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ANALYSIS AND PURIFICATION OF DOMOIC ACID FROM THE BLUE MUSSEL (*Mytilus edulis*), ITS RELATIONSHIP TO TOXICITY AND EFFECTS ON CALCIUM UPTAKE.

A Thesis

Submitted to the Graduate Faculty

in Partial Fulfillment of the Requirements

for the Degree of

Master of Science

in the Department of Anatomy and Physiology

Faculty of Veterinary Medicine

University of Prince Edward Island

Bryan Grimmelt

Charlottetown, P.E.I.

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ABSTRACT

The emergence of an unknown shellfish toxin in late 1987 in eastern Prince Edward Island initiated an extensive investigation by many laboratories culminating in the conclusion that the primary etiological factor was domoic acid (DA). Studies were performed relating mouse toxicity to domoic acid levels. Monitoring of eastern blue mussels (Mytilus edulis), contaminated with DA, involved mouse bioassays and quantitative analysis using high pressure liquid chromatography (HPLC). Mice undergo a typical scratching syndrome at sublethal as well as at lethal doses of DA. The onset of scratching behaviour and lethality in mice were inversely correlated to the dosage of DA. An LD50 (i.p.) value of 3.6 mg DA/kg mouse was calculated. Toxic mussels held in tanks containing uncontaminated sea water showed a decline in DA concentration in mussel tissue with time. In addition DA concentrations in mussels from two affected rivers declined with time under normal environmental conditions. The bulk of the DA and the toxicity was located in the digestive glands. These results support the conclusion that domoic acid was the primary causative factor in the shellfish poisonings from P.E.I. mussels in late 1987. An extraction method for DA which resulted in enhanced recovery of DA was developed by utilizing a Bligh and Dyer lipid extraction procedure and recovering the DA in the aqueous phase. Since DA has limited commercial availability, a new method of purification was developed which involved dissection of the digestive glands, lipid extraction, lyophilization, ultrafiltration, and reverse phase HPLC. The DA appeared to be pure based on analytical HPLC, spectrophotometry, and amino acid analysis. Effects of DA on calcium uptake in the central nervous system of guinea pigs were investigated. Domoic acid did not stimulate calcium uptake in synaptosomes or synaptoneurosomes from guinea pig brains. L-glutamate and depolarizing levels of potassium did cause a stimulation of calcium uptake in these preparations. These results suggest that DA does not directly open calcium channels "in vitro".

ACKNOWLEDGEMENTS

I would like to express my sincere thanks to Dr. M.S. Nijjar for his encouragement and confidence in me and the project during the past two years.

I would also like to thank the members of my advisory committee, namely, Drs: J.F. Amend, L. Bate, and J. Bellamy for helping to oversee the project.

This project was considerably enriched due to the help of Neil MacNair, Sandy Wagner, and Dr. Gerry Johnson of the Department of Pathology and Microbiology, AVC. Chapter two was the result of a collaborative effort, allowing data from mouse bioassays and HPLC analysis to be interwoven.

I would also like to thank Drs: William P. Ireland and Andrew Tasker for their many (**free!**) consultations and scholarly assistance; Heather Briand for her help with guinea pigs; and Roland Cusack for his editorial commentary.

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USE OF ANIMALS

Research involving the use of animals was performed in accordance with the guidelines of the Canadian Council of Animal Care.

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ABBREVIATIONS

AA:	Acetic acid
AUFS:	Absorbance units full scale
AVC:	Atlantic Veterinary College
°C:	Degrees celsius
$CaCl_2$:	Calcium chloride
CI:	Confidence interval
CNS:	Central nervous system
cpm:	Counts per minute
DA:	Domoic acid
DFO:	Department of Fisheries and Oceans
DHKA:	Dihydrokainic acid
EAA:	Excitatory amino acids
EDTA:	Ethylenediaminetetraacetic acid
FMOC:	9-fluorenylmethylchloroformate
g:	gram
HCl:	Hydrochloric acid
HPLC:	High Pressure Liquid Chromatography
h:	Hour
IDT:	Inverse of death time
i.p.:	Intraperitoneally
IST:	Inverse of scratching time
KA:	Kainic acid
KCl:	Potassium chloride
Kg:	Kilogram

KH_2PO_4:	Potassium phosphate
L:	Liter
ln:	Natural logarithm
LTP:	Long term potentiation
M:	Molarity
mg:	Milligram
$MgCl_2$:	Magnesium chloride
ml:	Milliliter
mM:	Millimolar
min:	Minute
N:	Normality
NaCl:	Sodium chloride
NaOH:	Sodium hydroxide
$NaHCO_3$:	Sodium bicarbonate
nm:	Nanometer
NMDA:	N-methyl-D-aspartate
NRC:	National Research Council
p:	Page
PA:	Phosphoric acid
P.E.I.	Prince Edward Island
ppm:	Part per million
psi:	Pounds per square inch
PSP:	Paralytic shellfish poison
QQ:	Quisqualic acid
RF:	Response factor
SSA:	Sulfosalicylic acid

s:	Second
s.d.:	Standard deviation
TFA:	Trifluoroacetic acid
TLC:	Thin Layer Chromatography
μCi:	Microcurie
μg:	Microgram
μl:	Microliter
μM:	Micromolar
uv:	ultraviolet
X g:	Centrifugal force

SECTION 1

1 INTRODUCTION TO DOMOIC ACID

1.1 EMERGENCE OF PROBLEM

In late 1987 a mysterious toxin was traced to cultured mussels harvested from estuaries in eastern Prince Edward Island (P.E.I.). The symptoms in humans who consumed contaminated mussels involved vomiting, diarrhea, cramps, disorientation and memory loss, and resulted in the death of at least three and possibly four individuals. An intense effort to identify the toxin was carried out by a task force comprised of the Department of Fisheries and Oceans, Health and Welfare Canada, the Department of Environment, the National Research Council (NRC), the P.E.I. Department of Fisheries, and the Atlantic Veterinary College (AVC), (1). The toxin was identified as domoic acid, (DA, Figure 1), a little known neurotoxin with a structural similarity to kainic acid (KA). Domoic acid had previously been found in two species of red algae: *Chondria armata* from Japan, and *Alsidium corallinum* in the Mediterranean (2,3).

1.2 DESCRIPTION OF DOMOIC ACID:

Domoic acid possesses an imino ring structure

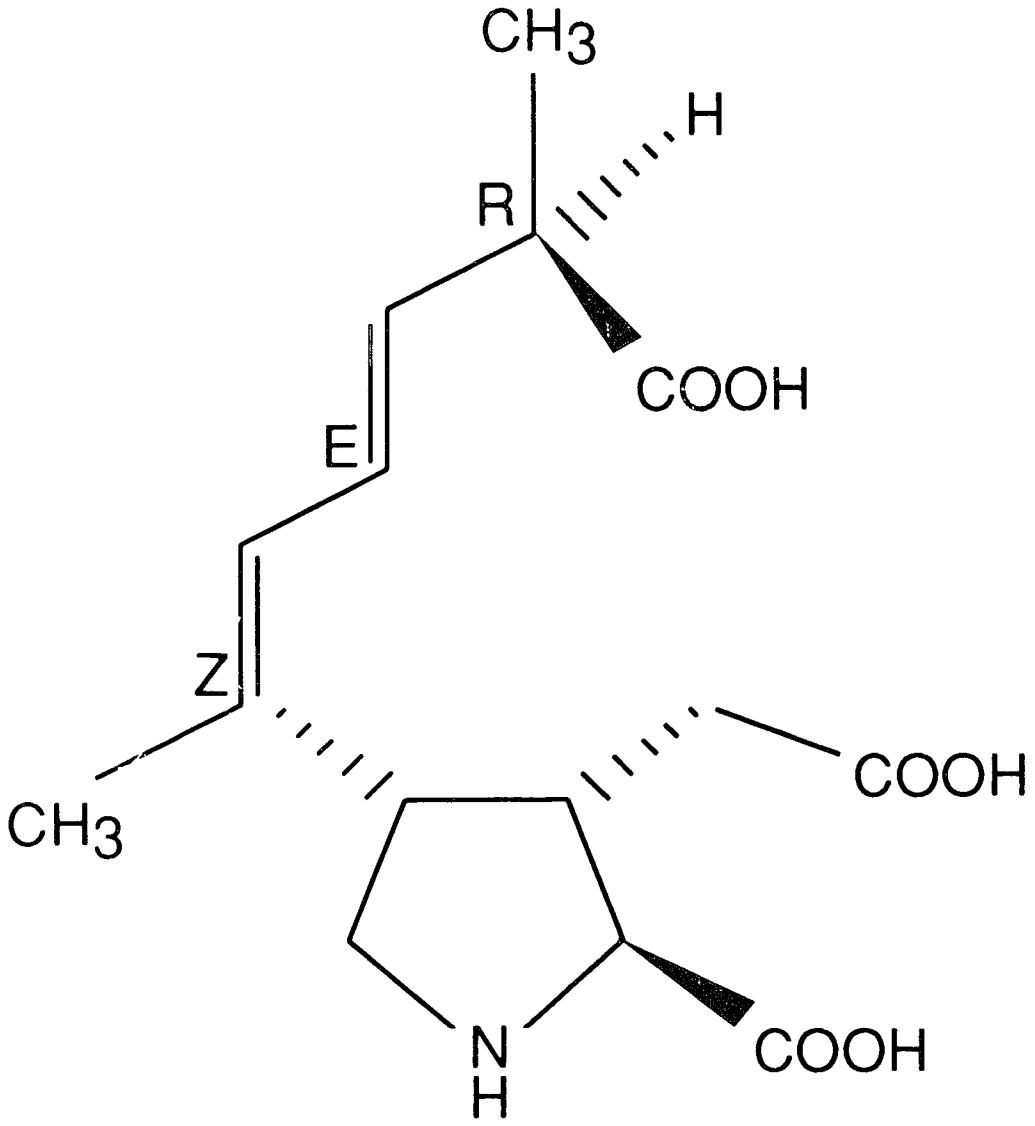


Figure 1. Structure of Domoic acid.

similar to pyroglutamic acid and proline, and is considered a conformationally restricted analogue of glutamic acid. It contains a total of three carboxyl groups along with its imino group, which cause it to be a zwitterion which is weakly acidic in water. Its pKa values are 2.10, 3.72, 4.93, and 9.82 (3). Domoic acid posesses an elaborate side chain consisting of two isoprene units with conjugated double bonds which are responsible for an absorption maximum of 244 nanometers. Its chemical name is (2S,3S,4S)-2-carboxy-4-isopropenylpyrrolidine-3-acetic acid. This indicates that a large number of stereoisomers of DA can potentially exist and indeed a number of them have been isolated (4). Due to the complex structure and rarity of DA it is unlikely to be synthesized by animals, which are much more limited than plants in their biosynthetic capabilities (5).

1.3 ANALYSIS AND PURIFICATION OF DOMOIC ACID:

Shortly after DA was determined to be the mussel toxin, a method for DA analysis based on reverse phase high pressure liquid chromatography (HPLC) was released (6). A similar method, outlined by the Atlantic Research Laboratory of the NRC, used 0.1% trifluoroacetic acid to

shorten the analysis time (1). Since DA is an imino acid, a number of methodologies for the analysis of imino acids can be adapted for the quantification of DA (7).

Due to the paucity of commercial DA, an economical purification method for DA could help to further research into the physiological effects of this molecule.

1.4 TOXICITY OF DOMOIC ACID:

Domoic acid is a potent neurotoxin which induces a profound and characteristic seizure disorder in mice (8). Symptoms observed in humans after consuming DA contaminated mussels varied from slight nausea and vomiting to muscle weakness, disorientation, and memory loss. In the 207 reported cases, 81% had nausea 54% vomiting, 42% diarrhea, 36% cramps, 35% headache, 32% loss of equilibrium, and 17% had loss of memory (9).

Domoic acid appears to possess the greatest toxicity relative to its isomers (10). This may be due to the cis relationship of the side chains and the pi electrons in the first double bond, since alpha-allokainate and dihydrokainate have relatively low potency (11).

1.5 NEUROCHEMISTRY:

1.5.1 Neurotoxicity:

Domoic acid, an anthelmintic used to expel pinworms (*Enterobium vermicularis* L.), was studied for its insecticidal properties (3,10). Since the invertebrate and vertebrate nervous systems share many properties, including L-glutamate as an excitatory neurotransmitter, it is not surprising that DA is also toxic to mammals, including humans. The mechanism of its neurotoxicity is generally considered to be the same as KA neurotoxicity (11).

Aspartic acid and glutamic acid are believed to be the primary excitatory neurotransmitters of the mammalian central nervous system (CNS), (11). Receptors for glutamic acid and aspartic acid have been classified according to their responsiveness to three different agonists: quisqualate (QQ), N-methyl-D-aspartate (NMDA), and KA (13). While L-aspartate is the most likely candidate for the endogenous neurotransmitter at the NMDA receptor, L-glutamate has affinity for all three receptors (13).

Kainic acid receptors occur in the cerebral cortex, hippocampus, cerebellum, retina, and the spinal cord (14). These receptors comprise about 10% of excitatory amino acid receptors and appear to be localized both pre-

and post-synaptically (15).

Kainic acid destroys post-synaptic cells while sparing afferent terminals on these cells (11,16). There is a high correlation between KA binding sites and vulnerability of neurons to KA (17, 18). Domoic acid has a greater affinity for the KA receptor than KA and increased neurotoxic potency (19). While DA binds at the KA receptor, it does not interact appreciably with other glutamate receptors (20, 21). Therefore, the current theoretical framework supports the idea that DA toxicity is most likely mediated through effects at KA receptors.

1.5.2 Excitotoxicity:

Domoic acid satisfies the criteria of an excitatory amino acid (EAA), (19, 20). A number of hypothesis have been proposed to explain the neurotoxic properties of EAAs. The excito-toxic hypothesis proposed by Olney suggested that the toxicity is a result of overstimulation of glutamic acid receptors (18). This was based on studies which showed correlations between toxicity and excitation with some excitotoxins (18). However, other scientists found a poor correlation between neurotoxic and excitatory effects of some agonists (22). Whereas NMDA and QQ caused powerful convulsions, they were relatively weak toxins (22).

Domoic acid and KA, which were much less efficacious in their tremorogenic properties, were more potent at causing neuronal degeneration (22). Accordingly neuronal excitation appears to be an incomplete explanation for DA toxicity. One speculation is that KA is taken up pre-synaptically and biotransformed into a toxic metabolite (18). Other possible mechanisms of KA and DA toxicity are through stimulation of glutamate release and/or inhibition of uptake mechanisms (18). This could result in increased levels of inter-synaptic glutamate which could cause the toxicity (18). Direct stimulation of post-synaptic KA receptors could also be a factor in DA toxicity (18).

The stimulation of post-synaptic KA receptors is insufficient for neurotoxicity, yet these receptors may be required for neurotoxic effects (18). Thus, a twofold mechanism was proposed to explain KA induced cell death (23). First, an interaction with pre-synaptic receptors could increase the extracellular concentration of endogenous excitatory amino acid neurotransmitters. Second, activity at post-synaptic receptors could potentiate the neurotoxicity of L-glutamate and/or L-aspartate (23).

1.5.3 EFFECTS OF DOMOIC ACID ON CALCIUM IN THE CNS:

1.5.3.1 Calcium uptake:

Calcium is intricately involved in the normal functioning of the nervous system (24). When an action potential reaches a nerve ending, one of its major effects is to increase the intracellular level of calcium through voltage-dependent calcium channels. This results in increased release of neurotransmitters (25). In a physiological situation, the extracellular calcium in brain is about 10^{-4} M, while the intracellular level is maintained at about 10^{-7} M. Intact cytostructure and metabolic energy are required to maintain this gradient. Increased intracellular levels of calcium can occur as a result of the opening of calcium channels in the cell membrane (26).

It is possible that DA opens presynaptic receptor-operated calcium channels. An influx of calcium would cause increased release of L-glutamate into the synaptic cleft resulting in enhanced toxicity of postsynaptic neurons. An extension of this hypothesis is that DA could also potentiate the opening of NMDA postsynaptic calcium channels which are responsive to glutamate and/or aspartate (27).

1.5.3.2 Calcium-induced toxicity:

The toxic effects of excitatory amino acids may be a result of calcium overload. There is a close connection between increased calcium influx and cell death (24). While KA and other neurotoxic amino acids caused increased calcium influx into rat brain cortical cells, non-neurotoxic acidic amino acids did not affect calcium uptake (28). Also, KA-induced neurotoxicity in cerebellar slices can be prevented by eliminating calcium from extracellular fluid in a tissue bath (27). These findings support the theory that KA results in increased calcium influx *in vivo* and that elevated calcium plays a role in KA toxicosis. A related phenomenon which is likely involved is the depletion of energy reserves in the post-synaptic neurons. The maintenance of a high calcium gradient outside neurons costs the cells a large amount of energy for calcium transport. Thus, prolonged depolarization could be one of the ultimate causes for chronically increased intracellular calcium, which may in turn augment the death rate of the neurons. Calcium may cause the degeneration of nerves through stimulation of calcium dependent proteases and lipases (28).

1.6 OBJECTIVES OF THE PRESENT STUDY:

The work presented in this thesis was focused on exploring the link between domoic acid contaminated mussel extracts and toxicity; establishing reliable methods of analysis and purification for this toxin; and investigating the question of whether domoic acid causes increased levels of presynaptic calcium in selected areas of the CNS of guinea pigs.

SECTION 2

2. RELATIONSHIP BETWEEN DOMOIC ACID LEVELS IN THE BLUE MUSSEL (*Mytilus edulis*) AND TOXICITY IN MICE

2.1 INTRODUCTION:

The emergence of domoic acid as an ecologically and economically important neurotoxin began in November 1987, when two cases of food poisoning in Montreal were linked to contaminated mussels harvested from Prince Edward Island (P.E.I.), (29). The symptoms in affected individuals included nausea, abdominal cramps, followed by neurological disorders such as confusion, loss of memory, ataxia and coma. After a concerted effort by many groups, the causative agent was determined to be domoic acid (DA), (1).

Domoic acid is considered to operate like its structural analogue, kainic acid (KA). Kainic acid is a potent excitotoxin which may elicit neurotoxic effects via an interaction at specific glutamic acid receptors (22).

During the early monitoring of mussel samples for toxicity, the use of mouse tests was the only means of screening samples for DA poisoning. The mice injected with contaminated mussel extracts underwent typical behavioural symptoms which included scratching and convulsions prior to death (8). The samples which showed

positive toxicity on mice were analyzed by high pressure liquid chromatography (HPLC) to measure the levels of DA. These data were used to investigate the relationship between toxicity in mice and concentration of DA in mussel extracts; examine the distribution of DA in mussel tissue; and to determine the changes in the level of DA in mussels from Cardigan river and St. Mary's bay over time. In addition an LD_{50} (i.p.) for DA in contaminated mussel extracts was calculated.

2.2 MATERIALS AND METHODS:

2.2.1 Materials:

Materials were purchased as indicated: acetonitrile HPLC grade (BDH Inc., Dartmouth NS); maleic acid (Sigma Chemical Company, St. Louis Mo.); acetic acid, phosphoric acid (Fisher Scientific Limited, Dartmouth NS)); 0.22 μm organic filters, 0.8 μm Millex filters. Deionized water was obtained by filtering distilled water through a Milli-Q™ system, (Millipore Corporation, Bedford MA). A standard solution of DA (117μg/ml) was provided by the Atlantic Regional Laboratory of NRC in Halifax.

2.2.2 Mussel extracts:

Mussel samples were collected from rivers in eastern

P.E.I. by the Department of Fisheries and Oceans (DFO, Charlottetown Branch). The locations of all the mussel samples were recorded. A sample of mussels collected from the Cardigan river and held in tanks flushed with Murray river water for 18 days was also collected. Extracts were prepared either by DFO or at AVC using a standard method for extraction of paralytic shellfish poison (30). Mussel meat was removed from the shells and the tissue was homogenized in a blender with an equal volume of 0.18N HCl. The pH of the homogenate was adjusted to 3.5 $\pm$ 0.5 with NaOH or HCl. The homogenate was boiled with constant stirring for 5-7 min and allowed to cool to room temperature. Distilled water was added to restore the original volume and the pH adjusted to 3.3 -3.8 using NaOH or HCL. The resulting mixture was centrifuged at 1500 X g for 5 min. The supernatant was removed and frozen until used in mouse bioassays and HPLC analysis.

2.2.3 Mouse bioassays[1]:

CD1 female mice weighing 17-29 g were used in determining the toxicity of the shellfish extracts. Mice

---

[1] The data from mouse bioassays was obtained in cooperation with Neil MacNair, Sandy Wagner and Gerry Johnson of the Fish Health Unit at AVC. Their help and camaraderie was very much appreciated.

were either purchased from Charles River Laboratories (Montreal, QB) or bred at AVC. Three mice were injected intraperitoneally with 1ml of each test sample and observed until death or for four hours. The weights, time of the first scratching behaviour, and time of death were recorded. Some extracts of intermediate toxicity were tested on groups of 6-12 mice, which allowed an LD_{50} (i.p.) to be calculated.

