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PHYCOTOXINS FROM DINOFLAGELLATES

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Abstract - Dinoflagellates produce many toxic substances throughout the world. Some of these poisons get into human food through the marine food chain to edible shellfish and fish and cause diseases such as paralytic shellfish poisoning and ciguatera poisoning. The structures of two poisons that cause paralysis have been determined and are (a) saxitoxin, the major toxin produced by Gonyaulax catenella and found in California sea mussels and Alaska butter clams and (b) 11-hydroxysaxitoxin sulfate, the major poison produced by Gonyaulax tamarensis and found in scallops and clams along the New England coast and the Bay of Fundy. These extremely poisonous substances cause paralysis by blocking the passage of sodium ions through nerve and muscle cell membranes. They are quite heat stable at ordinary cooking temperatures, making the food poisoning problem more acute. Poisons from other dinoflagellates produce ciguatoxin and maitotoxin which are believed to cause ciguatera poisoning. Another poison causes liver and kidney degeneration. These poisons have been partially characterized.

INTRODUCTION

As we observe and study the sources of the toxins and poisons found in many marine animals that we use for food, it is becoming more apparent that a great number of these disease producing substances have their origin in the marine dinoflagellates and reach humans and other animals by way of the food chain. Although many of these toxic substances cause food poisoning, they, like other toxic substances, are valuable in medical research because they have specific mechanisms of action. This paper describes several important poisons produced by the marine dinoflagellates. Out of about 1200 species of dinoflagellates only 10 or 12 are known to produce poisons.

POISONOUS DINOFLAGELLATES

The relationship between a particular dinoflagellate and poisonous mussels was first reported by Dr. Herman Sommer and his associates (1) at the University of California during an outbreak of poisoning in humans which caused sickness and death from eating sea mussels (Mytilus californianus) collected near San Francisco in the summer of 1927 and at times in later years. These investigators observed that a particular dinoflagellate was present in the water where the mussels were feeding at the time they became poisonous, and found that acidified water extracts of the organisms killed mice with paralysis similar to that caused by extracts of poisonous mussels. Sommer et al. (2) identified this organism as Gonyaulax catenella Whedon and Kofoid. To verify this observation Dr. Sommer placed nonpoisonous mussels in laboratory cultures of G. catenella and found that they soon acquired poisonous properties from feeding on the organisms. After removing the mussels from this culture and placing them in a culture of nonpoisonous organisms the poison in the mussels was destroyed or excreted within a week or 10 days. These experiments duplicated the natural occurrence of poisonous mussels and explained why they appeared only when the poisonous dinoflagellate bloomed, remained poisonous for a period of one to three weeks and then became safe again to eat as the poisonous dinoflagellate died out and nonpoisonous ones bloomed as food for the mussels. G. catenella has bloomed sporadically and has caused shellfish poisoning all along the Pacific coast of North America from central California to Japan and along the southern coast of Chile and South Africa.

The discovery of the relationship of G. catenella to poisonous mussels in California led to discoveries of other dinoflagellates that produced the paralytic poison and caused shellfish to become poisonous. Koch (3) found Pyrodinium phoneus Woloszynska and Conrad to be responsible for the extreme toxicity of Belgian mussels. Needler (4) and Prakash (5) established that the poison in scallops in the Bay of Fundy and in clams and mussels along

the North Atlantic coast of America and in the St. Lawrence estuary was caused by the dinoflagellate Gonyaulax tamarensis Labour. This organism caused outbreaks of shellfish poisoning along the northeast coast of England in 1968 and has bloomed sporadically in this area since that time. It also caused shellfish to become poisonous along the coast of New England in 1972 and has persisted in this area since that time. Prakash and Taylor (6) found the paralytic poison in another species, Gonyaulax acatenella Whedon and Kofoid, which occurs along the coast of British Columbia and has caused shellfish, particularly the Alaska butter clam, to become poisonous.

