PRODUCTION OF ANTIBODIES TO TOXINS INVOLVED IN CIGUATERA FISH POISONING

FIRDTF PROJECT

86/12 FINAL REPORT

RICHARD J. LEWIS, J. POTOMSKI & NOEL C. GILLESPIE

QUEENSLAND DEPARTMENT OF PRIMARY INDUSTRIES

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TABLE OF CONTENTS

SUMMARY

Sufficient pure ciguatoxin (0.5 mg of CTX-1) has been isolated from moray eel viscera to allow the immunisation of mice and screening of antibodies to ciguatoxin. We determined that *in vivo* immunisation techniques were most likely to yield antibodies using related toxins as models of ciguatoxin. The model toxins used included brevetoxin, okadaic acid and tetrodotoxin. Using the same approach we determined that the *in vitro* immunisation techniques investigated were inappropriate. It appears essential that ciguatoxin be conjugated to a carrier protein to allow production and screening of antibodies to ciguatoxin. The use of unconjugated toxin, either for immunisation or screening, appears inappropriate. The conjugation of ciguatoxin to a carrier protein has not been achieved despite considerable effort. Consequently, it was not possible to achieve the overall goal of the project. Until it is confirmed that ciguatoxin possesses the functional groups that allow the toxin to be conjugated to a carrier protein, work towards the development of antibodies to ciguatoxin cannot proceed.

During this project 595 potentially toxic fish samples were tested for toxin content. This included numerous samples of moray eel viscera and numerous portions of fish involved in ciguatera in Australia. During this project two new ciguatoxins (CTX-2, 0.3 mg; CTX-3, 0.1 mg) were also isolated from moray eel viscera. These toxins were determined to be less oxidised forms of ciguatoxin. Analysis of moray eel toxicity indicated that these fish may excrete ciguatoxin over time.

Using mouse bioassay, the efficiency of extraction of ciguatoxin was determined to be >50% in fish flesh spiked with ciguatoxin. Mouse assay is not suitable to detect low toxicity fish flesh samples but is useful as a confirmatory assay for fish with a toxicity high enough to demonstrate moderate to severe clinical effects.

Future studies are required to resolve questions on the chemistry of ciguatoxin. These studies are presently underway. With this information the project can proceed to the production of antibodies to ciguatoxin using the procedures and protocols developed during this project.

INTRODUCTION

Ciguatera (fish poisoning) is a significant problem facing the line fishery in the northern half of Australia. Outbreaks of ciguatera occur unpredictably in both time and geographic location. When an outbreak occurs it is often associated with media coverage that has an adverse impact on the sale of seafood. The possibility exists for victims of ciguatera to take legal action through the courts. To-date, such action has been unsuccessful or has resulted in out-of-court settlements. Ciguatera is now the single greatest factor affecting the marketing of reef fish and such species as the narrow-barred Spanish mackerel.

This project was initiated because of industry concerns about the impact ciguatera had on the marketing of seafood. At the time the project commenced one of the few management options available was the possible development of a simple, specific immunoassay which would allow testing of individual fish before sale. Consequently, this project was developed to produce an antibody upon which such a test could be developed. It was recognised that this was a high risk project because:

1. the structure of ciguatoxin was unknown.
2. the nature of the toxin (small size, high toxicity and lipid-solubility) could make it difficult to generate antibodies using standard procedures.
3. it would be difficult to accumulate sufficient ciguatoxin (it is present in fish in ppb or less).

In this report we outline the approaches taken and results obtained during this four year project which commenced in 1986. For clarity, the technical and related aspects of the project are included in Appendices I-IX. Appendix I is a study tour report assessing the Hokama Stick test for ciguateric fishes. This report was compiled after a QDPI funded visit to Professor Hokama's laboratory in 1986. For comparison, a summary description of the first commercial test kit (UBE Industries) available to detect polyether toxins is included (Appendix II). An internal review of the project that was completed in March, 1990 is included as Appendix III. Appendices IV-VII describe the technical details of the research undertaken during this project. Published papers concerning the management of ciguatera published during the project are included in Appendix VIII. For reference, a copy of the original application to FIRTA is provided (Appendix IX).

OBJECTIVES OF ORIGINAL APPLICATION

The following objectives are presented as a conservative assessment of the progress considered possible within a three year time frame, towards the ultimate objective - the development of a method of detection of ciguatoxin (CTX).

(i) Extract and purify sufficient ciguatoxin from toxic fish to service the requirements of the intended experimental program for toxin.

(ii) To develop using pure toxin an enzyme immunoassay for measuring anti-ciguatoxin antibody production.

(iii) Develop a method and schedule for immunisation to allow production of monoclonal antibodies.

In the formulation of these objectives, it has been assumed that considerable advances are shortly to be made in the derivation of the chemical structure of ciguatoxin. Recent communications from Professor Paul Scheuer at the University of Hawaii display optimism about the early completion of this work.

METHODS

Isolation, purification characterisation and assay of the ciguatoxins

Procedures for the collection of toxic moray eels and for the isolation and purification of the ciguatoxins are given in Appendix VI and VII. Techniques used were modifications of early procedures as well as incorporation of several new chromatographic steps that greatly facilitated procurement of pure ciguatoxin. The ciguatoxins were characterised by ^{1}H NMR (^{1}H nuclear magnetic resonance spectroscopy is a technique that indicates the environment of each hydrogen in a molecule, allowing its structure to be inferred), mass spectra (determines the mass of a compound eg. the mass of ciguatoxin is 1110 daltons) and binding to sodium channels. The efficiency of extraction of ciguatoxin from fish was assessed by testing extracts from spiked and unspiked samples of various fishes using mouse bioassay. The routine testing of fishes involved in ciguatera in Queensland was performed using the method of isolation developed for moray eels.

Conjugation of ciguatoxin to proteins

It appears essential that ciguatoxin must be conjugated to a carrier protein (typically a large, immunogenic protein eg. human serum albumin) both for immunisation and screening. Because it was suspected that ciguatoxin possessed a primary hydroxyl group, it was deemed possible to make a carboxylic acid derivative of ciguatoxin by reacting ciguatoxin with succinic anhydride (succinilation). Once a carboxylic acid derivative of ciguatoxin is produced it is then a relatively straightforward to conjugate ciguatoxin to a protein through this reactive functional group. Details of the succinilation reactions performed are given in Appendix V. Succinilations of ciguatoxin were attempted after establishing the reaction using microgram quantities of brevetoxin as outlined in Figure 1. The conjugation of a carboxylic acid derivative of brevetoxin and of okadaic acid was achieved by a routine procedure utilising a water-soluble carbodiimide cross-linking reagent. Preparation of the tetrodotoxin-protein antigen is described in section 15 of Materials and Methods of Appendix IV.

Immunisation and screening

Details of all methods used to produce monoclonal antibodies are provided in Appendix IV. Basically, two approaches to immunisation were employed. The first used standard *in vivo* procedures (Figure 2). This procedure requires injection of Immunogenic material into a mouse (eg. a toxin-protein conjugate). Since ciguatoxin is a relatively small molecule (hapten) it is unlikely to be immunogenic in the native state. This problem is conventionally overcome by conjugating the hapten of interest to an immunogenic carrier protein prior to immunisation. The high toxicity of ciguatoxin also precludes the injection of > 5 ng of material unless it is chemically modified. Because of the concern over toxicity, considerable effort was directed towards producing antibodies using *in vitro* immunisation techniques (see Figure 2) which avoids the problem of whole animal toxicity. A new procedure (European Patent Appl. # 87103975.6 developed by a U.S.A. research team) utilising unconjugated toxins for *in vitro* immunisation was also thoroughly investigated in this project.

Several screening procedures were tested to determine the appropriate method of exposing antigen to antibody. Enzyme linked immunosorbent assay (ELISA) procedures were developed using conjugated and unconjugated toxins.

RESULTS AND DISCUSSION

To accomplish the objectives of the project many approaches were investigated including several novel procedures (see Appendices IV-VII). All approaches, however, were based on indications from the literature or on a patent application. Assessment of the various antibody production and screening procedures was facilitated using model toxins (brevetoxin, okadaic acid and tetrodotoxin). This approach ensured that the valuable supply of ciguatoxin was not wasted on inappropriate methods.

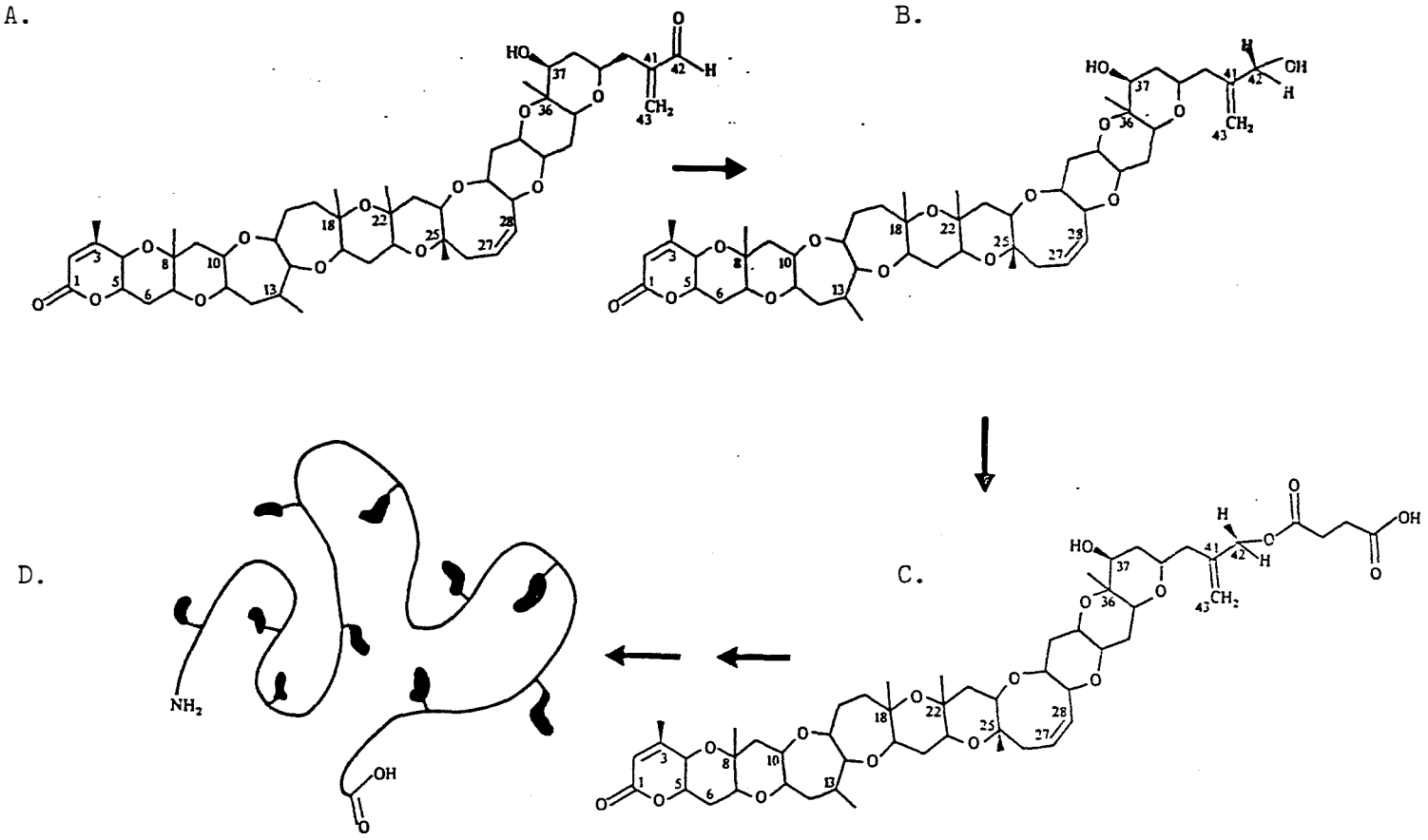

Figure 1. Procedure for production of a hapten protein conjugate (BTX-BSA). This procedure (from Baden, 1984) was successfully duplicated with 100 ug of BTX (brevetoxin) in our studies.

(A→B) sodium borohydride reduction to produce a primary hydroxyl derivative B. (B→C) succinilation to a carboxylic acid derivative C. (C→D) coupling of brevetoxin to a carrier protein (BSA).

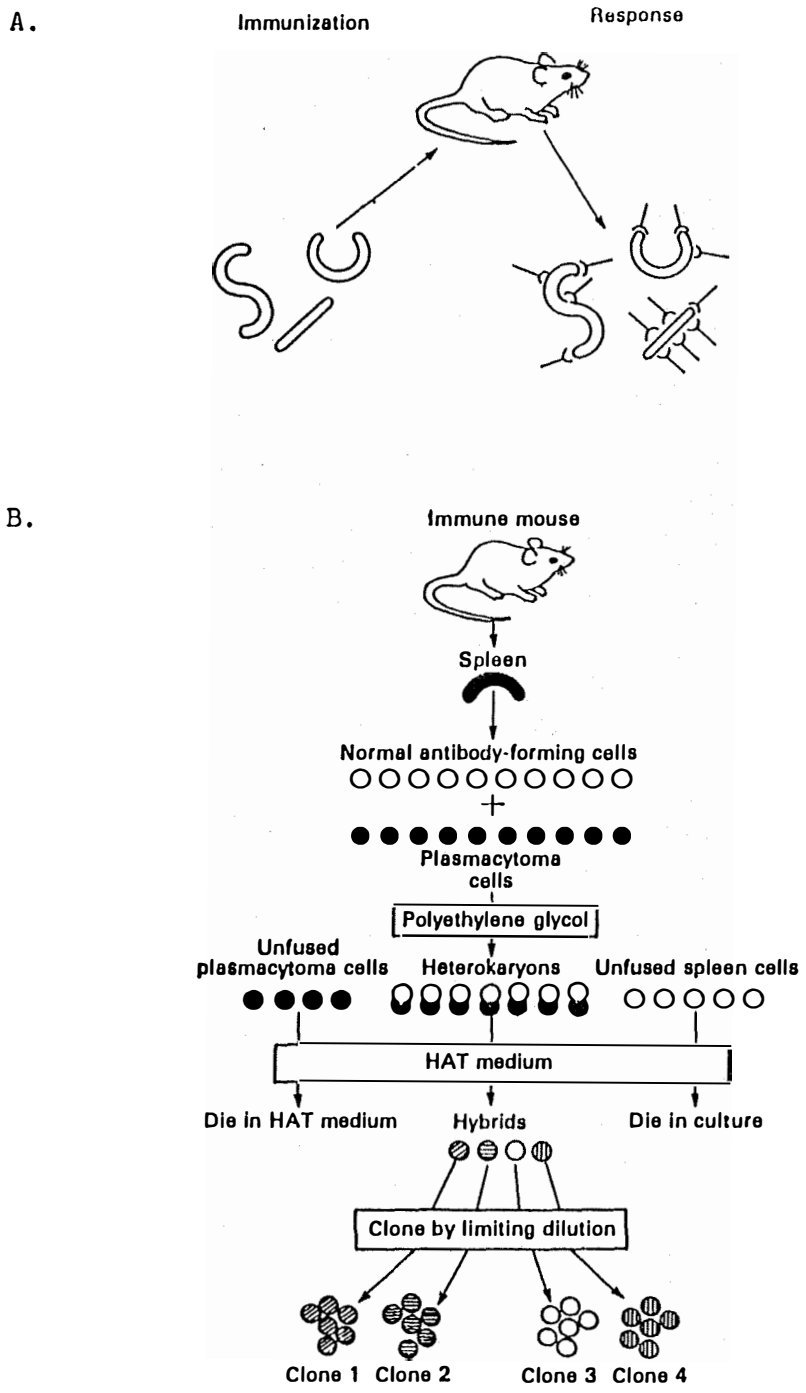

Figure 2. Method of immunisation and production of monoclonal antibodies (from Goding, 1986). A. Procedure for obtaining an immune response in vivo through injection of an immunogenic compound. Such a procedure produces a polyclonal antibody mixture that accumulates in the circulatory system. B. Generalised procedure for production and cloning of hybridomas in vivo. For in vitro immunisation spleens from unimmunised (or in some cases pre-immunised mice) are removed, dissociated and then exposed to antigen.

Isolation, purification and characterisation of the ciguatoxins

One of the difficulties associated with this project was that of obtaining sufficient pure ciguatoxin for antibody production and screening. Extraction of 50 kg of moray eel viscera collected from the Republic of Kiribati yielded 490 μg of ciguatoxin (named CTX-1). In addition, two less-polar congeners were also isolated and characterised for the first time (CTX-2 and CTX-3). CTX-1 had an LD_{50} of 0.25 μg/kg i.p. in mice and had purity > 95%. The less polar ciguatoxins were similarly purified but were less potent. Details of this study are presented in Appendix VI. The quantity of CTX-1 isolated is presently the single largest accumulation of pure toxin in the world. This quantity is considered sufficient to complete this project.

Concerns over the accuracy of the structure proposed for ciguatoxin (see Fig. 1 of Appendix XI) has necessitated its reinterpretation using ^{1}H NMR and mass spectral measurements of CTX-1 isolated in this study. This work has shown that the I-D NMR of CTX-1 is equivalent to spectra previously published. However, 2-D NMR techniques indicate that the structure of ciguatoxin could be interpreted in an alternative manner (unpublished result). This study is continuing. Spectral differences between CTX-1 and CTX-2 indicate that the structure of CTX-2 lacks one hydroxyl group and has altered stereochemistry. This latter difference means that CTX-1 and CTX-2 are likely to arise from separate precursors.

Quantification of the mouse bioassay for ciguatoxin in fish flesh

In the event of an antibody based test being developed it is essential that it be calibrated using alternative procedures. No study attempting to quantify the efficiency of extraction of ciguatoxin from fish flesh has been previously published. We established, using spiked samples, that extraction efficiency varied with fish species but was typically greater than 50%. The reproducibility within one fish was high. These results indicate that the mouse bioassay has some potential as a confirmatory test for ciguatoxin. This study also revealed that injection of extracts from non-toxic fish can cause reactions in mice not easily distinguishable from the effects of low toxicity fish. This indicates that sub-lethal responses of mice can give a false positive, a finding not previously recognised. The other disadvantage of the mouse bioassay is that it cannot be used for routine screening (it is too slow and too costly).

Testing fish implicated with ciguatera

During this project 595 potentially ciguateric fish were tested in an attempt to obtain ciguatoxin. This included the extraction of over 220 moray eels which provided the majority of the ciguatoxin purified during this project. In addition, numerous samples of fish involved in poisoning in Australia were tested but these yielded little toxin. The small quantity of ciguatoxin isolated from Australian fish will be chemically and chromatographically characterised and compared with the moray eel ciguatoxins.

Flesh samples of confirmed toxic and non-toxic fish have been collected and stored for later use in the evaluation of an assay for ciguatoxin. In addition, a portion of the extract from each fish have been stored as dried fractions under N_2 for later use. This represents a valuable collection for the validation of any prospective test for ciguatoxin in fish.

Conjugation

Because of its small size, ciguatoxin must be conjugated to a carrier protein [eg BSA (bovine serum albumin) or KLH (keyhole limpet haemocyanin)] before an antibody response to ciguatoxin can be obtained in mice. Early studies on the chemistry of ciguatoxin found that during attempted acetylation (a reaction similar to succinilation) of ciguatoxin toxicity was lost and that toxicity could be restored in alkaline conditions. From this result it was interpreted that ciguatoxin possessed a primary hydroxyl, although other interpretations would also be consistent. Recently, a structure of ciguatoxin has been proposed on the basis of interpretation of ^{1}H NMR and mass spectra (Murata *et al.*, 1990). The proposed structure included a primary hydroxyl. After ciguatoxin was purified we attempted to make a carboxylic acid derivative of 50 μg of ciguatoxin using succinic anhydride (succinilation).

Unfortunately, despite considerable effort the succinilation of ciguatoxin could not be achieved (Appendix V), calling into question the structure of ciguatoxin. Prior to attempts with ciguatoxin, a similar reaction was successfully performed on a microgram scale on the primary hydroxyl of brevetoxin. Until a carboxylic acid derivative can be attached to ciguatoxin (ie. by succinilation or similar reactions) ciguatoxin cannot be covalently conjugated to a protein - a step essential for antibody production and screening. Carboxylic acid derivatives of brevetoxin as well as of okadaic acid have been successfully conjugated to a range of proteins using the carbodiimide reaction (Baden *et al.*, 1984).

Immunisation and screening

Details of the results of hybridoma production and screening are given in Appendix IV. Several important results have been forthcoming from these studies. These include:

(i) non-conjugated lipid-soluble haptens are unsuitable as an immunogen for either *in vivo* or *in vitro* immunisation;

(ii) non-conjugated lipid-soluble haptens are unsuitable for use in a screening assay to detect specific antibodies;

(iii) methanol solutions >50% cannot be used to stick haptens to plastic 96 well plates as methanol exposure causes the plates to bind immuno-gamma-globulins (IgG's) non-selectively;

(iv) *in vitro* immunisation procedures investigated were not useful for antibody production;

(v) *in vivo* immunisation procedures utilising a model toxin (ie. tetrodotoxin) conjugated to a carrier protein can induce antibody production in mice;

(vi) problems of high toxicity, which limit the quantity of immunogen that can be administered, may be overcome by conjugation and also by the use of Freund's adjuvant.

During these studies we also tested antibodies that were raised to related toxins for cross-reactivity with ciguatoxin. Unfortunately, all antibodies tested to-date did not cross-react with ciguatoxin. These antibodies included: (i) a polyclonal to brevetoxin (see Appendix VI); (ii) a monoclonal to okadaic acid (using the UBE antibody described in Appendix II); (iii) and polyclonals to okadaic acid and palytoxin (Dr L. Levine, Brandeis University, unpublished result). This result confirms that the most appropriate means of obtaining antibodies that bind ciguatoxin is to immunise with ciguatoxin. Alternative methods using antibodies to different brevetoxins or to brevetoxins conjugated at different positions on the molecule may also yield useful cross-reacting antibodies (the brevetoxins appear to be closest to ciguatoxin in structure).

A clear direction for future research is indicated from these results. For antibody production we propose that *in vivo* immunisation procedures be used as the first choice. The immunisation and screening steps both require ciguatoxin to be conjugated to a carrier protein (a different protein for each procedure). *In vivo* immunisations can be successfully accomplished using microgram quantities of hapten. However, given the highly toxic nature of ciguatoxin, its potency will have to be significantly reduced after successful conjugation to a protein if *in vivo* immunisation is to work. This result awaits confirmation, but successful detoxification of tetrodotoxin indicates that it is possible. If ciguatoxin is not sufficiently detoxified, then further chemical modification of ciguatoxin or *in vitro* immunisation procedures could be pursued.

Excretion of ciguatoxin from fishes

Analysis of the toxicity of the viscera of moray eels from one site on the atoll of Tarawa, Republic of Kiribati, revealed a significant exponential decay in the level of ciguatoxin over time. This result was

interpreted to mean moray eels can excrete ciguatoxin (including the loss of ciguatoxin *per se* and possible metabolism to less toxic forms). The details of this result are given in Appendix VII. From this observation a model explaining the accumulation and excretion of ciguatoxin has been proposed. This result has obvious management implications. It is possible that toxic fish will become free of ciguatera within the life-time of the fish. The success in stemming the ciguatera problem in Spanish mackerel from Platypus Bay by closing this area to fishing could be attributed to an ability of Spanish mackerel to lose ciguatoxin. If Spanish mackerel did not lose ciguatoxin it would be expected that the ban on their capture in Platypus Bay would only result in the problem extending to areas outside Platypus Bay, which is contrary to our present observations.

FUTURE RESEARCH

Future research should be directed towards a better understanding of the chemistry of ciguatoxin with the view to obtaining a carboxylic acid derivate (see also Appendix III). Once this is achieved, studies can proceed to the production of antibodies and from there to a test. Assuming successful immunisation is achieved in the short term, a commercial test could be developed within several years. Biotechnology Australia has expressed interest in becoming involved in the project. The Australian Quarantine Inspection Service has indicated It may support such a project financially.

Despite not being able to obtain antibodies to ciguatoxin within the period of this project, we are still of the opinion that an antibody based test has the most potential for development of a routine screening procedure for suspect fish. A stick test for ciguateric fishes developed by Y. Hokama indicates the potential for such a test. This procedure has been modified and simplified (Hokama *et al.*, 1990) but awaits acceptance by the U.S.A. Food and Drug Administration. A review of an early version of the test (Appendix I) did not support its use to routinely screen fishes in Australia. Later versions have not been assessed, but they apparently utilise the same monoclonal antibody used in the earlier procedure. A commercial antibody based test has been developed to detect okadaic acid (Appendix II) with a sensitivity of 40 μg/ml. To detect levels of ciguatoxin in fish an even more sensitive test is required. This can be achieved in a modified test or if a higher affinity antibody is obtained. Antibody based screens have been developed to detect other haptens at levels well below 1 ppm (eg. Trucksess *et al.*, 1989). The use of antibody screening procedures as routine first-up screens is now becoming accepted by regulatory authorities in the U.S.A. Development of a test for ciguatoxin would provide a valuable management tool that would minimise the impact ciguatera has on the fishing industry.

ACKNOWLEDGMENTS

We thank Dr Ron Quinn for providing valuable guidance on succinilation reactions performed at his laboratory and Mike Holmes and Dr Geoffrey Pietersz for valuable discussion. We thank Miss Michelle Sellin for invaluable contributions to the extraction, purification and bioassay of the ciguatoxins. We thank Temakei Tebano for assistance with the collection of moray eels in the Republic of Kiribati and Raewyn Street, Heather Smythe, Sarah Brice and Hazra Thaggard for assistance isolating the ciguatoxins from fishes. We thank Drs L. Levine, K. Nakanishi, J. Aaskov, K. Ewings, H. Deeth, M. Capra and Mr M. Symons for input into the project. We thank the Queensland Department of Health for assistance collecting ciguateric fishes in Queensland, in particular Mr R. Warren. Drs R. Norton and J. MacLeod provided spectral measurements and Dr M. Poli performed binding studies detailed in Appendix VI. We thank Elaine Donovan for typing this report.

PUBLICATIONS

A number of publications have been published during the period of this project that relate to the management of ciguatera (Appendix VIII). Two papers that have been submitted for publication are included in Appendix VI and VII. A list of these publications is provided below.

GILLESPIE, N.C., LEWIS, R.J., PEARN, J., BOURKE, A.T.C., HOLMES, M.J., BOURKE, J.B. & SHIELDS, W.J. (1986) Ciguatera in Australia: Occurrence, clinical features, pathophysiology and management. *Med. J. Aust.* **145**, 584-590.

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LEWIS, R.J., CHALOUPKA, M.Y., GILLESPIE, N.C. & HOLMES, M.J. (1988) An analysis of the human response to ciguatera in Australia. In: *Proc. Sixth Int. Coral Reef Symp.* Townsville, Vol 3, pp. 67-72 (CHOAT, J.H., BARNES, D., BOROWITZKA, M.A., COLL, J.C., DAVIES, P.J., FLOOD, P., HATCHER, B.G., HOPLEY, D., HUTCHINGS, P.A., KINSEY, D., ORME, G.R., PICHON, M., SALE, P.F., SAMMARCO, P.A., WALACE, C.C., WILKINSON, C., WOLANSKI, E. & BELLWOOD, O., Eds). 6th International Coral Reef Symposium Executive Committee, Townsville.

LEWIS, R. AND GILLESPIE, N. (1988) Ciguatera. In: Venoms and Victims, (Pearn and Covacevich, Eds) pp. 31-36, Queensland Museum and Amphion Press, Brisbane.

LEWIS, R.J., SELLIN, M., STREET, R., HOLMES, M.J. & GILLESPIE, N.C. (in press) Excretion of ciguatoxin from moray eels (Muraenidae) of the central Pacific. *Third International Conference on Ciguatera Fish Poisoning,* Puerto Rico (see Appendix VII).

LEWIS, R.J., SELLIN, M., POLI, M.A., NORTON, R.S. & MACLEOD, J.K. (in preparation) Purification and characterisation of the major ciguatoxins from moray eel (Lycodontis javanicus, Muraenidae) (see Appendix VI).

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LEWIS, R.J. (1985) Ciguatera and ciguatoxin-like toxins in fishes, especially *Scomberomorus commersoni* from southern Queensland. PhD. thesis, University of Queensland, 175p.

MURATA, M., LEGRAND, A.M., ISHIBASHI, Y., FUKUI, M. & YASUMOTO, T. (1990) Structures and configurations of ciguatoxin from moray eel *Gymnothorax javanicus* and its likely precursor from the dinoflagellate *Gambierdiscus toxicus. J. Am. Chem. Soc.* **112**. 4380-4386.

TRUCKSESS, M.W., STACK, M.E., NESHEIM, S., PARK, D.L. & POHLAND, A.E. (1989) Enzyme-linked immunosorbent assay of aflatoxins B_1, B_2 and G_1 in corn, cottonseed, peanut butter, and poultry feed: collaborative study. *J. Assoc. Off. Anal. Chem.* **72**, 957-964.

APPENDIX 1

QUEENSLAND
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STUDY TOUR REPORT
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ASSESSMENT OF HOKAMA STICK TEST FOR CIGUATOXIC FISHES

R. J. Lewis
Fisheries Research Branch

Department of Primary Industries
Queensland Government

Queensland Department of Primary Industries
Study Tour Report QS88006

ASSESSMENT OF HOKAMA STICK TEST FOR CIGUATOXIC FISHES

R. J. Lewis
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Queensland Government

Queensland Department of Primary Industries
GPO Box 46
Brisbane 4001.

CONTENTS

LIST OF FIGURES

OBJECTIVES

The objectives of a study tour to Hawaii in October 1986 were:

- to evaluate the Hokama poke stick test for detecting ciguatoxin in fish;
- to obtain recent information on the chemistry and pharmacology of ciguatoxin; AND
- to obtain information from other ciguatera research groups in Hawaii.

INSTITUTIONS VISITED

Department of Pathology, University of Hawaii at Manoa

The laboratory of Professor Hokama was visited. This laboratory housed the monoclonal antibody facilities used in the production of the antibody to ciguatoxin. He had two full-time and two to three part-time technicians employed.

Professor Hokama had been producing antibodies to ciguatoxin since 1975. He had used these antibodies in a number of screening assays including radioimmunoassays (RIA), and enzyme-linked immunosorbent assays (ELISA). Early screening procedures he developed, using polyclonal antibodies, were moderately sensitive but were expensive and complicated to perform. False positives were a common problem encountered with early screening tests.

In 1985, Professor Hokama produced monoclonal antibodies to okadaic acid and ciguatoxin. The antibody to okadaic acid cross-reacts with ciguatoxin. This antibody was used in the stick test until the antibody to ciguatoxin was produced early in 1986. The antibody to ciguatoxin is less cross-reactive to other polyethers than is the antibody to okadaic acid and is now being used exclusively in the poke stick test. Features of the Hokama test will be described in detail in a following section. An abstract of a report suggesting mannitol may successfully treat ciguatera was obtained from Professor Hokama. The use of mannitol in the treatment of ciguatera is very exciting as it may provide for the first time an effective treatment for ciguatera. Mannitol is a plant sugar and is used therapeutically, for example, to reduce cerebral pressure by simple osmotic effects.

Department of Pharmacology, University of Hawaii at Manoa

Dr Miyahara's laboratory was visited. This laboratory had the facilities to evaluate the effects of toxins on isolated preparations from animals. Dr Miyahara was first to examine the action of maitotoxin (a water-soluble toxin from the likely progenitor of ciguatoxin, Gambierdiscus toxicus) on the heart. He had one technician employed on the project.

Dr Miyahara was investigating the effects of partially purified extracts of toxic fish. He found a variety of effects but the picture was confused by non-specific effects of impurities present in these extracts. I stressed the need for further purification before testing.

Previously with purified ciguatoxin Miyahara reported that all effects of ciguatoxin on the heart were the result of nerve stimulation. After discussing the results of my own study while at the Coral Reef Congress (Tahiti, 1985) he reported similar findings, that is, ciguatoxin had direct effects on the heart as well as the previously reported indirect effects. He was planning to continue analysing the effects of toxins on isolated preparations. I demonstrated the procedure of setting up a rat phrenic nerve-diaphragm preparation to the people at his laboratory.

Department of Chemistry, University of Hawaii at Manoa

At the laboratory of Professor Paul Scheuer, facilities for the extraction and purification of ciguatoxin from moray eels was available. Work on ciguatera here had been in progress since 1965 and the people at this laboratory had the greatest chance of unravelling the chemical structure of ciguatoxin. Professor Scheuer employed one post-doctoral student and one research assistant. Collaborators in the US mainland assisted with spectral determinations.

Professor Scheuer had only a small amount of ciguatoxin available. Approximately 0.8 mg of crystalline ciguatoxin had been lost in two separate unfortunate incidents. These losses set back attempts at structural elucidation by several years. He was still collecting moray eels from islands throughout the Pacific basin. Ciguatoxin was then extracted from the liver and viscera of toxic specimens. Slight modifications to previous purification schemes were being used and some features of the new scheme were to be incorporated into efforts by the Fisheries Research Branch of the DPI to extract ciguatoxin. Professor Scheuer was considering recommencing culturing Gambierdiscus toxicus in the hope of obtaining ciguatoxin from this source.

At the request of Professor Scheuer a lecture on ciguatera in Australia was given to members of the Chemistry, Pathology and Pharmacology faculties.

Hawaii Institute of Marine Biology

I visited the laboratory of Dr Rick York at Coconut Island. Dr York was maintaining his interest in ciguatera but research was limited by poor funding. He maintained strains of Gambierdiscus toxicus in culture but was not able to obtain ciguatoxin from his culture. Besides ciguatera research, he was heavily involved in aquaculture, mainly in an advisory capacity. He was experimenting with mixed rearing of tilapia, prawns and oysters, employing a partition between tilapia and prawns.

ASSESSMENT OF THE HOKAMA POKE STICK TEST

Outline of method

Basically this test employs a recently obtained antibody to ciguatoxin to detect ciguatoxin that adheres to bamboo sticks after insertion into fish flesh. From three to six bamboo sticks coated with typists' correction fluid are inserted into the flesh of a suspect fish. After the sticks are removed and dried they are rinsed in buffer and then incubated in antibody for 10 minutes. The sticks are washed again to remove antibody not firmly bound to the stick (presumably only antibody in a ciguatoxin-antibody complex remains). A final incubation in 4-chloro-napthol produces a colour reaction (to blue) proportional in intensity to the amount of ciguatoxin bound to the stick. Colour reactions above light blue (quantified against standard colour references) are considered to indicate ciguatoxin is present in fish flesh at a level sufficient to cause human intoxication. The whole procedure takes about 30 minutes per fish. During the visit, the precise details of the then latest method of using the poke stick were studied with several days of tuition provided by Hokama's staff. This training allowed stick test results obtained by the DPI to be directly compared with results obtained by Hokama. Appendix I provides details of this latest stick test protocol. It includes several modifications and improvements over the protocol provided for the collaborative study which was used previously by the Department.

Advantages

The Hokama poke stick test has several advantages for the detection of toxic fish.

- The procedure is relatively simple. A person with basic training and access to some laboratory equipment could apply the test successfully. There are plans to develop a kit which would allow a single fish to be tested.

- The procedure allows a fish to be tested within 30 minutes or about 4 minutes per fish when a large number are tested in one batch.

- Bamboo sticks coated with correction fluid (Pental opaquing fluid) are used in the test and appear to absorb ciguatoxin. The use of correction fluid was discovered by chance and it is not yet known how it works. Its use allows ciguatoxin to be easily fixed to the stick and then simply exposed to antibody.

In conclusion the stick test, as presented, is designed to meet the needs of the fish-eating public who wish to test a suspect fish before consumption. The antibody used in the Hokama test detects ciguatoxin. However, other polyether compounds can cross-react with the ciguatoxin antibody as described in the following section. A more specific antibody would greatly improve the value of the test. Production of a highly specific antibody to ciguatoxin is one of the aims of the ciguatera research group within the Department.

Disadvantages

While the poke stick test has several advantages many problems exist for the implementation of this test by the Department.

- The antibody produced in Hokama's laboratory is apparently not truly monoclonal and attempts at obtaining a true monoclonal have not yet been successful. Lack of a true monoclonal may increase the sensitivity of a test but could increase the cross-reactivity of the test over a true monoclonal antibody based test.

- The frequency of fish determined to be positive for ciguatoxin by the poke stick method appears much greater than the incidence of ciguatera in Hawaii. The false positives obtained with the poke stick test could arise for the reasons outlined below:

  (i) the cross-reactivity of the antibody used in the test is sufficient to detect non-toxic levels of polyether compounds other than ciguatoxin. This can occur because ciguatoxin is at least 500 times more toxic than other polyether compounds, including the dinoflagellate toxins, okadaic acid and brevetoxin. An extreme example was the finding that permanent freshwater samples of tilapia proved highly toxic according to the poke stick test but these fish have not been involved in human poisoning. A terrestrial source of non-toxic polyethers is suspected; or

  (ii) residual pieces of fish flesh left adhering to the bamboo stick are quite common, particularly with soft fleshed fish, for example, Spanish mackerel. If these pieces of flesh remain on the stick false positives often result. The enzyme reaction used in the ELISA assay could explain this result as the fish flesh contains the same class of enzyme. This enzyme apparently remains active in pieces of flesh possibly because the fixation step is not adequate to denature the enzyme in adhering flesh. The result is a colour reaction independent of the presence of ciguatoxin. The Department is searching for alternative enzyme reactions for the ELISA test. The most promising are B-galactosidase and urease, as these are apparently not found in fish flesh.

- The procedure as it stands requires fresh or frozen fish to be used (stored for not longer than 1 week and thoroughly thawed prior to testing). This factor could limit the usefulness of this test for the commercial screening of fish.

- Some preliminary work has been carried out by the Department using the antibody produced by Hokama's group in Honolulu in a microtitre plate assay. Samples of three fish (previously assessed by the Hokama poke stick test as being toxic, marginally toxic and non-toxic) were homogenised in 0.06 M carbonate buffer pH 9.6 to a protein concentration of 0.1 mg/ml. These samples and the Hokama antibody were used in checkerboard ELISA titrations to investigate:

(i) whether the toxicity of the fish could be distinguished;

(ii) the optimum titres and concentrations of the antibody and flesh in a microtitre assay; and

(iii) if the system worked in a microtitre plate assay.

Despite repeated experiments under varying conditions (for example, time, temperature, use of blockers) no reliable results could be achieved, although varying amounts of the antibody were detected. Sufficient controls were incorporated in the tests to enable a judgement to be made that although antibody was bound to the immobilised flesh, the binding was non-specific. Various other antisera, all conjugated to peroxidase, did not bind non-specifically to the immobilised extract of ciguatoxic fish. These results do not necessarily detract from the usefulness of this antibody in test conditions employed by Hokama but make it difficult to use the Hokama antibody for the commercial screening of fishes in Australia;

- The cost of materials for the test is approximately A$1.00 per fish. Large fishes (> 5 kg) need to be tested twice (in separate locations). The time required for one person to test a small fish is 30 min;

- While false negatives have not been reported with this test, recent experience in Professor Scheuer's laboratory indicated that a batch of highly toxic moray eels (tested by conventional mouse assay) produced borderline reactions with the stick test, while eels of low toxicity to mice produced a strong positive result by the stick test. Similar experiences have been obtained with Australian fish including trevally and Spanish mackerel, where toxicity has been assessed by mouse and chicken assay. Apparently the stick test reaction can saturate and then is 'self-inhibited' with increasing concentrations of ciguatoxin in fish flesh. This occurs over a narrow range of ciguatoxin concentrations. The presence of highly toxic and low toxic commercial fishes in Australian waters would warrant the use of two simultaneous tests optimised to test the high and low toxic specimens if the Hokama test was employed here.

GENERAL CONCLUSIONS

The Hokama poke stick test is a novel approach to the detection of ciguatoxin in fish. While the test has several advantages its disadvantages include:

(i) an unacceptably high incidence of false positives resulting in edible fishes being discarded;

(ii) the response to ciguatoxin is linear over a narrow range of ciguatoxin concentrations with highly toxic fish giving borderline reactions; and

(iii) the antibody obtained by Hokama was unable to be used in procedures applicable to the commercial screening of fish,

making it unsuitable for the commercial screening of Queensland fish.

Consequently, there remains an urgent need to develop a reliable means of identifying ciguatoxic fish in Australia under commercial conditions.

- On a previous study tour to Hawaii in 1985, Dr N. C. Gillespie indicated that Professor P. Scheuer would soon elucidate the structure of ciguatoxin. This had not been the case and recent losses of ciguatoxin (0.8 mg pure) have set back attempts to elucidate the structure of ciguatoxin by two years. Lack of detailed structural information for ciguatoxin means that more toxin will be required for the Department's research on the production of antibodies to ciguatoxin.

- Attempts to produce ciguatoxin in laboratory cultures of Gambierdiscus toxicus are in progress in Hawaii. Research on the problem by this Department is well in advance of Hawaiian research.

- During the trip the possibility of using mannitol in the treatment of ciguatera was revealed. This treatment was yet to be proven suitable for use in Australia but was apparently used effectively in the Marshall Islands. No research on the mechanism of action of mannitol was being undertaken in Hawaii.

- While research on ciguatera in Hawaii was expected to continue to make significant contributions to our understanding of the ciguatera problem, progress could be delayed through reduced financial support. For instance an anticipated US $1 million grant from the US Army to fund research by Professor Scheuer, Dr Hokama and Dr Miyahara was in doubt;

- The contact with ciguatera research groups in Hawaii provided valuable information for the Department which was not otherwise obtainable in Australia. Maintaining contact with these groups can only enhance the possibility of success in ciguatera research by the Department. Strong links with these groups were well established after several visits to Hawaii by ciguatera researchers in Australia. In fact, the Department's research in this field had gained wide recognition both in Hawaii and in the eastern United States.

RECOMMENDATIONS

- The DPI should continue its involvement in research to produce specific monoclonal antibodies to ciguatoxin. These are essential for the development of a test for ciguatoxin in fish which can be implemented on a commercial scale in Australia.

- Attempts to produce ciguatoxin in cultures of Gambierdiscus toxicus should be pursued by the Department. This approach may ultimately overcome the short supply of ciguatoxin for research, in addition to providing valuable information on the cause(s) of ciguatera outbreaks.

- The potential use of mannitol for the treatment of ciguatera should be thoroughly researched by the Department including experimental and clinical trials. Mannitol treatment could feasibly reduce the suffering (and even save the lives) of people affected by ciguatera poisoning in Australia.

ACKNOWLEDGEMENTS

This study tour was undertaken with the aid of financial assistance from Fisheries Research Fund, Queensland Department of Primary Industries. The scientific support and hospitality provided by Professor P. Scheuer, Professor Y. Hokama and Dr J. Miyahara is greatly appreciated. Thanks also to Miss G. Davidson for typing this report.

