CHAPTER 8

HISTOPHYSIOLOGICAL OBSERVATIONS ON THE CHOLINESTERASES IN THE LATERAL MUSCLES OF A MIGRATORY AND A NON-MIGRATORY FISH

That the presence or absence of butyrylcholinesterase differed according to species and was not a characteristic feature of a group of animals belonging to a particular habitat, was reported in the previous chapter. However, Lundin (1962) demonstrated the presence of S.W.F. (sea water fish) cholinesterase, which was said to possess butyryl-cholinesterase- like properties, besides acetylcholinesterase and myosincholinesterase in both marine and brackish water fishes. According to him, the function of S.W.F. cholinesterase might be concerned with ionic balance in the tissues of these fishes. The intracellular cholinesterase activity has also been shown to be necessary to maintain the 'sodium pump' in muscle cells (Van der Kloot, 1956; Varga, 1959). Barrnett and Palade (1959) had suggested its importance in the transverse impulse propagation into the muscle cell via the sarcotubular system.

In the light of the above mentioned observations and the fact that the muscles of H. ilisha and H. toli possess appreciable amounts of AChE, BuChE or both at the junctions as well as in the sarcoplasm (Chapter 7), it was thought worthwhile to investigate histochemically the changes in

cholinesterase activity in their muscles during different stages of maturity. The migratory (H. ilisha) and the non-migratory (H. toli) species of Hilsa were chosen for comparison and for correlating the enzymatic alterations with the accompanying electrolyte changes observed during migration (as reported in chapter 5). An attempt was also made to study the effect of different concentrations of added monovalent cations (Na^+ & K^+) on the level of cholinesterases at the neuromuscular and myotendinous junctions as well as in the sarcoplasm.

MATERIALS AND METHODS

The fishes were obtained as described in chapter 2. Immediately after collection from the net, the fishes were decapitated, bled and pieces of the lateral muscle from the middle region of the body (below the dorsal fin region) were fixed in cold 10% formol saline at 4°C for 6 - 8 hours. The method employed for demonstrating the activity of acetyl- and butyrylcholinesterase was the same as described in the previous chapter. In order to study the effect of added ions, chlorides of sodium and potassium of Analar grade were incorporated into the incubation media so that their final concentrations were between 0.05M to 0.6M in solution. The sections were incubated at 37°C in media with known amounts of sodium chloride and potassium chloride and then stained. Inspite of the fact that the salts used in the present study were analytical, yet they contained impurities in the form

of bivalent cations like Ba^{++}, Ca^{++} and Mg^{++}. Therefore, minor alterations in the enzyme activity may be attributed as an effect of these ions

The level of the esterases was assessed on the basis of the duration of the incubation time in which the optimum intensity of the enzymatic activity was observed.

RESULTS

The changes in the levels of acetyl- and butyryl-cholinesterases of the lateral muscles of H. ilisha and H. toli during different stages of their life cycle are summarized in Tables I and II respectively. On the other hand, the effects of added Na^{+} and K^{+} in increasing concentrations on the enzymatic activity in these stages are presented in Tables III to V.

In all the stages of maturity of H. ilisha, viz.,, the juveniles from the river, the immature fishes from the sea, mature and spent again from the river, both acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) were present. But the overall staining for AChE was more, except in the marine forms where the activity of both the esterases were more or less similar (Table I). The level of both esterases tended to decrease with maturity, because the highest was met with in juveniles, lesser in the immature fishes and least in mature and spent ones (Tables I & II).

Myotendinous junctions :

No significant difference in the AChE activity was

noticed at the myotendinous junctions of the red and white muscle fibres in all the stages. But the level of BuChE at these junctions was found to be comparatively lower in both the muscles of juveniles (Figs. 1,2,3,4) and in the white muscle of mature and spent H. ilisha collected from the freshwater zone (Figs. 6,7,8,9)

The white muscle of juveniles, with compactly arranged fibres, negligible intermuscular connective tissue and thin myosepta, possessed both the esterases in the form of dense linearly arranged regions on either ends of the fibres (Figs 1,2,3,4).

Amongst the red fibres it was only the superficial few which revealed enzymatic activity as described above for the white muscle, while the deeper ones had 'cap shaped' localizations, due to the better developed intermuscular connective tissue and widely separated adjacent myomeres. The pattern of activity in the fibres of two adjacent myomeres thus appeared in the form of an inverted 'Y' (Fig. 5).

In immature H. ilisha from sea and mature and spent forms from the river, the myotendinous junctions of red muscle fibres were the same ('cap shaped') as observed in the deeper region of the red muscle of juveniles (Figs. 6,7).

