RAPID AND FUNCTIONAL NEUROBIOCHEMICAL ASSAYS FOR MARINE ALGAL TOXINS USING MOUSE BRAIN SYNAPTONEUROSOMES

by

Laurence S. David

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Name: Laurence David

Degree: Master of Environmental Toxicology

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Rapid and functional neurobiochemical assays for marine algal toxins using mouse brain synaptoneurosomes.

Examining Committee:

Chair: Dr. E. Palsson. Assistant Professor

Dr. R.A. Nicholson, Associate Professor, Senior Supervisor
Department of Biological Sciences, SFU

Dr. C.J. Kennedy, Associate Professor
Department of Biological Sciences, SFU

Dr. M.M. Moore, Associate Professor
Department of Biological Sciences, SFU
Public Examiner

Date Approved: December 17, 2002

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Rapid and Functional Neurobiochemical Assays for Marine Algal Toxins using Mouse Brain Synaptoneurosomes

Author: _____
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Laurence S. David
(name)

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ABSTRACT

Regular monitoring of the levels of marine biotoxins in shellfish has become an essential activity in view of the serious human health consequences of contaminated shellfish consumption. The most commonly encountered marine biotoxins are the saxitoxin (STX) and brevetoxin (PbTx) types, responsible for paralytic shellfish poisoning (PSP) and neurotoxic shellfish poisoning (NSP) respectively. Traditionally, these biotoxins are quantitated by the mouse injection bioassay, which has come under criticisms for a number of reasons. As a potential replacement for the PSP toxin toxicity test, a rapid functional assay using mouse brain synaptoneurosomes has been recently developed and validated by comparison with mouse toxicity data (Nicholson *et al.*, 2002).

There were three aims of the present research. Firstly, to investigate the potential of veratrine as a substitute for veratridine to open sodium channels in the synaptoneurosomal PSP toxin assay. Secondly, to further validate the synaptoneurosomal PSP toxin assay using Atlantic mussels spiked with STX and examine the assay's ability to quantitate PSP toxin content of plankton samples. Thirdly, to examine the possibility of configuring and validating synaptoneurosomal assay for NSP toxin quantitation. Concentration-dependent inhibition by STX of veratridine-induced (IC_{50} = 4 nM) and veratrine-induced (IC_{50} = 2 nM) synaptoneurosomal depolarization was observed. Assays of spiked mussel extracts revealed a strong relationship between STX added and STX detected when either veratridine (r^2 = 0.998) or veratrine (r^2 = 0.997) was used. Veratrine therefore represents a valid alternative to veratridine in this assay. Low PSP toxin levels in plankton samples could also be detected.

A new method for quantitating NSP toxins using the synaptoneurosomal technique was developed. Two brevetoxin standards (PbTx-2) and (PbTx-3) showed detection thresholds of approximately 0.6 ng/assay (PbTx-2) and 1.0 ng/assay (PbTx-3) and EC_{50}s of 1.56 nM and 2.49 nM respectively. PbTx action is completely blocked by tetrodotoxin, a sodium channel-specific blocker. This pharmacological profile indicates that the assay is valid in terms of sodium channel pharmacology and receptor binding theory. When PbTx-2 assay results were correlated with PbTx-2 levels originally added to the mussel samples a regression coefficient of 0.973 was obtained. The mouse brain synaptoneurosomal technique was further validated by assaying brevetoxin contaminated oyster samples previously assayed by FDA laboratories using receptor binding assay (r^2 = 0.953), cytotoxicity assay (r^2 = 0.974) and HPLC/MS (r^2 = 0.605).

It takes 40 minutes to isolate synaptoneurosomes and individual assays take approximately 7 min (PSP toxins) and 30 min (NSP toxins). Since 20 assays can be performed with one mouse brain, there is real opportunity for reducing the number of animals used in the conventional PSP toxin and NSP toxin monitoring.

DEDICATION

To Con, Tophy and Troy.

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LIST OF ABBREVIATIONS

ASP – amnesic shellfish poisoning

BTX – batrachotoxin

CNS – central nervous system

CSF – cerebrospinal fluid

CTX - ciguatoxin

DA – domoic acid

dcSTX – decarbamoylsaxitoxin

DMSO – dimethylsulfoxide

EC_{50} – concentration producing 50% enhancement

IC_{50} – concentration producing 50% inhibition

IC_{100} – concentration producing 100% inhibition

LqTx – α-scorpion venom

MBSA – mouse brain synaptoneurosome assay

NSP – neurotoxic shellfish poisoning

PbTx - brevetoxin

PSP – paralytic shellfish poisoning

RBA – radioligand binding assay

STX – saxitoxin

se – standard error

STXOL - saxitoxinol

TTX – tetrodotoxin

VSSC – voltage-sensitive sodium channel

VTD – veratridine

VTN - veratrine

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Introduction

It is generally accepted that the incidence of problems associated with toxic algae is increasing. Possible reasons underlying this expansion include natural mechanisms of species dispersal (via currents and tides) to a host of human-related phenomena such as nutrient enrichment, climatic shifts, and transport of algal species via ship ballast water. Eutrophication, the enrichment of natural waters with plant nutrients, was initially known as a phenomenon in freshwater environments. Currently, this phenomenon is established as one of the leading causes of harmful algal blooms along coastal environments. At present, many coastal areas have experience order of magnitude enrichments with nitrogen (N) and phosphorus (P) compared to several decades ago (Riegman, 1998). Notably, the global nitrogen cycle is extraordinary to the extent to which it has been modified by human activity. Some of the anthropogenic activities that introduced more newly fixed N into the biosphere than the annual natural background fixation of 100Tg N were industrial N-fixation for fertilizer production, internal combustion engine emissions and the increased farming of legume crops. A portion of this newly fixed and mobilized nitrogen is transported to the atmosphere, but most is considered to be allocated in solution and consequently discharged to continental coastal areas (Reigman, 1998). Coastal marine environments, being the most productive in biological and economical

terms, are likely to be dramatically affected by anthropogenic nutrification. This can produce changes in algal biomass, changes in marine vegetation types that may lead to shifts in biogeochemical cycles, and reduced productivity of coastal areas. The latter is attributed to the mortality at higher trophic levels related to toxification and anoxia. The global increase in harmful algal blooms has been attributed in part to eutrophication (Smayda, 1990). This dense growth, or bloom of dinoflagellates is often described as a red tide.

Evidence for an apparent global increase in the distribution, frequency and intensity of harmful algal blooms has been evaluated recently by several authors (Anderson, 1989; Smayda, 1990). While the actual cause(s) of this phenomenon is still widely disputed, it is clear that toxic events associated with several marine biotoxin syndromes are now occurring in previously unaffected areas (Sundstrom *et al.*, 1990). Evidently, this has resulted in greater overall awareness of harmful toxic algae and their implications for food safety and associated public health issues. A worldwide increase in toxic phytoplankton blooms over the past twenty years has coincided with increasing reports of deaths of fish, birds and marine mammals, also of human diseases and deaths by unknown causes (Hokama *et al.*, 1990; Morris *et al.*, 1991; Dobi *et al.*, 1990; Anderson *et al.*, 1994). In North America particularly in the United States and Canada, shellfish toxicity has been a concern for hundreds of years and has become a recurrent annual problem even in locations which have not previously experienced these problems (Anderson *et al.*, 1994). An illustration of the distribution of marine algal toxins in coastal regions North America is given in Figure 1.1. As a result of this problem, government authorities have set up regulatory bodies to monitor shellfish farming in their

respective constituencies. In Canada, the federal government created the Canadian Shellfish Sanitation Program to address the risks posed by the consumption of bivalve shellfish. The three federal agencies responsible for this program are the Canadian Food Inspection Agency (CFIA), the Department of Fisheries and Oceans (DFO) and Environment Canada.

Several marine biotoxins are becoming increasingly important as etiologic agents of foodborne diseases. Among them are saxitoxins (responsible for paralytic shellfish poisoning, PSP), brevetoxins (responsible for neurotoxic shellfish poisoning, NSP), domoic acid (responsible for amnesic shellfish poisoning, ASP), and tetrodotoxin, ciguatoxin and okadaic acid (responsible for diarrhetic shellfish poisoning, DSP). These toxic algal blooms usually peak between May and October. Paralytic shellfish poisoning (PSP) is a worldwide problem. Blooms have occurred in New England, Canada, Northwestern United States, England, Norway, Brazil, Argentina, India, Thailand and Japan (Anderson, 1989). Saxitoxin and a range of structurally-related derivatives produced by three marine dinoflagellate genera (*Alexandrium, Gymnodinium* and *Pyrodimium*) are responsible for paralytic shellfish poisoning (PSP) in humans (Hall *et al.*, 1990; Usleber *et al.*, 1997). The name saxitoxin was given by Schuett and Rapoport in 1962 to the purified paralytic shellfish poison extracted from the Alaskan butterclam, *Saxidomus giganteus* (Evans, 1972). The major transvectors for PSP are the bivalve molluscs (mussels, clams, and oysters). PSP toxins are also found in certain crabs and snails that feed on coral reef seaweed. Transvectors accumulate the toxins in their digestive organs and soft tissues via filter feeding, an activity that apparently does not harm them. Once humans and other mammals, birds and fish consume these transvectors,

illness or fatalities may result. Herbivorous zooplankton represents the primary transvector that can directly transmit the toxin to fish and possibly other marine creatures that consume zooplankton (Baden, 1983). The usual assimilation route for humans is the consumption of raw or cooked contaminated shellfish.

Blooms of the dinoflagellate *Ptychodiscus brevis* Davis (previously *Gymnodinium breve* but reallocated to a new genus by Steidinger, 1979) have caused massive fish kills, bird kills, and human food poisoning along the Florida coast and the Gulf of Mexico (Atchison *et al.*, 1986; Baden, 1989; Risk *et al.*, 1982). These dinoflagellates contain a variety of extremely potent, lipid-soluble neurotoxins (Atchison *et al.*, 1986). Numerous attempts have been made since 1968 to isolate the toxins from cultured cells, however, discrepancies exist in the reported physical properties due mainly to the difficulties encountered with the separation and purification of individual toxin components (Lin *et al.*, 1981). Toxins from these dinoflagellates are now known as brevetoxins (Apland *et al.*, 1993; Atchison *et al.*, 1986; Lin *et al.*, 1981; Nakanishi, 1985; Stuart and Baden, 1988; Berman and Murray, 2000). Lin *et al* (1981) isolated two toxins, brevetoxin B and C as crystals and brevetoxin chemistry has been reviewed by Nakanishi (1985). A human intoxication syndrome known as neurotoxic shellfish poisoning (NSP) has been ascribed to the consumption of shellfish contaminated with brevetoxins (McFarren *et al.*, 1965; Morris *et al.*, 1991). Dinoflagellate-producing brevetoxins are primarily limited along to Gulf of Mexico particularly along the Florida coast (Baden, 1989) and more recently along New Zealand waters (Ishida *et al.*, 1995). Human intoxication results from the ingestion of contaminated shellfish or inhalation of brevetoxin-containing aerosols in seaspray (MacFarren *et al.*, 1965; Pierce, 1986).

Several intoxication cases have been described in which a different diatom genus *Pseudonitzschia* was involved (Mos, 2001). The first documented case occurred in 1987, when three people died and over a 100 became ill after eating mussels from Prince Edward Island, Canada (Bates *et al.,* 1998). Three poisoning victims died 11 – 24 days later due to septic shock pneumonia, while others recovered within 10 days after a period of confusion and nausea, but continued to show evidence of selective short-term memory loss (Todd, 1993). This prompted regulatory bodies to examine mussels from restaurant and stores and it was discovered that they contained considerable amounts of the neuroexcitatory toxin domoic acid. Domoic acid (DA) is a neurotoxic amino acid and produced by the diatom *Pseudonitzschia multiseries* in the marine environment (Dizer *et al.,* 2001). This diatom was originally known as *Nitzschia pungens* [later changed to *Pseudonitzschia multiseries* (Bacillariophyceae) (Hasle, 1995)] and was once thought to be an innocent diatom, free of all toxicity (Bates *et al.*, 1989). *Pseudonitzschia spp.* are high in abundance on the continental shelves of North America, the Gulf of Mexico, and the southwest Islands of Japan (Lange *et al.*, 1994; Dortch *et al.*, 1997; Horner *et al.*, 1998; Kotaki *et al.,* 1999). These organism occur sporadically in high numbers (10^4 – 10^6/L), usually between February and August (Lange *et al.*, 1994), and can accumulate in blue mussels (*Mytilus edulis*), razor clams (*Siliquata patula*), and dungeness crabs (*Cancer magister*) which then can be consumed by humans. The human toxic syndrome results specifically in a short-term or permanent memory impairment, so called amnesic shellfish poisoning (Bricelz and Shumway, 1998; Schmued and Slikker, 1999).

Since serious human health consequences can result from ingestion of PSP, NSP and ASP toxins, monitoring of shellfish for these substances is conducted on a continuous

basis in many parts of the world. One of the most widespread methods to detect toxins causative of PSP, ASP and NSP is the mammalian (rat, mouse) bioassay (Beani *et al.*, 2000; Nicholson *et al.*, 2002; AOAC, 2000; Kerr *et al.*, 1999; Truman and Lake, 1996). This approach, however, poses certain problems especially in terms of low selectivity and sensitivity, as well as for the high variability of the responses in different animal strains (Beani *et al.*, 2000). It has also been found to give occasionally unreliable and contradictory results i.e. false positives (Kerr *et al.*, 1999). Furthermore, this assay does not provide conclusive evidence as to the specific toxin in a sample extract, is costly, arguably inhumane and requires the use of large numbers of animals (Kerr *et al.*, 1999; Truman and Lake, 1996; Manger *et al.*, 1995). More reliable analytical methods with higher sensitivity, such as HPLC and liquid chromatography mass spectrometry, have also been developed. These methods have the advantage of quantifying already known toxins, but the former method needs several, not easy to find, standards, while the latter requires expensive and complex instrumentation. Recognizing these problems, various *in vitro* techniques have been evaluated as possible replacements for the mouse bioassay technique. Among the techniques developed include chemical, pharmacological and immunological analyses of marine toxins. Most of the pharmacological assays developed on the analysis of PSP were based on the known action of saxitoxin in binding to site 1 on voltage-gated sodium channels (Catterall *et al.*, 1979). The importance of understanding the pharmacological action of saxitoxin in the development of *in vitro* assays in some cases has led to good correlations with the standard mouse bioassays. Receptor binding assays have been developed (Weigele and Barchi, 1978; Doucette *et al.*, 1997) to analyze the extent of toxicity of PSP. This assay was found to have a strong

predictive value for toxicity determined by mouse bioassay. A more functional approach to toxicity testing such as cell cytotoxicity assay (Truman and Lake, 1996; Beani *et al.*, 2000; Manger *et al.*, 1995) has proven to be useful in the detection of saxitoxin, brevetoxin and other marine toxins. The results obtained from this type of assay also correlate well with the traditional mouse bioassay. Electrophysiological and immunological approaches (Usleber *et al.*, 1997; Kerr *et al.*, 1999) were also tested as replacement for mouse bioassay. However, potential drawbacks of the assays mentioned above include the routine use of radioligands which are very expensive and in the case of [^{3}H]-STX it is becoming increasingly difficult to purchase. Moreover, the times required for radiometric assay and analysis are usually lengthy and electrophysiological assays are not ideally-suited to routine high throughput screening. Enzyme immunoassay is highly sensitive and can be readily adapted for example, kit format, however, antibody production can be costly (Llewellyn *et al.*, 1998) and antibody specificity can be a problem when assaying the saxitoxin – group of toxins. Method for chemical analysis of saxitoxin using post-column derivatization liquid chromatographic method was described by Oshima (1995). This method has a greater advantage over other methods in its ability to quantitate each toxin in a crude sample of small size, however, this generally involves extensive sample work-up prior to analysis (Nicholson et *al.*, 2002).

A principal difficulty in establishing the presence of brevetoxins, and in quantifying the amounts present has been the lack of an analytical procedure possessing sufficient sensitivity (Trainer and Baden, 1991). Pharmacologically-based assays such as receptor binding assays (Van Dolah *et al.*, 1994; Stuart and Baden, 1988; Leighfield *et al.*, 1996; Baden *et al.*, 1988; Dechraoui *et al.*, 1999) have been extensively used in

estimation of NSP toxin content. This pharmacological assay is based on known mechanism of brevetoxin acting on site 5 of the α-subunit of the voltage-sensitive sodium channel (Poli *et al.*, 1986; Catterall, 1988; Catterall, 1992). The advantage of binding assays include their suitability in determining toxic potency of a sample containing multiple congeners, because within a toxin class, all congeners bind to the same molecular target in the cell with relative affinities that often correlate to their relative toxic potencies (Van Dolah *et al.*, 1994). However, their major limitation has traditionally been that they are time consuming and labour intensive. Another type of assay used in quantifying PbTx content is immunoassay (Baden *et al.*, 1988; Levine and Shimizu, 1992). This type of assay is attractive in that it is highly sensitive, specific for a given toxin, and can be potentially adapted to a field kit type format suitable for dockside or shipboard testing. However, immunoassays are based on toxin structure, and not on toxin efficacy, thus, a given antibody may not identify all active congeners (false negative) and may recognize inactive congeners (false positives)

1.2 Objectives of the study

In light of the continuous efforts to replace the mouse bioassay in the quantitation of marine biotoxins present in seafoods and significantly reduce requirement for *in vivo* testing, a key objective of this research was to develop a rapid and functional neurobiochemically-based assay for brevetoxin with a view to validate the assay using spiked samples and samples previously quantitated by other *in vitro* assays. It is also the aim of this research to further validate mouse brain synaptoneurosome assay with mussel

samples spiked with saxitoxin. The ultimate aim of this research is to strengthen our rationale for proposing synaptoneurosome-based tests in PSP and NSP toxin monitoring.

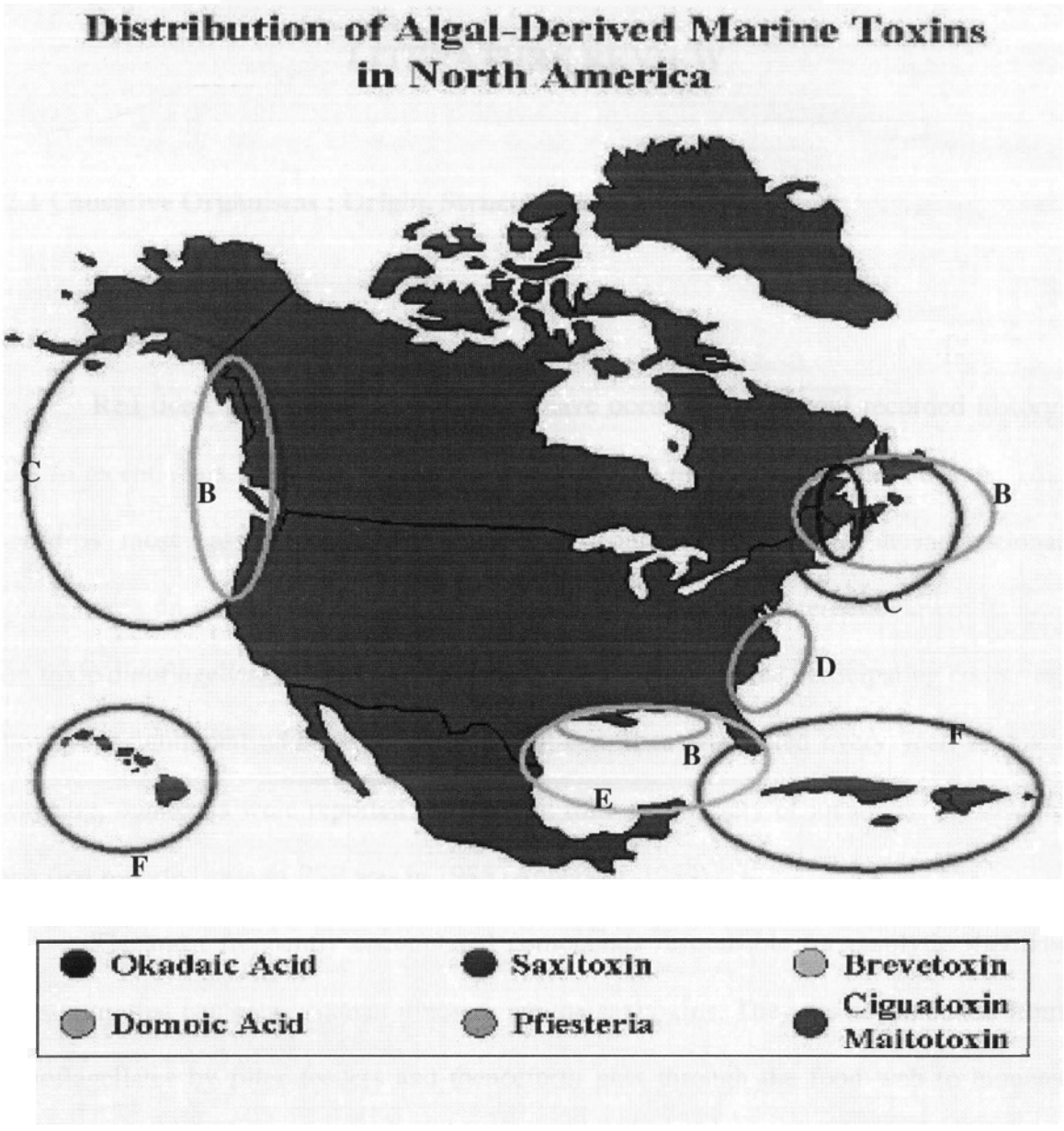


Figure 1.1. Distribution of algal-derived marine toxins in coastal regions of North America. (A, okadaic acid; B, domoic acid; C, saxitoxin; D, *Pfiesteria*; E, brevetoxin and F, ciguatoxin and maitotoxin). Taken from http://www.chbr.noaa.gov/CoastalResearch/, October, 2002. (Printed with permission from Kimberly Nowocin, NOAA, SC., U.S.A.).

CHAPTER TWO

LITERATURE REVIEW

2.1 Causative Organisms : Origin, Structure and Biology

2.1.1 Paralytic shellfish poisoning

Red tides, both toxic and non-toxic, have occurred throughout recorded history, but in recent years, there has been a global increase in the number of these events. This trend is most easily seen in the number of countries represented at international conferences on the subject (Anderson, 1989). In 1974, the first international conference on toxic dinoflagellate blooms was held and there were only three participating countries, however, significant addition of participating countries was noted every year. At each meeting, outbreaks were reported for the first time at a variety of locations. In Canada, the first reported case of PSP was in 1985 (Anderson, 1989).

The most frequently encountered compounds responsible for paralytic shellfish poisoning that can cause human illnesses are the saxitoxins. They are accumulated from dinoflagellates by filter feeders and thenceforth pass through the food web to humans (Hall *et al.*, 1990). Kodama (2000), suggested that besides the food web, another possible source of shellfish toxicity is through *de novo* synthesis of toxin derivatives by shellfish through digestion of algal cells or other ingested materials together with dinoflagellates. However, algae are widely recognized as the primary sources of biotoxins. They include three morphologically distinct genera of dinoflagellates and three species of cyanobacteria. The dinoflagellate genera are *Alexandrium, Gymnodinium* and *Pyrodinium*

(Hall *et al.*, 1990; Kodama, 2000; Lehane, 2000) and the cyanobacteria *Aphanazomenon flos-aqueae* (Hall *et al.*, 1990; Kodama, 2000), *Anabaena circinalis* and *Lyngbya wollei* (Kodama, 2000). The link between shellfish toxicity and dinoflagellates was first established through the efforts of Sommer and colleagues in their studies following the outbreak of PSP in the San Francisco bay area in 1927 (Hall *et al.*, 1990). The toxic dinoflagellate concerned was assigned to the genus *Gonyaulax* and named *G. catenella*. Several works on PSP on various regions has established that toxigenic gonyaulacoid dinoflagellates of similar morphology are a prime source of toxicity in many areas. These were assigned under genus *Gonyaulax* with a distinct subgroup but recent taxonomic revisions have variously reassigned the organisms to the genera *Alexandrium* (Balech, 1985). Among the groups of PSP organisms, the most common causative organism of paralytic shellfish poisoning in North America belongs to the genus *Alexandrium* (Figure 2.1). Environmental stress such as increase in temperature can affect the toxin production and reproduction of these dinoflagellates (Figure 2.2). As the temperature increases the chances of survival of motile cells is greater, leading to more active division of cells, hence, contributing to toxic algal blooms (Figure 2.2). In North America, two species of *Alexandrium* were documented as sources of saxitoxins. These two are *A. tamarense* and *A. fundyense* (Anderson *et al.*, 1994). These two closely related species are almost identical except that *A. fundyense* lacks ventral pore (Steidinger and Tangen, 1996). Both have epitheca that are broadly conical with slight shoulders and hypotheca is somewhat trapezoidal and posteriorly concave. *A. tamarense* is almost found in any coastal regions of the world whereas *A. fundyense* is endemic to the littoral region of the Bay of Fundy

and from the Canadian Atlantic to almost 41°N in the United States (Steidinger and Tangen, 1996).

The earlier works on the elucidation on the structure of PSP toxins have been reviewed by a number of investigators (Oshima *et al.,* 1977; Shimizu *et al.*, 1977; Hall *et al.*, 1990; Shimizu, 2000). The structure of saxitoxin was established first and this gave the basic framework to elucidate other PSP structures (Figure 2.3). The chemistry of saxitoxin was studied extensively by Wong *et al* (1971). Saxitoxin derivatives are formed by N-1, hydroxyl and 11-hydroxysulfate substitutions. Saxitoxin contains two guanidinium groups (Hall *et al.*, 1990; Lehane, 2000) and can be divided into two major categories: the STX series and the neoSTX series (Shimizu, 2000). The difference between the two groups is the presence or absence of N1-hydroxyl group (Figure 2.3a). This hydroxyl group has a profound effect on the stability and ionic character of the toxins. Another structural variation comes from the presence or absence of *O*-sulfate at C-11 and N-sulfate groups on carbamoyl groups (Figure 2.3b), which also greatly changes the physical and chemical character as well as pharmacological characters (Shimizu, 2000). Decarbamoyl saxitoxins have also been reported among tropical dinoflagellates and bivalves (Harada *et al.,* 1983) (Figure 2.3b). The blocking action of saxitoxin is attributed to the 7,8,9 guanidinium moiety not the 1,2,3 guanidinium moiety (Baden and Trainer, 1993).

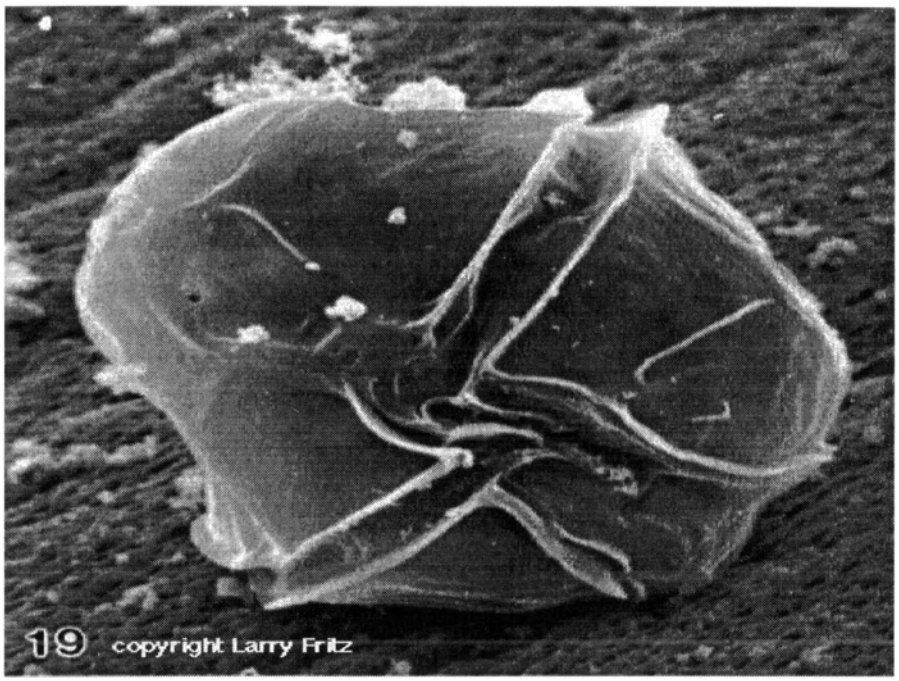


Figure 2.1. *Alexandrium sp.* (printed with permission from Dr. Lawrence Fritz, Northern Arizona University, Arizona, U.S.A.).

