FINAL REPORT

FISHING RESEARCH AND DEVELOPMENT

87/058: *A study of the protective mechanisms in fish for ciguatoxin and an assessment of their role in the therapy of human victims.*

88/029: *An assessment in an animal model of the ability of mannitol and other possible antagonists to reverse the neural symptomatology of ciguatera poisoning.*

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CIGUATERA POISONING

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EXECUTIVE SUMMARY

This is the final report for two Fishing Research and Development Grants, 87/058 and 88/029.

Both grants provided funds for research on Ciguatera poisoning and Ciguatoxin. Experimental studies were conducted on fish and mammals.

Ciguatoxin was shown to cause cellular and subcellular damage to the gills, gut and liver of fish, however damage was dose related and dependent on species. Attempts were made to visualise the attachment sites of ciguatoxin in the mammalian central nervous system using immunocytochemical techniques. No conclusive results emanated from these immunocytochemical studies.

Biochemical studies on skeletal muscle from the Spanish mackerel indicated that these fish when carrying ciguatoxin produced a soluble protein of molecular weight 37 400 - 40 600 Daltons. This protein could not be found in Spanish mackerel that did not carry ciguatoxin. This finding is of considerable importance to the Fishing Industry as it could form the basis of a reliable and cost effective test for the presence of ciguatoxin in fish flesh. There are currently no reliable test available for the detection of ciguatoxin in fish.

Feeding experiments in fish demonstrated that carnivorous fish can absorb ciguatoxin and that this toxin produces behavioural changes indicative of action on the nervous system. Ciguatoxin once absorbed from the gut was deposited in skeletal muscle. Precursors of ciguatoxin from *Gambierdiscus toxicus* had no observable effects on these fish and were not found in skeletal muscle after ingestion.

A series of experiments on rats indicated that mannitol had no effect on the alterations in nerve conduction induced by ciguatoxin and brevetoxin, another polycyclic ether of dinoflagellate origin. Improvements in the ciguatera syndrome after mannitol infusion reported in clinical trials have no basis in terms of fundamental nerve conduction parameters. This current study provided no evidence for reversal by mannitol of the changes in nerve conduction characteristic of ciguatera poisoning. However it was established that the local anaesthetic and anti-arrhythmic agent, lignocaine, in clinical doses reversed all of the changes in nerve conduction induced by ciguatoxin. It is suggested that clinical trials should be instigated for lignocaine treatment of the human ciguatera syndrome.

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1.0 Introduction

This final report details the outcomes of two Fishing Industry Research and Development Corporation Grants as given below:

> *87/058 A study of the protective mechanisms in fish for ciguatoxin (CTX) and an assessment of their role in the therapy of human victims.*
>
> *88/029 An assessment in an animal model of the ability of mannitol and other possible antagonists to reverse the neural symptomatology of ciguatera poisoning.*

Both of the above grants are specifically related to ciguatera poisoning and both will be discussed in this combined report. The format of the report is such that it gives an overview of ciguatera based on the literature, a general methods section in which the extraction and partial purification of CTX from fish is described and then sections that address how each of the specific research objectives of the initial proposals were met. At the end of the report significant outcomes and directions for future research are presented.

In terms of the initial grants, the specific research objectives are given below:

1. *To determine the principal sites of storage CTX in carrier fish. (87/058)*

2. *To attempt to elucidate the mechanisms of sequestration of CTX in the tissues of carrier fish. (87/058)*

3. *To examine the actions of CTX on selected tissues in a mammalian model of ciguatera poisoning in human. (87/058)*

4. *To evaluate the mechanisms of sequestration of CTX by fish as potential methods of therapy in humans. (87/058)*

5. *To use the ventral coccygeal nerve preparation of the intact anaesthetised rat to assess the efficacy of mannitol and other antagonists on the reversal of neurological manifestations of ciguatera poisoning. (88/029)*

2.0 General Overview of Ciguatera

Ciguatera poisoning is a form of food poisoning that occurs in humans after the consumption of particular specimens of a variety of tropical marine fish species. It is caused by the ingestion of small quantities of a very powerful toxin, CTX, that occurs in the tissues of the offending fish. The distribution of human ciguatera poisoning is confined mainly to the tropical regions of the world where it is a considerable cause of morbidity. Over 50,000 people may be affected each year in the tropics (Regalis, 1984).

Ciguatera poisoning has also been reported to be the most frequent form of food-borne disease of a chemical nature within the USA (Withers, 1982) with most cases emanating from Florida and Hawaii. Episodes of ciguatera poisoning have been documented from the Caribbean and the Pacific Regions (Bagnis et al., 1979; Holt et al., 1984). Cases of ciguatera poisoning occur annually along the tropical coast of eastern Australia (Capra and Cameron, 1991).

Although causative fish are restricted to tropical regions, cases of ciguatera poisoning have been reported from temperate regions as a result of individuals consuming toxic fish while in the tropics and then presenting with the ciguatera syndrome after air travel to a temperate country and also by the consumption of toxic tropical fish in temperate regions. Forty-four cases of ciguatera were reported from Canada in a period 1964-1985 both in victims who had consumed fish in the tropics and in those who consumed tropical fish purchased in Canada (Todd, 1985). The largest outbreak of ciguatera poisoning in Australia, occurred in 1987 in the temperate city of Sydney (Capra and Cameron, 1991). Sixty-three people were poisoned in this outbreak after eating Spanish mackerel *(Scomberomores commersomi)* marketed in Sydney but captured in a ciguatera endemic region, just below the southern margin of the Great Barrier Reef some 13OO km to the north of Sydney. In 1984 a further mass poisoning occurred in Sydney in which 40 people were intoxicated. The most dramatic case of mass ciguatera poisoning occurred in 1994 when 500 people in Madagas were poisoned with 98 deaths (Habermehl et al., 1994).

There is now a considerable amount of information on various aspects of ciguatera poisoning and CTX with regular publication of reviews and monographs (Juranovic and Park, 1991; Miller, 1991). Interest in the ciguatera syndrome is of long standing with the earliest European documentation coming from the West Indies in the sixteenth century (Withers, 1982). The first published records from the southern hemisphere were from Anderson (1776) who described an

episode of ciguatera poisoning among the crew of one of Captain Cook's ships in the waters of Vanuatu.

2.1 Toxin(s) Responsible for Ciguatera poisoning

Scheuer et al., (1967) suggested that the syndrome of human ciguatera intoxication that follows consumption of certain tropical fish was due to a single chemical entity, ciguatoxin (CTX). Subsequent chemical studies on the livers of toxic moray eels, *Gymnothorox javanicus* identified an extremely toxic compound (LD_{50} of 0.45g/kg ip mice) with a molecular weight of 1111.7 Daltons (Tachibana et al., 1987). The compound isolated was a polar and highly oxygenated molecule that belonged to a class of polyether compounds and showed similar chromatographic properties to the smaller polycyclic ether compound okadaic acid.

The complexity of the CTX molecule and its low concentration (0.5 to 10.0 ppb) in the tissues of toxic fish hindered the final elucidation of its structure. Legrand et al., (1989) on the basis of high resolution mass spectrometry published a probably molecular formula of $C_{60}H_{86}0_{19}$. Later in the same year a partial structure of CTX was published (Murata et al., 1989a). The full structure of CTX from fish and its likely dinoflagellate precursor molecule were subsequently published (Murata et al., 1989b; Murata et al., 1990). Murata et al., (1990) confirmed the previous molecular formula for CTX of $C_{60}H_{86}O_{19}$ and described a brevitoxin-like polyether molecule with 13 continuous-ether rings. A less oxygenated congener (gambiertoxin 4b) was isolated from *Gambierdiscus toxicus* the putative elaborator of CTX, which Murata et al., (1990) suggested may be oxidised as a detoxification step in carrier fish. Lewis et al., (1991) described three major ciguatoxins CTX-1, CTX-2 and CTX-3 from moray eels. CTX-1 was comparable to the ciguatoxin isolated from moray eels while CTX-2 and CTX-3 were less polar molecules that were 16 mass units less that CTX-1. The signs produced in mice by each of these toxins were similar, however, CTX-2 and CTX-3 induced hind limb paralysis that did not occur with CTX-1. Lewis and Sellin (1992) have now demonstrated the presence of CTX-1, CTX-2 and CTX-3 in three species of tropical fish, Spanish mackerel *(Scomberomorus commersomi)* coral trout *(Plectropomus* spp) and blotched javelin *(Pomadasys maculates).* The relative proportions of the three distinct toxins differed in each of the species.

Earlier concepts that the ciguatera syndrome in humans is due to a single toxic entity may now need modification. Lewis et al., (1991) and Lewis and Sellin (1992) have shown that variants of

the CTX molecule can occur in a number of species of tropical fish and that the ratios of the different forms of CTX are also variable. A recent review by Juranovic and Park (1991) acknowledges the possible involvement of five toxins (CTX, maitotoxin, scaritoxin, okadaic acid and prorocentrolid) in the ciguatera syndrome. Other toxins including palytoxin (Kodama et al., 1989) may contribute to the general ciguatera syndrome and may also afford an explanation for the variability of symptoms reported in humans by various authors (Capra and Cameron, 1991).

2.2 Origins and Transmission of CTX

Humans may fall victim to ciguatera poisoning after eating an individual specimen of any one of a number of marine tropical fish. Over 400 species of fish were listed worldwide by Halstead (1978) as potential carriers of CTX and hence possible sources of human intoxication. Halstead's figures are considered to be an overestimation and the number of potential carriers may be much lower. Data collected by Bagnis et al., (1985), over twenty years in French Polynesia, implicated approximately 100 species as potential carriers of CTX. In Australia several species of primarily carnivorous fish including mackerel, barracuda and coral trout are responsible for human intoxication (Gillespie et al., 1986). Three species of fish from northern Australia, red bass, (Lutjanus *bohar),* chinamen fish *(Symphorus nematophorus),* and paddletail *(Lutjanus gibbus),* have been shown to have a high rate of toxicity of 4%, 7% and 18% respectively from reefs in the vicinity of the coastal city of Cairns (Capra and Cameron, 1991). The incidence of toxicity in fish can vary both geographically and temporarily.

Although it has long been known that ciguatera poisoning follows the ingestion of certain specimens of tropical fish, the origin of the toxin remained obscure until quite recently. Examination of the diets of fish from a ciguatera endemic region of French Polynesia revealed large numbers of a dinoflagellate, *Diplopsalis sp,* extracts of which showed similar chromatographic, pharmacological and immunological properties to CTX derived from fish tissues (Yasumoto et al., 1977). This dinoflagellate, initially classified as *Diplopsalis sp* was subsequently described as *Gambierdiscus toxicus* (Adachi and Fukuyo, 1979). *Gambierdiscus,* an epiphyte found on a number of species of macroalgae, has now been identified in Hawaii (Taylor, 1979), the Caribbean (Miller et al., 1982) areas of the Pacific other than French Polynesia (Yasumoto et al., 1984) and Australia (Gillespie et al., 1985). Cultures of *Gambierdiscus* have produced maitotoxin (Gillespie et al., 1985) and mixtures purported to contain both maitotoxin and CTX (Durand et al., 1985). The structural studies of Murata et al.,

(1989a & b) and (1990) suggest that *Gambierdiscus* produces precursors (gambiertoxins) of CTX that are bioconverted to CTX(s) by biochemical mechanisms within fish. Variation in toxicity between different geographical localities may reflect variation in the numbers of *Gambierdiscus* or the presence of strains capable of producing greater concentrations of toxin. Bomber et al., (1988) worked with cultures of *Gambierdiscus* from different latitudes and found that clonal toxic potencies decreased with increasing latitudes. Holmes et al., (1991) have shown that the production of gambiertoxins by Gambierdiscus is strain dependent and propose that ciguatera only occurs when strains with the genetic capacity to produce gambiertoxins give rise to blooms. The presence of *Gambierdiscus toxicus* in non-toxic regions suggests that the strains are not capable of gambiertoxin synthesis and the appearance of toxicity within a previously non-toxic region may indicate colonisation of the region by toxin producing strains.

Gambierdiscus toxicus may not be the sole originator of toxins responsible for the ciguatera syndrome as *Ostreopsis sp.* (Ballantine et al., 1985) and *Prorocentrum concavum* (Tindall et al., 1984) have also been implicated. Recently the cyanobacterium *Oscillatoria erythreae* has been shown to be a potential source of CTX-like toxins (Hahn and Capra, 1992).

Ciguatoxin precursors are believed to enter the human food chain via browsing and grazing fish such as the surgeon fish, *Ctenochaetus* sp, and then move through various trophic levels. Gambiertoxin-like toxins and CTX-like toxins also enter the food chain via a variety of invertebrates (Hahn, 1991).

2.3 Pharmacological actions of Ciguatoxin

It has been long recognised that CTX has profound effects upon the nervous system and Li (1965), suggested that CTX acted primarily as an anticholinesterase. His studies however appeared to be based on impure extracts contaminated by other compounds and his assertions of an anticholinesterase activity were subsequently rejected, Rayner (1969). Rayner (1969) was the first to suggest that CTX caused a widespread effect on excitable tissues by increasing Na^+ permeability. There is now ample evidence to support the view that CTX acts on the Na^+ channels of excitable membranes and increases the permeability of these membranes to Na^+ (Rayner 1972; Bidard et al., 1984; Capra and Cameron, 1985; Lewis, 1985). In cultured neuroblastoma cells, CTX induces a membrane depolarisation that is antagonised by tetrodotoxin. The widespread neural effects of CTX would appear to be due to this fundamental

action of CTX on Na^+ channels within excitable membranes. The autonomic responses to CTX may be due to both presynaptic effects producing transmitter release and post synaptic effects of CTX on Na^+ channels in effectors (Lewis, 1985). Molgo et al (1990) have shown that CTX, in nanomolar concentration, acts on Na^+ channels of the neuromuscular junction to produce both pre- and postsynaptic effects. The inhibition of the actions of CTX by tetrodotoxin in both, *in* vitro neuromuscular preparation from whole animals (Moglo et al., 1990) and cell culture preparations (Moglo et al., 1991) indicate that the CTX exerts its primary action on Na^+ channels. Only preliminary evaluations of the pharmacological actions of the various forms of fish ciguatoxin have been made to date. All three fish ciguatoxins competitively inhibit the binding of brevetoxin to voltage dependent sodium channels (Lewis et al., 1991), suggesting qualitatively similar pharmacological actions for the currently described variants of ciguatoxin.

Pharmacological studies on the neural actions of CTX have been restricted, largely, to *in vitro* studies of isolated nerve and nerve muscle preparations. Such studies while providing considerable information on the action of CTX do not approximate the clinical situation that prevails in human victims. Capra and Cameron (1985) and Cameron et al., (1991a) used *in vivo* recordings from the ventral coccygeal nerve of anaesthetised rats as a model of human ciguatera intoxication. Rats given sublethal doses of CTX derived from toxic Spanish mackerel, *Scomberomorus commersoni*, displayed alterations in a number of nerve condition parameters. There was a significant slowing of both mixed and motor nerve conduction velocities and motor and mixed nerve amplitudes were significantly reduced. Both absolute refractory periods and supernormal periods were significantly prolonged together with an exaggeration of the supernormal response. The results of these *in vivo* studies indirectly suggest that CTX acts in intact mammals by prolongation of sodium channel activation.

While there is now a considerable literature on the effects of CTX on mammalian nerves, there are few studies on the actions of this toxin on fish nerves. CTX has been shown to be lethal to both fresh water fish (Lewis, 1992) and marine fish (Capra et al., 1988). However, marine fish are less susceptible to CTX than mammals and fish with a feeding regimen that leads to dietary exposure to *Gambierdiscus* are less susceptible to CTX than those with no exposure to the causative dinoflagellate (Capra et al., 1988). Individual tropical fish can carry sufficient CTX in their tissues to poison several humans without obvious signs of pathology. Neurophysiological studies (Flowers et al., 1987; Flowers, 1989) and $22Na^+$ efflux studies (Capra et al., 1987) on marine fish indicate that the Na^+ channels of fish are susceptible to CTX and that CTX exerts a similar effect on fish Na^+ channels to that on mammalian channels. Hahn et al., (1992) have

described the presence of a CTX induced soluble protein-CTX association in the muscle of toxic specimens of *Scomberomorus commersoni.* The induction of this protein and its association with CTX may be the basis of a sequestration mechanism that diminishes the binding of CTX to the target sites on the Na^+ channels of excitable membranes in fish.

2.4 The effects of Ciguatoxin on humans

There have been many reports on the clinical features and symptomatology of ciguatera poisoning in humans (Bagnis et al., 1979; Lawrence et al., 1980; Gillespie et al., 1986; Bagnis and Legrand, 1987; Capra and Cameron, 1991). After ingestion of toxic fish, the course of the affliction often follows a reasonably predictable pattern. The initial symptoms are usually gastrointestinal and develop at an early stage some three to twelve hours after the consumption of toxic fish. The gastrointestinal symptoms can include nausea, vomiting, diarrhoea and abdominal cramps. The usual time of onset of gastrointestinal symptoms is approximately six hours after ingestion. The severity of these symptoms is variable and may depend upon the toxicity and quantity of fish ingested. After the onset of gastrointestinal dysfunction, neurological symptoms usually begin to appear twelve to eighteen hours after consumption of toxic fish. These symptoms can include paraesthesia of the lips and extremities, arthralgia, myalgia, dental pain, convulsions, muscular paralysis, vertigo, severe headache, short term memory loss, temperature perception reversals, diaphoresis and pruritus. Some victims display psychological disturbances, manifest as anxiety and depression for months and sometimes years after intoxication. Cardiovascular symptoms which include bradycardia, tachycardia, arrhythmia and hypotension are also evident in a number of victims.

A feature of the ciguatera syndrome in humans is the variability in duration and severity of dysfunction in various victims of the toxin. Variations in the amount of toxin consumed and the physiological status of each victim may account for some of the variability of response. The recently established variations in the quantity of the variants of CTX in individual fish may also contribute to variations in clinical manifestation (Lewis and Sellin, 1992). An outbreak of ciguatera poisoning in Sydney, Australia in 1987 led to 63 reported cases of intoxication from possibly one to two toxic Spanish mackerel. A study of forty of these victims over a six month period (Capra and Cameron, 1991) revealed variations in severity of intoxication and in the duration of persistence of individual symptoms. The longitudinal study of the Sydney victims did reveal that symptoms persisted for longer periods than previously reported.

Six months after ingestion of the toxic fish, 20 of the 40 victims in the study displayed at least one symptom. One victim who had consumed one kg of toxic fish over a three day period had eight persistent symptoms after six months. Despite the often severe clinical manifestation of ciguatera poisoning, very few deaths were attributable to ingestion of fish containing CTXs until the high mortality reported by Habermehl et al., (1994).

The neurological effects of CTX are clinically prominent and account for the major discomfort of most victims of this toxin. Very few studies have been undertaken on the neuropathology of CTX in humans. Limited nerve biopsies of human with clinical ciguatera poisoning reveal striking oedema of the adoxonal Schwann cell cytoplasm (Allsop et al., 1986). A clinical study of nerve conduction parameter of the Sural and common peroneal nerves in 15 cases of acute ciguatera poisoning showed a significant slowing of sensory conduction velocity and prolongation of absolute and relative refractory periods and of the supernormal period (Cameron et al., 1991 b). The electrophysiological changes induced in humans by consumption of toxic fish are essentially similar to those in the rat as previously described. The use of the rat as a model for human intoxication may be useful in assessing potential therapies.

2.5 Summary

Ciguatera poisoning is often a severe form of food poisoning that follows the consumption of certain tropical fish. The syndrome of poisoning may be due to a variety of related polycyclic ether toxins that are produced in fish by minor modifications of precursor molecules elaborated by benthic dinoflagellates, in particular *Gambierdiscus toxicus.* The toxin(s) responsible for the syndrome have a fundamental action on the Na^+ channels of excitable tissues and appear to act both centrally and peripherally. The actions of CTX on Na^+ channels are qualitatively similar in humans, rodents and fish.

Two issues are now of prime importance in diminishing the human impact of ciguatera poisoning. These are the development of reliable and cost effective methods for detection of toxin in the flesh of fish and the introduction of effective therapeutic regimens for victims of the toxin(s). Considerable success in the treatment of certain ciguatera victims with intravenous infusions of mannitol has been reported (Palafox et al., 1992; Blythe et al., 1992).

3.0 Extraction and Purification of CTX

Several batches of extracted CTX were used in the course of the studies outlined below. Toxic and potentially toxic fish were acquired as donations from cases of human ciguatera poisoning and from commercial fisherman in Queensland (Hervey Bay and Cairns) and the Northern Territory (Gove). The fish were stored at -20 degrees Celsius prior to extraction and bioassay. The extraction method was based on the method of Nukina et al., (1984) as modified by Hahn (1991). Tissues of toxic fish were generally pooled for extraction. Species used included mackerel, barracuda, red bass, chinamen fish, stripey and trumpeter. Large quantities of toxic flesh were not available from any particular species.

The toxic extract used for pharmacological, physiological and histological experiments in this study was the fraction eluted form the silic acid column with chloroform: methanol 9:1 (Figure 1). The toxic extract was reduced by rotary evaporation and stored under anhydrous methanol at -20 degrees Celsius. When the toxin was required for use, the methanol was evaporated under a stream of nitrogen. The toxin was taken up in a vehicle consisting of a suitable physiological saline and 1% Tween 80 solution as an emulsifier. The extracts from the 9:1 chloroform: methanol are not pure CTX and it was not possible to determine a concentration of CTX in any particular extract. Estimates were made of the lethality of the toxin by limited bioassay in mice using death time. For ethical reasons minimal numbers of mice were used. Toxicity was generally expressed as an LD_{50} value or in terms of mouse units. LD_{50} values were calculated using the formula of Tachibana (1980) as given below:

$$\text{Log}_{10}(LD_{50}) = \text{Log}_{10}d - 2[\text{Log}_{10}(1+t^{-1})]$$

where d = dose in mg/kg

t = death time in hours

One mouse unit (mu) is defined as the LD_{50} value divided by 50 this then being the LD_{50} equivalent dose in a 20 gram mouse.

3.1 Algal Toxins

Toxins derived from cultures of *Gambierdiscus toxicus* were kindly provided to the QUT ciguatera group by Prof Donald Miller of the School of Medicine, Southern Illinois University, Carbondale,

Illinois USA. Prof Miller provided two fractions from extraction of cultured *G.toxicus* classified in terms of the chromatography. Green extract with an LD_{50} of 5mg/kg (ip mice) and a concentration of 1mg/mL in methanol and a Brown extract with an LD_{50} of 1.4mg/kg at a concentration of 1.75mg/mL in methanol.

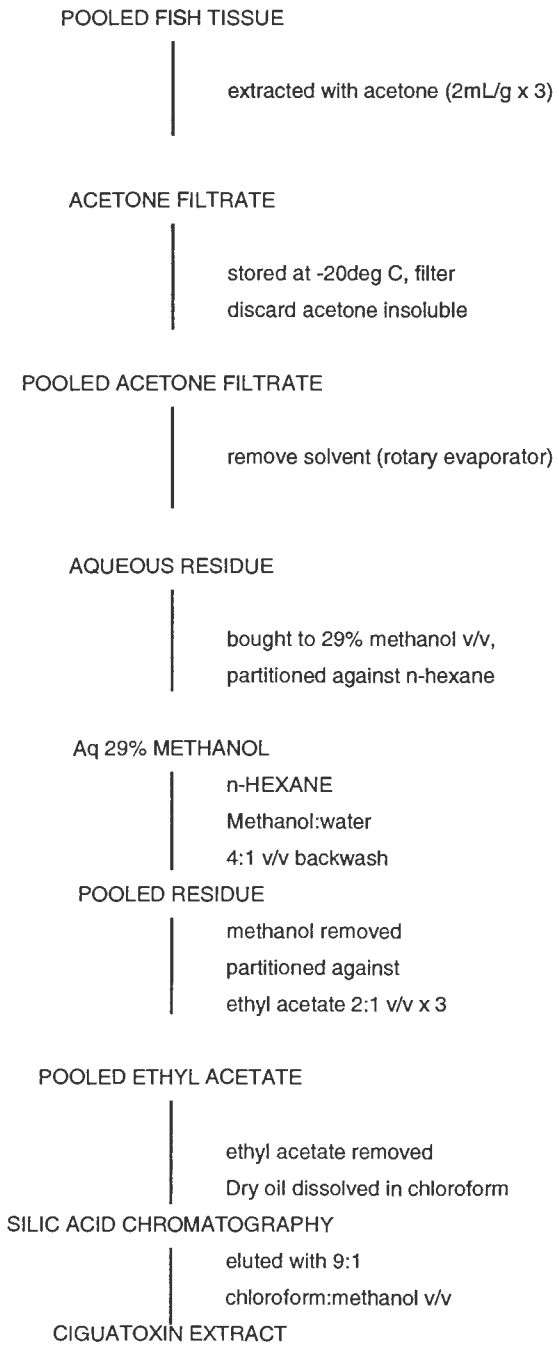


Figure 1: Flow diagram of the bulk extraction procedure for ciguatoxin

4.0 Addressing the Stated Research Objectives

The combined objectives of the two FRDC research grants supporting ciguatera research at QUT are given in Section 1.0. In the following sections of this report, the methods by which each of these objectives were addressed will be detailed. The research conducted at QUT over the past 10 years and supported, in part, by FRDC has fallen into two main categories: studies in fish and studies in mammals. The funds provided by FRDC in Grants 87/058 and 88/029 supported work in the fields of fish and mammalian studies and the results of this work will be presented under these headings.

5.0 Studies in Fish

5.1 General Introduction to Studies in Fish

There are very few reports on the action of CTX on fish. To date, this sparse literature presents a confusing picture.

The normally non-toxic surgeon fish, *Acanthurus xanthopterus*, can be fed CTX fish and acquire the toxicity without the signs of intoxication (Helfrich and Banner, 1963). When CTX was added to the ambient water of the guppy, *Lebistes reticulatus*, it produced death in a short time (Bagnis et al., 1980). The toxin in this case was absorbed via the gills, contrary to what would be the normal route encountered in nature. Davin et al., (1986) reported on the intoxication in blueheads, *Thalasomma bifascitum*, by the ingestion of ciguatera toxins associated with *G. toxicus* cultures. Later studies using piscivorous fishes showed abnormal behaviour and death after consumption of ciguatoxic barracuda (Davin et al, 1988). Capra et al., (1988), reported differences between two species of reef dwelling pomocentrids, *Pomocentrus wardii* and *Chromis nitida*. After i.p. injection of ciguatoxin it was found that both species were significantly more resistant to the toxin than were mammals. However, *P. wardii*, a browser which feeds on algae, was significantly more resistant than *C. nitida*, a planktivore. The difference in their resistance to the toxin reflects their different feeding habits. The browser, by its mode of feeding, can ingest *G.toxicus*, the putative source of ciguatoxin (Adachi and Fukuyo, 1979: Yasumoto et al., 1980) while the planktivore would rarely come in contact with this benthic dinoflagellate. The nerves of fish have been shown to respond to CTX in a manner that is qualitatively similar to the responses of mammalian nerves (Capra et al., 1988; Flowers et al., 1992).

It was this somewhat confusing picture that initiated the studies of the effects of CTX on fish in which the following two objectives were addressed:

- *To determine the principal sites of storage of CTX in carrier fish.*
- *To attempt to elucidate the mechanism of sequestration of CTX in the tissues of carrier fish.*

In order to address these objectives a number of related investigations on the effects of CTX on fish, and the distribution of CTX in fish were performed under the following general headings:

- Histological studies
- Toxicological studies
- Biochemical studies

5.2 Histological Studies on Fish

5.21 Introduction

Capra et al., (1988) found significant histopathological changes in the gills and gastrointestinal tract of two species of pomacentrids injected with CTX into the peritoneal cavity. These two structures appeared to be target sites for structural damage associated with the presence of CTX. It had previously been shown by Coombe et al., (1987), that CTX induced structural damage at the cellular level in the small intestine of laboratory mice.

In the initial research proposal to FRDC we intended to examine a range of tissues from fish exposed to CTX in order to assess the extent and site of toxin induced cellular damage. Several tissues from intoxicated fish were examined during the course of the study and the results are presented below. It was also our intention to use immunohistochemical techniques to attempt to localise the binding of CTX within specific tissues. Part of the project also involved the identification of binding sites within mammalian tissue. As histochemical and immunohistochemical techniques are in a more refined state for mammalian tissues we decided to begin these studies in mice. The immunohistochemical studies in mice (Section 6.1) were quite inconclusive and as these techniques could not be successfully used for mammals we

decided to abandon the study in fish. Instead we focussed on biochemical studies to gain some insight into the possible partitioning of CTX within fish and the results of these biochemical studies are given in Section 5.4.

5.22 Methods

All experimental work on histopathological changes was carried out at the University of Queensland, Heron Island Research Station (HIRS).

In the initial proposal two species of pomacentrid fish, *Pomacentrus wardi* and *Chromis nitida* were identified as test organisms. These fish could be easily captured by SCUBA divers working in shallow water (3-9m) on the edge of reefs. Fish were localised around individual coral heads and then sprayed with an alcoholic solution of the fish anaesthetic, quinaldine. Shortly after this program began we were alerted to the possible effects of quinaldine on the thyroid. Divers using quinaldine are exposed to the risk of cutaneous uptake particularly by the skin of the face and neck. Subsequent unpublished studies by Capra on the effect of quinaldine on mice indicated that this compound caused hyperplasia of thyroid follicles. The occupational risks associated with quinaldine use were considered to be unacceptable and alternative species and alternative methods of capture were explored. We subsequently found that reasonable numbers of the pomacentrid *Dascyllus aruanus* could be captured when a small coral head was lifted into a hoop net then into a boat from shallow water in the Heron Lagoon. Fish capture in this manner were held in aerated water on the collection boat prior to being placed in the HIRS aquarium in replenished sea water. Fish were held in the aquarium for periods up to three weeks and while held they were fed daily on aquarium fish food.

Fish were injected on a weight/weight basis with CTX in multiples of the mouse LD_{50}. Injections were performed using glass microelectodes attached to microsyringes. All injections were into the peritoreal cavity. Prior to injection, fish were weighed and placed in individually labelled holding aquaria. Immediately before injection, fish were anaesthetised in MS222 (ethyl m-aminobenzoate) made up in a 1:1000 sea water solution. All injections were carried out under a dissecting microscope at low magnification.

Tissues for histological examination were either taken from fish, at the point of death, given lethal doses of toxin or after fish had been euthanased in excess MS222 anaesthetic. Tissues were

fixed in 10% neutral buffered formalin (pH 7.2) for light microscopy or in Karnovsky's fixative (2.5% glutaraldehyde, 2% paraformaldehyde) for electronmicroscopy. Sections for both light and electronmicroscopy were prepared using standard methods (Blanton, 1994).

5.23 Results

Tissues were examined from both control and experimental fish. Control fish received injections of the saline and Tween vehicle only, or injections of an extract made in the same way as the CTX extract but from the flesh of the non toxic fish. Table 1 summarises the number of fish in each treatment for each species.

Experiment	Species		
	Pw	Cn	Da
Control	10	10	34
Toxin	17	28	78

Table 1: Number of fish for each treatment for each species Pw: *Pomacentrus wardi*; Cn *Chromis nitida*; Da *Dascyllus aruanus*

In both control and experimental groups, the intestine, gills and liver were examined for histological changes. The structure of the tissues from all three species were similar and there was no evidence of pathology in any of the tissues (Hibiya, 1982).

In initial series of experiments using *P wardi* and *C nitida* carried out in 1987 and 1988 , injection of two and four times the mouse LD_{50} per 5 g of fish resulted in the ultimate death of all fish. The time from injection to death (mins) was recorded and data for these series is presented in Table 2.

Species, dose X LD_{50}, year	Number of fish	Mean Death time (mins)	Standard Error of Mean (mins)
Pw, x2, 1987	2	264	81
Pw, x2, 1988	6	362	69
Pw, x4, 1987	5	268	16
Pw, x4, 1988	4	464	92
Cn, x2, 1987	8	253	69
Cn, x2, 1988	6	218	76
Cn, x4, 1987	9	183	63
Cn, x4, 1988	5	117	6

Table 2: Mean death times of *P wardi* (Pw) and *C nitida* (Cn).

For both sets of experiments (1987 and 1988) the same CTX extract, extract number 1 was injected into the fish. Overall for the two years combined the time to death of *P wardi* was significantly longer (1% level Dunnets t-test) than the time to death of *C nitida*. Within species there was no significant difference between the death times for the high dose (4 x LD_{50}/5g fish) and the low dose (2 x LD_{50}/5g fish) exposures in either year.

Samples of tissue were taken from the fish exposed to CTX in 1988 and assessed for histopathological signs. Most tissues examined from *P wardi* and *C nitida* displayed some pathological changes which are summarised in Table 3.

Species	Tissue		
	Liver	Intestine	Gill
P. wardi	100 (5)	60 (5)	100(5)
C. nitida	100 (7)	100 (7)	80 (7)

Table 3: Percentage of the sections examined that displayed some pathological changes for 1988 experiments, figures in parentheses represent the actual number of sections examined.

Damage to the intestines was restricted to the degeneration of the lamina propria, swelling of the villi tips and damage to the brush border. The damage was similar in both species (Figure 2). The changes that occurred in the gill included dilatation of the primary lamellae and stripping away of the surface epithelium from the secondary lamellae, similar changes occurred in both species (Figure 3). CTX at both levels of exposure induced slight histopathological changes in the livers of both species. The CTX induced changes included margination of nuclei, slight fatty changes and the loss of glycogen.

The use of *P wardi* and *C nitida* as test species was abandoned after the series of experiments reported above because of the occupational risks of exposure to quinaldine used to collect these fish. All subsequent studies were performed on *D aruanus.* Where as *P wardi* and *C nitida* were exposed to only one extract of CTX (extract number 1), *D aruanus* was exposed to several different extracts derived both from toxic fish flesh and cultured *G toxicus.* These extracts of CTX, extract number 1, extract number 2 and extract number 3 were used on *D aruanus* as well as two extracts (green extract and brown extract) derived from cultured *G toxicus* and provided to the QUT by Professor Don Miller of the School of Medicine, Southern Illinios University, USA. A much greater range of doses of CTX and algal toxins was given to *D aruanus* than to *P wardi* and *C nitida*. *D aruanus* experiments were conducted during three short field trips to HIRS in November 1988, April 1989 and November 1989. A summary of the toxin and control injections is given in Table 4.

