CHAPTER 7

LOCALIZATION OF CHOLINESTERASES IN THE RED AND WHITE MUSCLES OF THREE REPRESENTATIVE TYPES OF FISHES

Functional studies on vertebrate skeletal muscles have distinctly shown the existence of two types of muscle fibres, the twitch fibres, which show a propagated impulse and non-graded contractile activity, and slow fibres which do not diplay impulse activity but undergo a graded contraction (Kuffler and Williams, 1953 a, b; Ginsborg, 1960 a, b; Hess and Pilar, 1963). The twitch fibres show a "fibrillenstruktur" and receive a single nerve ending of 'en plaque' type while the slow fibres reveal "felderstruktur" and have multiple motor endings of 'en grappe' type (Krüger, 1949; Krüger and Günther, 1958; Ginsborg and Mackay, 1960; Hess, 1960, 1961, 1962).

In a series of studies Häggqvist (1960, 1962) demonstrated that the nerve fibres innervating a muscle are of two types, viz. small and large. The small fibres have 'en grappe' endings and large fibres have the 'en plaque' ones. Unlike in the case of 'motor plaques' of mammals or 'clusters of Kuhne' in frog, where the amyelinated portions of nerve fibres form a more or less compact ramification and are in contact with a single muscle fibre, in teleosts, its extensive ramification involve a number of muscle fibres (Supino, 1898). The motor innervation of the catfish, Ameiurus

nebulosus is formed by small myelinated fibres and these endings differ morphologically from those of the white muscle (Barets, 1952). Thus, as in higher vertebrates, the two types of muscle fibres in fishes too, have different types of innervation in addition to such differences as of colour, diameter, blood supply, mitochondrial content, enzyme activity etc. The motor innervation of deep lateral muscle in teleosts have been shown by Barets (1955) to differ from species to species. Thus, the present study was undertaken with a view to understand more about the innervation patterns and the localization of cholinesterases in the different fibre types of fishes.

MATERIALS AND METHODS

The lateral muscles of three representative fishes were studied; a migratory fish, Hilsa ilisha (Ham.), a non-migratory marine fish, Hilsa toli (Cuv. & Val.) and a fresh-water fish, Barbus stigma (Day). H. ilisha and H. toli were caught from the sea while B. stigma were maintained in aquaria under laboratory conditions. The fishes were decapitated, bled and pieces of the lateral muscle from the middle region (below the dorsal fin) were fixed in cold 10% formol saline (Gurr, 1956), for 4 - 6 hours at 4°C. The muscle pieces were washed thoroughly with distilled water, and sectioned longitudinally (20µ) on a freezing microtome. The method of Koelle and Friedenwald (1949) as modified by

Coupland and Holmes (1957) was employed for demonstrating cholinesterases, using the iodides of acetyl- and butyryl-thiocholine respectively as substrates. The sections were incubated at 37°C at a pH of 5.6 to 6.0 . The incubation time varied according to the concentration of the enzyme present in the tissue and the species concerned.

The control sections were treated with a 0.00003 M solution of eserine sulphate for 30 minutes at room temperature in order to inhibit both the esterases while a 10^{-6} M solution of di-isopropyl flurophosphate (DFP) was used to destroy the activity of butyrylcholinesterase alone.

Some of the formalin (10%) fixed muscle pieces of H. ilisha and H. toli were used for the demonstration of nerve fibres by the Urea - silver nitrate method (Gurr, 1956), employing paraffin sections of 10µ thickness.

RESULTS

Localization of Acetylcholinesterase (AChE) :

In H. ilisha and H. toli maximum activity of acetylcholinesterase (AChE) could be obtained between 12 - 14 hours and in B. stigma 2 - 4 hours. This observation is in accordance with those of Lundin (1962), that small fishes irrespective of species have high cholinesterase activity in their body muscles.

(A) Red muscle region :

The localization of AChE in red muscle is more or

less constant in all the three species studied (Figs.1, 3, 5).

Neuromuscular junctions :

The morphological patterns of the junction was observed in the form of closely distributed intrafibral nerve plexuses, each fibre receiving few to several nerve terminations (Figs. 1, 3, 5, 6). In most of the fibres the synaptic cups were interconnected thereby giving the appearance of a chain (Fig. 6). The nerve fibres formed plexuses of numerous chains linked together and enclosed a variable number of muscle fibres (Fig. 7). This type of innervation is similar to 'en grappe' type of innervation of catfish, tench and dogfish (Barets, 1961; Bone, 1964, 1966).

