Research Report No. 28

THE EFFECTS OF TOXIC BLUE-GREEN ALGAE ON AQUATIC ANIMALS

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John J. Sasner, Jr.

Department of Zoology

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Miyoshi Ikawa

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TECHNICAL COMPLETION REPORT

Project Number A-047-NH

Water Resource Research Center
University of New Hampshire
Durham, New Hampshire

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Annual Allotment Agreement No.: 14-34-0001-8031

The work upon which this publication is based was supported in part by funds provided by the Office of Water Research and Technology, Project Number A-047-NH, U.S. Department of the Interior, Washington, D.C., as authorized by the Water Research and Development Act of 1978.

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ABSTRACT

Toxic blue-green algae (Cyanobacteria) bloom in eutrophic, freshwater lakes and ponds in New England and have caused environmental, health, legal and recreational problems over the last 15 years. The most common offensive species were *Aphanizomenon flos-aquae* and *Microcystis aeruginosa* (a.k.a. *Anacystis cyanea*). Research has focused on: a) toxin accumulation and identification from natural blooms and laboratory cultivation, b) toxin assay, using the mouse bioassay and a new fluorometric method adapted for paralytic shellfish poisons, c) toxin characterization and purification, using solvent separation and molecular weight filters, and d) testing active extracts on whole animal and tissue preparations - to determine the specific sites and modes of action of aphantoxin (from *A. flos-aquae*) and FDF or microcystin (from *M. aeruginosa*).

Microgram quantities of aphantoxin reversibly blocked action potentials in amphibian and crustacean axons as mechanical activity in skeletal muscle. No effect was measured on transmembrane resting potentials or on miniature end-plate potentials. Aphantoxins may block excitability by affecting ion conductance pathways, as do toxins from several marine dinoflagellates and thus may be useful for basic studies on membranes.

Histological and ultrastructural studies showed that microcystin had no effect on neuromuscular systems, but caused liver damage in mammals. Liver sinusoidal epithelium and hepatocyte plasma membranes ruptured causing severe hemorrhaging within 1 hour in toxin-treated mice and rats. The toxin only affected endothermic vertebrates.

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INTRODUCTION

Freshwater blooms of toxic blue-green algae (Cyanobacteria) are common in many countries of the world. Animal, including human, involvement with toxicity problems has been reported for at least 12 countries, 4 Canadian provinces, and 10 of the United States (Schwimmer and Schwimmer, 1964, 1968; Moore, 1977; Collins, 1978). Although many microorganisms have been implicated with water fouling and animal kills, frequent offenders are Aphanizomenon flos-aquae, and Microcystis aeruginosa (also known as Anacystis cyanea). Representatives of these two 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 blue-green algae 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, 70; 1973-1975). In addition, at least one legal case in New Hampshire focused attention on sewage treatment effluent and toxic blue-green algal blooms in Kezar Lake, North Sutton, New Hampshire (W.A. Sundall, et al., versus Town of New London, 1977). The environmental parameters associated with toxic blooms in this state were described by Haynes

(1971) and in the New Hampshire Water Supply and Pollution Control Commission reports enumerated above.

Research supported by the New Hampshire Water Resource Research Center has focused on methods of a) toxin accumulation from natural blooms and laboratory cultivation, and identifying toxic from non-toxic forms, 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 whole animals and physiological preparations, to determine the specific sites and modes of action of the biotoxins from Aphanizomenon flos-aquae and Microcystis aeruginosa.

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. The toxins of interest in these studies meet most, if not all, of these criteria. Table 1 summarizes the "state of the art" concerning the chemical characteristics and physiological effects associated, thus far, with Aphanizomenon flos-aquae and Microcystis aeruginosa. The information on aphantoxin from A. flos-aquae and microcystin from M. aeruginosa was accumulated and synthesized in our laboratories at the University of New Hampshire over the last several years, with support from the New Hampshire Water Resource Research Center. Significant contributions were made by former graduate students** (indicated in text and Table 1 by "**") who matriculated to other laboratories and continued research on toxic microorganisms. The initial work on microcystin and other toxins from M. aeruginosa was done earlier in other laboratories and is summarized here.

