AN ABSTRACT OF THE THESIS OF

Daniel Brian Gant for the degree of Master of Science

in Fisheries and Wildlife presented on April 2, 1982

Title: NEUROMUSCULAR PHARMACOLOGY AND [^{125}I]-α-BUNGAROTOXIN BINDING CHARACTERISTICS OF THE PECTORAL MUSCLE OF THE BUFFALO SCULPIN, ENOPHRYS BISON

Abstract approved: Redacted for Privacy

Lavern J. Weber

The neuromuscular pharmacology of a marine teleost has had limited investigation. In order to characterize this system, in situ nerve-muscle experiments and receptor binding studies were performed on the buffalo sculpin, Enophrys bison.

The neuromuscular junction of the buffalo sculpin was characterized in situ by examining the effects of various neuromuscular blocking agents and acetylcholinesterase inhibitors (ACHE-I) on pectoral muscle response to indirect stimulation. The injection of both d-tubocurarine (350 μg/kg) and α-bungarotoxin (α-Butx) (1 mg/kg) resulted in a flaccid paralysis. The depolarizing agents, succinylcholine (30 μg/kg) and decamethonium (35 μg/kg) produced a spontaneous contracture.

The administration of the ACHE-I, diisopropylflurophosphate (DFP) and eserine in the sculpin resulted in responses which are contrary to those of classical mammalian cholinergic neuromuscular systems. In mammals, ACHE-I elicit two dose-dependent responses: (1) potentiation of twitch; (2) depression in twitch and a rapid increase in tetanic tension followed by immediate or partial relaxation. In the sculpin, twitch potentiation does not occur and the ability to maintain a tetanic response was not abolished after the animals had accumulations

of DFP (100 mg/kg) and eserine (10 mg/kg) which were lethal to the fish.

Characterization of the acetylcholine receptors at the sculpin neuromuscular junction was performed by incubating pectoral abductor muscle homogenates with [^{125}I]-α-Butx. α-Butx binds specifically to the cholinergic nicotinic receptor. Scatchard analysis of saturation data is linear, suggesting a single class of binding sites, and yields a K_D and Bmax of 69.5 ± 11 nM and .497 ± .28 pmol bound/mg protein, respectively. The kinetics of the interaction between the binding site and the [^{125}I]-α-Butx is also indicative of a single class of binding sites and an association constant (K_{+1}) of 1.08 ± .28 X 10^7 min^{-1} $mole^{-1}$ was generated. The [^{125}I]-α-Butx/receptor complex dissociates in a biphasic manner with a dissociation constant (K_{-1}) of 2.06 min^{-1} for the fast dissociating component and .34 min^{-1} for the slow. Inhibition constants were determined for various drugs, and an order of potency was determined: d-tubocurarine > acetylcholine > atropine > decamethonium > epinephrine.

The [^{125}I]-α-Butx binding illustrates the nicotinic nature of the binding site. However, when these results are compared with binding studies of mammalian skeletal muscle, there are difinite qualitative as well as quantitative differences between the two systems.

The <u>in situ</u> and receptor binding results indicate that the neuromuscular system of this marine teleost is not typical of mammalian cholinergic systems.

Neuromuscular Pharmacology and [^{125}I]-α-Bungarotoxin Binding Characteristics of the Pectoral Muscle of the Buffalo Sculpin (*Enophrys bison*)

by

Daniel Brian Gant

A THESIS

Submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Completed April 2, 1982

Commencement June 1982

APPROVED

Redacted for Privacy

Professor of Fisheries and Wildlife in charge of major

Redacted for Privacy

Head of Department of Fisheries and Wildlife

Redacted for Privacy

Dean of Graduate School

Date thesis is presented April 2, 1982

Typed by Nancy Brown for Daniel Brian Gant

ACKNOWLEDGEMENTS

I wish to thank Dr. Lavern J. Weber and Dr. John R. Smith for their friendship and guidance throughout the investigation and preparation of this thesis. I would also like to thank the other members of my graduate committee, Dr. James L. Hedtke and Dr. George W. Boehlert.

I am grateful for the support and friendship of many people at the Marine Science Center, especially Bob and Anita Stuart and Dave Strehlow. I am also grateful to Marilyn Guin for her assistance in acquiring the references for this thesis.

Finally, I wish to thank my parents, Paul and Genevieve, for their support and encouragement throughout my education.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

NEUROMUSCULAR PHARMACOLOGY AND [^{125}I]-α-BUNGAROTOXIN BINDING CHARACTERISTICS OF THE PECTORAL MUSCLE OF THE BUFFALO SCULPIN, *ENOPHRYS BISON*

Chapter I:

Effects of Neuromuscular Blocking and Acetylcholinesterase Inhibitors on Pectoral Fin Response to Indirect Stimulation

INTRODUCTION

The classical concept of the transmission of impulses across the neuromuscular junction involves the release of acetylcholine, which diffuses across the junctional cleft and binds to the receptor moieties on the endplate of the muscle. This interaction of transmitter and receptor causes an increase in the permeability of the endplate membrane to sodium and potassium ions resulting in a local potential charge across the muscle endplate. The action of acetylcholine is terminated by the hydrolytic activity of acetylcholinesterase (Katz, 1966).

Early investigators of the neuromuscular system of fish have concentrated on the physical properties of fish muscle. Takeuchi (1959) studied the electrical properties of pectoral fin muscles of the snakefish (*Ophiocephalus argus*) and found that the muscle consists of two types of fibers: red and white. Anderson et al. (1963) characterized two types of muscle fibers on the Atlantic hagfish (*Myxine glutinosa*), which could be distinguished by the speed of response to nerve stimulation. Bone (1964) also established that fish striated muscle are composed of red and white fibers, which are physiologically similar to the red and white fibers of frog skeletal muscle. Hidaka and Toida (1969) and Yamamoto (1972) showed that it was difficult to trigger an action potential in the red muscle of the goldfish (*Carassius auratus*), whereas an action potential was easily generated in white muscle.

Patterson and Goldspink (1972), as reported in Greer-Walker and Paul (1975), describe the red fibers of marine fish as smaller and more uniform in size than the white fibers, and they reported that the red fibers contained greater quantities of mitochondria, fats, and glycogen. The red fibers utilize aerobic respiration and the white fibers anaerobic.

Investigations into the pharmacological aspects of neuromuscular transmission in fish have been limited. The effects of phenol on transmission in the red muscle of Carassius auratus were examined by Kuba (1969). Phenol was found to increase the quantity of transmitter released. Hidaka and Kuriyama (1969) investigated the effects of catecholamines on cholinergic transmission in the pectoral fin of Carassius auratus. Their results indicated that norepinephrine acted on the prejunctional membrane and increased the amount of transmitter released, while epinephrine acted on the postjunctional membrane and increased the sensitivity of the membrane to the transmitter. The effects of tetanus toxin were investigated in Carassius auratus pectoral muscles, where it was found to block the nerve action potential and the spontaneous release of acetylcholine from presynaptic terminals (Diamond and Mellanby, 1971; Mellanby and Thompson, 1972).

An investigation of the effects of organophosphates on neuromuscular transmission in fish was conducted by Schneider and Weber (1974, 1975). Organophosphate compounds, which may enter the aquatic environment following their application as pesticides, are acetylcholinesterase inhibitors (ACHE-I). The ACHE-I act by covalently binding to acetylcholinesterase and result in an accumulation

of acetylcholine at cholinergic receptor sites. This accumulation produces effects equivalent to continuous stimulation in cholinergic fibers throughout the central and peripheral nervous systems (Taylor, 1980a). Schneider and Weber (1974, 1975) examined the significance of acetylcholinesterase to neuromuscular transmission in the pectoral abductor muscle of the black bass (Micropterus salmoides). They evaluated the effects of diisopropylflurophosphate (DFP) on acetylcholinesterase activity in the muscle, and correlated the attendant acetylcholinesterase inhibition with muscle response to nerve stimulation. Doses of DFP producing inhibition in excess of 97% were always accompanied by a well-maintained tetanic response, which is not typical of mammalian cholinergic systems. These results suggest that acetylcholinesterase is not as important for neuromuscular transmission in the black bass as in other vertebrates.

The general objective of this study was to characterize the neuromuscular pharmacology of a marine teleost, and to compare the system with that of the freshwater teleost as described by Schneider and Weber (1975). A more specific objective was to determine, in situ, if the neuromuscular system follows the classical cholinergic model, especially in the role of acetylcholinesterase.

MATERIALS AND METHODS

Buffalo sculpin (Enophrys bison), a marine teleost, were selected as the experimental animal, due to their abundance and easy maintenance. The sculpin were collected from Yaquina Bay, Oregon, by otter trawl and held in outside tanks with aerated running seawater at 6-14°C. They were fed chopped herring once weekly.

Sculpin weighing between 400 and 1000 grams were anesthetized with benzocaine (70mg/l). Each fish was then transected by cutting the spinal cord posterior to the bony plate of the head and anterior to the dorsal fin.

The transected fish was positioned on its right side with the left pectoral fin exposed. Seawater containing the anesthetic, maintained at the same temperature as in the acclimation tanks, constantly irrigated the gills. The branchial artery of the third gill arch was cannulated with PE 50 tubing, containing a heparinized (500 units) marine teleost saline solution. An incision was made posterior to the pectoral adductor muscle, leaving a small cavity between the anterior extremity of the trunk musculature and the adductor. Sensory nerves were then dissected away from supporting tissue and cut. The pectoral abductor and adductor motor nerve bundle was also dissected away from supporting tissue and vasculature. The area was bathed in mineral oil and the motor nerve bundle was decentralized by ligation. The motor nerve to the adductor muscle was separated from the bundle and severed. The pectoral fin was then cut away. A suture was attached to a muscle bundle of the dorsal section of the abductor and passed through a pulley to a linear motion transducer, interfaced with a Clevite Brush Mark 260 recorder. The abductor was held under a constant tension of

30 grams. An electrode was attached to the abductor nerve bundle distal to the ligation. The sculpin was placed on anesthetic-free seawater and allowed to recover for approximately 30 minutes.

In two experiments, blood pressure and heart rate were examined from the branchial artery using a Statham P23De pressure transducer.

The effects of various drugs on indirectly stimulated twitch and tetanus was studied. Twitch was produced by stimulating with a Grass SD9 stimulator the abductor nerve bundle at a frequency of .2 pulses per second and tetanus at a frequency of 15 pulses per second. The maximal voltage was determined for each preparation with a mean of 3.5 volts from all experiments.

The effects of the various drugs on opercular movements were noted on the recordings as changes in respiration. However, seawater was constantly irrigating the gills, and the elimination of opercular movements did not necessarily indicate the elimination of respiration.

The following drugs were injected intravenously (i.v.) via the branchial cannula: d-tubocurarine chloride and α-bungarotoxin (α-Butx), nicotinic antagonists; succinylcholine chloride and decamethonium bromide, depolarizing agents; eserine sulfate and DFP (also injected intraperitoneally) (i.p.), acetylcholinesterase inhibitors; atropine sulfate and atropine methyl bromide, muscarinic antagonists. All drugs were purchased from the Sigma Chemical Co. except for atropine sulfate which was from the J. L. Baker Chemical Co.

Drugs were administered in a marine teleost saline (KCl: 0.38g/l; NaCl: 7.25g/l; $CaCl \cdot 2H_2O$: 0.23g/l; $NaHCO_3$: 1.0 g/l; $MgSO_4 \cdot 7H_2O$: 0.23g/l; Trizma base: 0.80 g/l; Trizma HCl: 6.85 g/l; Glucose: 1.0 g/l) having an osmolality of 350 mOsm. The saline solution was also used to flush the cannula after each injection.

RESULTS

d-Tubocurarine

Preliminary experiments showed that 100 µg/kg d-tubocurarine resulted in an initial decline in twitch. A cumulative dose of 350 µg/kg was needed for complete neuromuscular blockade (Figure 1). Several experiments were then performed at each of the following doses of d- tubocurarine: 200 µg/kg; 250 µg/kg; 300 µg/kg; and 350 µg/kg. Of the four sculpins injected with 200 µg/kg, twitch initially declined approximately 25% in two fish, after 4 minutes. Of these two, twitch returned to its control level in one fish 15 minutes after injection and gradually declined in the other. Twtich rapidly disappeared in the third fish 2 minutes after injection, while twitch gradually declined in the fourth fish over a period of 30 minutes. In the three sculpin injected with 250 µg/kg d-tubocurarine, twitch declined 28%, 33%, and 57% after 5 minutes with a recovery of twitch in one fish and a gradual decline in the other two. A dose of 300 µg/kg d-tubocurarine resulted in reductions in twitch of 21%, 32%, and 56% after 10 minutes, and did not recover. Twitch was abolished after two minutes in one fish injected with 350 µg/kg d-tubocurarine, while it decreased more gradually in three other fish with twitch reductions of 47%, 48%, and 60% 10 minutes after injection.

α-Bungarotoxin

The dosage of α-Butx needed for a resultant decline in twitch was from 250-600 µg/kg for the three fish studied. In one fish, an initial decline in twitch was observed after 250 µg/kg α-Butx, and tetanus was not affected until after a cumulative dose of 500 µg/kg (Figure 2).

Figure 1. Effects of d-tubocurarine to a total dose of 350 µg/kg on indirectly stimulated twitch responses of the pectoral abductor muscle of the buffalo sculpin. Control = C. Chart speed is 25 mm/min.

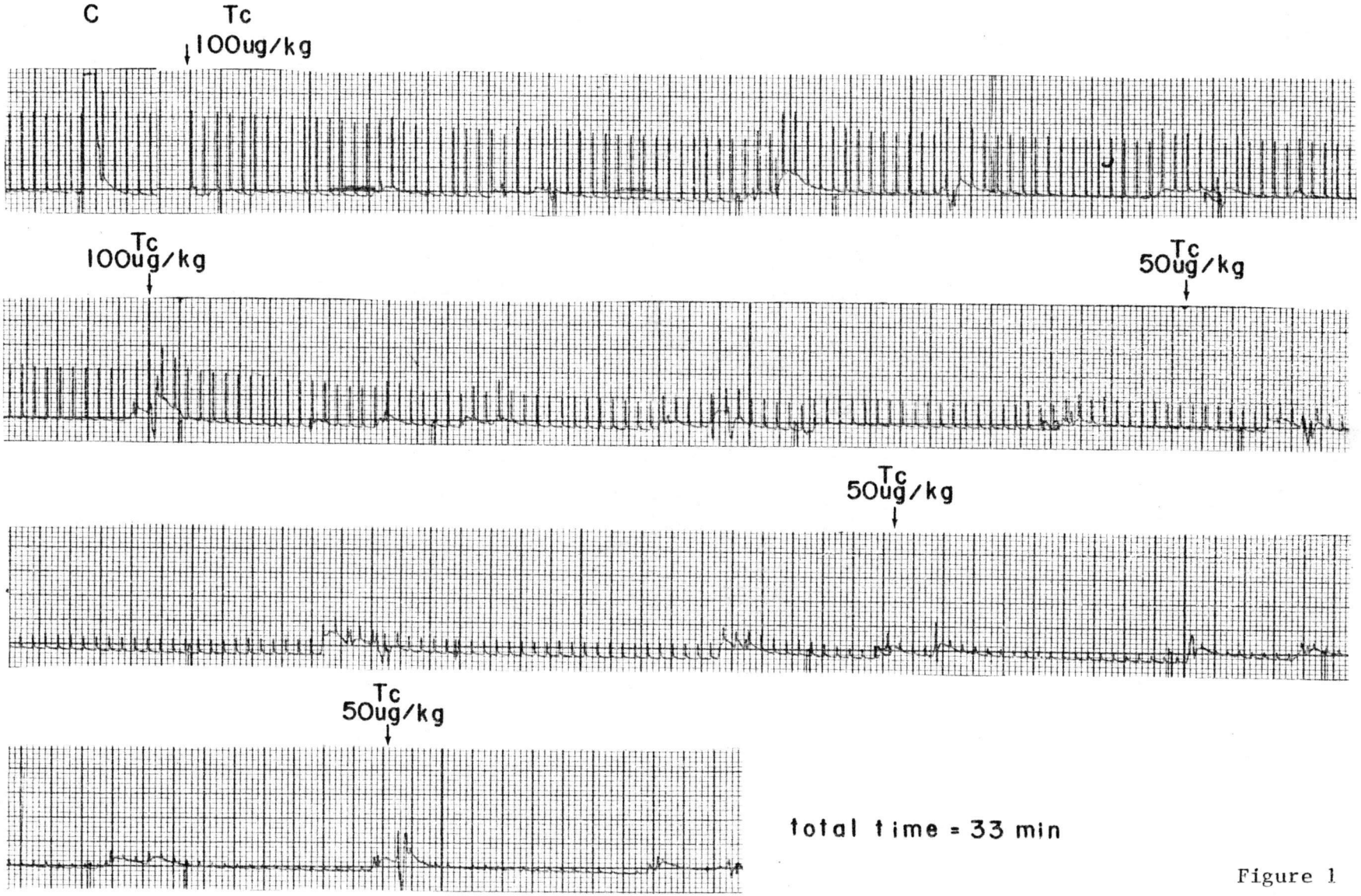

C
Tc
100ug/kg
Tc
100ug/kg
Tc
50ug/kg
Tc
50ug/kg
Tc
50ug/kg
total time = 33 min

Figure 1

Figure 2. Effects of α-Butx (250 μg/kg injections) to a total dose of 1 mg/kg on indirectly stimulated twitch and tetanic responses of the pectoral abductor muscle of the buffalo sculpin. α-Butx is preceded by succinylcholine (25 μg/kg) administration. Control = C. Tetanus = T. Chart speed is 25 mm/min. Time period above recording refers to the elapsed time between muscle stimulation.

