STUDIES ON APHANTOXIN FROM *APHANIZOMENON FLOS-AQUAE* IN NEW HAMPSHIRE

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ABSTRACT

Toxic Cyanobacteria (blue-green algae) bloom in eutrophic, freshwater lakes and ponds in New England and have caused environmental, health, legal and recreational problems over the past 15 years. Although several species have been implicated with animal kills and water fouling, a common offender was *Aphanizomenon flos-aquae*. Representative strains of *A. flos-aquae* bloom in New Hampshire intermittently, in both toxic (aphantoxins) and non-toxic forms. Research has focused on methods of: a) toxin accumulation from natural blooms and laboratory cultivation, b) toxin assay, using the mouse bioassay and a modified fluorometric technique developed for paralytic shellfish poisons, c) toxin characterization and purification, using solvent separation and molecular weight filters, and d) testing active extracts on nerve and muscle preparations to determine the specific sites and modes of action of aphantoxins.

Aphantoxin samples were passed through molecular weight filters (10,000 and 500 daltons), lyophilized and weighed, prior to physiological testing. Microgram quantities of toxin reversibly blocked compound action potentials in amphibian nerves as well as mechanical activity in skeletal muscle. No effect was measured on the transmembrane resting potential or on spontaneous miniature

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end-plate potentials (mepps). Tests on lateral and medial giant axons from crayfish gave similar results. The Na^+ dependence of the crayfish preparation was verified. The aphantoxins (4 µg/ml) reversibly blocked intracellular recordings of action potentials with no alteration of the resting potential. Amphibian and crustacean cardiac activity was blocked in diastolic arrest, while bivalve hearts were unaffected at increased dose levels. Aphantoxins may block excitability by affecting ion conductance pathways as do toxins from several marine dinoflagellates and may be useful in basic studies on membrane systems.

INTRODUCTION

Freshwater blooms of toxic Cyanobacteria (blue-green algae) are common in many countries of the world. Animal, including human, involvement with toxicity problems have been reported for at least 12 countries, 4 Canadian provinces, and 10 of the United States (Schwimmer and Schwimmer, 1964 and 1968; Moore, 1977; Collins, 1978). Although many microorganisms have been implicated with water fouling and animal kills, a frequent offender is *Aphanizomenon flos-aquae*. Representatives of this species occur in New England intermittently, in both toxic and non-toxic forms. Environmental problems may arise when these Cyanobacteria are involved in phytoplankton blooms. A critical time during bloom conditions occurs when dense cell masses decompose naturally or with the aid of algicides (e.g., copper sulphate) commonly used to enhance water quality. The decomposition products plus toxic cellular materials released into the water when the cells lyse may cause death or illness to mammals, birds, and fishes, and may reduce water quality for animal (including human) consumption and recreational purposes (Collins, 1978; Palmer, 1964; Schwimmer and Schwimmer, 1964).

Blooms of toxic Cyanobacteria occur in several freshwater lakes, ponds and reservoirs in New Hampshire, some of which are used for water supplies and/or recreational purposes. The environmental effects of these noxious blooms have caused concern from state water quality control agencies (New Hampshire Water Supply and Pollution Control Commission Staff Reports, Nos. 59, 62, 63, 64, 70; 1973-1975). In addition, at least one legal case in New Hampshire focused attention on sewage treatment effluent and toxic cyanophyte blooms in Kezar L., North Sutton, New Hampshire (W. A. Sundall et al., versus Town of New London, 1977). The environmental parameters associated with toxic blooms in this lake have been described by Haynes (1971) and in the reports enumerated above.

Biotoxins attract interest and attention from researchers because of their specificity, potency, and potential utility as

physiological and/or pharmacological "tools" (O'Brien, 1969). This is particularly true when the toxin's effects are reversible, i.e., when a system can be blocked and then restored to normal activity. Aphantoxins meet most, if not all, of these criteria.

