EFFECT OF SALINITY STRESS ON NEUROSECRE-TORY CELLS, PROTEIN AND FREE AMINO ACID CONTENT AND RATE OF AMMONIA EXCRETION OF THE PRAWN *PENAEUS INDICUS* H MILNE EDWARDS

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ABSTRACT

Stress-induced changes resulting from salinity exposure are sought in the neurosecretory cells of different neuroendocrine centres, in protein and free amino acid content in haemolymph, muscle, and hepatopancreas, and in rate of ammonia excretion in the prawn *Penaeus indicus*. Prawns acclimated to low saline medium (S ≈ 10‰) when transferred to high saline medium (S ≈ 40‰) showed little change in their neurosecretory cell histology. However, protein and free amino acid content in haemolymph, muscle, and hepatopancreas increased considerably. Simultaneously, the rate of ammonia excretion was reduced. Prawns acclimated to high salinity medium when transferred to low salinity exhibited changes in the above parameters in reverse to those findings in the former experiment. The probable reasons for such changes are discussed.

Many euryhaline animals thrive in media of varying salinities due to two types of adaptive mechanisms. The first, anisosmotic extracellular regulation, mainly involves ionic exchange between blood and external medium. The other is isosmotic intracellular regulation where amino acids are catabolized or synthesized for the same purpose (Schoffeniels and Gilles, 1970). Literature pertaining to extracellular and intracellular adaptations in response to external salinity changes have been reviewed for several euryhaline animals (Gilles, 1979). Duchateau and Florkin (1955) were the first to analyse the free amino compounds in the tissues of an animal subjected to osmotic shock. They showed in the muscle of chinese crab *Eriocheir sinensis* that free amino acids (FAA) account for about 40% of the total osmolarity of the tissue. In *Carcinus maenas* a significant drop in muscle FAA content has been reported when animals were exposed to 50% sea water (Duchateau *et al.*, 1959). In *E. sinensis*, Gregoire *et al.* (1962) showed a drastic fall in concentration of FAA in response to freshwater exposure. Similar observations have been reported in osmotic response of the mud crab *Panopeus herbstii* (Boone and Claybrook, 1977) and in penaeid shrimps *Penaeus kerathurus* (Richard and Ceccaldi, 1975) and *P. japonicus* (Spaargaren *et al.*, 1982).

Information with regard to protein variation, and its relation to the amino acid pool in response to osmotic stress, still appears to lack unanimity. Siebers *et al.* (1972) worked on the protein concentration in whole *Orconectes limosus* during acclimation from fresh water to saline water. Comparable studies have been made on similar aspects in *E. sinensis* (Gilles and Schoffeniels, 1969), *C. maenas, Astacus fluviatilis,* and *E. sinensis* (Gilles, 1977).

In conjuction with osmotic responses, significant changes in ammonia (NH_4^+ - N) excretion rate also were reported in many crustaceans (Spaargaren *et al.*, 1982; Regnault, 1984). However, salinity effects on other nitrogenous excretory products generally depend on the species and its particular osmoregulatory capability.

Although substantial evidences of neuroendocrine control in hydromineral regulation in decapod crustaceans have accumulated (Charmantier *et al.*, 1981), they lack consistency. Further, studies pertaining to changes occurring in neurosecretory cells in response to osmotic stress apparently are few, particularly in crustaceans.

Therefore, an attempt has been made here to determine the sudden effect of osmotic stress on neurosecretory cells of different neuroendocrine centres, and protein and FAA content in haemolymph, muscle, and hepatopancreas and on ammonia excretion rate of the prawn *P. indicus.*

MATERIALS AND METHODS

Seventy-two adult prawns of *P. indicus* (size range 80–100 mm), mostly in the intermoult stage, were collected from the wild and brought live to the laboratory. They were divided into two groups, each consisting of 36 animals. Group I was acclimatized in a fibreglass tank of 1 tonne capacity containing diluted sea water (S ≈ 10‰) for a period of 30 days with proper aeration and feeding. Group II was acclimatized to concentrated sea water having a salinity of 40‰. After acclimatization, groups I and II were each divided into two batches (A and B, C and D), each batch consisting of 18 animals. Each batch was again divided into six sub-batches, each consisting of three prawns.

To study the sudden effect of osmotic stress, animals belonging to B batch (acclimatized to diluted sea water) were transferred to sea water (S ≈ 40‰) in six plastic tubs each containing three prawns. The prawns of D batch, acclimatized to concentrated sea water, were transferred to dilute sea water (S ≈ 10‰) in six tubs. Animals of batches A and C were used as control by placing them in another two sets of six corresponding tubs. The experiment was conducted for 48 hr and during that period prawns were not fed but were continuously aerated.

Immediately after initiating the experiments, water samples from each tub were collected and the initial concentration of ammonia recorded. Animals of the first tub from all groups, A, B, C and D, were sacrificed first at 0 hr and the second from all groups after 3 hr and subsequently from the remaining tubs after 6, 12, 24 and 48 hr respectively. Before sacrificing the animals, they were weighed individually and haemolymph from each prawn was collected through pericardial cavity using chilled 1 ml hypodermic syringe previously rinsed with an anticoagulant (10% trisodium citrate). The haemolymph was delivered into small glass vials and kept in an ice water bath until further use. Simultaneously, water samples from all tubs were also collected at each time interval to record the ammonia concentration. After extracting the haemolymph, animals were then dissected, and their

muscle and hepatopancreatic tissues were isolated and analysed for protein and FAA content.

