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COMPARATIVE EFFECTS OF AQUATIC
BIOTOXINS ON CARDIAC SYSTEMS

by

FREDERICK P. THURBERG

B.A., University of Massachusetts, 1964

M.Ed., University of Massachusetts, 1967

M.S., University of New Hampshire, 1969

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In Partial Fulfillment of

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Doctor of Philosophy

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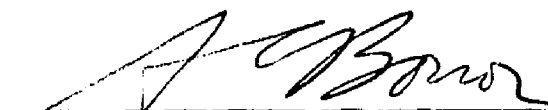
Department of Zoology

January 1972

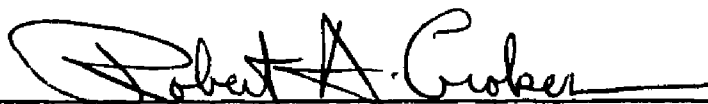
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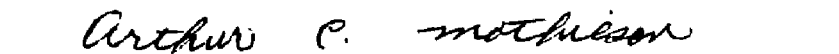
Arthur C. Borrer, Asso. Prof. of Zoology




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TABLE OF CONTENTS

LIST OF TABLES vi

LIST OF FIGURES vii

ABSTRACT viii

I. INTRODUCTION

1. Historical Perspective 1

2. Gymnodinium breve 6

3. Amphidinium carteri 8

4. Gonyaulax catenella 9

5. Aphanizomenon flos-aquae 10

6. Experimental Design 10

II. MATERIAL AND METHODS

1. Toxin Sources 12

 A. Gymnodinium breve 12

 B. Amphidinium carteri 13

 C. Aphanizomenon flos-aquae 14

 D. Other Toxins 14

 E. Controls 15

2. Bioassay of Toxic Material 15

3. Physiological Methods

 A. In Vivo Heart Preparations 17

 B. Isolated Heart Preparations 17

 C. Other Preparations 18

 D. Solutions 18

| | |
|---|----|
| III. RESULTS | |
| 1. Bioassay of Toxic Material | 24 |
| 2. Physiological Actions | 26 |
| A. <u>Gymnodinium breve</u> | 26 |
| B. <u>Amphidinium carteri</u> | 37 |
| C. <u>Gonyaulax catenella</u> and <u>Aphanizomenon</u> <u>flos-aquae</u> | 43 |
| IV. DISCUSSION | 49 |
| V. SUMMARY | 60 |
| VI. BIBLIOGRAPHY | 61 |

LIST OF TABLES

| | |
|---|----|
| 1. Summary of Extraction Data and Toxin Yield of Ten <u>Gymnodinium breve</u> Harvests | 22 |
| 2. Physiological Saline Solutions | 23 |
| 3. Bioassay of Crude <u>Gymnodinium breve</u> Toxin on Mice | 25 |

LIST OF FIGURES

| | |
|---|----|
| 1. Growth Curves of <u>G. breve</u> and <u>A. carteri</u> Cultures | 21 |
| 2. Effects of Aquatic Toxins on Crab and Bivalve Hearts | 28 |
| 3. Effects of Eserine and Aquatic Toxins on Crab and Bivalve Hearts | 31 |
| 4. Effects of Aquatic Toxins on <u>In Vivo</u> Frog Hearts . | 34 |
| 5. Effects of <u>G. breve</u> Toxins on Human Blood Serum Cholinesterase | 36 |
| 6. Effects of Aquatic Toxins on Mammalian Smooth Muscle and Bivalve Heart Preparations | 39 |
| 7. Effects of ACH, Mytolon, and <u>A. carteri</u> Toxin on Bivalve Hearts | 42 |
| 8. Effects of PSP-like Toxins on Crab Heart Mechanical Activity | 45 |
| 9. Effects of PSP-like Toxins on Crab Heart Electrical (ECG) Activity | 48 |

ABSTRACT

EFFECTS OF AQUATIC BIOTOXINS ON CARDIAC SYSTEMS

by

FREDERICK P. THURBERG

Toxins from three marine dinoflagellates, Gymnodinium breve, Amphidinium carteri, and Gonyaulax catenalla, and a freshwater blue-green alga, Aphanizomenon flos-aquae were obtained by laboratory culture, field collection and correspondence with other investigators. In vivo and isolated hearts of decapod crustaceans, Cancer irroratus, and Carcinus maenus, the bivalve molluscs, Mya arenaria and Mercenaria mercenaria and the grass frog Rana pipiens were exposed to these toxins, and mechanical and electrical activity were measured.

Gymnodinium breve toxin excited (increased frequency and tonus) crustacean hearts, depressed (reduced frequency and amplitude) frog hearts and had no effect on molluscan hearts. These observations and experiments with human blood cholinesterase and mammalian intestine suggest anticholinesterase-like activity as one action of G. breve toxin.

Amphidinium carteri toxin excited crustacean hearts and depressed molluscan and frog hearts. This choline-like action was further demonstrated with mammalian intestine preparations and the use of the choline blocking compound, mytolon chloride. Aphanizomenon flos-aquae and Gonyaulax catenella toxins depressed frog and crustacean hearts but had no effect on molluscan hearts. This evidence supports reported physiological and chemical similarities of these two toxins.

I. INTRODUCTION

1. HISTORICAL PERSPECTIVE:

Recent interest in aquatic biotoxins and their effects on biological systems is evidenced by the number of major publications during the last 10 years. The most ambitious undertaking was the 3 volume monograph by Halstead (1965-70). This work is a compilation of the history, chemistry and pharmacology of marine biotoxins, and it covers nearly all aspects of marine toxicology from 3000 B.C. to 1970. Baslow (1969) prepared an extensive survey of the potential of marine products in the drug industry, as well as a review of the chemistry and physiological actions of marine toxins. Nigrelli (1960) edited an extensive series of papers containing material on the discovery and action of active compounds from animals of several different phyla. Russell (1965) reviewed work on venomous and non-venomous poisons from a variety of marine sources, and Shilo (1967) detailed some of the research methodology in the study of aquatic toxins particularly those from Prymnesium parvum. Der Marderosian (1969) detailed much of the known chemistry and pharmacology of several marine toxins.

The terms biotoxin, venom, poison and drug are used extensively in the above reviews. I define biotoxin as a

substance of biological origin which causes harm or discomfort to some living organism other than itself. A biotoxin injected by a spine, fang or similar mechanical structure is a venom. The term poison is used to describe either of the above terms. A drug is a substance, including a toxin, that has been investigated using pharmacological or physiological methods. Drugs affect the structure or function of the living system in a predictable manner.

The above reviews and many shorter reports suggest the potential use of marine toxins as investigative tools in physiology. One of the best examples is tetrodotoxin (TTX), a poison extracted from the liver and ovaries of fish in the order Tetraodontiformes. Hagiwara and Nakajima (1965) demonstrated with TTX that the plateau phase of the frog cardiac action potential may be controlled by membrane permeability to calcium ions. The initial rise of the action potential is due to the influx of sodium ions (Brady and Woodbury, 1960) and it is blocked by TTX. The plateau phase is unaffected by the toxin. TTX blocks sodium conductance but has no effect on calcium dependent membrane systems (Kao, 1966), therefore the plateau was suspected of being calcium dependent. Magnesium ions inhibit calcium permeability and reduce the plateau. Thus evidence for calcium dependence in the electrical and mechanical activity of

vertebrate hearts was established. TTX is also an anticonvulsive drug and has been used as such for centuries (Feiger, 1968).

A cardiac extract from the hagfish (Eptatretus stouti), a primitive cartilaginous fish, exerts chemical control over the three independent hearts of this fish (Kennedy et al., 1967). When injected into dogs with impaired cardiac nervous control, hagfish cardiac extract stimulated heartbeat into a regular pattern for hours (Arehart, 1969). Thus it may facilitate conduction of impulses in cardiac tissue.

Erspamer and Anastasi (1962) prepared a type of cephalotoxin termed eledoisin from saliva of the octopus, Eledone noschata. It is a powerful vasodilator and controls high blood pressure in dogs and can also correct the irregular beat associated with heart attacks.

Holothurin, a poison extracted from sea cucumbers of the genera Holothuria, and Actinopyga, acts in a manner similar to digitalis, a cardiac stimulant used in diseases of the heart to correct lost compensation (Nigrelli and Jakowska, 1960; Arehart, 1969).

The toxin from Gonyaulax catenella, a dinoflagellate, causes paralytic shellfish poisoning (PSP) when accumulated by shellfish. After ingestion of such shellfish, the poison

is absorbed from the digestive tract, and it depresses respiration, alters conduction in the myocardium and reduces blood pressure (Kellaway, 1935). One milligram of this toxin is lethal but a diluted and controlled form of the poison might be useful in treatment of hypertension and other anomalies associated with cardiovascular systems. Poisons with similar physiological properties such as TTX and the toxin from the blue-green alga, Aphanizomenon flos-aquae, might also be useful in the treatment of these disorders.

Marine dinoflagellates have attracted attention because of dramatic effects of red tides throughout the world. At least 22 dinoflagellate species have been implicated in poisonings during red tide blooms (Halstead, 1965). Red tides, red water, or blooms of microorganisms occur under certain weather, salinity, and nutrient conditions, resulting in water being colored by their numbers. Although the term red tide is commonly used, the sea may also be brown, yellow, greenish, bluish, or even milky depending on the microorganism involved. Although most red tides are apparently harmless and disappear in a matter of hours or days, some create conditions resulting in animal mortalities and public health problems.

Two basic types of dinoflagellate poisoning are ecologically significant. Dinoflagellate poisons may be released by secretion or cell breakage due to mechanical agitation resulting in mortality of vast numbers of fish and other marine organisms. Red tides of Gymnodinium breve and Gonyaulax monilata cause animal mortalities in the Gulf of Mexico (Gunter et al., 1948; Davis, 1948; Connell and Cross, 1950; Howell, 1953; Gates and Wilson, 1960; Sievers, 1969). Likewise various species of Noctiluca on the coast of India and South Africa (Ryther, 1955), and other species of Gymnodinium on the coast of Japan (Nightingale, 1936) have been implicated in marine deaths.

The second effect of a poisonous red tide involves shellfish that feed on dinoflagellates during a bloom and concentrate the toxin within the digestive gland, mantle or siphons. Animals higher in the food chain, including man, are poisoned upon ingestion of the afflicted shellfish. Gonyaulax catenella is the best known source of shellfish poison and has caused serious public health problems on the Pacific coast of North America (McFarren et al., 1957; Schantz and Magnusson, 1964). Other dinoflagellates whose toxin is accumulated by shellfish include Gonyaulax tamarensis along the Canadian Maritime Provinces (Needler, 1949; Bond and Medcof, 1958; Prakash, 1963; Coulson et al.,

1968) and Pyrodinium phoneus in Belgium (McFarren et al., 1957). Gymnodinium breve, in the Gulf of Mexico, is one of the few organisms responsible for both shellfish poisoning and mass mortality of marine animals (Ray and Aldrich, 1965; McFarren et al., 1965).

The symptoms of shellfish poisoning in humans include peripheral paralysis and they may vary from a tingling or numbness of the lips, fingers, and toes, to a loss of voluntary movements, and eventual death by respiratory failure. In most cases a feeling of lightness or floating accompanies the symptoms (McFarren et al., 1957).

