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Analysis of Toxic Mussels (*Mytilus* sp.) from the Alaskan Inside Passage

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An analysis of the toxins present in two samples of PSP infested mussels from Haines and Elfin Cove, Alaska, is described. The toxins are extracted and fractionated by gel filtration and liquid chromatography (ion exchange). Both samples are found to contain gonyautoxin-I, gonyautoxin-II, gonyautoxin-III, gonyautoxin-IV, gonyautoxin-V, and a new PSP poison. In addition, the Haines sample contains minor amounts of neosaxitoxin and the Elfin Cove sample contains minor amounts of saxitoxin. In both samples, gonyautoxin-I is the predominant toxin. Some implications of these finding vis-a-vis PSP in the Alaskan butter clam are considered.

Paralytic shellfish poisoning (PSP), a severe and occasionally fatal form of food poisoning caused by the ingestion of certain shellfish which have been exposed to blooms of toxic dinoflagellates, is a recurring health hazard in certain areas of the world.

The potent neurotoxin, saxitoxin (**1**), has been isolated and identified as the responsible PSP toxin from: California mussels, *Mytilus californianus*; Alaska butter clams, *Saxidomus giganteus*; and the Pacific dinoflagellate, *Gonyaulax catenella* (Schantz et al., 1957, 1966).

In addition to saxitoxin, New England soft shell clams, *Mya arenaria*, and cultures of the Atlantic dinoflagellate *Gonyaulax tamarensis* were found to contain three related toxins: gonyautoxin-I (GTX_1), gonyautoxin-II (GTX_2), and gonyautoxin-III (GTX_3) (Shimizu et al., 1975).

The heterogeneity of the PSP toxins was confirmed and extended by the subsequent isolation of three additional toxins: gonyautoxin-IV (GTX_4), gonyautoxin-V (GTX_5), and neosaxitoxin (neoSTX) from *G. tamarensis* cells. Soft shell clams *Mya arenaria* exposed to a bloom of *G. tamarensis* were also found to contain GTX_4 and neosaxi-

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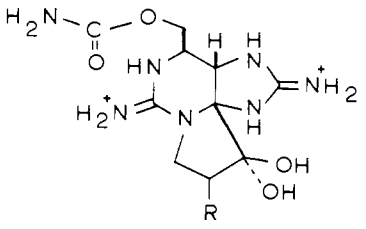
toxin in proportions different from those found in the dinoflagellate. Neosaxitoxin was also isolated as a minor toxic component of an Alaska butter clam sample (Oshima et al., 1977). Saxitoxin is the only toxin to have been isolated from the Pacific dinoflagellate *Gonyaulax catenella* and from PSP-infested California mussels.

The structure of STX (1) has been confirmed by X-ray crystallography (Schantz et al., 1975; Bordner et al., 1975). The structures of GTX_2 and GTX_3 have been proposed as **2** and **3** respectively (Shimizu et al., 1976).



1 R:H Saxitoxin

2 R:α-OH Gonyautoxin-II

3 R:β-OH Gonyautoxin-III

It is well documented that PSP occurring along the California and North Atlantic coasts is caused by blooms of *G. catenella* and *G. tamarensis*, respectively (Sommer et al., 1937; Needler, 1949; Prakash, 1963). However, the situation is less clear regarding the source of the poison in the Alaska butter clam which becomes toxic in irregular, isolated pockets and retains toxicity for prolonged or indefinite periods (Schantz and Magnusson, 1964).

Although cases of PSP resulting from the ingestion of infested Alaskan mussels have been reported (Meyers and Hilliard, 1955; Anderson, 1960, Edwards, 1956), no reports appear in the literature concerning the identity of the toxin(s) occurring in Alaskan mussels. Between June 30 and July 18, 1976, three separate incidents of PSP involving six persons were reported in southeastern Alaska. Two of these incidents were precipitated by ingestion of butter clams, the third incident was brought on by consumption of mussels (Morbidity and Mortality Weekly Reports, 1976). The present work presents an analysis of the toxins occurring in two samples of Alaskan mussels.