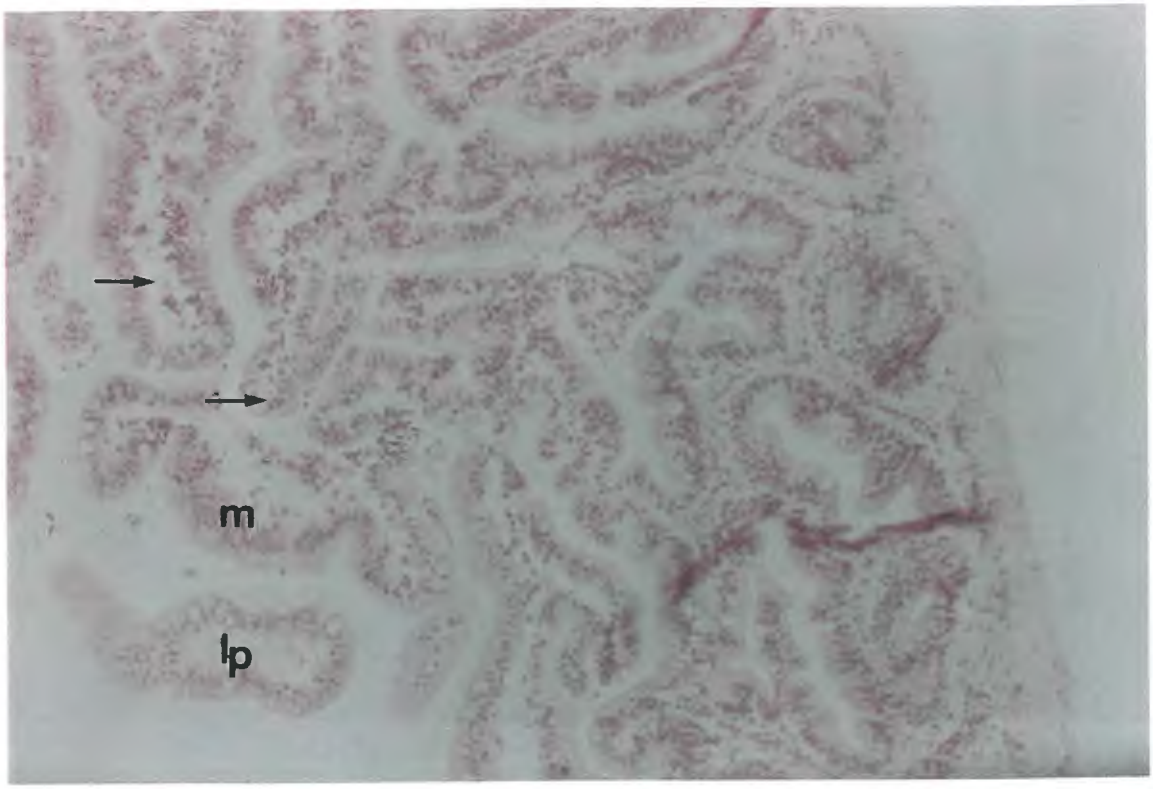


Figure 2: *C nitida*. Intestine stained with Haematoxylin and Eosin from a fish given 4 x LD_{50} / 5g of CTX. Note the disruption to the lamina propria (lp, arrow) and mucosa (m, arrow).

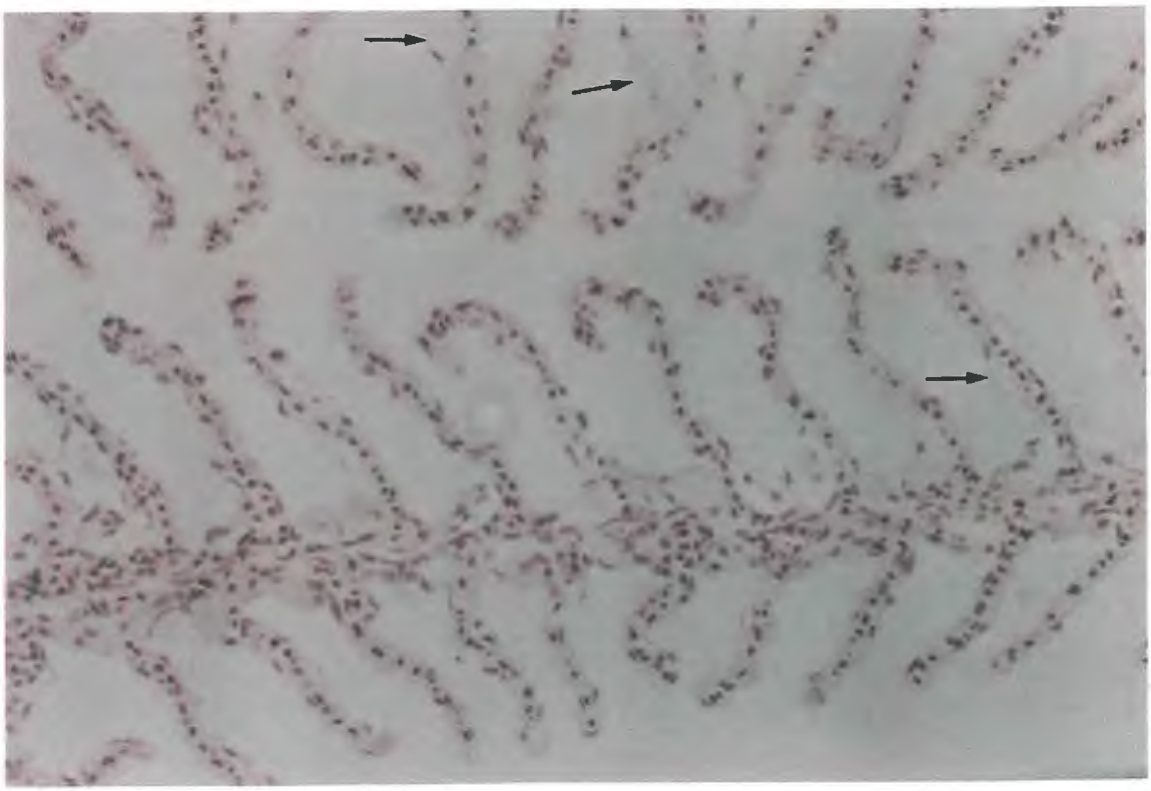


Figure 3: *P wardi*. Gill stained with Haematoxylin and Eosin from a fish exposed to 4 x LD_{50} /5g. Note the disruption to the secondary lamellae and the stripping away of the epithelium (arrows).

Toxin	Dose (LD_{50} /5g)	Number of fish	Number of Deaths
CTX Extact # 1	0.12	2	0
"	0.6	2	0
"	6	8	0
"	12	6	5
CTX Extract # 2	0.04	2	0
"	0.2	2	0
"	2	6	0
"	4	6	5
CTX Extract # 3	2	16	0
"	4	8	0
"	8	4	0
"			
Brown Extract	2	2	0
"	4	2	0
"	8	2	2
"	16	2	2
Green Extract	2	2	0
"	4	2	0
"	8	2	1
"	16	2	2
Control	6	6	0
Control Extract	18	18	0
Saline/Tween vehicle	10	10	0

Table 4: Dose regimen for *D aruanus* injected with CTX and *G toxicus* extracts.

In the three series of experiments of *D aruanus* only 17 out of 78 experimental fish died as a result of injection of high doses of CTX-fish extracts and *G toxicus* extracts (Table 4). Generally, CTX extracts had less effect on cell structure in *D aruanus* than in *P wardi* or *C nitida*. The percentage of sections with observable histopathology is shown in Table 5.

Extract	Tissue		
	Liver	Intestine	Gill
Extract # 1 and 2	30 (20)	10 (20)	0 (20)
Extract # 3	25 (30)	10 (30)	0 (30)
G.toxicus Green	70 (10)	10 (10)	0 (10)
G.toxicus Brown	80 (10)	10 (10)	0 (10)

Table 5: Percentage of the sections examined that displayed some histopathological change induced in *D.aruanus* by CTX and *G.toxicus* extracts. Figures in parentheses represent the actual number of sections examined.

Sections of both intestine and gills showed no pathological damage in *D aruanus* when examined by light and transmission electronmicroscopy. None of the CTX-fish extracts or the *G toxicus* extracts produced any discernible changes in the structure of the intestine or the gills. Unlike the gills and the intestine, histopathological changes were seen in the liver in *D aruanus* exposed to both CTX from fish and *G toxicus* toxic fractions. Changes that were apparent include nuclear margination and slight loss of fatty deposits (Figure 4) and loss of glycogen in Diastase-Periodic Acid Shifts (PAS) stained sections (Figure 5). Electron microscopy studies revealed loss of glycogen, lyposomal digestion of lipid droplets and margination of nuclei (Figures 6 and 7).

A number of behavioural observations were made on fish injected with CTX-fish extracts and *G toxicus* extracts. Deaths due to toxin injection only occurred at high dose concentration (Table 4). Higher dose levels produce discolouration, unilateral fin paralysis on the side of injection, sedentary listing loss of equilibrium and spastic swimming behaviour. Fish injected with low concentrations of toxins or given control extract or saline-Tween vehicle showed no abnormal behaviour.

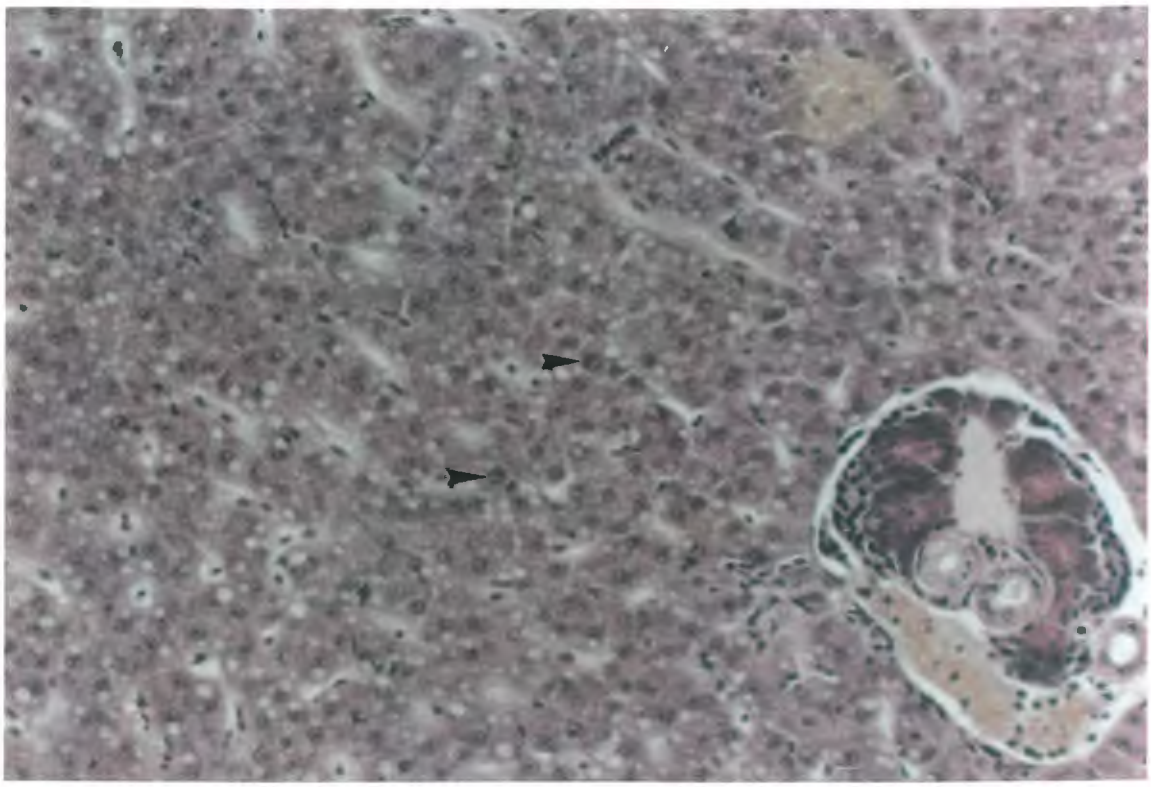


Figure 4: **Liver of *D aruanus* stained with Haematoxylin and Eosin exposed to 12 x LD_{50} / 5g of CTX extract number 2. Slight fatty changes and margination of nuclei are apparent (arrows).**

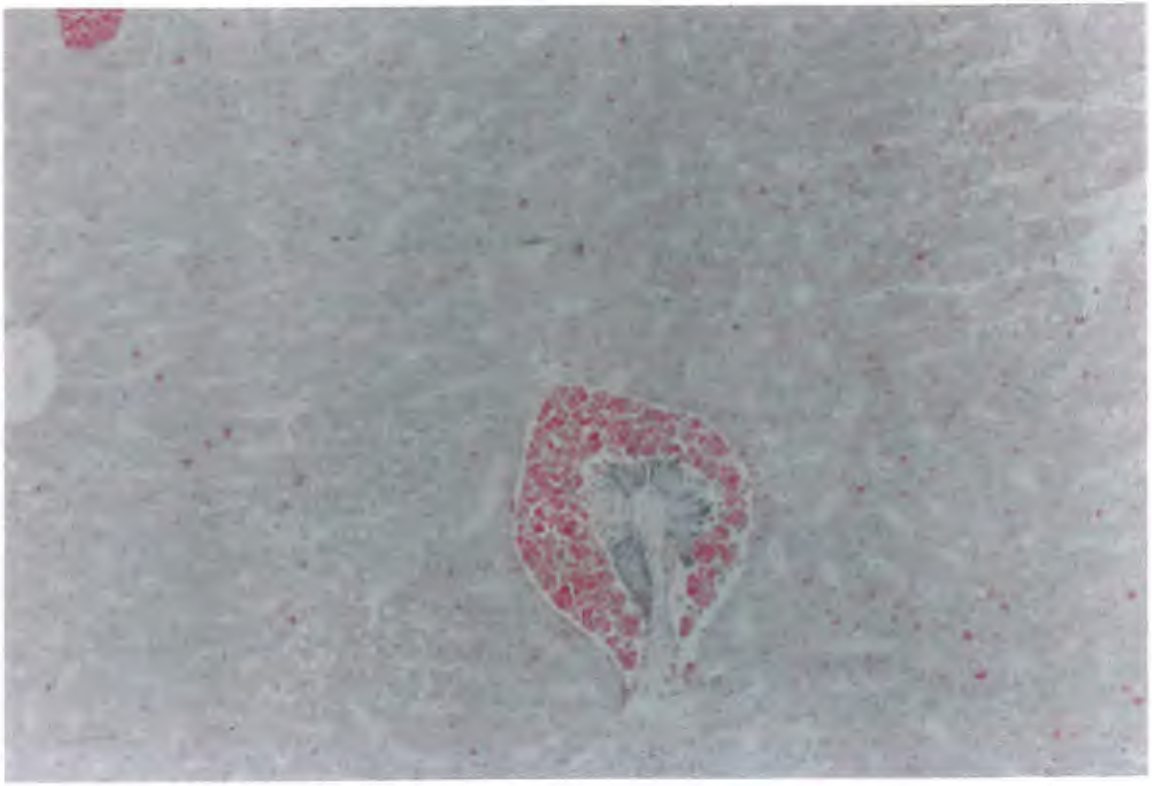


Figure 5: **PAS stained liver of *D aruanus* exposed to Brown extract from *G. toxicus* at LD_{50} 16/5g. Control liver stain bright red as for Melanomacrophages in the centre. Loss of staining in liver tissue indicates loss of glycogen.**

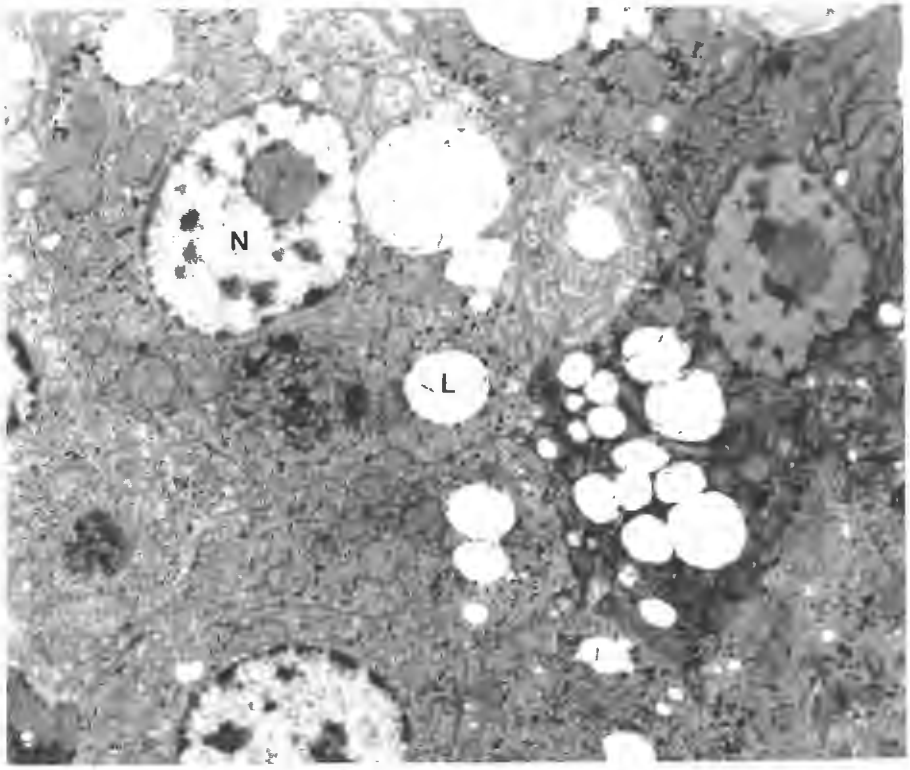


Figure 6: **Electron micrograph of the liver of *D aruanus* exposed to 4 x LD_{50} /5g of CTX extract number 3. The section shows a considerable loss of glycogen and oil droplets digested by lysosomes (L) and margination of nuclei (N).**

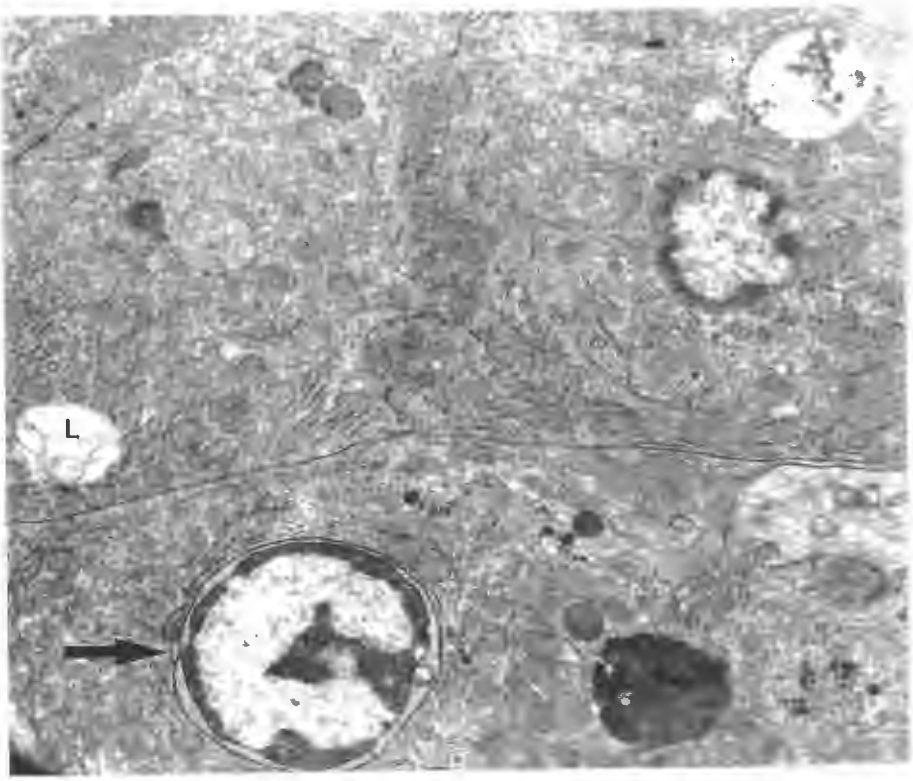


Figure 7: **Electronmicrograph of the liver of *D aruanus* exposed to 16 LD_{50} / 5g of *G.toxicus* Brown extract. Nucleolar margination (arrow) is evident. Lipid droplets have been digested by lysosomes (L) and little glycogen is stained.**

5.24 Discussion

Recorded death times in *P wardi* and *C nitida* indicate that these fish are susceptible to CTX but at levels in excess of the levels that cause death in mice. In terms of death times CTX was significantly more lethal to the planktivorous *C nitida* than it was to the algal browser *C nitida*. These results are in accordance with those of Capra et al (1988) who suggested that feeding mode may confer varying degrees of resistance to CTX. Fish that feed in a niche in which they are exposed to the elaborators of CTX as is the case with *P wardi* may have evolved mechanisms of resistance that are more developed than in fish that are not exposed to *G.toxicus* as is the case with the planktivore *C nitida*.

D aruanus is a planktivore with similar feeding habits to *C nitida* and as such it might be expected to be similar in its tolerance to CTX and G.toxicus extracts. This was not the case and *D aruanus* was able to withstand greater levels of toxin than *P wardi*. This result was surprising and may reflect differences in the CTX extracts or a biological difference in the response of each species to toxins. No experimentation was possible to resolve the possible reasons for these observed differences. As noted above we were forced to discontinue the use of *P wardi* and *C nitida* as test species because of potential harm to researchers from the collection method using quinaldine. The histological/histopathological studies performed on the three species are discussed below.

To gain an appreciation of how toxins might effect humans a number of mammalian models have been used. The principal model used is the mouse, although rats and other mammalian models have also been used. In this study the actions of CTX were assessed on fish tissues in the hope of gaining some insight into how this toxin acts on human tissues. In both fish and humans the normal route of ingestion of ciguatera toxins would be via the intestine following consumption of toxic fish or in the case of fish, toxic algae or toxic fish.

Previous studies on the effects of ciguatera toxins on fish include feeding experiments (Davin et al., 1986; Helfrich and Banner, 1963), and electrophysiological studies (Capra et al., 1988; Flowers et al., 1987; Flowers, 1989). Helfrich and Banner (1963) fed toxic flesh to surgeon fish, the fish absorbed and held the toxin, however showed no signs of pathology. Whereas Davin et al (1986) fed *G. toxicus* extracts to the bluehead, with numerous pathological and behavioural signs. These included behavioural changes whilst feeding and swimming, loss of equilibrium and

inactivity. Similar signs of intoxication were also noted in fish injected with the toxic extracts in this study and the study of Capra et al., (1988). In both the studies of Davin et al., (1986) and Capra et al., (1988) the response to the toxic extracts was dose dependent.

There have been several reported studies on the effects of ciguatera toxins and other similar marine toxins on mammals, primarily mice and rats. Banner et al., (1961) quoted a neuropathologist who stated that no pathology was observed in two mongeese maintained on ciguateric fish for over a month. Terao and his co workers (Tearo et al., 1986; Terao et al., 1988; Terao et al., 1989a; Terao et al., 1989b) have studied the effects of several marine toxins, including dinophysistoxin 1, pectenotoxin 1, goniodomina and maitotoxin, on mice and rats. All toxins produced pathology in the tissues examine. Maitotoxin, a toxin implicated in the ciguatera syndrome, cause atrophy of lymphoid tissues, a reduction of lymphocytes in circulating blood, reduced immunoglobulin M in serum and calcium increase in adrenal glands of mice (Terao et al., 1989b), and severe pathological changes in the heart and stomach tissues (Terao et al., 1988).

Coombe et al., (1987) examined the effect on mouse intestine of ciguatera toxin. Mice were injected ip with several doses of the toxin and all showed typical signs of intoxication. Pathological changes observed were, expansion of the lamina propria within the tips of the villi, nuclear degeneration and stripping of the brush border of the small intestine and extrusion of epithelial cells into the lumen of the large intestine.

Hassan (1990) examined, at both the light and electron microscope level, the effects of an algal derived maitotoxin on liver and brain cells of chick embryos. At the light microscope level necrosis of the liver cells was evident. Haemorrhaging was also described in both the liver and brain. These changes were dose dependant. At the ultrastructural level a high amount of cellular disruption was observed in both the liver and brain cells, which was again dose dependant.

The toxins used in the current study also produced intestinal pathology in mice (Coombe et al., 1987). Similar pathological changes were noted in the intestine of the fish (Capra et al., 1988). Similar changes to those in observed mice by Coombe et al., (1987) occurred in *P.wardi* and *C nitrida*. In *C nitida* changes in the intestine occurred in all sections examined whereas in *P wardi* only 60% showed pathology. However *D aruanus* displayed only very few pathological changes in the sections examined.

The histopathology of the gill tissues was difficult to assess due to the delicacy of the tissue. Capra et al., (1988) reported pathological changes in the gills of *P wardi* and *C nitida*. Similar patterns of histological damage were seen in the current study in which the gills of *P wardi* and *C nitida* were effected by the toxic extracts with 100% and 80%, respectively, of the sections examined displaying some pathological changes. However in *D aruanus* there were no signs of pathological change.

The histopathological observations in the liver were similar to those observed by Hassan (1990) in the liver of chick embryos. In chick embryo livers the tissue damage from the algal toxin included necrosis of the hepatocytes, haemorrhaging of the tissue and loss of architecture of the hepatocytes. In the fish livers damage included necrosis of the hepatocytes. The most noticeable change was the loss of glycogen in the liver, evident in both the light and electron microscope sections. This may have been due to metabolic disturbances during intoxication. Complete loss of glycogen was also noted in the liver of mice treated with the toxin from *Oscillatoria agardhii* (Meriluoto et al., 1989). In some of the electron microscope sections nucleolar margination was evident. Nucleolar margination is an indication of protein synthesis with the cell (Ghadially, 1985). Capra et al., (1988) postulated that when fish ingest ciguatera toxins they sequest or partition it in such a way so that it is not harmful to target tissues within the carrier animal. Any such partitioning mechanism will probably involve some protein binding mechanism (Section 5.4). The nucleolar margination would support an increase in protein production associated with the presence of toxin.

Fat degeneration which was also evident in the light microscope sections, was more obvious in the electron microscope sections. Such fat degeneration may result from impaired metabolism of lipids (Wheater et al., 1985). The fat degeneration evident in the liver may have been due to the metabolism of the extra load of lipid products introduced into the animal. As the toxin is very lipid soluble (Tachibana, 1980) the liver will be a primary target of metabolism of the extract and therefore the toxin may exert its effects on this tissue before other tissues.

The algal toxic extracts donated by Professor Miller when injected into the fish produced only slight changes in the tissues examined and these changes were restricted to the liver. Other algal, non-ciguatera, toxins examined histopathologically (Berg et al., 1988; Dabholdar and Carmichael, 1987; Meriluoto et al., 1989), have induced similar changes in mouse livers as were observed in the fish livers. Changes included complete loss of glycogen (Meriluoto et al., 1989) and necrosis of the liver cells (Berg et al., 1988). Unrelated toxins including snake venoms have

also produced similar histopathology in mice (Chaves et al., 1989) with necrosis of the liver hepatocytes.

5.3 Toxicological Studies in Fish

5.31 Introduction

In the previous section (5.2), it was established that injection of CTX and *G.toxicus* extracts causes death in small pomacentrid fish. Death times vary between species and may be related to the possible exposure of certain species to the dinoflagellate elaborator of the precursors to CTX. Injection of CTX or *G.toxicus* extracts intraperitoneally into fish may not mimic the route of uptake of toxins by fish in nature. A series of experiments was devised in which CTX and algal toxins were fed to a small tropical carnivorous fish, the stripey, *Lutjanus carponotatus.* These fish were examined for signs of intoxication and tissues from the fed fish were assessed for the presence of toxin.

5.32 Methods

Lutjanus carponotatus were chosen for this study based on availability, size, appetite and ease of handling. Approximately 40 specimens were fished from Heron Island Reef using debarbed No. 6 fish hooks on handlines baited with frozen pilchard. Captured fish were held in plastic bins containing seawater on board the boat. At the Heron Island Research Station the fish were transferred to a large cement pool (5 m x 5 m x 1 m) that was continuously replenished with raw seawater to a depth of 80 cm at a flow rate of 60 L/min. Prior to further handling, fish were observed for a minimum 7 day acclimation period without food. The pool was bisected with wire mesh; control *L. carponotatus* were allowed to swim freely on one side with various other reef fish specimens while the other side was partitioned into 15 numbered individual nylon mesh enclosures. Fifteen fish were selected at random from 40 captive specimens, weighed to the nearest gram and placed in the individual enclosures for a further 24 h prior to experimentation.

Extracted CTX of fish origin, green toxin extracted from cultured *G. toxicus* cells and lyopholised cultured *G. toxicus* cells supplied by Prof Donald Miller were administered orally to test fish during a 7 day time course. Toxicity of lyopholised *G. toxicus* cells was estimated to be between

5.4 and 6.3 mu/mg (Miller pers. comm.). A mouse unit is defined as the quantity of toxin required to kill a 20g mouse.

Each of the three toxins was administered orally to four *L. carponotatus* . Individual doses were placed in two No. 4 water-soluble capsules with 350 µL of absolute ethanol 5% Tween 80 and agar powder as a vehicle. Fish were administered three doses of toxin either by voluntary feeding or force-feeding at 48 h intervals. Five minutes prior to feeding times, 5 mL of pilchard body fluid was added to the holding pond. Free-swimming controls and experimentals were then fed a few pieces of pilchard to assess receptiveness to baits. If experimental subjects were receptive, both capsules containing the specified dose were placed in a pilchard bait and put into the appropriate enclosure. If the bait was not consumed within 1 min, the capsules were retrieved and the subject fish was force-fed. Fish initially unreceptive to bait were force-fed by briefly removing the subject fish from the water and injecting the capsules, 4 mL pilchard paste and 7 mL isotonic saline into the foregut with a syringe and modified pediatric enema tube attachment. After feeding, fish were observed continuously for 1 h for signs of regurgitation. Experimental controls consisted of fish allowed to feed voluntarily on baits and fish consistently force-fed pilchard paste containing water-soluble No. 4 capsules and vehicle. All fish were observed at regular intervals for signs of behavioural and physiological change. Fish were sacrificed at the end of the 7 day time course. Samples of blood (stabilised in heparin and separated into plasma and whole cell fractions by centrifugation), skeletal muscle, liver, spleen, gall bladder, gut tissue and intestinal tissue were promptly removed, frozen under liquid N_2 and stored at -60°C for biochemical examination. Fish carcasses were frozen and stored in conventional -20°C freezers until their skeletal muscle could be extracted and assessed for toxicity. For this series of experiments the amount of toxin fed to fish was expressed as a Fish unit (fu). The fu is derived from the mouse unit which is defined as the amount of toxin required to kill a 20 g mouse. The fu is defined as 25% of a mouse unit.

5.33 Results

L. carponotatus (n = 4) fed three administrations of CTX (2 f.u.) over a 7 day period showed signs of sedentary listing, discolouration, spastic swimming behaviour and tail kink after the second dose. Signs increased after the third application, although slight improvement was observed at the end of the 7 day trial period prior to preparation for biochemical evaluation. *L. carponotatus* fed green toxin (n = 4) and lyopholised *G. toxicus* (n = 4) totalling 15 f.u. and 10.5

f.u. respectively, over the trial period, showed no observable physiological or behavioural changes during the 7 day observation period. Experimental control fish (n = 3) were unaffected by treatment. (Table 6)

			Dosage (f.u.) at time t (hrs)			
Treatment	**Fish ID**	**Wt (g)**	**t=0**	**t=48**	**t=96**	**Signs**
CTX	1	172	2	2	2	sl, dc, ssb, tk
"	2	138	2	2a	2	" " " "
"	3	295	2	2*	2	" " " "
"	4	242	2	2	2	" " " "
Green	5	314	5	5	5	-
"	6	306	5	5	5	-
"	7	355	5	5	5	-
"	8	356	5a	5a	5a	-
G. toxicus	9	318	1.5	1.5	7.5	-
"	10	206	1.5	1.5	7.5	-
"	11	204	1.5	3	6	-
"	12	241	1.5	3	6	-
Control	13	269	_	_	_	-
"	14	236	_	_	_	-
"	15	320	_a	_a	_a	-

* regurgitated, dose unsalvagable, reapplied the following day

_ = fed bait containing capsule and vehicle only

a = accepted bait without force-feeding

sl = sedentary listing

dc = discolouration/darkened chromatophores

ssb - spastic swimming behaviour

tk - tail kink

Table 6: *L. carponotatus* oral toxin administration experiment protocol

Residues derived from 60 g samples of skeletal muscle from each fish were prepared individually for each administration group. Results from residues of one fish selected at random from each group, were negative. Remaining residues were pooled according to administration group and tested in a single mouse. Pooled residues from CTX-fed fish (1.4 mg, derived from 120 g skeletal muscle) resulted in a lethal dose estimated at 1.4 mu or 0.012 mu per gram of skeletal muscle. The total number of mouse units fed to these fish was 204 mu or 0.3 mu/g fish body weight.*L. carponotatus* accumulated at least 1/25 of the CTX they were fed in their skeletal muscle; possible toxin loss during extraction may have led to an underestimation of toxin accumulation. Pooled residues from fish fed Green toxin and lyopholised *G. toxicus* at

approximately twice the total dose used in CTX-fed fish did not affect mice during the 24 h bioassay observation period.

Toxicity Calculations

Toxicity calculations for pooled residues derived from CTX-fed fish:

$Log_{10}X = Log_{10}62.5 - 2(Log_{10}[1 + 9.4^{-1}])$ where X = 51.6 mg/kg

51.6 mg/kg x 1kg/50 mice = 1.02 mg/mouse LD_{50} or 1.02 mg/mu

1.4 mg lipid residue/1.02 mg/mu 1.4 mu from 120 g skeletal muscle

5.34 Discussion

Feeding trials in *L. carponotatus* (stripey) provide comparison of the effects of CTX and *G. toxicus* toxin(s) that previous studies involving ciguatera-related toxins have not described. While Banner et al., (1966) showed accumulation and retention of CTX in fish fed toxic fish flesh, it was purely a qualitative study. Feeding and subsequent extraction and quantification of CTX in *L. carponotatus* defined approximate oral effective dosages and rates of incorporation in skeletal muscle. Although GDT-derived toxins affected fish injected i.p., feeding experiments in *L. carponotatus* indicated that the potency of GDT is at least half that of CTX, a finding not discussed by Davin et al., (1986, 1988). *L. carponotatus* is also apparently unable to bioaccumulate or bioconvert GDT under these experimental conditions in quantities sufficient for detection in the mouse bioassay. Further toxicological studies focusing on primary consumer groups implicated as elaborators on the basis of diet and digestive strategy, and feeding experiments with animals such as the browsing mollusc, Apylypsia and browsing gastropods from reefal systems designed to elucidate specific links between toxins from *G toxicus* and CTX elaborate should clarify the "missing link" associated with ciguatera poisoning (Hahn, 1991).

5.4 Biochemical Studies on Fish

5.41 Introduction

CTX-protein associations *in vitro* have been described in the context of immunological studies (Parc et al., 1979; Emerson et al., 1983) and in cytoplasmic protein from fractionated liver cells of three marine teleosts (Vernoux et al., 1985). In view of demonstrated effects of CTX on marine animals, the widespread occurrence of CTX in the marine biota suggests that a CTX-neutralisation mechanism may exist in carrier species. Although a large proportion of toxin is associated with the liver and digestive tract of toxic fish, CTX is not restricted to these tissues (Tosteson et al., 1988; Vernoux et al., 1985). The following experiments were designed to explore specific CTX-protein associations in skeletal muscle of *Scomberomorus commersoni* (Spanish mackerel), a species often associated with outbreaks of ciguatera poisoning.

5.42 Methods

5.42.1 Specimens

Toxic (n = 2) and nontoxic (n = 10) specimens of *S. commersoni* were collected at various coastal locations between Bundaberg and Brisbane, Queensland. Toxic specimens originated from Hervey Bay. Toxic *S. commersoni* skeletal muscle samples (designated 'Turney' and 'Roy' after the individuals who provided the fish) contained approximately 1 LD_{50} dose equivalent per 8 and 13 g tissue respectively, determined by the CTX dose versus death-time curve (Tachibana, 1980).

Fresh-frozen 400 g fillets from each of nine nontoxic specimens were purchased from a Brisbane seafood distributor. Species identification and length and weight measurements of gutted, headed carcasses were taken prior to filleting (lengths: 740-940 mm; weights: 4.6-10.9 kg). Remaining portions of these fish were sold and consumed without incident.

Potential differences between control and toxic fish samples, attributed to freezer storage characteristics of the flesh, were anticipated. A nontoxic specimen of *S. commersoni* fished from Hervey Bay in 1986 was stored at -20°C prior to use as a control in these experiments (length 1900 mm; weight >35 kg). Liveweight and sex of fish were not available to this study.