Subsequently, to identify the changes occurring in neurosecretory cells, the neuroendocrine centres — optic, cerebral, thoracic, and abdominal nerve ganglia — were removed and fixed in Bouin's fluid. Paraffin sections were cut 4–6 μm thick and stained with Mallory's triple stain. Comparison of neurosecretory cell activity of experimental prawns was carried out with activity of those collected from the wild of isosaline condition (20‰).

Aqueous ammonia was determined by the phenol hypochlorite method of Solarzano (1969). The hourly excretion rate per gram body tissue was determined as described by Regnault (1984). Protein content in tissues and haemolymph was analysed by Biuret method (Gornall et al., 1949). Bovine serum albumin was used to prepare standard curve. Free amino acid was estimated by the method of Yemm and Cocking (1955). A mixture of glycine and glutamic acid was used as the standard.

Student's t test was used to test the significance of experimental results.

RESULTS

Ammonia

The rate of ammonia excretion was low in the prawns transferred to high saline medium (40‰) from diluted sea water (S ≈ 10‰). Differences in ammonia excretion rates between controls and experimental animals during different time intervals were not significant. Ammonia excretion rate was high in the prawns transferred to low saline from high saline medium. Significant differences in excretion rates of experimental animals were seen at 3, 6, and 12 hr but after that procedural compensation effect of stress was noticed and excretion rates returned toward normal levels (Fig. 1).

Free amino acids

Free amino acid content in the haemolymph of prawns transferred to high saline medium increased significantly throughout the experimental period (Fig. 2). Prawns transferred to low saline from high salinity medium also showed increased levels of FAA in haemolymph initially up to 6 hr. The level then decreased significantly throughout the experimental duration (Fig. 3).

Free amino acid content in muscle and hepatopancreas decreased significantly in experimental prawns in low saline medium when transferred from high salinity. The effect was more pronounced in hepatopancreatic tissue (Fig. 3). The levels of FAA in muscle and hepatopancreas were always high in prawns in high saline medium when transferred from low salinity (Fig. 2).

Protein

Protein content of the haemolymph, muscle, and hepatopancreas was found to be high in the prawns transferred to high saline medium from low salinity. Significant variations could be seen up to 12 hr. The percentage variations of protein content of the haemolymph throughout the experimental period ranged from 13 to 84, whereas that of muscle and hepatopancreas ranged from 10 to 67 and 4 to 53 respectively (Fig. 4).
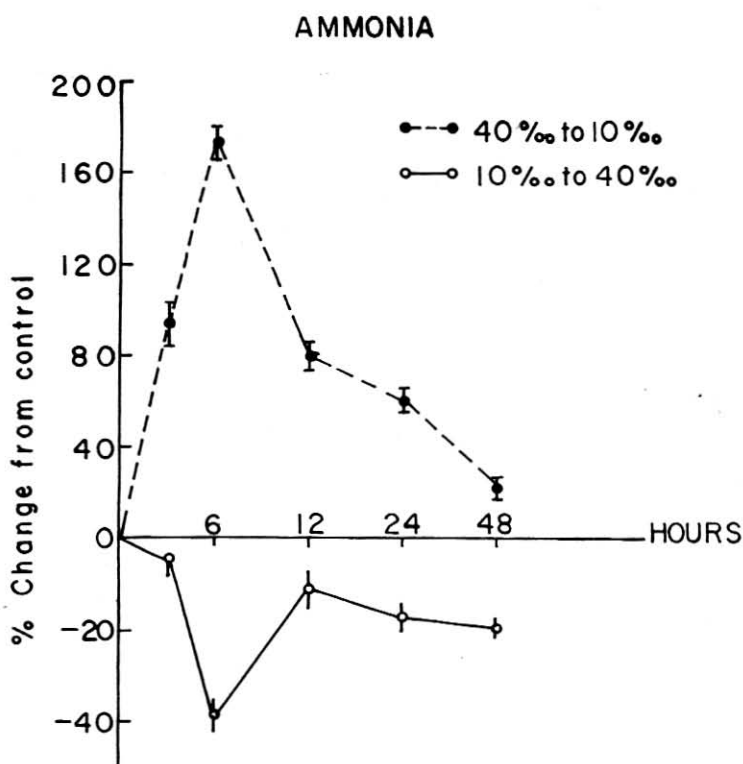
Fig. 1. Percentage changes in ammonia excretion rate of *P. indicus* transferred from low saline to high saline water (40‰) and high saline to low saline water (10‰) in comparison with controls

Haemolymph of the prawns transferred to low saline medium from high salinity showed low levels of protein throughout the experimental duration when compared to their correspondent controls. A decreasing trend of protein level was recorded and hence the highest percentage change recorded was at the 48th hr (Fig. 5). Similarly, the protein content in muscle and hepatopancreas was low in experimental prawns especially at 6, 12, and 24 hr (Fig. 5).