Aphanizomenon flos-aquae, a toxic freshwater blue-green alga, is of interest because it is similar to G. catenella in its physiological action and chemical composition (Jackim and Gentile, 1968; Sawyer et al., 1968). This species has been associated with recent phytoplankton blooms in several New Hampshire lakes enriched by sewerage.

2. GYMNODINIUM BREVE:

Gymnodinium breve, first associated with red tides and destruction of marine life in the Gulf of Mexico in 1947 (Wilson and Ray, 1956), can be maintained in completely defined artificial seawater (Aldrich and Wilson, 1960; Gates and Wilson, 1960; Abbott and Paster, 1970). Sasner (1965) and Sievers (1969) demonstrated its toxicity to whole

animals using crude extracts and whole cultures. McFarren et al. (1965) and Cummins et al. (1971) implicated G. breve in cases of human shellfish poisoning on the west coast of Florida. Red tides of G. breve also have produced local atmospheric conditions which are a source of eye and respiratory irritation, apparently due to an aerosol released by the concentration of organisms (Woodcock, 1948). Several investigators reported a number of toxic fractions from this species, (Cummins et al., 1968; Martin and Chatterjee, 1969; Sasner et al., in press). Extracts from G. breve cultures alter membrane potentials of excitable tissues, thus rendering them insensitive to stimulation (Sasner, 1965). Another fraction hemolyzes mammalian red blood cells (Paster and Abbott, 1969). Martin and Chatterjee (1969) indicated without describing data or methods, that one fraction may have anticholinesterase properties. McFarren et al. (1965) reported a ciguatera-like toxin from this dinoflagellate.

Purification of G. breve toxin has been difficult as evidenced by the variety of fractions and chemical characterizations reported. Martin and Chatterjee (1969) described the isolation of two fractions; substance I from interfacial material and substance II, described as a non-nitrogenous, phosphorous-containing optically active, pale yellow, low-melting solid. Cummins et al. (1968) also

demonstrated two toxic fractions by thin layer chromatography. Sasner et al. (in press) report a toxic substance, fraction IVa, with a carbonyl group and a molecular weight of 279. This molecular weight is significantly smaller than that of other active materials described from G. breve.

3. AMPHIDINIUM CARTERI:

Amphidinium carteri is a temperate water dinoflagellate. Dinoflagellates of the genus Amphidinium are toxic in high concentrations to organisms at higher trophic levels (Halstead, 1965; Russell, 1965). McLaughlin and Provasoli (1957) reported the toxicity of supernatants from centrifuged cultures of A. klebsii and A. rynchocephalum to fish (Lebistes reticulatus and Gambusia sp.). The activity of a substance released by A. carteri may be related to acetylcholine or an analog. Such compounds may act as a protective device against zooplankton (Wangersky and Guillard, 1960). Extracts from A. carteri cells kill terrestrial and marine bacteria (Duff, Bruce, and Antia, 1966). Red tides in Delaware Bay were attributed to members of this genus (Martin, 1927). Other Amphidinium species can discolor sand in subtidal areas (Herdman, 1924a and 1924b; McGeary, personal communication). A. carteri is smaller than A. klebsii and possesses a single chloroplast (Kofoid and Swezy, 1921; Hulburt, 1957).

4. GONYAULAX CATENELLA:

Gonyaulax catenella causes paralytic shellfish poisoning (PSP). Paralytic shellfish poisoning occurred on the coast of Vancouver, British Columbia as early as 1793, and in 1799 a group of Aleut hunters consumed a quantity of mussels and 100 men died in less than 2 hours (McFarren et al., 1957). In the mid-eighteen hundreds a yellowish foam appearing on the sea during PSP outbreaks was linked to the toxic shellfish, and in 1888 Linder (cited by Russell, 1965) suggested that the poison in shellfish was passed through the food chain. Sommer et al. (1937) found that the source of the toxin was G. catenella, filtered by molluscs. Other species of dinoflagellates are implicated in what is generally called paralytic shellfish poison. However, this term is generally accepted as G. catenella toxin, and it is treated as such in this study.

In the 1950's Schantz and his co-workers evaluated the chemistry of a toxin from Pacific coast mussels (mussel poison from Mytilus californianus), clams (sacitoxin from the Alaskan butter clam Saxidomus giganteus), and from G. catenella cultures. They demonstrated identical properties for the 3 toxins and published methods for their purification and characterization (McFarren et al., 1957; Schantz et al., 1958; Schantz, 1960).

5. APHANIZOMENON FLOS-AQUAE:

Aphanizomenon flos-aquae is a blue-green alga that blooms in some eutrophic freshwater lakes, and has been implicated in toxic situations (Prescott, 1948; Ingram and Prescott, 1954; Gorham 1964a and 1964b). Experiments by Sawyer et al. (1968) conclusively demonstrated the toxicity of this alga. Jackim and Gentile (1968) reported that its toxin may be similar, if not identical, to saxitoxin (PSP) as characterized by chromatography, infrared spectra, and color reagents.

6. EXPERIMENTAL DESIGN:

In general, biotoxins are of physiological interest because of their specificity, potency and because they initiate excitatory or inhibitory phenomena in excitable tissues. The comparative effects of the aquatic toxins described above were tested on the cardiac tissues of bivalve molluscs, decapod crustaceans and amphibians. The primary goal in this study was to determine whether mechanical and electrical records of cardiac activity in the presence of toxins would suggest mechanisms of action associated with either a) alteration in membrane permeability; b) the nervous control of contraction; or c) the transmitter system and its control. Preliminary experiments showed that the toxins caused differential effects in neurogenic and myogenic

hearts. Different chemical mediators and neuromuscular control mechanisms are present in the hearts chosen. The ultimate goal is to use such information in the determination of the specific site and mode of action of the poisons in biological systems.

II. MATERIALS AND METHODS

1. TOXIN SOURCES:

A. GYMNODINIUM BREVE

Dr. William B. Wilson of Texas A & M University Marine Station, Galveston, Texas provided a starter culture of G. breve. More than 250 unialgal cultures were maintained over a 30 month period in 1.2 liter volumes. The cells were cultured in 2.5 liter low form flasks (Corning #4422), maintained in incubators at $24 \pm 1^{\circ}$ C, and exposed to a 14 hour daily light period under 40 watt cool white fluorescent bulbs (500 foot candles). The culture medium was NH-15 (Gates and Wilson, 1960). New medium was inoculated from mature cultures to attain an initial cell count of 1000 ± 200 cells/ml. No stirring or aeration was employed. The cells were counted twice weekly with a Model F Coulter Counter and a growth curve was prepared as shown in Fig. 1A. When the cultures reached $15-22 \times 10^6$ cells/l (shown as "x" on the growth curve) the pH of the culture was adjusted to 5.5 with HCl. The cultures were extracted twice with ethyl ether in a separatory funnel, first with 150 ml of ether/l of culture then with 100 ml ether/l of culture, allowing separation after each extraction. The ether layer containing crude toxin was evaporated to dryness in vacuo and stored at -7° C. Table 1 summarizes ether extraction data from 10

harvests ranging from 63 to 127×10^7 cells. An average dry weight of 5.3 ± 0.4 mg of ether soluble material was attained per 10^6 cells. I obtained much lower values (2.0 - 4.0 mg per 10^6 cells) from cultures grown past peak density.

No attempt was made to maintain bacteria free cultures. The toxicity of G. breve is independent of associated bacteria (Ray and Wilson, 1957; Cummins and Stevens, 1970).

Dr. Miyoshi Ikawa, University of New Hampshire Biochemistry Department, obtained a purified form of the toxin, fraction IVa, by the method described in Sasner et al. (in press). Fraction IVa was made available for this study.

B. AMPHIDINIUM CARTERI:

Dr. Robert R. Guillard, Woods Hole Oceanographic Institution, supplied an unialgal culture of A. carteri. One hundred 12 liter cultures were maintained over a period of 15 months in 20 liter glass carboys. The carboys were positioned around vertically mounted, double, 40 watt, cool white fluorescent lights so that the center of each carboy received 250 foot candles. The cultures were maintained at a temperature of $24 \pm 2^\circ\text{C}$. Constant illumination and aeration were employed. NH-15 medium was inoculated to provide an initial concentration of $6-12 \times 10^6$ cells/l and the cultures were counted 5-6 times/week with a Model F Coulter Counter. The growth curve (Fig. 1B) represents cell counts

from 30 cultures. When the cultures reached a peak density ("x" on Fig. 1B), they were harvested by continuous flow centrifugation using a Sorvall RC2-B refrigerated centrifuge equipped with a Sorvall K2B continuous flow system and a SS34 rotor. The harvesting temperature was 15° C and the speed 3020 x g with a flow rate of 20 l/hr. Both the cell pellet and supernatant were tested for toxicity. The supernatant was tested immediately. The pellet was lyophilized in a Vertis lyophilizer and stored at -7° C. Dr. Miyoshi Ikawa provided a sample of 80-95% pure A. carteri toxin.

C. APHANIZOMENON FLOS-AQUAE:

Aphanizomenon flos-aquae cells were collected from unialgal blooms in Kezar Lake, North Sutton, New Hampshire, during the summers of 1968-70. Lake water continuously pumped into a 20 liter reservoir fed 2 Delaval continuous flow separators (225 l/hr at 5000 x g: total flow for both units). Every 2 hours, approximately 1 liter of algal concentrate was removed from the separators. Forty liters of such concentrate were frozen at -7° C and later lyophilized. The lyophilized material was allowed to extract in saline solutions by the method of Sawyer et al. (1968).

D. OTHER TOXINS:

Dr. Edward Schantz, Public Health Liason Office, Fort Detrick, Maryland, supplied a sample of pure Gonyaulax

catenella toxin (PSP). Calbiochem Corp., Los Angeles, California supplied the tetrodotoxin (TTX) used in comparative studies versus PSP and A. flos-aquae toxin.

E. CONTROLS:

Two types of controls were used. Uninoculated (no cells) NH-15 medium was extracted according to the procedures described above for G. breve and A. carteri cultures. In this way I was able to determine if the medium or extraction methods (including solvents) had any effect on the electrical or mechanical activity of cardiac tissues. I also lyophilized Spiroqira sp., a non-toxic green alga, collected from Kezar Lake prior to the A. flos-aquae blooms. This material was suspended in bathing medium and used to determine if it caused mechanical irritation of the hearts.

2. BIOASSAY OF TOXIC MATERIAL

Crude G. breve toxin was assayed on 40 non-inbred, white mice, Mus musculus, (19-21 g) and 60 killifish, Fundulus heteroclitus (4.0-8.5 g). Toxin was injected intraperitoneally (i.p.) at a concentration of 6 mg/0.2 ml saline (mice) and 6 mg/0.4 ml saline (killifish). Culture medium controls (described above) were used. Mouse toxicity was expressed in mouse units (MU) using McFarren's tables (McFarren et al., 1965). A G. breve mouse unit is defined as the LD50 effective in 20 g mice in 930 minutes. However, doses greater than 1 MU

are generally used to reduce the death time.

The activity of materials from Amphidinium carteri cells had not been quantitatively determined before this study. Three groups of 5 killifish (4.0-6.0 g) were placed into fingerbowls containing one liter of culture (100×10^6 cells/l) and reactions were noted. I placed 10 killifish (4.0-6.0 g) in groups of 2 and 3 in beakers containing 400 ml of supernatant from A. carteri cultures. Control animals (10 fish; 4.0-6.0 g) were tested in uninoculated NH-15 supernatant.

