The Purification and Characterization of the Poison Produced by *Gonyaulax catenella* in Axenic Culture*

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ABSTRACT: The paralytic poison produced by the dinoflagellate *Gonyaulax catenella* in axenic culture has been isolated in pure form. A study of its chemical, physical, and biological properties establishes that it is identical in chemical structure to saxitoxin, the poison isolated from toxic Alaska butter clams, and to the poison isolated from toxic California sea mussels.

The paralytic poison sometimes occurring in California mussels was found by Sommer and Meyer (1937) and Sommer *et al.* (1937) to be present only when the mussels were feeding on a particular dinoflagellate, *Gonyaulax catenella*. This organism has been found all along the Pacific Coast of North America and in particular along the coast of California where blooms of the organism sometimes produce red tides. Its occurrence has always resulted in several species of shellfish becoming very toxic.[1]

The work of Sommer *et al.* quoted above was the first to demonstrate the presence of a poison in *G. catenella* and to establish a definite relationship between this organism and the occurrence of a poison in the California mussels that causes paralysis and death in man and animals. Subsequently the poison in scallops from the Bay of Fundy which causes the same results has been associated with the occurrence of *G. tamarensis* (Needler, 1949; Prakash, 1963) and the poison in the Belgian mussels in the North Sea with the occurrence of *Pyrodinium phoneus* (Koch, 1939). The source of the poison in Alaska butter clams is not known but *G. catenella* may be involved (Schantz and Magnusson, 1964). Connell and Cross (1950) and Gates and Wilson (1960) have implicated *G. monilata* Howell in the mortality of fish in the Gulf of Mexico. Schradie and Bliss (1962) have reported that *G. polyedra* Stein produces a poison that in some respects appears similar to that produced by *G. catenella*.

Riegel *et al.* (1949) obtained concentrates of the poison from *G. catenella* collected from the Pacific Ocean off the California coast and found that many properties of the poison in crude extracts were similar to the properties of the poison from mussels. Burke *et al.* (1960) have studied the chromatographic behavior of crude extracts of *G. catenella* cells from axenic culture and found the poison to chromatograph similarly to mussel poison.

The poison occurring in California mussels and Alaska butter clams has been isolated in pure form and partially characterized (Schantz *et al.*, 1957, 1961; Mold *et al.*, 1957). Work on the chemical structure of the poison from clams (saxitoxin) was reported by Rapoport *et al.* (1964). This paper describes (a) the isolation, purification, and characterization of the poison from *G. catenella* cells obtained from axenic culture, and (b) a comparison of saxitoxin, mussel poison, and *G. catenella* poison that establishes them as identical.

Materials and Methods

A pure culture of *G. catenella* (free of bacteria) was obtained from Dr. L. Provaseli, Haskins Laboratories, New York City, N. Y. The culture medium was made up of sea water collected near Ocean City, Md., and was supplemented with 100 mg of KNO_3, 10 mg of K_2HPO_4, 1 mg of $FeCl_3$, and 0.05 mg of Na_2SiO_3/l. The pH was adjusted to 8.6 with NaOH and the medium

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[1] Blooms producing a red tide of *G. catenella* become apparent when the number of organisms reaches 20,000–30,000 or more/ml, but mussels may become too toxic for human consumption when the number has reached only 100–200/ml. When a bloom of *G. catenella* (which lasts about 2 weeks) recedes to a low level, the toxicity of the mussels soon disappears and within 2–3 weeks they are again safe for human consumption. Poisoning in humans from eating toxic shellfish has occurred in many places throughout the world, mainly in the areas around the North Sea, the Bay of Fundy, and along the coasts of South Africa, California, British Columbia, southeastern Alaska, and Japan. Symptoms of poisoning in man may begin about 30 min after toxic mussels are consumed, with a tingling sensation in the lips and finger tips followed by a progressive paralysis and death in 3–20 hr depending upon the dose. If one survives 24 hr, the prognosis is good. The medical aspects of poisoning in man are described by Meyer (1953).

autoclaved at 120° for 15 min.[2] The culture was initiated by inoculating 10 ml of medium with 1 ml of the stock culture containing about 20,000 organisms/ml and was then incubated at 13–15° for 12 days. This culture was used as the inoculum for 100 ml of the culture medium, which in turn was used as the inoculum for 1 l. of medium. It was incubated for 17 days at 13–15° in 2-l. Fernbach flasks. All cultures were incubated under continuous illumination with a bank of cool white fluorescent lights supplying 400 footcandles at the surface of the medium. *G. catenella* cells in the cultures were counted with a Howard mold count slide (Howard, 1922).