Neurosecretory cells

Depending on the size, shape, and staining characteristics, five types of neurosecretory cells have been identified in the neuroendocrine system of *P. indicus*. They are designated as giant cells and type A, B, C, and D cells. In the optic ganglion of the eyestalk, only B, C, and D cells were seen. In other neuroendocrine centres, such as the cerebral, thoracic, and abdominal ganglia, all types of cell have been noticed here. Major changes in neurosecretory cells in response to osmotic stress were observed in giant and A cell types (Table 1). In other cells the response was not prominent. Prawns transferred from dilute to concentrated sea water showed less change in their neurosecretory cell activity. The cytoplasm of giant and A cell types appeared granular with few vacuoles. The nucleus was distinct with or without nucleoli. The cellular profile was less disturbed. The secretory activity in general was found
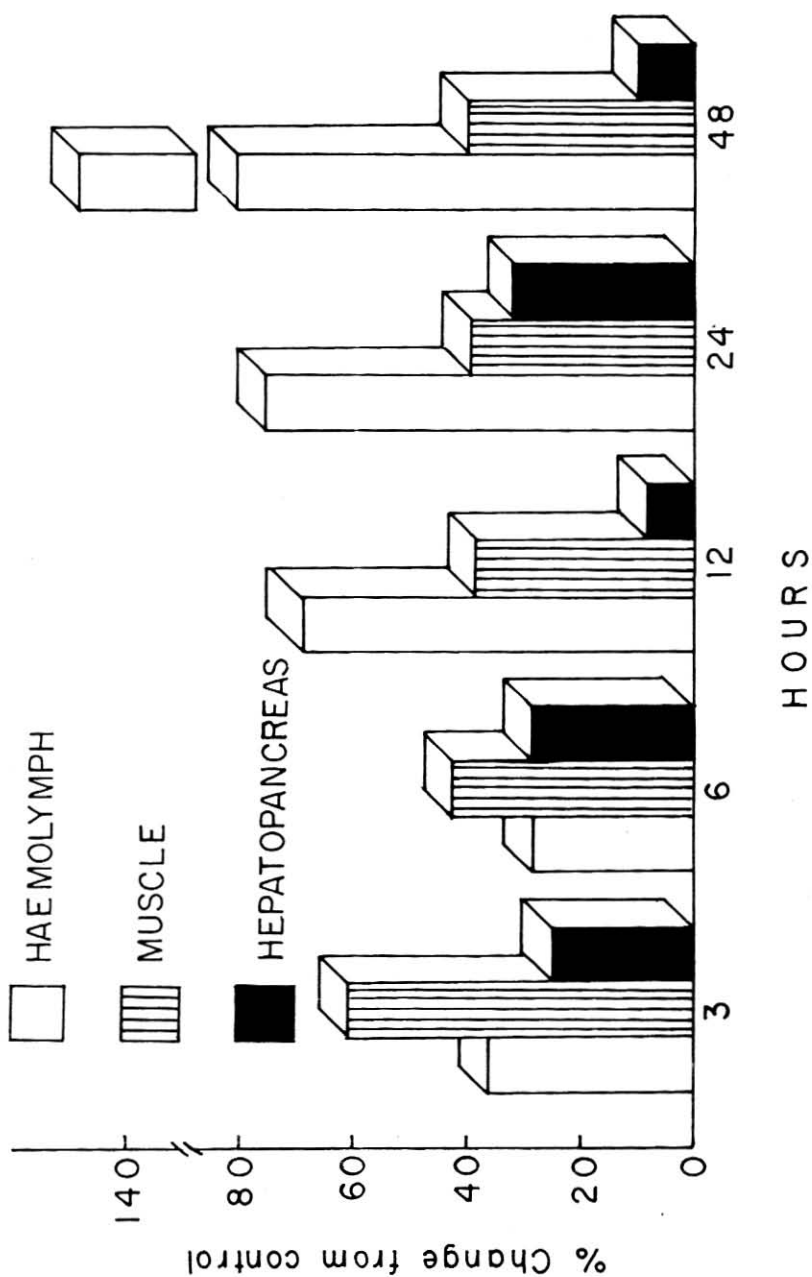
Fig. 2. Percentage changes in FAA content in haemolymph, muscle, and hepatopancreas of *P. indicus* transferred from low saline (10‰) to high saline water (40‰) in comparison with controls
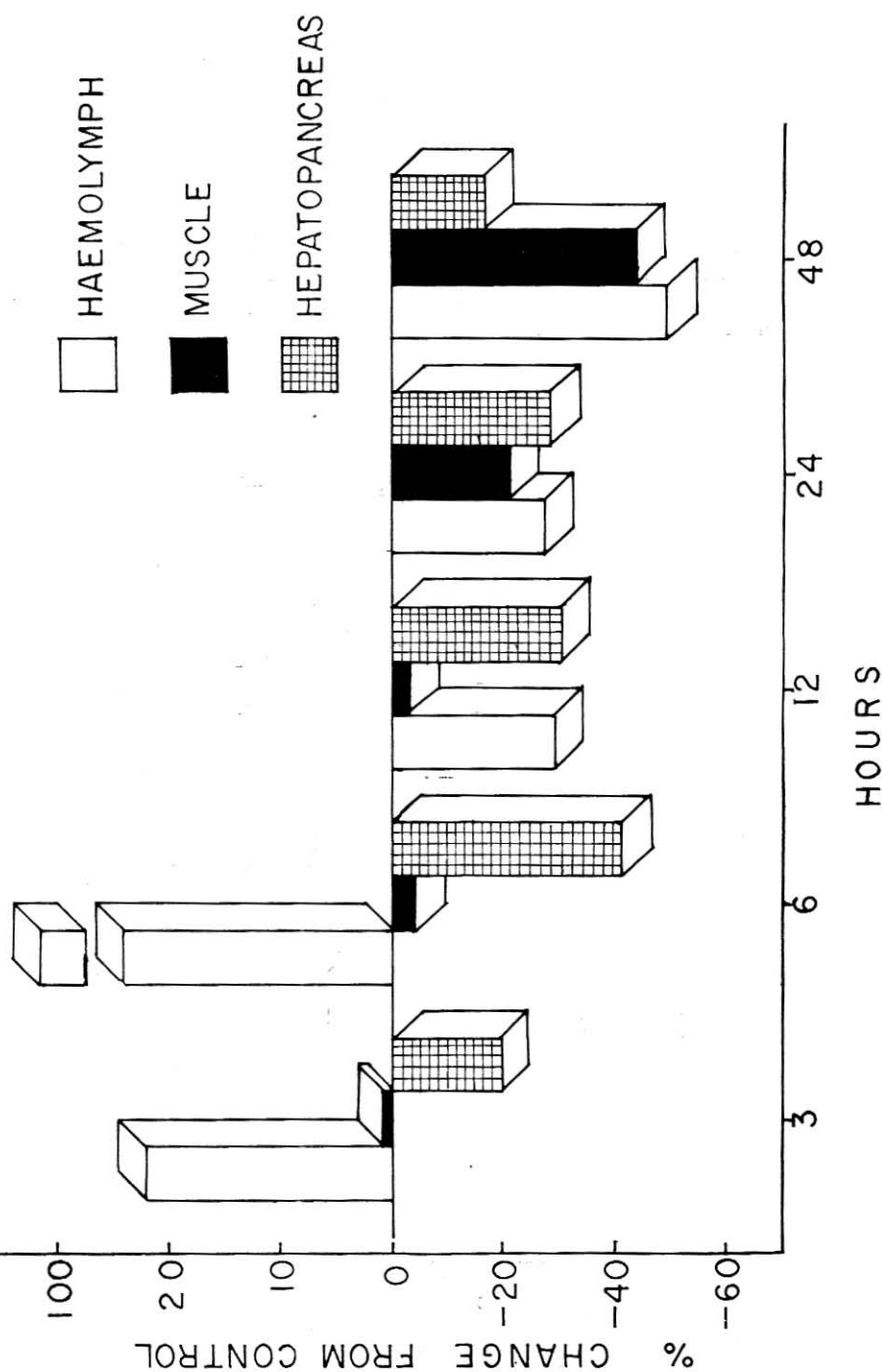