In 1968, Sawyer et al., demonstrated the presence of a very fast death factor (VFDF) from *A. flos-aquae* cells collected during blooms in two New Hampshire lakes. The toxin was dialyzable, heat and acid stable, alkali labile, and was soluble in water and ethanol, but non-soluble in less polar solvents. The same year (1968) Jackin and Gentile, using laboratory cultures, reported the partial purification and properties of aphantoxin. Three toxic fractions were obtained using acid extraction, preparative paper chromatography and silica gel column chromatography. The most potent of these gave three Weber reagent-positive spots, one of which corresponded in R_f value, reactions with Weber, ninhydrin and Jaffe reagents, and infrared spectrum with saxitoxin (STX). The latter (STX) is the paralytic shellfish poison (PSP) produced by marine dinoflagellates of the genus *Gonyaulax* and found in bivalves that act like "biological storage depots" for toxin accumulation in nature. The active material contained 1.5 to 2.0 µg per mouse unit (MU) which is equal to 500 to 667 MU/mg.

In 1973, Alam et al., reported the partial purification and properties of aphantoxin obtained from natural blooms in Kezar Lake, North Sutton, New Hampshire. Extraction and purification was done using acid, alcohol, and chloroform extractions and preparative high voltage electrophoresis. A toxic ninhydrin and Weber reagent positive zone was eluted and chromatographed on IRC-50 resin. An active fraction was eluted with acetic acid and purified further using preparative thin-layer chromatography (TLC). This resulted in a chromatographically homogeneous material with a potency of 745 MU/mg. Positive reactions of this material with Weber, diacetyl-α-naphthol, and Benedict-Behre reagents indicated that aphantoxin may be a substituted guanidine derivative. However, TLC in various solvent systems, color reactions given with various spray reagents, electrophoretic comparisons, and infrared spectra indicated that aphantoxin was not identical with saxitoxin (STX). Recent work by Alam et al. (1978), demonstrated that aphantoxin was a complex mixture of toxins containing saxitoxin and other related, but still unknown, substances. This mixture has not been completely characterized. In addition, Shoptaugh (1978) and Carter (1980) have shown that these toxic materials from *Aphanizomenon* may lend themselves to qualitative and quantitative analysis using alkaline-H_2O_2 oxidation and fluorometry; a method that is currently being tested with the aim of replacing the mouse bioassay.

Research at the University of New Hampshire has focused on the chemical properties, assay, and stability of the biotoxins from

Aphanizomenon flos-aquae. In addition, the physiological effects of the toxins were studied particularly as they effect neuromuscular systems (including cardiac) in vertebrate and invertebrate animals. Support for the research was provided by the New Hampshire Water Resources Research Center, G. Byers, Director.

MATERIALS AND METHODS

State agencies monitor algal bloom conditions regularly throughout New Hampshire and Vermont and their reports are available to us. We have routinely collaborated with the New Hampshire Water Pollution Control Commission on potential toxicity problems and on assessment of toxic cyanophytes prior to algicide treatment that could produce animal kills. In recent summers essentially unialgal blooms of Cyanobacteria occurred, intermittently, in Kezar Lake, Winnisquam Lake, Skatutakee Lake, Marsh Pond, Enfield Reservoir, Exeter Reservoir, several farm ponds and other freshwater environments. Cell concentrations generally exceeded 5×10^4 per milliliter, and during dense bloom conditions were $> 10^6$ per ml. An effective method for obtaining bulk quantities of material occurring during bloom conditions in remote locations employed DeLaval Separators, at lakeside, to spin and concentrate *Aphanizomenon* cells from large volumes of water. The crude materials were then stored in the frozen state, either wet or lyophilized. Samples retained potency under these conditions for more than seven years. Unialgal, but not bacteria-free, cultures were initiated from toxic clones of *Aphanizomenon* using serial dilution methods with solid (agar), then liquid media. The cultures were expanded to 20 liter carboys and grown in the synthetic, modified ASM-1 medium of Carmichael and Gorham (1974), under controlled conditions of temperature and illumination. Back-up cultures were maintained in incubators separate from the culture room to ensure against accidental equipment failure.