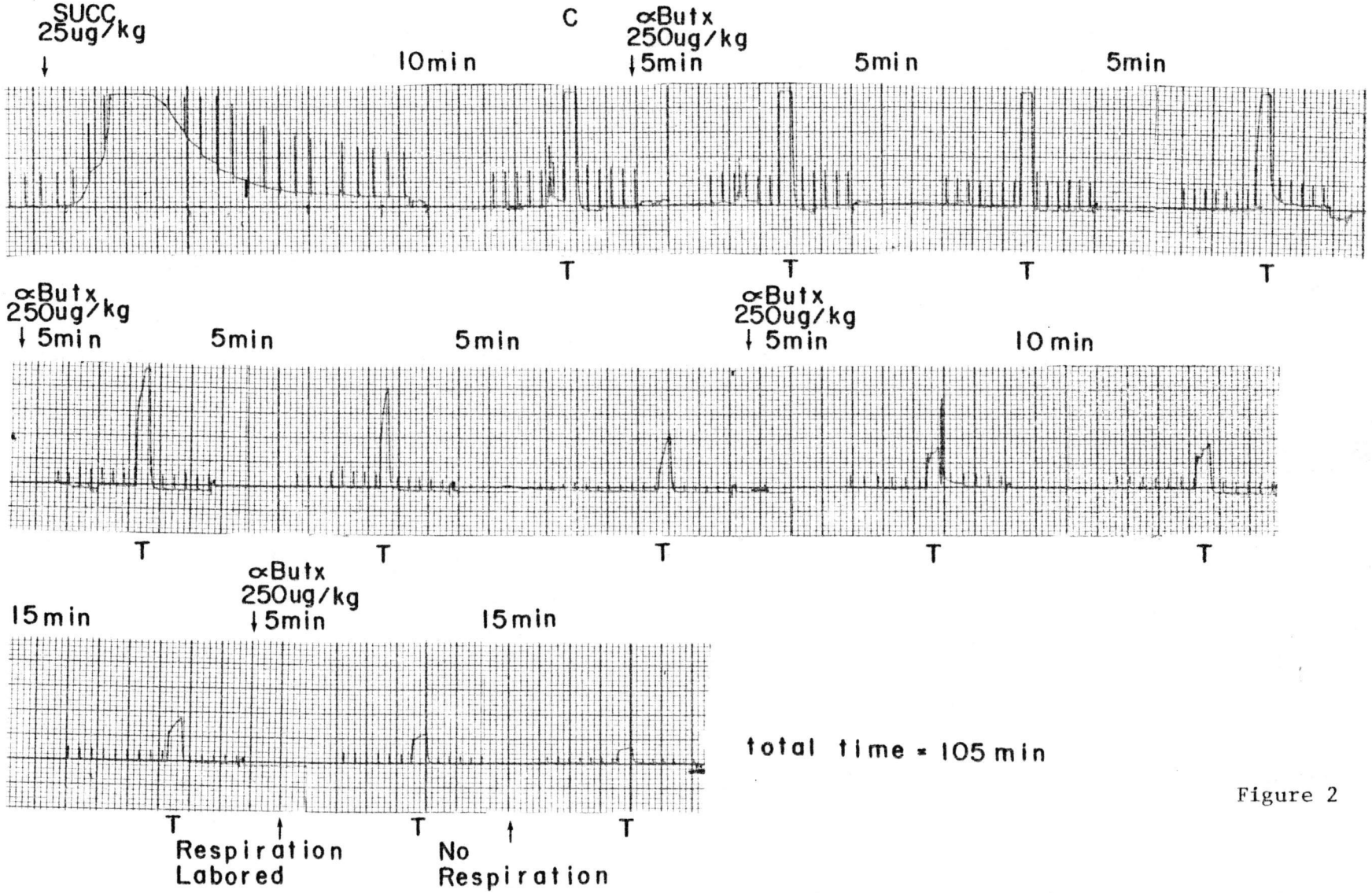

SUCC
25ug/kg
C
∝Butx
250ug/kg
10min
5min
5min
5min
T
T
T
T
∝Butx
250ug/kg
5min
5min
5min
∝Butx
250ug/kg
5min
10 min
T
T
T
T
T
∝Butx
250ug/kg
15min
5min
15min
T
Respiration
Labored
T
No
Respiration
T
total time = 105 min

Figure 2

In this fish, opercular movements became labored after 500 µg/kg α-Butx and ceased after 1 mg/kg.

Succinylcholine

The dosage of succinylcholine required to cause an increase in muscle tension was inconsistent for seven fish, as was the response to successive injections of the same dose. Initial increases in muscle tension required doses of 5 to 60 µg/kg and complete depolarization was achieved at doses ranging from 20 to 130 µg/kg. An example where 40 µg/kg was required for complete depolarization can be found in Figure 3. In several experiments where successive injections of succinylcholine elicited different degrees of depolarization, it was thought that tachyphylaxis may have been occurring. However, in an experiment where successive injections of the same dose were administered after the tension had returned to normal, no signs of tachyphylaxis were evident. In several experiments, twitch decreased as tension increased, while no change was evident in the tetanic response.

Decamethonium

Injections of 20 to 50 µg/kg decamethonium resulted in an initial increase in muscle tension, which was relatively consistent for three fish. Complete depolarization was achieved in one fish at a dose of 75 µg/kg (Figure 4). Twitch declined as tension increased and tetanus was not affected when the muscle was not completely depolarized.

Diisopropylfluorophosphate

An injection of 100 mg/kg DFP resulted in a decrease in twitch

Figure 3. Effects of succinylcholine on indirectly stimulated twitch responses of the pectoral abductor muscle of the buffalo sculpin. Control = C. Chart speed is 5 mm/min. Time period above recording refers to the elapsed time between muscle stimulation.

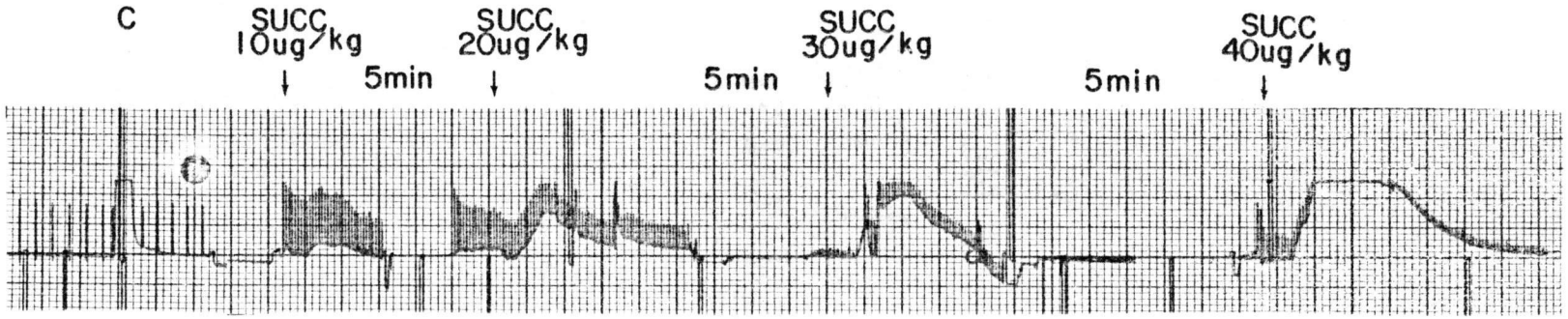

C
SUCC
10ug/kg
5min
SUCC
20ug/kg
5min
SUCC
30ug/kg
5min
SUCC
40ug/kg
total time = 41 min

Figure 3

Figure 4. Effects of decamethonium on indirectly stimulated twitch and tetanic responses of the pectoral abductor muscle of the buffalo sculpin. Control = C. Tetanus = T. Chart speed is 25 mm/min. Time period above recording refers to the elapsed time between muscle stimulation.

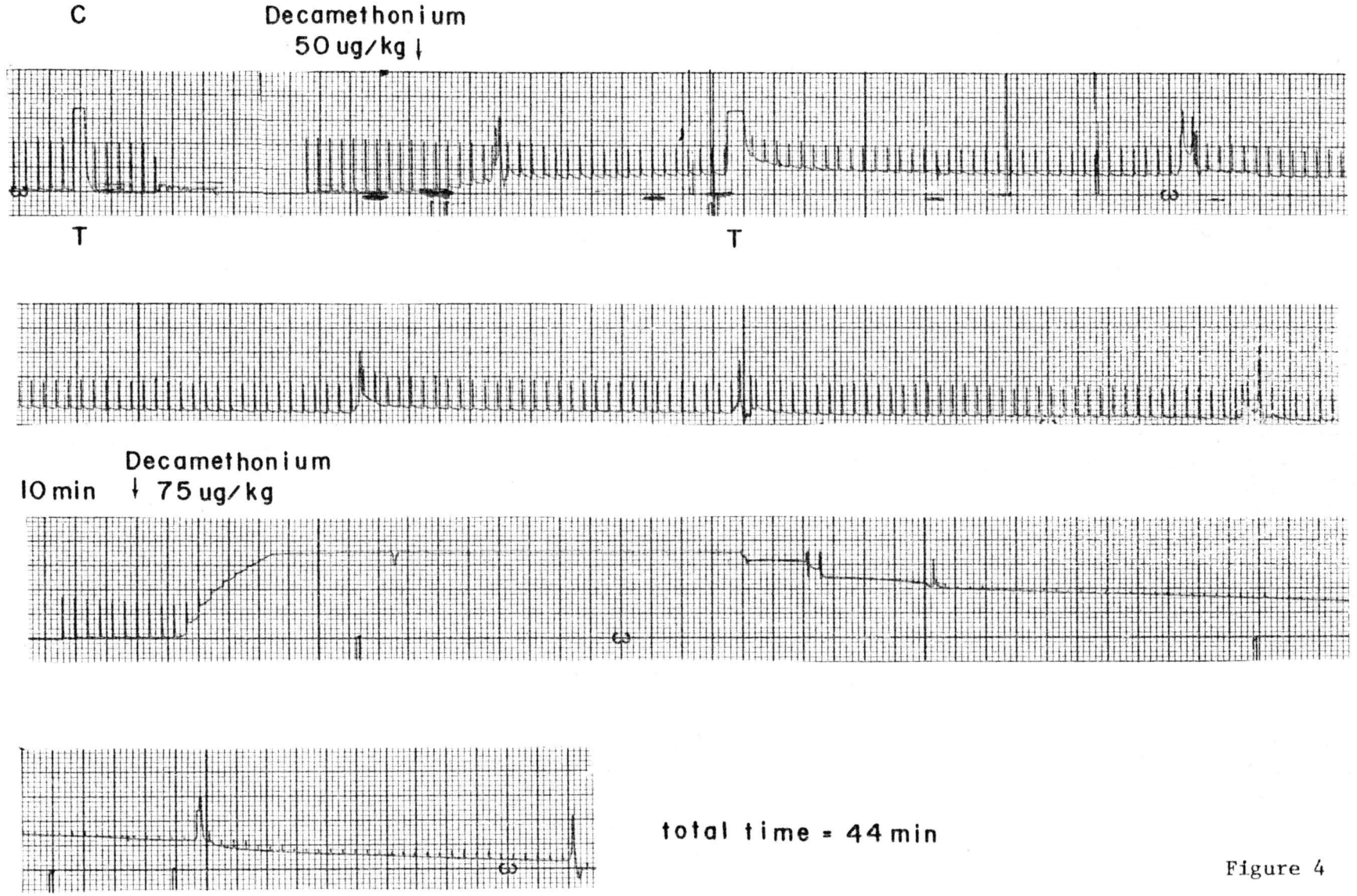

C
Decamethonium
50 ug/kg
T
T
Decamethonium
10 min
75 ug/kg
total time = 44 min

Figure 4

with the onset of tetanus slowing, but the height of tetanic contractions remained unaltered. Tetanus did decline after a cumulative dosage of 200 mg/kg (Figure 5). Opercular movements stopped approximately 10 minutes after the initial injection of 100 mg/kg DFP. Inspection of the gills after an injection of 100 mg/kg DFP revealed a gray coloration. Subsequently, blood samples were taken, and when .1 ml of blood was mixed with .1 ml of propylene glycol containing 20 mg of DFP, the blood turned gray and clotted. Blood samples mixed with propylene glycol without DFP remained red and clot free. Plasma samples, .1 ml, were then mixed with .1 ml of propylene glycol containing 20 mg of DFP which resulted in the plasma turning gray with the formation of a white precipitate. The plasma remained clear when mixed with only propylene glycol. Identical results were found when blood and plasma samples were combined with propylene glycol containing 10 mg DFP.

DFP was then injected intraperitoneally to preclude the problems associated with hemolysis. In two fish injected with 200 mg/kg DFP, both twitch and tetanus declined. In one fish, tetanus was almost completely abolished 48 minutes after DFP injection (Figure 6). In an experiment in which 5 mg/kg atropine sulfate was injected (i.v.) prior to the i.p. injection of 200 mg/kg DFP, both twitch and tetanus declined, but they were not completely abolished until a second injection of 200 mg/kg DFP was administered (Figure 7). In one fish, 10 mg/kg atropine sulfate (i.v.) resulted in a decrease in both twitch and tetanus. When this fish was injected with 200 mg/kg DFP, tetanus declined but returned to the control level in approximately 55 minutes.

Figure 5. Effects of two doses of DFP (100 mg/kg injections) on indirectly stimulated twitch and tetanic responses of the pectoral abductor muscle of the buffalo sculpin. Control = C. Tetanus = T. Chart speed is 25 mm/min. Time period above recording refers to the elapsed time between muscle stimulation.

Figure 5

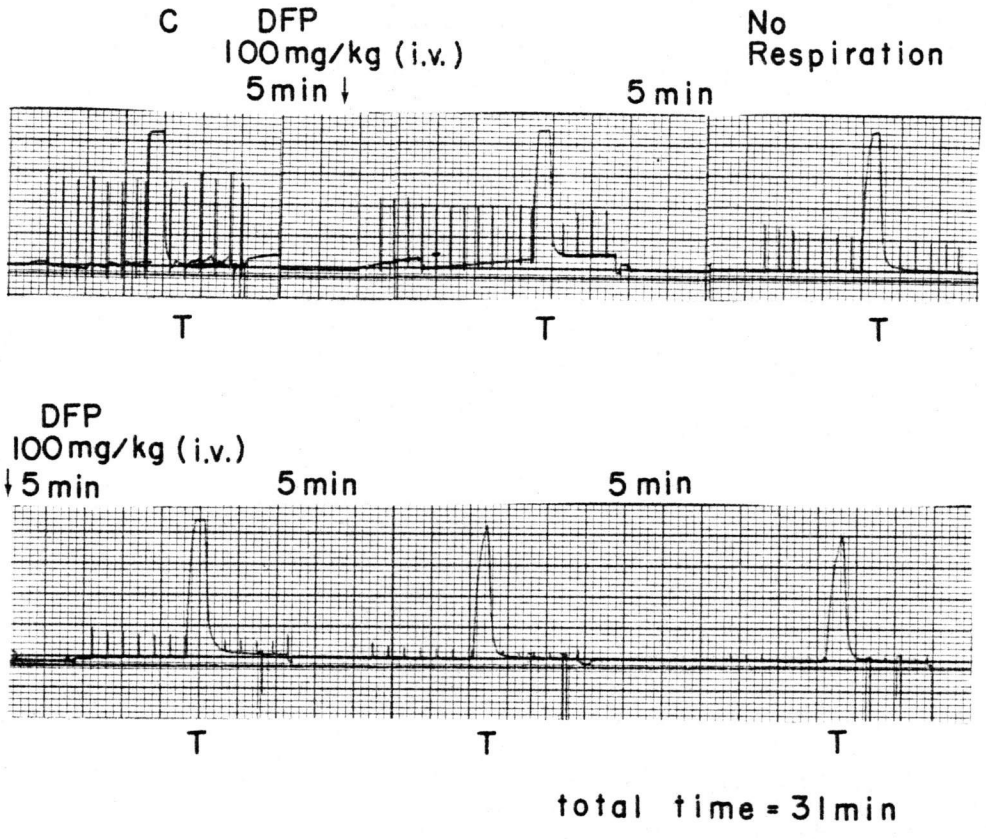

C
DFP
100 mg/kg (i.v.)
5 min
5 min
No
Respiration
T
T
T
DFP
100 mg/kg (i.v.)
5 min
5 min
5 min
T
T
T
total time = 31 min

Figure 6. Effects of 200 mg/kg DFP injected i.p. on indirectly stimulated twitch and tetanic responses of the pectoral abductor muscle of the buffalo sculpin. Control = C. Tetanus = T. Chart speed is 25 mm/min. Time period above recording refers to the elapsed time between muscle stimulation.

Figure 6

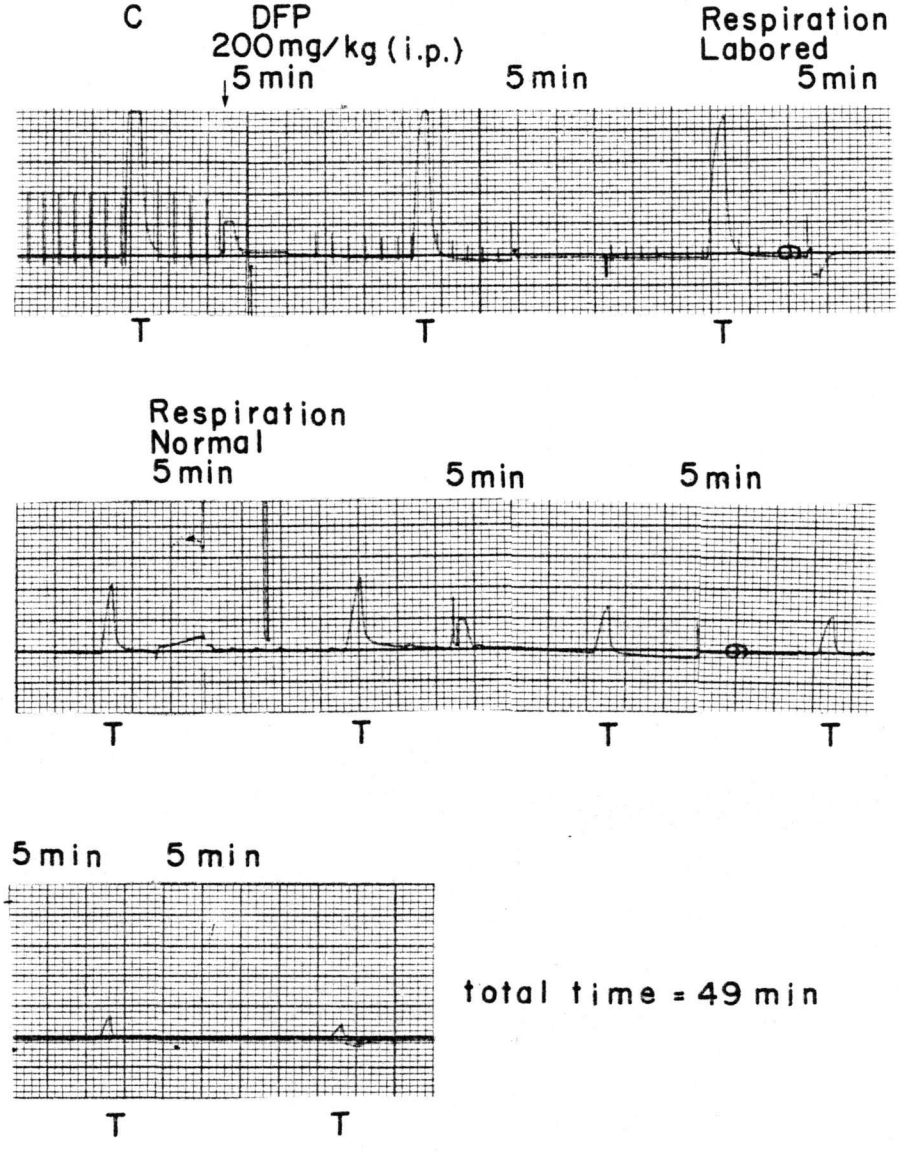

C
DFP
200mg/kg (i.p.)
5 min
5 min
Respiration
Labored
5 min
T
T
T
Respiration
Normal
5 min
5 min
5 min
T
T
T
T
5 min
5 min
T
T
total time = 49 min

Figure 7. Effects of two doses of DFP (200 mg/kg injections i.p.) on indirectly stimulated twitch and tetanic responses of the pectoral abductor muscle of the buffalo sculpin after pretreatment with atropine sulfate (5 mg/kg). Control = C. Tetanus = T. Chart speed is 25 mm/min. Time period above recording refers to the elapsed time between muscle stimulation.