Lyophilized A. carteri material was added to aquarium water (0.1 mg and 0.05 mg/ml water) containing 10 guppies (Lebistes reticulatus) and symptoms and death times were noted. Controls utilized lyophilized Spiroqira sp.

I assayed A. carteri on white mice by the same method described for G. breve using 50 mg and 25 mg/0.5 ml saline. Both NH-15 and Spiroqira controls were used.

The Gonyaulax catenella toxin employed was a bio-assay reference sample commonly used by public health and research laboratories in comparing shellfish toxicity. Toxicity of this sample was 5.5×10^6 MU/g.

Sawyer et al. (1968) reported that the minimum lethal dose of lyophilized A. flos-aquae cells was 10 mg/kg for white mice. I injected 20 mice by their method and

found the same toxicity level.

A. IN VIVO HEART PREPARATIONS:

I recorded mechanical activity from the hearts of the decapod crustaceans Carcinus maenas, and Cancer irroratus, the bivalve molluscs Mya arenaria and Mercenaria mercenaria (previously named Venus mercenaria), and frog (Rana pipiens) using Grass FT03C transducers, displayed on a Model 5 Grass Polygraph. Action potentials were recorded from frog heart muscle using a "hanging microelectrode" (Woodbury and Brady, 1956). Three Grass subdermal electrodes (EB2 platinum alloy) equally spaced around the heart monitored electrocardiograms (ECG's) that were displayed on the Grass Polygraph. Crustacean and molluscan hearts were exposed and bathed externally with both crude and purified toxins suspended in crab saline or seawater (see Table 2). Bivalve hearts were treated by injection directly into the ventricle. A plexi-glass chamber was used in this study. A coolant was circulated through an outer jacket surrounding an animal chamber, and it maintained a constant temperature in the animal bath. Frog heart preparations were treated by suspending crude and purified toxins in frog saline and injecting each via the ventral abdominal vein.

B. ISOLATED HEART PREPARATIONS:

Crustacean hearts were isolated by the method of

Welsh (1939) and suspended in a 10 ml volume chamber maintained in the constant temperature bath described above. Molluscan hearts were isolated by the method of Florey (1967) and maintained in the same bath and chamber as above.

C. OTHER PREPARATIONS:

Human blood serum cholinesterase levels were measured by the method of Rappaport et al. (1959) using a Sigma Cholinesterase Kit (Sigma Chemical Co., St. Louis, Mo.), and a Bausch and Lomb Spectronic 20 Spectrophotometer.

Sections of mouse intestine (2.5 cm long) were suspended in a 10 ml bath of aerated mammalian saline solution, maintained at 37° C. Test solutions were added to the bath and resulting tension development was measured with a Grass FT03C transducer displayed on a Model 5 Grass Polygraph.

All heart and smooth muscle experiments were performed on at least 10 preparations unless otherwise indicated.

D. SOLUTIONS:

The following commercially available chemicals were used in this study; acetylcholine (Ach) as acetylcholine chloride, eserine as eserine sulfate, atropine as atropine sulfate, benzoquinonium chloride (mytolon) and pyridine-2-aldosime methiodide (2-PAM). Various concentrations of each were prepared by dissolving the chemicals in appropriate physiological saline.

The saline solutions used in the preparation of the above chemical solutions as well as the toxin solutions are shown in Table 2.

FIGURE 1. GROWTH CURVES OF G. BREVE AND A. CARTERI
CULTURES

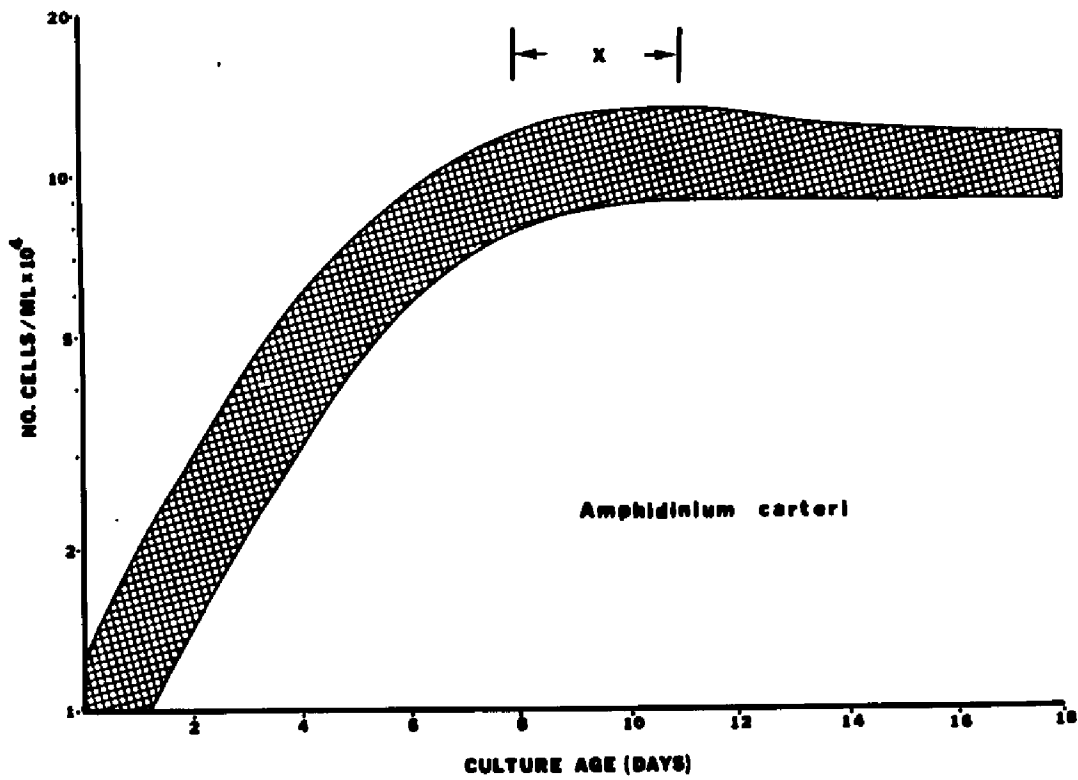
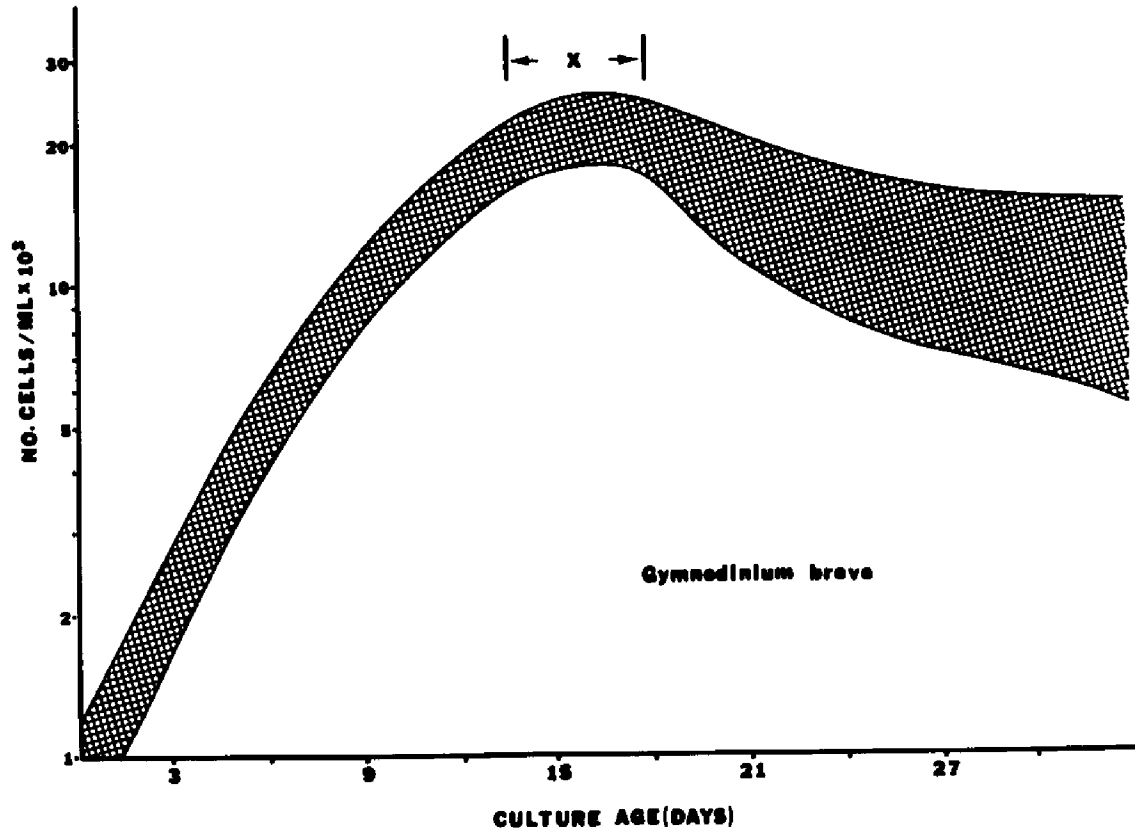


Table 1. SUMMARY OF EXTRACTION DATA AND TOXIN YIELD OF TEN GYMNODINIUM BREVE HARVESTS.

| Sample No. | No. of Liters Extracted | No. of Cells Extracted (X10 ⁶) | Ether Extracted Material (grams) | Material Extracted per 10 ⁶ cells (milligrams) |
|------------|-------------------------|--|----------------------------------|---|
| 1 | 4 | 73 | 0.4055 | 5.6 |
| 2 | 6 | 111 | 0.5980 | 5.4 |
| 3 + | 6 | 116 | 0.5845 | 5.0 |
| 4 | 4 | 75 | 0.3815 | 5.1 |
| 5 | 5 | 74 | 0.3700 | 5.0 |
| 6 * | 4 | 90 | 0.5135 | 5.7 |
| 7 *+ | 6 | 127 | 0.7363 | 5.8 |
| 8 | 4 | 88 | 0.4447 | 5.1 |
| 9 | 4 | 63 | 0.3338 | 5.3 |
| 10 *+ | 4 | 70 | 0.3610 | <u>5.2</u> |
| | | | Ave. | <u>5.3</u> |

* Indicates samples used for intraperitoneal injection into mice (6 mg in 0.4 mls Ringer)

+ Indicates samples used for intraperitoneal injection into killifish (Fundulus heteroclitus) (6 mg in 0.2 mls S.W.)

Table 2. PHYSIOLOGICAL SALINE SOLUTIONS

| Salt Solutions | Fish and Mollusc * | <u>Cancer</u> | <u>Carcinus</u> | Frog | Mammal |
|---|--------------------|---------------|-----------------|----------|----------|
| 0.54 M NaCl | 745.0 ml | 827.0 ml | 858.0 ml | 205.0 ml | 300.0 ml |
| 0.54 M KCl | 18.0 | 21.0 | 21.0 | 3.5 | 2.5 |
| 0.36 M CaCl ₂ ·2H ₂ O | 28.0 | 36.0 | 35.0 | 2.3 | 1.4 |
| 0.36 M MgCl ₂ | 146.0 | 68.0 | 51.0 | 4.6 | 3.1 |
| 0.44 M Na ₂ SO ₄ | 63.0 | 48.0 | 35.0 | - | - |
| 0.1 M NaH ₂ PO ₄ | - | - | - | 1.0 | - |
| Distilled Water | - | - | - | 783.6 | 693.0 |

* Sodium deficient molluscan saline was prepared by substituting osmotically equivalent amounts of LiCl or sucrose for NaCl.