We have recently found that Amicon and Millipore molecular weight filters were useful for "cleaning up" samples of aphantoxin (Shoptaugh, 1978). This method separated the aphantoxins from high and intermediate molecular weight contaminants. We have successfully passed small amounts of the aphantoxin through the 500 dalton filter, prior to lyophilization, in preparation for chemical and physiological studies.

Mice (B6D2F1/J) were obtained from the Jackson Laboratory, Bar Harbor, Maine. Standard methods employed for biotoxins from marine microorganisms were used for the bioassay of the active materials (Halstead, 1965; Prakash et al., 1971). The mouse unit (MU) for aphantoxin was the same as that used to evaluate amounts of paralytic shellfish poison (PSP) in marine bivalves, i.e., the amount of material that killed mice (18 to 22 g) in 15 minutes = 1 MU. When only small amounts of purified aphantoxin were availa-

able, assay was accomplished using electrophysiological methods by measuring action potentials and tension development in muscle preparations. The mouse bioassay was also used in preliminary tests to determine if freshwater bivalves (*Elliptio complanatus*) act as "biological storage depots" for aphantoxin accumulation, i.e., like marine bivalves exposed to *Gonyaulax* toxins. In addition, tests were run on the sensitivity of *Daphnia magna* to aphantoxins from laboratory cultures.

Shoptaugh (1978) found that aphantoxins form fluorescent derivatives when treated with H_2O_2 (like saxitoxin and other PSP derivatives) and a promising fluorescence assay for PSP and aphantoxin was developed (see Ikawa et al., 1980, these proceedings).

The toxic extracts from the field and laboratory were tested on standard nerve and nerve-muscle preparations, from mammalian, amphibian, and crustacean species. Control preparations were challenged with similarly treated material from non-toxic extracts. Compound action potentials were recorded externally from amphibian sciatic nerves and crayfish medial and lateral giant axons (sheathed and desheathed) using Ag-AgCl electrodes, Grass or Tektronix stimulator, and Tektronix preamp and dual beam oscilloscope. Transmembrane resting potentials and unicellular action potentials employed 3M KCl-filled glass microcapillaries, Grass P-16 Amplifier and Tektronix C8D. In the muscle tests dual multi-electrode assemblies were used for direct stimulation with recordings displayed on a dual beam oscilloscope or write-out recorder. Isotonic/isometric measurements on skeletal and cardiac muscles employed Grass FT-03C Mechano-electrical transducers. Previous work in this laboratory (Sasner, 1973) demonstrated the utility of the amphibian sartorious nerve-muscle preparation as an assay tool particularly because of its sensitivity to several aquatic biotoxins. This preparation provides a consistently reproducible system widely utilized in muscle physiology. The sartorious muscle is composed of long, parallel fibers, is thin enough to allow simple gas exchange when excised, and performs well at low temperatures where transient physico-chemical phenomena associated with excitability are slowed.

Kezar Lake is located in the rural community of North Sutton, New Hampshire. It is appropriate to include information about this particular lake because: a) it has provided starter cultures and toxic materials to several research groups participating in this conference, and b) it is one of few lakes where physical, chemical and biological parameters have been monitored for extended periods of time. This information was compiled by Terrence P. Frost, Ronald E. Towne and the late Harry J. Turner of the New Hampshire Water Supply and Pollution Control Commission. Their Staff Reports Nos. 59, 64 and 79 (N.H.W.S.P.C.C.) provide a comprehensive history of the recent stages of the eutrophication process in Kezar Lake as well as attempts at coping with annual blooms of toxic Cyano-

