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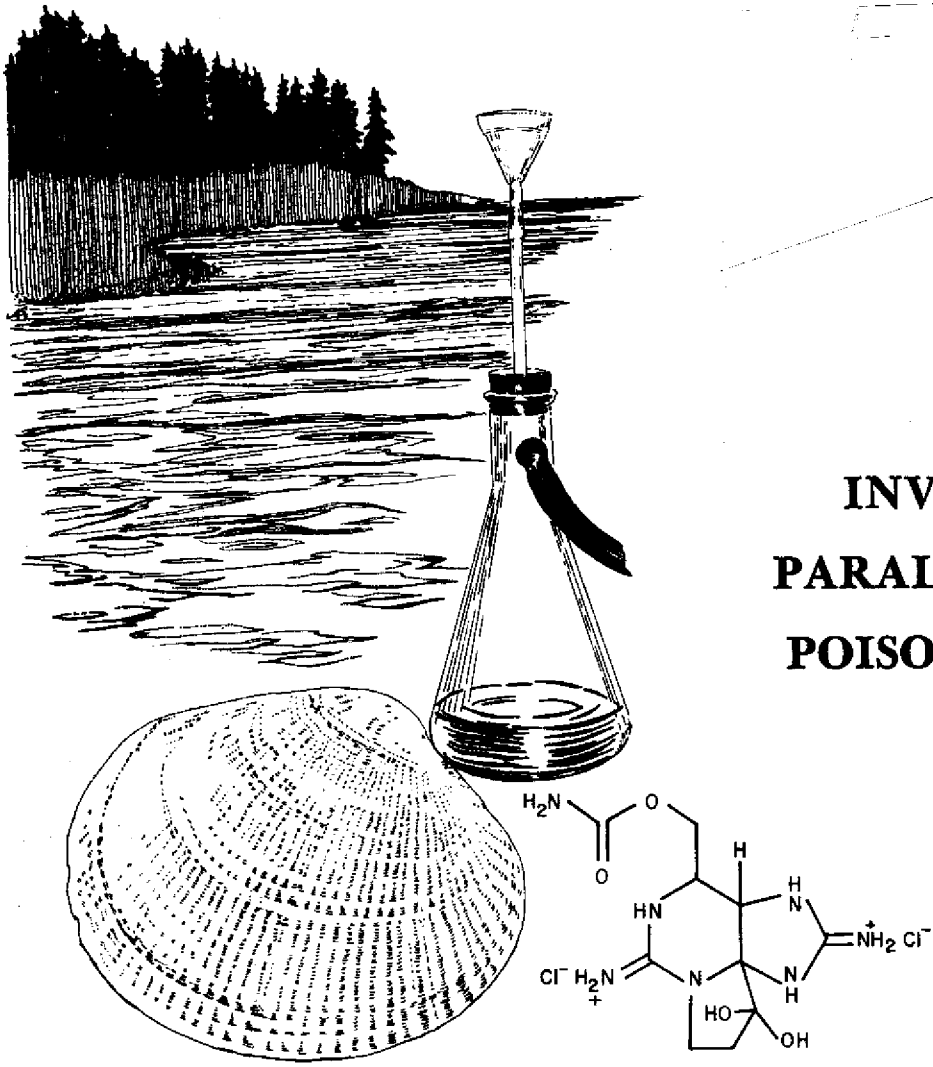

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ABSTRACT

Analysis of paralytic shellfish poisoning (PSP) in shellfish has traditionally been conducted by mouse bioassay. In an effort to provide a practical field assay procedure for PSP, a program to develop a chemical assay has been undertaken. Three chemical methods for the determination of saxitoxin, the best known of the PSP toxins, have been investigated. One of these, a colorimetric assay based on oxidative degradation of saxitoxin and quantification of the resulting guanidine by reaction with biacetyl, has been shown to provide a reliable test for standard saxitoxin. Application of the method to analysis of shellfish extracts suffers from interference problems, but these analyses may be successfully conducted after extensive chromatographic cleanup. Results from such analyses have led to the questioning of the long-held proposal that all the PSP toxicity in Alaskan shellfish is due to a single toxin, saxitoxin.

A limited PSP screening program in Southeast Alaska has confirmed previous reports of widespread toxicity in shellfish. The lack of correlation between toxicity and visible "red tides" calls for reinvestigation of the mechanism leading to PSP in Alaskan shellfish.

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CHAPTER I

INTRODUCTION

The Alaskan clam fishery represents one of the state's greatest undeveloped resources. From the southern tip of the Panhandle in Southeast to the Bering Sea, several species of clams are available in commercial quantities (Feder and Paul, 1974). It has been estimated that an annual sustainable harvest of nearly 50 million pounds shell weight is available (U.S. Department of the Interior, 1968) and that an actual annual harvest of about five million pounds (worth about $3 million) is a realistic expectation (Orth, et al., 1975).

One of the most serious obstacles in the way of development of this industry is the contamination of shellfish by paralytic shellfish poisoning (PSP). This phenomenon, which has worldwide distribution (Halstead, 1965), is particularly troublesome in North America (Quayle, 1969; Prakash, et al., 1971). It precipitated the closure of the Alaskan commercial clam fishery in 1947 and has dictated a very limited reopening.

The PSP phenomenon is characterized by the occasional contamination of bivalves with a toxin (or toxins) which renders the shellfish inedible to warm-blooded organisms. Human consumption of contaminated shellfish leads to a series of symptoms, including a tingling sensation, numbness in the face and extremities, partial paralysis, and--in extreme cases--death from respiratory failure. The PSP-causing toxins show similar physiological properties (Evans, 1975) and the best-studied toxin, saxitoxin, is known

to act by inhibiting the flux of sodium ions through membranes (Narahashi, 1975; Bull and Trevor, 1972).

Saxitoxin, the principal PSP toxin in Alaskan shellfish, is one of the most potent low molecular weight toxins known. A fraction of a microgram is fatal to a laboratory mouse, and a lethal human dose is about a milligram (Quayle, 1969). There are some indications that tolerance for the toxins varies among individuals. Although death can be prevented by sustained artificial respiration, there is no known antidote.

The outlines of the processes which lead to toxic shellfish have been elucidated (Schantz, 1971). Any one of several toxin-producing dinoflagellates may attain a population such that feeding shellfish consume large quantities of the organism. The toxin--which apparently has little effect on the shellfish (Twarog and Yamaguchi, 1975)--is concentrated by the bivalve, eventually rendering it toxic. Following the reduction of the dinoflagellate population, the shellfish cleanse themselves of the toxin at rates which are dependent upon the shellfish species involved.

While PSP contamination of shellfish is obviously dependent upon the dinoflagellate population, a microbial type of monitoring of shellfish beds does not provide a reliable method for toxicity determination. The presence of a "red tide" has often been utilized as a PSP indicator, but this is neither a necessary nor sufficient condition for PSP in shellfish. The population levels of toxic dinoflagellates which lead to a "red tide" greatly exceed those required to impart toxicity to shellfish. On the other hand, many innocuous organisms (e.g., *Noctiluca* sp.) produce non-toxic "red tides."

As a result of this situation, the U.S. Food and Drug Administration has charged the National Shellfish Sanitation Program (NSSP) with inspecting and certifying shellfish products in interstate commerce. To be acceptable, Alaskan shellfish must come from one of three certified beaches. The PSP monitoring is done by mouse bioassay (A.O.A.C., 1975), and the shellfish must have a toxicity level below 80 µg/100 g.* The assay is performed on processed meat, and contaminated products must be destroyed as no depuration procedure is available.

The Alaskan PSP problem is particularly acute. Toxic shellfish are found throughout Southeast Alaska into the Bering Sea. The butter clam (*Saxidomus giganteus*), which comprises the major resource in Southeast Alaska, unfortunately concentrates the toxin quite efficiently and retains it up to two years in indigenous waters (Quayle, 1969). Highly toxic butter clams are found throughout Southeast Alaska in areas having no records of "red tides."

Faced with these circumstances, it is obvious why a commercial clam fishery has not developed in Alaska. Shellfish would be harvested in remote areas with considerable expenditure of finances and effort without the assurance that the final product would be acceptable. Shipboard monitoring for PSP at the time of harvest would alleviate the problem, but the current mouse bioassay is not acceptable for this purpose. The assay suffers from a lengthy analysis time, low precision (especially for samples of marginal

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* This figure is arrived at indirectly, but corresponds to µg of saxitoxin in 100 g of shellfish meat.

toxicity), and the required maintenance of a large mouse colony. Clearly what is needed is a rapid, reliable alternative to the present mouse bioassay.

One proposed substitute for the mouse bioassay is a serological assay developed by Johnson and Mulberry (1966). Two variations of the procedure were reported, but they both suffer from instability of a required test component. Neither variation has received acceptance as an alternative to the mouse bioassay.

A second general approach to an alternative assay has been the development of chemical tests for saxitoxin. As a result of early efforts to isolate, purify, and characterize saxitoxin, several colorimetric detection methods are known. Mold, et al., (1957) reported positive colorimetric reactions between saxitoxin and Jaffé, Benedict-Behre, and Weber reagents. The Folin reagent also gives a positive test with saxitoxin (Price and Lee, 1971) as does Fast Blue B Salt (Proctor, et al., 1975). Of these detection methods, only one--the Jaffé test--has been modified for PSP assay. After extensive investigation by McFarren and coworkers (McFarren, et al., 1958; 1959; 1960) the Jaffé test was abandoned as an assay procedure because of low sensitivity and a lack of precision. The principal reason underlying its failure was its lack of specificity for saxitoxin and the resulting difficulty with interfering substances.

The elucidation of the structure of saxitoxin (Wong, et al., 1971) and its subsequent revision (Bordner, et al., 1975; Schantz, et al., 1975a) have provided investigators with the opportunity to design more specific chemical assays. Since the inception of our studies, three such tests (in addition to the ones outlined in this manuscript) have been developed. Bates and

Rapoport (1975) have developed a fluorometric assay for saxitoxin based upon an oxidative degradation product. The test is very sensitive (20x mouse bioassay) but requires a spectrofluorometer and strict adherence to experimental protocol. Various workers have experienced major difficulties with this method (E. J. Schantz, R. J. Bose, C. Yentsch, personal communications), and a revised procedure has been recently published (Bates, et al., 1978). A thin layer chromatographic-fluorometric method based upon the same chemical transformation employed by Bates and Rapoport has been reported (Buckley, et al., 1976). This method requires relatively pure toxin and does not have the sensitivity required for direct assay for PSP. It is, however, capable of detecting PSP toxins other than saxitoxin. A method based upon high performance liquid chromatographic separation followed by fluorometric detection of oxidation products of the PSP toxins has also been developed (Buckley, et al., 1978). This method has been primarily utilized in the analysis of isolated, partially purified toxins but has potential for application to routine shellfish assay.