APPENDIX

PROCEDURE FOR POKE STICK TEST

Collaborative Study: Stick Enzyme Immunoassay for Detection of Ciguatoxin and Related Polyether Toxins Directly from Fish Tissues

Submitted to: Dr. E.P. Ragelis, General Referee;
Association of Official Analytical Chemists

From: Yoshitsugi Hokama, Ph.D.,
Professor of Pathology, Associate Referee

I. Introduction:

The Stick Enzyme Immunoassay (stick-EIA) procedure for the detection of ciguatoxin and related polyethers (okadaic acid, brevetoxin, norhalichondrin A, etc.) was published in 1985 (1). This initial report utilized the sheep anti-ciguatoxin antibody used previously in the radioimmunoassay (2) and the direct solid phase enzyme immunoassay (3,4). The stick-EIA has been used extensively in our laboratory, but more recently with monoclonal antibodies prepared against purified ciguatoxin (5) and okadaic acid (7,8,9). The test has been of interest for use in large scale screening of commercial fishes from ciguatera endemic areas such as the Pacific and Caribbean and especially the states with tropical and sub-tropical climates. Other uses of the test include screening of contaminated fishing grounds and for routine examination for cyclic toxicity in various areas. There is a need for the acceptance of the test at a national and international level. For this reason the proposal is being submitted to the AOAC for its approval of a collaborative study. The international level of the study has been tentatively approved for collaborative study by the Aquatic Biotoxin working group of the IUPAC in Zurich, October 20-22, 1986.

This proposal presents a detailed plan for the assessment of the stick-EIA for the detection of ciguatoxin and related polyether toxins directly from fish tissues, utilizing monoclonal antibodies to CTX and related polyethers and a new stick coat (Pentel Opaquing Fluid). The plan covers: 1) objective; 2) details of procedure and materials; 3) statistical analysis; and 4) the list of the collaborative laboratories. The concept of the stick-EIA is presented in figure 1 with details.

The objective of this proposal is to evaluate the rapid stick enzyme immunoassay procedure for the detection of ciguatoxin and related polyether toxins directly from contaminated fish tissue. Eight laboratories interested in the stick test procedure have been selected (listed in part 7) for the collaborative study under the auspices of the Association of Official Analytical Chemists (AOAC), and the International Union of Purified Analytical Chemistry (IUPAC).

II. Stick Test Kit:

The following is a detailed set of instructions for the Stick Enzyme Immunoassay for Detection of Ciguatoxin and Related Polyether Toxins Directly from Fish Tissues. These instructions are intended for use only with the laboratory version of the stick test kit.

The following materials will be required, some of which are included in each stick test kit:

A. Materials supplied by participating laboratory.

3 - 50ml beakers

1 - 25ml volume opaque flask

5ml volume clear test tubes (12 x 75mm)

methyl alcohol (absolute)

hydrogen peroxide, 30%

funnel

25ml graduated cylinder

timer with second hand

test tube rack for 12 x 75mm test tubes

pipettor with 300ul (0.3ml) capacity

tape and/or marker to label tubes and/or sticks

absorbent paper wipes (Kimwipes)

B. Materials supplied by Laboratory of Associate Referee

Vial A - human serum albumin for Tris-buffer preparation

Tube B - lyophilized MonoAb to CTX

Vial C - 4-chloro-1-naphthol for substrate

Bottle D - Tris-buffer B, 10x stock

Tube E - positive control fish extract

Tube F - negative control fish extract

Item G - fish sampling sticks (bamboo sticks coated with Pentel Opaquing Fluid)

Item H - blank sticks (bamboo sticks coated with Pentel Opaquing Fluid-marked black)

Item I - Whatman #1 filter paper

Item J - dropping pipet for addition of 4-chloro-1-naphthol in substrate preparation

Item K - tube for fixative

Item L - color chart with white paper background

III. Reagents:

A. Preparation of final working reagents

1. Tris-buffer B preparation: dilute 10x stock (Bottle D) 1:10 with distilled H2O - 1 part 10x stock + 9 parts dH2O to make working dilution of Tris-buffer B. This buffer is used in preparation of Tris-buffer A and substrate, and for buffer washes during test procedure.

2. Tris-buffer A: add 5.0ml Tris-buffer B (working dilution from step 1) to vial containing 5.0mg HSA (Vial A) to make 5.0ml Tris-buffer A. This buffer is used only in reconstitution of lyophilized MonoAb.

3. 4-chloro-1-naphthol methyl alcohol solution: add 5.0ml methyl alcohol to vial containing 0.375g 4-chloro-1-naphthol for substrate (Vial C). This solution is used only in substrate preparation.

4. Control fish extract preparation: add 1.0ml methyl alcohol to each control fish extract tube (Tubes E and F). These controls are used only to check MonoAb anti-CTX activity.

B. Final working concentrations prepared as described in A

1. Tris-buffer A: 0.05M Tris (hydroxymethyl) aminomethane, pH 7.5 ± 0.05, with 0.1% human serum albumin and 0.01% sodium azide

2. Tris-buffer B: 0.05M Tris (hydroxymethyl) aminomethane, pH 7.5 ± 0.05, with 0.01% sodium azide

3. MonoAb anti-CTX-horseradish peroxidase conjugate: 5C8-Poly-anti-CTX-HRP in Tris-buffer A

4. Substrate: 0.3% hydrogen peroxide (H_2O_2) in Tris-buffer B, with 0.02% 4-chloro-1-naphthol

C. Storage of working reagents

1. Tris-buffer B: store tightly capped at 4°C.

2. Tris-buffer A: store tightly capped at 4°C.

3. 4-chloro-1-naphthol methyl alcohol solution: store tightly capped at -20°C, protect from light.

4. Control fish extracts: store tightly capped at -20°C. **CAUTION: MAY BE HIGHLY TOXIC.**

IV. Methods:

A. Preparation of official fish samples

1. Keep official fish samples frozen until ready to run stick test. Samples should be tested within one week of receipt.

2. Thaw samples completely.

3. For tissue samples; blot excess moisture with light pressure between absorbent paper towels.

4. For whole fish samples; cut slits into middle of fish (for small fish such as reef fish) or into the anterior and posterior ends of fish to allow stick access to flesh. Avoid cutting into the gut or any blood vessels. Wash knife between samples.

B. Preparation of fish sampling sticks

1. Insert coated end of a fish sampling stick (Item E) into flesh to a depth of one inch, rotate stick twice, remove and place stick into a test tube. Repeat process with 2 more sticks for a total of 3 sticks per slit in fish or tissue sample. Avoid sticking gut contents or bloody areas. Label set with sample ID.

2. Repeat step 1 for remaining samples.

3. Allow sticks to dry at room temperature for 15-30 minutes.

4. Pour approximately 20ml methyl alcohol into tube for fixative (Item K).

5. Immerse coated end of fish sampling sticks into methyl alcohol for 1 second. Return sticks to test tubes.

6. Repeat step 5 for remaining fish sampling sticks. Change methyl alcohol after every 24 sets of sticks. Use fresh methyl alcohol each time test is run.

7. Allow sticks to dry at room temperature for 5-10 minutes.

8. Arrange each set of sticks in alternate rows in test tube racks to facilitate handling.

9. Place one blank stick (Item H-marked black) into a test tube and add one to each set of fish sample sticks. **DO NOT FIX THE BLANK STICKS IN METHYL ALCOHOL.**

see figure 2. illustrations for steps A1 - A4 and B1 - B9.

C. Set-up of Test Solutions

1. MonoAb reconstitution: add 1.0ml of prepared Tris-buffer A to 1 tube lyophilized MonoAb anti-CTX (Tube B) to make 1.0ml working dilution 5C8-Poly-anti-CTX-HRP.

2. Substrate: using 25ml graduated cylinder, measure out 24.75ml of Tris-buffer B working dilution. Add 0.25ml of 30% H2O2 to the Tris-buffer B to make 25.0ml of H2O2-Tris-buffer B solution. Pour mixture into a bottle and add 3 drops of prepared 4-chloro-1-naphthol methyl alcohol solution to H2O2-Tris-buffer B mixture (using dropping pipet-Item J). Cap bottle tightly and shake vigorously for 5 minutes. Remove insoluble residue with filter paper (Item I) and funnel. Use clear filtrate. Keep in opaque flask.

3. Buffer washes: pour 50ml of Tris-buffer B working dilution, into each 50ml beaker.

4. Arrange MonoAb tube, substrate bottle, pipet, buffer washes, paper wipes, and test tube rack as shown in figure 3.

D. Stick Test System Pre-check

1. Substrate activity: check the activity of the prepared substrate each time fresh substrate is prepared.

   a. add 300ul of substrate to a clean test tube.

   b. dip an unused fish sampling stick (Item G) into MonoAb and remove excess antibody by running stick along side of tube.

c. place the stick into the tube containing 300ul of substrate and shake moderately.

d. an immediate color change to purple (4-5+) should occur.

e. if an immediate color change does not occur, prepare fresh substrate and re-test.

f. if results are still negative, prepare fresh MonoAb.

2. MonoAb activity and washing procedure: check antibody activity and washing procedure by running 1 positive control stick, 1 negative control stick, and 1 blank stick each time before testing fish samples. 1 blank stick is also run along with each set of fish sample sticks during testing to monitor washing steps.

   a. dip an unused fish sampling stick (Item G) into the positive control fish extract for 1 second, remove and place stick into a clean test tube, air dry. Repeat procedure for the negative control fish extract.

   b. run the three sticks through test solutions as in steps 1-10 of section E - Running Fish Sample Sticks.

   c. the positive control stick should give a reading of 1.5-2.0 or higher.

   d. the negative control stick should give a reading of less than 1.5.

   e. the blank stick should give a reading of no higher than 0.5.

   f. if the controls give a reading outside of the above ranges (positive < 1.5 or negative > 1.5), repeat the procedure with another control stick.

   g. if the results are still outside the ranges, check substrate activity.

h. if substrate is alright, prepare fresh MonoAb.

i. if the blank stick gives a reading of higher than 0.5, repeat the procedure with another blank stick paying careful attention to washing steps and MonoAb incubation time.

E. Running Fish Sample Sticks

Run Stick Test System Pre-check before beginning (paragraph IV-D)

1. Hold the first set of sticks (3 fish sample sticks plus 1 blank stick-marked black) and wash in buffer wash 1 for 5 seconds.

2. Blot excess buffer onto paper wipes (lay sticks down on blotter and press gently, DO NOT RUB).

3. Immerse sticks into MonoAb for 1 minute.

4. While sticks are incubating in MonoAb, add 300ul of substrate to each stick test tube with pipet.

5. After 1 minute, remove sticks from MonoAb (drain well by sliding along side of tube).

6. Wash sticks in buffer washes 2 and 3, 15 seconds each and blot after buffer wash 3 onto a separate paper wipe (in same manner as above).

7. Immerse sticks into tubes containing 300ul of substrate for 10 minutes with a gentle-moderate initial shaking for 5 seconds. Note: sticks do not haveto be placed back into exact original tubes.

8. After 10 minutes, read substrate according to color chart; hold tubes against white paper background of color chart and lift sticks out of substrate to obtain accurate readings. Do not score readings finer than half a unit (0.5, 1.5, etc.).

9. The color reactions are scored according to the chart as follows:

Color	Score
none, clear	0
slightly bluish	1.0
light bluish-purple	2.0
moderately bluish-purple	3.0
purple	4.0
intense dark purple	5.0

Note: the colors on the color chart included in the stick test kit (Item L) will not exactly match the colors obtained when running the test. The chart is to be used to indicate the gradation of color intensity obtained when running the stick test.

10. Average the scores of the 3 sticks and the toxicity of the fish tissue rated as follows:

Score (average of 3 sticks)	Rating
0 - 1.2	negative (<1.0ng toxin/gm tissue)
1.3 - 1.9	borderline(>1.5ng toxin/gm tissue)
2.0 - 5.0	positive (>5.0ng toxin/gm tissue)

see figure 4 illustrations for steps 1-10

*all values in negative category potentially contain CTX at concentrations less than 1.0ng/gm of fish tissue. values greater than 1.3 potentially contain CTX at concentrations greater than 1.5 ng/gm of fish tissue. Thus any mean score greater than 1.3+ value is not to be consumed.

V. Important points

A. Fish samples and sticks:

1. Do not test spoiled fish samples (cooked fish tissues and soups can be tested).

2. If fish is frozen, thaw completely before testing.

3. Avoid cutting into blood vessels or the gut.

4. Avoid sticking bloody areas or gut contents.

5. Do not use improperly coated sticks: cracked, etc.

B. Reagents:

1. Store all working and stock reagents according to instructions.

2. MonoAb may be used up to 3 days after reconstitution, provided it remains clear (does not become cloudy) and is kept refrigerated between use. Always check the activity before using.

3. Substrate must be made up fresh each time the test is run.

4. Do not expose substrate to UV or direct sunlight.

5. If substrate changes color spontaneously, do not use. Prepare a fresh solution.

6. Keep all working solutions between 5-25°C while test is being run.

C. Fish Sample Testing:

1. Replace paper wipes used for blotting sticks as they become saturated.

2. Do not immerse sticks in MonoAb for longer than 1 minute.

3. Washing steps are important. Wash thoroughly, but gently.

4. If blank stick gives a color reaction higher than 0.5, check coat on the stick. If coat is cracked, reject stick reading. If coat is intact, check washing procedure and MonoAb incubation time.

5. If sticks readings are consistently negative, periodically check test system.

6. If one out of three sticks gives a reading that differs from the other two by more than 2, check the stick coat. If the coat is cracked, reject the stick reading. If intact, average reading with others as usual.

VI. Statistical Analysis

The numbers of fish samples and the number of sticks per sample will be strictly adhered to and the data collected should be sufficient to evaluate both the repeatability and the reproducibility of the stick test procedure.

A. Repeatability within a laboratory will be assessed by the number of sticks per fish sample and will include a total of 6 fish samples. From the mean of each sample (based on 3 sticks per fish sample) the results will be categorized as negative (-), borderline (±), and positive (+) for each sample (see end-point reading under procedure).

B. Reproducibility between laboratories will be assessed by the means of each sample and the endpoint reading of -, ±, and +.

C. Any fish in the borderline and positive categories should not be consumed.

VII. List of Collaborative Laboratories and Addresses

1. Mr. Henry Sakuda, Director
   Division of Aquatic Resources
   Department of Land and Natural Resources
   State of Hawaii
   Honolulu, HI 96813

2. Mr. G. Kobayashi
   Chief of Laboratory
   Department of Health
   State of Hawaii
   Honolulu, HI 96813

3. Mr. P. Sardinas, Manager
   Palau Fishing Authority
   P.O. Box 586
   Koror, Palau 96940
   Western Caroline Islands
   U.S. Trust Territory

4. Dr. G. Yang
   Department of Health and Human Services
   Organic and Biological Chemistry Branch
   (HFF-454)
   Public Health Service
   FDA, 200 C Street, SW
   Washington, D.C. 20204

5. Dr. N.C. Gillespie
   Supervising Fisheries Biologist
   Southern Fisheries Research Centre
   P.O. Box 76
   Deception Bay Q 4508
   Australia

6. Prof. T. Yasumoto, Chairperson
   Faculty of Agriculture
   Tohoku University
   Tsutsumidori-Amamiyamachi 1-1
   Sendai, Japan

7. Dr. U. Raj, Director
   Institute of Marine Resources
   The University of the South Pacific
   Suva, Fiji

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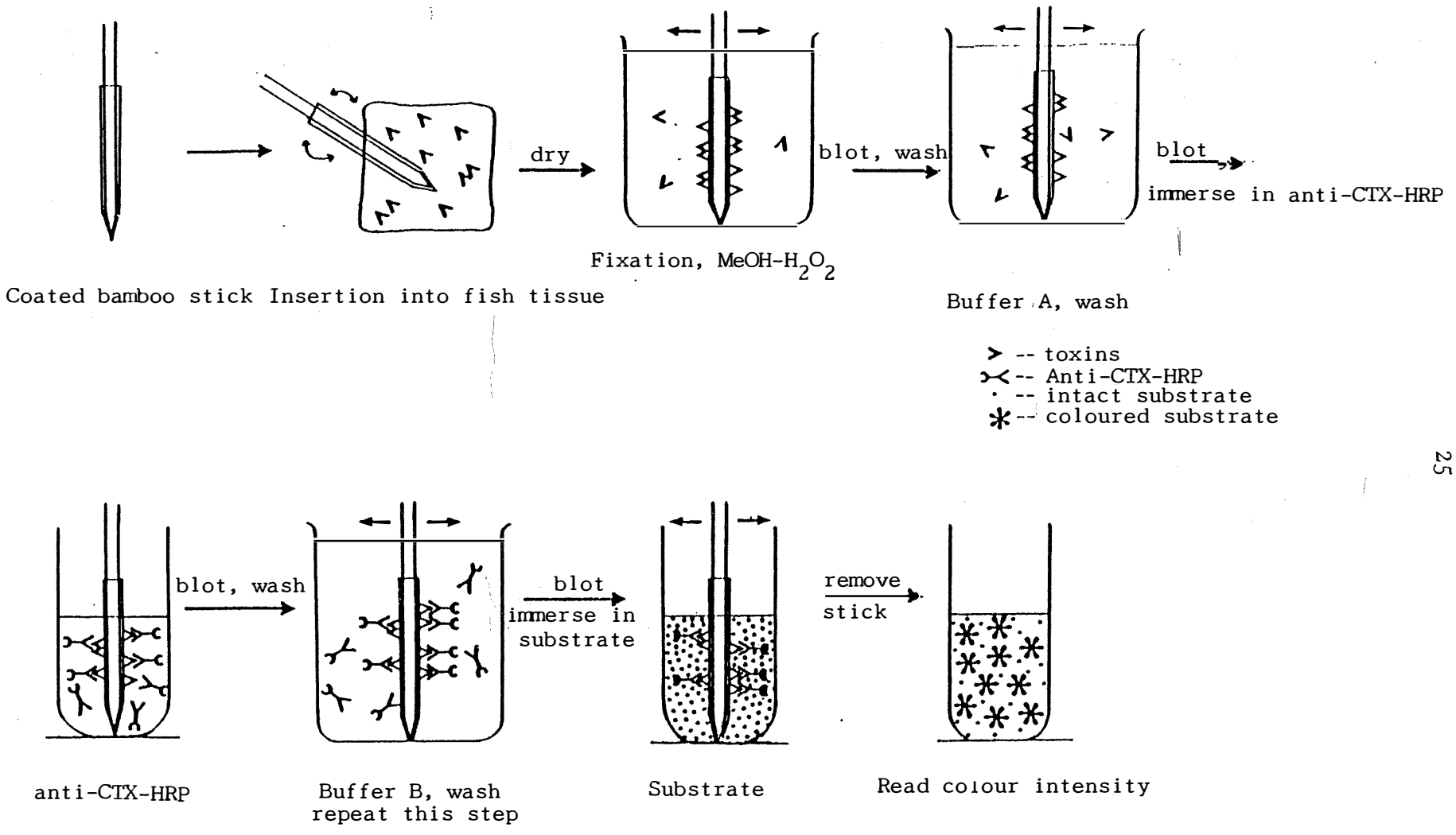
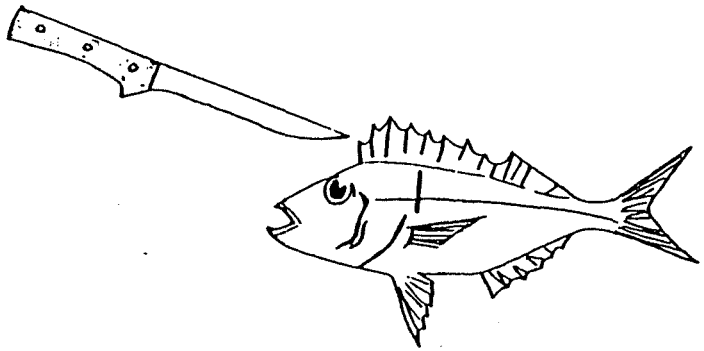
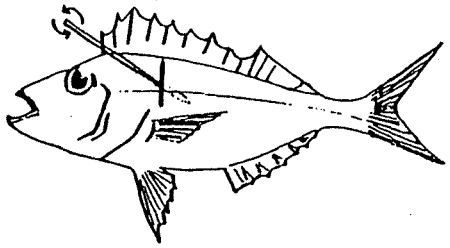


Figure 1. Diagrammatic scheme of the concept of the stick enzyme immunoassay.



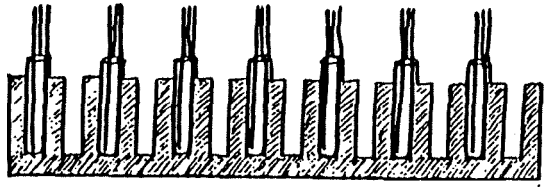
Cut a slit into the middle of the fish. Avoid cutting into any blood vessels or the gut.



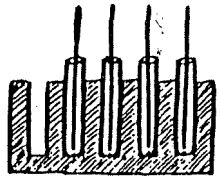
Insert the coated end of the stick into the flesh and rotate twice. Avoid bloody areas and gut.

Place the stick in a test tube and air dry 15 to 30 minutes.

Fix the sticks in MeOH for one second.



Return stick to test tube and air dry for five minutes. Arrange each set of sticks in alternate rows on the rack.



Add one blank stick in a tube to each set. Do not fix blank.

Figure 2. Preparation of fish samples and sticks.

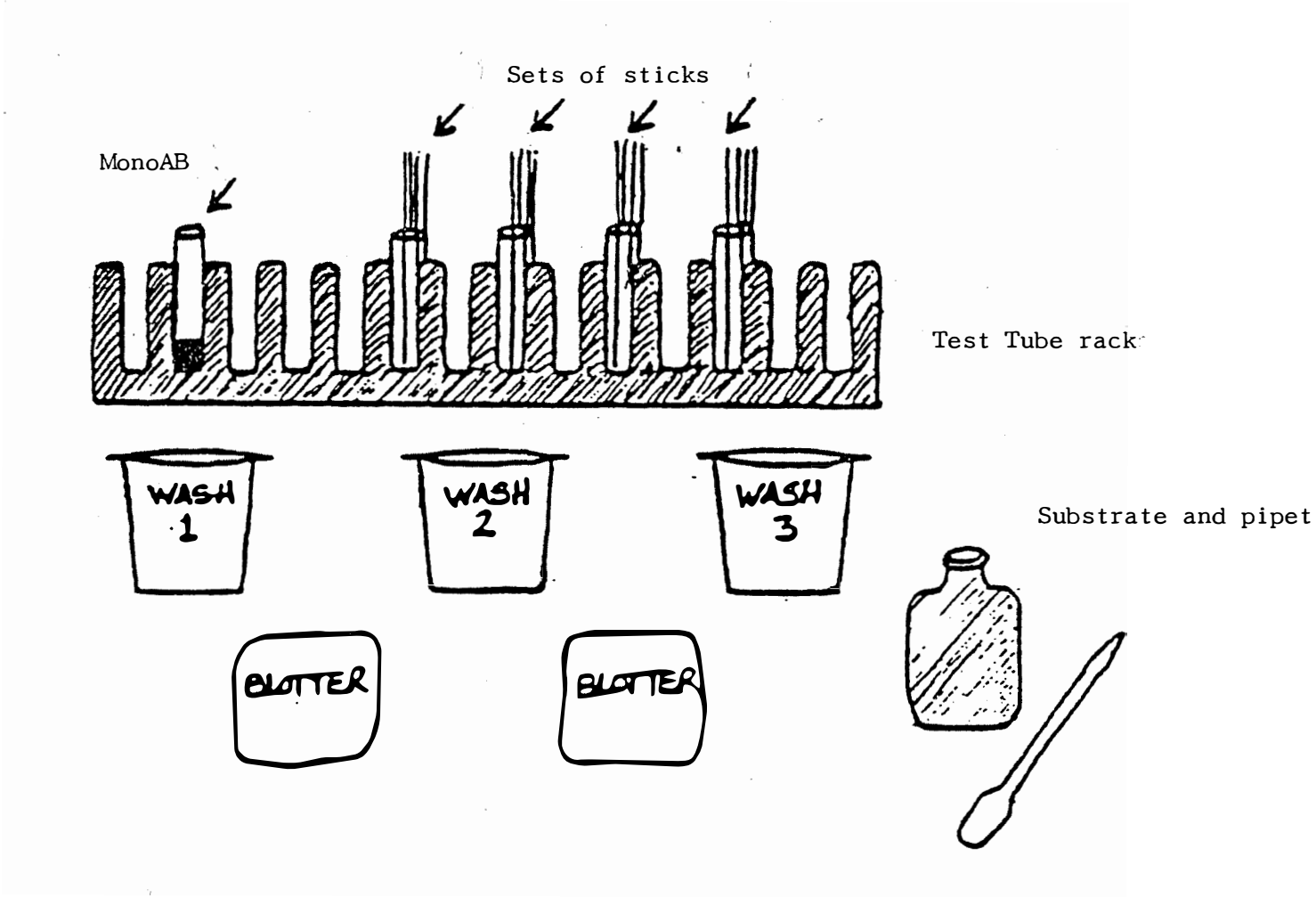
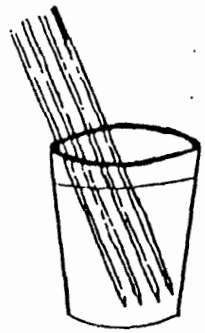
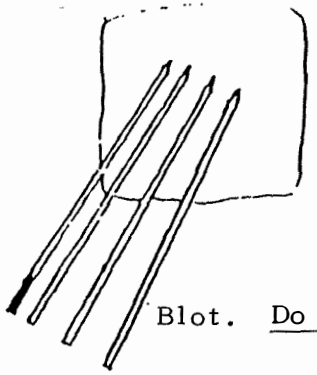


Figure 3. Set-up of supplies

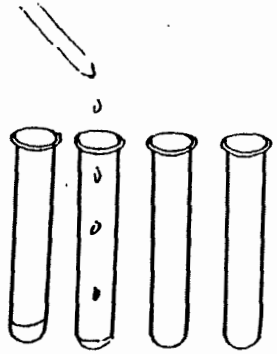


Buffer wash 1,
five seconds

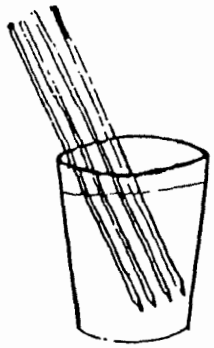


Blot. Do not rub.

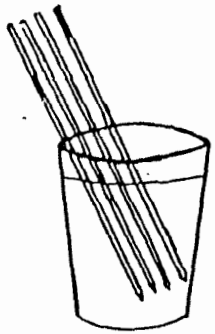
Immerse in MonoAB,
one minute



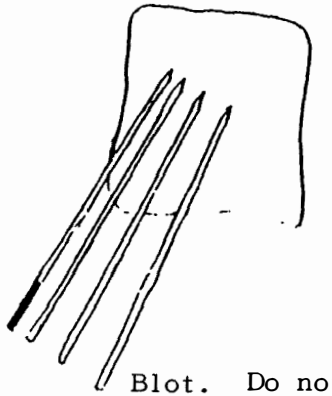
While sticks are in MonoAB,
add 300 ul substrate to each
tube.



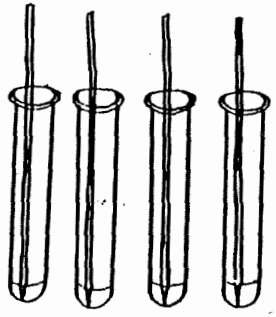
Buffer wash 2, 15 seconds



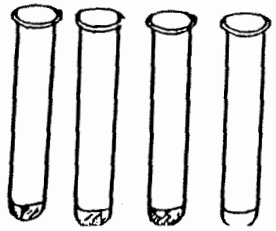
Buffer wash 3,
15 seconds



Blot. Do not rub.



Immerse sticks in tubes
containing substrate, shake
for five seconds, let stand
for 10 minutes



After 10 minutes read colour reaction
with colour chart

Figure 4. Fish stick test procedure.

IMMUNOASSAY OF DIARRHETIC SHELLFISH POISON
下痢性貝毒のイムノアッセイ

Diarrhetic shellfish poisoning (DSP) was first described in Japan as a new kind of a seafood disease. This is caused by eating mussells or scallops which are contaminated by poisonous plankton. At present, these poisons in shellfish are detected by bioassays using a large number of mice, which are very laborious and time-consuming.
UBE Industries, Ltd. has developed a monoclonal antibody specific to Okadaic acid (OA) and its derivative (DTX_1), which are major one among poisons, and immunoassay kit for detection of OA and DTX_1 using this antibody. This immunoassay kit (DSP-Check) enables more simple and easy, and time-saving detection of OA and DTX_1.

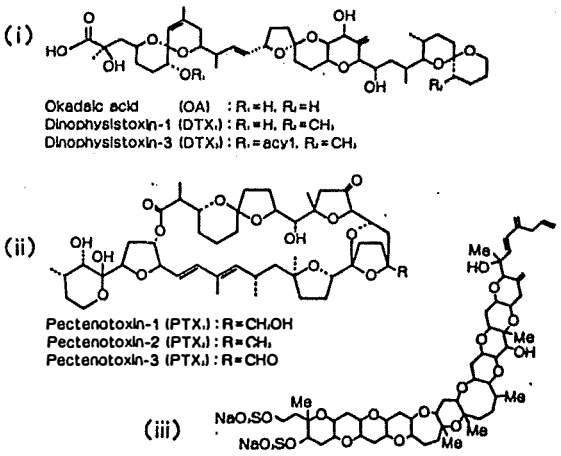
■ COMPONENTS OF DIARRHETIC SHELLFISH POISN

Prof. Yasumoto of Tohoku University has proved that Diarrhetic shellfish poisons consist of three major components.

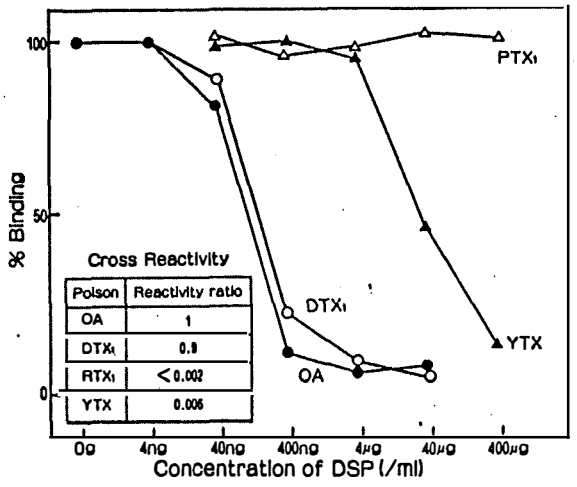
(i) Okadaic acid and its derivatives
(OA, DTX_1, DTX_3)

(ii) Pectenotoxin derivatives
($PTX_{1,2,3}$)

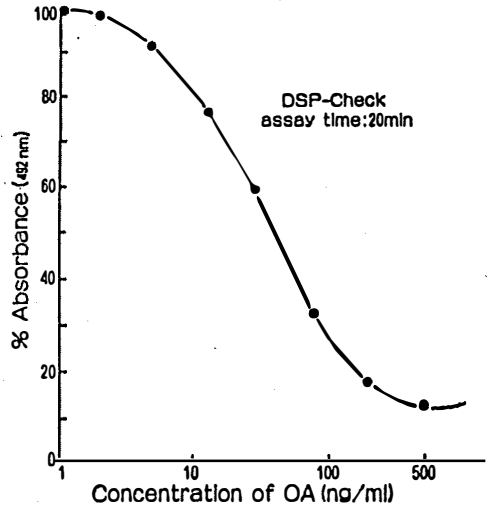
(iii) Yessotoxin
(YTX)



■ CROSS REACTIVITY OF MONOCLONAL ANTIBODY



■ STANDARD CURVE



APPENDIX III

Project Review By C.K. DIMMOCK, Principal Immunobiologist, Animal Research Institute, QDPI.

This project proposed to attempt to produce antibodies against ciguatoxin, the principal toxin involved in ciguatera fish poisoning. It was envisaged that success in this project could lead to the development of a rapid, specific method for the detection of individual toxic fish, before sale.

The project objectives were:

(1) Extract and purify sufficient ciguatoxin from toxic fish to service the requirements of the immunisation and screening experiments.

(2) To develop, using pure toxin, an enzyme immunoassay for measuring anti-ciguatoxin production.

(3) Develop a method and schedule for immunisation to allow production of monoclonal antibodies to ciguatoxin.

Objective (1) has been achieved as sufficient ciguatoxin has been collected and purified to allow objectives (2) and (3) to be completed.

Objectives (2) and (3) have not been completed but this is due essentially to circumstances beyond the control of the project personnel and is no reflection on their technical competence.

The form of the project proposal indicated that it was a high risk undertaking with no guarantee of success. At that time (1986) ciguatoxin was known to be a highly oxygenated lipid with structural features that included a number of ether linkages, olefines, hydroxyl and methyl groups, but the exact structure was unknown. Also, attempts in several overseas laboratories had failed to produce specific antibodies against ciguatoxin although immunoassays had been developed to detect polyether compounds. However, it was optimistically assumed that work on the derivation of the chemical structure of ciguatoxin being undertaken in Japan, was nearing completion and that the structure of ciguatoxin was such that it would be amenable to conjugation with a carrier protein for the immunological work. Hence, preliminary work to establish methodology for the production of monoclonal antibodies to this compound, was justified.

It now appears that this optimism was premature as the chemical structure of the toxin is still not absolutely defined, although a proposed structure has been published. Ciguatoxin itself is non-immunogenic and needs to be conjugated to a carrier protein for the production of antibodies and for antibody detection assays. The presence of a primary hydroxyl group, to allow the synthesis of a carboxylic acid derivative of ciguatoxin, is essential for the conjugation reaction. Despite guidance from recognised experts in synthetic and bio-organic chemistry at Griffith University and the University of Melbourne, Dr Lewis has, to date, been unsuccessful in achieving this reaction. This inability to conjugate ciguatoxin to a carrier protein has been the major inhibitory factor to success of the project. Thus, it appears that until there is no doubt that ciguatoxin has the structure that allows conjugation of multiple molecules of the toxin to a carrier protein, the work towards developing monoclonal antibodies to ciguatoxin cannot proceed.

A considerable amount of time and funds has been expended in attempting to use 'model' compounds to develop immunisation and antibody detection methods that could be applied subsequently to produce monoclonal antibodies to ciguatoxin. As it was considered that ciguatoxin may prove to be too toxic to allow use of the standard *in vivo* mouse immunisation technique the use of *in vitro* immunisation of mouse spleen cells was evaluated as an alternative. However, two well-known problems that were not overcome were the predominance of hybridomas secreting antibodies to the carrier protein, in the case of hapten-carrier immunisations and the predominance of hybridomas producing low affinity IgM antibodies unsuited to use for sensitive detection assays. Although with perseverance specific IgG antibodies may have resulted from use of this technique, experiments using the standard *in vivo* immunisation method were undertaken in the last year.

immunisation method were undertaken in the last year.

Brevetoxin, a closely related compound, though far less toxic than ciguatoxin, was conjugated to keyhole limpet haemocyanin and this conjugate was not toxic to mice. Immunised mice produced high titre IgG antibodies. However the specificity of these antibodies (hapten or carrier) has not been determined and no fusion experiments were undertaken as insufficient brevetoxin was available for this work.

Although no clearly defined direction was established, it appears that hybridomas secreting hapten-specific IgG antibodies could be produced by perseverance with either *in vitro* or *in vivo* immunisation. However, provided the toxicity of ciguatoxin was reduced by conjugation (ie. if conjugation can be achieved) the *in vivo* immunisation method would be more likely to succeed.

An ELISA procedure with the potential to detect toxin specific antibodies in hybridoma supernatants was established. Full evaluation of this technique is dependent on the aforementioned ability to conjugate ciguatoxin to a protein.

There is nothing more to be gained by using 'model' antigens in either immunisation or ELISA experiments as the critical issue is now whether or not ciguatoxin can be conjugated to a protein. In hindsight, employment of an experienced expert organic chemist on the project may have allowed the problems associated with the structure of ciguatoxin to be overcome. If this work were to continue, employment of such a scientist should be considered.

In summary, the development of a rapid, economical, specific test for ciguatoxin based on monoclonal antibodies is unlikely to be achieved in the near future.

APPENDIX IV

DETAILS OF PROCEDURES AND RESULTS OF IMMUNISATIONS AND SCREENING

Materials and Methods

1. Culture medium

Myeloma cells were growing in Rosewell Park Memorial Institute (RPMI-1640) medium containing 10% heat-inactivated foetal calf serum, supplemented with 2mM glutamine, 1mM sodium pyruvate, 100 units penicillin/ml and 100μg streptomycin/ml. All components were purchased from Flow Laboratories. Foetal calf serum (FOS) and aliquots of glutamine and antibiotics were stored at - 20°C.

2. Growth of myeloma cells

The cultures were maintained in a humidified incubator in an atmosphere of 5% CO_2 in air at 37°C. Cell growth rate was monitored daily and cell density was kept in the region of $5x10^4$-$5x10^5$cells/ml. the myeloma cells were not maintained in culture for more than three weeks; new cultures were started from frozen stocks regularly.

3. Cell counting and viability checks

Cell counting was done by the use of the Neubauer haemocytometer. The trypan blue dye exclusion test was used to determine viability of the cells.

4. Detection of contamination

Culture media were regularly tested for microbial contamination using brain-heart infusion broth and thioglycollate broth and cells were routinely screened for mycoplasma infection with the Du Pont Mycotrim-TC system.

5. Freezing of cells

The cells were suspended at about 10^6cells/ml in 90% FCS = 10% dimethyl sulphoxide (DMSO), frozen slowly at -72°C and stored in liquid nitrogen.

6. Preparation of conditioned media

(a) Thymocyte-conditioned medium (TCM)

A single-cell suspension of thymocytes was prepared from thymus glands obtained from 10 BALB/c mice (Animal Research Institute, Brisbane) of about 2 weeks age. The thymocytes were suspended at $5x10^6$cells/ml in a culture medium containing 20% FCS supplemented with $5x10^{-5}$M 2-mercaptoethanol (LKB, Sweden) and cultured in 175cm^2 flasks (Nunc, Denmark) at 37°C in a humidified 5%CO_2/95% air atmosphere for 48 hours. The culture supernatants were then harvested, centrifuged, filtered (0.2 um), and stored at -72°C.

(b) Mixed thymocyte conditioned medium (mTCM)

This medium was prepared in a similar manner to TCM but thymocytes were obtained from five BALB/c mice and five C57BL/6J mice 6-8 weeks old (Animal Resources Centre, Perth). The culture medium contained 20% FCS or 10% normal rabbit serum (NRS).

7. Myeloma cell lines

Immunoglobulin nonproducers Sp2/0 (Flow Labs) and X63/Ag8.653 (Walter Reed Army Research Institute, Washington, USA) were used in this study.

8. Feeder cells

Nonimmunized BALB/c mice spleen cells (10^5/ml) were used immediately after the fusion and for cloning of hybridomas.

9. Selective media

HAT medium contained 10^{-4}M hypoxanthine, $4x10^{-7}$M aminopterin, and $1.6x10^{-5}$M thymidine. HT medium was modified HAT medium, without aminopterIn. 50x stock solutions of HAT and HT were obtained from Flow Labs.

10. Polyethylene glycol (PEG)

PEG MW1500 (BDH) was used for the first few fusions but was replaced with PEG MW 4000 (merck, gas chromatography grade). PEG was dissolved in RPMI-1640 (without serum) and filtered (0.2μm). The pH was slightly alkaline.

11. Cell fusion and cloning

Myeloma cells were fused with immunised spleen cells using 50% PEG according to protocol described originally by Galfre and Milstein. Fused cells were suspended in a culture medium containing 20% FCS, 1xHAT and feeders. After 5-7 days, hybridomas were fed with a culture medium containing 20% FCS supplemented with 1xHAT. Non-fused myeloma cells and immunised spleen cells were used as controls. Cloning of hybridomas was done by limiting dilution method. Cells were plated at 10,3, and 0.5 cells per 200μl well in 96 well plates (Nunc). All wells were screened microscopically for the presence of single colonies and only single-clone wells were tested for antibody production.

12. *in vitro* immunisation of mouse spleen cells

(a) Immunisation with unconjugated okadaic acid (OA)

Immunisation of B-cells was carried out as described in the patent application (Research Corporation, New York, USA, European Patent Application, 1987). Spleen cells from two 6-8 weeks old BALB/c mice were suspended in 10ml RPMI-1640 medium containing 10% FCS. Splenocytes were added to a $80cm^2$ culture flask containing 1mg Escherichia coli 055:B5 lipopolysaccharide (Difco) or 1mg E. coli 0128:B12 lipopolysaccharide (Sigma), 10ml of TCM or mTCM and different amounts of okadaic acid dissolved in methanol (50μl). The cells were incubated for 48 hours in an incubator (5% CO_2, 37°C). The immunised cells were fused with X63/Ag8.653 myeloma cells ($2x10^7$ cells) suspended in HAT medium containing 20% FCS and distributed in four 24 well plates. In initial screening, hybridomas were selected for IgM production to eliminate non-secreting variants. In the second screening hybridomas were assayed by ELISA using okadaic acid conjugated to human serum albumin (OA-HSA) and human serum albumin (HSA) as coating antigens. Clones positive for OA-HSA were further analysed for specificity to okadaic acid.

(b) Immunisation with conjugated okadaic acid (OA-HSA)

Immunisation with OA-HSA was carried out as described for the immunisation with OA. The amount of okadaic acid used for immunisation was unknown since the yield of immunogen (OA-HSA) construction was not estimated. 160 μg of OA was covalently attached to 2.0 mg of HSA. Spleen cells were immunised with 19 6.3 and 2μg of okadaic acid-human serum albumin conjugate.

(c) Immunisation with keyhole limpet hemocyanin (KLH)

in vitro immunisation with KLH was carried out as described by Reading in 20 ml cultures in 80cm^2 tissue culture flasks (Nunc). Splenocytes (1x10^8 cells) from nonimmunized BALB/c mice (6-8 weeks old) were cultured for 5 days in the presence of 10µg/ml KLH. Thymocyte conditioned medium and mixed thymocyte-conditioned media were used at a concentration of 50% (v/v). Thymocytes were used at a density of 5x10^7 cells/ml. Adjuvant peptide (N-acetylmuramyl-L-alanyl-D-isoglutamine, Calbiochem) was used at a concentration of 20µg/ml. After immunisation, the cells were sedimented by centrifugation and used in hybridisation experiments.

(d) Immunisation with lipopolysaccharide (LPS)

Immunisation of mouse spleen cells with E. coli 055.B5 lipopolysaccharide (Difco) was performed as described by Reading. Splenocytes (10^7 cells/ml) from nonimmunized BALB/c mice, 7 weeks old, were suspended in a medium consisting of 5ml RPMI-1640 =20% FCS, 5ml TCM and LPS (3µg/ml). The immunisation was carried out in a 25cm^2 tissue culture flask at 37°C in a humidified CO_2 incubator for 5 days.

13. Screening of hybridomas

(a) Anti-OA screening

Hybrids were screened for specific antibodies by an enzyme-linked immunosorbent assay (ELISA). Commercially available monoclonal antibody to okadaic acid (UBE Industries, Ltd., Tokyo, Japan) was used to determine the optimum conditions of the assay. Usually, hybridomas were screened twice. 96-well polystyrene microtiter plates (Costar #3690) were coated with 50µl/well of either OA-HSA or HSA (3µg/ml) in 0.1M carbonate buffer pH 9.6 and incubated overnight at 37°C. The unabsorbed antigen was removed by washing the plates 3 times in wash buffer (phosphate buffered saline pH 7.2 with 0.05% Tween 20). The wells were then incubated with 0.25% fish skin gelatine in PBS (65µl/well) for 1 hour at 37°C. The plates were washed 3 times and hybridoma supernatants (50ul/well) were added to the plates and incubated for 1 hour at 37°C. For screening large numbers of culture supernatants, incubation proceeded at 4°C overnight. After incubation plates were washed 3 times and 50ul/well of a 1:20 000 dilution (in PBS= 0.05% Tween 20) of peroxidase-conjugated goat anti-mouse immunoglobulins (Cappel#3211-0231) was added and incubated for 1 hour at 37°C. The plates were washed 5 times, and 50ul/well of substrate, phenylenediamine was added, and Incubated for 30min in dark at room temperature. Four 2mg OPD tablets (Dakopatts) were dissolved in 12ml 0.1M citric acid-phosphate buffer, pH 5.0, containing 50ul 3% H_2O_2.solution. Substrate solution was prepared just before its use in the assay. The reaction was stopped by 25µl/well 3M HCl. The plates were read at 492nm (Titertek Multiskan MC, Flow Labs). The cultures whose medium resulted in $OD_{OA/HSA}/OD_{HSA}>2$ were considered to contain anti-okadaic acid antibodies.