Fig. 3. Percentage changes in FAA content in haemolymph, muscle, and hepatopancreas of *P. indicus* transferred from high saline (40‰) to low saline water (10‰) in comparison with controls
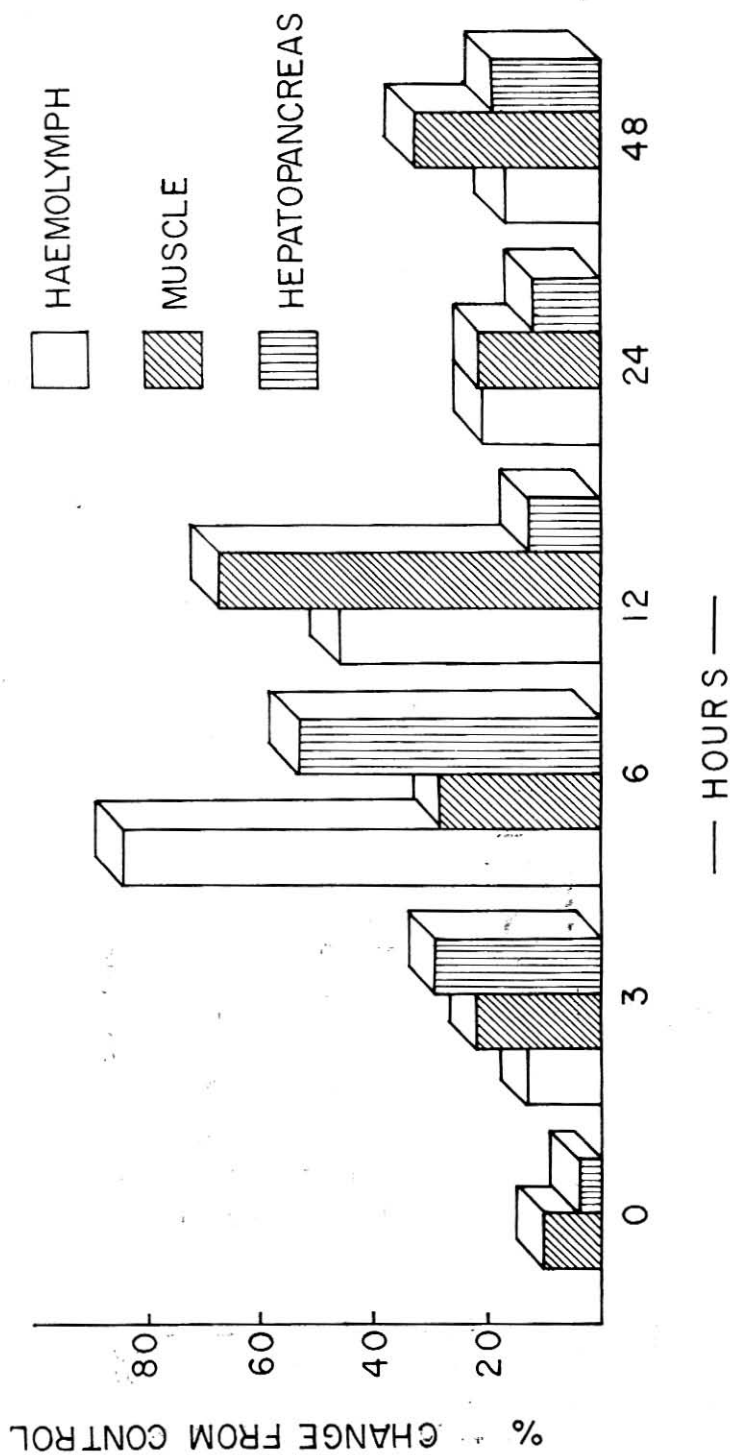
Fig. 4. Percentage changes in protein content in haemolymph, muscle, and hepatopancreas of *P. indicus* transferred from low saline (10‰) to high saline water (40‰) in comparison with controls
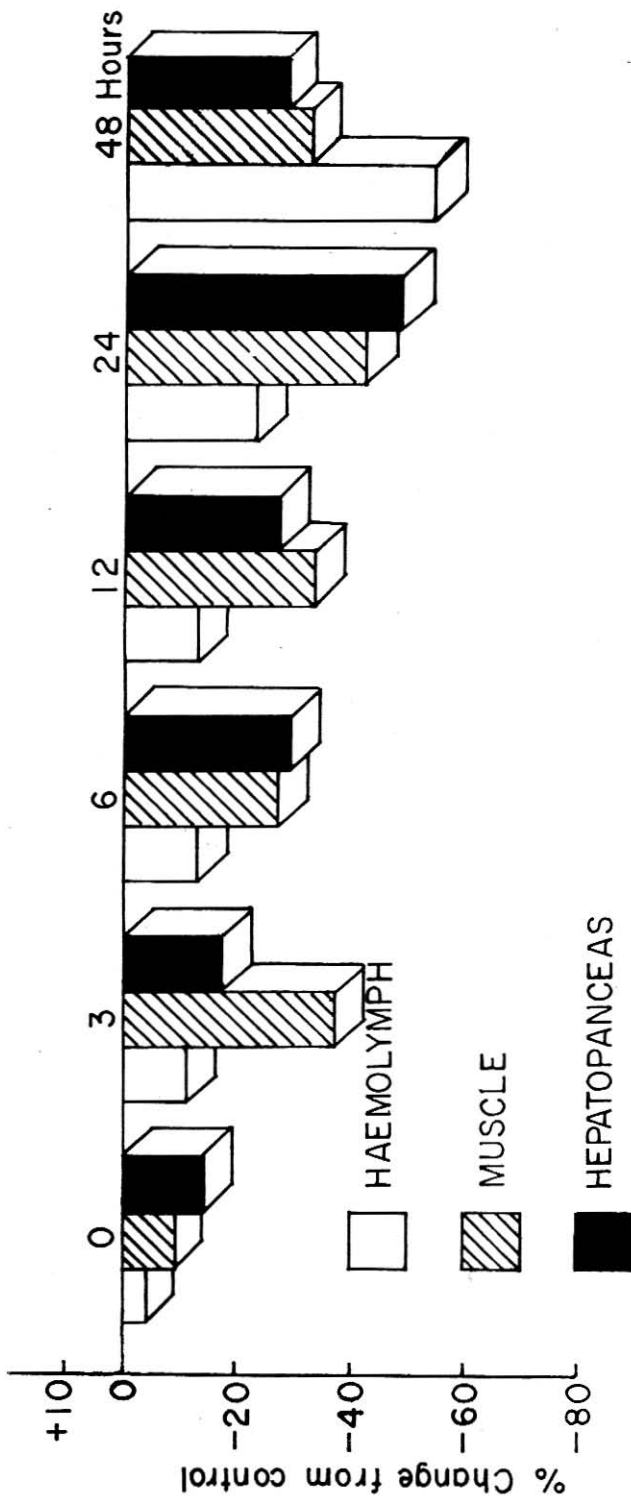
Fig. 5. Percentage changes in protein content in haemolymph, muscle, and hepatopancreas of *P. indicus* transferred from high saline (40‰) to low saline water (10‰) in comparison with controls