2.2.4 HPLC sample preparation:

The extract was prepared as described above and 5ml was centrifuged at 66,000 X g for 30 min. The resulting supernatant was deproteinized by one of the following two methods:

1) Sulfosalicylic acid (SSA) -50% w/v- was added to the supernatant in the ratio 1:10 and the denatured proteins were sedimented by centrifugation at 1500 X g for 10 min in a table top centrifuge (Beckman, Model TJ-6)) at room temperature. The resulting supernatants were diluted with phosphoric acid (PA) adjusted mobile phase, filtered through 0.8μm Millex-PF filters, and the pH of the filtrate was adjusted to 3.0.

2) The supernatant was, alternatively, deproteinized by

boiling for 10 min and centrifugation for 30 min at 66,000 X g in a refrigerated ultracentrifuge (Beckman, Model L8-70M). The resulting supernatants were diluted with PA adjusted mobile phase and filtered through 0.8μm Millex-PF filters using a plastic syringe. This resulted in clean preparations which allowed a good resolution of different components by HPLC. Comparable recoveries of DA were achieved after deproteinization by both techniques, although the measurements of SSA-precipitated supernatants were less sensitive (possessed a smaller signal to noise ratio) than the boiled supernatants.

2.2.5 Measurement of domoic acid by HPLC:

The shellfish extracts prepurified by one of the above two techniques were analyzed by reverse phase HPLC. The mobile phase was 12.5% acetonitrile in deionized water, pH adjusted to 3.0 with 85% PA (2 drops/L), filtered through 0.2μm organic filters, and then degassed by ultrasonification for 15 min. The column was Pecosphere® 5μm C18 -0.46mm i.d. (Perkin Elmer, Norwalk Conn) with a μBondapak® C18 cartridge guard column (Waters Division of Millipore, Mississauga ON). The flow rate was 0.75 ml/min. Domoic acid was monitored by v detection at 242nm. A standard curve was made using primary standard from NRC, and secondary standards were

analyzed after every five samples. The accuracy of the method was verified by an interlaboratory quality control study initiated by Health and Welfare Canada on June 16 1988. The HPLC system contained Gilson pumps (Model 302), a Gilson ultraviolet detector (Model 116), Gilson datamaster (Model 620) connected to 704 HPLC system manager software on an Apple II-C computer. The method required 12 to 15 min between samples. The retention time for DA was generally 7-10 min and was automated so that samples could be run overnight.

2.2.6 Tissue concentration of domoic acid:

Twenty mussels were randomly selected from a highly toxic collection of steamed mussels which had been shown to have 413μg/ml DA in a water extraction of whole mussels. The digestive glands of these mussels were dissected out, homogenized in an equal amount of water and extracted in parallel with a random sample of the rest of the mussel tissue by this method (section 3.2.2).

2.2.7 Statistical methods:

The inverse of the average death and scratching times were correlated to the DA concentrations in the extracts using Minitab software (Version 6.1.1 Minitab®

Inc. 1987). The amount of DA measured in the extracts was divided by the weight of the mouse for each test. The dosage (μg DA per g mouse) was also correlated to the inverse of the death time (IDT), and inverse of scratching time (IST). Regression analysis, nscores, and analysis of residuals (to test for normal distribution), were also done on minitab. Calculation of LD_{50} (i.p.) was done by using a program developed by Dr. William P. Ireland of AVC (Personal communication), which uses linear regression of a probit transformation of the death rate versus the log dose (31).

2.3 RESULTS:

2.3.1 Correlation between levels of DA and toxicity in mice:

Initial graphs of the average death time and onset of scratching versus the levels of DA measured in each extract showed an inverse relationship. The IST (Figure 2) , and IDT were then regressed against the DA concentrations.

In order to make full use of the data, the amount of DA measured in the extracts was divided by the weight of the mouse for each sample tested. The IDT (Figure 3) and IST were regressed against the DA concentrations per gram mouse. The correlation for the relationship between

IST and DA dosage (r=.904, n=114) was slightly higher than the correlation for the relationship between the averaged IST (for groups of three mice), and levels of DA (r=.859 ,n=34). This was also true for the correlations between IDT and dosage (r=.942, n=61), compared to averaged IDT and the DA levels (r=.934, n=16). The regression equations are further elaborated in appendix A.

2.3.2 LD_{50} (i.p.) for domoic acid contaminated mussel extracts:

The LD_{50} (i.p.), calculated by linear regression of probit transformed data, was 83.51 μg DA/mouse (n=9 groups) with a 95% confidence interval of 75.1 - 92.9. The correlation coefficient was 0.72. The average weight of the mice used was 23.5 $\pm$ 3.6 g (mean $\pm$ sd, n=134). This gave an LD_{50} value of 3.6 mg/Kg mouse (95% CI= 3.2-4.0).

2.3.3 Distribution of domoic acid in mussel tissue:

The toxicity, and the DA, in contaminated mussels was found predominantly in the digestive glands (Appendix A: Tables A.1 and A.2). On the average the digestive glands comprised 30% of the weight and contained 93.4 $\pm$

1.9 % (mean ± sd, n=4) of the total DA measured in these mussels.

2.3.4 Domoic acid levels in Cardigan River and St. Mary's bay:

The mussels harvested from various leases in Cardigan river and St. Mary's bay contained the highest levels of DA in Nov. 1987, which decreased to negligible levels by Jan 1988 (Figures 4, 5). The toxicity as measured by mouse bioassay decreased concurrently. The initial rise in DA occured before sampling began. Contaminated mussels from Cardigan river which had been flushed with uncontaminated Murray river water for 18 days showed no toxicity in mice and contained no measurable DA (detection limit = 2ppm).

2.4 DISCUSSION:

The LD_{50} (i.p.) value was calculated from the data on mouse bioassays collected between the period of December 1987 to May 1988. Since the amount of DA injected per mouse rather than the dosage was regulated, the LD_{50} (i.p.) is initially calculated in terms of μg DA per mouse. The correlations of IDT and IST with respect to levels of DA per mouse were only slightly lower than

the correlations obtained with regard to dosage. This suggests that the weight of each mouse is not as critical a factor in determining the toxic effects of the extracts as might be expected from weight alone. It is possible that heavier mice absorb relatively more of the toxin or that other compensating factors reduce the effect of weight on toxicity. There are no other LD_{50} (i.p.) values for DA available in the scientific literature. An unpublished communication by Health and Welfare Canada listed an approximate value of 5 mg/Kg which is consistent with the value reported here.

Strong relationships between the onset of scratching behaviour and death time with the amount of DA administered to mice were obtained (Figures 2, 3). The decline in shellfish toxicity from the Cardigan River and St. Mary's Bay was accompanied by a parallel decrease in DA concentration in shellfish extracts from the respective rivers over the months of November, 1987 until March, 1988 (Figures 4, 5). The toxicity and DA concentration in toxic mussels from the Cardigan River diminished to negligible levels when these mussels were flushed with non-contaminated water from the Murray River. These results demonstrate that DA and toxicity can be eliminated from contaminated mussels by flushing the toxic mussels with non-contaminated water.

The majority of the toxicity and 93.4% of the DA

was found in the extract from the digestive glands, whereas the remaining tissue contained low levels of DA and toxicity. This suggests that the toxin did not originate in the mussels, but was consumed by these organisms.

The results from this study demonstrate a significant connection between DA levels in mussel extracts and toxicity symptoms in mice as measured by two indices: onset of scratching behaviour and time of death. The primary tissue containing the toxin was shown to be the digestive gland. In addition the decline in toxicity with time coincided with decreased DA levels. An LD_{50} (i.p.), for DA contaminated mussel extracts in mice, of 3.6 mg/Kg was calculated from the available data. These observations support the conclusion of a nor-mussel origin to the toxin and DA as the primary causative agent of toxicity from consumption of mussels harvested from eastern Prince Edward Island in late 1987.

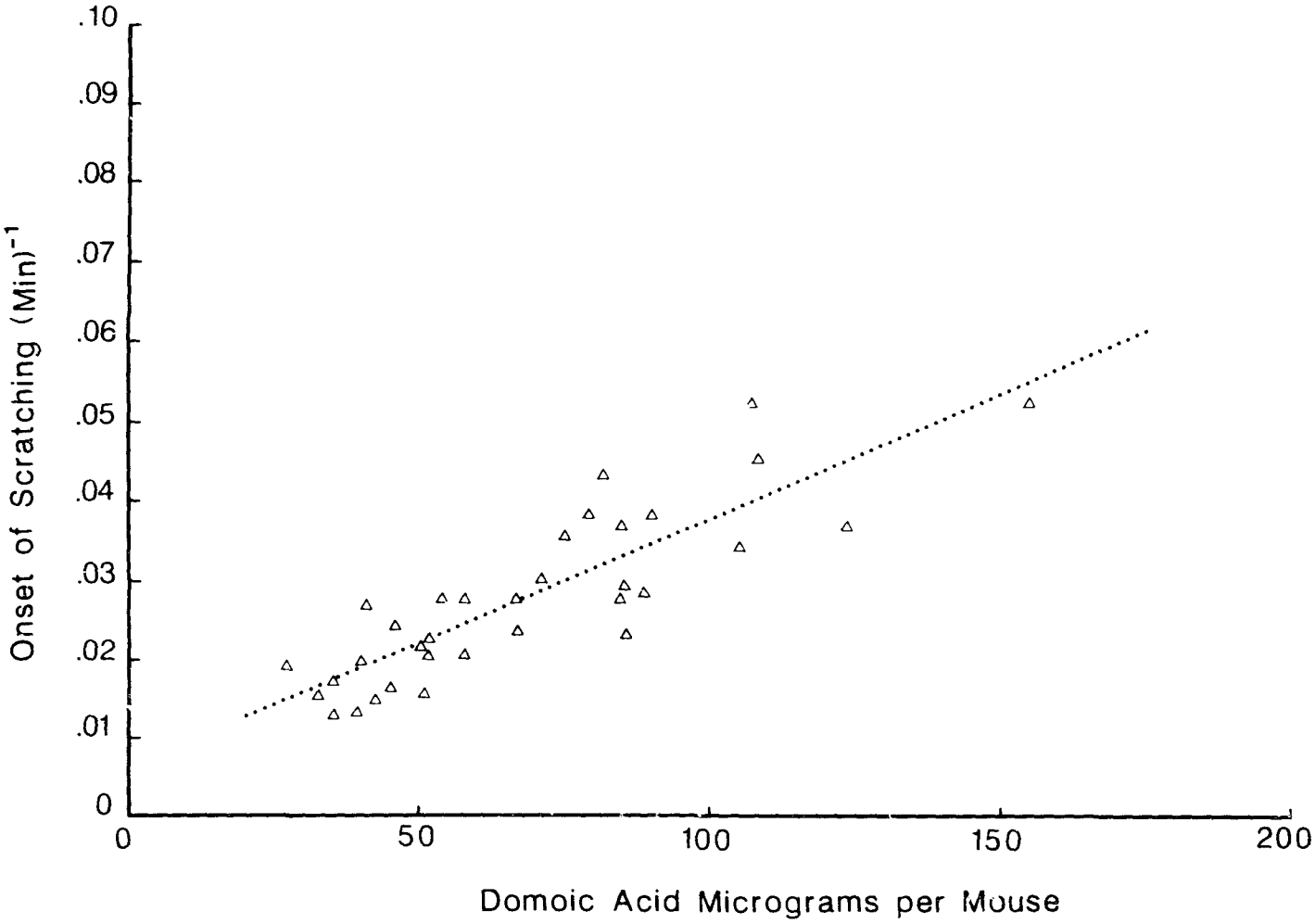


Figure 2. Inverse of onset of scratching time correlated to levels of domoic acid in mussel extracts. Each point represents the scratching onset of three mice injected intraperitoneally with domoic acid contaminated mussel extract.

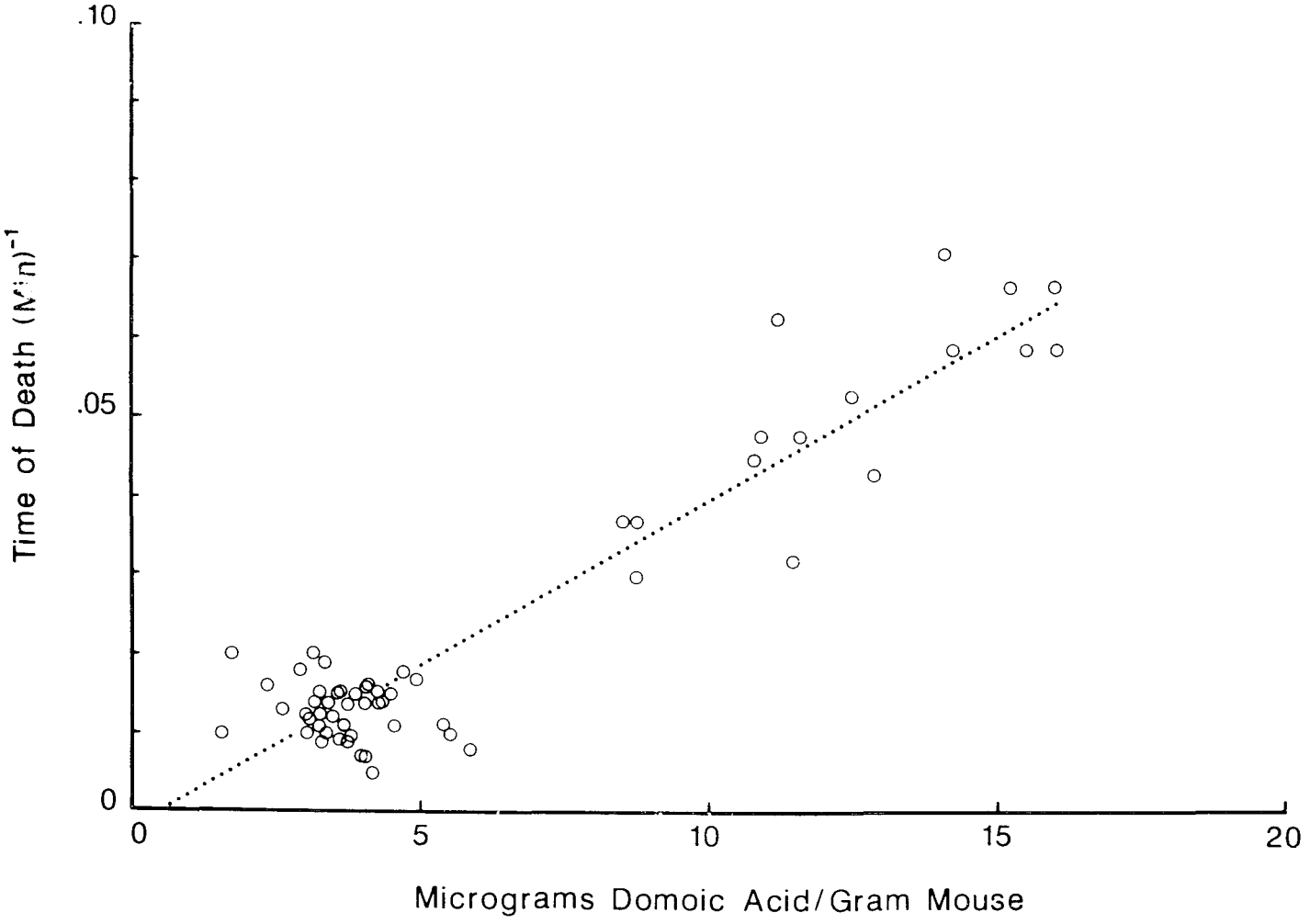


Figure 3. Inverse of death time correlated to dosage of domoic acid in mussel extracts. The line represents a least squares regression of the data. Each point represents one mouse injected intraperitoneally with domoic acid contaminated mussel extract.

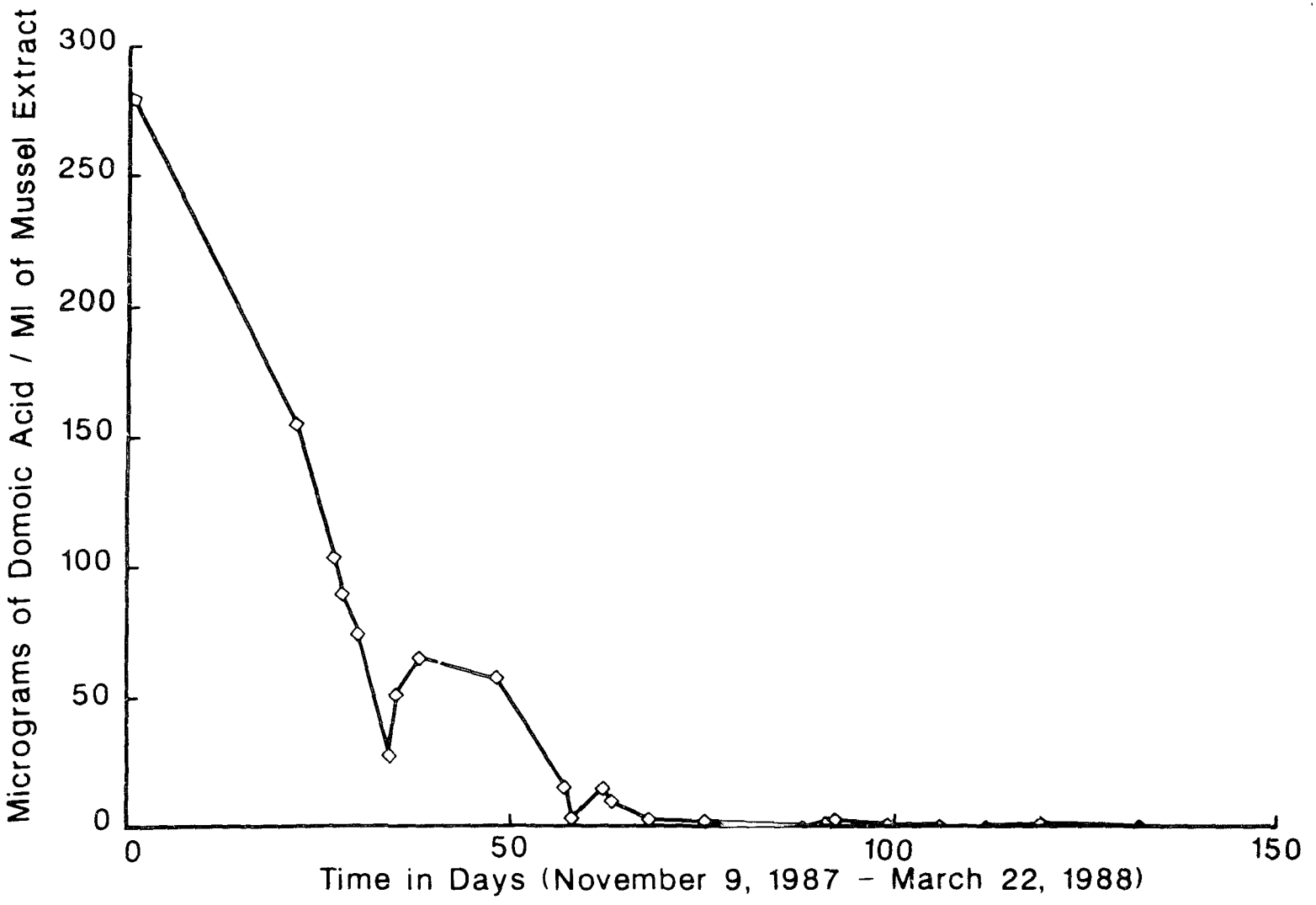


Figure 4. Decline of domoic acid levels in mussels collected from the Cardigan river.

