated for two weeks to defined environmental and nutritive conditions were gradually acclimated to different levels of salinity. Experimental salinities used were within the range occurring in the natural habitat of the animals.

Collection of *Sunetta scripta* and *Perna viridis* and their acclimation were done as given in 2.1.2. The three size groups of *Sunetta scripta* were then gradually transferred ($\Delta=5\times10^{-3}/3$ days) to 5, 10, 15, 20, 25, 30, 35 and 40×10^{-3} salinities and both the size groups of *P. viridis* were likewise transferred to 15, 20, 25, 30, 35 and 40×10^{-3} salinities. Higher salinities were obtained by adding sea water of higher salinity and dilution was done using deionized water. After attaining the desired levels of salinity as mentioned, they were maintained for a period of two weeks. Conditions of acclimation tanks and experimental tanks were the same except the salinity.

After the acclimation, six animals of same size groups were pooled, opened the valves and the mantle cavity fluid was drained

out. Any mantle cavity fluid remained was removed using an absorbent paper. The hemolymph was collected from the adductor sinus with a clean dry syringe. The pooled hemolymph was centrifuged at 6,000 rpm for 30 min. and the serum was separated from the blood cells. The serum was diluted with distilled water and was analyzed for Na^+ and K^+ by Flame Photometric method of Robinson and Ovenston (1951) using Flame Photometer (Elico, Type 22). The chloride content of the serum was estimated using Chloride Meter (Elico Chloride Meter, Model EE 34).

2.2.3 RESULTS

Concentration of Na^+ in different size groups of *Sunetta scripta* maintained in different salinities is given Table 11. Concentration of Na^+ in the blood is compared with that in the ambient medium (Fig. 29). In the three size groups studied, Na^+ in the blood is found to increase along with the concentration of the ion in the ambient medium ($P<0.001$). Correlation of ionic concentration of the hemolymph with that of the ambient medium is found to be positive in all size groups (large, 0.97; medium, 0.97; small, 0.95). Up to 35×10^{-3} salinity, large and medium size groups show a hyperionic regulation of sodium and in 40×10^{-3} salinity both show a hypoionicity. In the case of small size group a hyper regulation of Na^+ is noted up to 25×10^{-3} salinity followed by a hypo Na^+ regulation in 30 and 35×10^{-3} salinities. ANOVA shows that this variation between size groups is not significant at 5% level. In the three size groups, the hyper Na^+ regulation becomes more conspicuous below 15×10^{-3} salinity. The small size group,

maintains a higher hyperionicity than the medium and large size groups.

Concentration of K^+ in the hemolymph of *Sunetta scripta* in different levels of salinity is given in Table 12 and Fig. 30. It is noted that in the three size groups the increase in the ambient K^+ concentration is followed by the corresponding increase in the extracellular fluid, and is significant at 0.1% level ($P<0.001$). The positive correlation obtained for the three size groups for the above is as follows, large (0.98), medium (0.97) and small (0.99). All the three size groups express a hyper K^+ regulation in all the salinities studied, with highest regulation in small, followed by medium and large size groups, and is significant at 0.1% level ($P<0.001$). As in the case of Na^+, this hyperionic regulation is more pronounced below $15x10^{-3}$ salinity.

Extracellular chloride content of *Sunetta scripta* in different levels of salinity is given in Table 13 and Fig. 31. The figure shows that in the three size groups, hemolymph chloride ion concentration exhibits a positive correlation (large, 0.99; medium, 0.98; small, 0.99) with that of the ambient medium in all the salinities studied and is significant at 0.1% level ($P<0.001$). Hyperionicity of Cl^- in the blood is noted in all the salinities and is highest for small followed by medium and large size groups. The variation between size groups is found significant at 0.1% level ($P<0.001$). The hyper regulation of Cl^- as in the case of $Na^+$and K^+ is found to be more pronounced below $15x10^{-3}$ salinity.

The regulation of Na^+, K^+ and Cl^- in the hemolymph of *Perna viridis* in different salinities and size groups is given in Tables 14, 15 and 16 respectively. In the two size groups of this species

studied, the hemolymph concentration of Na^+ (Fig. 32) shows a positive correlation with that of the ambient medium (large, 0.99; small, 0.97) and is found significant at 0.1% level ($P<0.001$). As in the case of *Sunetta scripta*, small size group shows a hyper Na^+ regulation, but it is pronounced up to $25x10^{-3}$ salinity followed by a hypoionic regulation in 30 and 35 $x10^{-3}$ salinity and again a hyper Na^+ regulation in $40x10^{-3}$ salinity. In large size group as in the case of small a hyper Na^+ regulation is noted in $15x10^{-3}$, followed by a slight hypoionic regulation in $20x10^{-3}$, $35x10^{-3}$ and $40x10^{-3}$ salinities. In 25 and $30x10^{-3}$ salinities, the large size group shows a hyper Na^+ regulation. The variation noticed between size groups is not significant at 5% level.

In *Perna viridis*, as in the case of *Sunetta scripta* a positive correlation of K^+ in the blood with that in the ambient medium is noticed in both the size groups (Fig. 33) (large, 0.97; small, 0.96) and is significant at 0.1% level ($P<0.001$). The hyper regulation of K^+ is found to be more pronounced in the case of small size group than large and it is found to be statistically significant at 0.1% level ($P<0.001$).

The chloride ion concentration is maintained at a higher level in the body fluid of *Perna viridis* than that in the ambient environment (Fig. 34). As in the case of other ions, a positive correlation is noticed between the hemolymph and ambient concentrations in all the salinities studied in both size groups (large, 0.99; small, 1.0). This is found to be significant at 0.1% level ($P<0.001$). Here also, small size group maintained a higher hyperionic condition than large size group. The difference noticed between size groups is significant at 0.1% level ($P<0.001$).

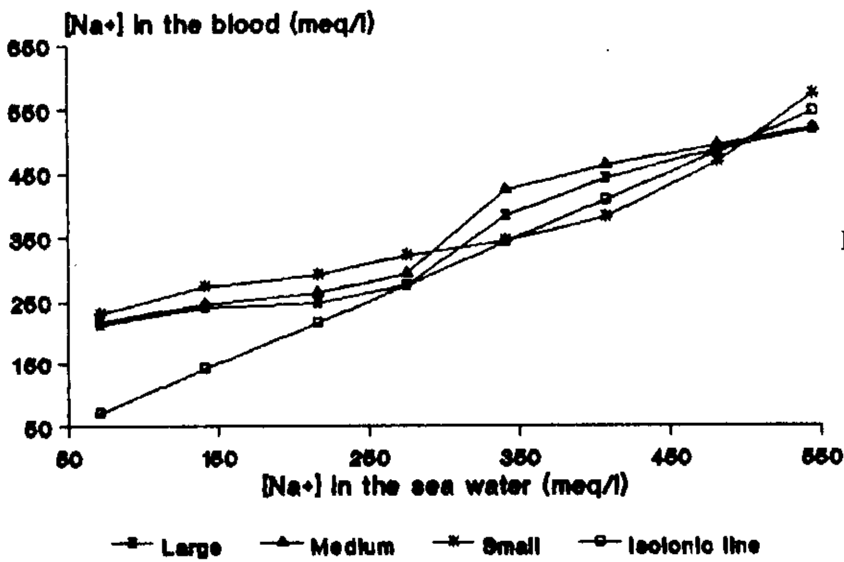

Fig. 29

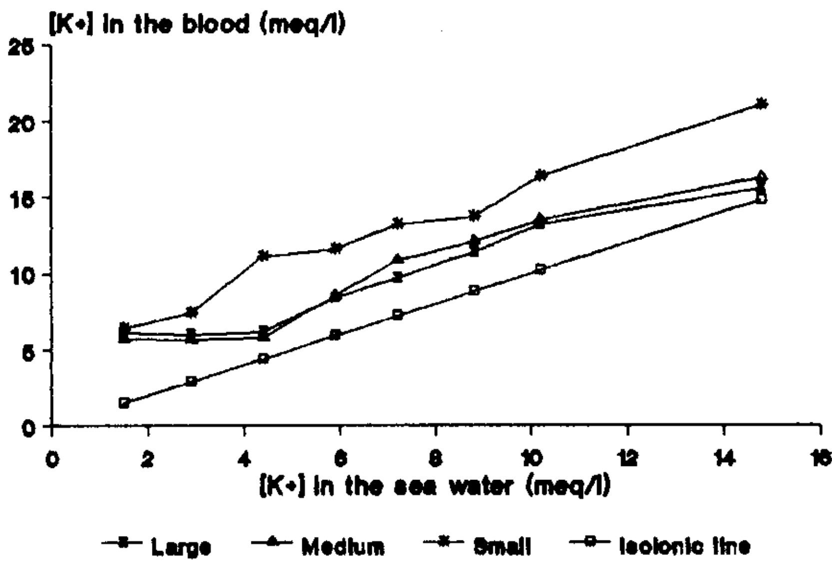

Fig. 30

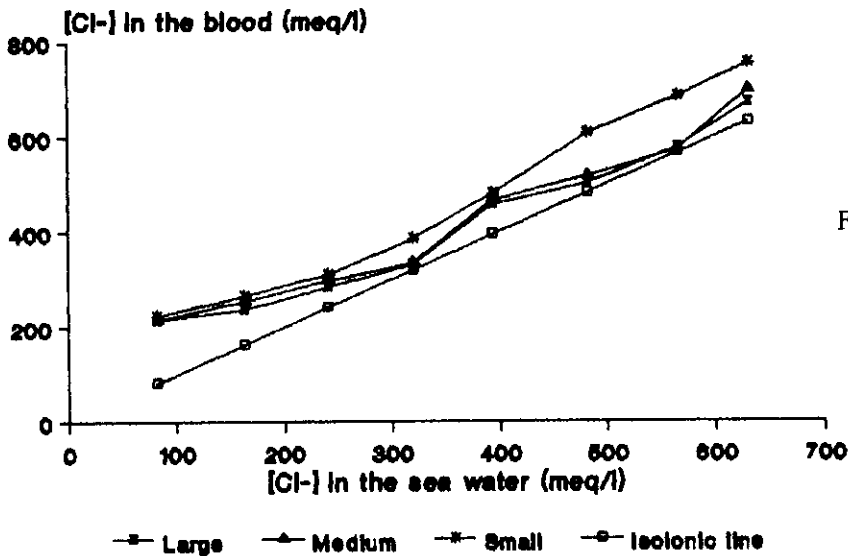

Fig. 31

Regulation of Na^+ (Fig. 29), K^+ (Fig. 30) and Cl^- (Fig. 31) in the hemolymph in relation to their concentration in the ambient sea water of 3 size groups of Sunetta scripta.

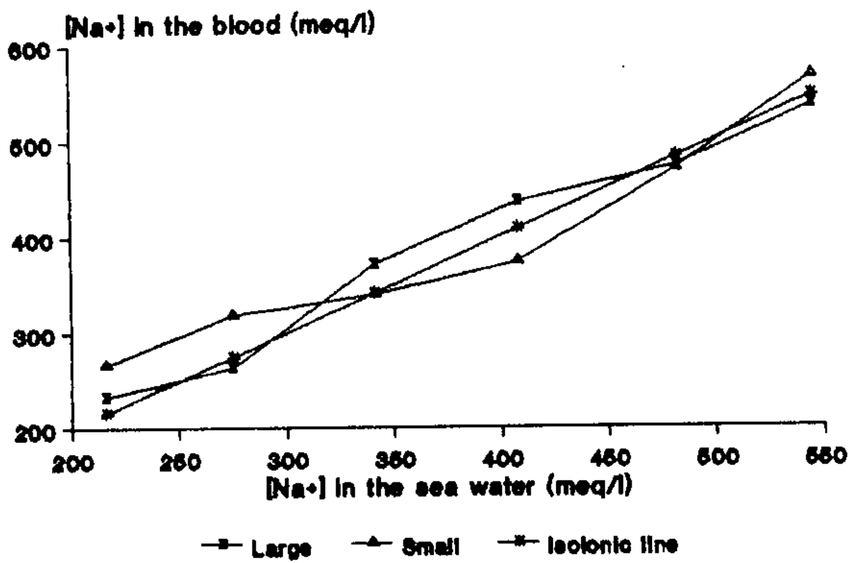

Fig. 32

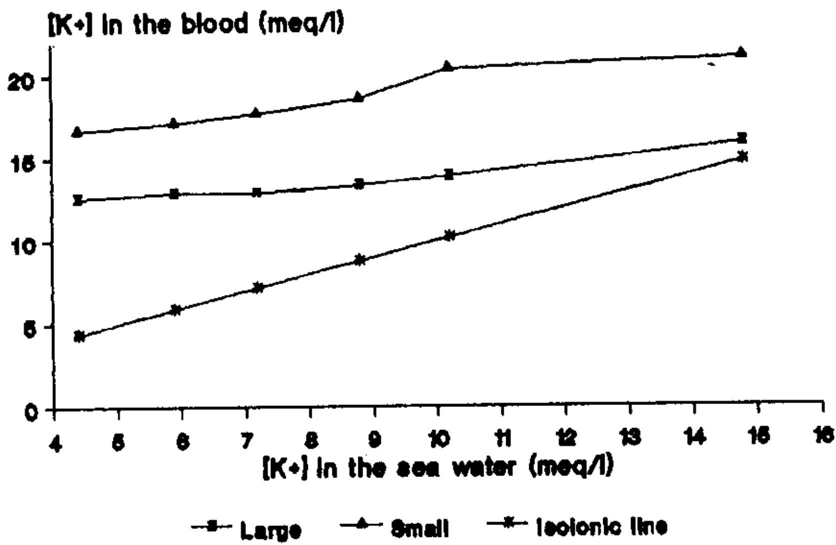

Fig. 33

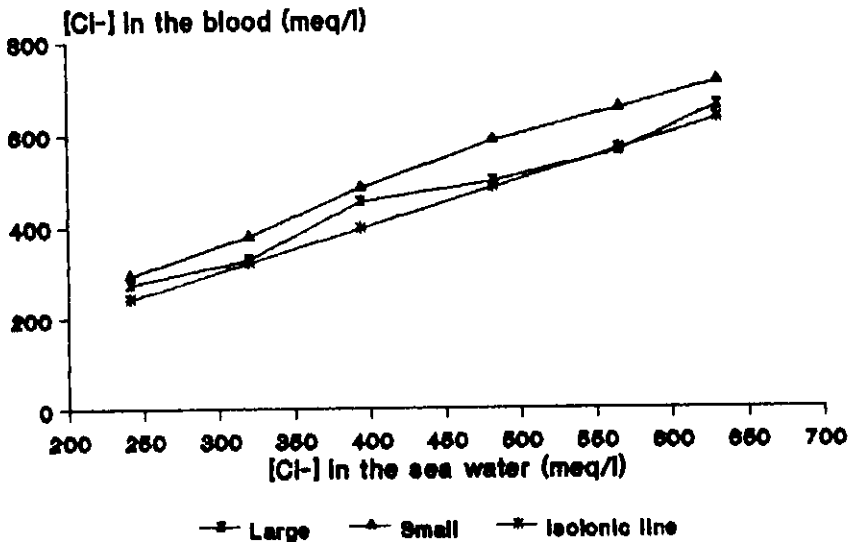

Fig. 34

Regulation of Na^+ (Fig. 32), K^+ (Fig. 33) and Cl^- (Fig. 34) in the hemolymph in relation to their concentration in the ambient sea water of two size groups of Perna viridis.

2.2.4 DISCUSSION

In most aquatic molluscs, extracellular fluid anisosmotic regulation is primarily affected by inorganic ions such as sodium and chloride. These two ions together with potassium form more than 90% of the total hemolymph ionic concentration in marine bivalves (Schoffeniels and Gilles, 1972; Gilles, 1975). As a matter of fact, the hyperosmotic state observed in fresh water forms and in many euryhaline forms acclimated to diluted media, as well as the hypoosmotic state observed in many marine species, can be accounted for by following the quantity of sodium and chloride ions present in the blood. In short, many workers have clearly indicated that the extracellular concentration of Na^+ and Cl^- follows the trend of blood osmotic pressure since they form the most abundant ions in the blood and ambient medium (Schoffeniels and Gilles, 1972; Rumsey, 1973; Gilles, 1975; Otto and Pierce, 1981; Burton, 1983; Deaton *et al.*, 1989; Deaton, 1992).

In all the size groups of *Sunetta scripta* and *Perna viridis*, the extracellular concentration of Na^+, Cl^- and K^+ is found to be a function of the corresponding ionic concentration in the ambient medium and varies along with the hypothetical isoionic line in different salinities. This is an indication that in both the bivalves osmotic pressure is not actively regulated but changes along with the ambient medium and these species can be considered as iono and osmoconformers.

Concentration of sodium, potassium and chloride ions in the hemolymph of different size groups of *S. scripta* and *P. viridis* is above the isoionic line except for Na^+ in few observations. The

negligible hypoionicity noted in the case of Na^+ is found to be adjusted by the other two ions. In total, a higher number of ions is noticed in the hemolymph than that in the ambient medium in all salinities. This is indicative of maintenance of a hyperosmotic extracellular fluid in both the species. Experiments of Robertson (1949, 1953) and Pierce (1970) have shown that the hyperosmoticity seen in marine molluscs is due to passive Gibbs-Donnan equilibrium. Even if this hyperosmoticity noticed in both the species is not due to a passive mechanism, the energy requirement for maintaining the same may not be very great since this hyperosmoticity is only marginally high and closely follows that of the ambient medium except in lower salinities. The small hyperosmotic condition may be an environmental requirement to induce a continuous influx of water which may be necessary to maintain minimal urine flow and secretion of mucus (Burton, 1983).

The hyperosmoticity noticed in both the species becomes more pronounced as the dilution of the ambient medium increases beyond $20x10^{-3}$ salinity. Conspicuous hyperosmoticity can be noticed in $15x10^{-3}$ salinity in all the size groups of both the species. The trend of ionic change in lower salinities is not available in the case of *Perna viridis* as the species cannot be maintained for experimental purpose below $15x10^{-3}$ salinity. The values collected for *Sunetta scripta* indicate that the hyperosmoticity progressively increases as the salinity decreases down to $5x10^{-3}$. *Sunetta scripta* can tolerate decrease in salinity very near to fresh water conditions (Thampuran *et al.*, 1982). The capability of the species to tolerate such low salinities may be mainly due to the increased hyperionic regulation of Na^+, K^+ and Cl^- as seen in

the present study. Similar results have been obtained for euryhaline molluscs by Schoffeniels and Gilles (1972), Rumsey (1973), Gainey and Greenberg (1977), Otto and Pierce (1981), Burton (1983), Salomao and Lunetta (1989), Deaton *et al.* (1989) and Deaton (1981, 1992). The increased extracellular hyperosmotic regulation noticed in diluted media not only provides necessary ionic environment for various life processes including cellular excitability but also reduces substantially the quantum of intracellular osmolytes that are to be mobilized to maintain isosmoticity of intracellular fluid.

According to Kinne (1971), different size groups of bivalves must be considered in any study on their osmoregulation. In general, smaller animals of most marine invertebrates have got better survival rate than fully grown adults in adverse environmental salinities (Kinne, 1971). In *Sunetta scripta* and *Perna viridis* small size groups maintain a higher extracellular fluid hyperosmoticity than larger size groups in all experimental salinities and the hyperosmoticity becomes more pronounced below 20×10^{-3} salinity. It appears that small size groups avail maximum advantage of extracellular-fluid anisosmotic regulation in diluted media and thereby become more independent of the osmotic variations of the external medium. Perhaps, this is a major reason for their increased survival rate in lower salinities. In the case of *Sunetta scripta* Thampuran *et al.* (1982) noted a higher survival rate for smaller size groups in lower salinities. This may be an important aspect, which helps to maintain a population of the species, even in environment where prolonged dilution is maintained. Studies on the scope for growth of the species have

shown that small size group has got a wider spectrum of salinity for scope for growth compared to medium and large size groups (Supriya, 1992). It has already been pointed out that the osmoregulation is a sensory mediated response altered with age in marine molluscs (Skinner and Peretz, 1989).