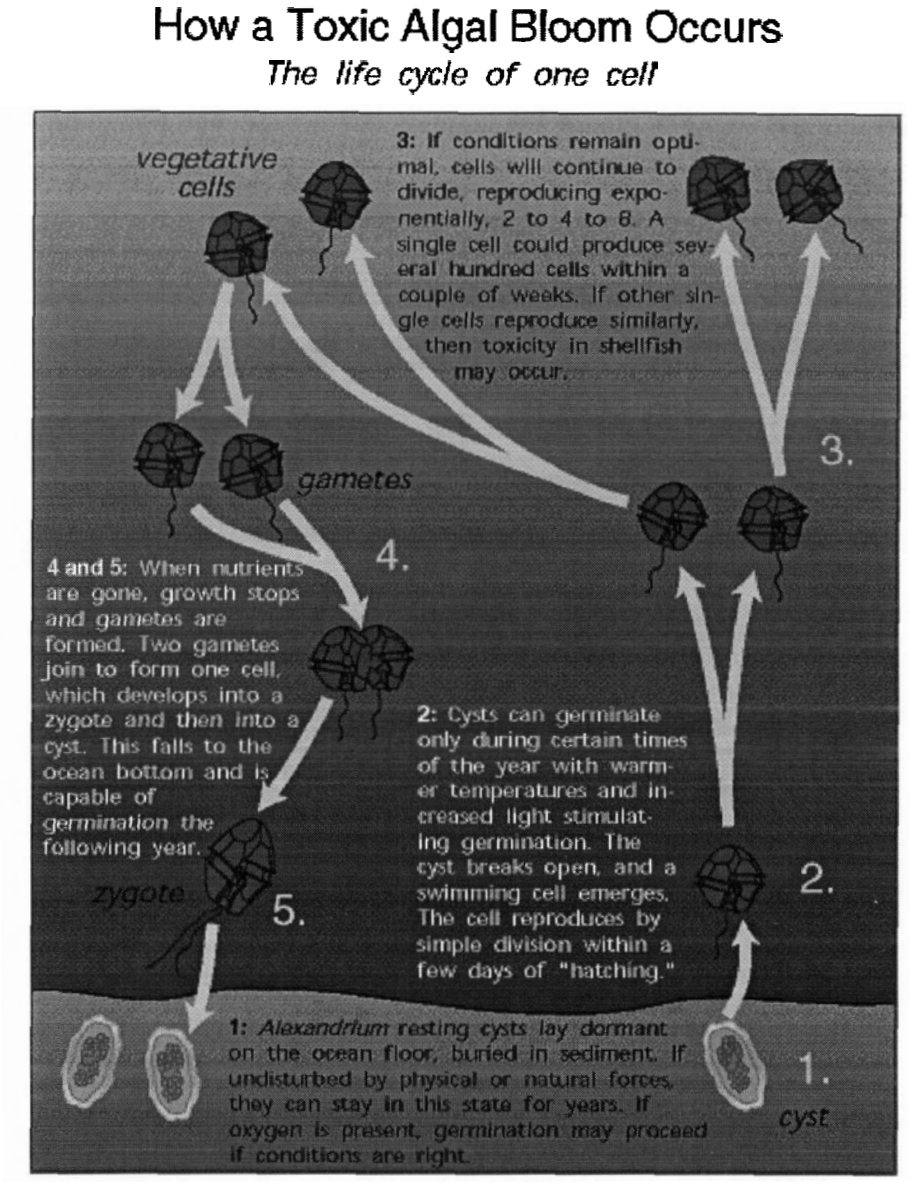
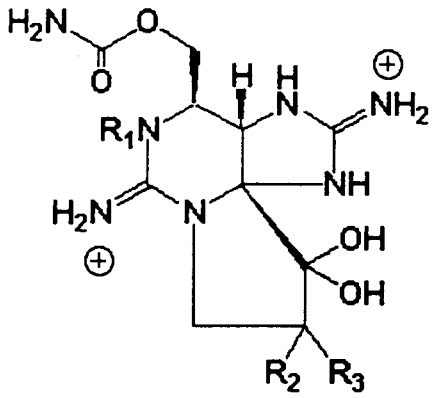


Figure 2.2. Illustration of how harmful algal bloom occurs. Taken from http://www.whoi.edu/redtide/whathabs/whathabs.html, October, 2002. (Printed with permission from Dr. Donald Anderson of Woods Hole Institution, Woods Hole, MA, U.S.A.).

a. Carbamoyl saxitoxins



STX	R_1	R_2	R_3
STX	H	H	H
GTX-II	H	H	OSO_3^-
GTX-III	H	OSO_3^-	H
NeoSTX	OH	H	H
GTX-I	OH	H	OSO_3^-
GTX-IV	OH	OSO_3^-	H

b. Decarbamoyl saxitoxins

Decarbamoyl Saxitoxins	R1	R2	R3
13	H	H	H
14	H	OSO_3^-	H
15	H	H	OSO_3^-
16	OH	H	H
17	OH	OSO_3^-	H
18	OH	H	OSO_3^-

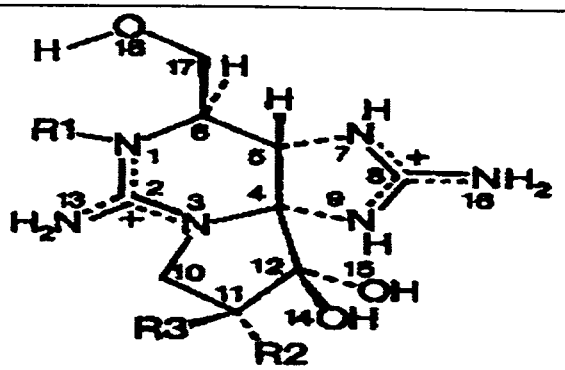


Figure 2.3. Chemical structure of saxitoxin and related analogues. (Hall *et al.*, 1990; Oshima, 1995).

2.1.2 *Neurotoxic shellfish poisoning*

The Gulf of Mexico has over 30 toxic microalgal species, but only one consistently produces dramatic fish kills and presents human health risks (Steidinger *et al.*, 1998). The causative microalga is *Gymnodinum breve* (synonym *Ptychodiscus brevis*), an unarmored dinoflagellate (Van Dolah, 2000; Steidinger *et al.*, 1998; Baden and Adams, 2000; Nakanishi, 1985). The toxins produce by marine dinoflagellate are generally called brevetoxins (Baden and Adams, 2000; Huang and Wu, 1989; Van Dolah, 2000; Steidinger *et al.,* 1998). These toxins can cause fish kills and other marine mortalities, cause filter feeding animals such as oysters and clams to become toxic to humans when consumed, leading to condition known as neurotoxic shellfish poisoning (NSP) and can also cause an airborne toxin (particulate irritant) in seaspray. *G. breve* (Figure 2.4) is a unicellular alga small dorsoventrally flattened cell with rounded epitheca and apical process or carina directed ventrally, hypotheca notched and slightly bilobed, cells are usually concave ventrally and convex dorsally (Steidinger and Tangen, 1996). There are two stages in the life cycle of *G. breve* (Figure 2.5). Both stages often represent successful adaptation strategies for survival and dispersal (Steidinger *et al.*, 1998). Steidinger (1975) suggested that the sexual life cycle with a benthic resting stage or a motile diploid nondividing stage represent a reservoir or seed population for bloom development. On the other hand, asexual reproduction is manifested by oblique binary division with an observed rate in culture of 0.2 to 1 division per day (Wilson, 1966; Shanley and Vargo, 1993). Figure 2.5 illustrates the detailed stages of *G. breve* life cycle. In the Gulf of Mexico where this organism is considered endemic, the background concentration ranges between 1 to 1000 cells per liter. Although cell concentrations are

higher at the surface, it can also be found down to > 50 m. This principally neritic species does not tolerate salinities in Florida bays and lagoons of less than 24% (Steidinger *et al.*, 1998). The occurrence of toxic bloom of *G. breve* has been reported since the 1800's along the Florida coast and this caused massive fish kills and neurotoxic poisoning among humans. Elsewhere in other parts of the world particularly in Spain, Japan, Greece, France, Israel and New Zealand similar phenomenon has been described (Steidinger *et al.,* 1998). In 2002, as mentioned in the paper by Plakas *et al., G. breve* was renamed as *Karenina brevis* in honor of Karen Steidinger, a leading Florida red tide scientist.

Numerous attempts have been made since 1968 to isolate pure toxins from cultured cells (Nakanishi, 1985) to enable elucidation of the structures of the molecules responsible for NSP. It was only relatively recently (1981) that Lin *et al.,* isolated certain PbTx toxins in crystalline form. Brevetoxins are suite of ladder-like polycyclic ether toxins divided into two types based on backbone structure, PbTx-1 type (type A) and PbTx-2 type (type B, Figure 2.6). Brevetoxin B congeners are the most abundant in nature (Van Dolah, 2000), with PbTx-2 and Pb-Tx-3 being the most prevalent in *G. breve*. Although the ring systems in the middle of the molecules differ somewhat, both PbTx-B and PbTx-A share a common lactone ring (head) and a conserved structure on the "tail" ring, both of which are required for their toxicity (Baden, 1989). Brevetoxin A congeners are more flexible (38 rotatable bonds) than those with the brevetoxin B backbone (31 rotatable bonds) which may play a role in their generally greater toxicity (Rein *et al.*, 1994). The planar and relative configuration of brevetoxin B was determined by x-ray crystallography, which identified an array of trans-fused rings, 1 lactone, and 10

ether rings leading to the ladder-like structure with a length of 30 Å , width of 6 Å and height of 6 Å (Lin *et al.*, 1981). The naturally occurring brevetoxins have the same molecular receptor site, the same overall interaction with voltage-sensitive sodium channels (VSSC), and all can be detected with varying efficacies by the same immunological and receptor-based assays (Baden and Adams, 2000). Numerous different derivatives of brevetoxins have also been produced by microsynthetic organic chemical derivatization. Within this cadre of synthetic derivatives are toxins which mimic all of the natural toxin effects, some of which have no effect on sodium channels, while some possess a specific subset of the overall effects seen with natural toxins (Baden and Adams, 2000). Generally, the brevetoxin molecule can be thought of as a long rigid cylinder with a slight bend in the middle. The molecule is composed of four regions: -a lactone ring (head), electrophilic regions thought to be requisite for activity, a spacer region (next six rings), a rigid region (last four) which is required for binding to the sodium channel, and a side chain (R-group) (Baden and Adams, 2000).

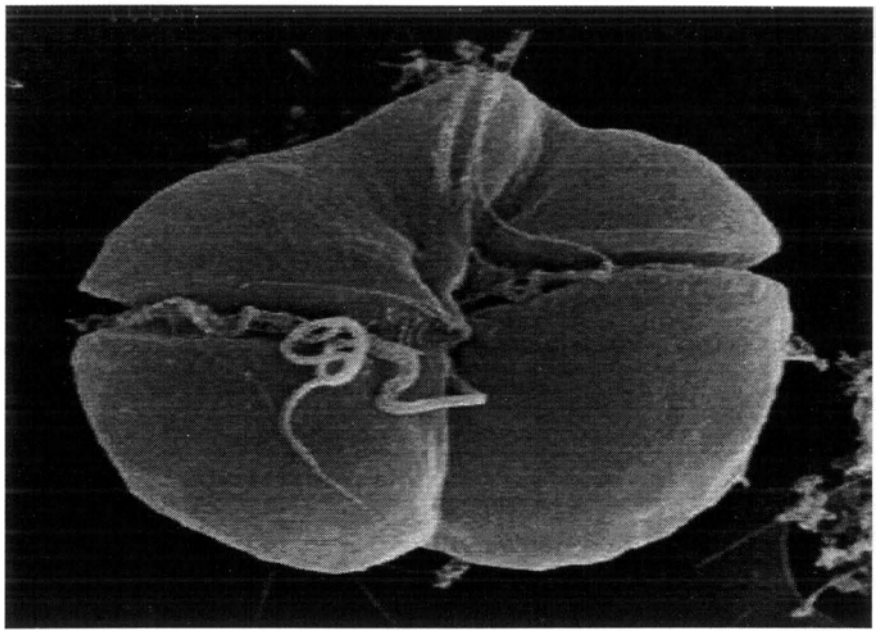


Figure 2.4 *Gymnodinium breve* (syn: *P. brevis*, *Karenina brevis*).Taken from Florida Department of Agriculture and Consumer Servives, 2002.

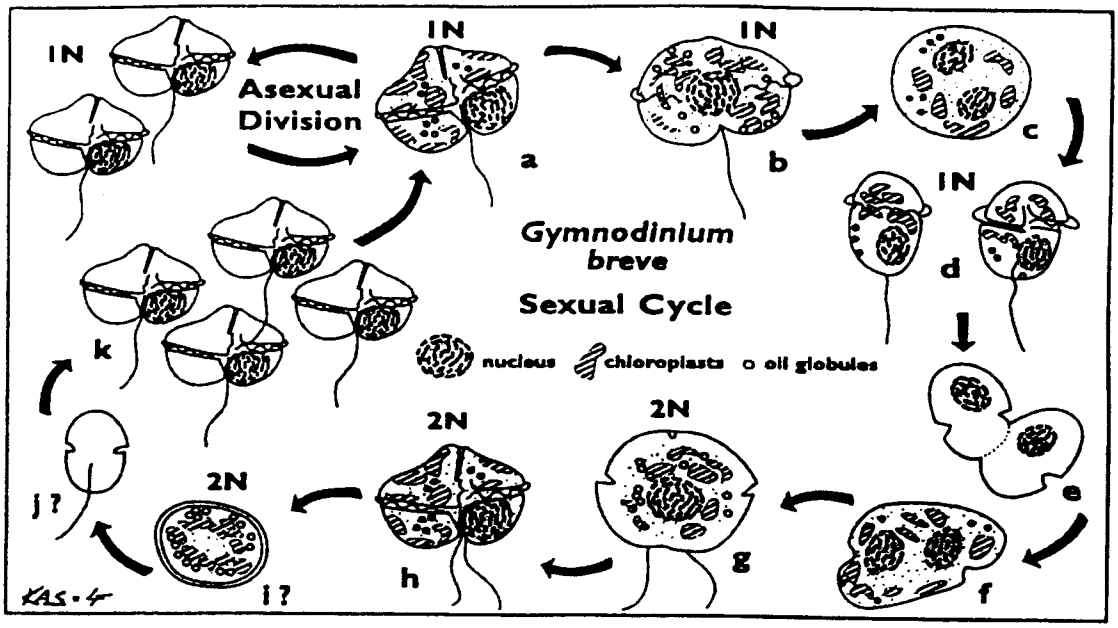
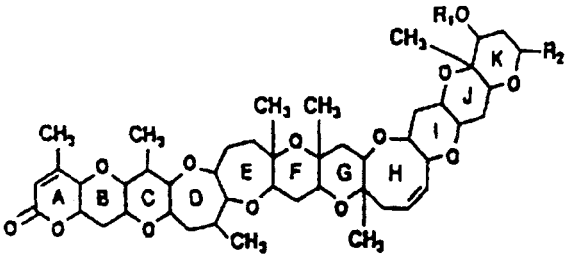
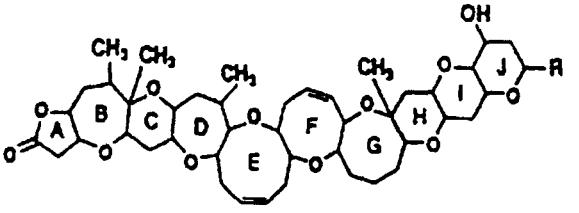


Figure 2.5 Life Cycle of *Gymnodinium breve.*(a) Vegetative 1N cell that is capable of mitotic division or producing gametes. (b) First stage in gamete formation with movement of nucleus to center. (c) Second stage of gamete formation with division of nucleus. (d) Third stage with production of isogametes. In heterothallic strains, there are + and – mating types that fuse. (e) In homothallic strains, gametes are the same mating type. (f) Cytoplasmic fusion precedes nuclear fusion. (g) Planozygote with 2N nucleus and 2 longitudinal flagella. (h) Motile planozygote. (i) Unconfirmed presumed hypnozygote. (j) Unconfirmed meiocyte. (k) Resultant 4 1N cells for meiosis II. (Steidinger *et al.*, 1998).





PbTx-2 and Derivatives:

PbTx-2: R_1=H R_2=$CH_2C(=CH_2)CHO$
PbTx-3: R_1=H R_2=$CH_2C(=CH_2)CH_2OH$
PbTx-5: R_1=Ac R_2=$CH_2C(=CH_2)CHO$
PbTx-6: R_1=H R_2=$CH_2C(=CH_2)CHO$(27,28 epoxide)
PbTx-8: R_1=H R_2=CH_2COCH_2Cl
PbTx-9: R_1=H R_2=$CH_2CH(CH_3)CH_2OH$

PbTx-1 and Derivatives:

PbTx-1: R=$CH_2C(CH_2)CHO$
PbTx-7: R=$CH_2C(CH_2)CH_2OH$
PbTx-10: R= $CH_2CH(CH_3)CH_2OH$

Figure 2.6 Structures of the PbTx-1 type and PbTx-2 type brevetoxins from *G. breve* (adapted from Poli *et al.*, 1995).

2.2 Pharmacological Profile of Marine Biotoxins used in this Study

2.2.1 *Saxitoxin and its Derivatives*

The saxitoxins (STXs) function by binding to a site on the extracellular surface of the voltage-activated sodium channel, interrupting the passive inward flux of sodium ions that would normally occur through the channel while it is in a conducting state (Hall *et al.,* 1990). The STXs are classic examples of marine toxins acting as occluders that act only from the external surface of the Na^+ channels to produce a slowly reversible, yet usually high affinity (Kd ~ 10^{-9} M) block of the channel (Strichartz and Castle, 1990). The α–subunit of the Na^+ channel contains the binding site for saxitoxin (Catterall, 1988; Suarez-Isla and Velez, 2000) and this binding site is named toxin site 1 (Catterall, 1980). Another marine toxin that acts on the same sodium channel site 1 receptor site is tetrodotoxin (TTX), which resides in the ovaries and liver of puffer fish of the suborder *Gymnodantes* and has been found recently in certain species of newt, frog and goby (Catterall, 1980). The mechanisms of action of both toxins have been extensively studied pharmacologically.

The guanidinium groups are prominent features of the structure of both saxitoxin and tetrodotoxin. Guanidine is one of the few cations that is an effective substitute for sodium in action potential generation (Hille, 1975). Kao and Nishiyama (1965) first proposed that the guanidinium moieties of tetrodotoxin or saxitoxin might enter the sodium channel like guanidine allowing the remainder of the toxin molecule to bind tightly to the ion channel, blocking further passage of ions. The 7,8,9-guanidinium moiety, and not the 1,2,3 one, is essential for the blocking action of STX (Baden and Trainer, 1993). The positively charged guanidinium group interacts with a negatively

charged carboxyl group at the mouth of the Na^+ channel on the extracellular side of plasma membranes of nerve and muscle cells, thus blocking the flow of Na^+ through the channel (Lehane, 2000). The entry of Na^+ through the nerve cell membrane is essential for impulse transmission and its blockade interferes with the signal transmission leading to paralysis.

In 1975, Hille presented the first binding model for saxitoxin in the sodium channel. The model (Figure 2.7) depicted the toxin molecule penetrating rather deep inside of the channel and plugging the channel, making an ion pair with a putative anionic site located near the bottom of the channel. The recognition of the high affinity interaction between saxitoxin and sodium channels played a major role in further pharmacological studies of the toxin and sodium channel function. The mouse bioassay has been the most common technique in detecting PSP toxins. This involves i.p. injection of a test solution, typically 1 ml, into a mouse weighing 17-23 g, and observing the time from injection to death (Hall *et al.*, 1990; AOAC Official Methods, 2000). This technique has revealed that sulfamate saxitoxins are less potent than the corresponding carbamate derivatives.

Binding assays for saxitoxin were conducted using tritium labelled STX in rat synaptosomes (Weigele and Barchi, 1978) and squid giant axon (Keynes *et al.*, 1984). These experiments relied on the ability of natural saxitoxin to compete with radiolabelled saxitoxin for the binding site in the sodium channel. This then, will give information on the specificity of the STX to the sodium channel of most excitable membranes. It has been reported that STX had high specificity to the sodium channel (Weigele and Barchi, 1978). In physiological studies STX has been shown to specifically and reversibly

eliminate the voltage-dependent sodium current at equilibrium dissociation constant (*Kd*) concentrations of 0.5 – 5.0 X 10^{-9} M at the site of action (Weigele and Barchi, 1978). Binding assays with tritiated saxitoxin are also useful in routine monitoring of paralytic shellfish poisoning brought about by harmful blooms. To further evaluate STX pharmacology, an experiment on the blockage of sodium conductance using rat sarcolemmal sodium channels incorporated into planar lipid bilayers was conducted by Hall and his co-workers (Hall *et al.,* 1990). These single channel studies were performed according to the methods by Moczydlowski *et al.* (1984) wherein they measure the current through single channels that had been incorporated into planar lipid bilayers treated with batrachotoxin (BTX) to shift their activation potential into accessible range. The sample toxins were then added and blocking events were recorded at different transmembrane potentials. These experiments showed that at 10 nM saxitoxin, blocking events at sodium channels tend to be relatively long (Hall *et al.*, 1990). It is significant to note in this finding that the duration of the blocked state, which corresponds to the dwell time of the toxin molecule at the binding site, provided information about the reversibility of binding for the toxin molecules that were bound.

The inhibition of sodium conductance by the saxitoxins and their effect on the physiology of laboratory animals have been reported by several investigators (Kao *et al.*, 1967; Nagasawa *et al.*, 1971; Chang *et al.*, 1993; Borison and McCarthy, 1977; Borison *et al.*, 1980). These effects include cardiovascular alterations and breathing problems, paralysis and coma as reported in clinical cases of intoxication. Details on the mechanism of intoxication by PSP are elaborated on in the toxicological profile of this review.

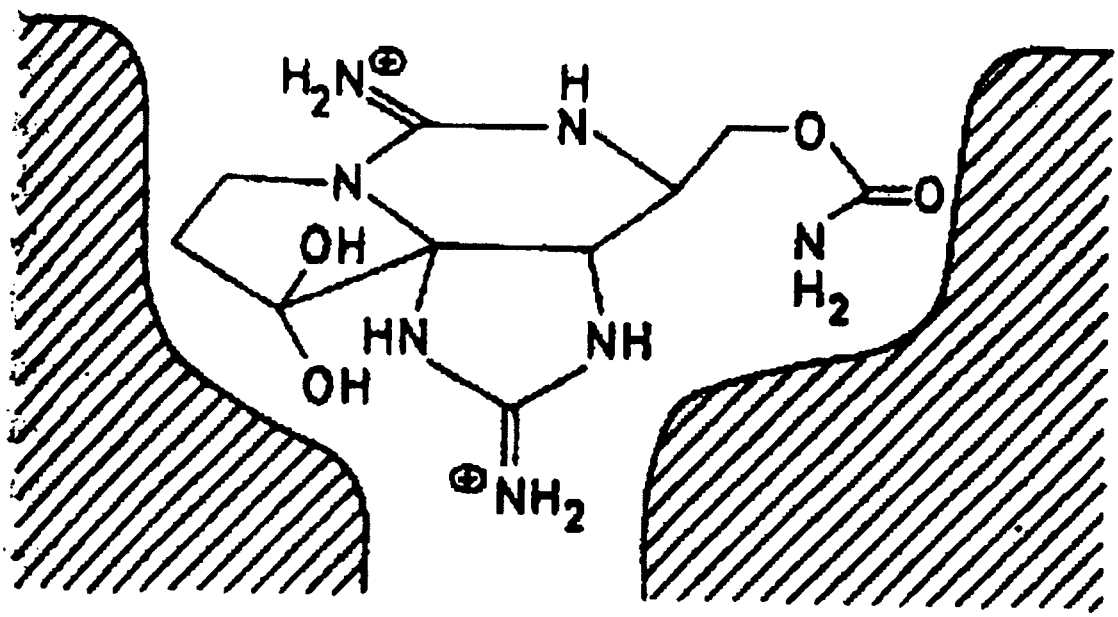


Figure 2.7 Model of the interaction of saxitoxin with its binding site on the Na^+ channel (Hille, 1975).

2.2.2 *Brevetoxin and its Derivatives*

Brevetoxins are lipid soluble toxins produced by *G. breve* (synonym: *P. brevis, K. brevis*) with molecular weights of approximately 900 and with approximately ten naturally occurring derivatives. These toxins are depolarizing substances that open voltage-gated sodium (Na^+) ion channels in nerve or muscle plasma membrane cell, leading to uncontrolled Na^+ influx into the cell (Baden, 1983). In early studies with brevetoxins, Catterall and Risk (1981), observed the allosteric interactions between toxins which bind to voltage-sensitive sodium channels (VSSCs). Significantly, further work by Poli *et al.* (1986), using tritiated brevetoxin (which was originally synthesized for the purpose of developing radioimmunoassays for the toxins in seafood), illustrated that the toxin bound to a site not previously described for any other sodium channel specific neurotoxin, and that the site was named site 5. Binding at this high affinity site was characterized as a 1:1 association with the α-subunit (*Kd* = 1.2 nM) was observed in all systems and the receptor exhibits a stokes radius of 55 ± 5 Å (Baden and Adams, 2000).

Naturally occurring brevetoxins and synthetically modified brevetoxins were examined for their ability to interact with site 5 of the VSSC and their toxicity with mosquito fish, *Gambusia affinis*. It was shown that all but three toxins studied retained some affinity for their receptor site (*Kd* in the range of 1-100 nM). Direct evidence on brevetoxin action of Na^+ fluxes has been demonstrated in neuroblastoma cells (Catterall and Risk, 1981; Catterall and Gainer, 1985) and rat brain synaptosomes (Poli *et al.*, 1986; Risk *et al.*, 1982). A purified fraction from T_4 (T_{47}; PbTx-2) was shown to cause a dose-dependent increase in $^{22}Na^+$ uptake in human neuroblastomal cell line (Risk *et al.*, 1979) and T_{46} (PbTx-3), a second active fraction purified from T_4, persistently enhanced the

activation of voltage-sensitive Na^+ channels by veratridine (Catterall and Risk, 1981). The brevetoxin molecule has been shown to bind to receptor site 5, with the tail end of the molecule located near the S5 – S6 extracellular loop of domain IV of the Na^+ channel α-subunit (Figure 2.8, Trainer *et al.*, 1994; Trainer *et al.*, 1991).

Pharmacological studies have also been conducted using other mammalian and invertebrate tissue preparations. Shinnick-Gallagher (1980) in her study with the toxin isolated from *G. breve* found that in the rat phrenic nerve diaphragm preparation concentrations of 2 μg/ml the toxin decreased the input resistance of the muscle membrane concomitantly with depolarization of the membrane. The toxin depolarized the membrane from –70.5 ± 3.5 mV to –61.8 ± 3.3 mV. The decrease in resistance accompanying membrane depolarization suggested that brevetoxin increased membrane permeability to Na^+. To further investigate brevetoxin's action on sodium channels, tetrodotoxin (TTX, a toxin known to block sodium channels), at concentration of 1 μg/ml was found to reverse membrane depolarization which, indicates that TTX antagonized the action of brevetoxin. Excitable tissue preparations from rat brain synaptosomes and [^{3}H]-PbTx were used by Trainer *et al.,* (1990) to investigate the specific binding of brevetoxins to the VSSC. Results showed that at 10 nM tritiated PbTx-3 IC_{50} (inhibitory concentration at competitor toxin required for 50% specific displacement of radioactive toxin) values for the brevetoxins range from 3.5 to 20 nM in rat synaptosomes. In their study with different derivatives of brevetoxins (named PbTx-1 to PbTx-7), they found that PbTx-1 and PbTx-7 had greater affinity than the other brevetoxins studied. On the contrary, PbTx-5 and PbTx-6 had a much lower affinity to site 5 of the sodium channels. The affinities of each toxin in each test system were considered to be based on structural

characteristics of the regions of the toxin which bound to the binding site (Trainer *et al.*, 1990). Of significance, the high affinities of PbTx-1 and PbTx-7, may be directly attributed to larger 8 and 9 membered rings and increased flexibility of their backbones which is about 90° bending capability (Nakanishi, 1985). Huang *et al.* (1984), on the other hand, suggested that the brevetoxin binding site lies in the hydrophobic portion of the channel, and since PbTx-1 and PbTx-7 are also the most hydrophobic among the toxins, their potency and affinity may also be due to solubility considerations.

Huang and Wu (1989), studied the pharmacological actions of brevetoxin using invertebrate axonal preparation specifically the giant axons in the circumesophageal connective of the crayfish (*Procambirus clarkii*). Their study revealed that brevetoxin depolarized nerve membranes in dose-dependent manner. External and internal application of PbTx-3 at a concentration of 1.1 μM induced depolarization which attained a maximum of 30 mV within 30 seconds, and then depolarization continued for 30 minutes. Under voltage clamp condition giant axons were used to investigate sodium currents induced by PbTx-3. Analysis of the current-voltage relationship indicated that brevetoxin-modified channels can be activated in the potential range from –160 to –80 mV when normal sodium channels remain closed. The associated sodium current was completely blocked by 300 nM TTX. Huang and Wu (1989), also studied the antagonistic effect of procaine which also blocks sodium channel. Application of PbTx-3 (1 μg/ml), immediately increased the holding current by 3.3 μA indicating simultaneous opening of a large number of sodium channels induced by the toxin. Procaine at 30 mM was applied to both inside and outside of the axon and caused a time-dependent suppression of the holding current, however, even at this relatively high concentration of procaine

suppression was incomplete as compared with the action of TTX, which abolished the induced current. These result suggested that the antagonism between brevetoxin and procaine may arise from a competitive interaction between the two agents acting on the same binding site or an allosteric interaction between separate binding sites bound with brevetoxin and procaine (Huang and Wu, 1989).

The investigations on the action of brevetoxin have shown that it induces sodium channels to open at resting membrane potentials, resulting in the depolarization of the nerve membrane. At the nerve terminal, depolarization of the membrane would lead to an enhanced release of transmitters. This basic action can account for a number of pharmacological actions of brevetoxin on various organ systems as well as CNS symptoms of intoxication (Huang and Wu, 1989). Some of the symptomology of intoxications due to exposure to brevetoxins are bronchoconstriction, cough, sneezing and asthma, which may not be Na^+ channel-mediated. These adverse effects are discussed in the following section of this review.

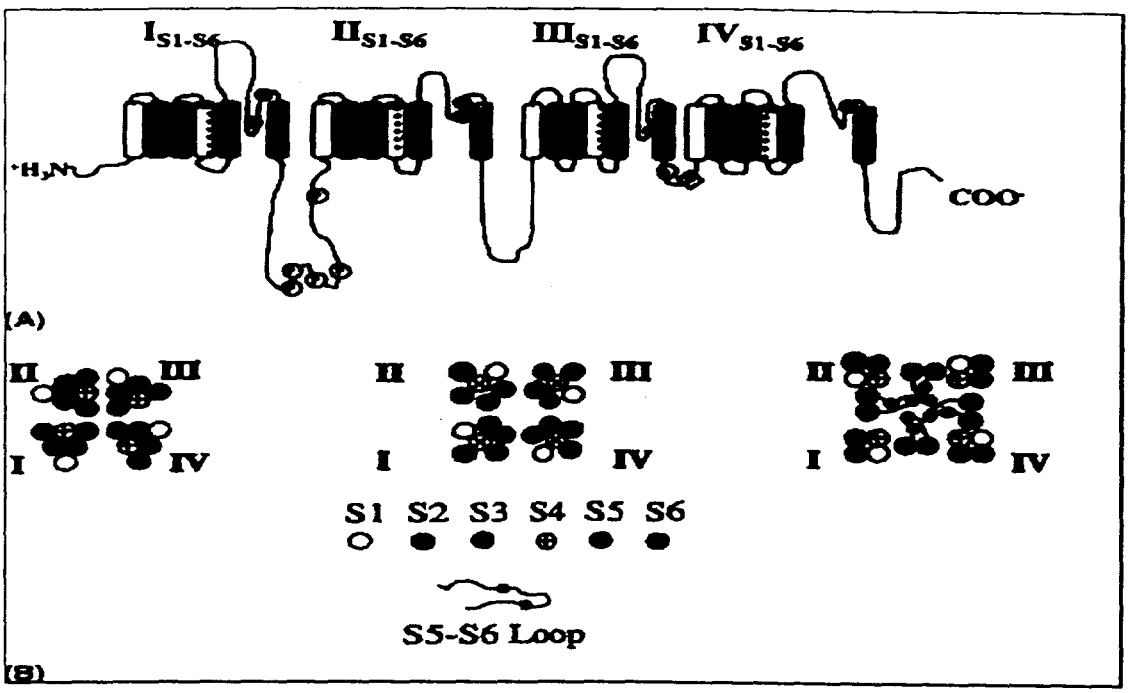


Figure 2.8. (A) Functional representation of the voltage-sensitive sodium channel α-subunit. The VSSC is composed of three separate proteins, α-subunit and two β-subunits. (B) Three models of the outside views looking inside of sodium channel α-subunit helices. Several schematics of how six-transmembrane α-helices within each of the four domains might be spatially organized to form an ion-conducting as postulated by Catterall, 1992. (Baden and Adams, 2000).