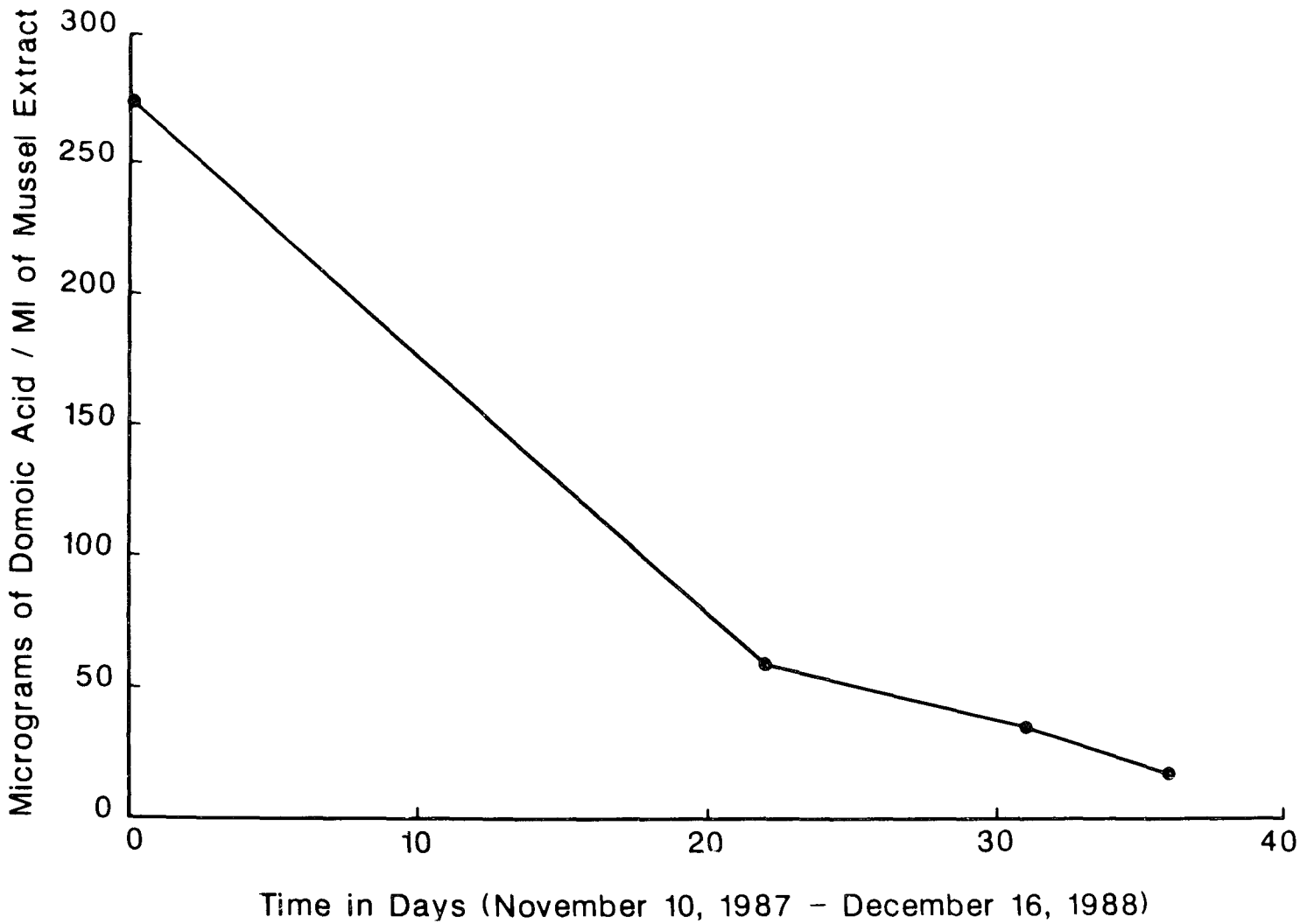


Figure 5. Decline of domoic acid levels in mussels collected from St. Mary's bay.

SECTION 3

3. A QUANTITATIVE METHOD FOR THE EXTRACTION AND PURIFICATION OF DOMOIC ACID FROM THE TISSUES OF THE BLUE MUSSEL (*Mytilus edulis*)

3.1 INTRODUCTION:

The identification of domoic acid (DA) as the primary toxin found in toxic blue mussels from eastern Prince Edward Island (P.E.I.), (1), in late 1987, led to a need for quantitative analysis and purification of the compound. Due to the novelty of the toxin, initial extraction methods for DA were based on procedures suitable for paralytic shellfish poison (PSP), (30). This involved boiling a tissue homogenate in an acidic medium. Since DA is unstable in acidic conditions, a water extraction method was recommended to increase the stability and yield of DA (32).

A method to extract DA from mussel tissue is needed to allow a clean preparation to improve the yield of DA during purification. This study compared acid, water, and lipid extraction procedures in terms of their DA yield and protein composition.

The aim of this research was to test for reliable methods for measurement of DA, and to find a quantitative procedure for the extraction and purification of DA from mussel tissue.

3.2 MATERIALS AND METHODS:

3.2.1 Materials:

Materials were purchased as indicated: YM2 ultrafiltration membranes (1000 dalton cutoff) and stirred cells (Amicon Division of W.R. Grace & Co., Danvers MA); chloroform, methanol, acetonitrile, isopropanol, trifluoroacetate were HPLC grade (BDH Inc., Dartmouth NS); precoated cellulose plates, acetone, acetic acid, ethyl acetate, phosphoric acid (PA), (Fisher Scientific Limited, Dartmouth NS); collidine, ninhydrin, dihydrokainic acid, amino acid standard kit, domoic acid, coomassie blue (Sigma Chemical Company, St. Louis Mo.); n-butanol (Johns Scientific Inc., Toronto ONT); 9-Fluorenylmethyl Chloroformate (Aldrich Chemical Company Inc., Milwaukie Wisc); boric acid (Anachemia, Montreal QC); domoic acid (Diagnostic Chemicals, Charlottetown P.E.I.). Deionized water was obtained by filtering distilled water through a Milli-Q™ system (Millipore Corporation, Bedford MA).

The HPLC system was described before (section 2.2.5, p 15). A Shimadzu fluorometer (model # RF 535) was used for detection of derivitized amino acids.

3.2.2 Extraction of domoic acid from mussel tissue in water:

The tissue was homogenized in an equal volume of water, and the homogenate was boiled for ten min. The resulting mixture was allowed to cool at room temperature for five min, centrifuged for ten min at 1500 X g in a table top centrifuge (Beckman, Model TJ-6), and the supernatant was decanted. The residue was re-extracted twice by addition of a half volume of water, vortexed vigorously, and recentrifuged (32).

3.2.3 Extraction of domoic acid from mussel tissue in acid:

A standard procedure for extraction of PSP was used (30). The tissue was homogenized in an equal volume of 0.18 N HCl; the pH of the homogenate was adjusted to 3.5 with HCL, boiled for seven min, allowed to cool at room temperature, and centrifuged for ten min at 1500 X g. To study the amount of DA which was not extracted by this method, two further extracts were made by adding one half volume of water and treating as in the water extraction method.

3.2.4 Extraction of domoic acid from mussel tissue with chloroform-methanol:

This procedure was based on the method of Bligh and Dyer (33). The tissue was homogenized in an equal volume of deionized water using a Ten Broeck glass homogenizer for small volumes and a Brinkman Polytron homogenizer for larger volumes. This homogenate was mixed with three volumes of methanol:chloroform (2:1,v/v), and vortexed for two min. One volume of chloroform was added followed by vortexing for one min; one volume of water was added and the solution was vortexed again for one min. The resulting mixture was centrifuged for ten min at 1500 X g. The top aqueous phase containing DA and other water soluble components was removed and the methanol was evaporated under a stream of nitrogen. A synthetic upper phase consisting of chloroform:methanol:water 3:48:47 was added to the bottom phase to make up to the original volume for the second and third extraction steps (34).

3.2.5 Estimation of proteins:

Proteins were measured by the method of Bradford (35). The coomassie blue reagent was made by dissolving 100mg of coomassie blue in 50ml of 95% ethanol. This was mixed with 100ml of 85% PA and the volume adjusted to 1L

using deionized water. A stock standard solution of bovine serum albumin (500 μg/ml) was made in deionized water and stored at 4°C. A set of protein standards, (5-25 μg protein/tube), was analyzed with each set of unknown samples. Samples and standards were made to 50μl with deionized water; 2.45ml of coomassie blue reagent was added to the tubes, vortexed and the absorbance read at 596nm within five min. The absorbance was measured using a Hewlett Packard Diode Array Spectrophotometer (Model 8452) and a standard curve was prepared concurrently using HP 89511 software.

3.2.6 Measurement of domoic acid by HPLC:

The reverse phase HPLC method used for measuring DA was described before (section 2.2.5, p 15).

During the development of the purificatic scheme for DA it was found that PA, which was used to acidify the mobile phase, is extremely difficult to remove and resulted in gummy residues. Acetic acid (AA) and maleic acid were tested as alternative acids for adjusting the pH of the mobile phase because they can be later separated from DA by ultrafiltration. A comparison was made between the response factors, (ratio of area or height to concentration of DA), of the three mobile phases for quantifying DA. The pH was adjusted to 3.0

with one of a) 85% PA (2 drops/L), b) maleic acid (150mg/L), or c) AA (19ml/L).

3.2.7 Diode array spectrophotometry:

It was desirable to find an alternative method for quantifying DA because of the time limitations on the HPLC. A standard curve was made using diode array spectrophotometry and comparisons were made between the HPLC method and the spectrophotometric method for quantifying DA in crude mussel extracts and in semipurified samples. Domoic acid purchased from Diagnostic Chemicals Ltd was used for the comparative studies. Standards were diluted in water and the same standards were used for the spectrophotometric and HPLC determinations on the same day. Comparisons of the different mobile phases were also made on the same day.

Standards and solutions containing DA were scanned over a range of wavelengths on a Hewlett Packard 8452A Diode array Spectrophotometer with HP-89500 software. Quantitation was performed at 242nm.

3.2.8 Purification of domoic acid:

Digestive glands from highly toxic steamed mussels were dissected away from the rest of the tissue and

stored frozen at -80°C until they were used. The digestive glands were homogenized and extracted for lipids as described above (Section 3.2.4). The lower phase was washed once with synthetic upper phase. A stream of nitrogen was blown into the pooled aqueous phases to remove the methanol. The remaining extract was lyophilized and resuspended in two ml of deionized water. This was ultrafiltered through 1000 dalton filters with two rinses of two ml of water, and the filtrate reconcentrated by lyophilization. The lyophilized sample was dissolved in 1N HCl to give a concentration of 10mg DA/ml. The pH was monitored with pH paper to ensure that the pH was approximately 1. The solution was injected (100μl/injection) onto reverse phase HPLC, mobile phase containing 12.5% acetonitrile in H_2O, flow rate 0.75 ml/min and one min fractions collected. The fractions containing DA were crystallized by lyophilization and tested for purity by analytical HPLC, spectrophotometry, and by amino acid analysis.

3.2.8.2 Lyophilization:

The samples were frozen at -80°C with the exposure of maximum surface area and lyophilized over night in a LABCONCO bench top freeze dryer connected to a two stage hign vacuum pump.

3.2.8.3 Ultrafiltration:

Ultrafiltration was performed in a plexiglass stirred cell at 5°C by applying nitrogen pressure of 30 psi. It took approximately 3h to ultrafiltrate a 5ml sample with two-2ml rinses.

3.2.9 Amino acid analysis:

Amino acids in the different HPLC fractions were monitored by one dimensional thin layer chromatography (TLC) using 250μm thick precoated cellulose plates. The solvent system was n-butanol:acetone:acetic acid:water (35:35:10:20,v/v), (36). An aliquot from each of the HPLC fractions was spotted onto the TLC plates which were developed for 90 min, dryed, and stained with ninhydrin (0.2g/100ml) - collidine (2.5ml/100ml) in isopropanol. The fractions containing DA and its putative isomer were analyzed by HPLC using a fluorescence detector. Samples were derivitized with 9-fluorenylmethyl chloroformate (FMOC) prior to analysis by HPLC. Initially 200μl of sample was mixed with 10μl of 2.3 ppm dihydrokainic acid (internal standard), and 50μl of borate buffer (pH 6.2). To this solution 250μl of FMOC (15mM in acetonitrile) was added and the mixture allowed to react for exactly 45s with continuous vortexing. The reaction was stopped by

the addition of 500µl of ethyl acetate and this mixture vortexed for 10s. The upper phase, containing underivitized FMOC, was removed and the lower phase was washed twice more with 500µl of ethyl acetate ea[illegible]ime. The bottom layer was analyzed by HPLC using a [illegible]ent system with solvent A (0.1% trifluoroacetic acid (TFA) in deionized water) and solvent B (0.1% TFA in acetonitrile). The column and the HPLC system are as described above (section 2.2.5, p 15), except that no guard column was used. The gradient was: 30%B at time 0, 50%B at 15 min, 100%B at 17 min, held until 22 min, 30%B at 24 min and held for a further 6 min until the next injection. The flow rate was 1.0ml per min. The derivitized amino acids were detected with excitation set at 265nm and emmission at 305nm (a 280nm cut off filter was placed in the light path). Some of the underivitized aromatic amino acids and methionine could be detected with the ultraviolet detector set at 220nm. The retention times of these amino acids in the reverse phase system with uv-detection of DA, (on a day when DA eluted at 9 min), were 3,4 DiOH phenylalanine - 3.21, methionine - 3.53, tyrosine - 3.98, phenylalanine - 7.36, histidine - 16.01, tryptophan - 22.85. Tryptophan was a contaminant of the putative DA isomer and was quantitated by an external standard method using peak area.

3.2.10 Measurement of toxicity by mouse bioassays:

Mouse bioassays were used to estimate the toxicity of important HPLC fractions. The mouse bioassay was the same as that described before (section 2.2.3, p13), except that the crystallized HPLC fractions tested and the DA from Diagnostic Chemicals Ltd were dissolved in 0.85% saline, 10mM citrate (pH 3.5), to maintain the osmolality and allow a small amount of buffering capacity. The control mice received injections of the same vehicle.

3.2.11 Statistical methods:

Analysis of variance and Student's-t tests were used to test for, and isolate, differences in the extraction methods and response factors (RF) for DA quantitation. Statistical calculations were performed on Minitab software (version 6.1.1 Minitab Inc. 1987).

3.3 RESULTS:

3.3.1 Methods for domoic acid quantitation:

It was found that the mobile phase made with AA gave significantly larger RFs, than the maleic acid and PA containing mobile phases (Table I). Although the RF for

the maleic acid adjusted mobile phase was similar to the PA solution, the baseline noise was much increased due to strong ultraviolet absorption of maleic acid.

The use of height for quantifying DA was limited due to the necessity of diluting the unknowns into a very narrow range (Figure 6). The standard curve did not follow Beers law because the regression line intercepts the Y-axis above zero.

3.3.2 Spectrophotometric measurement of DA:

The standard curves for the HPLC measurement using area were comparable to the spectrophotometric standard in terms of sensitivity, although the former has more variability at low concentrations of DA (Figures 6, 7). Two comparisons were made to determine the usefulness of diode array spectrophotometry for estimating DA. Contaminated mussel extracts containing 10-250μg/ml DA as determined by HPLC were diluted ten fold and an uncontaminated extract was diluted to the same proportions and used as a blank. This resulted in unreliable estimations of DA at levels below 100ppm because the uv contamination proved to be variable from extract to extract such that their was no proper blank for correcting for the non-DA absorbance at 242nm (Table II). Partially purified extracts which had been subjected

to HPLC separation of DA did show reliable comparisons between the two methods (Table III, correlation coefficient=.998).

3.3.3 Comparison of extraction methods:

It was found that a lipid extraction of mussel digestive glands allowed for the removal of lipids in the chloroform phase, and proteins in the interphase while giving a dramatic increase in the recovery of DA from the tissue (Table IV) when compared with a single water extract (Students-t test, $p<.01$). It was necessary to homogenize the tissue in order to effect a high recovery of DA (data not shown). Most of the DA is obtained in two lipid extracts, while the pellet from the second water extract still contains considerable amounts of extractable DA (Table IV). There was a significant difference in the amount of proteins and DA in the first extracts (ANOVA, $p<.01$). These differences were significant in the lipid extract versus the acid and water extracts (Students-t test, $p<.01$), but not between the acid and water extracts. There was a significant increase in DA recovery of the water extract over the acid extract (Students-t test $p<.01$).

3.3.4 Recovery and purity of domoic acid:

The purity of DA was monitored by HPLC, spectrophotometry and amino acid analysis. The uv-vis spectrum obtained from the purified DA was indistinguishable from DA purchased from Diagnostic Chemicals and Sigma Chemical company (Figure 8). Analytical HPLC showed that the DA from Diagnostic Chemicals and Sigma Chemical company possessed a small amount of contamination eluting prior to DA which was not seen in the fractions purified at AVC (Figure 9). Thin layer chromatography showed abundant amino acids in the mussel extract and all of the preparations at subsequent steps until the HPLC step. In the first 12 (one min) fractions of HPLC, all of the detectible amino acids eluted in the first four min. The fraction designated as pure DA (fraction 7, figure 10) gave a yellow streak on the TLC plate. Histidine and tryptophan bind with high affinity to the column and elute later than 12 min. HPLC fluorescent analysis of amino acids showed no detectible contamination of 500 ppb of the purified DA with other amino acids (detection limit 4 ppb), and gave a similar profile as DA purchased from Diagnostic Chemicals. Domoic acid from Diagnostic Chemicals contained one extra peak eluting right after DA which corresponded to the contaminant found in the uv-

absorbance method. This contaminant is considered a putative isomer of DA due to its similar chromatographic properties and absorbance spectrum. Out of four purification trials only one gave a relatively pure peak of the putative DA isomer, (the others were all contaminated with DA). The profiles of the important fractions are presented in figure 10, and the spectrum of fraction 5 is presented in figure 11. Fractions 7 and 8 had no detectible contamination, whereas fraction 5 was contaminated with tryptophan (21% by weight) which can account for the absorption peaks at 220nm and 196nm (Figure 11).

There was good recovery at all stages of purification except for the final HPLC step which resulted in only 29% pure DA in one fraction (Table V). However over 90% of the total DA that was injected onto the HPLC was recovered and the contaminated fractions can be lyophilized and used for subsequent purification.

3.3.5 Toxicity of HPLC fractions:

Fractions 7 and 8, (Figure 10), were highly toxic to mice at a level of 150 μg/ml. The toxicity was comparable to the DA purchased from Diagnostic Chemicals (Table VI). Fraction 5, the putative DA isomer, showed

no toxicity symptoms when injected in a mouse at a level of 200 μg/ml.

3.4. DISCUSSION:

3.4.1 Analytical methods for domoic acid:

It is necessary that DA is protonated so that it will bind to the column long enough for interfering substances to wash through. Although the RFs for the PA and maleic acid mobile phases were indistinguishable they were significantly increased for the AA containing mobile phase. This was not a function of differences in retention times because changing the retention time by itself through changing the flow rate did not affect the RFs. The results show that all of the mobile phases are reasonable for quantifying DA so that it is not necessary to change mobile phases when changing from preparative to analytical chromatography. However, it is necessary to run standards for each change in mobile phase and for each day. Other factors, such as the physical status of the detector, also affect the RFs. Since AA was found to be of limited use for preparative purposes, the increased RFs do not warrant using AA instead of PA for analyzing DA because approximately 74ml of AA was needed to adjust the pH of 4L of mobile phase to 3.0, whereas only eight drops of PA was needed. Furthermore, AA at

this level is distinctly odorous and its use should be avoided outside of fumehoods. Maleic acid itself has a strong absorbance at 242nm so that it increased the baseline noise compared to the other acids. When the HPLC was being regularly used it required a 30-60 min rinse at 1-2ml/min with the new mobile phases to re-equilibrate for use.

Both area and height could be used for calculating the levels of DA. The analysis of DA on the basis of height gave slightly more precision (less variability) than the analysis on the basis of area. However, it was of limited use since it was necessary to dilute the sample to a concentration within the range of standards used. High variations in area were found at low levels of DA (less than 2ppm) and were caused by the integrator drawing questionable baselines due to a low number of data points detected. At higher levels however, the area was found to be strongly correlated with DA.

The use of a diode array spectrophotometric method for measuring DA is desirable because an increased number of samples can be processed and the system can also be automated. The advantage of diode array spectrophotometry is that if an appropriate blank is available then all of the interfering substances are subtracted out of the chromatogram and accurate values can be obtained. A standard curve of DA in water showed

similar sensitivity to the HPLC method. Unfortunately there is a significant amount of uv-absorbing material in the mussel samples which interferes with the measurement of DA. An attempt was made to counteract this absorbance by using a blank from a control mussel extract. However, there was too much variability in the amount of interfering material from mussel to mussel for the method to yield accurate values (Table II). The spectrophotometric method was useful for measuring the DA in partially purified material (Table III), and if a convenient means for cleaning up the extracts becomes available, this method will offer advantages over HPLC for routine monitoring of DA levels in shellfish.

3.4.2 Extraction methods:

The quantification of DA by the acid and water extraction procedures results in a large underestimation of the total DA found in the digestive glands of mussels (Table IV). The acid extraction procedure is more limited than the water extraction method. However, three extraction steps are necessary to recover 90% of the DA by the water extraction method (Table IV). For monitoring needs the water or acid extraction methods are reasonable procedures for obtaining a rough estimate of DA because they do not require chloroform, methanol and

fumehoods. The lipid extraction is useful for purification and may be valuable for research which requires higher quality clean up methods. The lipid extracts contained lower proteins than the water extracts. This is significant for purification purposes because an extra deproteinization step was unnecessary, allowing the total yield of DA to be increased.

3.4.3 Purification of domoic acid:

After extracting lipids and evaporating methanol the resulting solution is still too crude for HPLC as this plugs up the precolumns making repeat injections impossible. Ultrafiltration through 1000 dalton filters cleaned up this dark green solution considerably and yielded a golden clear solution which could be injected onto the HPLC.

During initial attempts to purify DA the mobile phase was acidified with PA. It was found that there was no easy means of removing the PA which caused a gummy residue. Hydrochloric acid is unacceptable for adjusting the pH of the mobile phase because the chloride ion can degrade the stainless steel used in many of the components of HPLCs. Other acids were used, notably AA and maleic acid. Acetic acid was inconvenient because it is a weak acid. Maleic acid was used because it is

relatively strong and could be removed by ultrafiltration through 500 dalton filters which retain DA. A procedure using maleic acid and 500 dalton ultrafilters turned out to be unreliable because the amount of DA retained by the filters varied and three washes were necessary to insure the removal of all of the maleic acid. This resulted in very poor recovery of DA at this stage. It was found that when the extract was adjusted to a pH of 1 with HCL, it was not necessary to use an acid in the mobile phase to resolve DA to from the other substances.