Myotendinous junctions :

The localization of AChE was obtained at either end of the muscle fibres which are attached to the myosepta. In H. ilisha and H. toli the myosepta being very wide and fibres loosely arranged, the myotendinous junctions were seen as 'cap shaped' structures (Figs. 1, 3). In Barbus, on the other hand, since the myoseptae were limited and fibres closely packed, the activity of the enzyme was obtained in the form of dense lines at both the extremities of the muscle fibres (Fig.5). In all the three fishes investigated the localizations of the enzyme activity under high magnification were seen to be formed of groups of ovoid cups of variable diameter.

(B) White muscle region :

The superficial and deep zones of white muscle of

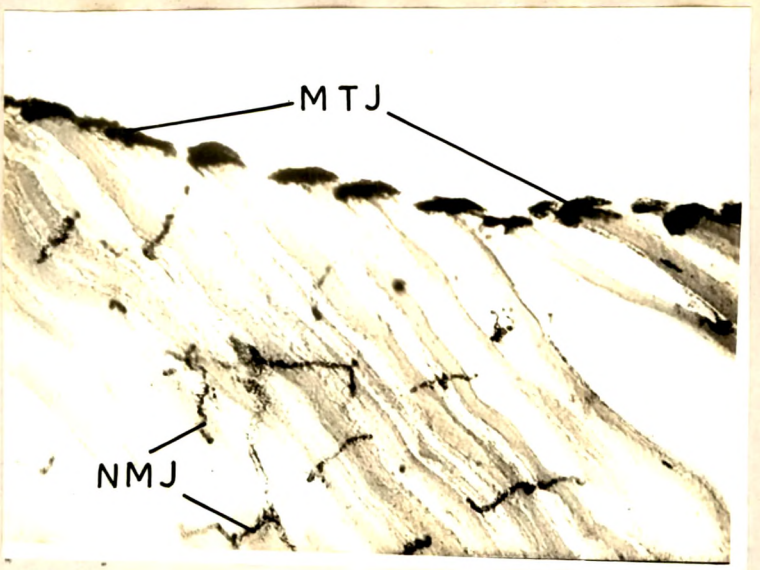

Fig. 1

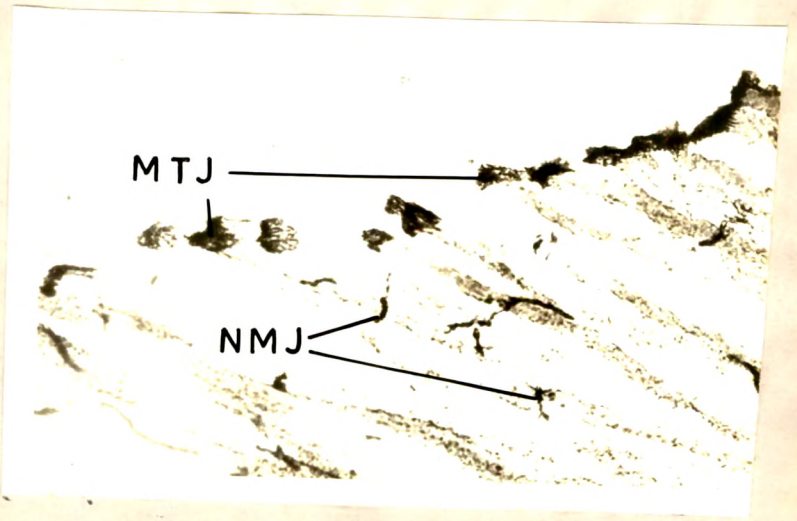

Fig. 2

Figs. 1 & 2. Red fibres (L. S.) of Hilsa ilisha showing the localization of acetyl- and butyrylcholinesterase activity. M.T.J.- myotendinous junctions; N.M.J.- neuromuscular junctions. 144 X.