The above organisms are the only identified species that produce the paralytic type of poison and cause shellfish to become poisonous, but there are poisons produced by some unidentified species that cause a similar paralysis (7). Other dinoflagellates produce different poisons that cause diseases in humans from eating shellfish and fish. Recently Yasumoto et al. (8) in Japan have found a dinoflagellate (not classified at present) in ciguatera-endemic areas in the torrid zone that produces two toxins that are believed responsible for the disease in humans called ciguatera. This disease, due to food poisoning, is acquired by eating certain fishes and eels such as red snappers, sea basses, sharks, etc. that have fed on smaller fishes that have consumed the toxic dinoflagellate. Ciguatera poisoning is no doubt the largest public health problem involving seafood poisons. Nakazima (9) and Okaichi and Imatomi (10) have reported another dinoflagellate, Exuviaella mariae-lebouriae (Prorocentrum minimum var. mariae-lebouriae), occurring in certain areas around Japan that has caused short-necked clams to become poisonous and when these were consumed by humans, produced a disease resulting in fatty degeneration of liver and kidney tissue. Another important dinoflagellate, Gymnodinium breve, has caused devastating red tide blooms in the Gulf of Mexico, particularly along the west coast of Florida and produces a poison that is toxic to fish, chicks and mice. The tremendous fish kills due to this organism along the Florida coast have caused severe environmental problems due to the decaying fish and polluted water in these areas. There are other dinoflagellates that produce poisons and have not been involved in shellfish or fish poisoning. Table 1 lists the poisonous dinoflagellates, their usual distribution and some of their properties.

TABLE 1. Known poisonous dinoflagellates

Dinoflagellate	Usual distribution	Poison
Gonyaulax catenella	North Pacific coasts, California to Japan, Chile, South Africa	Causes PSP[1] Structure determined (11)
Gonyaulax tamarensis[2]	Coasts of New England, Canada, countries along North Sea	Causes PSP Structure determined (12)
Gonyaulax acatenella	Coast of British Columbia	Causes PSP Poison not isolated (6)
Pyrodinium phoneus	North Sea	Causes PSP Poison not isolated (3)
Gonyaulax monilata	Gulf of Mexico	Toxic to fish but not warm blooded animals. Not isolated (13)
Gonyaulax polyedra	Coasts of southern California	Poison reported but not verified (14)
Gymnodinium breve	Gulf of Mexico	Toxic to fish, chicks and mice, partially purified (15)
Gymnodinium veneficum	English Channel	Toxic to fish and mice (16)
Exuviaella mariae-lebouriae[3]	Japan	Causes degeneration of liver and kidney tissue. Partly characterized (9,10)
Dinoflagellate not identified	Torrid zone	Reported likely cause of ciguatera poisoning. Partially characterized (8).

1. PSP, paralytic shellfish poisoning.
2. Called G. excavata in cases.
3. Synonymous with Prorocentrum minimum var. mariae-lebouriae.
Some types of paralytic shellfish poisoning recently have been reported along the coast of Brazil and Venezuela (7).

NATURE AND PROPERTIES OF DINOFLAGELLATE POISONS

Paralytic type shellfish poison

Gonyaulax catenella, G. tamarensis and G. acatenella all produce paralytic neuropoisons that produce their effects by blocking the passage of an impulse along a nerve axon or muscle fiber. The block is due to the poison (saxitoxin and related poisons) binding to the sodium channel in the muscle or nerve cell membrane. The action is similar to that of tetrodotoxin from the puffer fish. In humans the first symptoms include a tingling sensation and numbness in the lips, tongue and finger tips and may be apparent within a few minutes after eating poisonous shellfish. As the illness progresses respiratory distress and muscular paralysis become severe and death, apparently as a result of respiratory paralysis, occurs within 2 to 24 hours depending upon the size of the dose. If one survives 24 hours the prognosis is good.

My studies on the purification and characterization of the poison produced by G. catenella began with Dr. Herman Sommer at the University of California and Dr. Byron Riegel at Northwestern University and their colleagues in 1945. Purification of the poison (now called saxitoxin) was accomplished, after several years of research, by extracting the poison with water at pH 2 with hydrochloric or sulfuric acid, from the ground dark gland or hepatopancreas (which contained 95% of the poison) of sea mussels, Mytilus californianus, collected along the Pacific coast near San Francisco. This extract was chromatographed on carboxylic acid exchange resins (Amerlite XE-64 or CG-50) with dilute acetic acid and finally on acid washed alumina in absolute ethanol (17,18). The poison was assayed quantitatively with mice by a procedure developed by Dr. Sommer. One mouse unit (MU) is defined as the minimum amount of poison that will kill a 20-gram white mouse in 15 minutes when one ml of the extract or serial dilution of it at pH about 4 is injected intraperitoneally. The specific toxicity of the purified poison was found to be 5500 MU/mg solids (19). One MU is equivalent to 0.18 µg. Larger amounts of poison will kill in shorter times. Death times of 4, 6, 8 and 15 minutes are equivalent to 2.5, 1.6, 1.3 and 1 MU, respectively. The dose may be calculated directly with the equation, log dose = (145/t) - 0.2, where t is the time in seconds and death occurs between 240 and 480 seconds. The purified poison (saxitoxin) is a white solid, soluble in water, slightly soluble in methanol and ethanol but insoluble in most organic solvents particularly those immiscible with water such as ethyl and petroleum ether and chloroform. It has two titratable groups, pK_a 8.2 and 11.5. The molecular formula is $C_{10}H_{17}N_7O_4 \cdot 2\,HCl$, molecular weight 372 (20). The poison reacts with the Benedict-Behre reagent (trinitrobenzoic acid) and the Jaffe reagent (trinitrophenol) to produce blue and orange red derivatives, respectively (color reagents for creatinine) that are approximately equivalent on a molar basis to the colors produced when creatinine reacts with these reagents. Reduction of the poison with hydrogen in the presence of a catalyst results in the uptake of one mole of hydrogen per mole of poison at atmospheric pressure and room temperature. This reaction destroys the toxicity and also eliminates the color reaction with the Benedict-Behre and Jaffe reagents (18,20).