bacteria. The lake has an area of approximately 180 acres with a maximum depth of 8.0 meters and an average depth of 3.7 meters. Over the years Kezar Lake was a popular recreational site and in 1934 Wadleigh State Park was established on the southeastern shore. During the early 1930's, the New London sewage treatment plant began discharging secondary waste-water into Lion Brook a few miles upstream from the lake. The nitrogen and phosphate concentrations greatly increased in the lake and this additional nutrient load accelerated the natural eutrophication processes. Copper sulphate treatment of microorganism blooms were successfully administered during the early 1960's to combat *Anabaena* blooms. Similar attempts at controlling *Aphanizomenon* blooms in the mid-1960's were not as effective and in a couple of instances produced massive fish mortalities. One particular treatment resulted in tons of dead fish (mostly perch). During the late 1960's the recreational utility of Kezar Lake diminished, property values decreased and the New Hampshire State Tax Commission reduced property appraisals by 30 percent. Attempts at mixing and destratification of the lake with large air compressors was moderately successful for several years and the recreational utility of Wadleigh State Park increased. Nutrient stripping and the addition of advanced waste-water treatment at the New London sewage plant reduced the discharge levels of phosphorus. However, toxic blooms still occur in Kezar Lake. The last few summers bloom periods of *Aphanizomenon* were shortened and replaced by *Microcystis aeruginosa* (= *Anacystis cyanea*). Thus the history of cyanophyte blooms in this lake includes *Anabaena*, *Aphanizomenon* and *Microcystis*; the organisms of major concern in these conference proceedings.

RESULTS AND DISCUSSION

The effects exhibited by whole organisms, either injected with or bathed in toxic samples of *Aphanizomenon flos-aquae*, were qualitatively similar to those reported for several marine poisons. The characteristic symptoms in mice challenged with aphantoxins include spastic twitching, irregular ventilation, gaping mouth, coordination loss, violent tremors and subsequent death by respiratory failure. Mouse bioassay of bivalve tissue (*Elliptio complanatus*), either collected during a bloom or fed *A. flos-aquae* from lab cultures, produced these same symptoms. Twarog and Yamaguchi (1975) showed that *Elliptio* were more sensitive to saxitoxin and tetrodotoxin than several marine bivalves and our preliminary studies with aphantoxin may support these findings. Laboratory fed *Elliptio* were used because of the scarcity of these animals in Kezar Lake, where they were formerly abundant. Examination of bivalve gut contents showed a large percentage of broken *Aphanizomenon* cells indicating at least partial digestion of the algal material. After a two day exposure to lab cultures, the bivalves were themselves

Table 1. Summary of Toxin Characteristics from *Aphanizomenon flos-aquae*

Name(s)	Aphantoxin; very fast death factor (VFDF); endotoxin.
Form in Nature and Lab Culture	Single trichromes, 25 to 70 cells long.
Method of Collection	DeLaval Separators at lakeside concentrate *Aphanizomenon* from large volumes of water.
Chemical	H_2O and ETOH soluble, $CHCl_3$ insoluble; acid and heat stable, alkali labile, low mol. weight < 500, guanidine derivative, may contain saxitoxin (STX) + 3 unknown substances; forms fluorescent derivatives upon alkaline-H_2O_2 oxidation.
General Effects	Mammals and Fish: spastic twitching, coordination loss, respiratory irregularity; freshwater bivalves may store aphantoxin like PSP in marine molluscs. Some planktonic crustaceans (*Daphnia*) paralyzed -- reversible.
Bioassay (i.p.)	1 MU = amount of toxin to kill 20 ± 2 g mice in 15 minutes. Similar to mouse bioassay for paralytic shellfish poison (PSP) from marine bivalves (*Tonyaulax*).
Dose Range (mammals)	Lyophilized cells = 10 mg/kg; purified = 745 MU/mg.
Excised Tissues	0.4 to 100 µg/ml, to elicit a muscle block.
Nerve-Muscle Action Potentials	Blocked in desheathed nerves and muscle -- reversible.
Resting Trans-Membrane Potential	No effect.
Muscle Mechanical Activity	Vert. skeletal-block to indirect then direct stimulation; Vert. and Crustacean heart block -- diastolic arrest; cardiac A.P. reduced, reversible; Mollusc heart -- no effect at increased doses (x 100).
Site of Action	Nerve and muscle membranes.
Mode of Action	Ion conductance pathways blocked.
References	Sawyer et al., 1968; Alam, 1972, 1973, 1978; Gentile, 1971; Thurberg, 1972; Sasner, 1973; Sasner & Ikawa, 1975; Shoptaugh, 1978; Carter, 1980.