2.3 Toxicological Profile of Marine Biotoxins

2.3.1 *Saxitoxin and its Derivatives*

Paralytic shellfish poisoning (PSP) is the accepted epidemiological description of the toxic and potentially fatal syndrome in human caused by the ingestion of a complex of organic compounds known as saxitoxins (STXs) or the related guanidinium compound tetrodotoxin (TTX) and their analogues (Kao *et al.*, 1986; Hall *et al.*, 1990). The most frequent human intoxication is through the consumption of shellfish that have exposed to toxic phytoplanktons such as *Alexandrium, Gymnodinium* and *Pyridinium.* In humans, the threshold level of saxitoxin to exhibit its toxic effect is 80 μg STX equivalent per 100 g of tissue (AOAC Official Method, 2000). Saxitoxin is a neurotoxin that blocks neuronal transmission by binding to the voltage-gated sodium channel (Catterall *et al.*, 1979; Andrinolo *et al.*, 1999; Lagos and Andrinolo, 2000; Strichartz and Castle, 1990; Shimizu, 2000). The binding site is located on the outer opening of the sodium channel thereby acting only from the exterior surface of the channel. Mild to moderate levels of intoxications include a state of generalized malaise, facial paresthesia, asthenia, dystonia, ataxia, dyspnea, hypotension, tachycardia, vomiting, dysphagia, headache and gastrointestinal disturbance (Lagos and Andrinolo, 2000). Serious intoxication can be in the form of respiratory arrest, cardiovascular shock, paralysis and coma. Lethality may result from paralysis of the muscles of the diaphragm and thorax and respiratory failure which can occur within 2 – 24 hours, depending on the dose.

The toxin's mechanism of action is well known at the molecular level but there are still unresolved questions about its toxicokinetics in mammals. This prompted scientists to investigate the utility of animal models in evaluating the toxin's mode of

toxicity. Andrinolo *et al.,* (1999) in their study with anaesthesized cats found that at 2.7 μg/kg of STX administered intravenously, the mean arterial pressure falls in the first 10 minutes to about 50% of the control but was recovered to its original level in 50 minutes and then maintained constant during the four hours experimental period. However, upon increasing the dose to 10 μg/kg, a dramatic decrease in blood pressure was observed with irreversible arrythmia and finally cardiac arrest. To explore further the behaviour of STX at higher doses, it was necessary to help the animal overcome shock. They administered an intravenous bolus of 500 μg of dobutamine (an adrenergic agonist) immediately after STX which was followed by continuous intravenous infusion of 2.5 μg/min/kg dobutamine. Using this regime Andrinolo *et al.,* (1999) observed that at high doses cats showed an initial drop in arterial blood pressure similar to low doses. Furthermore, two groups of investigators (Nagasawa *et al.,* 1971; Borison *et al.*, 1977) in their studies with the cardiovascular effect of STX, found that as little as 2 – 3 μg/kg injected intravenously produced a fall in arterial pressure to about 50% from the normal level. However, when doses of STX are smaller than 1 μg/kg, STX has hypertensive effect, as a compensatory response to the vasodilatory action. This response disappears completely when doses higher than 1.5 μg/kg are administered (Kao *et al.*, 1967; Nagasawa *et al.*, 1971). Gessner *et al.*, (1997), in their study with PSP-intoxicated human patients, found that almost all patients suffered both diastolic and systolic hypertension. The mechanism of action of PSP-induced hypertension is unclear because the dominant autonomic influence on vasculature is sympathetic (i.e. resulting in an increased heart rate and rise in blood pressure) and sodium channel block of vasomotor nerves should lead to hypotension (Gessner *et al.,* 1997). Likewise, because vascular smooth muscle contains primarily

calcium channels (Godfraind, 1994), a direct effect seems unlikely. Possible explanations for the occurrence of hypertension among patients maybe a non-specific response to intoxication related stress or the effect of other unrelated and unmeasured toxins like polyether toxins that have calcium channel agonist activity (Yasumoto and Murata, 1990).

Progressive failure of respiratory function has always been recognized as the most life-threatening element of PSP intoxication (Long *et al.*, 1990; Borison *et al.*, 1980). Borison *et al.*, (1980), in their studies with cats, it was found that at 3.3 μg/kg of STX caused motor paralysis of respiration. This result is consistent with the findings of Andrinolo *et al.*, (1999) that at 2.7 μg/ of STX/kg injected intravenously is enough to produce respiratory failure in the first few minutes of injection. This was evident as the loss of activity in the diaphragmatic electromyogram (EMG) and makes artificial respiration essential. Rhythmic phrenic discharge persisted but at a slower rate than the control. Regular cardiac beat was observed at low dose of STX, hence, diaphragm blockade clearly represents a direct peripheral effect of STX. With intravenous administration of high doses of STX, in addition to a variety of peripheral cardiorespiratory effects, saxitoxin appears to have a profound influence on the central rhythmogenic network (Chang *et al.*, 1993; Borison *et al.*, 1980). A mechanism by which the rate and rhythm of central discharge can be modified by peripheral paralytic chemical action has not been established, hence, a reasonable conclusion is that the slowing of phrenic respiratory discharge frequency is due to the direct effects of STX on the central nervous system (Lagos and Andrinolo, 2000).

The effect of STX on central cardiorespiratory control was also evaluated. Investigation of such effects has been approached by introducing STX into the cerebrospinal fluid (CSF). Borison *et al.*, (1977), studied the effect of small quantitities of STX introduced directly into the CSF that surrounds the brainstem. Results showed a sequential disruption to respiration involving initial slowing of ventilatory frequency, increasing apneustic activity, loss of spontaneous function, and finally a depression of responsiveness to electrical stimulation of the respiratory center. Moreover, when STX is administered directly into CSF it produces dissociation of respiratory control components, since it causes slowing of the breathing frequency (which is under central control) independently to changes in the peripheral controlled inspiratory volume (Lagos and Andrinolo, 2000). Andrinolo *et al.*, (1999) found significant concentrations of STX in whole brain (1.81 ng/g of wet weight) and medulla oblongata (2.50 ng/g wet weight) when elevated levels of STX in blood are reached, as were the cases of cats intoxicated with high doses of toxin (10 μg of STX/kg). This result shows that STX is capable of traversing the blood brain barrier. Their findings support the published data on the central respiratory effects of STX when applied topically in the lateral cerebral ventricle and brain stem (Borison *et al.*, 1977; Jaggard and Evans, 1975), intraperitoneal administration (Chang *et al.*, 1993) and by intravenous infusion (Kao *et al.*, 1967; Borison *et al.*, 1980). These further reinforced the notion of Lagos and Andrinolo (2000) that, at high doses of toxin the STX-induced lethality involves both central and peripheral cardiorespiratory system components.

The receptor potencies of synthetic analogues of saxitoxin and stereoselectivity of decarbamoyl saxitoxin have been studied by Strichartz *et al.*, (1995). Competition

binding studies were used to assay for the stereopotency ratio of decarbamoylsaxitoxin (dcSTX) and it was observed that synthetic dcSTX had about 4% of the inhibitory potency of natural dcSTX. All of the synthetic analogues studied were shown to inhibit the binding of [^{3}H]-STX. It is noteworthy that synthetic analogues of saxitoxin had affinities for the sodium channel that decrease in orderly manner with structural changes.

Paralytic shellfish poisoning (PSP) toxins have been detected in the urine of dosed animals. The first evidence of this came from studies using dogs as the experimental model (McFarren *et al.*, 1960). In 1993, Hines *et al.*, used a tritiated (reduced) form of STX (dihydrosaxitoxin or saxitoxinol [H^3]STXOL), to study toxin metabolism and elimination in mammals. They found that when [H^3]STXOL is administered to rats intravenously, 60% of the injected radioacticity was excreted in the urine after 4 hours and post-injection radioactivity was not detectable in faeces. Stafford and Hines (1995) quantified the amount of STX in urine samples and reported that approximately 19% of the STX intravenously administered to rats was excreted during the first four hours. In 1999, Andrinolo *et al.*, using cats as experimental model observed that STX was only eliminated via urine and toxin could not be detected in any of the bile samples analyzed. The mechanism by which STX is eliminated and cleared via urine has been studied by Lagos and Andrinolo (2000) using a compound as known as inulin. Inulin is a substance that gains access to the urine only by glomerular filtration and is neither absorbed nor secreted by renal tubules, thus, inulin clearance is used as reference to compare with the clearance of any substance because its clearance is equal to the rate of glomerular filtration. Their results showed that at a dose of 2.7 μg of STX/kg, the STX renal clearance was 3.99 ml/min x kg^{-1} which correlated very well with the reported inulin

clearance in cats of 3.24 ml/min x kg^{-1} by Fettman *et al.*, (1985). This suggests that in cats with normal cardiovascular parameters and diuresis, the STX excretion mainly involves glomerular filtration (Andrinolo *et al.*, 1999).

Andrinolo *et al.*, (1989) studied the distribution of saxitoxin in cats. Using 10 μg of STX per kg, they detected the presence of STX in tissues such as brain, spleen, liver and medulla oblongata. The highest amount of STX was found in the spleen (6.32 ng/g) and liver (4.9 ng/g). Significant amounts were also detected in the brain (1.8 ng/g) and medulla oblongata (2.5 ng/g) and these studies were the first quantitative data on PSP toxins in the central nervous system. In the study by Hines *et al.*, (1993), using [^{3}H]STXOL injected into rats, results revealed that only one parent radioactive peak after HPLC analysis of urine samples. This suggested that [^{3}H]STXOL excreted was not metabolized by rats. Andrinolo *et al.*, (1999), using post column derivitization HPLC method demonstrated that no other toxin product was detectable in the body fluids and tissue samples analyzed within four hour of the experiment. Moreover, incubation of STX for 24 hours at 25°C in the presence of cat liver 100,000 x g supernatant did not show any chemical enzymatic transformation since 100% of the STX used in the incubation were recovered (Andrinolo *et al.*, 1999). However, conflicting results were observed by Gessner *et al.*, (1997). In their study, they reported a difference between toxin composition in mussels and human serum samples of intoxicated persons in Kodiak Island, Alaska in 1994. Their results suggested that human metabolism of PSP may occur. These cases need more investigation since the difference in toxin profiles could be due to the compartamentalization of the PSP toxins as a result of binding with different affinities to receptors and other components in the tissue. Also the clean-up and

extraction procedures used for samples prior to analysis could explain composition difference (Lagos and Andrinolo, 2000).

2.3.2 *Brevetoxin and its Derivatives*

Neurotoxic shellfish poisoning is a term applied to an illness resulting from the ingestion of shellfish exposed to blooms of the dinoflagellate *Karenina brevis* (synonym: *Ptychodiscus brevis*, *Gymnodinium breve*) (McFarren *et al.*, 1965; Morris *et al.*, 1991; Plakas *et al.*, 2002). The toxins produced by these dinoflagellates known as brevetoxins were studied extensively (Lin *et al.*, 1981; Baden, 1989) including its characterization in shellfish (Morohashi *et al.*, 1995). Test on the toxic effects of algal extracts and unextracted cultures on whole organisms were performed by Sievers (1969) using annelids, crustacea, molluscs and fish. The material is toxic to mice, causes human eye and respiratory irritation and has been implicated in producing symptoms of poisoning in humans via the ingestion of oysters and clams collected on the west coast of Florida (McFarren *et al.*, 1965; Cummins *et al.*, 1971). Ingestion typically results in sensory abnormalities, cranial nerve dysfunction and gastrointestinal symptoms (Franz and LeClaire, 1989). Inhalation of seaspray containing toxins may cause cough, rhinorrhea, watery eyes and sneezing in normal and wheezing asthmatic patients (Asai *et al.*, 1984).

The *in vivo* physiological effect of *G. breve* toxins are both cardiovascular and neurological in nature (Baden and Adams, 2000). Brevetoxin induces central depression of respiratory and cardiac function. In studies using anaesthetized, artificially ventilated dogs and cats, purified brevetoxin produced dose-dependent acute apnea, bradycardia and transient hypotension (Ellis *et al.*, 1979; Borrison *et al.*, 1980). It was shown that at a

dose of 0.1 mg/kg, brevetoxin induced a vagal reflex (Bezold-Jarisch reflex) on intravenous injection that could be reversed by atropine, vagotomy or ganglionic blocking drugs such as hexamethonium (Ellis *et al.*, 1979; Borison *et al.*, 1980; Borison *et al.*, 1985). These findings have been interpreted in terms of the toxin acting reflexly on vagally innervated receptors to evoke a peripheral Bezold-Jarisch effect and also acting centrally to cause marked disturbances to respiratory and cardiovascular control.

Baden *et al.*, (1982), in their study with anaesthetized guinea pigs, found that T17 (toxin fraction isolated from *P. brevis*) at i.v. doses ranging from 0.001 to 0.080 mg/kg increased resistance to pulmonary inflation. Upon direct administration of T17 to the pleural surface, a reduced pulmonary inflation was noted as indicated by bronchoconstriction. In most cases, bronchoconstriction was accompanied by rhinorrhea, lacrimation, salivation, urination and defecation. These findings on brevetoxin-induced bronchoconstriction were further supported by Richards *et al.*, (1990), in their study on airway smooth muscle cells from adult dogs. Richards *et al.*, (1990) concluded that induction of bronchoconstriction by brevetoxin was due to the depolarizing effect of endogenous acetylcholine which is released from peripheral cholinergic nerve terminal on the airway smooth muscle cell.

Franz and LeClaire (1989) studied the respiratory effects of brevetoxin and saxitoxin in awake guinea pigs. Their study revealed that both toxins caused lactic acidosis of unknown etiology which was compensated by administration of minute amounts of brevetoxin (PbTx-3). The mean time to respiratory failure was 25.0 ± 4.8 minutes for guinea pigs challenged with PbTx-3. It was also noted that just before respiratory failure, brevetoxin-intoxicated animals typically breathed with a frequency of

200 per minute and tidal volume of 2.0 – 2.5 ml; apnea occurred suddenly, lasting for 15 – 45 seconds, and this was followed by several minutes of slow variable (gasping) ventilation before death.

In 1972, Sasner *et al.,* used invertebrate neurogenic and myogenic hearts to examine the effect of a toxic fraction derived from cultured *G. breve*. They found that when the toxin (5 μg in 100 ml seawater) was applied to neurogenic hearts (from *Cancer irroratus* and *Carcinus maenas*) *in vivo,* it caused an increase in contractile frequency and development of prolonged, though irregular tension. On the other hand, myogenic hearts (from *Mercenaria mercenaria*) showed no alteration of mechanical activity even when doses 20x higher than this were injected directly into the ventricle. It has been argued that the difference in the activity of brevetoxin between these two groups of invertebrates may be due to the action of acetylcholine. Acetylcholine is the naturally occurring accelerating principle in crustacean hearts (Sasner *et al.*, 1972). Significant amounts of acetylcholine was reported in crustacean hearts (Smith and Glick, 1939). Molluscan heart is little affected by substances having anticholinesterase activity (Welsh and Taub, 1948) and anticholinesterase in this tissue is low (Smith and Glick, 1939). Moreover, Kao (1966) suggested possible explanations for the apparent immunity of marine bivalve molluscs to both saxitoxin and *G. breve* toxins; including (a) molluscan biochemical systems have the ability to bind or "inactivate" the different dinoflagellate poisons, (b) molluscan excitable tissues offer diffusion barriers to dinoflagellate toxins and (c) molluscan excitable membranes may be dependent on ionic mechanisms which differ from those which control vertebrate and some crustacean membrane systems, i.e. calcium dependent mechanisms.

Several groups of investigators have studied the metabolism of brevetoxins in shellfish (Morohashi *et al.*, 1995; Ishida *et al.*, 1995; Poli *et al.*, 2000; Dickey *et al.*, 1999). Ishida *et al.* (1995) isolated a C-42 *N*-taurine conjugate of PbTx-2 (originally designated as BTX-B1) in the cockle *Austrovenus stutchburyi*. On the other hand, an oxidized (sulfoxide) *S*-cysteine conjugate (BTX-B2) in the greenshell mussel *Perna canaliculus* and biosynthetic routes from either PbTx-2 or PbTx-3 were proposed (Murata *et al.*, 1998). These metabolites were consistent with the findings of Plakas *et al.*, (2002) using LC/MS analysis of water exposed to PbTx-2, where parent PbTx-2 was not recovered, instead only metabolites of *m/z* 1018 and 1034 were found. The structures were verified by accurate mass measurements along with MS/MS fragmentation. In another study by the same investigators rapid accumulation of the toxin was observed in oysters exposed to pure PbTx-3 but there was no evidence of metabolism since none of the metabolites identified previously in field exposed oysters were detected and parent PbTx-3 (*m/z* 897) was found using the LC/MS technique (Plakas *et al.*, 2002).

Elimination of PbTx from oyster was also examined by Plakas *et al.*, (2002) and confirmed the presence of metabolites in oysters after waterborne exposure to pure toxins (20 μg/L for 72 hours). In oysters dosed with PbTx-3, using LC/MS only parent PbTx-3 was detected. Unchanged PbTx-3 was largely eliminated within the first two weeks after dosing, and declined to trace levels by eight weeks. After dosing with PbTx-2, the metabolite profile by LC/MS was qualitatively the same as described in exposure with higher dosage. Levels of the metabolite PbTx-3 were highest immediately after dosing and declined in parallel fashion to those of parent compound in PbTx-3 dosed oysters. The cysteine conjugate increased in concentration between two and four weeks, and was

subsequently eliminated. The sulfoxide metabolite did not change appreciably in concentration over the time course of the study.

2.4 Na^+ Channel and Excitable Membranes

Excitation and electrical signalling in the nervous system involves movement of ions through the ionic channels. These ionic channels are integral membrane proteins that allow the passage of a number of physiologically significant cations (Na^+, K^+, Ca^{2+}) and anions (Cl^-) in and out of the cells. Each channel may be regarded as an excitable molecule, as it is can be specifically responsive to cellular stimuli and/or mechanical deformation (Hille, 1992). Since cell membranes are highly impermeable to charged species, ion channels function as catalyst of ion transfer through cell membranes (Hille, 1992). This translocatory function is called gating, which actually means simple opening or closing of the pore. Between these two, the open pore has the important property of selective permeability allowing some restricted class of small ions to flow passively down their electrochemical activity gradients at a rate that is very high (> 10^6 ions per second) when considered from a molecular viewpoint (Hille, 1992). This high throughput rate is considered a diagnostic feature which distinguishes ionic channel mechanisms from those of other ion transport devices such as the Na^+-K^+ pump.

Among the four ionic channels, the sodium channel is the most popular target of many neurotoxins. Phylogenetic studies indicated that sodium channels appeared first phylogenetically in the jellyfish (Suarez-Isla and Velez, 2000). Among invertebrates, sodium channels are expressed mainly in neuronal cells and in chordates, they can also be found in muscle cells. In fact they are widely distributed at relatively high concentrations

in axons and muscles. One good example is the mammalian heart muscle which contain approximately 10^5 sodium channels in a mean cell surface area of 10 μm^2.

In mammalian brain, the Na^+ channel consists of heterotrimeric complex of α (260 kd), β_1 (36 kd) and β_2 (33 kd) subunits (Catterall, 1988; Marban *et al.*, 1998; Suarez-Isla and Velez, 2000). All these subunits are glycosylated on their extracellular surfaces (Catterall, 1988; Catterall, 1992). The β_1 subunit is linked noncovalently to the α-subunit whereas β_2 subunit is linked through disulfide bonds to the α-subunit (Messner and Catterall, 1985; Catterall, 1992). Though the Na^+ channel consist of various subunits but only the α-subunit is required for function (Marban *et al.*, 1998; Suarez-Isla and Velez, 2000; Catterall, 1992). The sodium channel exists in three functionally distinct states: resting, active and inactivated. Activation controls the rate and voltage-dependence of the sodium permeability increase following depolarization. Inactivation, on the other hand, controls the rate and voltage-dependence of the subsequent return of sodium permeability to the resting level during a maintained depolarization. Inactivating and resting states are nonconducting, however, channels that have been inactivated by prolonged depolarization are refractory unless the cell is repolarized to allow them to return to the resting state (Catterall, 1992). These fundamental properties of sodium channels were established through the pioneering work of Hodgkin and Huxley (1952) in their studies using voltage-clamp techniques.

Seven groups of neurotoxins acting at different sites on the sodium channels and their physiological effects are shown in Table 2.1. Schematic diagram on neurotoxicant/modulator/therapeutic binding sites on the voltage-sensitive sodium channel is shown in Figure 2.9. Neurotoxin receptor site 1 is located near the extracellular

opening of the ion conducting pore of the sodium channel (Narahashi, 1974; Catterall, 1980; Catterall, 1986; Aidley, 1998; Kao, 1986). It binds the water soluble heterocyclic guanidines of tetrodotoxin (TTX) and saxitoxin (Catterall, 1992). The binding of saxitoxin and tetrodotoxin results in inhibition of sodium conductance. Blockade of neuronal transmission brought about by binding of these toxins to their receptor site on the voltage sensitive sodium channel is actually slowly reversible (Lagos and Andrinolo, 2000). A polypeptide toxin from the marine snail *Conus geographicus* was also shown to inhibit sodium channels by binding at the same receptor site (Yanagawa *et al.*, 1986; Moczydlowski *et al.*, 1986; Ohizumi *et al.*, 1986). Multiple arginine residues in this polypeptide toxin may serve to bind to the same subsites as the guanidinium moieties of tetrodotoxin and saxitoxin (Catterall, 1992).

Receptor site 2 is located within the transmembrane pore of the sodium channel and is involved in voltage-dependent activation and inactivation. It binds several lipid-soluble toxins including grayanotoxin, batrachotoxin and the alkaloids veratridine and aconitine (Catterall, 1980; Catterall, 1984). Competitive interactions of these four toxins at neurotoxin receptor site 2 have been demonstrated by influx studies and by direct measurements of specific binding of [^{3}H]-batrachotoxin A 20 α-benzoate to sodium channels (Catterall, 1980). These toxins cause persistent activation of sodium channels at the resting membrane potential by blocking sodium channel inactivation and shifting the voltage-dependence of channel activation to more negative membrane potentials (Catterall, 1980; Catterall, 1984; Catterall, 1992).

Neurotoxin receptor site 3 binds polypeptide toxins purified from North African scorpion venoms or sea anemone nematocysts. These toxins slow or block sodium

channel inactivation (Catterall, 1980) and enhance persistent inactivation of sodium channels by the lipid-soluble toxins acting at neurotoxin receptor site 2 (Catterall, 1980; Catterall, 1984; Catterall, 1992). Studies by Catterall (1984; 1992) showed that the affinity for binding of ^{125}I-labelled derivatives of the polypeptide toxin to the site 3 is reduced by depolarization and that the voltage-dependence of scorpion toxin binding is correlated with the voltage-dependence of sodium channel inactivation. These findings indicate that scorpion toxin and sea anemone toxin to bind to voltage-sensing or gating structures involved in the activation of sodium channels and to slow or block the normal coupling of the channel inactivation (Catterall, 1992).

Neurotoxin receptor site 4 binds a second class of scorpion toxins that are homologous to those that bind at receptor site 3 (Couraud *et al.*, 1982). These are prominent in the venoms of New World scorpions like *Centuroides sculpuratus* and *Titurus serrulatus*. These toxins achieve their effects by shifting the voltage-dependence of sodium channel activation to more negative potentials without modifying sodium channel inactivation (Cahalan, 1975; Couraud *et al.*, 1982). Their binding to sodium channel is unaffected by toxins that bind at neurotoxin site 3 and this indicates that this second class of scorpion toxins binds to a fourth receptor site on the sodium channel (Catterall, 1992; Catterall, 1984). They have been designated β-scorpion toxins to distinguish them from the α-scorpion toxins that bind at site 3 and slow sodium channel inactivation (Catterall, 1992; Catterall, 1984).

Two classes of novel lipid soluble polyether toxins with molecular masses in the range of 1,000 Da known as brevetoxins and ciguatoxins are known to bind at neurotoxin receptor site 5 (Catterall and Gainer, 1985; Catterall and Risk, 1981; Lombet *et al.*, 1987;

Poli *et al.*, 1986; Sharkey *et al.*, 1987). These toxins cause repetitive firing of nerves, shift the voltage dependence of sodium channel activation to more negative potentials and block inactivation. These actions resemble those of neurotoxins which act at receptor site 2, however, in radioactive toxin binding experiments brevetoxins do not displace toxins which bind specifically to receptor sites 1 and 3 (Catterall and Gainer, 1985). Furthermore, brevetoxin was shown to allosterically enhance the binding and action of neurotoxins that act at neurotoxin receptor sites 2 and 4 (Catterall and Risk, 1981; Sharkey *et al.*, 1987). These data confirmed that they exert their effect on sodium channel activation and inactivation at a fifth receptor site on the sodium channel (Catterall, 1992). This region of the Na^+ channel is involved in voltage-dependent gating (Baden and Adams, 2000). The site 5 locus has been further characterized by direct binding studies using a radiolabelled derivative of brevetoxin (Poli *et al.*, 1986), and the ciguatoxins have been shown to inhibit brevetoxin binding by interacting competitively at this site (Lombet *et al.*, 1987).

Neurotoxin receptor site 6 has been recently identified by direct binding studies of a new family of peptide conotoxins, designated δ-conotoxins, represented by δ-TXVIA derived from the marine snail-hunting species *Conus textile* (Fainzilber *et al.*, 1994). The δ-conotoxins have been shown to inhibit sodium channel inactivation by binding to a new extracellular receptor site, designated receptor 6 (Gordon, 1997). Receptor site 7 may be equivalent to receptor site 6 (Baden and Adams, 2000) which binds pyrethroids as group of neurotoxic insecticides.

Sodium channels are relatively sparsely distributed in excitable membranes (Catterall, 1984) and are responsible for the increase in sodium permeability during the

initial rapidly rising phase of the action potential in nerve, neuroendocrine, skeletal muscle and heart cells (Catterall, 1992). Like any other cells, these electrically excitable cells maintain a high intracellular K^+ concentration and a low intracellular Na^+ concentration relative to the extracellular fluid through the energy-dependent pumping of these cations by Na^+-K^+-ATPase. All excitable cells have a resting membrane potential, the entire cytoplasm is electrically more negative than the external bathing fluid by 30 to 100 mV (Hille, 1981) since their surface membranes are specifically permeable to K^+ (Catterall, 1992). However, electrically excitable cells are distinguished by having voltage-sensitive ion channels in their surface membranes that respond to membrane potential changes with large regenerative increases in permeabilities to specific ions on a fine scale of milliseconds. Hence, a change in membrane potential of excitable cells relies on changes in permeability and transport of different ions across excitable membranes. In the case of the Na^+ channel, an increase in sodium permeability leads to depolarization (Catterall, 1992) making the inside of the cell more positively charged (Hille, 1981). On the other hand, an increase in potassium permeability leads into repolarization or hyperpolarization making the inside of the cell more negative thus restoring the resting potential.

Table 2.1. Neurotoxin receptor sites associated with the Na^+ channel (Adapted from Catterall, 1988 ; Catterall, 1992; Gordon, 1997; Baden and Adams, 2000).

Site	Toxin	Effect
1	Tetrodotoxin Saxitoxin μ-Conotoxins	Inhibition of ion conductance
2	Veratridine Batrachotoxin Aconitine Grayanotoxin	Persistent activation
3	α-Scorpion toxins Sea anemone toxins	Inhibition inactivation Enhance persistent activation
4	β-Scorpion toxins	Shift in voltage dependence of activation
5	Brevetoxins Ciguatoxins	Repetitive firing Shift in voltage dependence of activation
6*	δ-Conotoxins	Delay inactivation
7*	Pyrethroids	Delay inactivation Shift in voltage dependence of activation

*Actual number of site is equivocal (Baden and Adams, 2000)

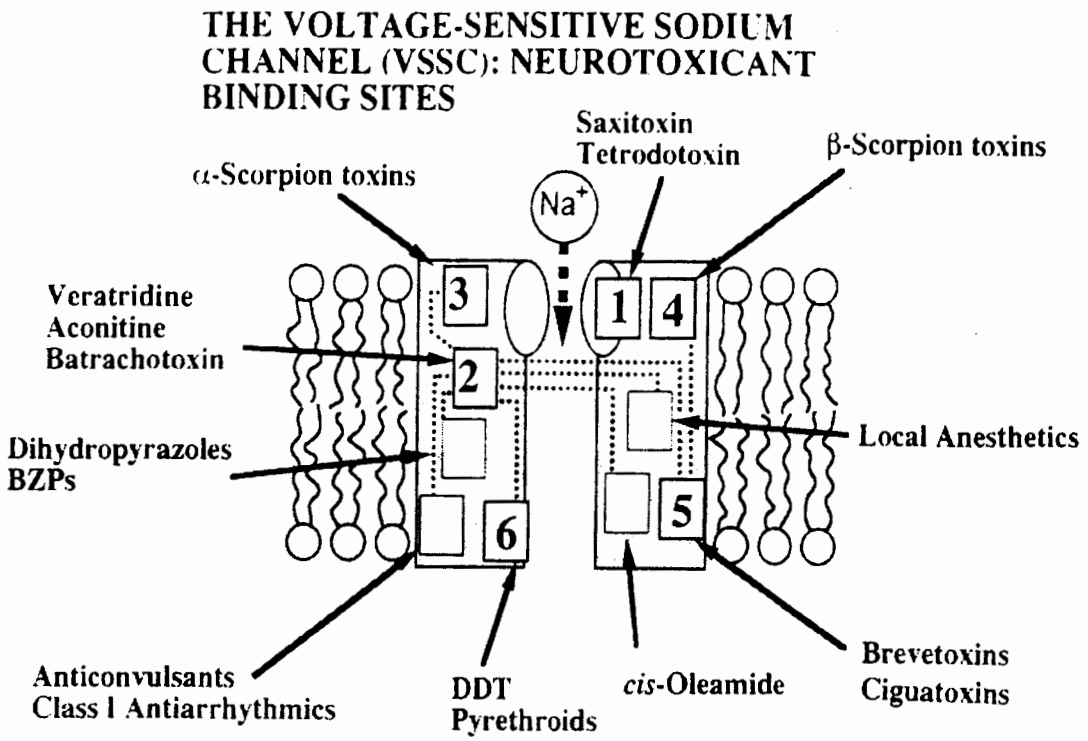


Figure 2.9. Schematic diagram on neurotoxicant/modulator/therapeutic binding sites on the voltage-sensitive sodium channel

2.5 Synaptoneurosomes and their Role in Neurobiochemical Assays

The utility of mammalian membrane preparations particularly the synaptoneurosomes has been described by several investigators (Schwartz *et al.*, 1985; Harris and Allan, 1985; Nicholson *et al.*, 2002). Synaptoneurosomes consist of pre-synaptic sac (synaptosome) attached to a resealed post-synaptic sac (neurosomes) which enclose their original contents of cytoplasm (Hollingsworth *et al.*, 1985). These two vesicular components were estimated to have a diameter of approximately 0.6 μm and 1.0 μm respectively.