The original work on the chemical structure of the poison was not completely successful because of difficulties in obtaining a suitable crystalline derivative for crystallographic studies. In our laboratory at the University of Wisconsin we finally obtained a suitable crystalline derivative by reacting the poison with p-bromobenzenesulfonic acid and in cooperation with crystallographers at Iowa State University, Ames, Iowa, the structure was established as illustrated in Figure 1 (11) and later verified by others (21).

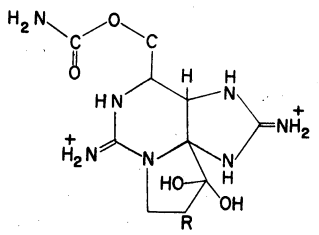

Fig. 1. Structure of saxitoxin and related poisons. Saxitoxin (from G. catenella), R is H (Ref. 11); Gonyautoxin (from G. tamarensis), R is OH (Ref. 22); 11-hydroxysaxitoxin sulfate (from G. tamarensis), R is OSO_3^- (Ref. 12).

Our studies showed that the reduction, either catalytically or with sodium borohydride and concurrent loss of toxicity referred to above, reduced one of the hydroxyl groups of the hydrated ketone with the elimination of a molecule of water (23). Treatment of saxitoxin with 7.5 M hydrochloric acid at 100°C for three hours removed the carbamyl groups leaving a hydroxyl group in its place (24). This derivative had about 60% of the toxicity of saxitoxin and established a means of making other derivatives of saxitoxin with toxic activity or ones containing radioactive elements.

Early investigations in our laboratory on the poison produced by G. tamarensis and found in scallops from the Bay of Fundy showed the presence of at least two different toxins: one was a weakly basic poison accounting for 80 to 90% of the total toxicity and came off the carboxylic acid exchange resins from pH 7 down to 4, whereas the remainder came off from pH 3 down to 2, similar to the more basic saxitoxin (29). Investigators at the University of Rhode Island reported (22) that the major poison from clams collected along the New England coast and from cultured G. tamarensis cells was 11-hydroxysaxitoxin which they called gonyautoxin (Fig. 1 where R is OH). Studies in our laboratory at the University of Wisconsin did not agree entirely with those at the University of Rhode Island because of the difference in the weakly basic nature of the poison compared to that expected of the proposed structure. We isolated the weakly basic poison from scallops and from cultured G. tamarensis and found, on the basis of NMR spectra, chemical analyses and properties, that the structure was the sulfate ester of 11-hydroxysaxitoxin as indicated in Figure 1 where R is OSO_3^- (12). The strongly negative or acidic nature of the sulfate group offset the basic or strongly positive nature of the guanidinium group resulting in the weakly basic nature of the poison. When the sulfate group was hydrolyzed from the poison to 11-hydroxysaxitoxin, it had normal basic properties like saxitoxin. The structure of the poison (saxitoxin) from California sea mussels, Alaska butter clams and G. catenella from central California are identical (25). Some fresh water blue-green algae such as Aphanizomenon flos-aquae produce a poison with biological properties similar to saxitoxin (26).

Other types of dinoflagellate poisons in shellfish and fish

Purification and characterization of poisons from other dinoflagellates is being carried on in many laboratories. Okauhi and Imatomi (27) have isolated two toxic substances from Prorocentrum var. mariae-lebouriae and have partly characterized it. When people consume short neck clams that have fed on this dinoflagellate they experience anorexia, abdominal pain, nausea, vomiting, constipation and headache within the first few days. These symptoms are followed by hemorrhagic spots on the skin, bleeding from the mucous membranes and acute yellow atrophy of the liver which often results in death. Yasumoto et al. (8) have isolated fat soluble and water soluble toxins from a new dinoflagellate found in ciguatera-endemic areas. The fat soluble toxin has been identified as ciguatoxin which Scheuer et al. (27) at the University of Hawaii had previously isolated from the moray eel and partially characterized.