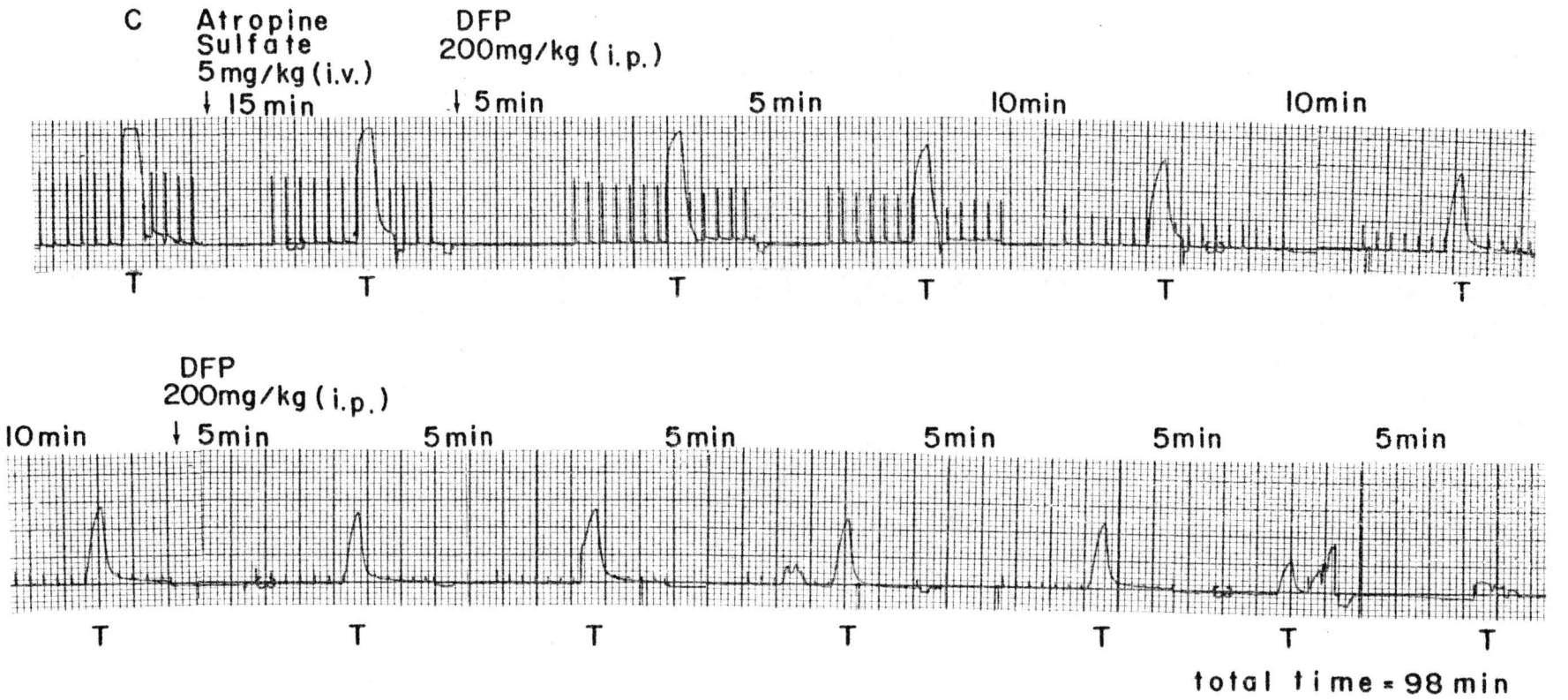

C
Atropine Sulfate 5mg/kg (i.v.)
↓ 15 min
DFP 200mg/kg (i.p.)
↓ 5min
5 min
10min
10min
T
T
T
T
T
T
DFP 200mg/kg (i.p.)
10min
↓ 5min
5min
5min
5min
5min
5min
T
T
T
T
T
T
T
total time = 98 min

Figure 8

Eserine

A total dose of 10 mg/kg eserine resulted in a decline in twitch, but there were no pronounced effects on tetanus (Figure 8). Opercular movements also became labored at 10 mg/kg eserine and ceased after a total dose of 12.5 mg/kg. The onset of tetanic contraction slowed at 12.5 mg/kg eserine. Similar results were seen with three other fish. In some animals tetanus did decline, but always after opercular movements had ceased and may be due to the deteriorating condition of the animal.

Atropine sulfate (5 mg/kg), injected prior to eserine to protect the animal against the systemic effects of eserine, elicited a slight decline in both twitch and tetanus. The five sculpin pretreated with atropine sulfate did not show a significant decline in twitch until they were subject to 20 mg/kg eserine. Tetanus was not affected with a total dose of 50 mg/kg eserine (example Figure 9). Opercular movements became labored at 20 mg/kg eserine, but they were still present with a total dose of 50 mg/kg. Additional protection against eserine was not seen with fish injected with 7.5 and 10 mg/kg atropine sulfate.

Similar results were seen with a fish injected with .125 μg/kg methyl atropine. The fish was able to maintain opercular movement until a total dose of 40 mg/kg eserine was administered (Figure 10). In this fish, twitch increased after the initial injection of 10 mg/kg eserine, but declined after successive injections. The onset of tetanic contraction slowed after a total dose of 40 mg/kg eserine, but the height of tetanic contraction was not affected even after a total dose of 60 mg/kg. No additional protection was seen in a fish pretreated with .250 mg/kg methyl atropine. The dose of atropine sulfate and

Figure 8. Effects of eserine (2.5 mg/kg injections) to a total dose of 20 mg/kg on indirectly stimulated twitch and tetanic responses of the pectoral abductor muscle of the buffalo sculpin. Control = C. Tetanus = T. Chart speed is 25 mm/min. Time period above recording refers to the elapsed time between muscle stimulation.

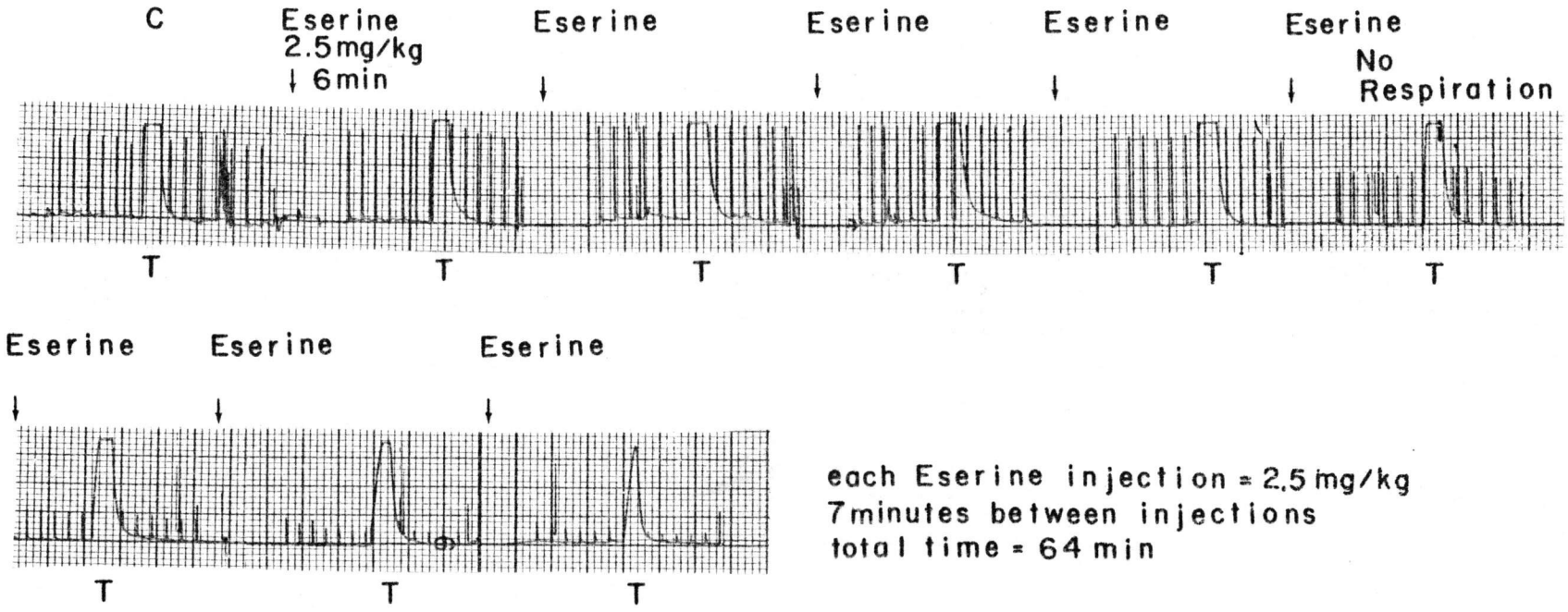

C
Eserine
2.5mg/kg
6min
Eserine
Eserine
Eserine
Eserine
No
Respiration
T
T
T
T
T
T
Eserine
Eserine
Eserine
T
T
T
each Eserine injection = 2.5 mg/kg
7minutes between injections
total time = 64 min

Figure 8

Figure 9. Effects of eserine (10 mg/kg injections) to a total dose of 50 mg/kg on indirectly stimulated twitch and tetanic responses of the pectoral abductor muscle of the buffalo sculpin after pretreatment with atropine sulfate (5 mg/kg). Control = C. Tetanus = T. Chart speed is 25 mm/min. Time period above recording refers to the elapsed time between muscle stimulation.

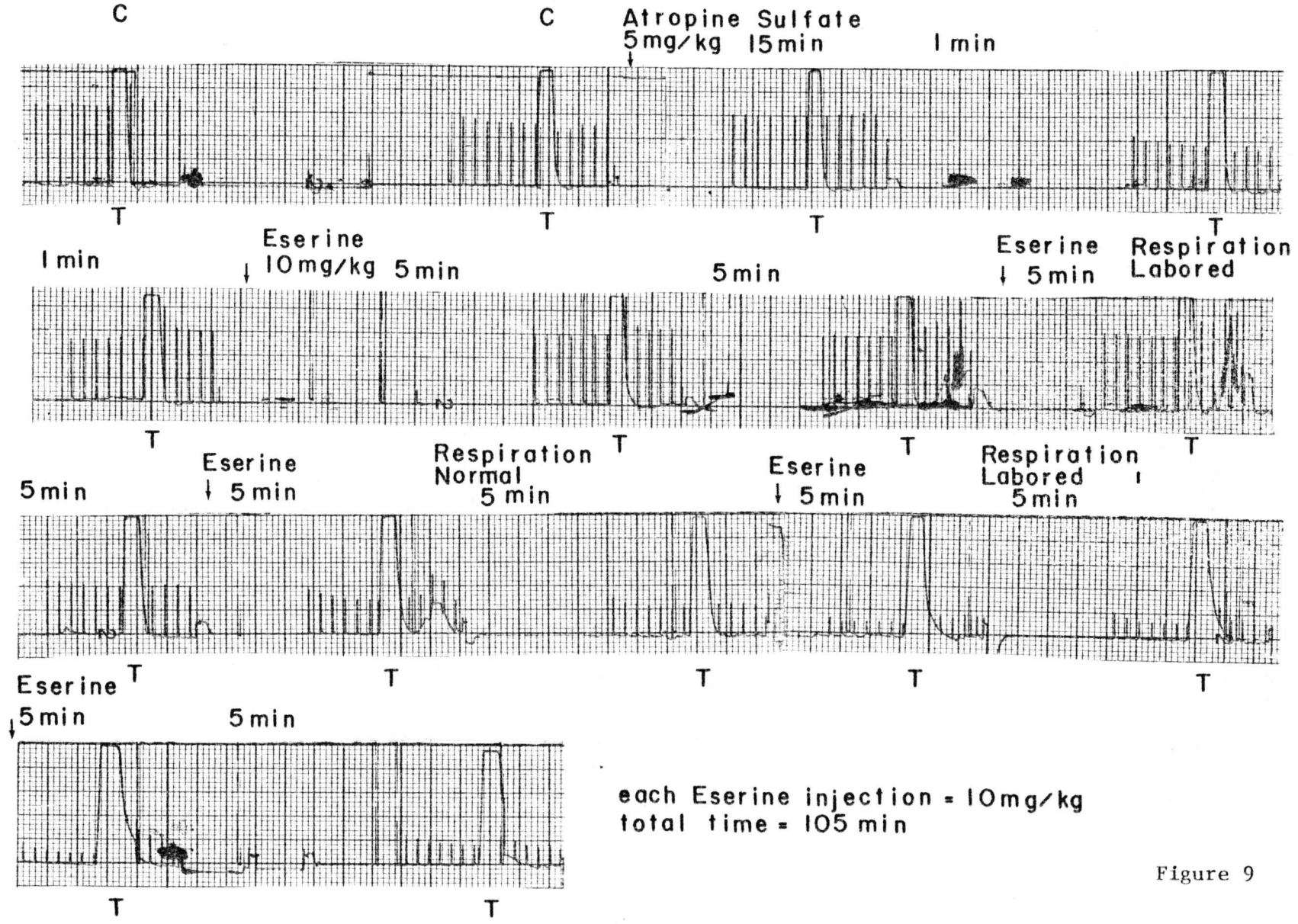

C
C
Atropine Sulfate
5mg/kg 15min
1 min
T
T
T
T
1 min
Eserine
10mg/kg 5min
5min
Eserine
5 min
Respiration
Labored
T
T
T
T
5min
Eserine
5 min
Respiration
Normal
5 min
Eserine
5 min
Respiration
Labored
5min
T
T
T
T
T
Eserine
5min
5min
T
T
each Eserine injection = 10mg/kg
total time = 105 min

Figure 9

Figure 10. Effects of eserine (10 mg/kg injections) to a total dose of 60 mg/kg on indirectly stimulated twitch and tetanic responses of the pectoral abductor muscle of the buffalo sculpin after pretreatment with methyl atropine (125 μg/kg). Control = C. Tetanus = T. Chart speed is 25 mm/min. Time period above recording refers to the elapsed time between muscle stimulation.

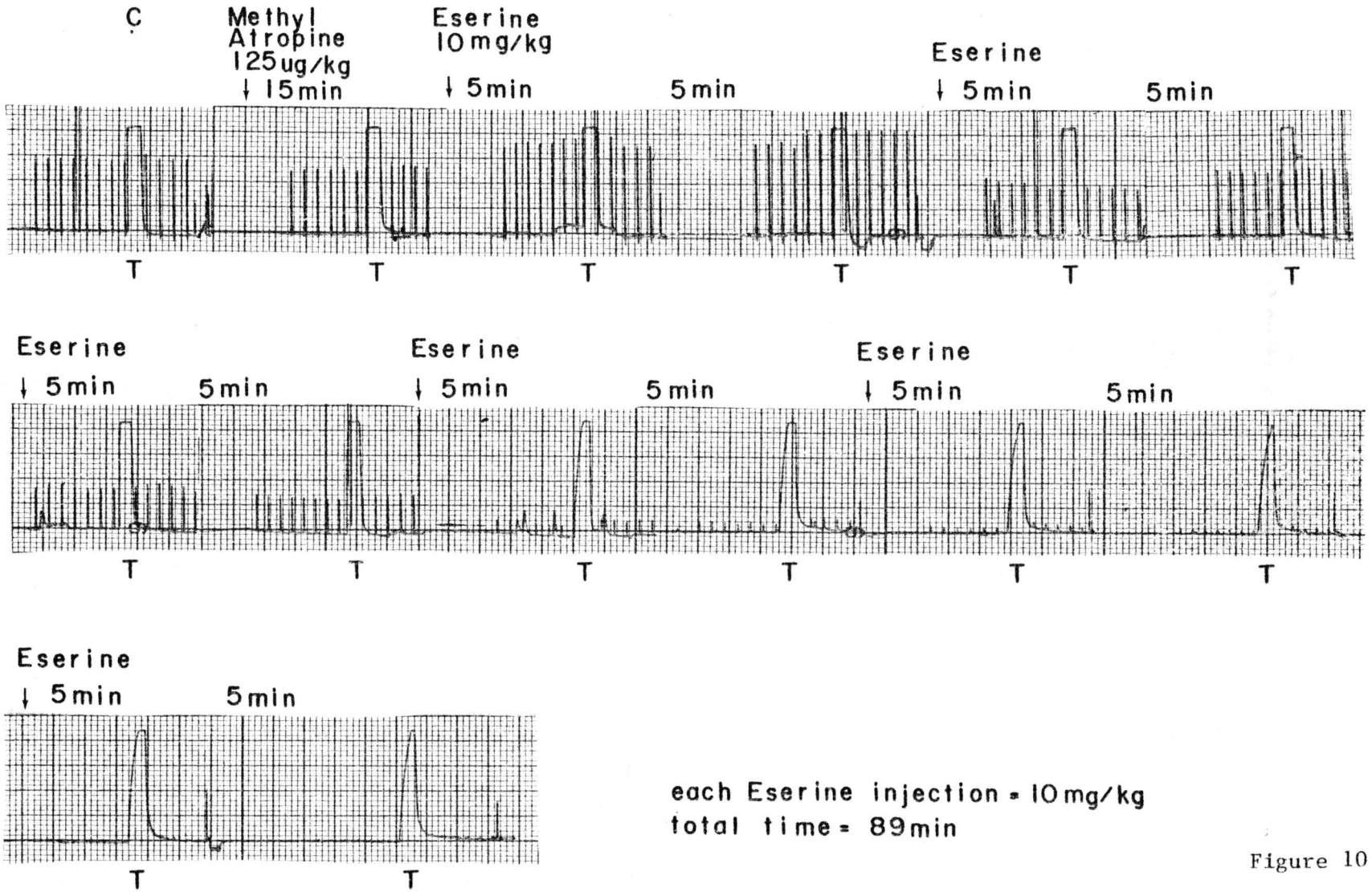

C
Methyl Atropine 125ug/kg
↓ 15min
Eserine 10mg/kg
↓ 5min
5min
Eserine
↓ 5min
5min
T
Eserine
↓ 5min
5min
Eserine
↓ 5min
5min
Eserine
↓ 5min
5min
Eserine
↓ 5min
5min
each Eserine injection = 10mg/kg
total time = 89min

Figure 10

methyl atropine used to protect the sculpin against the systemic effects of ACHE-I was that concentration which produced an initial decline in twitch.