affected by the toxin in a manner similar to soft-shell clams after exposure to massive concentrations of the "red tide" dinoflagellate *Gonyaulax tamarensis*. The *Elliptio* exhibited flaccid paralysis of the foot and mantle tissue; a condition that was reversed by replacing the *Aphanizomenon* culture with clean medium. Since freshwater bivalves are consumed by humans in certain parts of California, further research in this area may be of more than academic interest. It was estimated that 50,000 to 100,000 pounds per year of the freshwater bivalve (*Corbicula fluminea*) are available in the California marketplace without prior testing for consumer safety (Jerome Jenkin, Pacific Shellfish Company, personal communication).

The cladoceran *Daphnia magna* was also affected when placed in aerated cultures of *Aphanizomenon*. Within a few minutes, the characteristic movements of the second antennae were reduced, erratic and subsequently blocked. This paralysis of the swimming appendages caused the *Daphnia* to settle to the bottom of the container and perish in approximately 24 hours. Animals removed to clean water after exposure for 12 to 16 hours recovered within 24 hours. Zooplankton were usually absent in plankton samples collected from the upper one meter, during natural blooms of *Aphanizomenon*.

Table 1 presents a summary of the chemical and physiological characteristics of the toxins from *Aphanizomenon flos-aquae*. This information was compiled using unextracted lyophilized materials and partially purified samples, after passage through 500 dalton molecular weight filters. The acute sensitivity of neuromuscular systems and the potency of aphantoxins were demonstrated in both vertebrate and invertebrate preparations. Figure 1 shows the isometric tension developed in amphibian sartorious muscle challenged with 50 μg aphantoxin per milliliter. The mechanical response to indirect (via nerve) stimulation decreased faster than to direct (via muscle) stimulation, indicating a greater sensitivity of the nerves or a greater diffusion barrier in the muscle tissue. The effects of the toxin were readily reversible and showed a dependency on the exposure time in toxin. No change in the transmembrane resting potential of the sartorious muscle fibers was recorded during tension reduction and recovery. In addition, no effect was measured on spontaneous miniature end-plate potentials (mepps). Sheathed sciatic nerves were not affected by the toxin in large doses, whereas partially desheathed nerves were blocked with 10 μg per milliliter within a few minutes. The decrease in amplitude of compound action potentials in toxin-treated sciatic nerve preparations could have resulted from either a graded blockage of many units or a progressive but complete blockage of individual axons (Figure 2). To gain insight into these possibilities, we chose the medial and lateral giant axons from crayfish (*Cambarus* sp.) and measured action potentials with both extracellular and intracellular electrodes. Sheathed and partially desheathed axons were prepared and arranged in a chamber divided into three parts (A, B,