MATERIALS AND METHODS

Toxic Mussels. A. *Haines Sample.* Toxic mussels (*Mytilus* sp.) were collected near Haines on the Inside Passage of the Alaskan Alexander Archipelago (59° 11′ N, 135° 23′ W). At the time of harvest (Aug 1976), the mussels were assayed for 6312 μg of toxin/100 g of meat using the AOAC method. The mussels were kept frozen at −20 °C.

B. *Elfin Cove Sample.* Toxic mussels (*Mytilus* sp.) were collected at Elfin Cove on the Alaskan Alexander Archipelago (58° 11′ N, 136° 20′ W) on July 19, 1977, and were kept frozen at −20 °C.

No data are available concerning the presence of toxic dinoflagellates at the time when the samples were harvested; however, there were no reports of unusual water colorations or phosphorescence.

The Haines sample originated in an area from which several cases of PSP had recently been reported (Morbidity and Mortality Weekly Reports, Dec 3, 1976).

Processing. The Haines sample (73.5 g of shucked, undrained meat) and Elfin Cove sample (62.5 g of shucked, undrained meat) were processed identically. The bodies were covered with 30 mL of 95% EtOH previously acidified to pH 2.35 with concentrated HCl and homogenized in a Virtis "45" homogenizer. The homogenate was centrifuged and the residue reextracted (30 mL × 4) with 80% EtOH (pH 2.5). The supernatants were combined, concentrated to 30 mL, and washed (×3) with $CHCl_3$ (20 mL). After backwashing the $CHCl_3$ layers, the combined aqueous layers were concentrated in vacuo to 40 mL, and the pH was adjusted to 5.8 by the careful addition of about 1 mL of 1 N NaOH. Mouse bioassay of these preparations indicated the total toxicity of the Haines sample to be 13 250 mouse units and the total toxicity of the Elfin Cove sample to be 6800 mouse units.

Gel Filtration Chromatography. The samples were chromatographed on a Bio-Gel P-2 (Bio-Rad Laboratories) column (3 × 43 cm) eluted by gravity first with distilled water (825 mL) to remove the bulk of the impurities and subsequently with 0.03 N AcOH (360 mL) to elute the bound toxin. After elution of the void volume (75 mL), 20-mL fractions were collected per 16 min.

Thin-Layer Chromatography (TLC) and Spot Testing. TLC was performed on Silica Gel 60 precoated plates (E. Merck Laboratories) in pyridine–ethyl acetate–water–acetic acid (75:25:20:15, v/v). Spot testing (2–4 μL) was conducted on Silica Gel 60 precoated plates without solvent development. The developed chromatograms or spot tests were viewed under long-wave UV light after spraying the plate with H_2O_2 and heating (Buckley et al., 1976).

Ion-Exchange Chromatography. Spot positive fractions from the Bio-Gel P-2 chromatography were combined, lyophilized, and redissolved in 5 mL of glass-distilled water. The samples were charged on a Bio-Rex 70 (H^+) (<400 mesh) (Bio-Rad Laboratories) column (1 × 55 cm) which was sequentially eluted with: (1) 0–0.03 N AcOH linear gradient (200 mL total), (2) 0.03–1.3 N AcOH linear gradient (200 mL total), (3) 1.3 N AcOH (50 mL). Fractions (4.5–5.5 mL) were collected per 8 min. The chromatography was conducted using a Milroyal pump at about 40 psi.

PSP Analyzer Analysis. Spot positive fractions from the Bio-Rex 70 chromatography were analyzed by mouse bioassay, TLC, and by fluorescence monitoring using the PSP analyzer previously described (Buckley et al., 1978). Analysis using the PSP analyzer was conducted using 50-μL aliquots from each fraction.

