Fall 1972

CHEMICAL STUDIES ON TOXINS FROM GYMNODINIUM BREVE AND APHANIZOMENON FLOS-AQUAE

MAKTOOB ALAM

CHEMICAL STUDIES ON TOXINS FROM GYMNODINIUM BREVE AND APHANIZOMENON FLOS-AQUAE

BY

MAKTOOB ALAM

M.Sc., University of Karachi, 1964

A THESIS

Submitted to the University of New Hampshire

In Partial Fulfillment of

The Requirements for the Degree of

Doctor of Philosophy

Graduate School

Department of Biochemistry

November 1971

This thesis has been examined and approved.

Miyoshi Ikawa

Thesis director, Miyoshi Ikawa, Prof. of Biochemistry

Edward J. Herbst

Edward J. Herbst, Prof. of Biochemistry

Gerald L. Klippenstein

Gerald L. Klippenstein, Asst. Prof. of Biochemistry

Paul R. Jones

Paul R. Jones, Prof. of Chemistry

Kenneth K. Andersen

Kenneth K. Andersen, Prof. of Chemistry

November 19, 1971

date

ACKNOWLEDGEMENTS

I wish to express sincere thanks to Dr. Miyoshi Ikawa for his unreserved patience, encouragement and guidance throughout the course of this work. I also wish to express my gratitude to Dr. Kenneth Andersen and Dr. John Uebel for their suggestions and help in recording the ORD, NMR and mass spectra. I further wish to acknowledge the generous help of Dr. John Sasner and the help of Mrs. Pam Phair in culturing and maintenance of Gymnodinium breve, and Dr. Ronald Pero of National Institute of Environmental Health Sciences for high resolution mass spectral analysis.

TABLE OF CONTENTS

LIST OF TABLES vi

LIST OF FIGURES vii

ABSTRACT ix

PART I. GYMNODINIUM BREVE TOXIN

I. Introduction 1

II. Materials and Methods 5

1. Culturing of Gymnodinium breve 5

2. Extraction and purification of crude toxin 5

3. Column chromatography 8

4. Thin layer chromatography 9

5. Phosphorus determination 9

6. Ultra-violet and visible spectrum 10

7. Infrared spectral measurement 10

8. Nuclear magnetic resonance spectrum 10

9. Mass spectral analysis 11

10. Optical rotatory dispersion measurement 12

11. Toxicity studies 12

12. Molecular weight determination 12

III. Results 14

1. Growth of Gymnodinium breve 14

2. Extraction and purification of crude toxin 14

3. Column chromatography 18

4. Thin layer chromatography 19

5. Toxicity studies 19

6. Phosphorus determination 23

7. Ultra-violet, visible, infra-red and NMR spectra 27

8. ORD and mass spectrum 34

9. Molecular weight determination (Osmometric)..... 39

10. Acid base stability and nature of toxin..... 39

IV. Discussion..... 44

V. Summary and Conclusions..... 53

VI. Bibliography..... 54

PART II. A. FLOS-AQUAE TOXIN

I. Introduction..... 58

II. Materials and Methods..... 61

1. Concentration and collection of Aphanizomenon..... 61

2. Isolation and Purification of Crude toxin..... 61

3. High voltage electrophoresis..... 62

4. IRC-50 column chromatography..... 62

5. Thin layer chromatography..... 63

6. Cellulose column chromatography..... 64

7. Hydrogenation of crude toxin..... 64

8. Toxicity studies..... 65

9. Infra-red spectrum..... 65

III. Results..... 67

1. Isolation and purification of the toxin..... 67

2. High voltage electrophoresis..... 67

3. IRC-50 column chromatography..... 70

4. Thin layer chromatography..... 70

5. Experiments with cellulose column chromatography..... 74

6. Toxicity studies..... 74

7. Physical and chemical properties..... 79

IV. Discussion..... 88

V. Summary and Conclusion..... 91

VI. Bibliography..... 92

LIST OF TABLES

Part I. *G. breve* toxin:

1. Yields and toxicity of G. breve toxin at various stages of purification..... 16
2. Optical density at 830 nm of standard phosphorus solution and of purified *G. breve* toxin..... 26
3. Characteristic infra-red absorption frequencies of *G. breve* toxin dissolved in CCl_4..... 31
4. Optical and specific rotation of *G. breve* toxin solution (in methanol) at various wave-lengths..... 35
5. Mass-charge ratio (m/e), relative abundances and tentative formulae for the mass spectral analysis of *G. breve* toxin..... 38
6. ΔR values for the standard benzil solution in chloroform..... 40
7. Some physico-chemical and toxicological properties of *G. breve* toxin..... 46
8. Elemental analysis, emperical and molecular formulae and molecular weight of *G. breve* toxin..... 48
9. Possible molecular formulae and theoritical and experimental elemental composition of *G. breve* toxin..... 51

Part II. *A. flos-aquae* toxin:

1. Yields and toxicity of *A. flos-aquae* toxin at various stages of purification..... 68
2. Thin layer chromatography of *A. flos-aquae* toxin and saxitoxin..... 82
3. Color reactions of *A. flos-aquae* toxin..... 86

LIST OF FIGURES

Part I. *G. breve* toxin:

1. Scheme of purification of *G. breve* toxin..... 6
2. Growth curve of *G. breve* cultures..... 15
3. Thin layer chromatography of *G. breve* toxin..... 21
4. Fold increase in toxicity of *G. breve* toxin at various stages of purification..... 22
5. Minimum lethal dose of *G. breve* toxin at various stages of purification..... 24
6. Calibration plot for organic phosphorus determination..... 25
7. Ultra-violet and visible absorption spectrum of *G. breve* toxin..... 28
8. Infra-red spectrum of *G. breve* toxin..... 30
9. 60 MHz. NMR spectrum of *G. breve* toxin..... 32
10. Optical rotatory dispersion spectrum of *G. breve* toxin..... 36
11. High resolution mass spectrum of *G. breve* toxin..... 37
12. Calibration curve for molecular weight determination by osmometric method..... 41
13. Flow diagram for the determination of the nature of *G. breve* toxin..... 43

Part II. *A. flos-aquae* toxin:

1. Second high voltage electrophoresis pattern of *A. flos-aquae* toxin..... 71
2. Thin layer chromatography of *A. flos-aquae* toxin at various stages of purification..... 72
3. Fractionation of *A. flos-aquae* toxin on IRC-50 column..... 73
4. Fractionation of *A. flos-aquae* toxin on cellulose CC41 column... 75
5. Thin layer chromatography of combined fractions from CC41 column..... 76
6. Fold increase in toxicity of *A. flos-aquae* toxin at various stages of purification..... 77

LIST OF FIGURES (cont'd)

7. Toxicity of A. flos-aquae toxin at various stages of purification..... 78

8. IR spectrum of A. flos-aquae toxin..... 80

9. Thin layer chromatography of A. flos-aquae and saxitoxin..... 83

10. Thin layer chromatography of A. flos-aquae and saxitoxin..... 84

11. Thin layer chromatography of A. flos-aquae and saxitoxin..... 85

12. High voltage electrophoresis of A. flos-aquae and saxitoxin..... 87

ABSTRACT

CHEMICAL STUDIES ON TOXINS FROM

GYMNODINIUM BREVE AND APHANIZOMENON FLOS-AQUAE

by

MAKTOOB ALAM

Part I: Gymnodinium breve toxin

The dinoflagellate, Gymnodinium breve Davis, produces a toxin(s) which is lethal to marine organisms during "red tide" blooms in the Gulf of Mexico. This investigation concerns the isolation and chemical characterization of the toxin(s). Unialgal cultures of G. breve were grown in an artificial sea water medium, and the toxic material was isolated by extracting the culture with ether. The crude extract was further purified by partitioning between solvents. The partially purified toxin on column chromatography gave a toxic pale yellow glassy residue (ca. 0.1 mg per liter of culture medium), which on thin layer chromatography in different solvent systems showed only a single spot.