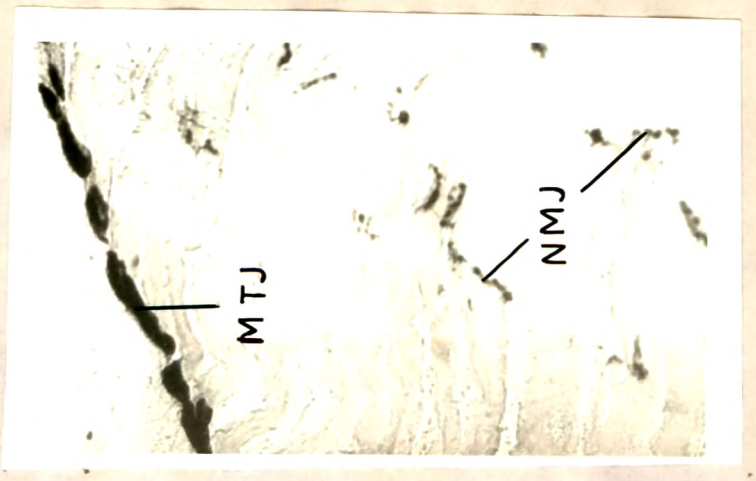

Fig. 3

Fig. 4

Figs. 3 & 4. Red fibres (L. S.) of Hilsa toli stained for acetyl- and butyrylcholinesterase activity. Note the absence of BuChE in Fig. 4. N.M.J.- neuromuscular junctions; M.T.J.- myotendinous junctions. 144 X.

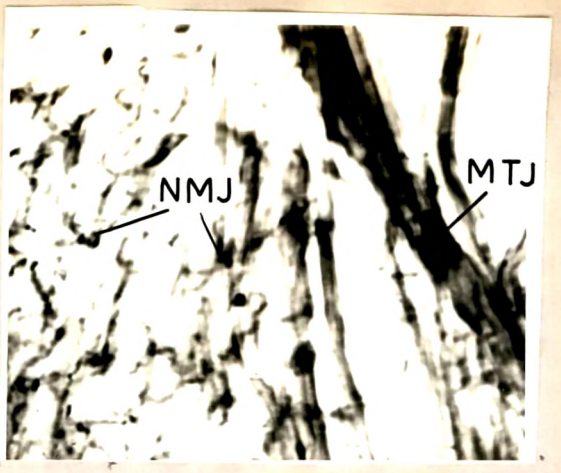

Fig. 5

Red fibres of (L. S.) Barbus stigma showing the localization of AChE. M.T.J.- myotendinous junction; N.M.J.- neuromuscular junction. 144 X.

Fig. 6

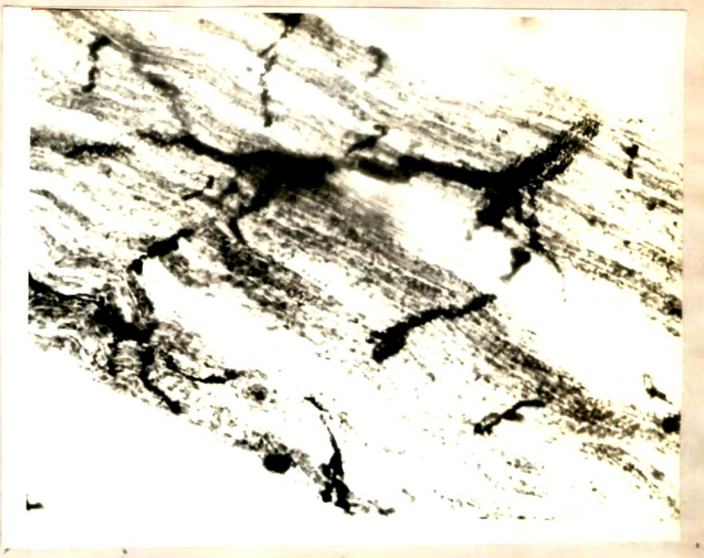

Fig. 7

Fig. 6. Magnified view of red fibres (L. S.) of H. ilisha showing the nature of neuromuscular junctions. Stained for AChE. 567 X.

Fig. 7. L. S. stained for AChE, showing nerve plexuses enclosing a number of red fibres in H. ilisha. 144 X.

H. ilisha and H. toli showed different patterns of innervation. In the superficial part both myotendinous and neuromuscular localizations were observed as in the red muscle (Figs. 8, 9, 12). The plexuses were however, less dense, irregularly distributed and fewer as compared to red muscle fibres (Fig. 9). The fibres in the deeper region on the contrary, revealed a total absence of neuromuscular junctions. The enzyme activity was exclusively localized at the myotendinous junctions on either ends of the fibres (Figs. 14, 15, 16).

In the white muscle of Barbus, the localization of AChE was different from that of the other two fishes. Firstly, no distinction between the superficial and deep zones could be observed; secondly, as in the red muscle region, the motor terminations were distributed almost regularly in the muscle fibres, thus each fibre was multiply innervated (Fig.13). However, the innervation was not so dense as in the red muscle region.

