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Identification and Characteristics of Acetylcholinesterase from Fish (*Micropterus salmoides*) Pectoral Muscles (38248)

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Most organophosphate agents produce 2 consistent and dose dependent results on intact neuromuscular preparations: first, a potentiation of the twitch response, and second, an abolition of the tetanic response. Complete depression of the twitch may not always occur (1), however, modification of twitch and tetanus and abolition of the tetanic response are consistently cited in the literature.

Investigators are in conflict regarding the mechanism by which organophosphorous anticholinesterase agents produce these effects. Some workers (2–5) maintain that the action of these drugs is primarily a direct prejunctional one that gives rise to secondary postjunctional effects. Others support Masland and Wigton's (6) original concept that cholinesterase inhibition is the primary action and that both pre- and postjunctional actions result from transmitter preservation. Bowman and Webb (7) have concluded that the fundamental actions of the anticholinesterase agents can be explained adequately on the basis of cholinesterase inhibition.

Stimulated by interest in the comparative aspects of muscle physiology and innervation, recent advances have been made in understanding the neuromuscular systems of fish. Bone (8) reviewed the innervation of striated muscle in both primitive and advanced fish, while several investigators have concentrated on the mechanical and biophysical properties of muscle and neuromuscular transmission in the fins of certain fish (9–13).

Despite interest in the physical properties of the fish neuromuscular system, little effort has been directed toward pharmacological investigation of the nature of junctional transmission in fish. Most investigators have assumed the presence of the classical cholinergic mechanism and Hidaka and Toida (11) have supported that assumption with their studies on electrical potentials in the isolated pectoral muscles from *Carassius auratus*.

Tentatively accepting the framework of a cholinergic mechanism, we investigated the effects of the organophosphate, diisopropylfluorophosphate (DFP), on *in vivo* neuromuscular activity of pectoral fins in black bass (*Micropterus salmoides*). We were unable to demonstrate modification of the indirectly stimulated twitch or tetanus with doses from 20 mg/kg to 600 mg/kg (14). These results supported earlier work in which twitch height was unaffected by 1 mg/kg eserine in curarized rainbow trout (15). The absence of a response to these relatively high doses of DFP and eserine appeared to be in conflict with accepted theories of cholinergic transmission.

The objective of the present work was to provide information on the *in vitro* occurrence and relative characteristics of cholinesterase enzymes presumed to be a component of the pectoral fin motor unit. This was considered to be a necessary prerequisite to investigations of the availability and function of cholinesterase *in vivo* and, ultimately, an explanation for the apparent lack of DFP modification of neuromuscular transmission.

Materials and Methods. Wild largemouth

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black bass (*Micropterus salmoides*) weighing between 100 and 300 grams were collected by seine and placed in concrete holding ponds until used. Prior to experimentation, fish were netted from the holding pond and transferred to the laboratory where they were maintained without food for at least 2 days but no longer than 7 days before use. Water temperature in the laboratory ranged from 12 ± 1° in the winter to 16 ± 1° in the summer.

The pectoral muscle, abductor pectoralis superficialis (16) was excised, washed in cold Courtland saline (17), blotted and weighed. The muscle was sliced into small pieces and frozen immediately in liquid nitrogen. A double mortar system was utilized in which a small precooled mortar containing the tissue was placed in a larger mortar containing dry ice. A small amount of dry ice was added to the frozen tissue which was pulverized with a precooled pestle. More dry ice was added and the mixture was triturated until a uniform powder was obtained. The frozen powder was transferred to a 100 ml beaker and placed on ice. The dry ice was allowed to evaporate and the homogenized tissue was brought to the desired volume in cold buffer (0.034 *M* sodium bicarbonate, 0.116 *M* sodium chloride and 0.00131 *M* magnesium chloride, equilibrated with 95% N_2–5% CO_2 to achieve a pH of 7.4). In all instances the homogenate was used immediately after preparation.

Protein was measured by the Biuret method (18). Samples were allowed to incubate overnight. Petroleum ether extraction and centrifugation preceded spectrophotometric measurement. A Beckman D. U. spectrophotometer was used and samples were read at 540 nm against a control. Duplicate samples were run on each homogenate, and human serum was used as a standard.

Esters tested as enzymic substrates included acetylcholine chloride (ACh), acetylmethylcholine chloride (MeCh), butyrylcholine chloride (BuCh) and benzoylcholine chloride (BzCh).

Compounds tested as inhibitors were diisopropylfluorophosphate (DFP), eserine sulfate and 15,-bis-(4-allyldimethylammoniumphenyl) pentan-3-one dibromide (284 C51).

The manometric technique was used to determine esterase activity and the procedure was similar to that outlined by Augustinsson (19). In all experiments the total volume of reaction mixture was 3.0 ml, which included 2.0 ml of tissue homogenate in bicarbonate buffer (initial pH 7.4), 0.6 ml of buffer or inhibitor depending on the experiment and 0.4 ml of substrate or buffer in controls. All Warburg runs were carried out at 25 ± .02°, and the gas phase was 95% N_2–5% CO_2.