Even though the involvement of K^+ in the total hemolymph osmolarity is much less than that of the Na^+ or Cl^-, the hyperionic state of this ion may be contributing to the hyperosmoticity seen in the bivalve molluscs. Hyper K^+ regulation of similar nature has been noted in other bivalve molluscs also (Robertson, 1949, 1953; Schoffeniels and Gilles, 1972; Veiga *et al.*, 1988; Salomao and Lunetta, 1989). Burton (1983) has pointed out the advantage of hyper regulation of potassium ion as less expensive and also that this ion may be entering into the system through food. When both the species are compared, a higher hyper K^+ regulation is noticed in *Perna viridis* than that in *Sunetta scripta* in all salinities.

TABLE NO : 11

Extracellular-fluid [Na^+] regulation of three size groups of *S. scripta* in different levels of salinity.

Concentration of Na^+ is expressed in meq/litre.

SEAWATER SALINITY ($x 10^{-3}$)	SEAWATER [Na^+]	[Na^+] IN THE BLOOD OF DIFFERENT SIZE GROUPS		
		LARGE (40±2mm)	MEDIUM (30±2mm)	SMALL (20±2mm)
05	071	212 ± 07	217 ± 09	231 ± 06
10	141	241 ± 08	245 ± 05	272 ± 08
15	216	248 ± 06	262 ± 07	292 ± 06
20	275	274 ± 09	293 ± 06	321 ± 08
25	341	382 ± 10	422 ± 08	343 ± 07
30	408	441 ± 05	461 ± 09	381 ± 06
35	482	487 ± 09	493 ± 11	467 ± 08
40	545	517 ± 08	520 ± 08	572 ± 05

Values are the mean of eight different observations ± SD.

Each observation is from a sample of six animals pooled together.

ANALYSIS OF VARIANCE

SOURCE	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARE	VARIANCE RATIO	SIGNIFICANCE
SIZE GROUPS	808.50	2	404.25	0.517	NS*
CONC.OF Na^+	284731.75	7	40675.96	51.989	$P<0.001$
ERROR	10953.50	14	.782.39		
TOTAL	296493.75	23			

NS* . Not significant at 5 per cent level.

TABLE NO : 12

Extracellular-fluid [K^+] regulation of three size groups of *S. scripta* in different levels of salinity.

Concentration of K^+ is expressed in meq/litre.

SEAWATER SALINITY (x 10^{-3})	SEAWATER [K^+]	[K^+] IN THE BLOOD OF DIFFERENT SIZE GROUPS		
		LARGE (40±2mm)	MEDIUM (30±2mm)	SMALL (20±2mm)
05	1.5	6.1 ± 0.5	5.7 ± 0.3	6.4 ± 0.6
10	2.9	6.0 ± 0.6	5.6 ± 0.4	7.4 ± 0.3
15	4.4	6.2 ± 0.5	5.8 ± 0.5	11.1 ± 0.5
20	5.9	8.4 ± 0.4	8.5 ± 0.4	11.6 ± 0.5
25	7.2	9.7 ± 0.8	10.8 ± 0.5	13.2 ± 0.5
30	8.8	11.4 ± 0.5	12.1 ± 0.5	13.7 ± 0.3
35	10.2	13.2 ± 0.6	13.5 ± 0.4	16.4 ± 0.7
40	14.8	15.6 ± 0.4	16.3 ± 0.6	21.1 ± 0.9

Values are the mean of eight different observations ± SD.

Each observation is from a sample of six animals pooled together.

ANALYSIS OF VARIANCE

SOURCE	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARE	VARIANCE RATIO	SIGNIFICANCE
SIZE GROUPS	46.01	2	23.00	23.985	P<0.001
CONC.OF K^+	348.35	7	49.76	51.888	P<0.001
ERROR	13.43	14	0.96		
TOTAL	407.78	23			

TABLE NO : 13

Extracellular-fluid [Cl^-] regulation of three size groups of *S. scripta* in different levels of salinity.

Concentration of Cl^- is expressed in meq/litre.

SEAWATER SALINITY (x 10^{-3})	SEAWATER [Cl^-]	[Cl^-] IN THE BLOOD OF DIFFERENT SIZE GROUPS		
		LARGE (40±2mm)	MEDIUM (30±2mm)	SMALL (20±2mm)
05	082	213 ± 10	215 ± 08	222 ± 08
10	163	236 ± 08	254 ± 06	265 ± 09
15	241	284 ± 08	296 ± 08	310 ± 06
20	320	331 ± 06	335 ± 09	384 ± 07
25	394	456 ± 09	466 ± 08	478 ± 10
30	482	502 ± 11	518 ± 07	607 ± 07
35	566	577 ± 07	572 ± 09	686 ± 12
40	631	671 ± 09	699 ± 11	755 ± 09

Values are the mean of eight different observations ± SD.

Each observation is from a sample of six animals pooled together.

ANALYSIS OF VARIANCE

SOURCE	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARE	VARIANCE RATIO	SIGNIFICANCE
SIZE GROUPS	13420.50	2	6710.25	12.143	$P<0.001$
CONC.OF Cl^-	677775.31	7	96825.05	175.222	$P<0.001$
ERROR	7736.19	14	552.58		
TOTAL	698932.00	23			

TABLE NO : 14

Extracellular-fluid [Na^+] regulation of two size groups of *P. viridis* in different levels of salinity.

Concentration of Na^+ is expressed in meq/litre.

SEAWATER SALINITY ($x10^{-3}$)	SEAWATER [Na^+]	[Na^+] IN THE BLOOD OF DIFFERENT SIZE GROUPS	
		LARGE (65±2mm)	SMALL (35±2mm)
15	216	233 ± 07	267 ± 08
20	275	263 ± 09	318 ± 08
25	341	371 ± 10	339 ± 07
30	408	436 ± 09	373 ± 09
35	482	473 ± 09	470 ± 06
40	545	535 ± 10	565 ± 07

Values are the mean of eight different observations ± SD.

Each observation is from a sample of six animals pooled together.

ANALYSIS OF VARIANCE

SOURCE	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARE	VARIANCE RATIO	SIGNIFICANCE
SIZE GROUPS	36.71	1	36.71	0.037	NS
CONC.OF Na^+	126241.38	5	25248.28	25.224	$P<0.001$
ERROR	5004.79	5	1000.96		
TOTAL	131282.88	11			

TABLE NO : 15

Extracellular-fluid $[K^+]$ regulation of two size groups of *P. viridis* in different levels of salinity.

Concentration of K^+ is expressed in meq/litre.

SEAWATER SALINITY ($\times 10^{-3}$)	SEAWATER $[K^+]$	$[K^+]$ IN THE BLOOD OF DIFFERENT SIZE GROUPS	
		LARGE (65±2mm)	SMALL (35±2mm)
15	4.4	12.6 ± 0.5	16.7 ± 0.7
20	5.9	12.9 ± 0.5	17.1 ± 0.8
25	7.2	12.9 ± 0.6	17.7 ± 0.5
30	8.8	13.4 ± 0.4	18.6 ± 0.4
35	10.2	13.9 ± 0.7	20.4 ± 0.8
40	14.8	15.9 ± 0.4	21.1 ± 0.9

Values are the mean of eight different observations ± SD.

Each observation is from a sample of six animals pooled together.

ANALYSIS OF VARIANCE

SOURCE	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARE	VARIANCE RATIO	SIGNIFICANCE
SIZE GROUPS	75.00	1	75.00	196.344	$P<0.001$
CONC.OF K^+	21.65	5	4.33	11.335	$P<0.001$
ERROR	1.91	5	0.38		
TOTAL	98.56	11			

TABLE NO : 16

Extracellular-fluid [Cl^-] regulation of two size groups of *P. viridis* in different levels of salinity.

Concentration of Cl^- is expressed in meq/litre.

SEAWATER SALINITY ($x\ 10^{-3}$)	SEAWATER [Cl^-]	[Cl^-] IN THE BLOOD OF DIFFERENT SIZE GROUPS	
		LARGE (65±2mm)	SMALL (35±2mm)
15	241	274 ± 10	292 ± 08
20	320	328 ± 08	377 ± 06
25	394	453 ± 08	482 ± 07
30	482	498 ± 10	585 ± 05
35	566	563 ± 09	655 ± 06
40	631	659 ± 07	712 ± 08

Values are the mean of eight different observations ± SD.

Each observation is from a sample of six animals pooled together.

ANALYSIS OF VARIANCE

SOURCE	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARE	VARIANCE RATIO	SIGNIFICANCE
SIZE GROUPS	8965.42	1	8965.42	20.025	$P<0.001$
CONC.OF Cl^-	234589.75	5	46917.95	104.794	$P<0.001$
ERROR	2238.58	5	447.72		
TOTAL	245793.75	11			

CHAPTER 3

INTRACELLULAR–FLUID ISOSMOTIC REGULATION

FREE AMINO ACIDS AND OTHER NPS

FREE AMINO ACIDS AND OTHER NPS

3.1 INTRODUCTION

Intertidal bivalves are not known to regulate their blood osmotic pressure substantially when exposed to osmotic stress and many of them are well known osmoconformers. They often escape from abrupt changes in ambient salinity by behavioural adaptation like valve closure or burrowing into the sediment (Akberali and Trueman, 1985; Deaton, 1992). But this avoidance mechanism due to its inherent limitations will not help the animal to overcome the adversities for prolonged periods and often it is used as an interim measure for gradual internal adjustments. Some species, in addition to their behavioural avoidance, were found to regulate their blood osmotic pressure in lower extremes of their salinity tolerance through regulation of inorganic ions (Davenport, 1979; Burton, 1983; Deaton, 1981; Deaton *et al.*, 1989; Salomao and Lunetta, 1989). Since hemolymph osmolarity is not subjected to considerable regulation and fluctuates with ambient salinity, the cells of the bivalves have to cope with this variation in their external medium (Hoyaux *et al.*, 1976; Leader *et al.*, 1986; Jeuniaux, 1988).

Isosmoticity between the cell and the extracellular fluid was not a concept acceptable to earlier physiologists. But recent studies clearly indicated that the cell is in osmotic equilibrium with the hemolymph for minimizing the osmotic transfer of water across the cell membrane. This necessitates regulation of osmotic effectors in the cells of osmoconformers concomitant with their

variation in the hemolymph. Through this intervention the cell maintains a more or less constant cell volume. Thus, regulation of intracellular osmolytes is the mechanism that underlies the salinity tolerance of euryhaline bivalve molluscs (Lange, 1963; Schoffeniels and Gilles, 1972; Gilles, 1975; Matsushima *et al.*, 1987; Deaton *et al.*, 1989; Deaton and Greenberg, 1991).

Although the intracellular fluid and hemolymph of poikilosmotic marine bivalves are in osmotic equilibrium, the osmotic constituents of the two systems are different. The osmotic constituents in the hemolymph are mainly inorganic ions (Schoffeniels and Gilles, 1972; Gilles, 1975; Burton, 1983; Jeuniaux, 1988), whereas the main osmotic effectors in the cells are free amino acids and their derivatives, potassium ions, bicarbonate ions and phosphate compounds. (Shumway *et al.*, 1977; Gilles, 1982; Pierce, 1982; Bishop *et al.*, 1983; Somero and Bowlus, 1983; Jeuniaux, 1988). Among the intracellular osmolytes, free amino acids including taurine are found to be the principal regulatory osmolytes, although inorganic ions do play a minor role (Lange, 1963; Pierce, 1971a; Pierce and Greenberg, 1972, 1973; Hoyaux *et al.*, 1976; Matsushima *et al.*, 1987).

Several works have shown that metabolically expensive nitrogenous osmolytes especially free amino acids and their derivatives not only make dominant contribution to intracellular osmolarity in molluscs but also are the major *varied* osmolytes during osmotic adaptation to different levels of salinity (Baginski and Pierce, 1977; Greenwalt and Bishop, 1980; Henry and Mangum, 1980b; Zurburg and De Zwaan, 1981; Matsushima *et al.*,

1987; Taylor and Andrews, 1988; Pierce *et al.*, 1992). Considering their importance in the intracellular-fluid isosmotic regulation, several authors have investigated the effect of salinity on the free amino acid pool of euryhaline bivalves. A linear relationship between salinity and total ninhydrin positive substances (NPS) has been observed pointing their significance in the process (Lange, 1963; Pierce and Greenberg, 1973; Hoyaux *et al.*, 1976; Heavers and Hammen, 1985; Matsushima *et al.*, 1987; Deaton *et al.*, 1989).

In fact, the ability of the cell to function under wide variations in the concentration of these amino acids or their derivatives suggests that the intracellular microenvironment is not substantially altered by their concentration gradient. This may be the reason why the concentration of readily available and metabolically cheap inorganic ions such as sodium and chloride are not allowed to vary substantially in the cell in the process of intracellular-fluid isosmotic regulation (Yancey *et al.*, 1982; Somero and Bowlus, 1983).

The goal of the solute regulatory system is the establishment of *compatible* solute microenvironment, for protein structure and function (Somero and Bowlus, 1983). Thus it appears that amino acids and their derivatives are acting as compatible solutes under wide variations in their concentration without disrupting the important kinetic and structural characters of enzymes (Fersht, 1977) and other macromolecules. Hence, the ability of the bivalve molluscs to conform osmotically to variations in salinity depends upon the limit of tolerance and mobilization of compatible solutes by the intracellular system

(Somero and Bowlus, 1983).

Previous studies on the pattern of intracellular osmolyte accumulation have shown considerable variation between species, age groups, tissues of individual and during the course of osmotic acclimation (Hoyaux *et al.*, 1976; Baginski and Pierce, 1977; Zurburg and De Zwaan, 1981; Ivanovici *et al.*, 1981; Matsushima *et al.*, 1987; Pierce *et al.*, 1992). This suggests the possibility of multiple regulatory mechanisms and one must inquire about the factors that determine which mechanism is appropriate for a given context. But the relative importance of any regulatory mechanism, irrespective of their accessibility from metabolic pathways or their metabolic cost is focussed on the establishment of a hospitable intracellular microenvironment (Somero and Bowlus, 1983).

Even though a number of works have been conducted on intertidal bivalves in this country so far no attempt has been made to study the role of intracellular organic osmolytes in the salinity tolerance of the species. The role of NPS pool in the intracellular-fluid isosmotic regulation and the qualitative and quantitative nature of the components in the NPS pool are investigated for the first time in different tissues of various size groups of *Sunetta scripta* and *Perna viridis* acclimated to different levels of salinity. The study is expected to give information regarding the compatible solute system in different tissues and in different size groups of both the species. It is also believed to fill the lacuna in understanding the physiological and biochemical adaptations of both the species and

their size groups in various levels of salinity.

3.2 MATERIALS AND METHODS

Different size groups of *Sunetta scripta* and *Perna viridis* were acclimated to $35x10^{-3}$ salinity (control) for four weeks to defined environmental and nutritive conditions as given in 2.2.2. All the size groups of *Sunetta scripta* were then gradually (Δ=$5x10^{-3}$/3days) submitted to hypoosmotic stress by exposing them to $15x10^{-3}$ salinity (test) for a period of four weeks. In the same way, both the size groups of *Perna viridis* were also exposed to hypoosmotic stress by keeping them in $20x10^{-3}$ salinity (test). The environmental and nutritive conditions were same in both the test and control except the salinity.

Test and control samples (pooled, n=20) of adductor muscle, foot muscle, mantle tissue and hemolymph were removed from different size groups of both the species. The excised tissues were quickly blotted and weighed. The tissues were then transferred to acid washed test tubes containing 80% ethanol, heated at 85-90°C for 15 min., cooled and homogenized (Matsushima *et al.*, 1987). The homogenates were centrifuged at 20,000g for 30 min. and the supernatants saved. Hemolymph taken was also deproteinised (80% ethanol), centrifuged as above and the supernatant was collected. Methyl ethyl ketone containing 5% 6 N HCl was added to the extracts to precipitate the inorganic ions (Shumway *et al.*, 1977). The supernatants collected after centrifugation were lyophilized (Lyophiliser, Yamato, Japan) and

the resultant residues were dissolved in appropriate volume of double glass distilled water (sample). Aliquots of the solutions were estimated for total NPS (Yemm and Cocking, 1955) and expressed as micromoles per gram wet weight or per ml in the case of hemolymph (leucine eq.)

Preliminary separation of amino acids from the samples of adductor, foot, and mantle was carried out by ion-exchange chromatography using a strong cation exchange resin, Dowex 50x8% DVB 200-400 mesh H^+ form (Sigma) by the method of Jayaraman (1981) and Moore and Stein (1951) with slight modification. Suspended the Dowex 50 in 4 N HCl (12.5 g/L) for 15 min. Filtered the suspension through a Whatman filter paper kept in a Buchner funnel and washed till neutral pH with double glass distilled water. Suspended the resin in double glass distilled water, allowed to settle and decanted the light particles. The resin was filtered again and then equilibrated with 0.2 N Citrate buffer, pH 2.2 (Duggan, 1957). This equilibrated suspension was used to set up a column (60x0.9 cm) and about 250 ml of the citrate buffer (pH 2.2; 0.2 N) was allowed to pass through it. Considering the exchange capacity of the column, sample dilution was made and the pH of the sample was adjusted to 1.0. Carefully introduced 2 ml of the sample on the top of the column and was allowed to percolate through it. When the level just reached the top layer of the column, the flow was stopped. The amino acids including taurine were then bound to the column and were eluted by gradually increasing the pH of the eluant buffer. This was done by connecting the column to a pH gradient generating device (LKB 93906661). The generated gradient

was allowed to pass through the column at a flow rate of 1 ml per 3 min. Eluant fractions were collected in 1 ml aliquots in serially numbered test tubes using an automatic fraction collector (LKB 2112 Redirac Fraction Collector).

Definite volumes of all the fractions were analyzed quantitatively using ninhydrin (Yemm and Cocking, 1955). The color formed was read at 570 nm except for proline which was read at 440 nm in a Spectrophotometer (Hitachi Model 200-20). An elution graph was drawn with the serial number of eluant fraction versus their absorbance. The area of each peak was quantified using internal standard technique (Williams and Wilson, 1981). Glycine, taurine, alanine and proline were quantified using their individual standards and the quantification of other NPS was done using a leucine standard.

A group of fractions which formed a single peak were pooled, lyophilised and dissolved in 10% isopropanol and the purity and identity of the peaks were tested using ascending thin layer chromatography employing silica gel coated (dry layer thickness, 0.15 mm) glass plates (20x20 cm) (Niederwieser, 1972; Brenner and Niederwieser, 1967). The following solvents were used for the purpose (Brenner *et al.*, 1965).

A. n-Butanol - Glacial acetic acid - Water 4:1:1 v/v

B. Chloroform - Methanol - 17% (g/g) ammonia 2:2:1 v/v

C. Phenol - Water 3:1 g/g

D. 96% (v/v) Ethanol - Water 7:3 v/v

E. n-Propanol - Water 7:3 v/v

F. 96% (v/v) Ethanol - 34% (g/g) ammonia 7:3 v/v

G. n-Butanol - Glacial acetic acid - Water 12:3:5 v/v

The combination of solvents A and C and in particular B and C were used for two dimensional thin layer chromatography. In both the cases the solvent C was used for the second dimensional development. All other solvents were used for one dimensional separation. Quantitative separation by this technique was done for those peaks which carried more than one NPS on ion-exchange analysis. Thus the concentration of individual amino acids was calculated and expressed as micromoles per gram wet weight.