Besides the neuromuscular and myotendinous localizations, diffuse sarcoplasmic AChE activity was observed in both the types of fibres; the intensity of staining was more in the red fibres than in the white ones. Incubation for more time than the optimum period enhanced the intensity of staining in the sarcoplasm, while at the two types of junctions it became diffuse.

Localization of Butyrylcholinesterase (BuChE) :

In H. ilisha the localization of BuChE at the two

Fig. 8

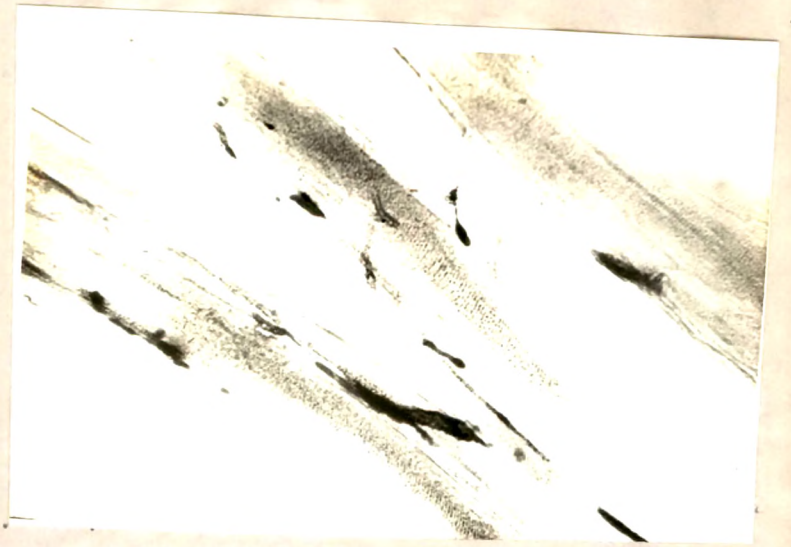

Fig. 9

Figs. 8 & 9. Superficial white fibres of H. ilisha showing acetylcholinesterase activity at myotendinous and neuromuscular junctions. 225 X.

Fig. 10

Fig. 11

Figs. 10 & 11. Superficial white fibres (L. S.) of H. ilisha showing BuChE activity at myotendinous and neuro-muscular junctions. 144 X.

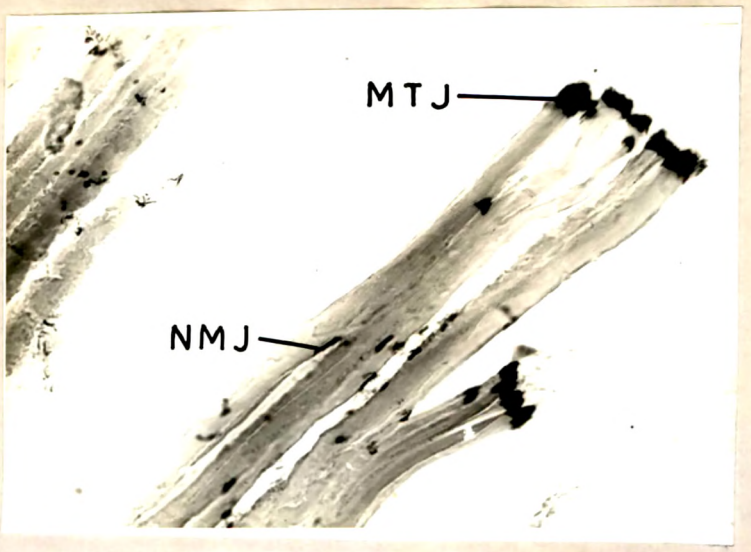

Fig. 12. Superficial white fibres (L. S.) of H. toli showing AChE activity . M.T.J.- myotendinous junctions; N.M.J.- neuromuscular junctions. 144 X.