Atropine sulfate did not effect blood pressure or heart rate with accumulations up to 12.5 mg/kg. Blood pressure became erratic in one fish injected with 2.5 mg/kg methyl atropine. Heart rate was not affected.

DISCUSSION

Neuromuscular Blocking Agents

d-Tubocurarine is a competitive neuromuscular blocking agent which acts by binding to the nicotinic receptor sites and thereby blocks the acetylcholine/receptor interaction (Taylor, 1980b). The administration of d-tubocurarine into the sculpin resulted in a flaccid paralysis, which is also characteristic of mammalian preparations.

The dose of d-tubocurarine needed for neuromuscular blockade in the sculpin, approximately 350 μg/kg, is close to the dose reported by Schneider (1974) for the black bass (Micropterus salmoides), 275 μg/kg. In mammalian preparations, the doses of d-tubocurarine required to produce a 90% block in indirectly elicited twitch in rats and cats are 70 and 400 μg/kg, respectively (Bowman, 1964), and 217 μg/kg is the mean concentration needed in man (Maclagan, 1976). Hence, the dose of d-tubocurarine required for blockade in the sculpin is higher than that reported in rats, but is comparable to the dose needed in other mammalian species.

α-Butx binds to the nicotinic receptor sites and results in a neuromuscular block similar to d-tubocurarine, but is irreversible. α-Butx does not block cholinergic transmission other than in skeletal muscle. The muscarinic acetycholine receptors in smooth muscle and nicotinic receptors in autonomic ganglia are not blocked (Chang, 1979). This peripheral blockade produces a flaccid paralysis, and results in death by respiratory failure in most animal species.

In the sculpin, 500 μg/kg α-Butx resulted in labored opercular

movement, and the animal died after an accumulation of 1 mg/kg over 80 minutes. This dose is high when compared to other animals. α-Butx injected subcutaneously (s.c.) in mice has a LD_{50} value of 140 µg/kg and a LD_{50} of 110 µg/kg when injected i.p. (Eterovic, et al. 1975). Lee and Tseng (1969) report a LD_{50} of 55 µg/kg for α-Butx when injected intramuscularly (i.m.) in the pigeon. Lee and Tseng (1966) injected rabbits (i.v.) with 1 mg/kg α-Butx and saw respiratory arrest within 10 minutes. They also state that To and Tin (1943) reported an i.v. minimal lethal dose of 25 µg/kg for α-Butx in rabbits.

Several authors mention the long latency period seen with α-Butx (Chang and Lee, 1963; Jiménez-Pornas, 1968). However, α-Butx was injected s.c. in those studies and since α-Butx was injected i.v. into the sculpin the onset of effect should have been relatively fast. Lee (1982, Personal Communication) states that there should be no latency period when α-Butx is injected i.v.

Depolarizing Agents

Succinylcholine and decamethonium interrupt neuromuscular transmission by producing a prolonged depolarization of the muscle endplate region (Taylor, 1980b). The administration of these agents to the sculpin resulted in spontaneous muscle contracture which was dose dependent in duration and magnitude. Succinylcholine had a shorter duration than decamethonium, which may be related to the rapid hydrolysis of succinylcholine by cholinesterase in the plasma and liver as reported in mammals (Taylor, 1980b). A similar contracture was seen in the black bass by Schneider (1972, unpublished)

after the injection of depolarizing agents. Schneider also saw inconsistent tension responses to succinylcholine, as were seen in the sculpin.

Avian tonic fibers also react to the depolarizing drugs with a spontaneous contracture, and a distinct twitch (Zamis and Head, 1976). When 100 µg/kg is injected i.v. into the adult hen, it produces a contracture of the gastrocnemius muscle and a flaccid paralysis of the pectoral muscle (Zamis, 1954; Zamis and Head, 1976). Ginsborg (1960) found that the muscle fibers in the hen which respond with a contracture contain multiple innervated fibers, and those which respond with a flaccid paralysis contain mostly focally innervated fibers. These multiple innervated fibers respond to depolarizing agents with a contracture almost identical to that produced by tetanic stimulation of the nerve. Amphibian tonic fibers also respond to depolarizing agents with a contracture (Kuffler and Vaughan-Williams, 1953). The results of the sculpin experiments suggest that tonic fibers are present in the pectoral muscles, which is supported by the work of several investigators (Takeuchi, 1959; Hidaka and Toida, 1969), who found tonic fibers in the pectoral muscles of other marine teleosts.

However, the depolarizing neuromuscular block in cats and man results in a flaccid paralysis (Zamis and Head, 1976), compared to the spontaneous contracture seen in the sculpin. Injections (i.v.) of 40 µg/kg decamethonium and 50 µg/kg succinylcholine are necessary to produce neuromuscular blockade in cats (Bowman, 1964), which are similar to the doses required in the sculpin. In other mammalian species: monkey, dog, rabbit, and rat, the depolarizing agents

produce a blockade which combines features of both the depolarizing and competitive blocking agents, and has been described as a "dual" mechanism by Zamis (1953). In these animals, decamethonium initially acts as depolarizing agent producing a flaccid paralysis, but during the blocking process their action develops a curare like effect which can be antagonized by acetylcholine.

Acetylcholinesterase Inhibitors

The effects of DFP and eserine on the neuromuscular system of the sculpin are similar to those reported by Schneider and Weber (1975) for the black bass. They found that although twitch decreased, tetanic response was usually not altered. In the sculpin, an i.v. injection of 100 mg/kg DFP was required for tetanus to decline, with the toxic effects seen as a decrease in opercular movement preceding this tetanic decline. This dose is greater than the values reported in mammalian studies. Faff et al (1973) studied the effects of DFP in an *in situ* sciatic nerve-anterior tibialis muscle in rats, and saw depressions of indirectly elicited tetanus within 16 minutes after an injection (i.v.) of 2.0-2.5 mg/kg DFP.

To minimize the systemic effects of DFP toxicity, atropine sulfate was injected prior to the DFP. Atropine acts by antagonizing the muscarinic actions of acetylcholine at the central nervous system and structures innervated by postganglionic cholinergic nerves, but has little effect on the nicotinic receptor site at the neuromuscular junction (Weiner, 1980). The decline in twitch seen in some experiments after atropine was administered may have been due to atropine blocking the ion conductance modulator of the nicotinic

receptor (Eldefrawi et al. 1977). When 5 mg/kg atropine sulfate was injected prior to DFP, the rate of opercular movement did not appear to be affected at a cumulative dose of 400 mg/kg (i.p.) DFP, whereas the tetanic response declined but was not abolished. When Schneider and Weber (1975) pretreated the black bass with atropine sulfate, tetanus was not affected with up to an accumulated dose of 500 mg/kg DFP (i.v.).

The apparent hemolytic activity of the DFP noticed after i.v. injection may have affected the distribution of DFP to the pectoral muscle. However, i.p. injections were used in the above experiments to preclude the problems associated with hemolysis. It is of interest that at this dose (100 mg/kg) DFP was not found to cause hemolysis in Schneider and Weber's (1975) work nor was hemolytic activity mentioned in any of the literature surveyed, which may indicate that this is a species specific reaction.

The effects of increasing doses of eserine on twitch and tetanus were similar to those of DFP; twitch decreased, with no initial twitch potentiation, and the muscle was able to sustain a tetanic response. Tetanus did decrease in some experiments, but the decrease occurred after opercular movements became labored. This may suggest that the toxic effects of eserine precede the neuromuscular effects in the sculpin. Schneider (1972, unpublished) also found that 10 mg/kg eserine had no effect on tetanus in the black bass. These results are contrary to those of classical mammalian cholinergic systems. In mammals, ACHE-I result in two dose dependent responses in indirectly stimulated phasic muscles: (1) potentiation of twitch, and (2) depression in twitch and a rapid increase in tetanic tension followed

immediately by partial or complete relaxation (Hobbiger, 1976). Loomis and Salafsky (1964) studied the effects of soman, a potent ACHE-I, on rats and they did not observe complete depression of twitch until a dose of soman was administered which resulted in bradycardia.

The responses of avian and amphibian muscle are similar to those seen in the sculpin. Brown and Harvey (1938) failed to obtain twitch potentiation or tetanic depression when they investigated the effects of eserine on indirectly stimulated twitch and tetanus of the gastrocnemius muscle of the domestic fowl. Studies on frog twitch fibers have reported that twitch potentiation due to ACHE-I is not a constant phenomenon and often only occurs under special conditions; and the tonic fibers are able to maintain a prolonged tetanus after inhibition of acetylcholinesterase (Hobbiger, 1976).

Atropine sulfate and methyl atropine were administered prior to the eserine to determine whether the toxicity was centrally mediated. Atropine sulfate can cross the blood brain barrier, methyl atropine is a quaternary ammonium derivative, modified by the addition of a second methyl group to the nitrogen, and is not easily transported across the blood brain barrier (Weiner, 1980). Since both atropine sulfate and methyl atropine displayed protective abilities, this suggests that the benefits are derived from peripheral rather than central action. Schneider (1974) observed similar results with the black bass, but expressed uncertainty about the nature of the blood brain barrier in fish. A study by Klika (1980) has found that the blood brain barrier in the rainbow trout (Salmo gairdneri) has exclusion mechanisms and ultrastructural configuration similar to those seen in mammals. Several researchers indicate that the

lethality of ACHE-I arises via respiratory paralysis due to central nervous system effects, (Karlzman and Ohta, 1981) but this does not appear to be the case in teleosts.

Chapter II:

Characterization of the Acetylcholine Neuromuscular Receptors in the Pectoral Abductor Muscle of the Buffalo Sculpin

Introduction

Characterization of the neuromuscular junction receptors can be done by performing detailed studies on the acetylcholine receptors in the skeletal muscle. In receptor labeling studies, a radioactive form of the transmitter, hormone, or biologically active agonist or antagonist is bound to membrane or cell preparations of the target tissue (Bennett, 1978). The binding of radioactive ligands to the high affinity binding sites in these preparations usually follows the kinetics and mathematical models similar to those of enzyme-substrate interactions. Saturation, kinetic, and pharmacological criteria are used to support the hypothesis that a binding site represents a certain receptor. From these tests, parameters such as number, distribution, and identification of binding sites can be attained (Burt, 1978). This information would be useful for comparing the neuromuscular system of fish to those of other vertebrates, and perhaps provide insights into the nonclassical cholinergic responses of the fish neuromuscular system.

Lee (1967, 1972) discovered that the venoms of snakes belonging to the families Elapsidae and Hydrophidae contain basic polypeptide neurotoxins which bind specifically to the cholinergic nicotinic receptor. One such agent is α-bungarotoxin (α-Butx) which is purified from the venom of the elapsid snake Bungarus multicinctus (many banded krait), of the Far East. α-Butx is used in many receptor binding studies because it forms an irreversible bond with

the nicotinic receptor, producing an antagonistic action similar to curare (Lee, 1972).

The primary structure of α-Butx consists of 74 amino acids in a single chain, crosslinked by five disulfide bridges, and it has a molecular weight of 8000 (Mebs, et al. 1971). Audioradiographs of mouse diaphragm muscle labeled with [^{3}H]-α-Butx have revealed that the toxin is associated with the postjunctional fold, while labeling elsewhere is negligible (Barnard, et al. 1973). This offers evidence for the specificity of this toxin for the nicotinic cholinergic receptor.

The most extensive investigations using radiolabeled ligands in nicotinic receptor binding studies involve the receptor of the electric organ of eels and rays. These organs are regarded as modified skeletal muscle (Bennett, 1970). Changeux, et al. (1970) discovered that α-Butx blocks the binding of decamethonium to a protein receptor isolated from the electric organ of Electrophorus electricus. They also found that d-tubocurarine protects against α-Butx binding to the protein receptor.

Studies of the acetylcholine receptors of vertebrate skeletal muscle can be difficult due to the low number of cholinergic receptors present in muscle (Dolly, 1979). For example, electric organs provide a rich source of receptor tissue (500-1000 pmol/gram of tissue), compared to the number of nicotinic receptors from the rat diaphragm (1.4-4 pmol/gram of tissue) (Colquhoun and Rang, 1976). Various quantitative studies on the labeling of skeletal muscle have been carried out. Miledi and Potter (1971) compared the binding properties of rat diaphragm and from sartorius muscle, and found that

the number of toxin molecules bound per endplate was 60 times greater in the frog than in the rat. Colquhoun and Rang (1976) studied the binding of [^{125}I]-α- Butx to rat skeletal muscle homogenates, and measured the inhibitory effects of various blocking and depolarizing agents. They reported that the binding can be blocked by d-tubocurarine, and suggested that the depolarizing drugs may inhibit binding of the toxin by causing desensitization, which is associated with a change in receptor conformation. Other studies have dealt with the increase in α-Butx binding sites in chronically denervated muscle (Miledi and Potter, 1971; Chang, et al. 1973; Froehner et al. 1977). The magnitude of this increase in binding sites outside the neuromuscular junction, extrajunctional receptors, and the time course of development vary with species and muscle type (Dolly, 1979). The binding sites in rat skeletal muscle may increase 10- to 20-fold within two to three weeks after denervation (Miledi and Potter, 1971).

Several review articles on receptor binding studies in vertebrate skeletal muscle have appeared in the literature: Rang, 1974; Barnard et al. 1975; and Dolly, 1979. However, no receptor binding studies of fish skeletal muscle have been reported.

The objectives of this study were to characterize the cholinergic receptors in the skeletal muscle of the buffalo sculpin using receptor binding analysis, to compare these results with cholinergic systems in other animals, and to determine the correlation between the dosage of drugs needed to initiate a response, in situ and their in vitro binding activity.

MATERIALS AND METHODS

The sculpin were pithed and the pectoral abductor muscles were dissected away. For every 1 ml of final homogenate, 3 grams of abductor muscle were minced and homogenized in 20mM, pH 7.5, phosphate buffer containing protease inhibitors (5mM EDTA, .1mM phenylmethylsulfonyl fluoride [PMSF]). The muscle was homogenized with a Brinkman Polytron PT10 homogenizer at a setting of 7 for approximately 1 minute. The homogenate was filtered through gauze and then centrifuged at 50,000xg (5°C) for 10 minutes. Pellets were collected and resuspended in a 20mM, pH 7.5, phosphate buffer (.4mM NaCl, .1mM EDTA, .1mM PMSF) and centrifuged at 10,500xg (5°C) to remove large particles from the sample. The supernatant was centrifuged at 50,000xg (5°C) for 10 minutes, the pellets were resuspended in 20 mM phosphate buffer (.1M NaCl, .1mM EDTA, .1mM PMSF), and centrifuged at 50,000xg (5°C) for 10 minutes. The pellets were then resuspended in a 20mM phosphate buffer (.1M NaCl, .1mM EDTA, .1mM PMSF) such that the resulting suspension represented 3 grams of pectoral muscle per 1 ml. Sodium azide (.02g/100ml) was added to the final buffer if the homogenate was to be stored at 4°C. In most experiments, the muscle homogenate was prepared the day prior to the receptor binding assay.

Fifty µl of tissue membrane suspension were mixed with 50 µl of 50 mM, pH 7.5, phosphate buffer (.1 mg/ml bovine serum albumen [BSA]) containing 2.5 pmol of [^{125}I]-α-Butx (2.5 X 10^{-8}M). After 30 minutes incubation at room temperature, each sample was diluted with 5 ml of 20mM, pH 7.5, phosphate buffer (.4M NaCl, .1mM EDTA, .1mM PMSF, .01 mg BSA/100 ml) and filtered through a 2.4 cm diameter filter

(Whatman GF/B), under 15 pounds/square inch pressure. The filters were rinsed twice with the same buffer, placed in liquid scintillation vials, and allowed to dry overnight. Scintillation fluor (Ready Solv-HP, Beckman Instruments Inc.) was added to the vials and approximately 4 hours was allowed for filter saturation. The radioactivity bound to the filters was counted on a Beckman LS8000 liquid scintillation spectrophotometer. The general assay was based on the procedure of Potter (1980).

Protein was determined by the Lowry et al. (1951) method, using BSA as the standard. The mean protein concentration was 225 µg/50 µl of homogenate. The amount of free [^{125}I]-α-Butx remaining on the filter was determined in all experiments by running the assay with a concentration of BSA equal to the protein concentration of the muscle homogenate.

Nonspecific binding was determined by incubating 50 µl of homogenate with 10 µl (1.6×10^{-5}M) unlabeled α-Butx for 30 minutes prior to the addition of the labeled toxin. Total binding minus nonspecific binding equals the amount of [^{125}I]-α-Butx bound specifically. This is based on the assumption that the specific interaction of the toxin is stable and irreversible, while nonspecific binding reaches equilibrium within 30 minutes and dissociates rapidly (Lukasiewicz and Bennett, 1978). Three replicates were prepared for experiments representing total binding and two replicates were prepared for nonspecific binding experiments.

Denervation experiments were performed on seven sculpin in an attempt to increase the number of [^{125}I]-α-Butx binding sites. The sculpin were anesthetized with benzocaine (70 mg/l seawater) and

placed on their right side with the left pectoral fin exposed. Seawater containing the anesthetic constantly irrigated the gills. An incision was made posterior to the pectoral adductor muscle, leaving a small cavity between the anterior extremity of the trunk musculature and the adductor. The left pectoral fin was then denervated by cutting the adductor and abductor nerve bundle. The cavity was closed with silk suture, and the animals were returned to the holding tanks. Muscle homogenates were prepared as usual in approximately 3 weeks after denervation. The right pectoral abductor served as the control.