Neuromuscular junctions :

The neuromuscular junctions of juveniles were similar to the ones described in the previous chapter. But the localization of cholinesterases was neither distinct

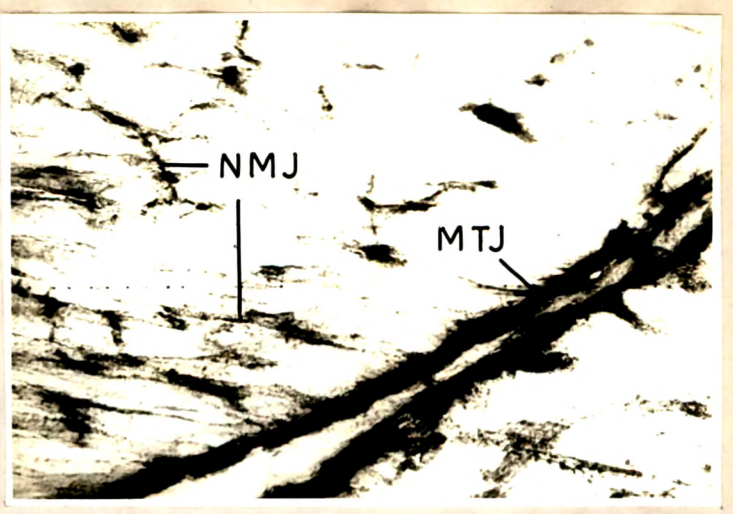

Fig. 1

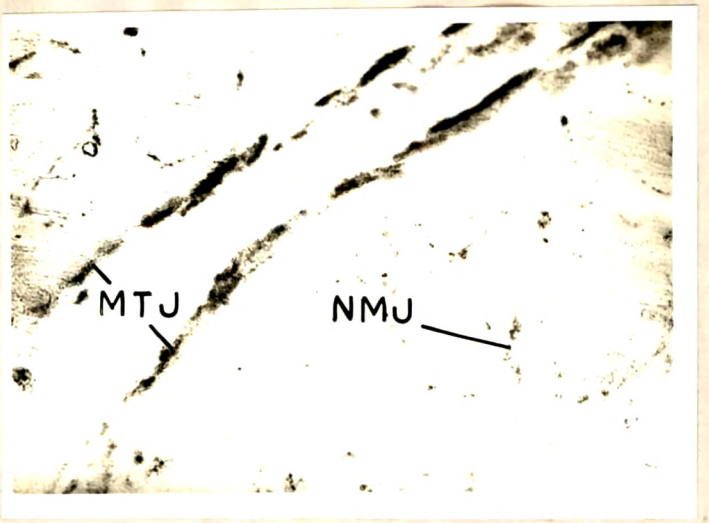

Fig. 2

Figs. 1 & 2. Red fibres (L. S.) of juveniles of H. ilisha collected from the freshwater zone showing AChE and BuChE activity. Note the less intense staining for BuChE. M.T.J.= myotendinous junctions; N.M.J. = neuromuscular junctions. 144 X.

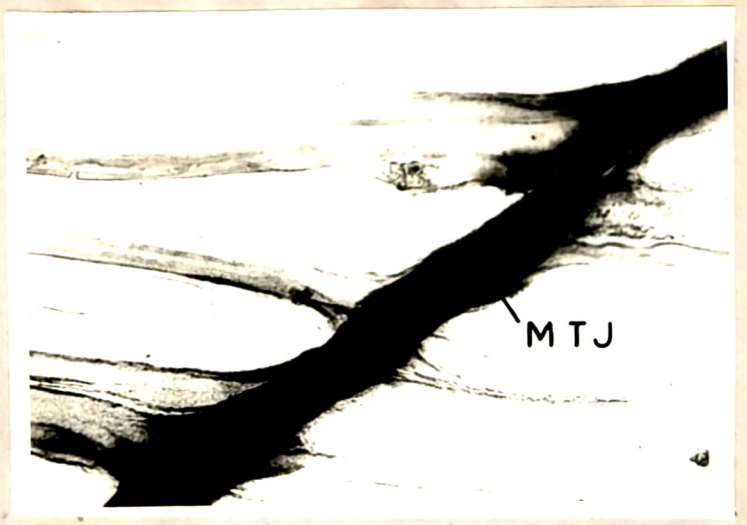

Fig. 3

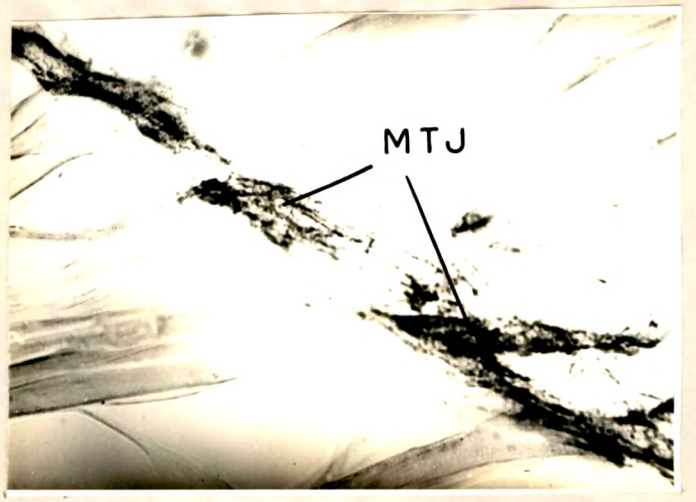

Fig. 4

Figs. 3 & 4. White fibres (L. S.) of juveniles of H. ilisha showing AChE and BuChE activity. Note the less intense staining for BuChE and the presence of myotendinous (M.T.J) junctions only. 144 X.