Certainly at the outset of our study there was no viable alternative to the mouse bioassay for analysis of PSP. In recent years several possibilities have come forth, yet each of them suffers from some disadvantages, particularly when one considers their inconvenience in a field situation. Our efforts to develop chemical assays for saxitoxin have been guided by the requirements for a practical field assay: minimal equipment, simple and rapid operations, and reliable data acquisition. This manuscript deals with our attempts to develop such a method.

CHAPTER II

METHODOLOGY

A. Materials

Amberlite IRP-64 ion-exchange resin (Rohm and Haas Co.) was obtained in the H^+ form. Before use, small mesh particles were removed by repeated suspension in distilled water, discarding the material which was still in suspension after five minutes. The resin was then stored in a pH 5.2 acetate buffer (1.0M) for use in the H^+ form.

The Na^+ form of the resin was prepared by treating the H^+ form with 1.0 N NaOH. The resin was reconverted to the H^+ form using 1.0 N HCl. This resin was rinsed and stored in a pH 5.6 acetate buffer.

Acid washed alumina was prepared by stirring chromatographic grade alumina (Fisher A-540) with 2-3 vol of 1.0 N HCl. This procedure was repeated until the pH remained below 0.4. After stirring for 24 hr, the small mesh particles were removed by repeated suspension in distilled water, discarding the material still in suspension after 5 min. The material was then dried for 6 hr at 110°C. Immediately prior to use the acid-washed alumina was activated for 24 hr at 260°C.

A standard solution of saxitoxin dihydrochloride (100 µg/ml) in aqueous ethanol was kindly supplied by Dr. J. E. Campbell (U.S. Food and Drug Administration, Cincinnati, Ohio).

A stock solution containing 1 mg/ml guanidine hydrochloride (Sigma G-4505) in water was prepared and diluted 1:10 to make a working

solution of 0.1 μg/μl guanidine hydrochloride. The working solution was prepared fresh daily.

A 17% (vol/vol) solution of H_2O_2 was prepared from a stock solution containing 30-35% H_2O_2. The strength of the working solution was determined by titration with 0.05 N $KMnO_4$ (Kolthoff, et al., 1969). Both solutions were stored at 0-5°C in the dark.

A solution of 5% (wt/vol) *α*-naphthol (Sigma N-1000) in propanol was freshly prepared every 2-3 days and stored at 0-5°C in the dark. The compound was used as received from the supplier. Only white crystals were used. When the naphthol discolored by oxidation it was purified by sublimation or steam distillation.

An aqueous solution of 0.1% (vol/vol) 2,3-butanedione (Sigma D-3634) in water was prepared and kept at 0-5°C in the dark.

Absorbance measurements were made on a Cary-14 or a Beckman DB spectrophotometer at a wavelength of 555 nm in 1-cm glass cuvettes.

Scanning fluorometry was accomplished with a Turner Model 111 filter fluorometer and a scanning door (Turner Model 110-710). The fluorometer was fitted with a Turner 110-851 lamp (254 nm), Corning 7-54 and Turner 110-815 exciting filters, and a Wratten 2A analyzing filter. Output was relayed to a Sargent Model SR strip chart recorder and peak areas were calculated by planimetry.

Shellfish samples from Southeast Alaska were collected in three cruises: May, 1975 aboard the University of Alaska vessel R/V *Acona* as well as August, 1976 and October, 1977, aboard the U.S. Park Service vessel M/V *Nunatak*. Non-toxic butter clam samples were collected at Sadie Cove and McDonald Spit, both near Homer in South-central Alaska. All specimens were harvested by hand at low tide.

Bioassays were conducted according to the standard procedure (A.O.A.C., 1975). Periodically samples were crosschecked by the Alaska Department of Health and Social Services, Juneau, Alaska.

B. Methods

Colorimetric assay

Aliquots of saxitoxin (standard solutions of saxitoxin dihydrochloride or material contained in chromatography fractions) or guanidine were placed in a series of test tubes. To each of these samples was added: 1 ml 85% ethanol, 0.03 ml 0.5 N HCl, and 0.10 ml 17% H_2O_2. The samples were then placed in an oven (110°C) and evaporated to dryness. To the dried residues were added: 2 ml 57.5% ethanol, 0.1 ml 2N NaOH, 0.1 ml α-naphthol solution, and 0.1 ml 2,3-butanedione solution. The contents of the tubes were mixed by gentle swirling and the color reaction was allowed to proceed for 1 hr at room temperature. The absorbance of the samples at 555 nm was then determined against distilled water as the reference.

Extraction of clam tissue

A 100 g portion of frozen clam meat was weighed in a tared beaker and then transferred to a blender jar containing 50 ml of an acidified solution of aqueous ethanol (15% ethanol, pH 2.0 with HCl). The meat was thoroughly ground at low speed until a homogenous mixture resulted. An additional 50 ml of acidified ethanol and 2.5 g of trichloroacetic acid were added and the mixture was heated to room temperature. The extract was decanted into 50-ml centrifuge tubes and spun down for 15 min at 3000 rpm. The supernatant was then decanted and stored at 0-5^{o}C.

Chromatography on ion-exchange resins (small scale)

Columns of the ion-exchange resins were prepared by packing disposable Pasteur pipets with ca. 3 ml (settled volume) of the resin in an aqueous acetate buffer (pH 5.6 or 5.2) slurry. The dimensions of the resulting resin bed were ca. 5 X 60 mm. The columns were then washed with 10 ml pH 5.6 buffer (for the Na^{+} form) or pH 5.2 buffer (for the H^{+} form).

Crude extract (2.0 ml) or standard toxin (20 µg) was diluted to 3 ml with a pH 5.6 buffer, applied to the top of the Na^{+} column, and the solution allowed to run through until even with the top of the resin bed. The column was washed with 15 ml water, 5 ml pH 4.0 acetate buffer, and 15 ml water. The toxin-containing material was eluted from the column with 6 ml 0.5 N HCl which was collected

in two 3-ml fractions. These fractions were further processed (*vide infra*) separately but toxin was present only in the first fraction.

The pH of a fraction from the first column was adjusted to pH 5.2 by the addition of 1 ml pH 5.2 buffer and approximately 0.2 ml 2 N NaOH. The sample was then applied to the H^+ Amberlite column and allowed to run through until even with the level of the resin bed. The column was washed with 15 ml pH 5.2 buffer and 15 ml water. The toxin-containing material was eluted with 6 ml 0.5 N HCl collected in two 3-ml fractions. The solvent was evaporated from the fractions in a vacuum oven (100°C; 380 torr). Again, both fractions were further processed (*vide infra*) separately but toxin was found only in the first fraction.

Chromatography on alumina

Columns were prepared by the addition of small portions of the dry absorbent to a Pasteur pipet filled with 85% (vol/vol) ethanol. Enough material was used to give a bed depth of ca. 4.5 cm.

The dried residue of a fraction of the H^+ Amberlite column was taken up in 0.5 ml 85% ethanol and applied to the top of an alumina column. The column was then developed with 4 ml 85% ethanol which was collected in four 1-ml fractions. Analysis of each fraction by the colorimetric method demonstrated that all the toxin was collected in the first two fractions.

Chromatography on H^+ ion-exchange resin (large scale)

A slurry of the H^+ form of Amberlite IRP-64 was pressure-packed into a 5 ml disposable pipet pH 5.2 acetate buffer, giving about 6 ml of packed volume. Crude extract (10 ml), adjusted to pH 5 with NaOH, was applied to the column. Following washing (under ca. 2 psi pressure) with pH 5.2 acetate buffer (15 ml) and distilled water (20 ml), toxin was eluted with 0.5 N HCl. After addition of the HCl, the pH of column effluent was monitored by pH paper and the toxin-containing fraction (2.0 ml) was collected immediately after the pH change in column effluent.

Isolation of interfering peptide

An aliquot (5.0 ml) of an extract of non-toxic (<50 µg/100 g, mouse bioassay) butter clams was chromatographed on H^+-IRP-64 as described above. The fraction corresponding to saxitoxin-containing eluate was analyzed by thin layer chromatography (Silica Gel 60 or Aluminum Oxide 60G; 85% ethanol/0.5 N HCl, 25/0.1). The major constituent of the mixture was isolated by preparative layer chromatography (same conditions) followed by extraction with developing solvent.

Hydrolysis of interfering peptide

The major constituent was dissolved in 6 N HCl (50 µl) and sealed in a capillary tube. The tube was heated at 110°C for 24 hr, cooled,

and the contents analyzed for amino acids by two-dimensional thin layer chromotography (Silica Gel 60; 1. ethanol/H_2O, 7/3; 2. chloroform/methanol/60% NH_4OH, 2/2/1) followed by ninhydrin spraying.

Sephadex column chromatography

Sephadex G-10-120 was allowed to swell in distilled water overnight. It was then washed with successive portions of 0.1 N NaOH, H_2O, 0.1 N HCl, H_2O, 0.1 N NaOH, H_2O and stored in pH 6.0 acetate buffer. A column was prepared by packing the equilibrated Sephadex in a disposable Pasteur pipet (ca. 3 ml settled volume). A 3 ml saxitoxin-containing fraction from an Na^+ Amberlite column was applied and the column eluted with pH 6.0 buffer. Saxitoxin-containing fractions were identified by the colorimetric test.

Silica gel column chromatography

A slurry of silica gel in 57.5% ethanol containing 0.2% 0.5 N HCl was packed in a disposable Pasteur pipet. Standard saxitoxin or the saxitoxin-containing fraction from a Na^+ Amberlite column (evaporated to dryness) in 400 µl of the acidic ethanol solvent was applied to the column. Elution with the acidic ethanol gave a set of 2.0 ml fractions which were evaluated for saxitoxin by the colorimetric procedure.

Activated charcoal column chromatography

Acid-washed (10% HCl) charcoal (Nuchar C-115-N) was packed into a chromatographic column in a disposable Pasteur pipet (0.5 M HCl).

A toxin-containing fraction (or fraction to which standard toxin had been added) was applied to the column and development was attained by elution with 0.5 N HCl, H_2O, and then increasing fractions of ethanol in H_2O. Chromatography fractions were monitored by the colorimetric test.