to be diminished or low in the majority of such cells (Fig. 6.2, 6.5, 6.8). No significant changes could be seen in cellular structure of such cells compared with those of prawns of isosaline conditions (S ≈ 20‰) (Fig. 6.3, 6.6, 6.9). Prawns transferred from high saline to dilute sea water did show increased secretory activity in their neurosecretory cells. A large number of vacuoles appeared in the cytoplasm of these cells with less granules in them. Many nucleoli were seen in the enlarged nucleus indicating enhanced activity of the cells (Fig. 6.1, 6.4 and 6.7).

Table 1. Histological changes observed in the neurosecretory cells of prawn *P. indicus* in relation to salinity stress

Neuroendocrine centres	Neurosecretory cells	Isosaline condition (20‰)	Salinity pressure	
			(40‰)	(10‰)
Cerebral ganglion	Giant neurons	Nucleus is oval and possesses two nucleoli, cytoplasm is dense due to granules. Cell diameter range 85–100 μm.	Nucleus with many nucleoli, granular cytoplasm less dense with few vacuoles. Cell diameter range 80–100 μm.	Nucleus with many nucleoli, granular cytoplasm with many vacuoles, cells irregular in shape. Cell diameter range 80–105 μm.
	A type	Nucleus is oval with prominent nucleoli, dense cytoplasm. Cell diameter range 64–72 μm.	Nucleus with many nucleoli, more vacuoles and less dense cytoplasm. Cell diameter range 60–68 μm.	Nucleus with many nucleoli, many vacuoles in the cytoplasm, irregular cell shape. Cell diameter range 63–75 um.
	B type	The cells are oval with prominent nucleus but without distinct nucleolus, dense cytoplasmic granules, cell diameter range 40–54 μm.	Cells are oval but without nucleoli, irregular cell boundary, few vacuoles in the cytoplasm. Cell diameter range 38–45 μm.	Cells are irregular without nucleus and nucleoli, no cell boundary. Cell diameter range 40–52 μm.
Thoracic ganglion	Giant neurons	Monopolar, multinucleolated, rounded nucleus with dense cytoplasmic granules. Cell diameter range 90–125 μm.	Cells without proper cell boundary, dense cytoplasmic granules. Cell diameter range 90–98 um.	Cells are irregular, many nuclei, more vacuoles in the cytoplasm and cell enlarged. Cell diameter range 90–140 μm.
	A type	Prominent cytoplasmic granules and nucleus with their boundaries, distinct nucleoli, no vacuoles in the cytoplasm. Cell diameter range 60–68 μm.	Prominent cytoplasmic granules with few vacuoles. Irregular cell margin. Cell diameter range 60–65 μm.	Cytoplasmic granules with many vacuoles, lack of nucleus and nucleoli. Cell diameter range 45–55 μm.

Neuroendocrine centres	Neurosecretory cells	Isosaline condition (20‰)	Salinity,pressure	
			(40‰)	(10‰)
	B type	Oval-shaped, more dense cytoplasmic granules with prominent nucleus and single nucleoli. Cell diameter range 30–42 µm.	Not much response, cell diameter range 30–40 µm.	Large nucleus with many vacuoles in the cytoplasm, cells enlarged and irregular. Cell diameter range 36–48 µm.
Abdominal ganglion	Giant neurons	Nucleus with prominent nucleoli, dense cytoplasm, cells are compact. Cell diameter range 80–95 µm.	Many nucleoli, irregular cell boundary, few vacuoles in the cytoplasm. Cell diameter range 80–105 µm.	Many nucleoli, irregular cell boundary. Many vacuoles in the cytoplasm. Cell diameter range 85–106 µm.
	A type	Nucleus with prominent nucleoli, dense cytoplasm. Cell diameter range 55–60 µm.	Cells without nucleus, few vacuoles in the cytoplasm. Cell diameter range 50–58 µm.	Cells without nucleus, irregular cell boundary. More vacuoles in the cytoplasm. Cell diameter range 55–70 µm.
	B type	Cells with dense cytoplasm, prominent nucleus, nucleoli regular cell boundary. Cell diameter range 35–44 µm.	Not much response. Cell diameter 35–44 µm.	Cell cytoplasm with many vacuoles, disturbed nucleus profile. Cell diameter range 38–50 µm.

Each observation is the mean of 30 paraffin sections.

DISCUSSION

The results of the present investigation show that sudden extreme change in salinity has greater impact on ammonia excretion rate of prawn *P. indicus* than on the excretion rate of controls. The ammonia excretion rate appeared to be inversely proportional to the salinity concentration at least in the initial stages of the experiment.

The findings reported here are in agreement with observations made for many euryhaline crustaceans exposed to different salinity gradients (Jeuniaux and Florkin, 1961). Haberfield *et al.* (1975) while working with *C. maenas* also reported increase in ammonia excretion rates with decrease in salinity. It was demonstrated that active sodium uptake in hyposmotic medium could be a factor for increase in ammonia output (Pressley *et al.*, 1981). Ammonia excretion is mainly dependent upon nitrogenous metabolic activity (synthesis or breakdown of peptides and proteins) in the tissues in response to osmotic stress (Gilles, 1977; Richard, 1982). According to Regnault (1984), rate of excretion was doubled in *Crangon crangon* when salinity dropped from 34‰ to 14‰ and the rate was again reduced as the salinity returned to 34‰. Spaargaren *et al.* (1982) also found a higher excretion rate of ammonia in *P. japonicus* when salinity was reduced from 31 to 21‰. In the present study in *P. indicus* ammonia excretion rate was very high especially after 3, 6, and 12 hr when

salinity dropped from 40 to 10‰. After 24 hr it returned to normal values. In our earlier studies (Diwan and Laxminarayana, 1989) on *P. indicus* we reported that isosmotic stability is achieved by the species to the new media within 48 hr and that may be one of the reasons that ammonia excretion rate is reduced after 24 hr. In higher salinity ammonia excretion rate was found to be reduced in *P. indicus*. Such a decrease may be due to reduction in the catabolic processing of amino acids as observed for many other euryhaline crustaceans (Gilles, 1979).