Specific Experimental Design and Data Analysis

1. Saturation Experiments

a. Equilibrium Constant (K_D) and Maximum Specific Binding (Bmax)

Various concentrations of [^{125}I]-α-Butx ranging from 1 to 23 nM were incubated with the muscle homogenate and the amount of toxin bound was measured after incubation for 30 minutes. The K_D and Bmax were determined by using Scatchard analysis (Bylund, 1980).

$$\frac{B}{F} = -\frac{1}{K_D} B + \frac{Bmax}{K_D}$$

B = Amount of bound ligand
F = Amount of free ligand

In Scatchard analysis, the amount of [^{125}I]-α-Butx bound at each concentration is multiplied by a factor which takes into account the specific activity of the [^{125}I]-α-Butx, the reaction volume, and the efficiency of the scintillation counting. A plot of bound [^{125}I]-α-Butx /Free [^{125}I]-α-Butx versus bound [^{125}I]-α-Butx yields a line with a slope equivalent to the negative reciprocal of the K_D and an abscissa

intercept equivalent to Bmax.

2. Kinetic Experiments

a. Association Rate Constant (K_{+1})

The kinetics of the association between the [^{125}I]-α-Butx and the binding site were analyzed by measuring the amount of binding at various times after the toxin and homogenate were mixed together. The K_{+1} was calculated using second order kinetics, where only the initial rate of binding is considered.

$$K_{+1} = \frac{1}{t\ (L_T - Bmax)} \times \ln \frac{Bmax\ (L_T - B)}{L_T\ (Bmax - B)}$$ (Bylund, 1980)

Bmax = maximal number of binding sites determined by the saturation experiments

B = amount bound at time t

L_T = total amount of [^{125}I]-α-Butx added

t = time of incubation

The K_{+1} was calculated after one minute of incubation.

b. Dissociation Constant (K_{-1})

Ten µl of buffer containing unlabeled α-Butx (8.3×10^{-6}M) was added to the homogenate and [^{125}I]-α-Butx after they had incubated for 30 minutes. Analysis of the loss of radioactive complex measured as a function of time was performed using the formula: $\ln (B/B_o) = -K_{-1} \cdot t$. Where B_o is equal to the amount of [^{125}I]-α-Butx bound before the addition of the unlabeled toxin, and B is the amount of toxin bound at time t. A plot of $\ln (B/B_o)$ versus time has a slope of $-K_{-1}$ (Bylund, 1980). A control to determine if the observed decrease in binding was a consequence of proteolysis of the [^{125}I]-α-Butx or other nonspecific events rather than the dissociation of the ligand/receptor complex was performed by adding buffer instead of the unlabeled toxin.

3. Inhibition Experiments

a. IC_{50}

The concentration of drug which inhibits 50% of the [^{125}I]-α-Butx binding (IC_{50}) was determined by incubating the homogenate with 10 µl of various concentrations of inhibitors for 30 minutes prior to the addition of the labeled toxin. Plots of % bound versus concentration of inhibitor were converted into linear relationships using logit transformation. The IC_{50} values were calculated by linear regression analysis of the logit plots (Bylund, 1980).

b. Inhibition Constant (K_I)

The K_I or equilibrium dissociation constant for the inhibitor was calculated using the formula: $K_I = \frac{IC_{50}}{1 + \frac{^{125}\text{I-α-Butx added}}{K_D}}$.

Drugs used in the inhibition experiments: acetylcholine chloride (in presence of 1 X 10^{-6}M eserine), cholinergic agonist; L-epinephrine bitartrate, adrenergic agonist; d-tubocurarine chloride and atropine sulfate, cholinergic antagonist; succinylcholine chloride, depolarizing agent.

All drugs were obtained from the Sigma Chemical Co. except for atropine sulfate which was from the J. L. Baker Chemical Co. The [^{125}I]-α-Butx was purchased from New England Nuclear. The mean specific activity of the [^{125}I]-α-Butx was 17.1 uCi/µg. Once reconstituted, the [^{125}I]-α-Butx was stored at -20°C and used within 60 days.

RESULTS

The saturation curve for the binding of [^{125}I]-α-Butx to the pectoral abductor homogenates is shown in Figure 11. All binding sites appear to be occupied at a [^{125}I]-α-Butx concentration of 2.27 X 10^{-8}M, and binding was approximately 85% specific (n=5). Scatchard analysis (Figure 12) of this data is linear, suggesting a single class of binding sites and yields a K_D and Bmax of 69.5 ± 11 nM and .497 ± .28 pmol bound/mg protein, respectively, in the muscle homogenate.

The association kinetics of the interaction between the muscle homogenate and the [^{125}I]-α-Butx displays regular second order kinetics (Figure 13). The K_{+1} generated using second order kinetics is equal to 1.08 X 10^7 ± .28 min $^{-1}$mol $^{-1}$(n=3). When this data was plotted according to the integrated form of the second order rate equation, the plot was linear and indicative of a single class of binding sites.

Results of the dissociation experiments indicate that the receptor-toxin complex consists of two distinct components (Figure 14). By extrapolating the slow dissociating component back to time zero, the slow component comprises approximately 21% of total binding and the fast component approximately 79%. No comparable reduction was seen in the buffer control, therefore the fast rate of dissociation was not due to proteolysis. Plots of $\ln(B/B_o)$ versus time for each of the three experiments completed are shown in Figure 15 with the K_{-1} constants for both components. The first and second trials are similar in that complete dissociation of the fast component occurs after approximately 60 minutes, whereas the fast component dissociates

Figure 11. Binding saturation curve for [^{125}I]-α-Butx binding to pectoral abductor muscle homogenates of the buffalo sculpin. Total, specific, and nonspecific binding (fmol/mg protein) are plotted against [^{125}I]-α-Butx concentration (nM).

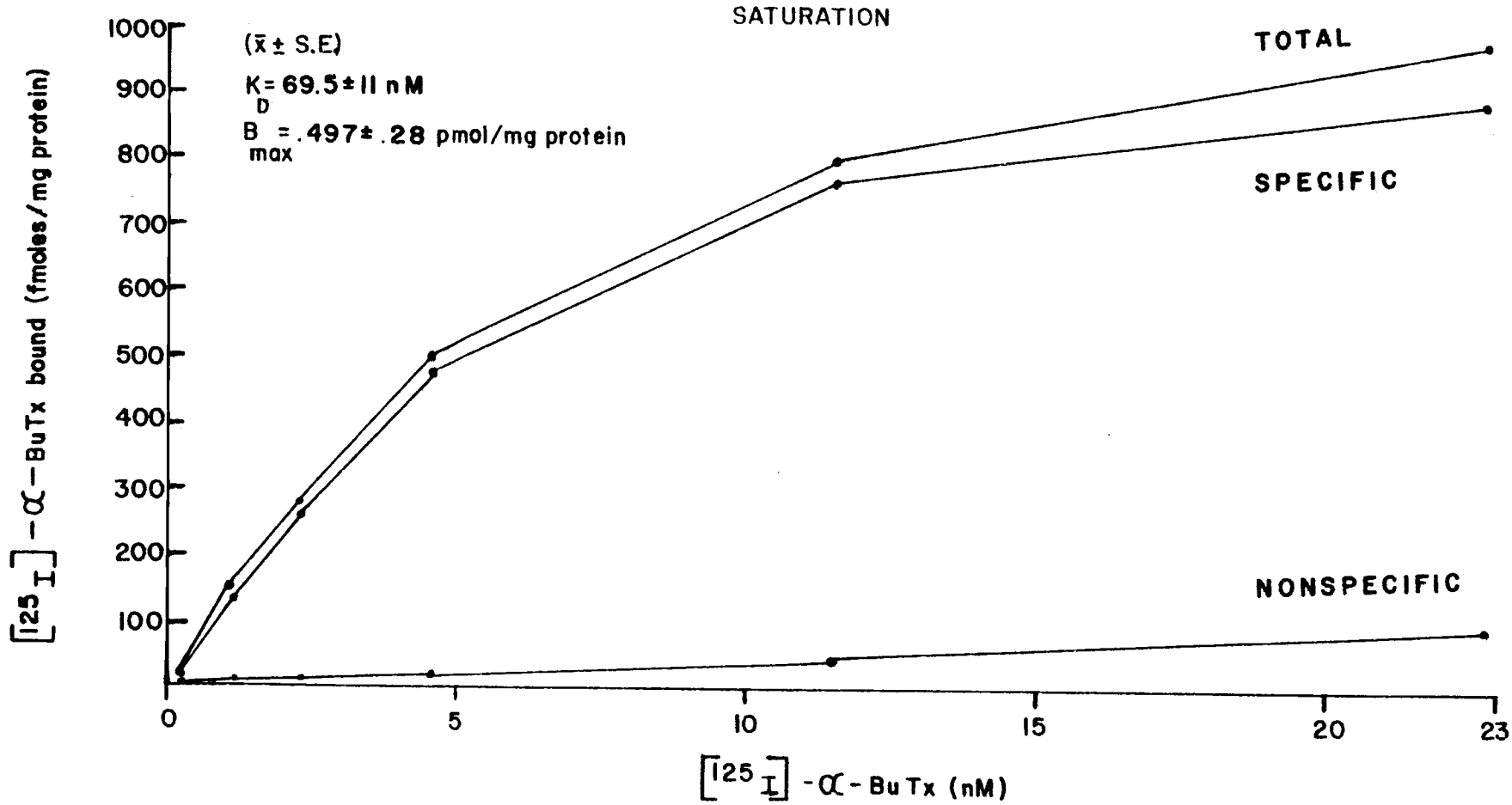

SATURATION
(x̄ ± S.E)
K_D = 69.5 ± 11 nM
B_max = .497 ± .28 pmol/mg protein
TOTAL
SPECIFIC
NONSPECIFIC
[125 I] -α-BuTx bound (fmoles/mg protein)
1000
900
800
700
600
500
400
300
200
100
0
5
10
15
20
23
[125 I] -α-BuTx (nM)

Figure 11

Figure 12. Scatchard plot of saturation data for [^{125}I]-α-Butx binding to pectoral abductor muscle homogenates of the buffalo sculpin. [^{125}I]-α-Butx bound/free X 10^{-3} is plotted against [^{125}I]-α-Butx concentration (nM). The line was fitted by linear regression analysis.

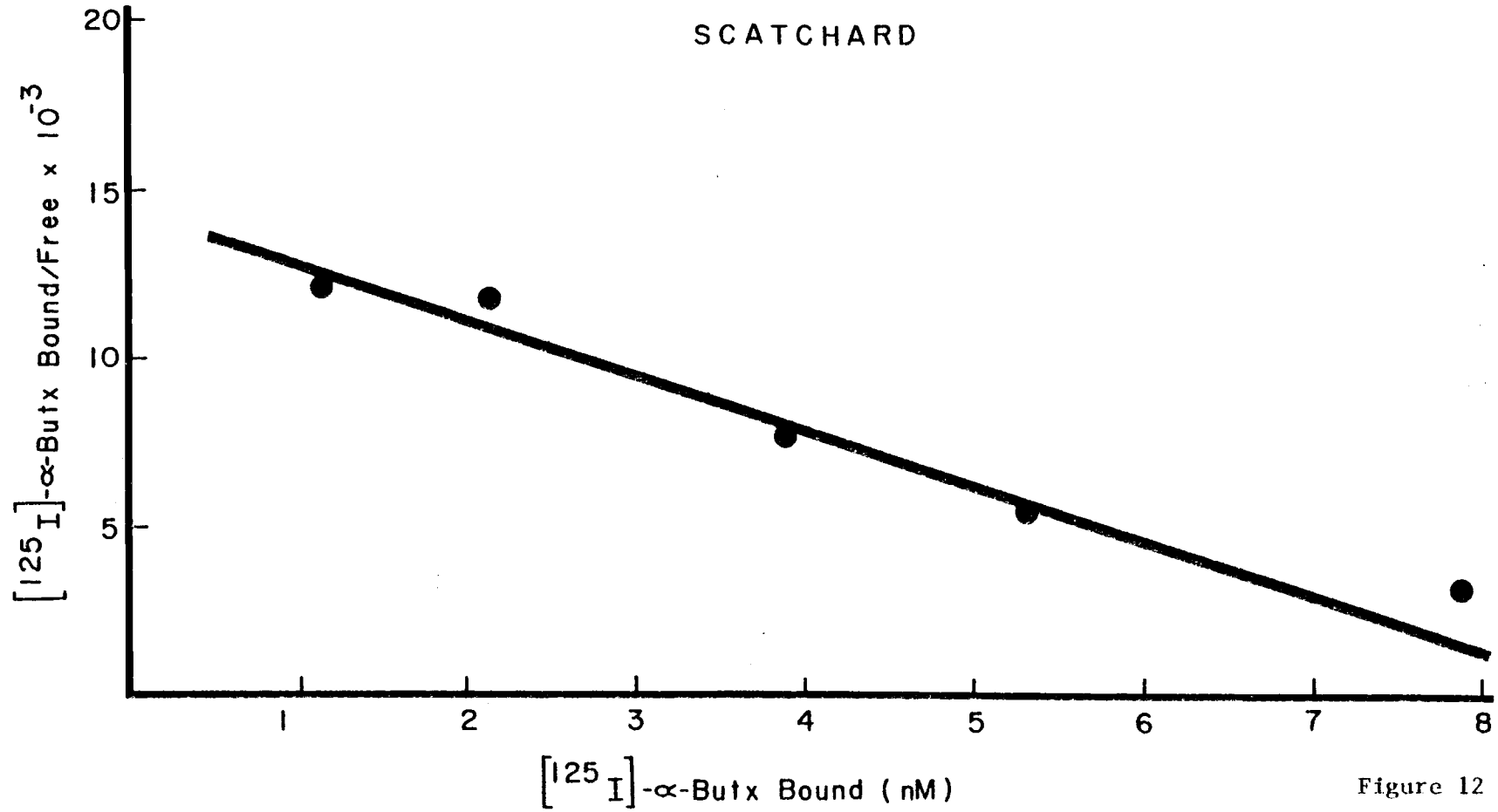

SCATCHARD
20
15
10
5
[125 I]-α-Butx Bound/Free x 10-3
1
2
3
4
5
6
7
8
[125 I]-α-Butx Bound (nM)

Figure 12

Figure 13. Association kinetics of [^{125}I]-α-Butx/receptor complexes. Specific binding of [^{125}I]-α-Butx (pmol/mg protein) to pectoral abductor muscle homogenates of the buffalo sculpin is plotted as a function of time of exposure to labeled toxin.

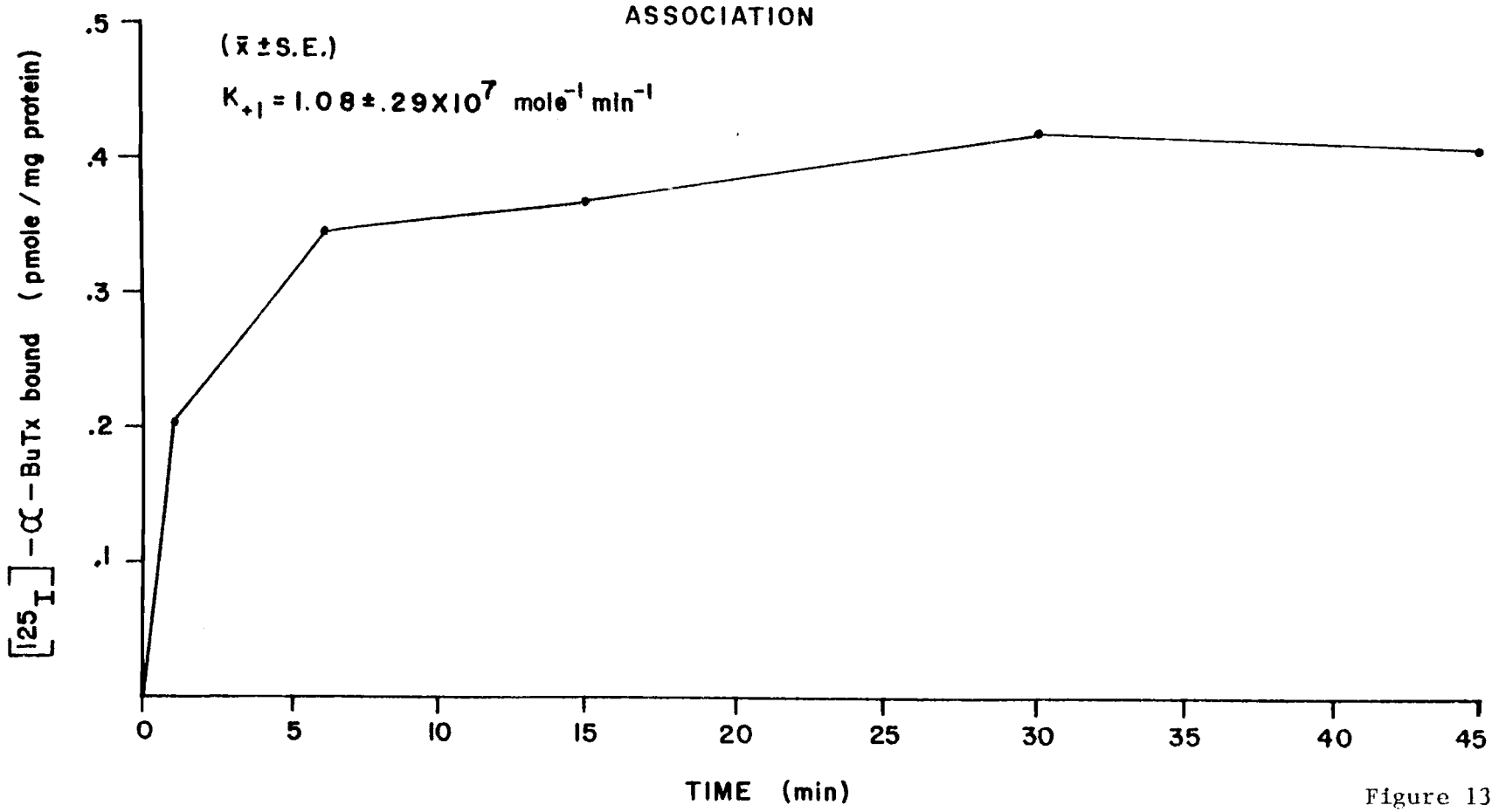

ASSOCIATION
($\bar{x}$ ± S.E.)
$K_{+1} = 1.08 \pm .29 \times 10^7$ mole^{-1} min^{-1}
[^{125}I]-α-BuTx bound (pmole / mg protein)
.5
.4
.3
.2
.1
0
5
10
15
20
25
30
35
40
45
TIME (min)

Figure 13

Figure 14. Dissociation kinetics of [^{125}I]-α-Butx/receptor complexes. Displacement of specific binding (%) after addition of unlabeled α-Butx is plotted against time (hours).