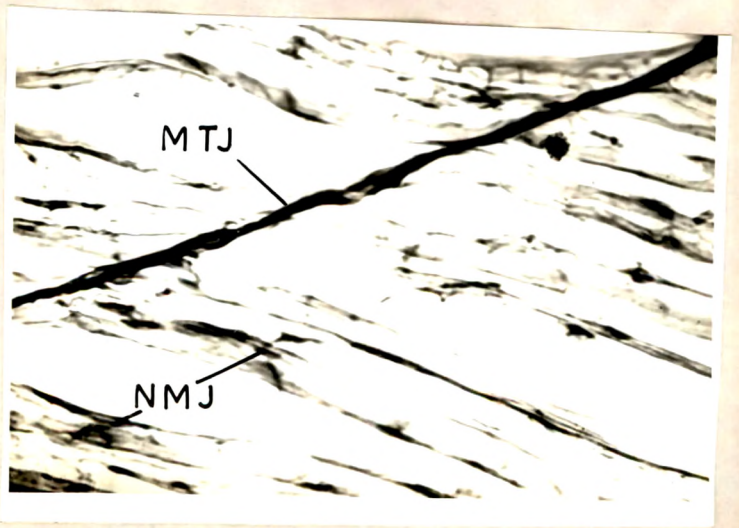

Fig. 13. White muscle fibres (L. S.) of Barbus stigma showing AChE activity. M.T.J.- myotendinous junctions; N.M.J.- neuromuscular junctions. 144 X.

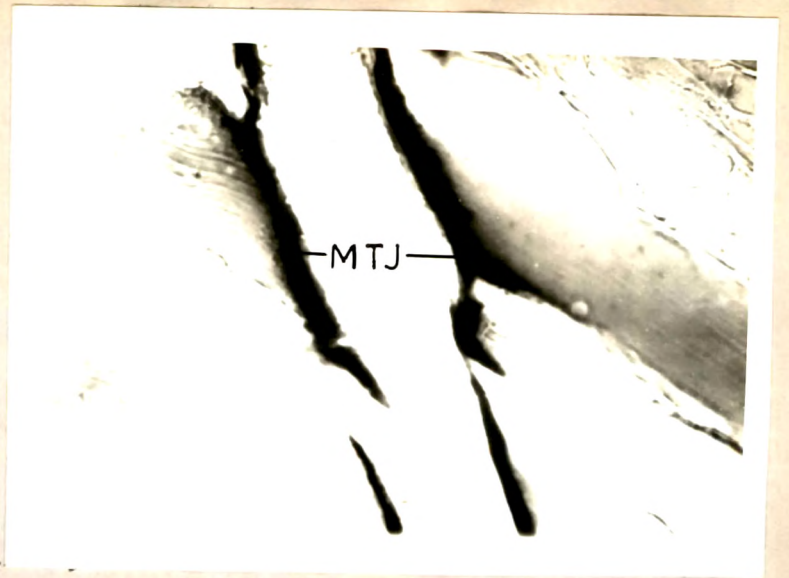

Fig. 14

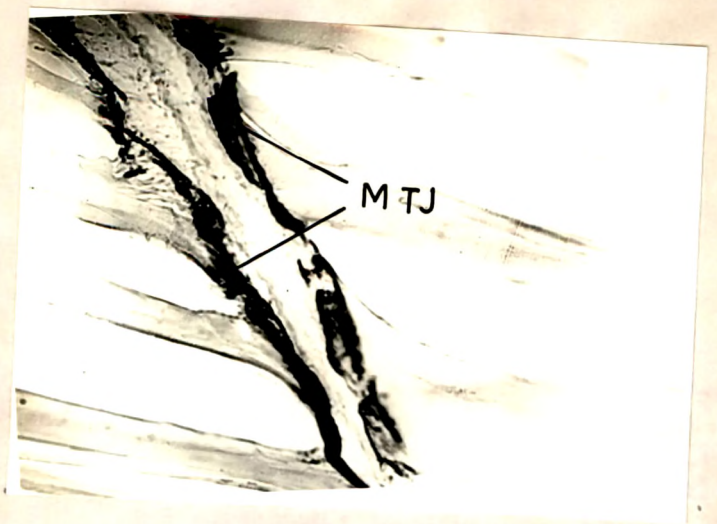

Fig. 15

Figs. 14 & 15. Deep white fibres (L. S.) of H. ilisha showing AChE and BuChE activity at the myotendinous (M.T.J.) junctions. Note the absence of neuromuscular junctions. 144 X.