5.42.2 *S. commersoni* skeletal muscle preparations

All protein separation steps were performed between 0 and 4°C in aqueous buffer consisting of 0.05 M Tris (Hydroxymethyl)-aminomethane-HCl, 0.1 M KCl, 0.5 mM EDTA (ethylenediaminetetraacetic acid) adjusted to pH 7.4 (Figure 8). Equal quantities of toxin entered the homogenate irrespective of the total weight of sample tissue (Turney [8 g/mu = 128 g; Roy [13 g/mu] = 208 g; control = 208 g). Samples were homogenised separately in 3:1 volume (mL):weight (g) extraction buffer using a glass and stainless steel Waring tissue homogeniser on high for 60 sec. The crude homogenate, in polycarbonate centrifuge tubes, was spun in a GSA rotor at 10 000 rpm (10 240 x *g*) for 30 min in a Sorvall Superspeed centrifuge. The pellet was then rehomogenised (3:1 v/w) and recentrifuged. Remaining insoluble material was stored at -20°C. Pooled supernatant (total soluble protein, Supernatant 1) was brought to 30% $(NH_4)_2SO_4$ saturation by addition of solid $(NH_4)_2SO_4$ crystals. Resulting precipitate was removed by centrifugation at 10 000 rpm (10 240 x *g*) for 15 min. The pellet was stored at -20°C. Supernatant 2 was brought to 60% $(NH_4)_2SO_4$ by addition of $(NH_4)_2SO_4$ crystals and centrifuged at 10 000 rpm (10 240 x *g*) for 15 min to remove resulting precipitate. This second pellet and remaining supernatant (Supernatant 3) were stored at -20°C prior to toxicity testing. Supernatants 1, 2 and 3 for each sample were subsampled for later protein determination and electrophoretic analysis. Insoluble material, pellets 1 and 2, and supernatant 3 were extracted and assessed for CTX.

5.42.3 Gel permeation LPLC of skeletal muscle soluble protein and CTX

There was insufficient Turney flesh for low pressure liquid chromatography (LPLC) study. Roy skeletal muscle was homogenised, fractionated by $(NH_4)_2SO_4$ and chromatographed on Sephacryl S-200 (Pharmacia) to determine the size of protein(s) associated with toxin. Samples were homogenised as described above, except that in one experiment fresh 0.2 mM phenylmethylsulfonyl fluoride (PMSF) was included in the buffers as a protease inhibitor (Moss and Fahrney, 1978). Aliquots of fractions from PMSF-containing homogenates were retained for comparison with equivalent material obtained by the usual procedure or by centrifugation with a Beckman high speed centrifuge using a JA 20 rotor at 16 000 rpm (20 070 x *g*) for 1 h or a JA 18 rotor at 15 000 rpm (24 700 x *g*) for 3 h. To fractionate the resulting supernatant, a 0-50% $(NH_4)_2SO_4$ cut was chosen on the basis of SDS Disc-PAGE patterns and the distribution of assessable toxin in previous 0-30% and 30-60% $(NH_4)_2SO_4$ cuts.

assessable toxin in previous 0-30% and 30-60% $(NH_4)_2SO_4$ cuts.

In one experiment, dialysed and clarified material from the 0-50% $(NH_4)_2SO_4$ cut was concentrated approximately eight-fold using a stirred Amicon Diaflo (model 402) ultrafiltration cell, fitted with a PM10 membrane and operated at 85 psi (high purity N_2 gas) at 4°C for approximately 59 h. As the solution was concentrating, material precipitated and accumulated on the membrane, reducing flow. When this occurred, the contents of the cell were clarified [Sorvall SS-34 rotor at 15 000 rpm (20 580 x *g*) for 1 h] in order to improve flow through the membrane. Materials accumulated on the membrane and precipitated during ultrafiltration were combined with insoluble materials from the resuspended 0-50% $(NH_4)_2SO_4$ pellet for toxicity assessment.

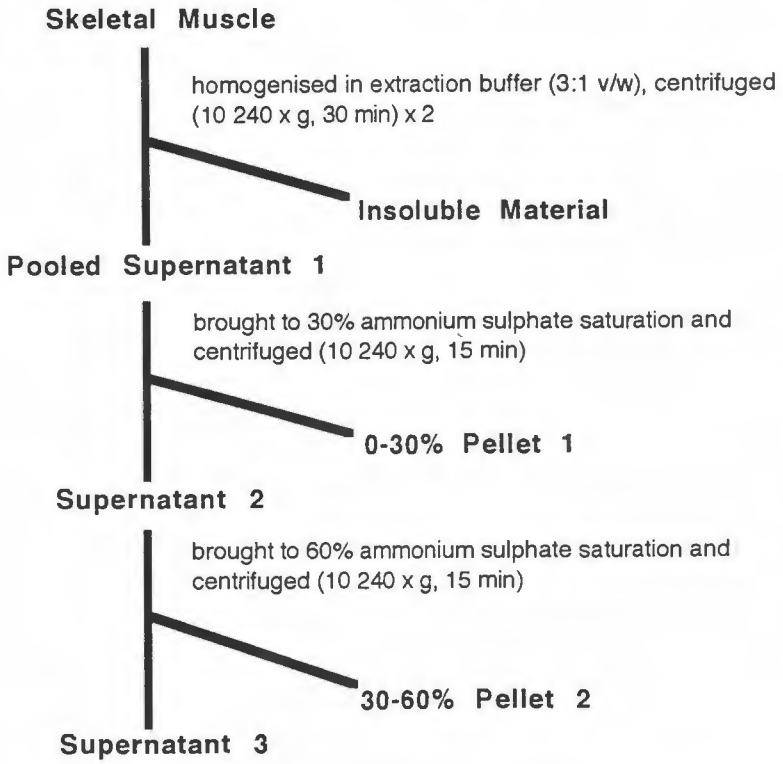


Figure 8. Skeletal muscle protein $(NH_4)_2SO_4$ precipitation purification method

An LKB LPLC column (2.6 cm x 100 cm) was packed with Sephacryl S-200 in extraction buffer at a flow rate of 100 mL/h at 4°C. The column was equilibrated at the same flow rate overnight using either standard extraction buffer or extraction buffer containing PMSF (0.2 mM). Approximately 20 mL aliquots of samples were chromatographed at 80-100 mL/h and eluate,

collected as 4 mL fractions, was monitored at 280 nm with a Uvicord UV detector and single channel chart recorder or a Bio-Rad EM-1 UV monitor and chart recorder (model 1325). Peak fraction absorbances were confirmed with a LKB MS222 spectrophotometer, and corresponding fractions from several applications of one sample were combined for electrophoretic analysis and toxicity assessment.

Molecular weight estimates of fractions were made from a standard graph of the ratio of elution volume to void volume (V_e/V_o) versus $\log_{10}$ (molecular weight) prepared from chromatography of blue dextran (Pharmacia, 2 x 10^6 Daltons), bovine serum albumin (Sigma Fraction V, 68 500 Daltons), trypsinogen (Sigma, 24 000 Daltons), cytochrome C (Sigma, 12 400 Daltons) and nicotinamide adenine dinucleotide (Sigma, NAD^+, 663 Daltons).

Column profiles were divided into zones based on the amount of toxin loaded onto the column, the number of peaks eluted, and the assumption that, given non-specific protein association, CTX would be uniformly distributed throughout the profile. All fractions within a zone were pooled, extracted with solvent and assessed for toxicity. This strategy was necessary to reduce the number of mouse bioassays and to ensure that an assessable amount of toxin could be obtained from the fractions. Peaks eluted from column 1 in zone 2b were separated into three subzones - 2b(2), 2b(3) and 2b(4) - eluted from column 2 (Figure 9).

5.42.4 Protein concentration determinations

Protein concentrations were monitored at various steps utilising the Bio-Rad Protein Assay Kit (Coomassie Blue G-250 method). Unknown protein concentrations were calculated from mean (n = 2) absorbances at 595 nm on a Pharmacia Novaspec II digital spectrophotometer using a standard curve prepared with bovine serum albumin.

5.42.5 Electrophoresis of *S. commersoni* soluble proteins

Soluble proteins were sampled at various purification steps. Where necessary, samples were dialysed against extraction buffer at 4°C prior to protein concentration determination. Total protein estimates for each purification step were back-calculated by the formula:

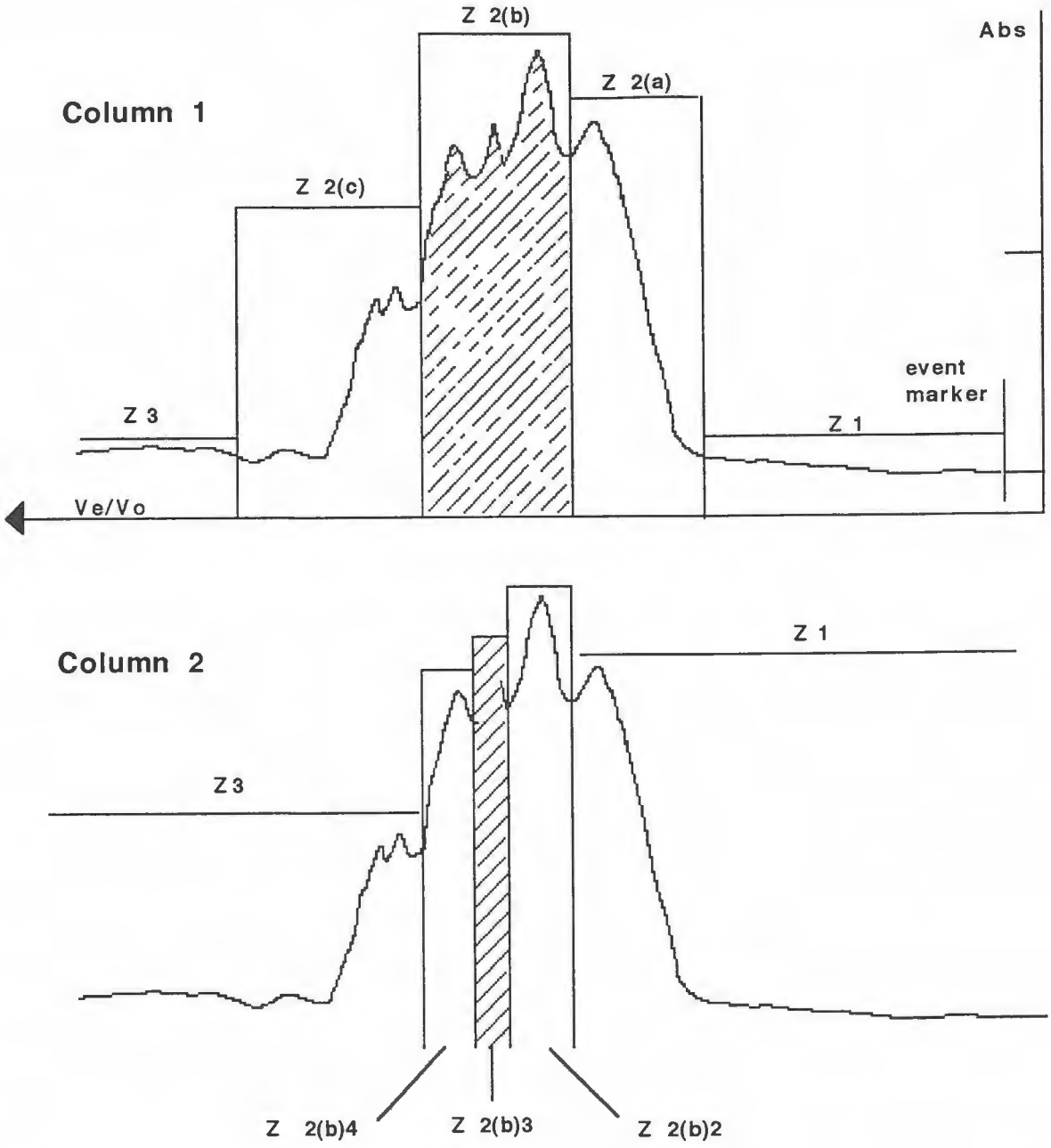
(mg protein/mL solution) (mL solution) = (mg protein)

5.42.6 Cellulose acetate electrophoresis

Electrophoretic separations of non-denatured soluble proteins were carried out using Helena Zip Zone Serum Protein (Titan III Serum Protein and Lipoprotein) cellulose acetate strips in Tris EDTA-boric acid buffer (ionic strength 0.025, pH = 8.2-8.6), or Tris-barbital-sodium barbital buffer (ionic strength 0.05, pH = 8.6-9.0). Protein solutions (2-8 µL) were loaded onto cellulose acetate strips and electrophoresed at 180 volts (6 mA per strip) for 15-30 min. Proteins were stained with 0.2% Ponceau S in 3% trichloroacetic acid and 0.2% Coomassie Blue G-250, and destained in methanol:acetic acid:water (2:1:7 v/v).

5.42.7 Polyacrylamide gel electrophoresis

SDS Disc-PAGE was performed according to the method of Laemmli (1970). Bio-Rad Mini-Protean II vertical slab gel apparatus were used to separate reduced skeletal muscle soluble proteins (6% stacking gels and 12% resolving gels) at 120 V (constant voltage) and initial current of 6 mA per gel. Approximately 4 µg of total protein was loaded into each lane. Protein bands were stained with 0.2% Coomassie Blue G-250, destained in methanol:acetic acid:water (2:1:7 v/v) and photographed on Polaroid black and white film. Prestained molecular weight standards (Bio-Rad Lab) used were: phosphorylase B (106 000 Daltons), bovine serum albumin (80 000 Daltons), ovalbumin (49 500 Daltons), carbonic anhydrase (32 500 Daltons), soybean trypsin inhibitor (27 500 Daltons) and lysozyme (18 500 Daltons).



Zones 1, 2 and 3, and corresponding subzones, represent pooled column elution fractions assessed for toxicity.

Figure 9. Representative Sephacryl S-200 column chromatographs of resolubilised 0-50% $(NH_4)_2SO_4$ precipitates for column 1 (soluble protein derived from 208 g skeletal muscle with standard extraction buffer) and column 2 (soluble protein derived from 624 g skeletal muscle with 0.2 mM PMSF extraction buffer)

5.43 Results

The 'insoluble material' pellets of skeletal muscle homogenate contained 56-72% of total assessable toxicity (Table 7). Remaining toxicity was precipitated from the supernatant between 0 and 60% $(NH_4)_2SO_4$ saturation. Roy and Turney supernatants yielded 3119 mg and 2224 mg protein respectively, in this cut. These precipitates contained 6.6% and 7.7% of total starting protein, and 44% and 28% of total sample toxicity. Control residues did not affect mice in the bioassay.

	Toxin content of:		
Source	**Unfractionated Skeletal Muscle (mu)**	**Insoluble Material (%)**	**Supernatant (%)**
Roy (208 g)	16.6	56	44
Turney (128 g)	15.2	72.4	27.6

Values for % toxicity are derived from the mean toxicity calculated for at least two mouse bioassay results.

Table 7: Distribution of CTX in *S. commersoni* fractionated skeletal muscle

Pooled eluates from the columns, represented by zones 1 and 3 in Figure 9, were nontoxic. Zone 2b (bracketed by V_e/V_o values 1.107-1.392) contained all assessable toxicity eluted from the column during the first experiment. Zone 2b consisted of three distinct absorbance peaks. Zone 2b(3) contained all assessable toxicity eluted from the column during the second experiment. Residues derived from zones 2b and 2b(3) elicited strong signs of intoxication, although mice did not die within the 24 h observation period.

Roy and Turney samples exhibited similar increases in specific activity (Table 8). Although there was a loss of toxin between 'flesh' and 'high speed supernatant', specific activity increased by an order of magnitude for both fish. Sephacryl S-200 chromatography of resuspended, clarified 0-50% $(NH_4)_2SO_4$ precipitate resulted in further increased specific activity and an overall 11.2 fold purification. Approximately 36% of total assessable toxicity was associated with insoluble material in the clarification pellet. V_e/V_o values defined by zone 2b bracket protein molecular weights between 25 300 and 70 500 Daltons (Figure 10). Toxicity remaining in the resolubilised 0-50% $(NH_4)_2SO_4$ cut eluted from the column in zone 2b(3). V_e/V_o values defined by zone 2b(3) bracket protein molecular weights between 35 500 and 59 500 Daltons (Figure 11).

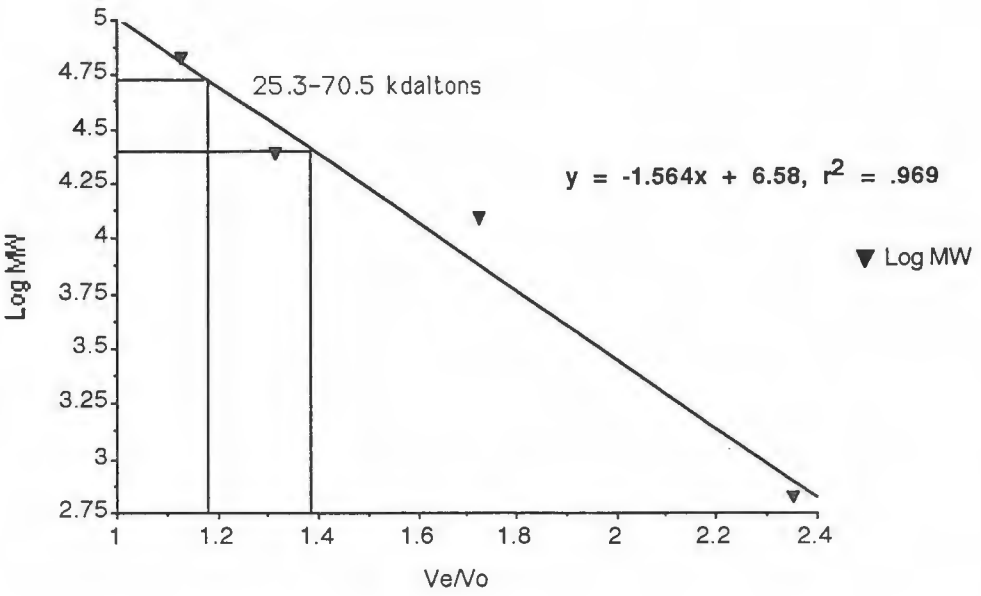


Figure 10: Molecular weight marker standards curve for column 1

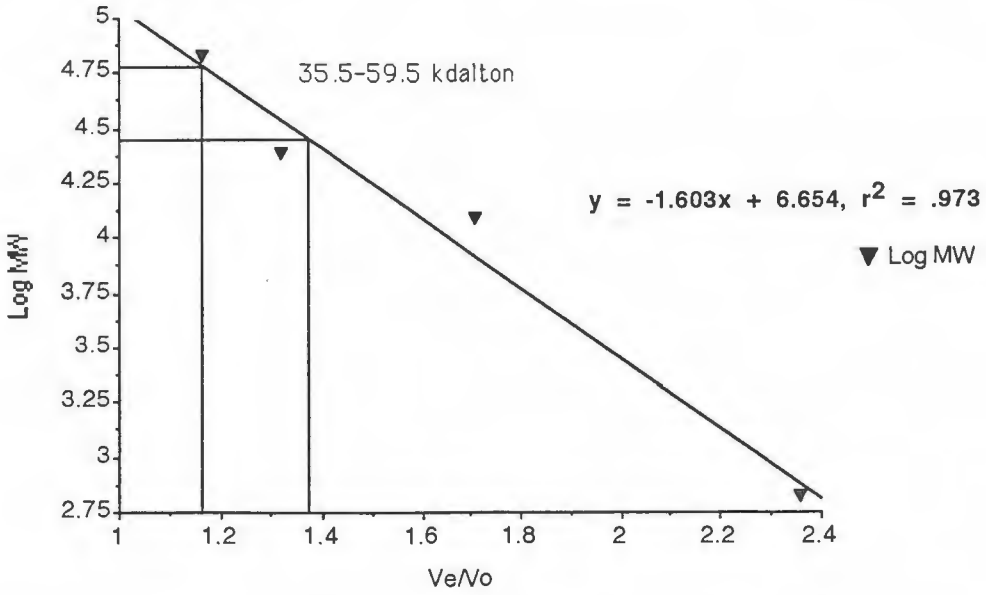


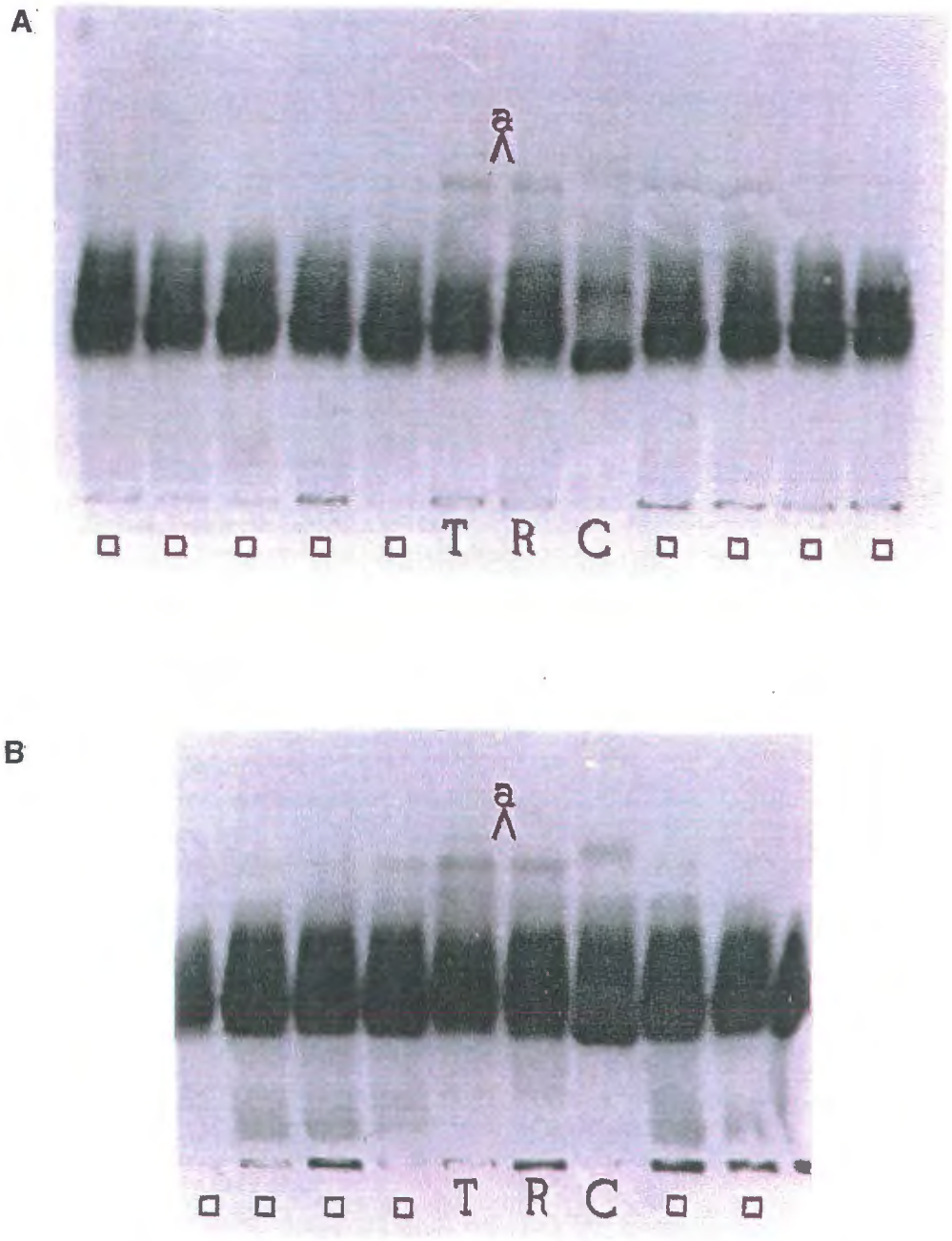
Figure 11: Molecular weight marker standards curve for column 2

Source	Fraction	Protein (mg)	Sp. Act. (mu/mg)	Tot. Act. (mu)	% Yield	Fold Purification
Turney (128 g)	flesh	28 928*	5.25×10^{-4}	15.2	100	1
	high speed supernatant	3499	1.20×10^{-3}	4.2	28	2.3
	0-60% pellet	2224	1.89×10^{-3}	4.2	28	3.6
Roy (208 g)	flesh	47 008*	3.53×10^{-4}	16.6	100	1
	high speed supernatant	6976	1.05×10^{-3}	7.3	44	3
	0-60% pellet	3119	2.34×10^{-3}	7.3	44	6.6
	resolubilised 0-50% $(NH_4)_2SO_4$ clarification pellet	2038	2.06×10^{-3}	4.2	25	5.8
	Sephacryl S-200 eluate (zone 2b)	647	3.74×10^{-3}	≤2.4	14	11.2
Roy (624 g)	flesh	86 784*	-	50†	100	1
	zone 2b(3)	-	-	≤ 2	4	-

Sp. Act. = Specific Activity
Tot. Act. = Total Activity
* Total protein estimates for skeletal muscle were based on New South Wales Fish Marketing Authority (1989) protein determination for skeletal muscle of Slimy Mackerel (22.1%) by weight.
† expected value

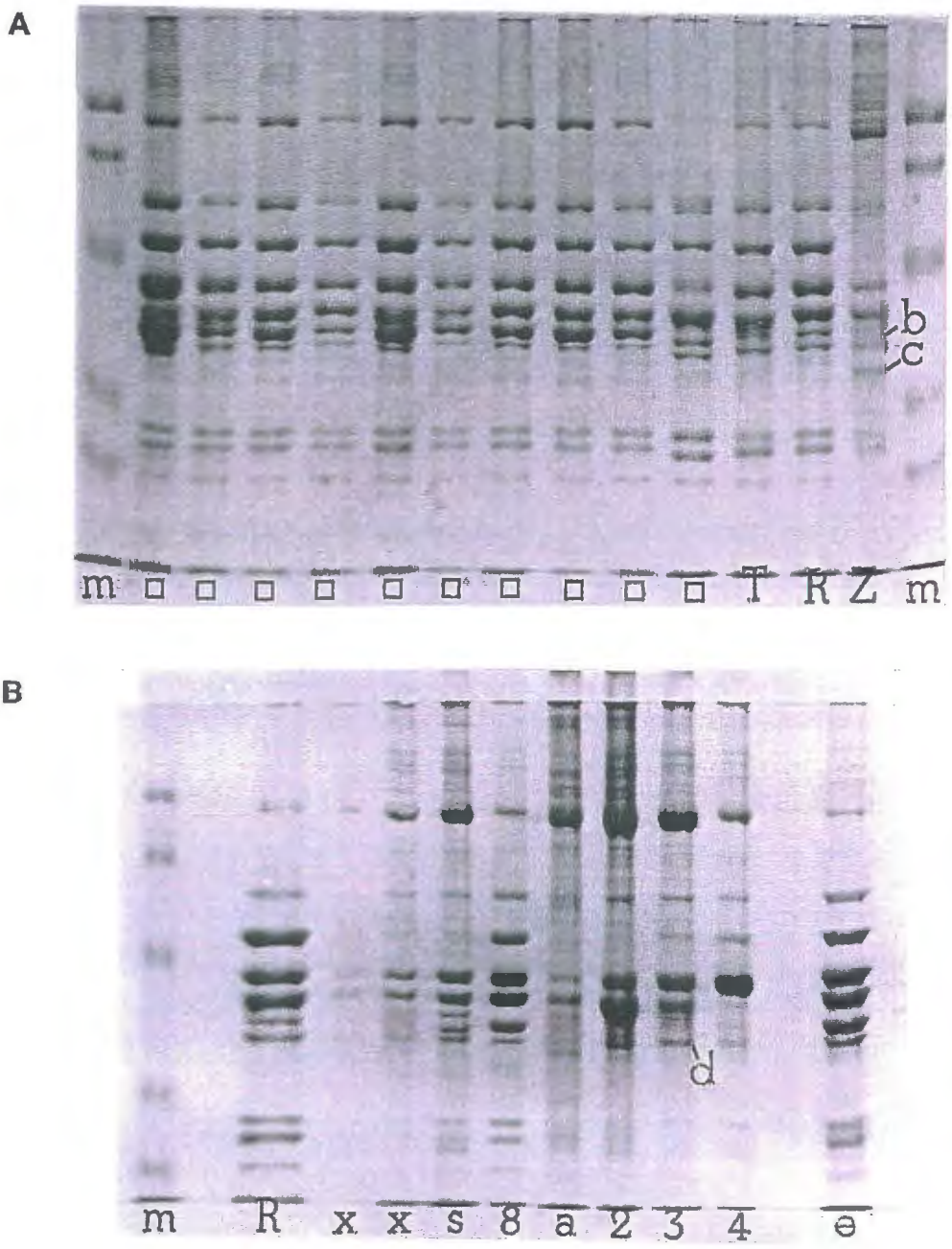
Table 8: Purification of CTX-protein fraction from *S.commersoni* skeletal muscle

Non-denaturing electrophoresis (Figure 12) revealed a protein band which appeared more strongly in Turney and Roy lanes than in control lanes. The band was absent in the lane for frozen control fish. SDS Disc-PAGE analyses of soluble protein fractions revealed unique bands [b and c in Figure 13 (A)] in Turney (T), Roy (R) and the zone 2b eluate (Z) from Sephacryl S-200; these bands were not visible in the controls (▫). Similar analyses of subzones in zone 2b showed the presence of a prominent band [d in Figure 13 (B)] in zone 2b(3) (lane 3) which also was visible faintly in zone 2b(4) (lane 4). It was not visible in control total soluble protein (lane 8), zone 2a eluate (lane a) or zone 2b(2) eluate (lane 2). The Rf values for bands b and d are equal (0.64). The migration distances of bands b, c and d correspond with molecular weight standards migration for proteins between 37 400 and 40 600 Daltons. Molecular weight range was derived graphically (Figure 14).



T = Turney total soluble protein
R = Roy total soluble protein
C = control freezer fish total soluble protein
= control *S. commersoni* total soluble protein
a = bands which appear more strongly in lanes T and R

Figure 12: Representative Helena cellulose acetate electrophoresis Titan III (A) and Lipo (B) strips



T = Turney total soluble protein
R = Roy total soluble protein
Z = zone 2b
m = molecular weight markers
= control *S. commersoni* total soluble protein
s = resolubilised 0-50% $(NH_4)_2SO_4$ precipitate post clarification
8 = control total soluble protein
a = zone 2a
2 = zone 2b(2)
3 = zone 2b(3)
4 = zone 2b(4)
e = zone 2c
x = Amicon filter precipitate
b, c and d = bands unique to proteins derived from toxic fish

Figure 13: SDS Disc-PAGE gels of *S. commersoni* soluble protein fractions

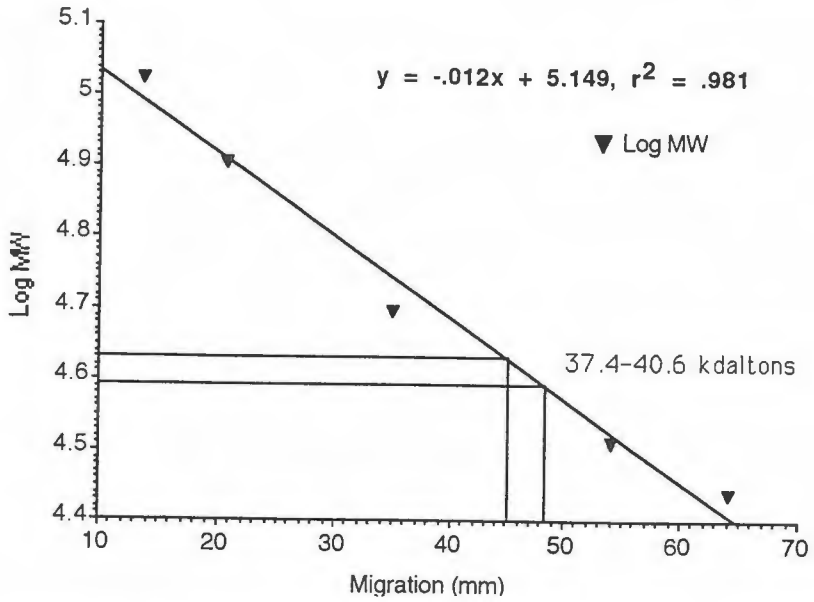


Figure 14: Molecular weight marker standards curve for SDS Disc-PAGE gels

5.44 Discussion

Studies of toxin distribution in fractionated liver cells of *Gymnothorax funebris* and *Seriola dumerili* (Vernoux et al. 1985) showed larger proportions of toxin in the supernatant than those observed for fractionated skeletal muscle of *S. commersoni*. A large percentage of toxin was nevertheless associated with soluble protein(s) derived from tissue homogenates in both cases. Relative toxin concentration differences associated with liver and skeletal muscle soluble proteins may be due to tissue toxin concentration differences (Vernoux and Talha 1989). If serum protein binds CTX, blood-rich organs such as liver and spleen would be expected to contain higher levels of toxin than other tissues. Subcellular toxin distribution studies suggest that CTX in these three marine carnivores is closely associated with $(NH_4)_2SO_4$ precipitable soluble protein(s). Viscera from toxic *S. commersoni* was not available for direct comparison. Toxin yield and specific activity differences between Turney and Roy soluble protein fractions may be attributed to individual sample toxicity (Turney was 1.6 times more toxic than Roy) and condition of the frozen samples (Turney, twice the freezer age of Roy, was subjected to frequent partial thawing and refreezing).

CTX eluted from Sephacryl S-200 with a protein fraction between 35 500 and 59 500 Daltons. Based on suggested structures and molecular weight estimates (Tachibana, 1980; Murata et al., 1989b) for CTX, the toxin alone would be expected to elute much later from the column. Toxicity

derived from zones 2b and 2b(3) implicated specific soluble protein-toxin associations, although lipophilic interaction between ciguatoxin and micelles, known to elute in this molecular weight range, could account for occurrence of toxin within this protein fraction. The toxicity of the initial insoluble pellet may be due to incomplete extraction of soluble proteins, lipophilic CTX interaction with membrane lipids or CTX association with membrane-bound protein. Toxicity loss in samples subjected to 0.2 mM PMSF buffer may be attributed to oxidative properties of PMSF; trebling the quantity of toxic fish extracted with 0.2 mM PMSF buffer increased processing time from 4 to 8 days. Percent yield loss in residue derived from zone 2b(3) was probably due to the combination of oxidative agents to which samples were exposed over this period. Cellulose acetate electrophoresis of skeletal muscle total soluble protein showed differences in Roy, Turney and freezer control fish samples consistent with denaturation due to long term storage (Umar and Qadri, 1982). Although lanes T and R do not appear to contain unique bands, band a is more distinct (Figure 5).

Two bands unique to Turney, Roy and Sephacryl S-200 column eluates of Roy soluble proteins are visible in SDS Disc-PAGE gels (bands b, c and d, figure 6). Bands b and c are more obvious in Turney than in Roy. Concentration differences between Turney and Roy total soluble proteins in these bands may be due to relative toxicity differences between the fish. Zone 2b proteins represent a 2.8 fold purification of total soluble protein. Although bands d and c are only visible in Turney, Roy and zone 2b lanes, they may not be exclusive to toxic fish. These gels do not imply that only toxic fish contain these bands. They are not visible, however, in the controls so were either not present, or present in concentrations too low to visualise in this system.

Sephacryl S-200 elution zones either side of zone 2b(3) did not contain assessable toxin. Zone 2b(4) contains a tailing of band d protein. If band d is associated with sample toxicity, the apparent lack of toxin in zone 2b(4) may be attributed to mouse bioassay minimum threshold detection limitations. These bands appeared in regions of the gels bracketed by carbonic anhydrase (32 500 Daltons) and ovalbumin (49 000 Daltons) molecular weight markers. Although these bands were not assessed individually for toxicity, their approximate sizes (between 37 400 and 40 600 Daltons) correspond with the molecular weight range that contained the toxin eluted from Sephacryl S-200 (35 500-59 500 Daltons). Concurrence of Sephacryl S-200 and SDS Disc-PAGE molecular weight estimates for protein fractions associated with toxicity may be interpreted as evidence of at least one monomeric protein, between 37 400 and 40 600 Daltons, associated with CTX in the skeletal muscle of *S. commersoni*. The quantity of soluble protein required for bioassay assessment precluded more specific size estimates utilising this

system and definition of possible toxin association (binding) with a particular protein. In view of reports of other proteins binding xeno-toxins (Olsson and Hogstrand 1987; Barber et al., 1988; Mahar et al., 1991), continuing investigations of this possibility for CTX are warranted. Larger LPLC facilities, more sensitive toxicity evaluation, and more toxic fish could be beneficial to future work. Further studies of CTX-protein association(s) should define more specifically the biochemistry of CTX partitioning and accumulation in marine teleosts.