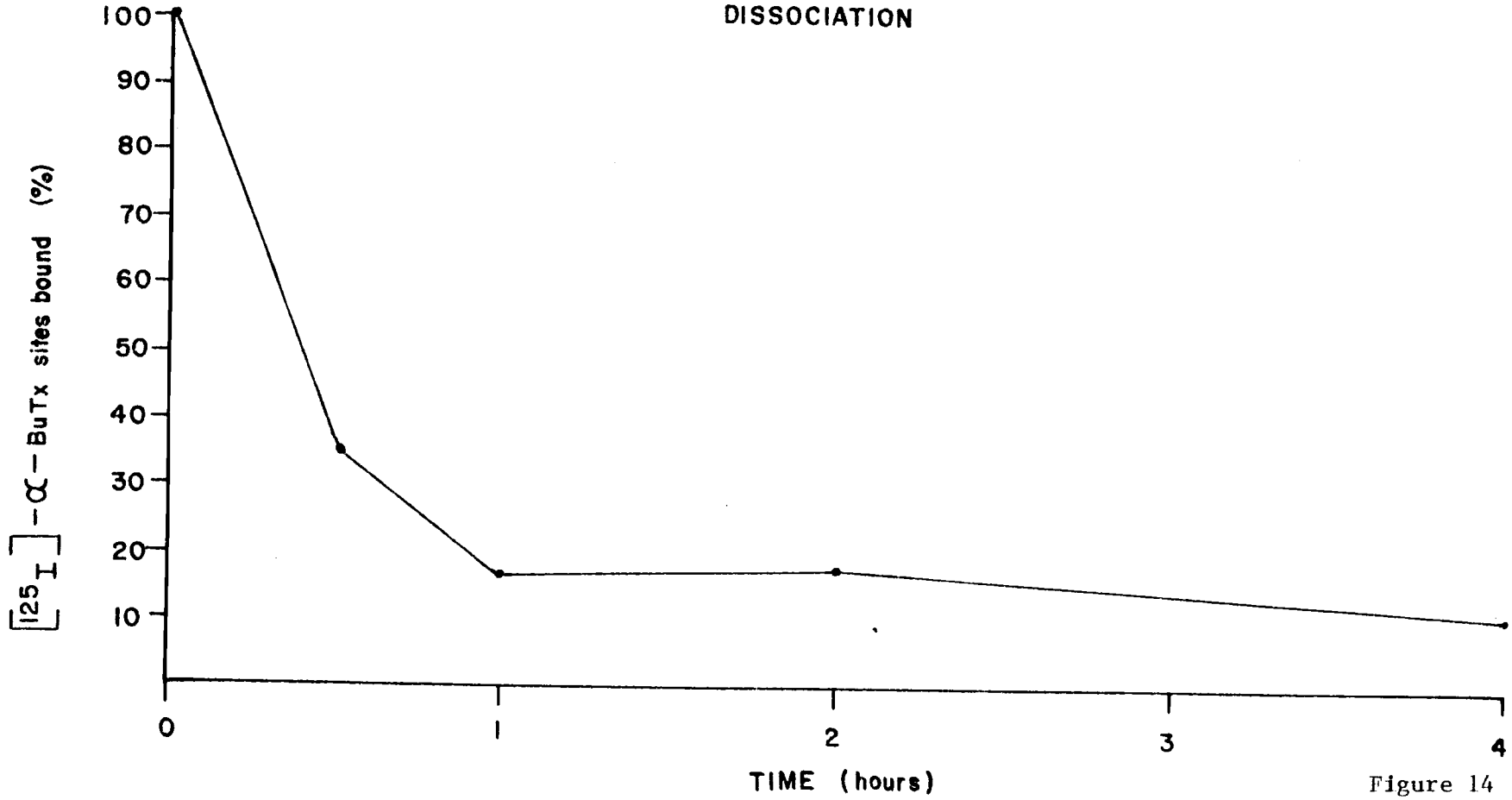
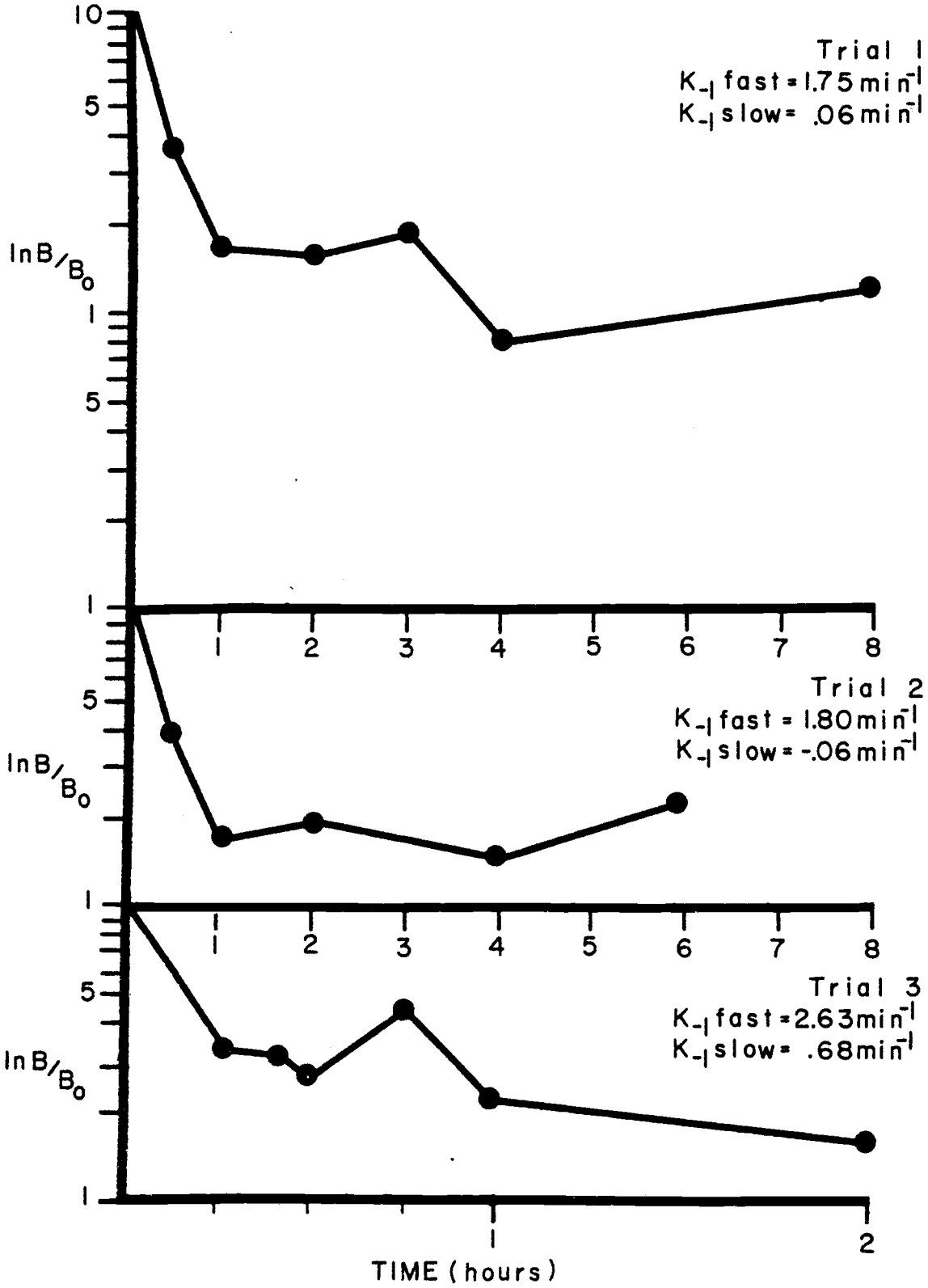

DISSOCIATION
[125I]-α-BuTx sites bound (%)
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
TIME (hours)

Figure 14

Figure 15. Dissociation kinetics of three individual experiments plotted as ln B (specifically bound [^{125}I]-α-Butx at time t)/B_o ([^{125}I]-α-Butx specifically bound before addition of excess unlabeled α-Butx) plotted against time (hours). K_{-1} for each experiment calculated for fast and slow dissociating components by linear regression analysis.

Figure 15


Trial 1
K-1 fast = 1.75 min-1
K-1 slow = .06 min-1
Trial 2
K-1 fast = 1.80 min-1
K-1 slow = -.06 min-1
Trial 3
K-1 fast = 2.63 min-1
K-1 slow = .68 min-1
ln B/Bo
10
5
1
5
1
2
3
4
5
6
7
8
TIME (hours)

in approximately 30 minutes in the third trial. When the dissociation constants are averaged, the K_{-1} is 2.06 min^{-1} for the fast component and .34 min^{-1} for the slow, as determined by linear regression analysis. The slow K_{-1} for the second trial was not included in this average due to it's positive slope.

When the K_D was determined using the formula: $K_D = \frac{K_{-1}}{K_{+1}}$, the K_D for the fast component is 5.7 X 10^{-7}M and 3.4 X 10^{-8}M for the slow.

The IC_{50} values determined in the inhibition experiments are in Table 1, and the inhibition plots are in the appendix. The acetylcholine inhibition experiments were pretreated with 1 X 10^{-6}M eserine to stop the hydrolytic activity of acetylcholinesterase. This eserine concentration did not result in a decrease in binding. d-Tubocurarine has the highest potency for the binding site, while epinephrine has the lowest.

Table 1. IC_{50} values for ligand inhibition of [^{125}I]-α-Butx binding to pectoral abductor muscle homogenates

Ligand	IC_{50} ± s.e. (uM)
Tubocurarine	22.5 ± 11
Acetylcholine (in presence of 1 X 10^{-6}M eserine)	77.6 ± 56
Decamethonium	127.0 ± 8
Atropine	227.0 ± 11
Epinephrine	>1000

n=4 for all experiments except acetylcholine where n=3.

Of the seven fish used in the denervation experiments, only two survived the three week period. In one experiment where the abductor muscle was denervated for 22 days, the Bmax of the denervated abductor was .329 pmol bound/mg protein compared to a value of .104 pmol bound/mg protein for the innervated abductor. In the other fish assayed 21 days after denervation, the Bmax was .158 pmol bound/mg protein for the denervated and .219 pmol bound/mg protein for the innervated abductor muscle.

DISCUSSION AND CONCLUSION

Albuquerque, et al. (1979) have reported Bmax values expressed as the number of binding sites per gram of tissue for several species (Table 2). The Bmax for the sculpin appears low when compared to the others. But, this value for the sculpin abductor muscle was calculated assuming 100% recovery of the [^{125}I]-α-Butx binding sites in the tissue homogenate, and the actual number of binding sites is probably much higher. The densities of binding sites vary greatly from a value of .6 pmol bound/gm of tissue for the sculpin pectoral abductor to 1100 pmol bound/gm of tissue for the electric organ of Torpedo. Most of the tissues in Table 2 have undergone purification procedures to decrease nonspecific binding. The high specific binding in the sculpin assay indicates that these further purification steps are not needed in this assay.

Table 2. Concentration of α-Butx binding sites in different tissues

Tissue	pmol α-Butx bound/gram of tissue
Electric organs	
*Torpedo	1100
*Electrophorus	35
Muscles	
*Rat diaphragm	3
*Rat diaphragm-denervated	60
*Frog sartorius	1
Sculpin pectoral abductor	.6

*Albuquerque et al. (1979)

The K_D determined from the Scatchard plot for the sculpin is greater than the values found in mammalian studies. Colquhoun and Rang (1976) combined [^{125}I]-α-Butx with denervated rat diaphragm muscle and report a K_D of 1 X 10^{-10}M, compared to the K_D of 6.95 X 10^{-8}M in the sculpin assay. Brockes and Hall (1975a) investigated the acetylcholine receptors of purified junctional regions of the rat diaphragm. Their experiment produced a Scatchard plot with two intersecting straight lines, which indicates two classes of binding sites. The components were classified according to their dissociation time, with the K_D of the fast component equal to 6.3 X 10^{-10}M and the slow equal to .6 X 10^{-10}M.

To have an equilibrium constant as large as the one determined by Scatchard analysis for the sculpin assay, the association constant would have to be slower and the dissociation constant would have to be faster ($K_D = \frac{K_{+1}}{K_{-1}}$) than those reported in other studies. The K_{+1} for the sculpin is 5X slower and the K_{-1} for the two binding sites are 2 to 3 orders of magnitude faster than those values reported by Colquhoun and Rang (1976) for the rat diaphragm. The two binding sites found in purified rat diaphragm by Brockes and Hall (1975a) have different K_{+1} and K_{-1} values. The K_{+1} for the sculpin is fairly close to their values. However, the fast K_{-1} component in the sculpin is 2 to 3 orders of magnitude higher than the value reported for the fast component by Brockes and Hall (1975a), and the K_{-1} for the sculpin slow component is over 3 orders of magnitude faster than the value reported for their slow component.

At this time it is difficult to explain the biphasic dissociation seen in the sculpin muscle assay. [^{125}I]-α-Butx appears to bind

competitively to the fast dissociating component, whereas classically α-Butx is said to be an irreversible antagonist. Several investigators have reported two binding sites in mammalian skeletal muscle (Brockes and Hall, 1975a; Chiu et al. 1973), but none have reported a binding site which dissociates as rapidly as that seen in the sculpin pectoral muscle. One possible explanation for the saturation and association experiments showing only one binding site could be the existance of two sites with the same rate of association but with different rates of dissociation. Maelicke, et al. (1977) studied interaction of the nicotinic receptor purified from the electric organ of Electrophorus, with a labeled cobra α-neurotoxin. Their results indicated the existance of two binding sites which appear to be paired; the association rate for each site is the same but the dissociation rates are different, depending on the degree of toxin saturation.

The IC_{50} for tubocurarine, 2.25×10^{-5}M, is from one to three orders of magnitude larger than values reported for mammalian studies. Colquhoun and Rang (1976) investigated the antagonistic action of d-tubocurarine on denervated rat diaphragm muscles, and an IC_{50} value of approximately 1×10^{-6}M can be estimated from their data. Brockes and Hall (1975b) examined the effects of d-tubocurarine on the binding of [^{125}I]-α-Butx to rat diaphragm extracts and an IC_{50} of 2×10^{-7}M can be estimated from their data. A similar study by Alper, et al. (1974) showed no difference between normal and denervated rat diaphragm muscle with IC_{50} values for d-tubocurarine of 1.2×10^{-8} and 1.1×10^{-8}M, respectively.

The K_I values for the sculpin and other species are in Table 3. K_I is a measure of the binding affinity of the inhibitor for the

Table 3. K_I values for various ligands inhibiting the binding of labeled α-Butx to the acetylcholine receptors in several animal species.

Ligand	Animal/Tissue	K_I	Reference
Tubocurarine			
	Sculpin-Pectoral Abductor	5.9×10^{-6}	
	Rat-Diaphragm	2.4×10^{-7}	Colquhoun and Rang 1976
	Rat-Diaphragm Denervated	1.1×10^{-8}	Alper et al. 1974
	Electrophorus-Electric Organ	1.7×10^{-7}	Weber and Chanqeux 1974
	Cat-Lower Leg Denervated Soluble Extract	3.4×10^{-7}	Dolly and Barnard 1974
	Frog-Sartorius	7.3×10^{-7}	Jenkinson 1960
Acetylcholine			
	Sculpin-Pectoral Abductor	2.1×10^{-5}	
	Rat-Diaphragm Denervated	4.7×10^{-5}	Colquhoun and Rang 1976
	Mammalian Skeletal Denervated Soluble Extract	1.0×10^{-6}	Dolly and Barnard 1977
Decamethonium			
	Sculpin-Pectoral Abductor	3.3×10^{-5}	
	Rat-Diaphragm Denervated	2.1×10^{-6}	Colquhoun and Rang 1976
	Mammalian Skeletal Denervated	5.0×10^{-7}	Dolly and Barnard 1977
Atropine			
	Sculpin-Pectoral Abductor	6.0×10^{-5}	
Epinephrine			
	Sculpin-Pectoral Abductor	$>2.6 \times 10^{-3}$	

binding site. The K_D constant calculated by Scatchard analysis was used to determine the K_I. The sculpin K_I for tubocurarine is at least one order of magnitude larger than other values. The sculpin K_I for decamethonium and atropine are also larger than the values reported in other animals.

The drug concentrations which reach the receptors in the in situ study can be approximated, since the blood volume of the sculpin is equal to 7.3% of its body weight (Sleet, 1981). Assuming that the drugs are equally distributed throughout the circulatory system, the drug concentration which results in approximately a 50% decrease in twitch can be determined (d-tubocurarine, 5.0×10^{-6}M; decamethonium, 2.2×10^{-6}M; atropine sulfate, 2.0×10^{-4}). These results are similar to their IC_{50} values, except for decamethonium which has an IC_{50} 15X greater than its neuromuscular blocking concentration. When the potency of drugs as neuromuscular blocking agents is compared to their inhibition of [^{125}I]-α-Butx binding, the order of potency is the same for both studies: tubocurarine > decamethonium > atropine sulfate. These preliminary results indicate that the receptor binding analysis may prove to be a useful tool for understanding the mechanism of action of various agents at the fish neuromuscular junction.

Of the two fish which survived until the end of the third week of denervation, only one showed an increase (3X) in Bmax. A 20 fold increase can occur in denervated rat diaphragm 20 days after nerve section (Chiu et al. 1973). In most receptor binding assays, researchers are working with purified receptors of the muscle endplates which may make the increase in Bmax more apparent. Although

the function of the extrajunctional receptors is not known, Brockes and Hall (1975a) suggest that they may be important for formation of the neuromuscular junction. Brockes and Hall (1975b) examined both junctional and extrajunctional receptors of the denervated rat diaphragm and conclude that both receptors are similar but are distinct molecules. One difference they did note was that d-tubocurarine was more effective in decreasing the rate of toxin binding to junctional rather than extrajunctional receptors.

When the in situ neuromuscular portion of this study is combined with the conclusion of Schneider and Weber (1975) it becomes apparent that the basic cholinergic characteristics of the skeletal muscles of the two teleosts are different from those of mammals. In both the sculpin and the black bass, the ability to maintain an indirectly elicited tetanus was not abolished even after the animals had received doses of ACHE-I which were lethal to the fish. In the sculpin, twitch potentiation was not observed after the administration of ACHE-I. These discrepancies could be explained by differences between teleost and mammalian acetylcholinesterase, particularly since fish are poikilotherms and the enzymes operate at different temperatures. However, Schneider and Weber (1974, 1975) have shown that bass do have true acetylcholinesterase, and greater than 97% of it can be inhibited and tetanus is still not depressed. It is apparent from the studies on both the sculpin and the bass that acetylcholinesterase is not as important for neuromuscular transmission in these species of fish as in other vertebrates. This implies that other mechanisms for termination of acetylcholine activity may exist.