Schwartz *et al.*, (1985) studied membrane chloride (Cl^-) permeability using synaptoneurosomes and found the preparation well suited to the measurement of pharmacologically relevant Cl^- transport associated with the benzodiazepine/GABA/Cl^- ionophore receptor complex. Several other group of investigators (Harris and Allan, 1985; Allan and Harris, 1986; Paul and Schwartz , 1986; Verdon *et al.*, 2000) described the usefulness of synaptoneurosomes in pharmacological studies of GABA (γ-aminobutyric acid) receptor mediated chloride transport. Recently, Nicholson *et al.*, (2002), utilized synaptoneurosomes in the development of novel neurobiochemical assay for PSP toxins. The assay correlated well with data from standard mouse toxicity bioassay (r^2 was between 0.84 and 0.86). These studies indicate synaptoneurosomes have utility in toxicological and pharmacological studies. The preparation of synaptoneurosomes is very straightforward and takes approximately 40 minutes to complete and when stored in ice, synaptoneurosomes remain viable in membrane potential response for at least 6 hours after preparation (Nicholson *et al.*, 2002).

The ease of isolation and versatility of synaptoneurosomes together with the fact that many investigations have demonstrated functional Na^+ channel to be present in this preparation suggest that this preparation will be useful in the development of novel neurobiochemical assays for marine algal toxins that cause economic impact to the shellfish industry and are hazardous to human health.

CHAPTER THREE

FURTHER VALIDATION OF A MOUSE BRAIN SYNAPTONEUROSOMAL ASSAY FOR PARALYTIC SHELLFISH POISONING (PSP) TOXINS

3.1 INTRODUCTION

The saxitoxins and other structurally related derivatives are known to cause various illnesses via ingestion of contaminated shellfish products. Manifestations of saxitoxin poisoning include oral paresthesia followed by cardiovascular dysfunction and respiratory problems. Some of the known symptomology associated with PSP in humans can be explained by the binding of saxitoxin and other derivatives to the neurotoxin receptor site 1 of the volatage-gated sodium channels. This binding leads to blockade of ion conduction that creates the impulse in excitable tissues such as peripheral nerve, muscles and the brain.

Detection of saxitoxin has been a challenge to the regulatory bodies. Currently, the standard assay in quantifying saxitoxin and related bioactives is through the mouse bioassay test. But this technique has received numerous criticisms because of its high cost and the use of several (3) animals to assay a single shellfish extract. Several *in vitro* systems have been explored as potential replacements of mouse bioassay. In this context, cytotoxicity studies, electrophysiological studies and radioligand binding approaches (Manger *et al.*, 1995; Weigele and Barchi, 1978; Doucette *et al.*, 1997; Kerr *et al.*, 1999; Beani *et al.*, 2000) have been pursued. Recently, a rapid and sensitive assay for PSP

toxins was developed by Nicholson *et al.*, (2002) using mouse brain synaptoneurosomes. In their study, the validity was tested by correlating synaptoneurosome-derived values of saxitoxin in mussel extracts with mouse toxicity bioassay data. The present study will therefore focus on approaches to further test its usefulness in quantifying PSP toxins. The first part of this study was designated to examine assay validation further using shellfish samples spiked with saxitoxins and extracts of plankton naturally contaminated with PSP toxins. The second objective was to determine absolute changes in membrane potential of synaptoneurosomes as this relates to saxitoxin concentration.

3.2 MATERIALS AND METHODS

3.2.1 Animals

Male CD1 mice weighing between 25 to 35 grams, were obtained from the Animal Care Center, University of British Columbia, Vancouver, B.C. Mice were housed in cages and maintained under a 12-hour light/dark cycle at the Simon Fraser University Animal Care Facility. Mice were fed with food (#5001 Rodent Lab Diet, PMI Nutrition International, St. Louis, MO., USA) and water *ad libitum*. Animal husbandry and all experimental procedures involving animals were carried out according to guidelines from the Canadian Council on Animal Care.

3.2.2 Chemicals

Veratridine and saxitoxin were purchased from Sigma-Aldrich Canada Limited (Oakville, Ontario). Rhodamine 6G was obtained from the Eastman Kodak Company (Rochester, New York). All other chemicals were of the highest purity available.

3.2.3 Preparation of Spiked Samples from Mussel Extracts

Canadian Food Inspection Agency, Burnaby, B.C. provided mussels extracts that had been demonstrated to be free of PSP toxins using the mouse bioassay. A total of 16 extracts were assayed using the mouse brain synaptoneurosomes assay. These extracts were spiked with saxitoxins (by Dr. R.A. Nicholson) and were assayed blind. Since mussel tissues were extracted in 0.1 N HCl, samples requiring volumes greater than 10 μL per assay (> 10 μL) were diluted with deionized water prior to assay.

3.2.4 Plankton Samples

Plankton samples were supplied by Dr. Gregory J. Doucette of NOAA, Charleston, South Carolina, U.S.A. Saxitoxin concentration for these samples were previously assayed using radioligand binding assay (RBA) by NOAA. These plankton extracts were used to assess the ability of the synaptoneurosome system to assay an alternative environmental sample containing low levels of PSP toxins.

3.2.5 Preparation of Synaptoneurosomes

The isolation of synaptoneurosome fractions from CD1 mouse brain was carried out using published protocols (Harris and Allan, 1985; Hollingsworth *et al.*, 1985; Verdon *et al.*, 2000; Nicholson *et al.*, 2002) with modifications. Mice were quickly

euthanized by cervical dislocation and decapitated. Whole brain (503.65 ± 23.19 mg) then was excised using forceps and then transferred into ice cold saline (132 mM NaCl, 5 mM KCl, 1.2 mM $Na_2HPO_4 \cdot 7H_2O$, 1.2 mM $MgCl_2 \cdot 6H_2O$, 10 mM glucose, 20 mM Tris adjusted to pH 7.4 with 1 M HCl). The brain was chopped into small pieces using razor blade in transverse and sagittal sections. Tissue fragments were then homogenized (6 to 8 strokes by hand) with 2.5 ml saline using a teflon/glass homogenizer with occasional pestle rotation. Saline (2.5 ml) was added to the homogenate and filtered though two layers of nylon mesh (about 100 μm). The filtered homogenate was further diluted with 12.5 ml saline and then centrifuged (Beckman J2HS; Beckman Instruments, Fullerton, CA) for 15 minutes at 1000 g. The supernatant was then discarded and pellet was resuspended in 10 ml ice cold saline by gentle vortexing. The resuspended material was then centrifuged again for 15 minutes at 1000 g. The supernatant was then discarded and the final synaptoneurosomal pellet was suspended with 900 μL of ice-cold saline. Aliquots (3 x 5 μL) were taken and stored at -80°C freezer for subsequent protein analysis, and synaptoneurosomes (57 μL) were apportioned into snap top plastic vials and held on ice until assay. All isolation procedures were done in the cold room (4°C).

3.2.6 Synaptoneurosomal Protein Determination

Assay of the protein in mouse brain synaptoneurosome samples was done using the modified Lowry *et al.* assay as simplified by Peterson (1977). In a snap top tube, 5 μL of synaptoneurosomes was added with Reagent A (25 ml 0.8 N NaOH, 25 ml 10 % SDS, 25 ml alkaline copper solution, 25 ml distilled water). Alkaline copper solution was made by mixing equal parts of 14 mM sodium potassium tartrate, 8 mM $CuSO_4$ and 1.89 M

Na_2CO_3. The mixture was vortexed and allowed to stand for 10 minutes. After 10 minutes, 0.5 ml Reagent B (10 ml Folin-Ciocalteau's reagent and 40 ml distilled water) was added with the first mixture. The resulting mixture was rapidly mixed and allowed to stand for one hour at room temperature. Absorbance was measured at 750 nm using Milton Roy Spectronic 3000 Array UV/Vis (Milton Roy, Acton, MA). Bovine serum albumin (BSA) was used as standard.

3.2.7 Fluorescence Quantitation of Saxitoxin Concentration

The rhodamine 6G technique was carried out using the protocol described by Auichi *et al.*, (1982) for synaptosomes and adapted by Verdon *et al.*, (2000) and Nicholson *et al.*, (2002) for synaptoneurosomes with modifications. Rhodamine 6G was used because it provides a highly quantitative measure of the extent of depolarization (Nicholson *et al.*, 2002) necessary to quantitate the amount of saxitoxin in the assay. All fluorescence measurements were carried out using a Perkin-Elmer LS-50 fluorescence spectrophotometer equipped with a temperature controlled (temperature was set at 35 °C) cuvette holder and linked to an IBM 50Z computer. Excitation and emission wavelengths were set at 520 nm and 550 nm respectively and slit widths were adjusted to 5 nm.

Standard curve for the assay was constructed first and was used in subsequent analysis of spiked samples and plankton samples. To a stirred quartz fluorescence cuvette containing 3 ml saline (132 mM NaCl, 5 mM KCl, 1 mM $CaCl_2 \cdot 2H_2O$, 1.2 mM $Na_2HPO_4 \cdot 7H_2O$, 1.2 mM $MgCl_2 \cdot 6H_2O$, 10 mM glucose, 20 mM Tris adjusted to pH 7.4 with 1 M HCl), the sodium channel activator veratridine (100 μM final concentration) was added with known amount of saxitoxin standard. Vehicle control (3 μL DMSO,

dimethyl sulfoxide) and blank control were used as references. Approximately 20 seconds after initiation of recording 1 μL of rhodamine 6G (0.05 μM final concentration) was added, followed by 50 μL (760 μg protein) of synaptoneurosomes (t = 100 seconds). Fluorescence intensity was quantified at 300 seconds. Fluorescence intensity values obtained with saxitoxin and veratridine combined were expressed as percentage of the fluorescence intensity with veratridine alone. To generate the saxitoxin standard curve, curve fitting and regression analyses were carried out using Prism 2.0 (Graphpad Software Inc.; San Diego, California).

Sixteen mussel extracts spiked with known amounts of saxitoxins were analyzed blind using the protocol described above. Blind analysis of spiked samples is necessary to ensure the integrity of the assay and is a very important component of food safety analysis. Spiked samples were initially tested using 1 μL and 3μL. From the initial test, if samples showed a complete block or strong inhibition dilution of the extracts were made and if samples showed weak or no inhibition of larger volume of the sample was used for further analysis. Where the volume of the extract exceeded 10 μL, appropriate adjustment on the volume of saline was done to ensure the consistency on the total volume and ionic strength of the assay medium. Percentage inhibition values obtained from this analysis were then related to the saxitoxin standard curve to estimate saxitoxin equivalents in appropriate concentration units (μg STX per 100 g of original shellfish tissue sample, nM, and ng/μL). In all cases average value of estimated replicates were employed for data analysis.

Nine plankton samples previously quantitated by radioligand binding assay were also analyzed using this assay and percentage inhibition values were also related to the

saxitoxin standard curve to estimate saxitoxin concentration in nM. Appropriate adjustment of the volume of saline was also used. Likewise, average values of estimated replicates were employed for data analysis.

3.2.8 Membrane Potential Assay

Estimation of the changes in membrane potential of synaptoneurosomes was carried out using the procedure described in 3.2.7 with modifications. Potassium ion equilibrium potential was employed to estimate the change in potential of the cell with different concentrations of potassium ions. Membrane potential was estimated directly from the relationship between fluorescence intensity and potassium ion equilibrium potential, and calculated using the reduced Goldman equation (Akerman *et al.*, 1987).

3.3 RESULTS

Fluorescence intensity readings for the standard saxitoxin are shown in Figure 3.2. The fluorescence intensity reading typically settles just above 150 units with saline containing 100 μM veratridine (VTD) whereas with the controls (both vehicle and blank) typically settle just below 100 units. As shown in Figure 3.2, standard saxitoxin produces a dose-related decrease (inhibition) of veratridine's response. At a concentration of 5 ng per assay, veratridine's response was reduced by approximately 50%. The fluorescence readings from mussel extracts spiked with saxitoxin (STX) is shown in Figure 3.3. Sample J completely blocked the veratridine response at endpoint. This sample was calculated to contain 20.94 ng STX per 1 μL assay that actually corresponds to the spiked amount of 20 ng/μL. The standard curve for saxitoxin is illustrated in Figure 3.3. The

curve is sigmoidal and the IC_{50} was estimated at 6 ng. Threshold levels of STX were evident at approximately 1 ng. Replicate values for single mouse preparation were averaged before incorporation into final calculation of averages to prevent pseudoreplication within the analysis.

Analyses of 18 samples (17 mussel extracts and 1 0.1 N HCl) for PSP toxin content using the mouse brain synaptoneurosome assay was again carried out blind and their concentrations, expressed in different units (ng/μL, nM and μg STX per 100 g tissue), are summarized in Table 3.1. Mussel extracts spiked STX ranging from 1.0 to 40.0 ng reveal a very close correspondence with mouse brain synaptoneurosome assay data. However, values below 1.0 ng STX showed a reduction to approximately half the actual spiked amount. Correlation coefficient (r^2) for the mussel extracts spiked with STX and the mouse brain synaptoneurosome assay is calculated at 0.9984 and a slope of 1.047 (Figure 3.5). Results showed that when the values are plotted, there is a slight shift in the lower portion of the graph from the line of best fit. However, when a separate regression analysis performed on this group of lower values these values (lower amount of STX) it resulted in a very good correlation (r^2 = 0.9946, Figure 3.5 inset). These lower values are in fact approaching the lower limit of the standard saxitoxin curve where the detection limit is approximately 1 ng/assay.

Nine plankton samples previously assayed with radioligand binding assay (RBA) were also tested using mouse brain synaptoneurosomes. The result of this preliminary sample set are summarized in Table 3.2. The result also showed a reasonable level of interassay agreement, although in some samples the mouse brain synaptoneurosome assay is estimating about half of the actual PSP toxin content as determined by RBA.

The effect of saxitoxin on (absolute) VTD-induced changes to membrane potential was also determined in this investigation. The dose-related changes are summarized in Figure 3.6. Results showed that as increasing STX concentration increases veratridine's ability to depolarize is decreased making the cytoplasm more negative.

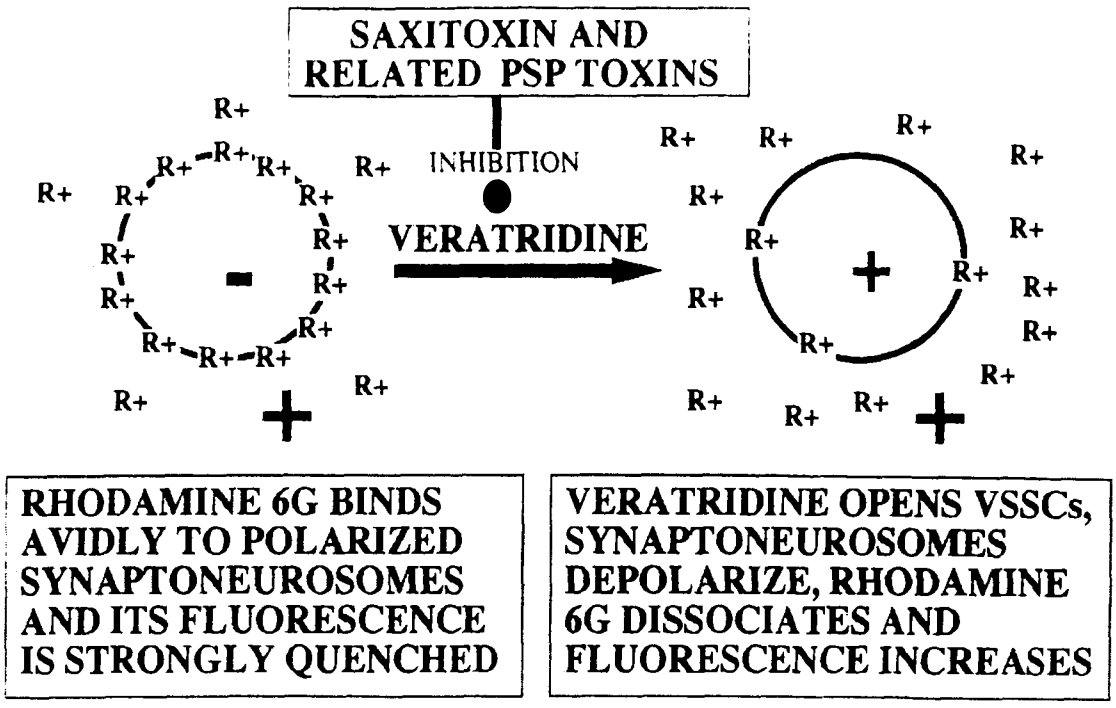


Figure 3.1. Schematic diagram on the binding of rhodamine 6G to the synaptoneurosomal membrane.

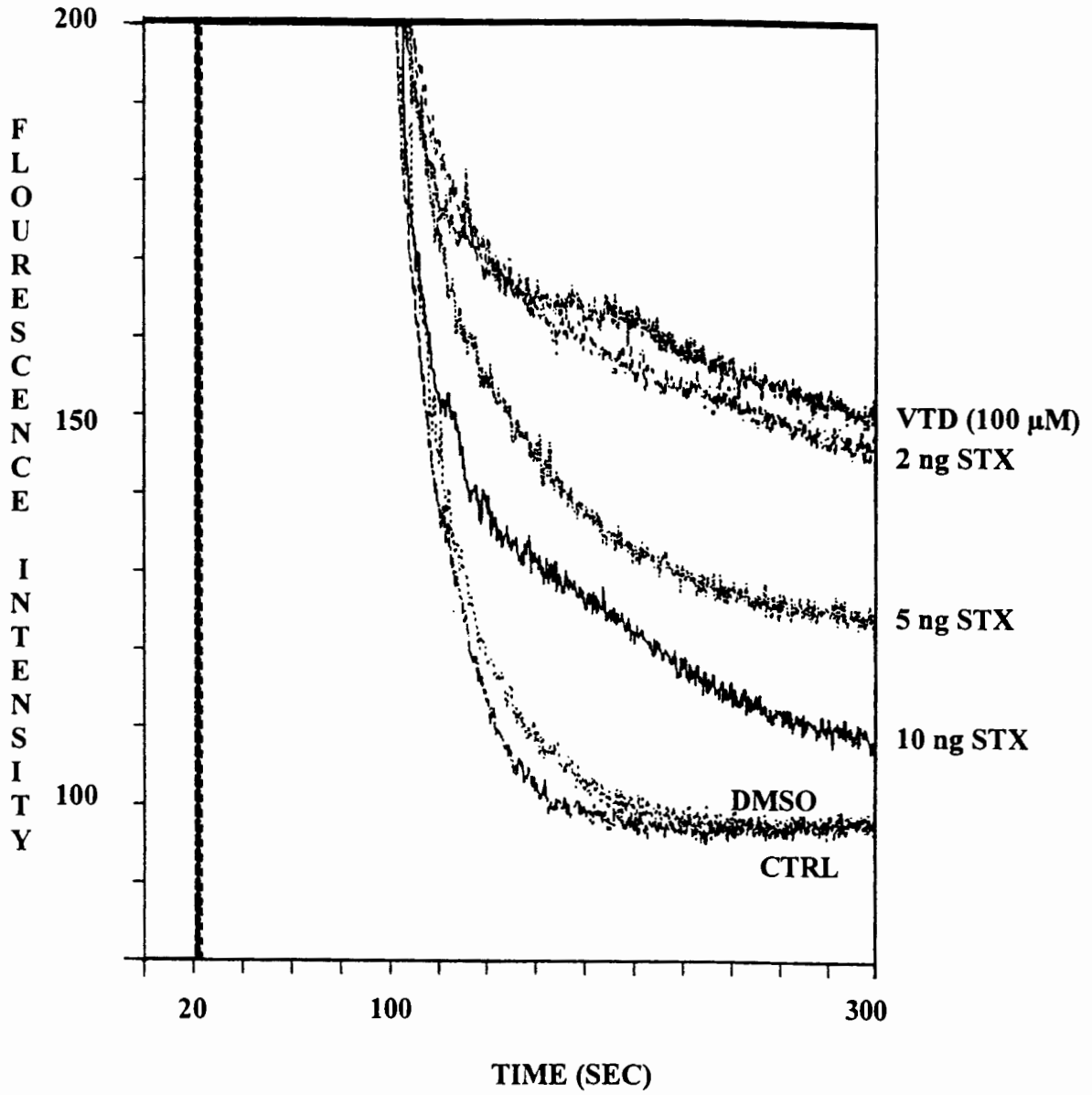


Figure 3.2. Typical rhodamine 6G fluorescence recordings illustrating the inhibition of veratridine (VTD)-induced fluorescence increase of mouse brain synaptoneurosomes by different amounts of standard saxitoxin (STX). T = 20 seconds, rhodamine 6G was added.

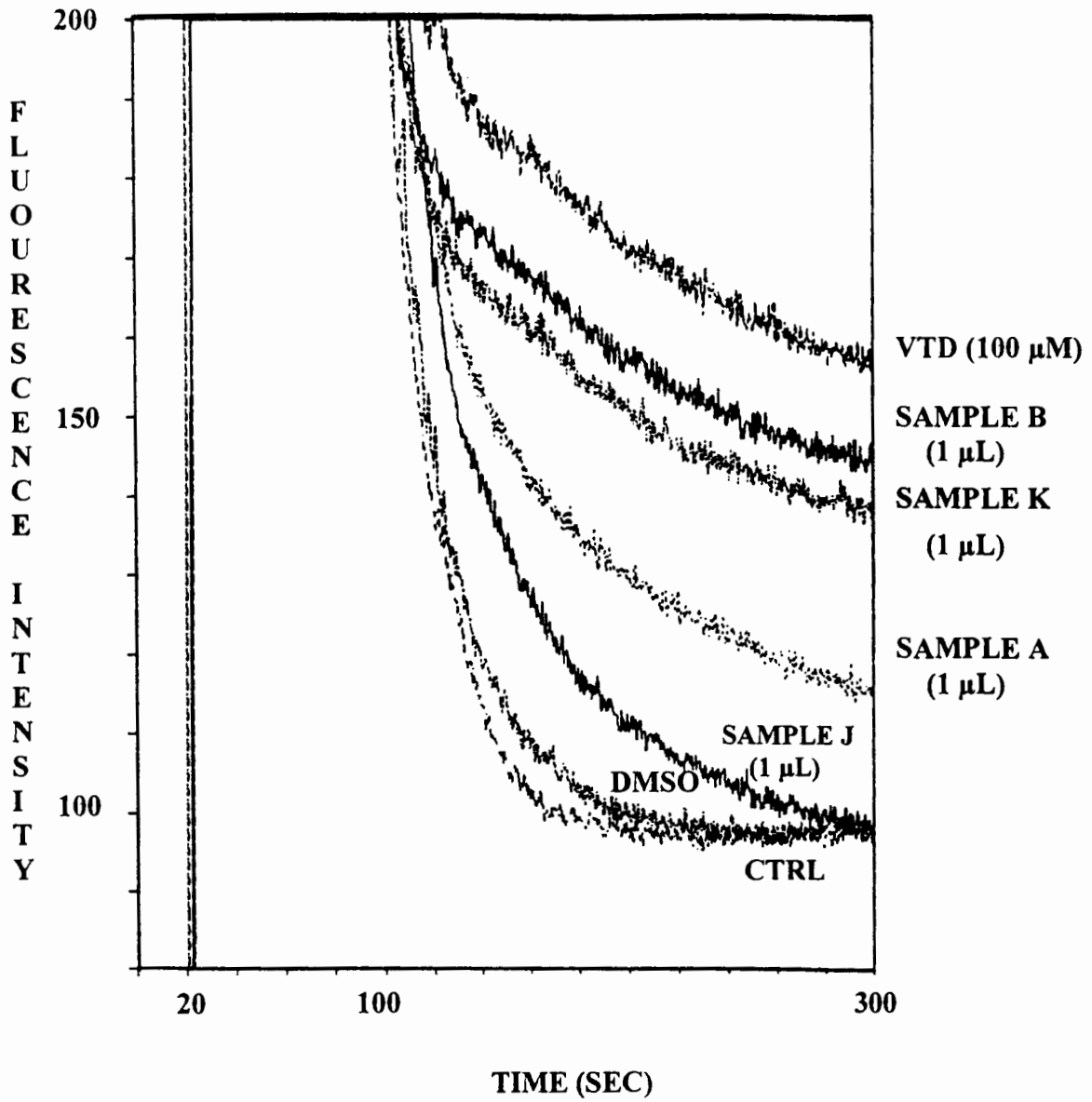


Figure 3.3. Rhodamine 6G fluorescence recordings illustrating the inhibition of veratridine (VTD)-mediated fluorescence increase of mouse brain synaptoneurosomes by four mussel extracts spiked with STX. Note that sample J (spiked with 20 ng STX) produced no changes to the fluorescence signal at endpoint compared with the control (non-depolarized) synaptoneurosomes indicating a maximum effect. Samples B (0.5 ng), K (2.5 ng) and A (7.5 ng). T= 20 seconds, rhodamine 6G was added.

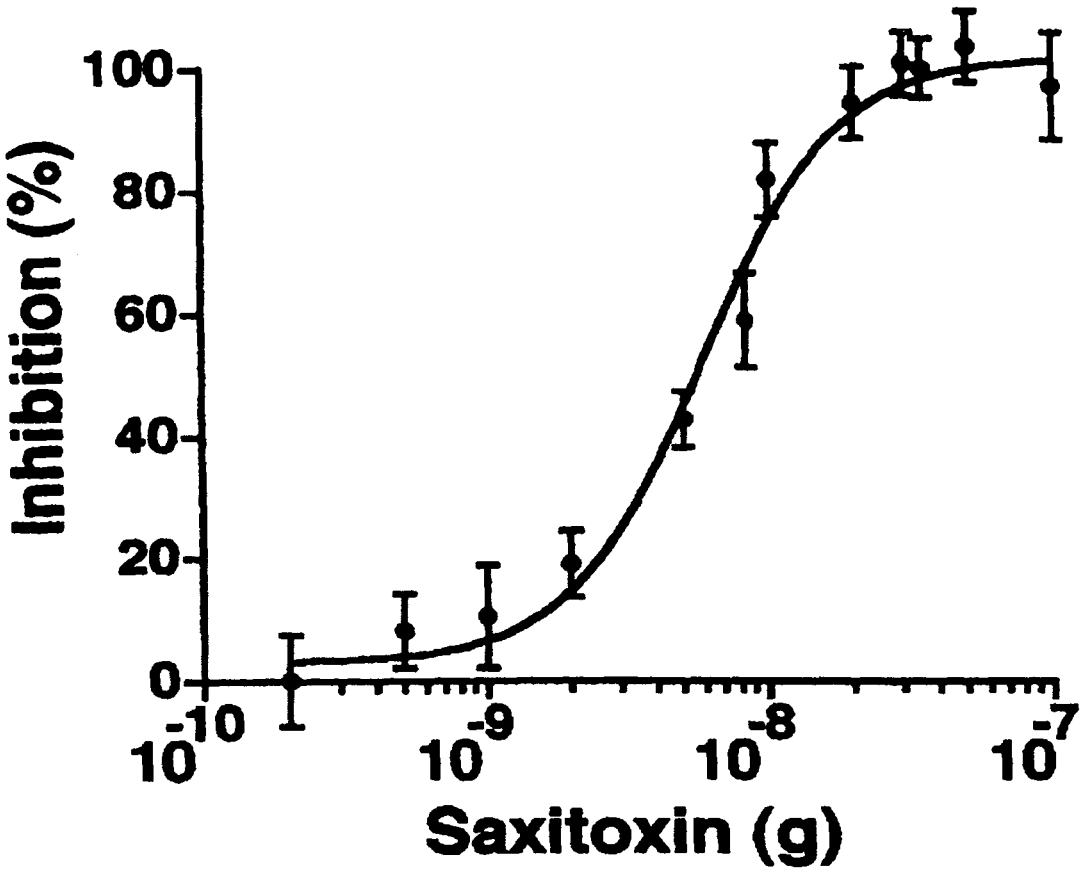


Figure 3.4. Calibration curve showing the relationship between amount of standard saxitoxin and inhibition of veratridine-induced fluorescence increase in mouse brain synaptoneurosomes. Values represent mean ± se of at least four independent experiments.

Table 3.1. Estimates of STX levels in STX-spiked mussel extracts using the mouse brain synaptoneurosome assay (MBSA) as compared to actual STX values.

Sample ID	SPIKED STX (ng/µL)	MBSA (ng/µL)	SPIKED STX (nM)	MBSA (nM)	SPIKED STX (µg STX/100 g)	MBSA (µg STX/100 g)
C	40	41.31	95374	98498	8000	8262
F	30	31.63	71531	75417	6000	6326
J	20	20.94	47687	49928	4000	4188
P	10	8.91	23844	21245	2000	1782
A	7.5	7.57	17883	18050	1500	1514
E	5	3.8	11922	9061	1000	760
K	2.5	2.33	5961	5556	500	466
O	1	1.02	2384	2432	200	204
B	0.5	0.25	1192	596	100	50
L	0.25	0.133	596	317	50	27
N	0.1	0.03	238	72	20	6
M	0.05	0.02	119	48	10	4
D	0.01	BDL*	24	BDL*	2	BDL*
G	0.005	BDL*	12	BDL*	1	BDL*
I	BLANK	BDL*	0	BDL*	0	BDL*
H	0.1 M HCL	BDL*	0	BDL*	0	BDL*

* BELOW THE DETECTION LIMIT.