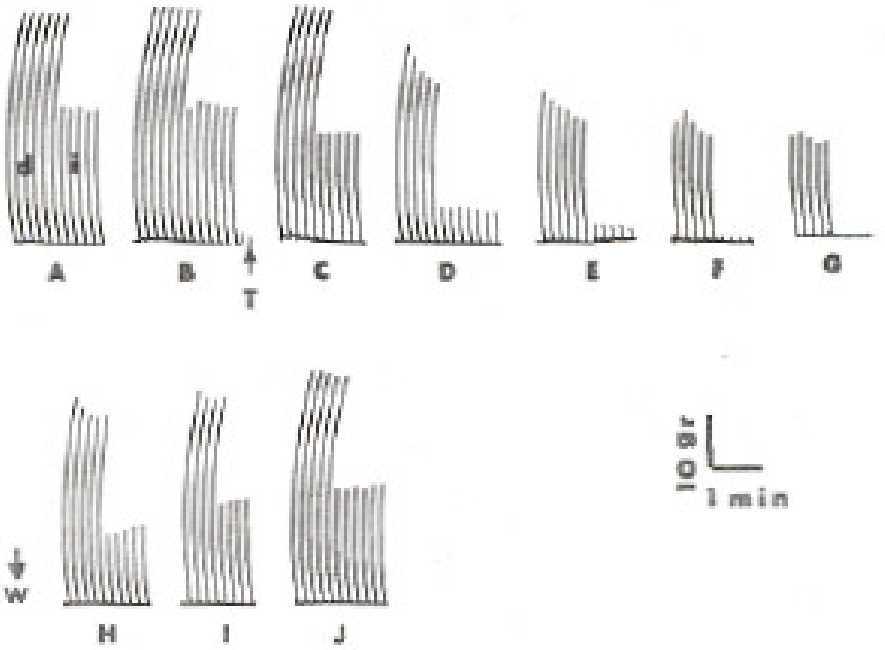

Fig. 1. Effect of aphantoxin on isometric twitches from the amphibian sartorius nerve-muscle preparation. Alternate series of stimuli: direct stimulation (d) of 5 msec duration, and indirect stimulation (i) of 50 usec duration. A = control; B = control 20 minutes after A; C, D, E, F, G = responses after 2, 5, 10, 15 and 20 min in aphantoxin (50 μg/ml); H, I, J = recovery after 15, 30, and 60 minutes in amphibian Ringer. All stimuli were given at 10 second intervals. Temperature 20°C.

and C), each separated by a paraffin and oil barrier. When the axons were stimulated at A, toxin treated in B, and action potentials recorded at C, the waveform shifted to the right and then abruptly disappeared. This blockage could be reversed 15 or more times in a single preparation. The shift to the right in the recorded action potential was interpreted as either a reduced conduction velocity or an increased latency time to reach firing threshold. When the toxin (4 μg/ml) and recording intracellular microelectrode were placed in the same chamber (B), the action potential amplitude gradually decreased, had a slower sodium-dependent rise time and was readily recoverable (Figure 3). The same results were obtained with both sheathed and desheathed axons. However, the former took approximately 5 times longer to block. In all records from toxin treated axons, the rise time from base line to peak amplitude increased 3 to 5-fold before complete block of the action potential occurred. The slope of the falling or recovery phase, however, remained essentially the same. In Figure 3, for example, the rise time increased from 0.5 msec to 1.8 msec,

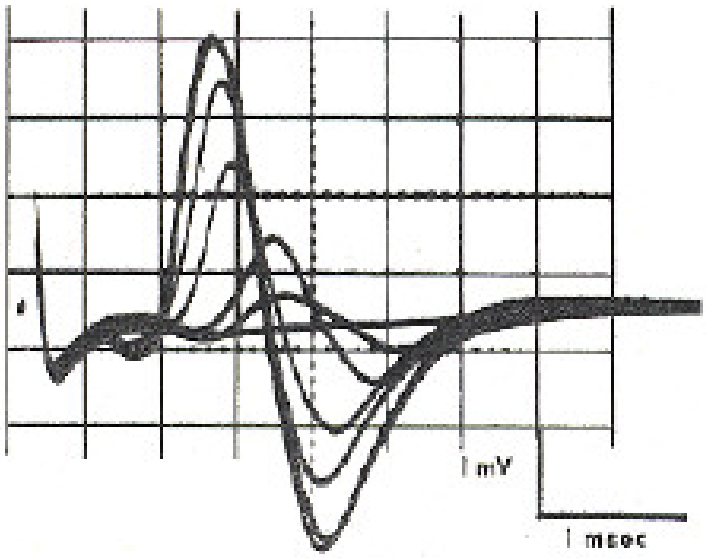

Fig. 2. Effect of aphantoxin on amphibian sciatic nerve compound action potentials. Nerve partially desheathed. Traces show progressive decline in amplitude and rise rate to complete block in 5 minutes. Wash in amphibian Ringer returned normal action potential in 15 minutes. Stimulus amplitude 3 V, duration 30 μsec. Grid scale 0.5 msec/division horizontal, and 1 mV/division vertical. Temp. 20°C.