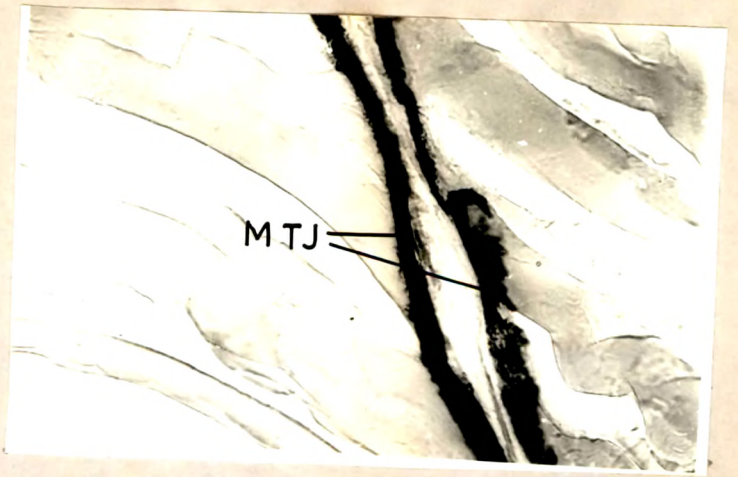

Fig. 16

Fig. 17

Figs. 16 & 17. Deep white fibres (L. S.) of H. toli stained for AChE and BuChE activity. Note the absence of BuChE. 144 X.

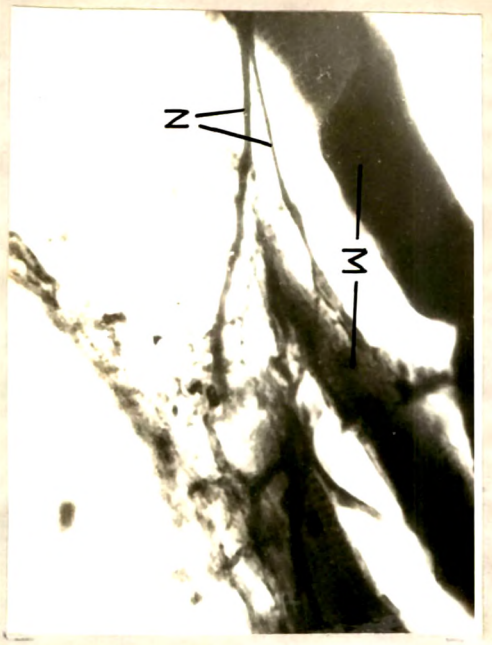

Fig. 18

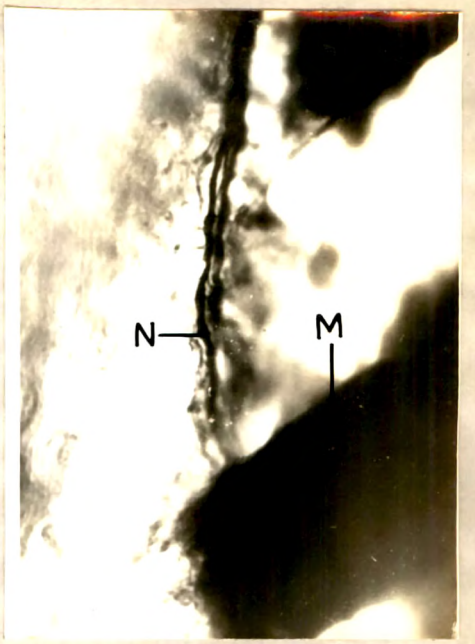

Fig. 19

Figs. 18 & 19. The red and white fibres (L. S.) of H. ilisha showing innervation at myotendinous junctions. Stained by Urea - silver nitrate method. M. = muscle fibre; N.= nerve. 567 X.

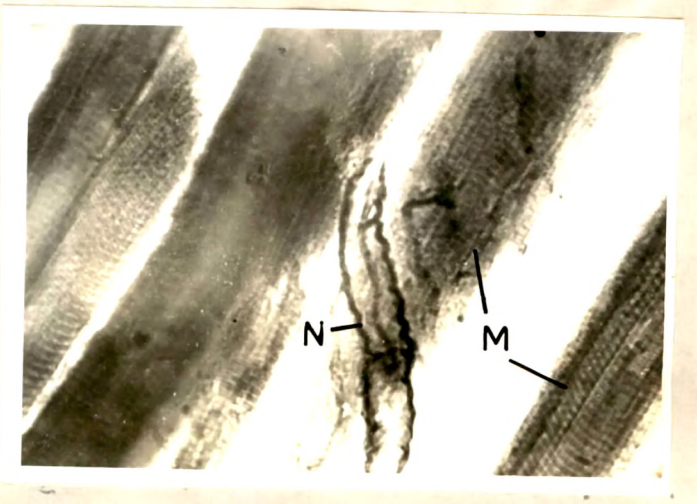

Fig. 20 L. S. of red fibres showing the nerve supply at neuro-muscular junction. Stained by Urea - silver nitrate method. M.= muscle fibre; N.= nerve. 567 X.

types of junctions as well as in the sarcoplasm of both the fibre types in a period of 14 - 18 hours was essentially similar to that of AChE activity (Figs. 2, 10, 11, 15). On the other hand, in H. toli and B. stigma the enzyme was completely absent from both the types of junctions(Figs. 4, 17). But on prolonged incubation (22 - 24 hours) little sarcoplasmic BuChE activity was discernible in the fibres of H. toli, but not of Barbus.