The [^{125}I]-α-Butx binding to the pectoral abductor muscle homogenates illustrates the nicotinic nature of this binding. As expected, d-tubocurarine, a nicotinic antagonist, was the most potent of the binding agents studied and epinephrine, an adrenergic agonist, was the least potent. However, it is notable that K_I and IC_{50} values for d-tubocurarine in the sculpin are up to three orders of magnitude higher than those reported in mammals. The high K_D and the apparent competitive binding of the [^{125}I]-α-Butx seen in the dissociation experiments also show that there are definite qualitative as well as quantitative differences between this system and the neuromuscular system of mammals.

The different [^{125}I]-α-Butx binding characteristics may indicate that the interaction between acetylcholine and the receptor is different than in mammalian systems. However, the K_I values for acetylcholine in the sculpin are close to those of mammalian studies which indicates that both receptors have similar affinities for acetylcholine.

This study also indicates that the sculpin is an interesting model to study the neuromuscular junction in fish, especially with regards to the effects of ACHE-I at this site. To further understand this system, additional comparative studies need to be completed.

BIBLIOGRAPHY

Albuquerque, E. X., A. T. Eldefrawi and M. E. Eldefrawi. 1979. The use of snake venoms for the study of the acetylcholine receptor and its ion conductance modulator. In: Snake Venoms. Ed. C. Y. Lee. Hndbch. d Exper. Pharmakol., Vol. 52. Springer-Verlag, Berlin. pp. 377-402.

Alper, R., J. Lowry and J. Schmidt. 1974. Binding properties of acetylcholine receptors extracted from normal and from denervated rat diaphragm. FEBS Lett. 48:130-132.

Anderson, P., J. K. S. Jansen and Y. Loyning. 1963. Slow and fast muscles in the Atlantic hagfish. Acta Physiol. Scand. 56:167-179.

Barnard, E. A., T. H. Chiu, J. Jedrzejcyzk, C. W. Porter and J. Wieckowski. 1973. Acetylcholine receptor and cholinesterase molecules of vertebrate skeletal muscles and their nerve junctions. In: Drug Receptors. Ed. H. P. Rang. University Park Press, Baltimore. pp. 211-224.

Barnard, E. A., J. O. Dolly, C. W. Porter and E. X. Albuquerque. 1975. The acetylcholine receptor and ionic conductance modulation system of skeletal muscle. Exp. Neurol. 48:1-28.

Bennett, M. V. L. 1970. Comparative physiology in electric organs. A. Rev. Physiol. 32:471-528.

Bennett, M. V. L. 1978. Methods in binding studies. In: Neurotransmitter Receptor Binding. Ed. H. I. Yamamura, S. J. Enna and M. J. Kuhar. Raven Press, New York. pp. 57-90.

Bone, Q. 1964. Patterns of muscle innervation in lower chordates. Int. Rev. Neurobiol. 6:99-147.

Bowman, W. D. 1964. Neuromuscular Blocking Agents. In: Evaluation of Drug Activities: Pharmacometrics, Vol. 1. Ed. D. R. Laurence and A. L. Bacharach. Academic Press, New York. pp. 325-351.

Brockes, J. P. and Z. W. Hall. 1975a. Acetylcholine receptors in normal and denervated rat diaphragm muscle I. Purification and innervation with ^{125}I-α-Bungarotoxin. Biochem. 14(10):2092-2099.

Brockes, J. P. and Z. W. Hall. 1975b. Acetylcholine receptors in normal and denervated rat diaphragm muscle II. Comparison of junctional and extrajunctional receptors. Biochem. 14(10): 2100-2106.

Brown, G. L. and A. M. Harvey. 1938. Reactions of avian muscle to acetylcholine and eserine. J. Physiol. 94:101-117.

Burt, D. R. 1978. Criteria for receptor identification. In: Neurotransmitter Receptor Binding. Ed. H. I. Yamamura, S. J. Enna and M. J. Kuhar. Raven Press, New York. pp. 42-55.

Bylund, D. B. 1980. Analysis of Receptor Binding Data. In: Receptor Binding Techniques. Society for Neuroscience, Short Course Syllabus. pp. 70-99.

Chang, C. C. 1979. The action of snake venoms on nerve and muscle. In: Snake Venoms. Ed. C. Y. Lee. Hndbch. d Exper. Pharmakol., Vol. 52. Springer-Verlag, Berlin. pp. 309-376.

Chang, C. C., T. F. Chen and S. T. Chuang. 1973. [^{3}H]-α-bungarotoxin as a specific labeling agent of cholinergic receptors. Br. J. Pharmacol. 47:147-160.

Chang, C. C. and C. Y. Lee. 1963. Isolation of neurotoxins from the venom of Bungarus multicinctus and their modes of neuromuscular blocking. Arch. Int. Pharmacodyn. Ther. 144:241-257.

Changeux, J. P., M. Kasai and C. Y. Lee. 1970. Use of snake venom to characterize the cholinergic receptor protein. Proc. Natl. Acad. Sci. U.S.A. 67:124-127.

Chiu, T. H., J. O. Dolly and E. A. Barnard. 1973. Solubilization from skeletal muscle of two components that specifically bind α-bungarotoxin. Biochem. Biophys Res. Commun. 51(1):205-213.

Colquhoun, J. M. and H. P. Rang. 1976. Effects of inhibitors on the binding of iodinated α-bungarotoxin to acetylcholine receptors in rat muscle. Mol. Pharmacol. 12:519-535.

Diamond, J. and J. Mellanby. 1971. The effects of tetanus toxin in the goldfish. J. Physiol. 215:727-741.

Dolly, J. O. 1979. Biochemistry of acetylcholine receptors from skeletal muscle. In: Neurochemistry and Biochemical Pharmacology. Ed. K. F. Tipton. International Review of Biochemistry, Vol. 26. University Park Press, Baltimore. pp. 257-309.

Dolly, J. O. and E. A. Barnard. 1974. Affinity of cholinergic ligands for the partially purified acetylcholine receptor from mammalian skeletal muscle. FEBS Lett. 46(1):145-148.

Dolly, J. O. and E. A. Barnard. 1977. Purification and characterization of an acetylcholine receptor from mammalian skeletal muscle. Biochem. 16(23):5053-5060.

Eldefrawi, A. T., M. E. Eldefrawi, E. X. Albuquerque, A. C. Oliveira, N. Mansour, M. Alder, J. W. Daly, G. B. Brown, W. Burgermeister and B. Witkop. 1977. Perhydrohistrionicotoxin: a potential ligand for the ion conductance modulator of the acetylcholine receptor. Proc. Natl. Acad. Sci. U.S.A. 74:2172-2176.

Eterovic, V. A., M. S. Herbert and E. L. Bennett. 1975. The lethality and spectroscopic properties of toxins from Bungarus multicinctus (Blyth) venom. Ann. Rev. Pharmacol. 8:299-318.

Faff, J., T. Rabsztyn and S. Rump. 1973. Investigations on the correlation between abnormalities of neuromuscular transmission due to organophosphates and activity of acetylcholinesterase in skeletal muscle. Arch. Toxikol. 31:31-38.

Froehner, S. C., C. G. Reiness and Z. W. Hall. 1977. Subunit structure of the acetylcholine receptor from denervated rat skeletal muscle. J. Biol. Chem. 252:8589-8596.

Ginsborg, B. L. 1960. Some properties of avian skeletal muscle fibers with multiple neuromuscular junctions. J. Physiol. (London) 154:581-598.

Greer-Walker, M. and G. A. Paul. 1975. A survey of the red and white muscle in marine fish. J. Fish. Biol. 7:295-300.

Hidaka, T. and H. Kuriyama. 1969. Effects of catecholamines on the cholinergic neuromuscular transmission in fish red muscle. J. Physiol. 201:61-71.

Hidaka, T. and N. Toida. 1969. Biophysical and mechanical properties of red and white fibers in fish. J. Physiol. 201:49-59.

Hobbiger, F. 1976. Pharmacology of anticholinesterase drugs. In: Neuromuscular Junction. Ed. E. Zamis. Hndbch. d Exper. Pharmakol., Vol. 42. Springer-Verlag, Berlin. pp. 487-581.

Jenkinson, D. H. 1960. The antagonism between tubocurarine and substances which depolarize the motor end-plate. J. Physiol. (London) 152:309-324.

Jiménez-Porras, J. M. 1968. Pharmacology of peptides and proteins in snake venoms. Ann. Rev. Pharmacol. 8:299-318.

Karczmar, A. G. and Y. Ohta. 1981. Neuromyopharmacology as related to anticholinesterase action. Fund. Appl. Toxicol. 1:135-142.

Katz, B. 1966. Nerve, Muscle, Synapse. McGraw-Hill Book Co., New York. 193 pp.

Klika, L. J. 1980. An investigation of the blood brain barrier in the rainbow trout (Salmo gairdneri). M. S. Thesis. Oregon State University. 35 pp.

Kuba, K. 1969. The action of phenol on neuromuscular transmission in the muscle of fish. Jap. J. Physiol. 19:762-774.

Kuffler, S. W. and E. M. Vaughan-Williams. 1953. Properties of the slow skeletal muscle fibers of the frog. J. Physiol. 121:318-340.

Lee, C. Y. 1972. Chemistry and pharmacology of polypeptide toxins in snake venoms. Ann. Rev. Pharmacol. 12:265-286.

Lee, C. Y. and L. F. Tseng. 1966. Distribution of Bungarus multicinctus venom following envenomation. Toxicon 3:281-290.

Lee, C. Y. and L. F. Tseng. 1969. Species differences in susceptibility to elapsid venoms. Toxicon 7:89-93.

Lee, C. Y., L. F. Tseng and T. H. Chiu. 1967. Influence of denervation on localization of neurotoxins from elapsid venoms in rat diaphragm. Nature 215:1177-1178.

Loomis, T. A. and B. Salafsky. 1964. Some effects of soman on neuromuscular function and on acetylcholinesterase in the rat. J. Pharmacol. Exp. Ther. 144:301-309.

Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall. 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. 193:265-267.

Lukasiewicz, R. and E. L. Bennett. 1978. α-Bungarotoxin binding properties of a central nervous system nicotinic acetylcholine receptor. Biochem. Biophys. Acta. 544:294-308.

Maclagan, J. 1976. Competitive neuromuscular blocking drugs. In: Neuromuscular Junction. Ed. E. Zamis. Hndbch. d Exper. Pharmakol., Vol. 42. Springer-Verlag, Berlin. pp. 421-486.

Maelicke, A., B. W. Fulpius, R. P. Klett and E. Reich. 1977. Acetylcholine receptor: responses to drug binding. J. Biol. Chem. 252(14):4811-4830.

Mebs, D., K. Narita, S. Iwanga, Y. Samejima and C. Y. Lee. 1971. Amino acid sequence of α-bungarotoxin from the venom of Bungarus multicinctus. Biochem. Biophys. Res. Comm. 44:711-716.

Mellanby, J. and P. A. Thompson. 1972. The effects of tetanus toxin at the neuromuscular junction in the goldfish. J. Physiol. 224:407-419.

Miledi, R. and L. T. Potter. 1971. Acetylcholine receptors in muscle fibers. Nature 233:599-603.

Patterson, S. and G. Goldspink. 1972. The fine structure of the red and white myotomal muscle fibers of the coalfish (Gadus virens). Z. Zellforsch. mikrosk. Anat. 133:463-474.

Potter, L. T. 1980. Methods for nicotine and muscarine receptors. In: Receptor Binding Techniques. Society for Neuroscience, Short Course Syllabus. pp. 123-139.

Rang, H. P. 1974. Acetylcholine receptors. A. Rev. Biophys. 7:283-399.

Schneider, P. W., Jr. 1972. Ph.D. Thesis Proposal. Oregon State University. 13 pp. (unpublished).

Schneider, P. W., Jr. 1974. Aspects of comparative neuromuscular physiology of the black bass (Micropterus salmoides) as evidenced by the action of diisopropylflurophosphate. Ph.D. Thesis. Oregon State University. 98 pp.

Schneider, P. W., Jr. and L. J. Weber. 1974. Identification and characterization of acetylcholinesterase from fish (Micropterus salmoides) pectoral muscles. Proc. Soc. Exp. Biol. Med. 146:1071-1075.

Schneider, P. W., Jr. and L. J. Weber. 1975. Neuromuscular function and acetylcholinesterase in pectoral abductor muscle of the black bass (Micropterus salmoides) as evidenced by the effects of diisopropylflurophosphate. J. Fish. Rev. Board Can. 32(11): 2153-2161.

Sleet, R. B. 1981. The relationship between the gut and water-electrolyte of a marine teleost, Enophrys bison (Girard). Ph.D. Thesis. Oregon State University. 114 pp.

Takeuchi, A. 1959. Neuromuscular transmission of fish skeletal muscles investigated with intracellular microelectrode. J. Cell. Comp. Physiol. 54:211-220.

Taylor, P. 1980a. Anticholinesterase Agents. In: The Pharmacological Basis of Therapeutics. 6th ed. Ed. A. G. Gillman, L. S. Goodman and A. Gillman. MacMillan Publishing Co., Inc., New York. pp. 100-119.

Taylor, P. 1980b. Neuromuscular Blocking Agents. In: The Pharmacological Basis of Therapeutics. 6th ed. Ed. A. G. Gillman, L. S. Goodman and A. Gillman. Macmillan Publishing Co., Inc., New York. pp. 220-234.

To, S. and S. Tin. 1943. Toxicologische untersuchungen betreffs des Giftes von Bungarus multicinctus. J. Formosan Med. Assoc. 42:(suppl.)8, 1.

Weber, M. and J. P. Changeux. 1974. Binding of Naja nigricallis [^{3}H]-toxin to membrane fragments from Electrophorus and Torpedo electric organs: II. Effect of cholinergic agonists and antagonists on the binding of tritiated α-neurotoxin. Mol. Pharmacol. 10:15-34.

Weiner, N. 1980. Atropine, Scopolamine and Related Antimuscarinic Drugs. In: The Pharmacological Basis of Therapeutics. 6th ed. Ed. A. G. Gillman, L. S. Goodman and A. Gilman. MacMillan Publishing Co., Inc., New York. pp. 120-137.

Yamamoto, L. 1972. Electrical and mechanical properties of the red and white muscles in silver carp. J. Exp. Biol. 57:551-567.

Zamis. E. 1953. Motor end-plate differences as a determining factor in the mode of action of neuromuscular blocking substances. J. Physiol. (London) 122:238-251.

Zamis. E. 1954. The interruption of neuromuscular transmission and some of its problems. Pharmacol. Rev. 6:53-57.

Zamis. E. and S. Head. 1976. Depolarizing Neuromuscular Blocking Drugs. In: Neuromuscular Junction. Ed. E. Zamis. Hndbch. d Exp. Pharmakol., Vol. 42. Springer-Verlag, Berlin. pp. 365-486.

APPENDIX

Figure 16. d-Tubocurarine inhibition of [^{125}I]-α-Butx binding to pectoral abductor muscle homogenates of the buffalo sculpin. Specific [^{125}I]-α-Butx sites bound (%) are plotted against concentration of d-tubocurarine preincubated with homogenate.

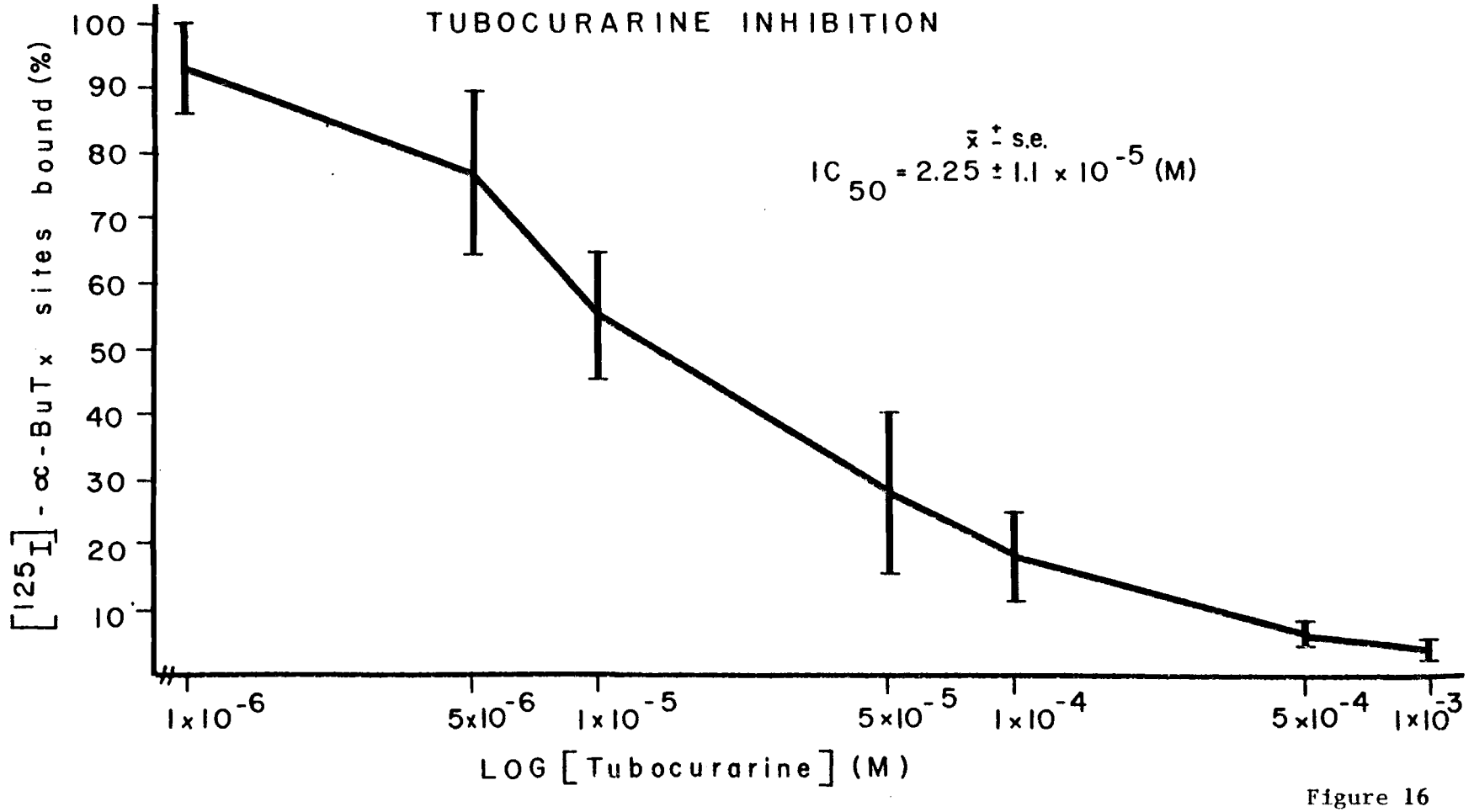

TUBOCURARINE INHIBITION
$\bar{x} \pm$ s.e.
$IC_{50} = 2.25 \pm 1.1 \times 10^{-5}$ (M)
[125I] - α-BuTx sites bound (%)
100
90
80
70
60
50
40
30
20
10
1x10-6
5x10-6
1x10-5
5x10-5
1x10-4
5x10-4
1x10-3
LOG [Tubocurarine] (M)

Figure 16

Figure 17. Acetylcholine inhibition of [^{125}I]-α-Butx binding to pectoral muscle homogenates of the buffalo sculpin. Specific [^{125}I]-α-Butx sites bound (%) are plotted against concentration of acetylcholine preincubated with homogenate.