There was one compound which eluted about one min earlier than DA and was considered a putative isomer of DA, based on its uv spectrum and chromatographic properties (Figure 11). The greatest variability in each purification process was the separation of DA from this compound. There was always one fraction which was a mixture of DA and its putative isomer (eg. fraction 6, Figure 10), while the very next two fractions appear to be pure DA. Although the recovery of pure DA is relatively low, because a significant amount is co-eluted with this compound, the contaminated DA can still be purified by subsequent HPLC. The flow rate was reduced to 0.75ml/min in order to get more pure DA for each HPLC run.

The putative isomer which was found in relatively pure form in fraction 5 (Figure 10) produced no toxicity

symptoms when injected intraperitoneally into one mouse at a level of 200μg/ml. Although this fraction was contaminated with tryptophan at a level of 21% by weight, this test indicates a much lower toxicity for the isomer than for DA. This is consistent with the findings of decreased insecticidal activity of isodomoic acids A, B, and C, by Maeda and his colleagues (10) and of the reported ten fold lower toxicity of the other isomers (36). The spectrum for fraction 5 showed two peaks with maximum absorption at 220nm and a second maximum at 244nm. This is consistent with the finding that this peak is contaminated with tryptophan because tryptophan also absorbs maximally at 220nm (Figure 11). The fact that the third absorption peak maximum is 244nm supports the hypothesis that this peak also contains an isomer of DA which retains the conjugated double bonds.

The results of TLC showed that the bulk of ninhydrin-positive amino acids have relatively weak affinity for the reverse phase column under the conditions used and elute in four min or less. The amino acids which remain on the column for longer periods of time were not found in the pure DA fractions by uv detection at 220nm or by the fluorescence derivitization method.

The HPLC profiles for DA purchased from Diagnostic Chemicals Ltd, and Sigma Chemical Company showed a small

amount of contamination with the putative isomer. This contamination was also seen in the fluorometric HPLC method where the FMOC-derivitized putative isomer elutes after the derivitized DA. In fact the DA from Sigma Chemical Company was likely from the same original batch as that purchased directly from Diagnostic Chemicals (Jeff Rallings, personal communication).

The most economical HPLC method tested for DA quantification is reverse phase chromatography with PA used to adjust the mobile phase to pH 3.0. Diode array spectrophotometry is useful for monitoring the purity of DA, and for measuring DA in reasonably pure samples, but requires the development of an efficient purification step before it can be widely applicable to quantification in shellfish extracts. The purification procedure for DA appears to be result in the pure compound as evidenced by its uv spectrum, HPLC profile, and amino acid analysis. This method can potentially be scaled up to allow for increased quantities of DA.

TABLE I: COMPARISON OF RESPONSE FACTORS FOR DIFFERENT MOBILE PHASES FOR DOMOIC ACID QUANTIFICATION ON HPLC

	Height R.F.	Area R.F.
PA	21.5 ± .5[a]	72800 ± 700[a]
MA	21.5 ± 1.5	72600 ± 2500
AA	25.1 ± .8[b]	78500 ± 2500[b]

PA = Phosphoric acid; MA = Maleic acid; AA = Acetic acid, 1ppm of DA was used for the comparison of RFs. Sensitivity was 0.01 AUFS. All values are mean ± sd, n=3, a vs b $p<.01$

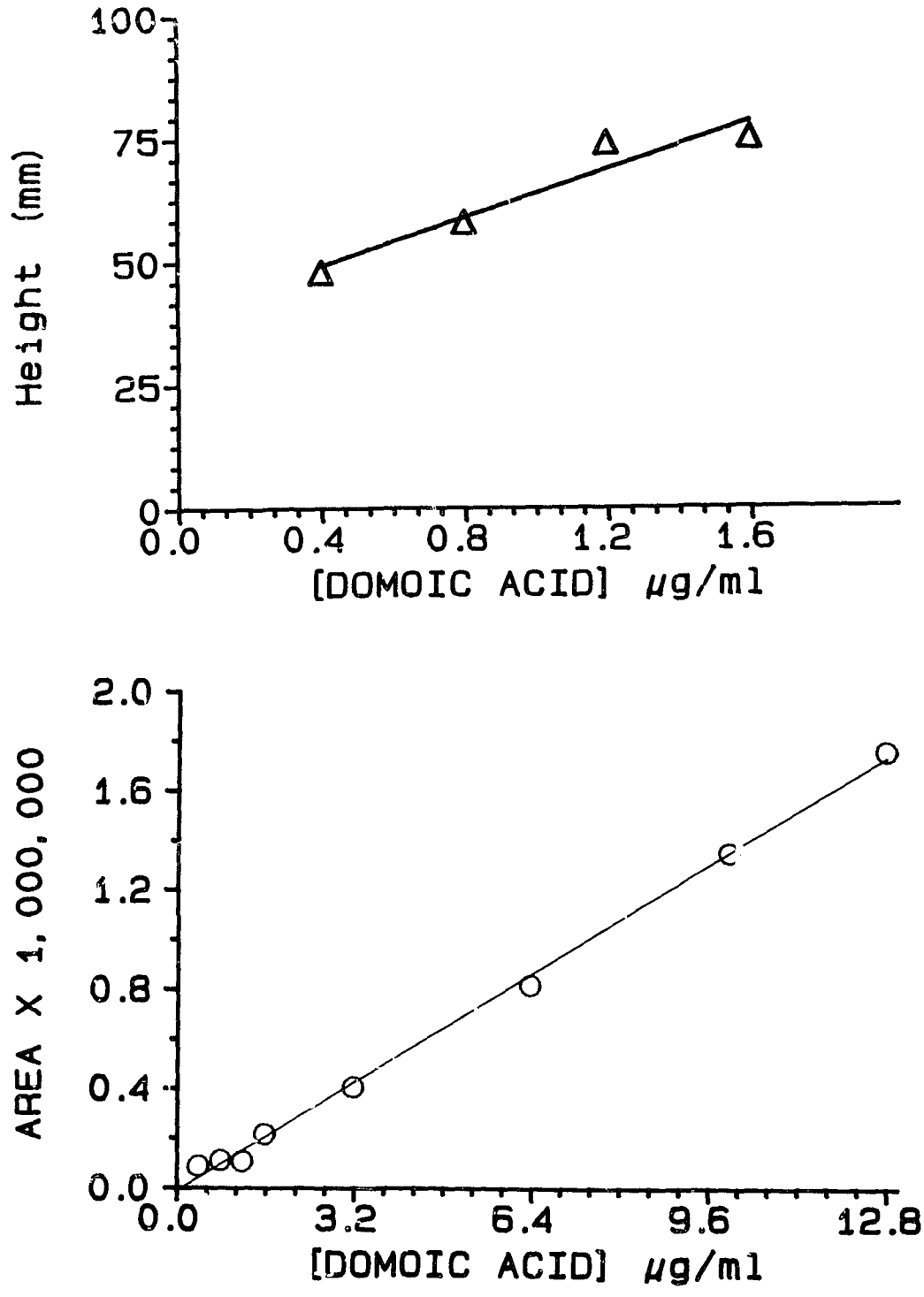


Figure 6. HPLC Standard curves for domoic acid.
A = Height vs concentration
B = Area vs concentration

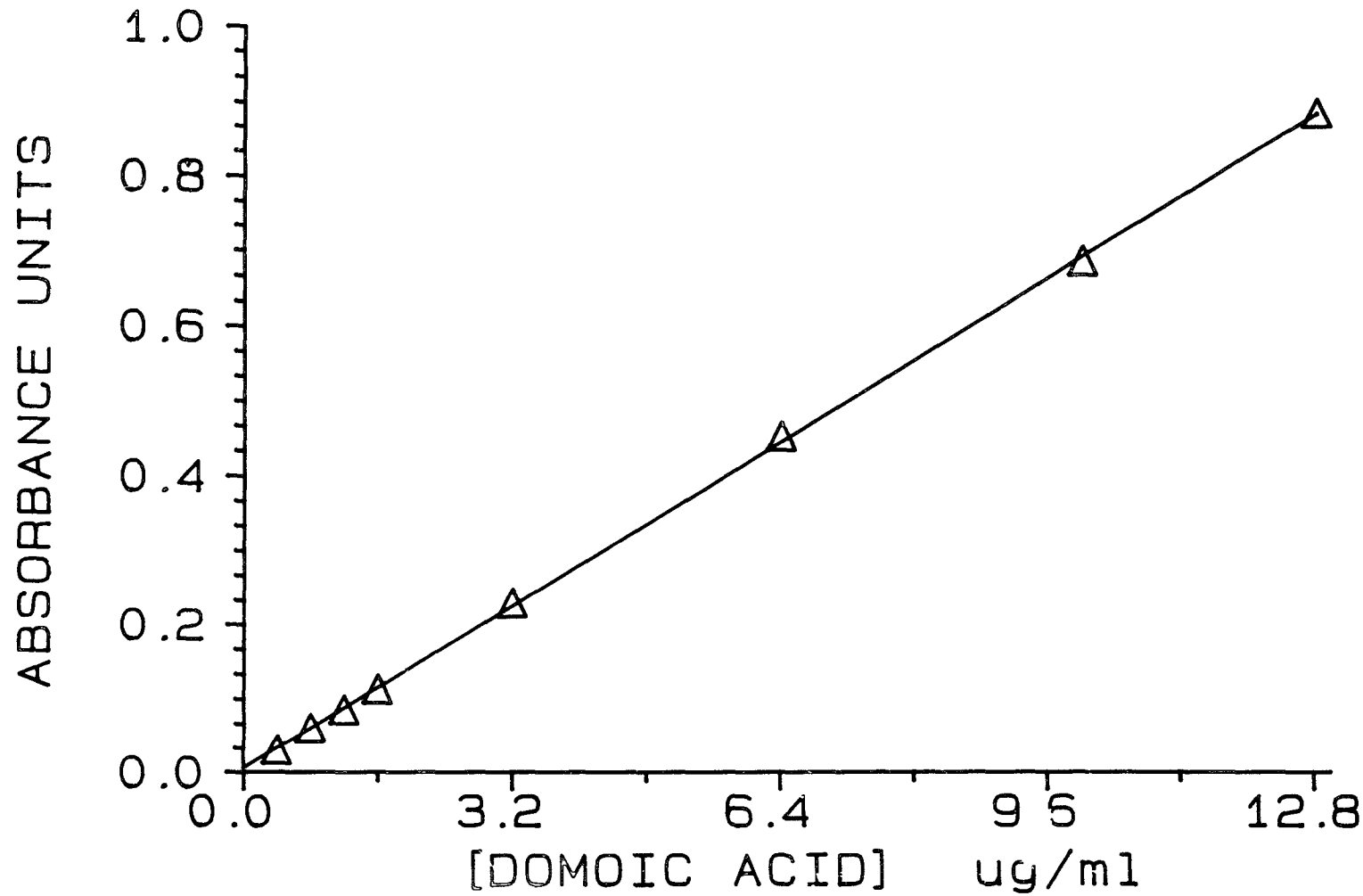


Figure 7. Spectrophotometric standard curve for domoic acid.

TABLE II. CONCENTRATIONS OF DOMOIC ACID IN MUSSEL EXTRACTS DETERMINED BY SPECTROPHOTOMETRY AND HPLC.

	[DOMOIC ACID] μg/ml	
AVC SAMPLE #	HPLC	SPECTROPHOTOMETRY
259	7	13
211	11	46
197	44	121
194	51	109
196	54	103
193	68	148
110	92	112
242	337	269
244	372	317
243	404	350

TABLE III: MEASUREMENT OF DOMOIC ACID IN HPLC FRACTIONS COLLECTED FOR PURIFICATION, BY HPLC AND SPECTROPHOTOMETRY

	[DOMOIC ACID] μg/ml	
FRACTION #	HPLC	SPECTROPHOTOMETRY
11	1.5	3.6
12	13.8	12.2
13	107	111
14	21	28
15	2.8	5
16	1.1	0.7

Prepurification treatment (prior to HPLC) is described in section 3.2.8; HPLC fractions were collected at 0.5 min. intervals starting at 1.0 min.

TABLE IV: RECOVERY OF DOMOIC ACID FROM THE DIGESTIVE GLANDS IN THE BLUE MUSSEL (Mytilus edulis), BY LIPID, WATER, AND ACID EXTRACTION PROCEDURES.[a]

Extract	[Domoate] µg/ml	% total	Proteins µg/ml
Whole Homogenate	1492	100	
L-1	1145 ± 141	77	3259 ± 503
L-2	180 ± 189	12	604 ± 53
L-3	25 ± 1	2	229 ± 46
W-1	726 ± 49[b]	49	4611 ± 270[b]
W-2	338 ± 20	29	3960 ± 345
W-3	253 ± 8	16	2025 ± 317
A-1	513 ± 52[b]	34	4182 ± 367[b]
A-2	338 ± 20	23	3239 ± 586
A-3	253 ± 8	17	2285 ± 469

a: Values are mean ± sd, (n=3). L=lipid extraction (section 3.2.4); W=water extraction (section 3.2.2); A=acid extraction (section 3.2.3).
b: $p < .01$ versus L.

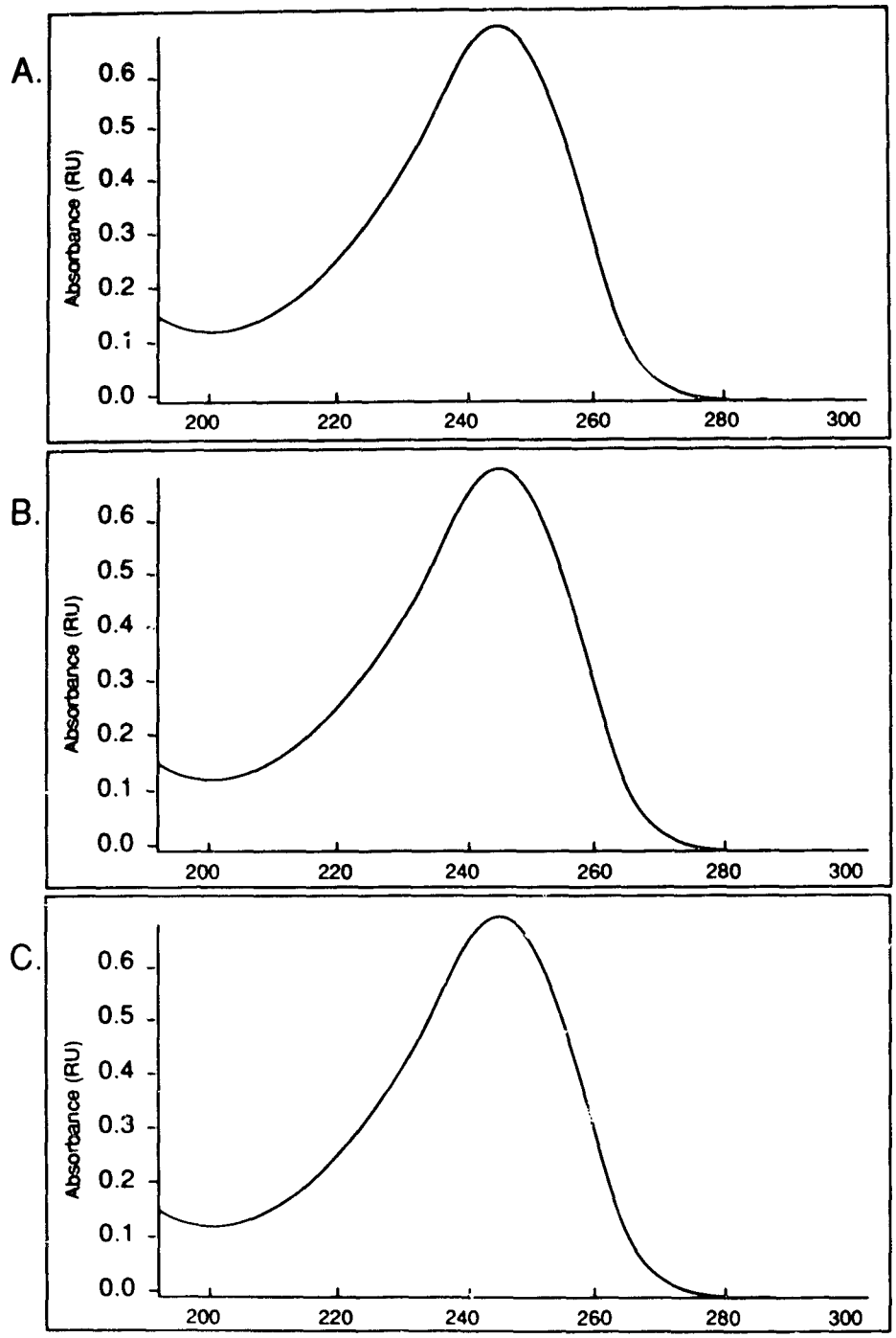


Figure 8. Ultraviolet spectra of domoic acid from different sources at 10 µg/ml,
A = Diagnostic Chemicals lot # 1 (cat# GH28);
B = Sigma lot # 108F0206 (cat# D-6152);
C = AVC, in house, (conditions in text).

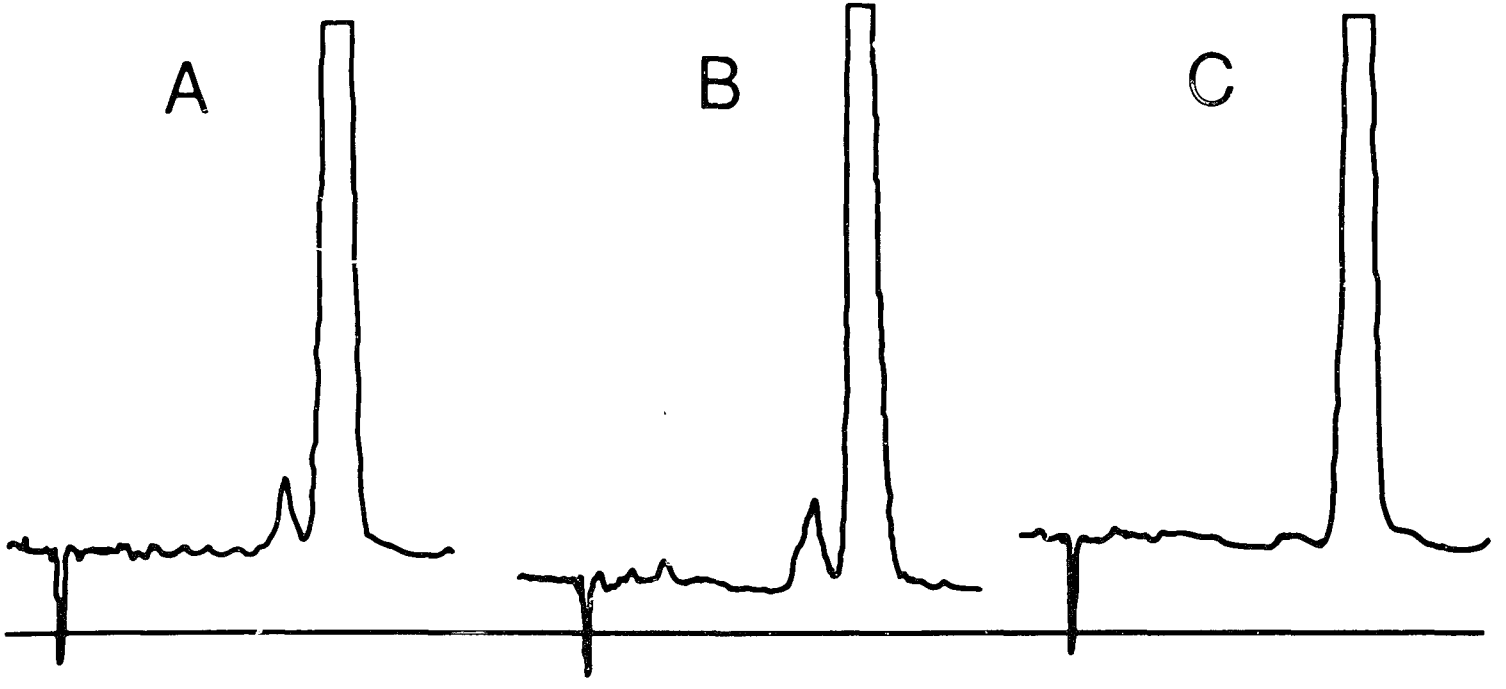


Figure 9. HPLC chromatograms of domoic acid from different sources at 10 µg/ml, (sens. = .01)
A = Diagnostic Chemicals lot # 1 (cat# GH28);
B = Sigma lot # 108F0206 (cat# D-6152);
C = AVC, in house, (conditions in text).