III. RESULTS

1. BIOASSAY OF TOXIC MATERIAL:

Ether soluble extracts from Gymnodinium breve cultures showed a potency in mice of 1.5 MU/mg. The dosage level used for i.p. injection was 6.0 mg/0.5 ml saline and the survival time 8-10 minutes (see Table 3). Sasner et al. (in press) reported that the purified fraction IVa had a potency of 67/MU/mg, thus it is 45 times more active in mice than the crude, ether soluble extracts. The average survival time of killifish was 25 ± 8 minutes after i.p. delivery of 6.0 mg of ether soluble extract per 0.2 ml saline. All of the samples listed in Table 1 showed a potency consistent with the above values. However when extractions were made from G. breve cultures maintained past peak density (> 18 days) variable toxicity to mice was recorded (see Fig. 1 and Table 3). The symptoms of G. breve poisoning in mice and fish include respiratory irregularity, muscular spasms, loss of coordination, and motor paralysis.

The active material from Amphidinium carteri is apparently stored within the dinoflagellate cells. Killifish appeared unaffected in the presence of intact cells (100×10^6 cells/liter) for periods greater than 24 hours. However, fish placed in the supernatant from broken cells lost equilibrium and coordination in 7-12 minutes and died within 31-49

Table 3. BIOASSAY OF CRUDE GYMNODINIUM BREVE TOXIN ON MICE

| SAMPLE 6 | | SAMPLE 7 | |
|-----------|------------|-----------|------------|
| Mouse No. | MU*/mg | Mouse No. | MU/mg |
| 1 | 1.6 | 11 | 1.5 |
| 2 | 1.6 | 12 | 1.7 |
| 3 | 1.6 | 13 | 1.5 |
| 4 | 1.7 | 14 | 1.5 |
| 5 | 1.6 | 15 | 1.6 |
| 6 | 1.6 | 16 | 1.5 |
| 7 | 1.7 | 17 | 1.5 |
| 8 | 1.6 | 18 | 1.5 |
| 9 | 1.6 | 19 | 1.5 |
| 10 | <u>1.6</u> | 20 | <u>1.4</u> |
| | Ave. 1.6 | | Ave. 1.5 |

| SAMPLE 10 | | Samples Extracted from Cultures Past Peak Density | |
|-----------|------------|---|------------|
| 21 | 1.6 | 31 | 1.3 |
| 22 | 1.5 | 32 | 1.2 |
| 23 | 1.5 | 33 | 0.7 |
| 24 | 1.8 | 34 | 0.5 |
| 25 | 1.8 | 35 | 0.0 |
| 26 | 1.5 | 36 | 0.0 |
| 27 | 1.3 | 37 | 0.1 |
| 28 | 1.5 | 38 | 0.2 |
| 29 | 1.5 | 39 | 1.0 |
| 30 | <u>1.4</u> | 40 | <u>1.1</u> |
| | Ave. 1.5 | | Ave. 0.6 |

* Potency in Mouse Units (MU) per mg was calculated using the tables given by McFarren et al. (1965).

minutes. When guppies were introduced into water containing 0.1 mg/ml of lyophilized A. carteri cellular material, they survived for only 12-17 minutes while $\frac{1}{2}$ of this dose caused death within 18-32 minutes. Lyophilized material introduced i.p. into mice caused loss of hind limb coordination in 10-15 minutes followed by severe convulsions. Mice receiving 25 mg of dried cells survived for 82-141 minutes while 2 times this dose level caused death within 37-46 minutes.

The control animals used in the bioassay studies on Gymnodinium breve and Amphidinium carteri were not affected by the methods employed nor did they show any characteristics that have been described for the test animals.

2. PHYSIOLOGICAL ACTIONS

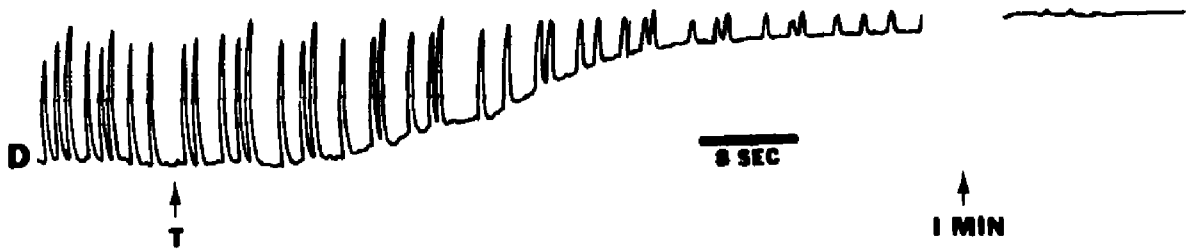
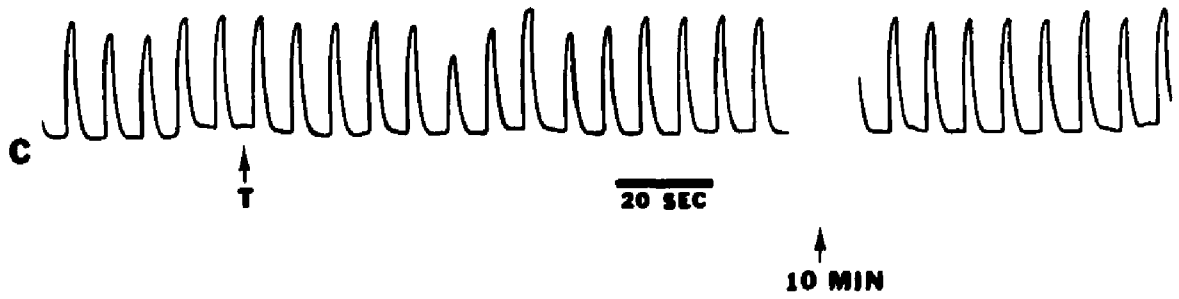
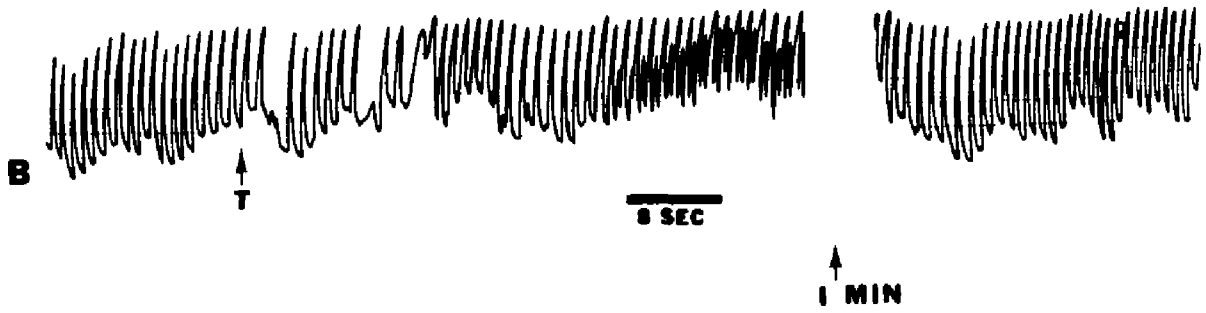
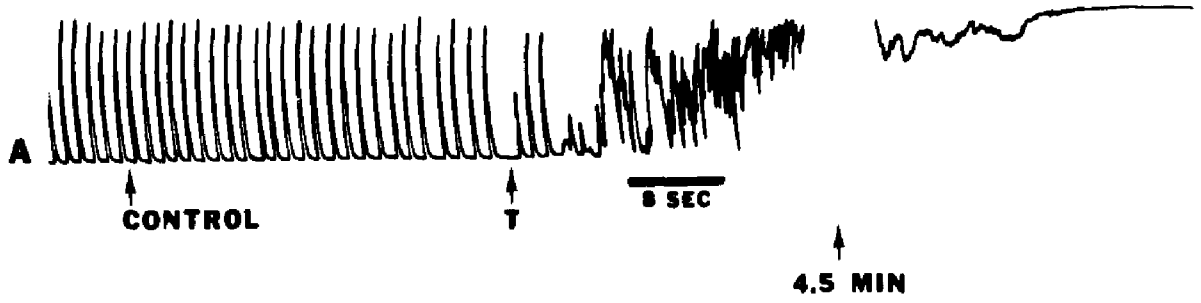
A. GYMNODINIUM BREVE:

The neurogenic hearts of the crabs responded to fraction IVa (5 μ g/0.1 ml crab saline) and crude ether extracted material (100 μ g/0.1 ml saline) with an increase in frequency and prolonged irregular tension development (Fig. 2A). In addition, Ach and eserine topically applied to the crustacean hearts caused a similar mechanical response (Fig. 3A).

FIGURE 2. EFFECTS OF AQUATIC TOXINS ON CRAB AND BIVALVE

HEARTS

- A. Mechanical activity of Cancer irroratus heart in vivo; (T) G. breve fraction IVa. (5 µg/0.1 ml) Temp. 14 C
- B. Mechanical activity of Cancer irroratus heart in vivo; pretreated with 2-PAM (10 mg/ml) before toxin (T) same as in A. Temp. 14 C
- C. Mechanical activity of Mercenaria mercenaria heart in vivo; G. breve fraction IVa (100 µg/0.1 ml) injected into ventricle (T). Temp. 14 C
- D. Mechanical activity of Cancer irroratus heart in vivo; (T) A. carteri (10^{-2} g/ml) crude toxin, 0.1 ml. Temp. 14 C



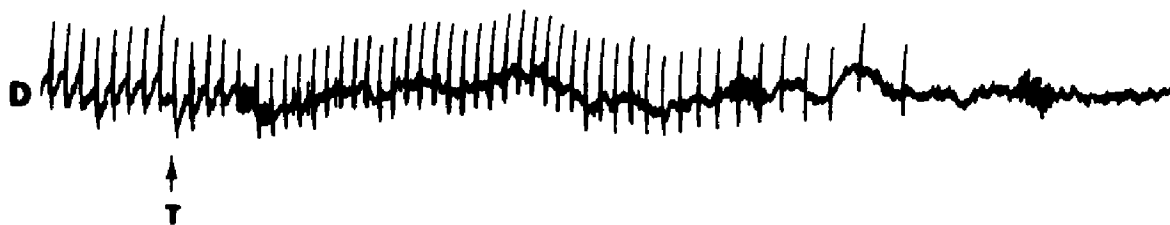
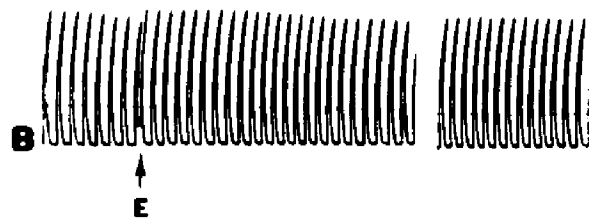
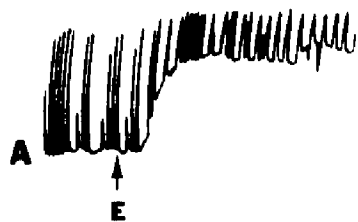
Pyridine-2-aldosime methiodide (2-PAM) reverses cholinesterase inhibition from organophosphate compounds such as nerve gas and insecticides. Pretreatment of 5 Cancer hearts (in vivo) with 2-PAM (10 mg/ml) appeared to nullify the effects of G. breve toxin (Fig. 2B).