3.3 RESULTS

Concentration of total NPS in the adductor muscle, foot muscle and mantle tissue of all the three size groups of *Sunetta scripta* acclimated to salinities of $35x10^{-3}$ and $15x10^{-3}$ and two size groups of *Perna viridis* acclimated to salinities of $35x10^{-3}$ and $20x10^{-3}$ is given in Tables 17 and 18 respectively.

The concentration of NPS in the hemolymph is found to be very low compared to other tissues. A significant reduction in the quantity of total NPS in the lower levels of salinity is noticed in all other tissues of all the size groups of both the species (Fig. 35 and 36). Between the tissues, higher concentration of total NPS is noticed in the adductor muscle, whereas the foot and mantle tissues maintain a lower concentration of NPS in all the size groups of both the species. The variation in the total NPS content between foot and mantle tissues is only marginal. In general, it appears that the total intracellular NPS become less

prominent as the animal becomes older. This is true in the case of both the species in all the levels of salinity. Between the species, higher concentration of total NPS is observed in all the three tissues of both the size groups of *Perna viridis* in $35x10^{-3}$ salinity. The magnitude of variation in the total NPS content between upper and lower levels of salinity is found to be greater for smaller size groups of both the bivalves.

The qualitative and quantitative nature of the components of the NPS pool in different tissues of various size groups of *Sunetta scripta* and *Perna viridis* acclimated to different levels of salinity is given in Tables 19, 20, 21, 22 and 23 and Figures 37 to 51. The concentration of the component amino acids is expressed in micromoles per gram wet weight. In different tissues of various size groups of both the species, the major free amino acids which constitute the NPS pool are taurine, glycine, alanine, proline, aspartic acid and glutamic acid. These free amino acids are not only the abundant intracellular osmolytes present in these species, but also the most *varied* ones during the ambient salinity variations. The presence of other amino acids like arginine, lysine, serine, threonine and valine is also noticed but they seem to have quantitatively less significant role in the isosmotic intracellular regulation. Concentration of few unidentified components of NPS pool appears to play a role in the regulatory process. This pool is composed of 2 to 4 NPS and are collectively expressed as miscellaneous (Misc).

Conspicuous quantitative variation is observed in all the components of the NPS pool between the two levels of salinity in

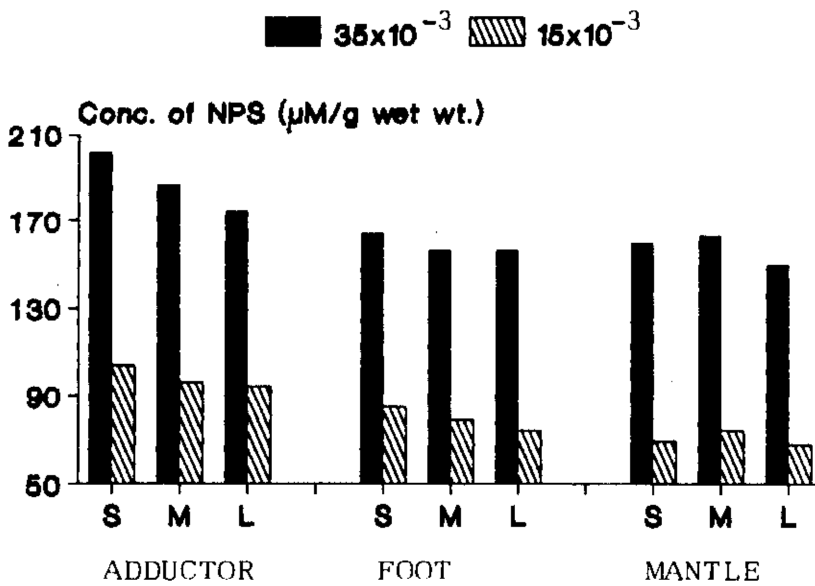

Fig. 35. Conc. of total NPS in various tissues of small (S), medium (M) and large (L) size groups of S. scripta acclimated to salinities of $35x10^{-3}$ and $15x10^{-3}$.

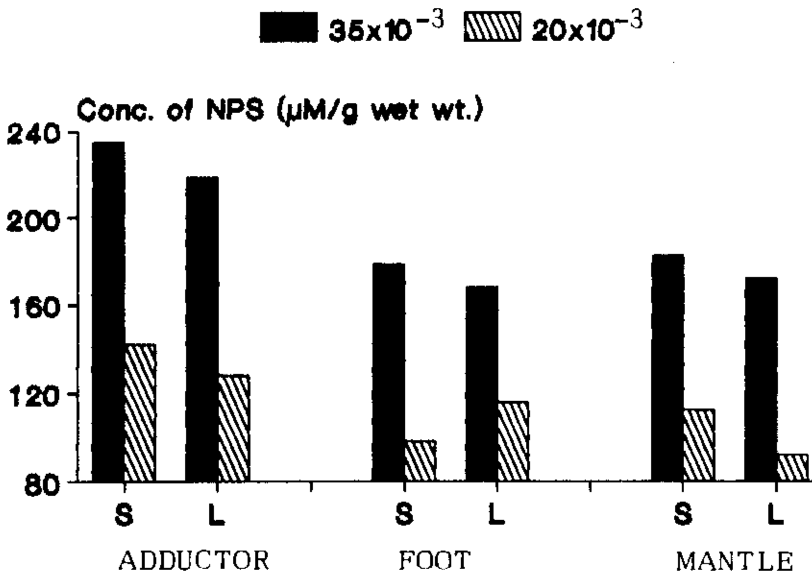

Fig. 36. Conc. of total NPS in various tissues of small (S) and large (L) size groups of P. viridis acclimated to salinities of $35x10^{-3}$ and $20x10^{-3}$.

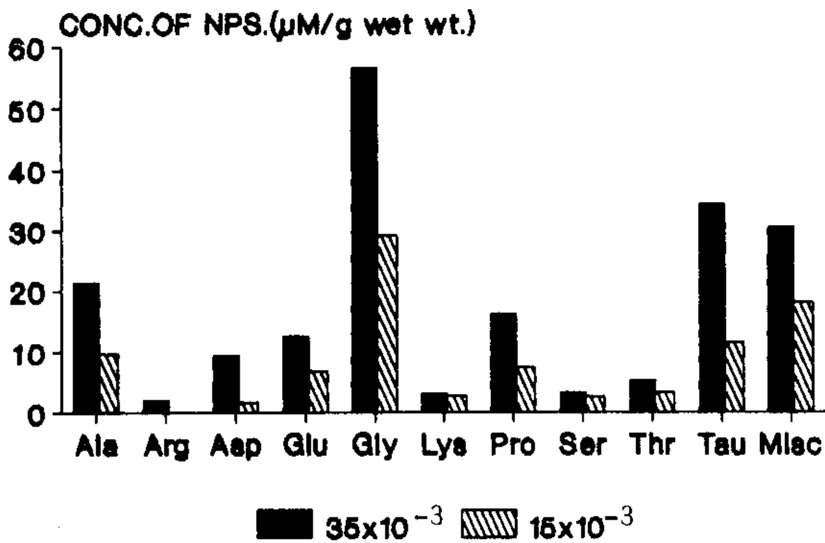

Fig. 37. Adductor

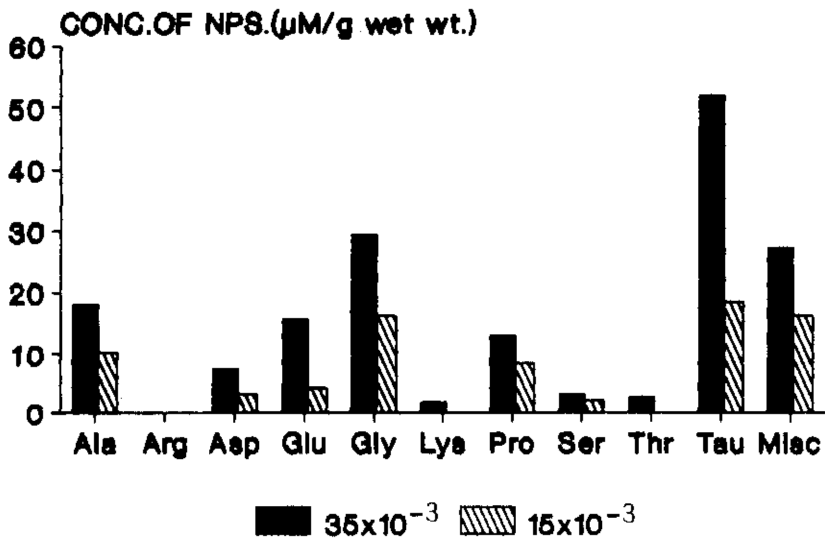

Fig. 38. Foot

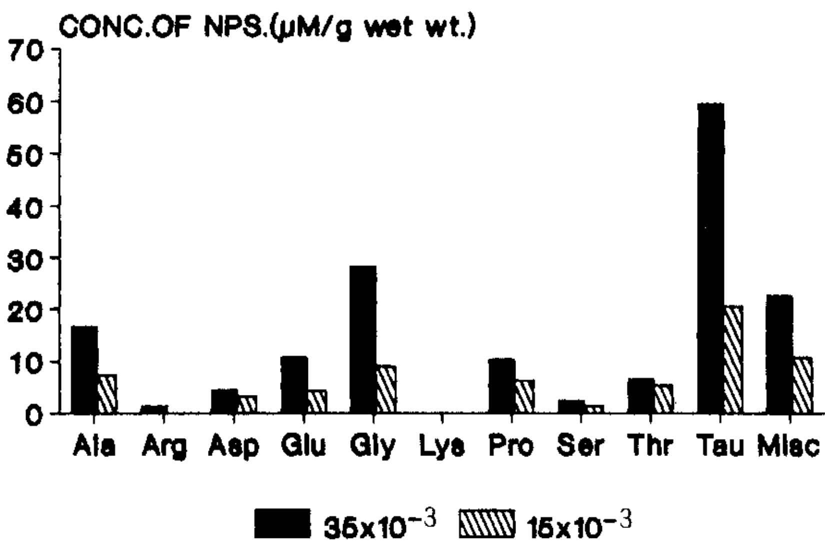

Fig. 39. Mantle

Concentration of free amino acids in various tissues of S. scripta (Small, 20 $\pm$ 2 mm) acclimated to salinities of 35×10^{-3} and 15×10^{-3}.

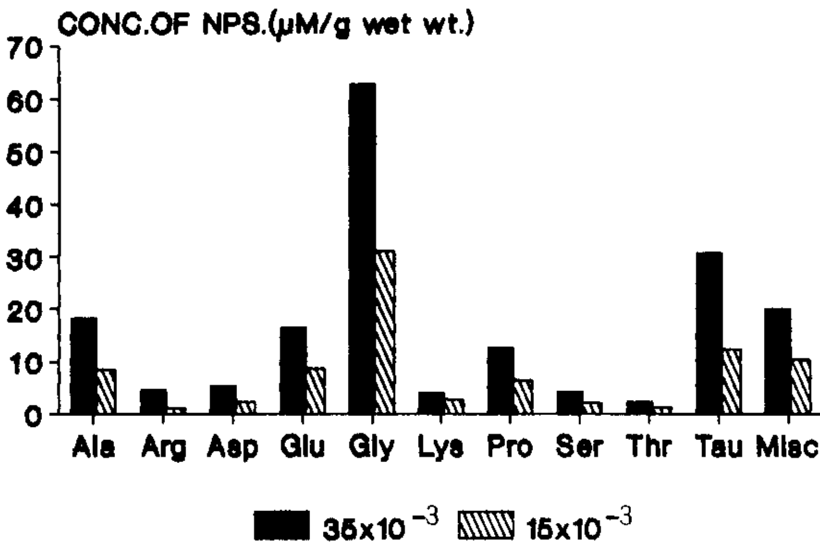

Fig. 40. Adductor

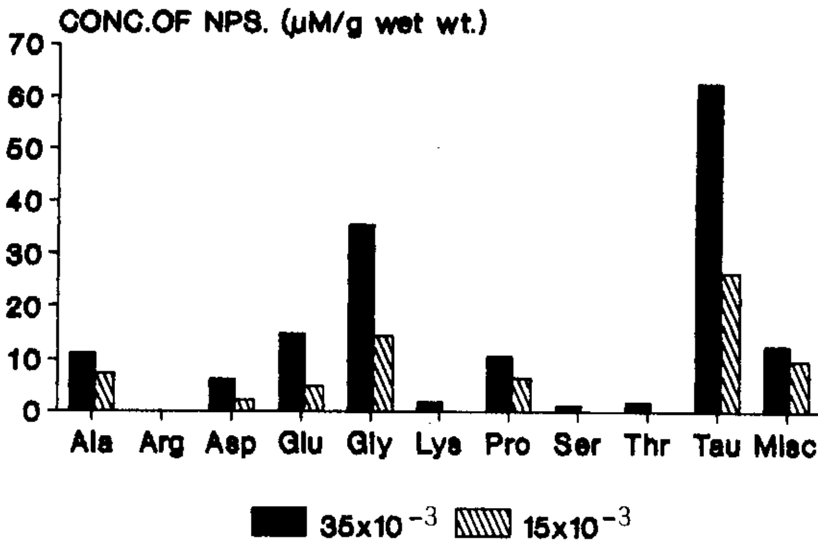

Fig. 41. Foot

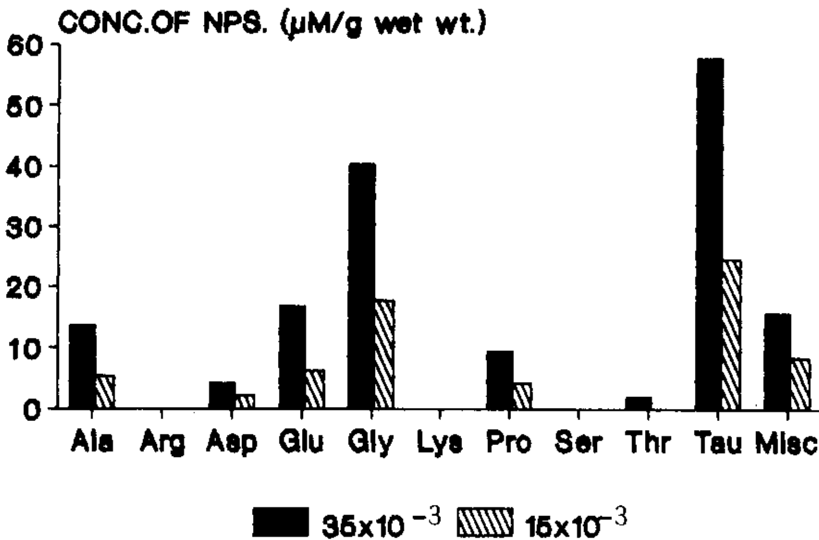

Fig. 42. Mantle

Concentration of free amino acids in various tissues of *S. scripta* (Medium, 30 $\pm$ 2 mm) acclimated to salinities of 35×10^{-3} and 15×10^{-3}.

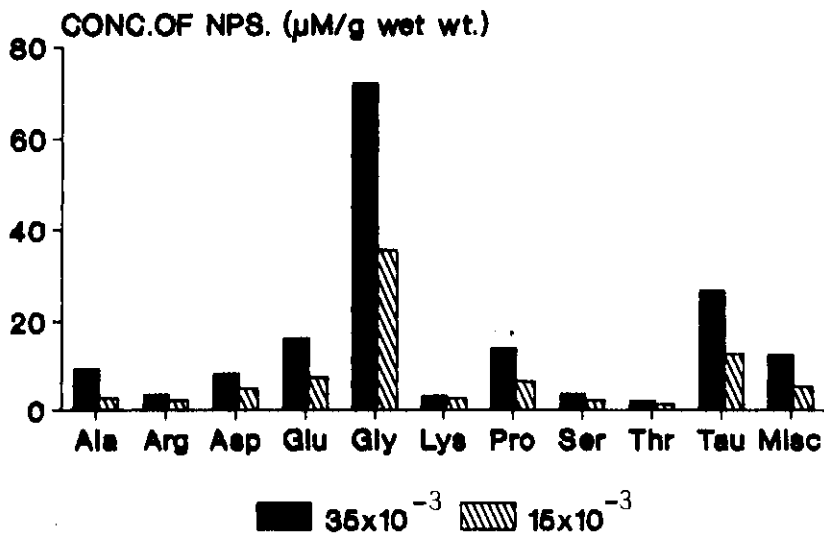

Fig. 43. Adductor

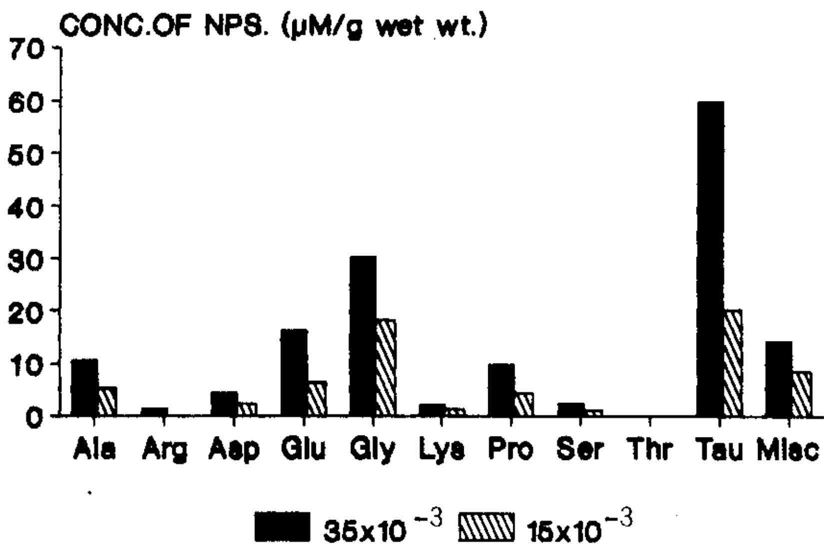

Fig. 44. Foot

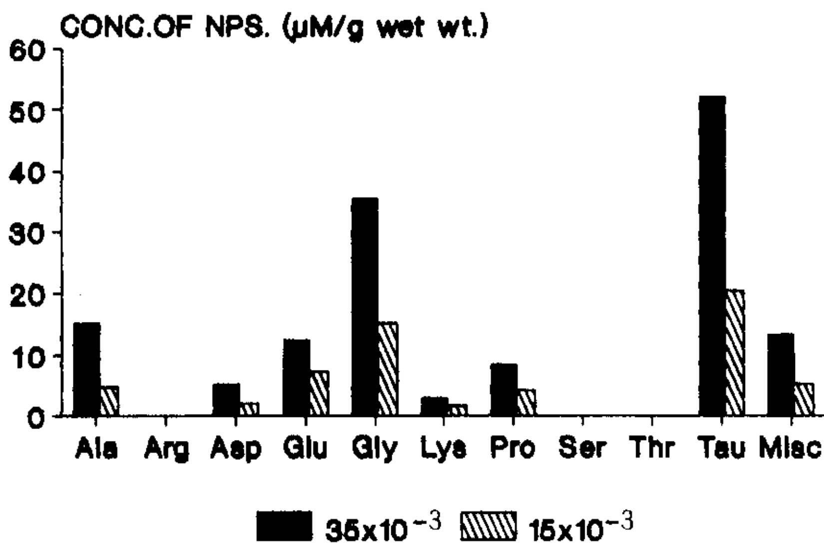

Fig. 45. Mantle

Concentration of free amino acids in various tissues of S. scripta (Large, 40 ± 2 mm) acclimated to salinities of 35×10^{-3} and 15×10^{-3}.