6.0 Studies in Mammals

Ciguatera poisoning can often have a severe impact on its human victims causing gastrointestinal and neurological symptoms. The apparent low level effect of CTX on fish led us to speculate that an understanding of how fish dealt with this toxin may prove to be of benefit to the treatment of human victims. The information presented for fish in Section 5 has shown that fish are susceptible to CTX and that this susceptibility can vary with species. In the Spanish mackerel, at least, there appears to be biochemical mechanisms that are initiated by the presence of CTX that may be protective to the fish. When these FRDC sponsored studies began we intended to examine the effects of CTX on mammalian tissues to provide a comparison with the studies in fish tissues. Previous work with mice by our group at QUT (Coombe et al., 1987) demonstrated that CTX produces damage to the small intestine in a manner similar to the actions of CTX on fish intestine (Section 5.2). It has also been reported that structural alterations may occur in the Schwann cells associated with peripheral nerves in human victims of ciguatera poisoning (Allsop et al., 1986). In this part of the report the two remaining research objectives from 87/058 will be addressed and the objective of 88/059 will also be addressed under the following subheadings:

- Histological studies in mice
- Fish protective mechanisms and human therapy
- Mannitol and ciguatera poisoning

6.1 Histological studies in mice

6.11 Introduction

As noted above, CTX when injected into laboratory mice (Coombe et al, 1987) produced histopathological changes in the small intestine. Terao et al (1991) failed to find any changes in the intestines of mice injected with CTX but found histopathological changes in cardiac muscle cells, medullary cells of the adrenal glands and changes in penile tissue. Variations in the effects of CTX on intestinal tissue may reflect differences in the toxins used in each study. What is clear however, is that CTX can cause damage to a range of tissues in the mouse and presumable other mammals including humans. In this section the following objective from 87/058 is addressed:-

To examine the actions of CTX on selected tissues in a mammalian model of ciguatera poisoning in humans.

As it is established that CTX can cause cellular damage to a range of tissues in mammals, the above research objective was addressed not by continuing to document structural damage to tissues and organs but by attempting to visualise the binding of CTX to specific target sites in mammalian tissue. An attempt was made to visualise attachment sites for CTX within the central nervous system of the laboratory mouse by using immunocytochemical methods.

Immunological studies in associated with ciguatera poisoning have indicated an affinity between IgG and ciguatoxin (Emerson et al., 1983; Hokama et al., 1983; Kimura et al., 1982). Although this non-specific binding of ciguatoxin to IgG has introduced difficulties in the production of a specific antibody (Emerson et al., 1983), it may be useful in the localisation of the binding sites of ciguatoxin in animal tissues. The animal or selected tissues could be loaded with ciguatoxin either *in vitro* or *in vivo*, and then any ciguatoxin may be able to be visualised using an immunohistochemical method involving IgG. This was attempted by using the labelled avidin-biotin technique (Guesdon et al., 1979).

CTX has a range of neurological effects that appear to be related to its action within the central nervous system. Included among these are the commonly observed ataxic behaviours seen in mice exposed to CTX. In this study the cerebellum was chosen as a potential site of CTX attachment and action. If CTX exerts physiological action on the CNS, then prior to the physiological and behaviour manifestations, it could be expected that CTX would bind to target sites. The role of the cerebellum within the brain is to control the precise timing of muscular contractions for coordinated, smooth muscle movement. The cerebellum was chosen as the site of study for the following reasons: it was easily removed whole from the animal, it has a grey matter layer containing three sections: a molecular layer, a Purkinje cell layer and a granular layer and also for its role in fine movements. As noted, in mice exposed to ciguatera toxins ataxia is frequently observed. The cerebellum is the centre for coordination of fine movement, if its functions are disrupted ataxia will result.

The Purkinje cells within the cerebellum are neurons receiving both excitatory and inhibitory impulses (Ham and Cormack, 1979). The Purinkje cells have large dendritic processes that extend into the molecular layer and axons that extend into the granular layer of the cerebellum.

Any binding of ciguatoxin to these structures may be able to be visualised with immunocytochemical techniques.

6.12 Methods

The cerebellum was dissected from male and female Quackenbush stain mice, euthanased in a CO_2 atmosphere. Once the cerebellum was removed the vermis was cut away and cut in half through the median line, then each half was used in subsequent procedures.

The vermis was either exposed to toxin before preservation of the tissue (pre-preservation) or after preservation of the tissue (post-preservation). Preservation of the tissue was achieved either by freezing or formalin fixation. Formalin fixation was used in the pre-preservation exposure of tissues, while freezing was used in both pre and post preservation exposure.

Controls were performed for both pre- and post-preservation methods. In the pre-preservation experiment blocks of the vermis were exposed to either Krebs solution alone or Krebs solution with control extract-Tween 80. Controls in the post-preservation experiment included exposure of cut sections to control extract in buffer-Tween or to buffer-Tween alone.

Blocks of vermis approximately 2 mm^3 were incubated in 1 mL of Krebs physiological saline in 1% Tween and bubbled with 95 % O_2 and 5 % CO_2. Initially only Krebs solution was used to establish a maximum time period of incubation where minimal damage to tissue was incurred. Once a time course was established, exposure to toxins was carried out. Time periods of 15, 30, 45 and 60 minutes were used.

Immediately after excision from the animal or incubation in Krebs solution tissues were immersed in a cryoprotectant (OCT, Tissue Tek, Miles Inc.), in a mould with a cryostat chuck in place. The whole assembly was then immersed in liquid Nitrogen until completely frozen. Once frozen the tissue block was placed in the cryostat to equilibrate from -196 °C to -20 °C. After freezing and equilibration the blocks were cut using a cryostat (Tissue Tek 2). The blocks were faced in to remove the OCT before the tissue face. Once faced in sections were cut a 8um and placed on clean microscope slides. The sections were allowed to dry at room temperature before staining. Before immunostaining a circle was cut around the tissue using a diamond pencil, to restrict the movement of reagents, and then the section was hydrated by washing in water. After hydration

the sections were rinsed with buffer, then treated at room temperature with 10uL toxin diluted in buffer-2% Tween 80. for time periods of 10. 20, 30 and 60 minutes. Control slides were exposed to buffer-Tween for the same time periods.

When frozen sections were not used, cerebellar tissue was fixed in 10% neutral buffered formalin and tissues were processed and sectioned using standard histological techniques. Before inmmunostaining a circle was cut around the tissue section in the glass slide with a diamond pencil and sections were hydrated with complete removal of wax.

The method of immunostaining used for both the pre and post preservation exposure was the same, using the labelled avidin-biotin technique of Guesdon et al., (1979) (Figure 15). Biotinylated swine anti-rabbit immunoglobin IgG and Avidin (horseradish) peroxidase (Dakkopatts) were used. The buffer used throughout the methods was Tris-HCL 0.05M pH 7.4.

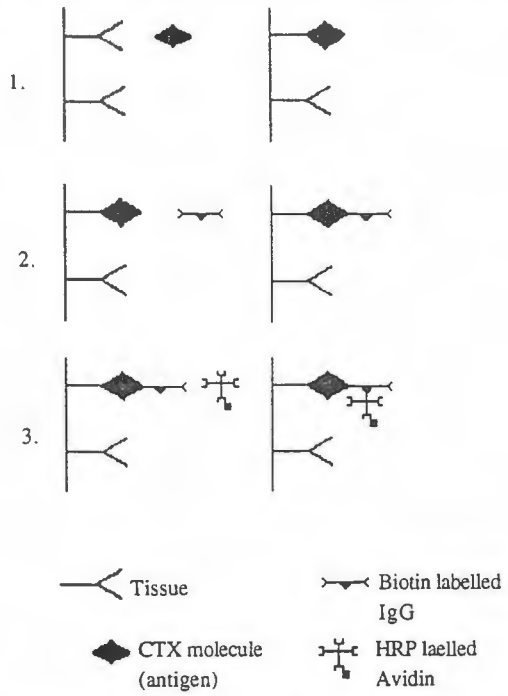


Figure 15: The Labelled antibody methods of staining. 1. The tissue is loaded with the antigen (CTX molecule), 2. then the biotin labelled IgG is attached and 3. located with an avidin labelled Horse radish peroxidase visualised by the diaminobenzidine method. (adapted from Guesdon et al., 1979).

After hydration and/or exposure to toxin the section was rinsed with several changes of buffer, then treated with fresh 3% hydrogen peroxide for 3-5 minutes. This was to destroy any

endogenous peroxidase present in the tissue. After rinsing with buffer and draining, the sections were treated with biotinylated swine anti-rabbit IgG diluted with buffer 1:400 for 30 minutes at room temperature in a petri dish lined with moist filter paper, to prevent drying of the section. They were then washed for 5 minutes in 4 changes of buffer, then treated with the avidin peroxidase diluted with buffer 1:400 for 30 minutes at room temperature in the petri dish. After washing in buffer as before, the sections were treated in the dark with fresh diaminobenzidine (DAB) solution for 10-20 minutes at room temperature inverted in a petri dish. The sections were then washed in water for 5 minutes and counter stained with Mayers haematoxylin for 30 seconds, washed , cleared and mounted.

The DAB solution was prepared directly before use in a fume hood by mixing 10mL buffer, 0.1 mL fresh 1% hydrogen peroxide and 5mg of DAB-4HCL (Sigma). Other stains carried out in conjunction with the immunostain were rapid (frozen) and routine (paraffin) haematoxylin and eosin stain and luxol fast blue stain. Luxol fast blue was used to stain the myelin sheaths of the nerve axons present in the tissue. This stain was used for both paraffin and frozen sections.

The toxins used in this part of the study were CTX extract #1 and CTX extract #3. CTX extract #1 was added to 1mL incubation volumes of Krebs, Tween solution and tissues were incubated for varying times. CTX extract #1 was used at full strength while a 1:10 dilution CTX extract #3 was used.

6.13 Results

Control and experimental mouse brains were examined after both types of preservation, for all treatments. Tables 9 and 10 indicate the numbers of sections examined for all treatments.

	Preservation	
Treatment	Frozen	Paraffin
Control*	3	2
Krebs-control	16	12
Control extract	8	8
Toxin	-	4

Table 9: The number of sections examined using the pre-preservation method of exposing the tissue.

Treatment	Frozen
Control*	8
Buffer-Tween	16
Control extract	16
Toxin	16

* These tissues were preserved immediately after excision from the animal

Table 10: The number of sections examined using the post-preservation method of exposing the tissue.

The very poor morphology exhibited by the pre-preservation frozen control sections (Figure 16) established that any exposure to toxin of material treated in this manner would yield no information on the possible binding sites within the vermis. Figure 17 shows the complete loss of architecture in a section of cerebellum exposed to CTX for 20 minutes. Because of the lack of recognisable architecture in CTX exposed frozen sections, it was decided to use paraffin sections utilising the same exposure regimen. When this method showed little immunocytochemical staining post-preservation techniques were attempted with frozen sections but these two were unsuccessful.

Formalin fixed tissue pre- and post- preservation control sections that were not incubated showed good morphology (Figure 18). The morphology of the cerebellum was well preserved and easily recognisable. The three layers, the molecular, granular and Purkinje cell layer were easily distinguishable. When cerebellar tissue was incubated relatively good morphology was attainable for incubations up to 30 minutes in duration. Therefore time series of 10. 15, 20 and 30 minutes were chosen for exposure of tissues to control extract and toxin. Sections prepared by immunostaining for controls and control time series experiments displayed very little background staining (Figure 19).

The experimental sections displayed very little immunocytochemical deposits other than that noted for the control sections as background staining. The frozen sections showed a complete loss of architecture and could not be used. The paraffin sections however, displayed better morphology, but gave no indication of any evidence of the localisation of bound toxin molecules (Figure 20).

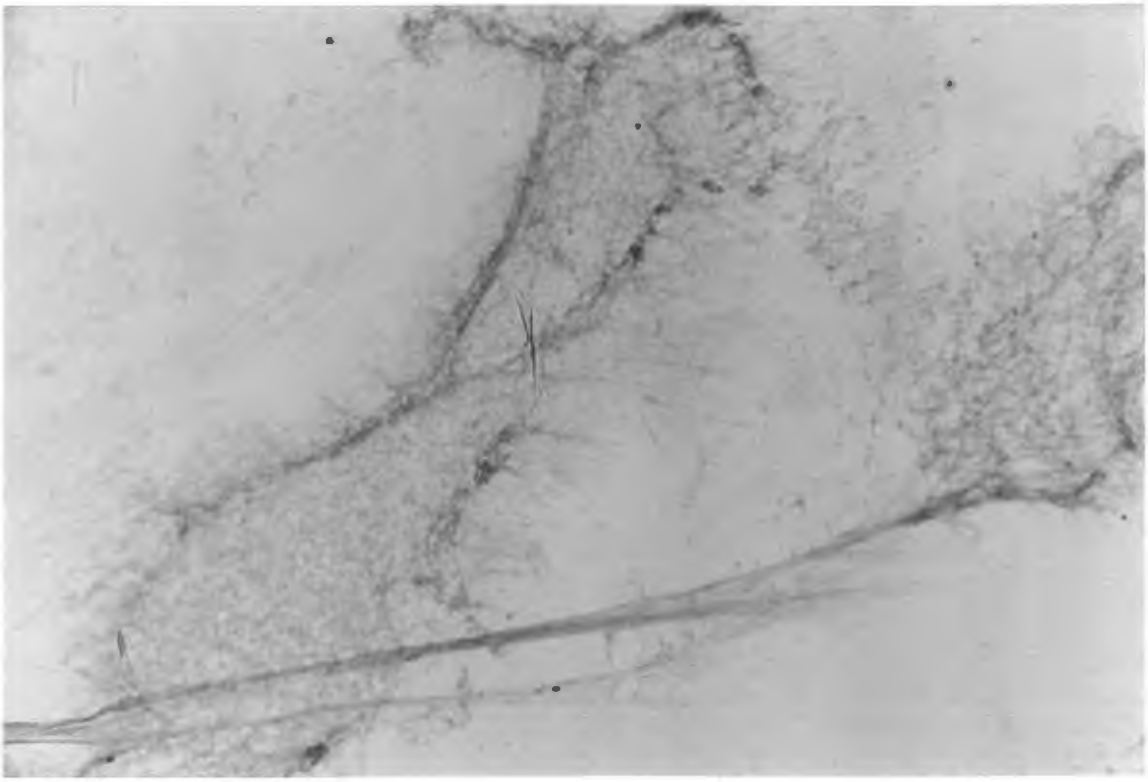


Figure 16: Cerebellum (frozen section) exposed to control extract and immunocytochemically stained. Note the disruption to the morphology and the lack of background staining.

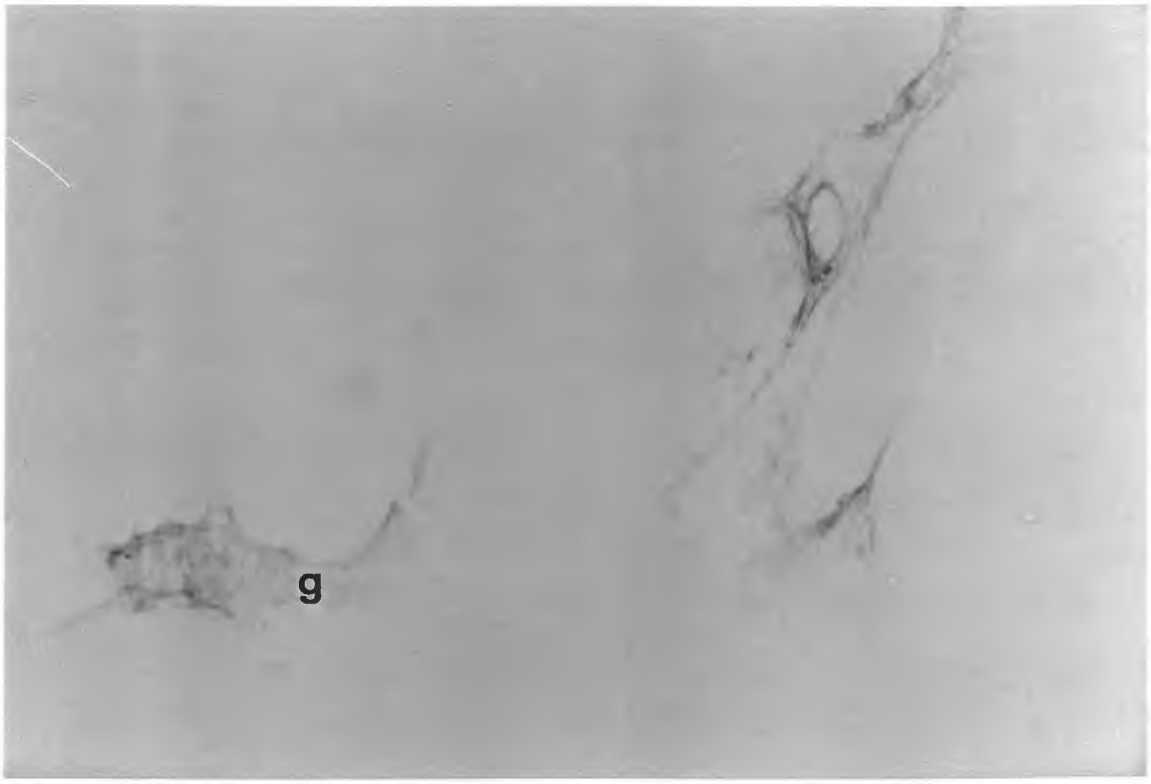


Figure 17: Cerebellum (frozen section) exposed to CTX for 20 minutes. Note the complete loss of architecture of the tissue. There is very little brown deposit and hence immunostained cells in this poor quality section. The granular layer is probably shown above (g).

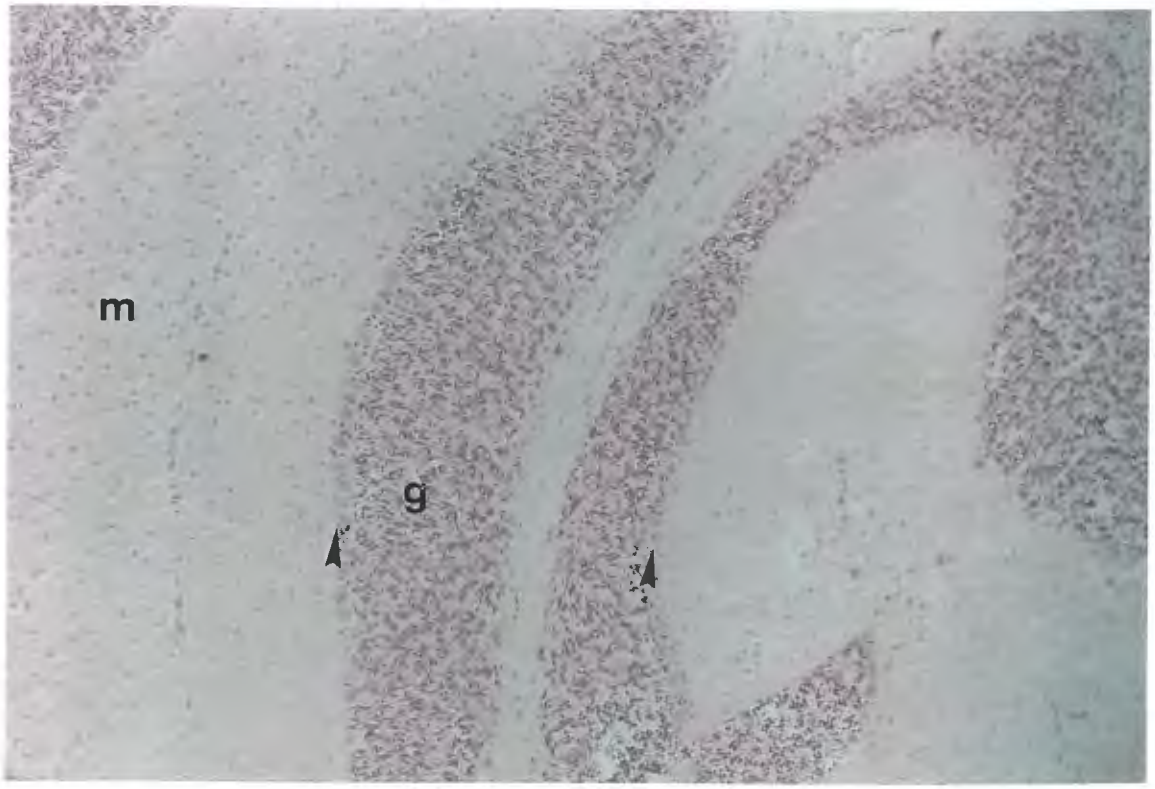


Figure 18: **Cerebellum (control section) stained with Haematoxylin and Eosin. Note the layers of the cerebellum: the molecular layer (m), the granular layer (g) and the Purkinje cell layer (arrow).**

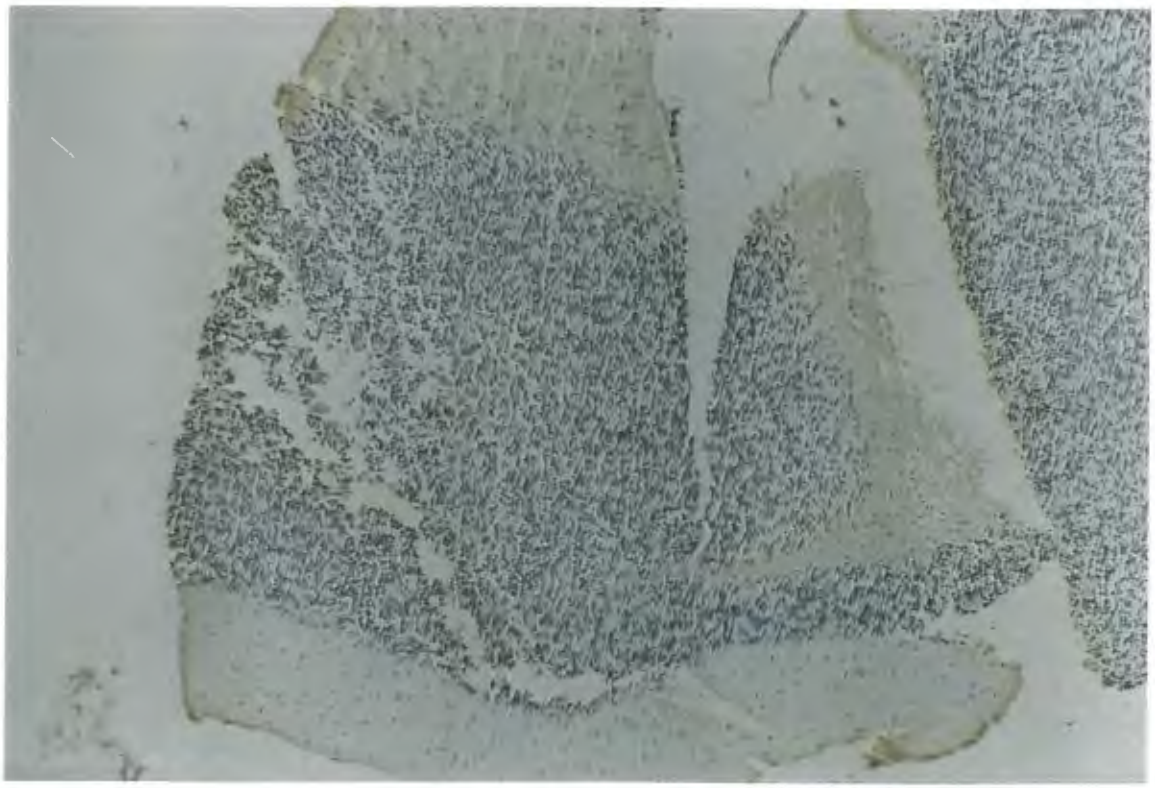


Figure 19: **Cerebellum control extract exposure for 20 minutes. This is a paraffin section stained by the immunocytochemical method. Not only light brown staining around the periphery of the tissue.**

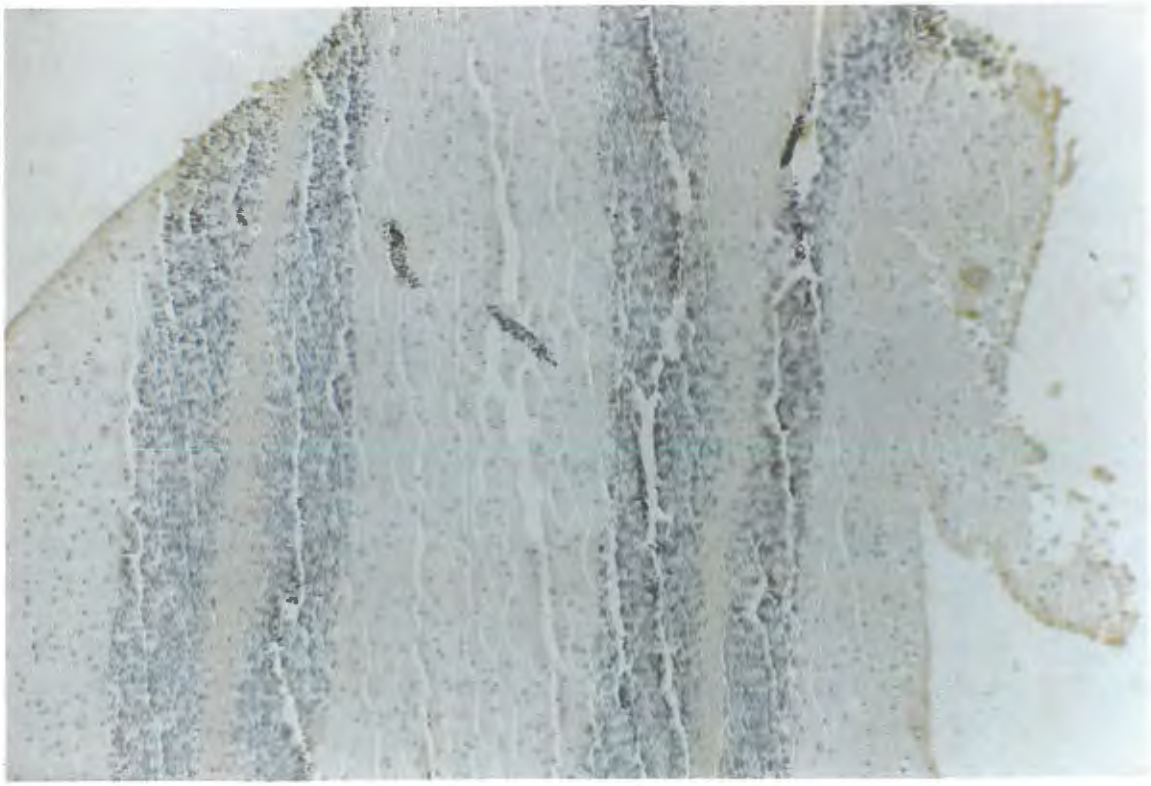


Figure 20: **Cerebellum exposed to CTX for 30 minutes and immunocytochemically stained. If CTX was present a heavy brown staining would have been apparent. Only background staining similar to control sections is apparent.**

6.14 Discussion

The theoretical basis of visualisation of ciguatera toxin was that the toxin molecule would interact with specific binding sites in the brain tissue. The specific sites might be expected to be associated with the NA^+ channels (Capra, 1985; Capra et al., 1987) within the nerve fibres of the cerebellar tissue, including the Purkinje cell dendrites and axons. It was predicted that the toxin molecule would bind to the Purkinje cell dendrites and axons, particularly at the synapses where a larger number of voltage independent ion channels occur (Berne and Levy, 1983). The biotinylated IgG would then link to the bound ciguatera toxin molecule. If this was to occur immunocytochemical localisation of the bound biotinylated IgG could be used as an indicator of the presence of ciguatera toxins. The staining methods used in this study would lead to brown deposits in the sections that could be visualised by light microscopy. The staining method chosen was the labelled avidin-biotin method (Guesdon et al., 1979). The species from which the immunoglobin was taken was swine anti-rabbit, as far removed from mouse and fish as possible to reduce non-specific staining. An avidin-biotin method was chosen because of the high affinity between avidin and biotin and the high sensitivity of the methods (Coggi et al., 1986)

As so little toxin was available a decision was made to conduct this study *in vitro*. By using *in vitro* techniques central nervous system tissue could be exposed to reasonable concentrations of toxin without using large amounts of toxin.

One of the difficulties in the application of immunocytochemistry to the visualisation of bound toxin molecule is the choice of the method of tissue processing and at what point in the procedure to expose the tissues to the toxin. As ciguatera toxin is very lipid soluble (Tachibana, 1980) and may be extracted during routine paraffin processing, it was initially decided to avoid this type of processing. Therefore, frozen sections were used, with studies using control extract exposure of the tissue before preservation. However as these sections were of very poor morphology, paraffin sections were subsequently used and this greatly improved the morphology. Unfortunately these sections showed no immunostaining when exposed to toxin. A post-preservation method using frozen sections was subsequently attempted but no usable results were obtained.

In frozen and paraffin control sections that were not incubated in toxin or control extract, good tissue morphology was apparent with both haematoxylin and eosin and luxol fast blue staining.

The paraffin immunocytochemical sections displayed better morphology than the frozen sections. Both paraffin and frozen control sections displayed little background staining. Sections derived from tissues incubated in Krebs solution alone or Krebs solution with control extract also showed little background staining, although frozen sections showed very poor tissue morphology. The poor morphology of these incubated frozen sections led to the use of paraffin sections.

There was little difference between the ciguatera toxin immunohistochemical sections and the control sections indicating no observable conjugation of the ciguatera toxin molecule to the brain tissue. There are however, problems with paraffin processing associated with immunohistochemical techniques that should be considered. Routine formaldehyde fixation and paraffin processing effects may cytoplasmic components, including immunoglobins (Bosman and Kruseman, 1979). Proteins are cross-linked by the formaldehyde, and this may have an effect on the binding sites of the ciguatera toxin. This problem was minimised by exposing the tissue to the toxin prior to fixation. The formaldehyde fixation of the tissue may have effected the bound toxin by altering the availability of antigenic sites on the bound toxin. Failure to completely remove paraffin before immunostaining may physically prevent access of immunochemicals to sites within the section and complete deparaffinisation must be completed before staining.

The frozen immunochemistry sections displayed extremely poor morphology and preservation of the tissue when compared to control sections stained at an earlier date. Control sections stained concurrently with the experimental sections, however displayed similarly poor morphology. Again there was no visible differences between control sections and their equivalent experimental sections. Although the majority of light microscopy immunohistochemical work is performed on frozen sections, the method is known for its poor preservation of tissues (Polak and Van Noorden, 1987). The control sections not exposed to the long incubation periods during an immunostaining procedure has the potential to cause tissue damage, as observed with these sections.

The lack of indications of toxin binding may reflect a lack of bound toxin or the inability of the methodology to identify bound toxin. If the toxin molecule doesn't bind before exerting its physiological effects then it can not be visualised by these methods. The molecule may not bind to the tissue at all. The methods used in this procedure relied upon the assumption, that ciguatera toxin molecules are bound to the tissue in such a configuration that their IgG binding site was available for binding. The ciguatera toxin molecule is relatively small and therefore binding to tissue may obscure the portion of the molecule that has been associated with its

affinity for IgG (Emerson et al., 1983). If the binding occurs within the Na^+ channel, as now seems to be the case, it is not an unexpected result for no IgG linking and hence visualisation to occur.

An unrelated species of IgG was used, and was unsuccessful. Use of a specific monoclonal antibody to ciguatoxin may have produced results that identified a specific binding site. Hokama and coworkers (1984; 1985a; 1985b; 1986) have tried other assays using monoclonal antibodies to the ciguatera toxin molecule with varying success. Monoclonal antibodies to ciguatera toxin were not available for this study.

The immunocytochemical technique was deliberately performed on isolated tissues because it was felt that significantly more toxin could be delivered to the tissues than could be achieved by *in vivo* dosing. As no definitive indication of toxin binding was achieved by the *in vitro* method, further *in vivo* studies in intact animals may be indicated, but difficult given the small quantities of CTX available for research.

6.2 Fish Protective Mechanisms and Human Therapy

It was our initial intention to assess protective mechanisms in fish from a physiological and biochemical perspective to assess how such protection may form a basis for the therapy of human victims. In our proposal for 87/058 we presented the following objective:

> *To evaluate the mechanisms of sequestration of CTX by fish as potential methods of therapy in humans.*

The initial work commenced on 87/058 was directed towards toxicological and histopathological studies in fish. The results of these studies indicated that fish are susceptible to CTX and potential precursor toxins from *G. toxicus*. The level of toxin required to produce overt signs of intoxication and death in fish was greater than that required in mammal. Our work with three species of pomacentrids indicated that there are also species related variations in the susceptibility to CTX in fish. Studies by Capra et al., (1988), Flowers (1989) and Flowers et al., (1992) have shown that the nerves of both carrier and non carrier species of fish are effected by CTX in a manner that is qualitatively similar to the actions of this toxin on rat (Cameron et al., 1991a) and human (Cameron et al., 1991b) nerves. The histological studies on fish have also

shown that the liver, intestine and gills of fish can be damaged at the cellular level by CTX and the precursors of CTX extracted from *G.toxicus*.

The toxicological and histological aspects of the study of the effects of CTX on fish indicate that although fish may be more resistant to CTX they are effected by this toxin. Many outbreaks of ciguatera poisoning in humans, including the 1987, large scale outbreak in Sydney (Capra and Cameron, 1991) have been traced to individual or small numbers of toxic fish. The question remains as to how large fish of 20-40kg can contain sufficient CTX to severely poison many humans. Do such fish show pathology? Are these fish perhaps easier to capture because their nervous system is compromised in a manner similar to humans? Are they able to withstand large quantities of toxin because they have evolved specific mechanisms to combat the CTX within their own tissues? The toxicological and histological studies reported in this document give little insight into possible defence mechanisms in fish. On the other hand the biochemical studies reported in the Spanish mackerel, provide strong evidence for a successful defence mechanism. Protein biochemistry studies have shown that within the flesh of toxic fish there is at least one monomeric soluble protein of 37 000 to 40 600 molecular weight that appears to be associated with CTX. A similar protein was not found in non toxic fish. We speculate that this soluble protein binds to CTX and thus prevents CTX from attaching to critical target sites such as the site deep within the Na^+ channel of the exciteable tissues.

CTX is a highly lipid soluble and small compound (MW approx 1100) that could easily pass through the lipid layers of cell membranes and bind to sites deep within ion channels. If CTX was bound to a soluble protein with a MW in the order of 40 000 Dalton then it would not penetrate the lipid layer in the membranes of target nerve cells and hence not bind to critical sites in the Na^+ channel.

The isolation of a CTX specific protein in fish has considerable significance for the detection of CTX in fish and less significance for the therapy of human victims of CTX. Ciguatera poisoning in a developed country like Australia is very much an "elective" disease. An individual who does not eat fish has zero risk of experiencing ciguatera poisoning. Likewise those who choose to eat only temperate water species have zero risk of poisoning. People who choose to eat tropical species experience a finite risk of poisoning and this risk increases with the consumption of large specimens of certain defined tropical species. However many people are either prepared to risk poisoning or are ignorant of the possibility of poisonig when they consume a range of tropical fish. When poisoning occurs and in particular when large numbers of people are simultaneously

poisoned the fishing industry in general suffers considerable adverse publicity. Victims of poisoning are also able to litigate against retail and wholesale vendors of fish under common law and specific Acts.

One of the great hopes of the industry and retailers in particular, is the development of a reliable and low cost method of testing fish prior to sale and human consumption.