Inhibitory data are expressed as pI_{50}, the negative logarithm of the molar inhibitor concentration required for 50% inhibition of activity. Inhibitors were preincubated with homogenates for 30 min prior to addition of substrate. Esterase activity is expressed as moles of substrate hydrolyzed per mg protein/hr.

Results. The rate of enzyme hydrolysis of ACh increased with enzyme concentration in a linear fashion (Fig. 1). All subsequent analyses were conducted with an homogenate having a concentration of 1 mg (wet wt) of muscle per 59 ml of buffer. Aliquots of the homogenates yielded approximately 5.5 mg protein per flask.

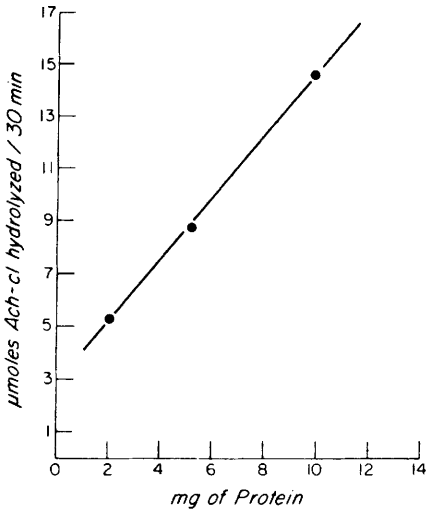

FIG. 1. Hydrolysis of ACh-Cl, 10 m*M*, as a function of pectoral muscle homogenate concentration.

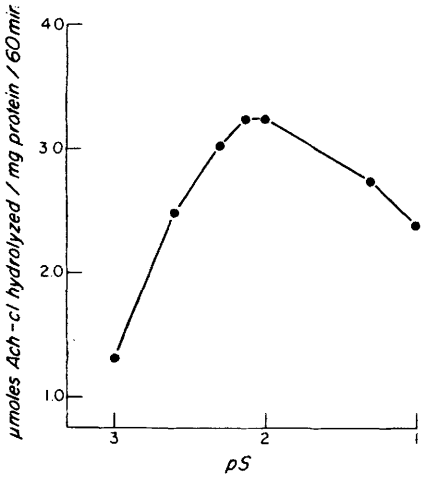

FIG. 2. Activity-substrate concentration curve for hydrolysis of acetylcholine. (pS, negative logarithm of molar substrate concentration. Each point is the mean of at least six experiments.)

Hydrolysis of ACh by the muscle AChE exhibited inhibition by excess substrate. The typical bell-shaped activity-substrate concentration curve gave a maximum rate of hydrolysis at a substrate concentration of 10 m*M* (Fig. 2). Specific activity at the optimum substrate concentration was approximately 3.2 μmoles of ACh hydrolyzed per mg protein per hr. The Michaelis constant and V max, calculated from the double reciprocal plot, were 2.47×10^{-3} *M* and 4.56 μmoles ACh hydrolyzed per mg protein/hr, respectively. Potter (20) gives Michaelis constants for ACh with AChE for various preparations and conditions from 2.8×10^{-4} to 5×10^{-3} *M*.

Specific activity of the homogenates was shown to have an inverse relationship with total wet wt of the fish. Analysis of the data indicated a linear relationship at the 0.01 significance level and an R^2 value of 0.68. Maximum reaction velocities and specific activities varied somewhat around the mean values given above, as expected from the inverse size-activity relationship.

Hydrolysis of the choline esters studied indicated the following relationship: ACh > BuCh ≃ MeCh (Table I). Hydrolysis of BuCh and MeCh were both less than one-fourth that of ACh, and BzCh was too low to be measured accurately.

TABLE I. Relative Rates of Hydrolysis of Choline Esters by Bass Pectoral Muscle Homogenates.

Substrate[a]	Rate of hydrolysis[b]
ACh	3.24
BuCh	0.78
MeCh	0.69
BzCh	negligible

[a] All ester concentrations 10 m*M*.
[b] Moles hydrolyzed per mg protein per 60 min.

The carbamate, eserine, was a potent inhibitor of muscle AChE as indicated by the pI_{50} of 6.75, while DFP and the selective AChE inhibitor 284C51 gave pI_{50}'s of 5.57 and 5.62, respectively (Table II).

Discussion. AChE's (EC 3.1.1.7, acetylcholinesterase, acetylcholine acetylhydrolase) are generally distinguished on the basis of inhibition by excess substrate and low concentration of eserine. These esterases hydrolyze ACh faster than BuCh at optimum substrate concentrations and they hydrolyze MeCh but not BzCh (21). By these criteria the esterase activity in homogenates of bass pectoral muscle appears to be predominately that of an AChE.