The infra-red spectrum indicated the presence of a carbonyl group and the absence of hydroxyl and aromatic groups. The absence of an aromatic chromophore was also supported by the NMR spectrum, which indicated the presence of six methyl and eight methylene protons not in the neighbourhood of an electron withdrawing group. The ultra-violet spectrum indicated that the toxin might be contaminated with very small amount of pigment. A molecular weight of 279 was determined

both by mass spectrometry and osmometry in chloroform. The mass spectrum also indicated the absence of an aromatic group. The formula $C_{19}H_{21}NO$, calculated on the basis of the m/e ratio from the mass spectrum, was also supported by the number of protons in the molecule calculated by integrating the NMR signals. On the basis of present information it is difficult to assign a definite formula to the toxin from Gymnodinium breve.

Part II: Aphanizomenon flos-aquae

Poisoning by blue-green algae is virtually world wide in occurrence. A serious outbreak of poisoning by blooms of Aphanizomenon flos-aquae occured in Kezar Lake, North Sutton, New Hampshire in 1966. Similar algal blooms were also noted in other lakes in the state during the summer months of 1966-1970. The purpose of this investigation was to isolate and characterize the toxic compound(s) from natural blooms of A. flos-aquae. Thick algal concentrates were obtained by continous centrifugation of lake water during the height of A. flos-aquae blooms. The toxin was purified by two different methods, one involving high voltage electrophoresis, IRC-50 resin column chromatography, and preparative thin layer chromatography, and a second method involving cellulose column chromatography followed by repeated thin layer chromatography. The toxin was obtained as a pale yellow powder in a chromatographically pure form. The A. flos-aquae toxin was a very basic substance. It differed from saxitoxin in its Rf values on cellulose MN plates in several solvent systems and its behavior to several spray reagents. The partially hydrated form of the toxin had a different infra-red spectrum than does saxitoxin. On the basis of this and other information it is concluded that the A. flos-aquae is different from saxitoxin.

PART I

GYMNODINIUM BREVE

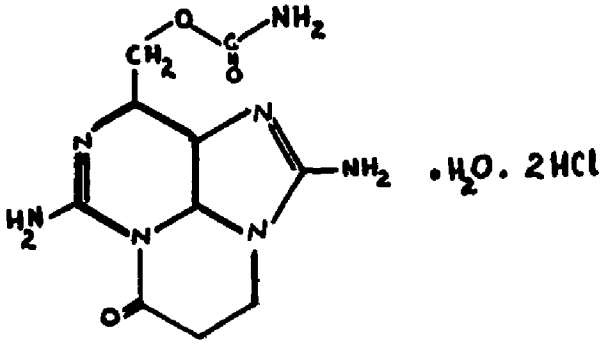
INTRODUCTION

Quite often in the aquatic environment the delicate balance of nature is upset by a phenomenon commonly known as "red tide". This term is used to indicate the massive growth or bloom of various algae or protozoa which causes a coloration of surface water over a vast area. This coloration is not always reddish, as the term "red tide" would indicate, but can be brown, yellow or green depending on the nature of the pigments in the organism and also on the depth and concentration of the causative organism. A great many of the microorganisms which are responsible for "red tide" are dinoflagellates, which can normally be found in all the oceans at all times of the year. Among all form of marine life the dinoflagellates are probably second in abundance only to diatoms, and in many cases may greatly outnumber them. Together with diatoms they constitute the bulk of the so-called phytoplankton - the community of unicellular autotrophic organisms which is the basis of life in the sea. The biological community in which the "red tide" occurs may be profoundly affected by the tremendous growth of the microorganisms causing them. Ballantine and Abbott (1) have suggested that the deleterious effects of massive microorganism blooms may be due to so-called secondary conditions, such as, imbalance of nutrients, oxygen deficiency, hydrogen sulfide production from decomposing organic matters, and increased bacterial concentrations. On the other hand toxic substances produced by these organisms may kill other members of the marine community directly.

Sommer and Meyer (2) were first to demonstrate that the dinoflagellate Gonyaulax catenella was responsible for the poisonous

California mussels that cause paralysis and death in man. Later other workers have reported that Gonyaulax tamarensis, Gonyaulax monilata, Gonyaulax acatenella, and Gonyaulax polyedra also produce toxin. In addition to species of Gonyaulax, other species of dinoflagellates such as Gymnodinium veneficum and Gymnodinium breve have also been implicated in toxin production. Blooms of G. breve have been responsible for the mass mortality of fish in the Gulf of Mexico during summer months.