while the recovery times were approximately 1.3 msec in all traces. The rising or depolarizing phase of the action potential is associated with a transient increase in sodium ion conductance. The membrane permeability to sodium ions increases upon stimulation and these cations flow inward, down concentration and electrical gradients toward the sodium equilibrium potential, E_{Na^+}. This is the familiar positive feedback loop called the Hodgkin cycle. The resting impermeability of the membrane to sodium ions returns, breaking the cycle, and potassium ions flow outward, down concentration and electrical gradients toward E_{K^+}, and restoration of the transmembrane resting potential, E_{mem}. The data from crayfish giant axons suggests that aphantoxin alters the depolarizing or sodium-dependent phase of the action potential and has little or no effect on the potassium-dependent repolarization phase. The sodium dependency was verified by challenging the axons with Na^+ free Ringer, in which case records similar to Figure 3 (with aphantoxin) were obtained. Calcium-free Ringers only slightly affected the action potential waveform and the addition of toxin decreased the amplitude and rise rate, as in Figure 3. Aphantoxin treatment did not alter the transmembrane resting potential (-80 mV) or the membrane resistance.

In comparative studies the myogenic hearts of amphibians and molluscs and the neurogenic hearts of crustaceans may offer

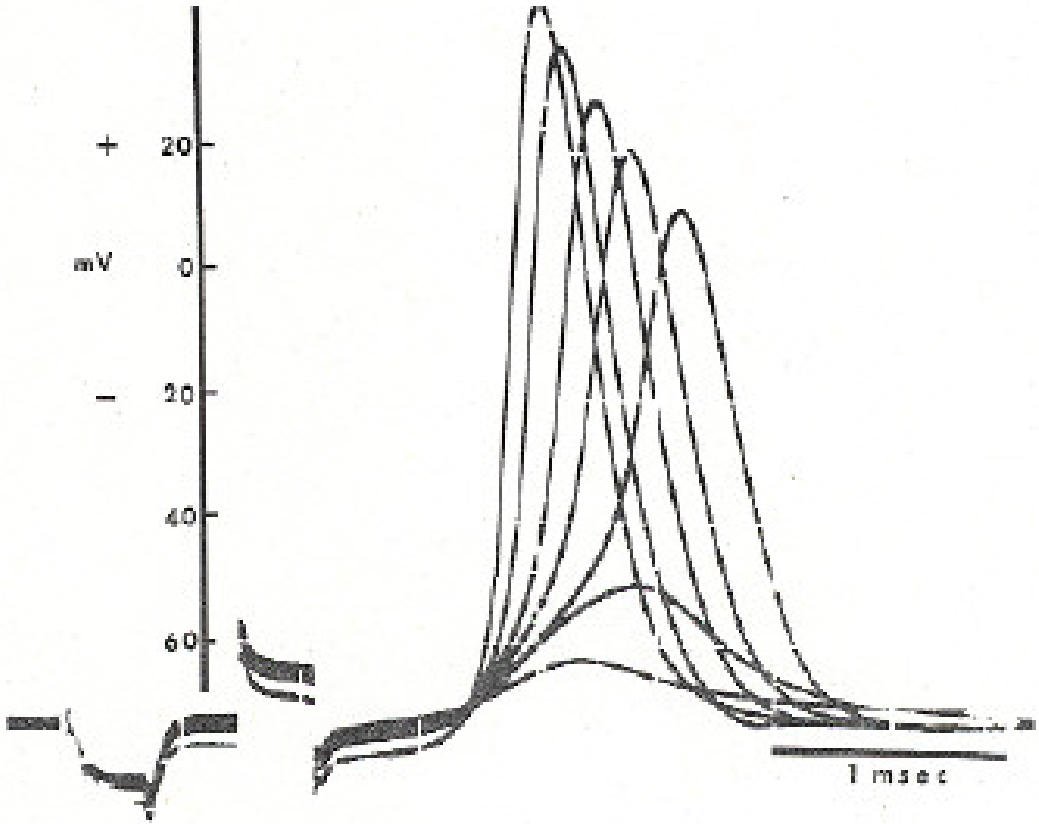

Fig. 3. Effect of aphantoxin on action potentials from crayfish medial giant axons. Desheathed preparation. Toxin (0.8 μg/ml) and intracellular recording electrode in same chamber. Top trace control, progressive decline in amplitude and rise rate after 4, 10, 14, 17, 20 and 25 minutes in toxin. Complete recovery within 15 minutes after wash in crayfish Ringer. Stimulus amplitude 2 times threshold (2 V); duration 250 μsec pulse width.

useful preparations for the testing of biotoxins. Thurberg (1972) showed that the marine poisons tetrodotoxin (TTX) and saxitoxin (STX) and the freshwater aphantoxin (4 μg/ml) all produced reversible diastolic arrest in the hearts of the crab, *Cancer irroratus*. None of these toxins, however, altered the normal mechanical or electrical activity of bivalve hearts (*Mercenaria mercenaria*), even at increased dose levels (100 X). The sodium dependence of the bivalve heart was verified by substitution with sucrose and lithium. This lack of sensitivity to sodium blocking toxins has not been explained. Amphibian heart preparations were slowed, then reversibly blocked in diastolic arrest by aphantoxin (Sawyer et al., 1968).