Gymnodinium breve toxin caused an increase in electrical activity of Cancer hearts recorded in vivo (Fig. 3C). Fraction IVa (5-10 $\mu\text{g/ml}$) produced a response in isolated Cancer hearts, similar to that shown in Fig. 2A, i.e. an abrupt increase in tension and frequency. Lower dosage (1 $\mu\text{g/ml}$) produced some increase in frequency but no tension development; below this level heart activity was unaltered. Toxin (fraction IVa, 10 $\mu\text{g/ml}$) applied to isolated hearts pretreated with 2-PAM (1.5 mg/ml) caused only slight alteration in frequency. Concentrations of G. breve toxins that provoked only increases in heart frequency were reversible in action by seawater washing. Any concentration that caused increased tension development was irreversible in action, and led to systolic arrest.

Topical application of crude (100 $\mu\text{g}/0.1$ ml saline) and fraction IVa (5 $\mu\text{g}/0.1$ ml saline) toxin on molluscan hearts (Mya and Mercenaria) in vivo, caused no alteration of mechanical activity. Fraction IVa, injected directly into the ventricles of Mercenaria (Fig. 2C) also provoked

FIGURE 3. EFFECTS OF ESERINE AND AQUATIC TOXINS ON CRAB
AND BIVALVE HEARTS

- A. Mechanical activity of Cancer irroratus heart in vivo; Eserine (E) dripped onto heart, 10^{-3} g/ml. Temp. 14 C
- B. Mechanical activity of Mercenaria mercenaria heart isolated. Eserine concentration in bath: 10^{-3} g/ml. Record gap equals 10 minutes. Temp. 15 C
- C. Electrical activity (ECG) of Cancer irroratus heart in vivo; G. breve crude toxin (T) 1 mg/0.1 ml; initial rate equals 50 spikes per min. Temp. 14 C
- D. Electrical activity (ECG) of Cancer irroratus heart in vivo; A. carteri crude toxin (T) 1 mg/0.1 ml. Rate and temp. same as (C).



no change in cardiac activity. The anticholinesterase eserine, applied in a similar manner to Mercenaria hearts, likewise caused no alteration of the heartbeat (Figure 3B). Isolated Mercenaria hearts continued normal heartbeat when bathed for 1 hour with fraction IVa at a concentration of 200 $\mu\text{g/ml}$.

Injection of fraction IVa (0.5 $\mu\text{g/g}$ body wt.) into the ventral abdominal vein of 15 frogs slowed heart rate without significantly altering the action potential (Fig. 4C). Crude toxin (5-10 $\mu\text{g/g}$ body wt.) produced a similar response. Acetylcholine (0.15 ml, 10^{-4} g/ml) likewise slowed the heart rate. After toxin injection, the body musculature underwent violent fibrillations. Higher doses (2.5-5.0 $\mu\text{g/g}$ body wt.) of fraction IVa caused a cessation of mechanical activity in diastole. Similar amounts of fraction IVa injected into previously atropinized frog hearts (1.0 μg atropine sulfate/g body wt.) did not affect cardiac activity. Saline flushing of frog hearts treated with both crude and fraction IVa toxins returned frogs hearts to normal activity.

Figure 5 shows results of serum cholinesterase studies using human blood cholinesterase and crude and fraction IVa G. breve toxin. The line shown on the figure is a cholinesterase calibration curve and the point labeled

FIGURE 4. EFFECTS OF AQUATIC TOXINS ON IN VIVO FROG HEARTS

- A. Simultaneous recording of ventricular action potentials (top) and mechanical activity (bottom) of frog heart. A. carteri crude toxin (T) added via ventral abdominal vein, 5 mg. Temp. 20 C
- B. Same as (A) with 10 mg crude A. carteri toxin added.
- C. Frog ventricular action potential; G. breve fraction IVa toxin (T) added via the ventral abdominal vein (0.5 μ g/gram body weight). Temp. 20 C

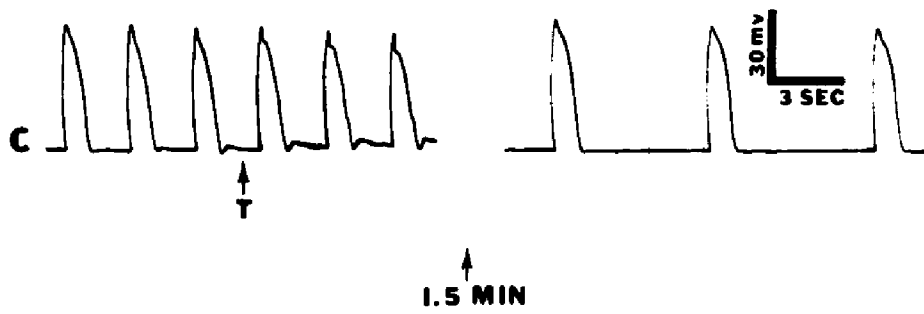
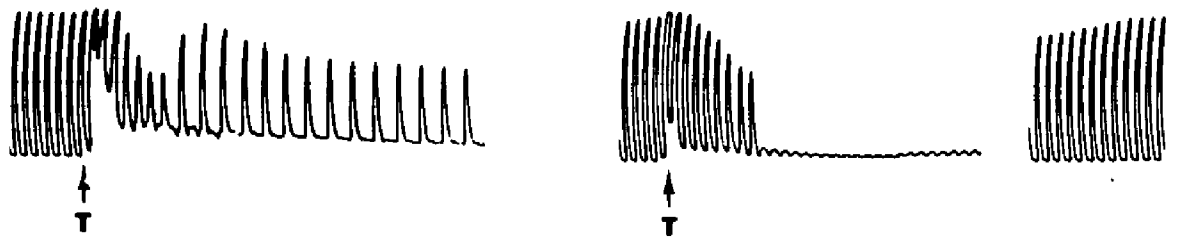
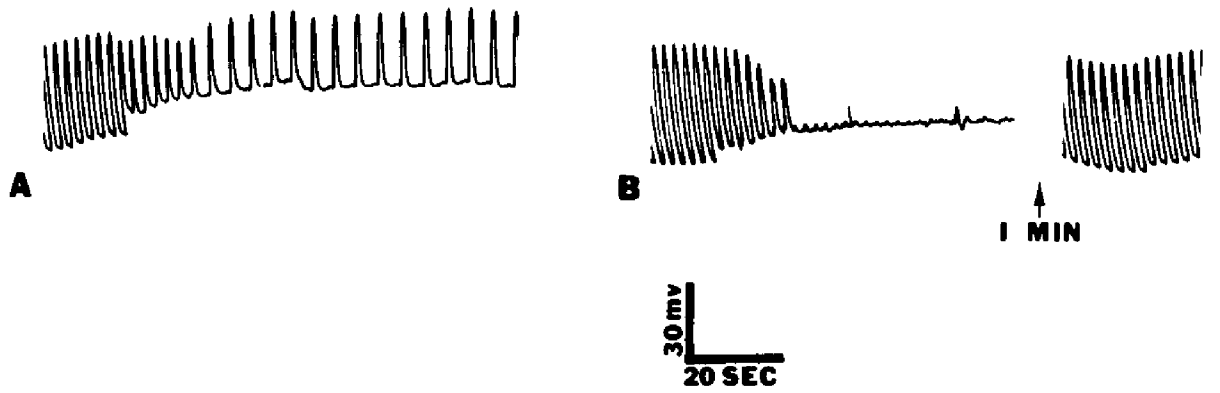
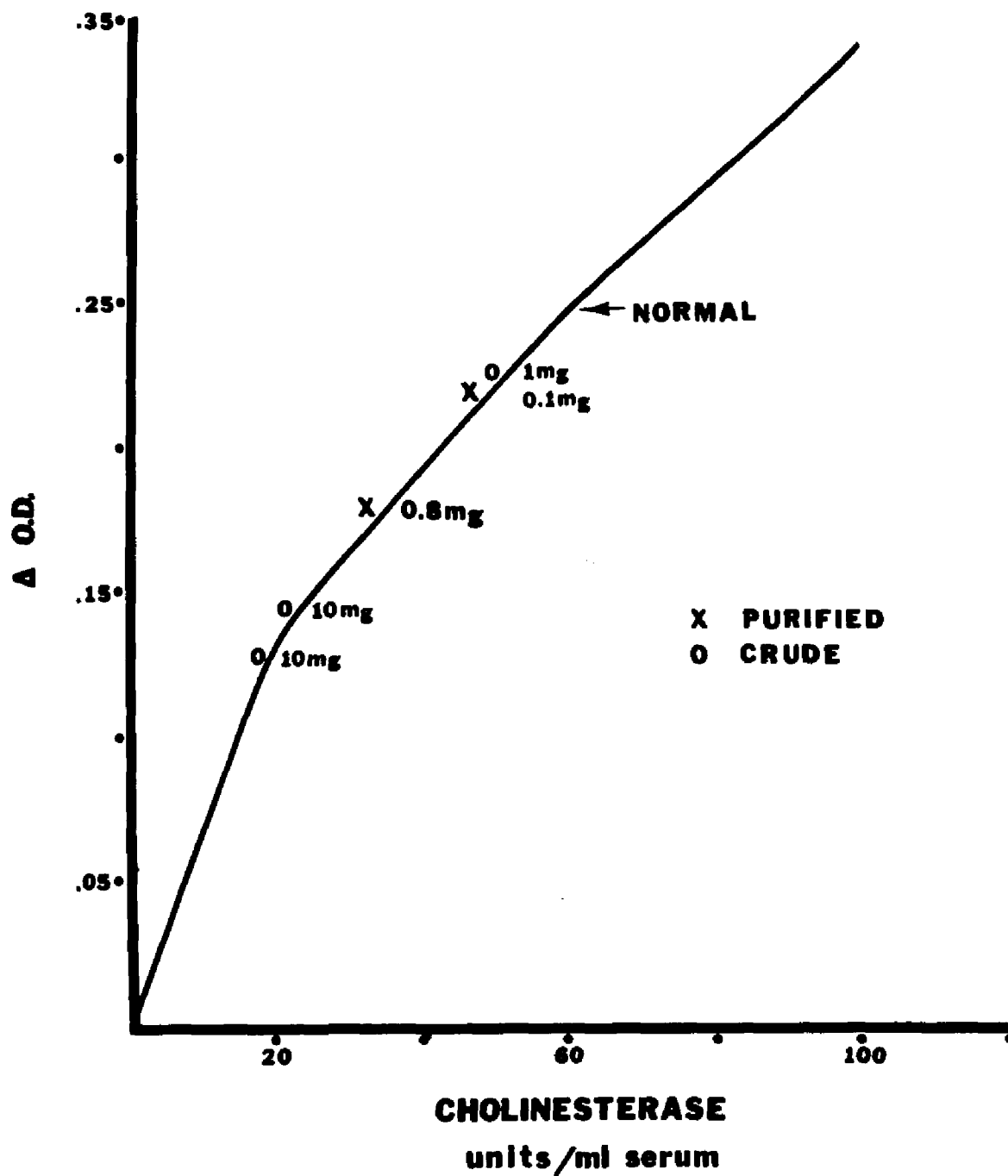


FIGURE 5. EFFECTS OF G. BREVE TOXINS ON HUMAN BLOOD SERUM
CHOLINESTERASE



normal is my normal serum cholinesterase value. The term OD is the optical density of an experimental sample subtracted from a blank. Cholinesterase is measured in Rappaport units defined as the amount of cholinesterase that will hydrolyze 1 μ mole of acetylcholine (Ach) in 30 minutes at 25° C. This value is represented by figures along the horizontal axis. Each experimental point on the curve is the average of duplicate tests. Crude G. breve toxin in concentrations of 1 mg/0.2 ml serum and 10 mg/0.2 ml serum, reduced cholinesterase levels by 90% and 30% of the normal values respectively. Purified fraction IVa was 10 times as potent as crude toxin in reducing cholinesterase activity.