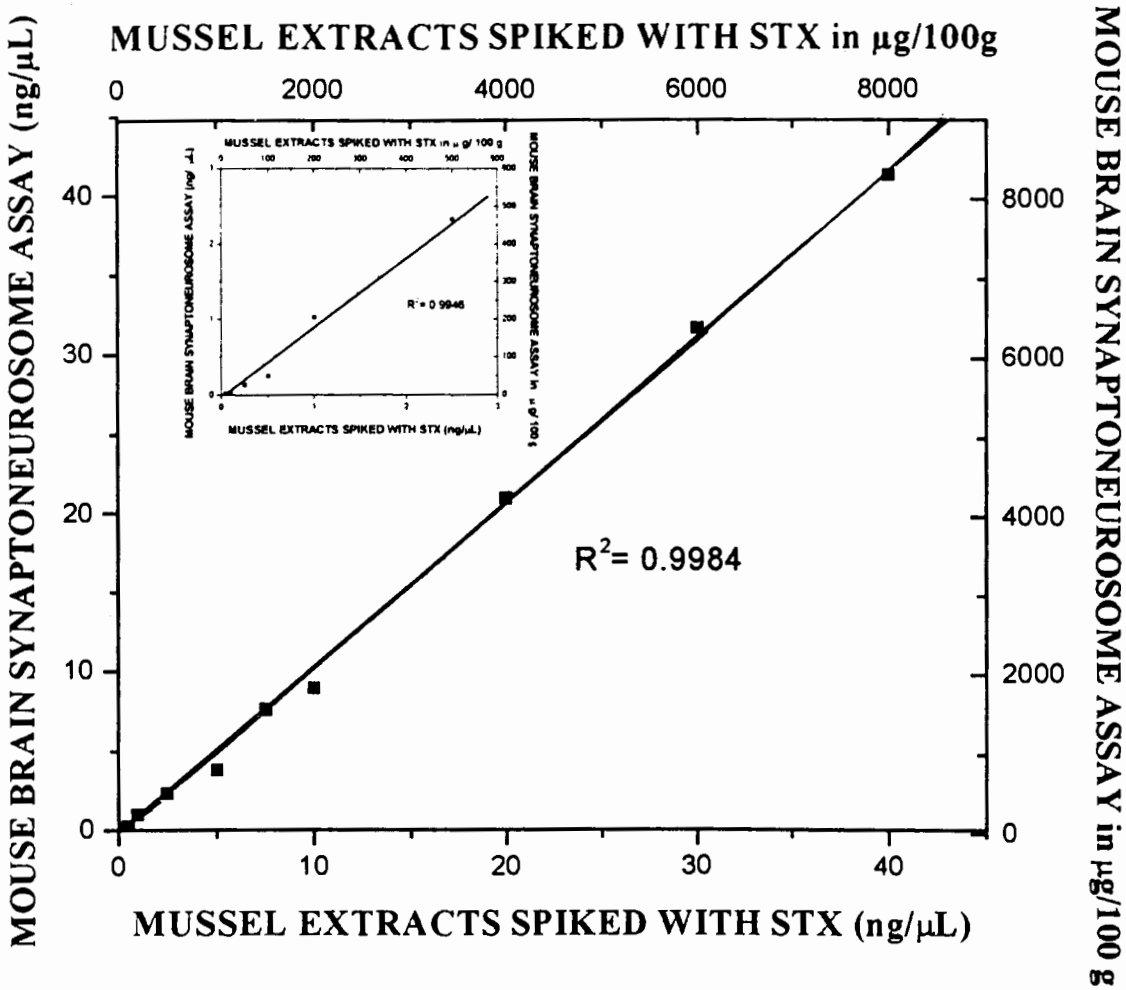


Figure 3.5. Correlation of PSP toxin levels in mussels spiked with saxitoxin and saxitoxin quantitated with mouse brain synaptoneurosome assay. Inset: relationship between assay and the spiked STX values at low concentrations. Slope = 1.047.

Table 3.2. Comparison of PSP toxin levels from plankton samples quantified with radioligand binding and mouse brain synaptoneurosome assays.

SAMPLE NUMBER	APPROXIMATE RADIOLIGAND BINDING ASSAY VALUE (nM STX equivalent)[1]	MOUSE BRAIN SYNAPTONEUROSOME VALUES (nM STX equivalent)
9	673	384
11	265	112
15	762	261
4	30	BDL[2]
5	90	21
44	160	107
31	0	BDL[2]
37	0	BDL[2]
38	0	BDL[2]

[1]Samples kindly provided by Dr. Gregory Doucette of Marine Biotoxins Program, Charleston Laboratory, NOAA, Charleston, SC, USA.
[2]BELOW THE DETECTION LIMIT

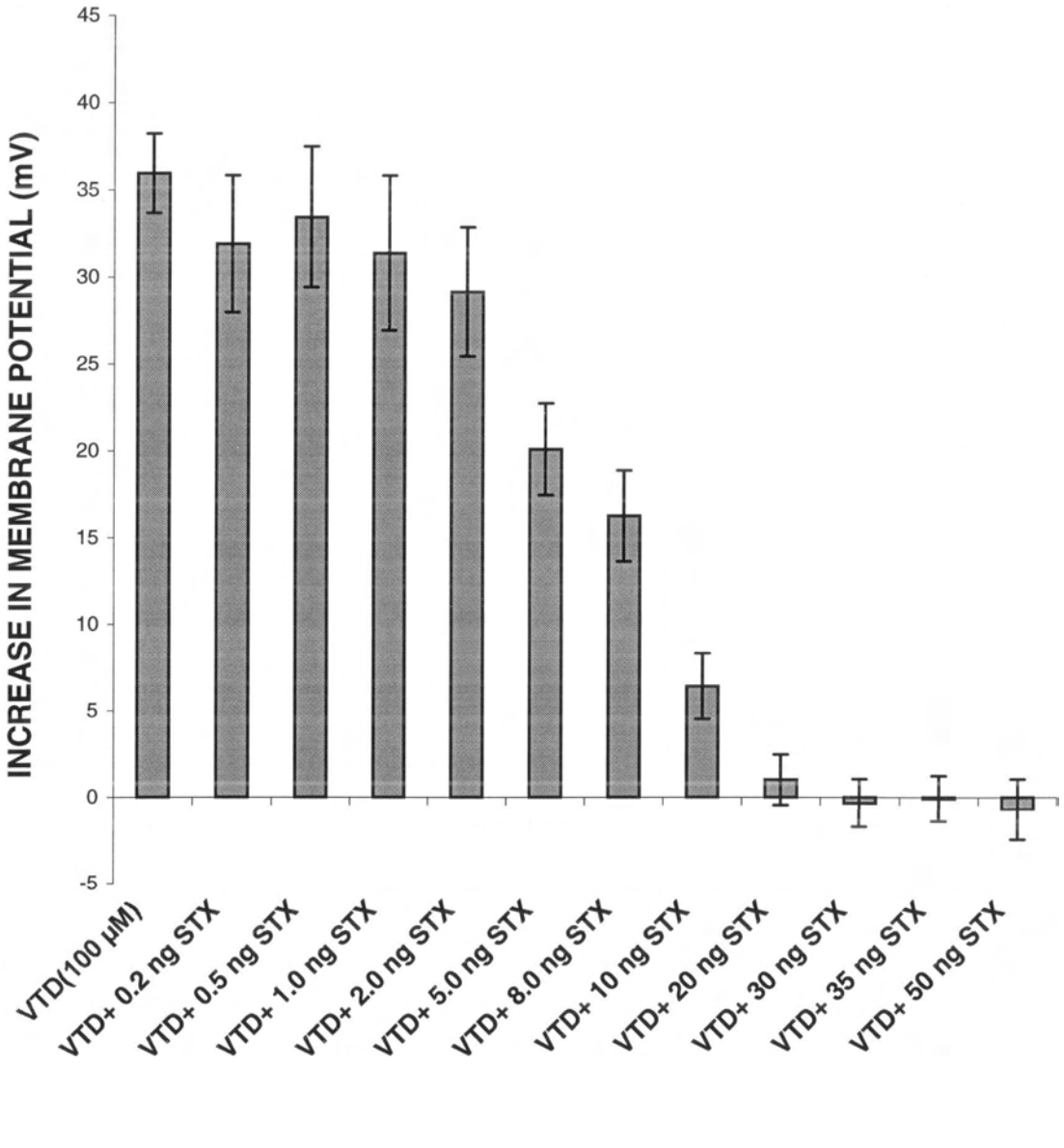


Figure 3.6. Concentration-related inhibition by STX of veratridine-induced depolarization. Values are represented as mean ± se of at least four independent determinations. Resting potential of mouse brain synaptoneurosomes is –77.85 ± 2.05 mV.

3.4 DISCUSSION

A number of assays have been developed in the search for a replacement to the conventional mouse toxicity bioassay. This type of assay brought several criticisms as already discussed (section 1.1). In this chapter, a functional *in vitro* neurobiochemical assay is described which offers a very rapid and dependable pharmacological technique for estimating PSP toxins in shellfish and plankton samples. The technique has been previously described by Nicholson *et al.,* (2002) and compared to the with mouse toxicity bioassay. A very good agreement was found on both mussels and other shellfish samples (r^2 = 0.84 and 0.86 respectively). In this part of the study, an important way to further validate the technique is to investigate the capability of the mouse brain synaptoneurosome assay to predict saxitoxin content of marine mussel extracts spiked with standard saxitoxin. The technique relies on the ability of the fluorescent dye rhodamine 6G to monitor membrane potential (Figure 3.1). The extent of depolarization can be estimated by increases in fluorescent intensity above the control. The lipophilic cation of rhodamine 6G binds to the negatively charged synaptoneurosomes. Free in solution, rhodamine 6G has a huge fluorescence response, however, with the addition of synaptoneurosomes and the subsequent binding of the lipophilic cation to the synaptoneurosomes, the fluorescence output is reduced. Hence, the greater the binding of synaptoneurosomes to the rhodamine 6G, the lower the fluorescence signal is. Sodium channel activators such as veratridine increase the fluorescence intensity consistent with less binding of rhodamine 6G as positively charged sodium ions enter the intracellular compartment. The change in the charge of the synaptoneurosome to more positive explains the reduced association of rhodamine 6G with synaptoneurosomes. The addition

of saxitoxin which is known to block sodium ion conductance, concentration-dependently reduces the increase in fluorescence that can be induced by veratridine. The resulting fluorescence intensity can be expressed in terms of percentage inhibition of the VTD response thereby quantifying the saxitoxin content in the assay.

The results clearly demonstrate a concentration-related change in fluorescence intensity with different amounts of both standard saxitoxin (Figure 3.2) and different mussel extracts spiked with STX (Figure 3.3). When small volumes of mussel extracts spiked with STX were used, the actual value of STX from samples that were within the standard curve were easily detected and estimated, but if the fluorescence intensity registered complete block, appropriate dilutions were made to bring them on to the standard curve. On the other hand, for samples that contained low levels of STX, large volumes of extracts were used to check if they could inhibit the veratridine-induced depolarization response. It was noted when assaying very large volume, they gave fluorescence responses above the veratridine alone. This was likely due to the increase in acidity of the assay medium causing synaptoneurosome to be less able to hold normal potentials, hence reducing rhodamine 6G binding to the synaptoneurosomes. To prevent such occurrences, dilution of the samples with deionized water prior to assay was carried out. Samples diluted with water gave far better fluorescence intensity response compared to with the more acidic extracts. Observations on the concentration-response relationship of STX supports the previously noted pharmacological potencies and effects of these neurotoxins in their action on voltage-sensitive sodium channels (VSSCs). Further, since their effects are opposing suggest that they may be acting on different sites on the sodium channel. This has previously been reported for those toxins where saxitoxin acts on

neurotoxin receptor site 1 and veratridine on neurotoxin receptor site 2 (Catterall, 1988; Catterall, 1992).

At least four independent experiments were used in establishing the saxitoxin standard curve (Figure 3.4). The standard curve is typically sigmoidal which makes it useful in estimating the PSP contents in the assay. The IC_{50} and a just IC_{100} (quantity of saxitoxin producing 50 and just 100% inhibition of the veratridine signal) were estimated at 6 ng and 90 ng respectively. These values correspond extremely well with the previous studies of Nicholson *et al.,* (2002) supporting the reproducibility of the assay. Moreover, the threshold of detection per assay (approximately 1 ng) is also consistent with their findings. The threshold of detection in this technique is slightly higher than the sensitivity of the rat primary culture (Beani *et al.*, 2000), neuroblastoma tissue culture (Jellet *et al.*, 1992), cytoxicity (Truman and Lake, 1996; Manger *et al.*, 1995) assays. Though these assays are slightly more sensitive than the present technique, one noteworthy disadvantage is the time needed to observe response. A 24 hour incubation period is required in tissue culture and cytotoxicity assays before results become available. In mouse brain synaptoneurosomes assay, an individual assay can be completed in 6 minutes.

Comparative saxitoxin values obtained from mouse brain synaptoneurosomes assay with the actual spiked STX values are tabulated in Table 3.1. These data sets correspond very well from the highest concentration (40 ng/μL) down to 1 ng/μL. With the lower concentrations (0.05 ng/μL to 0.5 ng/μL), a good correlation was also noted, however, the calculated values from the mouse brain synaptoneurosomes were estimated as approximately half of the actual spiked STX values. Since this represents the least

sensitive part of the saxitoxin standard curve, it's not unexpected to observe such a pattern. However, the overall efficacies of the spiked samples in the assay are very well correlated (Table 3.1). Underestimation of values was also observed by Usleber *et al.* (1997) on their study on their comparative study on ELISA and mouse toxicity bioassay. Recognizing such weakness of the assay with lower amount of saxitoxin, it is of paramount importance to cautious interpretation of the PSP content. It is then suggested to apply correction factor based on slope of the line whenever interpreting STX content of samples. My results suggest that, the technique can detect STX in extracts down to 0.05 ng per assay which corresponds to 10 µg STX/100 g tissue showing that the synaptoneurosome assay is clearly more sensitive than mouse toxicity bioassay (detection limit of 40 µg STX/100g tissue). Among the sixteen samples analyzed, only two samples that contained spiked STX were assessed as being below the detection limit. These samples contained traces of STX (0.01 and 0.005 ng/assay) and were not detected by the synaptoneurosome technique.

Regression analyses of the sixteen samples assayed using the mouse brain synaptoneurosome assay and the actual values of spiked STX in mussel extracts showed a value of 0.9984 (Figure 3.5). This demonstrates a very good correlation between the actual STX present and the STX detected from the assay. As mentioned previously, the technique underestimate the actual values of the less concentrated STX, however, correlation studies on these values with the spiked STX still gave a very good correlation (r^2 = 0.9946, Figure 3.5 inset). These robust correlations between the synaptoneurosome technique and the actual STX values of the spiked samples, support the contention that saxitoxin and its toxic congeners can be adequately quantitated by this technique. This

also indicates that this configuration of sodium channel assay is very well suited to the assessment of PSP hazard associated with shellfish consumption.

The versatility of the assay has also been tested using plankton samples. Nine plankton samples quantitated using radioligand binding assay (RBA) were analyzed. Results from this preliminary study are summarized in Table 3.2. Radioligand assay or receptor binding assay (Doucette *et al.*, 1997) is characterized by using radiolabelled saxitoxin ([^{3}H]-STX) and requires longer incubation before obtaining result. Results from the analysis of the plankton samples showed that the mouse brain synaptoneurosome assay is also capable of assaying environmental samples other than mussels or other shellfish.

Veratridine (100 μM) was routinely used to induced depolarization of synaptoneurosomes. This alkaloid is known to act on the sodium channel by activating the VSSC enabling an increase in movement of the sodium ions from the extracellular space into the cytoplasmic compartment. With an increase in entry of sodium ions, the resting negative potential inside of the cell will shift into more positive. Results from this study showed that veratridine alone increases the potential of synaptoneurosomes by 35 mV and STX-related changes in the ability of veratridine to depolarize synaptoneurosomes are illustrated in Figure 3.6. The change in potential strongly corresponds to the established saxitoxin standard curve where at low amounts of saxitoxin a minimal percentage of inhibition of veratridine's response was also evident. However, at higher amounts of saxitoxin, there is no apparent change in membrane potential from the resting potential (-77.85 ± 2.05 mV) compared to the large potential by

100 μM veratridine alone. The results described in this section support the idea that this assay is valid in terms of saxitoxin/sodium channel pharmacology.

CHAPTER FOUR

POTENTIAL USE OF VERATRINE AS SUBSITUTE FOR VERATRIDINE IN THE MOUSE BRAIN SYNAPTONEURSOMAL ASSAY FOR PARALYTIC SHELLFISH POISONING (PSP) TOXINS

4.1 INTRODUCTION

It has been long established that certain veratrum alkaloids exert potent excitatory effects on nerve fibers (Ohta *et al.*, 1973). Veratrum alkaloids originate from perennial veratrum plants belonging to the family *Liliaceae*. The indigenous North American species *Veratrum viridae* Aiton, (American Hellebore) was introduced into medical practice in the 1850's as treatment for hypertension and eclampsia (Wintersteiner, 1953). Besides this North American species, the European or White Hellebore, *Veratrum album* L. and the Central American plant *Veratrum sabadilla* (*Schoenocaulon officinale* Gray) are also documented as medicinal sources in the United States Pharmacopoeia. Veratrum alkaloids are classified into two main groups – unconjugated alkaloids or alkamines and the conjugated alkaloids. The latter is further subdivided into two subgroups, the glucosidic alkaloids composing an alkamine and one molecule of D-glucose and the ester alkaloids that contain one or more of the hydroxyl functions of an alkamine which may be acylated (Wintersteiner, 1953). The magnitude of the problems involving the isolation of pure forms of the individual ester alkaloids occurring in any one species have been clearly recognized. It is therefore not surprising that during 1950's, only five representatives of the subgroup ester alkaloids had been isolated (veratridine, cevadine,

proveratrine, proveratridine and germerine) and most were distinguished by relatively low solubility and good crystallizing properties. However, only with the advent of modern isolation techniques did it become possible to isolate components from these complex mixtures.

Recognizing the medical importance of veratrum alkaloids, several investigators studied their physiological effects (Shanes, 1952; Honerjäger, 1973; Ulbricht, 1972; Ulbricht, 1965), toxicity in insects (Allen *et al*, 1944; Ikawa *et al.*, 1945) and their pharmacology (Witt and Swaine, 1957; Ohta *et al.*, 1973, Herr and Akcasu, 1960). Among the ester alkaloids, veratrine (mixture of alkaloids such as veratridine, cevadine, cevadilline and sabadine) has been extensively studied and are known to cause membrane depolarization in amphibian muscles (Witt and Swaine, 1957) and squid and crayfish giant axons (Ohta *et al.*, 1973). Of the alkaloids reported to be present in veratrine, cevadine and veratridine are present in greatest quantity (Henry, 1939) and have been investigated extensively (Ikawa *et al.*, 1945). Witt and Swaine (1957), in their study found that veratridine can depolarize frog sartorious muscle fiber by 47.2 ± 1.8 mV. Wright and Tomita (1966) in their study with crustacean axon found that veratrine also caused a prolonged depolarization of the membranes. At higher concentrations, veratrine caused a reversible depolarization of the membrane and conduction block. In studies to characterized veratrum alkaloids present in veratrine, Shanes (1952), concluded that veratrine is composed of approximately equal amounts on a gram weight basis of veratridine and cevadine.

The fact that veratrine can also induce depolarization of membranes and is significantly cheaper (25 mg, $ 21.40) than veratridine (25 mg, $ 183.80), it is the

objective of this section of the study to investigate veratrine as a substitute for veratridine as a means of assaying PSP toxins in more economical ways. The utility of a small number of animals in mouse brain synaptoneurosomes assay arguably offers a more economical, ethical and reliable approach assaying PSP contents in plankton and mussel samples. The investigation on the potential use of veratrine in place of veratridine and validation of this modified assay with spiked samples are the main objectives of this section of this study.

4.2 MATERIALS AND METHODS

4.2.1 Chemicals and Animals

All animals used in this part of the study treated similarly as described in section 3.2.1. Veratrine and saxitoxins were purchased from Sigma-Aldrich Canada Limited (Oakville, Ontario). Rhodamine 6G was obtained from the Eastman Kodak Company (Rochester, New York). All other chemicals were of the highest purity available.

4.2.2 Preparation of Spiked Samples

Mussel extracts devoid of saxitoxin were provided by Canadian Food Inspection Agency (CFIA), Burnaby, B.C. Sixteen samples were spiked with different amounts of saxitoxin and were analyzed blind. Mussel tissues were originally extracted in 0.1 N HCl. In cases where extracts needed to be extensively diluted, dilutions were done in deionized water.

4.2.3 Preparation of Synaptoneurosomes and Protein Analysis

The isolation of synaptoneurosomes from male CD1 mice was carried out using the protocol mentioned in section 3.2.5. Likewise, synaptoneurosomal proteins were determined using the procedure mentioned in section 3.2.6.

4.2.4 Fluorescence Quantitaion of PSP Content Using Veratrine

The rhodamine 6G technique was used in quantifying synaptoneurosomal depolarization as described in the previous chapter. Initially a veratrine concentration that depolarized to the same extent as veratridine was identified. This veratrine concentration was used for subsequent construction of the standard curve and analysis of spiked samples. To a stirred quartz cuvette containing 3 ml saline (132 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1.2 mM $Na_2HPO_4 \cdot 7H_2O$, 1.2 mM $MgCl_2 \cdot 6H_2O$, 10 mM glucose, 20 mM Tris adjusted to pH 7.4 with 1 M HCl), veratrine (0.135 µg/µL final concentration) was added together with known amount of saxitoxin standard. Two sets of control were used, vehicle control (3 µL DMSO), and blank (saline). Approximately 20 seconds after intiation of recording, 1 µL of rhodamine 6G (0.05 µM final concentration) was added. At t = 100 seconds, 50 µL of synaptoneurosomes (760 µg protein) was added. After subtraction of control values fluorescence intensity readings were recorded at 300 seconds. Fluorescence intensity values obtained with saxitoxin and veratrine combined were expressed as percentage of fluorescence response with veratrine alone. The saxitoxin standard curve was fitted using Prism 2.0 (Graphpad Software Inc.; San Diego, California).

Sixteen mussel extracts spiked with STX were analyzed blind. Samples were coded with letters and were analyzed in random fashion using the rhodamine 6G technique. Blind analysis of spiked samples is essential to ensure the integrity of the STX assay which is a paramount importance in food safety. Spiked samples were initially tested using 1 μL and 3 μL extracts. From the initial test, if samples showed a complete block or strong inhibition dilutions of extracts were made. If samples showed weak or no inhibition, larger volume of samples were used for further analysis. Where the volume of the extract exceeded 10 μL, appropriate adjustment on the volume of the saline was done to ensure consistency of the total volume of the assay. Percentage inhibition values obtained from these analyses were then related to the saxitoxin/veratrine standard curve to estimate saxitoxin equivalents in different samples (as nM, ng/μL, and μg STX per 100 g of shellfish tissue sample). In all cases, average values of replicate assays were used for data analysis.

4.2.5 Membrane Potential Assay Using Veratrine as Depolarizing Agent

Changes in membrane potential of synaptoneurosomes were carried out using the procedure mentioned in the preceding section with modifications. Changes in membrane potential produced by the interaction of veratrine and saxitoxin were estimated by using the potassium ion calibration. In separate experiments, varying concentrations of potassium ions were used to set different membrane potentials and fluorescence intensities recorded. Membrane potential was determined from the relationship between the fluorescence intensity and potassium equilibrium potential, the latter calculated using the reduced Goldman equation (Akerman *et al.*, 1987).

4.3 RESULTS

In Figure 4.1, the fluorescence intensity reading for saline with veratrine (0.135 μg/μL) typically settles just below 150 units and the control (DMSO and saline) settles just below 100 units. As illustrated in this figure, standard saxitoxin produces a concentration-related inhibition of veratrine's response. At 3.0 ng of saxitoxin, veratrine's response was reduced approximately by 50% which is lower than 5.0 ng if veratridine is used as reference. Side by side (same assay) estimates with veratrine and veratridine were also conducted to confirm this differential. Analysis of eighteen mussel extracts was carried out blind. Fluorescence readings of four representative samples are shown in Figure 4.2. Results showed that sample A (2.5 ng spiked STX) produced approximately 50% inhibition of veratrine response which correspond very well to the IC_{50} of the standard saxitoxin. The standard curve for saxitoxin/veratrine assay is shown in Figure 4.3. The curve is also sigmoidal like the standard saxitoxin/veratridine curve, although, the veratrine curve rises more abruptly. Threshold detection was estimated at approximately 1 ng per assay.

Eighteen samples (17 STX-spiked mussel extracts and a 0.1 N HCl blank) were analyzed for PSP toxin contents using veratrine as the sodium channel-specific depolarizing agent. The results are summarized in Table 4.1. Spiked samples between 10 ng/μL to 40 ng/μL underestimated by the synaptoneurosomal assay when veratrine was used as an activator. However, spiked samples below this range were more accurately estimated for STX content. Compared with the veratridine/saxitoxin mouse brain synaptoneurosome assay, the veratrine/saxitoxin mouse brain synaptoneurosome assay detected spiked STX even at 0.01 ng/assay. Regression analysis of the veratrine/saxitoxin

mouse brain synaptoneurosome assay and the spiked mussel extracts revealed a r^2 of 0.9977 (Figure 4.4). Moreover, the regression coefficient for the lower part of the line showed a r^2 of 0.9937 (Figure 4.4, inset).

The concentration-related inhibition by STX of the veratrine-induced increase in membrane potential is shown in Figure 4.5. At 0.01 ng/assay to 0.05 ng/assay, the increase in membrane potential is similar to the veratrine alone response. Complete inhibition of the veratrine-induced increase in membrane potential is manifested at 10 ng/assay and 20 ng/assay.

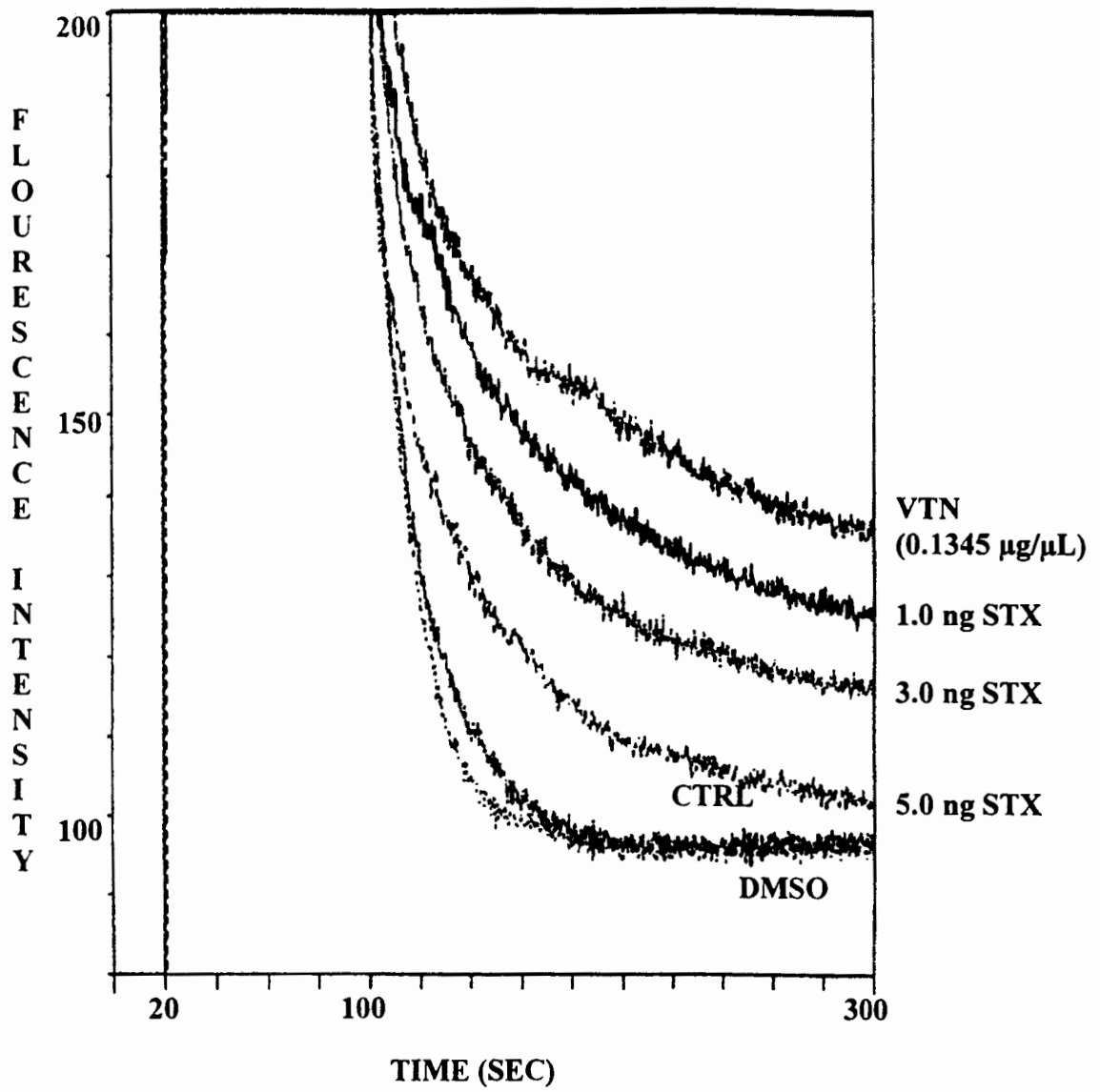


Figure 4.1. Typical rhodamine 6G fluorescence response illustrating the inhibition of veratrine (VTN)-induced increase in fluorescence of mouse brain synaptoneurosomes by different amounts of standard saxitoxin (STX). T = 20 seconds, rhodamine 6G was added.

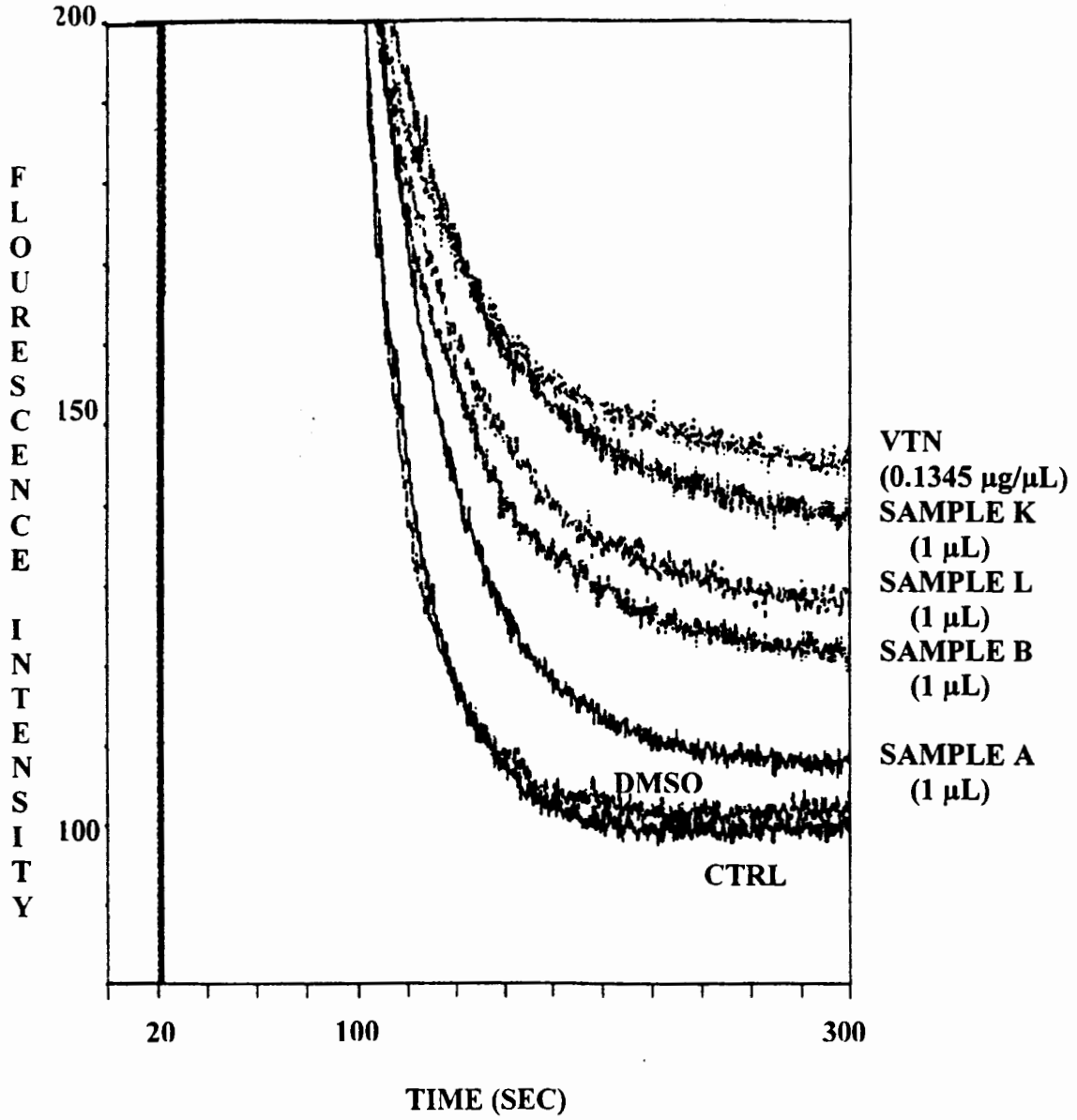


Figure 4.2. Rhodamine 6G fluorescence recordings illustrating the inhibition of veratrine (VTN)-mediated increase in fluorescence of mouse brain synaptoneurosomes by four mussel extracts spiked with STX. T= 20 seconds, rhodamine 6G was added.