In some studies "blotto", bovine serum albumin and bovine gamma globulin were used to block remaining protein-binding sites on EIA plates. "Blotto" consisted of 5%(w/v) non-fat powdered milk in PBS plus 0.01% Anti-foam A (Sigma). Bovine serum albumin (1%w/v) and bovine gamma globulin (1%w/v) were dissolved in phosphate-buffered saline pH 7.2. In some screening assays concentrated NaCl solution (25µl/well) was added to the plates followed by hybridoma supernatants (25µl/well). Okadaic acid- poly-L-lysine conjugate (OA-PLL) and poly-L-lysine (MW 30,000-70,000, Sigma) were prepared and used as OA-HSA and HSA.]

(b) Anti-brevetoxin (BTX) screening

Hybrids were screened for anti-BTX antibodies 6-10 days after fusion by the following procedure. EIA polyvinyl microtiter plates (Costar #2595) were coated with 100µl/well of BTX (0.5µg/ml) dissolved in methanol. The plates were incubated overnight at room temperature, blocked with 1%FCS (100µl/well) for 1 hour at room temperature and washed three times with PBS. Hybridoma supernatants (100µl/well) were added to the plates and incubated for 2 hours at 37°C. After incubation, plates were washed three times and 100µl/well of biotinylated antibody

to mouse IgG, diluted 1:1000 in PBS= 1%FCS, (Amersham RPN 1001) was added and plates were incubated for 1 hour at 37°C. Following incubation, plates were washed three times and 100µl/well of streptavidin-biotinylated-peroxidase complex (Amersham RPN 1051), diluted 1:1000 in PBS= 1%FCS, was added and plates incubated for 1 hour at 37°C. Plates were washed three times and 100µl/well of substrate, o-phenylenediamine (OPD) was added, and incubated for 30min in dark at room temperature. Four 2mg OPD tablets (Dakopatts) were dissolved in 12ml 0.1M citric acid-phosphate buffer, pH 5.0 containing 50ul of 3% H_2O_2. The reaction was stopped by 25µl/well 3M HCl and plates were read at 492nm (Titertek Multiskan Mc).

(c) Anti-KIH screening

EIA polyvinyl micro-plates (Flow Labs #77-172-05) were coated overnight at 4°C with 10µg/ml of KLH in carbonate-bicarbonate buffer, pH 9.6. The coating solution was removed and the plates were blocked with PBS=1%FCS for 2 hours at room temperature. The plates were washed with PBS-Tween solution and hybridoma supernatants were added to the plates and incubated overnight at 4°C. After the wells were washed, peroxidase-conjugated goat anti mouse IgG=IgM=IgA (Cappel) diluted 1:40 000 in PBS= 1%FCS was added and the plates were then incubated for 2 hours at 37°C. After being washed, the plates were developed with substrate solution described elsewhere (see point 13a).

(d) Anti-LPS screening

The screening was performed as for antibodies to KLH but the plates were coated with 10µg/ml of LPS in PBS, pH 7.2.

14. ELISA procedure for detection of mouse IgM

EIA plates (Costar # 3690) were coated overnight at 4°C with rabbit anti-mouse Igm (Fc specific) antiserum (Nordic Immunological Labs), diluted 1:1600 in carbonate-bicarbonate buffer, pH 9.6. The coating solution was removed, and the plates were blocked with PBS= 20%FCS for an hour at room temperature. Following the washing step, hybridoma culture supernatants were incubated for 2 hours at 37°C. After being washed, the plates were developed with biotin-streptavidin system (Amersham) as described above (see point 13a).

15. Preparation of tetrodotoxin (TTX) antigen

The antigen (TTX-HCHO-BSA) was prepared by reacting 9.5mg BSA in 1ml of 0.1M sodium citrate buffer pH 4.8 was reacted with 1mg TTX in the presence of 13.5µl of formaldehyde (37%). The coupling reaction was carried out at room temperature for 3 days and subsequently at 4°C for 1 day. The reaction mixture was run on a PD-10 column (Sephadex G-25M, Pharmacia) and concentrated on a Centricon-10 (Amicon).

16. *in vivo* immunisation

Immunisation was carried out as aseptically as practicable. Stable emulsions were formed using two 2ml syringes connected with an adaptor (Scientific Glass Engineering).

(a) Immunisation with OA-HSA

Six weeks old female BALB/c mice (ARI) were subcutaneously immunised with 42 ug of OA-HSA emulsified in Freund's complete adjuvant (2.2µg of OA provided a coupling efficiency of 65%). Six weeks later, the mice were boosted s.c. with the same amount of conjugate emulsified in Freund's incomplete adjuvant.

(b) Immunisation with TTX-BSA

Seven weeks old female BALB/c mice (ARI) were subcutaneously immunised with 135µg of

TTX-BSA (50x TTX LD_{50} provided coupling efficiency was 70%) or with 270µg of the antigen (100x LD_{50}) emulsified in Freund's complete adjuvant. Six weeks later, the mice were boosted s.c. with 163µg of conjugate emulsified in Freund's incomplete adjuvant.

(c) Immunisation with BTX-KLH

A BALB/c mouse 8 weeks old was primed with KLH and rested for 3 months. After that time the animal was immunised with BTX-KLH conjugate (emulsified in Freund's complete or incomplete adjuvant) over the next 3 months. The titre of antiserum to BTX, as determined by ELISA, was 1:10 000.

17. Binding of tritium labelled BTX to EIA plates

The polyvinyl 96-well plates (Titertek # 77-172-05, Flow; Costar # 2595) were coated with an oil-base, enamel paints (9 brands) for 1 hour at room temperature. The excess paint was removed and the plates were left for 1 day at room temperature. ^{3}H-BTX dissolved in methanol was applied to coated and uncoated wells and the plates were incubated overnight at room temperature. After that time, the plates were washed with PBS-Tween buffer in the same way as in ELISA. Individual wells were cut out and added to tubes containing 2ml of Optifluor 9packard) or PCS (Amersham), mixed for 1 minute, left overnight at room temperature and counted in a beta-scintillation counter (Rackbeta II, LKB).

18. Conjugating ^{3}H-BTX to methylated bovine serum albumin (MBSA)

MBSA was prepared by dissolving 200mg BSA in 20ml absolute methanol and adding 0.168ml concentrated HC1. This solution was left for 3 days in the dark at room temperature. MBSA precipitate was collected by centrifugation, washed twice with methanol, dissolved in water and neutralised with 1M NaOH. 5µg ^{3}H-BTX in 1.5ml 75% methanol was mixed with 75µg MBSA (15µl) and left at room temperature for 3 days. This solution was run on a PD-10 column and the radioactivity of ^{3}H-BTX-BMSA was counted in a beta-scintillation counter.

RESULTS

1. Binding of tritium labelled BTX to EIA plates

This investigation was prompted by the results of Hokama *et al.*, who used enamel paint coated beads coated with CTX for the screening assay. Unfortunately, antiserum to BTX was not available to allow the optimum conditions of BTX coating to be determined. The technique was found very cumbersome and attachment of BTX to microtitre plates coated with different brands of paints and liquid paper was difficult to assess due to random and high background. Surprisingly, unpainted wells exhibited better BTX absorption properties. There was no significant difference between absorption of the toxin to PVC Flow and PVC Costar plates. It was impossible to assess the exact amount of BTX absorbed to the plates. After extraction with Optifluor, significant amounts of BTX were still extracted with PCS. As a result of these studies 1µg/ml of BTX was selected, with wide margin of safety, as a coating concentration.

2. Conjugating ^{3}H-BTX to methylated bovine serum albumin (MBSA)

MBSA is an excellent adjuvant by its ability to complex with negatively charged substances (proteins, polynucleotides) due to its high positive charge (Sueoka and Cheng, 1962). However, it was found that only 2% of BTX was conjugated to MBSA. The very low efficiency of this reaction could have been due to requirements of having a methanol solution to solubilise BTX and the fact that brevetoxin is weakly charged.

3. *in vitro* immunisation

(a) Immunisation with LPS

Fusion efficiency of four experiments was 0; 2; 18 and 25%.
Specific efficiency of these experiments was 0%.
The low fusion efficiency was attributed to the changes in the pH in the post fusion period. At that time a manual CO_2 incubator was used by several people.

(b) Immunisation with KLH

KLH was chosen as a trial antigen as we intended to use it as an immunogenic carrier for ciguatoxin. The results of the study are shown in Tables 1-2. Fusion efficiency was 100% in all experiments. Specific efficiency was high and was dependent on the supportive media and the adjuvant. Of particular significance was the relatively high numbers of hybridomas producing IgG when TCM and mTCM (20% FCS) was used. Contrary to some reports in the literature media containing foetal calf serum were more effective than medium containing normal rabbit serum in terms of specific efficiency.

TABLE 1

The effect of adjuvant peptide (N-acetylmuramyl-L-alanyi-D-isoglutamine) on the *in vitro* immunisation response.

Adjuvant concentration (µg/ml)	fusion efficiency[a] (%)	specific efficiency[b] (%)
0	100	0
20	100	8.9

[a] Fusion efficiency = (no. wells with hybrids/no. wells seeded) x 100
[b] Specific efficiency = (no. wells with specific antibody/no. wells with hybrids) x 100

TABLE 2

The effect of thymocyte-conditioned medium, mixed thymocyte-conditioned media and thymocytes on the *in vitro* immunisation response.

Addition to splenocytes	Fusion efficiency[a] (%)	Specific efficiency$_b$ (%)	IgM secretors (%)	IgG secretors (%)
None	100	13.5	100	0
Thymocyte-conditioned medium	100	28.1	93.8	6.2
Mixed thymocyte-conditioned medium (20% FBS)	100	25.0	93.8	6.2
Mixed-thymocyte-conditioned medium (10% NRS)	100	19.8	100	0
Thymocytes	100	21.9	100	0

[a] Fusion efficiency = (no. wells with hybrids/No. wells seeded) x 100

[b] Specific efficiency = (no. wells with specific antibody/no. wells with hybrids) x 100

(c) Immunisation with unconjugated okadaic acid

The novel technique for production of MAbs was recently patented (Research Corporation, N.Y., 1988). The method consists of a brief immunisation of B-cells with an unconjugated hapten in the presence of an effective amount of mitogen (LPS). It is claimed that monoclonal antibodies to toxic and non-toxic haptens were produced. Furthermore it is claimed that monoclonal antibodies produced by this technique have unique properties. They can be specific not only for a particular hapten but also specific to a portion of the hapten or a particular functional group thereon. Thus these MAbs have the unique ability to be successfully utilised in a hapten sandwich assay and be able to detect the presence of extremely small amounts, as low as parts per trillion, of a hapten in a test sample. These new developments were very relevant to our project and consequently, we decided to implement this technique.

Details of 12 experiments are shown in Table 3. The number of hybridoma colonies was dependent upon the dosage of antigen and the incubation time. As described in the patent application, 50μg of okadaic acid per stimulation and 2 days incubation time were found to be the optimum. If immunisation was extended for more than two days, the number of hybridomas decreased significantly. All hybridoma wells from 4 fusions contained high level of IgM (OD> 1.4). About 1% of hybridoma wells when tested in ELISA with OA-HSA gave optical density near double that of the controls (HSA). These hybridomas were cloned by limiting dilution and retested. There was no increase in specificity to okadaic acid. No monoclonal antibodies specific to okadaic acid were produced by this technique. Two literature searches (1988/89) did not find any conformation of the results claimed in the patent.

TABLE 3

in vitro immunisation with unconjugated okadaic acid

OA dosage (ng)	Incubation Time (days)	Medium	LPS	Hybridomas per well	IgM production[a] (%)
100	2	TCM	Difco	5-10	100
100	2	TCM	Difco	5-10	100
1000	2	mTCM	Difco	toxicity	ND
100	3	TCM	Difco	toxicity	ND
100	2	TCM	Sigma	toxicity	ND
50	2	TCM	Difco	10-15	ND
50	2	TCM	Sigma	10-15	ND
50	3	mTCM	Difco	1-2	ND
50	2	TCM	Difco	15-20	100
50	2	TCM	Sigma	15-20	100
50	2	mTCM	Difco	15-20	ND
50	2	mTCM	Sigma	15-20	ND

[a] IgM production = (no. wells with IgM/no. wells with hybrids) x 100
[b] Not determined

(d) Immunisation with conjugated okadaic acid (OA-HSA)

Toxicity was observed at a dosage of 19μg (no growth) and 6.3μg (only about 50 colonies) of an okadaic acid-human serum albumin conjugate. 2μg OA-HSA per stimulation caused strong blast cell formation and about 3000 hybridoma colonies were produced from 2 fusions. No hybridomas secreting okadaic acid-specific antibody were detected.

Problems were experienced with screening hybridomas produced by *in vitro* immunisation. These problems were due to high level of non-specific immunoglobulins reacting with the components of the screening assay (blocking reagents, plastics, carriers). These were especially acute when highly sensitive biotin-streptavidin system was used. A wide range of blocking reagents and plastics were tested. 0.25% fish skin gelatine (non-immunogenic protein) was found to be the most suitable blocking reagent. OA-PLL and PLL gave very strong non-specific binding. Salt used in high concentrations did not solve the problem. As a result of these studies we chose an ELISA based on affinity purified anti-immunoglobulins.

4. *in vivo* immunisation

(a) Immunisation with OA-HSA and TTX-BSA

Sample bleeds will be taken from the mice and tested for anti-TTX antibodies (the project ended before screening for OA-HSA antibodies was completed). Results of this screening are given in Table 4. It is clear an immune response developing antibodies to TTX was achieved in each of 4 mice. This was achieved using amounts of TTX (in a conjugated form) equivalent to 50 and 100 lethal units per mouse of native TTX. These results show that immune response was achieved with as little as 20μg of toxin and indicate that a high antibody titre can be achieved with small molecular weight toxins.

TABLE 4

Screening for TTX antibodies produced against TTX-BSA *in vivo*[a].

Mouse	1st Dose (LD_{50} units)	Booster (LD_{50} units)	Titre producing a response 2-fold that of background	Background response
	50	60	1/1600	0.09
2	50	60	1/1600	0.09
3	100	60	1/200	0.20
4	100	60	1/1600	0.10

[1] see section 16b for details of methods.

(b) Immunisation with BTX-KLH

This study was undertaken to detect possible difficulties with the immunisation and screening assay. About 500-600 hybridoma colonies grown in 45 wells were screened for anti-BTX antibodies. The optical density for a negative control was 0.06; for positive control 1.9. Ten wells with hybrids gave an optical density more than twice that of negative control. Twelve hybridomas secreting monoclonal antibodies were established. These MAbs, when tested in ELISA with different concentrations of BTX, gave uniformly high optical density. This non-specificity was due to effect of methanol on PVC plates. Immunoglobulins were bound strongly to methanol treated PVC plates and this binding was not blocked out by blockers (1%FCS, 1%BSA, blotto, 0.25% gelatine). As a result of this study, the concept of using ELISA plates coated with unconjugated hapten was abandoned.

APPENDIX V

PROCEDURES FOR THE PRODUCTION OF TOXIN-PROTEIN CONJUGATES.

Reactions 1 and 2 were performed at Griffith University under the guidance of Dr Ron Quinn and reaction 3 was performed at Southern Fisheries Centre (SFC) after initial discussions with Dr Geoffrey Pietersz (University of Melbourne). Reaction 4 was performed at SFC.

1. Succinilation using a model toxin in pyridine

Brevetoxin was reacted with a 10-fold excess of succinic anhydride in pyridine at 80°C with stirring for 2 hours. Yield of product was ~ 80% using ~ 100 µg sample (successful 2 of 3 attempts). Pyridine was freshly distilled in a closed system under N_2 over sodium wire. All other reagents dried under high vacuum before the reaction transfers were accomplished under N_2.

2. Succinilation using ciguatoxin in pyridine

Ciguatoxin (CTX-1, 50 µg) was reacted with 10-fold excess of succinic anhydride in pyridine at 80°C with stirring for 2 hours. No desired product was detected either by high performance liquid chromatography or radioactivity and only 25% and 80% of original ciguatoxin was recovered (2 attempts). Pyridine was prepared as above and succinic anhydride was either ^{14}C labelled or cold. The presence of succinic anhydride was confirmed by HPLC on completion of experiment, indicating water had not degraded all the succinic anhydride to unreactive succinic acid.

3. Succinilation of ciguatoxin using a catalyst

CTX-1 (10 µg) was reacted with a 10-fold excess of succinic anhydride in acetonitrile for 24 hours at room temperature. Dimethyl aminopyridine with or without triethylamine (used as supplied) were used in catalytic amounts. Acetonitrile was dried on a alumina column and the reaction performed under a N_2 blanket. Yield of the desired product and loss of ciguatoxin were monitored by HPLC and mouse bioassay. No reaction to the carboxylic acid derivate was observed (3 attempts). The presence of succinic anhydride and catalysts in the reaction mixture were confirmed by HPLC. Small losses of ciguatoxin occurred during each attempted reaction. In one reaction the conversation of CTX-1 to more polar product was observed. However, this product was rapidly converted to ciguatoxin when conditions were made more alkaline by the addition of more catalyst.

4. Coupling of brevetoxin derivatives and okadaic acid to proteins

The carboxylic acid derivative of brevetoxin and okadaic acid were routinely attached to a variety of proteins using water-soluble carbodlimides (Baden *et al.*, 1984).

APPENDIX VI

TITLE: Purification and characterisation of the major ciguatoxins from moray eel (Lycodontis javanicus, Muraenidae)[+]

AUTHORS: Richard J. Lewis,*[1] Michelle Sellin,[1] Mark A. Poli,[2] Raymond S. Norton[3] and John MacLeod[4]

LABORATORIES:

[1]Southern Fisheries Research Centre, QDPI, Deception Bay, Qld, 4508, Australia.
[2]Division of Pathophysiology, USAMRIID, Fort Detrick, Frederick, MD 21701, USA.
[3]School of Biochemistry, University of New South Wales, Kensington 2033, Australia.
[4]Research School of Chemistry, Australian National University, Canberra, ACT 2601, Australia.

RUNNING HEAD: Ciguatoxins from moray eel

SEND PROOFS TO: Southern Fisheries Research Centre
PO Box 76
Deception Bay, Queensland, 4508, Australia

[+]This study was supported by the Fishing Industry Research and Development Council, Australia.

ABSTRACT

Viscera (48.3 kg) from moray eels (Lycodontis javanicus) collected in a ciguatera endemic area were extracted and the ciguatoxins characterised. Three major ciguatoxins, CTX-1, CTX-2 and CTX-3, were isolated and purified to homogeneity on reverse phase H.P.L.C. Several minor toxins were also detected. CTX-1 (490 μg) was comparable to ciguatoxin isolated previously from moray eels by both NMR and FAB mass spectroscopy (MH^+ m/z = 1111.6). CTX-2 (280 μg) and CTX-3 (100 μg) were less polar ciguatoxins not previously characterised. CTX-2 and -3 differed from CTX-1 by 16 mass units, suggesting they were less oxygenated ciguatoxins. ^1H NMR revealed that the hydroxyl at C54 in CTX-1 was absent in CTX-2. An additional change in chemistry of CTX-2 compared to CTX-1 was also suggested on the basis of ^1H NMR. Such a difference between CTX-1 and CTX-2 would indicate that these toxins arise from different precursors. The i.p. LD_{50} values for CTX-1, -2 and -3 were 0.25, 2.3 and 0.9 μg/kg, respectively. The signs induced in mice by the ciguatoxins were similar except that CTX-2 and -3 induced hind-limb paralysis that was absent with CTX-1. Each ciguatoxin was orally potent. Each ciguatoxin competitively inhibited the binding of [^3H] brevetoxin-3 to voltage-dependent sodium channels with relative potencies qualitatively (but not quantitatively) comparable to mouse lethality. This study reveals that the relatively small chemical differences between CTX-1, CTX-2 and CTX-3 underlie significant structure-activity and pharmacokinetic differences.

INTRODUCTION

Ciguatera is the disease caused by the consumption of fishes contaminated by the ciguatoxin class of lipid-soluble toxins. It is characterised by short-term gastrointestinal and longer-term neurological disturbances (Gillespie et al., 1986). Ciguatera originates from tropical and sub-tropical waters where it can be a significant health problem, particularly in the atoll island countries of the Pacific Ocean. Although the disease was first attributed to a single entity named ciguatoxin (Scheuer et al., 1967), on the basis of clinical studies the involvement of several toxins in the ciguatera syndrome has not been discounted (Bagnis et al., 1974; Lewis et al., 1988a; Kodama and Hokama, 1989). Chromatographic studies on the toxic component of fishes have revealed the presence of several less-polar toxins that may be involved in ciguatera (Chungue et al., 1977; Lewis and Endean, 1984; Nukina et al., 1984; Vernoux and Talha, 1989; Legrand et al., 1990).

The structures of ciguatoxin (Murata et al., 1990) and its likely precursor from Gambierdiscus toxicus (gambiertoxin-4b) proposed by NMR and mass spectral measurements are shown in Figure 1. Ciguatoxin is a cyclic polyether resembling the brevetoxin class of toxins. In this paper we report the isolation and characterisation of the three major ciguatoxins found in moray eel viscera for which we propose the names CTX-1, CTX-2 and CTX-3. On the basis of comparison with the proposed structure of ciguatoxin and its congener gambiertoxin-4b (Murata et al., 1990), we propose a structure for CTX-2 (Figure 1).

EXPERIMENTAL PROCEDURES

Isolation and Purification of the CiguatoxIns. Viscera including livers were removed from moray eels (Lycodontis javanicus) collected from a ciguatera endemic region of Tarawa (1.3°N, 173°E) in the Republic of Kiribati, central Pacific Ocean. The lipid-soluble toxins were isolated as summarised in Figure 2 and portions of this material applied to wide-bore columns packed with T.L.C. grade silica gel (60 H, Merck). Typical columns were 10 cm diameter and packed to 4-5 cm bed height with ~ 150 g silica gel. The columns were prewashed with one W_b (W_b = volume of solvent proportional to the dry weight of the silica gel bed, on a g = ml basis) of methanol and 2 W_b of chloroform-methanol (c:m) 97:3 before sample application. After elution (vacuum assisted) with c:m mixtures of increasing polarity (97:3, 2 W_b; 95:5, 2 W_b; 9:1, 4 W_b; 8:2. 2 W_b and 0:1, 2 W_b) the major toxin containing fraction (c:m 9:1) was subjected to a further five different chromatographic steps (Sephadex LH-20, Pharmacia; TSK HW40S, Fractogel, Merck; 5 µm PRP-1 (150 x 4.1 mm), Hamilton; 5 µm C-18 (250 x 4 mm), Li ChroCART, Merck), before obtaining homogeneous ciguatoxins. Details of these steps including solvents, relative elution volumes and yields are indicated in Figure 2. Eluants were pumped through these columns using a 6000 A pump (Waters Associates) and HPLC eluants (PRP-1 and C-18) were monitored at 215 nm with a 481 detector (Waters Associates). Samples of the isolated ciguatoxIns were reapplied to HPLC columns (PRP-1 and C-18) eluted with different polarity solvents to confirm that the eluting material was homogeneous. A photodiode array detector (Waters Associates) was used to determine the u.v. profile for the ciguatoxins and to establish additional criteria of purity. Toxic zones were located and quantified by lethality to mice as described below.

Mouse Bioassay. Fractions to be tested were first dried under vacuum and further freed of solvent under a stream of N_2. Fractions were then resuspended in 0.5 ml of 5% Tween 60 saline and injected i.p. (and in some cases peroral) into 18-21 g Quackenbush strain mice (either sex) up to a maximum dose of 1 g of dried fraction weight per kg mouse body weight. Mice were housed at 23 ± 2°C and observed over 7 days and signs and times to death recorded. Rectal body temperatures were intermittently measured with a Comark electronic thermometer. Total lethality is expressed in mouse units (MU). One MU is

defined as the LD_{50} dose for a 20 g mouse. Fractions were considered non-toxic if injection of a maximal dose was not lethal. The lethality of extracts containing a mixture of the ciguatoxins were estimated using a dose vs death-time equation obtained for partially purified ciguatoxin (Lewis et al., in press):

$$\log (\text{dose}) = 2.3 \log (1 + t^{-1}) \quad (1)$$

where dose is in MU, and t = time to death in hr.

Separate dose vs time to death relationships were determined for the pure ciguatoxins. These results were analysed using the approaches of Molinengo (1979) and Tachibana (1980).

^{1}H NMR spectroscopy.

NMR spectra were recorded at 500 MHz on a Bruker AM-500 equipped with an Aspect 3000 computer and a process controller. The probe temperature (25°C) was maintained with a Bruker B-VT 1000 unit and 5nm outside diameter spinning sample tubes were used. Spectral acquisition parameters were as follows: sweep width 5208 Hz, 8192 data points, 60° radio frequency pulses, 2.8 - 3.25 recycle time and 250-512 accummulations. Data were processed and plotted on an IRIS 4D/20 workstation using the program FTNMR from Hare Research, Woodinville, WA. Resolution enhancement was effected by a Lorentzian-Gaussian transformation, and data were zero-filled to 16,384 points prior to former transformation. Chemical shifts are expressed in ppm downfield from TMS, but were measured using the pyrldine-d_5 resource at 7.21 ppm as internal standard.

FAB mass spectra.

High resolution fast atom bombardment mass spectra (FAB-MS) and FAB-MS/MS were obtained at the Australian National University facility.

Binding Studies. Rat brain membranes were prepared according to the procedure of Davio and Fontelo (1984). [^{3}H] PbTx-3 (14.4 Ci/mmole) was prepared by Dr D.G. Baden (University of Miami, FL) by the reduction of PbTx-2 with [^{3}H] sodium borohydride, as previously reported (Poli et al., 1986). Purity was greater than 99%, as determined by HPLC analysis. A stock solution of 1.3 µg/ml in methanol was stored at -10°C and dilutions made immediately prior to use. The assay was performed in a binding medium consisting of (mM): HEPES, 50.0 (pH 7.4); choline chloride, 130.0; glucose, 5.5; magnesium sulfate, 0.8 and potassium chloride, 5.4. The binding medium contained 1 mg/ml bovine serum albumen (BSA) and 0.01% Emulphor EL-620. Emulphor EL-620 (GAF Corp., New York) is a nonionic emulsifier used to aid in solubilising hydrophobic toxins. Rat brain membranes (125 - 150 µg) suspended In 0.1 ml binding medium were added to 1.5 ml polypropylene microcentrifuge tubes containing [^{3}H] PbTx-3 (0.5 nM final concentration) and increasing concentrations of CTX-1, CTX-2 or CTX-3 in 0.9 ml binding medium. After incubation for 1 hr at 4°C, samples were centrifuged for 5 minutes at 15,000 x *g*. Supernatants were aspirated and the pellets quickly washed twice with several drops of ice-cold wash medium containing 5.0 mM calcium chloride, 0.8 mM magnesium sulfate, and 1 mg/ml BSA. The pellets were transfered to scintillation vials and the bound radioactivity measured with a Beckman liquid scintillation counter (Beckman Instruments, Inc., Fullerton, CA). Data points are the means of triplicate assays. Non-specific binding was measured in the presence of a saturating concentration of unlabelled PbTx-3 (1 µM) and subtracted from total binding to yield specific binding. To obtain double reciprocal plots, control binding of [^{3}H] PbTx-3 (10^{-8} - 10^{-9}M) was compared with similar binding in the presence of two doses of each of the ciguatoxins. Free [^{3}H] PbTx-3 was determined by counting an aliquot of the supernatant prior to aspiration. The cross reactivity of CTX-1, CTX-2 and CTX-3 was determined to a polyclonal antibody to PbTx-3 raised in goats (Poli, et al., ?). Best-fit curves of Inhibition of binding to rat brain membranes were generated by Fig P (Biosoft, Milltown, NJ).

Materials Used. The solvents, acetonitrile, isopropanol, chloroform, methanol (Waters Associates, Lane Cove, Australia) n-hexane and water (Mallinckrodt, Clayton, Australia) were HPLC grade. Pyridine-d_5 (Cambridge Isotope Laboratories) containing 99.96% deuterium was used immediately after opening.

All other reagents were A.R. grade or equivalent.

Hazardous Procedures. The ciguatoxins isolated are extremely toxic to mammals, particularly humans. Care must be exercised to avoid exposing the eyes, nose and lips to the toxins, especially highly purified material. Drying from an aqueous medium should be done without the formation of an ice plug to avoid the production of a loose powder that can cause severe throat irritation (a burning, menthol-like effect) lasting for 24 hr (without any systemic effects). There were indications that CTX-3 and one of the minor toxins were volatile.

RESULTS

Isolation and purification of ciguatoxins. Ciguatoxins were isolated from 48.3 kg of moray eel viscera and purified as described in Figure 2. The ciguatoxins eluted as a single toxic fraction, which separated into the more polar CTX-1 and the less polar CTX-2 and CTX-3 on reverse phase HPLC (Figure 3) (the nomenclature used reflecting the order of elution from a PRP-1 column). The elution of CTX-1 on a Merck C-18 column was comparable with that previously reported for ciguatoxin (Legrand et al., 1989). On the basis of total toxicity the yield of ciguatoxins was estimated to be >85% at each chromatographic step. CTX-1, -2 and -3 were obtained as white amorphous solids in yields of 490 μg, 280 μg and 100 μg, respectively. Purity of each ciguatoxin was determined to be >95% by HPLC. Purity was further confirmed analytically using a range of solvent systems on PRP-1 HPLC. Monitoring HPLC eluants with a photodiode array u.v. detector revealed that the ciguatoxins possessed only end absorption (see Figure 3). For CTX-1, comparing the u.v. profiles during upslope and downslope of the eluting peak with the profile at the apex (100%) revealed a high degree of similarity (98% and 99%, respectively), confirming this peak was homogeneous. The ciguatoxins are chromatographically distinct from the more polar maitotoxins produced by G. toxicus (Holmes et al., 1990). The ratio of the 3 ciguatoxins was constant over a period when moray eels were becoming progressively less toxic. If moray eels excrete ciguatoxin, as has been recently proposed (Lewis et al., in press) this result would indicate these ciguatoxins may be lost from eels at similar rates.

Several minor toxins (presumably ciguatoxins) were also detected in side fractions from the PRP-1 column. In addition, a volatile toxin was detected in a side fraction from the C-18 column and a toxin causing maitotoxin-like signs (Lewis et al., 1988b) was detected in the methanol eluate from the low pressure silica gel column. Each of these minor toxins were obtained in low yield (on a MU basis) and were not further characterised. Minor toxins were not detected in side fractions from Sephadex LH-20 or TSK HW40S columns or in the isopropanol wash of the PRP-1 column.

^{1}H NMR. Figure 4 shows the 500 MHz NMR spectra of CTX-1 and CTX-2 in pyridine-d_5. The spectrum

for CTX-1 was comparable with the spectra of ciguatoxin obtained previously (Nukina et al., 1984; Murata et al., 1990). The NMR spectrum of CTX-2 (Figure 4b) resembled that of CTX-1, with differences in spectra interpreted on the basis that the structure proposed for ciguatoxin and gambiertoxin-4b are correct (Murata et al., 1990). The absence of a signal at 6.53 ppm indicates that OH-54 in CTX-1 was absent in CTX-2. This was supported by the observation that the signal for H-54 also appeared altered in CTX-2 (1.76 and 2.10 ppm) compared to CTX-1 (4.86 ppm). In addition, the chemical shifts for the three methyls in this portion of CTX-2 (rings K, L, M) were shifted varying amounts (58, 1.33; 59, 1.21 and 60, 0.95 ppm) compared to gambiertoxin -4b (58, 1.29; 59, 1.28 and 60, 0.97 ppm) assigned in Murata et al. (1990). The chemical shift of H-48 in CTX-2 was also observed to have shifted 0.4 ppm upfield to 3.63 ppm compared to gambiertoxin -4b (revealed in a HOHAHA spectrum of CTX-2, unpublished result). These latter results indicate further structural differences (presumably stereochemical in nature) between CTX-1 and CTX-2, in addition to the loss of OH-54. Full spectral analysis are in preparation.

Mass Spectra. Details of the HR-FAB mass spectra obtained are shown in Table 1. CTX-2 and CTX-3 differ from CTX-1 by the loss of 16 mass units. CTX-1 can lose up to 2 H_2O, while CTX-2 and CTX-3 clearly show the loss of only one H_2O. CTX-2 was considerably less easily detected in FAB mass spectroscopy compared to CTX-1 and 3.

Mouse Bioassay. The i.p. injection of CTX-1, CTX-2 and CTX-3 into mice induced signs typical of ciguatoxin (Lewis and Endean, 1984; Lewis et al., 1988b), except that CTX-2 and -3 produced marked hind-limb paralysis rarely seen with CTX-1 (Table 2). CTX-1 had an i.p. LD_{50} of 0.25 μg/kg and the order of potency was CTX-1 > CTX-3 > CTX-2 (Table 2). The dose vs time to death relationships for the major ciguatoxins are shown in Figure 5. The response of male and female mice to CTX-1 were comparable. The ciguatoxins differed in the minimum time to death, with the less polar toxins taking longer to kill mice (Table 2). Low doses of CTX-2 caused particularly long times to death of up to 4 days compared to CTX-1 which typically kills mice in less than 24 hr. CTX-1 had similar minimum and maximum times to death as the ciguatoxin from Spanish mackerel and barracuda (Lewis and Endean,

1984). The equations describing the dose vs time to death relationships for the purified ciguatoxins are:

$$\text{CTX-1;}\ \log(\text{dose}) = 3.3 \log(1 + t^{-1}) \quad (2)$$

$$\text{CTX-2;}\ \log(\text{dose}) = 2.4 \log(1 + t^{-1}) \quad (3)$$

$$\text{CTX-3;}\ \log(\text{dose}) = 3.9 \log(1 + t^{-1}) \quad (4)$$

where dose is in MU, and t = time to death in hr. For CTX-1, CTX-2 and CTX-3, one MU is 5 ng, 46 ng and 18 ng of toxin, respectively. Each mouse injected with a lethal dose of CTX-1, CTX-2 or CTX-3 rapidly developed hypothermia. Peroral injection of 2 MU of each of the ciguatoxins was lethal to mice.

Binding Studies. Each of the CTX's inhibited the binding of [^{3}H] PbTx-3 to rat brain membranes (Figure 6). The ED_{50} values for CTX-1, CTX-2 and CTX-3 were 0.23, 0.85 and 0.43 ng/ml, respectively. Double reciprocal plots revealed that this inhibition was competitive, with all regressions intercepting at a theoretical infinite dose of [^{3}H] PbTx-3 (Figure 7). Hill coefficients for [^{3}H] PbTx-3 binding ranged from 0.9 to 1.1 either in the absence or presence of the CTX's. The CTX's did not cross-react with a polyclonal antibody directed to PbTx-3 (Figure 8). The affinity of the CTX's for the antibody was at least 300-fold less than the affinity of the antibody for PbTx-2.

DISCUSSION

Three ciguatoxins, CTX-1, CTX-2 and CTX-3, have been isolated from moray eel viscera and characterised. These toxins, which include two previously unreported ciguatoxins (CTX-2 and CTX-3), were related both chemically and biochemically. The relative yields by weight of pure CTX-1, CTX-2 and CTX-3 were 1 : 0.6 : 0.2, indicating that each of these toxins is present in considerable amounts in moray eels. However, the relative yields for these toxins on the basis of total mouse toxicity were 1 : 0.06 : 0.06, indicating that CTX-1 is the principal toxin. The relative potency of these ciguatoxins in humans must be established to determine the relative role of each in the ciguatera syndrome.

One-dimensional ^{1}H NMR (500 MHz) and FAB mass spectral measurements revealed that CTX-1 was comparable to ciguatoxin isolated by Murata et al. (1990). Mass spectra indicated that CTX-2 and CTX-3 each had one oxygen less than CTX-1. The sensitivity for CTX-2 in positive ion FAB mass spectra was poor compared to CTX-1 and CTX-3. ^{1}H NMR spectra of CTX-2 clearly indicated that the loss of a hydroxyl was at the C54 position. Compared with CTX-1, a change in stereochemistry in ring K, L or M (see Fig. 1) was indicated on the basis of observed shifts for hydrogens in this region.

The ciguatoxins are all highly potent toxins in mice by either the i.p. or oral route. The ratio of the potency (LD_{50}^{-1}) of CTX-1, CTX-2 and CTX-3 in mice is 1 : 0.11 : 0.28. This order of potency does not reflect the order of elution on reverse phase HPLC. CTX-2 is an order of magnitude less potent in mice than CTX-1, indicating that the loss OH-54 and/or the altered stereochemistry may have important consequences for toxicity. Binding studies determining the ability of the ciguatoxins to displace [^{3}H] PbTx-3 binding to sodium channels showed the same order of potency as seen for the ciguatoxins in mice. However, the differences in the binding inhibition potencies (1 : 0.27 : 0.53) were not as large as the differences in toxicity in mice. This suggests that pharmacokinetic effects, in addition to differences in absolute sodium channel binding affinity, contribute to the marked differences in mouse toxicity. Structural differences between the ciguatoxins may also contribute to the differences in the signs and times to death observed in mice for the different ciguatoxins. CTX-2 and CTX-3 cause longer times to death than CTX-1, and unlike CTX-1 they induce hind-limb paralysis reminiscent of the gambiertoxins (Holmes et al., in press). The delayed effects of the less-polar ciguatoxins are reminiscent of the delayed effects that can follow the consumption of parrotfish (Bagnis et al., 1974) which also contain less polar ciguatoxins (Chungue et al., 1977).

Ciguatoxin is a potent sodium channel activating toxin (Bidard et al., 1984; Gillespie et al., 1986; Lewis & Endean, 1986). Lombet et al. (1987) showed that CTX-1 ciguatoxin inhibits [^{3}H] PbTx-3 binding and that this inhibition appeared competitive at the single dose of ciguatoxin used. In this study we have shown that the three ciguatoxins inhibit the binding of [^{3}H] PbTx-3 to voltage-dependent sodium channels and have confirmed that the ciguatoxin class of toxins are competitive inhibitors of brevetoxin

binding. This result indicates that all three ciguatoxins share a common binding site with the brevetoxins. Despite the apparent close chemical and biochemical similarities between the ciguatoxins and brevetoxins, no cross-reactivity was found between the ciguatoxins and a polyclonal antibody to PbTx-3. Antibodies produced against related compounds that cross-react with the ciguatoxins would be useful in the development of an assay for ciguatoxins in fish. Cross-reacting antibodies may be obtained using PbTx-1 which has a different backbone from PbTx-3. Alternatively, immunisation with a hapten-protein complex coupled through different portions of the brevetoxins may yield cross-reacting antibodies. Antibodies that cross-react with ciguatoxin and more readily available compounds (eg the brevetoxins) would allow the establishment of a competitive ELISA assay for detecting ciguatoxin in fishes.

The clguatoxins in fishes are likely to be derived from less oxygenated precursors named gambiertoxins found in G. toxicus (Murata et al., 1990; Holmes et al., in press). Oxidation of the gambiertoxins to the clguatoxlns is likely to proceed through the cytochrome system in the livers of fish. If CTX-2 does indeed have altered stereochemistry it would indicate that the origin of this ciguatoxin is not gamblertoxin-4b (Murata et al., 1990) and that the different ciguatoxins found in fish do not all originate from a single precursor.

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Table 1. HR-FAB mass spectra of the major ciguatoxins.

Toxin	MH^+ (m/z)	Sensitivity	Fragment ions[a]
CTX-1	1111.6	+ + + +	$MH^+ - H_2O$, $MH^+ - 2H_2O$
CTX-2	1095.4	+	$MH^+ - H_2O$ ($MH^+ - 2H_2O$)
CTX-3	1095.5	+ + +	$MH^+ - H_2O$

[a] Indicated are the fragment ions detected after low and high energy collisions to decompose the ciguatoxins from a glycerol or a 3-nitrobenzyl alcohol matrix. Assignment in parenthesis could not be confirmed.

Table 2 **Responses[a] of mice to i.p. injection of CTX-1, CTX-2 or CTX-3.**

Toxin	LD_{50} (μg/kg)	Minimum[b] time to death (min)	Maximum time to death (hr)	Diarrheoa	Hyper-salivation	Lachry-mation	Hind[d] limb paralysis
CTX-1[c]	0.25	37	<24	10/10	3/10	4/10	0/10
CTX-2	2.3	53	100h	6/6	5/6	4/6	6/6
CTX-3	0.9	60	>24hr	6/7	3/7	1/7	5/7

[a] Data from mice used for figure 4.
[b] Estimated using the method described by Molinengo (1979).
[c] For CTX-1 it was determined that the LD_{50}, death time relationship and signs were comparable for ♂ and ♀ mice.
[d] In separate experiments occasional mice displayed hind limb paralysis after CTX-1.

FIGURE LEGENDS

Figure 1.

Structure of CTX-1 and CTX-2 and gambiertoxin-4b. The structures of CTX-1 and CTX-2 are proposed on the basis of NMR and mass spectra by comparison with the structure proposed for ciguatoxin and gambiertoxin-4b (Murata et al., 1990).

Figure 2.

Purification scheme used to isolate the three major ciguatoxins from the viscera of moray eels. Toxic fractions were detected using the mouse bioassay and u.v. detection (215 nm). Yields at each step are indicated.

a. Toxin yield is expressed in mouse units (MU)

b. side fractions containing a mixture of the three major ciguatoxins

c. side fraction causing maitotoxin-like effects in mice

d. elution volume/column bed volume (V_e/V_b)

Figure 3.

HPLC separation of CTX-1, CTX-2 and CTX-3. Shown is a typical separation of a fraction (0.2 mg) containing a mixture of CTX-1 (8 μg), CTX-2 (5 μg), CTX-3 (2 μg) by PRP-1 reverse phase HPLC monitored at 215 nm (eluant acetonitrile - H_2O, 1:1). Bars indicate the zone collected for each of the ciguatoxins. Insert A shows a final purification step for CTX-1 (0.4 μg) which is seen (at 215 nm) eluting from the Merck C-18 column at 17 minutes (eluant acetonitrile - H_2O, 1:1). Insert B shows the u.v. profile for CTX-1 (0.5 μg applied to a PRP-1 column) measured with a photodiode array detector at the apex of the eluting peak. HPLC conditions are described in "Methods". Absorbance units are indicated on all vertical scales.

Figure 4.

^{1}H NMR spectra at 500 MHz of CTX-1 (490 μg) and CTX-2 (280 μg) in pyridine-d_5 at 25°C. (A) CTX-1. (B) CTX-2.

Figure 5.

Relationship between i.p. dose in mice and time to death for CTX-1, CTX-2 and CTX-3. (▽, o) CTX-1 in ♀ and ♂ mice, respectively. (□, Δ) CTX-2 and CTX-3, respectively, in ♂ mice. Solid lines indicate best fit curves obtained as described in "Methods".

Figure 6.

Inhibition of [^{3}H] PbTx-3 binding to rat brain membranes by CTX-1, CTX-2 and CTX-3. The ED_{50} values are CTX-1, 0.23 μg/ml; CTX-2, 0.85 μg/ml; CTX-3, 0.43 μg/ml. Data are presented as % bound (B) of maximal binding (Bo) of [^{3}H] PbTx-3 (mean of triplicates).