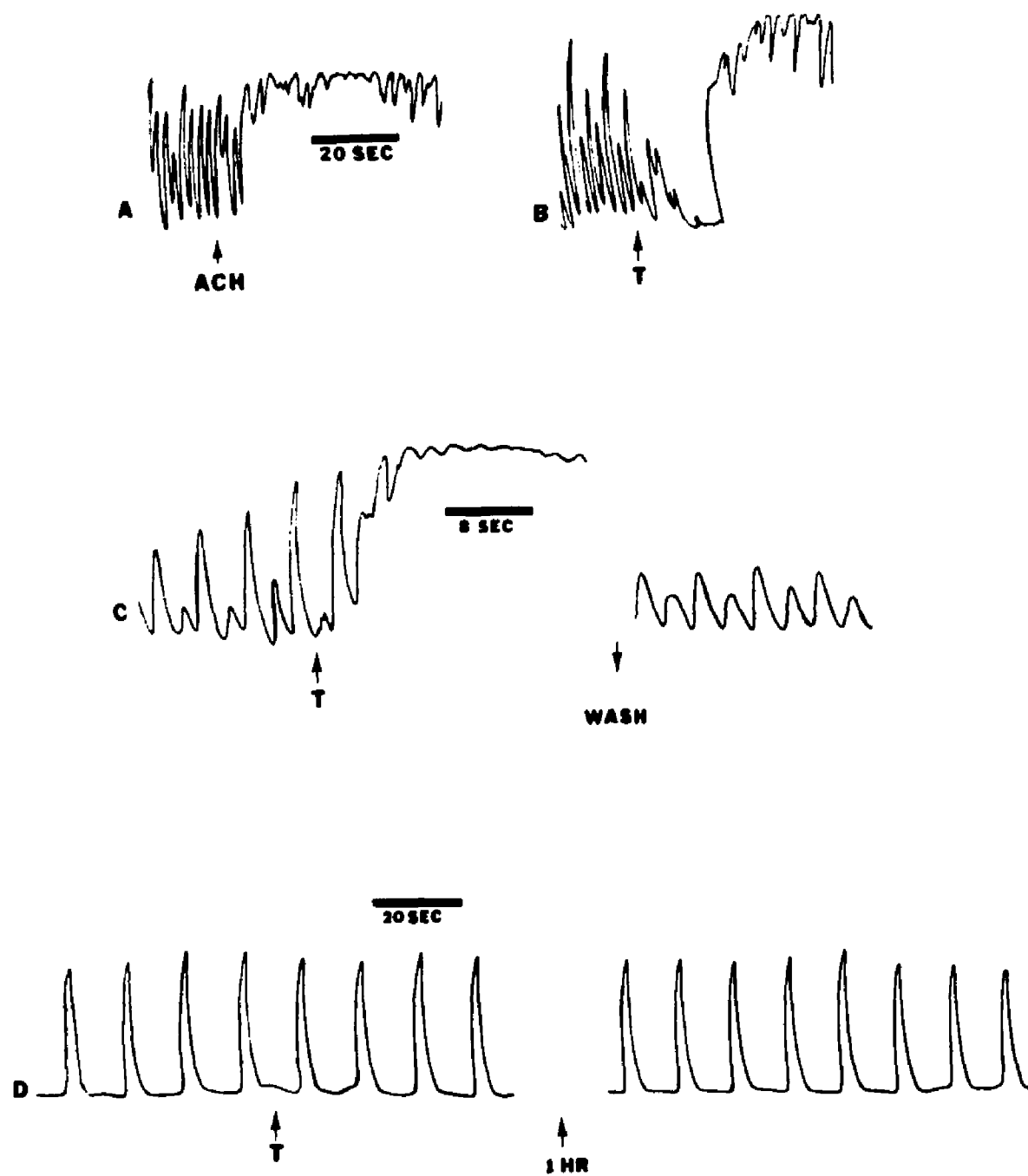
Addition of fraction IVa (1 μ g/ml) to the bathing medium of 5 mouse intestine smooth muscle preparations resulted in immediate tension development equal to that observed by direct application of 3 μ g/ml Ach (Fig. 6A and B). Washing with fresh saline reversed the effect.

B. AMPHIDINIUM CARTERI:

A five minute exposure to purified A. carteri toxin (2.2×10^{-5} g/ml) or crude toxin (1×10^{-2} g/ml) decreased frequency and amplitude of beat in isolated Mercenaria hearts. Diastolic arrest occurred after 20 min exposure to both toxins (Fig. 7C). Mercenaria hearts reacted in a

FIGURE 6. EFFECTS OF AQUATIC TOXINS ON MAMMALIAN SMOOTH
MUSCLE AND BIVALVE HEART PREPARATIONS

- A. Mouse intestine; ACH, acetylcholine (3 μ g/ml) Temp. 37 C
- B. Mouse intestine; T, G. breve fraction IVa (1 μ g/ml) Temp. 37 C
- C. Mouse intestine; T, A. carteri crude toxin (5 mg/ml) Temp. 37 C
- D. Mechanical activity of Mercenaria mercenaria heart in vivo T, Gonyaulax catenella pure toxin 50 μ g/ml. The same result is obtained with Tetrodotoxin (50 μ g/ml and Aphanizomenon flos-aquae toxin (4 mg/ml). Temp. 14 C



similar manner when bathed *in vivo* with crude toxin (2×10^{-3} g/ml). Washing the hearts with seawater partially or fully reversed the effect of this toxin.

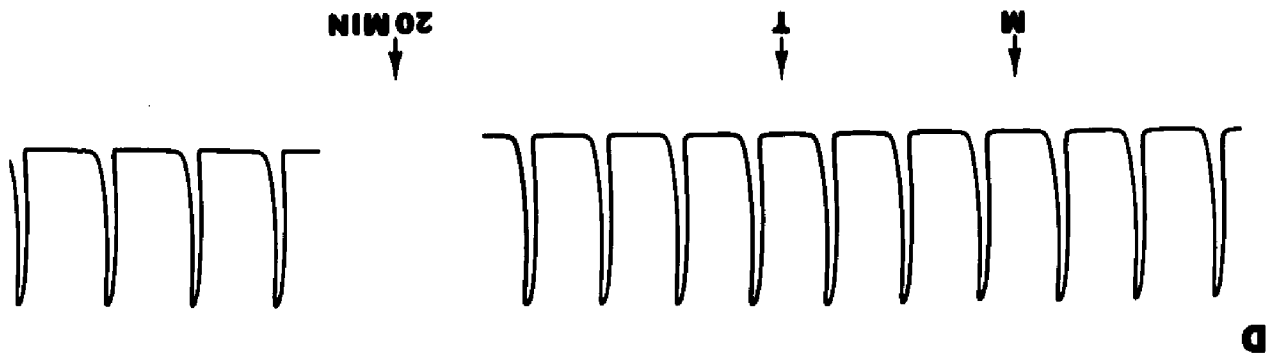
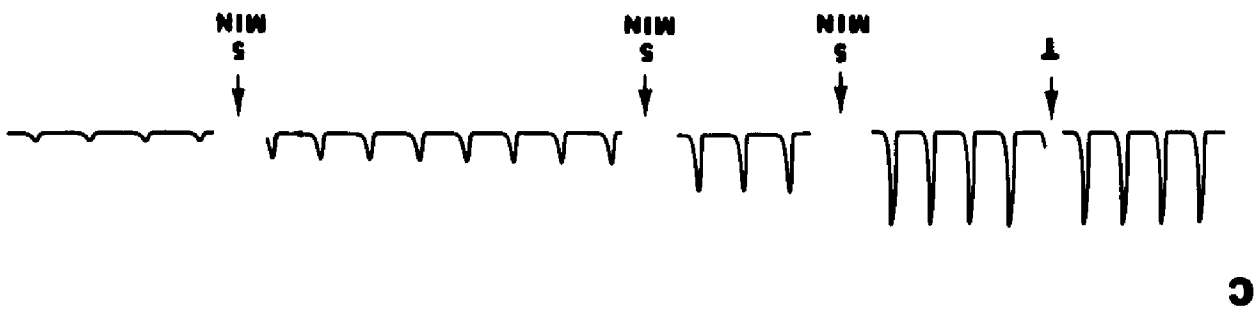
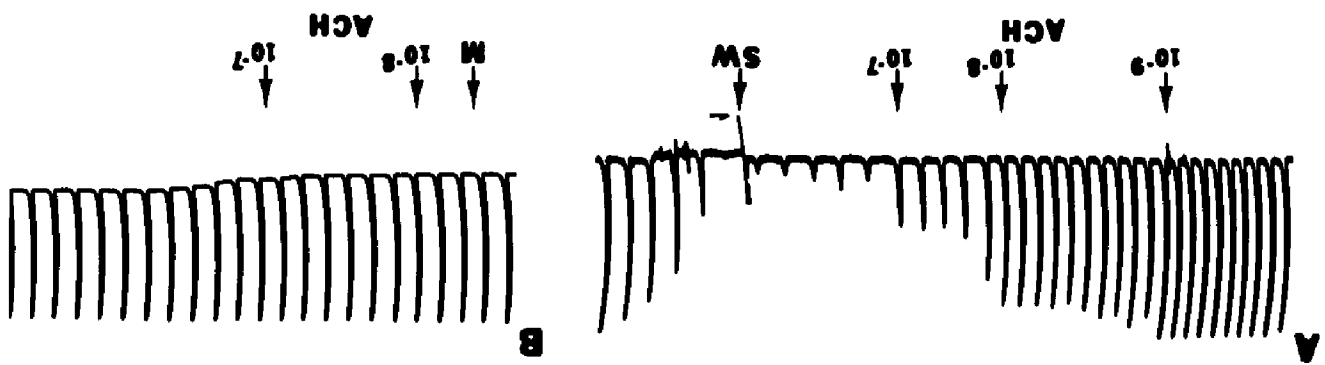
Isolated Mercenaria hearts were pretreated with a choline antagonist, Mytolon (benzoquinonium chloride) (1×10^{-5} g/ml). These pretreated hearts were not affected by Ach (Fig. 7B) nor by A. carteri toxin applied to concentrations equal to those described above (Fig. 7D).