Toxicities of all preparations were determined by injecting serial dilutions of the poison solution intraperitoneally into white mice weighing between 18 and 22 g and then measuring the death time to determine the potency as described by Schantz *et al.* (1958). The results were expressed as mouse units (MU) per milliliter or with the total solids per milliliter (specific toxicity) as MU per milligram of solids. Basically, the mouse unit is defined as the amount that will kill a 20-g mouse in 15 min (Sommer and Meyer, 1937).

The carboxylic acid exchange resins (Amberlite XE-64) used in steps 2 and 3 of the purification procedure were exchanged with sodium at least once before use. The acid-washed alumina used in step 4 was prepared and used as described by Schantz *et al.* (1957).

The poisons used for comparison with *G. catenella* poison were obtained from the Alaska butter clam, *Saxidomas giganteus*, and from the California mussel, *Mytilus californiaus* Conrad, and purified as described by Schantz *et al.* (1957).

Infrared measurements were made in KBr pellets, using a Perkin-Elmer Model 12C spectrophotometer. Absorbance in the ultraviolet was measured in 0.001 N HCl with a Model DU Beckman spectrophotometer. Titrations were carried out with a Radiometer TTT1C equipped with PHA630T scale expander using 3 ml of a solution of the poison at a concentration of 9 mg/ml in 0.001 N HCl (carbon dioxide free) and with 0.25 N sodium hydroxide under an atmosphere of nitrogen. Optical rotation was measured in 0.001 N HCl in a 20-cm tube with a Rudolph No. 80 polarimeter. Reduction with hydrogen was carried out in a Warburg respirometer at 1 atm of hydrogen pressure, using platinum black adsorbed on charcoal (10%), as described by Schantz *et al.* (1961). Diffusion coefficients were measured in the Northrop diffusion cell (Northrop and Anson, 1929) by observing the rate of diffusion of the biological activity through the sintered glass disk of this apparatus. Color tests were applied as described by Mold *et al.* (1957). Chemical degradations were carried out in an identical manner on purified samples (25 mg each) of the three poisons (from *G. catenella*, mussels, and clams) as described for saxitoxin by Schuett and Rapoport (1962) and Rapoport *et al.* (1964).

Purification and Characterization. The purification was carried out as follows: STEP 1. The cultured cells from the 17-day culture (about 30,000 cells/ml) were carefully collected by filtration on a fast-flowing filter paper (Eaton-Dikeman 615). The paper containing the cells was ground in a Waring blendor with sufficient water and dilute HCl to make a thick slurry at pH 2–3. The clear yellow-green filtrate obtained by filtering the slurry with suction constituted a crude extract of the poison and usually contained between 20 and 100 MU of poison/ml.

STEP 2. The crude extract was passed through a column of Amberlite XE-64 in the sodium form to remove the poison. About 100 g of the resin were used/1,000,000 MU of poison in the crude extract. The poison was fractionally eluted from the resin with 0.3 M acetic acid. A recovery of at least 90% was achieved with a toxicity of 400 or more MU/mg of solids. The fractions containing the bulk of the poison in 0.3 M acetic acid were pooled, concentrated under vacuum to remove most of the acid, and adjusted to pH 4.5.