RESULTS AND DISCUSSION

Analysis of the toxins from the two samples of mussels indicated that in both samples the majority of the toxin present consisted of a mixture of gonyautoxin-I, -II, -III, -IV, and -V. In both samples, gonyautoxin-I was the predominant toxin. Both samples showed the presence of minor amounts of a new unknown PSP toxin (UNK), the elution of which immediately preceded the gonyautoxins (Figures 1 and 2).

In the Haines sample, neosaxitoxin was identified as a minor component while saxitoxin was not observed. In the Elfin Cove sample this was reversed, saxitoxin occurred as a minor component and neosaxitoxin was absent.

The predominance of GTX_1 as the major toxin present in both mussel samples was established by TLC of all active fractions. The low PSP analyzer peak height response for GTX_1 is consistent with previously observed results. It has been shown (Buckley et al., 1978) that STX, GTX_2, GTX_3, and GTX_4 produce almost equal amounts of fluorescence (peak height) on the analyzer, GTX_1 and neoSTX give almost zero response, and GTX_5 gives a peak height response several times greater than does STX. The low analyzer response relative to the toxicity of the STX containing fractions in the Elfin Cove sample is due to differences in the volumes of fractions 86–90. The toxin

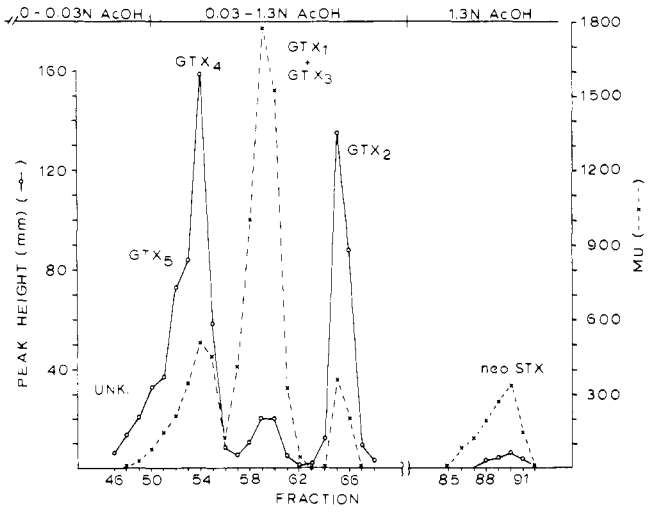


Figure 1. Toxicity (MU) and PSP analyzer response (peak height) of toxic fractions following Bio-Rex 70 chromatography of the Haines mussel sample. Upper line indicates elution solvent or gradient.

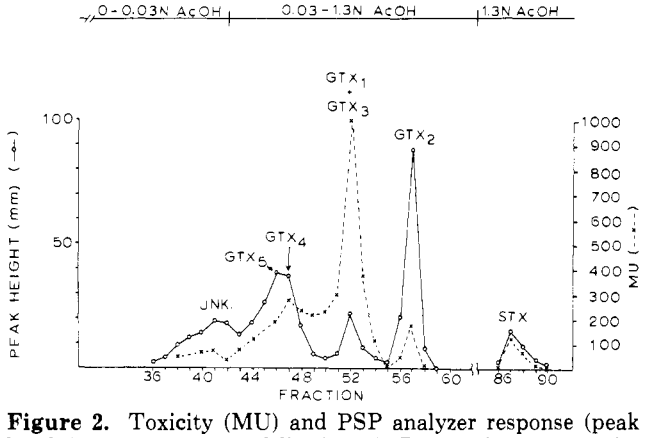


Figure 2. Toxicity (MU) and PSP analyzer response (peak height) of toxic fractions following Bio-Rex 70 chromatography of the Elfin Cove mussel sample. Upper line indicates elution solvent or gradient.

content and toxicity levels of all other fractions are consistent with the reported mouse test and PSP analyzer response.

Table I summarizes the chromatographic and electrophoretic behavior of the eight paralytic shellfish poisons.