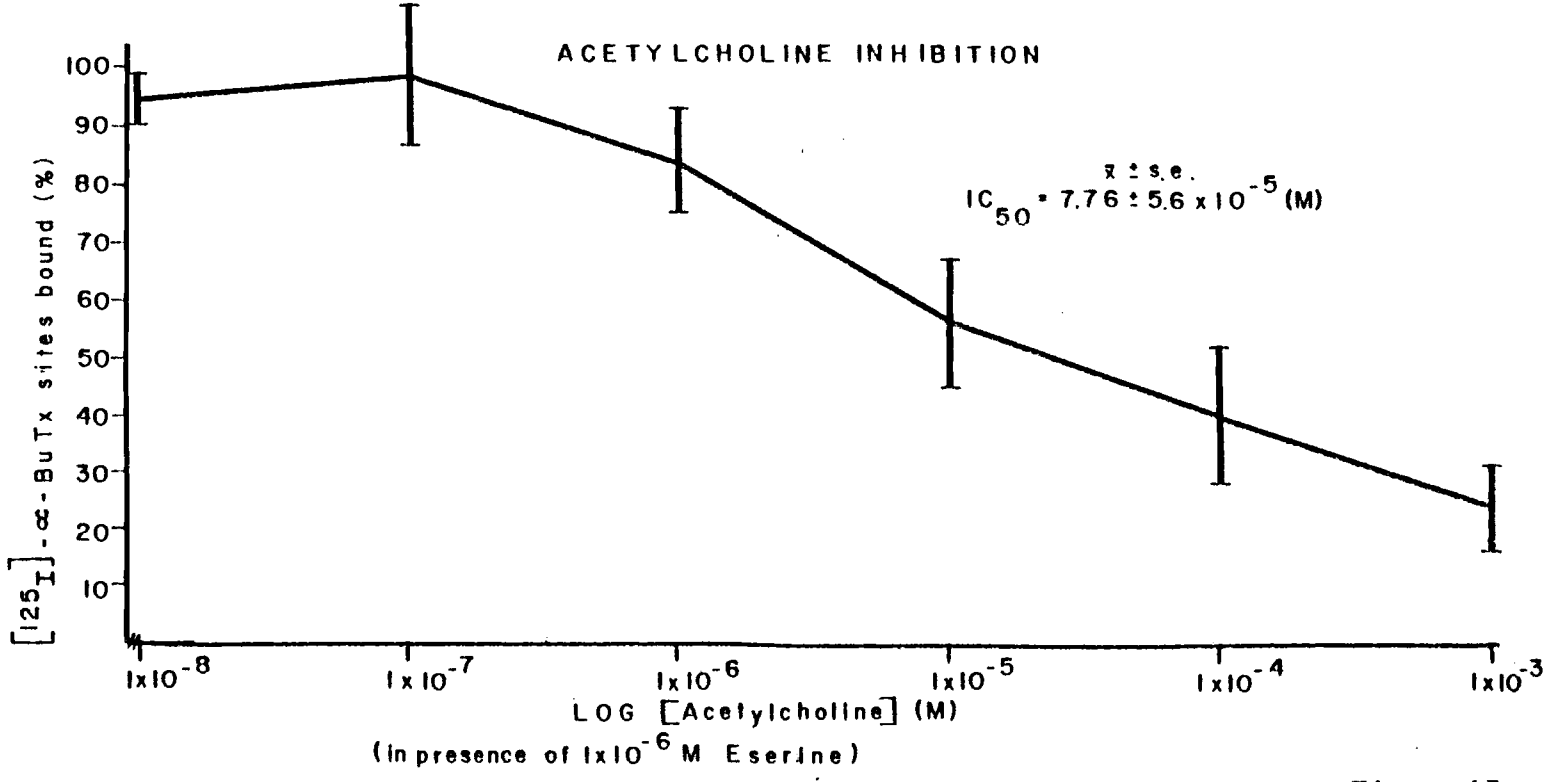

ACETYLCHOLINE INHIBITION
x̄ ± s.e.
IC50 = 7.76 ± 5.6 x 10-5 (M)
[125I]-α-BuTx sites bound (%)
100
90
80
70
60
50
40
30
20
10
1x10-8
1x10-7
1x10-6
1x10-5
1x10-4
1x10-3
LOG [Acetylcholine] (M)
(In presence of 1x10-6 M Eserine)

Figure 17

Figure 18. Atropine sulfate inhibition of [^{125}I]-α-Butx binding to pectoral abductor muscle homogenates of the buffalo sculpin. Specific [^{125}I]-α-Butx sites bound (%) are plotted against concentration of atropine sulfate preincubated with homogenate.

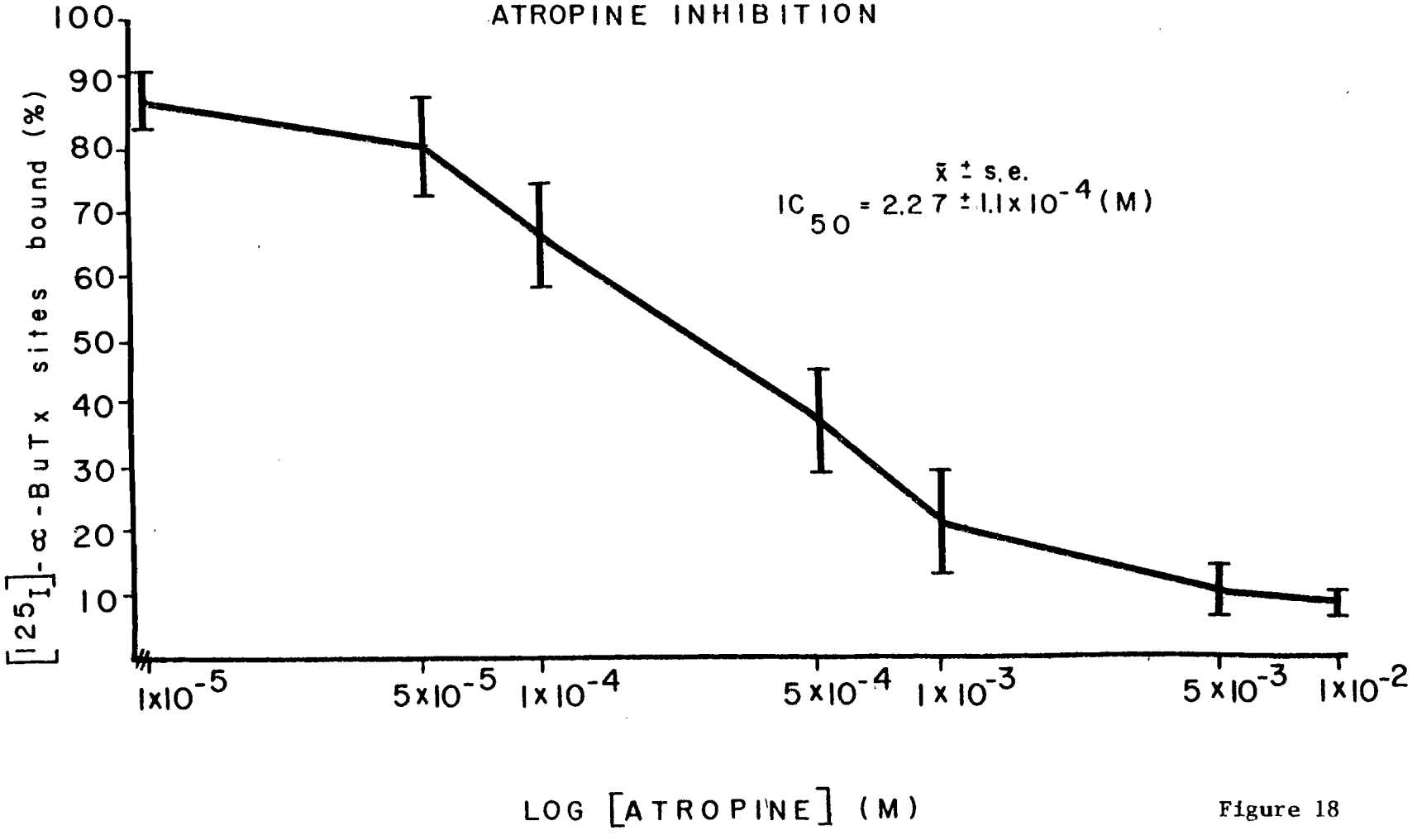

ATROPINE INHIBITION
100
90
80
70
60
50
40
30
20
10
[125I]-α-BuTx sites bound (%)
x̄ ± s.e.
IC50 = 2.27 ± 1.1 x 10-4 (M)
1x10-5
5x10-5
1x10-4
5x10-4
1x10-3
5x10-3
1x10-2
LOG [ATROPINE] (M)

Figure 18

Figure 19. Decamethonium inhibition of [^{125}I]-α-Butx binding to pectoral abductor muscle homogenates of the buffalo sculpin. Specific [^{125}I]-α-Butx sites bound (%) are plotted against concentration of decamethonium preincubated with homogenate.

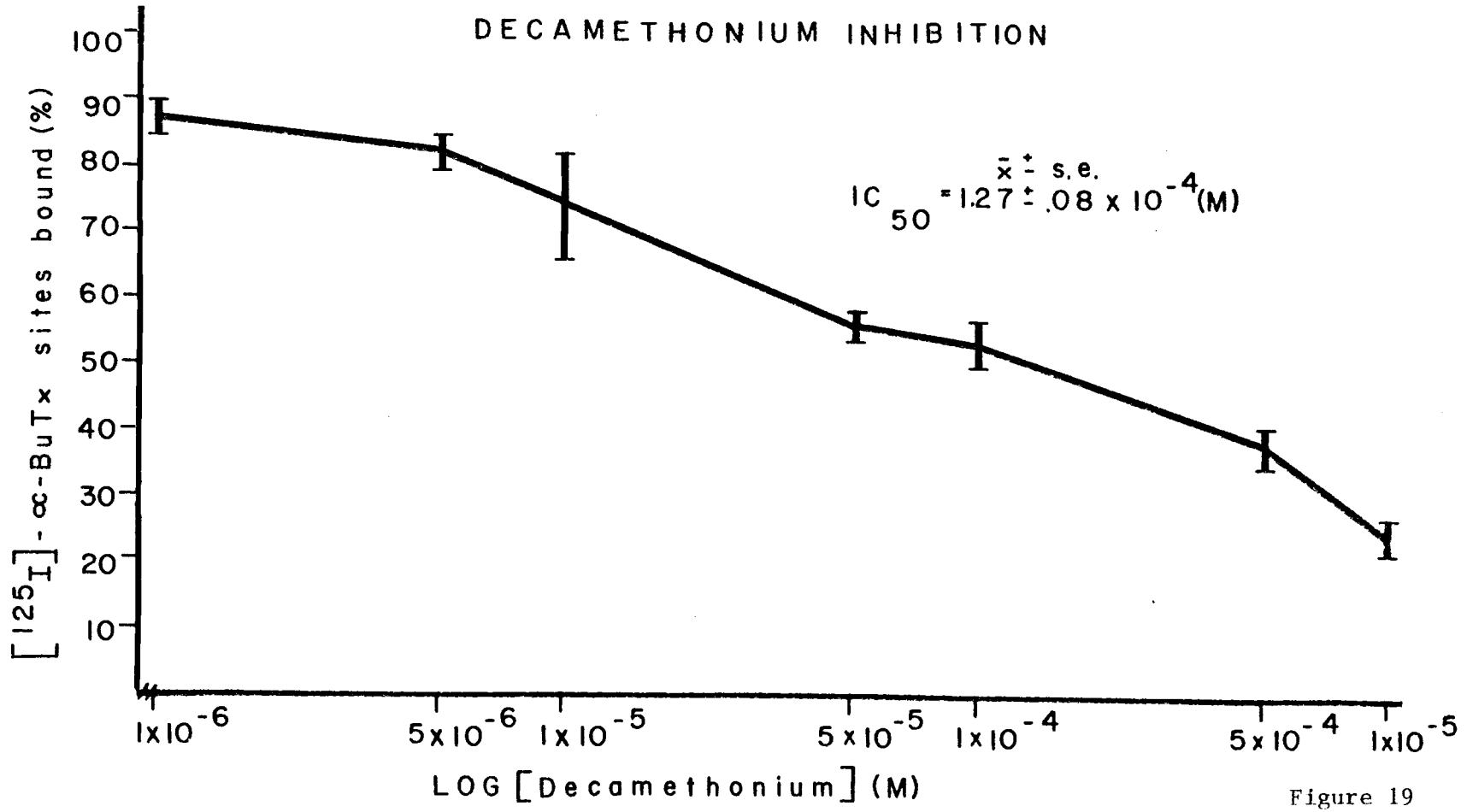

DECAMETHONIUM INHIBITION
$\bar{x} \pm$ s.e.
$IC_{50} = 1.27 \pm .08 \times 10^{-4}$(M)
[125I]-α-BuTx sites bound (%)
100
90
80
70
60
50
40
30
20
10
1x10-6
5x10-6
1x10-5
5x10-5
1x10-4
5x10-4
1x10-5
LOG [Decamethonium] (M)

Figure 19

Figure 20. Epinephrine inhibition of [^{125}I]-α-Butx binding sites to pectoral muscle homogenates of the buffalo sculpin. Specific [^{125}I]-α-Butx sites bound are plotted against concentration of epinephrine preincubated with homogenate.

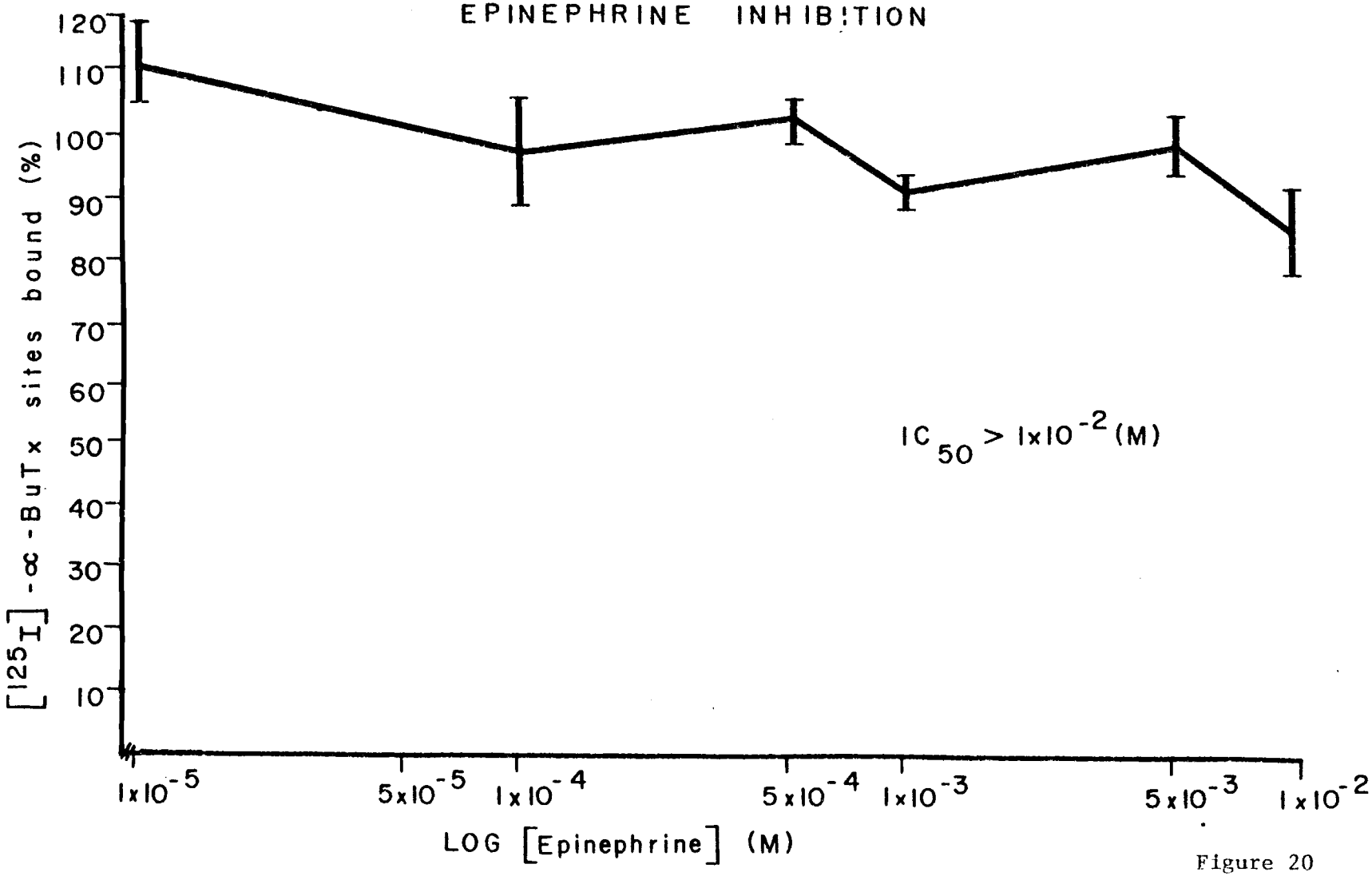

EPINEPHRINE INHIBITION
120
110
100
90
80
70
60
50
40
30
20
10
[125I]-α-BuTx sites bound (%)
IC50 > 1x10-2 (M)
1x10-5
5x10-5
1x10-4
5x10-4
1x10-3
5x10-3
1x10-2
LOG [Epinephrine] (M)

Figure 20