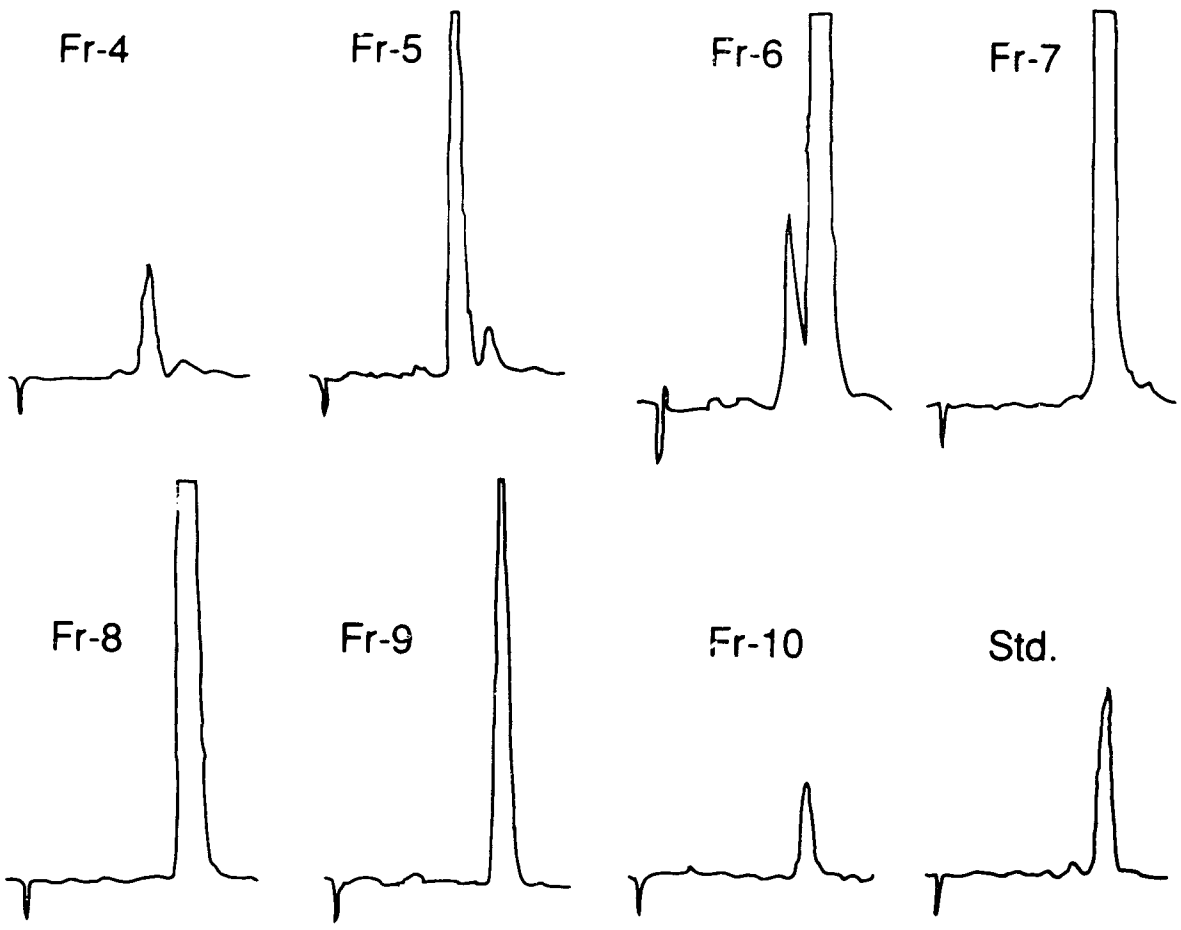
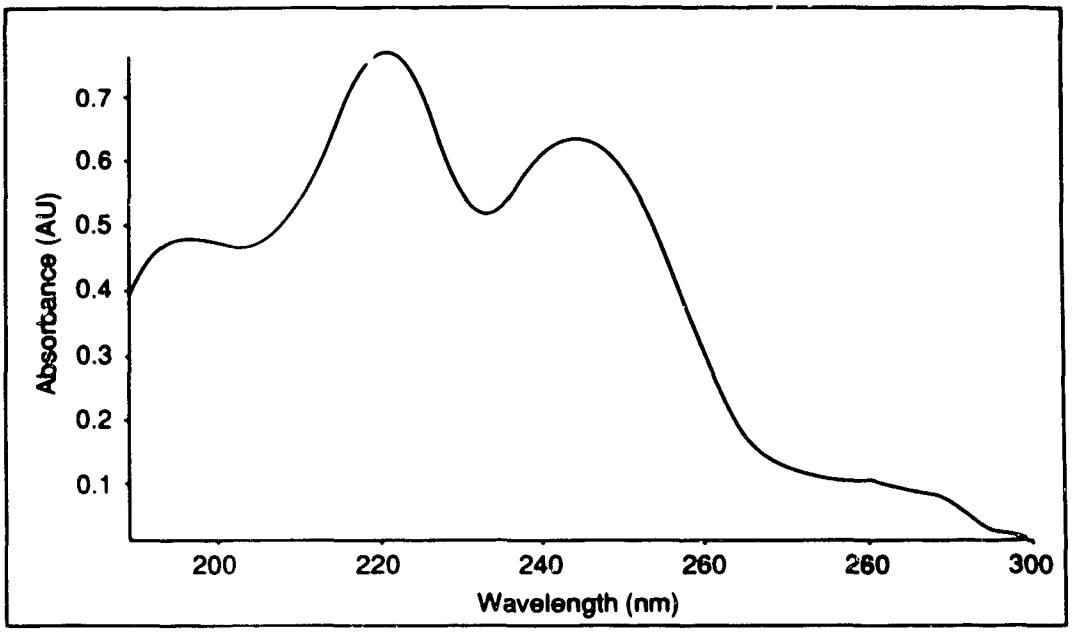


Figure 10. HPLC Chromatograms of fractions collected from domoic acid purification scheme (Section 3.4.3). These were 1 min fractions collected after a 5 min delay. The retention times for the predominant peak in fraction 5 was 6.44 min. And for the DA peak was 7.51 min.



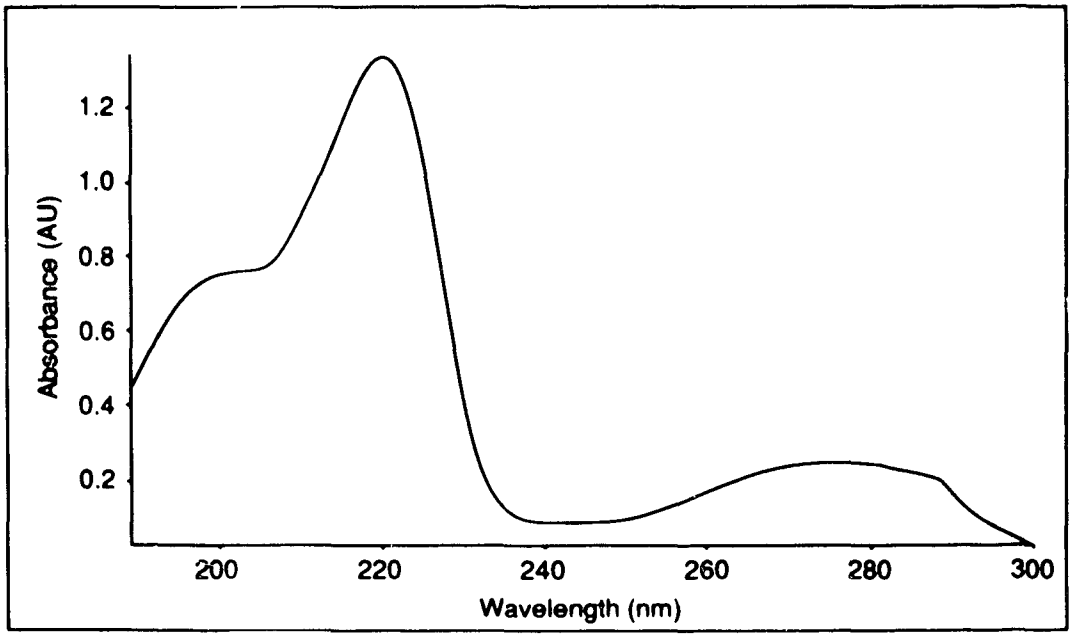


Figure 11. A = UV Spectrum of fraction 5 (10µg/ml) from HPLC (Figure 12) representing a putative domoic acid isomer contaminated with tryptophan.
B = UV Spectrum of tryptophan (10µg/ml)

TABLE V. RECOVERY OF DOMOIC ACID DURING PURIFICATION

	Percent of original
LIPID EXTRACT	87 ± 6
LYOPHILIZATION	90 ± 9
ULTRAFILTRATION	84 ± 5
SECOND LYOPHILIZATION	81 ± 11
"PURE" HPLC Fraction	29 ± 11
"IMPURE HPLC" Fractions	41 ± 8

(results are mean ± sd, n = 4)

TABLE VI. TIMES OF DEATH AND SCRATCHING ONSET FOR MICE INJECTED INTRAPERITONEALLY WITH FRACTIONS FROM HPLC PURIFICATION OF DOMOIC ACID

SAMPLE	Weight of mouse(g)	Scratching Time (min)	Death Time
DA,	14.4	10	36
150 μg/ml	14.7	9	34
from	13.8	12	lived
Diagnostic	17.4	22	35
Chemicals	17.0	12	17
Fr. 7 (Fig. 12)			
150 μg/ml	13.9[a]	-	20
	15.2	13	22
	13.6	14	19
Fr. 8 (Fig. 12)	18.3	11	30
150 μg/ml	17.7	16	36
	16.0	11	37
Fr. 5 (Fig. 12)	16.0	-	-
200 μg/ml			

a: This mouse showed atypical circling behaviour with no scratching.

SECTION 4

4. EFFECT OF DOMOIC ACID ON CALCIUM UPTAKE IN SYNAPTOSOME AND SYNAPTONEUROSOMAL PREPARATIONS FROM GUINEA PIG BRAIN:

4.1 INTRODUCTION:

Domoic acid (DA) is a potent neurotoxin, possessing a structure similar to kainic acid (KA), (12). It is more toxic and has greater affinity than KA for KA receptors (19). Kainic acid receptors are a specific subclass of L-glutamic acid receptors and are thought to make up approximately 10% of the glutamate receptors found in the mammalian central nervous system (CNS), (10). Kainic acid binding sites have been found in high numbers in the striatum, hippocampus, cerebral cortex and cerebellum where they are predominantly localized in synaptosomal membranes (14). These binding sites were found to be concentrated in terminal fields where KA acts as a potent neurotoxin (38). Kainic acid receptors are beleived to exist both presynaptically and postsynaptically (18).

It is possible that DA exerts its excitatory and neurotoxic effects, at least in part, through binding to pre-synaptic KA receptors resulting in increased release of L-glutamate (18). Calcium is an important intermediate in causing the release of glutamate from

presynaptic nerve endings (39). Pre-synaptic KA receptors may be linked directly to calcium channels (40). Domoic acid could therefore elicit some of its neurochemical effects by inducing calcium uptake pre-synaptically, and thereby causing glutamate or aspartate release (Figure 12).

The toxic effects of excitatory amino acids result in the death of post-synaptic neurons, a process which is dependent on the integrity of presynaptic inputs (11, 16). Elevated levels of calcium have been correlated with cytotoxicity in nerve cells treated with excitatory amino acids (EAA), (26). N-methyl-D-aspartate (NMDA) is an EAA which binds to a specific subclass of EAA receptors which are different from KA receptors and appear to be, at least in part, receptor-operated calcium channels (23). Since the NMDA calcium channel is blocked by low concentrations of magnesium, another possible mechanism of DA-induced neurotoxicity is that DA removes the magnesium blockade of the NMDA channel allowing increased influx of calcium.

The forebrains, hippocampi, and cerebella represent distinct structural and functional components of the mammalian CNS. These regions all possess large numbers of receptors for EAA (10). Since the biochemical effects of DA on one part of the brain could be different from its actions on other parts, it is important to

separate these different regions to study the neurochemical effects of this toxin.

Synaptosomes have been extensively used in neurochemical studies since Whittaker and De Robertis published methods for their preparation in the early 1960s. These preparations, (as disussed in reference 41), allow for more localized studies than whole cells because they consist predominantly of the presynaptic part of nerve cells.

Synaptoneurosomes are synaptosomes connected to neurosomes (resealed postsynaptic sacs). They have been used in a number of neurochemical studies involving cyclic AMP, phosphoinositide turnover and calcium flux (42, 43). These preparations are prepared by low speed centrifugation of brain homogenates, and are reported to consist of approximately 50% synaptoneurosomes (41).

The objective of this study was to determine if DA causes increased calcium uptake in synaptosomes from guinea pig forebrains, or in synaptoneurosomes from guinea pig cerebella or hippocampi.

4.2 MATERIALS AND METHODS:

4.2.1 Materials:

The materials were purchased as indicated: sucrose, copper sulfate, sodium potassium tartarate, bovine serum

albumin (fraction V), Folin reagent, N-methyl-D-aspartate, kainic acid, L-glutamate, quisqualate, sodium chloride, potassium chloride, potassium phosphate, magnesium chloride, dextrose, sodium bicarbonate, Tris-maleate (Sigma Chemical Company, St. Louis MO); radioactive calcium-45 (I.C.N. Radiochemicals, Cleveland OH); vacuum filtration apparatus and 0.45μM aqueous filters (Millipore Corporation, Bedford MA); ready safe scintillation fluid (Beckman Inc., Fullerton CA). Deionized water was obtained by filtering distilled water through a Milli-Q water system (Millipore). Guinea pigs of either sex weighing 300-400g had been purchased from Charles River Laboratories (Montreal, QC).

4.2.2 Preparation of synaptosomes:

Synaptosomes were prepared by the method of Whittaker (44), (Figure 15). Guinea pigs were killed by a sharp blow to the head. Forebrains, (the front part of a vertebrates brain, which includes the diencephalon as well as the telencephalon), were removed shortly after the death of the animals and the synaptosomes prepared on the day of the assays. Typically it took about 5h to prepare the synaptosomes (Figure 13). All procedures were done on ice or in refrigerated centrifuges set at 4^{o}C. The tissue was rinsed in isotonic saline and

weighed. Starting from a guinea pig cerebral cortex weighing from 2-3g, the final suspension contained approximately 20-30mg protein/ml. Preliminary studies establishing a framework for these experiments are presented in Appendix B.

4.2.3 Synaptoneurosome enriched preparations:

Synaptoneurosomes were freshly prepared by homogenizing the cerebellums or hippocampi in a medium containing 0.27M sucrose, 10mM tris-maleate, 1mM dithiothreitol. The 10% homogenates were centrifuged at 1,000 X g for 10 min and the pellet was diluted to 5ml with the homogenizing buffer. This was used for a crude synaptoneurosomal fraction.

4.2.4 Measurement of protein:

The amount of proteins in synaptosomes was determined by the method of Lowry (45). Determination of proteins by coomassie blue, a more conveniant method for measuring soluble proteins, was unreliable for synaptosomal proteins because of incomplete breakage of synaptosomes. Three separate ten-fold dilutions of the synaptosomes were made and 10μl of each of these diluted samples was added to 190μl of deionized water. Standards

were made with bovine serum albumin diluted to give a range of 5-25μg protein and processed along with the samples; 200μl of 1N NaOH was added to each tube, the samples vortexed and left for at least 30 min and usually overnight to ensure complete breakup of the synaptosomes. One volume of a 0.5% Copper-sulphate:($5H_2O$) - 1% Sodium potassium tartarate solution was mixed with 50 volumes of 2.0% sodium bicarbonate; 2.0ml of this mixture was added to each sample. Solutions were vortexed and allowed to sit for 10 min before adding 200μl of 1N Folin reagent. The reaction was allowed to continue at room temperature for 30 min. The absorbance was measured at 750nm with a diode array spectrophotometer (Hewlett Packard, model 8452A), utilizing HP 89511 UV/VIS quantitation software to draw a standard curve and to calculate the protein concentrations of the samples.

4.2.4 Determination of calcium uptake:

Calcium uptake in synaptosomes and synaptoneurosomes was measured by incubating the tissue in physiological medium containing radioactive calcium $^{45}CaCl_2$. The normal control medium contained in final concentration: 140mM NaCl, 4mM KCl, 1mM KH_2PO_4, 1mM $MgCl_2$, 10mM dextrose, 5mM $NaHCO_3$, 10mM Tris-maleate (pH 7.4), 1mM $CaCl_2$

(approximately 0.5μCi $^{45}CaCl_2$) (40). In experiments with synaptoneurosomes the normal medium contained: 125mM NaCl, 10mM dextrose, 5mM $NaHCO_3$, 20mM Tris-HCl (pH 7.4), 1mM $CaCl_2$ (approximately 0.5μCi ^{45}Ca). A total volume of 0.5ml was used for the experiments which included 20μl of either synaptosomes or synaptoneurosomes. All of the acids were dissolved in 1mM NaOH followed by the addition of an equal amount of 1mM HCl. The medium was preincubated at 30°C without synaptosomes for 2 min and the uptake started by adding synaptosomes which were kept on ice to maintain their stability. The reaction was stopped after one min by the addition of 2.5ml of ice cold stopping solution containing: 140mM NaCl, 2mM KCl, 1mM KH_2PO_4, 5mM $NaHCO_3$, 3mM EDTA, 10mM Tris-maleate (pH 7.4). Immediately upon stopping the reaction the suspension was filtered through 0.45μm aqueous filters, followed by three rinses with 2.5ml of rinsing solution which contained 130mM NaCl, 5mM KCl, 0.01mM $CaCl_2$ (cold), 2.5mM $MgCl_2$ and 40mM Tris-maleate pH (7.4), (46). The filters were soaked for at least 1h in ice cold 250mM KCl to bind cationic sites on the filters and were passed through a beaker of distilled water before being used. The vacuum filtration apparatus was rinsed with distilled water between each sample. The filters were allowed to dry, scintillation fluid was added, and the samples analyzed for radioactive decay in a scintillation counter

(Packard model 2000CA). Each day the experiment was run, two-10μl aliquots of the radioactive calcium solution were counted concurrently with the experimental samples. This measurement was the basis for calculating the amount of calcium taken up by the particles (see appendix B).

4.3 RESULTS:

4.3.1 Calcium uptake in synaptosomes:

Neither DA (1mM), KA (1mM), or NMDA (1mM stimulated calcium uptake in synaptosomes from the forebrain under the experimental conditions, although L-glutamic acid (1mM) caused a significant increase in calcium uptake (Figure 14). Results presented in Figure 14 are normalized means from 2 experiments using separate guinea pigs. Each experiment was performed in triplicate. Actual control values were 1.02 and 1.27 nmoles Ca++/mg/min. Calcium uptake was significantly stimulated by 1mM L-glutamic acid and by 70 mM potassium (Students-t test, $p<.01$), but was not altered by 1mM DA, 1mM KA or 1mM NMDA. Experiments using lower concentrations of EAA are not reported as no effects were observed.

4.3.2 Calcium uptake in synaptoneurosomes:

Calcium uptake in hippocampal and cerebellar

synaptoneurosomes was significantly increased for the test tubes containing 1mM glutamic acid, or 70mM potassium, (Figures 15-20). Neither KA (1mM), DA (40-400μM), QQ (100μM), or NMDA (1mM) showed any significant change in calcium uptake in any of the experiments with synaptoneurosomes (Figures 15-20). Domoic acid (200μM) did not potentiate calcium uptake by 1.0mM NMDA in hippocampal or cerebellar synaptoneurosomes (Figures 19, 20).

4.4 DISCUSSION:

No stimulation of calcium uptake by DA in either synaptosomes or synaptoneurosomes was found under the experimental conditions used. L-glutamate caused a significant increase in calcium uptake in synaptosomes. This effect was more pronounced in synaptoneurosomes. Since synaptosomes themselves may contain some postsynaptic sacs these results are consistent with the hypotheses that L-glutamic acid increases calcium uptake via a post-synaptic receptor.

Other investigators have shown that L-glutamate causes increased calcium influx into synaptoneurosomes from immature rat brain (47). They also found an extremely low potency and efficacy of KA and NMDA in causing increases in internal calcium. These results

also support the conclusion that DA and KA are not acting directly at either a pre-synaptic or a post-synaptic KA receptor to cause calcium uptake. The specific receptor mediating L-glutamate induction of calcium uptake in synaptoneurosomes was proposed to be the QQ receptor (47). The data presented here do not support this conclusion.

Physiological systems are characterized by dynamic equilibria, and elicit changes via perturbations in which there is a tendency to re-establish these equilibriums. There is a low level release and rapid uptake of glutamate occurring continuously at glutamatergic neurons in the central nervous system (48). Any compound which interferes with the ability of this system to return to a manageable concentration of intersynaptic glutamate is likely to be toxic. It is possible that DA and KA act indirectly *in vivo* to result in toxic levels of calcium in postsynaptic neurons. The neurotoxicity of KA and presumably DA is dependent on intact glutamatergic innervation, and like L-glutamate results in the destruction of post-synaptic neurons while sparing axons of passage (18). Most of the KA binding sites are found on synaptosomal membranes (14). These neurotoxins may operate by stimulating glutamate release or blocking the reuptake mechanisms resulting in increased levels of glutamate which could be the ultimate

cause of increased calcium uptake and toxicity in the intact nervous system (18).