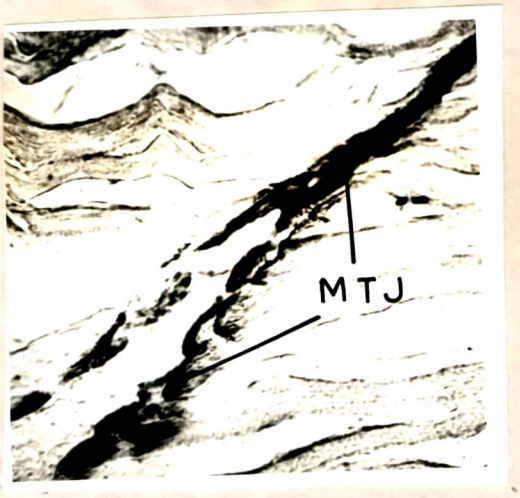

Fig. 5

The myotendinous junctions (M.T.J.) on red fibres (L. S.) of two adjacent myomeres showing the activity of AChE in the form of an inverted "Y". The activity on deeper fibres are "cap shaped". 144 X.

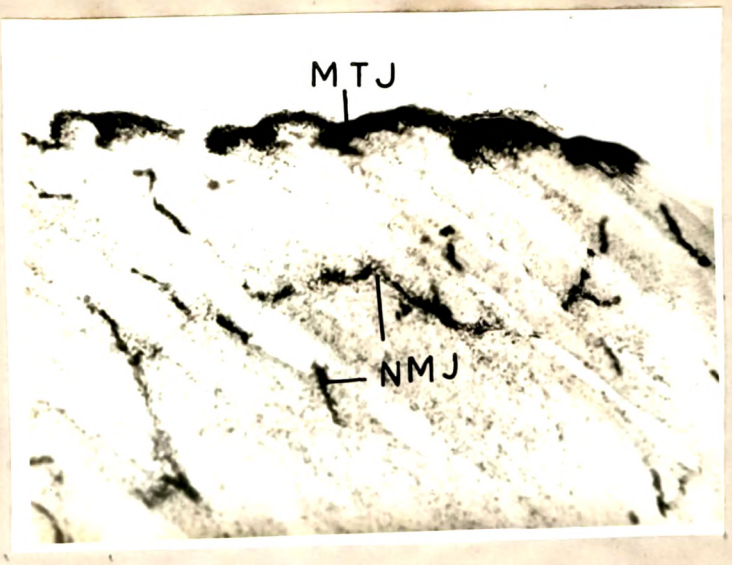

Fig. 6

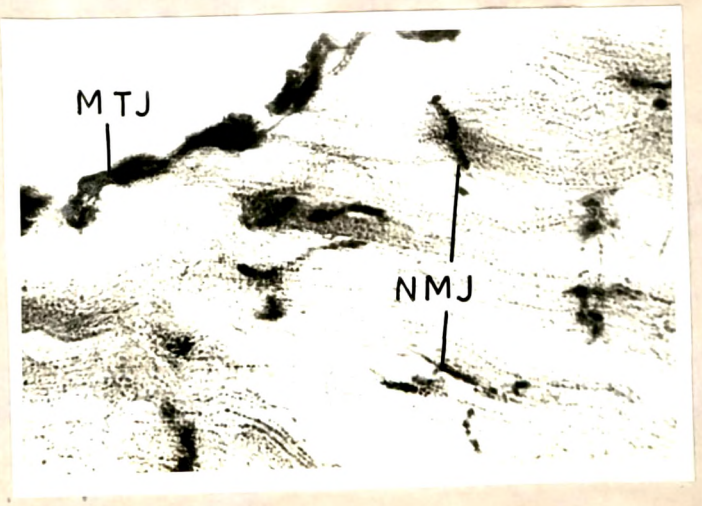

Fig. 7

Figs. 6 & 7. Red fibres (L. S.) of mature H. ilisha collected from the freshwater zone showing the localization of AChE and BuChE. M.T.J.= myotendinous junctions; N.M.J.= neuromuscular junctions. 225 X.