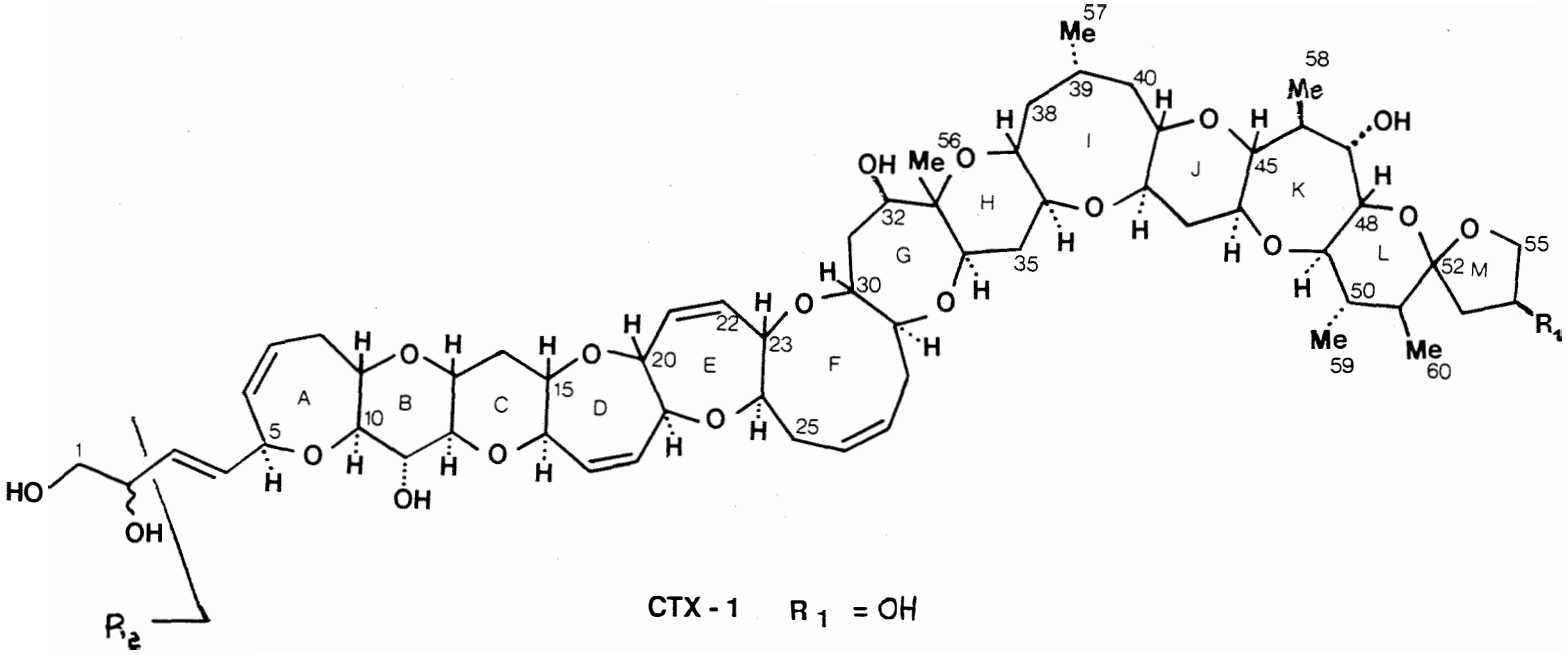
Figure 7.

Double reciprocal plots showing competitive inhibition of [^{3}H] PbTx-3 binding by CTX-1, CTX-2 and CTX-3. (A) shows the influence of CTX-1 (O, 0.125 μg/ml; □, 0.25 μg/ml). (B) shows the influence of CTX-2 (O, 0.5 μg/ml; □ 0.9 μg/ml). (C) shows the influence of CTX-3 (O, 0.15 μg/ml; □ 0.3 μg/ml). (●)Control [^{3}H] PbTx-3 binding. Bound and free concentrations of [^{3}H] PbTx-3 are measured in CPM.

Figure 8.

Influence of CTX-1, CTX-2, CTX-3 and PbTx-2 on the binding of ^{3}[H] PbTx-3 to a polyclonal antibody. (O) CTX-1, (□) CTX-2, (Δ) CTX-3 (▽) PbTx-2. Data presented as % bound (B) of maximal binding (B_o) of [^{3}H] PbTx-3 (mean of triplicates). The polyclonal used was raised against PbTx-3 in a goat.

FIG 1



CTX - 1 R_1 = OH

CTX - 2 R_1 = H

GT-4b R_1 = H

R_2 = $^1CH_2$=CH–

FIG. 2

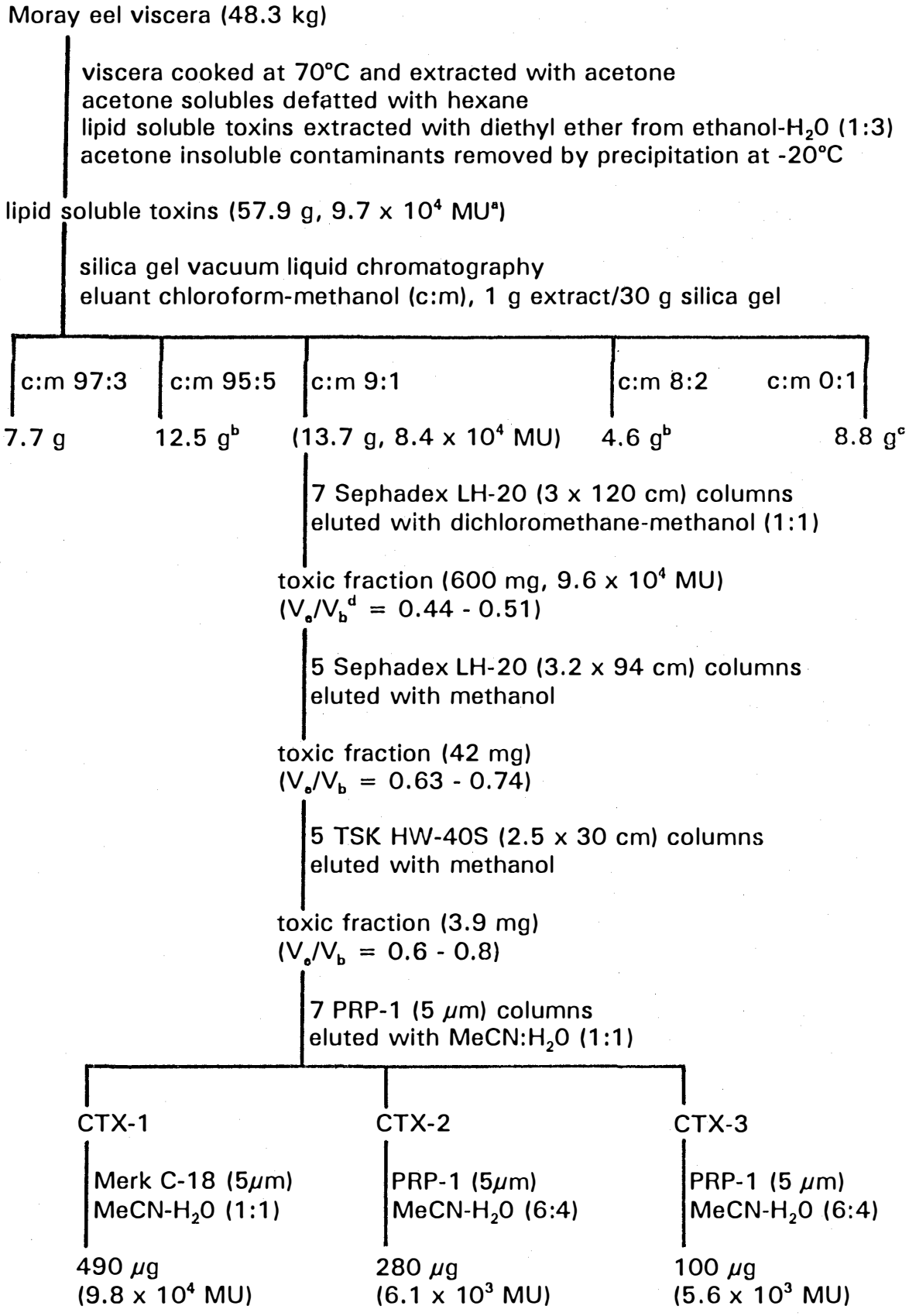

Moray eel viscera (48.3 kg)
viscera cooked at 70°C and extracted with acetone
acetone solubles defatted with hexane
lipid soluble toxins extracted with diethyl ether from ethanol-H2O (1:3)
acetone insoluble contaminants removed by precipitation at -20°C
lipid soluble toxins (57.9 g, 9.7 x 10^4 MU[a])
silica gel vacuum liquid chromatography
eluant chloroform-methanol (c:m), 1 g extract/30 g silica gel
c:m 97:3
c:m 95:5
c:m 9:1
c:m 8:2
c:m 0:1
7.7 g
12.5 g[b]
(13.7 g, 8.4 x 10^4 MU)
4.6 g[b]
8.8 g[c]
7 Sephadex LH-20 (3 x 120 cm) columns
eluted with dichloromethane-methanol (1:1)
toxic fraction (600 mg, 9.6 x 10^4 MU)
(Ve/Vb[d] = 0.44 - 0.51)
5 Sephadex LH-20 (3.2 x 94 cm) columns
eluted with methanol
toxic fraction (42 mg)
(Ve/Vb = 0.63 - 0.74)
5 TSK HW-40S (2.5 x 30 cm) columns
eluted with methanol
toxic fraction (3.9 mg)
(Ve/Vb = 0.6 - 0.8)
7 PRP-1 (5 μm) columns
eluted with MeCN:H2O (1:1)
CTX-1
CTX-2
CTX-3
Merk C-18 (5μm)
MeCN-H2O (1:1)
PRP-1 (5μm)
MeCN-H2O (6:4)
PRP-1 (5 μm)
MeCN-H2O (6:4)
490 μg
(9.8 x 10^4 MU)
280 μg
(6.1 x 10^3 MU)
100 μg
(5.6 x 10^3 MU)

Fig. 3

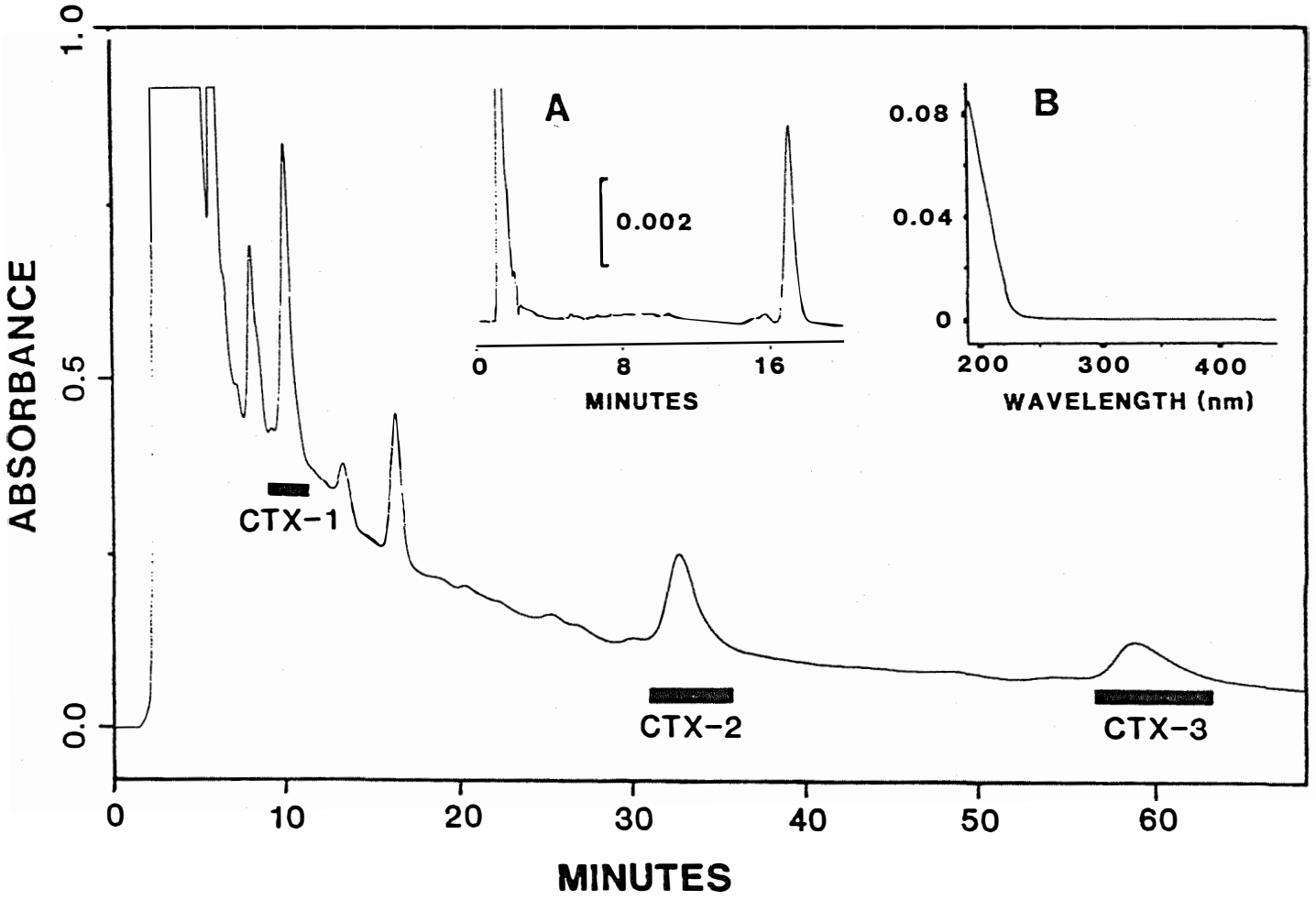

ABSORBANCE
1.0
0.5
0.0
A
0.002
0
8
16
MINUTES
B
0.08
0.04
0
200
300
400
WAVELENGTH (nm)
CTX-1
CTX-2
CTX-3
0
10
20
30
40
50
60
MINUTES

FIG. 4 A

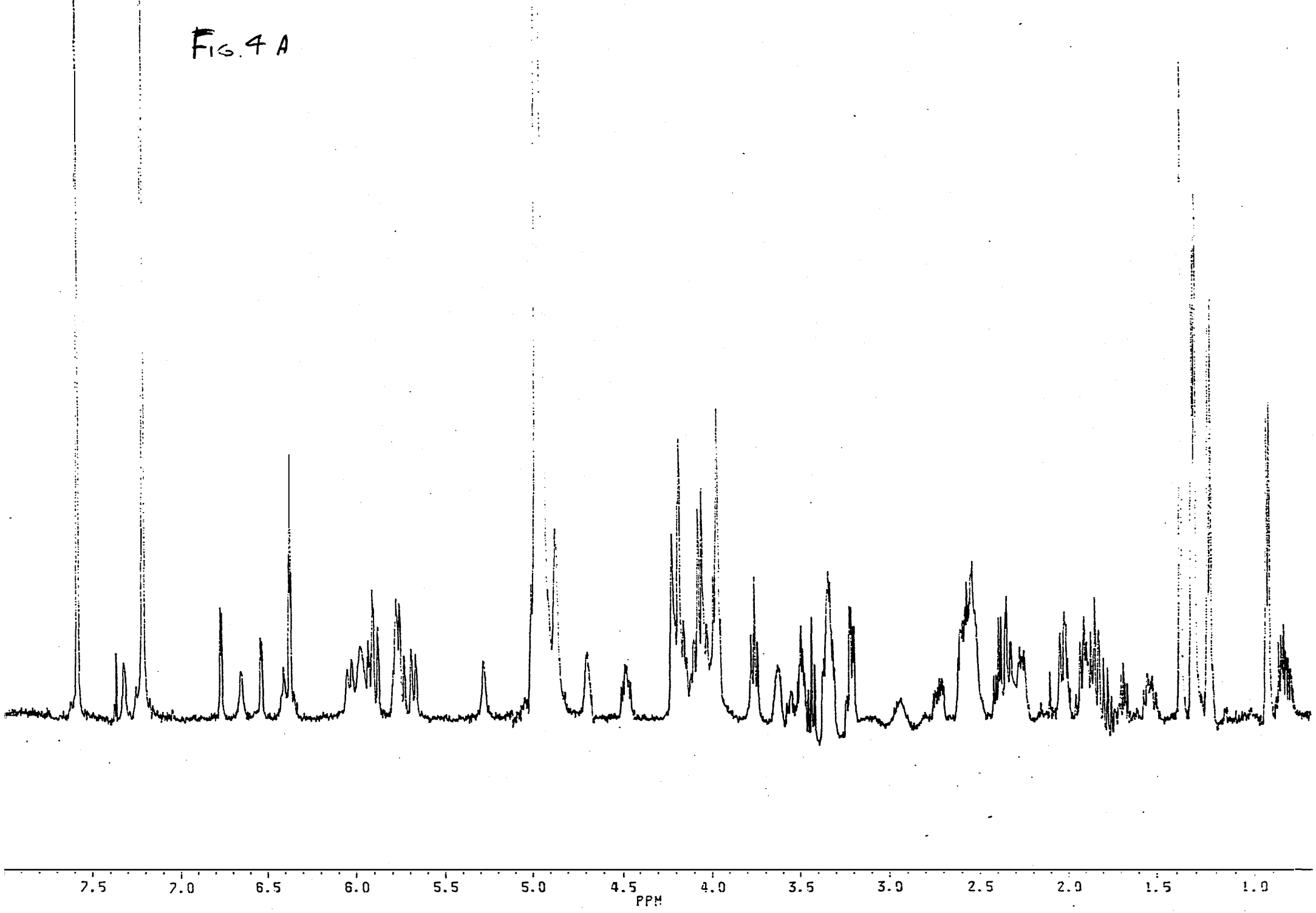
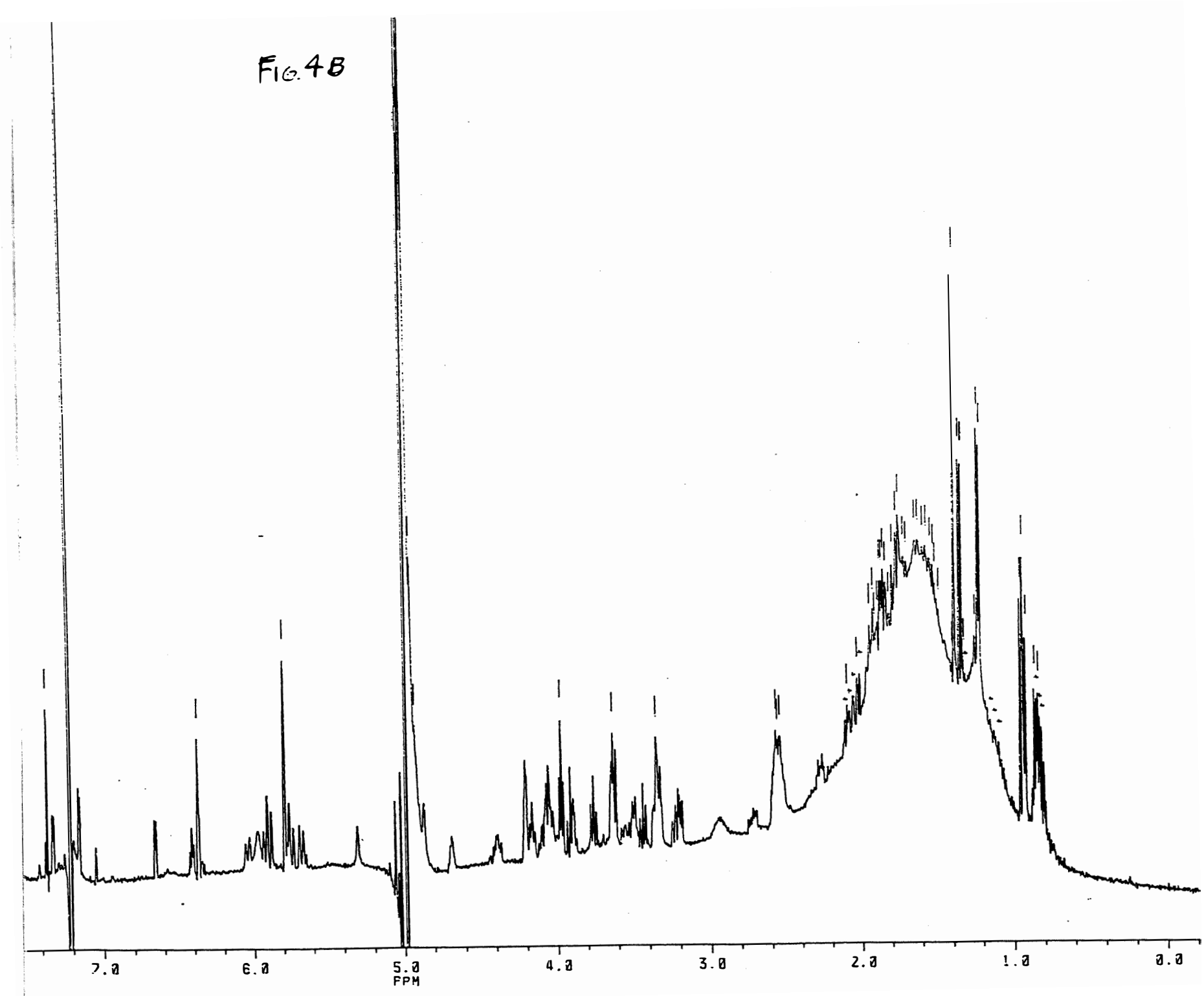


FIG. 4B



BRUKER

CTX210.001
GU PROG.
PRESAT.GU
DATE 6-10-90

SF 500.135
01 7151.000
SI 16384
TD 8192
SW 5208.333

PW 4.0
AQ .786
RD 30
NS 512
TE 298

02 9027.000
DP 65L FO

LB -3.000
GX 40.00
F1 10.199P
F2 -.204P
SR 4660.65

FIG. 5

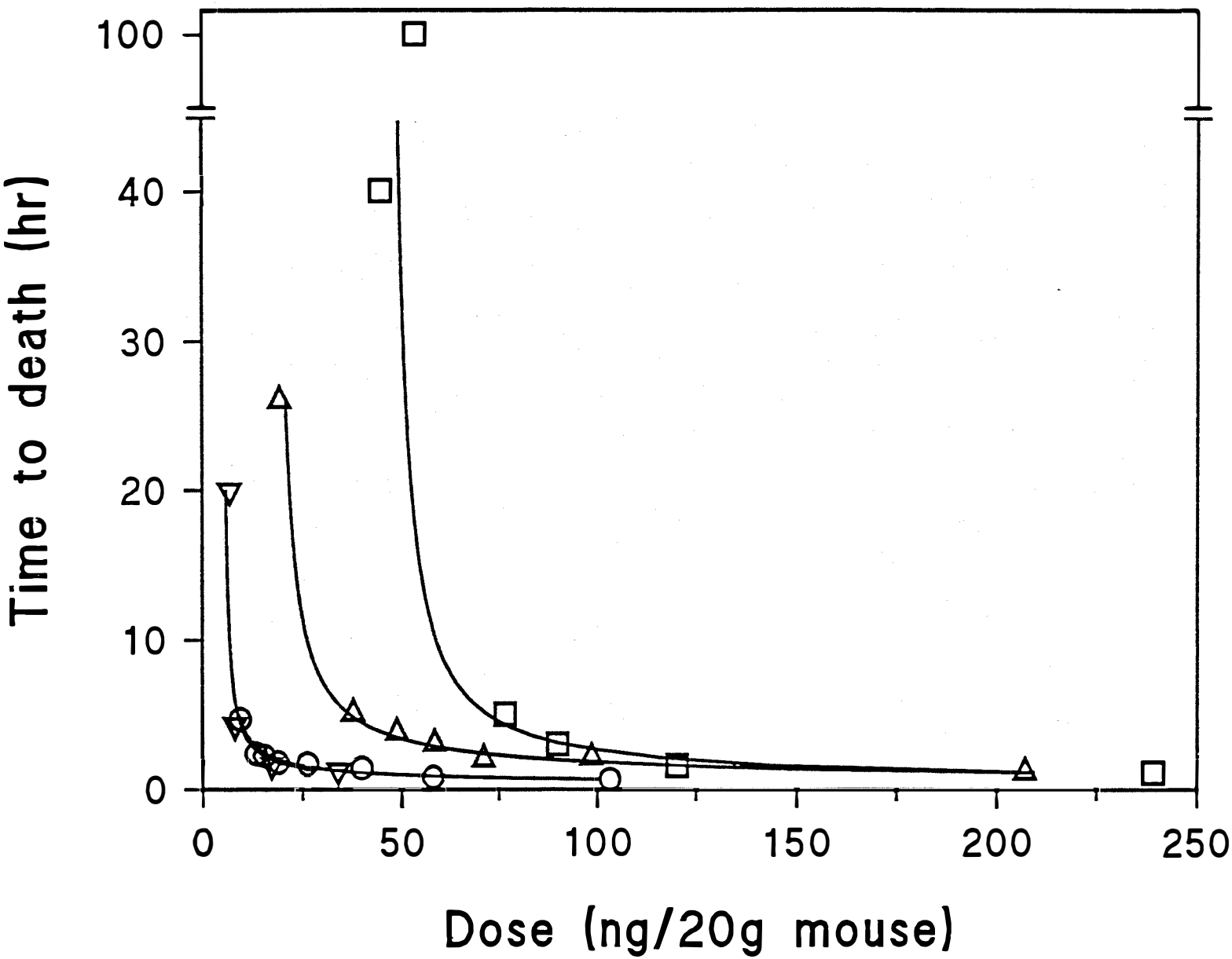

100
40
30
20
10
0
Time to death (hr)
0
50
100
150
200
250
Dose (ng/20g mouse)

FIG. 6

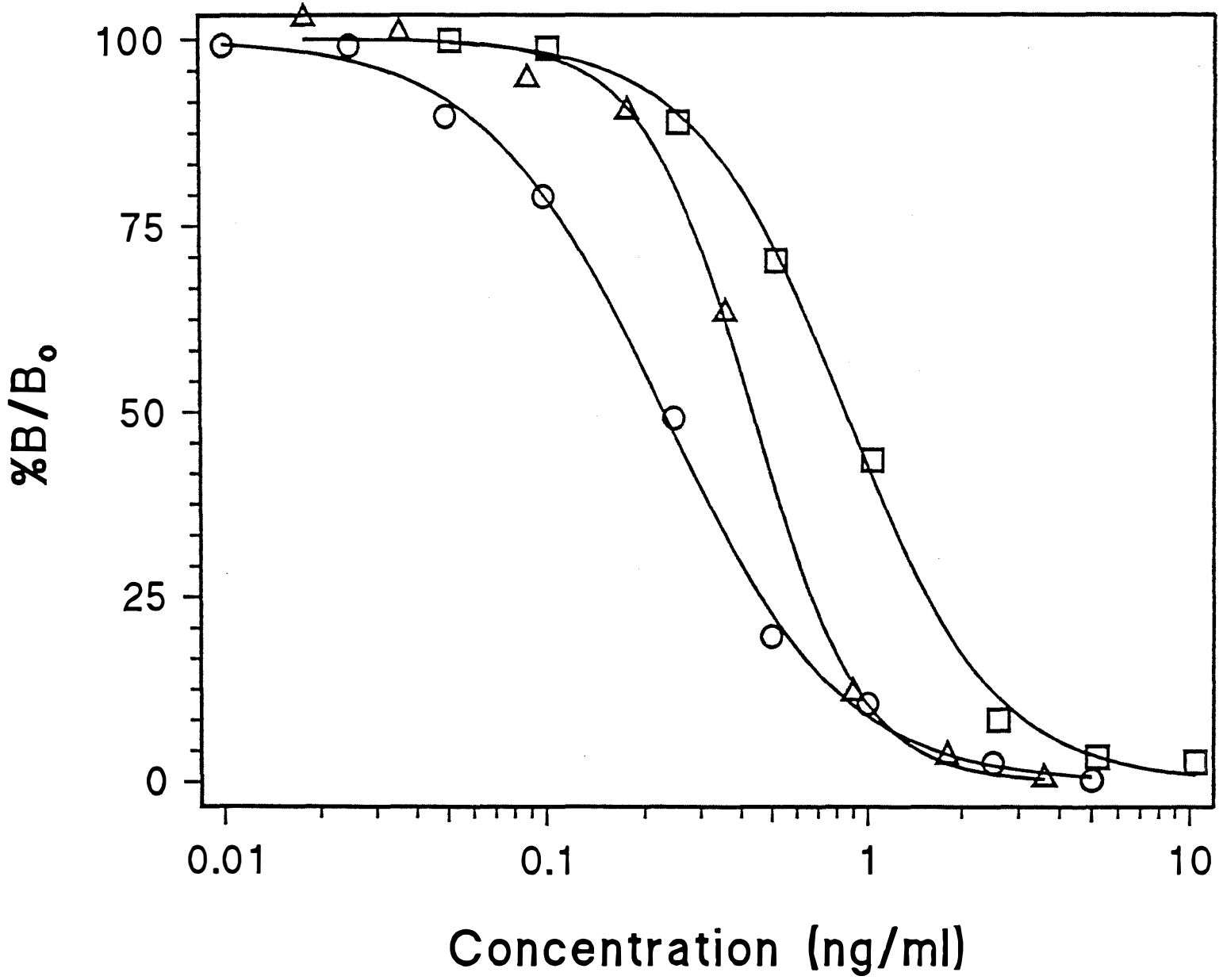


FIG. 7 A.

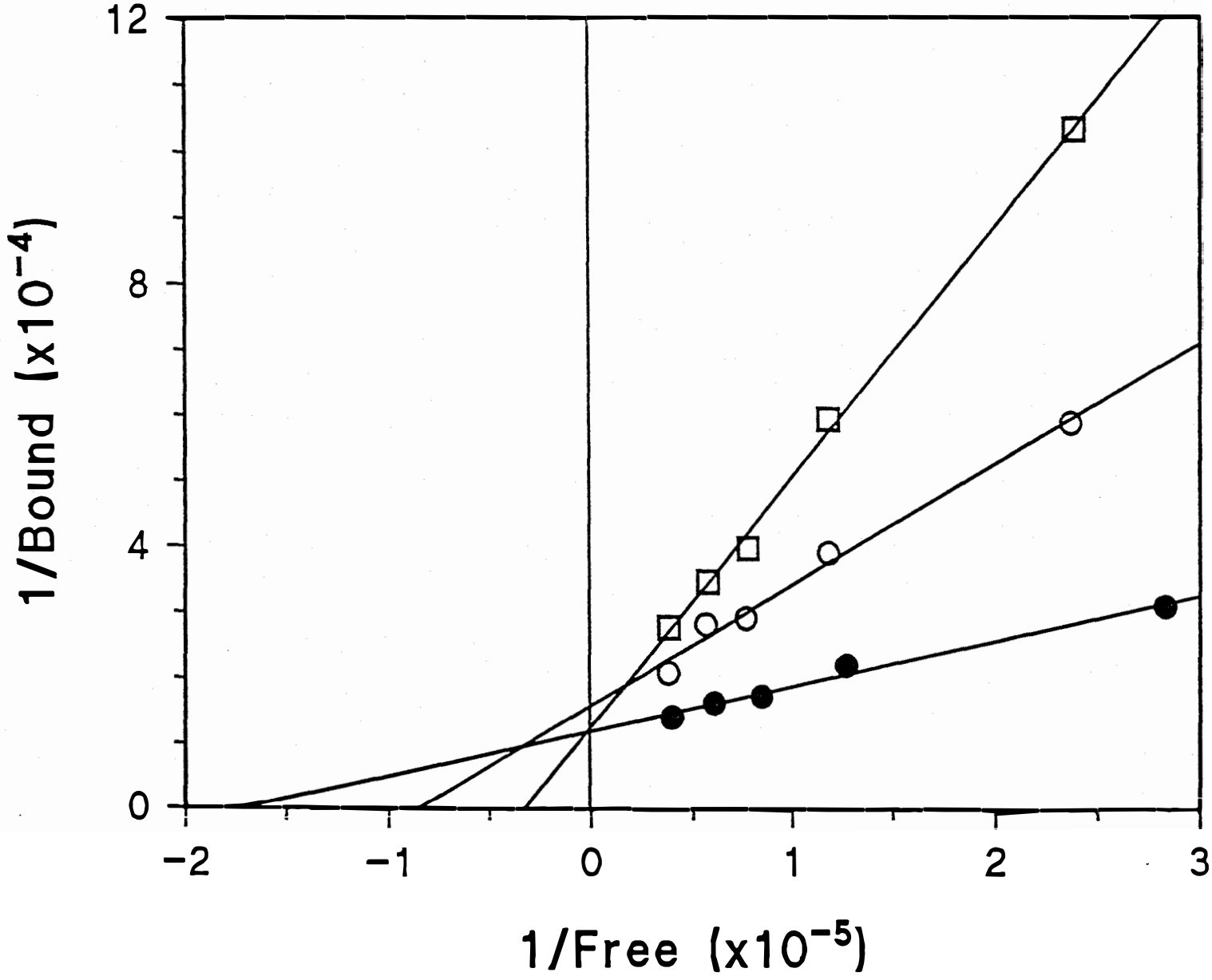


Fig. 7 B.

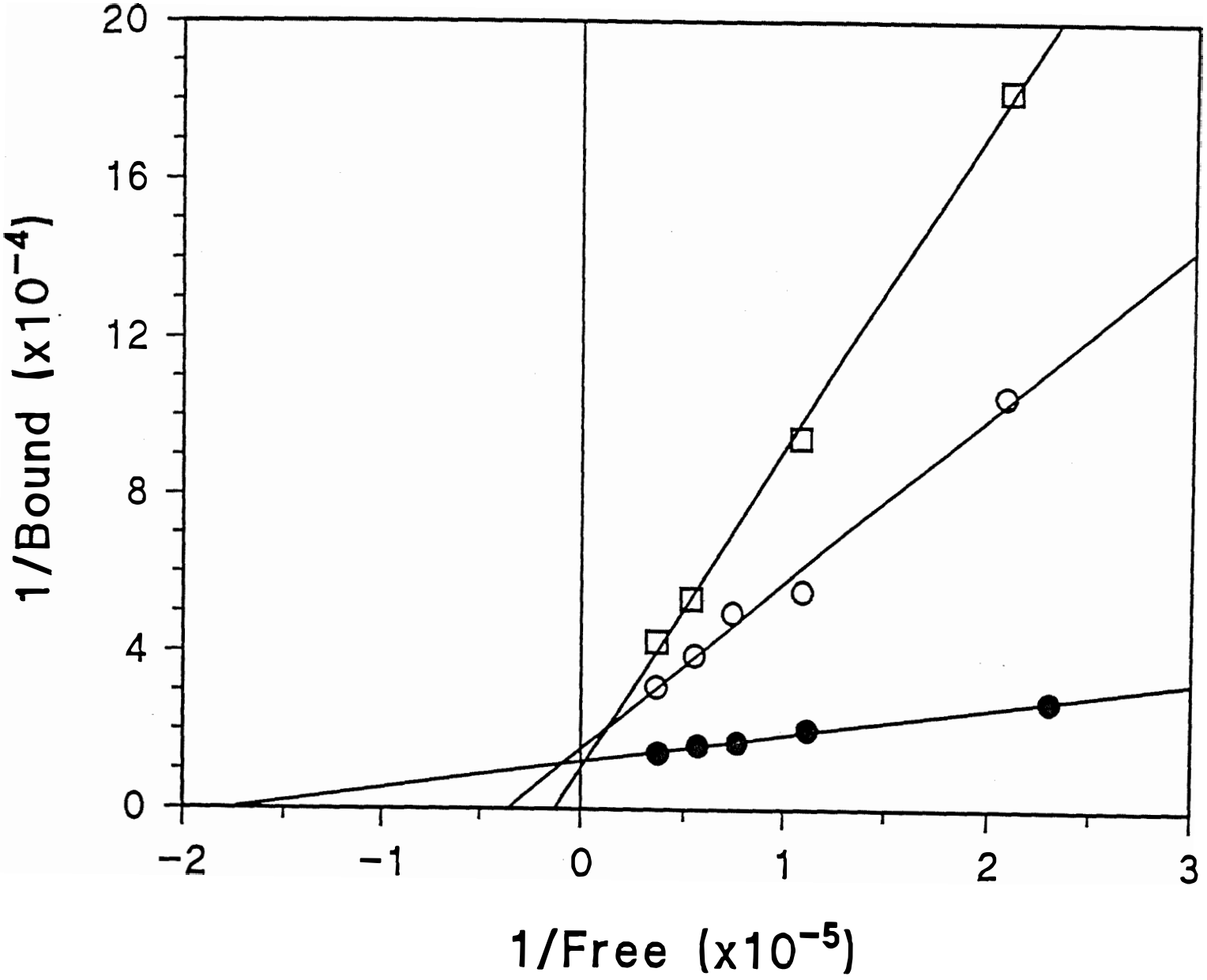


FIG. 7 C.

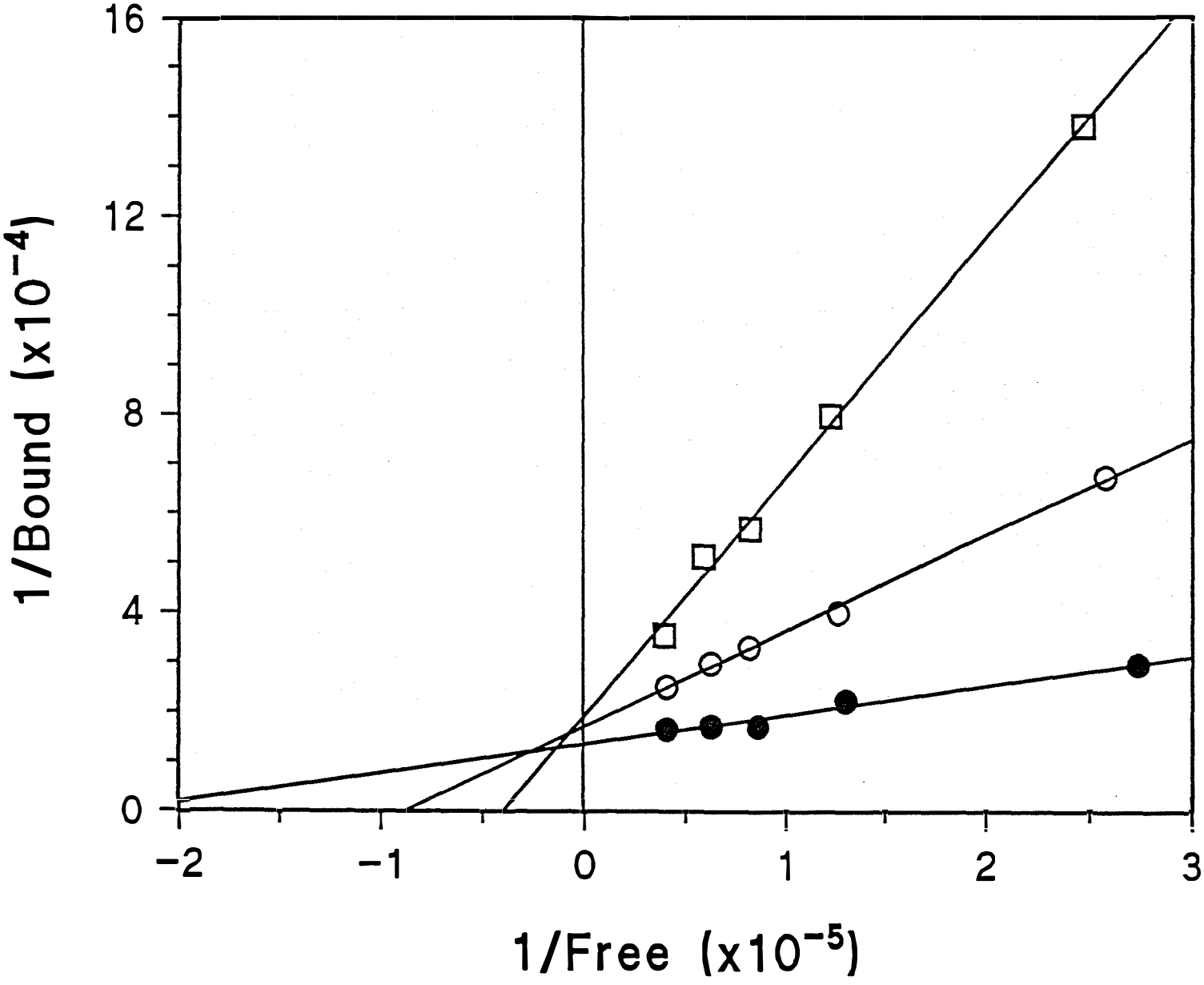
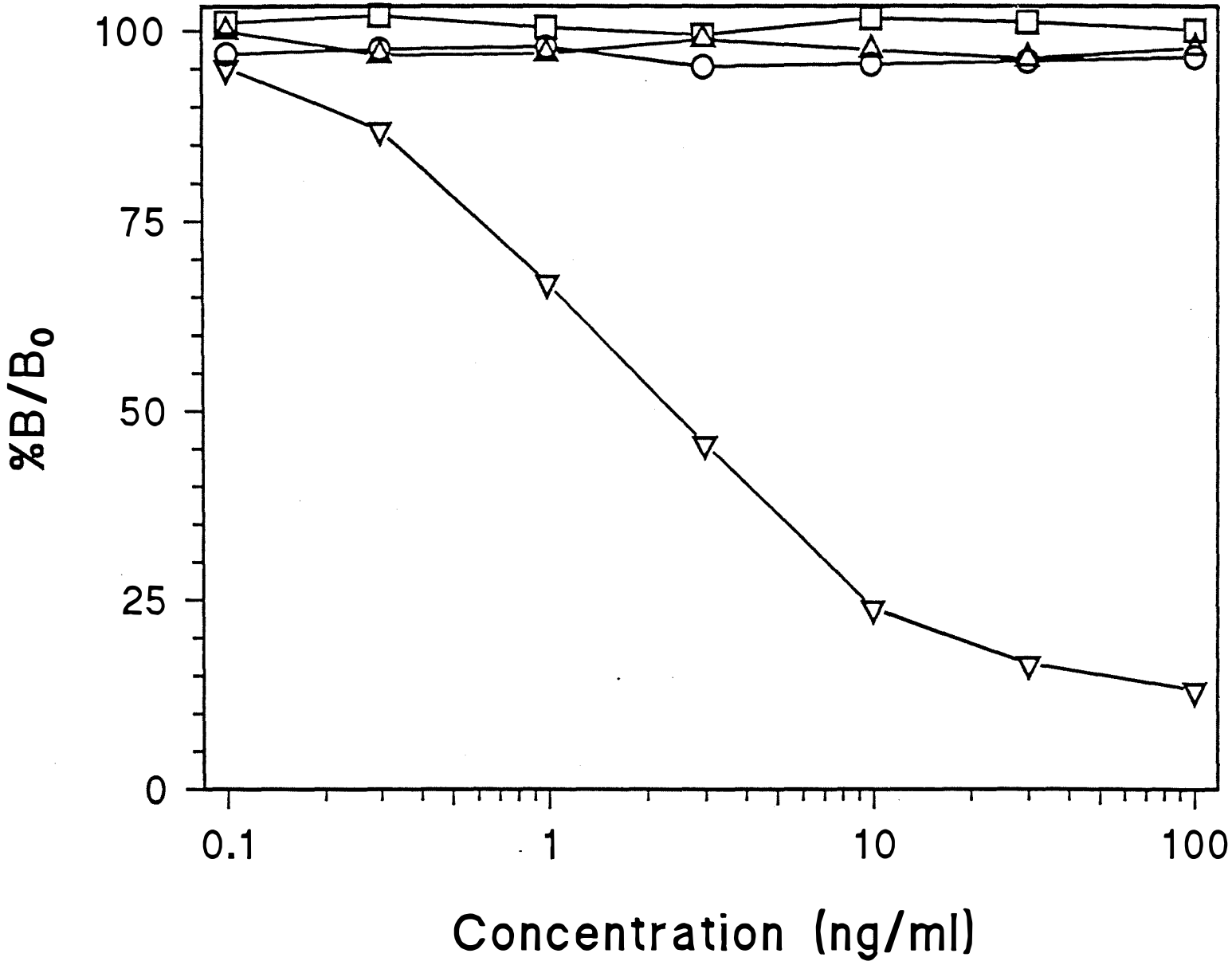


FIG. 8



APPENDIX VII

Excretion of Ciguatoxin from Moray Eels (Muraenidae) of the Central Pacific

by

R.J. Lewis, M. Sellin, R. Street, M.J. Holmes and N.C. Gillespie

Southern Fisheries Research Centre
PO Box 76
DECEPTION BAY QLD 4008

Running head: Excretion of ciguatoxin from fishes

ABSTRACT

Ciguatera is the disease in humans caused when otherwise edible fish become contaminated with ciguatoxin via the marine food chain. A longstanding dogma is that once contaminated, fishes retain the original level of toxicity for many years. In this study we analyze the pattern of change in toxicity of viscera of 218 moray eels (Lycodontis javanicus, Bleeker) collected from Teaoraereke, Tarawa in the Republic of Kiribati over a 500 day period. Our results indicate that the concentration of ciguatoxin (per g of viscera) declined exponentially over the 500 day period of this study ($p<0.001$). All 47 samples from Teaoraereke tested toxic to mice, indicating that recruitment of non-toxic eels could not explain this decrease in toxicity. The half-life for ciguatoxin efflux from the viscera of moray eels was determined to be 264 days. Over the 500 day period, the size of the viscera did not vary significantly. Interestingly, no relationship could be established between the toxicity of viscera and viscera weight. The half-life determined in our study assumes that ciguatoxin input into eels stopped prior to the start of our collections. The calculated efflux rate will underestimate the actual efflux rate if the input of ciguatoxin did not stop at this time but was merely reduced. The efflux rate of ciguatoxin is likely to vary between fish species (and perhaps even between individuals of a species) and may in part determine the ciguatera risk of a species. The decline in viscera toxicity parallels a reduction in the number of ciguatera cases in Tarawa. H.P.L.C. separated ciguatoxin from a minor, less polar form of ciguatoxin. The implications of these results are discussed and a model describing the ciguatera risk of a fish species is proposed.