No effect on calcium uptake in synaptosomes was found by KA or NMDA. This is consistent with the similar findings on rat striatal synaptosomes (49), but contrary to results presented for crude whole brain synaptosomes from rats (40). Since the latter experiments used a level of 5mM KA and found marginal increases, the results are of questionable physiological relevance. It is not obvious why there should be pre-synaptic receptors responsive to analogues of L-glutamate which induce glutamate release since this would represent a feed forward system. It is however clear that there are uptake mechanisms for glutamate which possess a structure complementary to DA and KA. Westerberg and colleagues found that cerebral ischemia in the rat hippocampus destroyed 90% of CA1 dendrosomatic neurons while preserving presynaptic terminals (50). This caused high losses of QQ and NMDA receptor binding but not of KA binding, suggesting that the KA receptors may be predominantly pre-synaptic (50). This leaves the possibility that the bulk of the KA binding is at the pre-synaptic receptors and/or the L-glutamate reuptake site. In fact it is possible that a significant component of the "KA receptor" is the glutamate reuptake site. If this is the case the high affinity of KA and

DA for this site could cause a blockage at the reuptake site (Figure 21). Since the normal termination of the excitatory message by L-glutamate relies on reuptake, an interference at this level could result in prolonged excitation and seizures.

Further research investigating the possible role of DA in blocking L-glutamate reuptake mechanisms and/or stimulation of presynaptic release is warranted.

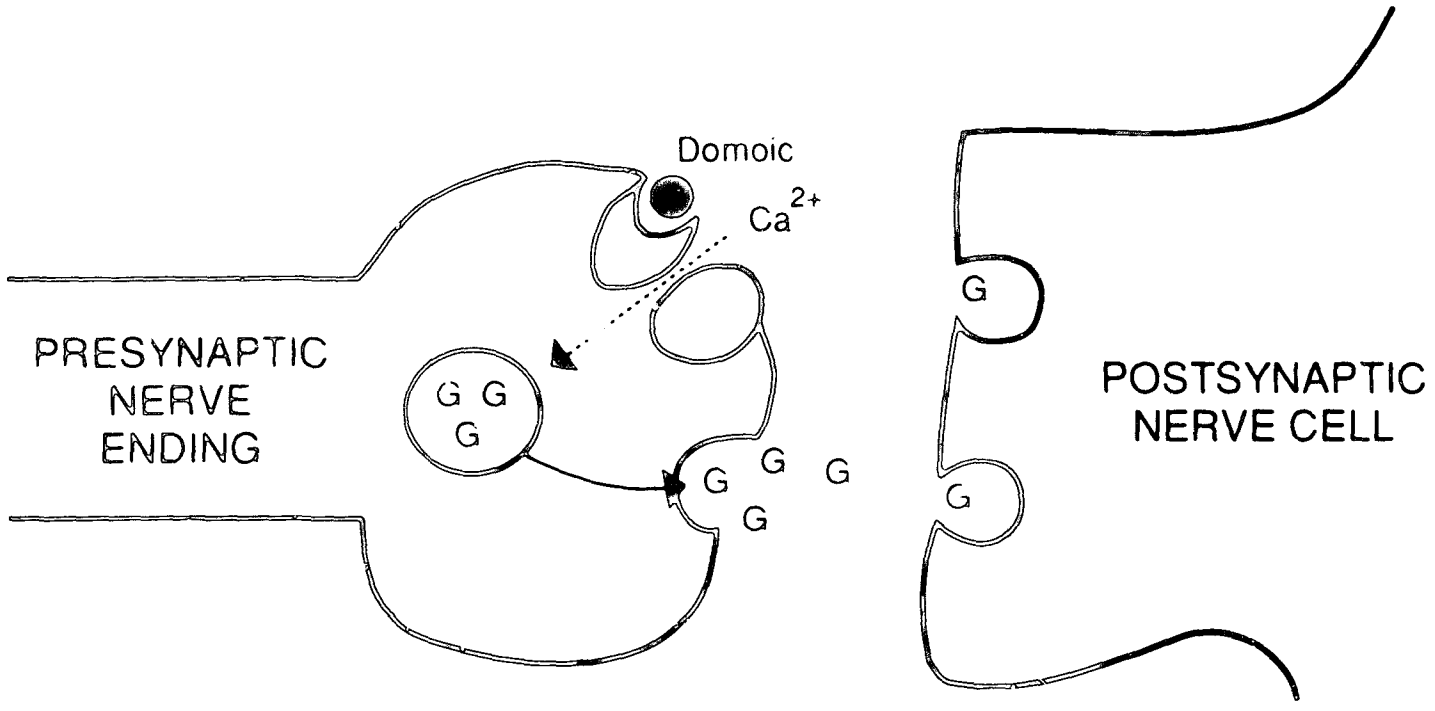


Figure 12. Theoretical scheme for domoic acid effects on pre-synaptic calcium channels. G = L-glutamate.

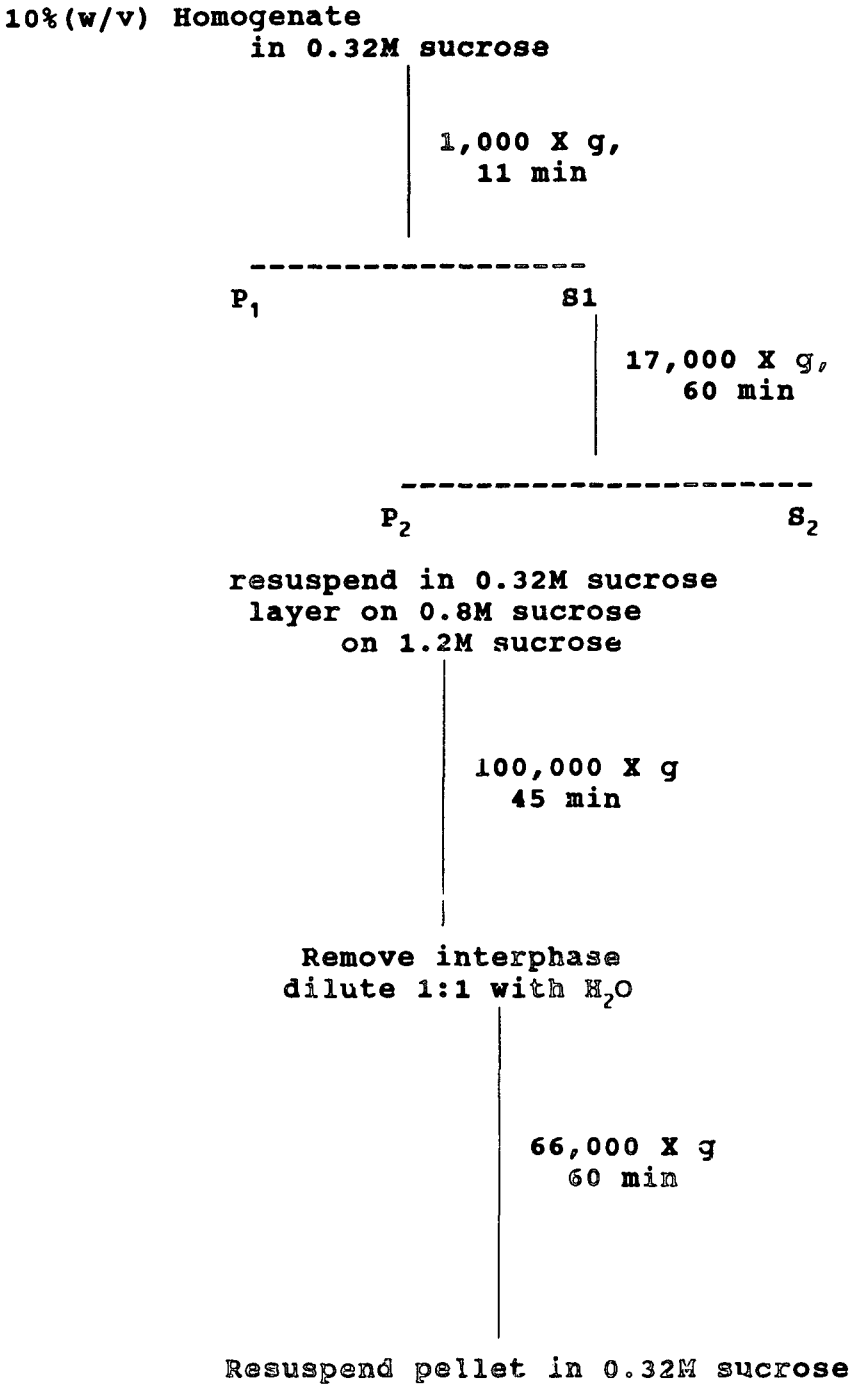


Fig 13. Protocol for the isolation of synaptosomes.
P=pellet, S= supernatant.

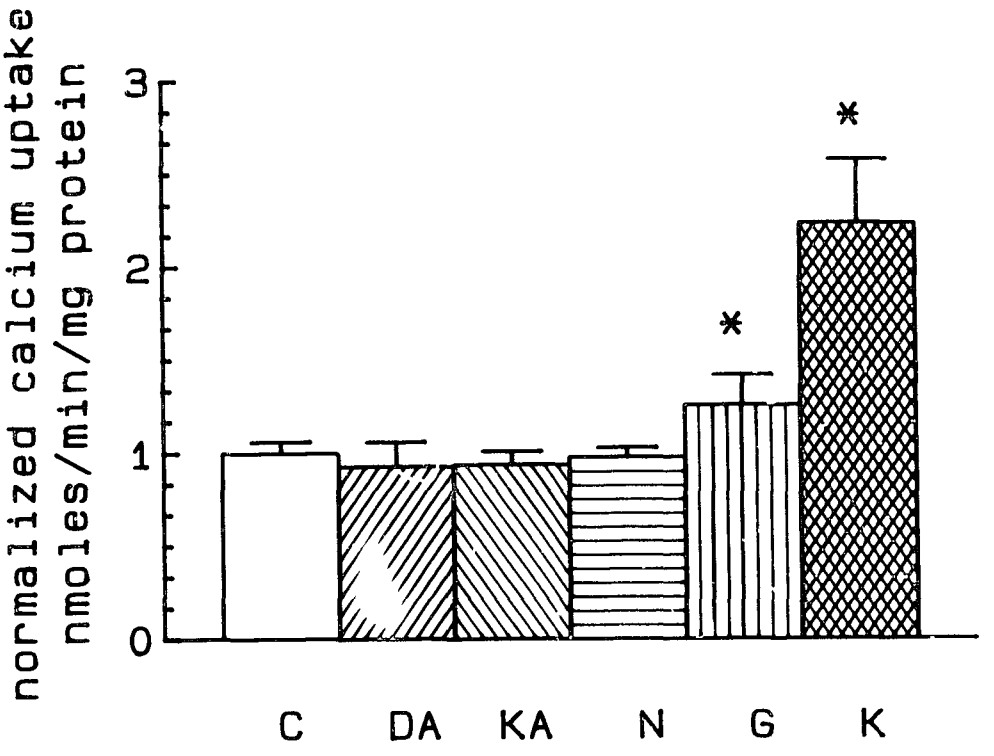


Figure 14. Effect of excitatory amino acids on calcium uptake in synaptosomes. Synaptosomes prepared from guinea pig forebrains were incubated in the absence (C) or presence of different excitatory amino acids i.e. 1mM domoic acid (DA); 1mM kainic acid (KA); 1mM N-methyl-D-aspartate (N); 1mM L-glutamic acid (G); 70mM potassium chloride (K). The medium contained 2mM [$^{45}CaCl_2$]. Results are based on two separate experiments each performed in triplicate. Bars represent normalized means $\pm$ sd, n=6 observations. * $p < .01$.

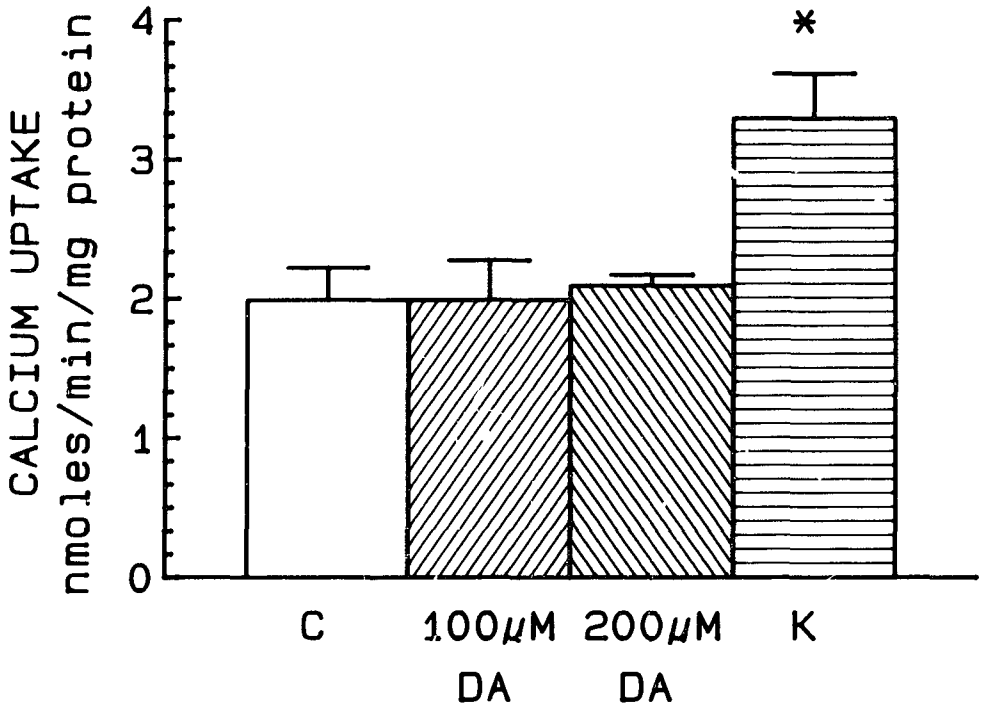


Figure 15. Effect of domoic acid and potassium on calcium uptake in synaptoneurosomes from guinea pig hippocampi. Synaptoneurosomes were incubated in the absence (C) or presence of either 100μM domoic acid (DA); 200μM domoic acid (DA); or 70mM potassium chloride (K). The medium contained 1mM [$^{45}CaCl_2$]. Bars represent means ± sd, n=3 observations. * $p < .01$.

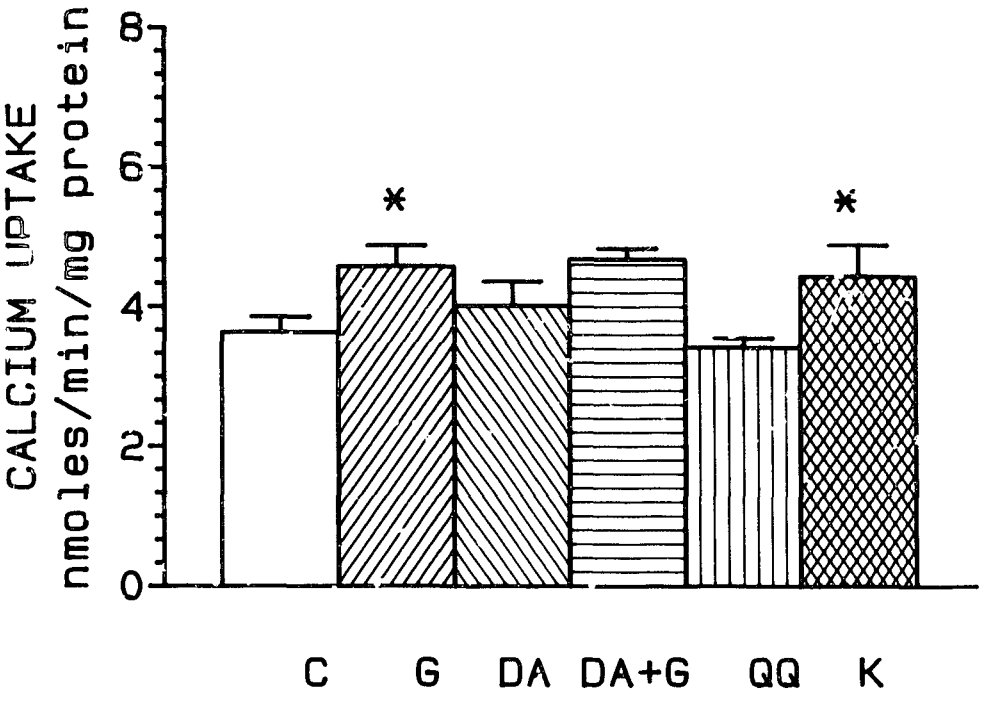


Figure 16. Effect of excitatory amino acids and potassium on calcium uptake in synaptoneurosomes from guinea pig hippocampi. Synaptoneurosomes were incubated in the absence (C) or presence of different excitatory amino acids i.e. 1mM L-glutamic acid (G); 80μM domoic acid (DA); 80μM domoic acid plus 1mM L-glutamic acid (DA+G); 100uM quisqualic acid (QQ); 70mM potassium chloride (K). The medium contained 1mM [$^{45}CaCl_2$]. Bars represent means $\pm$ sd, n=3 observations. * $p < .01$.

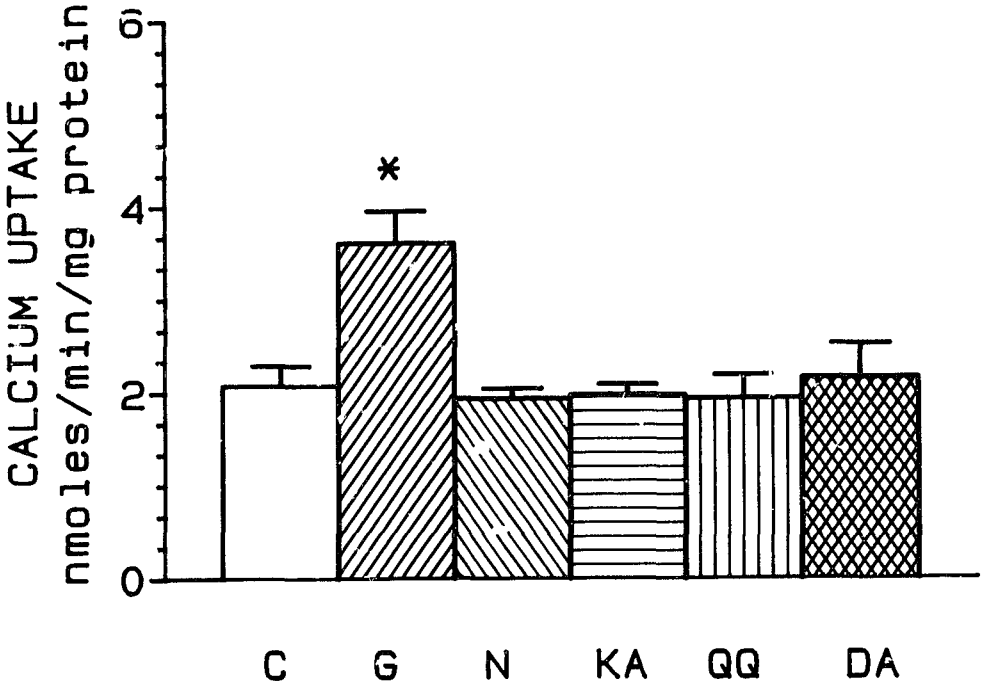


Figure 17. Effect of excitatory amino acid on calcium uptake in synaptoneurosomes from guinea pig hippocampi. Synaptoneurosomes were incubated in the absence (C) or presence of different excitatory amino acids i.e. 1mM L-glutamic acid (G); 1mM N-methyl-D-aspartate (N); 1mM kainic acid (KA); 100uM quisqualic acid (QQ); 400μM domoic acid (DA). Bars represent means $\pm$ sd, n=3 observations. * $p < .01$.