TABLE 1. SUMMARY OF TOXIN CHARACTERISTICS FROM *Aphanizomenon flos-aquae* & *Microcystis aeruginosa*

Species Characteristic ⟶	*Aphanizomenon flos-aquae*	*Microcystis aeruginosa* (a.k.a. *Anacystis cyanea*)
Name(s)	Aphantoxin; very fast death factor, (VFDF); endotoxin.	Microcystin, fast death factor, (FDF), endotoxin; & Slow death factor, (SDF), of assoc. bacterial origin; & Diarrheagenic factor, (DF).
Chemical	H_2O & ETOH soluble, $CHCl_3$ insoluble, acid & heat stable, alkali labile, low mol. wt. < 500, guanidine derivative, contains saxitoxin (STX) + 3 unknown substances, forms fluorescent derivative upon alkaline-H_2O_2 oxidation.	FDF-soluble in MeOH, EtOH & Na_2CO_3, cyclic peptide with ornithine & D-serine, enzyme resistant, mol. wts. 1300-2600-19,400. SDF-non-dialyzable, large mol. wt. material of unknown structure/composition. Ditto for DF,-diarrheagenic factor.
General effects (i.p.-mice)	Mammals & Fish-spastic twitching, coord loss, respiratory irreg; bivalves may store aphantoxin like PSP in marine molluscs?	FDF- lethargy, dyspnea, loss of coord, pallor. SDF- piloerection, dyspnea, intermittent convlusions. DF - intentinal disturbances.
Bioassay(i.p.)	1 MU=am't toxin to kill 20±2 g mice in 15 min	FDF- 1 MU=am't toxin to kill 20g mice in 60 min. SDF- 1 MU=am't toxin to kill 20g mice in 4 - 24 hrs.
Dose Range (mammals)	lyoph. cells = 10 mg/kg; purified=745 MU/mg	FDF- lyoph. cells=2mg/MU, 40-160 mg/kg; purif.=.1mg/kg SDF- 1-10 mg/kg
-excised tiss.	0.05 - 100 µg/ml	FDF- 0.5 - 5 mg/ml; SDF=?; DF=?
Nerve-Muscle Action potentials	blocked in nerves (desheathed) & muscle - reversible.	FDF- No immediate effect (>1 hr); SDF & DF = ??
Rest. potential	No effect	FDF- No effect; SDF & DF = ??
Muscle Mechan. Activity	Vert. skeletal-block to indirect then dir. stimulation; Vert & Crustacean heart block; cardiac A.P. reduced, reversible; mollusc heart-No effect at increased doses-(X 100)	FDF & SDF - slow flaccid paralysis; no immed. effect FDF- extravasation in liver, hepatocyte destruction, necrosis.
Site of Action	nerve & muscle membranes	FDF- hepatocytes; SDF = ?; DF = ?
Mode of Action	Ion Conductance Pathways - suggested	FDF- membrane integrity ?; SDF & DF = ??
References	Sawyer, et al.,(1968); Alam,** (1972,73,78); Gentile,**(1971); Thurberg,**(1972); Sasner, (1973); Sasner & Ikawa, (1975); Shoptaugh,**(1978); Carter,**(1980)	Bishop, et al.,(1959); Gorham, (1962 & 64); Murthy & Capindale, (1970); Gentile,**(1971); Kirpenko, et al, (1975); Sirenko, et al., (1976); Foxall,** (1980).