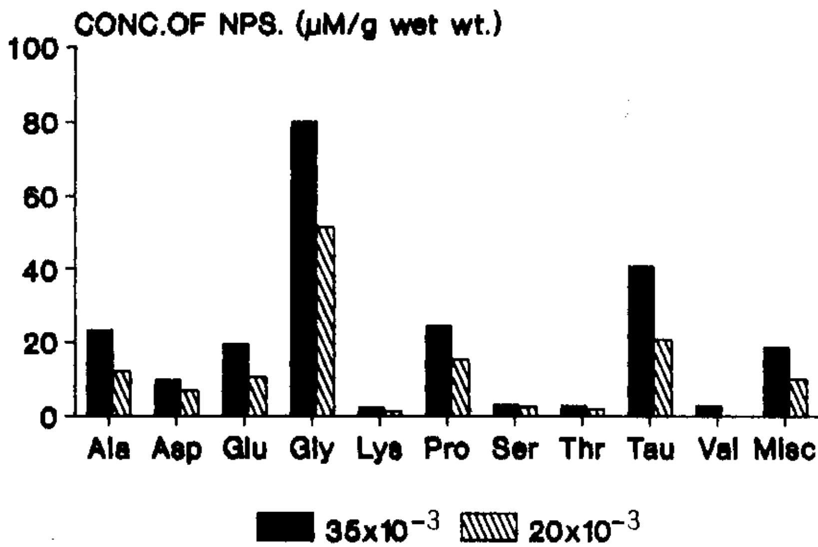

Fig. 46. Adductor

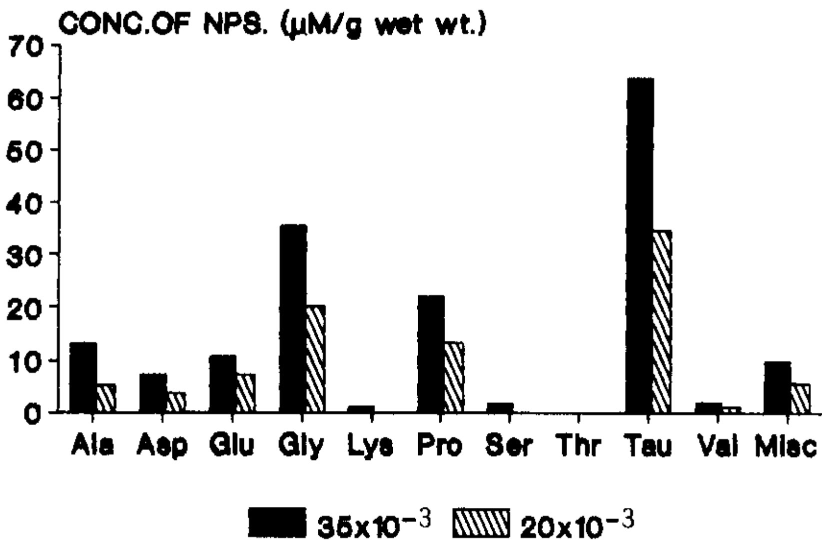

Fig. 47. Foot

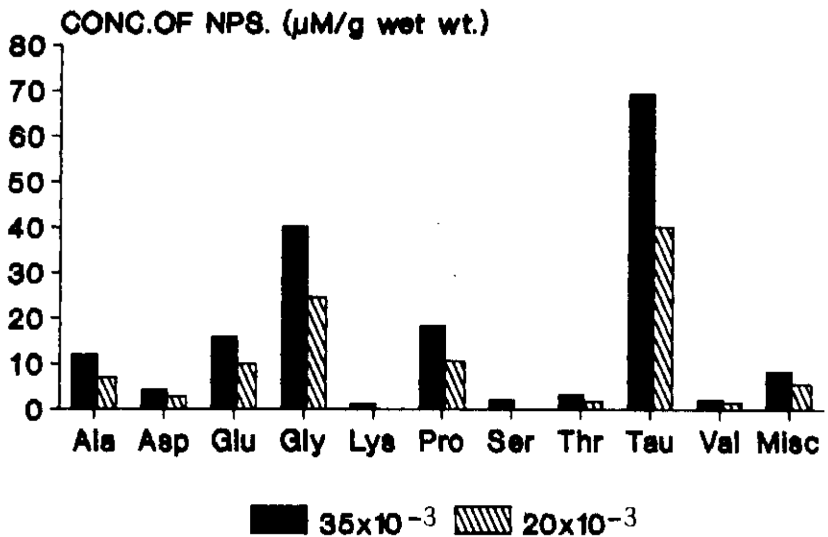

Fig. 48. Mantle

Concentration of free amino acids in various tissues of _P. viridis_ (Small, 35 ± 2 mm) acclimated to salinities of $35x10^{-3}$ and $20x10^{-3}$.

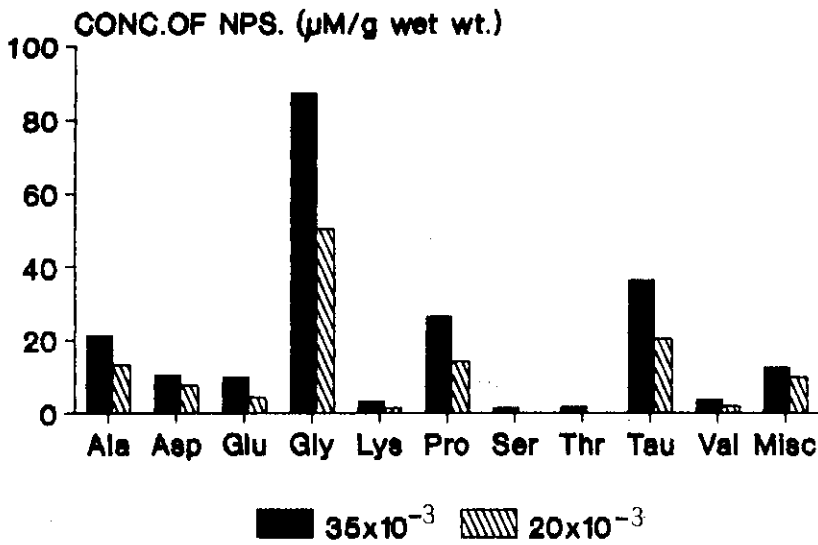

Fig. 49. Adductor

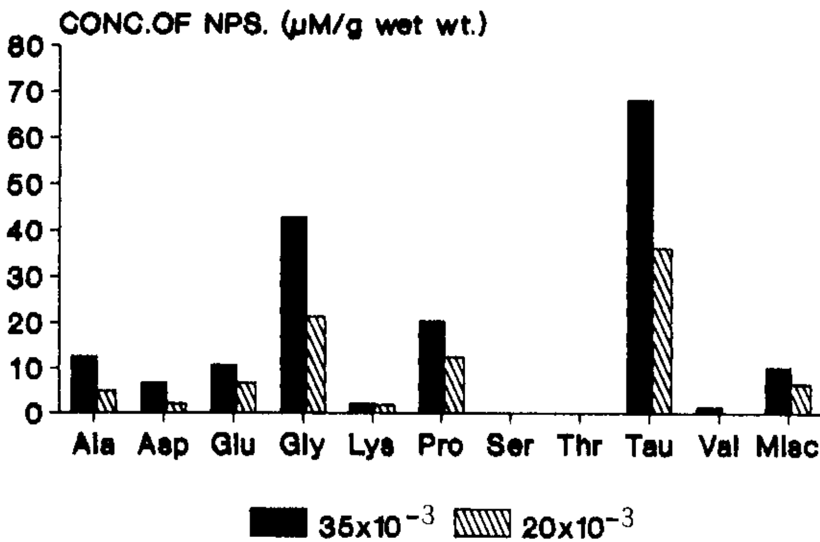

Fig. 50. Foot

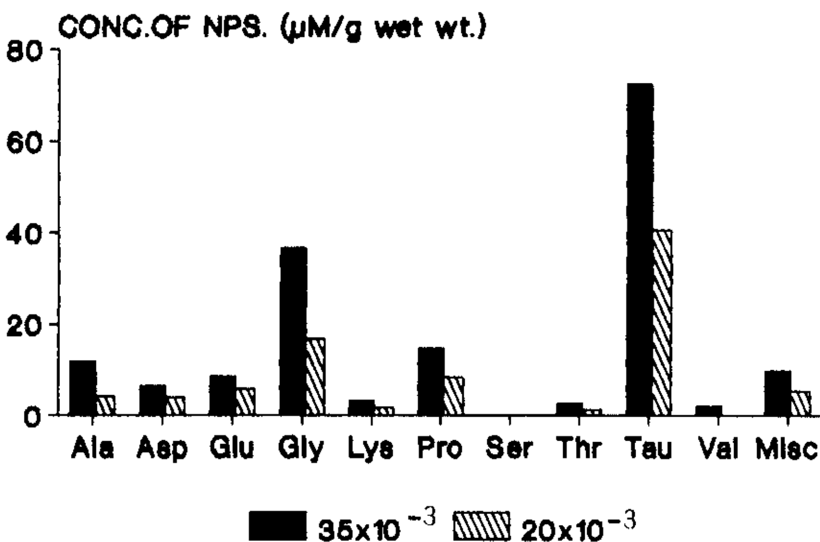

Fig. 51. Mantle

Concentration of free amino acids in various tissues of *P. viridis* (Large, 65 ± 2 mm) acclimated to salinities of 35×10^{-3} and 20×10^{-3}.

all the tissues in all the size groups of both the species. Pattern of accumulation of osmolytes in two levels of salinity is found to vary considerably between the species, size groups and tissues. High concentration of glycine is present in the adductor muscle whereas, a predominance of taurine is noted in the foot muscle and mantle tissue in all the size groups of both the species. High concentration of glycine is always accompanied by relatively low concentration of taurine and vice versa. In general, between species, high concentration of taurine is noticed in *Perna viridis*. The amino acid arginine which is present in *Sunetta scripta* is found to be absent in *Perna viridis*, whereas valine present in *Perna viridis* is totally absent in *Sunetta scripta*.

3.4 DISCUSSION

Intertidal zone is characterized by rapid and extensive changes in salinity. Salinity is one of the prominent ecological variables along the south west coast of India. Intertidal and shallow subtidal species of this region are subjected to wide variations in ambient salinity during south west monsoon. Therefore, both the experimental species, *Sunetta scripta* and *Perna viridis* are exposed to considerable variation in osmotic content of the ambient medium and must have adaptations to cope with it. Both the species express behavioural avoidance by valve closure which diminishes the amplitude of osmotic shock over relatively short periods in such situations (2.1). Present studies

show that extracellular-fluid anisosmotic regulation using inorganic ions is a major means only in lower extremes of salinity (2.2). The role of NPS in maintaining hemolymph osmolarity is negligible in different size groups of both the bivalves (Tables 17 and 18). In short, both the species are osmoconformers and their long term survival in any salinity must depend on isosmotic intracellular regulation.

The concentration of total NPS is found to increase or decrease in response to ambient salinity variations. Considering the experimental salinities, a conspicuous decrease in the concentration of total NPS in the cytoplasm is observed in the lower levels of salinity in all the tissues of all size groups of *Sunetta scripta* and *Perna viridis*. This reduction in the concentration of total NPS indicates their significant role in the intracellular-fluid isosmotic regulation. Previous studies have pointed out the importance of these nitrogenous solutes in marine bivalves in keeping their cell isosmotic with extracellular fluid and maintaining a constant cell volume (Lange, 1963; Florkin, 1966; Hoyaux *et al.*, 1976; Bayne, 1976; Henry and Mangum, 1980b; Matsushima *et al.*, 1987; Pierce *et al.*, 1992).

In a given salinity, total NPS content is found to vary between species, size groups and tissues. The variation of total NPS pool size in relation to salinity gradient is also not same in both the species, their size groups and tissues. Similar observation has been made in marine bivalves by Hoyaux *et al.* (1976), Amende and Pierce (1978), Henry and Mangum (1980b) and Zurburg and De Zwaan (1981). Pierce (1970, 1971a) and Matsushima

et al. (1987) have attributed the difference in NPS pool size to the capability of salinity tolerance of intertidal bivalves.

Of the two species studied, the concentration of total NPS is found to be greater in *Perna viridis*. In addition, the difference in NPS content in response to a similar quantum of osmotic variation is also higher in *Perna viridis* when compared to *Sunetta scripta*. But the salinity tolerance of *S. scripta* towards the lower extremes is found to be significantly greater than that of the mussel. The wider salinity tolerance of the clam, in spite of the smaller NPS content, can be due to greater efficiency of their behavioural avoidance mechanism and significantly higher extracellular ionic regulation in lower salinities. The observation made on *Perna viridis* and *Sunetta scripta* supports the view expressed by Gainey and Greenberg (1977) and Deaton and Greenberg (1991) that the main physiological mechanism which is of relevance in the salinity tolerance towards the lower extremes is extracellular ionic regulation.

Generally in molluscs high salinity tolerance has been noted in smaller size groups (Kinne, 1971). The same has been observed in *Sunetta scripta* (Thampuran *et al.,* 1982). In the present study when different size groups of both the species are taken to account, it is noticed that the smaller size groups maintain a higher concentration of NPS. In addition, the magnitude of variation of NPS between higher and lower levels of salinity is also greater in smaller size groups. In other words, in response to more or less similar quantum of osmotic stress, a higher change in the concentration of total NPS is noticed in smaller size

groups of both the bivalves. The higher concentration of total NPS of the smaller size groups may facilitate more expendable intracellular solutes as salinity drops from $35x10^{-3}$ to lower experimental salinities. This may be helping the smaller size groups to maintain their cellular volume more effectively using compatible nitrogenous osmolytes. Moreover, the smaller size groups also avail greater advantage of extracellular-fluid anisosmotic regulation using the inorganic ions. The above data suggest that for a species living in defined environmental, nutritive and physiological conditions, the size of the NPS pool is also significant in its salinity tolerance.

Among different tissues studied, high concentration of total NPS is noticed in the adductor muscle. The mantle tissue and foot muscle maintain almost a similar content of total NPS but lower than that of adductor muscle. This is true in all the size groups of both the species. The observation is in agreement with those of Amende and Pierce (1978) and Zurburg and De Zwaan (1981). Not only there is quantitative variation in the total NPS in different tissues, qualitative components also vary in their content. A conspicuous difference noticed is a higher content of glycine in the adductor muscle and taurine in foot muscle and mantle tissue. Whether the variation in the molar extinction coefficient produced by the components of NPS pool with ninhydrin (triketohydrindene), has got any expression in the quantitative evaluation of the NPS pool is a matter to be verified.

An important aspect to be noted in the variation of the concentration of total NPS between upper and lower levels of

salinity is the possible alterations it may cause in the biochemical environment of the cytoplasm. *Perna viridis* and *Sunetta scripta* are able to survive in a higher salinity of $35x10^{-3}$ and in lower salinities of $20x10^{-3}$ and $15x10^{-3}$ respectively for prolonged periods in the experimental conditions. The total intracellular NPS content shows a reduction in the lower salinities by 31-46% in *Perna viridis* and 46-56% in *Sunetta scripta*. This suggests that the intracellular milieu is maintained without serious impediment for various life supporting molecular interactions over this change in concentration. Hence, it is likely that every component of the NPS pool is subjected to scrupulous selection in order to make a compatible solute microenvironment in the cytoplasm.

The present study is, therefore, extended to find out the qualitative and quantitative nature of the compatible nitrogenous solute system in different tissues of various size groups of *Sunetta scripta* and *Perna viridis*. It is observed that both the bivalves utilize nitrogenous solutes such as free amino acids including taurine as the major intracellular osmolytes. The similarities among the osmotic system outweigh the variation noticed between the two species, their size groups and tissues. In all cases a few osmolytes make dominant contribution to the intracellular pool. Taurine, glycine, proline, alanine, glutamic acid and aspartic acid are not only the typically dominant solutes in the intracellular fluid of both the species, but also the most varied ones during the acclimation to ambient salinity variations. The presence of arginine, lysine, serine, threonine and valine is

observed but they seem to have comparatively limited role as intracellular osmotic pressure effectors.

Majority of the osmolytes present in the cytoplasm of these molluscs are non-essential amino acids. This is in agreement with other studies on marine bivalves (Bricteux-Gregoire *et al.*, 1964a,b; Gilles, 1972b; Hoyaux *et al.*, 1976; Baginski and Pierce, 1977; Henry and Mangum, 1980b; Zurburg and De Zwaan, 1981; Bishop *et al.*, 1983). The study suggests that the major intracellúlar osmotic effectors such as taurine, glycine, proline, alanine, glutamic acid and aspartic acid form the compatible solute microenvironment in both the bivalves and the biochemial environment is not altered over conspicuous variation in their concentration. Hence, these osmoprotectants are playing a significant role in the euryhaline nature of *Sunetta scripta* and *Perna viridis*. Compatibility of such solute system in marine molluscs and the nonperturbable environment they offer for action of enzymes and other macromolecules over their wide variations in concentration have been discussed by Yancey *et al.* (1982), Somero and Bowlus (1983).

Glycine is an important intracellular osmoprotectant in both the intertidal bivalves studied. Similar observation made by Hoyaux *et al.* (1976) suggests that the relatively high amount of intracellular glycine may be characteristic of bivalves of littoral area. Though the abundance of this simplest amino acid is noticed, its contribution to different tissues is not uniform. In the three tissues studied in all the size groups of both the species, the importance of glycine as a principal osmolyte is

noticed in adductor muscle, whereas, the taurine claims the status in mantle tissue and foot muscle. Even though such a generalization may need more data, similar observation in marine bivalves has been made by Amende and Pierce (1978), Henry and Mangum (1980b) and Zurburg and De Zwaan (1981).

Taurine (2-aminoethanesulfonic acid) is a β-amino acid with a molecular mass of 125 daltons. Overall, taurine is found to be the most abundant and varied osmolyte in these two bivalves. It appears that the intracellular taurine content increases or decreases with corresponding changes in ambient salinity. This does not imply that the contribution of taurine to the total NPS content is always the same. It can be noticed from the concentration of taurine in species, size groups and tissues. Similar findings have been made by Lange (1963) and Zurburg and De Zwaan (1981) in marine bivalves and pointed out the importance of taurine with regard to the maintenance of intracellular volume. In both the bivalves, the higher concentration noticed in the case of taurine indicates the prominent role of this amino acid in the intracellular-fluid isosmotic regulation. The role of taurine in the process has been indicated by Awapara (1962) when he observed a high content of taurine in marine molluscs and little or no taurine in their fresh water or terrestrial counterparts. It is also noteworthy that in euryhaline bivalves taurine concentration maintains a positive correlation to the habitat salinity (Allen, 1961; Lange, 1963). Lange (1963) also suggests that taurine exerts a sparing effect on the use of essential amino acids as intracellular osmolytes. This is true for different size groups of

both the bivalves. The study shows the importance of taurine as a principal osmoprotectant in both the species.

High taurine content noticed in both the species points to the fact that the high salinity acclimated ones can be used as a potential source of this bioactive substance. A substance present in such abundance inherently suggests an integral role in physiological processes (Wright *et al.*, 1986). Taurine has been implicated in diverse functions in many vertebrate and invertebrate species, they include osmoregulation (Lange, 1963; Thurston *et al.*, 1980), neuroinhibition (Thurston *et al.*, 1980), cardiac rhythm control (Huxtable, 1978), membrane stabilization, prevention of retinitis pigmentosa, role in human nutrition, thermoregulation, detoxification of attenuating toxic compounds and antioxidation (Wright *et al.*, 1986), abolition of cell swelling and increased cell viability (Pasantes-Morales *et al.*, 1984) and hypolipidemic action (Gandhi *et al.*, 1992). These suggest a potential field for studies on taurine in marine molluscs.