Hexametaphosphate precipitation

A portion of crude clam extract (3.0 ml) was mixed with 0.06 M hexametaphosphate (1.0 ml) and the mixture heated to 90^{o} for 10 min. After cooling, the mixture was centrifuged and the supernatant treated with pH 5.2 acetate buffer (2.0 ml) and NaOH to bring the pH to 5.2. This solution was analyzed for saxitoxin by ion-exchange chromatography and the colorimetric assay.

Adsorption of saxitoxin by melanin

Commercial melanin granules (Sigma Chemical Co.) were washed with 0.5 N HCl and pH 5.2 acetate buffer and air-dried. To 10.0 mg of dry melanin in a screw cap vial were added pH 5.2 acetate buffer (1.0 ml) and saxitoxin (0.20 ml standard solution or 0.5 N HCl fraction from an H^{+} ion-exchange column). The reaction was periodically shaken over a 30 min period and the mixture transferred to a 5.0 ml syringe fitted with a Millipore filter (0.4 μm). The mixture was filtered and the melanin washed with 1.0 ml buffer, 2 ml water, and 4 ml 0.5 N HCl. The washes were analyzed by the colorimetric test and mouse bioassay.

Attempted rapid HCl destruction of interfering peptide

The peptide obtained by thin layer chromatography (TLC) of 3.0 ml of crude clam extract was dissolved in 1.0 ml 0.5 N HCl and evaporated to dryness (110°C) over a one-hour period. The reaction mixture was analyzed for residual peptide by TLC.

Papain digestion of interfering peptide

The peptide obtained by TLC of 3.0 ml of crude clam extract was added to the aqueous mixture: 0.5 ml 0.4 M citrate buffer (pH 5.6), 0.1 ml EDTA in H_2O (0.001 M), 1.5 ml cysteine in H_2O (0.25 M), 50 µl papain (0.24 mg/ml), and NaOH (to pH 5.5). The mixture was incubated at 25°C and analyzed by TLC and colorimetric assay at 0.5 and 1.0 hr.

Trypsin digestion of interfering peptide

The peptide obtained by TLC of 3.0 ml of crude clam extract was dissolved in 0.20 ml pH 5.6 acetate buffer and the pH adjusted to 7.0 (NaOH). A solution of trypsin in 0.001 N HCl (0.10 ml, 1.0 mg/ml) was added and the reaction incubated at 40-50°C for 1 hr. The reaction mixture was analyzed by TLC and colorimetric assay.

TLC analysis of saxitoxin: The modified Buckley test

The following samples (5 µl each) were spotted on a 5x20 cm cellulose chromatography plate (E.M. Laboratories, 5772-9H): toxin-containing

fraction from a large H^+ ion-exchange chromatography column, a mixture of standard toxin and an aliquot of the above fraction, and standard toxin spotted on top of an aliquot of the toxin-containing fraction from ion-exchange chromatography. Following development, the plate was oven-dried (30 min, 110°C). The plate was sprayed with H_2O_2 (10%) and heated (110°) for 30-60 min. Visualization of saxitoxin was accomplished with a hand-held lamp (366 nm).

Dansylation assay

Aliquots of standard saxitoxin (10 to 70 µl) or chromatographic fractions were taken to dryness (110°C) in Reactivials (Pierce Chemical Co.). Carbonate buffer (100 µl, pH 10.2) was added to dissolve the residue. An equal volume of dansyl chloride in acetone (1.0 mg/ml) was added and the reaction agitated on a Vortex mixer. After standing until disappearance of the yellow color (ca. 30 min) an aliquot (1.0 µl) was spotted on a polyamide sheet (Cheng Chin, 7.5x7.5 cm). The plate was developed by two-dimensional chromatography (eluent 1: water/formic acid; 200/3; eluent 2: methyl acetate/aq. NH_3; 9:1). After air drying the saxitoxin-derived spots were visualized by a hand-held lamp (254 nm) and quantitatively analyzed by scanning fluorometry.

CHAPTER III

RESULTS AND DISCUSSION

A. Colorimetric Assay

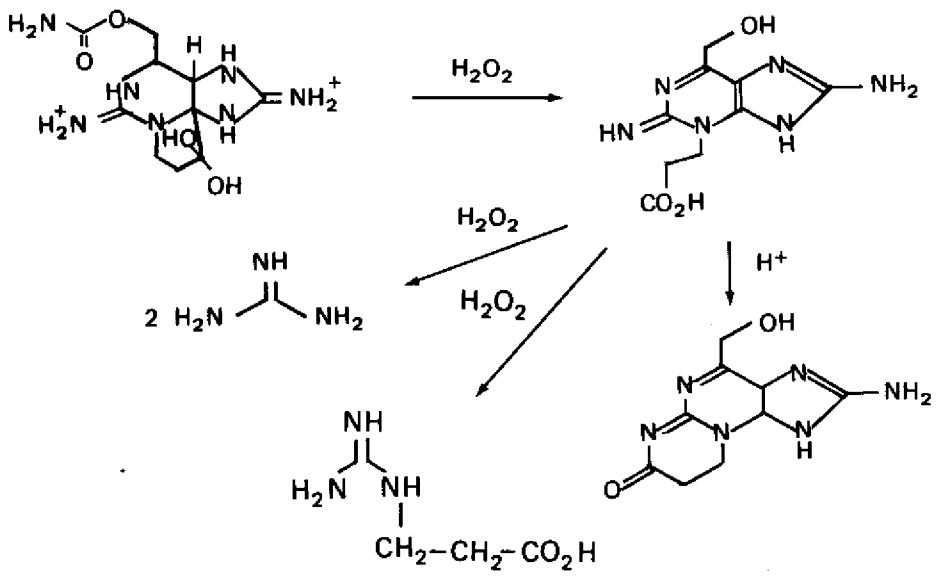
This colorimetric assay for saxitoxin is based upon the reaction of α-diketones with guanidine and other compounds containing the guanidino group (Micklus and Stein, 1973; Roos and Siest, 1970). Such compounds are formed by the oxidation of saxitoxin with hydrogen peroxide under a variety of conditions (McFarren, et al., 1961; Wong, et al., 1971; Schantz, et al., 1961; Bates and Rapoport, 1975) as outlined in Figure 1.

With the outlines of such an assay available from the literature, we turned our attention to this specific application. Being guided by the goal of developing a field assay, we began evaluating experimental parameters in light of the following set of requirements: simplicity of operations, speed, precision, and sensitivity.

Our initial efforts were directed toward optimizing the procedure for colorimetric analysis of guanidine. This requires reaction of guanidine with an α-diketone in basic alcoholic solvent in the presence of α-naphthol (which increases both the stability and colorimetric intensity of the resulting complex). We, therefore, sequentially evaluated α-diketones, alcoholic solvents, pH, and time-dependence of the reaction.

Our initial choice of diketone was phenanthrenequinone. However, subsequent experiments with biacetyl (butane-2,3-dione) indicated that it provided more sensitivity and a more stable colored complex. Propanol

FIGURE 1. Oxidation of Saxitoxin.



solvent appeared to give slightly more sensitivity than other simple alcohols, and pH's in the range 8-11 gave approximately the same results. The time-dependence of the reaction is presented in Figure 2. Further analysis of the reaction showed the absorbance to be essentially constant from one to six hours after initiation.

With an optimized colorimetric test for guanidine in hand, we turned our attention to oxidative production of guanidine from saxitoxin. In the initial stages of these studies it became clear that even trace amounts of H_2O_2 inhibited colored complex formation from liberated guanidine. Although excess H_2O_2 could be removed by a variety of methods (evaporation, NaI followed by $Na_2S_2O_3$, Cd), it was found that guanidine formation required conditions approximating evaporation (actually evaporating or heating at $100^{\circ}C$ with high H_2O_2 concentrations for prolonged periods). Thus reaction and destruction of excess H_2O_2 are accomplished in a single, but somewhat time-consuming, step. The yield of guanidine was found to be fairly sensitive to pH, with slightly acidic conditions effecting the transformation in best yield.

The saxitoxin molecule contains two latent guanidine residues (Figure 1). Under the oxidizing conditions employed in this method, 1.30 mole of reaction product (calculated as guanidine hydrochloride) were obtained for every mole of saxitoxin, resulting in a yield of 65%. This yield was consistently obtained when using standard toxin. These data are illustrated in Figure 3 which is a composite curve for saxitoxin and guanidine from data obtained by different personnel using two different instruments. The reproducibility of this procedure is noteworthy. The precision of the test is even greater in a single analysis as exemplified in Figure 4.

FIGURE 2. Time-dependence of Colorimetric Analysis of Guanidine. Analysis of 10 µg Guanidine with Biacetyl.