To our knowledge this is the first report of the occurrence of the gonyautoxins in any North American Pacific Coast shellfish. The toxin profile is somewhat reminiscent of the pattern previously reported in mussels and short-necked clams collected at Oase Bay, Mie, Japan, in 1975, which had been exposed to a dinoflagellate bloom tentatively identified as *Gonyaulax catenella* (Hashimoto et al., 1976; Oshima et al., 1976).

Although the unknown new toxin has not previously been observed in North American or Japanese shellfish, its elution position and R_f value suggest that it is identical with the new toxin recently observed in PSP infested mussels, *Mytilus edulis*, which caused a massive PSP epidemic in a wide area of Europe (Zwahlen et al., 1977; Hsu et al., 1977).

In the past it has been generally assumed that the saxitoxin occurring in Alaskan butter clam siphons originated in *G. catenella*. However, it has also been observed (Schantz and Magnusson, 1964) that there are certain difficulties inherent in such an assumption. Among these difficulties are: (1) the low counts of *G. catenella* in Alaskan waters and the lack of peak growth periods which coincide with clam toxicity, (2) similar counts of *G. catenella* in waters around toxic and nontoxic clam beds, (3) the ability of the butter clam siphon to retain toxicity the year round while other shellfish depurate themselves soon after the abatement of a bloom, and (4) the fact that the poison is localized in the butter clam siphon while other shellfish characteristically concentrate poison in the hepatopancreas.

Table I. Chromatographic and Electrophoretic Behavior of Paralytic Shellfish Poisons

Poison	Elution order from Bio-Rex 70 column	TLC[a] R_f	Relative electrophoretic mobility[b]
UNK	1	0.57	0.08
GTX_5	2	0.50	0.42
GTX_4	3	0.77	0.23
GTX_1	4	0.81	0.34
GTX_2	5	0.74	0.68
GTX_3	6	0.66	0.51
neoSTX	7	0.72	0.66
STX	8	0.61	1.00

[a] Silica gel 60 plates developed in pyridine-ethyl acetate-acetic acid-water (75:25:15:20). [b] Electrophoresis conducted for 1.5 h on cellulose acetate membrane strips at 250 V using a pH 8.5 veronal buffer electrolyte solution (Fallon and Shimizu, 1977).

These observations would seem to invite an investigation into the nature of the poisons to be found in other Alaskan shellfish, although it has further been observed (Schantz and Magnusson, 1964) that toxicity in mussels proximate to toxic butter clams is frequently negligible or of a very low order.

Our findings establish that the toxins present in two samples of Alaskan mussels are quite different from the toxin (STX) occurring in the butter clam. Two possible explanations for this difference are (1) the possibility that the gonyautoxins are converted to saxitoxin by the butter clam (in vivo) or (2) that only saxitoxin is accumulated by the butter clam despite its low initial concentration.

It is apparent that the ecologically complex questions surrounding the origin of PSP in Alaskan butter clams and other shellfish will require continued investigation.

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Conversion of Aflatoxin B_1 to Aflatoxin D_1 in Ammoniated Peanut and Cottonseed Meals

Louise S. Lee* and Alva F. Cucullu

Cultured and spiked peanut and cottonseed meals, high in aflatoxin, were ammoniated under laboratory conditions paralleling those employed for the model ammoniation of pure aflatoxin B_1, as well as under conditions used in commercial detoxification with ammonia. In the model reaction, 10% aflatoxin B_1 remained after ammoniation, and 10% was converted to aflatoxin D_1. In all ammoniation studies conducted on spiked and cultured peanut and cottonseed meals, an average of 0.36% aflatoxin B_1 was converted to aflatoxin D_1, and an average of 0.37% of the B_1 remained unreacted.