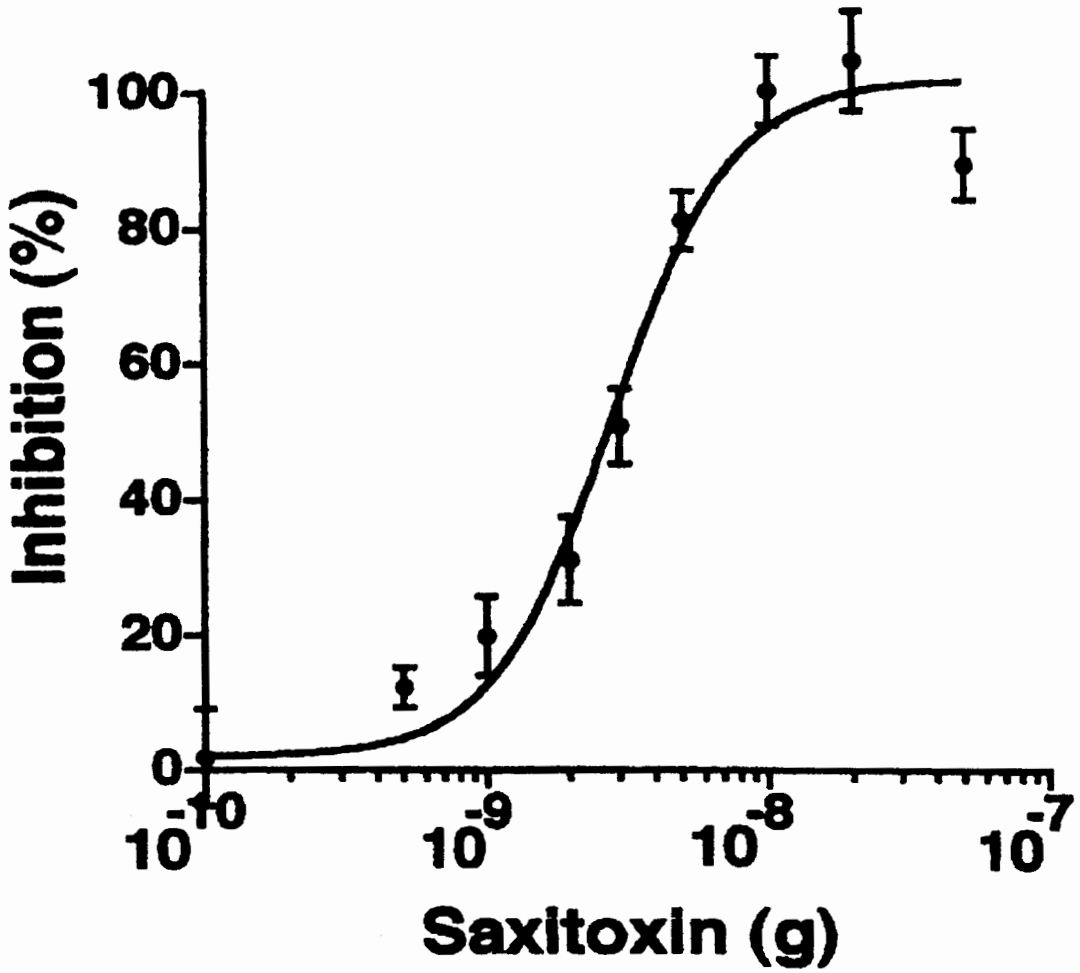


Figure 4.3. Calibration curve showing the relationship between amount of standard saxitoxin and inhibition of veratrine-induced increase in fluorescence of mouse brain synaptoneurosomes. Values represent as mean ± se of at least three independent experiments.

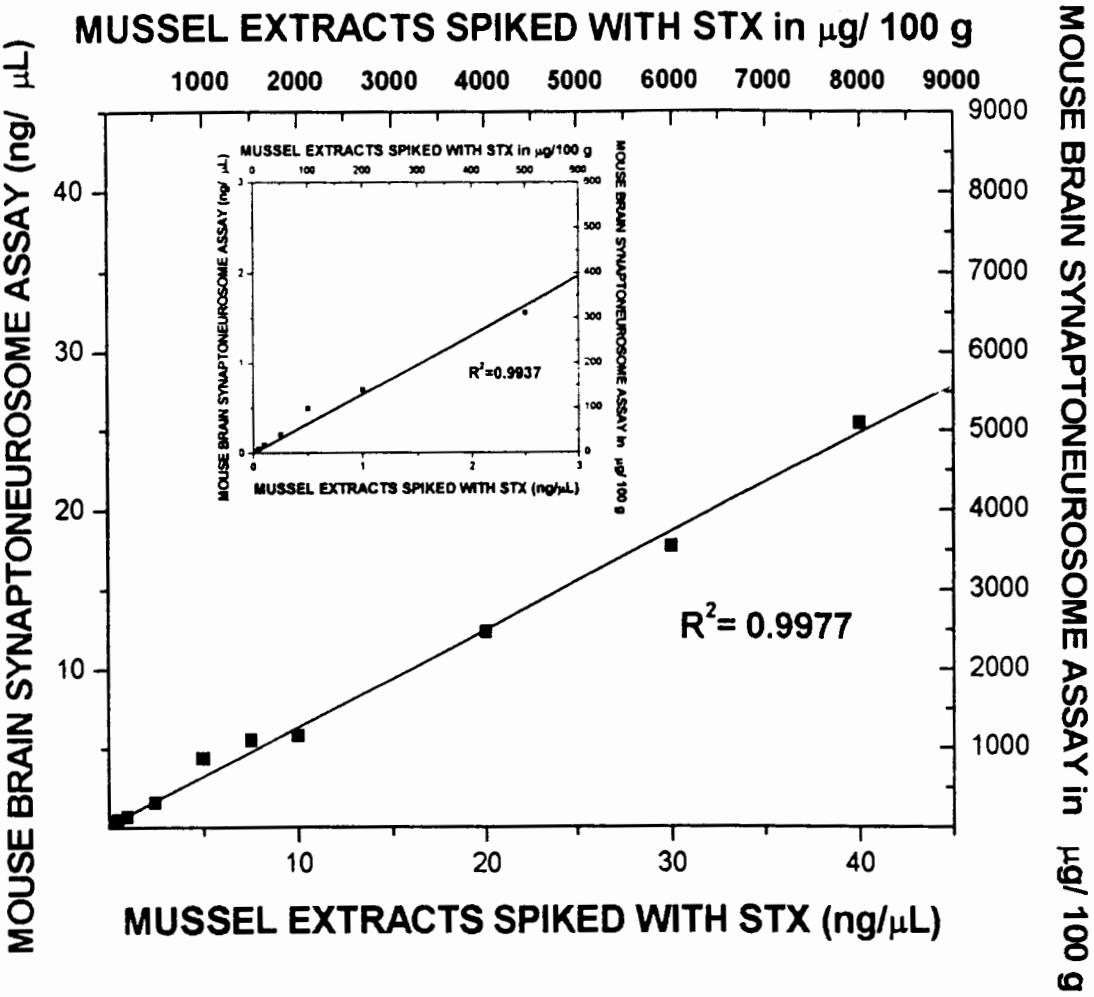


Figure 4.4. Correlation of STX content from mussel extract and STX toxin levels quantitated using mouse brain synaptoneurosomes with veratrine as the activator. Inset: relationship between assay and the spiked STX values at low concentration. Slope = 0.65

Table 4.1. Estimates of STX levels in STX-spiked mussel extracts using the mouse brain synaptoneurosome assay (MBSA) and veratrine as depolarizing agent, as compared to actual STX values.

Sample ID	SPIKED STX (ng/µL)	MBSA (ng/µL)	SPIKED STX (nM)	MBSA (nM)	SPIKED STX (µg STX/100 g)	MBSA (µg STX/100 g)
E	40	25.44	95374	60658	8000	5088
J	30	17.69	71531	42179	6000	3538
C	20	12.35	47687	29447	4000	2470
F	10	5.75	23844	13710	2000	1150
P	7.5	5.5	17883	13114	1500	1100
A	5	4.35	11922	10372	1000	870
B	2.5	1.56	5961	3720	500	312
L	1	0.70	2384	1669	200	140
K	0.5	0.49	1192	1168	100	98
O	0.25	0.203	596	484	50	41
H	0.1	0.092	238	219	20	18
D	0.05	0.043	119	103	10	9
G	0.01	0.015	24	36	2	3
M	0.005	BDL*	12	BDL*	1	BDL*
I	BLANK	BDL*	0	BDL*	0	BDL*
N	0.1 M HCL	BDL*	0	BDL*	0	BDL*

* BELOW THE DETECTION LIMIT.

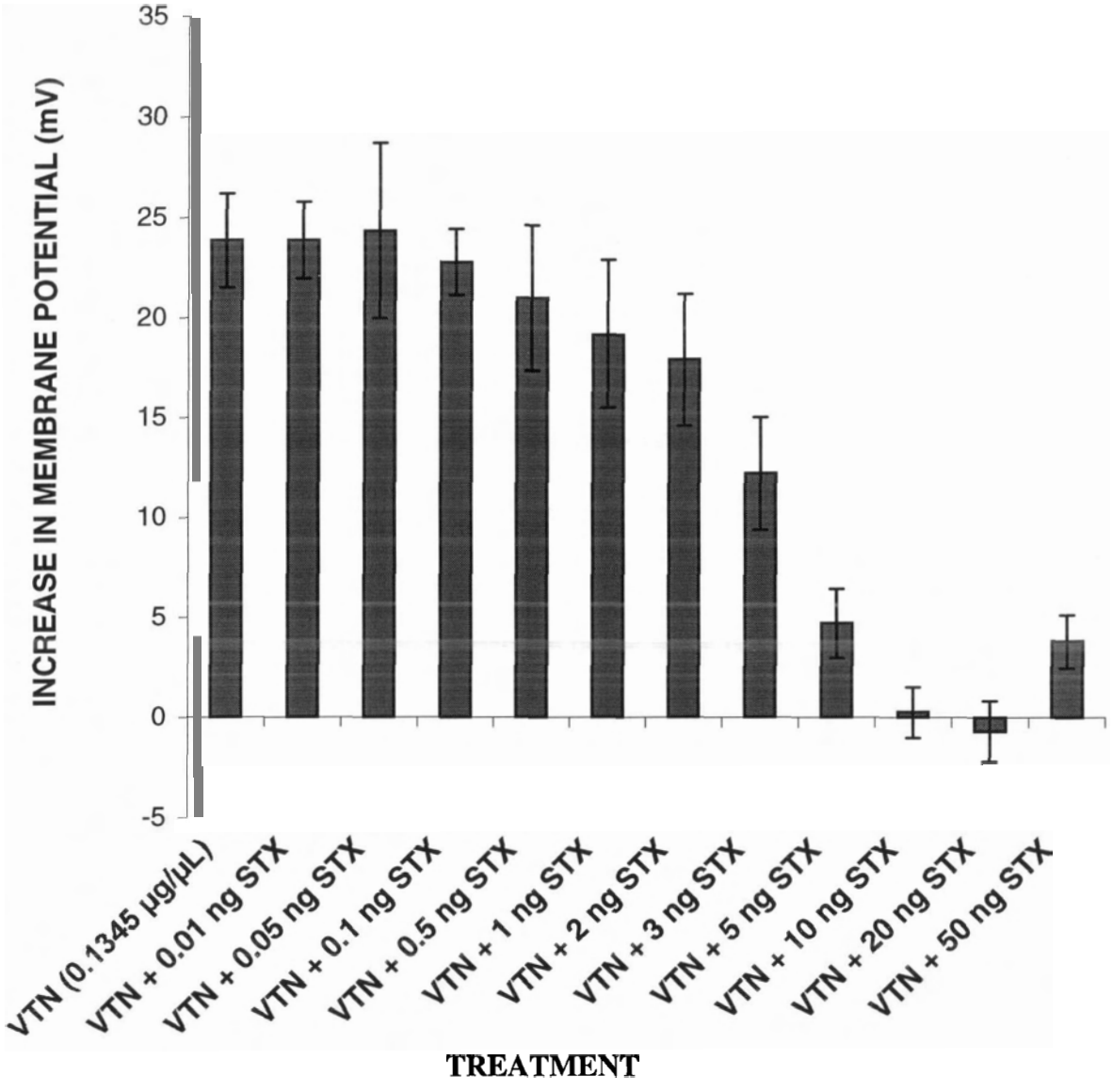


Figure 4.5. Concentration-related inhibition by STX of veratrine-induced depolarization. Values represent mean ± se of at least three independent determinations. Resting potential for mouse brain synaptoneurosomes is -74.84 ± 1.02 mV.

4.4 DISCUSSION

The usefulness of veratridine and mouse brain synaptoneurosomes as assay to quantititate saxitoxin has been previously indicated through comparison with mouse toxixity bioassay data (Nicholson *et al.*, 2002) and by using spiked samples (Chapter 3). In this chapter, the objective was to investigate an alternative depolarizing agent. The depolarizing agent selected was a mixture of veratrum alkaloids known as veratrine. Veratrine induced depolarization in amphibian muscles (Witt and Swaine, 1957) and on squid and crayfish giant axons (Ohta et al., 1973). *In lieu* of veratridine, veratrine was investigated as potential replacement in mouse brain synaptoneurosome assay. My results demonstrated that there were reproducible reductions in fluorescence intensity when increasing quantities of STX were combined with a fixed concentration of veratrine. This applied to both standard saxitoxin additions (Figure 4.1) and mussel extracts spiked with STX (Figure 4.2). The threshold detection of the assay is estimated at approximately one nanogram which is similar to the estimated threshold of the veratridine/saxitoxin assay. Moreover, the STX IC_{50} using veratridine was noted at approximately 6 ng/assay whereas for veratrine the STX IC_{50} was estimated at 3 ng/assay, a 2 fold higher sensitivity of the veratrine/saxitoxin as compared with veratridine/saxitoxin. This finding was further verified by performing side by side (same assay) analysis at STX IC_{50} with each activator on three separate occasions.

The threshold of detection using veratrine as depolarizing agent is still lower than the sensitivity of the rat primary culture assay by Beani *et al.* (2000) of 4 µg/100 g tissue and neuroblastoma tissue culture bioassay of Jellett *et al.* (1992) of 2 µg/ 100 g tissue. The sensitivity of detection using the present technique is also less sensitive than the

cytotoxicity assays (Truman and Lake, 1996; Manger *et al.*, 1995). Although the previously mentioned assays were more sensitive than the synaptoneurosomal technique using veratrine, one disadvantage is the long assay incubation of at least 24 hours prior to quantitation. In the present technique, an individual assay will only require 6 - 7 minutes to provide an estimate of STX present in a sample.

Higher amounts of saxitoxin ($\geq$50 ng per assay) were investigated for their ability to inhibit veratrine-induced depolarization. The findings revealed that instead of reaching the plateau of 100 % inhibition, 50 ng saxitoxin showed a 89.5 $\pm$ 5.17 % inhibition. The fact that inhibition did not maintain itself at 100 % suggests that there is something affecting the ability of saxitoxin to block the veratrine's response under these conditions. One possibility is that there could be conformational modulation in the sodium channels brought about by the interaction of a compound or compounds (present in the veratrine alkaloid mixture) with saxitoxin causing the latter to alter its binding to mouse brain sodium channels. Another possible explanation is the interference in the assay brought about by direct chemical interaction of saxitoxin with some of the components of veratrine. These assumptions are best investigated when neurobiochemical assays can be performed specifically with each of the individual compounds present in veratrine and it is suggested that that other components of veratrine should be tested to have a clear understanding of their pharmacology in this system .

Sixteen samples were analyzed using the synaptoneurosomal technique configured using and veratrine as a depolarizing agent and the saxitoxin values are displayed in Table 4.1. A very good correspondence was noted on all values (r^2 = 0.9977). The synaptoneurosomal estimates of STX correspond well with the actual

spiked STX values. Saxitoxin values from spiked samples ranging from 10 ng/assay to 40 ng/assay were underestimated by approximately half in the assay, however, values lower than 10 ng/assay were estimated by the assay quite accurately (Table 4.1). One significant observation was that though the detection limit was 0.5 ng/assay, during analysis the assay was able to detect STX in extracts down to 0.01 ng/assay (2 µg STX/100 g tissue) which is comparable to the sensitivity of neuroblastoma tissue culture assay by Jellet *et al.* (1992).

Regression analyses of the sixteen samples determined using the mouse synaptoneurosome technique and veratrine with the actual STX values was calculated at 0.9977 (Figure 4.4). This good overall correlation value supports the idea that veratrine can be used as potential substitute for the more expensive veratridine in estimating the amounts of STX present in shellfish samples. However, a potential concern is the potential underestimation on higher amounts of saxitoxin. As suggested in the previous chapter, in order to alleviate this problem, again it is suggested to apply correction factor based on the slope of the line. However with the robust correlation between the two data sets, it is apparent that veratrine can be used as substitute for veratridine in the mouse brain synaptoneurosome assay.

As mentioned previously, veratrine causes membrane depolarization. From a resting potential of 74.84 ± 1.02 mV, veratrine (0.135 µg/µL) depolarized synaptoneurosome by 23.86 ± 2.35 mV. Examination of the ability of saxitoxin to block veratrine-induced increases in membrane potential was undertaken revealing a concentration-related inhibition by STX (Figure 4.5). This membrane potential analysis corresponds very well to the established veratrine/saxitoxin standard curve (Figure 4.3)

suggesting the compatibility of the technique both in terms of percentage inhibition of depolarization and increase in membrane potential.

CHAPTER FIVE

DEVELOPMENT AND PRELIMINARY VALIDATION OF MOUSE BRAIN SYNAPTONEUROSOME ASSAY FOR NEUROTOXIC SHELLFISH POISONING (NSP) TOXINS

5.1 INTRODUCTION

The occurrence of neurotoxic shellfish poisoning (NSP) has historically been limited to the west coast of Florida where blooms of dinoflagellate, *Gymnodinium breve* proliferate offshore and are subsequently carried inshore by wind and current conditions (Steidinger, 1998). Massive fish kills and shellfish toxicity have been reported during these outbreaks (McFarren *et al.,* 1965; Cummins *et al.*, 1971; Baden, 1983; Poli *et al.*, 1995). These problems have also been reported along the coast of North Carolina and Texas. In 1993, an outbreak of NSP has been reported in New Zealand (McKenzie *et al.,* 1995). The toxin responsible for NSP are suite of ladder-like polycyclic ether toxins collectively called brevetoxins (PbTx for *Ptychodiscus brevis*, formerly called *Gymnodinium breve* then reinstated, Van Dolah, 2000). Recently, this taxonomic classification has been renamed *Karenina brevis* as mentioned in the paper by Plakas *et al.,* (2002). Toxic syndromes of oral brevetoxin exposure in humans accidentally exposed to contaminated shellfish include nausea, cramps, paresthesias of the lips, face and extremities, weakness and difficulty in movement and if severe paralysis, seizures and coma (Berman and Murray, 1999). However, though severe cases of human intoxication have been reported no deaths have been reported. Brevetoxins are known to interact

specifically with site 5 on the α-subunit on the voltage sensitive sodium channels. This interaction causes a shift in the voltage dependence of channel activation to more negative potentials, inhibits sodium channel inactivation, and thereby produces neuronal depolarization at resting membrane potentials (Catterall and Gainer, 1985; Poli *et al.*, 1986; Sharkey *et al.*, 1987).

The economic impact on the shellfish industry and concerns over food safety had prompted regulatory bodies to conduct continuous monitoring on the occurrence of neurotoxic shellfish poisoning. Regulatory guidelines mandate a legal limit for brevetoxin of is 80 μg of toxin per 100 g of shellfish tissue (Hunt and Tufts, 1979; Trainer and Baden, 1991). Several investigations have used various techniques to quantify the brevetoxins. *In vitro* studies such as radioligand binding assays, radioimmunoassays, mouse neuroblastoma cell assays, enzyme immunoassays, and HPLC/ MS (Van Dolah, *et al.*, 1994; Leighfield *et al.*, 1996; Whitney and Baden, 1996; Whitney *et al.*, 1997; Baden *et al*, 1988; Levine and Shimizu, 1992; Trainer and Baden, 1991; Stuart and Baden, 1988; Dechraoui *et al.*, 1999; Dickey *et al.*, 1999) were used to detect the amounts of brevetoxin.

The main objective of this chapter is to develop an *in vitro* neurobiochemical assay to quantify the brevetoxins. The assay will be validated using mussels spiked with known amount of PbTx-2 and by assays of extracts naturally contaminated with brevetoxins from oyster samples previously assayed by other laboratory. This will involve the use of mouse brain synaptoneurosomes and the known pharmacological action of brevetoxin to potentiate veratridine's response in the sodium channel as observed by Sharkey *et al.*, (1987) and Lombet *et al.*, (1987).

5.2 MATERIALS AND METHODS

5.2.1 Animals and Biological Samples

Mice used in this phase of study were treated as described in section 3.2.1 of this study. Mussels were purchased from a local market and extracts spiked for analysis as described in 5.2.3. Oyster homogenates were kindly provided by Dr. Robert Dickey of FDA, Dauphin Island, Alabama.

5.2.2 Chemicals

PbTx-2 was obtained from Dr. Nakanishi of Columbia University, New York, U.S.A. and PbTx-3 was kindly provided by Dr. Robert Dickey of FDA, Dauphin Island, Alabama. Veratridine and Tetrodotoxin (TTX) were purchased from Sigma-Aldrich Canada Limited (Oakville, Ontario). Rhodamine 6G was obtained from the Eastman Kodak Company (Rochester, New York). All other chemicals were of the highest purity available.

5.2.3 Preparation of Spiked Samples

Investigation of appropriate conditions for extraction of mussel tissues and subsequent for spiking of samples for analysis was carried out. Initially, mussels were homogenized in standard saline (132 mM NaCl, 5 mM KCl, 1 mM $CaCl_2 \cdot 2H_2O$, 1.2 mM $Na_2HPO_4 \cdot 7H_2O$, 1.2 mM $MgCl_2 \cdot 6H_2O$, 10 mM glucose, 20 mM Tris adjusted to pH 7.4 with 1 M HCl) and were spiked with known amount of brevetoxin. However, during the analysis of these spiked samples large inconsistencies and obvious deterioration of

samples was observed. Since DMSO was used as solvent in preparation of brevetoxin standard curve, DMSO was examined as solvent to homogenized mussel tissues. 9.21 grams of Altantic mussels were homogenized using 9.21 ml of DMSO in a polytron homogenizer (Brinkman Instruments, Rexdale, Ontario, Canada). The homogenate was centrifuged at 4,000 rpm (Beckman J2HS; Beckman Instruments, Fullerton, CA) for 15 minutes at 18°C. The supernatant was passed sequentially through a 100 μm mesh, Whatman No. 1 filter and then finally through a Whatman GF/B filter yielding a slightly opalescent, yellow colored extract. Part of this extract (300 μL) was spiked at nominal concentration of 100 ng/μL PbTx-2 and then the remainder was used to make serial dilutions of the stock. A total of ten samples were analyzed blind using the newly developed synaptoneurosomal assay for brevetoxins.

5.2.4 Preparation of Oyster Extract Samples

Six 1.0 gram oyster homogenates were provided by Dr. Robert Dickey of FDA, Dauphin Island, Alabama, U.S.A. Each sample were in a 13 x 1000 mm screw cap test tube; was centrifuged for 10 minutes using IEC CL centrifuge (Needham Hts. Massachussets, U.S.A). Volumes of the supernatant were estimated and aliquots (50 μL) of each sample were removed for subsequent brevetoxin estimation. These samples were tested to further validate the brevetoxin assay.

5.2.5 Preparation of Synaptoneurosomes and Protein Analysis

Isolation of synaptoneurosomes from male CD1 mice was carried out using the protocol outlined in section 3.2.5, and synaptoneurosomal proteins were determined using the method described in section 3.2.6.

5.2.5 Fluorescence Quantitation of Brevetoxins.

The rhodamine 6G technique was conducted based on the protocol described by Auichi *et al.*, (1982) for synaptosomes and adapted by Verdon *et al.*, (2000) and Nicholson *et al.*, (2002) for synaptoneurosomes with modifications. The technique is used to measure the amount of saxitoxin (Nicholson *et al.*, 2002) and is used in this phase of the study to quantitate the amount of brevetoxin in the assay. All fluorescence measurements were carried out using a Perkin-Elmer LS-50 fluorescence spectrophotometer equipped with temperature controlled (temperature was set at 37°C) cuvette holder and linked to an IBM 50Z computer. Excitation and emission wavelengths were set at 520 nm and 550 nm respectively and slit widths were adjusted to 5 nm.

Standard curve for both derivatives of brevetoxin B (PbTx-2 and PbTx-3) assays were first established and were used to subsequently quantitate brevetoxin levels in spiked mussel samples and oyster extracts. Into a stirred quartz fluorescence cuvette containing 3 ml saline (132 mM NaCl, 5 mM KCl, 1 mM $CaCl_2 \cdot 2H_2O$, 1.2 mM $Na_2HPO_4 \cdot 7H_2O$, 1.2 mM $MgCl_2 \cdot 6H_2O$, 10 mM glucose, 20 mM Tris adjusted to pH 7.4 with 1 M HCl) and 50 µL of synaptoneurosomes (760 µg protein), known amounts of PbTx was added. The sodium channel activator veratridine (100 µM final concentration) and vehicle control (1.0 µL DMSO) and blank were used as references. Approximately 480 seconds after initiation of recording 1.0 µL of rhodamine 6 G (0.05 µM final

concentration) was added and was followed by sodium channel activator veratridine (1.67 μM final concentration) at t = 1000 seconds. Fluorescence intensities were quantified at the base and peak of fluorescence after the addition of veratridine (usually between 1000 and 1100 seconds). The difference in between base and peak fluorescence following the addition of low concentration of veratridine (1.67 μM) were used to calculate the potentiating effect of brevetoxins on this standard veratridine response. Percentage enhancement relative to 100 μM veratridine alone was used as an upper reference point. Curve fitting and regression analyses were carried out using Prism 2.0 (Graphpad Software Inc.; San Diego, California).

Some assays were also conducted with TTX a neurotoxin that specifically blocks sodium channels. Tetrodotoxin (16.67 μM) and 20 ng PbTx-2 were added to saline before initiation of fluorescence readings, at t = 480 seconds, rhodamine 6G was added. After 520 seconds, veratridine (1.67 μM) was added. In another test, 50 ng STX was used *in lieu* of tetrodotoxin (TTX). TTX and STX completely blocked depolarization (observed as an increase in fluorescence) due to 1.67 μM VTD alone and the VTD (1.67 μM) + 20 ng PbTx-2 combination.

Ten mussel extracts samples spiked with brevetoxin (PbTx-2) were used to initially validate the assay. Analysis of spiked samples was carried out blind. Initially samples were tested with 1 μL and 3 μL. From the initial test, if samples showed more than 87% increase in response with 1.67 μM VTD, appropriate dilutions of the extracts were made to bring the percentage increase within the standard curve. On the other hand, if samples showed less than 10% increase in response, larger volume of samples was used for subsequent analysis. Appropriate adjustment on the volume of saline was done

to ensure consistency on the total volume of assay. The above mentioned percentages , 10% and 87%, represent the points where the curve started to rise and peaked respectively.

Six oyster homogenates were used to test PbTx-3 mouse brain synaptoneurosome assay. Analysis on the NSP toxin contents of oyster extracts previously estimated by FDA, Dauphin Island, Alabama, U.S.A. was also carried out blind. Samples were initially tested using 3 µL extracts and from the result of initial run, appropriate volumes were selected to bring the percentage enhancement with 1.67 µM VTD within the PbTx-2 and PbTx-3 standard curves. As in the case of assayed spiked samples, if the sample showed more than 90% increase in response, appropriate dilution of the samples was made to bring down the response within the established standard curve.

5.2.6. Membrane Potential Assay for Veratridine Potentiation with Brevetoxin

Estimation on the membrane potential of synaptoneurosomes was done using the methodology described in section 5.2.5. The change in membrane potential was estimated after establishing the potassium ion equilibrium calibration as described in the previous chapters. The increase in fluorescence intensity brought about by potentiation of PbTx and VTD was estimated from the established potassium ion equilibrium potential and calculated using the reduced Goldman equation (Akerman *et al.*, 1987).

5.3 RESULTS

The utility of mouse brain synaptoneurosome in the neurobiological assay of brevetoxins, the causative agent for neurotoxic shellfish poisoning was investigated.

Typical fluorescence traces used to construct brevetoxin standard curves is illustrated in Figure 5.1. Fifty microliters of synaptoneurosomes (760 μg protein) was used in all assays. The peak fluorescence reading for 100 μM VTD alone was just below 150 fluorescence units and for 1.67 μM VTD alone was just below 110 fluorescence units. The DMSO control showed no increase in fluorescence, confirming that there is no depolarization of synaptoneurosomes. PbTx-2 dose-dependently enhanced the fluorescence response of 1.67 μM VTD (Figure 5.1).

At least four independent experiments were conducted to establish the standard curve for brevetoxin, PbTx-2 (Figure 5.2). The curve is sigmoidal. Results showed that the EC_{50} (concentration producing 50% enhancement) was estimated at 4.2 ng/assay (1.56 nM). The curve started to rise at about 0.6 ng/assay and peak at about 30 ng/assay. These two values represent the lower and upper limit of the curve that served us reference in the estimation of NSP content of the samples analyzed.