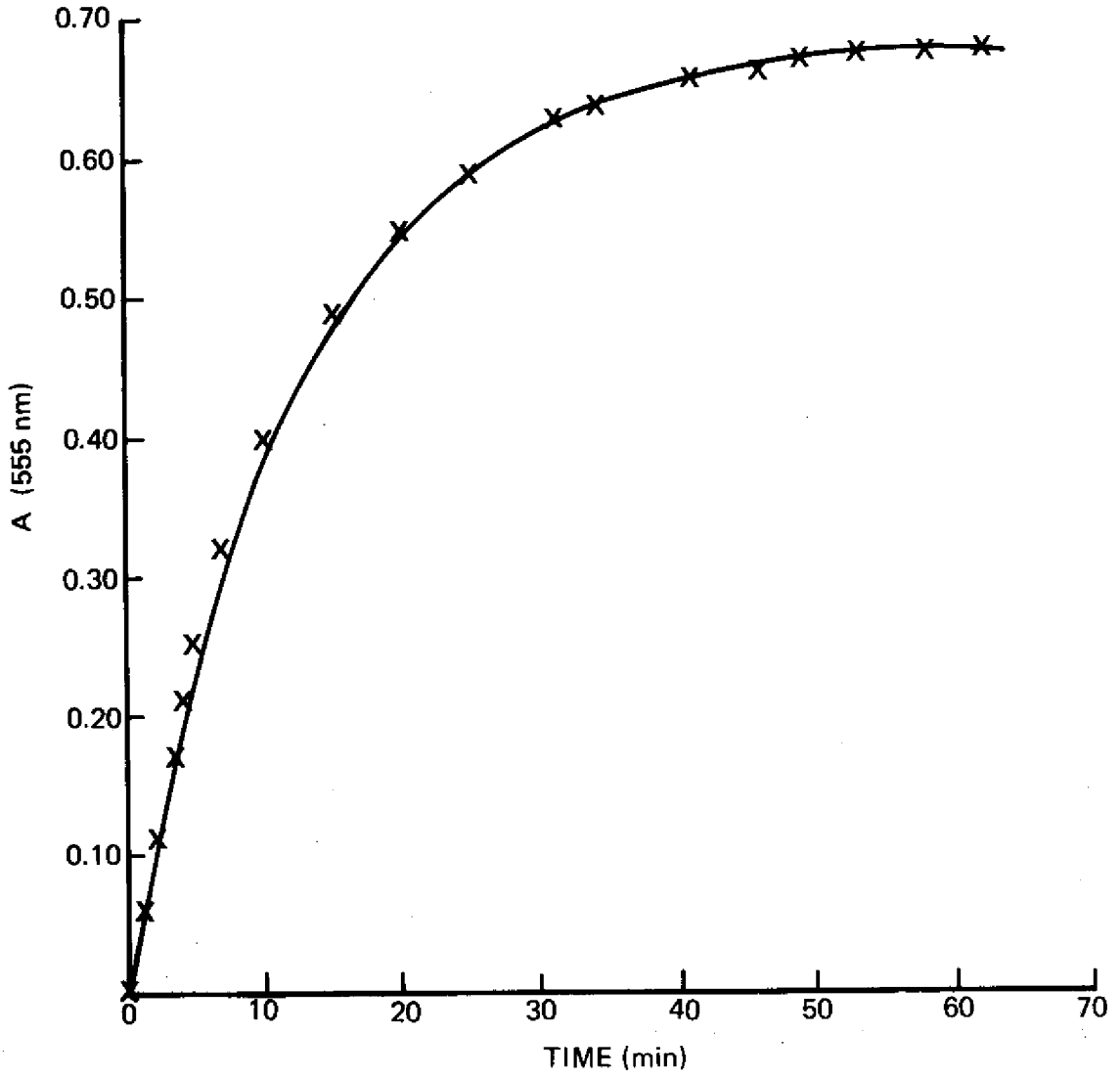


FIGURE 3. Composite Curve for Colorimetric Analysis of Guanidine and Saxitoxin.

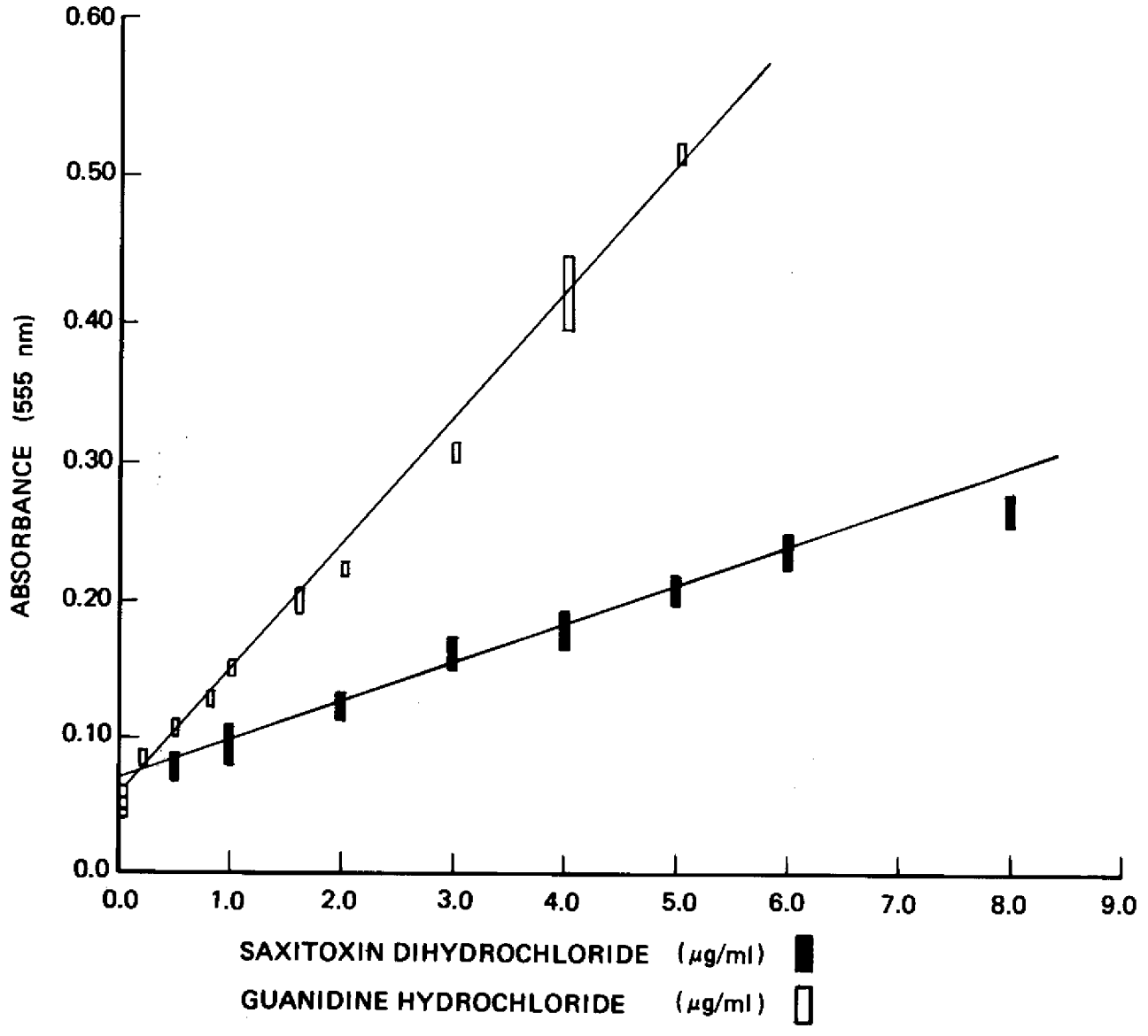


FIGURE 4. Single Colorimetric Determination of Saxitoxin.

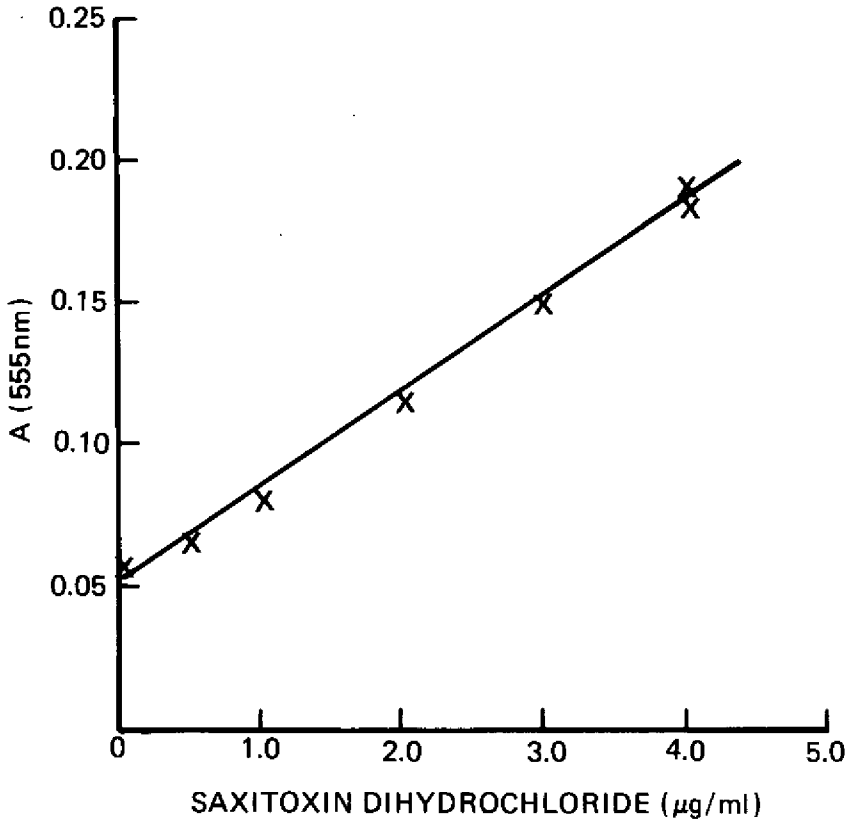


Table 1. Representative Results from Colorimetric Saxitoxin Analysis of Alaskan Clams

Experiment	Species	Sample	Date of Collection	Mouse Assay (µg/100g)	Colorimetric assay (µg/100g)
1		Saxitoxin standard		2000	1830 ± 400
2	Butter clam (siphons)	Porpoise Island	5/23/75	7300 ± 1400	2720 ± 660
3		#2 & saxitoxin (2000 µg/100g)			4780
4	Butter clam (siphons)	Farragut Bay	5/25/75	274	127 <50
5	Butter clam (siphons)	Porpoise Island	8/24/76	2710 ± 406	2268 2125
6	Butter clam	Porpoise Island	8/24/76	465	300
7	Butter clam	Tenakee Springs	8/25/76	66 ± 16	2334 1634
8	Great Alaskan tellin	Bering Sea (*Smaragd* Haul #58)	7/22/77	89 ± 25	1400
9	Surf clam	Bering Sea (*Smaragd* Haul #58)	8/1/77	<50	<50

The application of this test to analysis of saxitoxin in clam extracts (Gershey, et al., 1977) has, however, proved more difficult. Analyses of crude clam extracts by chromatographic cleanup and colorimetric testing have been characterized by inconsistency. Representative results from a variety of samples are presented in Table 1. These results show that the colorimetric test has about the same precision as does the mouse bioassay. However, inspection of the table reveals that the chemically determined saxitoxin levels are in agreement with those determined by bioassay in only a fraction of the samples. If one takes the bioassay data as correct, the chemically determined concentration may be either high or low.