SUMMARY OF KNOWLEDGE IN THE FIELD

Aphanizomenon flos-aquae

In 1968, Sawyer et al., demonstrated the presence of a very fast death factor (VFDF) from A. flos-aquae cells collected during bloom conditions in two New Hampshire lakes (Kezar Lake and Winnisquam Lake). The toxin was dialyzable, heat and acid stable, alkali labile, and was soluble in water and ethanol, but insoluble in less polar solvents. The same year (1968) Jackim and Gentile**, using laboratory cultures, reported the partial purification and properties of aphantoxin. This work was a "spin-off" from studies initiated in our laboratory earlier. Three toxic fractions were obtained using acid extraction, preparative paper chromatography and silica gel column chromatography. The most potent of these gave 3 Weber reagent-positive spots, one of which corresponded in R_f value, reactions with Weber, ninhydrin and Jaffe reagents, and infra-red 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 ug per mouse unit (MU), which is equal to 500 to 667 MU/mg. In other words, one mg of the toxin had the potency to kill more than 500 twenty-gram mice.

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 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 aphantoxins may be substituted guanidine derivatives. However, TLC in various solvent systems, color reactions given with various spray reagents, electrophoretic comparisons, and infra-red spectra indicated that aphantoxins were not identical with saxitoxin (STX). More recent work by Alam** et al. (1978), showed that aphantoxins are a complex mixture containing saxitoxin and other related, but still unknown, substances. This mixture has not been completely characterized and current research proposals are pending for this purpose. In addition, Shoptaugh** (1978) and Carter** (1980) have shown that aphantoxins may lend themselves to qualitative and quantitative analysis using alkaline-H_2O_2 oxidation and fluorometry, a method that we are currently exploring with the aim of replacing the expensive, and often inaccurate, mouse bioassay.

The effects exhibited by whole organisms, either injected with or bathed in toxic samples of A. flos-aquae, are qualitatively similar to those reported for the marine dinoflagellate poisons (PSP) cited above. The characteristic symptoms of aphantoxins in mice and fish include respiratory irregularity, spastic twitching, gaping mouth, loss of coordination, violent tremors, and subsequent death by respiratory failure. The mouse unit (MU) for aphantoxins is the same as that used to evaluate amounts of paralytic shellfish poisons (PSP) in marine bivalves, i.e., the amount of material (i.p.) that will produce survival times of 15 minutes in 20±2 gram mice = 1 MU. Aphantoxins are potent nerve and muscle blocking agents that reversibly destroy action potential conduction in these tissues without affecting the transmembrane resting potential (Sawyer et al., 1968; Alam** et al., 1973; Sasner and Ikawa, 1975). The effects on nerve and muscle preparations are suggestive of biotoxins which block excitability by altering ionic mechanisms associated with activity in excitable systems. This type of effect is similar to that described for saxitoxin (STX) and tetrodotoxin (TTX - puffer fish poison), both of which are useful "tools" important in basic studies on membrane function (Kao, 1966, 1972; Narahashi, 1972).

Microcystis aeruginosa - (a.k.a. Anacystis cyanea)

This species is the source of at least 3 different active materials: 1) microcystin or fast death factor (FDF); 2) a slow death factor (SDF) that may arise from associated bacteria; and 3) a diarrheagenic factor.

Microcystin (FDF)

In 1959, Bishop et al. reported the isolation of a fast death factor from laboratory grown cells of M. aeruginosa. The toxin was extracted from the cells with methanol, ethanol, or 0.1 N sodium carbonate. The dialyzable material in the extract was purified by paper electrophoresis to yield a toxic peptide (later named microcystin by Konst et al., 1965). The toxic peptide had a LD_{100} of 0.466 $\pm$ 0.013 mg/kg when injected intraperitoneally (i.p.) into mice. The active material did not form a N-2,4-dinitrophenyl amino acid, and its resistance to attack by proteolytic enzymes indicated the presence of a cyclic compound. The presence of D-serine was indicated by the disappearance of serine when the hydrolysate of the peptide was treated with D-amino acid oxidase. None of the other amino acid components were affected, indicating they were in the L-configuration.