A number of experiments have been conducted to study the effect of osmotic shocks on isolated tissues and nerve axons to show that the amino acids of intracellular origin participate in adjustment of intracellular fluid osmolarity or cell volume regulation (Gilles, 1977). Decrease in intracellular amino acid content under hyposmotic stress is interpreted as one due to enhanced oxidation and an increased efflux through the cell membrane (Gerard, 1975). In the current study decrease in FAA level, noted in muscle and hepatopancreas in low saline condition, could be due to similar reasons. But in the haemolymph initially (up to 12 hr) there was an increase in FAA that could be a result of enhanced catabolic process of stored tissue proteins. A significant decrease after 24 hr may be due to enhanced oxidative deamination of amino acids but corresponding increase in ammonia level was not observed in the present study. Increased efflux of amino acids from tissues to blood in euryhaline species undergoing hyposmotic stresses is indicated in several studies and increase in blood amino acid content is concomitant with its decrease in the tissue (Gilles, 1977). The present observation is in agreement with the reports of the above workers. Richard and Ceccaldi (1975) also found that under hyposmotic exposure of shrimp, *P. kerathurus*, FAA content in the muscle declined, but in hepatopancreas it increased. Boone and Claybrook (1977) also reported decreased levels of FAA in haemolymph, gills, muscle, and hepatopancreatic tissues under low saline conditions. Claybrook (1983) was of the opinion that decrease in the FAA pool could be due to amino acid excretion or incorporation into protein, or peptides catabolism either by total oxidation or by conversion to other constituents. Studies of Chaplin *et al.* (1970); Huggins *et al.* (1975), and Gilles (1979) on incorporation of labelled amino acids in different crustaceans suggested that adaptation to dilute media is accompanied by stimulated utilization of amino acids.

In the tissues exposed to hyperosmotic shock Gilles (1974) demonstrated a lack of immediate major increase in amino acid concentration, but found decreased amino acid oxidation activity. Gilles suspected that increase of amino acid levels in tissues submitted to hyperosmotic shocks depends on the time scale, and such increases may be due to decreased output from the intracellular pool (endogenous resources). Increased FAA levels in haemolymph, muscle, and hepatopancreas under hypersaline conditions noted here may be a result of decrease in amino acid oxidation or active synthesis of peptide molecules and also decreased efflux of FAA, as reported by Gilles (1974). This is further shown with decreased excretion rate of ammonia. Gilles and Schoffeniels (1969) have found increased levels of FAA in isolated nerve axons from freshwater-adapted *E. sinensis* when placed in hypersaline media. Siebers *et al.* (1972) analysed FAA of whole *Carcinus* and crayfish *O. limosus* as well as of haemolymph, 0.5–10 days after transfer from 11 to 38‰ salinity, and found that the total FAA per crab recorded an increase within 12 hr followed by gradual 60% rise within 10 days. Gilles (1977) has reported slight decreases in FAA content in haemolymph and simultaneous increase of the same in tissues level in *E. sinensis* under hyperosmotic stress. This decrease in FAA of haemolymph is due to increased uptake of FAA from blood by the tissue. In the present study under hyperosmotic conditions although
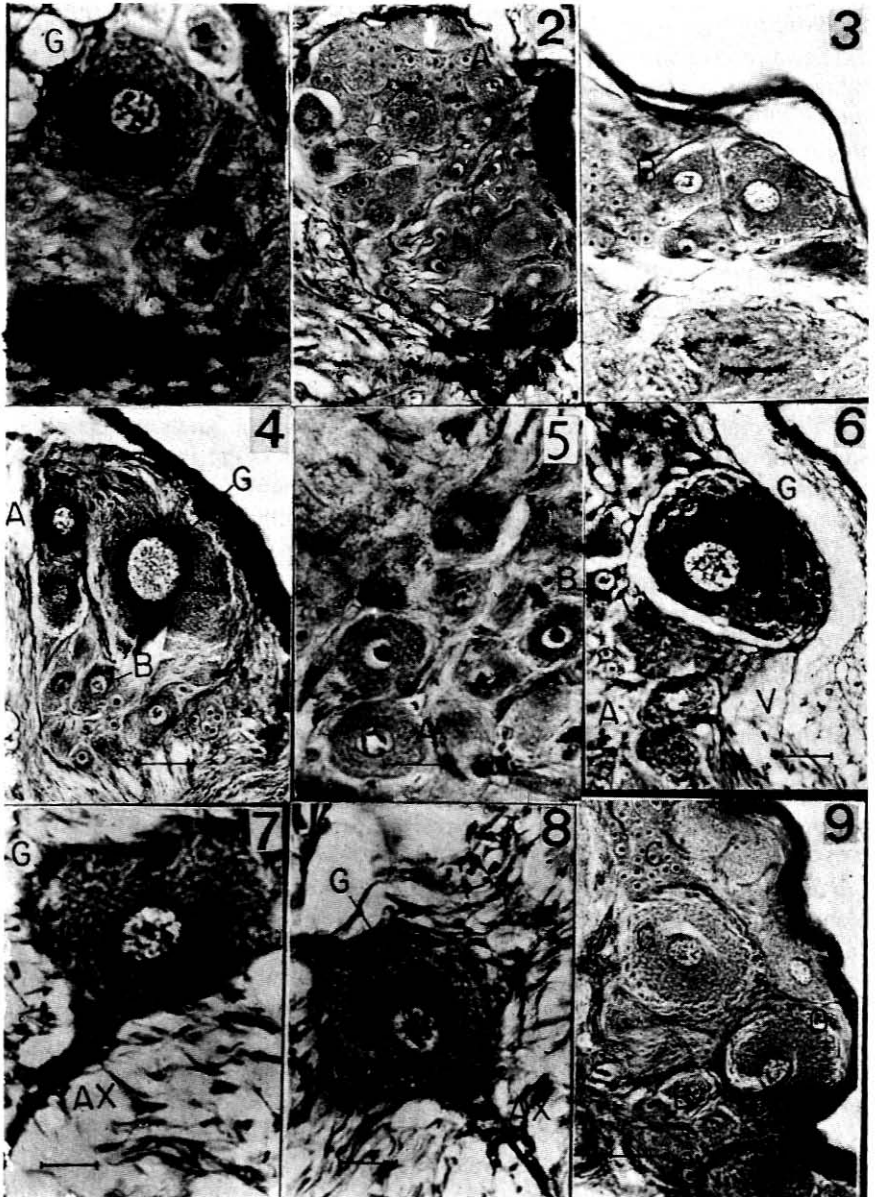
Fig. 6(1) Transverse section of the cerebral ganglion showing giant (G) and A types of neurosecretory cells of prawns transferred from higher saline water (40‰) to lower saline water (10‰). Note diffused cytoplasm. Scale bar = 30 μm × 400. (2) Transverse section of cerebral ganglion showing A and B type of neurosecretory cells of prawns transferred from low saline water (10‰) to high saline water (40‰). Note granular cytoplasm with prominent nucleoli in many cells. Scale bar = 30 μm × 200. (3) Transverse section of cerebral ganglion showing A and B type of neuroscretory cells of prawns in 20‰ saline water.Note granular cytoplasm in the cells with prominent nucleus and nucleoli. Scale bar = 30 μm × 200. (4) Longitudinal section of thoracic ganglion showing predominantly giant A and B neurosecretory cells of prawns transferred from high saline water (40‰) to low saline water (10‰). Note diffused cytoplasm with uneven cell boundaries, enlarged nucleus with diffused nucleoli. Scale bar = 30 μm × 200. (5) Longitudinal section of thoracic ganglion showing A and B type of