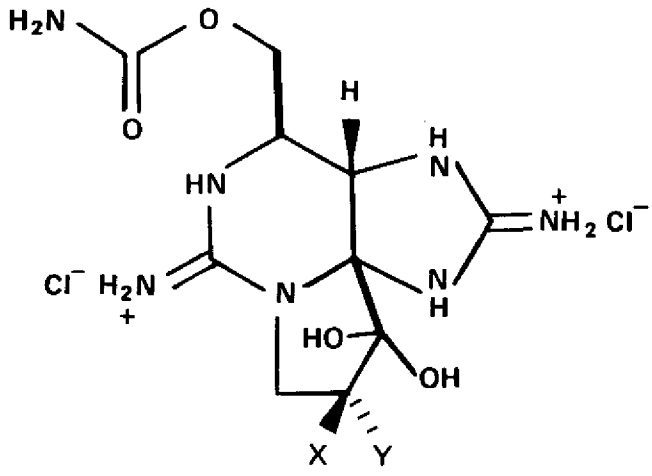
It has been demonstrated that consistent chemical and mouse bioassay determinations are attainable if the extracted toxin is sufficiently purified. For example, a large scale extraction of saxitoxin from butter clams followed by extensive purification (Schantz, et al., 1957) produced a chromatography fraction which gave the analyses displayed in Table 2. The problems associated with analyses of crude clam extracts, therefore, seem to reside in the extraction and cleanup stages of the analysis.

Table 2. Analysis of partially purified extract of butter clams (*Saxidomus gigantea*) from Porpoise Island.

Procedure	Saxitoxin concn (μg/ml)
Mouse assay	135
Colorimetric test (1)	130
Colorimetric test (2)	144

We believe that there are two possible explanations for the colorimetric assay being lower than the mouse bioassay. The first explanation is that one occasionally experiences loss of toxin during chromatography. We have found by mouse assay and colorimetric assay (entries 1 and 3, Table 2) that our sequence of chromatographic purifications normally results in about 90% recovery of <u>added</u> saxitoxin. The actual recoveries vary by ca. 10%, and occasionally standard saxitoxin is completely lost on a column. Our experience is consistent with the findings of other investigators (Schantz, et al., 1957).

A second possible explanation for the colorimetric assay being lower than the mouse bioassay is that the bioassay analyzes for total toxin while the colorimetric test analyzes just saxitoxin. Recent work has shown that PSP cannot always be traced to a single toxin, but that instead it can be the result of several chemically distinct toxins which exhibit similar physiological properties (Shimizu, et al., 1977; Oshima, et al., 1976; 1977). There have been seven PSP-related toxins identified to date (Oshima, et. al., 1977), and the structures of three of them are now known: saxitoxin (I), gonyautoxin-II (II), and gonyautoxin-III (III).



I. X = Y = H

II. X = H, Y = OH

III. X = OH, Y = H

All seven toxins have been identified in *Gonyaulax tamarensis* cultures (Oshima, et al., 1977), and they are also found in PSP-contaminated shellfish from the eastern coast of the United States (Shimizu, et al., 1977).

However, the Alaskan PSP problem is thought to originate from *G. catenella* (Schantz and Magnuson, 1964), and until recently all evidence has pointed to a much less complex situation in which all toxicity of Alaskan shellfish is accounted for by saxitoxin (Schantz, et al., 1975b; Schantz, et al., 1966; Bates and Rapoport, 1975). There are two recent publications which indicate that the Alaskan PSP problem may involve more than just saxitoxin. First, a recent report concludes that *G. catenella* may produce a set of toxins, not just saxitoxin (Oshima, et al., 1976). Second, a sample of butter clam siphons from specimens collected at Porpoise Island have been found to contain both saxitoxin and neosaxitoxin. Neosaxitoxin, one of the seven toxins produced by *G. tamarensis*, accounted for about 10% of the toxicity in this sample (Oshima, et al., 1977).

While it appears that all of these toxins are chemically very similar and thus may all release guanidine under our oxidation conditions, it seems likely that some of the toxins do not elute in the same chromatographic fraction as saxitoxin (Shimizu, et al., 1975). Thus, the colorimetric test may assay correctly for saxitoxin, with the discrepency between colorimetric and mouse assay accounted for by related toxins which are excluded by the chromatographic procedure. Evidence for support of this proposal can be obtained from Table 1. Comparison of the results from experiments 2 and 3 shows that only about 40% of the material quantified by bioassay is detected in the colorimetric test. However, the colorimetric test detects

all (103%) of the standard saxitoxin added to the sample. Furthermore, greater than 90% of standard saxitoxin was detected when it was chromatographed and colorimetrically evaluated (experiment 1, Table 1). Barring procedural difficulties, one can propose that only 40% of the toxicity in the sample for experiment 2 is saxitoxin and that the remaining toxicity resides in other PSP toxins.

The determinations in which the colorimetric assay overestimates the total toxicity have, in our hands, been more common than those in which it underestimates total toxicity. Since the interfering material gives a positive colorimetric test without prior oxidation, one can in principal eliminate the problem by analyzing an unoxidized aliquot of each sample. However, our attempts with this procedure have determined that the unoxidized blank often produces a background reading corresponding to over 1000 μg of saxitoxin per 100 g of shellfish (e.g., experiment 7, Table 1). This clearly represents a background reading too high for subtraction from samples containing marginal (<100 μg/100 g) toxicity. We therefore have undertaken an extensive search for an efficient cleanup procedure. We have investigated a broad range of chromatographic, precipitation, digestion, and adsorption techniques.

Our initial efforts in this regard were designed to chemically define the interfering substances in enough detail to postulate reasonable remedies. Our experiments were based upon the observation that saxitoxin elutes from ion-exchange columns with a band of colored material. Thin layer chromatographic analysis of this colored band from non-toxic butter clams allowed isolation of a major component with chromatographic properties similar to

saxitoxin. This component proved to be a peptide which contains arginine. Apparently the arginine residues in the peptide provide it with chromatographic properties similar to saxitoxin and the contained gaunidino moieties produce (even without oxidation) a colored complex with biacetyl.

Extensive efforts to modify the present chromatographic system have not resulted in satisfactory removal of the peptide. The use of more gradual gradients in elution from ion-exchange columns effects partial separation of peptide from saxitoxin but also causes the saxitoxin to be eluted in a much larger volume of solvent. In the course of these studies it was found that the Na^+ Amberlite column could be eliminated by employing a single larger H^+ Amberlite column. Thus 10 ml of crude extract can be chromatographed on a 6 ml packed volume column of H^+ Amberlite and the saxitoxin eluted in a 2 ml volume. Complete separation of the interfering peptide and saxitoxin could not be consistently accomplished on an alumina column, even though alumina serves as a satisfactory adsorbant for thin layer chromatographic analysis.

At this point we turned our attention to replacement of the alumina column with an alternate procedure which would remove the interference. A number of chromatographic systems have been investigated for further cleanup of the saxitoxin eluted from the Amberlite column.

1. Saxitoxin can be eluted from Sephadex G-10-120 with pH 6.0 acetate buffer. However, incomplete separation from the interfering peptide is effected and saxitoxin often elutes in a rather broad band (for a related system see Tanino, et al., 1977).

2. Saxitoxin can likewise be eluted from silica gel with acidic aqueous ethanol. While it is removed from the column in a tight band, it is not separated from the interfering peptide. For example, one analysis of a butter clam extract showed 50% of the color from an Amberlite fraction to be due to peptide (the remaining 50% being due to saxitoxin) and 34% of the color still being due to peptide after subsequent silica gel chromatography.

3. An investigation of chromatography of saxitoxin-containing fractions from Amberlite on charcoal essentially verified the results of Sommer, et al., (1948). Good recoveries (65% to 85%) of toxin are attainable, but the toxin elutes in a rather large volume of solvent and the exact retention volumes vary from run to run. Furthermore, separation from the interfering peptide is incomplete.

4. Preparative thin layer chromatography on alumina resolves saxitoxin and the interfering peptide. However, a portion of the saxitoxin must be sacrificed due to the visualization method (Buckley, et al., 1976), and the remainder is inefficiently (ca. 50%) removed from the alumina.

Having failed to find a convenient chromatographic procedure for the required purification of saxitoxin, we turned our attention to alternate methods for reducing interference from the peptide. Experiments with hexametaphosphate as a protein-precipitating reagent (Spinelli and Koury, 1970) showed that trichloroacetic acid is more effective in reducing interference than is

hexametaphosphate. In addition, precipitation of protein with hexametaphosphate reduces the amount of saxitoxin present in the supernatant. Thus, one can see from Table 3 that blanks are high in both cases and that 45% to 60% of the saxitoxin is recovered from an Amberlite column in comparison with the normal 65% to 80% recovery.

In an attempt to selectively bind saxitoxin to a solid matrix, we investigated the saxitoxin-melanin interaction (Price and Lee, 1972). Up to 40 μg of saxitoxin can be bound to 10 mg of melanin in 15 minutes in pH 5.2 acetate buffer, and 60% to 80% can be recovered in a single wash with 0.5 N hydrochloric acid. However, the partial binding of impurities (including the interfering peptide) and their resultant elution again cause interference in the colorimetric assay.

A final attempt at reducing interference was based upon chemical modification of the interfering peptide. Three different approaches to hydrolyzing the peptide resulted in no satisfactory method. Attempted rapid hydrolysis with HCl followed by ion-exchange chromatography gave less than 10% reduction of total protein and butadione-positive material in the saxitoxin-containing fraction.* Enzymatic hydrolyses with papain (which cleaves peptides at lysine and arginine) and trypsin (which cleaves peptides at arginine) both gave, following chromatography, less than 30% reduction in total protein and butadione-positive material.

*Note, however, that heating with 6 N HCl at 110° for 24 hr effects hydrolysis. We have not yet been able to find a less time-consuming procedure.

Table 3. Colorimetric Analysis of Saxitoxin-Spiked Clam Extracts Treated with Hexametaphosphate and Trichloroacetic Acid[a]

Precipitation Method	Absorbance of Unoxidized Blank	Absorbance of Oxidized Sample	Net Absorbance	Percent Recovery[b]
Hexametaphosphate	.755	.940	.185	49
(1.76×10^{-3} M)	.618	.850	.232	61
Hexametaphosphate	.747	.923	.176	47
(3.53×10^{-6} M)	.668	.837	.169	45
Trichloroacetic Acid (0.5 M)	.427	.677	.250	67

a. Following precipitation the samples were chromatographed on an H^+ Amberlite Column.

b. Based upon net absorbance = 0.375 for the added saxitoxin.

B. The Modified Buckley Test

As a result of evaluating various methods for monitoring saxitoxin elution from chromatography columns, we were led to an investigation of the detection method developed by Buckley, et al., (1976). We were struck by its simplicity and began considering ways to increase its sensitivity to the levels required for shellfish analysis.