If anyone of the three tissues of all the size groups of both the species is taken into account, the main difference noticed in the total NPS pool is quantitative, and the qualitative components of the NPS pool do not vary considerably. But striking qualitative difference can be noticed between different tissues. Whether it is due to functional requirement of the tissue is a matter to be probed. It is also likely that some of the dominant amino acids are performing functions other than being simple osmolytes, as noted in the case of taurine. An important step in

understanding the physiological or biochemical role of a solute in an organism is to ascertain where that organism pools that solute, which can be determined by the evaluation of its concentration in various tissues, organs and age groups. The work presented here is an attempt to implicate this basic aspect and hopefully provides avenues for future research.

TABLE NO : 17

Concentration of total NPS in various tissues of three size groups of *S.scripta* acclimated to salinities of 35×10^{-3} and 15×10^{-3}. Concentration is expressed in micromoles per gram wet weight (or per ml in the case of hemolymph).

SIZE GROUPS	TISSUE	CONCENTRATION OF NPS		VARIATION BETWEEN THE SALINITIES
		35×10^{-3}	15×10^{-3}	
SMALL (20±2mm)	ADDUCTOR	201.5 ± 24.6	104.1 ± 27.2	97.4
	FOOT	164.1 ± 26.2	084.8 ± 24.3	79.3
	MANTLE	159.1 ± 31.5	069.4 ± 13.4	89.7
	HEMOLYMPH	0.171 ± 0.06	0.195 ± 0.04	0.024
MEDIUM (30±2mm)	ADDUCTOR	186.5 ± 31.7	096.1 ± 28.3	90.4
	FOOT	156.3 ± 27.2	079.4 ± 16.4	76.9
	MANTLE	162.7 ± 23.2	074.5 ± 15.4	88.2
	HEMOLYMPH	0.180 ± 0.02	0.184 ± 0.04	0.004
LARGE (40±2mm)	ADDUCTOR	174.3 ± 25.6	094.3 ± 18.6	80.0
	FOOT	156.2 ± 19.7	074.5 ± 15.6	81.7
	MANTLE	149.5 ± 13.2	068.2 ± 16.4	81.3
	HEMOLYMPH	0.093 ± 0.02	0.115 ± 0.03	0.022

Values are the mean of ten different observations ± SD
Each observation is from a pooled sample of twenty clams.

TABLE NO : 18

Concentration of total NPS in various tissues of two size groups of *P.viridis* acclimated to salinities of 35×10^{-3} and 20×10^{-3}. Concentration is expressed in micromoles per gram wet weight (or per ml in the case of hemolymph).

SIZE GROUPS	TISSUE	CONCENTRATION OF NPS		VARIATION BETWEEN THE SALINITIES
		35×10^{-3}	20×10^{-3}	
SMALL (35±2mm)	ADDUCTOR	234.5 ± 16.4	142.1 ± 16.8	92.4
	FOOT	178.2 ± 29.7	098.6 ± 18.9	79.6
	MANTLE	182.6 ± 24.8	112.5 ± 21.7	70.1
	HEMOLYMPH	0.238 ± 0.02	0.242 ± 0.03	0.004
LARGE (65±2mm)	ADDUCTOR	218.4 ± 16.6	128.1 ± 23.8	90.3
	FOOT	168.2 ± 27.1	115.8 ± 22.6	52.4
	MANTLE	171.9 ± 14.3	092.1 ± 26.5	79.8
	HEMOLYMPH	0.163 ± 0.02	0.164 ± 0.02	0.001

Values are the mean of ten different observations ± SD

Each observation is from a pooled sample of twenty mussels.

TABLE NO : 19

Concentration of free amino acids in various tissues of *S. scripta* (Small, 20±2 mm) acclimated to salinities of 35×10^{-3} and 15×10^{-3}. Concentration is expressed in micromoles per gram wet weight.

FREE AMINO ACIDS (FAA)	ADDUCTOR MUSCLE				FOOT MUSCLE				MANTLE TISSUE			
	35×10^{-3}		15×10^{-3}		35×10^{-3}		15×10^{-3}		35×10^{-3}		15×10^{-3}	
	CONC	%	CONC	%	CONC	%	CONC	%	CONC	%	CONC	%
Ala	21.2	10.9	9.7	10.5	18.1	10.7	10.3	13.1	16.6	10.3	7.3	10.9
Arg	2.1	1.1	0.0	0.0	0.0	0.0	0.0	0.0	1.4	0.9	0.0	0.0
Asp	9.4	4.8	1.6	1.7	7.5	4.4	3.1	3.9	4.5	2.8	3.1	4.6
Glu	12.5	6.4	6.8	7.3	15.6	9.2	4.2	5.3	10.5	6.5	4.2	6.2
Gly	56.6	29.2	29.1	31.4	29.3	17.3	16.1	20.4	28.2	17.4	8.9	13.2
Lys	3.1	1.6	2.7	2.9	1.7	1.0	0.0	0.0	0.0	0.0	0.0	0.0
Pro	16.2	8.3	7.5	8.1	12.9	7.6	8.4	10.7	10.1	6.2	6.2	9.2
Ser	3.2	1.6	2.5	2.7	3.1	1.8	2.1	2.7	2.3	1.4	1.4	2.1
Thr	5.1	2.6	3.2	3.5	2.6	1.5	0.0	0.0	6.5	4.0	5.2	7.7
Tau	34.2	17.6	11.4	12.3	51.9	30.6	18.5	23.5	59.3	36.6	20.5	30.5
Misc	30.5	15.7	18.1	19.6	27.2	16.0	16.2	20.5	22.6	14.0	10.5	15.6

Values are the mean of three different observations.

Each observation is from a pooled sample of twenty clams.

TABLE NO : 20

Concentration of free amino acids in various tissues of *S.scripta* (Medium, 30±2 mm) acclimated to salinities of 35×10^{-3} and 15×10^{-3}. Concentration is expressed in micromoles per gram wet weight.

FREE AMINO ACIDS (FAA)	ADDUCTOR MUSCLE				FOOT MUSCLE				MANTLE TISSUE			
	35×10^{-3}		15×10^{-3}		35×10^{-3}		15×10^{-3}		35×10^{-3}		15×10^{-3}	
	CONC	%	CONC	%	CONC	%	CONC	%	CONC	%	CONC	%
Ala	18.2	10.1	8.4	9.6	11.2	7.1	7.2	10.1	13.5	8.5	5.3	7.7
Arg	4.6	2.6	1.2	1.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Asp	5.2	2.9	2.3	2.6	6.3	4.0	2.2	3.1	4.2	2.6	2.2	3.2
Glu	16.4	9.1	8.6	9.9	14.7	9.3	4.9	6.9	16.7	10.5	6.3	9.2
Gly	62.8	34.8	31.2	35.8	35.6	22.5	14.3	20.1	40.2	25.2	17.7	25.8
Lys	4.1	2.3	2.8	3.2	1.8	1.1	0.0	0.0	0.0	0.0	0.0	0.0
Pro	12.5	6.9	6.4	7.3	10.6	6.7	6.4	9.0	9.3	5.8	4.2	6.1
Ser	4.2	2.3	2.2	2.5	1.2	0.8	0.0	0.0	0.0	0.0	0.0	0.0
Thr	2.3	1.3	1.4	1.6	1.7	1.1	0.0	0.0	2.1	1.3	0.0	0.0
Tau	30.6	16.9	12.3	14.1	62.4	39.5	26.5	37.2	57.7	36.2	24.6	35.8
Misc	19.8	11.0	10.4	11.9	12.6	8.0	9.7	13.6	15.6	9.8	8.4	12.2

Values are the mean of three different observations.

Each observation is from a pooled sample of twenty clams.

TABLE NO : 21

Concentration of free amino acids in various tissues of *S. scripta* (Large, 40±2 mm) acclimated to salinities of $35x10^{-3}$ and $15x10^{-3}$. Concentration is expressed in micromoles per gram wet weight.

FREE AMINO ACIDS (FAA)	ADDUCTOR MUSCLE				FOOT MUSCLE				MANTLE TISSUE			
	$35x10^{-3}$		$15x10^{-3}$		$35x10^{-3}$		$15x10^{-3}$		$35x10^{-3}$		$15x10^{-3}$	
	CONC	%	CONC	%	CONC	%	CONC	%	CONC	%	CONC	%
Ala	9.2	5.4	2.6	3.1	10.6	7.0	5.2	7.7	15.2	10.5	4.6	7.6
Arg	3.4	2.0	2.3	2.8	1.4	0.9	0.0	0.0	0.0	0.0	0.0	0.0
Asp	8.2	4.8	4.7	5.6	4.5	3.0	2.3	3.4	5.2	3.6	2.1	3.5
Glu	16.3	9.5	7.4	8.9	16.2	10.8	6.4	9.4	12.4	8.6	7.2	11.9
Gly	72.1	42.0	35.6	42.7	30.2	20.0	18.3	27.0	35.4	24.5	15.2	25.1
Lys	3.2	1.9	2.6	3.1	2.1	1.4	1.4	2.1	2.9	2.0	1.7	2.8
Pro	14.2	8.3	6.4	7.7	9.7	6.4	4.4	6.5	8.3	5.7	4.2	6.9
Ser	3.6	2.1	2.2	2.6	2.3	1.5	1.2	1.8	0.0	0.0	0.0	0.0
Thr	2.1	1.2	1.4	1.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Tau	26.8	15.6	12.8	15.4	59.6	39.6	20.2	29.8	52.1	36.0	20.3	33.5
Misc	12.4	7.2	5.3	6.4	14.1	9.4	8.5	12.5	13.2	9.1	5.3	8.8

Values are the mean of three different observations.
Each observation is from a pooled sample of twenty clams.

TABLE NO : 22

Concentration of free amino acids in various tissues of *P. viridis* (Small, 35±2 mm) acclimated to salinities of 35×10^{-3} and 20×10^{-3}. Concentration is expressed in micromoles per gram wet weight.

FREE AMINO ACIDS	ADDUCTOR MUSCLE				FOOT MUSCLE				MANTLE TISSUE			
	35×10^{-3}		20×10^{-3}		35×10^{-3}		20×10^{-3}		35×10^{-3}		20×10^{-3}	
(FAA)	CONC	%	CONC	%	CONC	%	CONC	%	CONC	%	CONC	%
Ala	23.1	10.2	12.1	9.1	13.2	7.9	5.2	5.7	11.7	6.6	6.9	6.6
Asp	9.7	4.3	6.9	5.2	7.2	4.3	3.8	4.2	4.2	2.4	2.7	2.6
Glu	19.3	8.5	10.8	8.1	10.7	6.4	7.2	7.9	15.7	8.9	9.9	9.5
Gly	80.1	35.4	51.4	38.6	35.6	21.3	20.1	22.1	40.1	22.7	24.7	23.8
Lys	2.1	0.9	1.4	1.1	1.2	0.7	0.0	0.0	1.1	0.6	0.0	0.0
Pro	24.3	10.7	15.3	11.5	22.1	13.2	13.4	14.7	18.2	10.3	10.6	10.2
Ser	3.2	1.4	2.6	2.0	1.8	1.1	0.0	0.0	2.1	1.2	0.0	0.0
Thr	2.8	1.2	1.9	1.4	0.0	0.0	0.0	0.0	3.2	1.8	1.8	1.7
Tau	40.7	18.0	20.5	15.4	63.8	38.1	34.5	38.0	69.4	39.4	40.2	38.7
Val	2.7	1.2	0.0	0.0	1.9	1.1	1.1	1.2	2.1	1.2	1.5	1.4
Misc	18.4	8.1	10.2	7.7	9.8	5.9	5.6	6.2	8.4	4.8	5.6	5.4

Values are the mean of three different observations.

Each observation is from a pooled sample of twenty mussels.

TABLE NO : 23

Concentration of free amino acids in various tissues of *P. viridis* (Large, 65±2 mm) acclimated to salinities of $35x10^{-3}$ and $20x10^{-3}$. Concentration is expressed in micromoles per gram wet weight.

FREE AMINO ACIDS (FAA)	ADDUCTOR MUSCLE				FOOT MUSCLE				MANTLE TISSUE			
	$35x10^{-3}$		$20x10^{-3}$		$35x10^{-3}$		$20x10^{-3}$		$35x10^{-3}$		$20x10^{-3}$	
	CONC	%	CONC	%	CONC	%	CONC	%	CONC	%	CONC	%
Ala	21.1	9.9	13.2	10.8	12.4	7.1	4.7	5.1	11.5	6.9	4.2	4.8
Asp	10.3	4.8	7.6	6.2	6.7	3.8	2.1	2.3	6.2	3.7	3.8	4.3
Glu	9.7	4.6	4.2	3.4	10.6	6.1	6.7	7.3	8.5	5.1	5.7	6.5
Gly	87.2	40.9	50.2	41.0	42.8	24.6	21.3	23.2	36.7	21.9	16.8	19.2
Lys	3.1	1.5	1.2	1.0	2.1	1.2	1.8	2.0	3.2	1.9	1.7	1.9
Pro	26.5	12.4	14.1	11.5	20.3	11.7	12.5	13.6	14.7	8.8	8.3	9.5
Ser	1.2	0.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Thr	1.7	0.8	0.0	0.0	0.0	0.0	0.0	0.0	2.7	1.6	1.2	1.4
Tau	36.4	17.1	20.4	16.6	68.1	39.1	36.2	39.5	72.3	43.1	40.6	46.4
Val	3.7	1.7	1.8	1.5	1.2	0.7	0.0	0.0	2.1	1.3	0.0	0.0
Misc	12.5	5.9	9.9	8.1	10.1	5.8	6.4	7.0	9.7	5.8	5.2	5.9

Values are the mean of three different observations.

Each observation is from a pooled sample of twenty mussels.

CHAPTER 4

MECHANISMS IN THE REGULATION OF OSMOLYTES

INTRACELLULAR FREE AMINO ACIDS

INTRACELLULAR FREE AMINO ACIDS

4.1 INTRODUCTION

Intracellular-fluid isosmotic regulation is the cardinal biological process that protects an organism against the lethal effects of osmotic stress (Le Rudulier, 1984). It is well known that free amino acids including taurine form the major part of the measured intracellular NPS in marine bivalves. Similar observation is noticed during the present study in both the bivalves experimented (3). NPS play a significant role in adjusting the cellular osmolarity to changing osmotic concentrations of hemolymph values in both the species (3). The process of cellular salinity tolerance using NPS reduces the salinity related fluctuations in intracellular electrolytes and averts perturbations of molecular interactions that support life. In general, the involvement of free amino acids including taurine in the regulation of cell volume and its importance in bivalves are well recognized. However, cellular regulation of these osmolytes in euryhaline species is not simple and remains to be clarified (Somero and Bowlus, 1983; Heavers and Hammen, 1985; Hayashi, 1987).

Possible involvement of different regulatory mechanisms under hypoosmotic and hyperosmotic stress has been pointed out by Gilles (1975), Zurburg and De Zwaan (1981), Bishop *et al.* (1983), Somero and Bowlus (1983) and Hayashi (1987). The mechanisms involved in the hypoosmotic stress are comparatively simple and well understood (Zurburg and De Zwaan, 1981). They include removal of nitrogenous osmolytes from the cell and their enhanced catabolism. The higher NPS content of the cell during hyperosmotic

stress has been attributed to active uptake, *de novo* synthesis and proteolysis. Regulation of NPS pool in response to ambient salinity variations employing the different mechanisms has been supported by various studies, but the relative importance of these processes depends on the nutritional, physiological and environmental conditions of the animal (Riley, 1980).

Mobilization of amino acids across the cell membrane is passive during hypoosmotic adjustment and active during hyperosmotic regulation. Large chemical gradient prevailing between ambient medium and body fluids favours the loss of amino acids to surrounding medium (Wright and Secomb, 1986). Such a loss of amino acids has been suggested to constitute a significant fraction of total nitrogen excreted by bivalves (Hammen, 1968; Bayne, 1973; Bayne and Scullard, 1977). Bayne (1975) and Wright and Secomb (1986) have demonstrated that the major constituent of this loss is taurine. Bayne (1973) suggested that the energy drain due to loss of amino acids can be an average of 10% of an animal's metabolic rate which can increase up to 63% under nutritional and temperature stress, and the same may apply to osmotic stress also.

The active uptake of amino acids in marine bivalves from very dilute external medium has been well documented by Bamford and McCrea (1975), Rice *et al.* (1980), Ferguson (1982), Manahan *et al.* (1983), Jorgensen (1983), Wright and Secomb (1984, 1986), Matsushima and Hayashi (1988), Manahan (1989) and Wright and Pajor (1989). Many bivalves make use of intestinal and extraintestinal free amino acids as potential sources for epithelial amino acid transport during hyperosmotic stress (Wright and Secomb, 1986). In many studies the relative importance of this mechanism is

primarily attributed to taurine regulation.

Taurine is found to be an important osmolyte in most of the euryhaline bivalves studied. Similar observation is made in the two experimental species now under study (3). However, high levels of taurine in the tissues of marine bivalves cannot be accounted for by their ability to synthesis taurine (Bishop *et al.*, 1983; Wright and Secomb, 1986). They also lack the ability to catabolize taurine (Awapara, 1976; Bishop *et al.*, 1983; Wright and Secomb, 1986). In addition, no protein source can also be attributed for the high content of taurine. Hence it is suggested by many workers that much of the taurine is probably obtained from the diet, intestinal symbionts and through active uptake from the ambient medium (Pierce and Amende, 1981; Manahan *et al.*, 1983; Bishop *et al.*, 1983; Wright and Secomb, 1986; Wright, 1987). Uptake of taurine has been reported from very dilute external concentration against an apparent chemical gradient in excess of six million to one (Wright and Secomb, 1986). Hence taurine regulation during osmotic stress seems to be mainly controlled by membrane related processes. Although not prominent as in the case of taurine other amino acids may also avail this process (Crowe *et al.*, 1977; Stewart, 1978; Manahan *et al.*, 1983; Matsushima and Hayashi, 1988).

Metabolic regulation of NPS pool in response to salinity variations of external medium involves changes in glycolysis, TCA cycle reactions, transaminases and metabolite shuttling between cellular compartments (Karam *et al.*, 1987).

Many workers have observed an elevation of ammonia excretion in bivalves along with the reduction of NPS pool during their

acclimation to lowered salinity. This indicates catabolism of amino acids during intracellular-fluid isosmotic regulation (Bishop *et al.*, 1983; Hayashi, 1987). Mitochondrial transport and oxidation of amino acid osmolytes may contribute to cell volume regulation during hypoosmotic stress (Ballantyne and Storey, 1983, 1985; Moyes and Ballantyne, 1987). Since considerable energy loss accompanies the removal of amino acids through membrane related process (Bayne, 1973; Bayne and Scullard, 1977; Wright and Secomb, 1986), the cell may prefer the metabolic regulation of those amino acids which have high metabolic turnover (Heavers and Hammen, 1985). In this context it is noteworthy that the level of taurine which has got a low metabolic turnover is adjusted mainly through membrane related process (Wright and Secomb, 1986). This exerts a sparing effect on the loss of essential free amino acids (Lange, 1963; Gilles, 1972a; Heavers and Hammen, 1985; Wright and Secomb, 1986).

Synthesis of amino acids from the precursors and intermediates of metabolism and augmentation to the NPS pool during hyperosmotic stress in euryhaline bivalves have been suggested by Chen and Awapara (1969), Bishop *et al.* (1983), Karam *et al.* (1987) and Moyes and Ballantyne (1987). Biosynthesis has also been reported by Baginski and Pierce (1975), Livingstone *et al.* (1979), Zurburg and De Zwaan (1981), Bishop *et al.* (1983), Somero and Bowlus (1983) and Karam *et al.* (1987) and concluded that acclimation to high intracellular salinity in molluscs depends on synthesis of amino acids in which anaerobic pathways are involved.