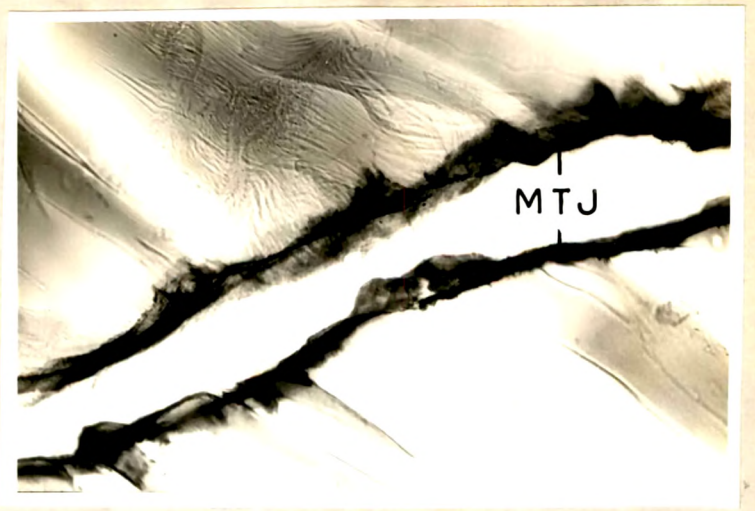

Fig. 8

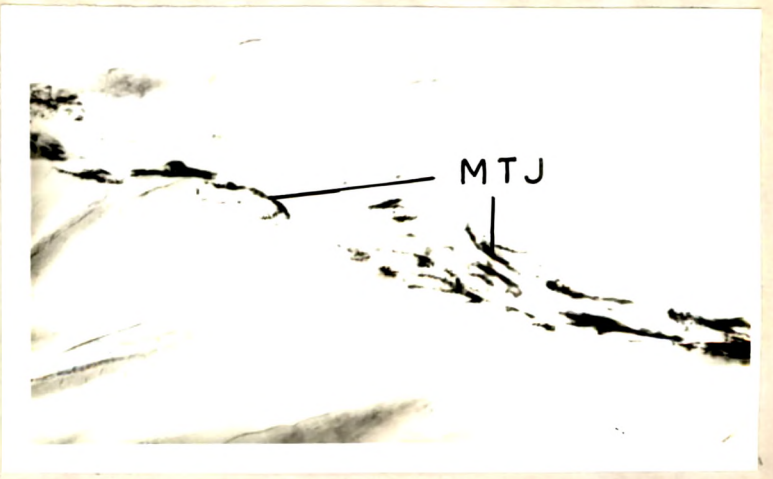

Fig. 9

Figs. 8 & 9. Deep white fibres (L. S.) of mature H. ilisha collected from the freshwater zone stained for AChE and BuChE. Note the less intense staining for BuChE and the absence of neuromuscular junctions in both. M.T.J.= myotendinous junctions. 144 X.

nor clear as in the later stages of the life cycle (Figs.1,2).

The white muscle region of juveniles could not be distinguished into superficial and deeper zones as the neuromuscular junctions were absent in both these regions. However, some of the superficial fibres showed patches of AChE activity in the median part, which did not reveal the characteristic features of neuromuscular junctions.

A higher level of diffuse sarcoplasmic AChE activity (myosincholinesterase) was found to be located in the red fibres of juveniles than in the later stages, while BuChE staining showed negative results in the white fibres.

In the immature, mature and spent fishes the morphological structure of neuromuscular junctions and sarcoplasmic cholinesterase activity were the same as described in chapter 7 (Figs. 6,7).

Effects of ions on AChE :

Na^+ and K^+ in low concentration (0.05M) caused a slight activation of enzyme in all stages. A slightly higher concentration (0.1M) brought about a decrease in enzymatic activity at the neuromuscular junctions and the sarcoplasm in juveniles and immature forms (Fig. 10, 11), but did not affect much either the mature or spent fishes since their ChE activity was almost the same as normal.

A more or less complete loss of activity at the neuromuscular junctions and the sarcoplasm was recorded at ionic concentrations of 0.2M and 0.4M in juveniles and immature

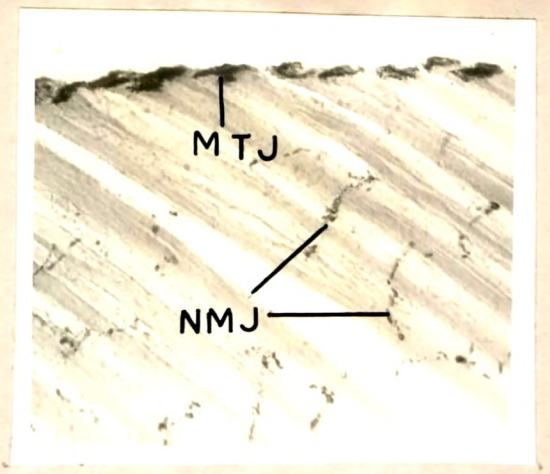

Fig. 10

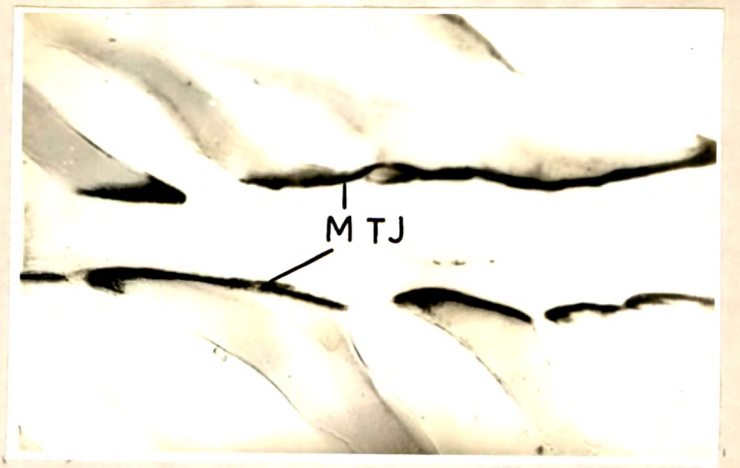

Fig. 11

Figs. 10 & 11. Red and white fibres (L. S.) of immature H. ilisha treated with 0.1M concentration of monovalent cations (Na^+ and K^+) and stained for the demonstration of AChE activity. Note the reduction in the enzyme level. 144 X.

Table I. Changes in cholinesterase activity during different stages of maturity of *H. ilisha*.