A standard curve for PbTx-3 was also established with at least three independent experiments. Results showed the curve is also sigmoidal (Figure 5.3) and very similar in profile to the PbTx-2 standard curve. However, the EC_{50} was estimated at 6.7 ng/assay (2.49 nM). This value is higher than the EC_{50} for PbTx-2 of 4.2 ng/assay.

Preliminary validation of the novel neurobiochemical and pharmacological assay for NSP monitoring was undertaken through analysis of mussel extracts spiked with PbTx-2. As mentioned in the materials and methods, the analysis was performed blind to ensure the integrity of the assay and honest interpretation of the results obtained. The results from the analysis of these spiked samples are tabulated in Table 5.1. Among the ten samples analyzed, 50% of the mussel samples were overestimated by the PbTx-2

mouse brain synaptoneurosome assay with remaining samples being estimated more accurately. Regression analysis of the estimated values from the PbTx-2 mouse brain synaptoneurosome assay and the actual spiked brevetoxin values showed a very good correlation (r^2 = 0.9730, Figure 5.4a). The slope of regression was estimated at 1.08. Correlation between PbTx-2 equivalents in spiked mussel extracts and estimated PbTx-2 using MBSA fitted in log scale is shown in Figure 5.4b. This graph shows all the data points from both PbTx-2 equivalents in spiked samples and the estimated PbTx-2 values, r^2 and slope, however, were obtained from linear plot.

Validation of PbTx-3 mouse brain synaptoneurosome assay was done via analysis of oyster homogenates previously quantitated by FDA, Dauphin Island, U.S.A. Six homogenates were assayed using the current technique and the estimated contents were compared with their estimated values. Results showed that the assay correlates very well with their receptor binding assay (Figure 5.5; r^2 = 0.953), and cytotoxicity assay using mouse neuroblastoma (Figure 5.6; r^2 = 0.974). Though cytotoxicity assay showed good correlation, a toxicity ratio of at least 10:1 (cytotoxicity to MBSA) was observed. A moderate correlation (r^2 = 0.605) was obtained from HPLC mass spectrometry (Figure 5.7). Regression analyses of brevetoxin estimates from oyster samples using both PbTx-2 and PbTx-3 standard curves showed very good correlation (Figure 5.8; r^2 = 0.9977). Moreover, the brevetoxin content of oyster homogenates using the receptor binding assay and the estimated brevetoxin (PbTx-3) content using PbTx-2 mouse brain synaptoneurosome assay showed very good coefficient value of 0.9333 (Figure 5.9).

The pharmacological basis of the assay was examined using other neurotoxins specifically acting on the sodium channels (Figure 5.10). Results showed that 1.67 μM

VTD alone increased synaptoneurosomal membrane potential by 5.4 ± 0.23 mV whereas when combined with 20 ng PbTx-2, a potentiated response (27.7 ± 3.03 mV) was observed (Figure 5.10). In contrast, 20 ng PbTx-2 alone did not increase synaptoneurosomal membrane voltage-sensitive sodium channels. When used by themselves (TTX and STX), gave no change in membrane potential since although these neurotoxins block Na^+ conductance, Na^+ channels are already closed in the resting state. However, when these toxins were applied with VTD and PbTx-2 combination, no increase in membrane potential was observed (Figure 5.10), indicating strong antagonistic effects on sodium channel activation by both STX and TTX.

Dose-related enhancement in increase in membrane potential for brevetoxin and veratridine is illustrated in Figure 5.11 and 5.12. Results showed that with the addition of 1.67 μM VTD at varying concentration of PbTx, a dose-related potentiation of membrane depolarization is achieved, which supports the idea that these toxins act independently on sodium channels to promote synaptoneurosomal depolarization.

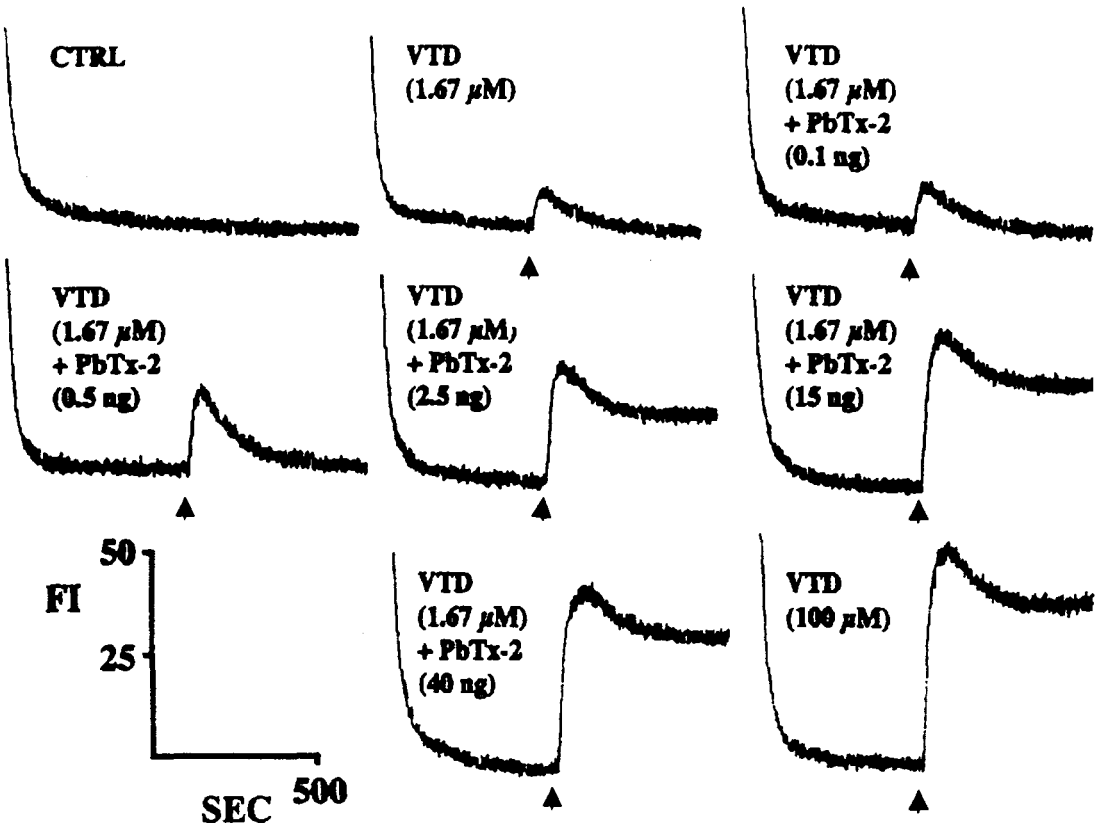


Figure 5.1. Rhodamine 6G fluorescence recordings illustrating potentiation of VTD's effect by different amounts of brevetoxin (PbTx). Potentiation is evident immediately after VTD (1.67 μM) is added. Synaptoneurosomes were pre-incubated with brevetoxin. (▲ = point at which 1.67 μM VTD was introduced in the assay)

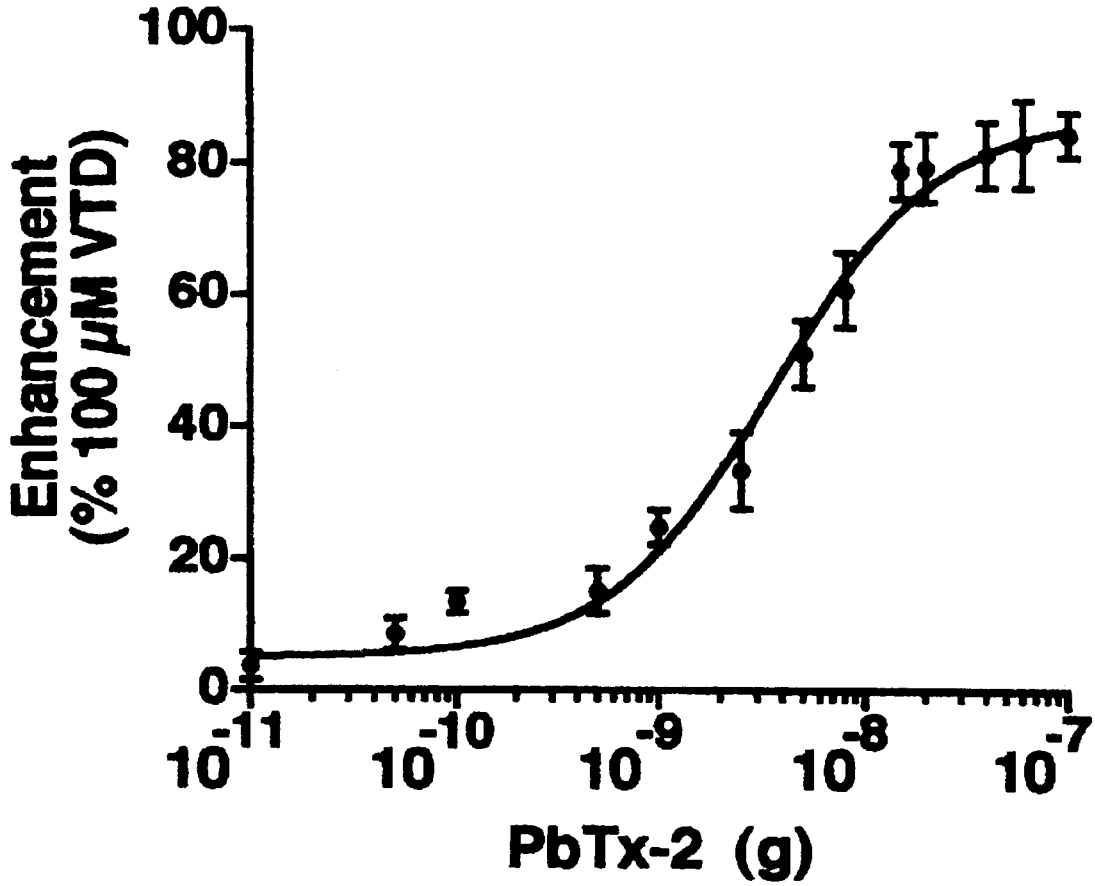


Figure 5.2. Standard calibration curve for PbTx-2 showing the relationship between the amount of standard brevetoxin and enhancement of veratridine-induced depolarization of mouse brain synaptoneurosomes. Values represent means ± se of at least four independent experiments.

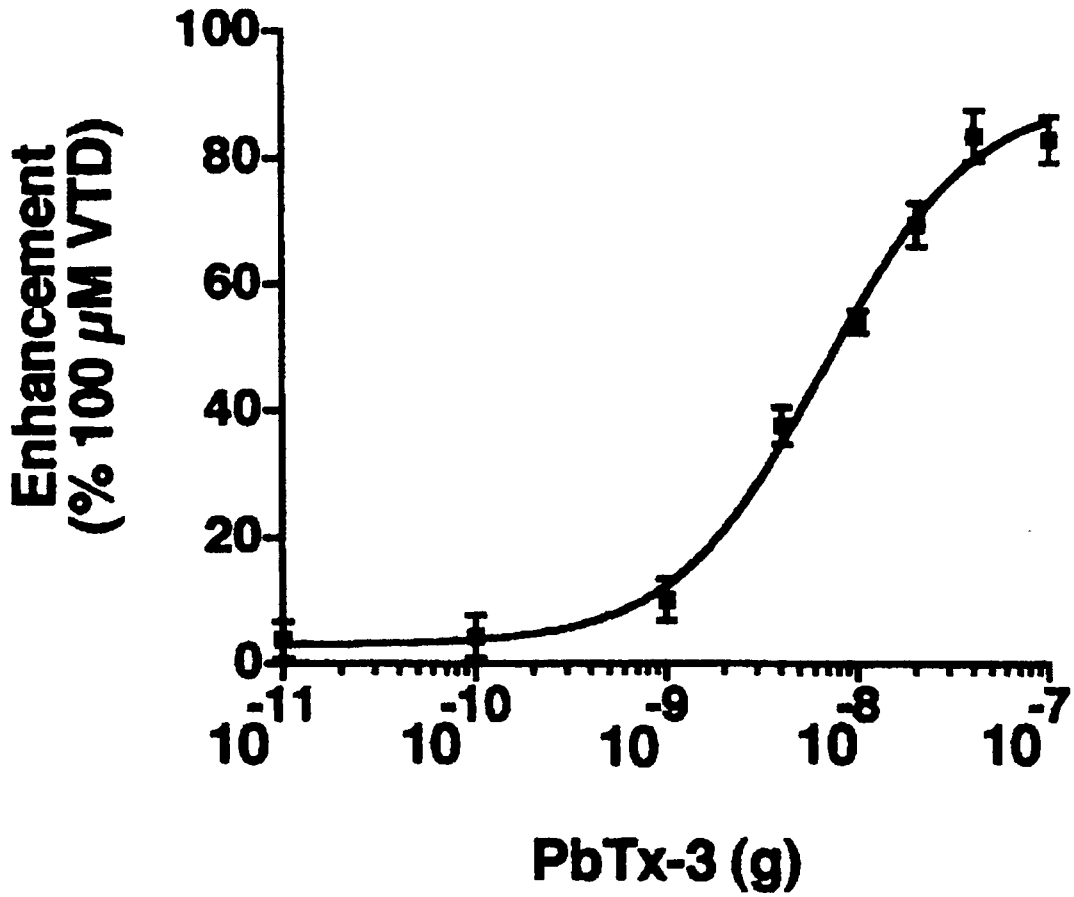


Figure 5.3. Standard calibration curve for PbTx-3 showing the relationship between the amount of standard brevetoxin and enhancement of veratridine-induced depolarization of mouse brain synaptoneurosomes. Values means ± se of at least three independent experiments.

Table 5.1. Estimates of brevetoxin levels in PbTx-2 spiked mussel extracts using mouse brain synaptoneurosome assay (MBSA) as compared to actual PbTx-2 values.

SAMPLE ID	SPIKED PbTx – 2 (ng/μL)	PbTx-2 MBSA (ng/μL)	SPIKED PbTx-2 (nM)	PbTx-2 MBSA (nM)
C	100	115.86	111,719	129,438
B	40	20.82	44,688	23,260
D	20	13.82	22,344	15,440
A	10	12.91	11,172	14,423
G	2	4.74	2234	5295
H	0.4	0.75	447	838
E	0.2	0.5	223	559
J	0.04	0.02	45	22
I	0.02	0.01	22	44
F	0.004	0.004	4	4

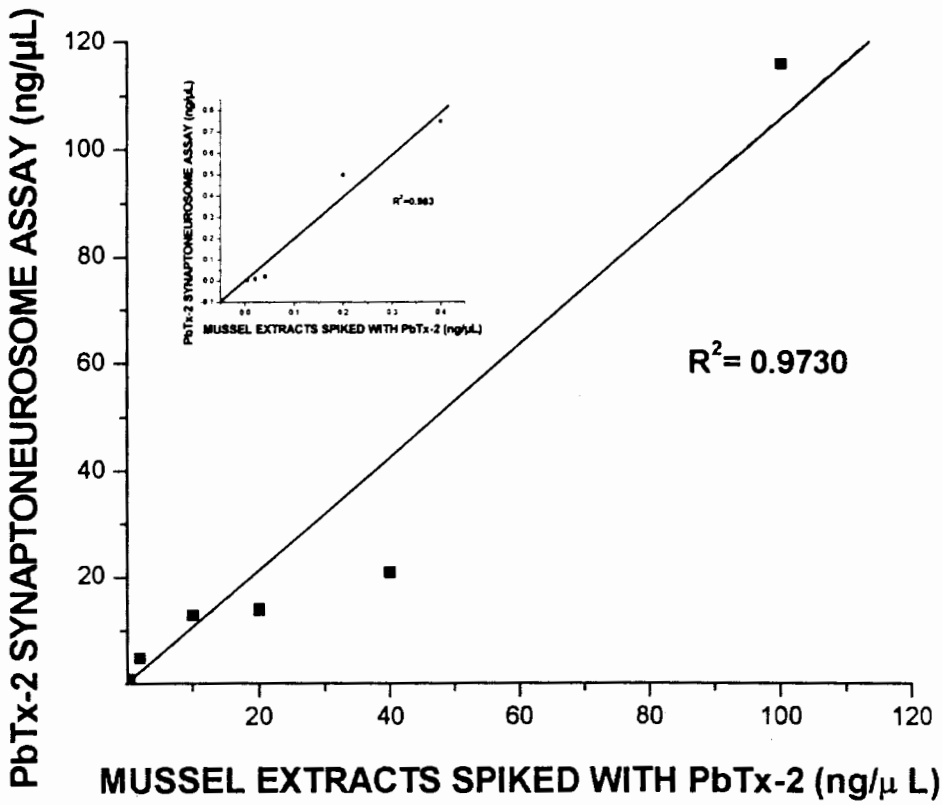


Figure 5.4a. Correlation of PbTx-2 equivalents in mussel extracts spiked with standard brevetoxin and PbTx-2 estimated with PbTx-2 mouse brain synaptoneurosome assay. Inset: PbTx-2 equivalents representing the lower part of the graph. (Slope = 1.08).

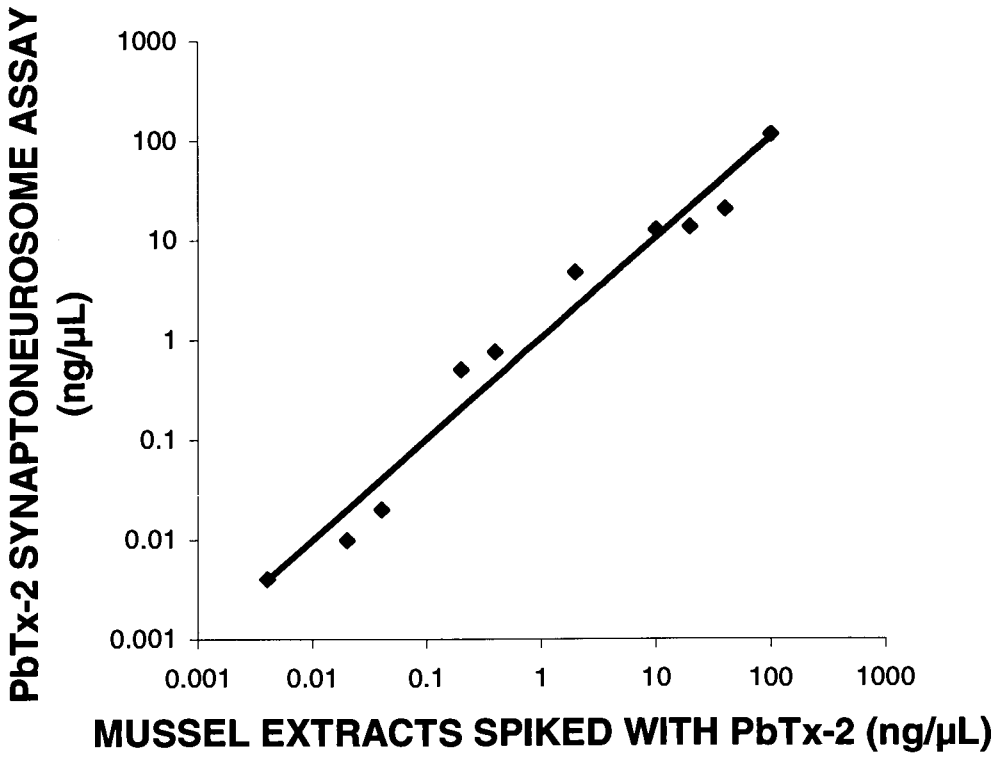


Figure 5.4b. Correlation of PbTx-2 equivalents in mussel extracts spiked with standard brevetoxin and PbTx-2 estimated with PbTx-2 mouse brain synaptoneurosome assay fitted in log scale. (R^2 = 0.9730 and Slope = 1.08 from linear scale).

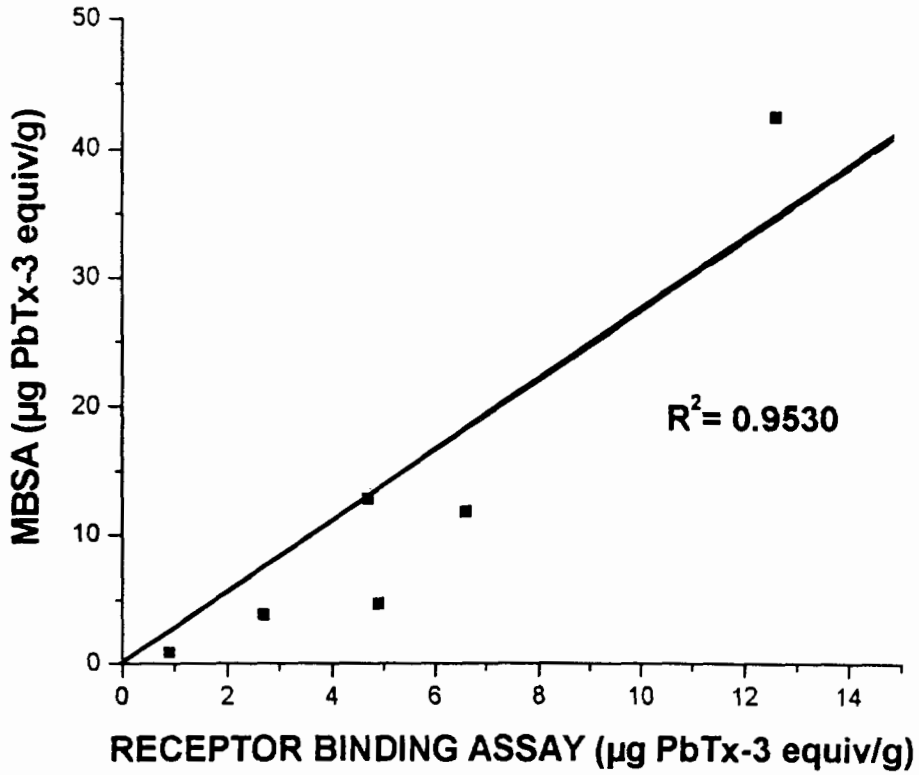


Figure 5.5. Correlation of PbTx-3 equivalents obtained from receptor binding assay and PbTx-3 mouse brain synaptoneurosome assay. Receptor binding assay data provided by Dr. Robert Dickey, FDA, Alabama, U.S.A. (Slope = 3.6).

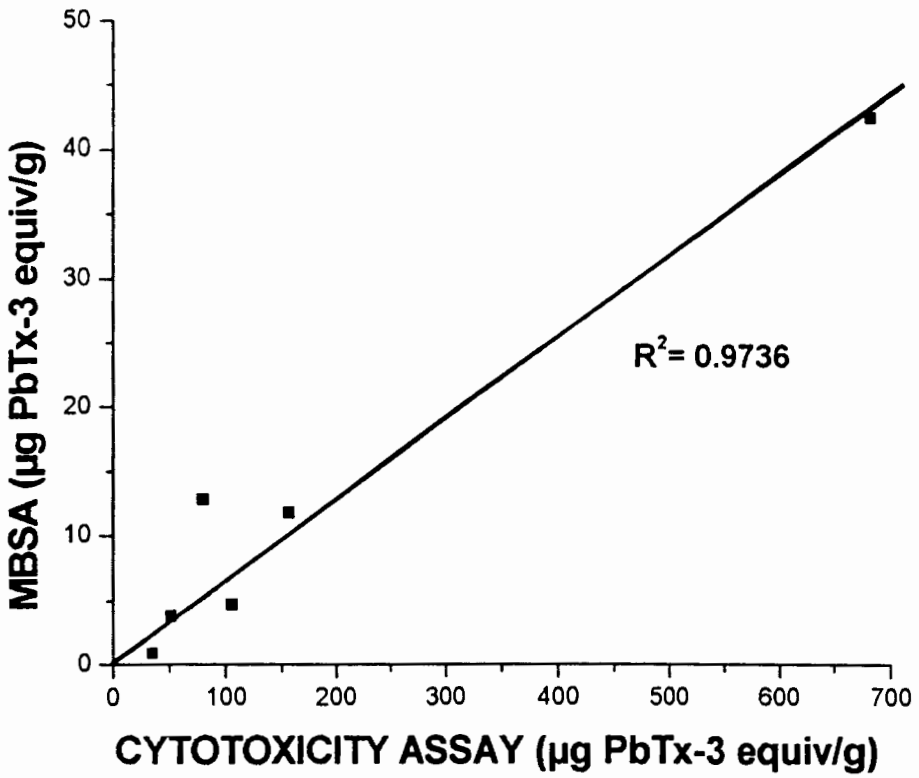


Figure 5.6. Correlation between cytotoxicity and PbTx-3 mouse brain synaptoneurosome assays. Cytotoxicity data provided by Dr. Robert Dickey, FDA, Alabama, U.S.A. (Slope = 0.06).

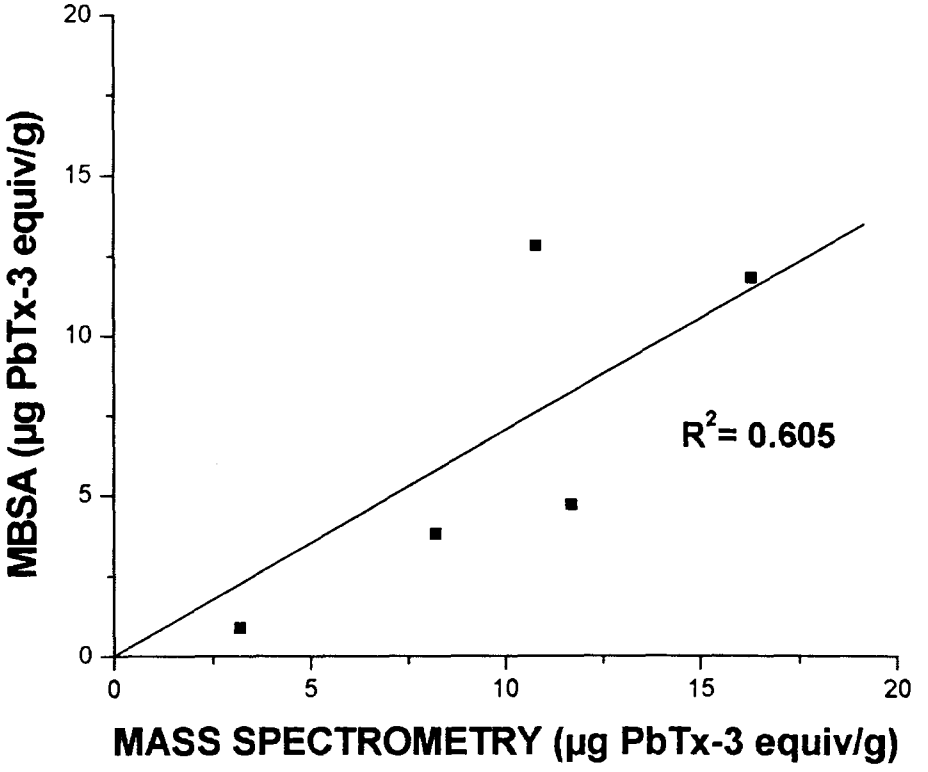


Figure 5.7. Correlation of PbTx-3 equivalents measured by mass spectrometric analysis and the mouse brain synaptoneurosome assay. Mass spectrometric data provided by Dr. Robert Dickey, FDA, Alabama, USA. (Slope = 0.84).

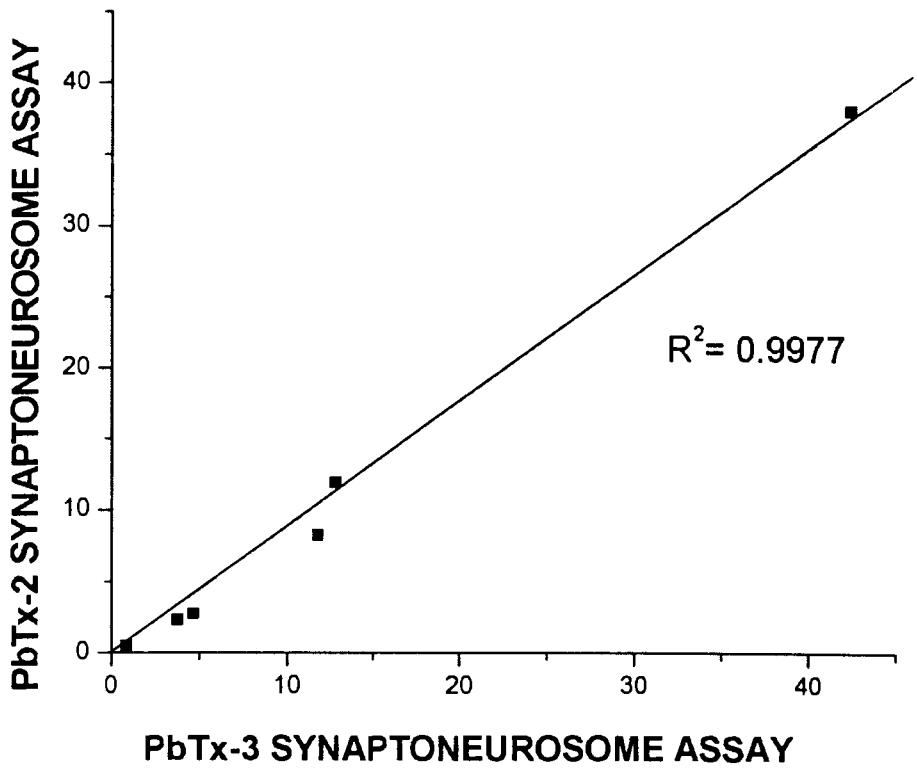


Figure 5.8. Correlation of brevetoxin estimates from oyster samples using standard curves for PbTx-2 and PbTx-3.

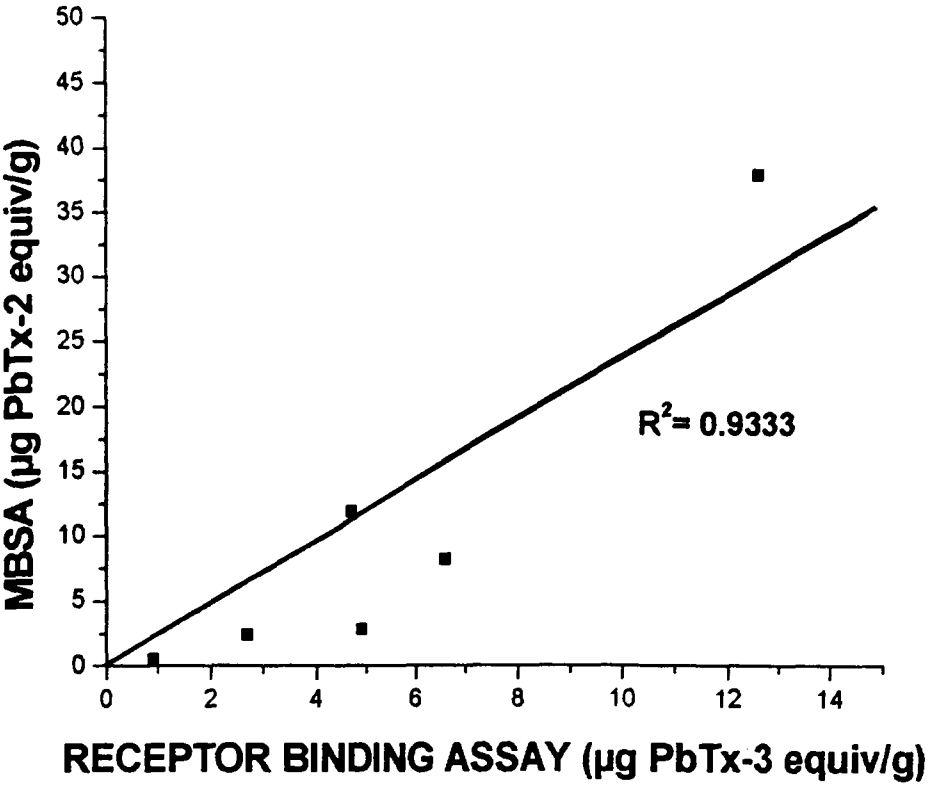


Figure 5.9. Comparative estimates of PbTx-3 equivalents using receptor binding assay with the estimated PbTx-3 content using the PbTx-2 calibration curve. Receptor binding data provided by Dr. Robert Dickey.