STEP 3. The poison in the pooled fractions was adsorbed on a column of XE-64 in the acid form, followed by chromatographic elution of the poison with 0.3 M acetic acid. The fractions having a toxicity of 1000 or more MU/mg of solids were pooled and rechromatographed in the same manner. Usually the specific toxicity at this point was about 3000 MU/mg. The fractions possessing a toxicity of 3000 MU or more were pooled, evaporated under vacuum to remove most of the acetic acid, and acidified with hydrochloric acid to about pH 2. The solution was again evaporated under vacuum to remove all of the acetic acid and to convert the poison from the acetate to the chloride salt. The pH was maintained between 2 and 3 until all traces of acetic acid were distilled from the solution. The solution was then treated with small amounts of well-washed Amberlite IR45 to bring the pH to 4.5, concentrated under vacuum at 30 to 35° to bring the total solids to about 5 mg/ml, and lyophilized.

STEP 4. The dried poison was dissolved in absolute ethanol and chromatographed on acid-washed alumina as described for the purification of mussel poison. The combined fractions from the chromatography on alumina with a specific toxicity of 5000 or more and a specific rotation of 128° or more were taken as the purified poison. The over-all yield was about 50% based on the poison content of the crude extract.

Rechromatography of the poison from these fractions on the alumina did not significantly increase the specific toxicity or change the specific rotation of the poison. Chromatography of the preparation on Whatman No. 1 paper strips using solvent systems of phenol–water (4:1) and *t*-butyl alcohol–acetic acid–water (2:1:1) (Mold *et al.*, 1957) showed that all substances detectable with the Jaffe reagent and by assay in mice moved as a single band with an R_F of 0.26–0.30. Continued studies with this poison clearly indicated that the substance isolated by the above procedure was highly

[2] Some sources of sea water support better growth of this organism when traces of certain vitamins such as thiamin and B_{12} are added (personal communication, Dr. L. Provasoli. See also Hutner and McLaughlin, 1958).

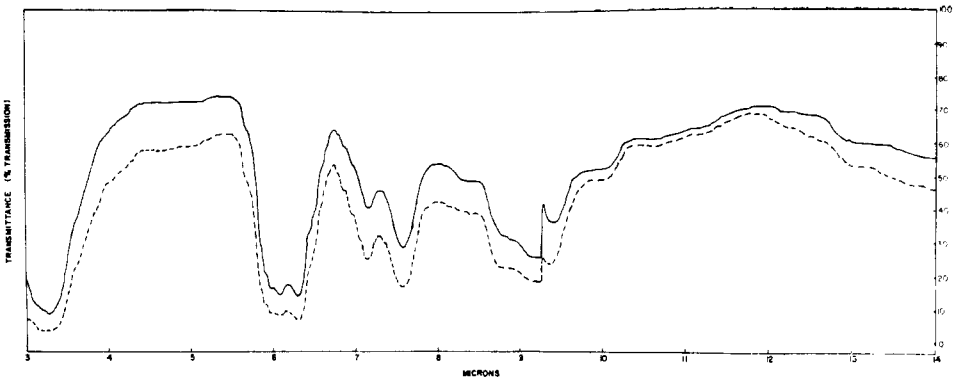

FIGURE 1: Infrared spectra of purified *G. catenella* poison (solid line) and purified mussel poison (broken line) in KBr pellets. The concentration of mussel poison was made slightly less than *G. catenella* poison so that both curves would be distinct. The break in the curves at 9.3 μ is caused by a change from one photocell to another. The spectrum of clam poison (saxitoxin) is identical with that of mussel poison.

purified and similar in its chromatographic behavior to purified mussel poison.

Because of the similarities of this poison to mussel and clam poisons, studies were undertaken to compare this poison with purified clam and mussel poisons. The results of these studies are summarized in Table I. All chemical reactions and physical measurements on the three poisons and on the degradation products of these poisons were identical in every respect, indicating that their structures must be identical. The infrared spectra of purified mussel poison and purified *G. catenella* poison are shown in Figure 1. Reduction of the poisons with hydrogen resulted in a loss of 95–100% of the toxicity when 1 mole of the gas was consumed for each mole of poison. The infrared spectra of the reduced poison (nontoxic) showed some loss of absorption at 5.6 and 8.7 μ, which is identical with that obtained with the reduced clam and mussel poisons. The specific toxicity of the three poisons was identical within experimental error of the bioassay. The specific optical rotation was also the same for all poisons within the error of the measurements.

TABLE I: Comparison of Properties of Poison from Cultured *Gonyaulax catenella* Cells with Poison from Mussels and Clams.