The toxin (molecular weight about 600) is considered to be a lipid containing a quaternary nitrogen, one or more hydroxyl groups and a cyclopentanone moiety. The water soluble toxin is identical to maitotoxin which also is associated with ciguateric fishes such as snappers, sturgeon fishes, groupers, sea basses and eels found in the Caribbean area and in the torrid zone of the Pacific area. Ciguatera poisoning is a disease complicated with a variety of symptoms consisting of nausea, vomiting, metallic taste, dryness of the mouth, abdominal cramps, diarrhea, headache, prostration, chills, fever and general muscular weakness. This weakness may progress to the point where the patient is unable to walk. Recovery is slow and mortality rates are not high. In cases, death has resulted from various complications, mainly cardiovascular collapse. Another poisonous dinoflagellate that has been studied extensively is Gymnodinium breve. The poison produced by this dinoflagellate causes extensive fish kills along the west coast of Florida and reports of shellfish poisoning in humans with symptoms somewhat like ciguatera poisoning have been made (28). Progress in work on the chemical and physical nature of the poisons has been slow. Research groups using different methods of purification have isolated poisons that are different. This difficulty would indicate that there are several poisons present or that the purification procedure alters the molecular structure. All of the poisons are lipid soluble. Recently Baden et al. (29) and Risk et al. (30) have isolated lipid soluble neurotoxins from cultured G. breve cells that are non-aromatic and non-proteinaceous with molecular weights of about 800.

PUBLIC HEALTH ASPECTS OF DINOFLAGELLATE POISONS

The public health problem due to poisons produced by certain dinoflagellates is, for the most part, food poisoning in humans. In years past shellfish poisoning was a local public health problem in areas where the poisonous dinoflagellates bloomed and people collected the shellfish for food. Now that commercially harvested shellfish and fish that could contain the toxins are shipped by air to various parts of the world, the food poisoning problem could become more widespread unless proper care and control are maintained. These poisons are difficult to control because of the unpredictable and sporadic occurrence of the organism producing them. Shellfish that feed on toxic as well as nontoxic microorganisms usually show

no visible signs in their appearance when feeding on the toxic ones because they have a mechanism which binds the poison in the dark gland or hepatopancreas. After the poisonous dinoflagellate has disappeared, the shellfish excrete or destroy the poison within a week or two and are safe again to eat. The person collecting shellfish for food, therefore, has no way to distinguish poisonous shellfish from edible ones except by some type of animal assay for the poison or observing the onset of sickness in persons that have consumed the poisonous shellfish. Somewhat the same problem is involved with the ciguateric fishes. The very palatable Alaska butter clam presents another problem because 60 to 80 percent of the poison (saxitoxin) is bound in the siphon, an organ of low metabolic activity, and a year or more is required for the poison to be eliminated. If the clams were exposed to a source of the poison each summer they could remain poisonous for long periods.

Many government agencies carry out assays for the paralytic poison on clams and mussels in areas where they are collected for food from May to October. If shellfish become dangerously poisonous, warnings are posted and publicized. The most practical means of controlling shellfish poisoning is by direct sampling and assaying of shellfish by mouse assay in areas where they are harvested commercially and where picnickers commonly collect them for food. Education of the public regarding the danger of the sporadic occurrence of poison and its cause is important, especially in areas where shellfish poisoning is common. The United States Food and Drug Administration has set the maximum acceptable level for paralytic poison in fresh, frozen or canned shellfish at no more than 400 MU or about 80 μg per 100 grams of edible portion. This amount or less has not been known to cause sickness.

Estimates of the amount of paralytic shellfish poison to cause sickness and death have been made from accidental poisonings. Information obtained along the California coast from deaths due to poisonous sea mussels that fed on G. catenella, indicates that death occurred with a dose of about 20,000 MU or between 3 and 4 mg. Data gathered in Canada along the St. Lawrence Estuary on poisonous clams that fed on G. tamarensis, indicate that death occurred in some individuals with a dose of 5,000 MU or about one mg. Death usually occurs within 2 to 24 hours depending upon the magnitude of the dose. There is no known antidote for the paralytic shellfish poison, but artificial respiration, which should always be employed when respiratory distress becomes apparent, is believed to have saved lives, particularly in cases where a borderline dose was consumed. The dose that causes human sickness with other dinoflagellate poisons is not known at present.

Because of its action of blocking specifically the passage of sodium ions through nerve and muscle cell membranes, one of the dinoflagellate poisons, saxitoxin produced by G. catenella, has become a valuable tool in medical research on nerve transmission. A great deal of the saxitoxin we have purified in our laboratory has been donated for this type of research.

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