To date the only hopeful tests for CTX have been based on the development of antibodies for CTX which is a small non protein molecule. In order to raise antibodies to CTX it is first necessary to couple the toxin to a suitable protein. Attempts have been made by Prof Hokama of the University of Hawaii and Prof Park of the University of Louisiana to produce antibodies to CTX and to develop simple colour change diagnostic kits for the detection of CTX in fish. These diagnostic kits have proved to be unreliable and give both false positive and false negative results. The major difficulty in the development of a simple diagnostic test for CTX has been the difficulty in successfully raising antibodies for CTX. The results of the FRDC sponsored work at QUT indicate that, at least, in the Spanish mackerel which is a commercially important species, unique and diagnostic proteins occur in fish carrying CTX. It would be much easier to develop a diagnostic antibody based detection but for this induced protein rather than for CTX. While the development of a CTX test based on the presence of a CTX induced protein is certainly feasible, the use of such a protein for human therapy would seem unlikely. If the functional portion of the CTX-induced protein could be sequenced it would be possible to use modern techniques of molecular biology to clone this protein and then use it as a pseudo-antibody for victims of ciguatera poisoning. At the present time the costs involved in such a therapeutic regimen would far out weigh its usefulness. On the other hand the costs involved in using the induced protein to develop a sensitive antibody based test would not be excessive and would benefit the Tropical Fishing Industry.

6.3 Mannitol and Ciguatera Poisoning

6.31 Introduction

The final objective of the combined studies on ciguatera is:

To use the ventral coccygeal nerve preparation of the intact rat to assess the efficacy of mannitol and other antagonists on the reversal of neurological manifestations of ciguatera poisoning.

In this Section the analysis and comparison of effects of CTX and brevetoxin (PbTx) on the mammalian peripheral nervous system will be described. .These two structurally related polyether toxins bind to the same site (site 5) on the voltage-dependent sodium channel (Bidard et al., 1984).

Capra and Cameron (1985) and Cameron et al., (1991a, 1991b) documented modifications to the parameters of nerve conduction by CTX in the peripheral nerves of mammals. Results from *in vivo* laboratory experiments on rats corresponded with alterations to nerve conduction recorded in humans following the consumption of ciguatoxic fish. Similar studies relating to the effects of PbTx on the mammals *in vivo* have not appeared in the literature.

When researching the pharmacological basis of therapeutic intervention in CTX intoxication it is preferable that these studies be carried out on *in vivo* preparations. This aspect of research employing whole animal mammalian preparations has been severely hampered by the limited availability of CTX. Due to the reported similarities between PbTx and CTX, PbTx was introduced into the current study in the belief that it could prove to be a useful pharmacological model for mimicking the electrophysiological changes effected by CTX. If this could be established, then the existing difficulty of limited availability of CTX could perhaps be alleviated.

Over the years, a number of therapeutic agents have been administered to victims of ciguatera poisoning. These have included atropine, calcium, dopamine, various vitamins, steroids and antihistamines (Banner et al., 1963; Bagnis, 1968; Russell, 1975). All have been given in an attempt to achieve clinical improvement, largely without any documented success. More recently several compounds have been reported to induce a reduction of symptoms; tocainide (Lange et al., 1988) and mannitol (Palafox et al., 1988; Pearn et al., 1989).

Lange et al., (1988) documented clinical improvement in three victims of chronic ciguatera poisoning following the oral administration of tocainide, a lignocaine analogue. Lignocaine is a local anaesthetic agent that is capable of producing a reversible dose-dependent conduction block in all types of nerve fibres (Ritchie and Greene, 1991). Local anaesthetics interact directly with the sodium channels, preventing the increase in the influx of sodium (Strichartz and Ritchie,

1987). They exert their effects by occluding the sodium channel and also influencing the gating properties of the channel (Hondeghem and Miller, 1987). Several *in vitro* studies have reported a lignocaine-induced reversal of effects produced by CTX (Legrand et al., 1985a; Lewis, 1985c, 1988; Flowers, 1989). Sodium channels in an activated state have a much higher affinity for local anaesthetics than those in the rested state (Hondeghem and Miller, 1987). This perhaps should make them ideal therapeutic agents for ciguatera poisoning where CTX is maintaining the channels in their open state.

Mannitol is an osmotic diuretic agent used widely in the clinical situation for a range of conditions including glaucoma and cerebral oedema (Weiner, 1991). This pharmacologically inert compound is not metabolised and when administered intravenously remains largely confined to the extracellular space where it exerts its osmotic effect. Mannitol was instigated as a therapy for ciguatera because an association was made between ciguatera symptomatology and cerebral oedema (Palafox et al., 1988). The proposed mechanism of action of mannitol when used as a therapeutic agent for ciguatera, ranges from either direct stabilisation of the cell membrane, a scavenger for the hydroxyl groups of CTX or by direct reduction of axonal oedema (Palafox et al., 1988; Pearn et al., 1989; Stewart, 1991).

Palafox et al. (1988) initially treated twenty-four patients with acute ciguatera poisoning. Mannitol (1g/kg) was administered intravenously over 30 minutes along with standard intravenous fluid replacement. A dramatic improvement was reported to occur in all patients in this study including the following striking description; "two patients in coma and one in shock responded within minutes, with full recovery after infusion". The following year Pearn et al., (1989) administered mannitol to twelve victims of ciguatera. These authors indicated that mannitol at a dose of 1 g/kg was effective in achieving clinical improvement whereas a lower dose (0.5 g/kg) was not. Although treatment failures as well as increased morbidity have been observed to occur following mannitol infusion these have been attributed to underdosing and overdosing respectively (Palafox pers. comm.). The above reports have been empirical in nature and relied on subjective description of symptoms by the victims. The exception to this is the coma reversal reported by Palafox et al., (1988), in which case mannitol was given in conjunction with standard fluid replacement. It could be argued that the initial cause for coma in these patients was in fact dehydration which was subsequently relieved by the fluid replacement rather than the effect of mannitol per se.

Although only a small number of patients have been studied, mannitol has so far failed to reverse CTX-induced nerve conduction abnormalities in humans (Blythe pers. comm., International Ciguatera Workshop, Bribie Island, 1993). In human atrial trabeculae *in vitro*, mannitol failed to reverse the CTX-induced positive inotropy (Lewis et al., 1992). Furthermore mannitol has been unsuccessful in saving animals injected with CTX in the laboratory (Lewis et al., 1993). In electronmicroscopic studies of single fibres, mannitol has been observed to reduce the CTX-induced increase in surface area in Schwann cells and motor nerve terminals (Molgo pers. comm., International Ciguatera Workshop, Bribie Island, 1993).

This FRDC supported study utilised the intact rat as a model for the human nervous system. Nerve conduction studies were performed in order to assess the effects of mannitol and lignocaine when used as a therapeutic agent for intoxication with either PbTx or CTX.

Two major series of experiments were undertaken in this part of the study. The most comprehensive series assessed both the antagonism of CTX and PbTx by mannitol and the antagonism of PbTx by lignocaine. These experiments are reviewed first as Series 1. A second series of experiments evaluated the antagonism of lignocaine on the response of nerves to CTX (Series 2).

6.32 Methods (Series 1)

6.32.1 Drugs

In experiments conducted on rats the following drugs and toxins were used; PbTx, CTX, lignocaine, mannitol and leptan. CTX was prepared as detailed in Section 3.0 and PbTx was purchased from Latoxan, France. Lignocaine used was from Astra and mannitol (20% w/v Osmitrol) from Travenol. The drug used for all anaesthesia was Leptan from Parnell Laboratories. This neuroleptanalgesic contains Fentanyl (0.4 mg/ml) and Droperidol (20 mg/ml). Leptan was purchased from ProVet Supplies and records kept of its use.

6.32.2 Physiological Saline

Physiological (normal) saline had been employed for all previous CTX-related pharmacological studies conducted within the QUT laboratory. This vehicle was utilised for all CTX experiments

performed in this section of the current study. Physiological saline (normal saline) was composed of NaCl 9.0 grams in 1 litre distilled water and stored at 4°C.

Based on reports in the literature (Templeton et al., 1989; Poli et al, 1990), a phosphate-buffered physiological saline was utilised in all PbTx experiments (Table 11). The pH was 7.1. The saline was stored at 4°C.

Composition	Concentration
NaCl	8.0
$2NaHPO_4$	1.15
NaH_2PO_4	0.2
KCl	0.2

Table 11: Composition of the phosphate-buffered physiological saline in grams per litre.

6.32.3 Animals

Male Wistar rats weighing between 150 and 200 grams were purchased from the Central Animal Breeding House at the University of Queensland. Following delivery to the Queensland University of Technology the animals were kept at the registered animal holding facility until required for use. Following the experiments rats were euthanased with intravenous Lethobarb (purchased from ProVet Supplies) and disposed of through the university-recognised pathological waste disposal company. In the graphs presented in the results section, each point was derived from a minimum of five rates. In total 75 rats were used in this series of experiments.

6.32.4 Preparation for Nerve Recordings

The ventral coccygeal nerve was used for all electrophysiological studies on the rat (Figure 21). Positioning of the electrodes was achieved by measuring the tail from anus to tip and dividing this measurement into three equal portions. Two silver surface stimulating electrodes were then positioned 1 cm apart at the distal third of the tail, and two stainless-steel needle recording electrodes 1 cm apart at the proximal third of the tail. A flexible foil earth plate was sited midway

Figure 21: **Position of electrodes for recording from the ventral coccygeal nerve in rats.**

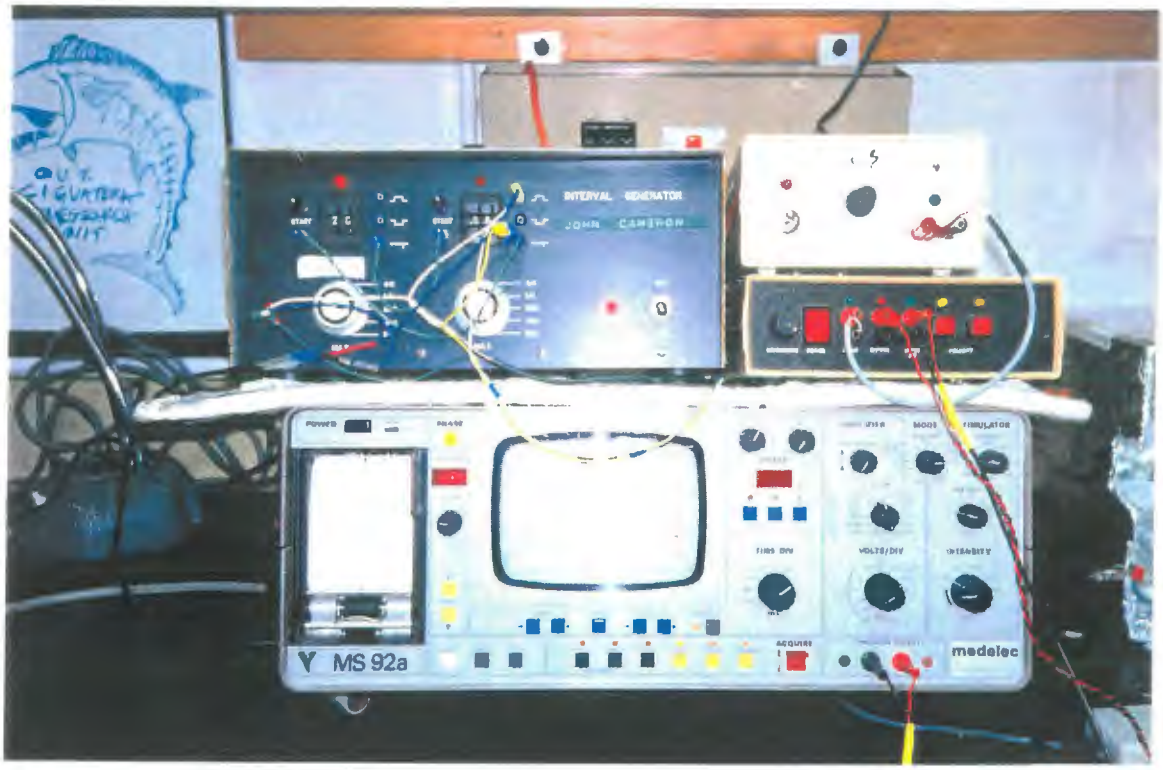


Figure 22: **Electronic equipment used for simulating and recording in electrophysiological studies on rats.**

between the stimulating and recording electrodes. The ventral surface of the tail was thoroughly cleaned prior to positioning the electrodes. Electrode gel was placed under both the stimulating and the earth electrodes. These electrodes were then strapped in position with Leukosilk. Care was taken to avoid excessive tightness as this could abolish the supernormal response (Gilliatt and Willison, 1963). Attention was paid to prevent the spread of gel along the tail as well as to keep the tail dry in order to achieve peak conductance. Throughout recording procedures the tail temperature was monitored by a thermistor placed on the tail and maintained at 36 - 37°C via the thermostatically controlled heated mattress. The tail was covered with cotton wool to prevent heat loss.

6.32.5 Electrophysiological Equipment

A Medelec MS92a clinical electromyography unit was used to stimulate and record nerve action potentials (Figure 22). Single and paired supramaximal stimuli were used in which square wave pulses with an intensity of 150-200V and a duration of 0.1ms were delivered. A supramaximal stimulus is defined as one which is 25% above the voltage causing a maximal nerve action potential. Filter settings for the MS92 were EMG 2Hz-10Hz. The rate of stimulation was 1 pulse per second. Potentials were averaged for 4-8 times prior to recordings being collected. Compound nerve action potentials and conduction velocities were assessed as well as the absolute and relative refractory periods.

An additional stimulator with variable voltage was employed for supernormal studies. This stimulator was connected to the MS92a and triggered by an interval generator (Figure 22). In supernormal studies paired stimuli were used in a technique based on that described by Parkin and Le Quesne (1982). The first stimulus, regarded as the conditioning stimulus is denoted as S1. This supramaximal stimulus was adjusted to ensure it elicited a maximal response in the nerve. The second stimulus, S2 was adjusted to evoke a response which was approximately one-third that of the maximal response. At each interstimulus interval studied, the amplitude of S2 when delivered alone was recorded to confirm stability of the preparation. The amplitude of the response to S2 when preceded by S1 (CS) was then expressed as a percentage of the amplitude of the response to S2 when delivered alone (UCS). Nerves were regarded to be supernormal when the percentage response to the conditioned stimulus in comparison with the response to the unconditioned stimulus (CS/UCS%) exceeded 100 (Figure 23). Supernormality

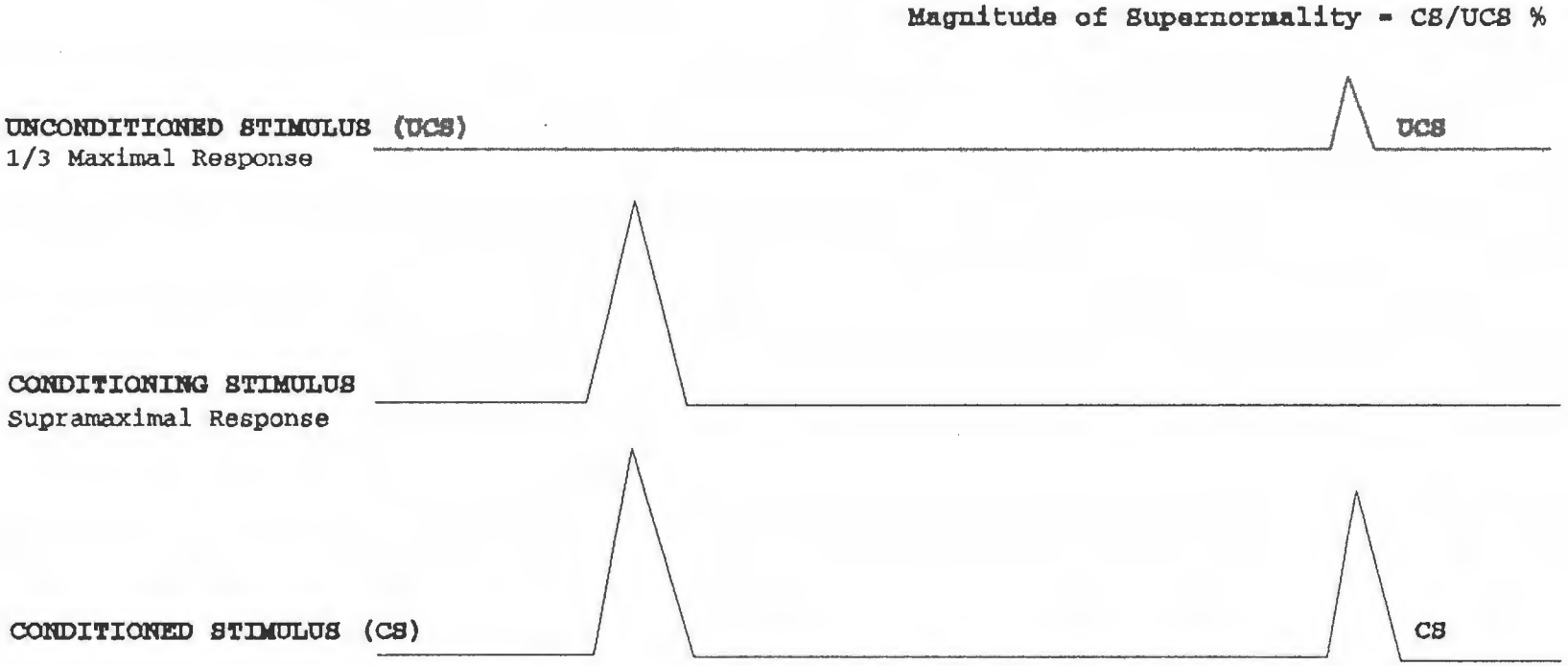


Figure 23: A schematic representation of the supernormal response. At each stimulus is delivered to produce a response in the nerve which is ⅓ of the maximal response. During the supernormal period, the amplitude of the neural response to this stimulus becomes greater when conditioned by a preceding supermaximal stimulus.

was examined at interstimulus intervals of 6, 8, 10 20, 60 and 100 ms. With several of the treatment groups supernormality was also measured at 500, 1000 and 2000ms.

6.32.6 Treatment Group Protocol

Experiments conducted followed two general formats. All intravenous infusions were delivered by a Harvard Infusion Pump.

6.32.6.1 Format #1

This general format was used for the phosphate-buffered saline control group, as well as experiments utilising PbTx when administered intravenously (brevetoxin, brevetoxin/lignocaine, brevetoxin/mannitol).

Prior to these *in vivo* studies each animal was anaesthetised with intramuscular Leptan. The initial dose was 420 µl/kg, with smaller incremental doses being given throughout the experiment to maintain an adequate and ethical level of anaesthesia.

Following satisfactory anaesthesia the femoral vein was cannulated by a venous cut-down procedure. The venous cannula was composed of polythene tubing (Portex PP10 with an inside diameter of 0.28 mm) to which a 30 gauge blunted needle had been inserted in the distal end to allow for syringe connection. The length of the cannula was 20 cm. All cannulation procedures were completed in 10 minutes with minimal to nil blood loss. Following cannulation the wound was sutured with surgical silk to prevent fluid loss.

After cannulation the animal was immediately placed on a custom-designed heated mattress. The mattress was manufactured from a matrix of coiled narrow-diameter copper tubing through which thermostatically controlled water was passed and re-circulated. Reusable cold/hot packs, Medi-Paks from Medi-Ice Pak Australia, were placed over the top of the copper to achieve an even heat distribution. The copper was earthed for the duration of electrophysiological recordings. During recordings this entire assembly was placed inside an earthed foil lined (Faraday) cage.

Following cannulation in the manner described, three groups received intravenous PbTx (15 µg/kg) which had been taken up in a vehicle comprised of 1% Tween 80 in phosphate-buffered saline - total volume 200 µl. This treatment was then delivered as an infusion over 15 minutes. A fourth group (saline control), received the 1% Tween 80 in phosphate-buffered saline vehicle only - total volume 200 µl.

Subsequent to the initial infusion, two of the PbTx-treated groups were administered with a further treatment as follows:

(a) One group received intravenous lignocaine (500 µg/kg) in physiological saline - total volume 200 µl, delivered over 30 minutes.

(b) A second group received intravenous mannitol 20 % (1 g/kg), also delivered over 30 minutes.

In all four treatment groups described above, nerve conduction studies were performed one hour after commencement of the initial treatment infusion (i.e. brevetoxin or saline vehicle). For each of these experimental treatment groups, studies were carried out on eight individual rats. An outline of the time frame for each experiment is given below in Procedure Tables 12, 13 and 14.

Time (mins)	
0	intramuscular leptan
15	cannulate femoral vein
45	i.v. 1% Tween 80 in phosphate buffered saline
	total volume 200 µl - given over 15 minutes
60	end infusion
105	record nerve conduction parameters
120	animal euthanased

Table 12: Time Frame for saline control group

Time (mins)	
0	intramuscular leptan
15	cannulate femoral vein
45	i.v. brevetoxin (15 µg/kg) in 1 % Tween 80 in phosphate buffered saline, total volume 200 µl - given over 15 minutes
60	end infusion
105	record nerve conduction parameters
120	animal euthanased

Table 13: Time frame for brevetoxin treated group

Time (mins)	
0	intramuscular leptan
15	cannulate femoral vein
45	i.v.i. brevetoxin (given as above)
60	end infusion i.v.i. mannitol 1 g/kg over 30 minutes OR i.v.i. lignocaine 500 µg/kg over 30 minutes
90	end infusion
105	record nerve conduction parameters
120	animal euthanased

Table 14: Time frame for brevetoxin/mannitol and brevetoxin/lignocaine treated groups.

6.32.6.2 Format #2

Experiments conducted with this general format included the following; intraperitoneal PbTx (with and without mannitol) as well as intraperitoneal CTX (with and without mannitol).

In these experimental groups conscious animals received either brevetoxin or ciguatoxin by intraperitoneal injection. The dose of toxin is set out in Table 15. Recordings were performed on six animals in the PbTx-treated group, and five in the CTX-treated group.

Toxin	Dose
CTX:-	⅔ LD_{50} in 1% Tween 80 in physiological saline - total volume 200 µl. (NOTE: The LD_{50} value for a rat was calculated from the mouse LD_{50} value, based on the weight of the rat.)
PbTx:-	75 µg/kg in 1% Tween 80 in phosphate-buffered saline - total volume 200 µl.

Table 15: Doses for intraperitoneal administration of ciguatoxin and brevetoxin.

Each animal was observed and signs of intoxication were recorded until 2 hours after administration of the toxin when the animal was anaesthetised and the femoral vein cannulated as described in the previous section relating to format #1. Table 16 sets out the time frame for experiments conducted with treatment groups in format #2. After cannulation the animal was placed on the heated mattress assembly and nerve conduction parameters were recorded. This became the pre-mannitol recording. This recording was carried out two and a half hours after injection of the toxin.

Three hours after toxin administration, a mannitol infusion (1 g/kg) was commenced. Mannitol was administered over 45 minutes in these experiments. This was done in an attempt to overcome seizures noted in rats given mannitol over a 30 minute period. Nerve conduction parameters were then recorded a second time (post-mannitol recording).

Time (mins)	
0	intraperitoneal toxin
120	anaesthetise & cannulate
155	record nerve conduction parameters (pre-mannitol recording)
180	i.v.i. mannitol (1 g/kg) over 45 minutes
240	record nerve conduction parameters (post-mannitol recording)

Table 16: Time frame for format #2.

6.32.7 Control Experiments

In addition to the treatment groups set out in formats #1 and #2, experiments were performed on four separate experimental control groups. For each control group recordings were carried out on 8 animals.

6.32.7.1 Saline Control

The methodology for saline control experiments can be found in the previous section relating to format #1.

6.32.7.2 Control

Animals were anaesthetised with intramuscular Leptan (420 µl/kg). Following satisfactory anaesthesia being achieved, each animal was placed on the heated mattress assembly and nerve conduction parameters recorded. This control group did not receive a vehicle.

6.32.7.3 Lignocaine Control

The animal was anaesthetised with intramuscular Leptan (420 µl/kg). 10 - 15 minutes later the femoral vein was cannulated. Lignocaine (500 µg/kg) was then administered intravenously as an infusion over 30 minutes. Nerve conduction parameters were recorded 10 minutes later.

6.32.7.4 Mannitol Control

Following anaesthesia with Leptan and cannulation of the femoral vein the animal was given intravenous mannitol 20 % (1 g/kg) as an infusion delivered over 45 minutes. Nerve conduction parameters were recorded 10 minutes later.

6.32.8 Physiological Data Collection

All rats in treatment groups labelled as format #1 had respiratory and electrocardiograph data collected. After being placed on the heated mattress assembly, a skin suture was placed superficial to the diaphragm. This was then connected to a Grass force displacement

transducer. Three surface electrocardiographic electrodes were applied to the torso of the rat in order to trace the electrical output of the heart. Respiratory and cardiac traces and recordings were obtained by way of a BIOPAC (MP100) physiological workstation. Continuous visual monitoring of respiratory and cardiac traces was possible on the Apple Macintosh computer connected to the MP100. All data collected was then stored on disk. Recordings were commenced prior to the start of the infusion of the toxin (or vehicle) and continued until just prior to performing nerve conduction studies.

6.32.9 Statistical treatment of Data

For each treatment group the mean, standard deviation and standard error were calculated. Comparisons were then made between the means of all possible pairs of treatment groups by way of an analysis of variance (ANOVA). The post-hoc test, Fisher's Protected Least Significant Difference (PLSD) was then carried out in order to estimate which pair(s) of means were significantly different. These post-hoc comparisons were performed at the 95% level of significance. A p value of <0.05 was considered as a significant difference between treatment groups. In this section p values will be reported for all significant results. In the case of supernormality where p values may vary between the range of interstimulus intervals measured, the range of p values obtained will be reported.

All statistical analyses were performed on StatView, a computerised statistical package for Macintosh computer systems. During the process of initial data analysis intercomparisons between treatment groups were also carried out using an unpaired *t*-test. While results were comparable to Fisher's the latter test was found to be the more robust.

6.33 Results (Series 1)

6.33.1 Signs

Groups of rats given both PbTx and CTX intraperitoneally were observed for the development of signs of intoxication for 2 hours prior to anaesthetising. Signs observed in the ciguatoxin-treated rats included ataxia, lacrimation, diarrhoea, tachypnoea, loss of righting, piloerection, hypersalivation and hind leg weakness.

In rats given PbTx a dose-dependent respiratory response was noted. A respiratory arrhythmia similar to Cheyne-Stokes breathing was observed. The onset of this breathing pattern began approximately 15 minutes after administration of the toxin and continued until anaesthesia. The pattern consisted of 8-20 normal respirations then 8-12 extremely rapid shallow respirations followed by a short period of long sighing respiratory movement. Other signs noted in brevetoxin-treated animals included irritability, increased activity, an increase in defaecation and diarrhoea.

6.33.2 Controls

When comparisons between all four control groups were performed, no differences in either the refractory periods or the conduction velocity could be demonstrated.

In supernormal studies all groups of control nerves manifested a supernormal response at 6 ms, the earliest interstimulus interval measured. Supernormality peaked at around 8 ms in control groups. By 100 ms nerves were no longer supernormal (Figure 24).

With the exception of the lignocaine control, there were no significant differences in the magnitude of supernormality when all control groups were compared. Lignocaine control produced a significant decrease ($p=0.0111$) in supernormality below other control groups at the interstimulus interval of 10 ms (Figure 24). For all additional intervals, the supernormal response elicited by lignocaine control was not significantly different to other control groups.

6.33.3 Brevetoxin

The administration of intravenous PbTx (15 µg/kg) significantly prolonged both the absolute ($p=0.0057$) and the relative ($p<0.0001$) refractory periods when compared to control nerves (Figures 25A, 25B). The conduction velocity was significantly slowed ($p=0.0149$) in animals treated with PbTx (Figure 26A). PbTx did not alter the amplitude of the nerve action potential when compared to control (Figure 26B).

The magnitude of supernormality was significantly increased in this group of PbTx-treated animals when compared to control values (Figure 27). This increase occurred at all interstimulus intervals tested (6ms $p<0.0001$; 8ms $p=0.0009$; 10ms $p=0.0129$; 20ms $p=0.0001$; 60ms $p<0.0001$; 100ms $p<0.0001$). In the PbTx group the peak in magnitude occurred at 6 ms (mean

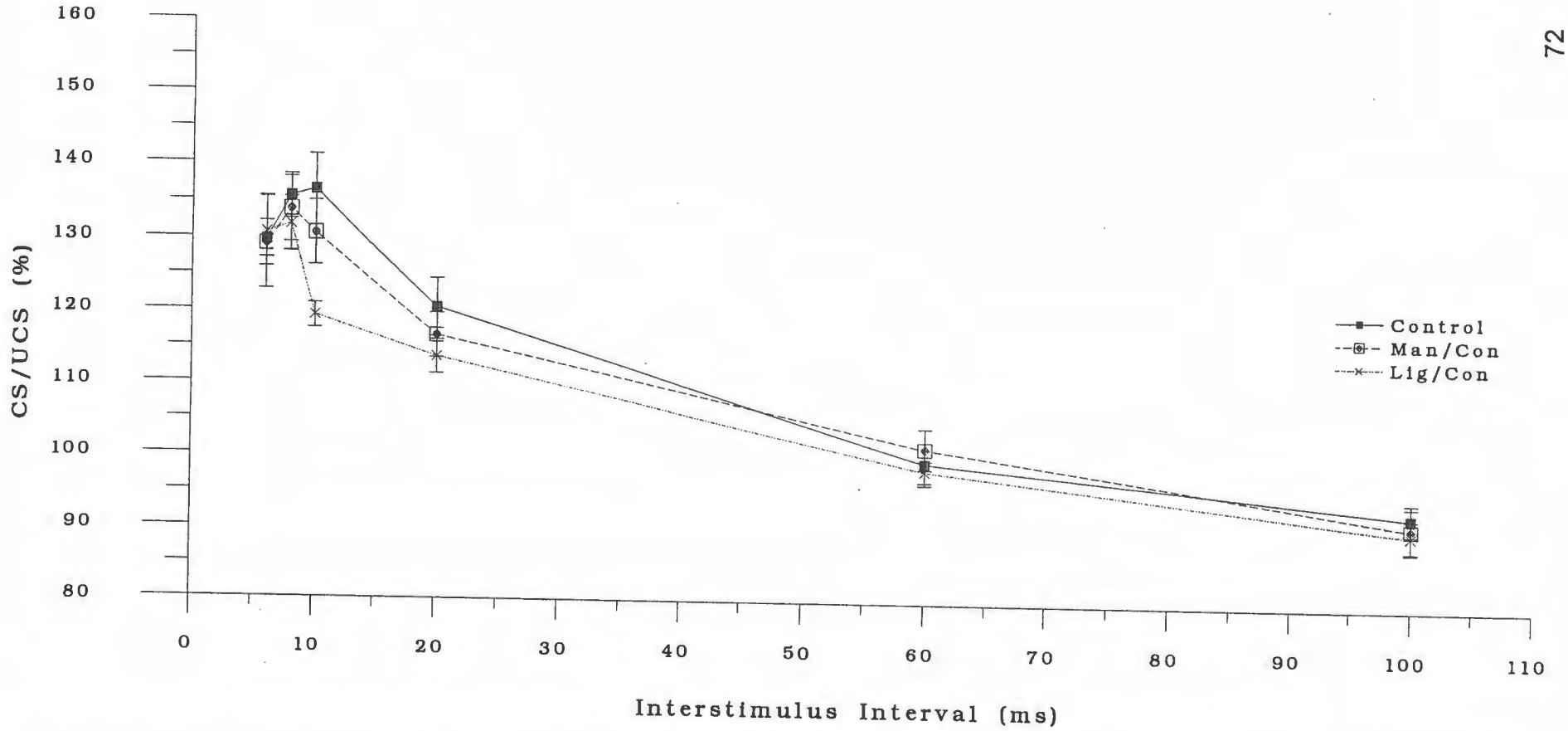
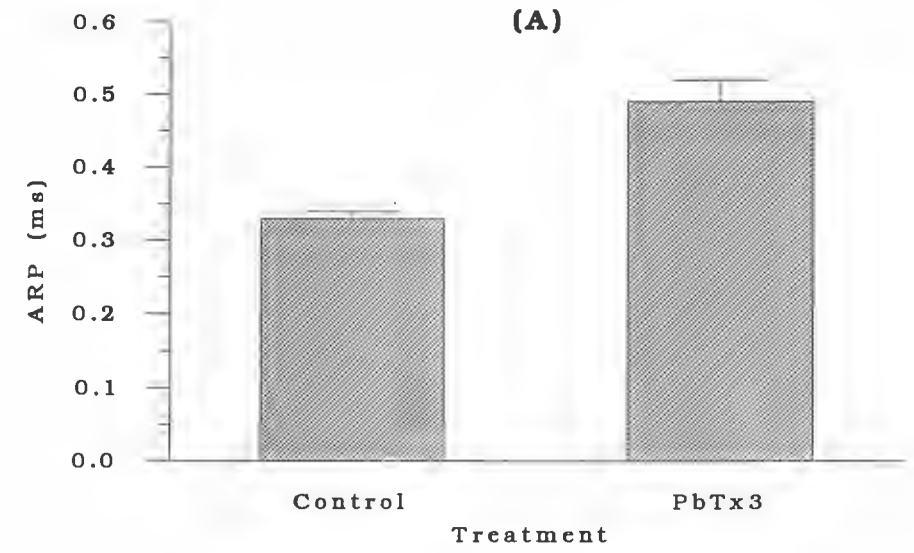


Figure 24: Graph of the percentage change in amplitude of the conditioned stimulus over the unconditioned stimulus (CS/UCS%) versus the interstimulus interval recorded *in vivo* from the ventral coccygeal nerve in rats. Comparisons are made between three groups of experimental controls. One group was given i.v. 1% Tween in PBS (Control). A second group received i.v. mannitol (Man/Con). The third group received i.v. lignocaine (Lig/Con). Error bars indicate the standard error of the mean.

Figure 25: Graphs (A) and (B) present the means of the absolute (ARP) and relative (RRP) refractory periods respectively. Rats treated with i.v. brevetoxin (PbTx3, 15 *u*g/kg) are compared to a control group (treated with i.v. 1% Tween in PBS). *In vivo* recordings were performed on the ventral coccygeal nerve. Error bars indicate the standard error of the mean.