INTRODUCTION

Humans in tropical and sub-tropical areas that eat fish are at risk from ciguatera. This risk stems from the ability of otherwise edible fish to become contaminated with ciguatoxin through the marine food chain (Randall, 1958; Scheuer et al., 1967; Murata et al., 1989). The structure of ciguatoxin in moray eels has been elucidated (Murata et al., 1989). Ciguatoxin apparently arises from a less-polar form of ciguatoxin produced by wild Gambierdiscus toxicus which is oxidised (three times) to ciguatoxin as it passes through the marine food chain (Murata et al., 1989).

Many species of herbivorous, detritivorous, omnivorous and carnivorous fishes have been implicated with ciguatera (Cooper, 1964; Halstead, 1967; Helfrich et al., 1968; Randall, 1979; Gillespie et al., 1986; Bagnis et al., 1988). However, certain fish ie. Lutjanus bohar (Forsskål), Lycodontis javanicus (Bleeker), Scomberomorus commersoni (Lacepede), Sphyraena spp. and Epinephelus spp. are most likely to cause ciguatera in particular areas. Outbreaks of ciguatera are often biphasic in nature with an initial upsurge followed by a gradual decline in the incidence of ciguatera in an area (Helfrich and Banner, 1968; Bagnis et al., 1988). In this paper we report the potential for the excretion (decay) of ciguatoxin in fish and we propose a model that can account for increases or decreases in ciguatera risk within and between fish species. This conclusion stems from an analysis of toxicity of viscera of a demersal fish (Lycodontis javanicus) collected from Tarawa, Republic of Kiribati, an area where ciguatera has been endemic for several decades (Tebano and McCarthy, unpublished results).

METHODS

Eel collection

Moray eels (Lycodontis javanicus) were captured in fish-baited cage traps set at various locations on ocean reefs adjacent to the island of Tarawa (Fig. 1) in the Republic of Kiribati. The viscera (including liver) of each eel was removed and stored frozen prior to air dispatch to Brisbane, Australia. On arrival in Brisbane viscera were still in either a chilled or frozen state. Viscera were pooled (n=1-18) to a convenient sample weight for extraction (0.3 to 1.0 kg). Each sample of viscera contained only viscera of similar size to allow the relationship between size and toxicity to be determined. The date of arrival of viscera in Brisbane was recorded as the collection date and represented the collection of eels up to one month prior to this date. During this study, eels captured from the ocean reef adjacent to the villages of Teaoraereke, Bikenibeu, Bariki and Betio (Fig. 1) were found to be similarly toxic. However, a pooled sample of viscera from five eels from the ocean reef adjacent to Tanea did not contain detectable ciguatoxin (20 mg of diethyl ether fraction injected). The flesh of one eel from Teaoraereke tested non-toxic. This study reports the analysis of viscera toxicity of 217 eels (47 pooled samples) collected from the ocean reef adjacent to Teaoraereke in the centre of the toxic zone on Tarawa. Eels were obtained from nine collection dates over a 500 day period.

Extraction of ciguatoxin

Viscera samples (n=47) were thawed and cooked in a plastic cooking bag and then refrozen prior to extraction. Cooking denatured proteins that otherwise hampered homogenisation in acetone. Frozen samples were chopped into small pieces and minced in a hand-operated meat mincer to yield a fine slurry. Ciguatoxin was extracted from the viscera with acetone for 15 minutes using an air powered homogeniser (Ystral) fitted with 20 T shaft with a medium viscosity generator with protruding cutters. Each viscera sample was extracted twice with acetone at room temperature. A third extraction with acetone yields <0.15% of total toxicity (Lewis and Endean, 1984a).

Partial purification of ciguatoxin

Two liquid-liquid partitioning schemes were compared to determine the most efficient method for isolating ciguatoxin. Both schemes first extracted a 90% aqueous methanol phase with hexane (Fig. 2). Method I then used the standard diethyl ether-water partitioning, while method II (Fig. 2) used a modification of this method (25% ethanol added to the aqueous phase) suggested by Vernoux et al. (1985). For this purpose a minced sample of viscera was divided into two equal portions for extraction. After four diethyl ether extractions using Method I a total of 0.9 g of lipid and 1,160 mouse units (m.u.) of ciguatoxin was recovered. A further four diethyl ether extractions yielded 0.3 g and 930 m.u. of ciguatoxin and an additional extraction of the aqueous phase using method II yielded 2.1 g and 145 m.u. of ciguatoxin. Extraction of the other portion by method II alone (three diethyl ether extractions) yielded 7.0 g and 2,750 m.u. of ciguatoxin. Consequently, method II (Fig. 2) was used for all assays reported here. This method also has the additional advantage of a rapid separation of the diethyl ether-25% aqueous ethanol phases compared with an often difficult separation of the diethyl ether-water phases (method I). Much of the high yield of lipid impurities obtained using Method II could be removed with -20°C acetone precipitation (acetone

precipitate contained ~ 5% of lethality). The hexane and water phases contained no detectable ciguatoxin (at 1 g/kg dose) although the high yield of impurities in these fractions precluded a sensitive assay.

Characterisation of toxin content of viscera

A portion of the diethyl ether fraction was purified to homogeneity by low pressure and high pressure liquid chromatography (H.P.L.C.). Fast atom bombardment (F.A.B.) mass spectral measurements were performed on two toxic fractions separated by H.P.L.C.

Bioassay

A portion (1-5 mg) of each diethyl ether fraction was suspended in 0.5 ml 5% Tween 60 saline and assayed in duplicate by i.p. injection into 20 ± 2 g Quackenbush mice of either sex. For each mouse the signs and the time to death were recorded. The relationship between dose and time to death was used to quantify each fraction (adapted from data of Lewis and Endean, 1984a) and is approximated by:

$$\log \text{m.u.} = 2.3 \log (1 + T^{-1}) \qquad 1$$

where, m.u. = number of mouse units of ciguatoxin injected
T = time to death in hr.

One m.u. is the LD_{50} dose of ciguatoxin for a 20 g mouse. Based on the data of Tachibana (1980) one m.u. = 9 ng ciguatoxin. Hexane and 25% aqueous ethanol soluble fractions were intermittantly assayed in mice at doses up to 1 g/kg (dose weight/mouse body weight).

Statistical analysis

Data are expressed as the mean±1 population standard deviation. Linear regression analysis were performed on unweighted data or on data weighted with the number of fish per sample.

RESULTS

Moray eel collection

Whole viscera from 217 moray eels were collected over a 500 day period from one ciguatera-prone site on Tarawa and pooled into 47 samples as described in Methods. The average weight of whole individual eels from a subsample of 38 eels was 3.6±2.3 kg with individual weights ranging from 0.6 kg to 10 kg. A total of 35.9 kg of pooled eel viscera were collected from the 217 eels. Figure 3 shows the average viscera weight for each of the 47 pooled samples. No significant change occurred in the average weight of viscera collected over the period of this study.

Viscera toxicity

A total of 99,200 m.u. of ciguatoxin was extracted from 35.9 kg of eel viscera. The average toxicity was 2.43 ± 1.69 m.u. per g viscera (n=47) and ranged from 0.59 to 7.3 m.u. per g. No significant regression was found between toxicity and average viscera weight (Fig. 4).

The toxicity of viscera (n=47) was found to decline significantly over the 500 day period of the collections (Fig. 5). This decline was significant for both weighted (using number of fish per sample) or unweighted data. The weighted negative linear regression of log toxicity vs time is approximated by:

$$\log y = 0.62 - 0.00114x \qquad (p<0.001) \qquad 2$$

where: y = toxicity (mouse units/g viscera)
x = days from start of collection.

The equation for the weighted data was chosen as the most appropriate as it takes into account the high variability in viscera toxicity. The exponential relationship for excretion (decay) was chosen as the simplest model to explain the observed decrease in toxicity. The slope of this regression estimates that the half-life for the excretion (decay) of ciguatoxin stored in the viscera is 264 days. Averaging the toxicity for each collection date (n=9) estimates the half-life for the decay of ciguatoxin is 316 days. All 47 samples contained detectable levels of ciguatoxin and no seasonal fluctuations in toxicity were evident.

Characterisation of the toxin content of viscera

Two toxins were isolated by reverse phase H.P.L.C. (Fig. 6). The more-polar toxin was the major component representing approximately 90% of total toxicity. This toxin chromatographed similar to ciguatoxin from the Spanish mackerel (Lewis and Endean, 1984b) and was regarded as ciguatoxin. The approximate MH^+ m/z of this toxin was determined to be 1111.3, similar to 1111.584 reported for ciguatoxin by Murata et al. (1989). The less-polar toxin induced signs in mice similar to ciguatoxin and had a MH^+ m/z of 1095.5, indicating it differs from ciguatoxin by the absence of one oxygen atom. Presumably one hydroxyl group on ciguatoxin is missing in this less polar toxin.

DISCUSSION

The concentration of ciguatoxin in the viscera of a population of moray eels collected from one site within the toxic area of Tarawa was monitored over a 500 day period (1987-1989), using a modified extraction procedure and the mouse bioassay. In this period there was a significant exponential decline in the concentration of ciguatoxin. The half-life for this decline in toxicity was estimated to be 264 days. We propose that this loss stems from excretion and/or decay of ciguatoxin. Excretion comprises the loss from eels of stored native ciguatoxin and decay comprises conversion of ciguatoxin to a less-toxic moeity within eels. The calculated half-life assumes that the concentration of ciguatoxin in the diet of eels reduced to zero before the commencement of eel collections. The actual half-life will be shorter if the concentration of ciguatoxin in the diet of eels reduced only partially prior to these collections. Parrotfish and surgeonfish taken from reefs near Teaoraereke eight months after the last eel collection contained ciguatoxin. As these fishes can be part of the diet of moray eels (Randall, 1979) it is likely the actual half-life for the excretion (decay) of ciguatoxin from eels is considerably shorter than 264 days. Case history data on fish poisoning (including ciguatera) collected by the South Pacific Epidemiological and Health Information Service from 1973 to 1989 (Fig. 7) indicate a general increase in the number of ciguatera cases over this period. The upsurge in

ciguatera in 1986/87 may reflect a transient increase in ciguatoxin production that could explain the high levels of ciguatoxin found in eels at the start of the collection period.

At least two other mechanisms could contribute to the observed reduction in eel toxicity. First, immigration of non-toxic eels may have occurred as a result of our collections depleting eel stocks. This is considered unlikely for several reasons: (i) all 47 samples contained significant levels of ciguatoxin; (ii) the collection site is in the centre of a large toxic zone; (iii) our collections did not influence eel size over the collection period (assuming viscera weight is proportional to whole weight as found for other fish species (Weatherley and Gill (1987)). Second, growth would contribute to a reduction in the concentration of ciguatoxin in the tissues of eels. Quantifying this effect of growth was not possible as data on the growth rate of moray eels were not available. To explain the reduction in concentration of ciguatoxin in eel viscera by growth alone these eels would need to have doubled in size in 264 days.

This study revealed that the concentration of ciguatoxin in the viscera of eels from Tarawa did not correlate with eel viscera weight. Lack of correlation presumeably extends to whole eel weight as typically viscera weight correlates with whole weight for fishes (Weatherley and Gill, 1987). Vernoux (1988) found a correlation between size and toxicity for Caranx latus but not for the closely related Caranx bartholomaei captured in the same area. L. bohar was found to have a correlation between size and toxicity irrespective of location (Helfrich et al., 1968; Banner, 1974).

A model incorporating the influence of key variables (excretion, growth, first-pass assimilation efficiency, concentration of ciguatoxin in diet, immigration and emmigration) is proposed to explain the ciguatera risk posed by fish species in a ciguatera-endemic area (Fig. 8). The rate of production of ciguatoxin (and related compounds) by benthic species including Gambierdiscus toxicus (Murata et al., 1989) determines the overall level of ciguatoxin in the system and consequently the overall level of ciguatera risk. Genetic and environmental factors are proposed as the key factors influencing ciguatoxin production (Gillespie et al., 1985; Bagnis et al., 1988; Lewis et al., 1988). This model assumes that fishes assimilate ciguatoxin from their diet, as indicated by feeding studies with Acanthurus xanthopterus Cuvier and Valenciennes (Helfrich and Banner, 1963). This is a basic requirement of the food chain hypothesis originally proposed by Randall (1958). However, no assessment of the efficiency of assimilliation of ciguatoxin from the diet has been undertaken for any fish species. The potential to excrete ciguatoxin has been investigated for a few species. L. bohar may not excrete ciguatoxin (Banner et al., 1966), while Sphyraena barracuda (Walbaum) may excrete ciguatoxin more rapidly than eels (Tosteson et al., 1988). Between species variability in excretion, assimillation and the dietary concentration of ciguatoxin are likely to explain most of the variability in ciguatera risk between species. For each species the magnitudes of the variables presented in the model may specifically determine the relationship between fish size and toxicity. Within species variability in these factors may explain why some individuals are more toxic than others.

This model can explain the different general patterns of ciguatera that are observed (Helfrich and Banner, 1968). Both genetic and environmental factors influencing the periodicity of ciguatera risk and the import of new genetic material (perhaps a ciguatoxin-producing strain of G. toxicus) may explain

the first appearance of ciguatera in an area. The rate of decline of ciguatera risk will be determined by factors including excretion, growth and mortality. This proposed model may be useful in the development of management strategies that may be implemented to reduce the ciguatera risk in an area.

Until procedures to detect of toxic fishes become available that are suitable for large scale screening, the use of a bioindicator species is desirable to measure the level of ciguatera risk in an area. Moray eels are a sensitive indicator of ciguatera and appear useful for the long term monitoring of ciguatera over the medium to long term. Herbivorous species are likely to be more useful bioindicators if details of short term changes in ciguatoxin production are required.

The eels from Tarawa contained at least two toxins. The major toxin is similar in molecular weight and chromatography to the major ciguatoxin from eels from French Polynesia (Legrand et al., 1989) and is similar in chromatography to ciguatoxin from Spanish mackerel (Lewis and Endean, 1984b). A less polar form of ciguatoxin has also been isolated from Tarawa eels. This toxin differs in molecular weight from ciguatoxin by the loss of one oxygen (presumably by the loss of one hydroxyl group). This toxin may be the same as the less polar toxins previously reported to occur in eel viscera (Tachibana, 1980; Nukina et al., 1984; Legrand et al., 1989). The relationship between "ciguatoxins" from eels and the "ciguatoxins" found in the flesh and viscera of other fish species (Chungue et al., 1976; Lewis and Endean, 1984a; Vernoux and Andaloussi, 1986; Vernoux and Talha, 1989) remains to be established. Species specific differences in assimillation and conversion of the less polar ciguatoxin produced by G. toxicus (Murata et al., 1989) may explain the different composition of "ciguatoxins" present in different fish species. The presence in fish of a class of ciguatoxins which have no detectable toxicity should not be discounted.

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FIGURE LEGENDS

Figure 1

Map of the southern half of Tarawa, Republic of Kiribati. The dashing indicates the outer barrier reef. The areas reported toxic in 1983 are indicated by the dotted line (adapted from Tebano and McCarthy, unpublished). Eels were collected initially from outer reefs adjacent to the five villages indicated. Only results of toxicity testing of eels collected on outer reefs adjacent to Teaoraereke from September 1987 to January 1989 were used in this study.

Figure 2

Scheme used for the extraction and partial purification of ciguatoxin. Fractions marked by an asterisk were tested for toxicity in mice.

Figure 3

Average weight of moray eel viscera collected over a 500 day period. Data represent the average viscera weight (sample weight ÷ number of fish per sample) for each of 47 samples obtained during nine collections. Viscera weight did not change significantly over time. Eels collections commenced in September, 1987.

Figure 4.

Sample toxicity (mouse units per g viscera) vs. average viscera weight. Data represent the toxicity of the 47 samples collected during the study period.

Figure 5.

Toxicity (mouse units per g viscera) of eel viscera over a 500 day period. Note log scale for y axis. Numbers adjacent to each data point indicate the number of fish pooled for that sample. Samples of 3 or less are unmarked for clarity. Toxicity declined significantly over the 500 day period ($n=47$, $p<0.001$). Eel collections commenced in September 1987.

Figure 6.

H.P.L.C. elution profile of a semi-purified fraction containing 120 mouse units (m.u.) of ciguatoxin (CTX). Chromatography was performed on a Hamilton PRP-1 reverse phase column (5μm, 150 x 4.1 mm) eluted with acetonitrile-water (1:1) at 0.5 ml/min and monitored at 206 nm. Ciguatoxin was eluted at 8.4 minutes as a homogeneous peak indicated by the dark bar. A less-polar toxin eluted at 27 minutes as a broad homogeneous peak. The MH^+ m/z for ciguatoxin and the less-polar toxin was 1111.3 and 1095.5, respectively. The approximate number of m.u. is indicated for both toxins.

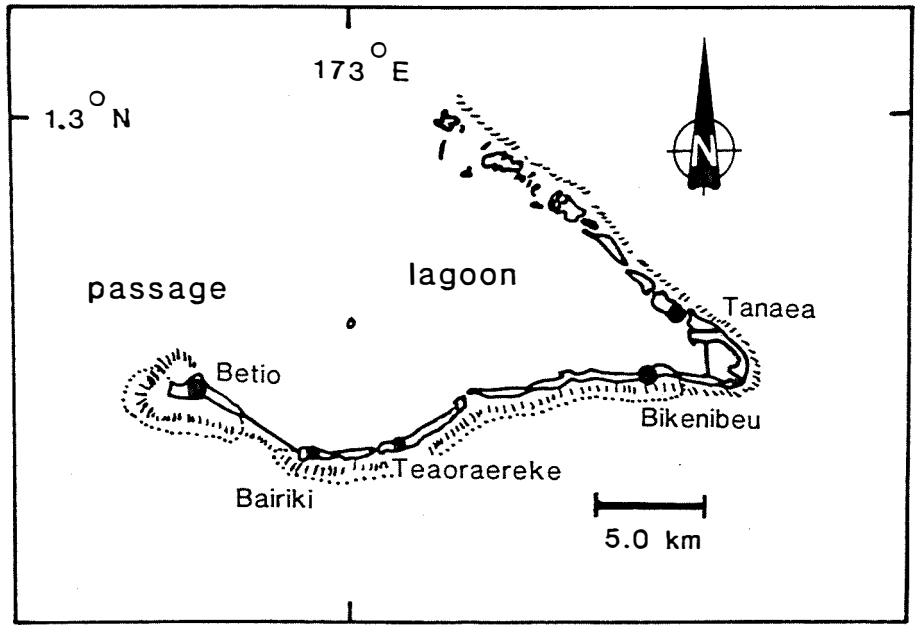
Figure 7.

Annual incidence of fish poisoning in the Republic of Kiribati, 1973 to 1989. Data provided by the South Pacific Commission Epidemiological and Health Information Services and includes mostly cases of ciguatera as well as other forms of fish poisoning including histamine poisoning.

Figure 8.

A model describing the ciguatera risk of herbivorous (including detritivorous and omnivorous fishes) and carnivorous fishes in an area producing ciguatoxin. The risk from ciguateric fishes is driven by the rate of ciguatoxin (CTX) production. This rate is increased (+) or decreased (-) by the influence of both genetic and environmental factors. The horizontal arrows indicate the transfer of ciguatoxin through the food chain from the benthos to herbivorous and carnivorous species. Factors influencing the concentration of ciguatoxin within and between fish species are indicated.

c = concentration of CTX in the total diet of a particular species of fish
a = first pass efficiency of assimilation of CTX from the diet into the tissue of a particular fish species
o,i = rate of emmigration (including mortality) of particular toxic fish species and immigration of non-toxic fish species, respectively.
e = rate of excretion (decay) of stored ciguatoxin from a tissue of a particular fish species (leading to a reduced CTX concentration).
g = growth of individual fish species (leading to a dilution of CTX in fishes).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Bairiki
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Fig. 1.

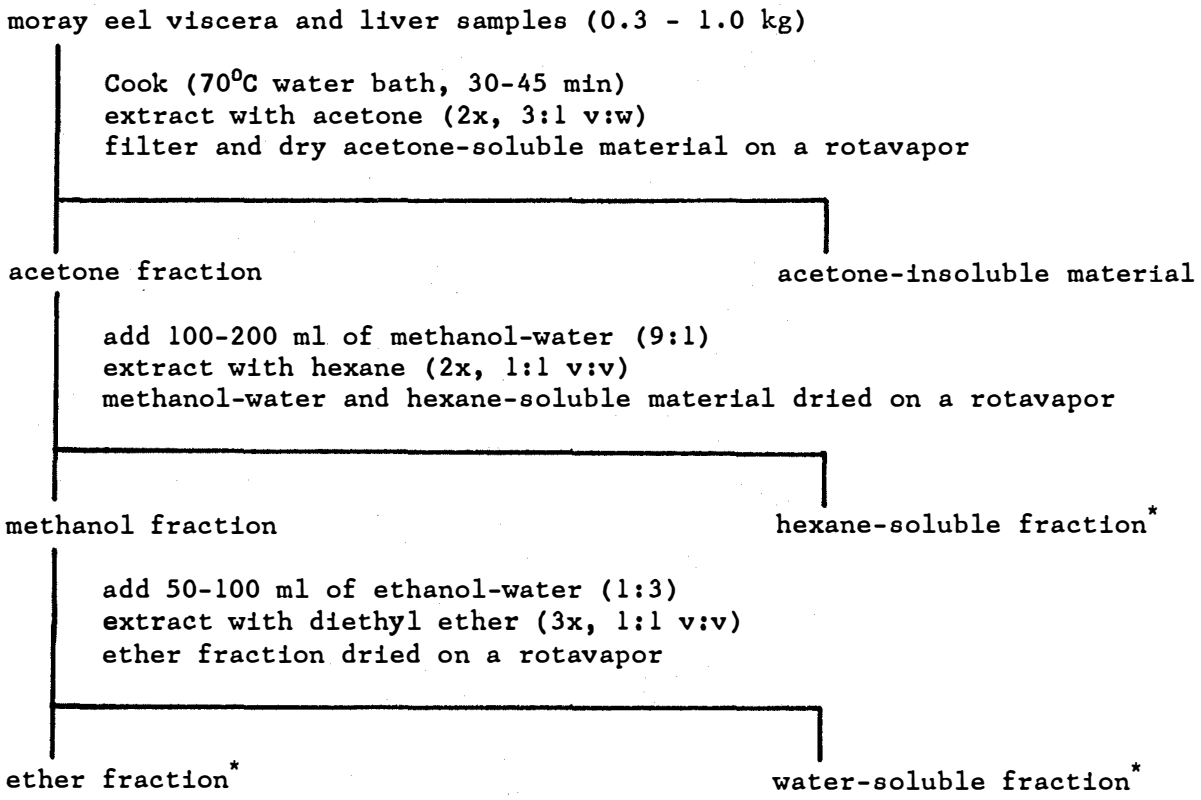

moray eel viscera and liver samples (0.3 - 1.0 kg)
Cook (70°C water bath, 30-45 min)
extract with acetone (2x, 3:1 v:w)
filter and dry acetone-soluble material on a rotavapor
acetone fraction
acetone-insoluble material
add 100-200 ml of methanol-water (9:1)
extract with hexane (2x, 1:1 v:v)
methanol-water and hexane-soluble material dried on a rotavapor
methanol fraction
hexane-soluble fraction*
add 50-100 ml of ethanol-water (1:3)
extract with diethyl ether (3x, 1:1 v:v)
ether fraction dried on a rotavapor
ether fraction*
water-soluble fraction*

Fig. 2

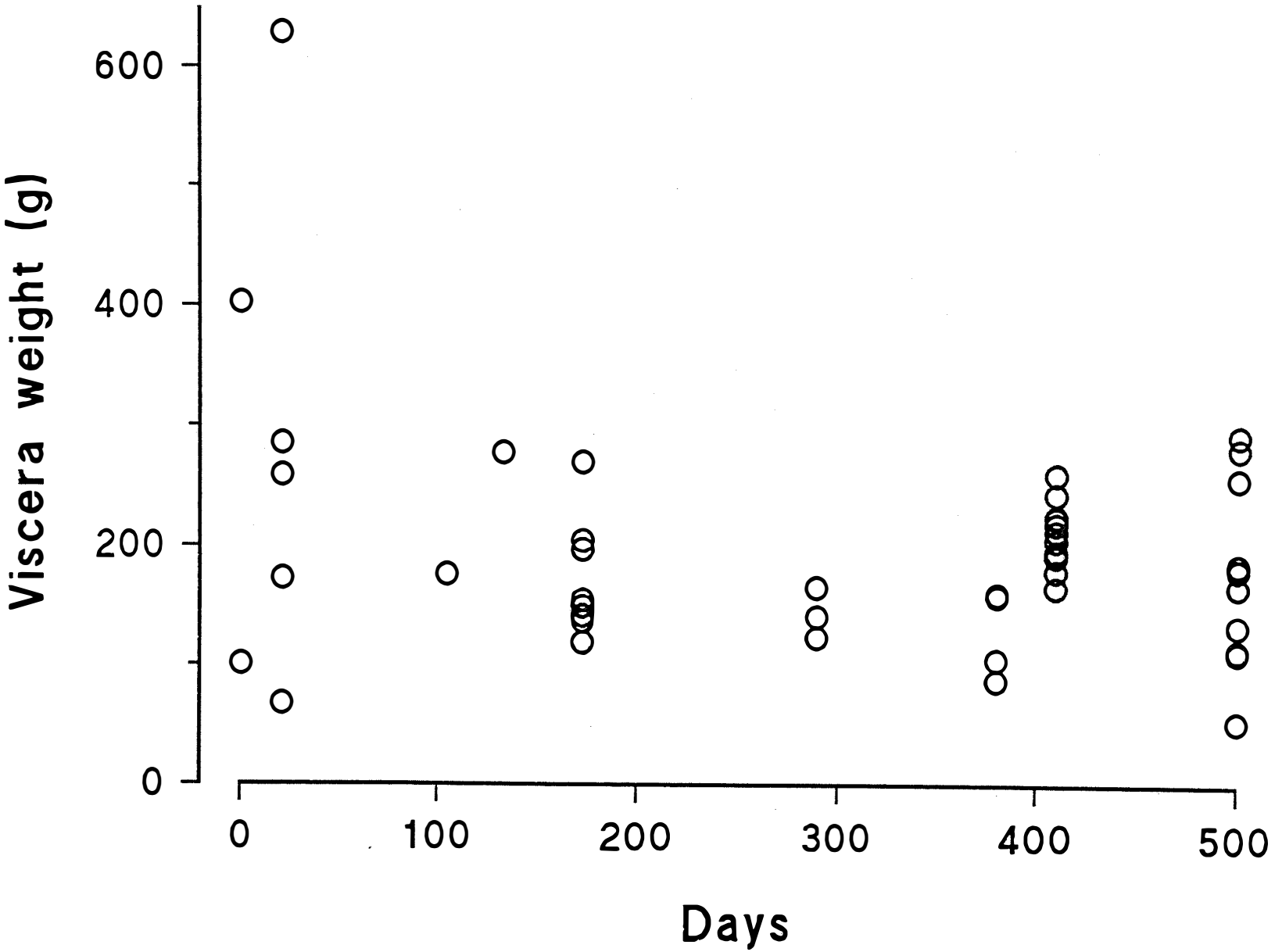


Fig. 3

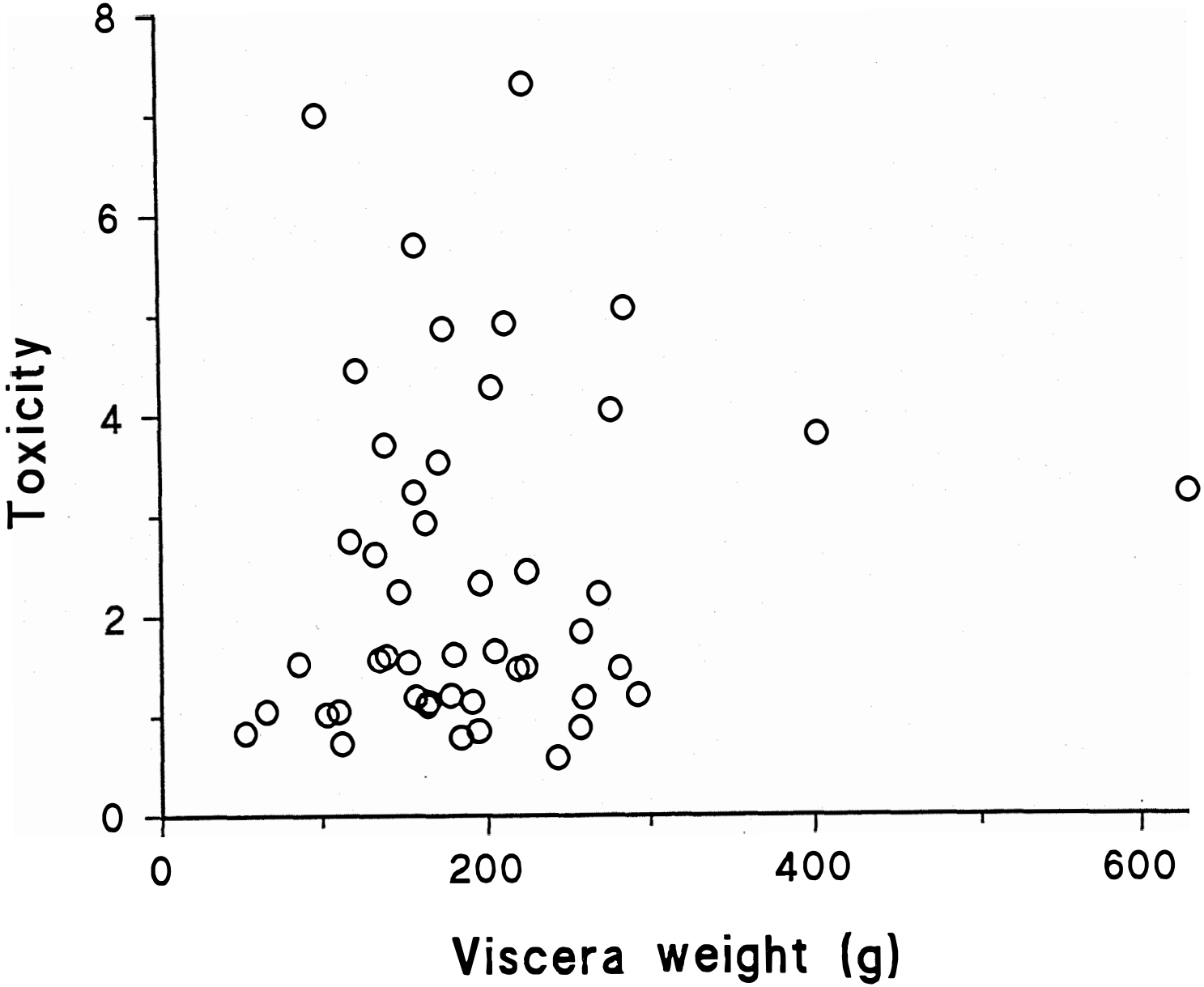


Fig. 4.

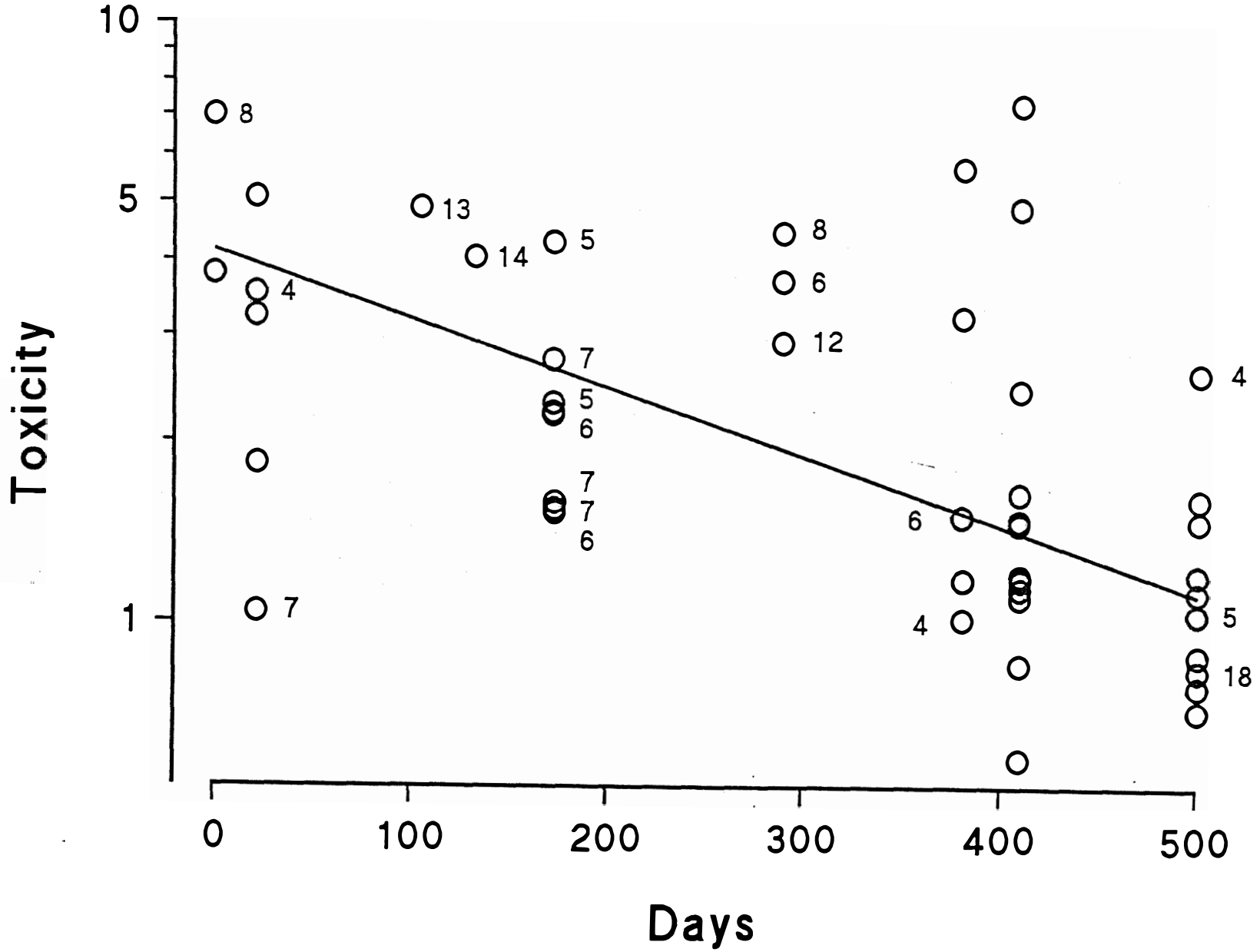


Fig. 5

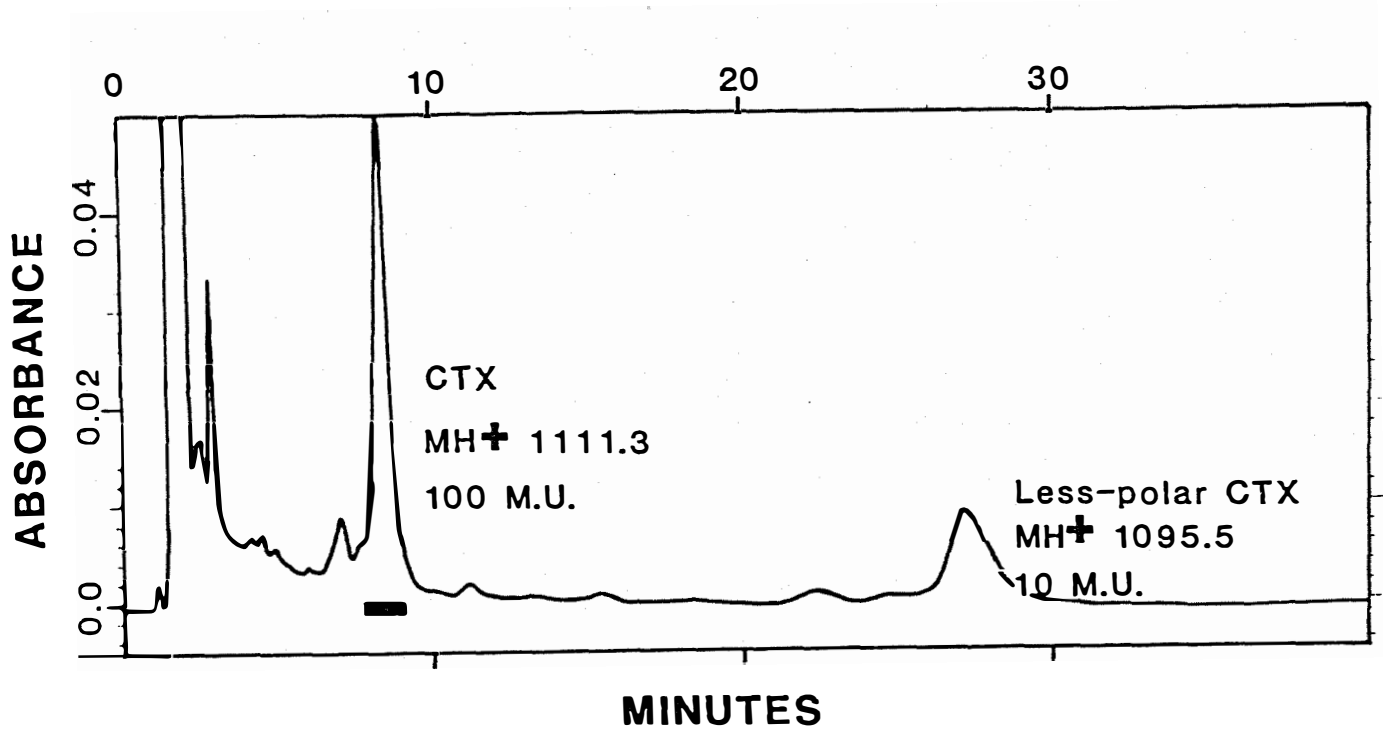
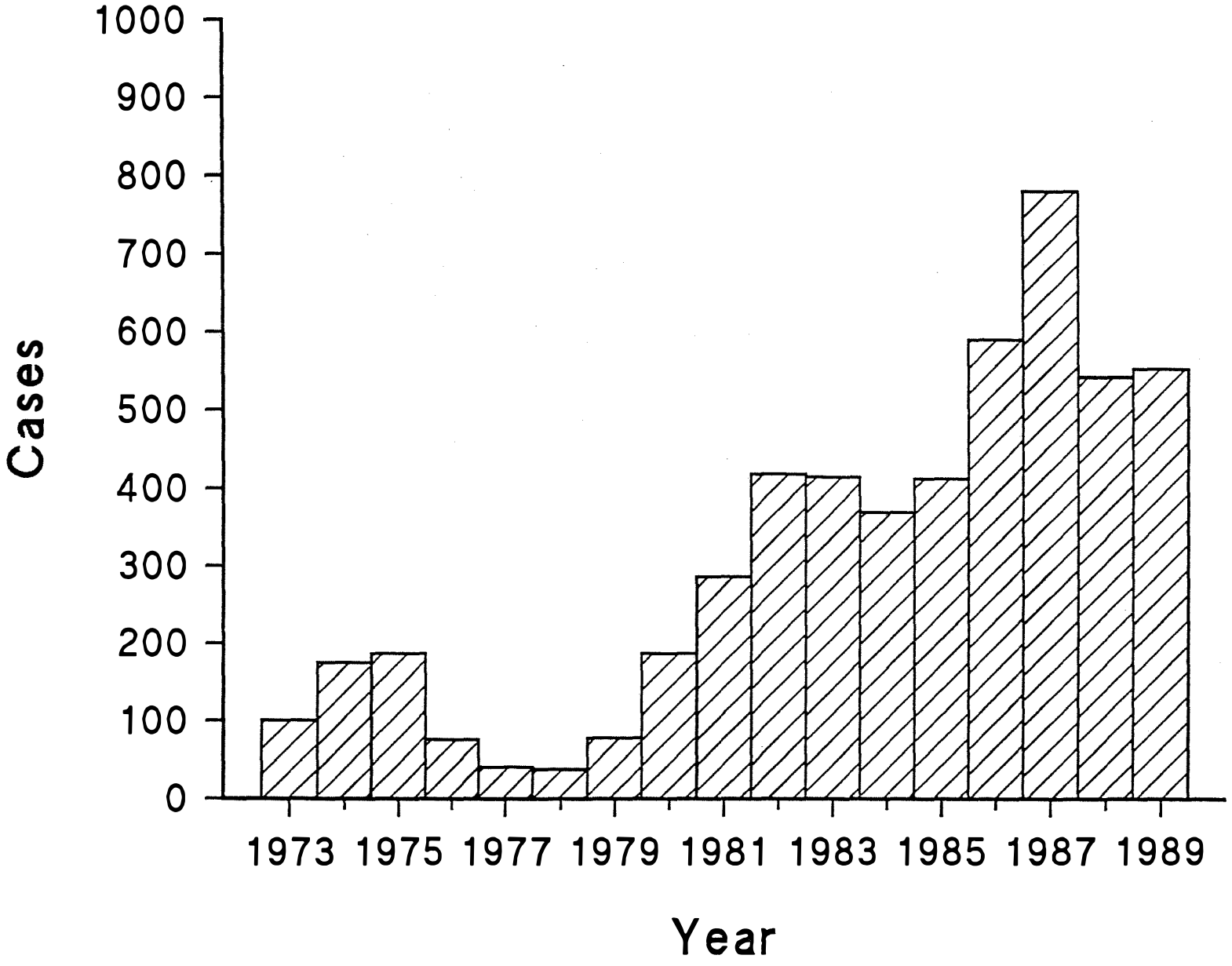

0
10
20
30
ABSORBANCE
0.0
0.02
0.04
CTX
MH+ 1111.3
100 M.U.
Less-polar CTX
MH+ 1095.5
10 M.U.
MINUTES

Fig. 6


1000
900
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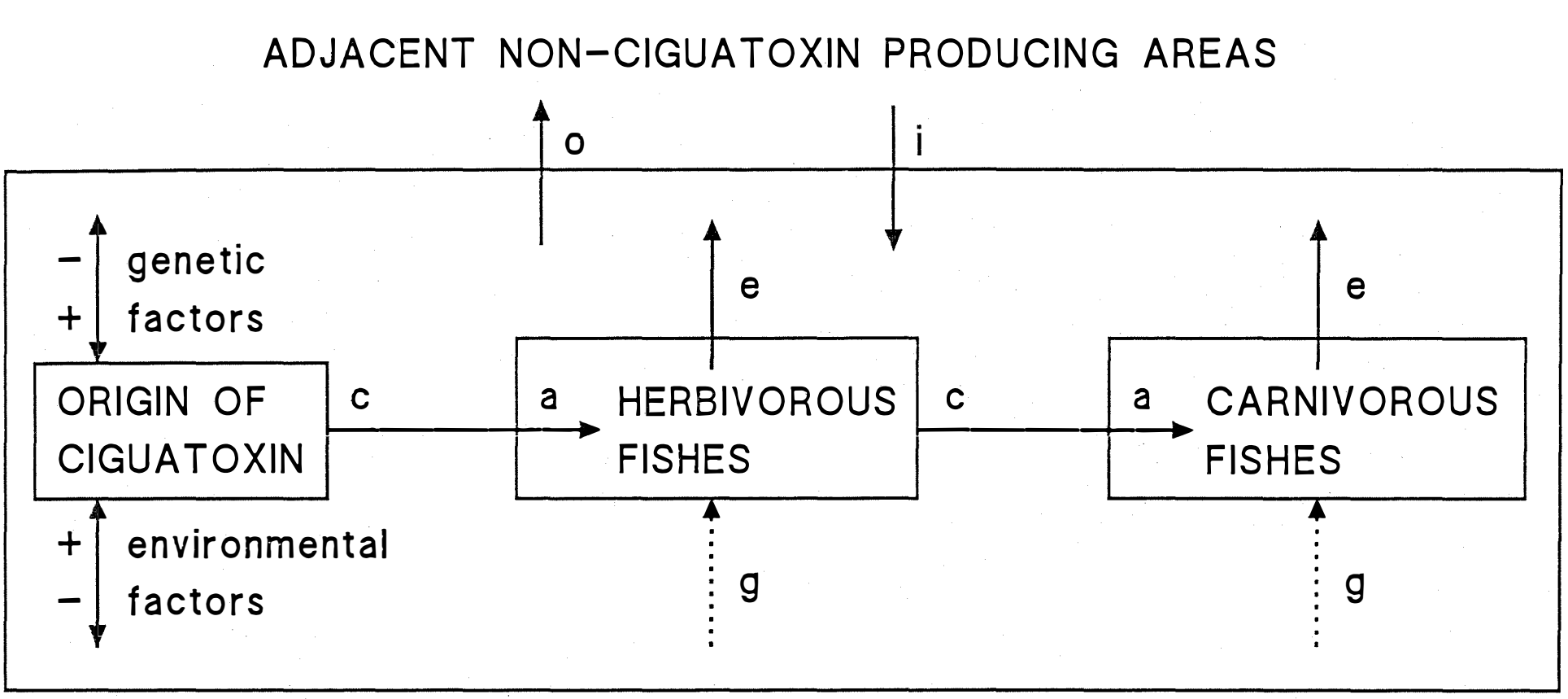
Cases
1973 1975 1977 1979 1981 1983 1985 1987 1989
Year

Fig 7


ADJACENT NON-CIGUATOXIN PRODUCING AREAS
o
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ORIGIN OF CIGUATOXIN
c
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CARNIVOROUS FISHES
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- factors
g
g

Fig. 8

APPENDIX VIII

Publications (4) relating to the management of ciguatera

Ciguatera in Australia

Occurrence, clinical features, pathophysiology and management

Noel C. Gillespie, Richard J. Lewis, John H. Pearn, Anthony T.C. Bourke, Michael J. Holmes, John B. Bourke and William J. Shields

ABSTRACT Ciguatera is a type of food poisoning that results from eating certain tropical fish which have become toxic. It is common in Australia: 175 outbreaks, which involved 527 people, were reported in Queensland between 1965 and 1984. It seems restricted to certain areas around the Australian coastline. Most reports have involved the narrow-barred Spanish mackerel, *Scomberomorus commersoni*, most of which were caught in southern Queensland waters. Up to 2100 cases may have occurred in north Queensland between 1965 and 1984, which were not recorded by the writers. The symptoms of ciguatera in Australia are similar to those reported elsewhere in the South Pacific. Ciguatoxin acts by increasing the permeability of excitable membranes to sodium ions. This type of membrane is found throughout the body in nerve tissue as well as in heart and skeletal muscle. The treatment of ciguatera remains symptomatic and supportive only. Major advances in treatment for ciguatera and detection of ciguatoxin await the means of producing additional ciguatoxin.