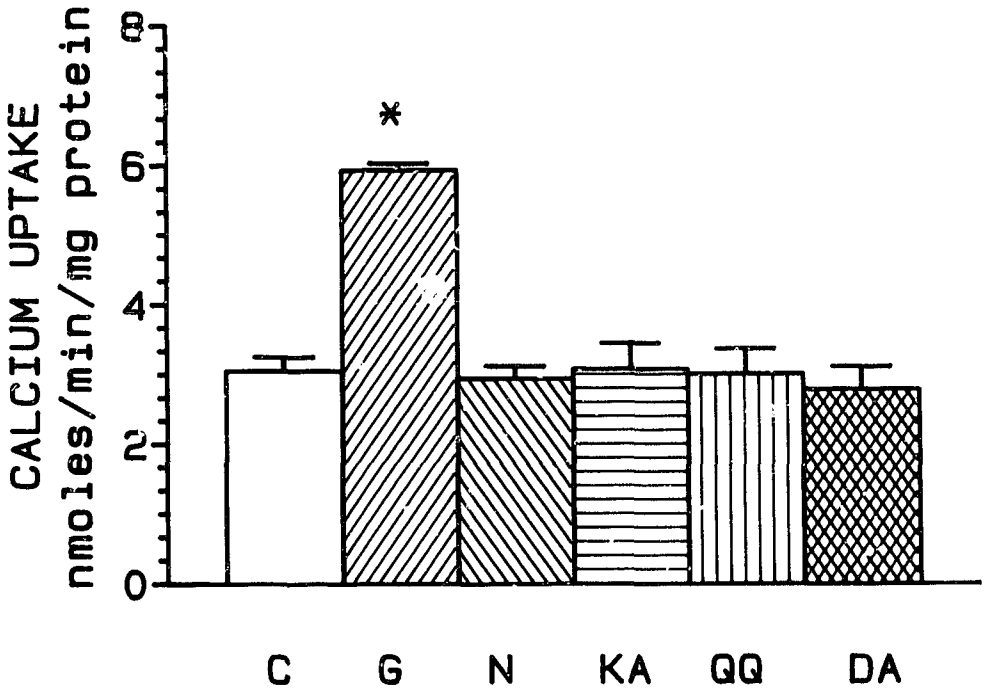


Figure 18. Effect of excitatory amino acids on calcium uptake in synaptoneurosomes from guinea pig cerebellum. Synaptoneurosomes were incubated in the absence (C) or presence of different excitatory amino acids i.e. 1mM L-glutamic acid (G); 1mM N-methyl-D-aspartate (N); 1mM kainic acid (KA); 100μM quisqualic acid (QQ); 400μM domoic acid (DA). Bars represent means $\pm$ sd, n=3 observations. * $p < .01$

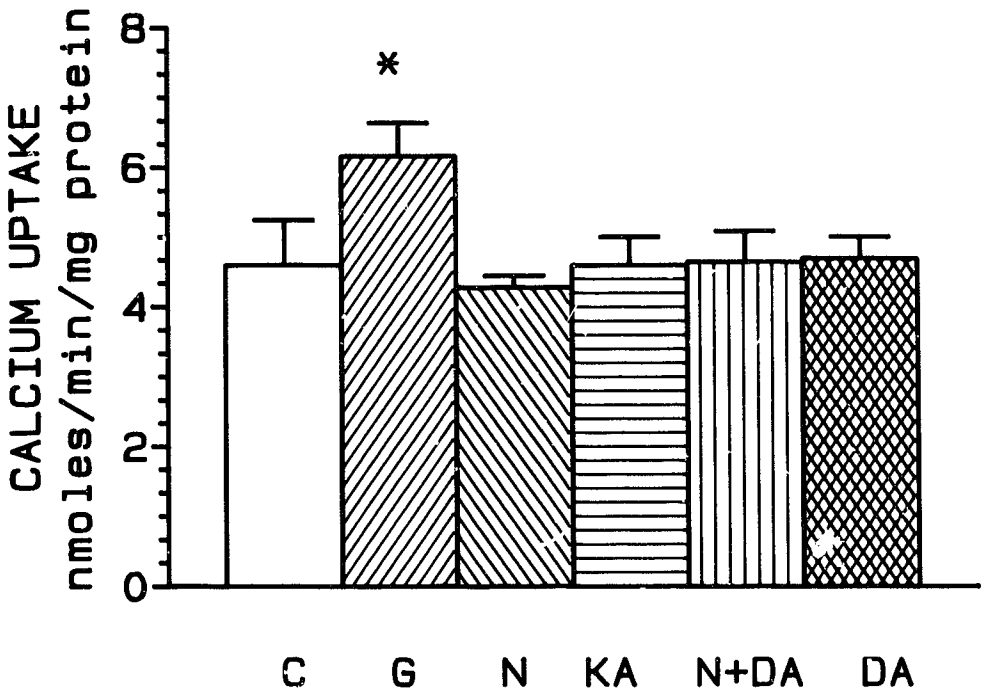


Figure 20. Effect of excitatory amino acid ands on calcium uptake in synaptoneurosomes from guinea pig cerebellum with 35 mM potassium. Synaptoneurosomes were incubated in the absence (C) or presence of different excitatory amino acids i.e. 1mM L-glutamic acid (G); 1mM N-methyl-D-aspartate (N); 1mM kainic acid (KA); 1mM NMDA plus 200μM domoic acid (N+DA); 200μM domoic acid (DA). The medium contained 35mM potassium chloride. Bars represent means $\pm$ sd, n=3 observations. * $p < .01$

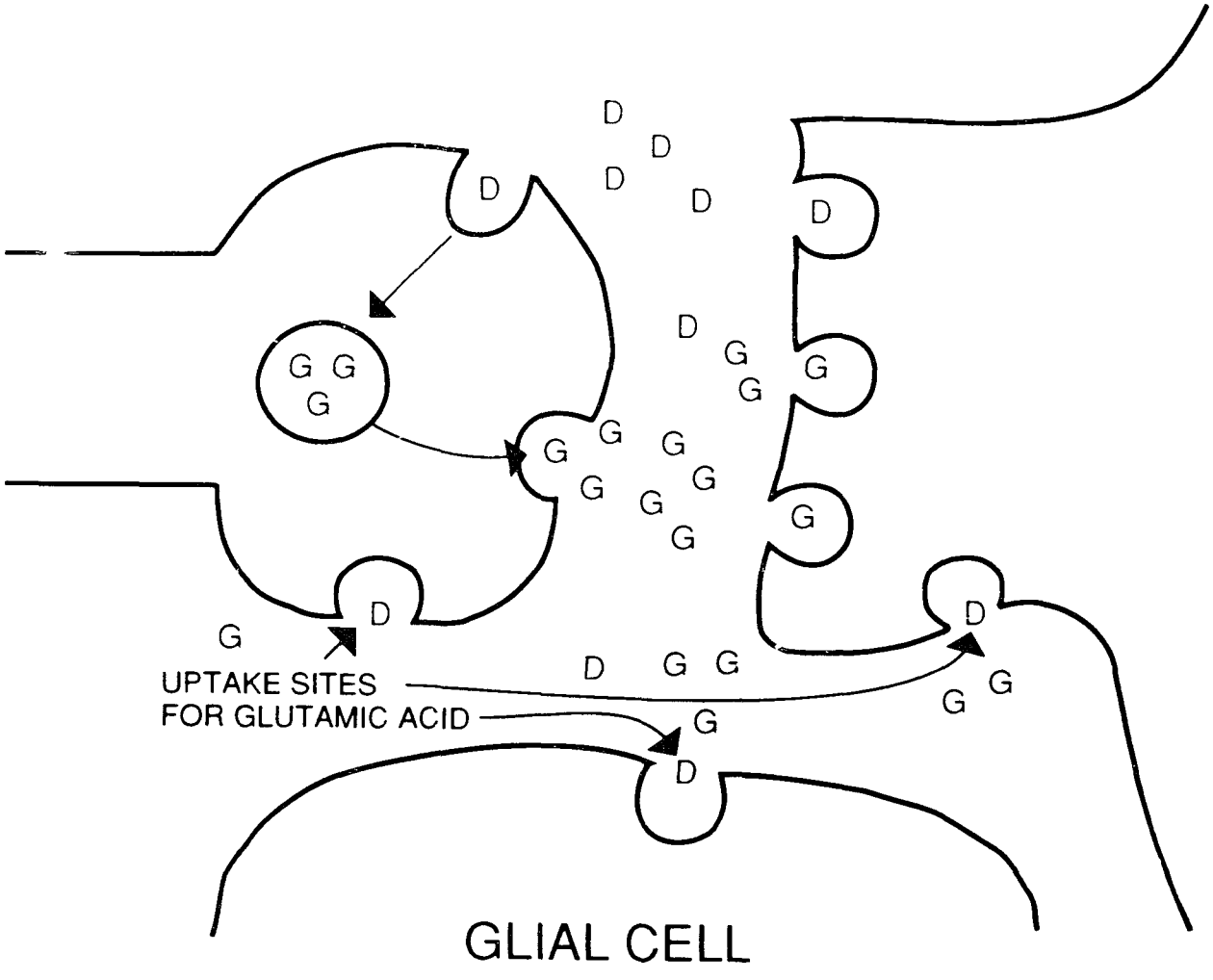


Figure 21. Hypothetical scheme for domoic acid blocking reuptake sites for L-glutamate. G = L-glutamate.

SECTION 5

5. GENERAL DISCUSSION:

5.1 SOURCE OF DOMOIC ACID:

Shellfish have been known to concentrate toxins such as lead, mercury, and hydrocarbons which can accumulate in their tissue at 1000 times the level which occurs in the environment (51, 52, 53). In addition natural toxins produced by phytoplankton such as paralytic shellfish poison and diarrhetic shellfish poisons have been shown to concentrate in mussels and cause toxicity in humans who consumed these mussels (54). These incidents are invariably associated with algal blooms (55, 56).

Unlike most sources of food used by humans, consumption of shellfish often includes eating the digestive tract. This means that all of the undigested matter in the mussel is eaten along with the organism. Since DA was concentrated in the digestive glands of mussels, this was consistent with a non-mussel origin to the toxin. Early observations of contaminated mussels revealed that they had much larger and greener digestive glands than non-toxic mussels suggesting that they had been consuming large amounts of phytoplankton. Contaminated mussels possessed four times more chlorophyll-A, an indicator of algal biomass, than non-toxic mussels (57).

Dr. Louis Hanic, (Department of Biology U.P.E.I.), reported blooms of the pennate diatom *Nitzschia pungens* in the Cardigan River during the same time period that large amounts of DA were found in mussels in 1987 and again in 1988 (L. Hanic, Personal communication). Other investigators have demonstrated that pure cultures of *N. pungens* from P.E.I. river waters contained DA and suggested that phytoplankton are the source of the toxicity (7). Although the number of algal species which have the capacity to produce DA is unknown, these results support the conclusion that DA in eastern P.E.I. mussels was produced by *N. pungens* during an algal bloom which appears to be an annual event.

5.2 ANALYSIS OF DOMOIC ACID:

The spectrum of analytical methods available for the measurement of DA is still in the process of being developed by a number of scientists. A sensitive amino acid method has been adapted for the measurement of DA, and is useful for seawater or samples that are low in the other amino acids (7). Proline, in particular, elutes very close to DA in this method. It is also important to find another internal standard for this method because the putative DA isomer was found to co-elute with the internal standard, dihydokainic acid, in

the system used and can introduce an error in the results by giving a false high value for the internal standard. The putative DA isomer was invariably associated with DA in mussel samples analyzed by HPLC. A cleanup method perhaps based on anion or cation exchange chromatography is needed in order to make this technique more widely applicable.

The reverse phase HPLC method which was the predominant method used in this thesis, is limited to approximately 1.0 ppm with the current sensitivity setting of 0.01 AUFS. Finding a method to further clean up the sample should increase the signal to noise ratio allowing the sensitivity to be adjusted downwards or the injection amount to be increased.

The usefulness of diode array spectrophotometry for quantifying DA is dependent on an even higher quality clean up method for the samples because it does not possess the capacity, that HPLC methods do, to separate out interfering substances. A cleanup method developed for improving the HPLC methods may also be adapted for a spectrophotometric method. Therefore all of the analytical methods discussed are at present limited by a need for a better developed cleanup procedure.

5.3 PURIFICATION OF DOMOIC ACID:

Domoic acid, along with isodomoic acids A, B, and C had been purified from *Chondria armata* using reverse phase, DEAE cellulose, and sephadex gel chromatography although the method is not described in detail (4). A preparative HPLC method starting with a homogenate of whole mussels was recently published (8). This method used 1% acetic acid with an acetonitrile:water gradient and resulted in a yellowish oil which required repeated crystallization in order to get colourless needles (8). The same authors also reported a rather complex anion exchange method. The recoveries for these two methods were not reported.

The purification scheme for DA outlined in this thesis possesses the advantages of simplicity and high recovery. This method may be upgraded for obtaining greater quantities of DA by using preparative HPLC columns, although care must be taken that the resolution necessary for a high quality separation of DA from contaminants is maintained. If a large quantity of DA is purified, this will allow a larger number of purity tests to be performed on one batch while leaving a significant amount available for research. Also an anion or cation exchange chromatography system should be set up to allow a further purification step and/or an

additional means of purification and analysis for DA.

5.4 NEUROCHEMISTRY:

5.4.1 Effect of domoic acid on memory:

One of the effects of DA toxicity is an interference with the establishment of long term memories. The hippocampus is strongly associated with memory formation in mammals, including rats and primates (58). This brain region, where NMDA and KA receptors are involved in long term potentiation (LTP), is a major site of EAA toxicity (12, 59). Based on Hebbs general theory of synaptic plasticity (60), LTP is a specific neurochemical phenomenon which could be a means through which memories are temporarily encoded (61), while long term storage is in the process of being established. Increased calcium levels caused by L-glutamate or L-aspartate are hypothesized to increase the probability that an "a posterior" presynaptic signal will result in the firing of the postsynaptic neuron (62). The destruction of the pathways involved in LTP by DA represents a mechanism through which the impairment of memory by DA may operate.

5.4.2 Mechanisms of neurotoxicity:

The mechanism(s) by which DA causes toxic effects are only partially understood at this time. It is thought that DA produces toxicity analagous to KA, and that KA toxicity is mediated through KA receptors (21, 62). However, it should be remembered that DA possesses an elaborate side chain and an additional carboxyl group which may allow it to exert effects which are different from KA. Since DA finds its way into the brain it may also find its way into the interior of cells where it could interfere with the normal functioning of intra-neuronal signalling mechanisms. One of the potential sites of action is at the level of cyclic nucleotides. Cyclic GMP has been shown to be stimulated by KA (63, 64) and cyclic AMP appears to be affected by DA (unpublished results). Since calcium may regulate both of these cyclic nucleotides, effects on these messenger systems may be through a chronic perturbation of calcium levels either intracellularly or at the level of receptors.

It is still quite possible that DA causes elevated intracellular calcium in an "in vivo" situation where the nerve cells are still intact. Future experiments could be directed towards brain slices in order to test whether DA increases calcium or sodium uptake into intact neurons.

5.5 SUMMARY AND CONCLUSIONS:

The information from the data on mouse bioassays and HPLC analysis of mussel extracts demonstrated that the toxic mussel extracts contained large amounts of domoic acid (DA). The levels of DA were strongly correlated to toxicity in mice as measured by two indices: death time and onset of scratching behaviour. The bulk of toxicity was localized within the mussel tissue in parallel with the levels of DA. The decline in toxicity with time coincided with a decline in DA content. Therefore, the work presented in this thesis supports the conclusion that the primary toxin found in cultured mussels in eastern P.E.I. in late 1987 was DA.

The most reliable analytical method for DA was reverse phase HPLC with uv detection. The other methods were limited because of interfering substances. A purification procedure for DA from contaminated mussel tissue was developed which used tissue dissection, Bli and Dyer lipid extraction, lyophilization, ultrafiltration, and reverse phase HPLC.

The hypothesis that DA stimulates calcium uptake presynaptically was not supported by the results from the calcium uptake experiments. L-glutamate caused increased calcium influx into synaptoneurosome and synaptosome enriched preparations, whereas neither 1mM NMDA nor 1mM KA affected calcium uptake in these experiments.

APPENDICES

A. APPENDIX-A: REGRESSION EQUATIONS FOR PREDICTING LEVELS OF DOMOIC ACID BY MOUSE BIOASSAY

A.1 INTRODUCTION:

In this appendix the regression equations derived from the comparisons between levels of domoic acid (DA) and the inverse of the onset of scratching (IST) and the inverse of death time (IDT) of mice are developed to show their potential use and limitations for predicting levels of DA in toxic mussel extracts.

A.2 METHODS:

Statistical analysis was performed on minitab (Version 6.1.1 Minitab Inc. 1987).

A.3 RESULTS AND DISCUSSION:

A.3.1 Summary of correlations:

The correlation of average IDT with DA concentration in the extract is 0.934, n=16. The regression equation is:

$$IDT(min^{-1}) = -.00450 + .000189[\mu g/ml].$$

The t-ratio for the constant is not significant (t=-1.34), but is highly significant for the slope (t=5.82).

The correlation of the average IST with DA concentration is 0.859, n=34. The regression equation

is:

IST(min^{-1}) = .006308 + .000316[μg/ml].

The t-ratio for the constant and the slope is 2.57 and 9.50 respectively. Analysis of residuals and nscores revealed a normally distributed population.

Since the above data are based on tests which generally used three mice and the weights of all of these mice were measured this allowed the data to be analysed in terms of dosage i.e. μg DA/gr mouse.

The correlation of IDT with DA dosage is 0.942, n=61. The least squares regression equation is:

EQUATION 1: IDT = -.00173 + .00416[μg DA/gr mouse].

The t-ratio for the constant is non-significant (t=-1.24), but is highly significant for the slope, (t=21.54). Therefore, if the level of DA is zero then the possibility of death within the experimental time is very small.

The correlation of IST with DA dosage is 0.904, n=114. The regression equation is:

EQUATION 2: IST = .0145 + .00476[μg DA/gr mouse].

The t-ratios for the constant and the slope are 12.92 and 21.38 respectively. This suggests that if the level of DA is zero the mice may still scratch at an average time of 69 min.

Analysis of residuals and nscores revealed a normally distributed population validating the use of regression analysis.

A.3.2 Estimation of DA levels by scratching and death times:

The regression equations allow for a rough estimation of the level of DA in a sample after a mouse bioassay has shown positive signs of DA toxicity.

The regression equations can be rearranged to give:

ppm(DA) = (IST/.00476 - 3.05)(g mouse)

and equation 2 gives:

ppm(DA) = (IDT/.00416 - .41)(g mouse)

The death and scratching times can be substituted into the above formulas to allow a reasonable estimate of the level of DA in an extract. This was done for the comparison between the mussel hepatopancreas and remaining tissue for toxicity and DA levels and gave reasonable results (Tables A-1 & A-2).

TABLE A-I: TIMES OF DEATH AND SCRATCHING ONSET AND THEIR PREDICTED LEVELS OF DOMOIC ACID FOR DILUTED WHOLE HOMOGENATE[a] OF DIGESTIVE GLANDS OF TOXIC MUSSELS INJECTED INTRAPERITONEALLY INTO MICE

Weight of mouse	Scratching Time	(Predicted [DA] ppm)	Death Time	(Predicted [DA] ppm)
17.7	6.5	(518)	11	(380)
19.2	9	(390)	12	(377)
17.8	8	(413)	22	(187)
Avg.	7.8	440	15	315

a: Dilution was 3:1 to give 314 ppm DA as measured by HPLC.

TABLE A-II: TIMES OF DEATH AND SCRATCHING ONSET AND THEIR PREDICTED LEVELS OF DOMOIC ACID FOR WHOLE HOMOGENATE[a], OF MUSSEL TISSUE MINUS DIGESTIVE GLANDS OF TOXIC MUSSELS, INJECTED INTRAPERITONEALLY INTO MICE

Weight of mouse	Scratching Time	(Predicted [DA] ppm)	Death Time	(Predicted [DA] ppm)
18.1	19	(151)	45	(93)
16.0	22	(104)	61	(56)
18.8	-		16[b]	(275)
Avg.	20.5	132.5	41	141

a: Level of DA by HPLC was 89 $\pm$ 9 ppm, n=4
b: This individual mouse showed atypical hyperactivity with jumping.

APPENDIX B ESTABLISHMENT OF CALCIUM UPTAKE METHODOLOGY:

B.1 INTRODUCTION

The data presented in this appendix was used to establish a method for measuring calcium uptake. Experiments reported in the literature are repeated in order to substantiate the experimental technique.

B.2 METHODS:

B.2.1 Calcium uptake methodology:

The methods are the same as described in chapter 4 except that the guinea pig forebrains had been stored frozen at -80°C before synaptosomes were prepared. The experiments presented in this appendix used a level of 0.1mM $CaCl_2$ except for the last one where different levels were used. When the synaptosomes were stimulated with 70mM potassium chloride, the osmolarity was maintained by substituting an equivilent amount of KCl for NaCl. The rest of the solutions, apparatus and protocol were the same as those described (sections 4.2.2 and 4.2.5).

B.2.2 Electron microscopy:

A preparation of synaptosomes and of

synaptoneurosomes from frozen guinea pig forebrains were given to the electron microscopy lab (AVC) for electron microscopy. They were fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.3) overnight; postfixed in 1% osmium tetroxide in 0.1M phosphate buffer; dehydrated in ethanol and propylene oxide; embedded in an epon-araldite mixture; thin sections were cut and stained with uranyl acetate and lead and examined under an electron microscope.

B.3 RESULTS AND DISCUSSION:

The uptake of calcium in synaptosomes was directly proportional to the amount of protein present up to 0.6mg (Figure B.1). It was of value to verify that the calcium uptake was dependent on the amount of proteins because this allowed the proteins to be measured *a posterior* and the calcium uptake divided by the amount of proteins after they were measured. Although controls were run for each experiment meaning that the intra-experiment comparisons did not depend on processing the data, it was still valuable to do the calculations based on the amount of protein to test for consistency between experiments.

Potassium depolarization of synaptosomes resulted in a dramatic stimulation of calcium uptake (Figure B.2). This was useful for verifying the integrity of the synaptosomes and the calcium uptake methodology. Potassium depolarization is widely believed to open

calcium channels in synaptosomes. These results also showed that the amount of radioactive calcium inside the synaptosomes tends to equilibrate with time regardless of whether the channels are opened or not. Since a large difference between the control and the potassium stimulated synaptosomes was apparent after a 1 min incubation period, this was chosen as an appropriate interval to conduct the calcium uptake experiments.