Baslow and Nigrelli (22) gave AChE values for muscle homogenates of 15 different species of fish. Their values, assayed at 26° and pH 8.0 by electrometric microtitration, ranged from 4.1 to 28.2 mg ACh hydrolyzed by the enzyme contained in 100 mg tissue/hr. The range of values was correlated with physical activity of the fish. Our homogenates yielded 12.9 mg ACh

TABLE II. Inhibition of Bass Pectoral Muscle Acetylcholinesterase by Selected Compounds.

Inhibitor[a]	pI_{50}[b]
Eserine	6.75
DFP	5.57
284C51	5.62

[a] Inhibitors preincubated with enzyme for 30 min prior to addition of acetylcholine.
[b] Negative logarithm of the molar inhibitor concentration required for 50% inhibition of the enzyme.

hydrolyzed per 100 mg tissue/hr, within Baslow and Nigrelli's range for moderately active to active fish.

The observation of an inverse relationship between specific activity and size of fish is corroborated by Weiss (23, 24). He indicated an inverse relationship between specific activity of brain ChE and brain size in several different species. However, Alsen (25) found the specific activity of brain and skeletal muscle AChE of cod to be essentially independent of body weight.

The optimum substrate concentration for hydrolysis of AChE was 10 m*M*. This is the same optimum substrate concentration found for brain homogenates of bluegill and channel catfish (26) and for brain homogenates of cutthroat trout (27) using the manometric technique. Lundin (28) also observed a substrate optimum close to 10 m*M* for goldfish muscle AChE using an electrometric method.

The effect of the inhibitors DFP and eserine were similar to results obtained on other fish (Table III), although brain homogenates were usually the source for enzymic activity in other studies. Lundin's (28) pI_{50} values for DFP and 284C51 are considerably lower than the other values reported. However, Lundin's use of a freeze-dried, dialyzed supernate rather than a crude homogenate may partially explain the observed disparity.

These studies have indicated the presence of an AChE in bass pectoral fin muscle homogenates. The presence of substantial quantities of this enzyme gives tentative support to the assumption that the neuromuscular junction is, indeed, cholinergic. Kinetics as well as substrate and inhibitor specificities suggest that the enzyme has much the same character as that isolated from other fish brain and muscle homogenates. Furthermore, the bass pectoral fin AChE appears to be fully inhibitable by DFP *in vitro*. However, if the pectoral fin neuromuscular junction is cholinergic and if AChE plays a predominant role in transmitter termination as has been suggested by Katz and Miledi (29), then modification of normal neuromuscular function would be expected to result from anti-AChE action *in vivo*. Furthermore, such modification might be expected to increase as a function of increased AChE inhibition as implied by the dose-related effects typical of organophosphate anticholinesterases. That modification of twitch and tetanus did not occur with substantial doses of DFP *in vivo* (14) suggests abnormalities in DFP distribution or AChE availability or function in bass pectoral fin preparations.

TABLE III. Inhibition of Acetylcholinesterases in Fish by Selected Compounds.

Inhibitors	pI_{50}[a]	Enzyme source	Reference
Eserine	6.8	Cutthroat trout brain	Hogan, 1971
Eserine	6.6	Bluegill brain	Hogan and Knowles, 1968
Eserine	6.7	Channel catfish brain	Hogan and Knowles, 1968
Eserine	6.0–6.5	Goldfish body muscle	Lundin, 1959
Eserine	6.75	Largemouth bass pectoral muscle	
DFP	4.1	Cutthroat trout brain	Hogan, 1971
DFP	4.4	Bluegill brain	Hogan and Knowles, 1968
DFP	6.1	Channel catfish brain	Hogan and Knowles, 1968
DFP	7.0	Goldfish body muscle	Lundin, 1959
DFP	5.57	Largemouth bass pectoral muscle	
284C51	6.7	Goldfish body muscle	Lundin, 1959
284C51	5.62	Largemouth bass pectoral muscle	

[a] Negative logarithm of the molar inhibitor concentration required for 50% inhibition of the enzyme.

Summary. Acetylcholinesterase (EC 3.1.1.7) activity was investigated in bass pectoral fin muscle homogenates using a manometric technique. The enzyme was inhibited by excess substrate and had an optimum substrate concentration of 10 m*M*. Specific activity was 3.2 μmoles ACh hydrolyzed per mg protein/hr at 25°. The Michaelis constant and V max were 2.47×10^{-3} *M* and 4.56 μmoles ACh hydrolyzed per mg protein/hr, respectively. Specific AChE activity of the homogenates was inversely related to total wet wt of the fish with an R^2 of 0.68. Relative rates of hydrolysis for the choline esters studied were ACh > BuCh $\simeq$ MeCh. Hydrolysis of BzCh was negligible. The inhibitors eserine, DFP and 284C51 gave pI_{50}'s of 6.75, 5.57 and 5.62, respectively.

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