Place of collection	Stage in life cycle	Type of muscle	Acetylcholinesterase activity				Butyrylcholinesterase activity			
			Incubation time (hours)	Myotendinous junctions	Neuromuscular junctions	Sarcoplasm	Incubation time (hours)	Myotendinous junctions	Neuromuscular junctions	Sarcoplasm
River	Juveniles	Red M.	7-8	+++++	+++	++	16-18	+++	++	+
		White M.	7-8	+++++	-	+	16-18	+++	-	±
Sea	Immature	Red M.	12-16	+++++	++++	+	16-18	+++++	++++	+
		White M.		+++++	-	±		++++	-	±
River mouth	Mature	Red M.	16-18	+++++	++++	+	18-20	+++++	+++	+
		White M.		+++++	-	±		+++	-	±
River (Fresh water zone)	Mature & Spent	Red M.	22-24	+++++	++++	+	24-26	+++++	+++	+
		White M.		+++++	-	±		+++	-	±

The intensity of the positive reaction is denoted by the number of '+' signs.

'-' sign indicates negative staining.

Table II. Changes in cholinesterase activity during different stages of maturity of H. toli.

Place of collection	Stage in life cycle	Type of muscle	Acetylcholinesterase activity				Butyrylcholinesterase activity			
			Incubation time (hours)	Myotendinous junctions	Neuromuscular junctions	Sarcoplasm	Incubation time (hours)	Myotendinous junctions	Neuromuscular junctions	Sarcoplasm
Sea	Immature	Red M.	14-16	+++++	++++	+	22-26	-	-	+
		White M.		+++++	-	±		-	-	±
	Mature & Spent	Red M.	16-18	+++++	++++	+	22-26	-	-	+
		White M.		+++++	-	±		-	-	±
Bhadbhut (river)	Mature	Red M.	22-26	++++	+++	+	26-28	-	-	+
		White M.		++++	-	±		-	-	±

The intens ity of positive reaction is denoted by the number of '+' signs.

'-' sign indicates negative staining.

Table III. Effect of different concentrations of added Na^+ or K^+ on cholinesterases in mature and spent H. ilisha from river.

Concentration of Na^+ or K^+ in the medium	Type of muscle	Acetylcholinesterase activity				Butyrylcholinesterase activity			
		Incubation time (hours)	Myotendinous junctions	Neuromuscular junctions	Sarcoplasm	Incubation time (hours)	Myotendinous junctions	Neuromuscular junctions	Sarcoplasm
0.05M	Red M.	19-22	+++++	++++	+	24-26	+++++	+++	+
	White M.		+++++	-	±		+++	-	±
0.1M	Red M.	22-24	+++++	++++	+	24-26	++++	++	±
	White M.		+++++	-	±		++	-	±
0.2M	Red M.	24-28	++++	++	±	26-30	+++	+	±
	White M.		++++	-	±		+	-	-
0.4M	Red M.	24-28	+++	+	-	26-30	+	±	-
	White M.		+++	-	-		±	-	-
0.6M	Red M.	24-28	+	±	-	26-30	±	-	-
	White M.		+	-	-		-	-	-

The intensity of positive staining is denoted by the number of '+' signs.
'-' sign indicates negative staining.

Table IV. Effect of different concentrations of added Na^+ or K^+ on cholinesterases in immature *H. ilisha* from sea.

Concentration of Na^+ or K^+ in the medium	Type of muscle	Acetylcholinesterase activity				Butyrylcholinesterase activity			
		Incubation time (hours)	Myotendinous junctions	Neuromuscular junctions	Sarcoplasm	Incubation time (hours)	Myotendinous junctions	Neuromuscular junctions	Sarcoplasm
0.05M	Red M.	10-14	+++++	++++	+	14-16	+++++	++++	+
	White M.		+++++	-	±		++++	-	±
0.1M	Red M.	14-16	++++	++	±	16-18	++++	++	±
	White M.		++++	-	±		++++	-	±
0.2M	Red M.	18-20	+++	+	-	18-20	+++	+	-
	White M.		+++	-	-		++	-	-
0.4M	Red M.	20-24	++	±	-	20-24	++	±	-
	White M.		++	-	-		+	-	-
0.6M	Red M.	20-24	±	-	-	20-24	±	-	-
	White M.		±	-	-		±	-	-

The intensity of positive staining is denoted by the number of '+' signs.
'-' sign indicates negative staining.

Table V. Effect of different concentrations of added Na^+ or K^+ on cholinesterases in juveniles of of *H. ilisha*

Concentration of Na^+ or K^+ in the medium	Type of muscle	Acetylcholinesterase activity				Butyrylcholinesterase activity			
		Incubation time (hours)	Myotendinous junctions	Neuromuscular junctions	Sarcoplasm	Incubation time (hours)	Myotendinous junctions	Neuromuscular junctions	Sarcoplasm
0.05M	Red M.	6-7	+++++	+++	++	16-18	+++	++	+
	White M.		+++++	-	+		+++	-	±
0.1M	RedM.	10-11	++++	++	+	16-18	++	+	±
	White M.		++++	-	±		++	-	±
0.2M	Red M.	14-16	++	±	±	20-24	+	±	-
	White M.		++	-	-		+	-	-
0.4M	Red M.	16-20	+	-	-	20-24	±	-	-
	White M.		+	-	-		±	-	-
0.6M	Red M.	16-20	±	-	-	20-24	±	-	-
	White M.		±	-	-		-	-	-

Intensity of positive reaction is denoted by the number of '+' signs; '-' indicates negative reaction.

fishes respectively (Fig. 12,13,14,15), but the myotendinous areas still showed activity of the enzyme. At 0.6M concentration a total inhibition of the esterase at the myotendinous junctions of above mentiones fishes (juveniles and immature forms) and neuromuscular junctions of mature and spent fishes was effected (Fig. 16,17), while the myotendinous junctions of latter fishes showed activity at this concentration also.