The activity of both the esterases appeared simultaneously at both the junctions (neuromuscular and myotendinous) and in both the muscle types. No significant difference in activity was observed between the myotendinous junctions of the red and white muscles. On comparing the overall intensity of staining at neuromuscular and myotendinous junctions, it could be stated that the former had lesser activity than the latter in all the three fishes investigated.

Muscle sections stained for the demonstration of nerve fibres and nerve endings by the urea - silver nitrate method revealed the presence of nerves at both myotendinous (Figs. 18, 19) and neuromuscular junctions (Fig. 20).

DISCUSSION

The results of the present investigation indicate obvious peculiarities in the localization of cholinesterases at neuromuscular junctions of the red and white muscles. In all the three fishes studied, the red muscle fibres were

multiply innervated with numerous regularly arranged motor terminations, whereas, in the white muscle they were irregularly distributed or absent. This type of localization has been described by Barets (1952) as '*termination distribuees*', while Bone (1964, 1966) considers them to be of the "en grappe" type. According to Bone (1966) these "en grappe" terminations are formed by the axons passing into the red muscle from the myoseptal end of the fibres.

It was generally accepted that the slow tonic extrafusal fibres of vertebrates possessed "en grappe" motor end plates and the fast, tetanic fibres likewise, had "en plaque" nerve endings (Krüger and Günther, 1958; Kuffler and Williams, 1953; Ginsborg and Mackay, 1960; Hess, 1960, 1961, 1962). In the myotomal parietal muscle of hagfish, the slow fibres have been shown to possess distributed innervation by thin, varicose nerve fibres which extend the entire length of the muscle fibre, while fast fibres have an end plate area located at one end of the fibre (Andersen *et al.*, 1963; Alnaes *et al.*, 1964). According to Barets (1961) the motor innervation of red muscle was remarkably constant (multiple nerve endings) in a variety of teleosts studied by him. Thus the red, slow muscle fibres of fishes and of other vertebrates are both alike in their nature of innervation. It was however, realized from the present study, that the innervation at the neuromuscular areas of both red and white muscle fibres revealed striking similarities except that the nerve endings of the latter muscle were

irregular and sparse.

The presence of the so called "en grappe" type of nerve endings in the white muscle too (as in the red muscle), of Barbus and in a few superficial white fibres of the other two fishes, finds support from the work of Chinoy and George (1965) who have shown only one type of nerve ending, "en plaque", located in the red as well as white fibres of the pectoral muscle in a variety of vertebrates. Their findings indicated that in a mixed muscle like the pectoralis, the innervation of its component fibres was identical, but the concentration of the two enzymes varied depending on the functional adaptation of the muscle fibre in question.

Extreme variations in the innervation of the white muscle in teleosts were reported by Barets (1955). According to him, the myoseptal innervation is exceptional in teleosts. Out of all the fishes investigated, in only three species (Anguilla, Conger and Ameiurus) he could observe a disposition of nerve endings as seen in Hilsa, i.e. only a few superficial white fibres having neuromuscular junctions, they being completely absent from the deeper white fibres. On the other hand, in the other fishes studied by Barets (1955),the white muscle possessed nerve endings along the length of the fibre as in Barbus, where myotendinous junctions besides the neuromuscular were also observed.

In both red and white muscles of all the fishes investigated the AChE activity in myotendinous junctions was

present at both myoseptal ends of the fibre. On the contrary, Bone (1966) had shown innervation only at one end of the white muscle fibres of dogfish. Unlike in Barbus, in the deep white muscles of both species of Hilsa, only myotendinous innervations were observed. But Barets (1961) had pointed out that careful examination of muscle fibres revealed localizations of enzymatic activity near the extremity of the fibres similar to the ones at neuromuscular junctions in the catfish. In the present study, however, no such structures were discernible. In goldfish too, cholinesterase activity was not only confined to the myotendinous regions, but also along the shafts of the muscle fibres as confirmed by electron microscopy (Mackay and Peters, 1960).