In addition to the synthesis of amino acids, endogenous

protein breakdown in response to salinity changes has been attributed as another source of amino acids during hyperosmotic volume regulation (Lange, 1972; Moore *et al.*, 1980; Deaton *et al.*, 1984; Stickle *et al.*, 1985; Hawkins and Hilbish, 1992). Depolymerization of dietary proteins, storage proteins and endogenous proteins are the different sources of free amino acids. Of the three processes, the proteolysis of endogenous proteins is supposed to be mediated by lysosomes. It has been noticed that the lysosomal system is highly developed in the digestive cells of marine bivalves (Owen, 1972; Bayne, 1976). This lysosomal system is known to respond to variations in salinity (Bayne *et al.*, 1978; Moore *et al.*, 1980).

Previous studies have indicated that the lysosome mediated proteolysis in response to hyperosmotic stress is responsible for the generation of free amino acids for intracellular osmoregulation in bivalves (Moore *et al.*, 1980; Bayne *et al.*, 1981; Moore, 1982; Stickle *et al.*, 1985; Deaton, 1987). This function appears to involve permeability or fluidity changes in lysosomal membrane (Moore *et al.*, 1980; Bayne *et al.*, 1981; Moore 1982; Stickle *et al.*, 1985).

Variations of salinity in the ambient medium is accompanied by variations in the concentration of inorganic ions especially sodium, potassium and chloride. This in turn, is almost totally reflected in the concentration of these ions in the hemolymph of marine bivalves (2.2). Reflection of this variation of inorganic ions in the intracellular fluid is comparatively small and the cell may be actively regulating their concentration in order to avoid their perturbing effects over a critical level (Yancey *et*

al., 1982; Somero and Bowlus, 1983). However levels of intracellular sodium, potassium and chloride ions undergo appreciable changes in concentration in bivalve molluscs with acclimation to different levels of salinity (Bricteux-Gregoire *et al.*, 1964a,b; Pierce, 1982; Pierce and Politis, 1990; Pierce *et al.*, 1992). Changing intracellular levels of monovalent ions may be important signals in initiating changes in the rates of metabolic turnover of osmotically compatible amino acids (Gilles, 1969; Gomez-Puyou *et al.*, 1972; Moyes and Ballantyne, 1987). In addition, the variations can also bring about changes in lysosomal membrane stability (Moore *et al.*, 1980; Bayne *et al.*, 1981; Moore, 1982; Stickle *et al.*, 1985). In short, various workers have suggested that it is the change in concentration of inorganic ions to which cell is first submitted during acclimation to different levels of salinity which largely controls the intracellular-fluid isosmotic regulation in euryhaline bivalves. Intracellular ionic concentration may control the activity of key enzymes implicated in the amino acid metabolism and also the stability of lysosomal membrane.

The present study therefore is an attempt to understand the effect of different concentrations of ions on the activity of key enzymes involved in the metabolism of amino acids and the lability of lysosomal membrane under various concentration of NaCl and KCl. Since intracellular concentration of sodium, potassium and chloride ions is subjected to variation under osmotic stress, the influence of the above salts was examined in both the experimental species, *Sunetta scripta* and *Perna viridis*.

4.2 MATERIALS AND METHODS

Glutamate dehydrogenase (GDH), EC 1.4.1.2 : Tissues (adductor, foot and mantle) were removed from the bivalves and homogenized in 5 mM Tris-HCl buffer (pH 7.8) containing 0.1% (v/v) Triton X-100. The homogenate was centrifuged at 20,000g for 30 min.in a refrigerated centrifuge at $0^{o}C$. The supernatant was saved and used for the enzyme assay (Hayashi, 1987). The enzyme activity was assayed following the method of Reiss *et al.* (1977) and Hayashi (1987). 2.6 ml of reaction mixture consisted of 100 mM Tris-HCl (pH 7.8), 10 mM α-ketoglutarate, different concentrations (0, 50, 100, 200, 300, 400 and 500 mM) of NaCl or KCl, 100 mM ammonium acetate, 0.2 mM NADH, 1 mM ADP and enzyme preparation. The reaction was initiated by the addition of enzyme and was performed at $30^{o}C$. Change in absorbance at 340 nm was measured using spectrophotometer. A standard graph of NADH was prepared and the activity of GDH was expressed as mg NADH oxidized per hour per gram protein present in the extract. Protein content was estimated by the procedure of Lowry *et al.* (1951) using bovine serum albumin as standard.

Serine hydrolyase (SH), EC 4.2.1.13 : Removed adductor, foot and mantle tissue from the bivalves and homogenized in 0.1 M phosphate buffer (pH 8.0). The homogenate was centrifuged at 20,000g for 30 min. in refrigerated centrifuge at $0^{o}C$. The supernatant collected was employed as the enzyme sample. The activity of SH was assayed following the method of Suda and Nakagawa (1971). 0.1 ml of 0.5 mM pyridoxal phosphate, 0.5 ml of 0.1 M phosphate buffer (pH 8.0) containing 2 mM EDTA and 0.2 ml of

enzyme extract were incubated at 37°C for 5 min. To this was added 0.2 ml of serine previously warmed to 37°C containing different concentrations (0, 50, 100, 200, 300, 400 and 500 mM) of NaCl or KCl. The reaction mixture was again incubated at 37°C for 5 min. 0.5 ml of 10% trichloroacetic acid was pipetted into all the tubes. This mixture was placed in ice bath for 10 min. and the precipitate formed was removed by centrifugation. 0.5 ml of this supernatant was mixed with 0.5 ml of 0.033% (2 N HCl) 2,4-Dinitrophenylhydrazine. The mixture was kept at room temperature for 5 min. 2 ml of 2 N NaOH was added to all the tubes and the extinction was read at 520 nm within 5 min. Amount of pyruvate formed was calculated from the standard graph and the specific activity was expressed as mg pyruvate formed per hour per gram protein. Protein was assayed following the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Lactate dehydrogenase (LDH), EC 1.1.1.27 : Preparation of enzyme samples from the tissues was done using 50 mM phosphate buffer (pH 7.5) as given for SH. The LDH activity was assayed by the method of Bergmeyer and Bernt (1974). 3.15 ml of reaction mixture consisted of 50 mM phosphate buffer (pH 7.5), 0.6 mM pyruvate, different concentrations (0, 50, 100, 200, 300, 400 and 500 mM) of NaCl or KCl, 0.18 mM NADH and enzyme preparation. The reaction was initiated by the addition of enzyme and was performed at 30°C. The activity was determined from the rate of oxidation of NADH. The change in extinction at 340 nm was measured in a spectrophotometer. A standard graph of NADH was prepared and the activity of the enzyme was expressed as mg NADH oxidized per hour per gram protein present in the sample. Protein content was

estimated by following the procedure of Lowry *et al.* (1951) using bovine serum albumin as standard.

Aspartate aminotransferase (AST), EC 2.6.1.1 : Preparation of the enzyme sample from the tissues was done as given for lactate dehydrogenase. The activity of AST was determined following the method of Reitman and Frankel (1957). 0.5ml of buffered substrate (13.3 mg of aspartate and 14.6 mg of α-ketoglutarate in 50 ml of 0.1 M phosphate buffer, pH 7.4) containing different concentrations (0, 50, 100, 200, 300, 400 and 500 mM) of NaCl or KCl was warmed for 3 min. at 37°C. 0.3 ml of enzyme sample was added to all test tubes except the blank and incubated for 60 min. at 37°C. The enzyme activity was stopped by the addition of 1 ml of 1 mM 2,4-Dinitrophenylhydrazine (chromogen). The mixture was shaken and allowed to stand for 20 min. at room temperature. The color reaction was stopped by the addition of 5 ml of 0.4 N NaOH. After 15 min. the hydrazone formed was measured spectrophotometrically at 510 nm. The pyruvate released was determined from a standard graph prepared using sodium pyruvate. Specific activity of AST was expressed as mg pyruvate formed per hour per gram protein present in the extract. Protein content was estimated by following the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Stability of lysosomal membrane in different concentrations of NaCl and KCl was determined following the method of Philip and Kurup (1977, 1978) and Rao and Sisodia (1986) with slight modifications. Digestive tubules of both the bivalves were removed and gently homogenized in 0.4 M sucrose containing 2 mM mercaptoethanol at 4°C and the homogenate was centrifuged at 8000g

for 10 min. in a refrigerated centrifuge. The supernatant saved was again centrifuged at 23,000g for 30 min. at 0°C. The sediment obtained (lysosome rich fraction) was suspended in 0.4 M sucrose containing 2 mM mercaptoethanol. This suspension was incubated at 30°C in presence of different concentrations (0, 50, 100, 200, 300, 400 and 500 mM) of KCl or NaCl for 30 min. Intact lysosomes were then removed by centrifuging at 23,000g for 30 min. in a refrigerated centrifuge at 0°C. The supernatant was collected and assayed for acid phosphatase released from the lysosomes following the method of Anon (1963). 0.5 ml of the substrate (400 mg p-nitrophenyl phosphate in 100 ml distilled water) and 0.5 ml of 100 mM citrate buffer (pH 4.5) were incubated for 3 min. at 37°C. The reaction was initiated by the addition of enzyme sample and was incubated for 30 min. at 37°C. The reaction was stopped by the addition of 2 ml of 0.25 N NaOH. The yellow color of p-nitrophenol formed in the alkaline medium was read at 410 nm. The concentration of p-nitrophenol liberated by the action of the enzyme was determined from the calibration curve prepared. Simultaneously the protein content was estimated following the method of Lowry *et al.* (1951) using bovine serum albumin as standard. Specific activity of acid phosphatase as mg p-nitrophenol liberated per hour per gram protein was calculated.

The relation between the concentration of salts (NaCl and KCl) and the enzyme activity or lability of lysosomes was tested statistically using correlation analysis. The variation of this effect among NaCl and KCl was tested using analysis of variance (ANOVA).

4.3 RESULTS

Effect of NaCl and KCl on the activity of glutamate dehydrogenase (GDH) from *Sunetta scripta* and *Perna viridis* is given in Tables 24 and 25 and Fig. 52 and 53 respectively. In the case of *S. scripta*, the activity of GDH is found to be positively correlated with NaCl (0.99) and KCl (0.94) concentration. GDH activity from *P. viridis* also showed a positive correlation with NaCl (0.99) and KCl (0.99). Enhancement of GDH activity with KCl concentration is however found to be greater than that with NaCl in both the bivalves and is statistically significant ($P<0.001$).

The activity of serine hydrolyase from *S. scripta* showed a decrease with increase in NaCl and KCl concentration (Table 26 and Fig. 54). In the presence of KCl the decrease in activity is conspicuous up to 300 mM and above that the enzyme showed a positive correlation in activity. The inhibitory effect of KCl is found to be significantly higher compared to NaCl below 300 mM ($P<0.001$). The trend of activity exhibited by the enzyme in presence of salts is similar in the case of *P. viridis* also (Table 27 and Fig. 55), When both the species are taken into account, KCl-induced reduction in activity is more pronounced in *S. scripta* whereas the NaCl claims the status in *P. viridis*. The lowest activity of SH from *P. viridis* with NaCl is noticed at 300 mM followed by an increase which attains near control activity at 500 mM. Variation in the effect of NaCl and KCl on the enzyme activity is found to be statistically significant ($P<0.001$).

In the case of *S. scripta*, the activity of lactate dehydrogenase (LDH) is found to decrease with increase in

concentration of salts (Table 28 and Fig. 56). Thus a negative correlation has been observed with NaCl (-0.99) and KCl (-0.95). However the inhibitory effect is found to be greater for KCl compared to NaCl in the lower concentrations which is found to be significant (P<0.001). In the case of *P. viridis* also (Table 29 and Fig. 57) a negative correlation is observed with NaCl (-0.9). However, the LDH activity is found to increase with KCl concentration up to 400 mM followed by a decrease in activity. The overall correlation is noted to be positive (0.76).

Effect of NaCl and KCl on the activity of aspartate aminotransferase (AST) from *S. scripta* and *P. viridis* is given in Tables 30 and 31 and Fig. 58 and 59 respectively. The enzyme activity in *S. scripta* showed a steady decrease with increase in NaCl concentration up to 300 mM, followed by an increase which reached control value at 500 mM. However the enzyme activity showed an increasing trend with KCl concentration till 400 mM and at 500 mM a decrease is observed, altogether exhibiting a positive correlation (0.6). Unlike in *S. scripta*, both the salts exert an enhancing effect on the AST activity in the case of *P. viridis*. The positive correlation observed with NaCl is 0.98 and with KCl is 0.9. However the NaCl-induced activation of the enzyme from *P. viridis* is found to be significantly higher when compared to that due to KCl (P<0.001).

The effect of NaCl and KCl on the membrane stability of lysosomes from *S. scripta* and *P. viridis* are given in Tables 32 and 33 and Fig. 60 and 61 respectively. Lability of lysosomal membrane indicated by the activity of acid phosphatase released from lysosomes showed a steady increase with NaCl and KCl

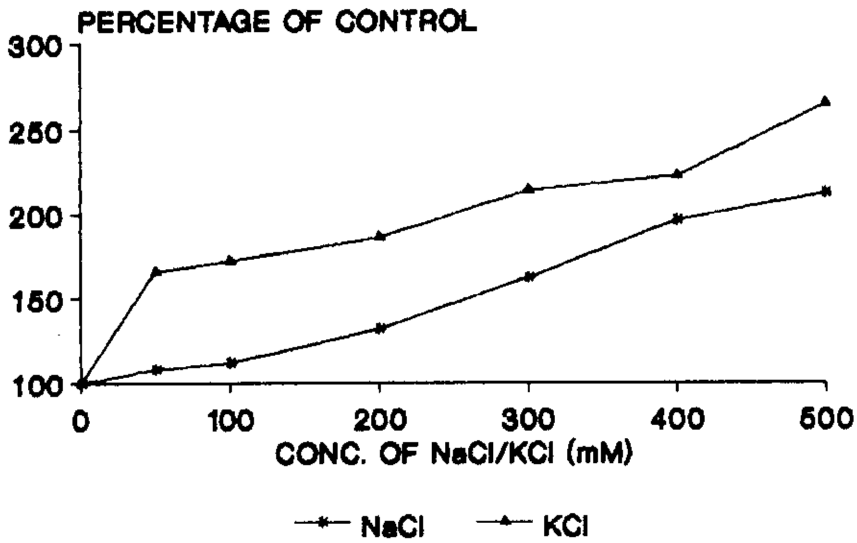

Fig. 52. *S. scripta*

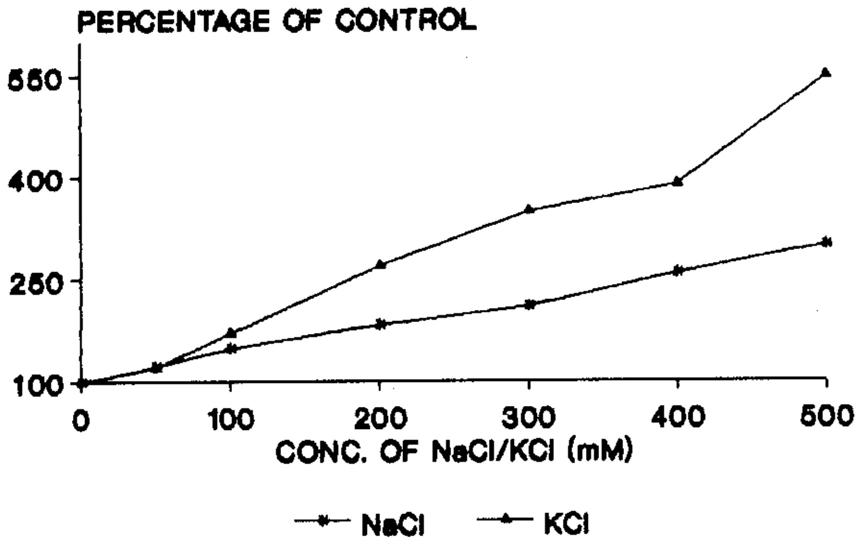

Fig. 53. *P. viridis*

Activity of GDH modified by different levels of NaCl and KCl concentration.

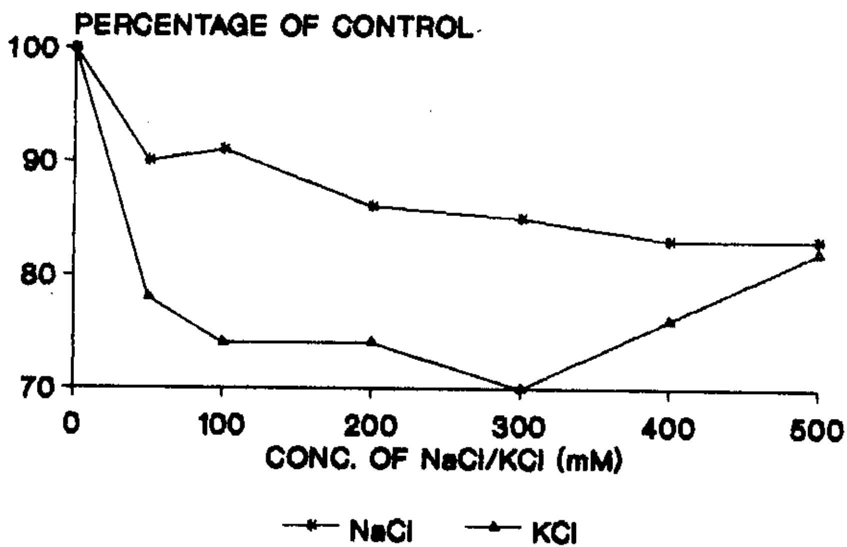

Fig. 54. S. scripta

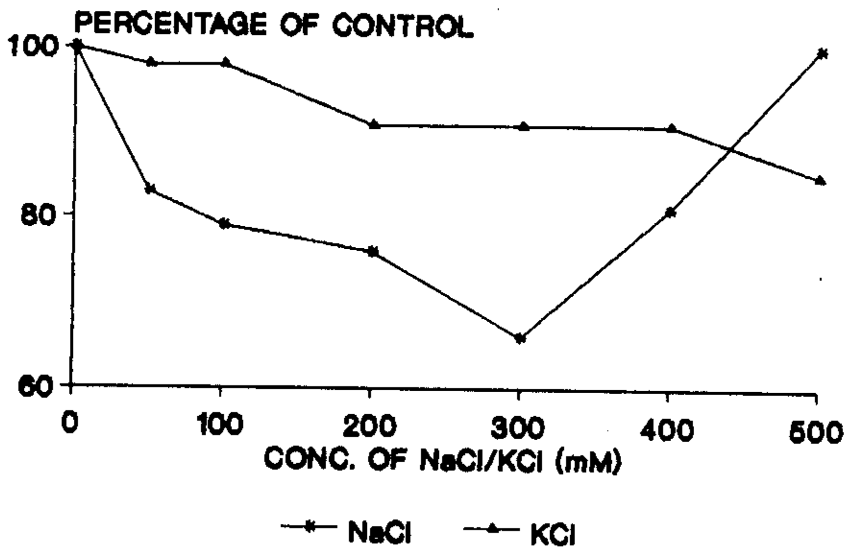

Fig. 55. P. viridis

Activity of SH modified by different levels of NaCl and KCl concentration.