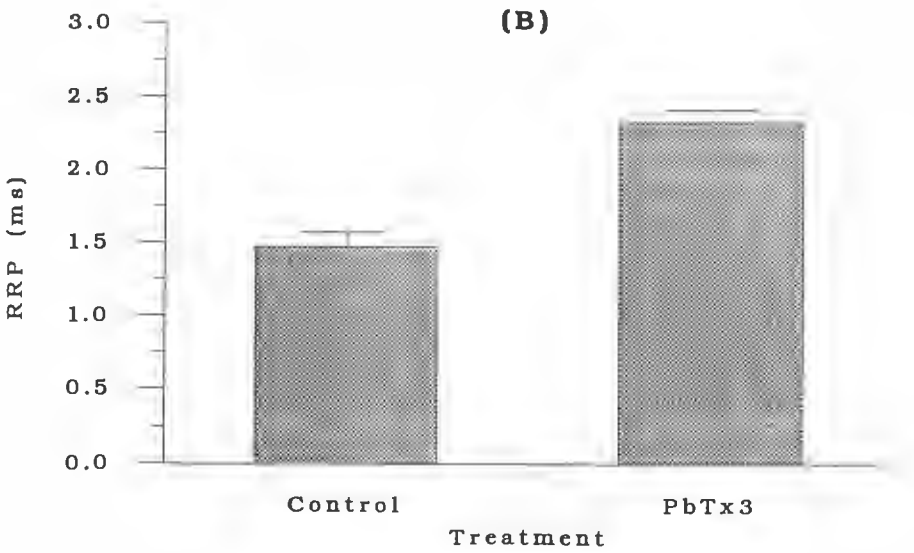
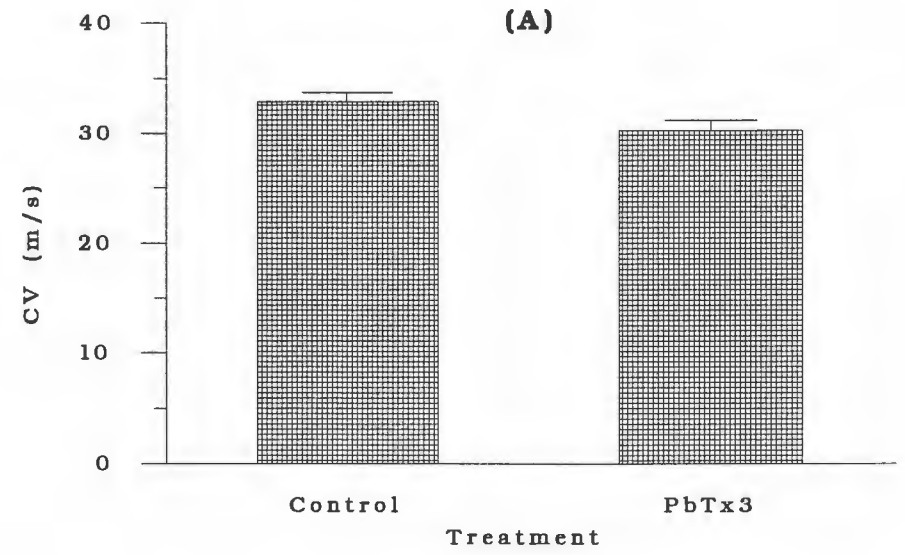
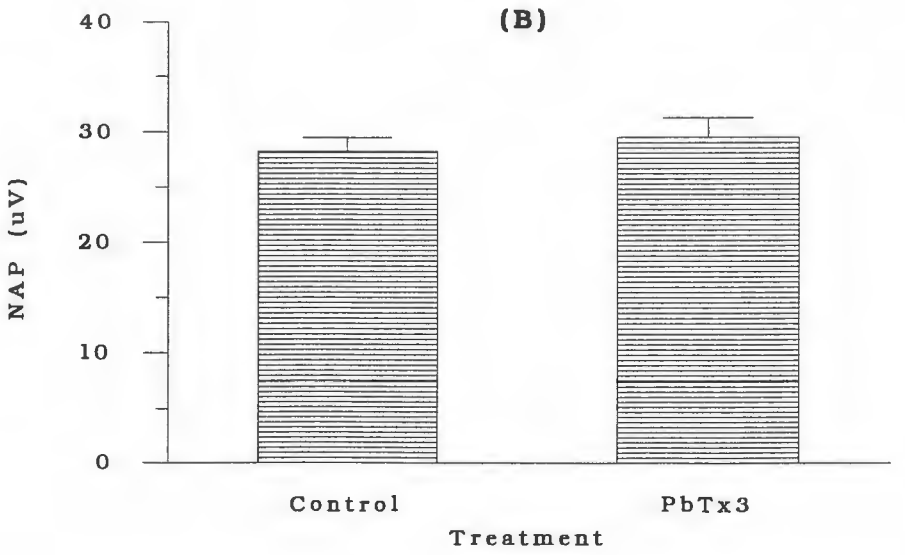


Figure 26: Graphs (A) and (B) present the means of the compound nerve conduction velocity (CV) and action potential (NAP) respectively. Rats treated with i.v. brevetoxin (PbTx3, 15 *u*g/kg) are compared to a control group (treated with i.v. 1% Tween in PBS). *In vivo* recordings were performed on the ventral coccygeal nerve. Error bars indicate the standard error of the mean.





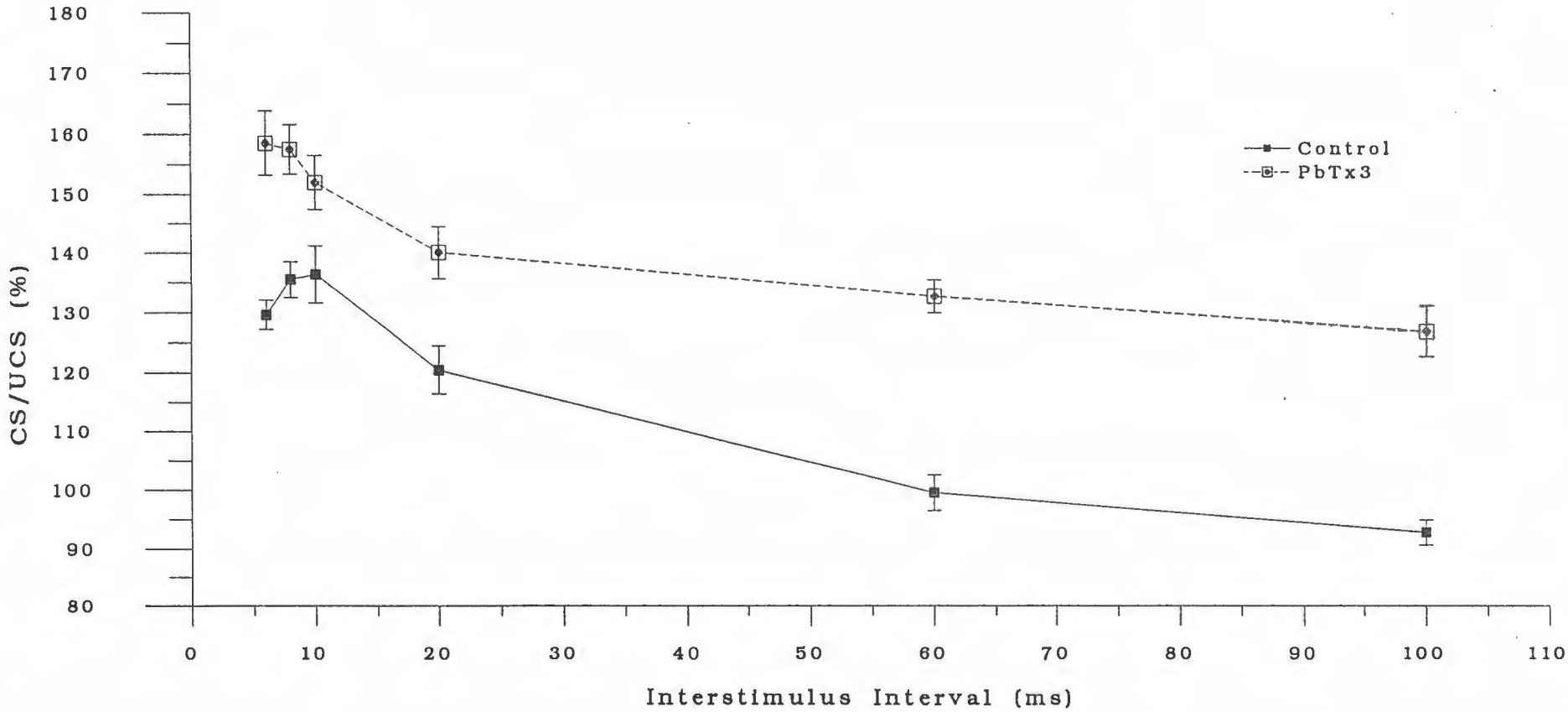


Figure 27: Graph of the percentage change in amplitude of the conditioned stimulus over the unconditioned stimulus (CS/UCS%) versus the interstimulus interval recorded *in vivo* from the ventral coccygeal nerve in rats. Rats treated with i.v. brevetoxin (PbTx3, 15 *u*g/kg) are compared to a control group (treated with i.v. 1% Tween in PBS). Error bars indicate the standard error of the mean.

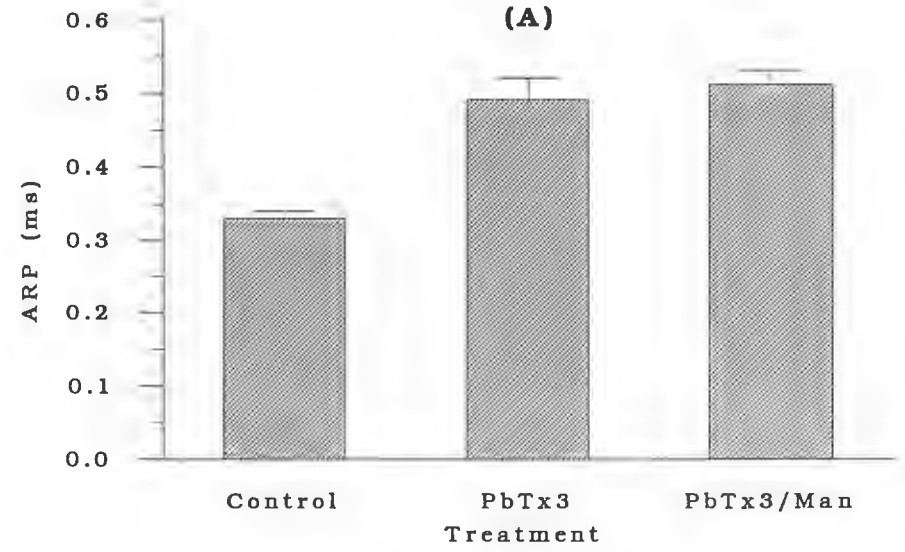
158.6 CS/UCS%) although this was virtually unaltered at 8 ms (mean 157.6 CS/UCS%). This was followed be a very gradual decline in the magnitude of the supernormal response, with supernormality found to be still present (mean 110.8 CS/UCS%) at the interstimulus interval of 1000 ms. In the control group, the onset of supernormality was at the interstimulus interval of 6 ms, and by 60 ms these nerves were no longer supernormal. PbTx therefore increased the duration of the supernormal period.

6.33.3.1 Brevetoxin and Mannitol

In this experiment animals were given intravenous infusions of PbTx (15 µg/kg) followed by mannitol (1 g/kg) as described in Table 14. In these PbTx/mannitol treated animals, both the absolute ($p=0.0018$) and the relative ($p<0.0001$) refractory periods were significantly prolonged (Figures 28A, 28B) while the conduction velocity was significantly slowed ($p=0.0340$) when compared to control animals (Figure 29A). There was no significant difference in the amplitude of the nerve action potential when the treated group was compared to control (Figure 29B). When results obtained from the PbTx/mannitol treated animals were compared with those of animals treated with PbTx alone, there were no significant differences in either the refractory periods, compound nerve conduction velocity or action potential (Figures 28A,B; 29A,B).

In animals treated with PbTx/mannitol the magnitude of supernormality was significantly increased above control at all interstimulus intervals measured (6ms $p<0.0001$; 8ms $p=0.0007$; 10ms $p=0.0042$; 20ms $p=0.0003$; 60ms $p<0.0001$; 100ms $p<0.0001$). When compared to animals treated with PbTx alone, the PbTx/mannitol group displayed no alteration in the magnitude of the supernormal response at all interstimulus intervals recorded until 100 ms. (Figure 30). In these two treatment groups, due to the prolonged nature of the supernormal period, recordings were also performed at the interstimulus intervals of 250, 500 and 1000 ms. In the PbTx/mannitol treatment group, a significant decrease in the magnitude of supernormality ($p=0.0335$) existed at the interstimulus interval of 1000 ms when compared to the group of animals that were given PbTx alone.

Figure 28: Graphs (A) and (B) present the means of the absolute (ARP) and relative (RRP) refractory periods respectively. One treatment group of rats received i.v. brevetoxin (15 *u*g/kg) (PbTx3). A second group received i.v. brevetoxin (15 *u*g/kg) followed by i.v. mannitol (1 g/kg) (PbTx3/Man). Comparisons are made to a control group (treated with i.v. 1% Tween in PBS). *In vivo* recordings were performed on the ventral coccygeal nerve. Error bars indicate the standard error of the mean.



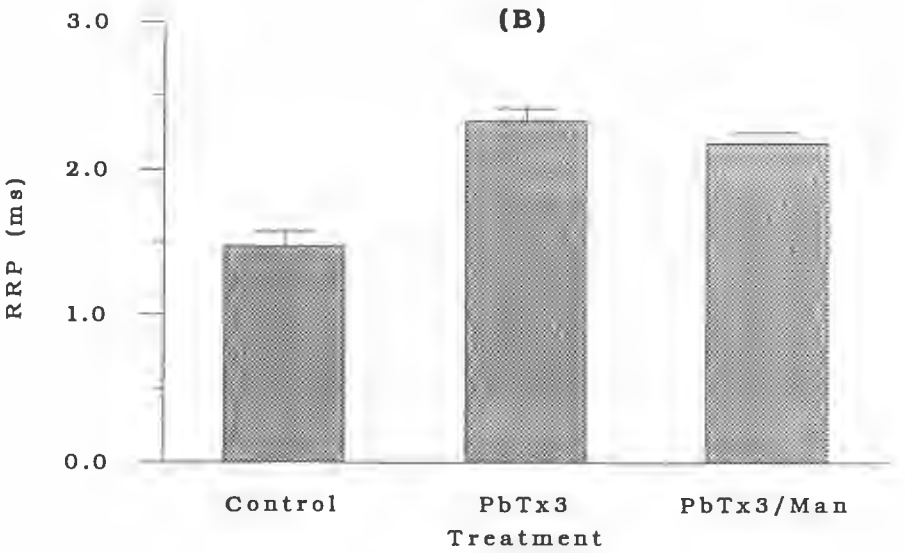
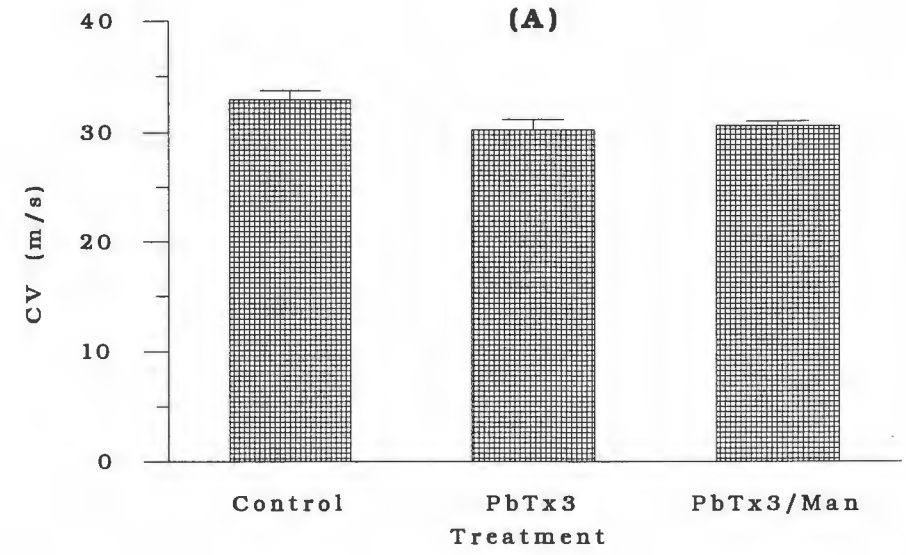
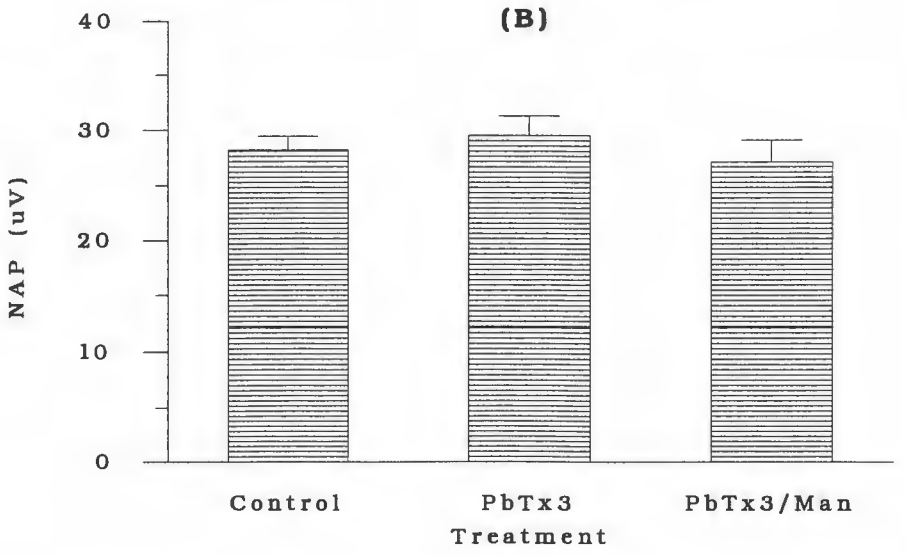


Figure 29: Graphs (A) and (B) present the means of the compound nerve conduction velocity (CV) and action potential (NAP) respectively. One treatment group of rats received i.v. brevetoxin (15 *u*g/kg) (PbTx3). A second group received i.v. brevetoxin (15 *u*g/kg) followed by i.v. mannitol (1 g/kg) (PbTx3/Man). Comparisons are made to a control group (treated with i.v. 1% Tween in PBS). *In vivo* recordings were performed on the ventral coccygeal nerve. Error bars indicate the standard error of the mean.





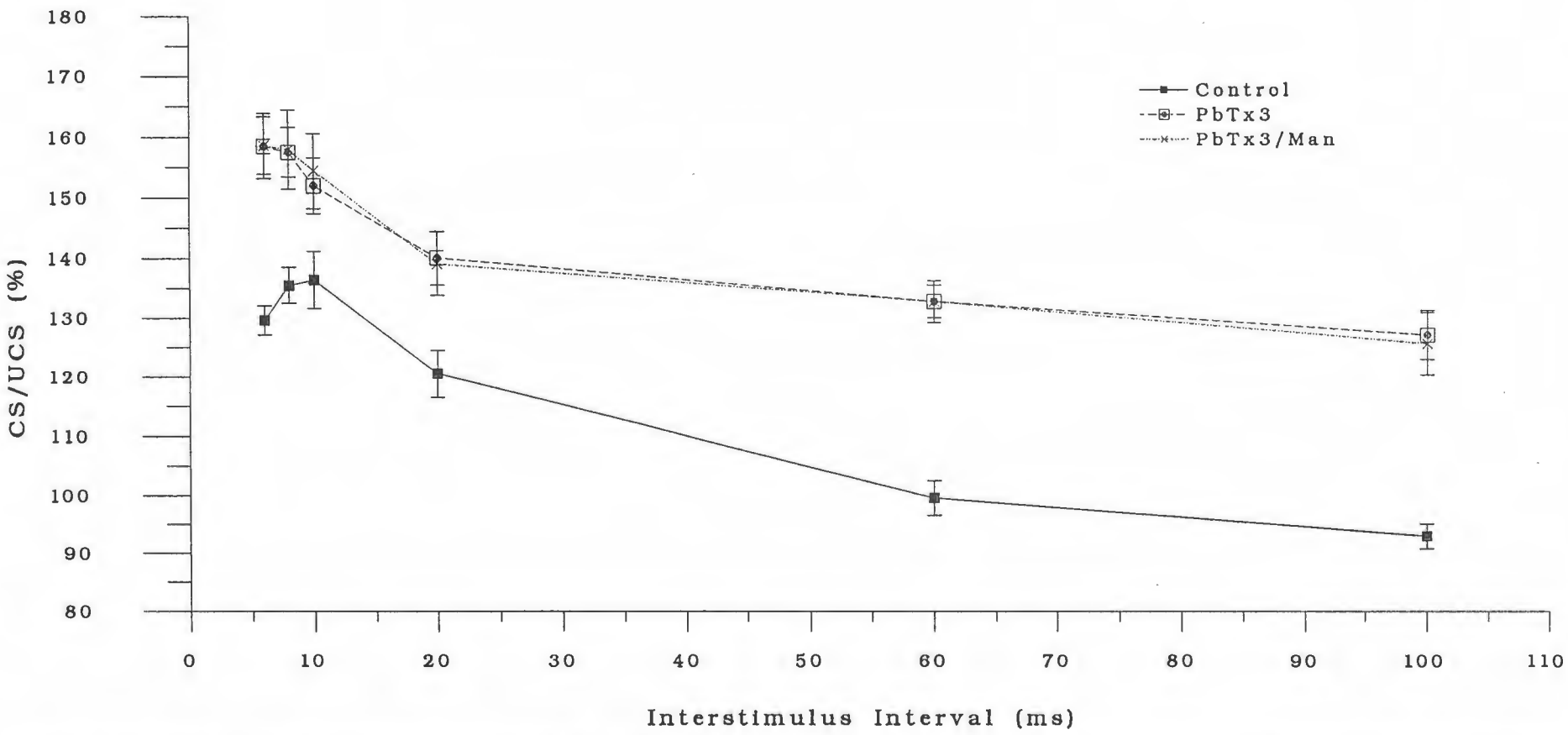


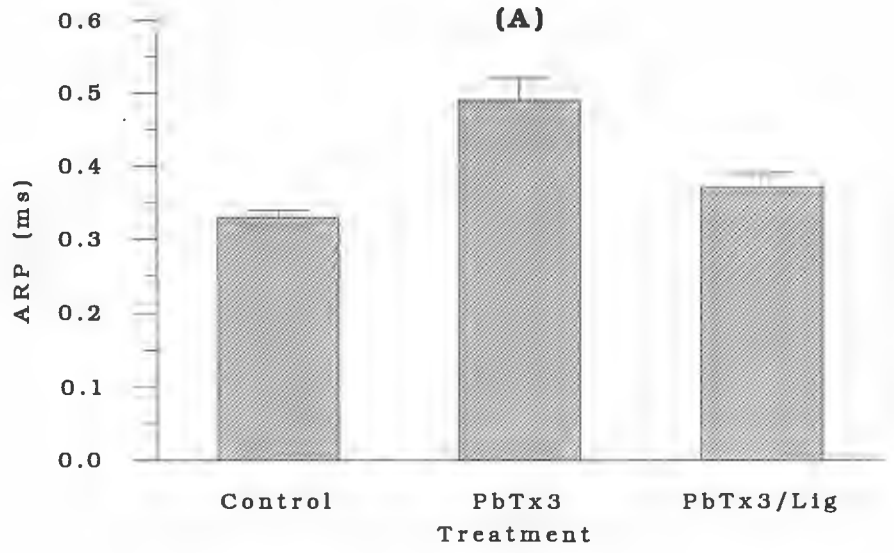
Figure 30: Graph of the percentage change in amplitude of the conditioned stimulus over the unconditioned stimulus (CS/UCS%) versus the interstimulus interval recorded in vivo from the ventral coccygeal nerve in rats. One treatment group received i.v. brevetoxin (PbTx3, 15 *u*g/kg). A second group received i.v. brevetoxin (15 *u*g/kg) followed by i.v. mannitol (1 g/kg) (PbTx3/Man). Comparisons are made to a control group (treated with 1% Tween in PBS). Error bars indicate the standard error of the mean.

6.33.3.2 Brevetoxin and Lignocaine

In this experiment animals were intravenously administered PbTx (15 µg/kg) followed by lignocaine (500 µg/kg). Both the absolute ($p=0.0438$) and relative ($p<0.0001$) refractory periods in the PbTx/lignocaine group were significantly less than those of animals given PbTx alone (Figure 31A, 31B). The conduction velocity in the PbTx/lignocaine group was significantly faster ($p=0.0001$) when compared to PbTx-treated animals (Figure 32A). When this PbTx/lignocaine group were compared to control animals no significant differences were detectable in either the refractory periods, compound nerve conduction velocity or action potential (Figures 31A,B; 32A,B).

The magnitude of supernormality in animals treated with PbTx/lignocaine was significantly less than in animals treated with PbTx alone. This decrease in supernormality in the PbTx/lignocaine group occurred at all interstimulus intervals examined (p range = 0.0070-<0.0001). When compared to control animals, neither the magnitude nor duration of the supernormal response was significantly different to that of the PbTx/lignocaine group (Figure 33).

Figure 31: Graphs (A) and (B) present the means of the absolute (ARP) and relative (RRP) refractory periods respectively. One treatment group of rats received i.v. brevetoxin (15 *u*g/kg) (PbTx3). A second group received i.v. brevetoxin (15 *u*g/kg) followed by i.v. lignocaine (500 *u*g/kg) (PbTx3/Lig). Comparisons are made to a control group (treated with i.v. 1% Tween in PBS). *In vivo* recordings were performed on the ventral coccygeal nerve. Error bars indicate the standard error of the mean.



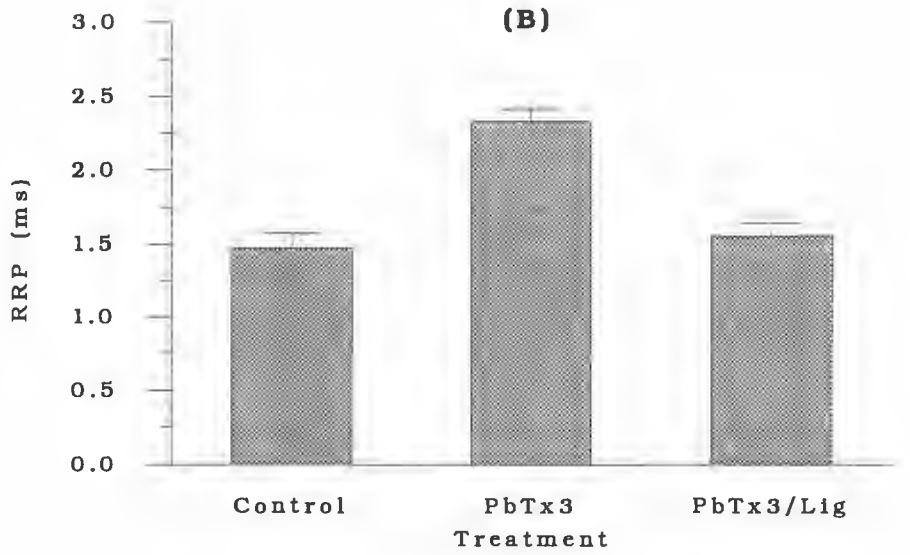
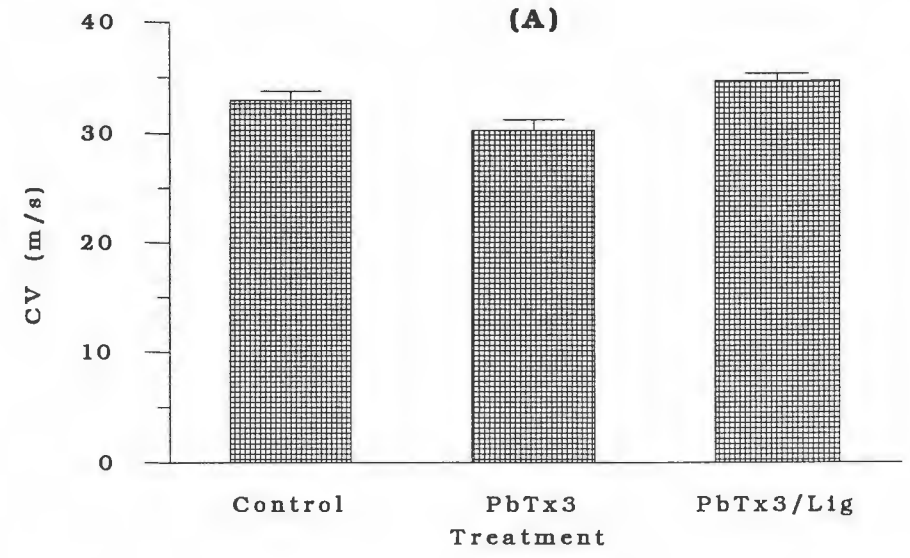
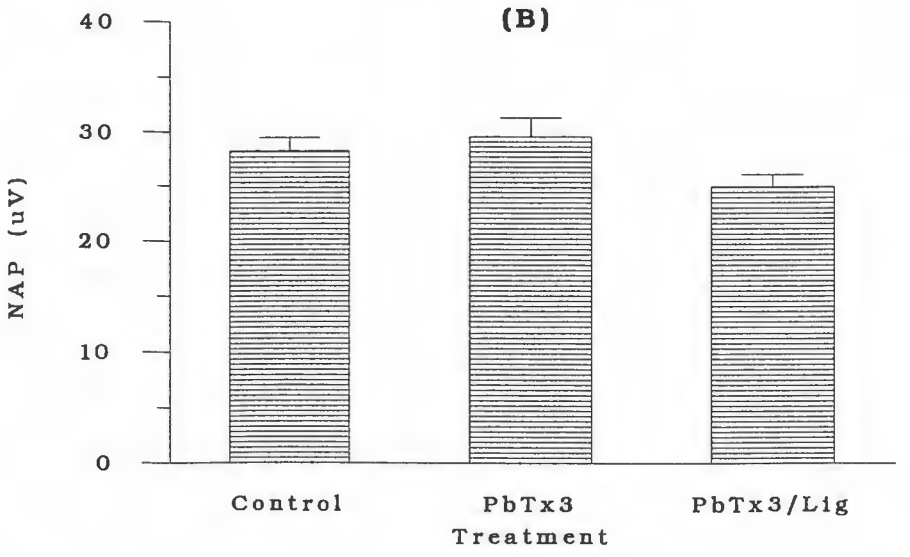


Figure 32: Graphs (A) and (B) present the means of the compound nerve conduction velocity (CV) and action potential (NAP) respectively. One treatment group of rats received i.v. brevetoxin (15 *u*g/kg) (PbTx3). A second group received i.v. brevetoxin (15 *u*g/kg) followed by i.v. lignocaine (500 *u*g/kg) (PbTx3/Lig). Comparisons are made to a control group (treated with i.v. 1% Tween in PBS). *In vivo* recordings were performed on the ventral coccygeal nerve. Error bars indicate the standard error of the mean.





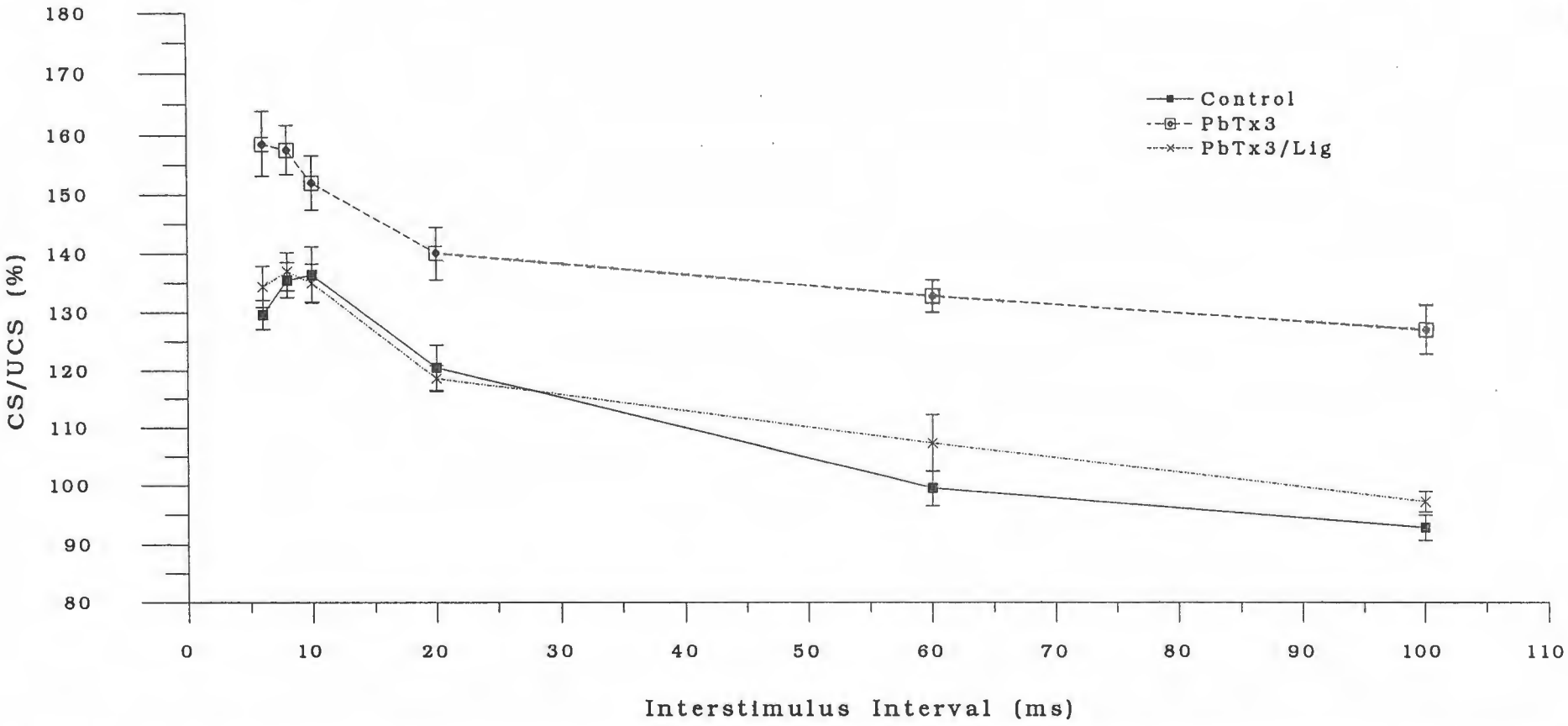


Figure 33: Graph of the percentage change in amplitude of the conditioned stimulus over the unconditioned stimulus (CS/UCS%) versus the interstimulus interval recorded in vivo from the ventral coccygeal nerve in rats. One treatment group received i.v. brevetoxin (15 *u*g/kg) (PbTx3). A second group received i.v. brevetoxin (15 *u*g/kg) followed by i.v. lignocaine (500 *u*g/kg) (PbTx3/Lig). Comparisons are made to a control group (treated with 1% Tween in PBS). Error bars indicate the standard error of the mean.

6.33.3.3 Brevetoxin Pre and Post Mannitol

In this treatment group animals were given intraperitoneal PbTx. Nerve conduction studies were then performed before and after the administration of intravenous mannitol

6.33.3.4 Pre-Mannitol Recording

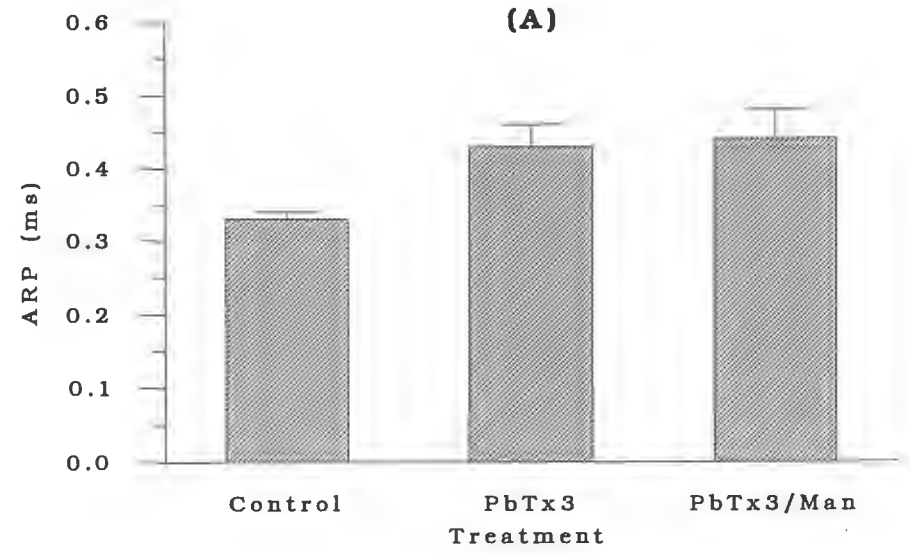
Although both the absolute and the relative refractory periods were prolonged following the administration of intraperitoneal PbTx the differences were not statistically significant when compared to control animals (Figures 34A, 34B). No difference could be detected in the conduction velocity of the PbTx group when compared to controls (Figure 35A).