Property	Clam Poison	Mussel Poison	*G. catenella* Poison
Bioassay (MU/mg)[a]	5200	5300	5100
Specific optical rotation	+128°	+130°	+128°
pK_a	8.3; 11.5	8.3; 11.5	8.2; 11.5
Diffusion coefficient	4.9×10^{-6}	4.9×10^{-6}	4.8×10^{-6}
Absorption in ultraviolet and visible[b]	None	None	None
N content (Kjeldahl)	26.8	26.1	26.3
Sakaguchi test[c]	−	−	−
Benedict–Behre[c]	+	+	+
Jaffe test[c]	+	+	+
Reduction with H_2	Dihydro derivative, nontoxic	Dihydro derivative, nontoxic	Dihydro derivative, nontoxic

[a] All bioassay values are within experimental error of the value 5500 ± 500 MU/mg solids reported previously for clam and mussel poisons (Schantz *et al.*, 1958). [b] Infrared absorption of *G. catenella* poison was identical with that of clam and mussel poisons. See Figure 1. [c] Tests carried out as described by Mold *et al.* (1957).

Elemental analyses of the purified *G. catenella* poison as the dihydrochloride salt showed 31.8% carbon, 5.5% hydrogen, and 26.3% nitrogen. The diffusion coefficient of the poison (4.8×10^{-6} cm^2 sec^{-1}) indicated a molecular weight of 300–400. Titration of the poison showed two titratable groups; one with a pK_a at 8.2 and the other at 11.5–11.7. The molecular weight calculated from the titration was 386. This titration indicated the chlorine content to be 18.4%. If it is assumed that oxygen makes up the remainder of the elemental composition (18.0%), the molecular formula should be $C_{10}H_{17}N_7O_4 \cdot 2$ HCl (Calcd: C, 32.26; H, 5.11; N, 26.36; O, 17.21; Cl, 19.06) with a molecular weight of 372. This value is exactly that obtained for the clam and mussel poisons (Schantz *et al.*, 1957).

Reduction of the three poisons by heating under reflux with concentrated HI in glacial acetic acid in the presence of red phosphorus (Schuett and Rapoport, 1962) and subsequent purification by ion exchange (Dowex 50, H^+) and sublimation led to a 50–57% yield in each case of 3-methyl-6,7-dihydro-5H-pyrrole-[1,2-*c*]pyrimidin-1-one, mp 100–102° alone and on mix-

ing. The three products showed identical ultraviolet [$\lambda_{max}^{CH_3OH}$ 219 mμ (ε 8100), 279 (6700); $\lambda_{max}^{CH_3OH,H^+}$ 305 mμ (ε 9600)], infrared, and nuclear magnetic resonance absorption, and identical paper chromatographic behavior (butanol–acetic acid–water, 4:1:5, R_F 0.48; amyl alcohol–pyridine–water, 7:7:6, R_F 0.71).

Oxidation with alkaline hydrogen peroxide (1 M NaOH, 1% H_2O_2) of poison from each of the three sources was carried out for 6 hr at room temperature. Subsequent purification of the oxidation products by ion exchange chromatography on the hydrogen form of Dowex 50 showed the same purine[3] from each poison (Rapoport *et al.*, 1964), $C_9H_{10}O_2N_6 \cdot HCl$, melting above 320° with decomposition. The three products showed identical ultraviolet [$\lambda_{max}^{CH_3OH}$ 208 mμ (ε 24,700), 237 (14,200), 261sh (5,700), 325 (23,200)], infrared, and nuclear magnetic resonance absorption, and identical behavior in the two paper chromatographic systems described above (R_F 0.23 and 0.54, respectively).