Adaptation of the method to direct shellfish analysis requires a ten-fold concentration of toxin and removal of substances which interfere with the thin layer chromatographic (TLC) separation. The ion-exchange column used in the colorimetric test provides a partial solution. As described above, one can achieve a five-fold concentration and partial purification by a single pass through the column. Our experimentation with the TLC systems described by Buckley, et al., (1976) showed saxitoxin to produce a somewhat diffuse spot, and we hoped that modification of the TLC system could effectively produce the further "concentration" required in the form of a tighter spot.

A wide range of TLC systems has been investigated toward this end (Table 4). The best of these (system 1, Table 4) provides the approximate "concentration" required. When done carefully, this analysis provides a crescent-shaped brightly fluorescent spot corresponding to saxitoxin (visual detection limit ca. 25 ng). A modified procedure involving an alternate sequence of manipulations (plate spotting, H_2O_2 oxidation, plate development) results in less sensitivity for saxitoxin than the procedure described here.

This chromatographic system confirms the presence of impurities in the saxitoxin obtained from ion-exchange chromatography. While the interfering substances weakly fluoresce under the conditions described, they do not preclude detection of saxitoxin. In fact, their presence appears to enhance saxitoxin-derived fluorescence; thus we may be observing chromatography and visualization of a saxitoxin-containing complex.

While the impurities do not preclude saxitoxin detection, they do produce somewhat variable Rf's for saxitoxin (ca. 0.6 in system 1, Table 4) and interference in fluorometric plate scanning. The analysis system is therefore capable of detecting but not quantifying saxitoxin in shellfish extracts. Several extracts have been analyzed by this method. An extract of nontoxic butter clams from Homer, Alaska (<50 µg/100 g. mouse bioassay) gave a negative test while extracts of toxic butter clams from Funter Bay (400 µg/100 g, mouse bioassay) and Bartlet Cove (200 µg/100 g, mouse bioassay) gave positive tests. The analysis of Bartlet Cove clams was conducted aboard the U.S. Park Service M/V *Nunatak* and required ca. 3 hr.

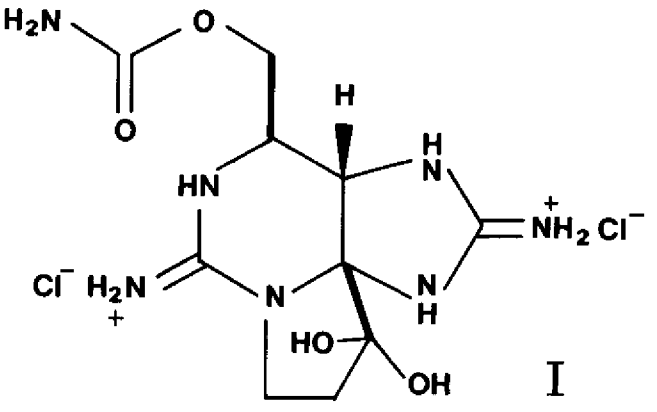
TABLE 4. Thin Layer Chromatographic Systems for Saxitoxin Analysis

	Solvent	Support	STX R_f	Comments
1.	pyridine-ethyl acetate-H_2O-acetic acid (75:25:30:15)	pre-coated cellulose glass plates (E.M. Labs)	0.61	In general this is the best solvent system; nice separation.
2.	t-butyl alcohol-acetic acid-H_2O (50:25:25)	"	0.39	Good solvent system, but travels too slowly.
3.	ethanol-H_2O-acetic acid (100:40:25)	"	0.38	Good separation, but not as good as system 1.
4.	85% ethanol-H_2O-acetic acid-acetone (20:5:5:1)	"	0.88	Problem with reproducibility due to volatile acetone.
5.	t-butyl alcohol-ethanol-acetic acid-H_2O (25:100:25:40)	"	0.34	Good separation.
6.	pyridine-ethyl acetate-H_2O-acetic acid (75:25:30:15)	pre-coated silica gel glass plates (E.M. Labs)	0.66	In general this seems to be the best solvent system.
7.	t-butyl alcohol-acetic acid-H_2O (50:25:25)	"	0.49	Good separation, but too slow.
8.	ethanol-H_2O-acetic acid (100:40:25)	"	0.62	Good separation.
9.	t-butyl alcohol-ethanol-acetic acid-H_2O (25:100:25:40)	"	0.60	Good separation, but slower than 1 or 3.
10.	85% ethanol-H_2O-acetic acid-acetone (20:5:5:1)	"	0.75	Very good separation.
11.	acetone-ethyl acetate-H_2O-acetic acid (15:5:7:3)	"	0.31	Interferences present.
12.	n-propanol-ethyl acetate-H_2O-acetic acid (15:6:7:3)	"	0.18	Interferences present.
13.	acetone-ethyl acetate-H_2O-acetic acid (10:5:12:3)	"	0.31	Interferences present.
14.	5.2 acetic acid buffer-acetone-ethyl acetate (15:10:5)	"	>.95	
15.	5.2 acetic acid buffer-acetone-ethyl acetate-85% ethanol (15:15:5:7)	"	0.30	Buffer seems to interfere with spots.
16.	ethanol-acetic acid-H_2O (100:40:25)	"	0.39	

C. Dansylation of Saxitoxin

Reaction of amino acids and amines with dansyl chloride (1-dimethylamino-naphthalene-5-sulfonyl chloride) and fluorometric analysis of the products is an accepted procedure for low-level analyses of these substances (Seiler, 1970; Seiler and Wiechmann, 1970). A convenient procedure involves reaction of an aqueous solution of substrate with an acetone solution of dansyl chloride, thin layer chromatographic separation of an aliquot of the reaction mixture, and *in situ* scanning fluorometric analysis of the separated mixtures. As little as 0.1 nmole of dansylated amino acid can be detected by this method (Seiler, 1970).

Consideration of the application of this analytical method to saxitoxin determination leads to the conclusion that three sites within the saxitoxin molecule (I) may be reactive. Dansylation of the carbamate functionality is known to produce dansyl amine due to carbamate hydrolysis prior to



I

dansylation (Frei and Lawrence, 1971). Thus the side chain of saxitoxin will produce, upon dansylation, analytically useless dansyl amine. The two substituted guanidine residues within the tricyclic saxitoxin nucleus are capable of reacting with dansyl chloride and should do so at high pH (Seiler and Wiechmann, 1970). The pKa's for saxitoxin are 8.24 and 11.60 (Schantz, et al., 1966), suggesting that pH greater than 10 is required for bis-dansylation.

With these considerations as a background we initiated an investigation of the feasibility of a dansylation-based analysis for saxitoxin. A wide-ranging examination of reaction, chromatography, and detection conditions resulted in the test described here.

It was quickly determined that saxitoxin indeed does react with dansyl chloride to produce fluorescent materials other than dansyl amine. Chromatographic analysis of the reaction mixture revealed dansyl hydroxide, dansyl amine, and a compound which appeared only in reactions to which standard saxitoxin had been added. Fluorometric analysis of the chromatographic plates or visualization with a hand-held lamp indicated that fluorescence was more intense under 254 nm radiation than under the more commonly used 360 nm (Seiler and Wiechmann, 1970). Although it has been reported that radiation of less than 300 nm decreases the fluorescence of dansyl amino acids (Airhart, et al., 1973), we have found this wavelength to give greater sensitivity (detection limit ca. 5 ng) in our application.

With a satisfactory detection system in hand we next turned to development of a convenient chromatographic system. After evaluation of several adsorbants (silica gel, alumina, polyamide) and a wide variety of solvent

systems, we chose the two-dimensional polyamide thin layer chromatographic system (Woods and Wang, 1967) described in the Methodology section. Careful examination of all plates reveals two fluorescent products derived from saxitoxin. Only the more intense of the two is analyzed in this procedure. This system, although the best we have found to date, has several flaws. Irregular solvent fronts and streaking of materials preclude fluorometric evaluation of about 10% of the developed plates. Visual examination of these "flawed" chromatograms with a hand-held lamp reveals the presence of dansylated saxitoxin, but the spot is distorted to the point that scanning fluorometry leads to spurious results.

Reaction parameters have been evaluated using the analysis system described above. For convenience, reaction temperature was held constant ($25^{o}C$) and the effect of pH on reaction time was investigated. It was found that all the dansyl chloride was consumed (determined by disappearance of yellow color within 20 min of mixing) and that the fluorescence from dansylated saxitoxin maximized at this point for all runs in the pH range of 8 to 11. Representative plots are shown in Figures 5 and 6. The response of fluorescent intensity of dansylated saxitoxin to pH is shown in Figure 7. The fluctuations in intensity with respect to pH in this range are due to several factors. The gradual rise in fluorescence from pH 8.5 to 10 is probably due to the increased fraction of saxitoxin in the basic form (the protonated acidic form being unreactive to dansyl chloride) leading to the increased ability of saxitoxin to compete with water (or hydroxide ion) for dansyl chloride. At pH's greater than 10 the fluorescent intensity due to dansylated saxitoxin decreases for at least two reasons: (1) hydroxide ion is now in high enough concentration to compete with

FIGURE 5. Time-dependence of Saxitoxin Dansylation at pH 10.2 (Saxitoxin = 50 µg/ml)

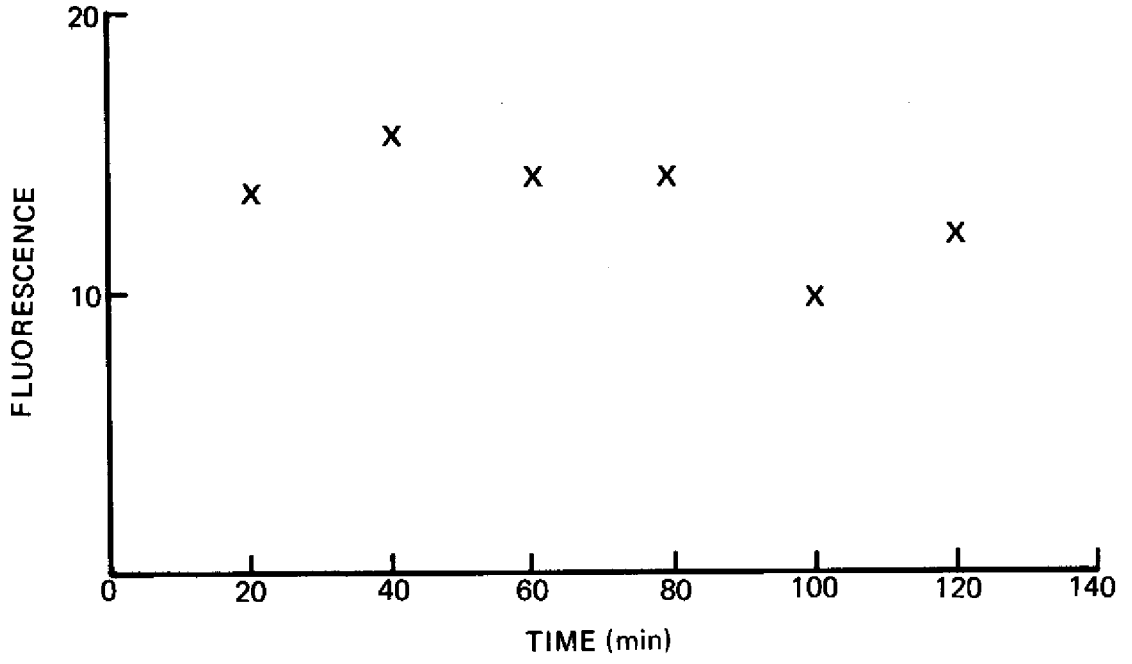


FIGURE 6. Time-dependence of Saxitoxin Dansylation at pH 8.5 (Saxitoxin = 50 µg/ml)