The amplitude of the compound nerve action potential in PbTx-treated animals was not significantly different to control values (Figure 35B). Following the administration of intraperitoneal PbTx the magnitude of supernormality was increased above the control at all interstimulus intervals (Figure 36). The increases were only significant at the interstimulus intervals of 60 and 100 ms ($p < 0.0001$). The peak in magnitude of supernormality was at 8 ms (mean 146.1CS/UCS%) in animals treated with intraperitoneal PbTx. The nerves in this PbTx-treated group were still supernormal at 1000 ms (mean 106.2 CS/UCS%) confirming that intraperitoneal PbTx caused an increase in the duration of supernormality.

6.33.3.5 Post-Mannitol Recording

A second series of nerve conduction studies was performed on this group (intraperitoneal PbTx) following the administration of mannitol. These post-mannitol recordings indicated there were no significant changes in any of the conduction parameters (refractory periods, conduction velocity, magnitude and duration of supernormality) when compared to pre-mannitol recordings (Figures 34A,B; 35A,B; 36).

Figure 34: Graphs (A) and (B) present the means of the absolute (ARP) and relative (RRP) refractory periods respectively. *In vivo* recordings were performed on the ventral coccygeal nerve in rats following the administration of i.p. brevetoxin (75 *u*g/kg) both before (PbTx3), and after (PbTx3/Man) i.v. mannitol (1 g/kg). Comparisons are made to a control group (treated with i.v. 1% Tween in PBS). Error bars indicate the standard error of the mean.



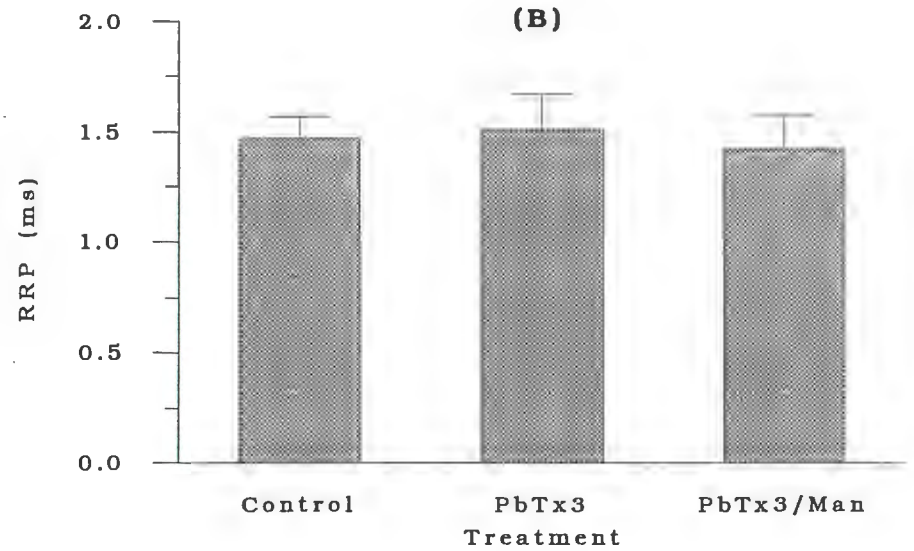
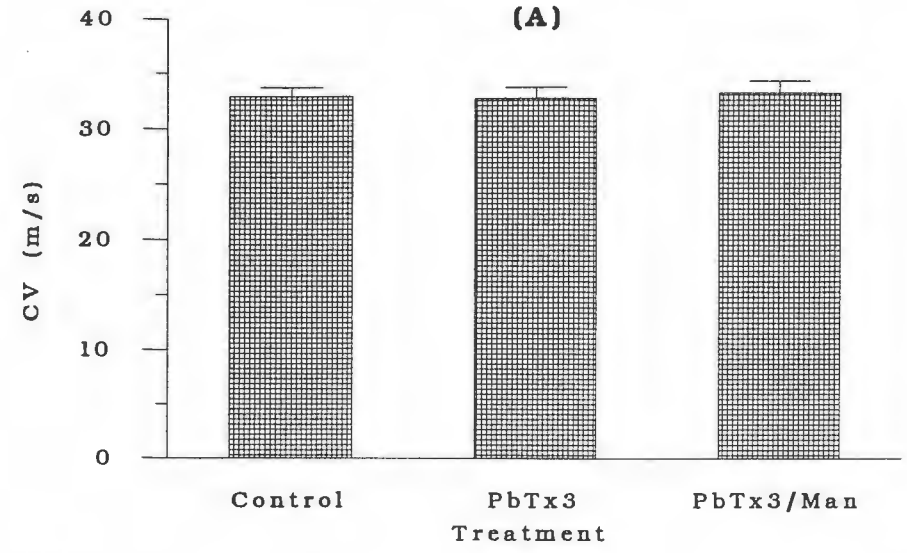
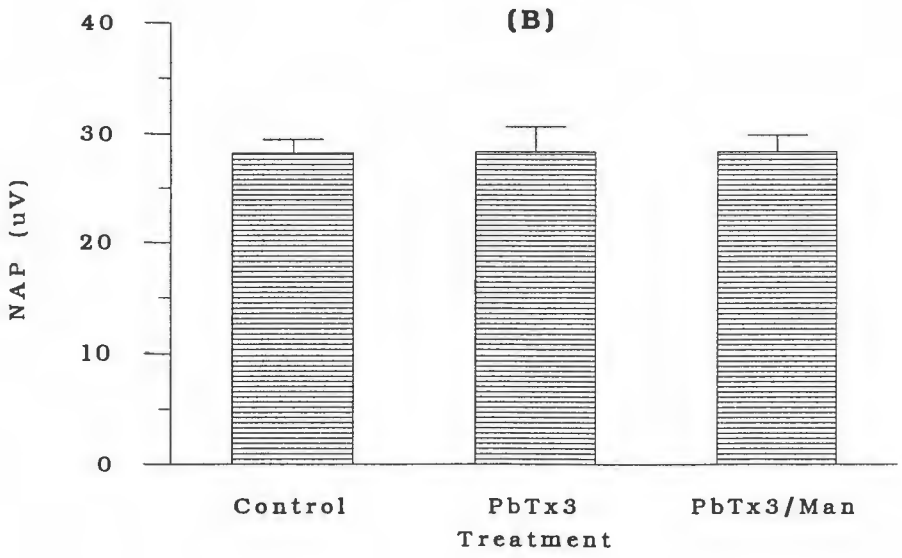


Figure 35: Graphs (A) and (B) present the means of the compound nerve conduction velocity (CV) and action potential (NAP) respectively. *In vivo* recordings were performed on the ventral coccygeal nerve in rats following the administration of i.p. brevetoxin (75 *u*g/kg) both before (PbTx3), and after (PbTx3/Man) i.v. mannitol (1 g/kg). Comparisons are made to a control group (treated with i.v. 1% Tween in PBS). Error bars indicate the standard error of the mean.





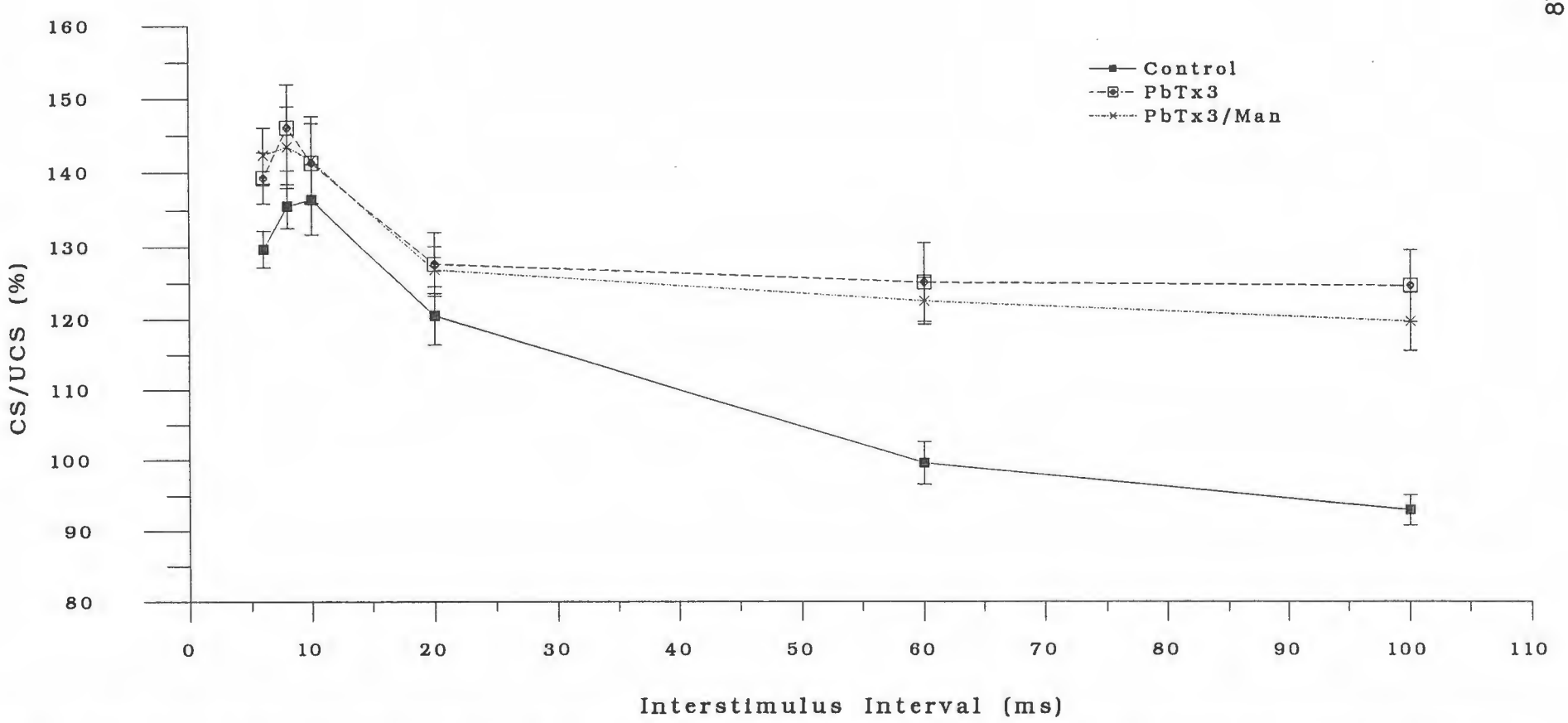


Figure 36: Graph of the percentage change in amplitude of the conditioned stimulus over the unconditioned stimulus (CS/UCS%) versus the interstimulus interval recorded *in vivo* from the ventral coccygeal nerve in rats. Following the administration of i.p. brevetoxin (75 *u*g/kg) recordings were conducted both before (PbTx3) and after (PbTx3/Man) i.v. mannitol (1g/kg). Comparisons are made to a control group (treated with i.v. 1% Tween in PBS). Error bars indicate the standard error of the mean.

6.33.4 Ciguatoxin Pre and Post Mannitol

Animals in this group were given intraperitoneal CTX, followed by intravenous mannitol. Conduction parameters were recorded both before and after mannitol (see Section 6.32.6.2).

6.33.4.1 Pre-Mannitol Recording

Following the administration of CTX but prior to the mannitol, the CTX group displayed a significantly prolonged absolute refractory period ($p < 0.0001$) when compared to the control group (Figure 37A). The relative refractory period in CTX-treated animals was not significantly different to control animals (Figure 37B). When compared to control, the compound nerve conduction velocity of CTX-treated animals was significantly slowed ($p=0.0078$) (Figure 38A). The amplitude of the compound nerve action potential in animals treated with CTX was not significantly different to control animals (Figure 38B).

In supernormal studies CTX significantly increased the magnitude of supernormality at all interstimulus intervals tested (6ms $p < 0.0001$; 8ms $p=0.0095$; 10ms $p=0.0227$; 20ms $p=0.0003$; 60ms $p < 0.0001$; 100ms $p < 0.0001$) when compared to control animals (Figure 39). The magnitude of the supernormal response peaked at the first interstimulus interval measured, 6 ms (mean 160.94CS/UCS%). Supernormality was still present in these CTX-treated animals at the interstimulus interval of 1000 ms (mean 107.1CS/UCS%), hence CTX increased the duration of the supernormal period.

6.33.4.2 Post-Mannitol Recording

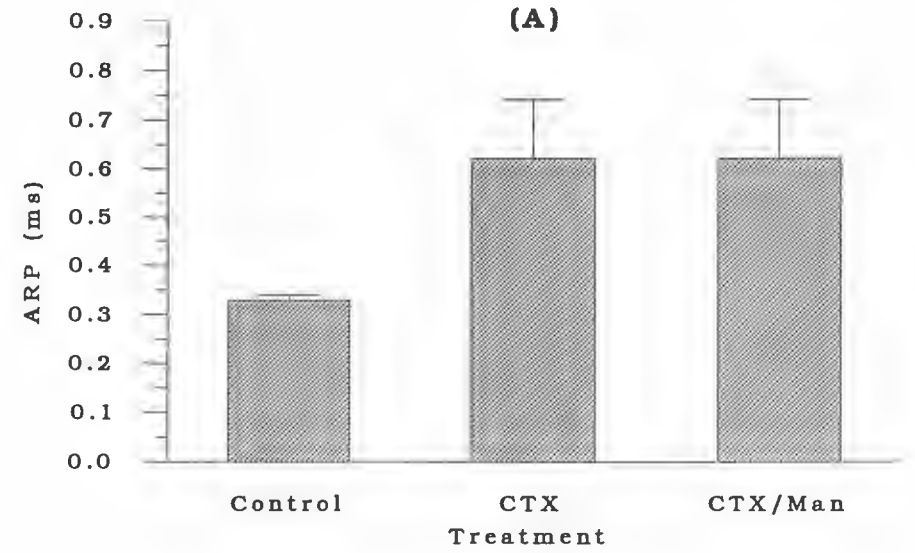
The absolute refractory period recorded after the infusion of mannitol remained prolonged and was not significantly different to the pre- mannitol recordings (Figure 37A). The relative refractory period was unchanged after mannitol when compared to pre-mannitol results (Figure 37B). The conduction velocity remained slowed in post-mannitol studies and was not significantly different to pre-mannitol studies (Figure 38A).

The magnitude of the supernormal response remained elevated in post-mannitol recordings (Figure 39). On comparison of pre- and post- mannitol recordings there were no significant differences in the magnitude of supernormality at any of the interstimulus intervals studied.

6.33.4.3 Comparison between Ciguatoxin and Brevetoxin

Modifications of nerve conduction occurring in animals treated with intravenous PbTx were compared to those occurring in animals administered with intraperitoneal CTX. Neither the absolute refractory period nor the conduction velocity were significantly different when these groups were compared. The relative refractory period was significantly longer ($p < 0.0001$) in the PbTx-treated animals when compared to the CTX-treated group. The magnitude of supernormality in PbTx-treated animals was not significantly different to that of the group treated with CTX at any of the interstimulus intervals measured (Figure 40).

Figure 37: Graphs (A) and (B) present the means of the absolute (ARP) and relative (RRP) refractory periods respectively. *In vivo* recordings were performed on the ventral coccygeal nerve in rats following the administration of i.p. ciguatoxin both before (CTX), and after (CTX/Man) i.v. mannitol (1 g/kg). Comparisons are made to a control group (treated with i.v. 1% Tween in PBS). Error bars indicate the standard error of the mean.



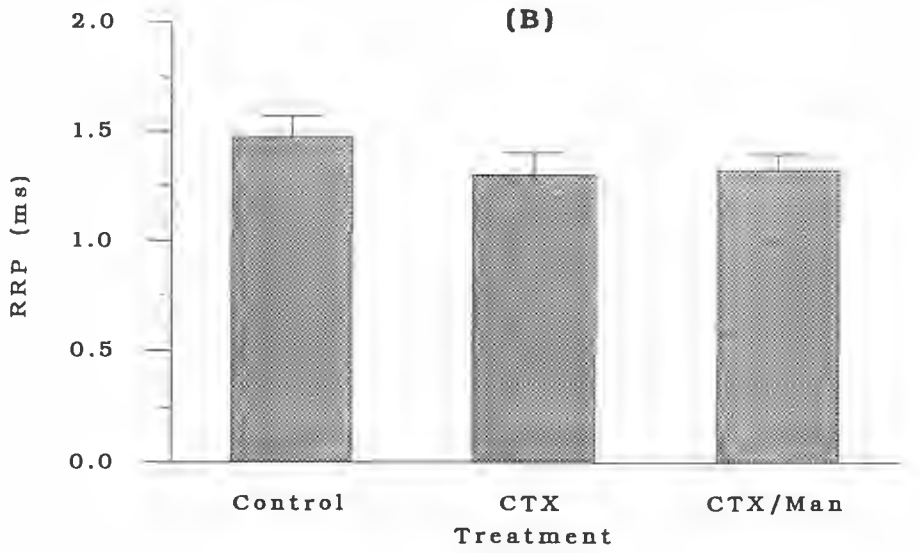
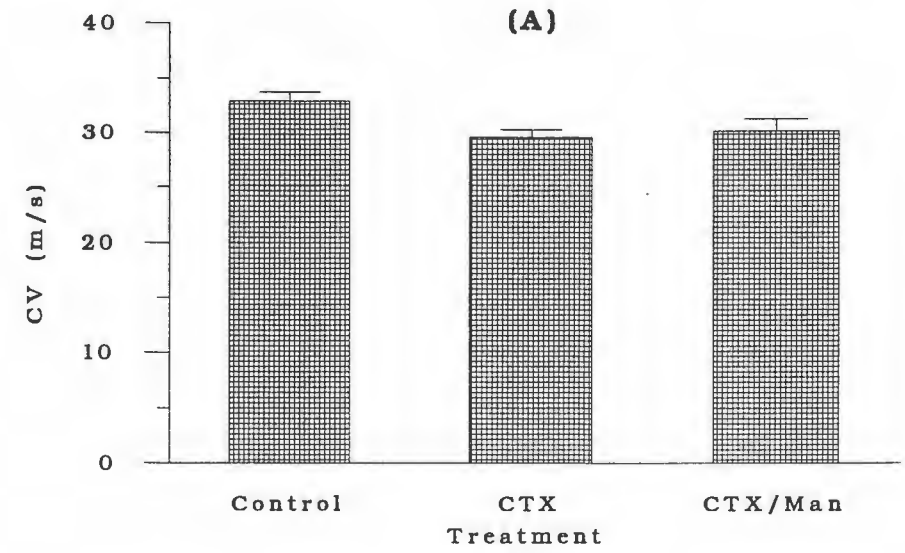
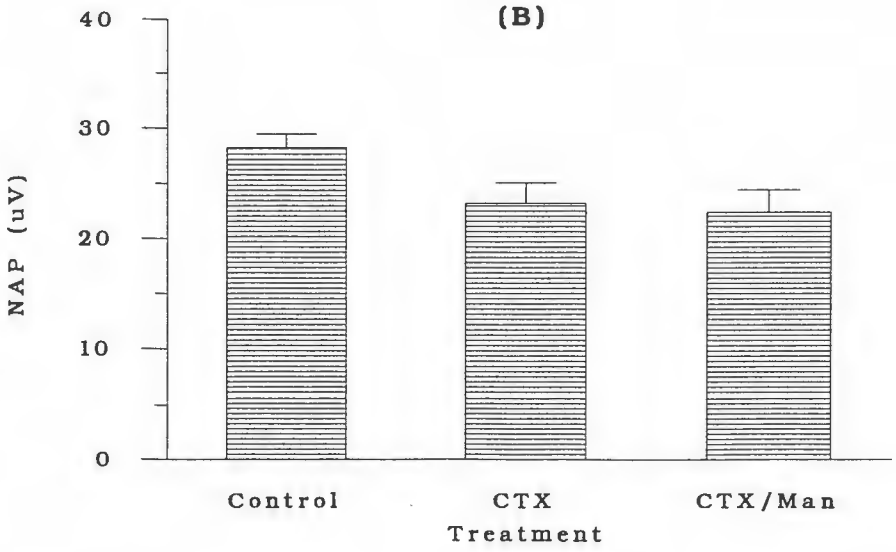


Figure 38: Graphs (A) and (B) present the means of the compound nerve conduction velocity (CV) and action potential (NAP) respectively. *In vivo* recordings were performed on the ventral coccygeal nerve in rats following the administration of i.p. ciguatoxin both before (CTX), and after (CTX/Man) i.v. mannitol (1 g/kg). Comparisons are made to a control group (treated with i.v. 1% Tween in PBS). Error bars indicate the standard error of the mean.





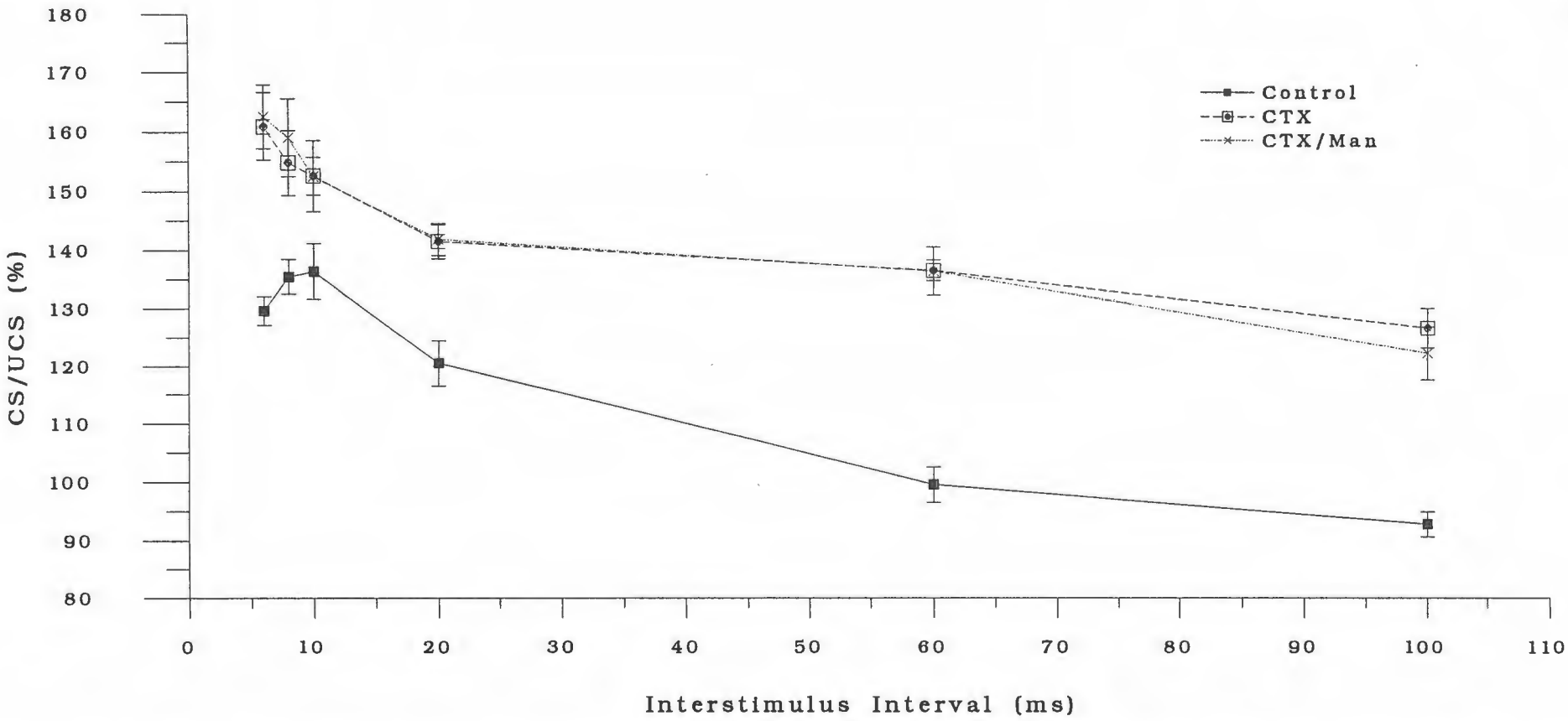


Figure 39: Graph of the percentage change in amplitude of the conditioned stimulus over the unconditioned stimulus (CS/UCS%) versus the interstimulus interval recorded *in vivo* from the ventral coccygeal nerve in rats. Following the administration of i.p. ciguatoxin recordings were conducted both before (CTX) and after (CTX/Man) i.v. mannitol (1 g/kg). Comparisons are made to a control group (treated with i.v. 1% Tween in PBS). Error bars indicate the standard error of the mean.

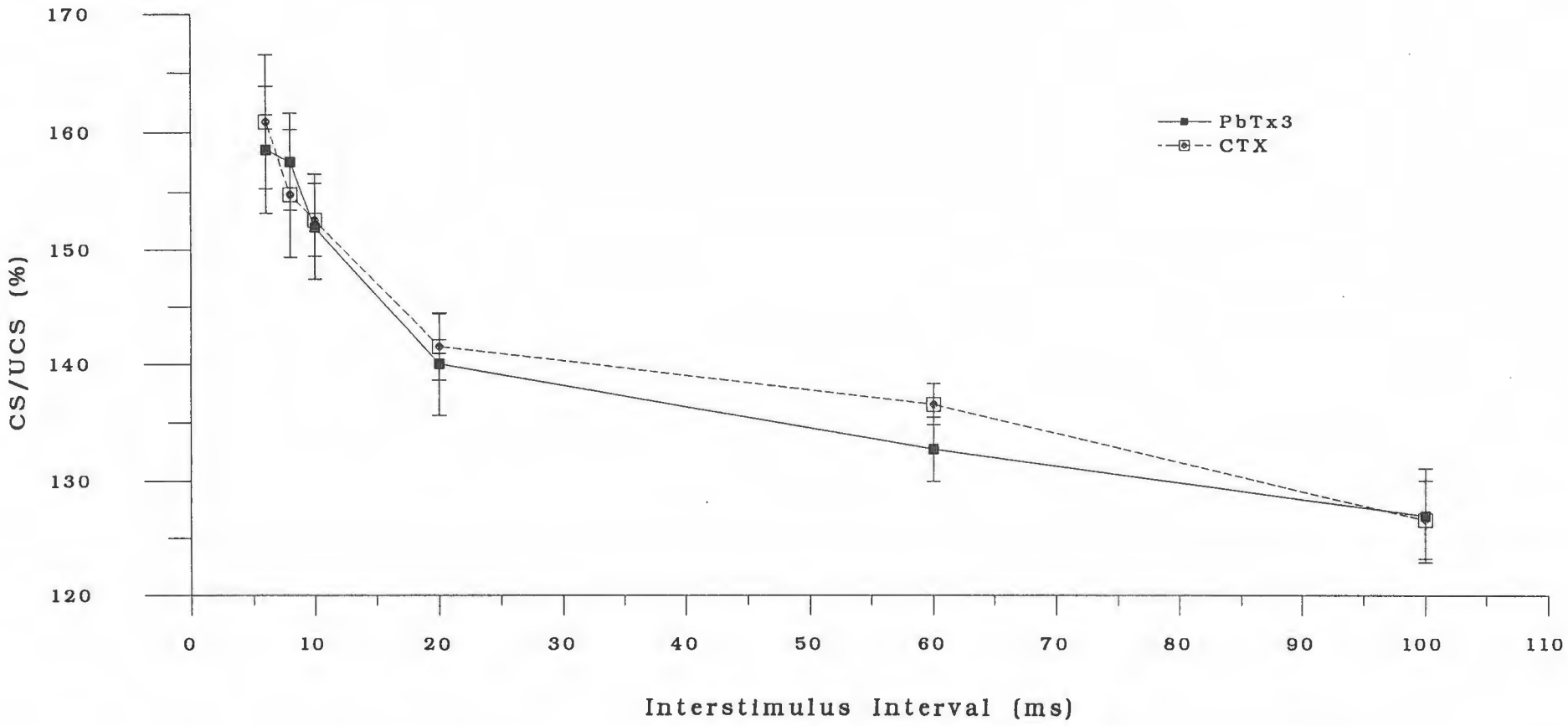


Figure 40: Graph of the percentage change in amplitude of the conditioned stimulus over the unconditioned stimulus (CS/UCS%) versus the interstimulus interval recorded *in vivo* from the ventral coccygeal nerve in rats. Rats treated with i.v. brevetoxin (PbTx3, 15 *u*g/kg) are compared with rats treated with i.p. ciguatoxin (CTX). Error bars indicate the standard error of the mean.

6.34 Physiological Recording from animals exposed to toxins

Control animals displayed normal sinus rhythm throughout the recording, with a heart rate of approximately 380 beats per minute. The average respiratory rate in control animals was 108 per minute (Figure 41). Lignocaine was administered intravenously to one rat at a dose of 1 mg/kg. Due to the development of cardiac arrhythmias the infusion was abandoned. The dose rate used in further rats of 500 µg/kg produced no such arrhythmias. Cardiac and respiratory rates in the lignocaine control group were no different to rates in the saline control group (Figure 42). Normal sinus rhythm was observed on the ECG throughout the experiments. Normal sinus rhythm was also observed in animals administered with intravenous mannitol. The cardiac and respiratory rates were no different to rates observed in the saline control group.

6.34.1 Brevetoxin

Prior to determination of the precise dose of PbTx, one rat was initially given 20 µg/kg by intravenous infusion. When approximately two-thirds of the toxin had been received, respirations became extremely irregular then developed further into Cheyne-Stokes breathing. No changes were noted on the electrocardiograph at this time. Five minutes later at the completion of the toxin infusion respirations ceased and arrhythmias commenced, simultaneously. The cardiac arrhythmia appeared to be ventricular tachycardia, or perhaps idioventricular rhythm. Intravenous lignocaine was administered with the result of reversion to sinus rhythm. Breathing recommenced temporarily but 3:1 heart block ensued followed by apnoea and asystole. Unfortunately due to malfunction in the computer software the data were not stored.

All animals administered with intravenous PbTx (15 µg/kg) underwent a period of bradypnoea and apnoea towards the end of the 15 minute infusion time. This was accompanied by an ensuing onset of cardiac arrhythmias. Most notably these included sinus bradycardias of varying rate, heart block as well as ventricular premature contractions (Figures 43, 44, 45, 46, 47)

6.34.2 General Observations in Mannitol Experiments

In many animals receiving intravenous mannitol, seizures were noted. These ranged from bursts of localised twitching in one or several extremeties through to generalised seizures. The onset varied but was as early as 5 minutes after commencement of the mannitol infusion. In an

attempt to stabilise this situation it was decided to slow the rate of the mannitol infusion. This strategy appeared to decrease the severity of the seizures however most rats could still be described as 'very twitchy'. The other symptom of note in mannitol experiments was a pronounced scrotal twitch.

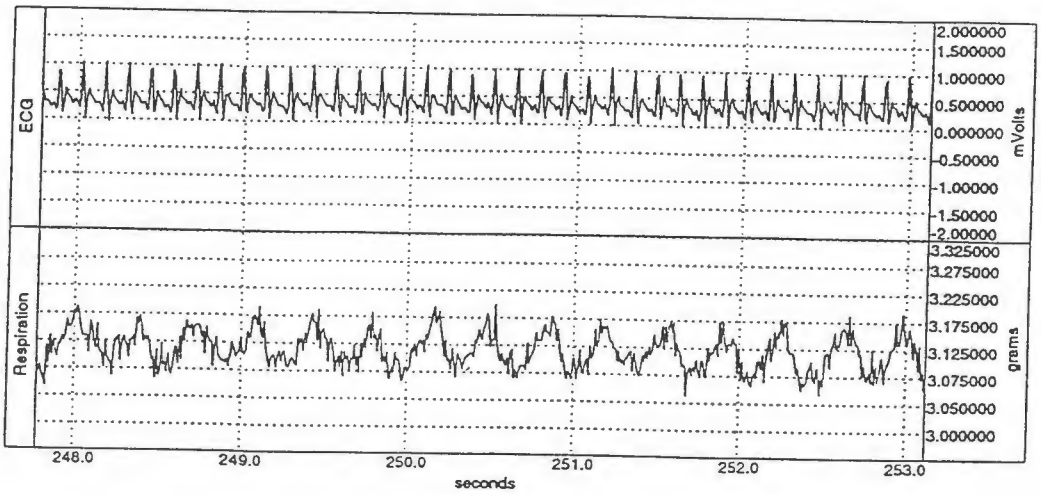


Figure 41: Electrocardiographic (ECG) and respiration trace recorded in an anaesthetised control rat. The recordings were collected during the administration of i.v. PBS and 1% Tween.

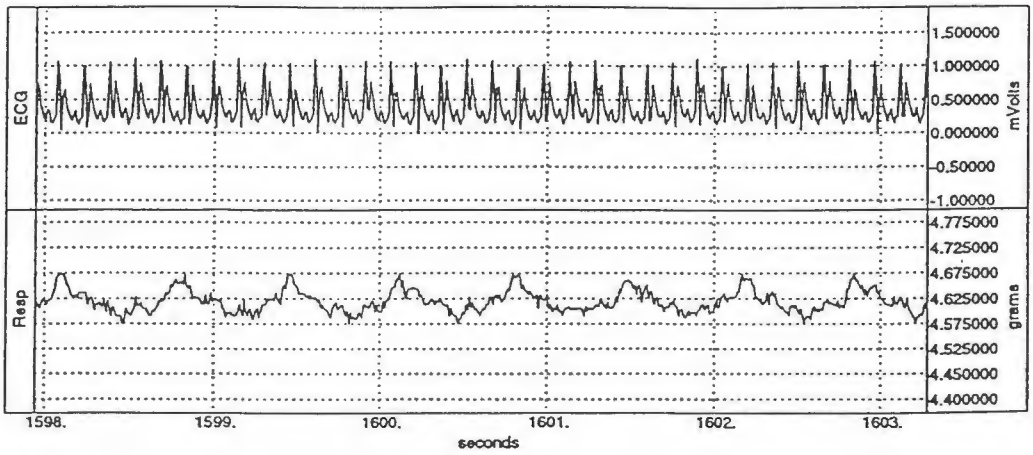


Figure 42: Electrocardiographic (ECG) and respiration trace recorded in an anaesthetised rat (lignocaine control) during the administration of intravenous administration of i.v. lignocaine (500 μg/kg).

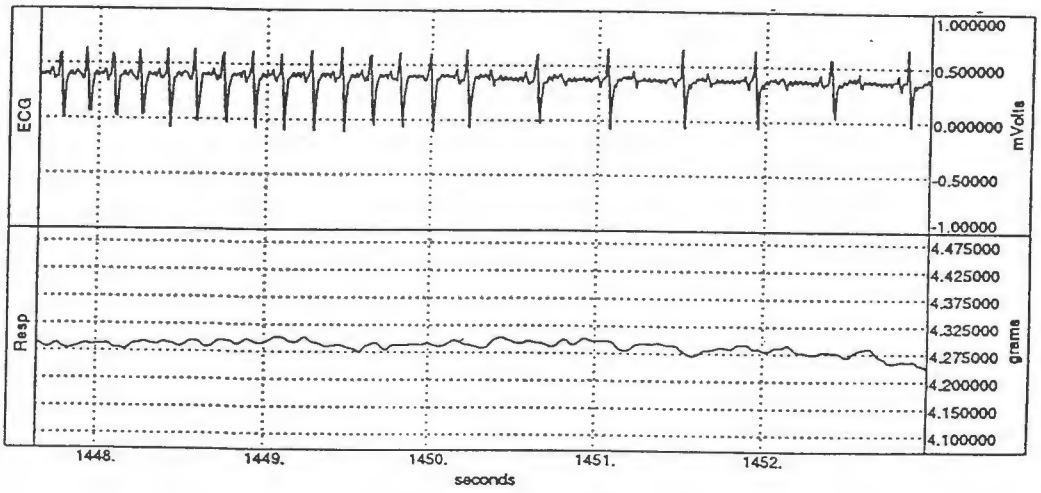


Figure 43: Electrocardiograpic (ECG) and respiration trace recorded during the administration of i.v. brevetoxin (15μg/kg). The recording shows the development of AV block corresponding to a period of apnoea. The recording was collected during the final minute of toxin administration.