(Med J Aust 1986; 145: 584-590)

Ciguatera is a distinctive type of food poisoning which sometimes follows the consumption of ordinarily edible tropical fish species. Such toxic fish specimens contain elevated amounts of ciguatoxin (a lipid-soluble polyether compound),[1] and possibly other closely related compounds,[2] and maitotoxin (a water-soluble toxin).[3]

The syndrome typically includes both gastrointestinal and neurological symptoms. Diagnosis is based on clinical symptoms only; it is sometimes supplemented (in ideal circumstances) by the bioassay of the fish involved. The clinical symptoms can vary considerably among individuals, even those who have been poisoned by the same fish. Incorrect diagnosis is common. At present, ciguatera is not a notifiable disease, so it is difficult to obtain a true indication of incidence. Nevertheless, reports of ciguatera since 1934 indicate that it has been endemic in Australia for a long time.[4-11] Most reports involve fish that were caught along the northern coast of Australia and include both demersal and pelagic species.

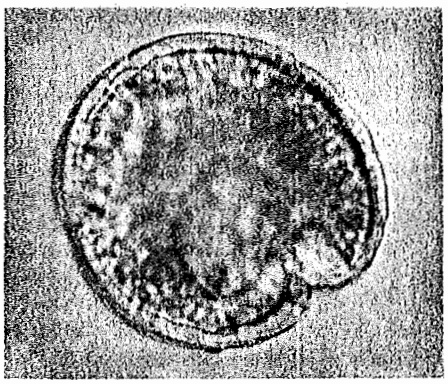

FIGURE 1: The dinoflagellate Gambierdiscus toxicus, *as it appears under a light microscope. This cell is approximately 70 μm in diameter.*

Described initially by Spanish explorers in the Caribbean,[12] ciguatera is now known to be widespread, affecting many tropical and subtropical coastal regions of the world. Whereas it has been reported along the coasts of tropical and subtropical Australia and the Florida coast of the United States, it is largely confined to islands in the true tropics.[13] In these areas, ciguatera outbreaks are sporadic and unpredictable, with a patchy distribution in both space and time.[13,14]

It has been demonstrated that ciguatoxin is elaborated at the base of the food chain on coral reefs;[15] it is probably produced by a benthic dinoflagellate, *Gambierdiscus toxicus* Adachi and Fukuyo,[16] which adheres to dead coral surfaces and bottom-associated algae (Figures 1-3). Herbivorous fish species, which graze on algae and detritus, ingest the

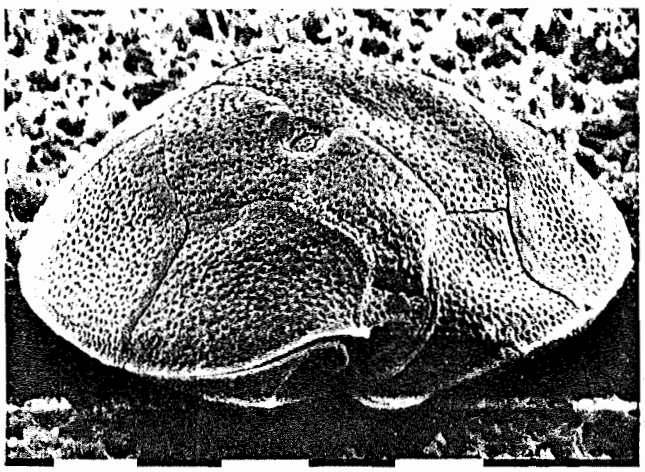

FIGURE 2: A scanning electronmicrograph of Gambierdiscus toxicus *(each white bar is equivalent to 10 μm).*

FIGURE 3: Gambierdiscus toxicus *attaches to bottom-fixed macroalgae on coral reefs and is ingested by herbivorous fish species.*

Queensland Department of Primary Industries, Southern Fisheries Research Centre, Deception Bay, QLD 4508.
Noel C. Gillespie, BSc, PhD, Assistant Director, Fisheries Research Branch.
Richard J. Lewis, BSc, PhD, Fisheries Biologist, Fisheries Research Branch.
Michael J. Holmes, BAppSc, Fisheries Technician.
John B. Bourke, CBLT, Assistant Senior Technician.
Department of Child Health, Royal Children's Hospital, Brisbane, QLD 4029.
John H. Pearn, MD, PhD, FRACP, FRCP(UK), Professor and Head.
Division of Environmental and Occupational Health, Queensland Department of Health, State Health Building, Brisbane, QLD 4000.
Anthony T.C. Bourke, MSc, MD, DrPH, FRCPA, FACPM, MASM, DTM&H, Epidemiologist.
William J. Shields, CertRSH, AAIHS, Chief Inspector of Foods.
Reprints: Dr N.C. Gillespie.

organism and its toxin, and the toxin becomes concentrated in large carnivores by way of the marine food web. Large predatory coral-reef fish are strongly-favoured culinary items and are distributed Australia-wide. For this reason, cases of ciguatera may occur in any part of the country. Tourists returning from Pacific Islands are also a source of clinical cases in this country.

The ingestion of seafood can also cause a number of other forms of poisoning in humans. Examples of these (excluding bacterial food poisoning) include paralytic shellfish poisoning and diarrhoetic shellfish poisoning, which are both derived from toxic dinoflagellate blooms;[17] puffer fish poisoning, which is due to tetrodotoxin;[18] and clupeotoxism, which is a major cause of fish-related fatalities in Fiji.[17,19] Of these icthyosarcotoxaemias, ciguatera is the only one that is known to occur commonly in Australia, where considerable research has now been carried out with respect to its origin, symptomatology, epidemiology and pharmacological properties. This article attempts to integrate this information with a view to enhancing the identification and treatment of ciguatera in Australia.

Clinical presentation

Table 1 shows the frequency of signs and symptoms that were displayed by 527 cases of ciguatera from 175 outbreaks between 1965 and 1985 which were confirmed by us. A significant proportion of these cases were collected by the Queensland Department of Health through the investigation of complaints of food poisoning; these were supplemented by data that were collected in 1980/1981 during a survey by the then Queensland Fisheries Service and by case reports that were obtained by Lewis during a study of the properties of ciguatoxin in Queensland.[20] Also included in Table 1 are the summarized results of an extensive analysis of the clinical symptoms from 3009 cases from French Polynesia and New Caledonia, which were collected between 1964 and 1977,[21] as well as those of a study of 792 cases in Fiji.[19]

Of the 219 Queensland cases for which data for the time of onset are available, 65% had an incubation period of between two and eight hours. Greater than 90% of patients had felt the effects within 12 hours of the ingestion of the fish. In a few cases the period involved was greater than 24 hours. Even within a group of persons who had consumed the same fish, the incubation period and symptoms were highly variable; some individuals did not experience any toxicity whereas others were seriously affected.

In almost all respects, the symptoms that were displayed by the Queensland cases are very similar to those recorded in other parts of the world, in particular the South Pacific.[19,21] Victims report a heterogeneity of symptoms and combinations of symptoms (Table 1), and differential diagnosis is difficult. Because many of the persistent symptoms are those of neurosis, depression, hysteria or frank malingering, the diagnosis depends particularly on an awareness of the potential for ciguatera in this country and a comprehensive clinical history that includes the ingestion of reef fish or certain mackerel or barracuda species.

TABLE 1: Frequency of signs and symptoms in cases of ciguatera from three areas in the Pacific region

Sign or symptom	Percentage of victims with finding		
	Queensland	Fiji[19]	French Polynesia and New Caledonia[21]
Gastrointestinal tract			
Diarrhoea	64.2%	51.2%	70.6%
Vomiting	35.0%	29.8%	37.5%
Abdominal pain	52.0%	58.9%	46.5%
Nausea	54.9%	—	42.9%
Loss of energy	90.3%	—	60.0%
Myalgia	83.3%	—	81.5%
Burning of skin on contact with cold water	76.1%	55.3%	87.6%
Pruritus	76.3%	35.1%	44.9%
Arthralgia	79.1%	69.3%	85.7%
Paraesthesia			
Hands	71.2%	—	—
Mouth	65.8%	51.7%	89.1%
Feet	63.5%	—	—
Headache	62.2%	—	59.2%
Ataxia	54.0%	—	37.7%
Chills	49.2%	42.2%	59.0%
Vertigo	44.9%	37.6%	42.3%
Perspiration	42.6%	34.0%	36.7%
Eye soreness	41.1%	—	22.4%
Dental pain	37.2%	—	24.8%
Tremor	30.5%	—	26.8%
Dyspnoea	28.3%	8.5%	16.1%
Neck stiffness	26.7%	—	24.2%
Paresis	26.5%	—	10.5%
Skin rash	25.9%	2.4%	20.5%
Dysuria	22.0%	—	10.0%
Salivation	9.9%	10.0%	18.7%

Among the first symptoms to appear are paraesthesia and numbness around the lips and tongue and tingling in the distal extremities. The presence of paraesthesia is considered to differentiate ciguatera from other forms of food poisoning or mild gastroenteritis.[22] A reversal of temperature sensation is often associated with the paraesthesia. In the clinical experience of one of us (J.H.P.), this is reported as an indescribable unpleasant dysaesthesia; it is certainly a hyperaesthesia with an altered quality such that even the feel of running water on one's skin (as when taking a shower) is unpleasant. These symptoms may last for several days or more. Gastrointestinal symptoms also occur at an early stage. Nausea is followed quickly by vomiting, watery diarrhoea and abdominal pain. These symptoms generally abate within 24 hours.

Other more general disturbances can persist for weeks or months (or even years) in severe cases in which the disease can produce long-term disability. These include loss of energy, arthralgia (especially of the knees, ankles, shoulders and elbows), myalgia, headache and pruritus. Pruritus seems to be more common in Australian cases than in those from other areas of the South Pacific (Table 1). The symptoms fluctuate characteristically, sometimes with a pseudodiurnal periodicity.

Cardiovascular disorders that are associated with ciguatera have only occasionally been reported in Australia, with bradycardia and hypotension being noted in some acute cases. In Queensland, patients with severe cases of ciguatera have been hospitalized to control the loss of body fluids, the respiratory distress or the cardiovascular abnormalities, or a combination of these.[20] Fatalities are rare; only one fatality, which was probably due to ciguatera, has been reported in Australia.[8]

An initial intoxication does not confer immunity. On the contrary, reports of sensitization to the toxin are common — patients who had been affected previously suffered recurrences of typical ciguateral symptoms after eating fish that did not produce symptoms in others.[13,19] In Queensland many such cases of sensitization have been noted. In other instances, individuals with a previous history of ciguatera poisoning have reported a rapid onset of symptoms, which is more typical of an allergic response, after eating fish species that are not normally considered to be ciguateric. The consumption of chicken is also involved sometimes. These cases add a confusing dimension to the diagnosis of

ciguatera-related ailments that cannot be explained by the present knowledge of the disease's toxins or pathophysiology. Equally mystifying are the effects of alcohol, the consumption of which caused a recurrence of ciguateral symptoms in 28% of the victims in Queensland. Alcohol consumption may also increase the severity of the initial illness.[23]

Histamine poisoning may be confused with ciguatera. Several cases of histamine poisoning have been recorded in Queensland; these resulted from the consumption of tailor (*Pomatomus saltatrix*) that had been stored with inadequate refrigeration (unpublished observations). Histamine poisoning can be distinguished from ciguatera by its rapid onset, short duration, and symptoms of flushing, sweating and oral burning (sometimes likened to a peppery taste). Good recovery is achieved by the administration of antihistamine agents such as diphenhydramine.[24]

Occurrence

A large number of cases of ciguatera are not reported to health authorities, so the true incidence of ciguatera in Australia is difficult to assess. We have confirmed 166 ciguatera outbreaks which involved 479 people and occurred between 1976 and 1984.

Reports of outbreaks have increased dramatically since 1976, when four outbreaks were reported. Since 1980, about 20 outbreaks were reported annually until 1984 when the number of reports rose sharply to 36 (Figure 4). Whether these figures reflect a trend towards an increasing incidence of ciguatera or increased public awareness is not known, but it is certain that the abovementioned reports represent only a proportion of the outbreaks that have occurred. Table 2 shows the broad geographical distribution of catches of toxic fish and the number of cases that were involved. It can be seen that the bulk of cases that were reported to us occurred in southern Queensland. Only 24 outbreaks, involving 40 persons, are on record for areas north of Yeppoon.

TABLE 2: Geographical distribution of ciguatera outbreaks based upon the location where the toxic fish were caught for the period 1965–1984

Location of catch	No. of outbreaks*	No. of persons affected
Southern Queensland (Coolangatta to Yeppoon, including Yeppoon)	46	218
Central Queensland (Yeppoon to Bowen, including Bowen)	2	11
Northern Queensland (Bowen to the Northern Territory)	22	29

*The catch location is not available for 105 outbreaks.

Many more cases occur in northern Queensland than are indicated by these figures. Barnes reported that he had recognized over 100 cases in a three-year period in the Cairns district alone and he thought that "these represented only a token of the true incidence".[7] Furthermore, Broadbent collected records for 750 cases in Townsville over a 10-year period between 1964 and 1974 (G. Broadbent, personal communication). According to this information, it is feasible that 2100 cases occurred in Cairns and Townsville over the period 1965 to 1985, most of which were not noted by us.

A telephone survey, which was carried out in Cairns and Maryborough in 1984, indicated that 2.5% and 1.8%, respectively, of the populations of these towns had been affected by ciguatera, which allows an estimate of annual incidence of about three per 10 000 head of population.[25] This suggests that the annual incidence in coastal areas of Queensland is similar to the 3.6 per 10 000 head of population that is the average reported for the South Pacific region.[17]

Areas that are known to produce ciguatoxic fish in Australia are shown in Figure 5. It can be seen that most toxic fish are caught from three broad areas: in southern Queensland, between Caloundra and

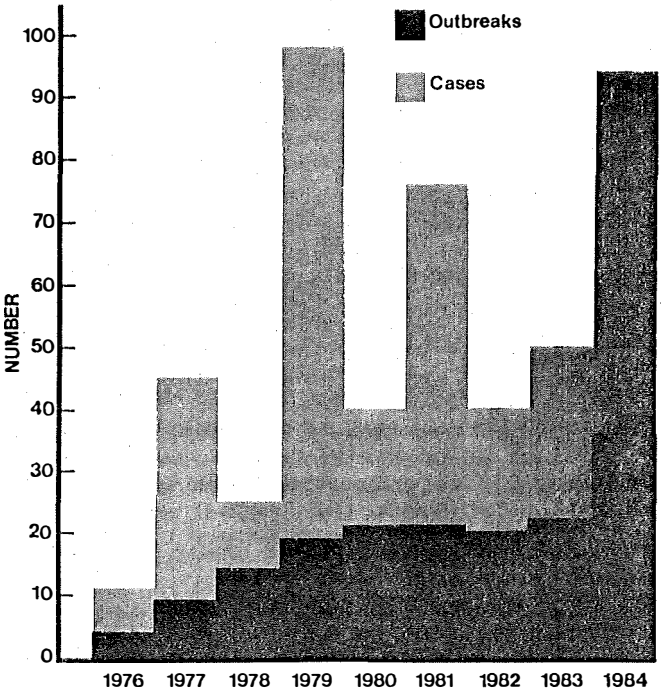

FIGURE 4: Annual reports of ciguatera in Queensland between 1976 and 1984.

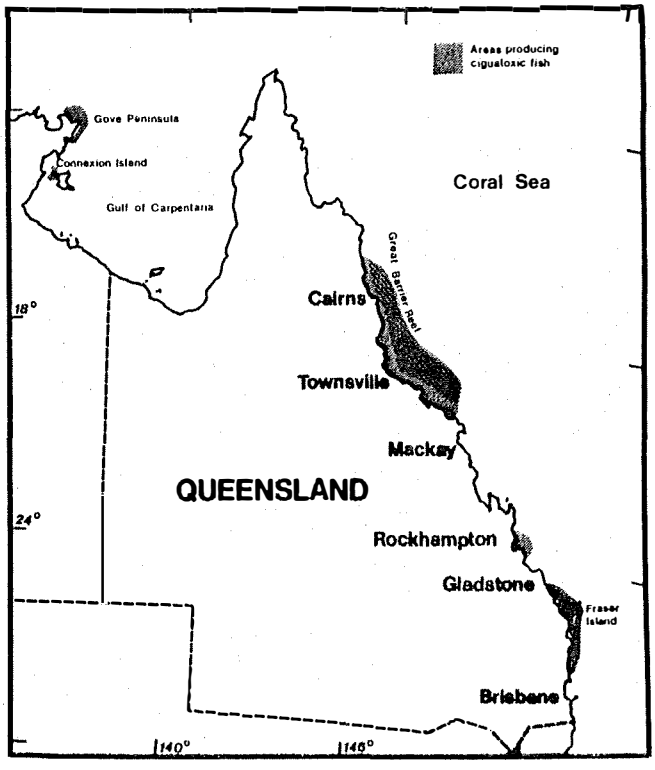

FIGURE 5: Areas in Australia in which ciguatoxic fish occur.

TABLE 3: The number of outbreaks and cases of ciguatera in Queensland between 1965 and 1984, by fish species

Fish species	No. of outbreaks	No. of cases
Scomberomorus commersoni (narrow-barred Spanish mackerel)	30	226
Scomberomorus spp. (mackerel, species unknown)	51	134
Sphyraena jello (barracuda)	13	29
Plectropomus spp. (coral trout)	18	27
Epinephelus fuscoguttatus (flowery cod)	5	14
Lutjanus sebae (red emperor)	6	13
Scomberomorus queenslandicus (school mackerel)	3	9
Scomberomorus munroi (spotted mackerel)	4	8
Scomberoides commersonianus (giant dart)	3	8
Epinephelus tauvina (spotted cod)	3	5
Lethrinus nebulosa (yellow sweetlip)	1	4
Seriola lalande (yellowtail kingfish)	1	4
Epinephelus spp. (cod, species unknown)	4	4
Caranx spp. (trevally, species unknown)	1	3
Cephalopholis miniatus (coral cod)	2	3
Lutjanus bohar (red bass)	3	3
Chelinus trilobatus (maori wrasse)	3	3
Scomberomorus semifasciatus (grey mackerel)	1	2
Seriola spp. (kingfish, species unknown)	1	2
Epinephelus lanciolatus (grouper)	1	2
Epinephelus merra (wire-netting cod)	1	2
Choerodon venustus (venus tusk fish)	2	2
Trachinatus spp. (dart)	1	1
Caranx ignobilus (lowly trevally)	1	1
Paracesio pedlryi (southern fuselier)	1	1
Lates calcarifer (barramundi)	1	1
Other	2	2
Unknown	12	14

Bustard Head; in northern Queensland, between Bowen and Port Douglas; and in the Northern Territory, near Gove. In southern Queensland the majority of toxic fish have been caught on the north-western shores of Fraser Island — a large sand island which fringes Hervey Bay. In northern Queensland toxic fish have been caught around the coral reefs of the Great Barrier Reef. Very few cases involving fish that were caught between Yeppoon and Bowen have been reported; this includes the large Swain Reef complex off the central Queensland coast. There is very little published information on the incidence of ciguatera in the Northern Territory. The only known ciguatera-prone areas are around the Gove peninsula — fish that have caused ciguatera have been caught near Bremer Island, the East Bremer Islands, Bonner Rocks, Miles Island and the Cape Arnhem area, and near Connexion Island, which is off Groote Eylandt.

FIGURE 6: Large narrow-barred Spanish mackerel, Scomberomorus commersoni, *have been responsible for the majority of ciguatera cases in southern Queensland.*

Toxic species

Halstead listed more than 400 species of fish as being potentially ciguateric.[26] However, it is felt that the true figure lies well below that.[13,26] The ciguatoxic species have been found to be limited to those fish that feed on algae or detritus on coral reefs, especially the surgeon-fish (Acanthuridae), the parrot-fish (Scaridae) and the larger reef carnivores that prey largely upon these herbivores.[13] It is the larger carnivores that are the most likely to become toxic and, ironically, it is these species that are most widely caught for human consumption in Australian waters. In Australia, most fish of this type do not actually contain sufficient ciguatoxin to cause human intoxication. The specimens that are usually involved in ciguatera normally contain low levels of toxin and are not a serious health threat. Highly toxic fish are rare in Australian waters, but, when encountered, they can represent a significant health risk.

Table 3 shows the fish species that were involved in 527 cases of ciguatera in Queensland between 1965 and 1984. While we recognize that these data may not be truly representative of the incidence throughout Queensland, the figures show that the narrow-barred Spanish mackerel (*Scomberomorus commersoni*; Figure 6) accounts for most of the cases. Other *Scomberomorus* spp. have also been responsible for a large number of cases. Most of these toxic mackerel were caught in southern Queensland. In northern Queensland a wide range of demersal species have been involved, principally cods, which belong to the genus *Epinephelus*, and the complex of *Plectropomus* spp., which are collectively known as coral trout (Figure 7).

The red bass (*Lutjanus bohar*), the chinaman-fish (*Symphorus nematophorus*) and the paddle-tail (*Lutjanus gibbus*) are recognized as high-risk species in Queensland and are not accepted for sale by the Queensland Fish Board. *Lutjanus bohar* (Figure 8) is recognized as one of the most highly toxic species throughout the Pacific,[27] even though it is commonly eaten by many Queensland fishermen and has been involved in few reported cases locally.

The viscera, particularly the liver, of cigua-

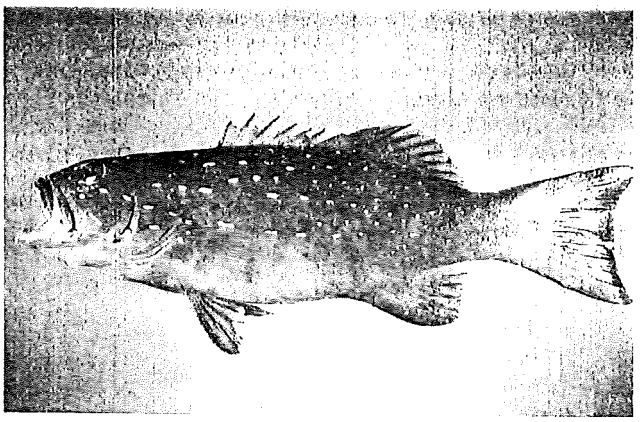

FIGURE 7: An example of one of the complex of Plectropomus *spp., collectively known as coral trout.*

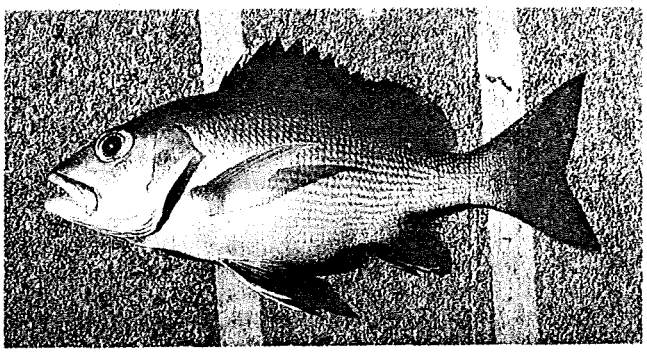

FIGURE 8: The red bass, Lutjanus bohar, is not accepted by the Queensland Fish Board because of a risk of ciguatera.

toxic fish are far more toxic than the flesh,[17] containing up to 50 times more toxin per unit weight than the flesh.[13] Consequently, the risk of ciguatera can be minimized by complete avoidance of the viscera and gonads of reef-fish species. In addition, one should avoid eating large specimens of suspect fish or very large portions of individual fish, at least until a catch is known to be safe. Repeated meals of a slightly toxic fish could also lead to the development of ciguateral symptoms.

Pharmacology of ciguatoxin

Research on the mode of action of ciguatoxin entered a new era in the early 1980s after the use of improved separation techniques which were able to remove biologically-active contaminants from crude extracts of ciguatoxin. Some early studies that used partially purified ciguatoxin concluded mistakenly that ciguatoxin possessed anticholinesterase activity. Digitalis-like activity was also detected in some ciguatoxic extracts; this further complicated efforts to elucidate the true nature of ciguatoxin's effect. Recent studies have confirmed that ciguatoxin has neither of these actions. It is now known that the action of ciguatoxin is to open voltage-dependent sodium channels in the cell membrane.[28-34] If ciguatoxin possesses additional actions other than that of modifying sodium channels, these remain to be identified.

The widespread distribution of sodium channels in nerve and muscle tissues can account for the variety of effects that are caused by ciguatoxin in humans. Sodium channels play a key role in the propagation of action potentials, thereby underlying the transfer of information in nerves and muscles. A possible location of ciguatoxin binding is illustrated in Figure 9, which shows ciguatoxin slotting between the membrane lipid and the sodium-channel protein phases. This location for ciguatoxin binding has been suggested by chromatographic studies which indicate that ciguatoxin possesses regions of moderate polarity and regions of low polarity. Therefore, ciguatoxin could span the lipid-protein phases as indicated in Figure 9 and have a high degree of stability in this location. Ciguatoxin binds to a unique part of the sodium channel.[35] Another dinoflagellate toxin, brevetoxin, also binds to a unique site on the sodium channel,[36] and it remains to be determined whether the sites for ciguatoxin and brevetoxin overlap.

In-vivo studies

The pharmacology of ciguatoxin has been investigated *in vivo* and *in vitro*. Whole animal studies allowed the target tissues that were affected by ciguatoxin to be pinpointed. It was found that ciguatoxin caused death by blocking phrenic nerve conduction, thus causing respiratory failure.[37] This nerve block was believed to be of central origin. However, the experimental protocol (that is, pentobarbital anaesthesia and nerve stimulation [50 Hz]) may have favoured the finding of a central respiratory block, and further studies are required to confirm the accuracy of this result.[32] Low doses of ciguatoxin cause mild bradycardia and hypotension in mammals. Sufficient ciguatoxin that has been injected intravenously into artificially-ventilated cats induces (within one hour) biphasic cardiovascular changes, which consist of initial bradycardia and hypotension (cholinergic origin) that are followed by tachycardia and hypertension (adrenergic origin),[38] and eventually causes arrhythmias and cardiac failure.[39]

Drugs that have been found to be effective

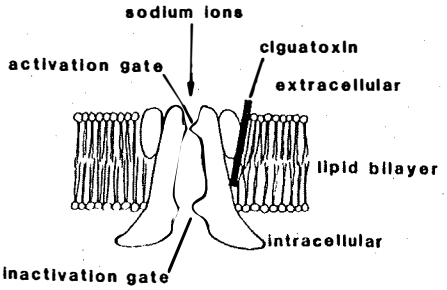

FIGURE 9: Diagrammatic cross-sectional view of a sodium-channel protein complex which spans the cell membrane (lipid bilayer). The channel is shown in the non-conducting state, in which it is generally found at resting membrane potentials. Upon cell depolarization the activation gate opens, allowing an influx of sodium ions. Shortly after, the inactivation gate closes, stopping the flow of ions and allowing cell repolarization. A possible location for ciguatoxin shows it spanning the lipid-protein phases.

(experimentally) at countering the initial cardiovascular effects of ciguatoxin are atropine, phentolamine, calcium gluconate and lidocaine.[38] It remains to be determined which drugs are effective against ciguatoxin-induced cardiac failure. Ciguatoxin does not cause detectable lesions in the brain.[32] It removes the ability of mice to regulate their body temperature, and experimental animals apparently become poikilothermic.[40] Ciguatoxin also enhances the amplitude and duration of the supernormal period as well as reducing the velocity of nerve conduction and increasing the absolute refractory period of rat peripheral nerve *in vivo*.[41]

In-vitro studies

The precise mode of action of ciguatoxin has been determined on a wide variety of isolated tissues. Ciguatoxin was found to cause phasic contractions of the smooth muscle of guinea-pig ileum and vas deferens at doses as low as 2×10^{-12} M and 2×10^{-10} M, respectively.[30,31] Blockers of nerve conduction and antagonists of the specific neural transmitter, which were released in each tissue, countered these contractions effectively, indicating that the ciguatoxin caused the autonomic nerves to fire spontaneously. A reduced response to repeated doses of ciguatoxin was observed in ilea, and the response of the nerves to nicotine was almost abolished after the administration of ciguatoxin.[30] Both observations are indicative of nerve-conduction block after the initial neural stimulation. Nerves that innervate cardiac tissues are also stimulated by ciguatoxin.[31,33,42] Interestingly, ciguatoxin (2×10^{-9} M) caused a total block of the rat phrenic nerve without any detectable nerve stimulation.[43] Voltage-clamp studies of ciguatoxin's action on frog myelinated nerves revealed that ciguatoxin opens voltage-dependent sodium channels at resting membrane potentials,[34] at least in this species.

Direct effects of ciguatoxin on cardiac, smooth and skeletal muscle tissues also have been reported. In 1972, Rayner first showed that ciguatoxin caused cell depolarization.[28] This effect was completely reversed by tetrodotoxin — a toxin which blocks the voltage-dependent sodium channel. Therefore, ciguatoxin must open sodium channels in muscle cells. This action of ciguatoxin can explain its blocking of striated muscle contractions,[41] and its potentiation of the response of the vas deferens to agonist drugs.[44] A block of the phrenic nerve and the diaphragm muscle, which was caused by ciguatoxin, occurred similarly in unstimulated and stimulated preparations.[32]

The cardiovascular effects that are associated with ciguatera deserve special mention as they are potentially lethal. Early in-vitro studies revealed that ciguatoxin had a prominent stimulatory effect on nerves that innervate the heart but had no apparent direct effect on the cardiac musculature. Indirect effects of ciguatoxin on the heart are

unlikely to account for cardiac failure on their own, and additional actions of ciguatoxin are indicated.[31] Ciguatoxin is expected to have direct cardiac effects because the sodium channels of nerves and muscles are quite similar. A recent study has confirmed that ciguatoxin has direct and indirect effects on the atria (at 10^{-10} M) and papillary muscles (at 10^{-9} M) of guinea-pigs.[11] The direct effect of ciguatoxin caused cell depolarization which was not reversed by washing and which resulted in a large increase in the systolic force of the heart. Tetrodotoxin reversed the increase in force and the cell depolarization, showing that ciguatoxin opened the sodium channels in the muscle cells of the heart. The established mechanisms of sodium/calcium exchange and calcium-induced release of calcium can explain the link between the sodium-dependent depolarization that was caused by ciguatoxin and the increased systolic force.[33] Ciguatoxin also reduced the conduction velocity and the rate of rise of the upstroke of the action potentials and it increased the stimulation threshold voltage, probably as a direct consequence of cell depolarization.[33]

The direct action of ciguatoxin in combination with its indirect effects could lead to cardiac failure. Arrhythmias, including extrasystoles, flutter and delayed after-contractions, are induced in isolated cardiac preparations by a combination of the direct and indirect effects of ciguatoxin.[32] It was found that the local anaesthetic agent lidocaine is a particularly effective antagonist of ciguatoxin's action,[45] but that digitalis-like drugs enhanced the toxic effects of ciguatoxin.[32] It was speculated that lidocaine blocks sodium channels that have been modified by ciguatoxin in preference to normal sodium channels.[32] However, procaine appeared to be a poor blocker of ciguatoxin-modified channels. Research is required to determine which lidocaine-like drugs are most effective clinically.

Treatment

The treatment of ciguatera remains symptomatic and supportive in spite of major advances in our understanding of the pharmacology of ciguatoxin, as described above. No controlled clinical studies have yet been reported. The treatment of ciguatera falls into two broad categories which require differing approaches. These are (1) the treatment of the mild and the long-term neurological effects of ciguatera and (2) the treatment of the rare, but severe, potentially lethal effects of ciguatera. Drugs that have been used with varying degrees of efficacy to treat the long-term effects of ciguatera include vitamin B_{12}, calcium gluconate and antihistamine agents,[16,46-49] and recently the use of amitriptyline has been advocated.[50,51] Debate continues as to whether vomiting and diarrhoea in the early stages of ciguatera should not be suppressed because they can rid the body of undigested toxin. An emetic agent may be useful when vomiting is not a symptom of ciguatera and when the diagnosis is made within four hours of the fish ingestion. Atropine may be useful to control persistent vomiting or diarrhoea, and the fluid and electrolyte balance should be monitored and maintained.[17] Severe cases of ciguatera may be accompanied by respiratory distress and cardiac disturbances. Artificial respiration may be necessary. Atropine can control bradycardia and hypotension,[17] but a drug which can prevent respiratory or cardiac failure in cases of ciguatera has not yet been found. Food items that can cause the recurrence of ciguateral symptoms,[52] in particular fish and alcohol, should be avoided during the early weeks of convalescence after ciguatera poisoning.

There is considerable need to find drugs that can treat ciguatera specifically. Such drugs would partition in the body similarly to ciguatoxin, they would block selectively the sodium channels that were opened by ciguatoxin and would have relatively few side-effects. According to the symptomatology, there are indications that ciguatoxin has a central action. The observation that the fetus of a ciguatera victim might have been affected by ciguatera indicates that ciguatoxin probably crosses the maternal–fetal blood barrier.[53] Potentially useful drugs include those that possess local anaesthetic activity,[32] of which there are many.[54] Local anaesthetic agents block the sodium channels by binding with different affinity to the sodium channel, depending upon the state of the sodium channel, that is, whether the activation and inactivation gates are open or closed (see Figure 9). It is conceivable that a local anaesthetic drug can be found that prefers to block the sodium channel in the state that remains after ciguatoxin modification. Further pharmacological and clinical studies will be required before the drugs which most effectively treat ciguatera are found.

Future developments

The general features of ciguatoxin have been known for some time; it is a polyether compound with a molecular weight of about 1112, it is lethal to mice at 0.45 $\mu g/kg$[55] and is heat stable, and therefore not affected by normal cooking procedures. Further progress in obtaining its chemical structure has been slow because of difficulties in obtaining sufficient toxin. Currently, it appears that the ciguatoxin in Queensland fishes is identical to ciguatoxin in fishes from other areas,[11,56] although preliminary immunological studies indicate that subtle differences might exist. The principal source of toxin for research are fish samples that have been involved in cases of human intoxication. In spite of the discovery in French Polynesia that the dinoflagellate *Gambierdiscus toxicus* contained ciguatoxin,[16] nobody has yet succeeded in consistently inducing this organism to produce the toxin in culture. The derivation of the chemical structure of ciguatoxin could allow its synthesis in sufficient quantities to allow research; this would lead to more effective treatment and detection procedures and allow further pharmacological evaluation.

At present, there is no reliable, practical method for detecting ciguatoxin in fish. The toxin itself does not affect the normal organoleptic qualities of the fish in any way. A variety of assays that have employed animals including cats and mongooses,[13] and chickens[57] or injection of extracts into mice[58,59] have been used for the detection of toxic fish. The prevalence of toxic fishes is very low, so any test should be sufficiently simple and reliable to allow routine application to individual fish. While none of the tests has met these criteria, immunoassay techniques may eventually enable the development of a simple routine test. Hokama et al. have recently described a simple poke-stick test that could be used to detect ciguatoxin.[60] This test, which is based on an enzyme-linked immunoassay for ciguatoxin, has demonstrated that a routine screening test that can be applied to individual fishes is feasible.

As well as the development of a simple test for the presence of ciguatoxin and improved treatment regimens, additional public safeguards may be forthcoming from improved reef management strategies. It is notable that the Great Barrier Reef between Bowen and Port Douglas is experiencing an increase in tourist-oriented developments. It is inevitable that these developments will cause a certain amount of disturbance to the reef, through the action of anchors, diver activities and minor harbour works. Ciguatera outbreaks have often occurred after the disturbance of coral reefs by man-made or natural causes. Whereas every instance of reef disturbance has not necessarily been followed by an upsurge in fish toxicity,[61] a causal link between disruptive activities and the appearance of ciguatera in a reef ecosystem has been demonstrated.[62] In order to minimize the incidence of ciguatera through management of human activities on reefs it will be necessary to have a much greater understanding of the origin of ciguatoxin and the mechanism by which it accumulates in reef fishes than is presently available.