In previous work (Sasner and Ikawa, 1975) we have described aphantoxin as a non-depolarizing, reversible, membrane blocking agent that may alter ion conductance pathways associated with

excitation. This hypothesis is currently being tested using voltage-clamp methods on individual axons. The goal in these studies is to measure the physical characteristics of the axon membrane and transmembrane current flow on voltage clamped cells challenged with purified aphantoxin (Ehrenstein, 1976; Adelman and French, 1976). The specific aim is to clamp the transmembrane voltage and measure the inward (Na^+) and outward (K^+) currents to determine whether the toxin blocks specific ion channels or all cation flow, as it blocks excitability. The specific site and mode of action should be revealed by measuring the current density, I_{mem}, (mA/cm^2) as a function of clamped membrane potential, E_{mem}, (mV).

The results of these studies hold more than just academic interest for biologists because of the potential utility of aphantoxin. Saxitoxin (STX) and tetrodotoxin (TTX) are currently used in basic research as "tools" in the study of Na^+ dependent membrane systems (Kao, 1966; Evans, 1972; Narahashi, 1975). Aphantoxin may be equally important in this regard. The most significant role of STX and TTX involves the specific but reversible blockage of action potential conduction in a veriety of vertebrate and invertebrate nerve and muscle preparations. There are, however, more subtle differences between these two marine toxins. These differences are related to: a) dose-survival relationships in injected animals, b) resistance of amphibian nerves (*Taricha*) and puffer fish nerves (*Tetradon*) to TTX but not STX, c) recovery time of nerve-muscle preparations after poisoning, and d) the differential effect on evoked end-plate potentials , i.e., STX causes gradual decrease, while TTX produces abrupt blockage. If our hypothesis is correct, and aphantoxin specifically alters the ion conductance properties of the membrane, then more extensive comparative work will be done to determine if the cyanobacterial toxin is more STX-like or more TTX-like. In addition, it would be important to include the variety of toxins from the "red tide" dinoflagellate, *Gonyaulax tamarensis*, since these materials are similar in their effects on membrane systems (Evans, 1975; Narahashi, 1975; Shimizu, 1978).

ACKNOWLEDGMENTS

This research was supported through the New Hampshire Water Resources Research Center of the University of New Hampshire by a grant from the Office of Water Research and Technology, United States Department of the Interior as authorized under the Water Research and Development Act of 1978, Public Law 95-467.

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