Effect of ions on BuChE :

The pattern of activation and inhibition by Na^+ and K^+ in different concentrations on BuChE activity in marine immature forms was more or less similar to that of AChE (TableIV).

In the other stages (juveniles, mature and spent forms from river) 0.05M concentration did not have an appreciable effect on the enzymatic activity; 0.1M affected the neuromuscular areas and sarcoplasm more than myotendinous; 0.2M mostly inhibited the activity at the former sites; 0.4M brought about a complete depletion of the enzyme from all sites in juveniles. In mature and spent forms except in the myotendinous junctions of red fibres (which were completely inhibited at 0.6M) in all other sites complete inhibition took place at 0.4M concentration.

It could be inferred from Tables III to V that 1. the monovalent cations ($Na^+$and K^+) in low doses revealed a slight activation of AChE in all stages of maturity, 2. the AChE activity was more resistant to the effects of ions than

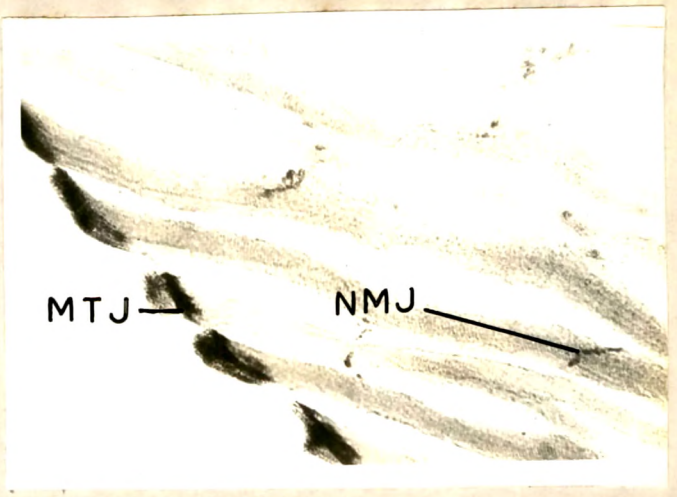

Fig. 12

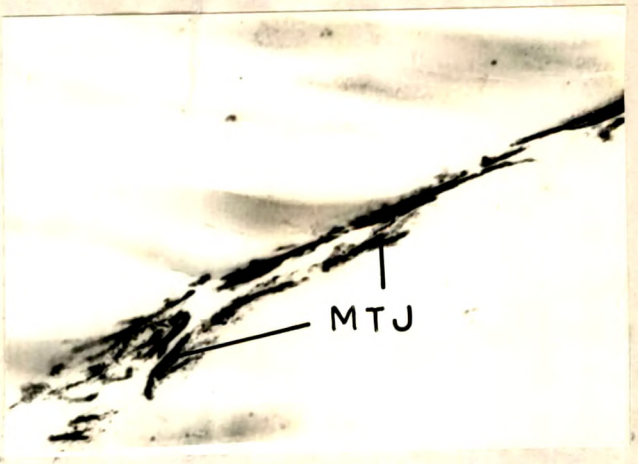

Fig. 13

Figs. 12 & 13. Red and white fibres (L. S.) of immature H. ilisha treated with 0.2M concentration of monovalent cations (Na^+ and K^+) and stained for the demonstration of AChE activity. Note that the inhibition of the enzyme is more at neuromuscular junctions (N.M.J.) than at myotendinous junctions (M.T.J.). Fig. 12- 225 X.; Fig. 13- 144 X.

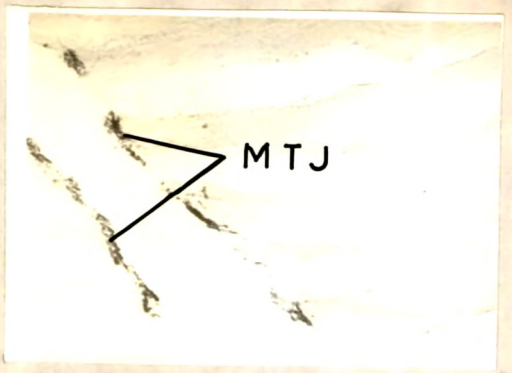

Fig. 14 Fig. 15

Figs. 14 & 15. Same as Figs. 12 & 13, but treated with 0.4M concentration of monovalent cations (Na^+ and K^+). Note the inhibition of the enzyme at neuromuscular junctions of red fibres. 144 X.

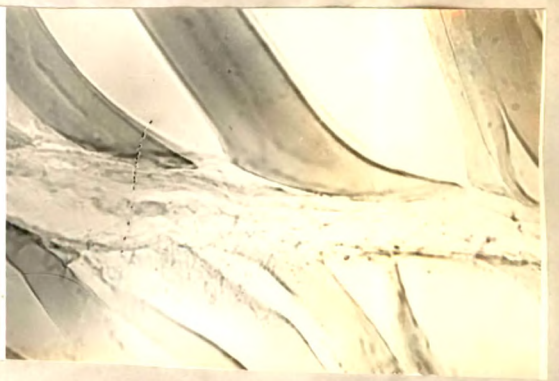

Fig. 16 Fig. 17

Figs. 16 & 17. Same as Figs. 14 & 15, but treated with 0.6M concentration of ions (Na^+ and K^+). Note the total inhibition of the enzyme activity at all sites. 144 X.