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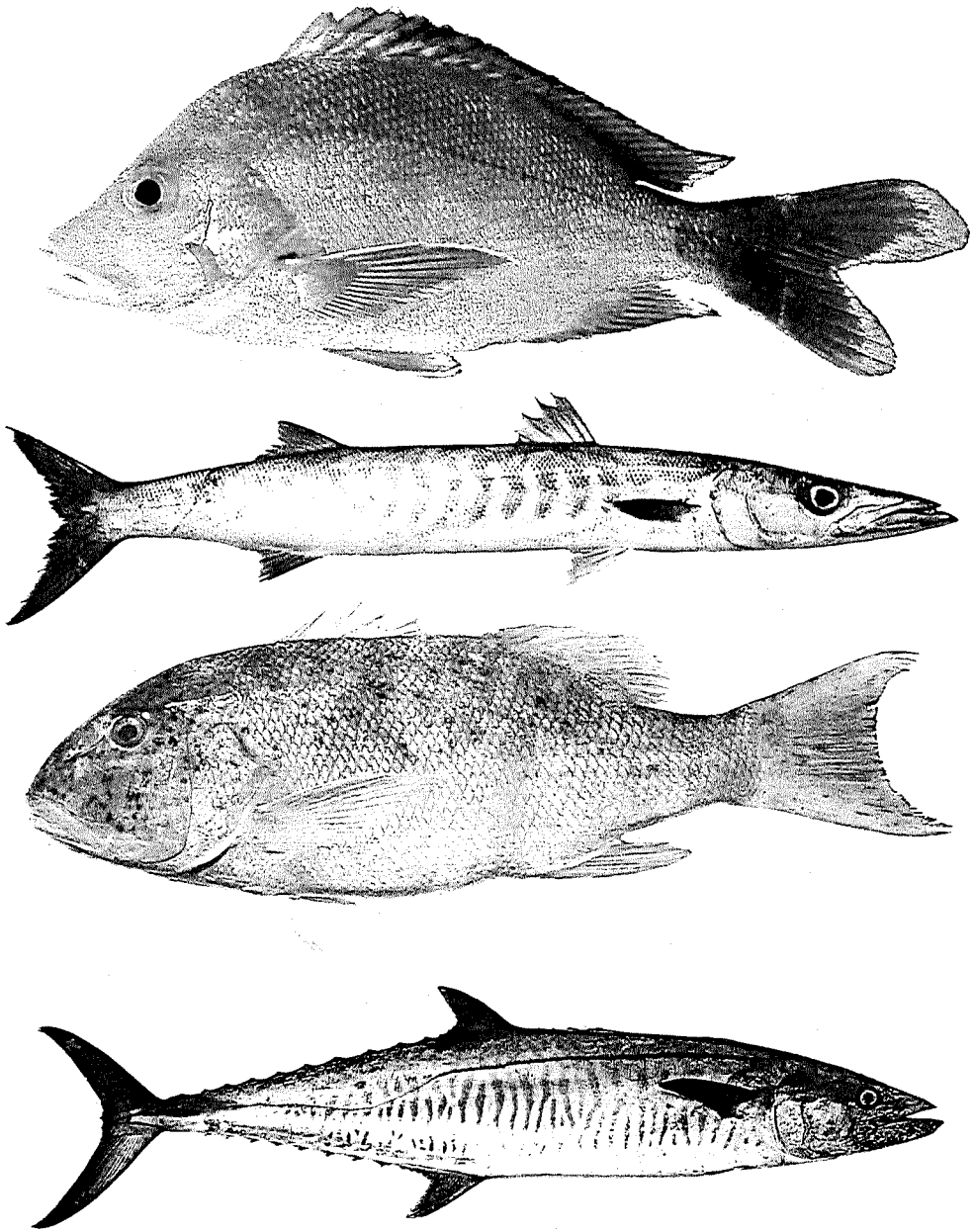


CIGUATERA POISONING

Noel Gillespie

Noel Gillespie, B.Sc. (Hons.), Ph.D., is Assistant Director of Fisheries Research in the Department of Primary Industries. His research interests focus on various aspects of ciguatera fish poisoning. He has also worked on the bacteriology of spoilage of fish and prawns and a number of other subjects related to seafood handling. (Deception Bay Fisheries Laboratory, Box 76, Deception Bay, Qld. 4508).

◄ Paddletail, *Lutjanus gibbus*.
Slender Barracuda, *Sphyraena jello*.
Chinaman Fish, *Symphorus nematophorus*. (Photographs: E. Grant).

Queensland School Mackerel, *Scomberomorus queenslandicus*.

Ciguatera is a term used to describe a disease caused by eating certain fishes associated with coral reefs. The principal toxin involved is a heat stable lipid-soluble compound named ciguatoxin (Scheuer *et al.* 1967). Other toxins including the lipid-soluble scaritoxin (Chungue and Bagnis 1976) and the water-soluble maitotoxin (Yasumoto *et al.* 1976) may also be involved.

The term ciguatera was originally applied to neurological and gastrointestinal disorders found in Cuba that resulted from eating the 'cigua', a turban shell *Turbo* (*Livona*) *pica* L., (Gudger 1930) and was then extended to cover similar symptoms produced by toxic fish. The occurrence of ciguatera was noted in the Pacific as early as 1606 when sailors with Spanish explorer de Quiros suffered from ciguatera in the New Hebrides (Banner 1976). The syndrome is now proven to be widespread affecting both tropical and subtropical coastal regions of the world, but is largely confined to islands in the true tropics (Banner 1976). In those areas where ciguatera occurs, toxic outbreaks are sporadic and unpredictable with a patchy distribution in both space and time (Banner 1976; Cooper 1964).

Ciguatera produces characteristic gastrointestinal and neurological symptoms, but is rarely fatal. Diagnosis is based only on clinical symptoms. These can vary considerably between individuals in their manifestation and incorrect diagnosis is common.

In Caribbean and South Pacific countries, ciguatera fish poisoning is often the most frequently reported food-borne disease. In these areas, ciguatera is not only a public health problem, but also has economic implications. It restricts the exploitation of easily available demersal reef fish which might otherwise be a source of much needed export income. This paper briefly examines the general nature of ciguatera and its occurrence in Australia.

SYMPTOMS

Bagnis *et al.* (1979) completed a very extensive analysis of clinical symptoms of ciguatera from 3009 cases from French Polynesia, collected between 1964 and 1977. Another larger study of 792 cases in Fiji has been reported by Narayan (1980). The frequency of signs and symptoms determined by these

workers is summarised in Table 1, which also shows the symptoms displayed by 476 cases of ciguatera reported to health authorities in Queensland, Australia, principally between 1975 and 1985. Records for only 42 cases in Queensland prior to 1975 are available.

Paresthesia, or numbness and tingling of the extremities and lips and mucosa of the mouth, is frequently considered a hallmark of ciguatera in the South Pacific and differentiates this form of intoxication from other forms of food poisoning or mild gastroenteritis (Bagnis *et al.* 1979). Certainly paresthesia is very common in those cases reported in Queensland (Table 1). Often associated with paresthesia is an apparent reversal of temperature sensation, whereby contact of the extremities and mouth with cold objects produces a very unpleasant, often acutely painful tingling or burning sensation. Contact with heat does not generally result in any adverse sensation (Bagnis *et al.* 1979). Other neurological changes such as joint pain, muscle pain, weakness and itchiness are extremely common in Queensland, often persisting for weeks or years in severe cases. The high incidence of pruritus or itchiness and certain other neurological symptoms in Queensland relative to French Polynesia (Bagnis *et al.* 1979) or Fiji (Narayan 1980) may suggest a difference in susceptibility to these symptoms or a slight difference in the toxins involved. Clinical cases in French Polynesia and New Caledonia include those caused by surgeonfish and parrotfish which contain different toxins in addition to ciguatoxin. On the other hand the data from Queensland is strongly influenced by cases involving the Narrow-barred Spanish Mackerel, *Scomberomorus commersoni*, in which ciguatoxin is the principal toxin (Lewis and Endean 1983a).

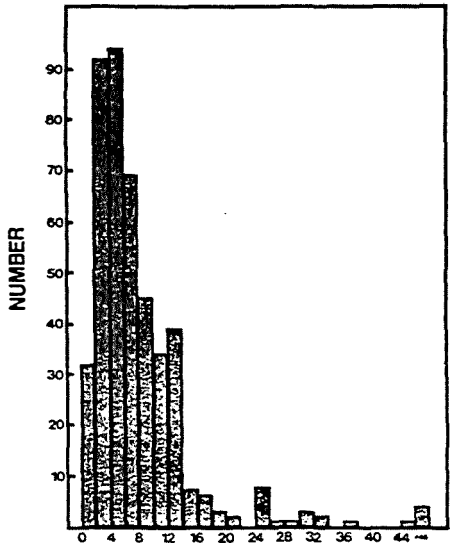
Gastrointestinal symptoms such as vomiting, diarrhoea, nausea and abdominal pain usually develop at an early stage and are usually the first symptoms to disappear (Lawrence *et al.* 1980). Cardiovascular signs and symptoms including hypotension and bradycardia have been noted in some acute cases (Morris *et al.* 1982; Bagnis *et al.* 1979).

Intoxication does not confer immunity and report of sensitisation to the toxin are common, victims reporting a recurrence after eating fish that do not produce symptoms in others (Banner

Table 1: Frequency of signs and symptoms in cases of ciguatera fish poisoning from three areas in the Pacific region.

Sign or Symptom	Percentage of patients with finding		
	Queensland (a) Australia	Fiji (b)	French (c) Polynesia New Caledonia
Numbness and tingling of hands	73.1	—	—
Numbness and tingling of feet	64.3	—	—
Numbness around the mouth	66.5	51.7	89.1
Burning of skin on contact with cold water	76.4	55.3	87.6
Joint pains	79.1	69.3	85.7
Muscle pains	84.8	—	81.5
Diarrhoea	65.2	51.2	70.6
Weakness	90.3	—	60.0
Headache	63.8	—	59.2
Chills	51.7	42.2	59.0
Abdominal pain	54.4	58.9	46.5
Itchy skin	76.9	35.1	44.9
Nausea	57.2	—	42.9
Dizziness	44.8	37.6	42.3
Difficulty walking	56.2	—	37.7
Vomiting	35.9	29.8	37.5
Sweating	44.5	34.0	36.7
Shaking	30.7	—	26.8
Dental pain	41.9	—	24.8
Neck stiffness	25.7	—	24.2
Eye soreness	42.8	—	22.4
Skin rash	26.5	2.4	20.5
Pain on urination	22.8	—	10.0
Salivation	11.6	10.0	18.7
Shortness of breath	28.4	8.5	16.1
Low blood pressure	—	1.2	12.2
Inability to move arms and legs	28.4	—	10.5

(a) Queensland Health Department data.
(b) Narayan (1980).
(c) Bagnis *et al.* (1979).



Distribution of time taken for the first appearance of ciguatera symptoms reported by 434 victims of ciguatera fish poisoning in Queensland 1976–1984 (Queensland Health Department data).

1976; Bagnis *et al.* 1979). In Queensland, 28% of victims report recurrence of symptoms after consuming alcohol. It is possible that alcohol consumption also increases the severity of the initial illness (Edmonds 1974).

The incubation period before onset of symptoms ranges from 1 to 36 hours but most victims develop signs within 5–12 hours. The associated figure shows the distribution of time of onset recorded in 434 cases recorded in Queensland. The fatality rate is very low and only one probable fatal case has been reported in Australia (Tonge *et al.* 1967). Most victims recover within 3–4 days but weakness, paresthesia, pruritus, muscle and joint pains can persist for extended periods: sometimes years.

TREATMENT

Until now, no specific antidote for ciguatera poisoning has been found. Consequently treatment is symptomatic and supportive only. During the first 24 hours after ingestion of toxic meals, the patient should be induced to vomit to eliminate unabsorbed toxin, either by using oral emetics or by inserting a finger down the throat. Recommendations for treatment of ciguatera by R. Bagnis have recently been described (Yasumoto *et al.* 1984). In mild cases systematic daily intravenous injection over 3 days of a cocktail consisting of calcium gluconate, vitamin B_6 and vitamin B_{12} as well as an antihistamine was suggested. If necessary, the same substances could be administered orally for a further three days. For severe hospitalised cases the treatment recommended included gastric lavage, ventilation assistance, intravenous injection of steroids (cortisone), non-respiratory depressants, analgesics, atropine, calcium gluconate and B vitamins. Intravenous infusion of fluids and nutrients was suggested in cases of extended paralysis.

OCCURRENCE OF CIGUATERA IN AUSTRALIA

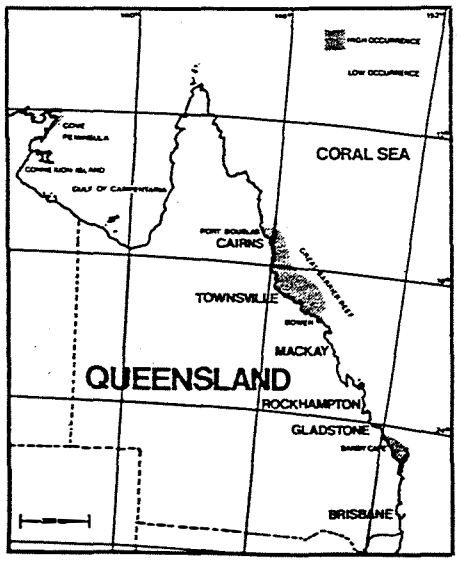
In the past, it was accepted that ciguatera was largely confined to islands in the true tropics and was not found along continental margins (Halstead 1967; Banner 1976). While there has been a general paucity of published records of ciguatera in Australia, reports over a long period (Whitley 1934, 1943; Cleland 1942; Barnes 1965; Tonge *et al.* 1967; Broadbent 1968; Gillespie 1980; Lewis and Endean 1983a) are sufficient to indicate that a long term endemic situation exists along the Queensland coast. The Northern Territory Department of Health also recognises a number of ciguatera prone areas near Gove Peninsula.

The true rate of ciguatera incidence in Australia is difficult to assess. While the Queensland Health Department has recorded 434 cases between 1976 and 1985, most of them involve Narrow-barred Spanish Mackerel caught in southern Queensland, and include only 27 cases involving areas north of Rockhampton. However Barnes (1965) indicated that in the Cairns district alone he had recognised over 100 cases in a three year period and these represented only a token of the true incidence. Furthermore, Broadbent (pers. comm.) collected records for 750 cases in Townsville over a 10 year period between 1964 and 1974.

In an attempt to determine the true rate of ciguatera incidence along the coast of Queensland, Capra and Cameron (1985) undertook a telephone survey of 5% of private telephone listing in Cairns and Maryborough.

Their results indicate that 2.5% and 1.8% respectively of the populations of these towns were affected by ciguatera, allowing an estimate of annual incidence of about 3 per 10,000 head of population (Capra and Cameron 1985). This suggests an annual incidence rate in Queensland similar to that which is general for the South Pacific region. Based on figures reported to the South Pacific Commission between 1973 and 1981 the mean annual rate of incidence in South Pacific countries is 3.6 per 10,000 head of population (Yasumoto *et al.* 1984).

Areas known to produce ciguatoxic fish in Australia are shown on the accompanying map. In Queensland, areas producing the greatest number of toxic fish are in southern Queensland, principally in the Hervey Bay area and in other coastal areas extending northwards to Bustard Head; and in far north Queensland, including reefs between Bowen and Port Douglas. In southern Queensland, ciguatoxic fish are principally caught near the mainland coast and the western shores of Fraser Island, a large sand island fringing Hervey Bay. In north Queensland toxic fish are caught mainly around coral reefs



Areas in northern Australia known to have produced fish responsible for cases of ciguatera. No reports are available for areas without shading.

on the outer edge of the Barrier Reef but inshore reefs have also been implicated (Queensland Health Department data). Very few cases involving fish caught between Yeppoon and Bowen have been reported and there have been no instances of ciguatoxic fish being caught on the Swain Reef complex off the central Queensland coast.

In the Northern Territory, there is very little published information on ciguatera incidence. The only problem areas known (see map) are in the Gove area with fish causing ciguatera having been caught near Bremer Island, East Bremer Islands, Bonner Rocks, Miles Island and the Cape Arnhem area at Gove and Connexion Island off Groote Eylandt (Northern Territory Health Department).

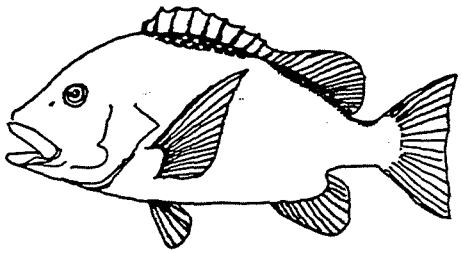
TOXIC SPECIES

Halstead (1967) listed more than 400 species of fish as being potentially ciguateric. However it is felt that the true figure must lie well below that (Yasumoto *et al.* 1984; Banner 1976). While it is difficult to generalise it has been found that ciguatoxic species are limited to those fish that feed on algae or detritus on coral reefs, especially the surgeonfish (*Acanthuridae*), parrotfish (*Scaridae*) and the larger reef carnivores that prey largely upon these herbivores (Banner 1976). It is the larger carnivores that become the most toxic and ironically it is these species which are most widely caught for human consumption in Australian waters.

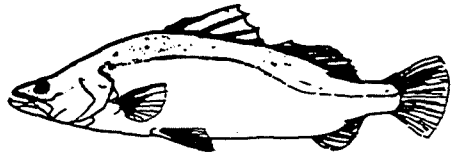
Table 2 shows the fish species involved in 476 cases of ciguatera poisoning reported to the Queensland Health Department, mostly between 1976 and 1984. The Narrow-barred Spanish Mackerel (*Scomberomorus commersoni*) accounts for the majority of cases reported, 25 fish being responsible for poisoning 213 people. Most of these fish were caught in southern Queensland. Other *Scomberomorus* spp. have also been responsible for a larger number of cases. In north Queensland a wide range of demersal species have been involved, principally cods belonging to the genus *Epinephelus* and the complex of *Plectropomus* spp. collectively known as coral trout. However, it is appropriate to mention that even in areas known for producing toxic fish, less than 1 in 1,000 fish may actually contain sufficient ciguatoxin to cause human intoxication. Excepting Barracuda (*Sphyraena*

Table 2: The number of outbreaks and people involved in ciguatera poisoning by fish species.

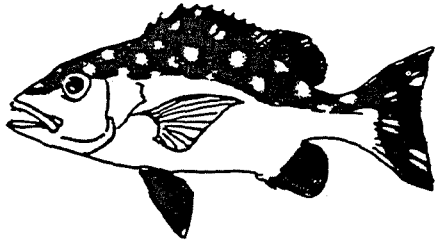
Fish Species	Common Name	No. of Outbreaks	No. of Cases
Scomberomorus commersoni	(Narrow-barred Spanish Mackerel)	25	213
S. queenslandicus	(School Mackerel)	6	20
S. munroi	(Spotted Mackerel)	4	8
S. semifasciatus	(Grey Mackerel)	1	2
Scomberomorus sp.	(Mackerel unspec.)	36	118
Plectropomus sp.	(Coral Trout)	17	27
Scomberoides commersonianus	(Giant Dart)	2	6
Trachinotus sp.	(Dart)	1	1
Lithrinus nebulosus	(Yellow Sweetlip)	1	4
Seriola lalande	(Yellowtail Kingfish)	1	2
Lutjanus bohar	(Red Bass)	2	2
Lutjanus sebae	(Red Emperor)	3	10
Sphyraena jello	(Barracuda)	7	25
Epinephelus lanceolatus	(Grouper)	1	2
Epinephelus fuscoguttatus	(Flowery Cod)	4	4
Epinephelus tauvina	(Spotted Cod)	2	3
Cephalopholis miniatus	(Coral Cod)	1	2
Epinephelus sp.	(Cod unspec.)	3	3
Caesio xanthurus	(Southern Fusilier)	1	1
Lates calcarifer	(Barramundi)	1	1
Other	—	1	1



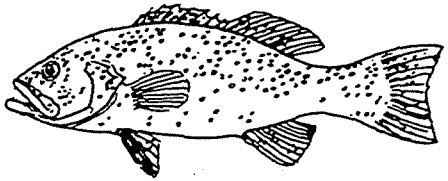
Lutjanus sebae RED EMPEROR



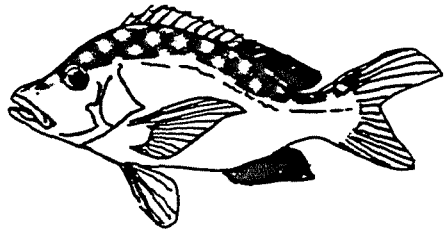
Lates calcarifer BARRAMUNDI



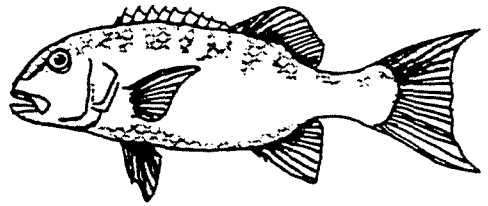
Lutjanus bohar RED BASS



Plectropomus leopardus CORAL TROUT



Lutjanus gibbus PADDLE-TAIL

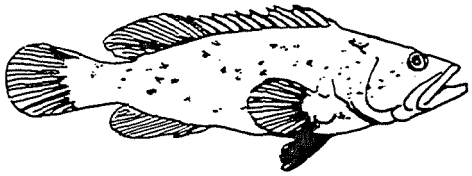


Symphorus nematophorus CHINAMAN-FISH

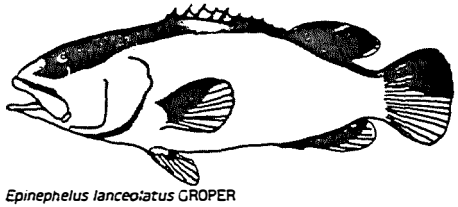
jello) and the Red Bass (*Lutjanus bohar*) most of the fish species listed in Table 2 are caught in commercial quantities and eaten without ill-effect.

The Red Bass. *Lutjanus bohar*. together with the Chinaman-fish. *Symphorus nematophorus*. and Paddle-tail. *Lutjanus gibbus*. are recognised as 'high risk' species in Queensland and not accepted by the Queensland fish Board. *Lutjanus bohar* is recognised as one of the most highly toxic fish species throughout the Pacific (Banner 1976) even though it is eaten with impunity in the Solomon Islands (author's unpublished results).

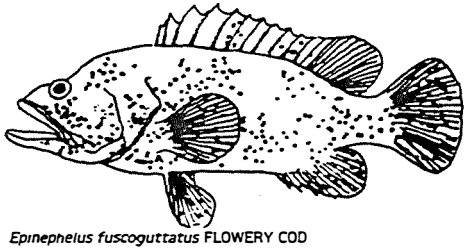
The viscera. especially the liver of those fish involved. are far more toxic than the flesh (Yasumoto *et al.* 1984). Helfrich *et al.* (1968) was able to show that the liver of *L. bohar* from Palymra contained 50 times more toxin per unit weight than the flesh. Yasumoto *et al.* (1977) found a much higher concentration of toxin in the liver than the flesh of a wide range of reef fish species.



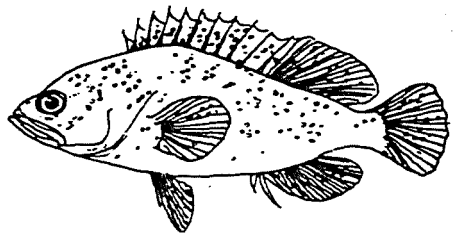
Epinephelus tauvina ESTUARY COD



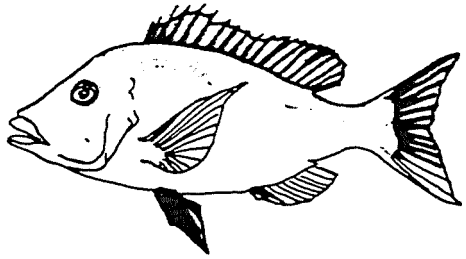
Epinephelus lanceolatus GROPER



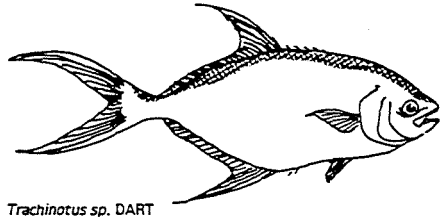
Epinephelus fuscoguttatus FLOWERY COD



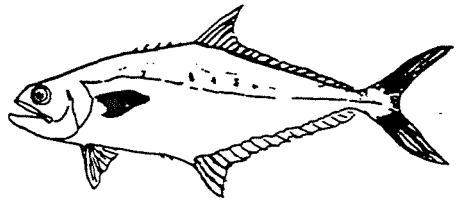
Cephalopholis miniatus CORAL COD



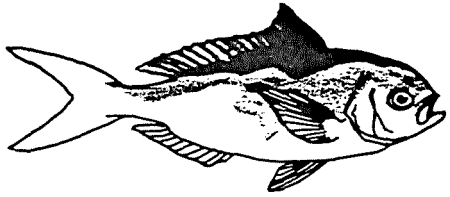
Lethrinus nebulosus YELLOW SWEETLIP



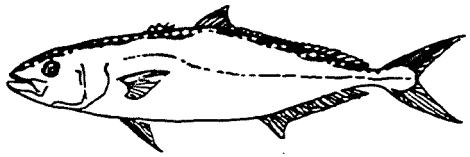
Trachinotus sp. DART



Scomberoides commersonianus GIANT DART

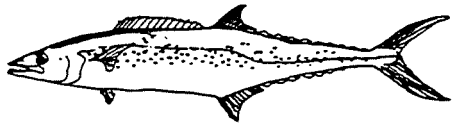


Paracaesio pedleyi SOUTHERN FUSILIER

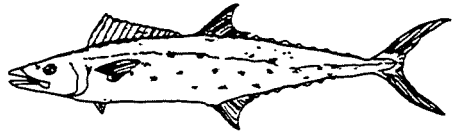


Seriola lalandi YELLOWTAIL KINGFISH

Sphyraena jello SLENDER BARRACUDA

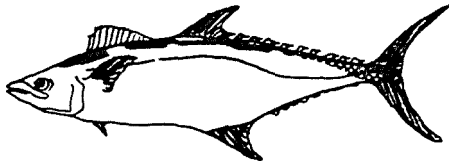


Scomberomorus munroi SPOTTED MACKEREL



Scomberomorus queenslandicus SCHOOL MACKEREL

Scomberomorus commersoni NARROW-BARRED MACKEREL



Scomberomorus semifasciatus BROAD-BARRED MACKEREL

CHEMICAL, TOXICOLOGICAL AND PHARMACOLOGICAL PROPERTIES OF CIGUATERA TOXINS

Ciguatoxin from the Moray Eel was isolated and characterised by Scheuer *et al.* (1967). The principal toxic component of Narrow-barred Spanish Mackerel (*S. commersoni*) and Barracuda (*Sphyraena jello*) responsible for poisoning in Queensland has been shown to be ciguatoxin (Lewis and Endean 1983a,b, 1984a,b). While the general features of ciguatoxin have been known for some time, progress in obtaining its chemical structure has been slow because of the difficulties in obtaining sufficient toxin. However Tachibana (1980) has shown the compound to be a highly oxygenated lipid compound of about 1112 molecular weight, lethal to mice at 0.45 μg/kg.

The primary action of ciguatoxin is to increase permeability of excitable membranes to sodium ions. This type of membrane is found throughout the body in nerve tissue as well as heart and skeletal muscle. The direct effect of ciguatoxin on these tissues are blocked by tetrodotoxin and calcium ions. Hence the use of calcium salts in treatment regimes.

Scaritoxin is ciguatoxin-like and found in the flesh of parrotfish (Chungue and Bagnis 1976; Chungue *et al.* 1977). It is a key component in poisoning involving parrotfish and is thought to closely resemble ciguatoxin in structure (Yasumoto *et al.* 1984). Little is known of its action but it is not obviously different to ciguatoxin (Yasumoto *et al.* 1984).

Maitotoxin is a water-soluble toxin lethal to mice at 0.13 μg/kg (i.p.) found in the viscera of surgeonfish (Yasumoto *et al.* 1971, 1976) and is regarded as one of the most potent known marine toxins (Ohizumi and Yasumoto 1983). Its action is to increase the permeability of excitable membranes to calcium ions (Takahashi *et al.* 1982).

MANAGEMENT OF THE PROBLEM

In Australia, ciguatera represents a problem of public relations for the tourism and fishing industries and of public health for health authorities. While it affects principally some parts of the Northern Territory and Queensland, coral reef fish species are also distributed widely from these areas to centres throughout

Australia. Several cases of ciguatera involving fish caught in north Queensland have been reported in Sydney and Melbourne.

Unfortunately there is presently no reliable, practical method for detecting ciguatoxin in fish. A variety of assays employing animals including cats and mongooses (Banner 1976), chickens (Vernoux *et al.* 1985), injection of extracts into mice (Banner *et al.* 1961; Hoffman *et al.* 1983), as well as brine shrimp (Granade *et al.* 1976) have been used for the detection of toxic fish. The incidence of toxic fishes is very low, so any test should be sufficiently simple and quantitative to allow routine application to individual fish. None of these tests have met these criteria. Immunoassay techniques may eventually enable the production of a simple routine test. Hokama *et al.* (1977) developed a sensitive radioimmunoassay for the detection of ciguatoxic fishes which proved too costly and time consuming for routine application. Subsequently a sensitive enzyme linked immunoassay procedure was developed (Hokama *et al.* 1983) that was much more simple to apply but lacked specificity to ciguatoxin, cross-reacting with a number of lipid-soluble polyether compounds including okadaic acid, brevetoxin and monensin. More recently, Hokama *et al.* (1985) has described a simple poke stick test that detects polyether compounds with a high degree of sensitivity. Using a bamboo skewer coated with Liquid Paper and an immunoassay based on monoclonal antibodies to okadaic acid, this method is the closest yet developed to the accepted ideal of a simple dip stick test to ciguatoxin. Unfortunately the present test cross-reacts with okadaic acid and possibly other polyether compounds that may occur in fish and is consequently prone to false positive reactions. Nevertheless Hokama *et al.* (1985) has demonstrated that a routine screening test that can be applied to individual fishes is within the bounds of feasability.

In the absence of a proven test, public safeguards against ciguatera can be improved by education of fisherman, wholesalers, retailers and consumers about simple precautionary measures. These include the avoidance of known high risk species, large specimens of all reef fish species and the viscera (especially liver and gonads) of all fishes as well as the adoption of the practice of eating only small portions of fish at each meal and avoiding repeated meals of the same fish.

It is notable that the Great Barrier Reef is experiencing an increase in tourist-oriented developments. It is inevitable that these developments will cause a certain amount of reef disturbance, through the action of anchors, diver activities and minor harbour works. Ciguatera outbreaks have often followed disturbance of coral reefs by man-made or natural causes. While every instance of reef disturbance has not necessarily been followed by an upsurge in fish toxicity (Banner 1974), Bagnis (1969) has demonstrated a causal link between disruptive activities and the appearance of ciguatera in a reef ecosystem. However, in order to minimise the ciguatera incidence through management of human activities on reefs it will be necessary to have a much greater understanding of the origin of ciguatoxin and the mechanism by which it accumulates in reef fishes than is presently available.

SUMMARY

Ciguatera in Australia results from eating fish caught in some areas along the coast of Queensland and the Northern Territory. Its incidence in those areas in which it is endemic appears to parallel that experienced throughout the South Pacific region. The severity of symptoms which can be experienced by victims creates a need to provide public safeguards. Because of its sporadic, unpredictable occurrence, absolute protection against ciguatera is not possible without a simple specific test which allows rapid screening of individual fish. However the risk can be minimised by adopting simple precautions. More research is required on the mechanisms through which ciguatoxin accumulates to dangerous levels in reef fishes.

ACKNOWLEDGEMENTS

Dr Tony Bourke and Mr Bill Shields of the Queensland Health Department provided invaluable assistance in obtaining and collating case records for Queensland. Messrs John Burke and Michael Holmes performed the analysis of Queensland data. Miss Kym McKauge prepared the illustrations.

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AN ANALYSIS OF THE HUMAN RESPONSE TO CIGUATERA IN AUSTRALIA

R.J. LEWIS[1], M.Y. CHALOUPKA[2], N.C. GILLESPIE[1] & M.J. HOLMES[1]

[1] Southern Fisheries Research Centre, Queensland Department of Primary Industries, P.O. Box 76, Deception Bay, 4508, Australia
[2] Maritime Estate Management Branch, Queensland National Parks and Wildlife Service, Brisbane, 4002, Australia

ABSTRACT

Ciguatera is prevalent in Australia. A database consisting of 617 cases of ciguatera collected over 23 years was analysed in an attempt to more precisely define the ciguatera syndrome in Australia. Nine of the 27 signs and symptoms occurred more often for consumers of toxic mackerel than for consumers of other toxic species. Persons living south of Gladstone were more likely to consume toxic mackerel species while non-mackerel species caused the majority of poisonings north of Gladstone. The time to onset of symptoms ranged from < 1 hr to > 70 hr with a mean of 6.4 hr. Neither age nor gender influenced the time to onset of symptoms. Persons living north of Gladstone had a significantly longer time to the onset of symptoms than persons south of Gladstone. Both the symptom profile and time to onset of symptoms support the conclusion that mackerel are on average more toxic than non-mackerel species, although the presence of different toxins in mackerel and non-mackerel species cannot be excluded as a possibility at this stage.

INTRODUCTION

Ciguatera is the disease caused by the consumption of fish whose flesh and viscera have been contaminated with ciguatoxin and/or structurally related toxins. In Australia ciguatera is a prevalent though underreported disease (Gillespie et al. 1986) often going unrecognised or misdiagnosed (Lewis 1987). Ciguatera is diagnosed if gastrointestinal signs and symptoms (i.e. diarrhoea, abdominal pain, vomiting, nausea) and/or neurological symptoms (i.e. paraesthesia, reversal of temperature perception) occur within three days of the ingestion of tropical or subtropical fishes. However, the individual response to ciguateric fishes is highly variable, even between consumers of the same fish (Gillespie et al. 1986, Lewis 1987).

A number of apparently structurally related toxins may be involved in ciguatera (Chungue et al. 1977, Yasumoto et al. 1977, Lewis and Endean 1984, Nukina et al. 1984, Vernoux and Abbab El Andaloussi 1986). These different toxins may explain part of the variability of the human response particularly between geographically distinct regions. For instance, cases of ciguatera in the Caribbean region present a high frequency of gastrointestinal symptoms and a low frequency of neurological symptoms (Lawrence et al. 1980, Engleberg et al. 1983, Holt et al. 1984) compared with ciguatera in the south Pacific (Bagnis et al. 1979). Even within the Pacific basin apparent differences exist in the human response, e.g. in the Gambier Islands parrotfish initially cause a typical ciguatera syndrome followed by a second phase of poisoning (Bagnis et al. 1974). This second phase was later attributed to scaritoxin (Chungue et al. 1977). The average dose of toxin consumed per person is also likely to differ within and between ciguatera endemic regions, adding further variability to the ciguatera syndrome.

This study represents a preliminary attempt to more precisely define the ciguatera syndrome in Australia. A database of 617 cases of ciguatera was analysed, revealing an influence of fish type and location on the frequency and time to onset of symptoms.

MATERIALS AND METHODS

Survey

Details of 617 cases of ciguatera were obtained in Queensland in the period from 1965 to 1987. These cases arose from 225 outbreaks with each outbreak involving a separate toxic fish. These data were collected through a survey implemented by the Queensland Health Department and the then Queensland Fisheries Service (1980/81) with additional records provided by Lewis (1978/85). Information including the symptoms present (see table 1), fish species consumed, time to onset of symptoms and location of consumption of fish were obtained either by personal interview or by persons suffering from ciguatera filling out a standard ciguatera questionnaire. The severity of the signs and symptoms was not recorded and all cases arising from the 225 outbreaks may not have been recorded.

Initial analysis of these data has been reported (Gillespie et al. 1986). The influences of location and fish type on the human response were analysed in this report. These data are not appropriate for determining the prevalence of ciguatera in Australia as the data represent an unknown fraction of ciguatera outbreaks. Many cases of ciguatera go unrecorded as a result of misdiagnosis, lack of recognition of mild cases, or diagnosed cases often not being referred to the appropriate authority i.e. the Queensland Health Department (Lewis 1987, see also Gillespie et al. 1986). To adequately address such questions a comprehensive epidemiological survey would need to be implemented.

Table 1: Percentage Prevalence of symptoms in survey and Relative Prevalence conditional on fish type (• = sign. $P < 0.05$).

SIGNS & SYMPTOMS	% PREVALENCE IN SAMPLE			RELATIVE PREVALENCE LIKELIHOOD	
		CONDITIONAL ON FISH TYPE:		CONDITIONAL ON FISH TYPE	
	ALL	MACKEREL	OTHER	ODDS RATIO	[95% CI]
Loss of Energy	89.9	89.4	91.0	0.83	[0.43,1.63]
• Myalgia	83.1	86.8	74.8	2.21	[1.36,3.60]
• Temperature Reversal	77.1	81.6	67.6	2.13	[1.35,3.36]
Pruritus	76.3	78.2	72.1	1.39	[0.89,2.17]
• Joint Pain	74.9	79.2	66.4	1.92	[1.21,3.05]
Paresthesia (Hands)	71.4	72.4	68.9	1.18	[0.79,1.75]
Paresthesia (Mouth)	67.3	69.5	61.5	1.43	[0.98,2.09]
Headache	63.3	62.4	65.8	0.86	[0.59,1.26]
• Diarrhoea	62.7	68.6	46.9	2.48	[1.71,3.59]
• Paresthesia (Feet)	61.4	65.8	49.2	1.99	[1.29,3.04]
Ataxia	54.3	56.1	51.2	1.22	[0.79,1.88]
• Nausea	53.9	58.0	45.3	1.67	[1.11,2.50]
Chills	50.8	51.9	48.4	1.15	[0.75,1.77]
• Abdominal Pains	50.5	54.7	39.1	1.88	[1.29,2.74]
Sweating	45.6	46.4	44.3	1.09	[0.71,1.67]
Vertigo	44.4	46.7	38.1	1.42	[0.97,2.09]
Eye Soreness	42.3	45.2	36.6	1.43	[0.93,2.19]
Alcohol Recurrence	37.3	34.1	44.2	0.65	[0.31,1.37]
• Dental Pain	36.6	43.5	24.1	2.43	[1.52,3.90]
• Vomiting	32.8	36.1	24.2	1.76	[1.17,2.66]
Dyspnoea	30.7	28.6	34.4	0.77	[0.48,1.22]
Tremor	29.3	30.8	26.7	1.22	[0.74,2.02]
Neck Stiffness	27.8	29.9	24.2	1.34	[0.80,2.23]
Skin Rash	26.9	27.1	26.6	1.02	[0.65,1.61]
Paresis	26.1	23.9	30.0	0.73	[0.45,1.21]
Dysuria	18.9	21.1	15.2	1.50	[0.85,2.65]
Salivation	10.3	10.9	9.2	1.22	[0.59,2.51]

Odds Ratio= Odds contracting ciguatera given that mackerel consumed/Odds contracting ciguatera given that 'other' fish consumed
95% Confidence Interval=exp(ln(Odds Ratio)±1.96(Standard Deviation of the Odds Ratio))

Data analysis

The data were examined using combinations of exploratory statistical graphics techniques (see Chambers et al. 1983). A more detailed statistical modelling of this data set will be presented elsewhere. Means were determined to be significantly different if the 95% confidence intervals did not overlap.

RESULTS

Geographic distribution

Outbreaks of ciguatera have occurred from the ingestion of fish caught at many localities along the coast of Queensland as well as around Gove in the Northern Territory (Lewis and Endean 1983, Gillespie et al. 1986). However, only outbreaks of ciguatera from Queensland are included in this study. Outbreaks were divided into those coming from southern (Zones I and II) and northern (Zone III) Queensland (figure 1). An outbreak involves one toxic fish and may give rise to one or more cases of ciguatera. Mackerel species (including Scomberomorus commersoni, S. queenslandicus, S. munroi and S. semifasciatus) were responsible for 82% of 151 cases in Zone I, 87% of 290 cases in Zone II and 30% of 112 cases in Zone III. Details

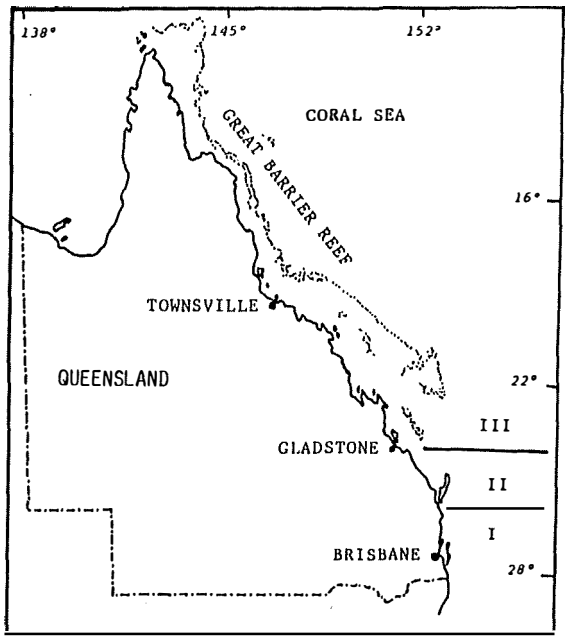

Figure 1 : Map of Queensland divided into three zones. Zone I = south of 26°S, Zone II = 26°-24°S, Zone III = north of 24°S.

of other species (mainly demersal reef fishes) implicated in ciguatera outbreaks in Australia are given in Gillespie et al. (1986). A Q-Q plot (see Chambers et al. 1983) of numbers of cases per outbreak for mackerel and non-mackerel species is shown in figure 2. The plot clearly demonstrates that for all outbreak sizes, mackerel were found to poison more people than non-mackerel species.

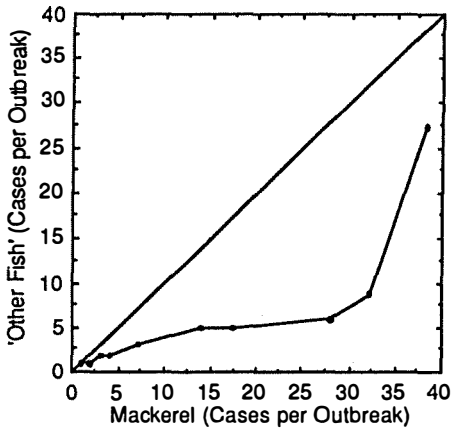

Figure 2 : Empirical Q-Q plot comparing distribution of cases/outbreak for mackerel and non-mackerel fish species. Q-Q plots compare the quantiles(similar to percentiles) of each distribution to indicate both the direction and magnitude of difference, if any, between two variables with similar units of measurement; if both variables the same then plot would lie along the x=y line included in the graph (see Chambers et al., 1983 for details).

Signs and symptoms of ciguatera

The percentage prevalence of 27 signs and symptoms of ciguatera is given in table 1. Overall, these data reflect the pattern described by Gillespie et al. (1986) and Bagnis et al. (1979). Separating outbreaks into those caused by mackerel and those caused by other species it was determined by odds ratio that 9 symptoms were more likely to occur after consumption of toxic mackerel ($P < 0.05$, table 1). These symptoms were myalgia, temperature perception reversal, joint pain, diarrhoea, paraesthesia of feet, nausea, abdominal pain, dental pain and vomiting. It is noteworthy that all four gastrointestinal signs/symptoms were significantly more likely to follow consumption of mackerel than other species.

Time to onset of symptoms

The onset of symptoms ranged from < 1 to > 70 hr with a mean of 6.4 hr (figure 3). The frequency distribution of the data was highly skewed. Table 2 presents a multiway arrangement of ciguatera cases, looking at the interactions of age and gender on time to onset of symptoms. Neither age nor gender were found to have a significant influence on the time to onset of symptoms ($P > 0.05$). However, there was a tendency for females to present with symptoms within 6 hr more often (54%) than males (47%). Males tended to present with symptoms in 6-12 hr more often (37%) than females (28%). People > 30 years tended to have a prolonged onset of symptoms (> 24 hr) more often (5.9%) than persons < 30 years (0.9%).

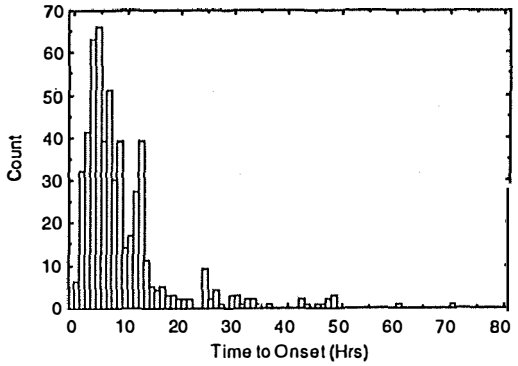

Figure 3: Histogram showing time to onset of symptoms

Figure 4 shows the effects of gender and location on time to onset of symptoms. Gender had no significant influence ($P > 0.05$) on time to onset of symptoms overall (figure 4a) or when the influence of location was assessed (figures 4c and d), i.e. the notches of these box plots overlap. However, cases in the southern region presented with symptoms earlier than cases in the northern region (figure 4b, $P < 0.05$), irrespective of gender. Other features of the box plots, including the outliers (circles indicating single records occurring outside the 90th and 10th percentile) and the top and bottom of the boxes (75th and 25th percentile) describe the spread of the data. Figure 5 shows a Q-Q plot of time to onset of symptoms for single and multiple person outbreaks. Outbreaks involving only one case of ciguatera were found to have a significantly longer time to onset of symptoms than multiple person outbreaks.