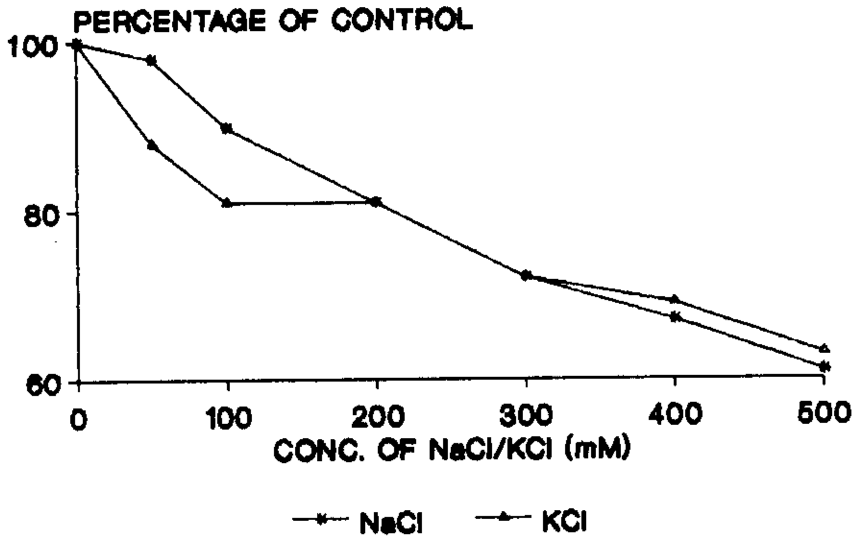

Fig. 56. S. scripta

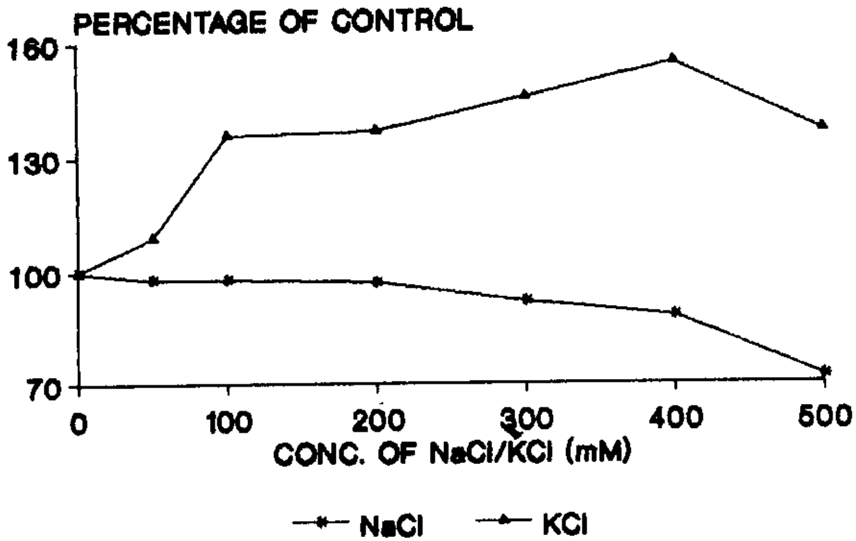

Fig. 57. P. viridis

Activity of LDH modified by different levels of NaCl and KCl concentration.

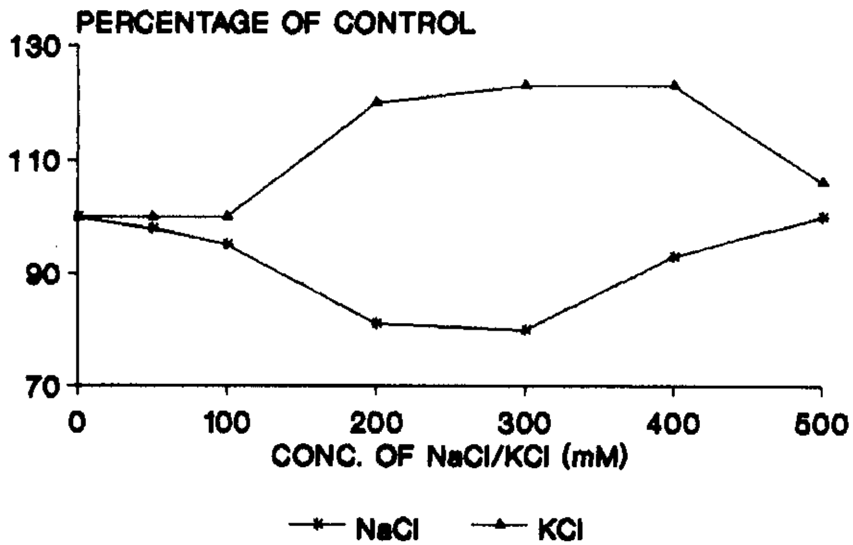

Fig. 58. S. scripta

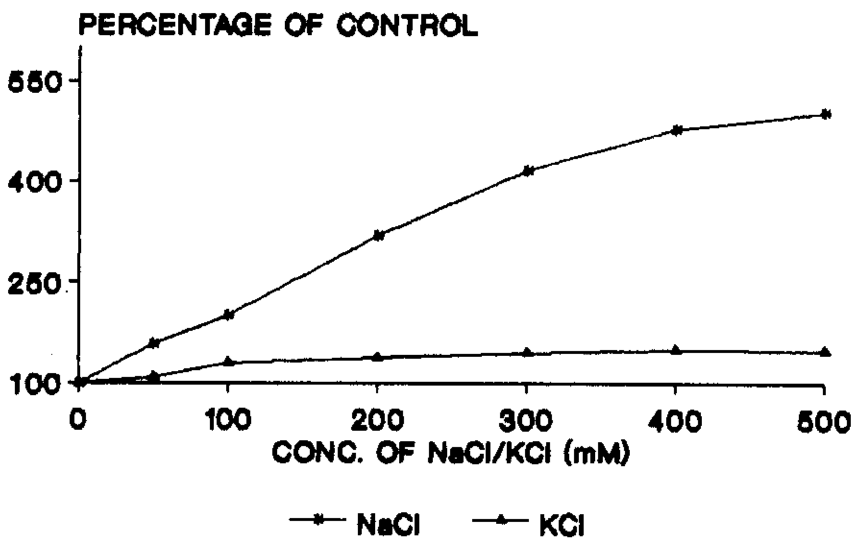

Fig. 59. P. viridis

Activity of AST modified by different levels of NaCl and KCl concentration.

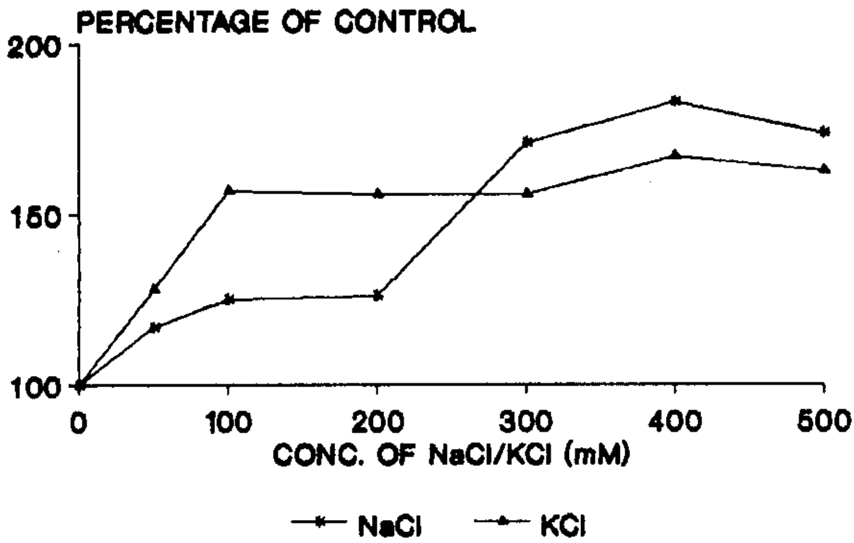

Fig. 60. S. scripta

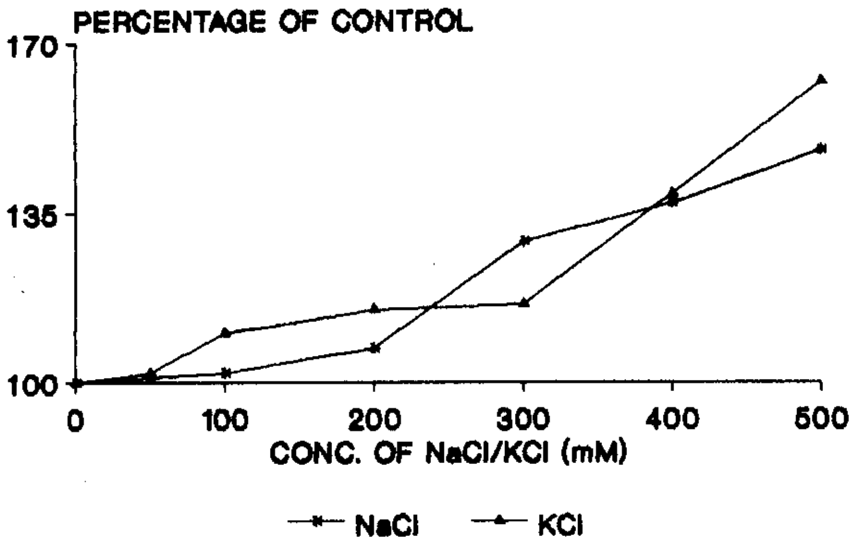

Fig. 61. P. viridis

Effect of different levels of NaCl and KCl concentration on the stability of lysosomal membrane.

concentration in both the bivalves. In the case of *S. scripta* a positive correlation is observed with NaCl (0.93) and KCl (0.75). Similar trend is also noticed in *P. viridis* with NaCl (0.97) and KCl (0.94). In both the bivalves the effect of NaCl and KCl on the lysosomes is almost similar and the variation is not significant at 5% level. When the bivalves were compared, under similar conditions the destabilization of lysosomal membrane is found to be greater in *S. scripta* in the lower concentrations of NaCl and KCl. The acid phosphatase released from lysosomes of both the bivalves when incubated with different concentrations (0, 50, 100, 200, 300, 400 and 500 mM) of NaCl and KCl did not show any difference in activity.

4.4 DISCUSSION

Control of intracellular osmolytes is the basis of osmoregulation and mechanism that underlies the salinity tolerance of the bivalves. It is an accepted fact that the dominant solutes within the living cells are different from that of extracellular fluid. In marine bivalves major intracellular osmolytes are restricted to few classes of organic molecules such as free amino acids and their derivatives. Therefore, intracellular osmotic adjustment of these animals depends upon the regulation of concentration of these molecules. The nitrogenous osmolytes did not appear to interfere with protein structure and function over a wide range of their concentration which may be the prime reason for their acceptability as intracellular osmolytes (Yancey *et al.*, 1982; Somero and Bowlus, 1983). Though not to the same magnitude

as that of organic osmolytes, all cells use inorganic ions to some degree to regulate cellular volume when they face external osmotic variations (Bricteux-Gregoire *et al.*, 1964a,b; Pierce, 1982; Jeuniaux, 1988; Pierce and Politis, 1990).

Even though many biochemical functions require specific inorganic ions, increase in concentration of these ions above a critical level often leads to perturbations of the intracellular microenvironment (Yancey *et al.*, 1982; Somero and Bowlus, 1983; Gilles, 1988). Yancey *et al.* (1982) and Moyes and Ballantyne (1987) have suggested that high intracellular concentration of inorganic ions during osmotic stress would seriously affect the metabolism, transmembrane potential, pH gradients and permeability of biological membranes to solutes. These deleterious effects necessitate an active intracellular regulation of inorganic ions within the physiological range. Since this physiological range is narrow, the cells necessarily have to depend upon the regulation and maintenance of nitrogenous osmolytes for intracellular-fluid isosmotic regulation. It appears that this regulation of NPS is triggered by variation in the concentration of inorganic ions to which the cell is subjected initially (Gilles, 1969; Gomez-Puyou *et al.*, 1972; Wickes and Morgan, 1976; Ballantyne and Storey, 1983, 1985; Moyes and Ballantyne, 1987).

For many euryhaline bivalves, metabolic regulation including protein turnover is a major means by which the intracellular concentrations of free amino acids and their derivatives are adjusted (Wickes and Morgan, 1976; Deaton *et al.*, 1984; Hayashi *et al.*, 1986; Deaton, 1987; Hayashi, 1987). Since metabolism is the sum total of enzyme catalyzed reactions, the metabolic regulation

of NPS can be achieved by varying the activity of the enzymes implicated in the process. The present study reveals that the activity of four such enzymes, GDH, SH, LDH and AST is affected by the different concentrations of inorganic ions, Na^+, K^+ and Cl^-. The study also shows that the stability of the lysosomal membrane is altered considerably by the concentrations of the above ions.

In general in the case of marine molluscs, the intracellular variation of the concentration of inorganic ions (Na^+, K^+, Cl^-), when submitted to osmotic stress was found to vary between 50-200 mM (Schoffeniels and Gilles, 1972; Gilles, 1975). The pattern of enzyme activity variation of the bivalves in different levels within this range of concentration of these ions is generally found to agree with the expected biosynthesis or catabolism of amino acid osmolytes. It is noteworthy that even minor variation (0-50 mM) is causing considerable acceleration or retardation of enzyme activity. Hence, the above observation may be taken as an indication of the effect of inorganic ions on the activity of the enzymes in vivo.

The existence and physiological importance of GDH as an ammonia forming or fixing enzyme in molluscan species was in doubt till 1970's (Campbell and Bishop, 1970). Since then GDH activity has been demonstrated in tissues of a number of species (Addink and Veenhof, 1975; Wickes and Morgan, 1976; Reiss *et al.*, 1977; Bishop *et al.*, 1983; Hayashi *et al.*, 1986; Hayashi, 1987). GDH activity towards the direction of glutamate formation noticed a steady increase with NaCl and KCl concentration. The Serine hydrolyase activity under similar conditions is found to decrease. Serine hydrolyase catalyzes the formation of pyruvate and ammonia

from serine. The activity could serve as the primary deaminating step at the end of a series of transaminases that couple the reaction to the triose metabolic pathways (Bishop, 1976; Bishop *et al.*, 1983; Zubay, 1988). This may be taken as an indication of reduction in deaminating activity with increase in NaCl and KCl concentration in both the bivalves. When the activity of both the enzymes is taken into account, the possible enhanced biosynthesis of amino acid osmolytes during hyperosmotic adaptation and their catabolism in hypoosmotic stress may be visualized. The augmented glutamate formation may lead to subsequent conversion of this amino acid to glycine, alanine, and proline. In this context, it is noteworthy that glycine, proline, alanine and glutamic acid form the major amino acid osmolytes in both the bivalves.

The fact that enhanced GDH activity in the direction of glutamate formation accompanied by reduction in SH activity in both the bivalves under the influence of inorganic ions is bound to reduce their ammonia disposal. The decrease in ammonia excretion during hyperosmotic acclimation has been noticed in marine bivalves (Emerson, 1969; Gilles, 1975; Bartberger and Pierce, 1976; Henry and Mangum, 1980b; Burcham *et al.*, 1983; Moyes *et al.*, 1985; Hayashi, 1987). Of the two bivalves studied, report available on *Sunetta scripta* also indicates the same result (Supriya, 1992). Therefore, the possible role of these enzymes may be the incorporation of ammonia for the intracellular mobilization of amino acid osmolytes with increase in ambient salinity.

The activity of these enzymes also suggests the possibility of catabolism of amino acids during hypoosmotic stress. The SH activity increases towards the lower levels of NaCl and KCl

concentration. Hayashi (1987) and Moyes and Ballantyne (1987) have suggested that GDH also is an important enzyme in the catabolism of amino acids in bivalves during hypoosmotic stress. Of the two enzymes, SH may be more important in the process of ammonia formation in bivalves (Hayashi *et al.*, 1986; Hayashi, 1987).

LDH activity decreases with increase in NaCl concentration in both the bivalves. But with increase in concentration of KCl, activity of LDH from *Sunetta scripta* showed a decrease whereas LDH from *Perna viridis* expressed an increase in activity. The reduction of LDH activity with increase in NaCl concentration in both the bivalves and with KCl concentration in *Sunetta scripta* points to increased accumulation of pyruvate. The pyruvate can give rise to glutamate through α-ketoglutarate and can act as precursor of alanine. The possibility of involvement of LDH in the energy metabolism by controlling the extramitochondrial ratio of $NADH/NAD^+$ is also worth consideration here. Due to the formation of reducing equivalents by the catabolism of amino acids, a higher enzyme activity is to be expected in lower salinities than in higher levels of salinity. The control of extramitochondrial reducing equivalents was found to be of importance in euryhalinity (Gilles and Jobsis, 1972; Gilles, 1975).

The enhanced LDH activity with KCl in *Perna viridis* needs further explanation. One possibility is that the animal never gets exposed to salinity lower than $15x10^{-3}$. The concentration of K^+ in the hemolymph of this bivalve remains more or less steady and shows minimum variation unlike that of *S. scripta* under variations of salinity. Therefore, the experimental concentration of K^+ to which the enzyme is subjected may not be relevant in vivo.

An increased AST activity is expected with increase in salinity or ionic concentration. But this is noticed only with KCl in both the species. With the increase in concentration of NaCl the enzyme activity is found to decrease in *S. scripta*, whereas it increases in *P. viridis*. AST appears to be an important enzyme in marine bivalves and high AST activity in different tissues of bivalves has been reported by various workers (Read, 1962; Du Paul and Webb, 1974; Addink and Veenhof, 1975; Wickes and Morgan, 1976). Though the aspartate concentration is never found to be very high in both the bivalves, aspartate generated can be a source of glutamate by the activity of AST and through glutamate to glycine, proline and alanine. Studies on the effect of ionic concentrations or salinities on the activity of this enzyme do not give a picture that can be generalized. According to Bishop *et al.* (1983) this may be due to the difference in species or other factors related to season, food, size groups, assay procedures and conditions of assay (pH, buffer, temperature, substrate and enzyme concentration). Therefore, decreased activity of AST from *S. scripta* with increase in NaCl concentration needs further studies.

Endogenous protein breakdown appears to be one of the prominent sources of NPS during hyperosmotic volume regulation (Deaton *et al.*, 1984; Hawkins and Hilbish, 1992; Weber *et al.*, 1992). It has been suggested that in marine bivalves, the proteolysis in response to hyperosmotic stress is lysosome mediated (Segal, 1975; Dean, 1977; Moore *et al.*, 1980; Bayne *et al.*, 1981; Moore, 1982; Stickle *et al.*, 1985; Deaton, 1987). In both the bivalves studied, lability of lysosomal membrane is found to increase with NaCl and KCl concentration. Activity of acid

phosphatase released from the lysosomes is taken as an index of permeability or fluidity changes associated with the membrane lability. It is interesting to notice that even very small variation in concentration of NaCl and KCl (0-50 mM) is found to affect the stability of lysosomal membrane. Concomitant with the destabilization, a reduced latency of lysosomal protease which is a direct consequence of the permeability of the lysosomal membrane is expected (Moore, 1985). Moore *et al.* (1980), Bayne *et al.* (1981) and Stickle *et al.* (1985) pointed out labilisation of lysosomes during high salinity adaptation in marine molluscs accompanied by an increased production of amino acids from proteins which was attributed to lysosome mediated proteolysis. Of the two bivalves studied, the labilisation of lysosomal membrane in the lower concentrations of NaCl and KCl is found to be greater in *S. scripta*. The salinity tolerance of *S. scripta* compared to *P. viridis* is higher and whether the expected lysosome mediated proteolysis has got any expression in their euryhalinity needs further study.

To sum up, the overall picture indicates the importance of intracellular variations of concentration of inorganic ions in regulating the activity of mitochondrial and cytoplasmic enzymes. The impact of inorganic ions on the lability of lysosomal membrane also suggests their role in lysosome mediated proteolysis. More information on the intracellular localization of enzymes and the ionic strength and composition therein, the qualitative and quantitative variation of inorganic ions in the cell in different levels of salinity, effects of concentration of inorganic ions on the enzyme activity and the modification of this by various

compounds are obviously needed to have a complete picture of intracellular osmoregulatory mechanisms. However euryhalinity does not result solely from the control of enzymatic or organelle system. The expression of euryhalinity depends upon behavioural, morphological, physiological and biochemical adaptations involved in the regulation of blood and cellular fluid osmolarity. Future studies in this line are rewarding in understanding the ecological potential of euryhaline species and the mechanisms of acclimation to environmental changes.