In 1970, Rama Murthy and Capindale reported the isolation of a toxic peptide from the same strain of M. aeruginosa used by Bishop et al. (1959). The toxin was also extracted using sodium carbonate, but further purification was done by column chromatography on DEAE-Sephadex, instead of paper electrophoresis. The product appeared to be a single component by chromatography and electrophoresis and had a LD_{100} of 0.1 mg/Kg (i.p. in mice). The toxic peptide migrated as a cation during electrophoresis at pH 9.1, whereas the product described by Bishop et al. (1959) migrated as a slow moving anion at this pH value. An amino acid analysis showed the presence of 7 amino acids in addition to the 7 amino acids reported earlier for microcystin. Although this peptide obviously differed from microcystin, the authors believed that both were possibly derived from the same toxic material. More recent evidence suggested a larger

(19,400 daltons) polypeptide (Kirpenko et al., 1975), although extracts in the latter study were made from mixed algal masses, instead of unialgal cultures as in the earlier studies. All reports dealing with the chemistry agree that the active material is a polypeptide. These reports, however, differ as to the amino acid composition and molecular size.

Gorham (1964) reported that the survival time for a minimum lethal dose of FDF in 20-gram mice was 30 to 60 minutes. For bioassay purposes, one mouse unit (MU) was defined as the amount of FDF required to kill 20-gram mice in 60 minutes after i.p. injection. Our lyophilized cell extracts from natural blooms in New Hampshire had a potency of 1 MU/mg, or 50 mg/Kg body weight in mice. The gross effects in mammals challenged with FDF were characterized by lethargy, dyspnea, pallor, labored ventilation and non-violent death. The primary site of action of FDF in adult mice is in hepatic tissues where the animal essentially bleeds to death (Foxall, 1980, see Appendix). Sublethal doses of toxin were reversible over time (Heaney, 1971) and there is evidence that a) young mice are more resistant to FDF than adults, and b) adults may be more resistant to lethal doses after pre-treatment with sublethal doses. The role of FDF (microcystin) in animal deaths has been attributed to effects at the organ, tissue, and cell levels of organization, e.g. acute hepatic insufficiency, cardiovascular damage, respiratory failure, and the depression of cellular metabolism in body tissues. Common characteristics associated with FDF include a) the liver as the target organ, b) the engorgement of the liver with blood causing up to 33% increase in mass, c) the destruction of hepatocytes, and d) the rapid onset of cell degeneration and necrosis (Foxall, 1980).

SDF - Slow Death Factor

Hughes et al. (1955) reported the presence of two different toxins isolated from a bloom of M. aeruginosa in Ontario, Canada. Using ultraviolet irradiation on unialgal cultures, they were able to cause fast deaths (1 hour) and slow deaths (24 to 48 hours) in mice. Further work showed the presence of a slow death factor (SDF)

in addition to the already known fast death factor (FDF) in the same algal samples, each with its own unique symptoms (Hughes et al., 1958). When lethal amounts of FDF were present, the effects of the SDF were obscured. Further confirmation of the SDF came from Thompson et al. (1957) when they found a strain of M. aeruginosa with no FDF, but only SDF. The latter was produced by bacteria associated with the cells. The SDF was defined as the toxin that produced survival time in mice ranging between 4 and 48 hours and was of bacterial origin (Gorham, 1962). Symptoms associated with SDF included intermittent lethargy, piloerection, and convulsions preceeding death. Thus far, we are not aware of any chemical or physiological studies associated with SDF from M. aeruginosa.

In addition to FDF and SDF, Aziz (1974) described a diarrheagenic material from a strain of M. aeruginosa isolated from a pond in Bangladesh. The active substance was a non-dialyzable fraction of whole cell lysate and caused fluid accumulation in the small intestine of guinea pigs. Outbreaks of diarrhea in the human population have often been reported from areas where there were blooms of this species and where no other obvious etiology could be found. Gorham (1962) suggested that SDF might be linked to cases of intestinal inflammation in connection with algal poisoning by M. aeruginosa. Chemical and physiological information on this active material, as with the SDF, is lacking.