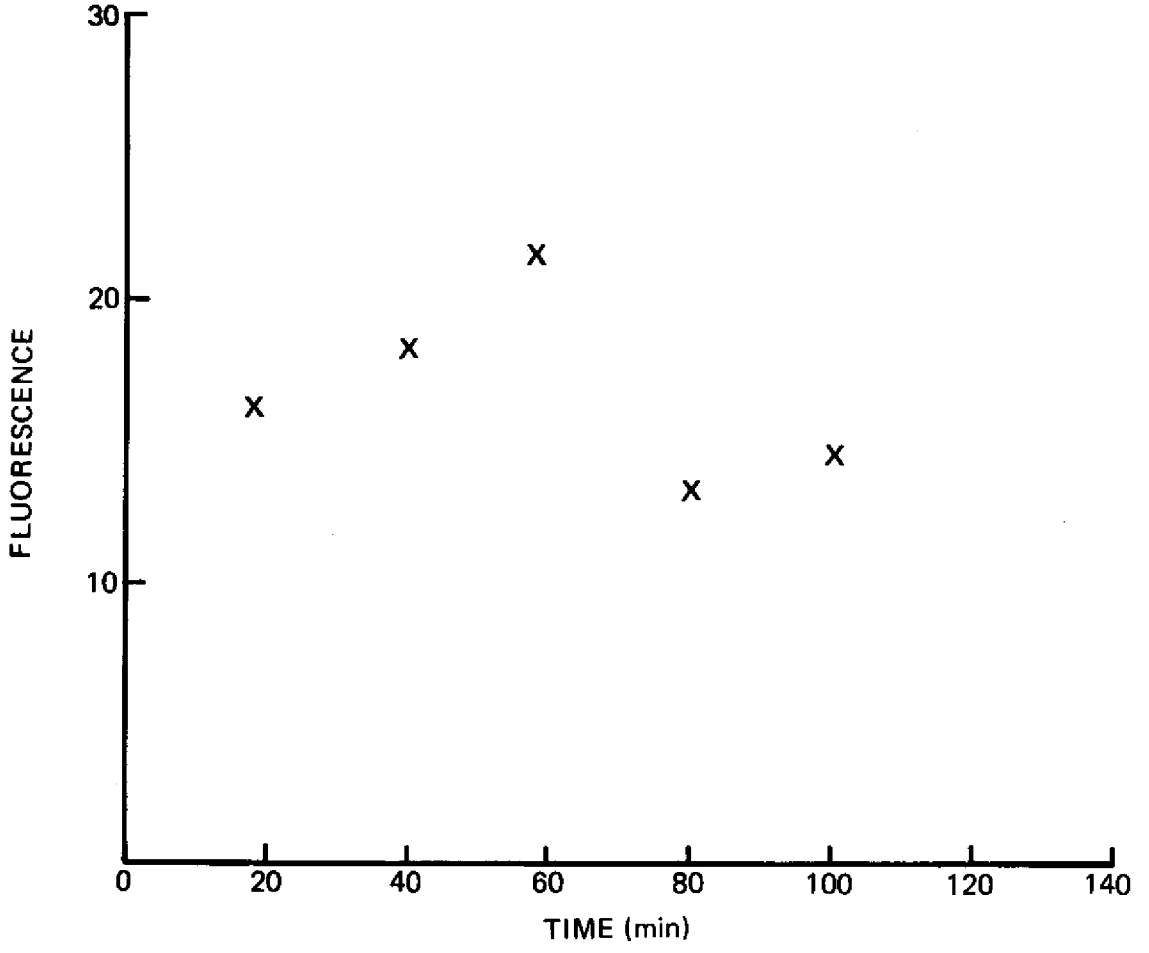


FIGURE 7. Dependence of Saxitoxin Dansylation on pH (Saxitoxin = 50 µg/ml).

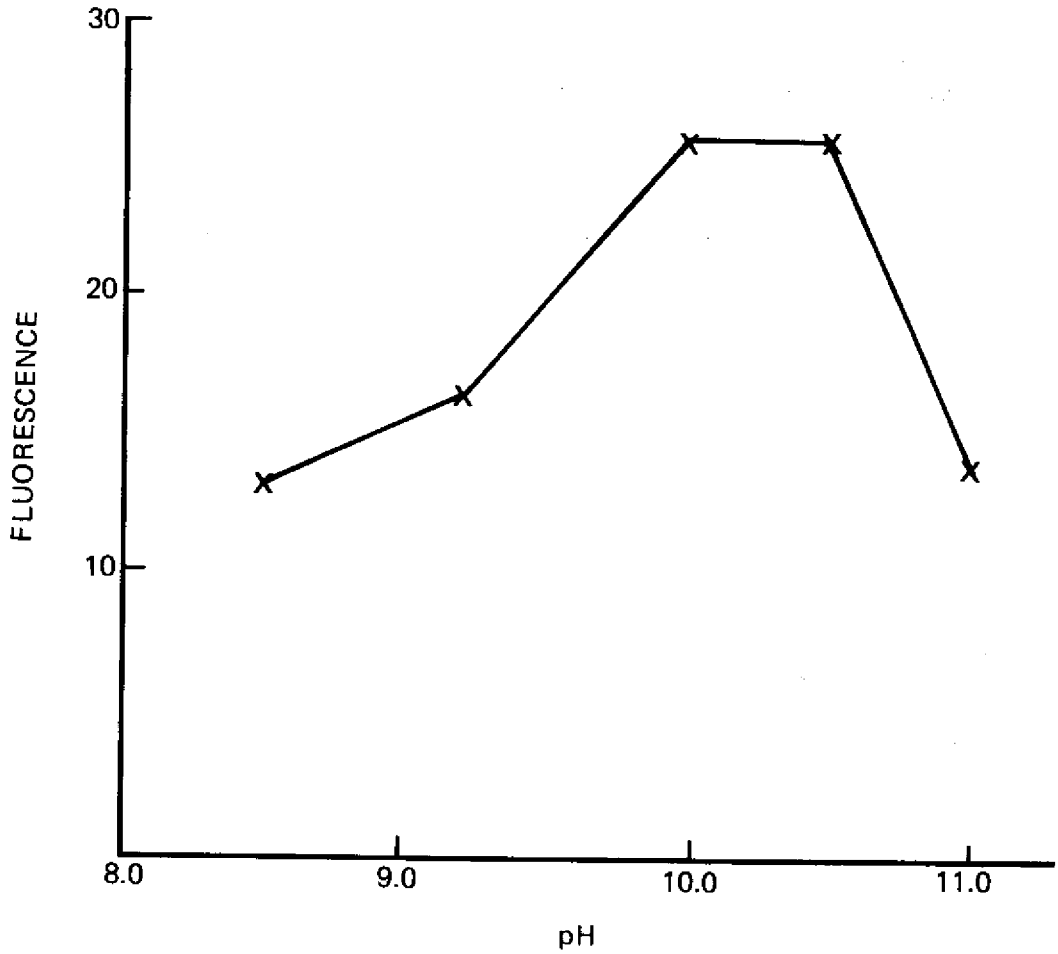
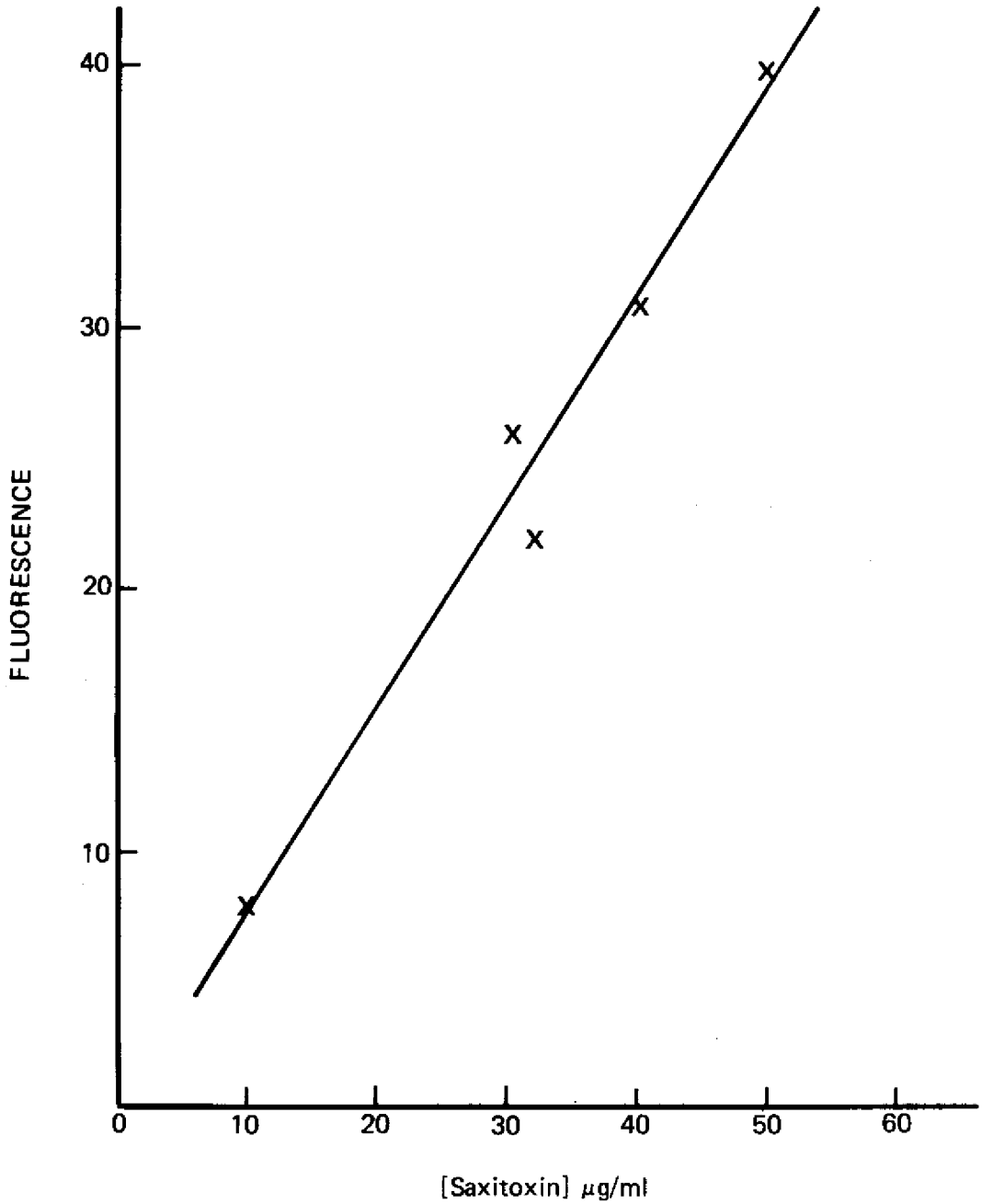