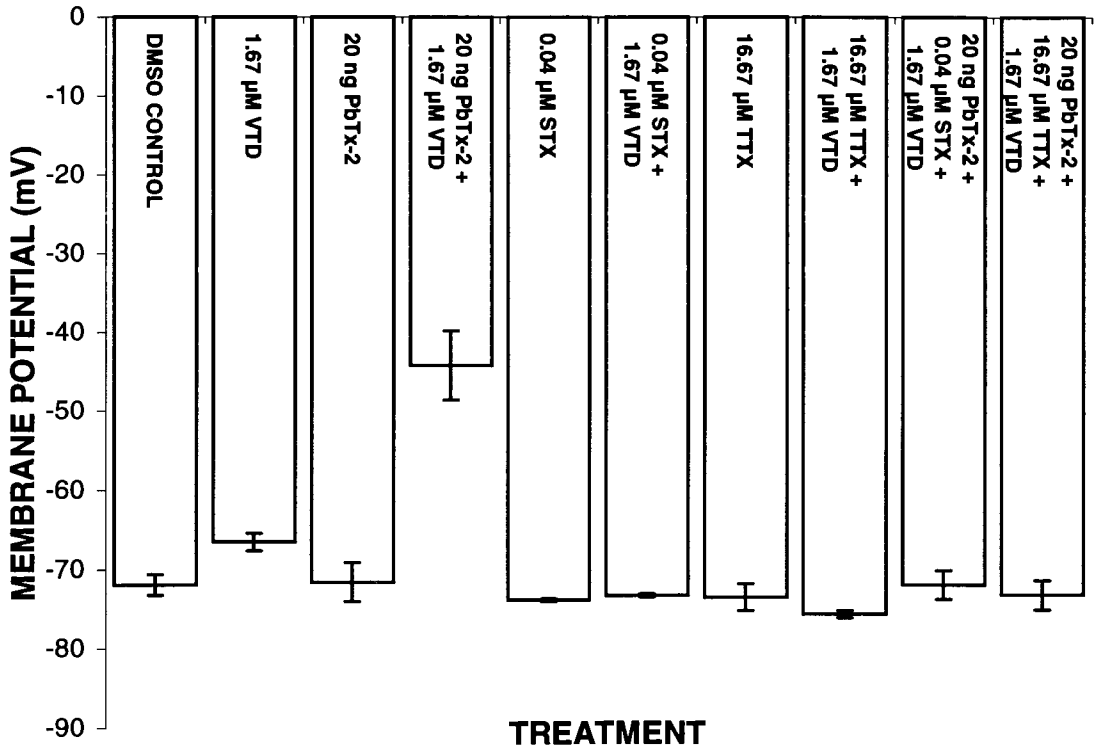


Figure 5.10. Potentiation of VTD-induced depolarization of synaptoneurosomes by maximum effect levels of PbTx-2 is totally blocked by the sodium channel blockers STX and TTX. Values expressed as mV of 3 – 10 independent experiments.

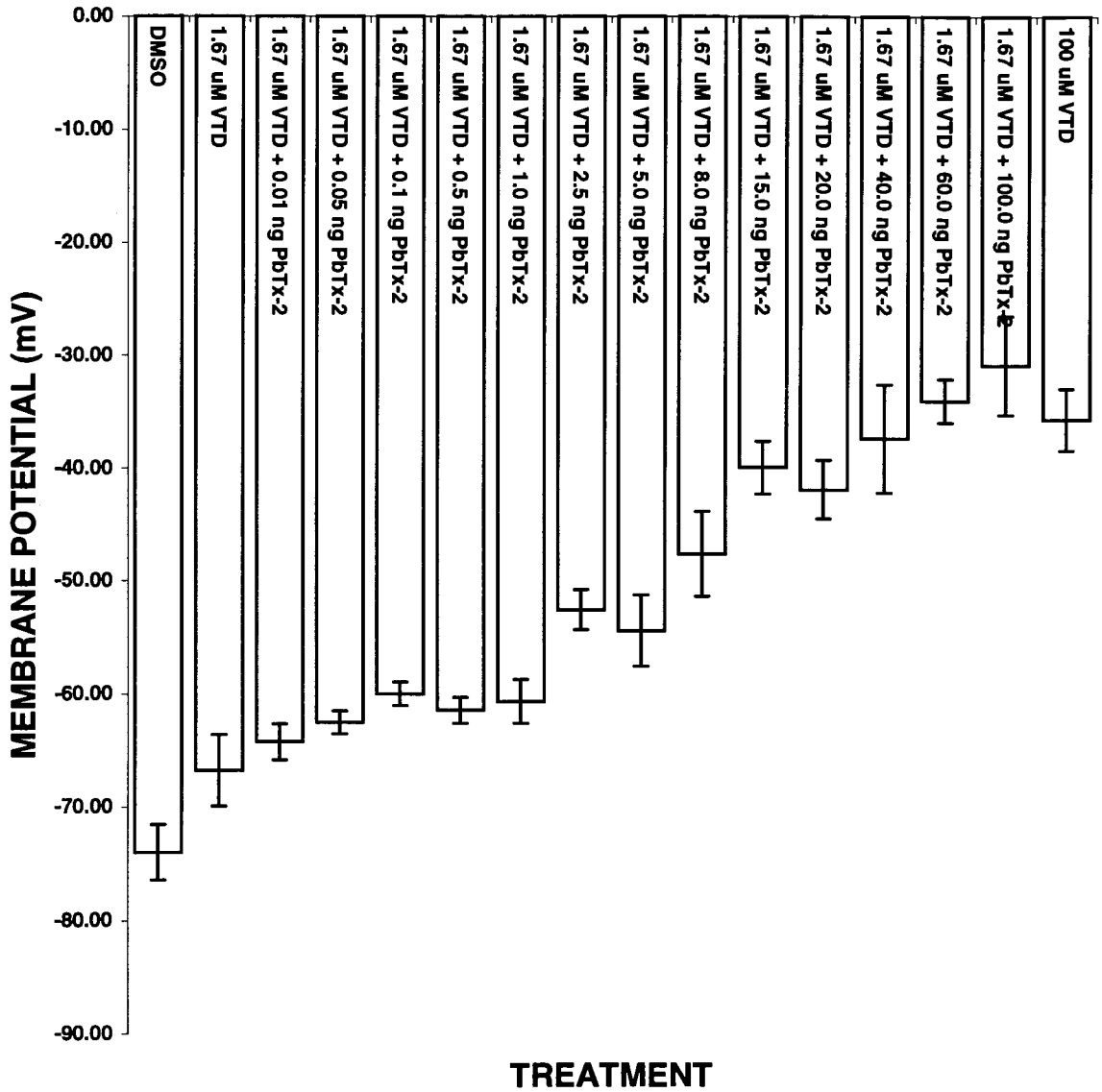


Figure 5.11. Dose-related enhancement of membrane depolarization with VTD (1.67 μM) by PbTx-2.Values are represented as mean ± se of at least four independent determinations.

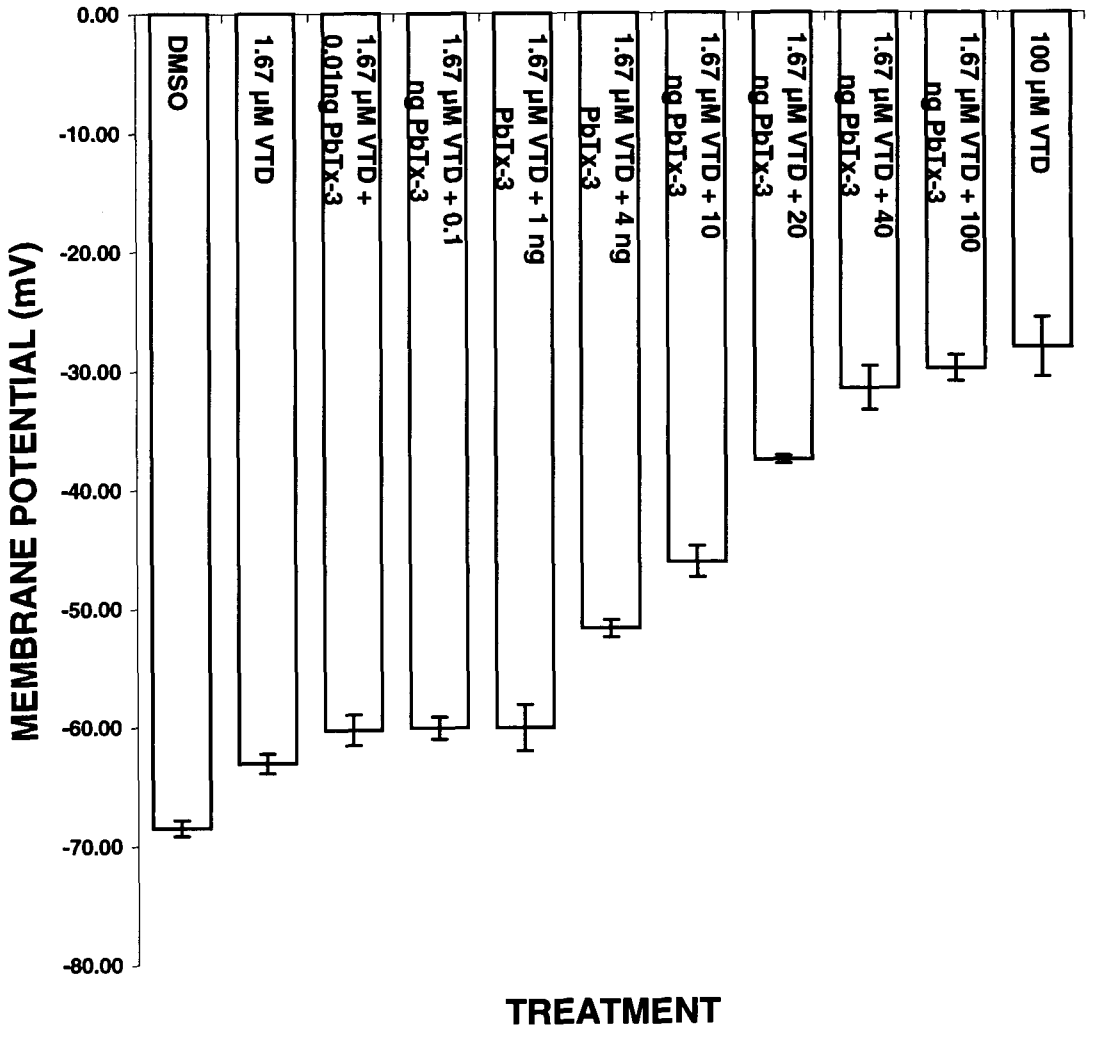


Figure 5.12. Dose-related enhancement of membrane depolarization with VTD (1.67 µM) by PbTx-3.Values are represented as mean ± se of at least four independent determinations.

5.4 DISCUSSION

The mouse brain synaptoneurosome assay described in this thesis represents a novel, and relatively rapid and reliable means of estimating brevetoxins (as PbTx-2 or PbTx-3 equivalents) in contaminated shellfish. Preliminary validation of the assay was investigated using mussel extracts spiked with brevetoxin while other experiments focused on quantitation of brevetoxin content of contaminated oysters. Values obtained from mussel samples showed a good agreement with the actual PbTx-2 spiked in all ten samples analyzed. Likewise, the oyster data derived by synaptoneurosome assay showed a high level of correlation with receptor binding and cytotoxicity data. A moderate linear correlation was also observed with HPLC/MS data. The synaptoneurosome assay relies on the ability of brevetoxin to enhance the fluorescence response of veratridine as measured with the fluorescent probe rhodamine 6G. Rhodamine 6G has been proven useful in similar *in vitro* assays. The two previous chapters described the used of this preparation in quantifying PSP content of mussel extracts. Assay of membrane potential using this fluoroprobe was previously described by Auichi *et al.*, (1982) and modified for synaptoneurosomes by Nicholson *et al.*, (2002). In the present experiments, rhodamine 6G was used as a voltage-sensitive probe to quantitate potentiation of VTD-induced depolarization of synaptoneurosome by brevetoxin. Veratridine is known for its ability to open sodium channels by binding to site 2 of the α-subunit. Brevetoxins bind with high affinity (Kd = 1 – 50 nM) to site 5 voltage-sensitve sodium channels (Van Dolah, 2000). Binding to this site alters the voltage sensitivity of the channel, resulting in opening of the channel under conditions in which it is normally closed whereas inhibition of channel inactivation results in persistent activation or prolongation of channel opening. Its action

on the sodium channel is similar to that of veratridine (Catterall, 1988; Catterall, 1992). Allosteric modulation of neurotoxin binding to voltage-sensitive sodium channels by brevetoxin was investigated by Sharkey *et al.*, (1987) and the results showed that the PbTx-2 increases the binding of [^{3}H]BTX-B. [^{3}H]BTX-B is also known to specifically bind at site 2 of the sodium channel, enhancing binding of radiolabelled β-scorpion toxin (site 4) and but has no effect on the binding of [^{3}H]-STX to site 1 or on the binding of [^{125}I]-LqTx on site 3. These results demonstrate that PbTx-2 allosterically modulates sites 2 and 4 by binding to site 5 on the sodium channel. Further, this also explains how brevetoxin enhances (potentiates) the effect of toxins like VTD which act on site 2 of the voltage-sensitive sodium channel.

The fact that PbTx potentiates the effect of VTD (Sharkey *et al.*, 1987; Lombet *et al.*, 1987; Dechraoui *et al.*, 1999), forms the basis for the development of the present assay for brevetoxin that satisfies the pharmacology and biochemistry of brevetoxin action on sodium channels. This approach was used to quantitate the NSP toxin content of shellfish samples in terms of PbTx-2 and PbTx-3 equivalents. In the process of establishing the standard curves for the brevetoxins, a series of experiments were carried out. Brevetoxins produced a dose-dependent enhancement of the VTD response in mouse brain synaptoneurosomes as illustrated in Figure 5.1. The potentiated response of VTD by PbTx is consistent with the finding of Sharkey *et al.*, (1987) and Lombet *et al.*, (1987) in binding experiments. In addition, Dechraoui *et al.*, (1999), in their experiment investigating cytotoxic effects of PbTx and VTD on neuroblastoma cells also showed a much more marked decrease in the viability of the cells when both neurotoxins were used.

PbTx-2 and PbTx-3 are amongst the more toxic components of brevetoxin extracts and have similar structural backbone type. Both possess to "B type" backbone which consists of one lactone ring and ten polycyclic ether rings (Poli *et al.*, 1995; Van Dolah, 2000; Baden and Adams, 2000). PbTx-3 only differs from PbTx-2 by the presence of two more hydrogens (H) on the side K ring chain of the tail region of PbTx-3. It is with this apparent similarity in structure that either standard curve is valid in estimating the NSP contents from shellfish samples in PbTx-2 and PbTx-3 equivalents.

Upon establishing the PbTx-2 standard curve, it was observed that the threshold for detection is approximately 0.6 ng per assay. The mouse brain synaptoneurosomal technique is more sensitive than the rat hippocampal slice preparation of Kerr *et al.*, (1999) and slightly more sensitive than the binding assay with rat brain synaptosomes (Whitney *et al.*, 1997). The EC_{50} of the present technique was estimated at 1.56 nM in the assay which indicates a lower the sensitivity using radioimmunoassay (Poli *et al.*, 1995), ELISA (Trainer and Baden, 1981) but a greater sensitivity than the excitotoxicity assay which uses rat cerebellar granule neurons (Berman and Murray, 1999) for both derivatives of brevetoxin. It was also noticeable in this technique that even high levels of brevetoxin do not significantly affect the resting potential of the synaptoneurosomes which is clearly illustrated by the minimal variation of fluorescence intensity readings compared to the vehicle control (Figure 5.1). This can be explained by the fact that brevetoxin binds preferentially to open Na^+ channels in most preparation and associates very slowly with the resting neuronal membrane, usually taking hours to reach the equilibrium (Whitney and Baden, 1996). However, with the addition of VTD will lead to immediate opening of the sodium channel, allow rapid access of brevetoxin to site 5 and

so facilitate the potentiating effect on membrane potential of the cell as previously reported. This dose-related enhance effect on synaptoneurosome's membrane potential provides basis for the current technique.

Similarly, at least three independent experiments were conducted to establish the PbTx-3 standard curve. The results showed that the curve is sigmoidal, the curve started to rise at about 10% and levelled off at about 87%. These results are similar in profile to the PbTx-2 standard curve. Since both toxins are of close structural similarity in terms of of the backbones (Poli *et al.*, 1995; Van Dolah, 2000; Baden and Adams, 2000) and the polycyclic H- I- J- K ring system and the A ring lactones which are critical for receptor recognition (Baden and Adams, 2000), it is therefore not unexpected for the two brevetoxin derivatives to have such pharmacological graphical profile. However, in terms of EC_{50} (enhancement concentration at 50%), PbTx-3 had higher EC_{50} (2.49 nM) which contradicts the previously reported potency of the two toxins. PbTx-2 was reported to have higher ED_{50} in rat brain synaptosome binding assay (Baden *et al.*, 1988), radioimmunoassay (Poli *et al.*, 1995), and cytotoxicity assay (Berman and Murray, 1999). Given that the membrane potential assay is a different functional test system, the interassay differences in the EC_{50}s for both toxins is not unexpected. Thus, synaptosome binding and radioimmunoassay rely on the affinity of the toxin to its receptor sites and do not take into consideration the other secondary mechanisms that may contribute to its potency. This secondary mechanism maybe in the form of conformational modulation of the sodium channel receptors and initiation and maintenance of secondary responses such as ion flux and membrane potential changes. Likewise, the EC_{50} for the cytotoxicity relies on the LDH activity which is not a clear representation of the sodium channel

pharmacology, but rather it more accurately represents cellular necrosis. The approximate detection limit for the PbTx-3 calibration curve is 1.0 ng/assay which is very close to the detection limit of PbTx-2. This supports the reproducibility and the potential of the assay to estimate NSP toxin levels in terms of either PbTx-2 or PbTx-3 equivalents. Moreover, the EC_{50}s for both derivatives correspond very well with the reported *Kds* of binding assays conducted by other investigators (Van Dolah *et al.*, 1994; Poli *et al.*, 1986) supporting the idea that our results are valid in terms of receptor binding theory and brevetoxin and sodium channel pharmacology.

All ten mussel extract samples spiked with PbTx-2 showed good correlation with the synaptoneurosomal technique. It is clear from the regression analysis (Figure 5.4a and b) that the PbTx-2 synaptoneurosome assay corresponds very well with the actual spiked PbTx-2. Among the ten samples, five samples were overestimated, four were underestimated and one was accurately estimated. However, when values from the assay and the actual values were plotted, the r^2 value is equal to 0.9730 and a slope of 1.08, which indicate that overall the assay is a valuable tool in predicting NSP contents of mussel samples. These variations are similar to those reported in other assays for PbTx (Dickey et al., 1999). For food safety reasons, a correction factor based on the slope should be applied on each of the estimated PbTx-3 levels in each sample.

Six oyster homogenates previously assayed by U.S. F.D.A. for the presence of PbTx-3 were assayed using the mouse brain synaptoneurosome assay and quantitated by using reference to PbTx-3 and PbTx-2 standard curves. Significantly, an excellent linear correlation was obtained in both receptor binding (r^2 = 0.9530) and cytotoxicity assays (0.9736). The fact that it has good correlation with the binding assay suggest strongly that

the current technique satisfied the toxins mechanism of interaction in the sodium channel. Although, the synaptoneurosome assay underestimates the PbTx-3 equivalents at about ten-fold, the fact that they have very good correlation, it is still safe to say that using a correction factor based on slope the technique can predict relative order of potency of the oyster extracts using a correction factor based on slope. Furthermore, overestimation of brevetoxin content was also observed between receptor binding and cytotoxicity assays (Dickey *et al.*, 1999). When the assay was correlated with HPLC mass spectrometry (HPLC/MS) a moderate linear correlation (r^2 = 0.605) was observed. This is not unexpected since this instrumental analysis require the separation and quantification of individual congeners to yield an estimate total toxin content (Van Dolah *et al.*, 1994), hence the possibility of compound degradation prior to analysis is high. Any discrepancy observed can be resolved by applying correction factor based on each slopes of each correlation.

Estimation of PbTx-3 equivalents using PbTx-2 standard curve was performed. The result proved that using either standard curves, a very good correlation on overall estimate on order of toxic potency was observed. Regression analysis on quantified oyster samples using both standard curves showed an excellent linear correlation (r^2 = 0.9977). The finding proved that one standard curve for toxins having the same backbone can precisely estimate the toxic potency of shellfish samples using mouse brain synaptoneurosome. The result from the regression analysis of the oyster samples estimated using the established PbTx-2 standard curve and the receptor binding assay of oyster samples with PbTx-3 further support the contention that the assay using either standard curves can accurately predict brevetoxins relative order of potency.

Tetrodotoxin (TTX) and saxitoxin (STX), neurotoxins known acting specifically on the voltage-sensitive sodium channels (VSSCs) were also examine in the synaptoneurosome assay. At low concentrations of VTD (1.67 μM) alone, there was small but consistent increase in fluorescence compared with the large (potentiated) increase in fluorescence response when 20 ng of PbTx-2 was combined with VTD. This response was completely blocked when either 50 ng STX or 16.67 μM TTX was added, similarly, when these toxins were used in *lieu* of PbTx in the assay, no increase in fluorescence was noted but a slight hyperpolarization was observed. Since TTX and STX hyperpolarize a few mV compared to the control (resting state) it indicates that there may be a very low level of sodium channel opening occurring in the resting state, allowing slight influx of sodium ions which creates a slightly more positive potential inside the synaptoneurosome. STX and TTX block sodium channels hence the potential shifts to a few mV in the negative direction. From these observations, the assay appears fully consistent with PbTx pharmacology supporting the contention that it is measuring an interaction with voltage-gated sodium channels, the prime target of toxicological relevance.

As mentioned previously, the preparation of synaptoneurosomes from mouse brain is straightforward and takes approximately 40 minutes to complete. An individual assay provides a result within 30 minutes. This is certainly faster than the longer incubation (> 1 hour) required for radioimmunoassay (Baden *et al.*, 1988; Poli *et al.*, 1995), receptor binding assay (Leighfield *et al.*, 1996; Baden *et al.*, 1988) and neuroblastoma cell assay (Dechraoui *et al.*, 1999; Dickey *et al.*, 1999). Moreover, the present technique is also faster than the high throughput pharmacologic assay of Van

Dolah *et al.*, (1994). It is noteworthy that, as has been proposed for the pharmacological assay of Van Dolah *et al.*, (1994), the present neurobiochemical assay for brevetoxin using synaptoneurosomes has the potential to be adapted into higher throughput. This can be achieved by adapting the assay to a 96 well microtitre plate format with integral automated fluorescence detection.

Aside from its potential as high throughput assay, the present technique also permits a minimum of twenty individual synaptoneurosomal assays to be performed using one mouse brain. Hence, an equivalent amount of data can be generated by fewer animals compared to mouse toxicity bioassay. In the preparation of mouse brain synaptoneurosomes, animals were euthanized by rapid cervical dislocation, hence, eliminating significant amount of distress and discomfort to the animals.

In this study, an alternative approach on quantifying NSP contents on shellfish samples was developed. This assay is based on a pharmacological interaction of brevetoxin with to the voltage-sensitive sodium channel (VSSC), the target of toxicological relevance. Pharmacological assays are particularly suitable for determination of toxic potency of a sample as proven through the analysis of mussel and oyster samples studied. Though some assays, for instance immunoassays are highly sensitive, specific for a given toxin and can potentially adapted to a field kit type format suitable for dockside or shipboard, however, these assays are based on toxin structure and not on toxin efficacy (Van Dolah *et al.*, 1994). Further, receptor binding assays, though they are based on pharmacology of brevetoxin, one noteworthy disadvantage is its longer incubation before a single result is obtained.

An assay based on preparation containing voltage-sensitive sodium channels like synaptoneurosomes looks promising not only because of its accurate reflection of composite toxin potency but also because this assay can be used to detect other sodium channel toxins. Hence, the assay developed in this study has potential to significantly help in routine monitoring of marine phycotoxins, thereby alleviating problems of human intoxication.

CHAPTER SIX

CONCLUSION

The results of the studies have demonstrated that mouse brain synaptoneurosomes demonstrate robust toxin-specific pharmacology and neurobiochemistry of saxitoxin and brevetoxin. The development and validation of assays using this mammalian brain preparation confirmed its potential in the rapid and reliable detections of these two important groups of marine neurotoxins. The relative ease of synaptoneurosomal preparation (taking approximately 40 minutes) and its strong viability (no deterioration for at least 6 hours after preparation) in predicting membrane potential response have proven to be useful in estimating toxin contents in the shellfish samples. The results from the battery of different biologic samples suggest the versatility of mouse brain synaptoneurosome in the assessment of toxin contents of the specific samples being assayed. However, it is premature to suggest that the synaptoneurosome preparation will effectively discriminate marine biotoxins in samples containing more than one toxic component. In the present technique, if STX is present in PbTx samples being analyzed it is likely the 1.67 μM VTD will be reduced alerting the investigator to contamination with a Na^+ channel blocker. But, given the fact that toxic algal blooms involving more than one toxin-producing species are relatively rare, the preparation should be useful in the assessment of toxic contents of shellfish or finfish phycotoxin contamination.

Previously the synaptoneurosomal technique has been validated using samples assayed with mouse toxicity bioassay (Nicholson *et al.*, 2002), in my study the first phase included further validation of the technique using spiked mussel samples and plankton

samples assayed with radioligand binding assay. I confirmed that the study that the synaptoneurosomal technique is a rapid (individual assay provides a result within six minutes) and a valuable tool in estimating PSP contents on both biological samples. The assay was also shown to be highly reproducible as the IC_{50} obtained from the current study is consistent with the IC_{50} calculated from the previous study by Nicholson *et al.*, (2002). During the course of analysis on biological samples I was able to establish that: (a) the technique can detect PSP content down to 21 nM (plankton samples) and 48 nM (mussel extracts); (b) the mouse brain synaptoneurosome assay can easily predict the the rank order of potency concentration of the test samples in STX equivalents; (c) the assay produced a very good correlation with the actual values of the spiked samples; and (d) results from spiked samples analysis showed that at very low saxitoxin values, the assay underestimated PSP content by approximately half the actual and the same was true for plankton samples. Hence, it is recommended for conservative reason to apply correction factor based on the calculated slopes. With these observations, it is clearly apparent that membrane potential assay using mouse brain synaptoneurosome is rapid, reliable and sensitive assay in examining samples intoxicated with PSP from contaminated shellfish.

This membrane potential assay using mouse brain synaptoneurosome relies on the ability of PSP toxin content of mussels to block veratridine-induced depolarization of synaptoneurosomes. Veratridine is an alkaloid derived from veratrum plants that is active primarily on site 2 of the voltage-sensitive sodium channels (Catterall, 1980; Catterall, 1988; Catterall, 1992). An investigation on the potential of another veratrum alkaloid that has similar pharmacological action but significantly less expensive has been undertaken. A mixture of veratrum alkaloids known as veratrine was evaluated.

Veratrine, about 9-fold cheaper than veratridine was shown to be a potential substitute. Significantly, the result from the membrane potential assay using veratrine showed very good correlation with the spiked mussel samples. The assay can detect PSP toxin levels down to 36 nM (3 μg STX per 100 g) shellfish tissue. Moreover, the assay using veratrine, was shown to be slightly more sensitive than the assay using veratridine. This was confirmed during "side by side" comparison on their IC_{50}s. However, since veratrine is a mixture of alkaloids of which veratridine is included, further studies is needed to confirm if other veratrum alkaloids from the mixture may have enhance effect on the veratrine that created such slight sensitivity. Overall, the result on this second phase of study proved the potential of veratrine as substitute for veratridine in estimating PSP toxin levels in mussel samples. It is also suggested based on the findings that further validation is needed in other biological samples to ensure that saxitoxin and its toxic congeners can be adequately quantitated by this technique and is highly suited for PSP hazard associated with shellfish consumption.

Perhaps the most significant contribution of this research is the development of a rapid, reproducible and reliable assay for brevetoxin. My results corroborated brevetoxin's known effect on voltage-sensitive sodium channels since it was inhibited by two neurotoxins whose only known action is sodium channel blockade.

Initially, a standard curve for PbTx-2 was established and this was validated against mussel samples spiked with known amount of PbTx-2. A very good agreement between the amount of PbTx-2 spiked and the estimate of the assay was obtained. Hence, PbTx-2 synaptoneurosomal assay proved that it could predict NSP toxin content from mussel samples.

Using the same technique, PbTx-3 (a closely related derivative of brevetoxin B) was also assayed. The standard curve was shown to have a similar profile to the established PbTx-2 standard curve. Moreover, using oyster samples previously quantitated using receptor binding, cytotoxicity and HPLC mass spectrophotometry were tested to validate the mouse brain synaptoneurosome assay. The results showed that the synaptoneurosomal technique correlates very well with both cytotoxicity and receptor binding assays and gives a moderate linear correlation with HPLC/MS. The observation that synaptoneurosomal results correlate very well with the receptor binding data also supports the idea that the results of the studies are valid in terms of brevetoxin/sodium channel pharmacology and biochemical binding to its receptor. The results from the present study also indicate that synaptoneurosomal technique can be applied to a variety of bivalve species important for shellfish industry.

Finally, with my results demonstrate that the CD1 mouse brain synaptoneurosomal technique has clear potential as a rapid, reliable and sensitive alternative to live animal testing for both PSP and NSP bioactives in shellfish tissues. It has also the potential to estimate toxins in plankton extracts. One would predict that this technique will also have potential to quantitate other marine toxins acting on site 1 (such as TTX) and site 5 (such as CTX) on the voltage-sensitive sodium channel.

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