TABLE NO : 24

Effect of NaCl and KCl concentrations on the activity of Glutamate dehydrogenase (EC 1.4.1.2) from the tissues of *S. scripta*.

CONC. OF NaCl/KCl (mM)	EFFECT OF NaCl		EFFECT OF KCl	
	OXIDATION OF NADH (mg/h/g protein)	% OF CONTROL	OXIDATION OF NADH (mg/h/g protein)	% OF CONTROL
00	1118 ± 54	100	1930 ± 076	100
50	1211 ± 40	108	4172 ± 138	166
100	1258 ± 60	112	3309 ± 053	172
200	1479 ± 12	132	3493 ± 119	186
300	1817 ± 60	162	4137 ± 076	214
400	2190 ± 60	196	4275 ± 088	222
500	2370 ± 40	212	5056 ± 076	264

TABLE NO : 25

Effect of NaCl and KCl concentrations on the activity of Glutamate dehydrogenase (EC 1.4.1.2) from the tissues of *P. viridis*.

CONC. OF NaCl/KCl (mM)	EFFECT OF NaCl		EFFECT OF KCl	
	OXIDATION OF NADH (mg/h/g protein)	% OF CONTROL	OXIDATION OF NADH (mg/h/g protein)	% OF CONTROL
00	1085 ± 37	100	346 ± 57	100
50	1322 ± 40	122	415 ± 40	120
100	1614 ± 48	149	587 ± 29	170
200	1981 ± 41	183	933 ± 66	270
300	2271 ± 27	209	1208 ± 66	350
400	2813 ± 74	259	1335 ± 65	390
500	3259 ± 52	300	1899 ± 22	549

Values are the mean of six different experiments ± SD.

TABLE NO : 26

Effect of NaCl and KCl concentrations on the activity of Serine hydrolyase (EC 4.2.1.13) from the tissues of *S. scripta*.

CONC. OF NaCl/KCl (mM)	EFFECT OF NaCl		EFFECT OF KCl	
	PYRUVATE FORMED (mg/h/g protein)	% OF CONTROL	PYRUVATE FORMED (mg/h/g protein)	% OF CONTROL
00	965 ± 20	100	974 ± 35	100
50	871 ± 13	90	761 ± 60	78
100	873 ± 08	91	723 ± 30	74
200	832 ± 12	86	723 ± 30	74
300	818 ± 09	85	685 ± 50	70
400	799 ± 19	83	740 ± 19	76
500	799 ± 19	83	799 ± 30	82

TABLE NO : 27

Effect of NaCl and KCl concentrations on the activity of Serine hydrolyase (EC 4.2.1.13) from the tissues of *P. viridis*.

CONC. OF NaCl/KCl (mM)	EFFECT OF NaCl		EFFECT OF KCl	
	PYRUVATE FORMED (mg/h/g protein)	% OF CONTROL	PYRUVATE FORMED (mg/h/g protein)	% OF CONTROL
00	2021 ± 64	100	1686 ± 47	100
50	1675 ± 36	83	1650 ± 29	98
100	1601 ± 28	79	1650 ± 29	98
200	1556 ± 40	76	1540 ± 47	91
300	1328 ± 24	66	1539 ± 29	91
400	1624 ± 32	81	1540 ± 47	91
500	2021 ± 64	100	1431 ± 30	85

Values are the mean of six different experiments ± SD.

TABLE NO : 28

Effect of NaCl and KCl concentrations on the activity of Lactate dehydrogenase (EC 1.1.1.27) from the tissues of *S. scripta*.

CONC. OF NaCl/KCl (mM)	EFFECT OF NaCl		EFFECT OF KCl	
	OXIDATION OF NADH (mg/h/g protein)	% OF CONTROL	OXIDATION OF NADH (mg/h/g protein)	% OF CONTROL
00	356.66 ± 7.90	100	392.46 ± 63.00	100
50	349.65 ± 4.20	98	343.38 ± 31.66	88
100	320.17 ± 5.95	90	318.84 ± 31.73	81
200	290.67 ± 5.95	81	318.84 ± 19.88	81
300	255.57 ± 5.79	72	282.13 ± 10.17	72
400	237.32 ± 7.94	67	269.76 ± 20.11	69
500	216.26 ± 3.14	61	245.22 ± 40.23	63

TABLE NO : 29

Effect of NaCl and KCl concentrations on the activity of Lactate dehydrogenase (EC 1.1.1.27) from the tissues of *P. viridis*.

CONC. OF NaCl/KCl (mM)	EFFECT OF NaCl		EFFECT OF KCl	
	OXIDATION OF NADH (mg/h/g protein)	% OF CONTROL	OXIDATION OF NADH (mg/h/g protein)	% OF CONTROL
00	314.98 ± 19.71	100	237.46 ± 11.10	100
50	307.25 ± 11.60	98	259.35 ± 27.90	109
100	307.25 ± 11.60	98	323.56 ± 21.16	136
200	305.32 ± 15.58	97	324.31 ± 12.53	137
300	290.36 ± 06.30	92	345.85 ± 27.96	146
400	276.33 ± 43.21	88	367.47 ± 17.72	155
500	226.09 ± 23.20	72	324.31 ± 12.53	137

Values are the mean of six different experiments ± SD.

TABLE NO : 30

Effect of NaCl and KCl concentrations on the activity of Aspartate aminotransferase (EC 2.6.1.1) from the tissues of *S. scripta*.

CONC. OF NaCl/KCl (mM)	EFFECT OF NaCl		EFFECT OF KCl	
	PYRUVATE FORMED (mg/h/g protein)	% OF CONTROL	PYRUVATE FORMED (mg/h/g protein)	% OF CONTROL
00	202.23 ± 04,03	100	206.86 ± 05.08	100
50	198.67 ± 06.41	98	206.57 ± 06.21	100
100	192.26 ± 00.00	95	206.86 ± 05.08	100
200	163.92 ± 11.14	81	248.23 ± 07.16	120
300	160.93 ± 04.03	80	254.14 ± 05.08	123
400	187.99 ± 12.08	93	254.44 ± 06.21	123
500	202.23 ± 04.03	100	218.68 ± 05.08	106

TABLE NO : 31

Effect of NaCl and KCl concentrations on the activity of Aspartate aminotransferase (EC 2.6.1.1) from the tissues of *P. viridis*.

CONC. OF NaCl/KCl (mM)	EFFECT OF NaCl		EFFECT OF KCl	
	PYRUVATE FORMED (mg/h/g protein)	% OF CONTROL	PYRUVATE FORMED (mg/h/g protein)	% OF CONTROL
00	108.69 ± 10.25	100	551.02 ± 14.06	100
50	171.50 ± 13.67	158	602.68 ± 19.88	109
100	217.39 ± 10.25	200	711.73 ± 58.53	129
200	347.41 ± 17.24	318	757.65 ± 19.88	138
300	451.69 ± 19.02	416	800.72 ± 07.03	145
400	519.32 ± 18.08	478	826.53 ± 19.89	150
500	545.89 ± 09.04	502	817.94 ± 07.03	148

Values are the mean of six different experiments ± SD.

TABLE NO : 32

Effect of NaCl and KCl concentrations on the membrane lability of lysosomes from the digestive cells of *S. scripta*.
(Acid phosphatase released from the lysosomes in 30 min.at 30°C)

CONC. OF NaCl/KCl (mM)	EFFECT OF NaCl		EFFECT OF KCl	
	pNITROPHENOL FORMED (mg/h/g protein)	% OF CONTROL	pNITROPHENOL FORMED (mg/h/g protein)	% OF CONTROL
00	97.02 ± 1.19	100	97.02 ± 1.19	100
50	113.74 ± 4.21	117	124.30 ± 2.90	128
100	120.99 ± 3.21	125	151.96 ± 2.26	157
200	121.80 ± 1.71	126	151.24 ± 3.02	156
300	165.91 ± 4.35	171	151.39 ± 3.06	156
400	177.84 ± 4.60	183	162.17 ± 2.79	167
500	168.93 ± 3.16	174	158.29 ± 5.21	163

TABLE NO : 33

Effect of NaCl and KCl concentrations on the membrane lability of lysosomes from the digestive cells of *P. viridis*.
(Acid phosphatase released from the lysosomes in 30 min.at 37°C)

CONC. OF NaCl/KCl (mM)	EFFECT OF NaCl		EFFECT OF KCl	
	pNITROPHENOL FORMED (mg/h/g protein)	% OF CONTROL	pNITROPHENOL FORMED (mg/h/g protein)	% OF CONTROL
00	28.31 ± 0.47	100	26.32 ± 0.57	100
50	28.46 ± 0.51	101	26.93 ± 0.31	102
100	28.89 ± 0.75	102	28.89 ± 0.57	110
200	30.33 ± 0.73	107	30.33 ± 3.63	115
300	36.39 ± 0.34	129	30.62 ± 0.94	116
400	38.88 ± 2.25	137	36.68 ± 0.56	139
500	41.89 ± 0.29	148	42.64 ± 3.04	162

Values are the mean of six different experiments ± SD.

CHAPTER 5

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

Littoral zone of the marine environment is subjected to considerable variations in salinity. Regulation of ions and water in the body fluids are the principal problems of life in this region. Even though the processes of ion and osmoregulation are different, the former affects the osmotic mobility of water and any study on the latter necessarily has to consider the ionoregulation.

Present study is an attempt to understand the behavioural, physiological and biochemical aspects of osmoregulation of two intertidal bivalve molluscs, *Sunetta scripta* and *Perna viridis*. Though both the species are euryhaline, the range of salinity tolerance of *S. scripta* is found to be wider towards the lower extremes than that of the mussel, *P. viridis*. In the present study *S. scripta* is found to tolerate a lower salinity of 5×10^{-3}, whereas the *P. viridis* could not be maintained below 15×10^{-3} salinity in the laboratory conditions for longer periods. Both the bivalves are found to tolerate an upper limit of 40×10^{-3} salinity for considerably long periods for the purpose of experiment. However, previous studies have indicated that the optimum salinity for both these bivalves is between 30×10^{-3} and 35×10^{-3}. Hence, for all the experiments, the bivalves were acclimated to 30×10^{-3} salinity.

Valve closure as a behavioural avoidance mechanism is observed in both the bivalves when exposed to extremes of their salinity tolerance range. In the case of *S. scripta*, the duration of valve closure in four levels of salinity follow the order 5>

10> 15> 40×10^{-3}. *P. viridis* when transferred to 15×10^{-3} salinity showed valve adduction, but it is for a comparatively shorter period than that of *S. scripta* in this salinity. Upon transfer to unfavourable salinities, both the bivalves closed their valves enclosing sea water from the acclimation tank. The salinity of this sea water in the mantle cavity gradually attained ambient salinity and this gradual change in the mantle cavity fluid is almost totally reflected in the hemolymph. It is also observed that the smaller size groups of both the bivalves avail this grace due to valve closure to a shorter period than larger ones in all experimental salinities. These results indicate that both the bivalves close their valves to tide over short-term adverse salinity fluctuations and the tenure of this temporary exemption is positively correlated to the unfavourableness of the ambient salinity. In addition, the valve closure is employed by these animals as a highly useful adaptation to avoid sudden exposure to osmotic shock. The gradual exposure of soft parts of the animal to altered ambient salinity also offers time to make necessary internal adjustments. Between species, *S. scripta* is found to depend on valve closure to a greater extent and this may be one reason which helps them to tolerate lower extremes of salinity. Decreased dependence of smaller size groups of the species to valve closure may be due to their increased metabolic rate which enable them to make faster internal adjustments. These adjustments may relieve the animal from the burden of penalties due to the curtailment that results from valve closure. This may be a reason for the increased survival rate of smaller size groups in adverse salinities than larger ones.

Studies on extracellular ionic regulation have considered the regulations of Na^+, K^+ and Cl^-. The Na^+ and Cl^- form the most abundant ions in the sea water and previous literature have indicated that they also contribute more than 90% of the osmotic effectors in the hemolymph of euryhaline bivalves. Hence, it is logical to consider that the pattern of variation of Na^+ and Cl^- in the hemolymph in different levels of salinity indicates the osmotic pressure changes in the hemolymph. The concentration of Na^+ and Cl^- in the extracellular fluid is found to follow the ambient concentration in all experimental salinities in both the bivalves. Considering the concentration of Na^+, K^+ and Cl^- together, a marginal hyperosmoticity can be observed in all experimental salinities except in the lower levels where the hyperosmoticity becomes conspicuous. In *S. scripta* this hyperosmoticity is found to increase steadily below $15x10^{-3}$ salinity and attains maximum in $5x10^{-3}$. In *P. viridis* significant hyperosmotic regulation can be seen in $15x10^{-3}$ salinity which is very close to the lower limit of salinity tolerance of the species. When different size groups of both the bivalves are considered, the smaller ones recorded higher hyperosmoticity than larger ones in all experimental salinities.

The above observations are in agreement with the general concept and point to the fact that both the bivalves are iono and osmoconformers and the osmoticity of extracellular fluid fluctuates with ambient variations. However, the marginal hyperosmoticity observed in both the bivalves may be important with regard to a continuous influx of water which may favour the secretion of mucus. The conspicuous extracellular-fluid

anisosmotic regulation seen in both the bivalves in the lower extremes of their salinity tolerance range is considered as a factor which contributes to their survival in these salinities. It is of interest to note that *S. scripta* which has a wider salinity tolerance towards the lower extremes is found to have higher power of extracellular-fluid anisosmotic regulation. Moreover, the smaller size groups of *S. scripta* which posses higher extracellular regulatory power, have an enhanced survival rate in low salinities. When compared to Na^+ and Cl^-, K^+ is maintained at a higher level in the hemolymph. This is observed in all size groups of both the bivalves. The high extracellular K^+ regulation is found to be greater in *P. viridis* compared to *S. scripta*. Maintenance of a more or less stable extracellular K^+ concentration in different levels of salinity is necessary to keep the intracellular level of potassium ions without much change.

A significant reduction in the intracellular concentration of NPS is noticed in all the tissues of different size groups of both the bivalves exposed to hypoosmotic stress. The contribution of NPS to the hemolymph osmolarity is found to be negligible, whereas, they contribute substantially to the intracellular osmolarity. It is of interest to note that the osmolyte system differ almost totally between extracellular and intracellular environment. Among different tissues studied in various size groups of both the bivalves, adductor muscle recorded highest concentration of NPS. The mantle tissue and foot muscle maintained almost similar quantum of NPS. Between species, higher NPS content is noticed in *P. viridis* but the species fail to tolerate lower salinities. Hence, the tolerance of *S. scripta* towards the lower

levels of salinity emphasize the importance of extracellular-fluid anisosmotic regulation and behavioral avoidance by valve closure. The smaller size groups of both the bivalves have a higher concentration of NPS and greater variation of NPS content when exposed to hypoosmotic stress compared to larger ones. This is attributed as one reason for their successful survival in diluted medium. If the above fact is accepted, it is likely that for a species living in defined environmental, nutritional and physiological conditions, the size of the NPS pool can be taken as an indication of its salinity tolerance. It can be concluded from these results that the intracellular-fluid isosmotic regulation is the basic mechanism that sustains the euryhalinity of both the bivalves. Added to this extracellular-fluid anisosmotic regulation and behavioural avoidance mechanism help *S. scripta* to withstand further dilution of the environment.

Results of qualitative and quantitative nature of the components of NPS pool revealed that taurine, glycine, proline, alanine, glutamic acid and aspartic acid are the major and varied amino acid osmolytes in all the tissues of all the size groups of both the bivalves. Presence of arginine, lysine, serine, threonine and valine is noticed but they are osmotically less significant. A conspicuous difference noticed between the tissues is the high content of glycine in the adductor muscle whereas taurine is the most abundant osmolyte in the foot muscle and mantle tissue. It is observed that major constituents of the intracellular NPS pool are low molecular weight nitrogenous osmolytes like glycine, taurine, alanine, proline, glutamic acid and aspartic acid. Previous studies have indicated the compatibility of such solute system in

marine molluscs and the nonperturbable environment they offer for various molecular mechanisms that support life. These nitrogenous osmolytes act as compatible solutes over wide variations in their concentrations and hence the euryhalinity of both the bivalves depends upon these intracellular osmotic pressure effectors. High content of taurine observed in bivalves acclimated to high salinity indicates that these bivalves subjected to such conditions can be used as a potential source of taurine which is an important bioactive substance of pharmacological, nutritional and industrial importance.

Although studies have been conducted on the source and the means of regulation of intracellular osmolytes by various workers, a definite conclusion is yet to come. The various processes suggested include regulation of metabolic pathways involved in the biosynthesis and catabolism of amino acid osmolytes, proteolysis and membrane related processes. Among these, attempt is made to study the metabolic regulation and lysosome-mediated proteolysis.

Glutamate dehydrogenase activity is found to increase towards the glutamate forming direction in response to increase in concentrations of NaCl and KCl in both the bivalves. The SH activity is found to decrease under similar conditions. These observations point to a situation which favour the biosynthesis of amino acids and decreased deamination during high saline conditions. The reduction of LDH activity with increase in KCl and NaCl concentration may result in an enhanced accumulation of pyruvate which can act as a source of α-ketoglutarate, substrate for GDH to produce glutamate. The enhanced LDH activity in the lower levels of NaCl and KCl concentration indicates a possible

role of this enzyme in diminishing the extramitochondrial reducing equivalents which can be high in the lower levels of salinity due to increased catabolism of amino acids. However, the enhanced LDH activity with KCl in *P. viridis* does not agree with the generalization. The activity of AST in different concentrations of NaCl and KCl does not give a picture that can be generalized. Except in the case of NaCl in *S. scripta*, activity of AST is found to increase. The enhanced AST activity indicates a situation in which the aspartate accumulated during hyperosmotic stress in both the bivalves is controlled to a compatible level by converting it to other amino acid osmolytes.

Endogenous protein breakdown is another source of amino acids to meet hyperosmotic stress. This proteolysis is supposed to be mediated by lysosomes. In vitro studies have indicated that the stability of lysosomal membrane decreases with increase in NaCl and KCl concentration. Decreased latency of lysosomal enzymes is associated with increased lability of lysosomal membrane. The in vitro experiments simulate a situation which the animal may face under hyperosmotic stress. This indicates that increase in concentration of inorganic ions may lead to a higher proteolytic activity. This may lead to the generation of amino acid osmolytes except taurine.

In short, the salinity tolerance of these bivalves is the net result of the interactions of behavioural, extracellular and intracellular mechanisms. The behavioural avoidance is a highly useful measure to tide over short-term adverse salinity fluctuations relieving the animal from employing the internal adjustment to a greater extent. However, when exposed to long-term

drastic variations in salinity, this mechanism acts as a control measure to convert the drastic change to a gradual one and protects the animal from the osmotic shock.

In both the bivalves, internal adjustment in relation to osmoregulation is observed at extracellular and intracellular levels. Power of osmoregulation seems to be a sum total of the mechanisms at both the levels. Extracellular-fluid anisosmotic regulation is noticed to be marginal in salinities closer to the optimum whereas, it becomes conspicuous in lower extremes of their salinity tolerance. Concomitant with the variations of the osmotic pressure effectors in the hemolymph an intracellular-fluid isosmotic regulation is in operation to prevent the osmotic flux of water. The intracellular osmolytes appear to be carefully selected to make the environment suitable for various life supporting molecular interactions. The low molecular weight nitrogenous osmolytes like free amino acids and their derivatives are the abundant and varied osmolytes in the cytoplasm of both the bivalves.

Present work on the effect of NaCl and KCl on the activity of key enzymes implicated in the amino acid metabolism suggests the involvement of enzymes and inorganic ions in the regulation of nitrogenous osmolytes. The study also indicates a role of inorganic ions in proteolysis mediated by lysosomes.

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