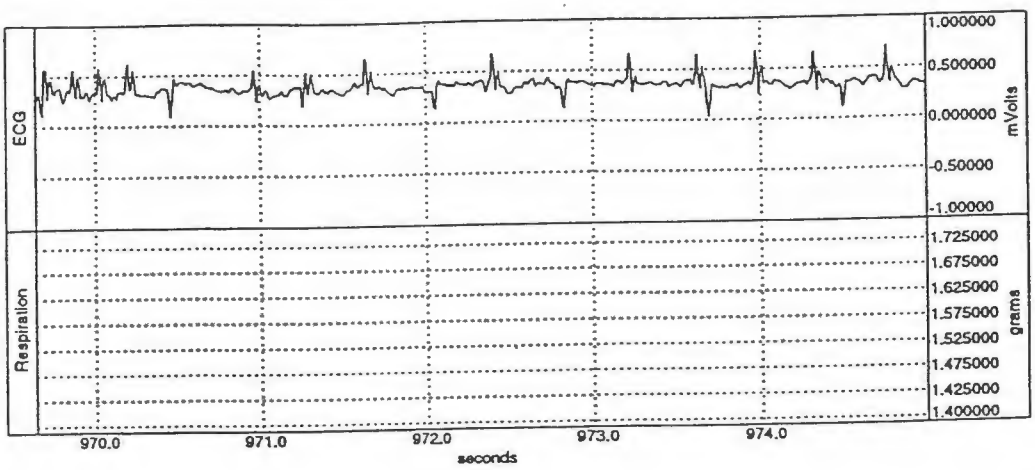


Figure 44: Electrocardiographic (ECG) trace recorded during the administration of i.v. brevetoxin (15 μg/kg). The cessation of respiration was accompanied by ventricular premature contractions.

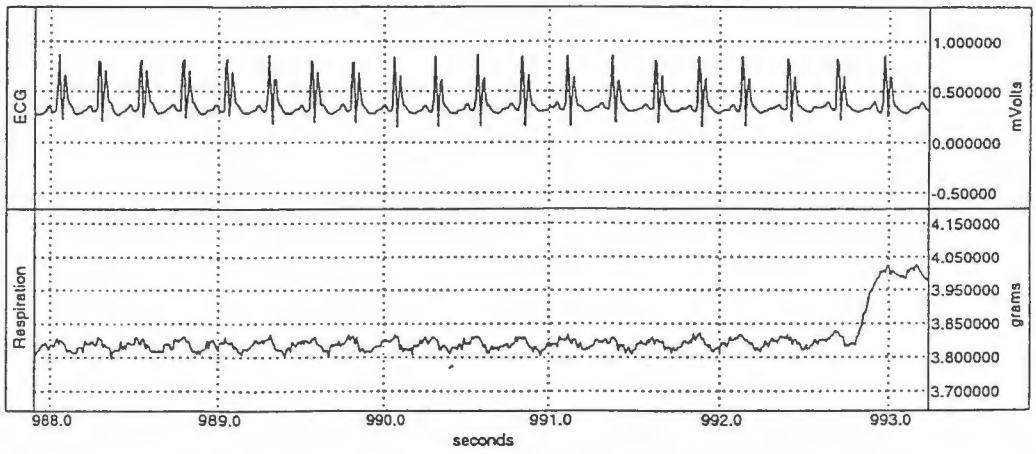


Figure 45: Electrocardiographic (ECG) and respiration trace recorded during the administration of i.v. brevetoxin (15 μg/kg). The recording shows sinus bradycardia corresponding with a period of apnoea.

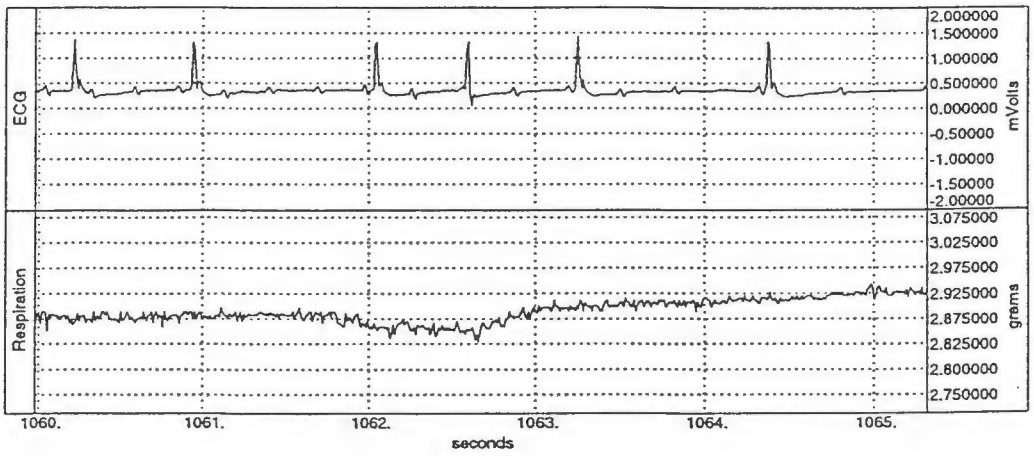


Figure 46: Electrocardiographic (ECG) and respiration trace recorded during the administration of i.v. brevetoxin (15 μg/kg). The cessation of respiration was accompanied by a period of pronounced AV block.

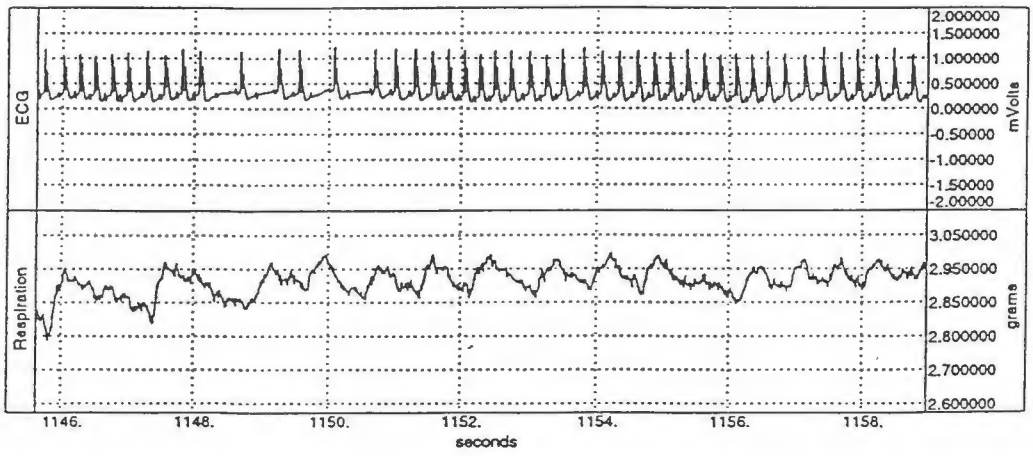


Figure 47: Electrocardiographic (ECG) and respiration trace recorded after the administration of i.v. brevetoxin (15 μg/kg). This recording was collected immediately following the completion of toxin administration demonstrates the return of sinus rhythm and respiratory movement.

6.35 Methods (Series 2)

This series of experiments was performed on male Wistar rates (150-200g). Twenty one rates were treated with an intraperitoneal injection of an aqueous CTX, saline, and 1% Tween-80. All CTX-treated rats manifested signs of acute ciguatera poisoning within 30 minutes of dosing. At 90 minutes after injection the CTX-treated rats were subjected to electrical studies.

A further 12 control rats were given an intraperitoneal injection (1mg/kg) of lignocaine solution and subjected to electrical studies after 30 minutes. These animals manifested no ill effects over this period of time.

Lignocaine was given as an intraperitoneal injection since this route provided a quick, simple and relatively atraumatic method of drug delivery and negated the need to anesthetize the animals for a second time to administer the drug as a slow intravenous infusion. An additional 10 CTX-treated rats (all manifesting signs of acute ciguatera poisoning) were given an intraperitoneal injection (1 mg/kg) of lignocaine, 1 hour after dosing with CTX. This group was then subjected to electrical studies 30 minutes after dosing with lignocaine.

Each animal was anesthetized with an intramuscular injection (420uL/kg) of Innovar Vet (S.K.F.) prior to electrical studies, and further smaller doses were given if the rat became restless during the electrical studies.

All electrical studies were performed on the ventral coccygeal nerve as described above.

6.36 Results (Series 2)

CTX induced significant changes in peripheral nerve conduction and excitability in the rat. A significant slowing in both mixed nerve and motor velocities together with a significant reduction in the nerve action potentials and motor action potentials was found (Table 17). The absolute refractory period in the CTX-treated group was also significantly prolonged, however, the relative refractory period was not significantly altered (Table 17).

The duration of the supernormal period was significantly prolonged to that of the control animals and extended up to 100ms (Table 17). These findings have been previously reported in the rat

and are almost identical to the electrophysiological abnormalities found in similar studies conducted during the acute phase of ciguatera poisoning in humans.

Lignocaine produced no significant effect on the rat nerve when the lignocaine-treated group was compared to the control animals apart from an isolated but significant reduction in the amplitude of the supernormal response recorded at the 10 ms interstimulus interval (Table 17). This latter effect was possibly a result of a partial blocking action by lignocaine on sodium channels at this particular time of high Na^+ activity.

When lignocaine was administered to CTX-treated animals, the electrophysiological disturbances recorded previously in the CTX treated group were no longer detectable except for a slight but significant prolongation of the absolute refractory period (Table 17).

Lignocaine therefore appeared to block the electrophysiological abnormalities induced by CTX on mammalian nerve in an *in vivo* preparation.

	CON	CTX	LID	LID/CTX
Conduction velocity of the mixed nerve(m/s)	35.7+-2.05	30.8+-0.80*	36.3+-0.95	36.3+-0.78
	[12]	[11]	[12]	[10]
Amplitude of the mixed nerve action potential(uV)	87.4+-9.54	51.8+-4.18↘	103.7+-17.68	62.7+-5.379
	[22]	[21]	[12]	[10]
Conduction velocity of the motor nerve(m/s)	31.3+-1.43	24.8+-0.61↙	31.0+-1.21	32.0+-1.10
	[12]	[11]	[12]	[10]
Amplitude of the more nerve action potential(mV)	5.8+-0.69	1.7+-0.20↙	5.7+-0.69	4.8+-0.59
	[12]	[11]	[12]	[10]
Absolute refractory period(ms)	0.5+-0.03	0.8+-0.06↙	0.6+-0.05	0.7+-0.09*
	[22]	[21]	[12]	[10]
Relative refractory period(ms)	1.3+-0.07	1.2+-0.07	1.3+-0.08	1.4+-0.16
	[22]	[21]	[12]	[10]
Supernormal response (CS/UCS,%) at a stimulus interval of 8 ms	127.4+-8.35 [12]	141.7+-8.97 [11]	110.9+-6.19 [12]	124.9+-9.97 [10]
Supernormal response (CS/UCS,%) at a stimulus interval of 10 ms	132.8+-5.54 [12]	147.0+-10.04 [11]	106.1+-5.58↘ [12]	117.6+-7.61 [10]
Supernormal response(CS/UCS,%) at a stimulus interval of 60 ms	96.0+-3.36 [12]	134.0+-7.65↙ [11]	89.6+-3.17 [12]	107.0+-5.80 [10]
Supernormal response(CS/UCS,%) at a stimulus interval of 100 ms	92.9+-4.54 [12]	126.7+-9.96↘ [11]	97.6+-3.66 [11]	102.9+-4.31 [10]

*The supernormal responses are expressed as the conditioned stimulus over the unconditioned stimulus as a percentage (CU/UCS [%]). Student's t-test was used to assess the significance of the differences between groups. Each value quoted is the mean value +- the standard error of the mean, *(0.05 >P>0.01), †(0.01>P>0.001), and ‡(P<0.001) versus control.*

Table 17: The effect of ciguatoxin, lignocaine, and ciguatoxin and lignocaine on the electrophysiological properties of the rat.

6.4 Discussion

The physiological/pharmacological activity of CTX has been studied by many workers using a variety of preparations and toxins of varying degrees of purity. This potent neurotoxin has been determined to be a sodium channel agonist. It exerts its effects on excitable membranes by increasing the permeability of sodium channels thereby producing an overall excitatory effect in excitable membranes. This has particular relevance for the human victims of ciguatera since the occurrence of the action potential in mammals appears to be caused by sodium channels alone, with potassium channels playing a very minor role (Chiu et al., 1979; Brismar 1980; Chiu and Ritchie 1980).

In the current study, the rat was selected as an *in vivo* animal model as it is small enough so as not to require the use of large amounts of toxin while at the same time allowing for satisfactory

in vivo nerve recordings. The rat has also been shown to be sensitive to CTX. Previous studies have demonstrated similar electrophysiological disturbances in rats following administration of CTX as those seen in the human subsequent to ciguatera poisoning (Capra and Cameron, 1985; Cameron et al., 1991a, 1991b).

In rats administered with intraperitoneal CTX, signs noted were similar to those reported by Cameron et al., (1991a). Some differences were noted between these and the signs induced by intraperitoneal PbTx. Chiefly, PbTx elicited a marked effect on the pattern of respiration in all treated rats. Alteration of respiratory pattern was also noted on respiratory traces from rats given intravenous PbTx. This effect on respiration has been reported by other workers. The CTX-induced signs of ataxia and hind leg weakness were not noticed in PbTx-treated rats. As PbTx is far less potent than CTX, this may reflect a dose-dependent difference.

6.4.1 Electrophysiology

6.4.2 Control Rats

Results reported for all conduction parameters in control groups were similar to those described by a number of other workers (Misumi, 1979; Parkin and Le Quesne, 1982; Takahashi and Le Quesne, 1982; Cameron et al., 1991a). For all parameters observed in the current study, no differences existed between control groups. One exception existed with the lignocaine control group in Series 1 and Series 2, where the magnitude of supernormality at the interstimulus interval of 10 ms was significantly less than that of all other control groups. The mechanism for this is unclear but perhaps reflects a lignocaine-induced block to the voltage-dependent sodium channel at this time of high sodium activity.

6.4.3 Brevetoxin

PbTx when administered intravenously (15 µg/kg) produced a significant prolongation of both the absolute and relative refractory periods and a decrease in the conduction velocity. The fast sodium inactivation is inhibited by PbTx (Huang et al., 1984; Atchison et al., 1986). Inactivation of the voltage-dependent sodium channel controls the rate of return of the membrane to resting level (Catterall, 1985). Inhibition of inactivation will therefore prolong the refractory periods.

The magnitude and duration of supernormality were significantly increased in this PbTx-treated group. This increase is consistent with that observed for pyrethroids, which was attributed to an increase in excitability of the voltage-dependent sodium channels (Parkin and Le Quesne, 1982; Takahashi and Le Quesne, 1982). PbTx shifts the voltage dependence of sodium activation, leading to depolarisation at resting potentials (Huang et al., 1984; Atchison et al., 1986).

When administered intraperitoneally (75 µg/kg), PbTx failed to slow the conduction velocity. Although the absolute and relative refractory periods in this treatment group were prolonged when compared with control values, the differences were not statistically significant. Both the duration and magnitude of supernormality were increased following intraperitoneal PbTx, however the increases in magnitude were only significant from the interstimulus interval of 60 ms onwards.

Similar changes in nerve conduction were found with both intravenous and intraperitoneal PbTx administration although these changes overall were less pronounced in the intraperitoneal group. This suggests a dose-dependent result as well as perhaps a method-of-administration difference in susceptibility. A further implication could relate to the difference in time frames of the two experiments. In summary there were several variables involved between groups, hence it is not possible to make valid comparisons between the two groups (intraperitoneal vs. intravenous).

6.4.4 Brevetoxin and Lignocaine

Lignocaine when used as a therapeutic agent following intravenous PbTx caused pronounced changes to all of the PbTx-modified conduction parameters. Following the intravenous administration of lignocaine (500 µg/kg) no significant differences could be detected in the refractory periods, conduction velocity or supernormality when compared with values for control rats. These results demonstrate that lignocaine reverses the abnormalities in conduction caused by PbTx. This reversal is identical to that found in the Series 2 experiments with CTX. Lignocaine at the dose given did not produce any cardiac arrhythmias.

Various *in vitro* studies have shown a reversal of CTX activity by lignocaine (Legrand et al., 1985a; Lewis, 1985a, 1985b; Flowers et al., 1992). *In vitro* studies demonstrated that PbTx-binding could be prevented but not reversed by procaine (Huang et al., 1984; Wu et al., 1985). Local anaesthetic agents such as lignocaine block the conduction of an impulse along axons.

They exert their action by direct interaction with the voltage-dependent sodium channel, binding at a site near the intracellular end of the channel, thereby leading to a reduction in the sodium current (Starmer et al., 1984; Hondeghem and Miller, 1987; Ritchie and Greene, 1991). This reversible block is more prominent in rapidly firing axons, assumed to occur because the molecule in its protonated form can only gain access to its binding site when the sodium channel is open (Ritchie and Greene, 1991).

6.4.5 Brevetoxin and Mannitol

Findings outlined above indicated that PbTx causes similar modifications to nerve conduction as for CTX. Furthermore comparable results were obtained when lignocaine was utilised as a therapeutic agent to both PbTx and CTX. In order to examine the effects of mannitol when given as a therapeutic agent for ciguatera poisoning, PbTx was initially used as a model.

Mannitol when administered intravenously 30 minutes after the infusion of PbTx failed to alter any of the PbTx-modified conduction parameters. These findings suggest that no direct antagonism between mannitol and PbTx exists within the sodium channel. These results are similar to those previously described for CTX. Lewis et al., (1992) reported that mannitol failed to antagonise the CTX-induced positive inotropy in isolated human cardiac tissue (Lewis et al., 1992). These results suggest that mannitol does not dislodge either CTX or PbTx from their binding site (site 5) on the voltage-dependent sodium channel.

When mannitol was given 3 hours following intraperitoneal PbTx it still failed to return any of the PbTx-modified parameters to control values. The time span in this experiment was designed to mimic the clinical situation thereby allowing for alterations to nerve pathology to occur. PbTx and CTX share the same binding site on the voltage-dependent sodium channel. They apparently induce similar ionic shifts. Although axonal oedema has been reported in ciguatera poisoning it is not known whether PbTx contributes to axonal oedema. It may therefore be difficult to directly infer that these PbTx-related findings will be similar to CTX.

6.4.6 Ciguatoxin

CTX led to a significant prolongation of the absolute refractory period and a slowing of the conduction velocity. The magnitude and duration of the supernormal period were increased.

These findings are similar to those reported by Capra and Cameron (1985a) and Cameron et al. (1991a, 1991b). CTX modifies many properties of the voltage-dependent sodium channel (Wu and Narahashi, 1988). The open state of the sodium channels is prolonged by CTX leading to an increase in sodium permeability and depolarisation of the membrane at resting potentials (Bidard et al., 1984). This prolonged activation of the sodium channels is reflected in the exaggerated supernormal response induced by CTX in these experiments (Parkin and Le Quesne, 1982).

Mammalian nerve conduction is almost totally dependent on sodium channel function (Chiu et al., 1979; Brismar, 1980). Neurotoxins that modify the sodium channel would then be capable of modifying a range of nerve conduction parameters in mammals. Examination of the refractory period is one parameter which is utilised to gain insight into the capability of the nerve membrane to swiftly reestablish a normal resting membrane potential following an action potential (Kimura, 1983). CTX affects a fraction of the inactivation current (Legrand et al., 1985a; Benoit et al., 1986). This prevention of total inactivation possibly contributes to the prolongation of the absolute refractory period. Closed inactivation gates after a depolarisation are an important factor in the refractory period (Bezanilla & Armstrong, 1977).

The mechanism for the CTX-induced slowing of the conduction velocity is unclear. It is possible that the nerve membrane could be partially depolarised in patches along the membrane. The impulse could therefore be slowed and blocked at times in some fibres which may lead to an overall net effect of slowing of the conduction velocity. Similar findings have been reported in human victims of ciguatera poisoning (Ayyar & Mullaly, 1978; Allsop et al., 1986; Cameron et al., 1991b).

6.4.7 Ciguatoxin and Mannitol

When mannitol was administered intravenously three hours after intraperitoneal injection of CTX the conduction parameters were unchanged when compared with those recorded in CTX-treated animals prior to mannitol. In this *in vivo* rat model mannitol therefore failed to reverse the CTX-modified conduction parameters.

The time frame for this experiment was chosen to ensure that CTX would have been attached to the nerve by this time. This time frame was also an attempt to address the issue of a mannitol-

induced reduction in axonal oedema. Axonal oedema has been reported on nerve biopsy in humans with ciguatera poisoning by several workers (Allsop et al., 1986; Sozzi et al., 1988). Recently Molgo (pers. comm., International Ciguatera Workshop, Bribie Island, 1993) using fluorescence techniques has demonstrated that the surface area of single fibres increases following the application of CTX.

Axonal oedema is most likely a secondary phenomonen to the rapid influx of Na^+ through the CTX-modified channels. The contribution of any such oedema to ciguatera symptomatology is not known. The role of nerve conduction in clinical neuropathies including those induced by neurotoxic substances has meanwhile been well documented (Baker & Lowndes, 1986; Morgan, 1989). More importantly a connection has been made between clinical disturbances such as paraesthesiae and an enhanced supernormal response (Ng et al., 1987). Cameron et al., (1991a, 1991b) have documented a significant enhancement in the supernormal response of mammals intoxicated by CTX.

A reduction in the surface area of the Schwann cell was noted following bathing the fibre in mannitol (Molgo pers. comm., International Ciguatera Workshop, Bribie Isalnd, 1993). While it cannot be claimed from results of this study that mannitol fails to reduce axonal oedema it is suggested that any such reduction does not impact on the CTX-modified nerve conduction parameters. If the effects of the toxin on nerve conduction are not reversed it is difficult to conceive that the symptomatology will be improved.

To date all reports of clinical improvement in ciguatera victims following mannitol have been of an empirical nature. Double-masked studies are currently being planned although results will still be judged according to subjective criteria (Palafox, pers. comm., International Ciguatera Workshop, Bribie Island, 1993). Nerve conduction studies are advantageous as a diagnostic tool in that they provide quantitative information about neurological function. Recently nerve conduction studies have commenced on human ciguatera victims following mannitol infusion. While the numbers completed are small, the findings have not been positive (Blythe pers. comm., International Ciguatera Workshop, Bribie Island, 1993).

Mannitol was given three hours after CTX in the present study. Perhaps experiments of a longer time frame are warranted. CTX-induced axonal oedema may not have occurred in this time span. The oedema described by Allsop et al. (1986) was documented following nerve biopsy performed several days after ciguatera intoxication. However Molgo (pers. comm., International

Ciguatera Workshop, Bribie Island, 1993) reported a maximal increase in nerve fibre surface area at three hours after immersing in toxin.

Post-mannitol recordings in the present study were conducted ten minutes after completion of mannitol. This is consistent with reports from clinicians that symptom improvement is immediate (occurring within minutes) and dramatic (Palafox et al., 1988; Pearn et al., 1989).

Mannitol is regarded to be a safe therapy for ciguatera poisoning. Few adverse reactions to mannitol have been reported in humans (one death has reportedly occurred as a result of mismanagement). The muscle contractions and seizures observed in rats do cause concern. In isolated preparations of human airways mannitol induced bronchoconstriction that was proportional to the magnitude of osmolarity (Jongejan et al., 1990). These authors implied that the smooth muscle contraction was due to mobilisation of intracellular calcium as well as cell shrinkage caused by mannitol.

Mannitol is capable of producing complex shifts in body fluids (Cloyd et al., 1986). Mannitol extracts water from cerebral fluid and possibly shrinks endothelial cells thereby opening the tight junctions of the blood-brain barrier (Rapoport et al., 1980; Lange, 1987). Structural changes to the blood-brain barrier as well as enhanced permeability of proteins have been documented in hyperosmolar states (Sterrett et al., 1974). It is conceivable that intracerebral osmotic shifts could be intensified by toxin-opened sodium channels. In an attempt to eradicate bias the rats in this study were not given fluid replacement. This may have contributed to the seizures noted in these animals. Cloyd et al., (1986) nevertheless documented similar mannitol pharamacokinetics in humans who were administered with maintenance i.v. fluids and dogs who were not given fluid replacement.

6.4.8 Ciguatoxin and Lignocaine

Lignocaine reversed the actions of CTX in a similar manner to the antagonism described above for PbTx.

Studies on neuroblastoma cells have demonstrated that CTX induces membrane depolarisation by increasing Na^+ permeability (Bidard et al, 1984). These studies also found that CTX caused

spontaneous oscilliations in the membrane polarisation levels, resulting in bursts of action potentials.

The effects of CTX on the rat nerve demonstrated in this study, in particular the exagerrated supernormal response, are compatible with a disturbance of sodium channel function(Parken and Le Quesne, 1982; Takahashi and Le Quesne, 1982).

Local anaesthetic agents have been shown to modify the electrical properties of nerve by increasing the threshold for excitation which results in a reduction in impulse conduction velocity and the rate of rise of the action potential. This leads to an overall decrease and ultimate abolition of the nerve action potential amplitude (Hondeghem and Miller, 1987). Local anaesthetics block Na^+ permeability through excitable membranes by occluding sodium channels and also influencing the gating properties of these channels. Lignocaine has been shown to diffuse through the nerve membrane in the neutral form and act in a charged protonated form on the axoplasmic side of the sodium channel.

The blockade of the sodium channel by local anesthetic agents is strongly voltage and time dependent and the sodium channel must first be open for a block to develop. Consequently, the effect of lignocaine is more marked in states where axons are rapidly firing, such as in ciguatera poisoning,than in resting axons.

Previous studies examining the effects of lignocaine on respiratory and cardiovascular systems of the cat and guinea pig have demonstrated that the effects of CTX were largely reduced or abolished following the administration of lignocaine (Legrand et al, 1985b).

There has been only one report of the use of an anesthetic agent in acute ciguatera poisoning in humans. Gross (1960), described a dramatic symptomatic improvement in 5 cases of acute ciguatera poisoning. These patients were inadvertently infused with procaine hydrochloride in place of the prescribed calcium gluconate. When the mistake was recognised and calcium gluconate was subsequently given, there was a deterioration in the clinical condition of each victim. The condition of each victim again improved when procaine was reinstituted. This accidental finding suggests that local anesthetic agents could plany an important role in the treatment of the acute symptoms of ciguatera poisoning.

Lange et al., (1988) reported a beneficial response to the administration of an oral form of lignocaine (tocainide) in 3 victims who were still complaining of neurological symptoms some 4 years after acute ciguatera poisoning. One explanation for this is that local anesthetic agents may also act by selectively blocking those sodium channels which have been chronically altered by CTX.

This controlled study in the rat has demonstrated quite significant reversal and improvement of neurophysiological disturbance in acute ciguatera poisoning with intraperitoneal lignocaine using doses approximately equivalent to those used to prevent acute cardiac arrhythmias (1 mg/kg) in humans.

Lignocaine is more conveniently and effectively administered via the intravenous route in man and is also well tolerated. Intravenous lignocaine would therefore appear to be a potentially effective therapeutic agent on both theoretical and experimental bases in the treatment of the neurological disturbance in acute ciguatera poisoning.

7.0 Conclusions, outcomes, and future directions

Both grants reported upon in this document were initiated with the ultimate view of providing better treatment for the human victims of ciguatera poisoning. Two fundamental approaches were taken. In one, the mechanisms used by fish to avoid intoxication with CTX were reviewed in hope that such mechanisms might provide some insight into how the progression of ciguatera poisoning in mammals (especially humans) might be modified. The second approach was to examine how possible therapeutic regimens, in mammals, modified the well established changes in the conduction parameters of mammalian peripheral nerves that are initiated by CTX.

7.1 Studies in Fish

7.11 Histological studies

Histological studies in fish have confirmed that CTX produces changes at the cellular level to a variety of tissues. Damage has been observed in the gills, gut and liver. The histopathological changes in fish appear to be species related with some species experiencing a higher degree of cellular and subcellular disruption than other species. The findings reported in the current study also relate to different batches of CTX and some of the variations observed may be due to differences in the composition of the toxins. Studies on CTX have been and continue to be limited by the supply of pure toxin of known composition. Small variations in the structure of CTX or its precursors may lead to variations in its physiological and structural effects. This study was no exception and relied on extraction of CTX from small amounts of toxic fish obtained from a variety of species originating from a wide geographic area.

No attempt was made to examine the location of CTX in fish tissues by immunocytochemical means when it was found that immunocytochemical studies attempted in mammals were inconclusive. Immunocytochemical localisation of CTX may not be possible until a CTX specific antibody has been developed and even then the tissue attachment of CTX maybe such that the antigen is unavailable for coupling to a labelled antibody.

Histological studies on fish have shown that fish tissues are susceptible to CTX and damage may follow CTX consumption. The effects of CTX on the liver indicates that fat metabolism is altered and that supplies of glycogen are diminished. Some hepatic changes are suggestive of an

increased protein synthesis. This latter result when considered in the context of the results of the biochemical studies in fish indicates that CTX may induce production of "protective liver proteins". While further general histological studies can be justified on academic grounds, specific studies combining histopathology and liver biochemistry may elucidate protein based, CTX protection mechanisms in fish.

7.12 Toxicological studies

Feeding experiments with *Lutjanus carponotatus* have shown that CTX when ingested by this fish is absorbed and begins to be stored in skeletal muscle. Not only is the CTX absorbed and stored but it produces behavioural changes in the fish that are indicative of the actions of CTX on nervous tissue. CTX obviously acts on the fish nervour system. Algal toxins produced by *G.toxicus*, on the other hand, induced no behavioural changes and were not deposited in skeletal muscle.

The feeding experiments reported for *L carponotatus* confirm that CTX can pass between trophic levels in the food chain. The non accumulation of *G.toxicus* toxins indicates that predominantly carnivorous species may not be able to bioconvert the precursors of CTX to CTX. In order to gain a greater knowledge of the movement of algal precursor toxins into the marine fish food chain it would be necessary to work with both herbivorous fish and marine invertebrates that contribute to the food intake of commercial carnivorous species. Further work on the biochemistry of toxin bioconversion and the passage of CTX(s) through trophic levels would assist in understanding the distribution of CTX in commercial fish species.

7.13 Biochemical studies

The most significant finding of the combined grants 87/058 and 88/029 came from biochemical studies in fish. A soluble protein with a molecular weight in the range of 37,400 to 40,600 Daltons was found to be associated with CTX in the skeletal muscle of Spanish mackerel which were shown to contain CTX. One of the fish in which this protein was found was supplied by the NSW Health Department and had caused poisoning during the 1987 outbreak of ciguatera poisoning in Sydney. The occurrence of this soluble protein in carrier fish appears to be induced by the presence of CTX, and by inference this protein may offer some protection to the fish from the membrane actions of CTX.

The significance of this finding is that the induced protein may form the basis of a simple test for the presence of CTX in fish. To date, attempts to develop tests for the presence of CTX have relied upon raising antibodies to CTX which is a small non protein molecule (molecular weight approx 1100 Daltons). To raise antibodies to CTX, the toxin must be conjugated to a protein. Tests developed using such CTX antibodies have been of little value and give an unacceptable number of both false positive and false negative results. The development of a simple colour change dipstick type test for an induced protein would be much more feasible than the current experimental tests based on CTX - antibodies. Further research in this area could have enormous benefits for the Fishing Industry in its attempts to ensure product safety. Product safety will become increasingly important from both marketing and legal liability view points.

7.2 Studies in Mammals

7.21 Histological studies

These studies were initiated in an attempt to localise the sites of CTX attachment within the Central Nervous System of mammals. The cerebellum of mice was chosen as an appropriate model. Attempts were made to use immunocytochemical methods to localise binding sites for CTX in the cerebellum. Unfortunately no conclusive results were obtained in this part of the total study. It is concluded that this study would only be feasible if and when a specific CTX antibody becomes available. Even if such an antibody were available, current evidence suggests that CTX binds deep within the Na^+ channel so that labelled antibody visualisation methods may not be feasible. The membrane itself may form a barrier to the successful binding of a CTX antibody to attached CTX molecules. Further study in this area would not seem to be justified, at present.

7.22 Fish protective mechanisms and human therapy

The histological and toxicological studies on fish provided no direct insight into mechanisms that may be useful for human therapy. As noted above the biochemical studies on fish provided information that is highly significant for potential testing of fish. At the present time the most cost effective way of minimising human intoxication may be the development of reliable tests for toxic fish, thus preventing ciguatera poisoning rather than attempting to cure the victims by some form of therapy based on fish protective mechanisms. Alleviation of symptoms may be possible using currently available therapeutic agents (Section 6.23). In the long term, molecular techniques may

allow the cloning of the fish protective protein to form some type of "pseudo-antibody' that can bind toxin in victims of ciguatera poisoning. Such an approach would not seem warranted at present given the lack of understanding of fish protective proteins. Further work on protective proteins and their role in CTX detection would seem to be more desirable at present.

7.23 Mannitol and ciguatera poisoning

The primary objective in this part of the combined study was to determine if mannitol, an osmotic diuretic, has any effect on the nerve conduction parameters that are altered by CTX. Extensive and well controlled experiments performed on rats, in this study, failed to show any reversal of the CTX induced changes in nerve conduction in the peripheral nerves of the rat. These CTX induced changes in rat nerves are essentially identical to the changes observed by the authors in the peripheral nerves of human victims of ciguatera poisoning. The reported mannitol induced reversals of symptoms in human victims of ciguatera poisoning has not been shown to have a physiological basis at the level of peripheral nerves. The actions of mannitol may be placebo in nature or they may be mediated centrally or by action on unbound toxin. Further work is needed if the physiological actions of mannitol on the ciguatera syndrome are to be elucidate.

Although no physiological basis for the actions of mannitol were established, two significant outcomes resulted from the project. Firstly, we established that the dinoflagellate toxin, brevetoxin, produced similar changes in nerve conduction parameters to ciguatoxin. Brevetoxin is commercially available and it may be useful in further elucidation of the physiological effects of CTX on humans and also in the screening and testing of potential therapeutic agents. Secondly, we found that the local anaesthetic agent, lignocaine, was capable of reversing all of the major CTX induced changes in nerve conduction parameters. Clinical studies on lignocaine and related agents such as Mexitil would seem to be warranted, and should be pursued.

7.24 General outcomes

Support from FRDC to the Queensland University of Technology Ciguatera Research Group over the past ten years has contributed to the successful completion of the theses and research papers listed below. There are still several research papers in preparation and these will be published over the next two years.

Theses and research papers produced at QUT (1985 - 1995) in the area of ciguatera poisoning:

- PhD Theses
  - Hahn, S.T. (1991) "Toxinology of Ciguatera".
  - Purcell, C.E. (1994) "The Neural Effects of Ciguatoxin and Related Toxins".
- Masters Theses
  - Flowers, A.E. (1989) "The Effects of Ciguatoxin on Teleost Nerves".
  - Blanton, C.G. (1991) "The Histopathological Effects of Ciguatera Toxins on Fish and Mammalian Toxins".
- Research Papers

  - Cameron, J. and Capra, M.F. (1991) Neurological studies on the effects of ciguatoxin on mammalian nerves. In D.M. Miller (Ed) *Ciguatera Seafood Toxins*. CRC Press, Boca Raton. 33-54.
  - Cameron, J., Flowers, A.E and Capra, M.F. (1991) Electrophysiological studies on ciguatera poisoning in man (Part II) *J.Neruol.Sci.* 101: 93-97.
  - Cameron, J., Flowers, A.E and Capra, M.F. (1991) Effects of ciguatoxin on nerve excitability in rats (Part I). *J.Neurol.Sci.* 101: 87-92.
  - Cameron, J. and Capra, M.F. (1993) The paradoxical disturbances of temperature perception in ciguatera poisoning. *Clin.Toxicol.* 31: 571-579.
  - Capra, M.F. and Cameron, J. (1991) Ciguatera in Australia. In D.M. Miller (Ed) *Ciguatera Seafood Toxins*. CRC Press, Boca Raton. pp 33-54.
  - Capra, M.F., Cameron, J., Flowers, A.E., Coombe, I.F., Blanton, C.G. and Hahn, S.T. (1988) *Proc.Sixth Coral Reef Symp.* 3: 37-41.
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