The localization of AChE at the myotendinous junctions of vertebrate skeletal muscles was investigated by Gerebtzoff (1959) and Schwarzacher (1960) who concluded that the enzyme present there showed no relationship to the nerve supply and that, neither motor nor sensory nerve endings were located in these areas. But studies on the deep lateral muscle of the catfish, Ameiurus nebulosus (Barets, 1952);on Amphibian muscle (Mackay and Peters, 1960) as well as the urea-silver nitrate staining in the present investigation have shown clearly the presence of nerves at both ends of muscle fibres. Giacomini (1898) reported that the myotendinous localizations ("basket endings") in teleosts were sensory in function, but Couteaux (1950) and

Barets (1952) showed their motor nature. Electrophysiological studies by Cuypers and Fessard (1954) adds support to the view that the nerves present at the myotendinous junctions of deep lateral muscle of catfish are motor in function. They have further shown that the muscle fibres with innervation at both their extremities were unable to propagate action potential for more than a very short distance.

Earlier studies (Häggqvist, 1960, 1962) had indicated that AChE was exclusively localized at "en plaque" nerve endings and BuChE at endings of "en grappe" type. But investigations of Klinar and Župančič (1962) and Silver (1963) employing selective inhibitors for these esterases in a variety of mammalian and avian skeletal muscles demonstrated that both enzymes were located at every nerve ending irrespective of the type of fibre. The studies of Chinoy and George (1965) confirmed the observations of the latter authors. Thus there is no evidence to support the earlier view that one morphological type of ending contains one enzyme and the second type of another.

In a comparative study of cholinesterases in the body muscles of fishes, Lundin (1962) concluded that BuChE was present only in marine and brackish water fishes, while the freshwater forms studied by him lacked the ability to hydrolyze butyrylcholine. On the other hand, Bokdawala (1965) demonstrated both the esterases in the lateral muscle of the freshwater fish, Cirrhina mrigala. AChE however, was

present in higher amounts than BuChE. Similarly, in dogfish, which is marine, only AChE could be demonstrated at the motor terminations of red and white muscles (Bone, 1966). The present investigation too, fails to support Lundin's (1962) view since it revealed the occurrence of AChE alone at the myotendinous and neuromuscular junctions of the marine fish, H. toli and in the freshwater one, B. stigma. In the marine migratory species of Hilsa, i.e. H. ilisha on the other hand, both the cholinesterases were found to be located at the two types of junctions and in the sarcoplasm of the fibres. Thus the presence or absence of BuChE differs according to species. It cannot be generalized as a characteristic feature of any particular type of nerve ending, muscle type or animals belonging to any particular habitat.

The presence of granular sarcoplasmic cholinesterases in both the muscle types of all the three fishes studied suggests that the cholinesterase activity of a muscle is due not only to its concentration at the end plates, but also its sarcoplasmic distribution. The presence of this myosin cholinesterase in the muscle fibres and its comparatively higher level in the red fibres than in the white ones, has been shown by several investigators (Kövér et al., 1957; Varga, 1959; Kovács et al., 1961; Kövér and Kovács, 1961). It has been suggested that myosin cholinesterase is concerned with propagation of impulse into

muscle cells via the sarcotubular system (Varga, 1959; Barrnett and Palade, 1959).

The density of innervation of muscle fibres according to Hess (1961) determines whether it is 'fast', 'slow' or intermediate types. The red fibres of fishes are found to correspond to the slow fibres of frog not only in their response to electrical stimulation but also in having multiple nerve endings (Takeuchi, 1959; Barets, 1961). Further, by electrophysiological studies of Barets (1961), it was possible to compare the deep white fibres of catfish (where the innervation is similar to that of H. ilisha and H. toli) with the fast ones of the frog. Thus indirectly, the fast fibres of these fishes could also be compared to such fibres in the frog. In tench (where the white fibres have innervation along the length of the fibre as in Barbus) the electrical activity of the white muscle is by and large, similar to the fast fibres of the frog (Takeuchi, 1959; Barets, 1961).

In the light of above mentioned observations and depending on the frequency of occurrence of nerve endings as well as electrophysiological properties, three types of fibres may be identified in the lateral muscle of fishes; 1. 'fast' fibres with myotendinous junctions only, as in the case of deep white muscle of H. ilisha and H. toli, 2. 'slow' fibres, with myotendinous and regularly arranged neuromuscular junctions as in the red muscle region of all

the fishes investigated, and 3. white fibres which could not be considered as typically 'fast', eg. white muscle of Barbus and also the superficial white muscle fibres of both species of Hilsa, where both myotendinous and neuromuscular junctions are present. In the third group, much variation exists due to differences in density of distribution of neuromuscular junctions.