Treatment of peanut and cottonseed meals with ammonia gas at elevated temperature and pressure is a commercially feasible approach to inactivation of aflatoxins that may occur as contaminants of these oilseed meals (Gardner et al., 1971). The success of this approach prompted us to initiate research to isolate and chemically characterize the products formed by ammoniation of aflatoxin B_1 (Lee et al., 1974; Cucullu et al., 1976). Lee et al. isolated the major product formed during a model reaction of pure aflatoxin B_1 with ammonium hydroxide under heat and pressure. They identified this compound as a nonfluorescent phenol of molecular weight 286 that retains the dihydrofurano group but lacks the lactone carbonyl moiety characteristic of aflatoxin B_1. This compound, which derives from opening the lactone ring during ammoniation and subsequent decarboxylation of the resultant β-keto acid, was termed aflatoxin D_1.

Cucullu et al. (1976) estimated a 10% yield of D_1 formed from B_1 during the model reaction after 3 to 4 h ammoniation. Less D_1 was formed after ammoniation at 1 and 8 h. The present report describes the conversion of aflatoxin B_1 to D_1 during ammoniation of peanut and cottonseed meals that are highly contaminated with aflatoxin, under the conditions of this model reaction and also under laboratory conditions that closely simulate those of commercial ammoniation. It also describes the method developed to determine aflatoxin D_1 in ammoniated peanut and cottonseed meals.

EXPERIMENTAL PROCEDURES

Method for Determination of Aflatoxin D_1. Samples of ammoniated peanut or cottonseed meals weighing either 1 or 20 g were soaked for 10 min in 40 mL of 0.1 N hydrochloric acid, and 210 mL of methyl alcohol was added. This acidic methanol solvent mixture is the same as that used by Pons and Franz (1977) for the quantitative extraction of aflatoxins from peanut butter. The samples were shaken on a Burrell wrist action shaker for 30 min and filtered. A 175-mL aliquot of each extract was collected, and 40 mL of a 20% lead acetate solution and 185 mL distilled water were added. Ten grams of acid-washed Hyflo Supercel filter aid was stirred into the solution, and the mixture was filtered. A 340-mL aliquot of each extract was then partitioned with two successive 50-mL portions of chloroform in a separatory funnel, and the chloroform extract was drained through a Butt tube containing the sodium sulfate–silica gel described by Pons and Franz (1977) for assaying aflatoxins in ammoniated cottonseed meals. Extracts were evaporated on a steam bath and further purified on a cellulose partition column prepared according to Pons et al. (1973), except that 11 × 500 mm columns and 4 g of cellulose were used. Interfering substances were removed by elution with 75 mL of hexane–benzene (5:1), followed by 75 mL of hexane–ether (4:1). Unreacted aflatoxin B_1 and the ammoniation product, aflatoxin D_1, were eluted from the column with 100 mL of hexane–chloroform (1:1).

Aflatoxin B_1 in the extract was quantitated according to Pons et al. (1968). Aflatoxin D_1 in the extract was further purified by preparative thin-layer chromatography (TLC) on silica gel G-HR plates developed in chloroform–acetone (9:1). Aflatoxin D_1, R_f 0.46, was eluted immediately from the gel with chloroform–methanol (1:1) into small vials fitted with Teflon-lined screw caps. After removal of the solvent, each sample was acetylated according to Stack et al. (1972). The resultant fluorescent acetate (Lee et al., 1974) was separated by TLC with chloroform–acetone (9:1) and quantitated by comparing the fluorescence intensities with the acetate derivative prepared from known aflatoxin D_1 spotted on TLC plates. Calculations were based on a sample weight of 0.6 of the original meal used, after correcting for the extract removed for estimation of aflatoxin B_1.

Percent recovery of aflatoxin D_1 was estimated by the addition of aliquots of a chloroform solution, containing 100 μg of aflatoxin D_1/mL, to 20-g samples of ammoniated aflatoxin-negative cottonseed meal. Meal samples were spiked with aflatoxin D_1 at levels of 10, 20, 40, 80, 120, 200,

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