The uptake is also a function of the concentration of calcium in the incubation medium (Figure B.3). There is a large variation in the level of calcium used in uptake experiments by different scientists ranging from at least 0.02mM to 1.25mM (40, 64, 65). For the experiments presented in chapter 4, a level of 1.0mM was chosen as a reasonable level which should allow for the detection of stimulatory or inhibitory effects on calcium uptake.

A test was also made to find an appropriate preincubation time (Figure B.4). It was decided to use a two min preincubation for the remainder of the experiments.

The electron micrographs did show entities that appear to be synaptosomes and synaptoneurosomes, although other cellular debris was also present (Figures B.5, B.6). Since the method used 0.45μM filters most of the smaller entities such as neuronal mitochondria should wash through the filters at the rinsing stage.

It was noticed that there was a decrease in the

calcium uptake in synaptosomes prepared from previously frozen brains compared with those prepared from freshly killed guinea pigs. It was decided only to use freshly killed guinea pig brains for the experiments presented in chapter 4.

B.4 CALCULATION OF CALCIUM UPTAKE:

Calcium uptake was calculated by measuring the counts per minute (cpm) in 10μl of either 10mM or 1.0mM $CaCl_2$ and relating this back to the amount of radioactivity measured in synaptosomes.

EXAMPLE CALCULATION: If 200,000 cpm was obtained for 10μl of 1.0mM $CaCl_2$ and a single test gave 2000 cpm for 0.300mg protein then the calculation proceded as follows:

1) 10×10^{-6} L X .001 moles/L = 1.0×10^{-8} moles
2) 1.0×10^{-8} moles / 200,000 cpm = 5.0×10^{-14} moles/cpm
3) 5.0×10^{-14} moles/cpm = 5.0×10^{-5} nanomoles/cpm
4) 5.0×10^{-5} nmoles/cpm X 2000cpm/0.92[b] = 0.109 nmoles
5) 0.109 nmoles / 0.300 mg = 0.363 nmoles/mg protein

The results were calculated in one step to eliminate rounding errors. The results were also expressed in terms of time, but since most of the experiments used a one min incubation this is equivalent to nmoles or pmoles per mg protein.

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[b] The value of 0.92 compensates for the efficiency lost because of the filter paper.

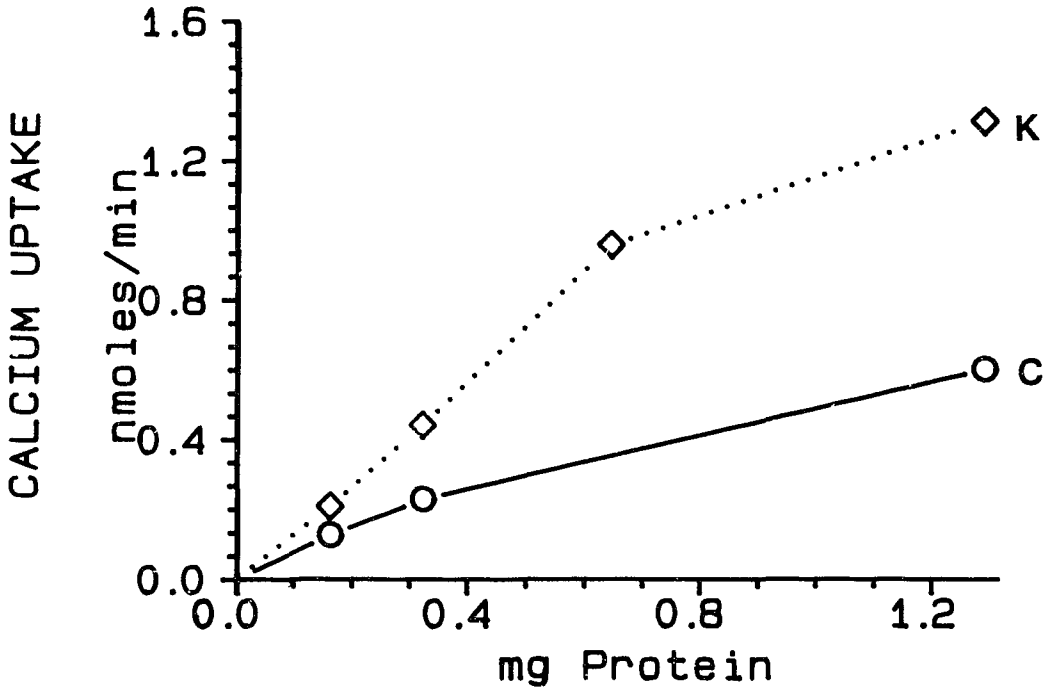

Figure B.1 Calcium uptake as a function of protein concentration in control (C) and potassium stimulated (K) synaptosomes.

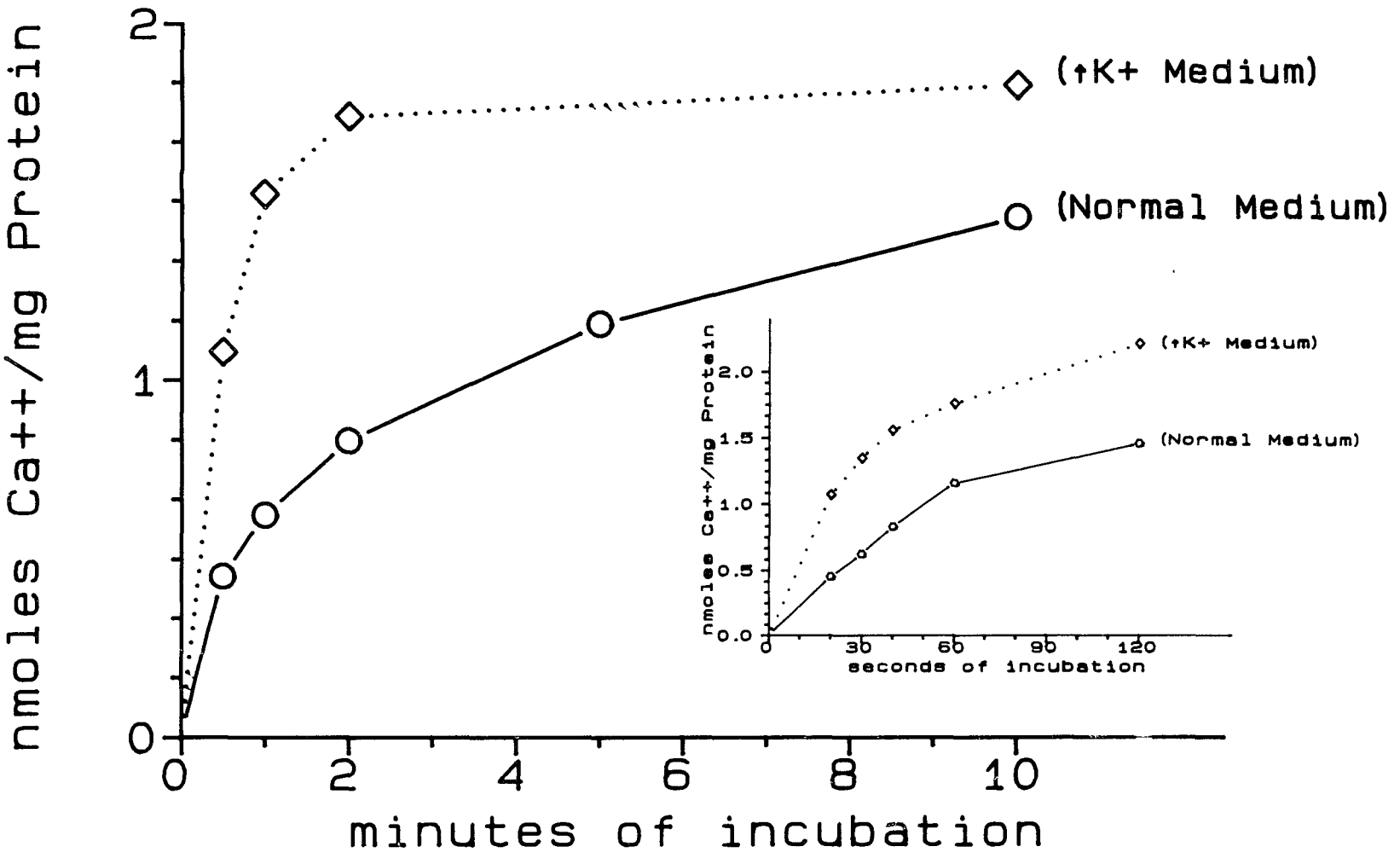

Figure B.2. Calcium uptake as a function of time in control and potassium stimulated synaptosomes.

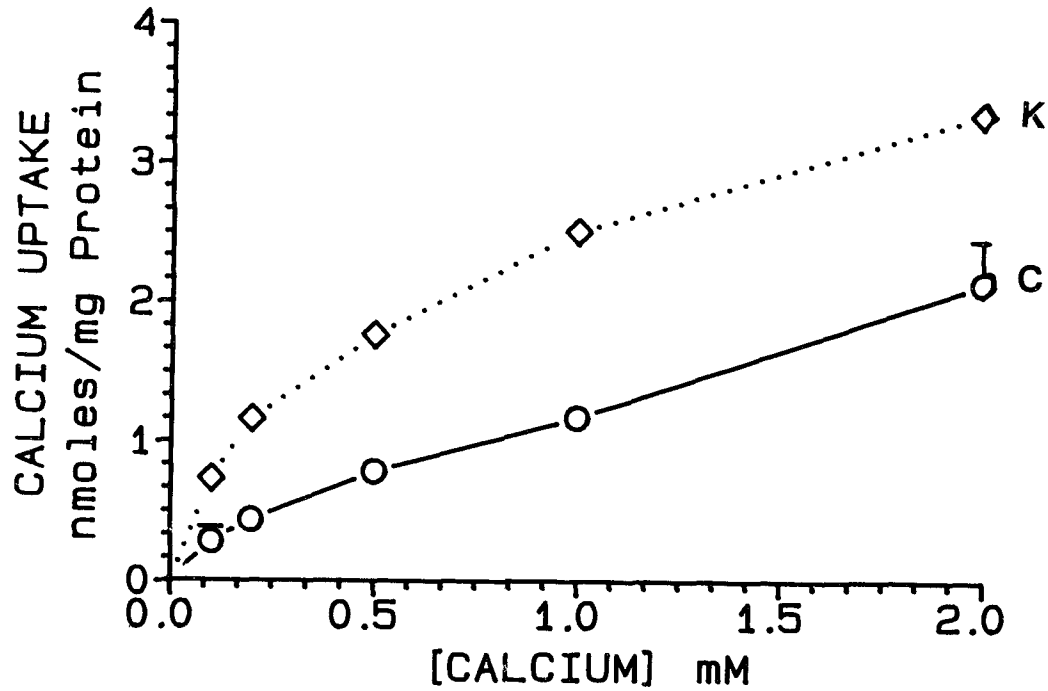

Figure B.3. Calcium uptake as a function of calcium concentration in control (C) and potassium stimulated (K) synaptosomes.

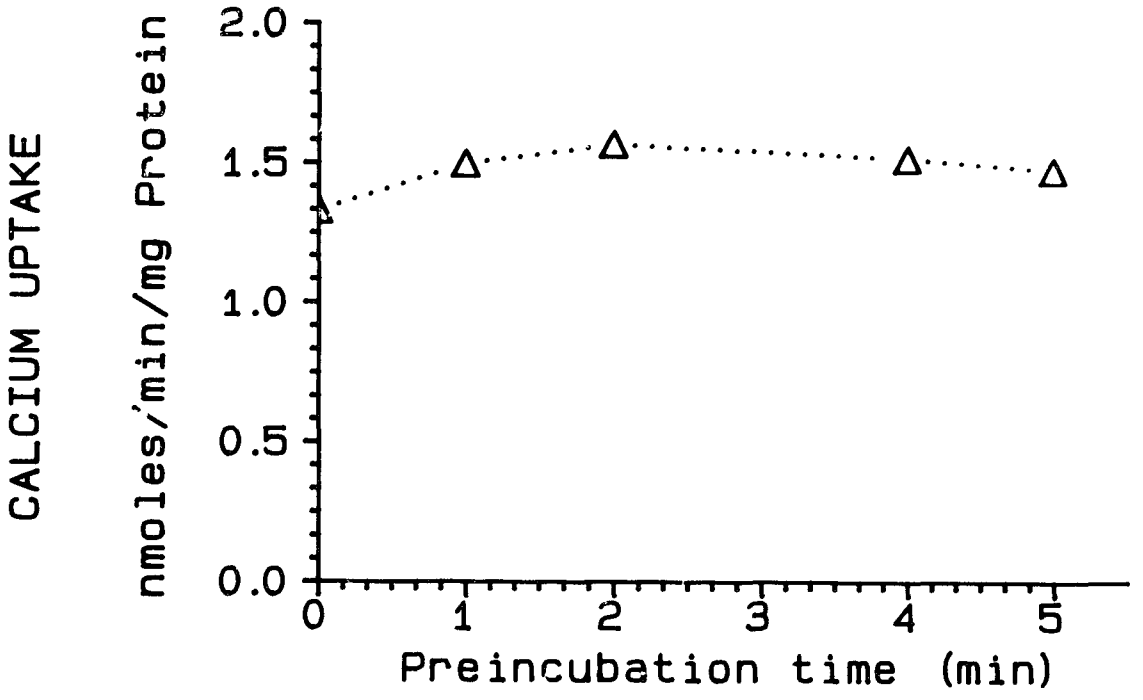

Figure B.4. Calcium uptake as a function of preincubation times in synaptosomes.

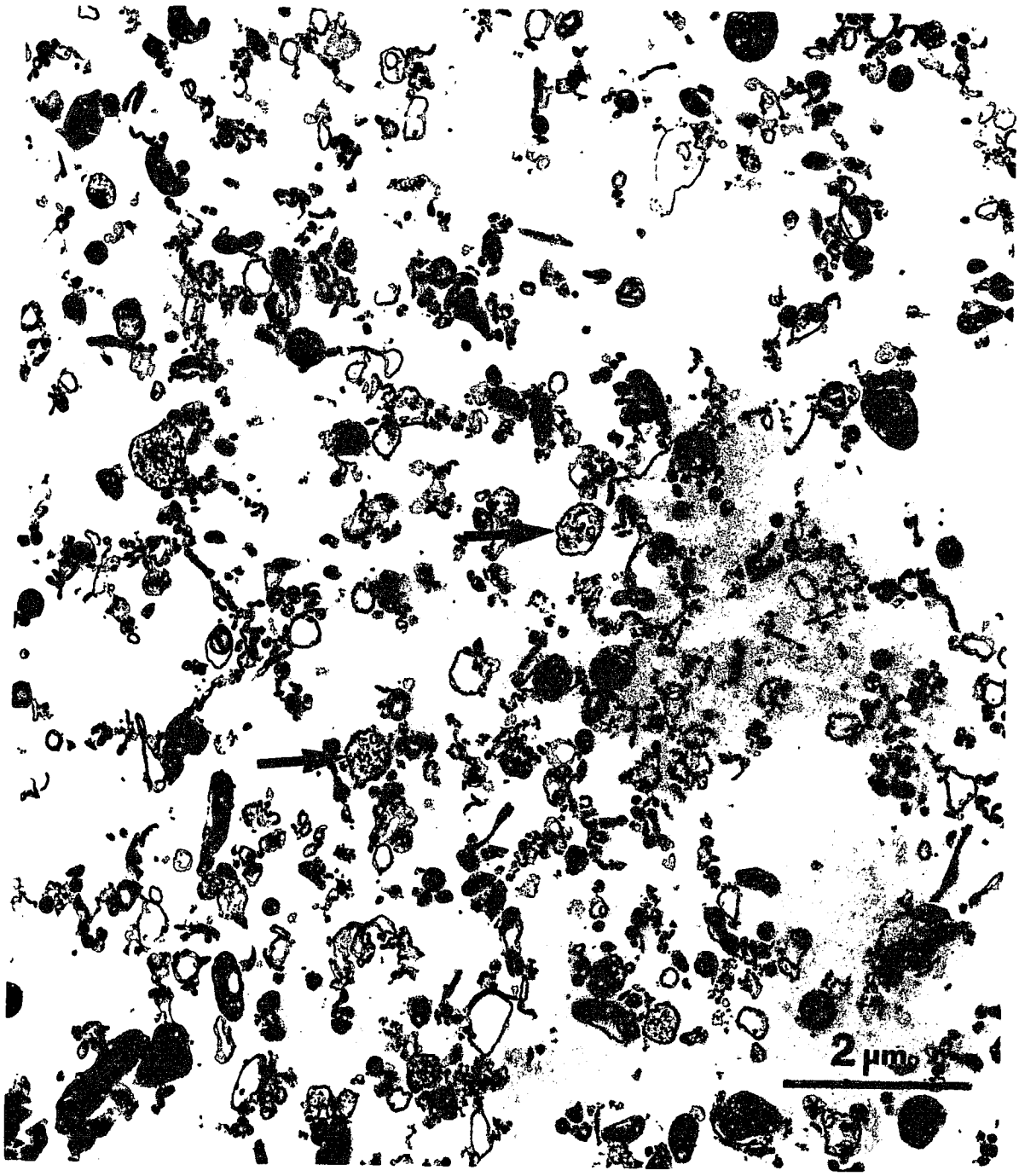

Figure B.5. Electron micrograph of synaptosomal preparation. Arrows point to putative synaptosomes.

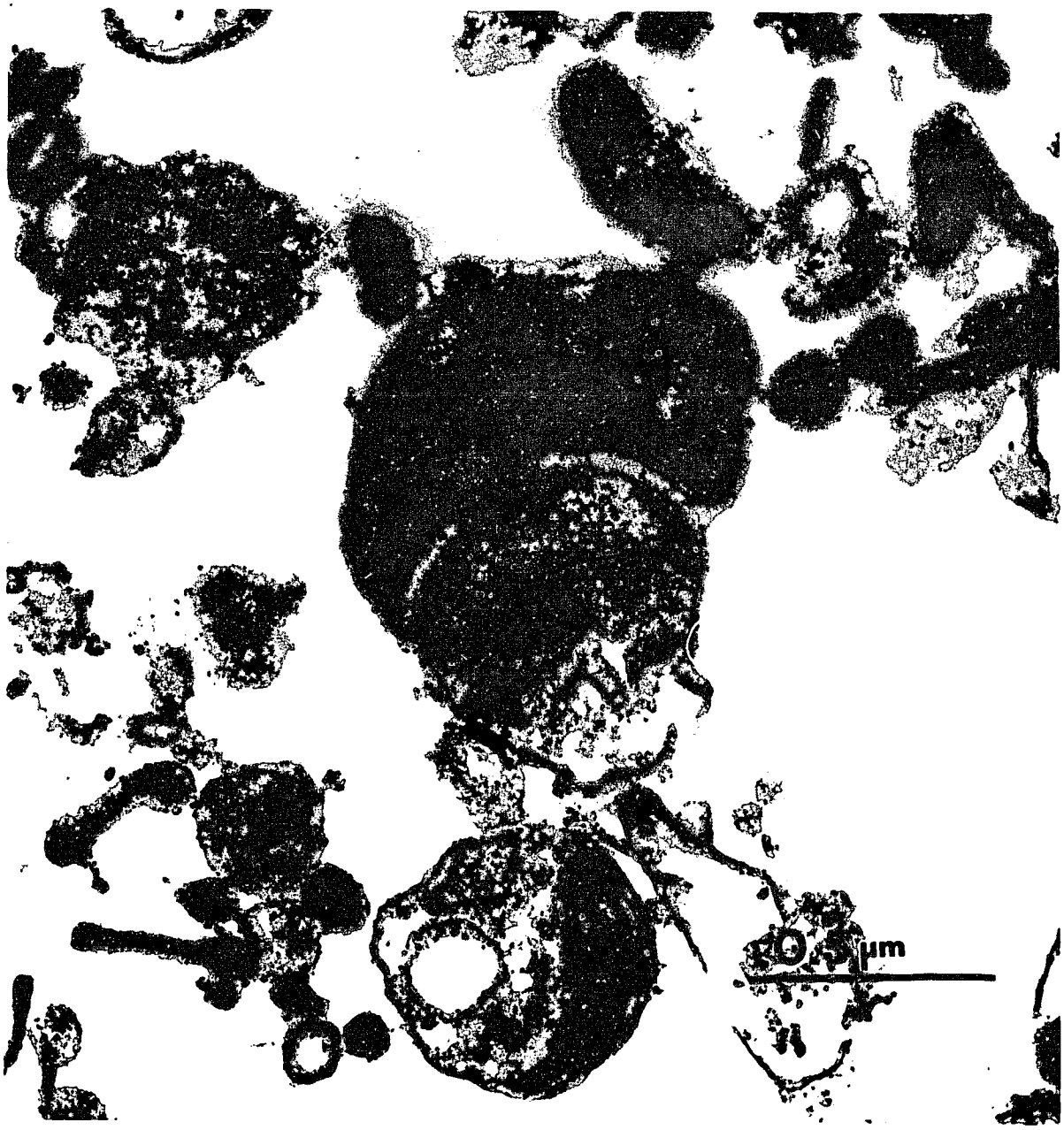

Figure B.6. Electron micrograph of a synaptoneurosomal preparation.

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