Discussion

The purification, characterization, and proof that the structure of the poison produced by *G. catenella* in axenic culture is identical with the clam and mussel poisons have answered several questions concerning the nature and origin of the shellfish poisons. First of all the identical nature of mussel and *G. catenella* poisons finally confirms the postulation of Sommer *et al.* (1937) and Sommer and Meyer (1937) that mussels become toxic by the consumption of *G. catenella*. Also the identical nature of *G. catenella* poison and clam poison (saxitoxin) constitutes supporting evidence that the poison in Alaska butter clams may originate in *G. catenella*. Although *G. catenella* has been observed in Alaskan waters, no direct correlation has been found between the occurrence of this organism or any other toxic dinoflagellate and the occurrence of poison in Alaska butter clams such as was found by Sommer and Meyer between the occurrence of *G. catenella* and the poison in California mussels. However, other complicating factors may be responsible for the failure to observe this correlation in Alaskan waters (Schantz and Magnusson, 1964).

The poison is found concentrated in the dark gland or hepatopancreas of mussels and in the siphon of the Alaska butter clams. Apparently no change in chemical structure takes place when the poison is bound in the hepatopancreas or the siphon because acid extracts of these organs and *G. catenella* yield the same poison. The production of the poison in axenic culture definitely indicates that the poison is a metabolic product of this dinoflagellate, and the bacteria normally associated with it in nature have no symbiotic effect on the production or structure of the poison.

Because of the identical chemical structure it is assumed that *G. catenella* poison should have the same physiological action in animals as mussel and clam poisons. No studies on the action of *G. catenella* poison have been reported. However, in the case of mussel poison, it has been shown that propagation of impulses in nerves and skeletal muscles are blocked without any depolarization (Evans, 1964; Kao and Nishiyama, 1965a,b; Dettbarn *et al.*, 1965; H. Grundfest, 1965, personal communication). Kao and Nishiyama (1965) have further shown that the block is due to some specific interference with an increase in sodium permeability normally associated with excitation, and that the resting membrane conductances attributed chiefly to potassium and chloride permeabilities are unaffected. The action is similar to that observed for tetrodotoxin and tarichatoxin (Kao and Nishiyama, 1965; Kao and Fuhrman, 1963; Nakamura *et al.*, 1965).

In contrast to the action of the shellfish poisons on cellular membranes, Ballantine and Abbott (1957) have demonstrated a toxin in the dinoflagellate *Gymnodinium veneficum* that causes a depolarization of nerve and muscle cell membranes of fish. Shilo and Aschner (1953), Shilo and Rosenberger (1960), and Yariv and Hestrin (1961) have found a variety of toxic substances produced in axenic cultures of the phytoflagellate *Prymnesium parvum* Carter, one of which (the ichthytoxin) has a particular effect against many gill-breathing species (fish, molluscs, and arthropods) and against gill-breathing stages of amphibia.

G. catenella poison and the shellfish poisons are among the most potent nonprotein poisons known and their unusual structure as pointed out by Rapoport (1964) should make them particularly interesting compounds for physiological studies.

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[3] The structure of this purine will be discussed in detail in a forthcoming publication.

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Structural Relationships in the Interaction of Adrenocorticotropin with Plasma Proteins*

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ABSTRACT: Reverse thin film dialysis and gel filtration have been used to study the interaction and binding of adrenocorticotropin (ACTH) with proteins. Comparisons have been made with a tricosapeptide analog. A high degree of interaction is shown to occur between both of these peptides and proteins. Albumin is particularly effective in binding the hormone peptides, and this binding has been found to be concentration dependent.

Consistent and striking differences are observed between the bound and unbound forms of these peptides with regard to their hormonal behavior. From these results, and a knowledge of the portions of the peptide chains which are required for activity, some conclusions are drawn regarding the amino acid sequences which may be primarily involved in binding. The results indicate the probable major site of binding of the tricosapeptide to be at amino acid residues 15–18. It is also shown that amino acid residues 24–39 have an important effect on the hormone–protein interaction.

Interaction or binding of peptide hormones with proteins is difficult to study experimentally in terms of specific molecular structure. This is particularly true if these studies are to be carried out with the very small relative amounts of a peptide hormone which approach the physiological level. Measurements must then rely upon labeling techniques or some sensitive biological assay. Labeled peptides, pure enough for studies of this kind, are difficult to prepare and are not readily available. Biological assay procedures, while often tedious and frequently lacking in precision, nevertheless are capable of yielding information not obtainable by other methods.

The family of biologically active peptides related to

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