there was an increase in FAA content of muscle and hepatopancreas, haemolymph showed no decrease in FAA levels.

Experimental evidences on the effect of osmotic stress on protein modifications in crustaceans are limited. Some studies have linked the alteration of protein content with that of total amino acids (Venkatachari, 1974) in response to changes in salinity. But Gilles and Schoffeniels (1969) saw no substantial variation of protein concentration in isolated axons of *E. sinensis* submitted to osmotic stress. So also Siebers *et al.* (1972) found no significant change in protein concentration of whole *O. limosus* during acclimation from fresh water to saline water. Hence, it seems that equilibrium between protein and amino acids has no significant role in the adjustment of the amino acid pool occurring during cell volume regulation. Gilles (1977) had determined the blood serum protein levels in *E. sinensis* acclimated for one month in sea water and fresh water and reported a high content of blood protein in freshwater-acclimated animals, in comparison with that in seawater-acclimated animals. Gilles (1977) reasoned that swelling of the muscle tissue in hyposmotic condition could be related to an increase in blood protein concentration. In the present investigation decrease in protein content in haemolymph, muscle, and hepatopancreas under hyposmotic stress is possibly due to increased protein catabolic process. This has been reflected in high rate of ammonia excretion especially during times of critical stress. But it was not possible to compare equilibrium between protein and amino acid content under such stress. Although there was substantial increase in amino acid content of haemolymph when protein content is decreased, decrease in protein content in muscle and hepatopancreas did not lead to an increase in amino acid content of these tissues at the same time under hyposmotic stress. In hyperosmotic condition, significant increase in protein content haemolymph, muscle, and hepatopancreatic tissue occurred in the present study.

Studies of neurosecretory changes in crustaceans in response to osmotic stress are meagre. Bosch (1976) has reported increased secretory activity of neurosecretory cells of *Artemia salina* when they are transferred from hyperosmotic condition to dilute sea water. In 30% sea water the neurosecretory activity of such cells was diminished (Bosch, 1976). Observations on neurosecretory cell activity in relation to osmotic responses in *P. indicus* are in agreement with the findings reported by Bosch (1976); they also support our earlier findings of neuroendocrine control of osmolal concentration (Diwan and Laxminarayana, 1989).

nucreosecretory cells of prawns transferred from low saline water (10‰) to high saline water (40‰). Note granular cytoplasm with distinct nucleus and nucleoli in many of the cells. Scale bar = 30 μm × 200. (6) Longitudinal section of thoracic ganglion showing giant A and B type of nucreosecretory cells of prawns maintained in 20‰ saline water. Note densely stained granular cytoplasm with few vacuoles. Scale bar = 30 μm × 400. (7) Transverse section of abdominal ganglion showing predominant giant neurosecretory cells of prawns transferred from high saline water (40‰) to low saline water (10‰). Note less dense diffuse cytoplasm with many vacuoles. Scale bar = 30μm × 400. (8) Transverse section of abdominal ganglion showing predominantly giant neurosecretory cells of prawns transferred to high saline water (40‰) from low saline water (10‰). Note granular cytoplasm with vacuoles. The nucleus and nucleoli are distinct. Scale bar = 30μm × 400. (9) Transverse section of abdominal ganglion showing A and B type of neurosecretory cells of prawns maintained in 20% saline water. Note densely stained granular cytoplasm with prominent nucleus and intact cell boundaries. Scale bar = 30μm × 400.

108

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