BuChE in all the stages except the immature forms from sea, where the effect was more or less same for both the esterases and 3. the enzyme was destroyed faster at the neuromuscular junctions than at the myotendinous junctions in all the the stages of H. ilisha

Specimens of H. toli (immature, mature and spent) from sea revealed the same staining pattern for AChE as immature H. ilisha, whereas, BuChE activity could not be demonstrated at either of the junctions, but only in the sarcoplasm of the red fibres. The enzymatic activity decreased with maturity and it was much lower in the river specimens than in the marine ones of the same stage of maturity (Table II).

The effects of ions in different concentrations was also investigated in all the stages of this fish and the results obtained were very nearly the same as those of immature H. ilisha from sea.

Cholinesterases in degenerating muscle fibres :

In the red muscle fibres of H. ilisha wherein degeneration had set in with replacement by connective tissue (Chapter 2), it was possible to see isolated muscle fibres (surrounded by connective tissue) bearing either neuromuscular or myotendinous junctions or both (Figs.18,19) depending on the portion of the fibre which had escaped degenerative changes. These fibres also exhibited greater intensity of sarcoplasmic staining than normal ones.

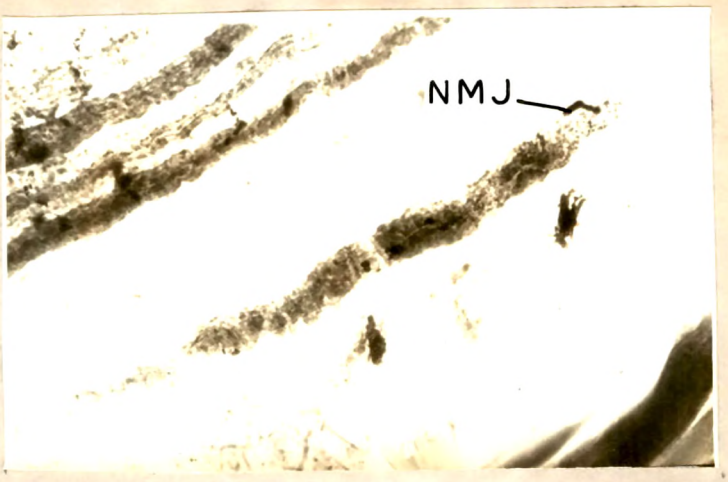

Fig. 18

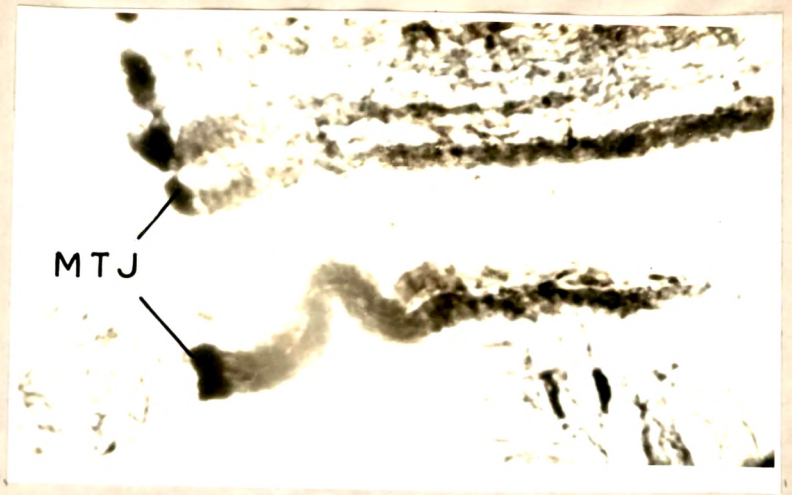

Fig. 19

Figs. 18 & 19. L. S. stained for AChE to show the neuromuscular (N.M.J) and myotendinous (M.T.J) junctions of degenerating red fibres in spent H. ilisha collected from the river. 144 X.

DISCUSSION

The presence of high levels of cholinesterase activity in muscle fibres during early development and in immature forms has been reported by a number of authors (Sawyer, 1943, 1944; Lundin, 1962; Chinoy, 1965), in fishes (*Fundulus heteroclitus* and *Lebistes reticulatus*), amphibians (*Amblystoma punctatum*)and birds (*Columba livia*). In the present investigation also, the overall activity of AChE in the juveniles of *H. ilisha* was much greater than in the later stages of life cycle.

Considerable amount of diffuse sarcoplasmic cholinesterase activity, myosincholinesterase (as observed in the juveniles of *H. ilisha* in the present investigation), was also reported to be a characteristic feature of developing muscle fibres whereas, the neuromuscular junctions remain indistinct with diffuse activity (Varga *et al.*, 1957; Kovács *et al.*, 1961). They gain complexity in structure and become differentiated only with development along with myosincholinesterase which reciprocally decrease with age to attain a constant level in adults.