Purified A. carteri (2.2×10^{-4} g/ml) topically applied to Cancer hearts (*in vivo*) prompted an immediate increase (2x) in frequency of beat. This effect is reversible by seawater washing. Crude A. carteri toxin (10^{-3} g/ml) caused the same effect, while 10^{-2} g/ml provoked an increase in tension with irreversible systolic arrest (Fig. 2D). Carcinus hearts reacted similarly. The ECG of Cancer reflected the increasing frequency of the heartbeat (Fig. 3D).

Frog hearts injected with 5 mg of crude A. carteri toxin displayed a decrease in frequency which is reversed by allowing the circulatory system to remove the toxin. Doubling the dose resulted in a diastolic arrest that also was reversible (Fig. 4A and B). The action potential did not change shape. At cardiac arrest the ventricle ceased beating and then became engorged with blood as the auricles

FIGURE 7. EFFECTS OF ACH, MYTOLON AND A. CARTERI TOXIN ON
BIVALVE HEARTS.

- A. Mechanical activity of Mercenaria mercenaria heart, isolated; ACH: acetylcholine; SW: seawater wash. Rate: 12 beats (contractions) per minute. Temp. 20 C
- B. Same as (A); M: mytolon chloride (1×10^{-5} g/ml) followed by Ach.
- C. Same as (A); T: A. carteri crude toxin (1×10^{-2} g/ml)
- D. Same as (A); Heart pretreated with (M) mytolon followed by (T) crude A. carteri (1×10^{-2} g/ml)



continued to beat for several minutes. The auricular action potential remained unchanged after the ventricular potential had ceased. Atropine sulfate (0.2 ml of 10^{-3} g/ml) injected prior to toxin application effectively blocked the action of crude toxin. Injection of 0.1 ml of purified A. carteri toxin (1.25×10^{-4} g/ml) failed to elicit a response. I had insufficient purified material to increase this dosage.

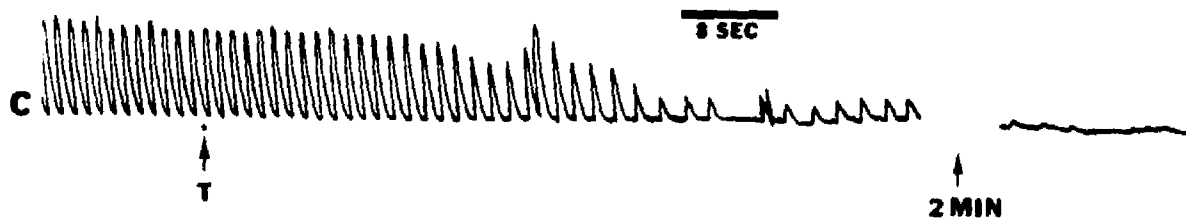
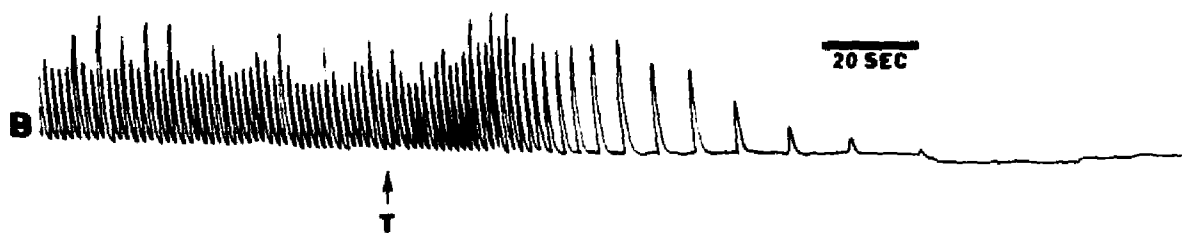
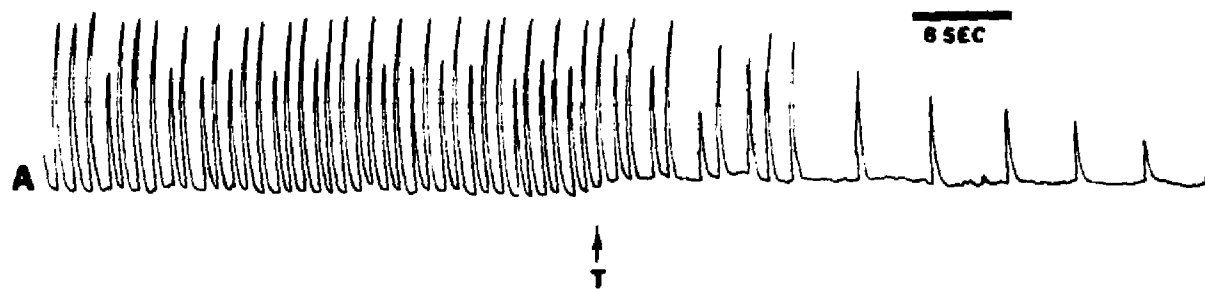
Pure A. carteri toxin doubled the frequency of contraction in 5 mouse intestine preparations within 5 minutes. This increased rate was accompanied by a 50% increase in amplitude and a much more regular series of spontaneous contractions (Fig. 6C). Crude toxin (0.5×10^{-3} g/ml) resulted in an immediate increase in frequency and tension development similar to that produced by treatment with 10^{-6} g/ml Ach. The results were reversible by flushing the chamber with fresh saline solution.

C. GONYAULAX CATANELLA AND APHANIZOMENON FLOS-AQUAE:

Toxins from G. catanella and A. flos-aquae slowed the beat of Cancer hearts, in vivo, and resulted in reversible diastolic arrest (Fig. 8A and C). TTX, which has pharmacological properties similar to G. catanella toxin, caused a similar effect in Cancer hearts (Fig. 8B). The similar cardiac depression brought about by all these

FIGURE 8. EFFECTS OF PSP-LIKE TOXINS ON CRAB HEART MECHANICAL
ACTIVITY

- A. Mechanical activity of Cancer irroratus heart
in vivo; Aphanizomenon flos-aquae toxin (T)
0.8 μg /0.2 ml. Temp 14 C
- B. Same as (A) with tetrodotoxin, 0.05 μg /0.1 ml.
- C. Same as (A) with Gonyaulax catenella toxin,
0.05 μg /0.1 ml.



toxins is evident in the ECG patterns (Fig. 9).

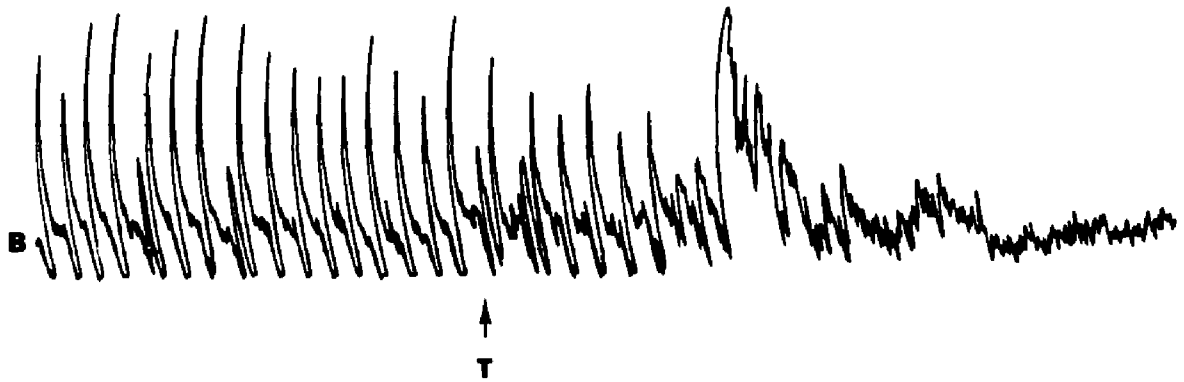
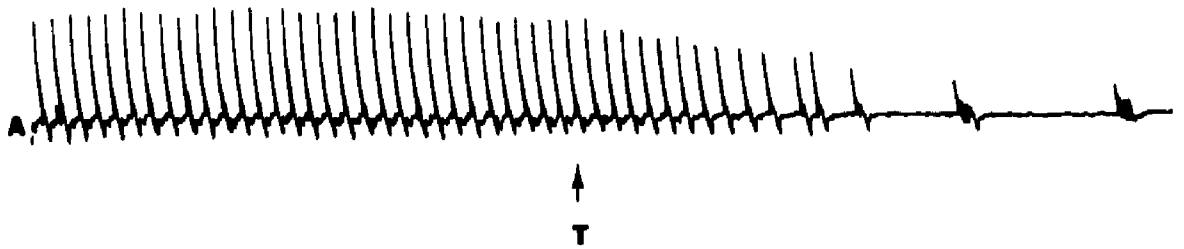
None of these 3 toxins provoked a response or alteration in mechanical activity in the myogenic bivalve hearts, despite application of higher (10x) concentrations than those employed on Cancer hearts (Fig. 6D). I analyzed the sodium dependence of Mercenaria cardiac tissue, since this organ was unaffected by these sodium-blocking toxins. Mercenaria hearts stopped beating immediately when bathed in sodium free media (sucrose or lithium substituted). Mercenaria hearts appear to be sodium dependent and their immunity to sodium blocking toxins remains unsolved.

FIGURE 9. EFFECTS OF PSP-LIKE TOXINS ON CRAB HEART

ELECTRICAL (ECG) ACTIVITY

- A. ECG of Cancer irroratus in vivo; Aphanizomenon flos-aquae toxin (T) 0.8 μg /0.2 ml. Temp. 14 C
- B. Same as (A) with tetrodotoxin (T), 0.05 μg /0.1 ml.
- C. Same as (A) with Gonyaulax catenella toxin (T), 0.05 μg /0.1 ml.

4 SEC



IV. DISCUSSION

Gymnodinium breve toxins excite decapod crustacean hearts, depress frog hearts and have no effect on bivalve molluscan hearts. The beat of the neurogenic crustacean heart originates from a ganglionic nervous tissue pacemaker. Electrical activity in the ganglion precedes the electrical and mechanical response in the cardiac tissue by 10-14 msec., and isolated pieces of the heart show contractions only when ganglion cells are present (Welsh and Maynard, 1951; Matsui, 1955; Maynard, 1955 and 1960). Ach accelerates decapod crustacean hearts and the presence of this compound and cholinesterase suggest that Ach may be the natural excitatory mediator (Welsh, 1938 and 1939b; Smith, 1947; Wiersma and Novitski, 1942; Bacq and Nachmansohn, 1937; Smith and Glick, 1939; Walop and Boot, 1950). Ach (10^{-9} g/ml) excites lobster (Homarus americanus) hearts and 10^{-8} g/ml causes some tonus (rise in base line) and increased frequency in the spiny lobster (Panulirus argus). Higher concentrations cause systolic arrest (Welsh, 1939b). Cancer crabs and green crabs (Carcinus maenas) also respond similarly to Ach (Davenport, 1941; Welsh, 1942; Smith, 1947). The anticholinesterase, eserine, induces effects similar to high concentrations of Ach. A dosage of 10^{-5} g/ml eserine

will increase crustacean heart sensitivity to Ach from to 10 to 100 fold (Welsh, 1939a and 1942; Davenport, 1941; Smith, 1947). The action of an anticholinesterase such as eserine presents evidence of a cholinergic system of excitation, however the excitatory neurohumor of crustacean hearts has not been fully resolved. Florey (1967) suggests that compounds other than Ach are involved, for example 5-hydroxytryptamine (5HT). The toxins of G. breve excite the crustacean heart in a manner characteristic of Ach and eserine. The mechanism of this action is unclear, however, with the lack of clear-cut evidence for a cholinergic excitatory system. The toxic action could result from a stimulated pre-synaptic release of Ach, 5HT, or some other excitor; from inhibition of cardiac ganglion cholinesterase and resultant Ach buildup; from increased post synaptic sensitivity to the excitatory transmitter; or any combination of these and other neuromuscular alterations.