SUMMARY OF THE RESEARCH

ACCUMULATION OF TOXIC MATERIALS

Field Collection

State agencies monitor algal bloom conditions regularly throughout New Hampshire and Vermont and their findings were readily 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 both Aphanizomenon flos-aquae and Microcystis aeruginosa have occurred intermittently in Kezar Lake, Marsh Pond, Enfield Reservoir, Exeter Reservoir, several farm ponds and other freshwater environments. Cell concentrations, in some cases, exceeded 5×10^7/liter. We have worked out an effective method for obtaining bulk quantities of cells during bloom conditions in remote locations. DeLaval Separators, at lakeside, were used to spin and concentrate Aphanizomenon cells from large volumes of water. The crude materials were stored in the frozen state, either wet or lyophilized. Samples have retained potency under these conditions for more than 7 years. Bulk samples of Microcystis were also collected from natural blooms. The method used was dependent on the buoyancy provided by gas vacuoles associated with the cell colonies. When the cells occurred singly or in small colonies, they were centrifuged, like the Aphanizomenon cells. When the cells comprised dense massive colonies, they were collected by skimming the surface water, concentrated further by flotation in separatory funnels and by centrifugation, and then stored, either wet or lyophilized, in the frozen state. Microcystin potency was unaltered after 8 years under these conditions.

Laboratory Cultivation

Unialgal, but not bacteria free, cultures were grown from toxic

clones of Aphanizomenon and Microcystis 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. Reserve cultures were maintained in incubators separate from the other culture stock to ensure against equipment failure and thus loss of toxic cells. The cells were collected using continuous-flow centrifugation and/or by the use of separatory funnels and stored as with the natural samples. Although our principal interest was in the study of toxins from natural blooms, we supplemented the natural biotoxin supplies with material from laboratory cultures.

Partial Purification of Toxins

Recent work has shown that Amicon and Millipore molecular weight filters were useful for "cleaning up" samples of aphantoxin (Shoptaugh**, 1978; Foxall**, 1980 - see appendix). This method separates the toxins from high and intermediate molecular weight contaminants and may even be useful for separating the low molecular weight microcystin from the higher molecular weight SDF. Small amounts of the aphantoxin have routinely been passed through the 500 dalton filters. This may prove to be a useful step in processing toxin before further purification with high pressure liquid chromatography (HPLC).

Assay Systems

For the bioasssy of the active materials, 18 - 22 gram mice (B6D2F1/J; Jackson Laboratory, Bar Harbor, Maine) and the standard methods developed for marine toxins, were used (Halstead, 1965; Prakash et al., 1971). When only small amounts of toxin were available, assay was accomplished using electro-physiological methods. In addition, we have found (Shoptaugh**, 1978) that aphantoxin forms fluorescent derivatives when treated with hydrogen peroxide (like STX and other PSP derivatives). A promising fluorescence assay for PSP and aphantoxin was developed (Shoptaugh**, 1978; Carter**, 1980).

Preliminary assay work with freshwater bivalves indicated that they may store aphantoxin like marine molluscs accumulate PSP.

Toxicity monitoring must be done on a large scale to support or refute our preliminary observations. Since freshwater bivalves are consumed by certain ethnic groups in California, this monitoring work may be of more than just academic interest. It was estimated that 50,000 to 100,000 pounds/year of Corbicula fluminea, (bivalve, Mollusc) are available in the California marketplace (Mr. Jerome Jenkin, Pacific Shellfish Company, S.F., personal communication).

Physiological Testing

The toxic extracts from the field and laboratory were tested on standard nerve and nerve-muscle preparations, including mammalian, amphibian, and crustacean species. Control preparations were challenged with similarly treated material from non-toxic extracts. All testing employed dual stimulating and recording systems. Compound action potentials were recorded externally from amphibian sciatic nerves using Ag-AgCl electrodes, and Tektronix stimulating and recording equipment. Transmembrane resting potentials and action potentials from medial and lateral giant axons from crayfish used KCl-filled glass, microcapillary electrodes, Grass P-16 Amplifier and Tektronix storage Cathode Ray Oscilloscopes. Muscle tests used dual multi-electrode assemblies for direct stimulation with recordings displayed on a dual beam oscilloscope or Grass Polygraph. Isotonic and isometric measurements on amphibian muscle used Grass FT-03C Mechano-electrical Transducers. Previous work in our laboratory (Sasner, 1973) demonstrated the utility of the amphibian sartorious nerve-muscle preparation as an assay tool because of its sensitivity to aphantoxin. 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 physio-chemical phenomena associated with excitability are slowed.