FIGURE 8. Analysis of Standard Saxitoxin by Dansylation at pH 10.2.



saxitoxin for dansyl chloride and (2) saxitoxin is being destroyed in the basic, oxygenated system (Schantz, et al., 1961).

The outcome of these experiments was the selection of a reaction time of 45 min at pH 10.2 for the dansylation of saxitoxin. Although the test is plagued by the occasional "flawed" chromatography plates, the curve shown in Figure 8 is typical for the analysis of standard saxitoxin. Precision at a saxitoxin concentration of 30 µg/ml is ± 15%.

Attempted application of the dansylation test to clam extracts has resulted in failure. With a detection limit of 5 ng from a 1.0 µl aliquot of reaction mixture applied to the thin layer chromatographic plate, the test is theoretically capable of detecting saxitoxin in shellfish contaminated with 1000 µg of saxitoxin per 100 g of meat (yielding an extract volume of 200 ml according to the standard procedure). Mixture of clam extracts fortified to this level (or greater) with standard toxin and the acetone solution of dansyl chloride results in rapid consumption of dansyl chloride (loss of color) and formation of a flocculent precipitate. Chromatographic analysis of the supernatant indicates dansylated material but nothing with the chromatographic mobility of dansylated saxitoxin. Apparently saxitoxin either cannot compete with other substrates for dansyl chloride or the dansylated saxitoxin is absorbed on the precipitate. When the dansylation test is applied to material passed through an Amberlite IRP-64 column, the same precipitation phenomenon is observed.

D. Field Sampling

While this project was not specifically designed to include large scale beach assessments for PSP, annual field trips to test assay procedures and to procure shellfish samples were conducted. As a result of these endeavors a moderate set of assay results have been accumulated. The results are presented in Table 5 and Figure 9.

Since the results are not from a thorough study, one must be conservative in drawing conclusions from them. However, it appears that PSP remains a problem in butter clams throughout Southeast Alaska. Furthermore, the data indicate recent inputs of toxin which cannot be related to massive "red tides." This situation underlines the need for a rapid, reliable test for toxicity if the shellfish resource of Southeast Alaska is to be commercially utilized.

Table 5. PSP levels as Determined by Mouse Bioassay at Selected Stations in Southeast Alaska

Station	Date	Species*	Toxicity µg/100g)
1. Porpoise Island	7/7/75	*S. giganteus*--siphons	4096
	8/24/76	*S. giganteus*-whole	826
		S. giganteus-siphons	2710
	10/13/77	*S. giganteus*-whole	99
		S. giganteus-siphons	2890
2. Bartlett Cove	8/23/76	*S. giganteus*-whole	794
		M. edulis-whole	80
	10/9/77	*S. giganteus*-whole	512
		M. edulis-whole	192
3. Farragut Bay	7/8/75	*S. giganteus*-whole	274
		S. giganteus-siphons	43
4. Tenakee Springs	8/24/76	*S. giganteus*-whole	124
		S. giganteus-siphons	2710
		M. edulis-whole	<40
		P. staminea-whole	<40
		C. nuttalli-whole	<40
5. Funter Bay	8/26/76	*S. giganteus*-whole	901
		M. edulis-whole	76
		P. staminea-whole	45
6. Hoonah	10/11/77	*S. giganteus*-whole	75
7. Salt Lake Bay	10/12/77	*S. giganteus*-whole	53

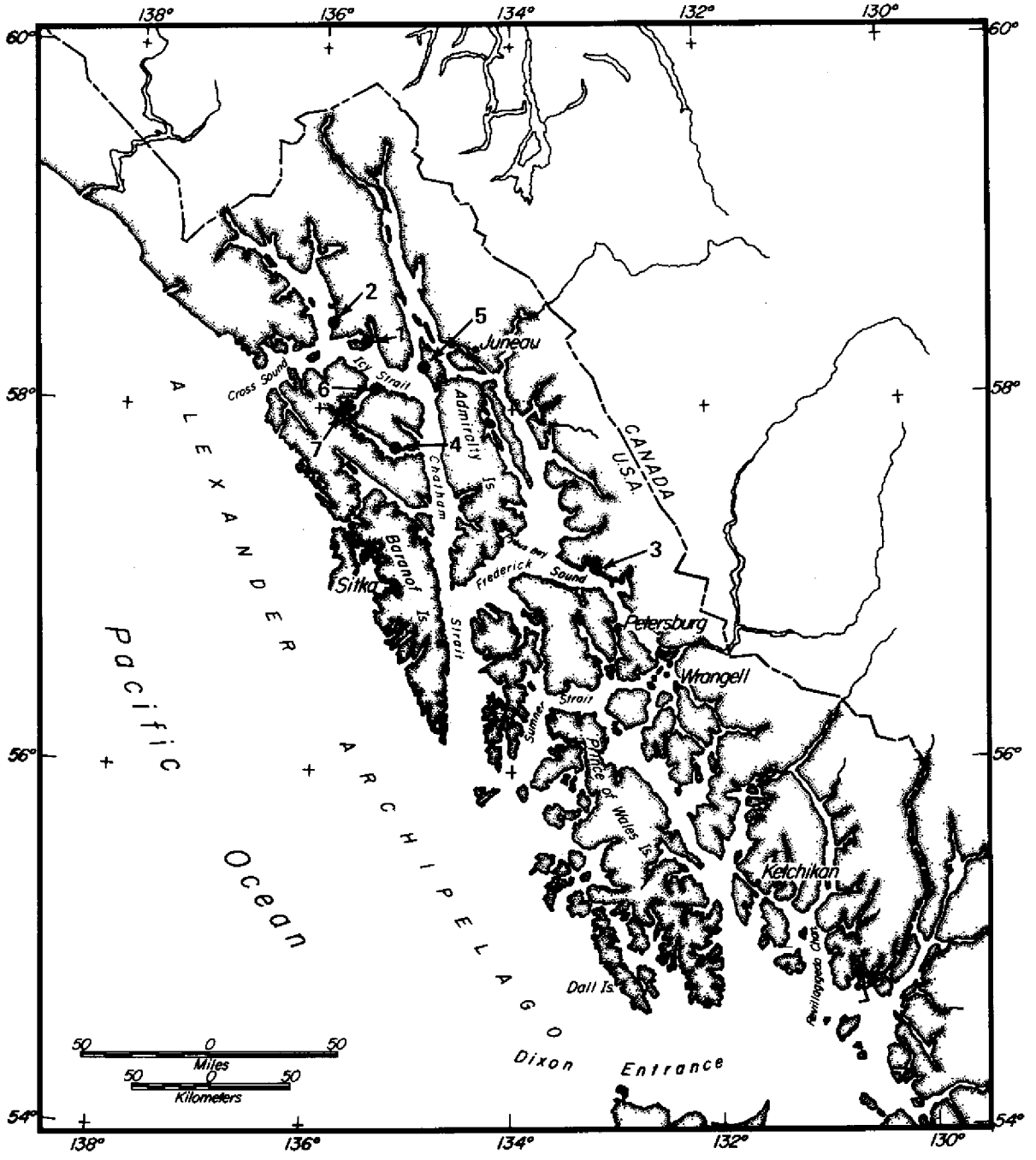
* *S. giganteus:* *Saxidomus giganteus* (butter clam)

M. edulis: *Mytilus edulis* (mussel)

P. staminea: *Prototheca staminea* (littleneck clam)

C. nuttalli: *Clinocardium nuttalli* (cockle)

FIGURE 9. Field Stations Sampled for PSP Determinations



CHAPTER IV

CONCLUSIONS

1. We have developed a quantitative assay for saxitoxin based upon its oxidative degradation and subsequent colorimetric analysis of the resultant guanidine. The procedure can be completed in two hours and is sensitive to as little as 0.5 μg of saxitoxin.

2. This colorimetric test can be applied to determination of saxitoxin levels in shellfish, but only with an extensive cleanup of the crude extract.

3. Attempts to develop a rapid, practical assay for saxitoxin in shellfish based on this method have been thwarted, to this point, by the presence of an interfering substance. This substance, which has chromatographic properties similar to saxitoxin, appears to be an arginine-containing peptide.

4. Several colorimetric saxitoxin analyses in shellfish extracts have shown lower levels than those determined by mouse bioassay. Although we have not yet totally eliminated the possibility that this difference is due to procedural difficulties, the discrepancy may be taken as evidence for the existence of more than one toxin in contaminated Alaskan shellfish.

5. A modification of the thin layer chromatographic-fluorometric method for saxitoxin assay developed by Buckley, et al., (1976) shows promise as a crude screening method for shellfish. However, a completely satisfactory TLC system has not yet been discovered.

6. Saxitoxin can be dansylated, but the reaction does not provide the basis for a practical saxitoxin assay.

7. The shellfish throughout Southeast Alaska continue to experience periodic inputs of toxin without the occurrence of "red tides." Since the dinoflagellate blooms associated with "red tides" are apparently not necessary for shellfish toxicity in Southeast Alaska, a thorough investigation of the processes leading to PSP in these waters is needed. In particular, the role of the cyst form of dinoflagellates needs investigation.

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