Cholinesterase inhibition resulting from organophosphate compounds, i.e., nerve gas and insecticides, can be reversed by 2-PAM when a potentially reversible complex exists between enzyme and inhibitor (Holmstedt, 1959). Crab hearts pretreated with 2-PAM were unaffected or quickly returned to normal when exposed to G. breve toxins. This

action strongly suggests that a reversible complex exists between the toxin and an enzyme affecting excitation in crabs. Alone, 2-PAM has no effect and can be taken internally as a nerve gas antidote, thus it is unlikely to block the release of an excitatory transmitter substance.

Bivalve hearts possess very low cholinesterase levels (Julien et al., 1938; Smith and Glick, 1939) thus anticholinesterases rarely or only slightly alter the heart-beat of such molluscs (Hill and Welsh, 1966; Welsh and Taub, 1948). The low level of acetylcholinesterase may be explained by low Ach levels (but high Ach sensitivity) and the possible removal of some Ach after action via the open circulatory system rather than hydrolysis. The toxins of G. breve, have no effect on this heart, nor do mytolon or eserine, two additional anticholinesterases utilized in this study. This action may be due to the lack of a cholinesterase substrate or to permeability barriers in the cardiac membranes that prevent such compounds from affecting this tissue.

The hearts of frogs and other vertebrates are myogenic with the beat originating in the sinus venosus (Noble, 1931; Prosser and Brown, 1962). Ach, released by the vagus nerve, inhibits the heart by reducing the amplitude

and rate and is then hydrolyzed by cholinesterase (Prosser and Brown, 1962). The toxins of G. breve also inhibit the heart with resultant reduction in amplitude and rate terminating in diastolic arrest. Sasner et al. (in press) have demonstrated fibrillations and spontaneous tension development in frog (Rana pipiens) striated muscle treated with G. breve fraction IVa toxin. These actions could be attributed to anticholinesterase activity, increased pre-synaptic Ach release, or post-synaptic depolarization. The inhibitory effects of G. breve toxins on frog hearts are blocked by atropine. Atropine competes with Ach for post-synaptic active sites thus suggesting cholinergic toxic action.

The reduction of human blood serum cholinesterase activity demonstrates an in vitro anticholinesterase effect of G. breve toxins. Both crude and fraction IVa toxins inhibit the action of serum cholinesterase on an Ach substrate. The testing procedure used in this study is also used in evaluating the anticholinesterase effects of certain insecticides on human blood cholinesterase (Goltz and Shaffer, 1966). Paster and Abbott (1969) reported a hemolytic effect of G. breve toxin. Thus there are at least two toxic actions of G. breve that effect the circulating

tissue of the circulatory system; one which acts on serum cholinesterase and one which hemolyzed red blood cells.

The experiments discussed above point to a number of possible actions for G. breve toxins. The common denominator, however, is an anticholinesterase-like activity. The possibility, in fact probability, exists that G. breve is a multi-action toxin with anticholinesterase activity and another action or actions, such as the post-synaptic activity reported by Sasner et al. (in press). It is not uncommon for a compound to have multiple actions. Mytolon, for example, possesses anticholinesterase activity and also competes for Ach receptor sites (Holmstedt, 1959).

Amphidinium carteri cell extracts, like the toxins from G. breve excites crustacean hearts. Unlike G. breve, however, it depresses bivalve molluscan heart activity. The active material from Amphidinium carteri is a choline compound thus the excitatory action on crustacean hearts is explained by its action on the choline sensitive excitory neurons. The depressant action on bivalve hearts is due to the cholinergic nature of the inhibitory neurons.

Bivalve molluscs hearts are myogenic, i.e. the beat originates within the cardiac muscle tissue rather than in a nervous tissue pacemaker. The beat of clam and mussel

hearts may originate anywhere in the heart and the contraction may be local or complete (Prosser & Brown, 1962). The hearts of bivalves are sensitive to Ach, and since this substance depresses (decreases frequency and amplitude) the heart, it has been suggested that the inhibitory regulator nerves are cholinergic.

The heart of Mercenaria is an excellent assay organ for Ach because of its extreme sensitivity to this compound (as low as 10^{-12} g/ml). Prosser (1940) noted that test solutions could drip directly onto the in vivo heart, but that the isolated heart, mounted in a chamber through which test fluid was perfused, was more sensitive. Sensitivity of the heart to Ach remains stable for hours when the preparation is kept below 20° C. Both natural and artificial seawater are satisfactory perfusion fluids as Ach will remain potent for 4 to 6 hours in either. Welsh and Taub, (1948) described the action of 12 choline derivatives and noted that all depressed mollusc cardiac activity but were less reactive than Ach. Stimulation of inhibitory nerve fibers produces the same effects as Ach (Krijgsman and Divaris, 1955) and gives additional support to the theory of a cholinergic inhibitory system.

The choline toxin of A. carteri, like Ach depresses

bivalve hearts by stimulating the inhibitory receptors. This action is further demonstrated by the use of the Ach blocking compound, mytolon. Mytolon blocks the responses of Mercenaria hearts to Ach by competing for active inhibitory sites (Luduena and Brown, 1952; Greenberg and Windsor, 1962). Amphidinium carteri inhibition was blocked in the present study by pretreatment of bivalve hearts with mytolon.

The action of A. carteri toxin on the frog heart is also characteristic of choline compounds. Winker et al. (1962) working with a choline substance from the digestive gland of two west coast sea hares, Aplysia californica and A. vaccaria, reported that frog hearts were stopped in diastole and returned to normal after removal of the toxin. Mendes, Abbud, and Umiji (1963) perfused amphibian hearts with a compound reported to possess Ach-like properties that was extracted from sea urchin (Lytechinus variegatus) pedicellariae. They reported that it slowed the heart to diastolic arrest and that atropine blocked its action. A. carteri toxin inhibits frog hearts in a manner characteristic of increased Ach (slows heartbeat to diastolic arrest) and is also blocked by atropine.

Analysis of the choline compound extracted from A. carteri by chemists from the UNH Biochemistry Department

indicated that it is an undefined choline compound but not Ach. Welsh and Taub (1948) reported that "choline affects the isolated Venus heart in a manner very much like that of Ach, except it is far less active". Assuming that the refined toxin is 80-95% pure (as it was estimated), A. carteri toxin is about 100 times less potent than Ach when assayed on bivalve hearts. Thus A. carteri toxin is in the same potency range as many choline compounds tested by Welsh and Taub (1948).

Both G. breve and A. carteri toxins increase activity at the synaptic area and produce an ECG reflecting increased activity of cholinergic systems. Interference apparently occurs within the cholinergic system at the neuromuscular junction. Toxin from the Portuguese man-o-war (Physalia physalis) produces a similar ECG in crab hearts with some interference between the neurons of the cardiac ganglion and the heart contractile cells (Lane and Larsen, 1965). When G. breve and A. carteri toxins were applied to mammalian intestine an initial spasm and cessation of peristalsis occurred. This is the same type of effect that choline sea hare toxin produces (Winkler et al., 1962) and that choline esters of various marine gastropods elicit (Wittaker, 1960). This further suggests the choline action of A. carteri toxin and points to cholinergic activity as one action of

G. breve toxin.

Tetrodotoxin and the toxin from G. catenella both block sodium conductance in neuromuscular tissues (Kao, 1966). Both toxins block conductance without affecting the transmembrane resting potential (Mosher et al., 1964; Schantz et al., 1966). Sawyer et al. (1968) reported that A. flos-aquae toxin acts similarly on neuromuscular tissue and Jackim and Gentile (1968) reported chemical similarities between G. catenella and A. flos-aquae toxins. In the present study, these three toxins slowee crustacean and frog hearts resulting in diastolic arrest. Electrical activity in each case was diminished. As previously reported, this action could be due to a blockage of sodium conductance. These toxins might block the passage of other ions also. Sawyer et al. (1968) reported that A. flos-aquae toxin reduces excitability in calcium dependent tissue (crayfish deep extensor abdominal muscle) thus it may also block this ion. Voltage clamp studies must be performed before a complete evaluation of the action of these toxins is possible.

Molluscan hearts were not affected by any of the PSP-like toxins. Kao (1966) suggested that bivalve immunity to PSP might be due to a calcium dependent membrane rather than a PSP susceptible sodium tissue. The present study

showed that the molluscan hearts tested were very sensitive to sodium deficiencies and were thus sodium dependent. Kao's hypothesis is apparently not valid for cardiac tissue in the bivalves tested. Another possible explanation of the toxin immunity may be a natural permeability barrier. Some of the tissue surrounding the heart may be impermeable to these PSP-like toxins thus protecting the heart (and perhaps other tissues) from toxic effects. A comparison of the membranes of frogs, molluscs and crustaceans may prove useful in solving the question of shellfish accumulation of highly toxic material.

This study has made additional information available on the nature of four aquatic biotoxins. Aphanizomenon flos-aquae toxin exhibited mechanical and electrical effects on cardiac systems similar to those effects provoked by PSP and TTX. This observation supports earlier reports of chemical similarities between PSP and A. flos-aquae toxins. The bivalve hearts tested were sodium dependent thus their immunity to PSP-like toxins could not be attributed to hearts controlled by some other ion. The toxins of G. breve and A. carteri exhibited different cholinergic actions. Both are good indicators of different levels of cholinergic action and might be useful biological tools in the study of such action.

Many cholinergic drugs are used in the biomedical field today. Ambenonium chloride and neostigmine, for example, are anticholinesterases used in the treatment of pathologic exhaustion of voluntary muscles. Belladonna derivatives (atropine etc.) and choline compounds are also used in the treatment of cholinergic disorders. Basic information presented in this study on the cholinergic toxins of G. breve and A. carteri may yield future biomedical applications for these and similar aquatic biotoxins.

V. SUMMARY

1. The activity of cellular material from Amphidinium carteri has been evaluated.
2. The action of A. carteri toxin is choline-like although it appears to be other than Ach.
3. Gymnodinium breve toxin acts in at least 2 ways on the circulatory system. One fraction (IVa) possesses anti-cholinesterase-like activity while a second fraction exhibits hemolytic activity.
4. The toxins of G. breve and A. carteri act in predictable ways on the cholinergic systems of myogenic and neurogenic hearts. These compounds may be good indicators of different levels and types of cholinergic activity.
5. The toxin obtained from Gonyaulax catenella and Aphanizomenon flos-aquae cause similar electrical and mechanical alterations of cardiac activity. This similarity supports previous reports of chemical similarities.
6. The bivalve hearts tested appear to be sodium dependent but they are not altered by "sodium blocking" toxins.
7. Basic physiological data is presented on the action of several aquatic toxins. Compounds with similar action are in use in the medical field today. Future research may demonstrate uses for these biologically active materials.

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