A correlation between the increase in cholinesterase activity and a number of causal factors have been suggested. The neurological (Nachmansohn *et al.*, 1946) or the general activity of the animal concerned (Sawyer, 1943; Baslow and Nigrelli, 1961) have been shown to influence enzymatic levels; thus greater the activity of animal, higher would be the

concentration of the enzyme in the muscle. It has been reported that the more active tonic fibres of the pectoralis muscle of pigeon possess greater AChE for sustained activity while the tetanic ones on the contrary, have greater BuChE for quick phasic action, as the pectoralis of the domestic fowl (Chinoy, 1965). In the light of these observations, it is logical to assume that the greater cholinesterase activity in the marine immature H. ilisha might be due to increased locomotor activities under migratory stress.

Upon maturation and spawning in both species of Hilsa, there was a reduction in the doncentration of esterases, as growth is known to bring about a decrease in cholinesterase levels. In marine H. toli (mature and spent), the reduction was comparatively lesser than in the corresponding stages of H. ilisha from the spawning grounds (freshwater zone). The greater reduction in enzymatic activity in the latter species might be due to its migration to freshwater accompanied by starvation, since malnutrition and starvation are known to bring about a significant reduction in BuChE (McCance et al., 1948). Fluctuations in the levels of cholinesterase in the pectoralis of starving pigeons has also been demonstrated by Chinoy (1965), wherein there was a decline in activity during the initial stages followed by a peak level on the 4th day, leading finally to 35% reduction on the 5th day. These fluctuations have been explained as due to the upsetting of the sodium pump in the muscle and the accompanying

ionic imbalance during inanition (Chinoy, 1965).

The concentration of the substrate is again an important factor determining its rate of hydrolysis by the respective cholinesterase (Davies and Greene, 1958; Krupka, 1964). Thus it has been reported for a number of fishes by Lundin (1962), that an optimal substrate (acetylcholine) concentration was necessary to bring about its maximum splitting. On comparing the values obtained for two of the best represented freshwater families (Cyprinidae and Salmonidae) with those of two marine ones (Gadidae and Pleuronectidae), it was realized that the former had a lower substrate concentration than the latter. On the basis of this finding it could be mentioned that in the migratory H. ilisha, the change from salt to freshwater, coupled with intense muscular activity under starvation might have resulted in the reduction of the esterases level in mature and spent fishes.

Since the activity of cholinesterase is a prerequisite for the working of 'sodium pump' in muscle, an inhibition of intracellular cholinesterase has been shown to block sodium extrusion (Van der Kloot, 1956). Therefore, it follows that any alteration of cholinesterase activity in the muscle of H. ilisha during its migratory ascent to freshwater would necessarily affect their ionic contents. It has been reported (Chapter 5) that the marine immature fishes contain a lower sodium and higher potassium level in their muscles as compared to the gravid and spent stages

from freshwater. But the total sodium, potassium content was much greater in the former fishes. Thus, the increase in sodium observed in the latter fishes may be due to the disturbance in its extrusion because of lowered cholinesterase levels. According to Mendel and Rudney (1945) it is the outflux of potassium which probably helps in maintaining optimal conditions for cholinesterase activity upon stimulation. A similar drop in potassium level has been recorded in H. ilisha after migration.

The incorporation of monovalent cations (Na^+ & K^+) in low doses revealed an activation of AChE, as has been reported by Nachmansohn (1940) and Chinoy (1965). Mendel and Rudney (1945) also reported that the relationship between enzymatic activity and substrate concentration was altered by the addition of salts to the medium, so much so, that a step-wise increase in the electrolyte (NaCl or KCl) concentration caused a correspondingly gradual shifting of the optimal enzyme activity to higher levels of the substrate. This factor might be the one maintaining enzymatic activity in mature and spent H. ilisha even at ionic levels as high as 0.6M. It has already been reported earlier that in freshwater fishes, substrate concentration for getting maximum splitting rate of the enzyme, is lower than in the marine fishes (Lundin, 1962) and in mature and spent H. ilisha from the river the substrate inhibition may be suggested as a reason fo lowering the cholinesterase levels. Therefore,

the addition of ions would favour optimum enzymatic activity, but now at a greater substrate level. In this context it should be mentioned that an optimum substrate concentration is necessary for its ready hydrolysis. Thus the presence of Na^+ and K^+ upto a certain level only could influence the enzyme activity in the different stages of H. ilisha, depending on whether they were salt- or freshwater fishes.

The source of the enzyme is another important factor in determining the effects of Na^+ and K^+ on cholinesterases (Glick, 1941). Thus, these ions may produce activation of the enzyme from one source and may not manifest an identical effect on the enzyme from another source as observed by him on serum cholinesterase activity of rabbit and horse. In the present investigation too, the disparity in activation and inhibition of the same enzyme from different sources (i.e. various stages of maturity) or from different sites (neuromuscular or myotendinous junctions or sarcoplasm) supports Glick's (1941) findings. Thus in the juveniles and immature stages as against the later ones, the cholinesterase was on the whole much more readily destroyed. In contrast, the BuChE of marine immature forms was more resistant than in the other stages. Among the junctions, however, it was the myotendinous, which resisted the effects of abnormal levels of ions better than the neuromuscular.