DISCUSSION

Ciguatera is the major obstacle to the safe consumption of tropical and subtropical fishes in Australia (Gillespie et al. 1986). Preliminary analysis of 617 case histories of ciguatera in Australia was undertaken in an attempt to more precisely define the features of this disease in Australia.

The fish species responsible for ciguatera was found to vary between locations in Australia. Mackerel species caused most cases south of Gladstone, while non-mackerel species caused most cases north of Gladstone. Within the mackerel group the principal species implicated is the Spanish mackerel, Scomberomorus commersoni (Lewis and Endean 1983, Gillespie et al. 1986, Lewis 1987). Platypus Bay, Fraser Island, is the source of most toxic Spanish mackerel and barracuda (Sphyraeno jello) and recently a ban has been imposed on the capture of these two species at this location. Outbreaks of ciguatera involving mackerel cause more cases per outbreak than outbreaks involving non-mackerel species. This

Table 2: Multiway Arrangement of Ciguatera Cases

SEX	AGE INTERVAL	TIME TO ONSET OF SYMPTOMS				TOTAL
		< 6 HOURS	6-12 HOURS	13-24 HOURS	>24 HOURS	
FEMALE	<10	6	7	1	1	15
	10-19	10	11	5	1	27
	20-29	26	11	5	0	42
	30-39	30	16	7	4	57
	40-49	24	8	5	5	42
	50-59	22	7	2	4	35
	≥ 60	10	5	2	0	17
	SUBTOTAL	128	65	27	15	235
MALE	<10	9	9	3	0	21
	10-19	8	13	3	2	26
	20-29	13	9	1	0	23
	30-49	25	15	2	3	45
	50-59	13	11	2	4	30
	≥ 60	11	8	0	1	20
	SUBTOTAL	105	84	20	16	225
TOTAL		233	149	47	31	460

may stem from mackerel involved in ciguatera being large (up to 20 kg, Lewis and Endean 1983) and that mackerel are often processed into a large number of portions (e.g. cutlets) prior to sale to the consumer. On the other hand, other species such as coral trout are often sold as whole fish, thereby limiting the distribution of these fishes.

The frequency of the signs and symptoms of ciguatera was clearly influenced by fish species. Nine of the 27 signs and symptoms occurred more often in cases of ciguatera from mackerel compared with non-mackerel species. These include one of the hallmarks of ciguatera, the peculiar reversal of temperature perception, and all the gastrointestinal signs and symptoms. The remaining 18 symptoms were not influenced by the species of fish consumed. An influence of dosage on the human response is suggested with mackerel being more toxic than non-mackerel. Information on the dose of ciguatoxin ingested is not available at this stage. The few mackerel implicated with ciguatera that have been assayed were mostly moderately to highly toxic (Lewis 1987). Comparable data for other species involved in ciguatera in Australia are not available. An alternative possibility is that more than one toxin is involved in ciguatera in Australia and that these different toxins induce different human responses. Laboratory studies have revealed a single toxin in the flesh of Spanish mackerel and barracuda (Lewis and Endean 1983, 1984). This toxin was not distinguishable from the toxin in reef fish using mouse bioassay (Lewis, unpublished observation). However, the occurrence of a second (minor) toxin in the viscera of barracuda (Lewis and Endean 1984), suggests additional toxins may be involved in certain instances.

The time to onset of symptoms of ciguatera in Australia can be very rapid (< 1 hr) with 93% of people reporting an onset of symptoms within 24 hr, similar to ciguatera in the south Pacific (Bagnis et al. 1979). The frequency of times to onset was not normally distributed. Anderson et al. (1983) report similar data for ciguatera in Hawaii. The present study revealed that age and gender did not have a significant influence on time to onset of symptoms. In 7% of cases onset was > 24 hr with 87% of these cases observed in persons > 30 years. It is probable that cases with delayed responses are often not diagnosed as being cases of ciguatera. Alternatively, some cases with long onset may be incorrectly diagnosed as ciguatera. In a recent, confirmed outbreak of ciguatera (from Pomadasys maculatus from Platypus Bay in May 1988) the age and time to onset were: 6 years, 25.5 hr; 8 years, 65 hr; 12 years, 44 hr; 32 years, 5.5 hr; 33 years, 2.5 hr, supporting the conclusion that ciguatera can have a delayed onset. The consumption of a low effective dose of ciguatoxin most likely explains the delayed onset of symptoms. Overall, the time to onset of symptoms was longer in outbreaks involving one person compared with outbreaks involving multiple cases of ciguatera. Most significant, however, was that the time to onset of symptoms was shorter in cases of ciguatera from southern (Zones I and II) compared to northern (Zone III) Queensland. This result probably stems from the large proportion of toxic mackerel species involved in ciguatera in southern Queensland compared with northern Queensland.

In conclusion, a major influence of fish type on ciguatera in Australia is indicated. Mackerel species appear to cause a more rapid onset and more symptoms (including both gastrointestinal and neurological) than do the other species involved in ciguatera in Australia. These differences are proposed to stem from an influence of dose, with a meal of toxic mackerel being on average more toxic

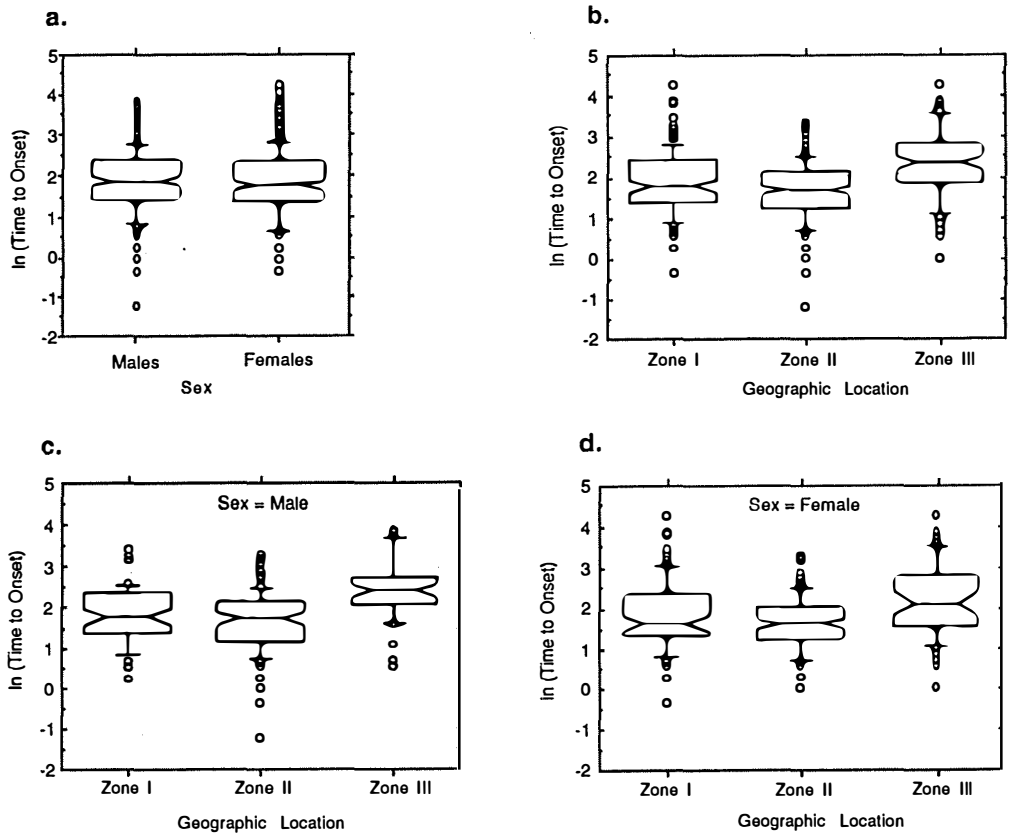

Figure 4a-d: Box plots of Time to Onset of Symptoms (hrs) in relation to **(a)** Sex ; (b) Geographic Location; **(c)** Males within Geographic Locations; **(d)** Females within Geographic Locations.

Features of these box plots include notches (ie., 95% simultaneous confidence intervals); top lid or hinge = 75 th percentile; bottom hinge = 25th percentile; bar across each box = 50th percentile or median; circles = outliers (see Chambers et al., 1983 for further details of schematic box plots).

than a meal of other toxic fish. Laboratory studies also reveal that the response of mice to ciguatoxin is dose-dependent (Hoffman et al. 1983, Lewis and Endean 1984, Sawyer et al. 1984). Only one toxin is apparently involved in ciguatera in Australia. This toxin induces a pattern of ciguatera similar to that reported for the south Pacific (Bagnis et al. 1979) but different from ciguatera in the Caribbean (see Introduction for refs). The involvement of minor toxins in ciguatera in Australia awaits confirmation but any involvement is expected to be small.

ACKNOWLEDGEMENTS

We thank Miss G. Davidson for typing of this manuscript and Mr S. Hyland for assistance with data transfer. Dr T. Bourke, Mr W. Shields, Mrs Y. Cossins and Mr J. Burke provided invaluable assistance in obtaining an collating case history records.

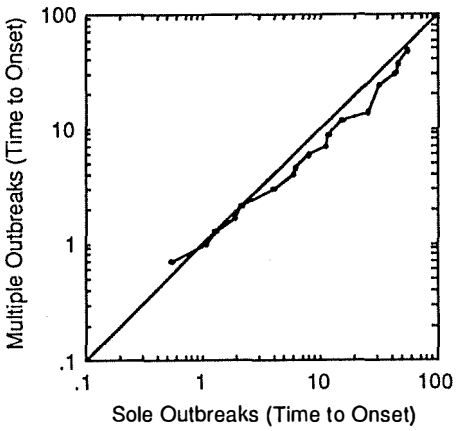

Figure 5 : Empirical Q-Q plot comparing distribution of time to onset of symptoms for two classes of outbreaks (note logarithmic scale; see Fig. 2 for further explanation of Q-Q plots).

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5. Ciguatera

RICHARD LEWIS AND NOEL GILLESPIE

Ciguatera is prevalent in Australia, many cases remaining undiagnosed. The syndrome of acute poisoning includes nausea and vomiting, diarrhoea, tingling about the mouth and hands and a peculiar reversal of temperature perception. The toxic effects may be chronic, lasting many months, and may recur. In most cases, the condition is self-limiting, complete recovery being the rule after several days' debility. The condition is caused by the eating of toxic fish. A recognized risk follows the eating of Moray Eel, Red Bass, Chinaman Fish and Paddletails. Fortunately these species are seldom consumed and few incidents incriminate these species. Analysis of over 100 outbreaks of ciguatera in Queensland has shown that the majority of cases follow the eating of mackerel, although vast quantities of this desirable fish are of course eaten quite safely. If poisoning is going to occur, in over half the cases this is obvious within six hours after ingestion. Prevention includes abstinence from known "high-risk" fish and moderation in the quantities eaten when there is any risk.

The disease of ciguatera is caused by the consumption of fish whose flesh and viscera have been contaminated with ciguatoxin and related toxins. In Australia, ciguatera is a prevalent, though unreported disease[1] which is often unrecognised or misdiagnosed[2].

A diagnosis of ciguatera must be considered if gastrointestinal signs and symptoms (i.e. diarrhoea, abdominal pain, vomiting, nausea) or neurological symptoms (i.e. tingling, reversal of temperature perception) follow by up to three days the ingestion of tropical or subtropical fishes. Ciguatera, like syphilis, is very variable in its effects. It can be a great mimic. The clinical picture is often confused because *individual* responses to the toxin(s) in ciguateric fishes may be highly variable, even among those who eat the same fish[1,2].

This puzzling and variable clinical syndrome may result from the fact that several toxins are involved[3-7]. These different toxins may explain part of the variability of the human response, particularly that seen in different geographic localities. For instance, cases of ciguatera in the Caribbean region present a high frequency of gastro-intestinal symptoms and a low frequency of neurological signs[8-10], when clinical patterns are compared with those encountered in victims who have eaten fish in the South Pacific[11]. Even within the Pacific basin apparent differences exist in the human response. In the Gambier Islands in the Central Pacific, for example, eating of toxic parrotfishes causes a typical ciguatera syndrome followed by a second phase of poisoning[12] due to the presence of scaritoxin[3]. The average dose of toxin per person is also likely to differ, adding

further variability to the ciguatera syndrome.

The origin of ciguatoxin remains an enigma. It is almost certainly of algal or bacterial origin. The currently-accepted "best candidate" is the microscopic dinoflagellate *Gambierdiscus toxicus*.

It is thought that this tiny organism is eaten by the smallest herbivorous fish, which in turn are eaten by small carnivores, and so on up the food chain until a human gourmet forms the end link. (There ain't no such thing as a free lunch)(Ed).

Australian fish which may cause ciguatera

A wide variety of fishes may on occasion contain sufficient toxin in their flesh to cause human illness. The fishes most often implicated are the Narrow-barred Spanish Mackerel (*Scomberomorus commersoni*) and large specimens of the beautiful Coral Trout (*Plectropomus maculatum*).

Large numbers of these fine table fish are eaten in Australia each year, with only the occasional fish being toxic. The risk of eating a toxic fish, at a casual fish meal, is approximately 1 in 5000 even in regions where ciguatera occurs. It is very important that the risk of ciguatera be not overstated.

The rank order of risk in eating the various fish species, remains unknown. However, three species of fish have an especially bad reputation for being toxic, and should not be eaten. These are the Red Bass (*Lutjanus bohar*), the Chinaman Fish (*Symphorus nematophorus*), and the Paddletail (*Lutjanus gibbus*). Moray Eels have a relatively high rate of being toxic, and currently form the best source for the collection of ciguatoxin for scientific research. Fortunately, for cultural reasons they are not eaten in Australia. Other fishes such as the puffer fish group (the "toad fish", members of the Family *Tetrodontidae*) are also toxic, but for a different reason. This latter fish type contains tetrodotoxin, a highly potent toxin not related to ciguatoxin. The toxic syndromes that result from eating this latter type of fish are quite distinct from those experienced after eating ciguatoxic specimens. The most commonly implicated fish species, causing ciguatoxin in Australia, are shown in Table 5.1.

Fish Species	Common Name	No. of Outbreaks
Scomberomorus commersoni	Narrow-barred Spanish Mackerel	36
Scomberomorus sp.	Mackerel (unspecified)	30
Plectropomus sp.	Coral Trout	20
Sphyraena jello	Barracuda	7
Scomberomorus queenslandicus	School Mackerel	7
Epinephelus fuscoguttatus	Flowery Cod	4
Scomberomorus munroi	Spotted Mackerel	4
Lutjanus sebae	Red Emperor	3
Epinephelus sp.	Cod (unspecified)	3
Lutjanus bohar	Red Bass	2

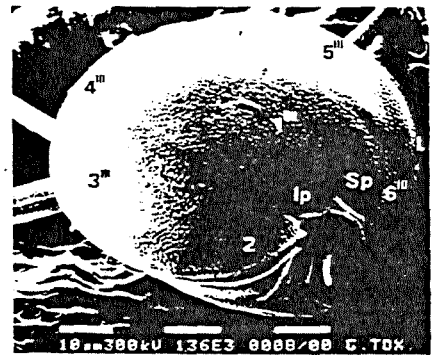
TABLE 5.1: Rank order of the "top ten" fish species responsible for outbreaks of ciguatera in Northern Australia, 1965-1988*.

* This is not the rank order of risk, as some species which may be *relatively* safe are eaten extensively.

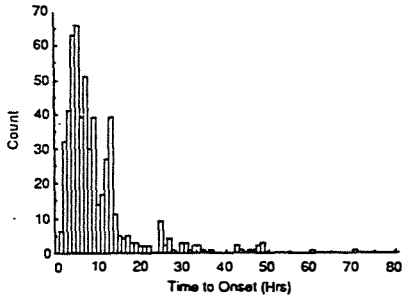
Geographic distribution

Outbreaks of ciguatera have occurred from fish caught at many localities along the coast of Queensland, as well as localities as far west as Gove in the Northern Territory[1]. Cases of ciguatera may be divided into those caused by fish caught from the southern zones of Queensland (Zones I and II), and from more northern zones (Zone III) in Queensland.

Narrow-barred Spanish Mackerel *Scomberomorus commersoni*. These specimens containing ciguatoxin were caught in Platypus Bay, Fraser Island, S.E. Queensland.



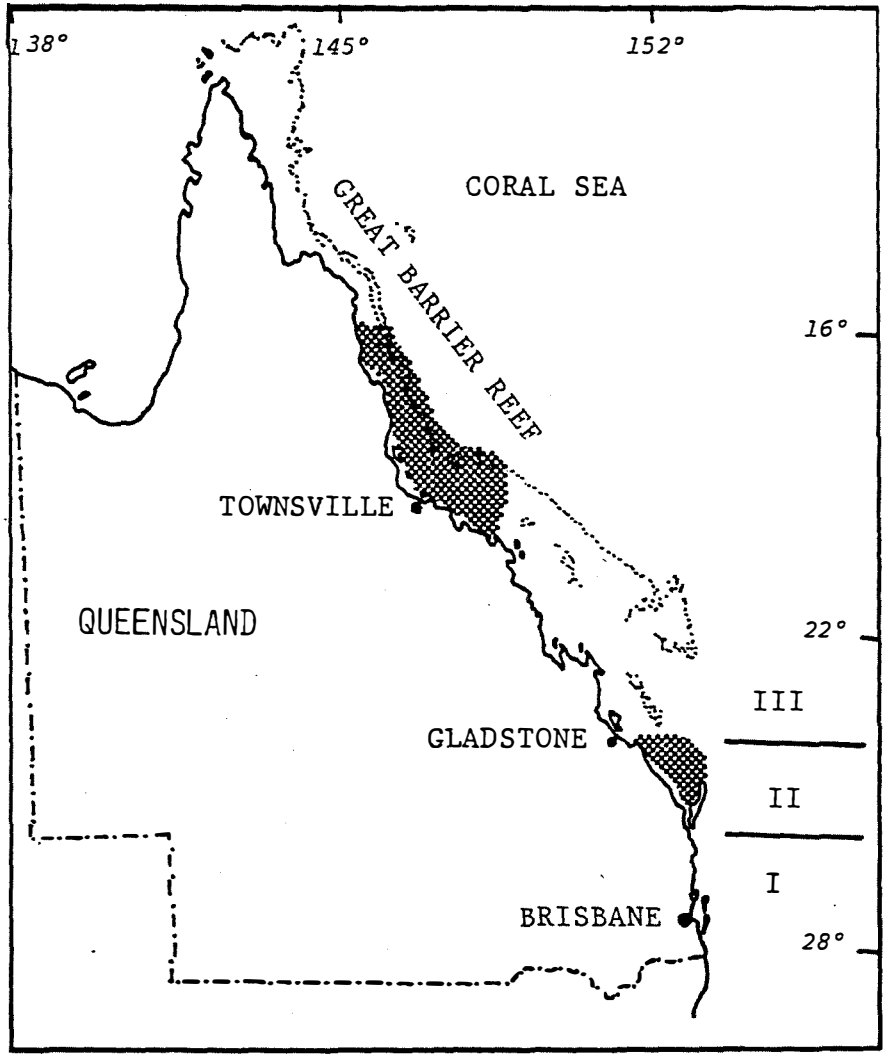
Scanning electron micrograph of *Gambierdiscus toxicus*. The organism has a dia-meter of approximately 70 µm. Numbers 1 to 6 refer to the thecal plates which protect the organism.



Histogram showing times between ingestion of toxic fish, and the onset of symptoms. Data from 400 cases of human ciguatera in Queensland 1965-1988.

Mackerel species were the culprit in some 82% of cases that have occurred from fish caught in Zone I, 87% of cases from Zone II, and 30% of cases in Zone III[1,13]. The non-mackeral species comprise mainly the demersal (bottom-living) reef fishes.

Tourists to islands in the Pacific are also at potential risk from ciguatera. There is no way of avoiding some risk, apart from abstinence — not really a practical



Known "ciguatera areas" in Queensland. In Zone II, Narrow- barred Spanish Mackerel are responsible for most outbreaks. In Zone III, reef fishes are frequently involved.

option if one is sampling the delights of the South Pacific centres. The best approach is to eat moderately if "risk" species are being consumed, to enquire about local knowledge, and to avoid totally the eating of Moray Eel and Red Bass.

Signs and symptoms

Ciguatera is a great mimic of other diseases, but the more severely poisoned present to the local first aid attendant or doctor with a full syndrome which is obvious to the experienced; and which, if it recurs, is *very* obvious to the victim.

The rank order of the commonest symptoms experienced by the unfortunate victims is shown in Table 5.2.

Many other symptoms, quite often of a bizarre nature, may also be experienced. Painful urination, for example, is experienced by some 15% of sufferers. The under-lying neurological basis of these bizarre features, remains quite unknown.

Two special features characterise ciguatera poisoning — the fact that mini-clusters rather than single cases usually occur, and the distressing chronic nature of the symptoms which may persist for many months[1,11]. Most victims, however, experience an illness course of several days only. Most enjoy complete recovery within a week and have no further disruption to their lives.

Symptoms	Mackerel	Other Fish
Loss of energy	90	91
Muscle pain	87	75
Reversal of temperature perception	82	68
Itching	78	72
Joint Pains	79	66
Tingling (hands)	72	69
Tingling (mouth region)	69	61
Headache	62	66
Diarrhoea	69	47
Unsteadyness (ataxia)	56	51
Nausea or Vomiting	58	45
Chills	52	48
Abdominal Pain	55	39
Sweating	46	44
Vertigo	47	38
Sore Eyes	45	37
Symptom Recurrence after alcohol	34	44
Aching Teeth	44	24
Shortness of Breath	29	34
Tremor	31	27
Neck Stiffness	30	24
Skin Rashes	27	27
Weakness	24	30
Salivation	11	9

TABLE 5.2: Frequency (percentage) of symptoms reported by ciguatera victims in Australia*.

* This syndrome profile has been compiled from over 600 victims poisoned following the eating of toxic fish.

The sub-acute and chronic symptoms include loss of energy and joint aches, in particular. A number of such sufferers are diagnosed as being neurotic, and differential diagnosis may be very difficult. However, the presence of sub-acute debility in an individual previously well is always a suspicious sign, if the symptoms date from an attack of peri-oral tingling and diarrhoea which in turn followed an "at risk" fish meal, there is little practical doubt about the diagnosis.

The time from ingestion of toxic fish to the onset of symptoms varies. Symptoms may occur within an hour of eating toxic fish, or may be delayed for up to 48 hours, or even longer. Higher doses of toxin are associated with the more rapid onset of symptoms. The average time from eating the toxic fish meal to the onset of symptoms is six hours. Gastro-intestinal symptoms — nausea and abdominal pain, and sometimes diarrhoea and vomiting — are usually the first to occur. Peri-oral tingling has been reported as a first symptom in some cases. Age and sex do not appear to influence the time delay before symptoms appear[13].

Conclusion

Ciguatera is a major obstacle to the safe consumption of many tropical and sub-tropical fishes in Australia, and indeed throughout the Pacific[11,15]. Unfortunately, it is too dangerous to eat otherwise magnificent fish from one particularly risky area in Queensland. Fraser Island is the source of most toxic Spanish Mackerel and Barracuda (*Sphyraena jello*) and recently a ban has been imposed on the capture of these two species at this location. Outbreaks of ciguatera involving mackerel cause more cases per outbreak than outbreaks involving non-mackerel species[13]. This may stem from the fact that mackerel which are involved in ciguatera tend to be large, up to 20 kilograms[14]. Mackerel are often processed into a large number of portions (e.g. cutlets) prior to sale to the consumer, and one toxic fish may thus poison many victims. On the other hand, non-mackerel species such as Red Bass may be caught by amateur fishermen and eaten (in ignorance) as a single whole fish which supplies a meal to just one individual or his family, or a small fishing party.

In practice, the chance of a prudent person's being poisoned is very low. As the clinical effects are dose-dependent[16,17], the chance of a prudent person being severely poisoned is even lower. Prudence implies not eating Moray Eel, Paddletail, Chinaman Fish or Red Bass, ever; moderation in the quantities eaten when there is any risk; and if there is any suspicion of other fish being ciguatoxic, it means giving a small portion to one individual (or the cat!) at a prior meal. If these simple rules are followed, the diner can enjoy fine fish meals, with confidence.

Acknowledgements

The authors thank Mr M.Y. Chaloupka, Mr M.J. Holmes, and Professor John Pearn for much helpful collaboration.

APPENDIX IX

FISHING INDUSTRY RESEARCH TRUST ACCOUNT

APPLICATION FOR GRANT 1986/87

1. TITLE OF PROPOSAL

Production of antibodies against toxins involved in ciguatera fish poisoning.

2. NAMES OF APPLICANTS

(i) Queensland Department of Primary Industries (QDPI)
(QDPI will administer the funds granted to the project)
(ii) Queensland Institute of Technology (QIT)
(iii) Griffith University (GU)

3. DIVISION, DEPARTMENT OR SECTION

(i) Division of Dairying and Fisheries (QDPI)
(a) Food Research Branch; (b) Fisheries Research Branch
(ii) School of Health Sciences (QIT)
(iii) School of Science (GU)

4. PROPOSAL

It is proposed to attempt to produce antibodies against ciguatoxin, the principal toxin involved in ciguatera fish poisoning, with a view to then developing a rapid specific method of detection. This will involve the extraction and purification of toxin from toxic fish, determination of means of eliciting an immune response to ciguatoxin in animals, development of assay procedures against antibodies to ciguatoxin and the final production of monoclonal antibodies using hybridoma techniques.

5. NAME OF PERSON RESPONSIBLE FOR PROGRAM

Mr J.G. Miller
Director
Division of Dairying and Fisheries
Queensland Department of Primary Industries
GPO Box 46
BRISBANE Q 4001 Phone: (07) 227 6435 Telex: AA 41620

6. QUALIFICATIONS OF PERSONNEL TO BE EMPLOYED ON PROGRAM

Project Leader

Dr N.C. Gillespie, B.Sc., Ph.D., Supervising Fisheries Biologist, QDPI Southern Fisheries Research Centre, Deception Bay, is responsible for co-ordinating the various aspects of the program. Dr Gillespie has been active in the ciguatera field for a number of years and has completed epidemiological and ecological studies on ciguatera fish poisoning in Queensland.

Associate Investigators

Dr J. Aaskov, B.Sc., Ph.D., Senior Lecturer in Immunology, School of Health Sciences, QIT, will provide input into the immunological aspects of the program and supervise sections of this work to be carried out at QIT.

Dr M.F. Capra, B.Sc., M.Sc., Ph.D., Senior Lecturer in Anatomy and Physiology, School of Health Sciences, QIT, has been developing systems for investigating the action of toxins on nerve preparations and will confirm the identity of toxins being purified for immunological investigations.

Dr R. Quinn, B.Sc., Ph.D., Lecturer, School of Science, Griffith University, has a strong background in marine product chemistry and synthetic organic chemistry and will direct the production of a conjugate with ciguatoxin.

Dr K. Ewings, B.Sc., Ph.D., Bacteriologist, QDPI Food Research Laboratories, Hamilton, is experienced in the production of monoclonal antibodies and will supervise the development of an assay procedure for ciguatoxin in consultation with Dr J. Aaskov (QIT).

Dr H. Deeth, B.Sc., Ph.D., Supervising Chemist, QDPI Food Research Laboratories, Hamilton, has a considerable background in lipid chemistry and will supervise the final purification of toxin fractions to be used in this work.

Other Staff

Mr M. Symons, B.Sc., Microbiologist, QDPI Food Research Laboratories, Hamilton, has had extensive experience in cell culture, hybridoma technology and immunology and will be physically involved in the development of assay procedures, etc.

Mr R. Clements, B.App.Sc., Temporary Serologist, QDPI Food Research Laboratories, Hamilton, has considerable experience in the production of monoclonal antibodies. He will be employed by QDPI until 31 December 1986, using State funds and FIRTA funds are requested to employ him for the remainder of the project. He is to work together with Mr Symons on the immunological investigations.

Technician QDPI - to be appointed. This appointee will be employed using State funds between January 1986 and 30 June 1986, prior to the commencement of this project to carry out initial extraction and purification of ciguatoxin. FIRTA funding is sought for the duration of the project to employ such a person to continue to obtain supplies of toxin for the purposes of this project.

Mr G. Street, B.Sc., Technician, Immunology Laboratory, QIT, has a considerable background in the production of monoclonal antibodies and will undertake some aspects of the work under the direction of Dr J. Aaskov.

Mr A. Flowers, B.App.Sc., Research Assistant, School of Health Sciences, QIT, will also be involved in the extraction of toxin and purification from fish, if necessary at the QIT.

7. OBJECTIVES

The following objectives are presented as a conservative assessment of the progress considered possible within a three year time frame, towards the ultimate objective - the development of a method of detection of ciguatoxin (CTX).

(i) Extract and purify sufficient ciguatoxin from toxic fish to service the requirements of the intended experimental program for toxin.

(ii) To develop using pure toxin an enzyme immunoassay for measuring anti-CTX production.

(iii) Develop a method and schedule for immunisation to allow production of monoclonal antibodies.

In the formulation of these objectives, it has been assumed that considerable advances are shortly to be made in the derivation of the chemical structure of ciguatoxin. Recent communications from Professor Paul Scheuer at the University of Hawaii display optimism about the early completion of this work.

8. JUSTIFICATION

Ciguatera fish poisoning is a widespread public health problem resulting from the consumption of fish caught in northern Australia. It is now the greatest single factor affecting the marketing of tropical reef fish and such species as narrow-barred Spanish mackerel. The value of the commercial catch for this fishery in the Great Barrier Reef region in 1979-80 was $6 million (Hundloe, 1985). The catch of the small boat fleet comprising the recreational fisheries of the Great Barrier Reef region in 1980 was 6.5 million kilograms of fish. Other fisheries affected but not included in the Great Barrier Reef region are the mackerel fisheries of Hervey Bay and the remainder of southeastern Queensland.

While public reaction to the risk of ciguatera has been a problem for the industry, the major danger at present is that a ciguatera victim can successfully seek compensation under the provisions of the Sale of Goods Acts of both Queensland and New South Wales.

A test case between a New South Wales ciguatera victim and a Sydney retailer is an indication of what may occur in the future. This case involves the intoxication of a Sydney businessman, who purchased a coral cod originally caught in north Queensland. His family suffered ciguatera poisoning after eating the fish. He has subsequently sought compensation from the retailer, who in turn has sought same from a north Queensland wholesaler. Legal opinion at present indicates that this action will be successful and a decision is likely to be made to settle out of court.

This will greatly affect insurance arrangements for public risk with all wholesalers, who in the absence of any simple procedure for assessing ciguatoxicity would be unprotected against the risks of similar actions in the future.

This will lead to reluctance amongst wholesalers and indeed any buyer

of fish to accept the risk of handling tropical fish species for sale. It is likely that distribution of reef fish from the Northern Territory and Western Australia would also be affected. The distribution of species other than reef species around Australia could also be disrupted.

It is therefore clear that, apart from minimising the impact of ciguatera by improving reef management practices and therapeutic methods for victims, that a rapid specific test for detecting ciguatoxin is desirable to protect fishermen, wholesalers and retailers from the risk of legal action.

Presently, there is no validated quantitative method for the determination of ciguatoxin. The chemical structure is largely unknown, although it is known that ciguatoxin is a highly oxygenated lipid with a molecular weight of 1111.7 (Tachibana, 1980). A feature of its structure is the number of ether linkages. It also has a number of olefins, hydroxyl and methyl groups.

Any method of detection must be very sensitive as even very toxic fish contain only minute quantities of toxin. Up until now the only way in which ciguatoxin has been able to be assayed has been through the use of bioassays involving the feeding of suspect fish to cats, and mongooses (Banner et al, 1961), injecting extracts into mice (Hoffman et al, 1983), mosquitoes (Chungue et al, 1984), and using brine shrimp (Granade et al, 1972). None of these procedures is sufficiently specific, sensitive or rapid to allow routine testing of fish.

A number of immunoassay procedures have been developed at the University of Hawaii by Hokama and his co-workers. Hokama et al (1977) initially reported a radioimmunoassay (RIA) procedure based on sheep antiserum to a conjugate between a relatively crude preparation of ciguatoxin and human serum albumin. While apparently seeming quite useful, the RIA was too costly and time consuming for routine testing and cross-reacted with a range of polyether containing lipid materials, with the resulting occurrence of "false positive" results.

Enzyme-linked immunoassays have been attempted by several authors but failed because of the lack of a high-titre antiserum (Berger & Berger, 1979; Chanteau et al, 1981). However, Hokama et al (1984) reported that it was possible to differentiate between clinically documented toxic and non-toxic fishes of several different species using an enzyme immunoassay, using the same antisera as employed in his RIA procedure. Emerson et al (1983) also reported that toxic fish could be distinguished from non-toxic samples using a rapid counter-immuno-electrophoresis method but found that the interaction between toxin extracts and antisera was not a true antigen-antibody reaction, since putative immune and non-immune sera reacted equally well.

More recently Hokama et al (1985) have reported the development of a poke-stick test for polyethers based on monoclonal antibodies against okadaic acid, a polyether compound possibly similar in structure to ciguatoxin. This method was recently the subject of a news release (November, 1985) in the "Weekend Australian" and was described as a test against ciguatoxin. However, as far as is known specific antibodies against ciguatoxin had not been produced successfully and the test is known to detect polyethers in general. This method has been submitted to the Association of Official Analytical Chemists

(AOAC) for validation. The Queensland Department of Primary Industries has sought to be included in this validation process.

Considering the failure up to this point in time to produce specific antibodies against ciguatoxin and the need, in terms of legal considerations, to be able to specifically detect ciguatoxin in Australian fish, there is an urgent need for the type of investigations proposed here. Similar investigations, financed by the USFDA, involving Dr T. Higerd from the University of South Carolina, J. McMillan from the College of the Virgin Islands and the National Marine Fisheries Service, are also planned to develop tests suitable for toxic fish caught in the Caribbean area. The fishing industry in Florida is facing similar problems through litigation to those being experienced in Queensland. Transference of methods developed for Caribbean fish will also be difficult because it is thought that ciguatera poisoning from that region may be due to several different toxins, including ciguatoxin.

9. LOCATION OF OPERATION

Toxic fish samples are being obtained from known ciguatoxic areas near Gove, in the Northern Territory and in Hervey Bay in southern Queensland. Extraction and initial purification of toxin from fish will be carried out at the Southern Fisheries Research Centre, Deception Bay. Final purification and production of antibodies will be carried out jointly by the Queensland Food Research Laboratories at Hamilton and the Queensland Institute of Technology.

10. PROPOSAL IN DETAIL

(a) Plan of Operation

(i) Method of Procedure

Preparation of pure toxin. Fish from known toxic areas are being captured by commercial fishermen and are being transported frozen and uneviscerated to Deception Bay, where they will be screened for toxicity using animal bioassay. The screening procedure being examined is one that employs only 8g of fish and it is thought that, with appropriate modifications, it might be suitable for use by Government testing laboratories until a more rapid test becomes available. Initially the viscera of toxic fish will be extracted and purified using the methods of Tachibana (1980), as later modified by Nukina and Scheuer (pers. comm.). Some ciguatoxin is now being produced at Deception Bay by laboratory cultures of *G. toxicus*. However, production is presently quite limited. If the factors controlling toxin production become more clearly understood over the next few months then *G. toxicus* may also be used as a source of toxin.

Production of anti-ciguatoxin antibodies. Considerable evidence exists that demonstration of a specific antibody response to pure ciguatoxin (a low molecular weight lipid) may be quite difficult. Production of specific antibodies to a low molecular, non-immunogenic compound such as ciguatoxin is possible provided it can be conjugated covalently to an appropriate immunogenic carrier via a known functional group. The hydroxyl groups reported to be part of the structure of ciguatoxin should be amenable to conjugation, provided

they are accessible to chemical coupling. The more information that is available on the chemical structure of ciguatoxin, the more likely it is that a reliable conjugate might be produced.

Before the presence of anti-ciguatoxin antibody can be detected, a suitable enzyme-linked immunoassay (ELISA) must be developed. This will have its own inherent difficulties, as it would appear that ciguatoxin will not adhere directly to the base of wells in conventional ELISA plates. Suitable carriers or binding procedures will need to be developed. Once a suitable system has been developed the presence of anti-ciguatoxin antibodies would be detected using conventional ELISA methods.

Once an assay procedure has been developed an immunisation schedule for rats or mice will be developed using pure or conjugated ciguatoxin with a view to eliciting an immune response. The development of monoclones of hybridomas producing anti-ciguatoxin antibody will then be carried out by conventional procedures.

Development of a stick test for ciguatoxin. A "desired outcome" for this project would be that the basis would be laid for the development of a rapid test kit for ciguatoxin. Once true monoclonal anti-ciguatoxin antibodies are available, the development of such a test would not be a lengthy process and funding for the development of the test would be requested when this occurs.

Other possible outcomes resulting from the availability of anti-ciguatoxin antibody. Promising work is now being undertaken at the US Army Medical Research Institute of Infectious Diseases at Fort Detrich, USA on the use of monoclonal antibodies to displace marine toxins such as saxitoxin and brevitoxin from their receptor sites on the Na+ channel in the treatment of cases of human intoxication. Any anti-ciguatoxin antibody produced in the course of this project could also be evaluated as to its efficacy as a possible therapeutic agent.

(ii) Facilities Available

QDPI Southern Fisheries Research Centre, Deception Bay. Dr Gillespie's laboratory is equipped with explosion-proof tissue homogenisers, rotary evaporators, chromatography columns, pumps, fraction collectors, and storage facilities for the screening, extraction and purification of toxic fish samples.

QDPI Food Research Laboratories, Hamilton. Dr Ewings' laboratory is equipped with biohazard cabinets, carbon dioxide incubators and automated equipment for ELISA readings, high speed and ultra-centrifuges, and high powered inverted microscopes. The Food Research Laboratory is equipped with HPLC equipment necessary for final toxin purification.

QIT School of Health Sciences, Brisbane. Dr Aaskov's immunology laboratory is also equipped with biohazard cabinets, carbon dioxide and associated equipment for the development and performance of ELISA assays. Dr Capra's laboratory is equipped with equipment required for confirming the identity of ciguatoxin through its action on various animal tissue preparations and can also undertake toxin purification.

Griffith University School of Science. Dr Quinn's laboratory is fully

equipped to carry out any synthetic chemistry involving the production of conjugate between ciguatoxin and some immunogenic carrier.

(b) Supporting Data

The research group assembled to undertake this project is well equipped to do so. Dr Gillespie and Dr Capra have both published in the field of ciguatera research and both have a significant research record in other fields.

Dr Aaskov has, since 1976, established his reputation in the area of arbovirus immunology - in particular the pathogenesis of Ross River virus infection in man - and has published 21 research papers in this area. His ELISA assay for the diagnosis of recent Ross River virus infection is now used to perform the majority of Australia's Ross River virus serology.

Dr Quinn has 15 years post-doctoral research experience in the area of organic chemistry of biologically active compounds, particularly marine products, and has published 32 research papers and 3 patents in his field.

The group at the Queensland Food Research Laboratories, including Dr Ewings and Mr Symons, have published 12 papers in the area of biochemical action and immunoassay of proteases in milks and have recently produced a monoclonal antibody to proteases from psychrotrophic bacteria. Mr Symons has had 7 years experience in the manufacture of monoclonal antibodies and brings a total of 20 years experience in serological and cell culture technology to the project.

Mr Clements, to be employed on the project, is completing a Masters degree in December 1985 involving the use of immunochemical techniques, including the development of enzyme immunoassay, immunodiffusion assays and monoclonal antibodies.

11. PROPOSED COMMENCEMENT DATE

1 July 1986.

ANTICIPATED COMPLETION DATE

30 June 1989.

12. FUNDS REQUESTED

	Year 1 $	Year 2 $	Year 3 $
(a) Total Salaries and Wages	39 881	57 825	60 708
(b) Total Operating Expenses	21 500	22 500	25 500
(c) Total Capital Items	3 000	3 000	3 000
Gross Total Cost	64 381	83 325	89 208

The very large salaries and wages component, together with staff

resources to be committed by both QDPI and QIT is a reflection of the labour intensive nature of this type of investigation, and the complex difficulties expected to be encountered. Without this level of committment, this program would become quite protracted with little chance of significant progress within 5 years.

13. FUNDS TO BE PROVIDED BY THE APPLICANTS

During 1985/86, before commencement of this project, the Queensland Department of Primary Industries committed $40 000 towards the acquisition of toxic fish samples and purification of toxin for use on this project. This includes funds for a temporary position to assist with screening and extraction of toxic fish samples. The Department is also committing funds to ensure that state of the art equipment is available for this work. In addition, Mr R. Clements is to be employed on State funds during 1986. After January 1987 it is hoped that FIRTA funds will become available to continue his employment. A 12 month period of study by Dr K. Ewings at the Walter Reid Institute of Medical Research is also to be supported by the Queensland Department of Primary Industries. Consequently, a considerable financial committment is being borne by the Queensland Department of Primary Industries prior to the project.

During the first year of the grant the major committment by all parties to the project will be the full time salaries of Dr K. Ewing, M. Symons, half of the salary of Mr R. Clements and the use of existing facilities of the QDPI Southern Fisheries Research Centre, the Queensland Food Research Laboratories, the QIT Immunology Laboratory in the School of Health Sciences, and the School of Science at Griffith University.

14. CO-OPERATING AGENCIES AND THEIR FUNCTION

Toxic fish are being obtained through the co-operation of commercial fishermen in Gove and in Hervey Bay.

15. IS SIMILAR WORK BEING UNDERTAKEN IN AUSTRALIA?

At present, there is no similar work in progress in Australia.

16. PLANS FOR REPORTING OR PUBLISHING RESULTS

Any monoclonal antibodies produced would be patented. Appropriate sections of the work would be published in refereed scientific journals and descriptive articles would be prepared for the "Australian Fisheries" magazine.

FUNDS REQUESTED

1986/87

Project: Production of antibodies against toxins involved in ciguatera fish poisoning.

	1986/87 $	1987/88 $	1988/89 $
(a) Salaries and Wages			
Graduate Scientist S-5 (R. Clements)	11 416	24 641	26 269
Laboratory Technician Division II	19 262	19 840	20 430
Total on costs (Workers' Compensation, Payroll Tax, etc.)	9 203	13 344	14 009
Total Salaries and Wages	39 881	57 825	60 708
(b) Operating Expenses			
(i) Travelling			
Fares	1 000	1 000	1 000
Allowances	1 000	1 000	1 000
(ii) Vessel charter, etc.	4 000	4 000	4 000
(iii) Acquisition of fish	2 000	2 000	2 000
(iv) Other operating expenses			
Chemicals and reagents	3 000	3 000	5 000
Glassware	1 000	1 000	1 000
Animals	2 000	2 000	2 000
Tissue culture medium	3 000	4 000	5 000
Column materials	1 500	1 500	1 500
Disposable containers	3 000	3 000	3 000
Total Operating Expenses	21 500	22 500	25 500
(c) Capital Items			
Miscellaneous	3 000	3 000	3 000
Total Capital Items	3 000	3 000	3 000
GROSS TOTAL COST	64 381	83 325	89 208

These figures do not include an allowance for inflation.

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