In previous work (Sawyer et al., 1968; Alam** et al., 1973; 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 can

only be tested by using voltage-clamp methods on individual axons from a variety of animals (squid, crustacean, polychaete and amphibian). The goal in such studies is to measure the physical characteristics of the axon membrane and transmembrane current flow on voltage-clamped cells challenged with 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 aphantoxin 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} in mA/cm^2) as a function of clamped membrane potential (E_{mem} in mV).

The specific equipment and methods to accomplish the above are not yet available at UNH. We are currently collaborating with another laboratory that is actively engaged in voltage-clamping studies. Collaborative arrangements have been made with Dr. William Adelman, Chief of the Laboratory of Biophysics, NINCDS, Woods Hole, Massachusetts, for voltage-clamp experiments. These should be done by September, 1980. The results of these unfinished studies hold more than just academic interest to 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, 1972; 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 variety of vertebrate and invertebrate nerve and muscle preparations. There are, however, differences between the 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 ion conductance properties of the membranes, then more extensive comparative studies should be done to determine if the

cyanobacterial toxin (aphantoxin) is more STX-like or more TTX-like in action. In addition, it is important to include the variety of toxins from the local marine dinoflagellates, Gonyaulax tamarensis, since these materials are similar in their effects on membrane systems (Evans, 1975; Narahashi, 1975; Shimizu, 1978).

The research on FDF (microcystin) from M. aeruginosa focused on the specific site and mode of action of these toxic peptides at the tissue, cell and sub-cellular level of organization. This species is commonly involved in freshwater blooms, and exerts its effects by causing liver damage in birds and mammals. Histological changes in centrilobular regions of hepatic tissue were noted after i.p. administration of FDF in mice. Hepatic sinusoids expanded, parenchymal cords disintegrated, and cells lysed resulting in extensive tissue damage and subsequent death within 1 hour. Ultra-structural studies showed that sinusoidal epithelium and hepatocyte plasma membranes ruptured with the release of cellular components that pooled with blood. Mitochondria appeared swollen, but there were no obvious distortions of other organelles. Extensive vesiculation of membrane fragments was observed. Hepatic damage caused hemorrhaging into the liver and cell debris accumulated and produced a significant increase in liver weight.

Primary cultures of hepatocyte micro-explants from pre and postnatal mice and rats were exposed to microcystin but in vitro effects were not observed. In vivo experiments demonstrated that young animals were not sensitive to the toxin, but developed sensitivity as they matured. Microcystin was shown to be very specific in its site of action, since it had no antibiotic activity against green algae, yeast, or bacteria and was non-toxic to certain zooplanktors, crayfish, amphibians and teleosts. Electrical or mechanical activity in isolated nerve, nerve-muscle and cardiac preparations also was not effected. The toxins employed in this study came from laboratory cultured cells and naturally occurring blooms and gave identical results in all experiments.

The research, thus far, has provided new information in several areas of biotoxicology and has related freshwater and marine research

on the chemistry and physiology of naturally occurring, but harmful, materials. These include: a) the chemical identification of aphantoxins and their relationship to marine dinoflagellate poisons from Gonyaulax tararensis (the New England "red tide" organism), b) the development of a new assay method for the qualitative and quantitative determination of biotoxins that lend themselves to fluorescent methods and which may replace the commonly used mouse bioassay method, c) the elucidation of food chain relationships, particularly with respect to bivalve accumulation of toxins, and d) the elucidation of the physiological effects of aphantoxins and FDF (microcystin) and the determination of their sites and modes of action.

The results of our research will be presented and later published in the Proceedings of the upcoming Environmental Protection Agency Conference - entitled "The Water Environment; Algal Toxins and Health" to be held in Dayton, Ohio in June-July 1980.

APPENDIX

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