The toxin from G. catenella (paralytic shellfish toxin commonly known as saxitoxin) has been isolated and studied in greater detail than any other dinoflagellate toxin. Schantz et al. (3) isolated and characterized the toxin from poisonous Alaskan butter clams, California mussel, and G. catenella itself and showed that they were the same toxic compound. It had a molecular weight of 372 (as the hydrochloride) and the formula $C_{10}H_{17}N_7O_4.2HCl$. A tentative structure for saxitoxin has been suggested by Rapoport (4).



Saxitoxin is one of the most toxic non-proteinaceous substances known to man (5000 ± 500 lethal mouse units per mg), and at the present time no effective antidote against it is known. The poison is not antigenic, but recent studies have shown that it can be used as a hapten to immunize animals against the toxin (5).

The toxin from G. tamarensis has been studied by Evans (6). He reported the presence of two toxins, one closely resembling saxitoxin in its behaviour on Amberlite ion-exchange resin and its biological effects, and the other behaving quite differently on the ion-exchange resin. The latter appeared to be a small molecule comparable in size to saxitoxin. Its biological effects were similar, but not identical, to those of saxitoxin. Like saxitoxin and tetrodotoxin (a toxin from puffer fish) it inhibited the inward movement of sodium ions across electrically excitable membranes. The chemical nature of this toxin is still unknown. G. acatenella occurs along the coast of British Columbia and has caused clams to become poisonous (7). This organism produces a toxin similar to G. catanella and G. tamarensis, but the exact chemical composition is still unknown. G. polyedra often blooms along the coast of Southern California and Schradie and Bliss (8) have reported that this organism produces a toxin which has some properties similar to those of saxitoxin. However, Schantz (5) reported that G. polyedra produces toxin only under specific conditions, and that it has never been known to cause shellfish to become poisonous. G. monilata often blooms to "red tide" proportions in the Gulf of Mexico and produces a toxin which is toxic to fish but not to chickens (9, 10, 11). This toxin has not been isolated in pure state.

Abbott and Ballantine (12) reported a toxin from G. venificum which was toxic to fish and mice. No further chemical work has been done on this toxin. Within the past several years the toxin from G. breve has been studied by a number of workers. Starr (13) described its toxicity to fish, and Ray and Aldrich (11, 14) its toxicity to chicks. McFarren et al.(15) described the occurrence of a ciguatera-

like poison in oysters from the Gulf of Mexico and in G. breve itself. Cummins et al.(16) using thin layer chromatography reported the isolation of two chromatographically different toxins from a Soxhlet extract of G. breve cultures, and Spikes et al.(17) developed a method involving ether extraction followed by thin layer chromatography for the purification of G. breve toxin. Martin and Chatterjee (18) described the isolation of two toxic fractions from "red tide" blooms and laboratory cultures of G. breve; a substance I from the interfacial material and a substance II from the chloroform layer. Substance II was described as a non-nitrogenous phosphorus-containing optically active pale yellow low melting solid with a molecular weight of 650. Cummins and Stevens (19) reported the isolation and chemical characterization of a slow acting toxin from G. breve cultures with a molecular weight of around 2000. Trieff et al.(20) using repeated thin layer chromatography of an ether extract of G. breve cultures reported the isolation of a toxin which had an average molecular weight of 468. The toxin however was impure as shown by mass spectral data. In a later communication Trieff et al. (21) reported a dry column chromatographic (DCC) method for the purification of G. breve toxin. The toxin purified by this method had a molecular weight of 520, while toxin purified using a combination of dry column chromatography and thin layer chromatography had a molecular weight of 694.

MATERIALS AND METHODS

Culturing of Gymnodinium breve

The G. breve starter culture used in this study was originally obtained by Dr. J. J. Sasner, Jr. of the Department of Zoology, University of New Hampshire, from Dr. W. B. Wilson, Texas A and M University Marine Station, Galveston, Texas. G. breve cultures were grown in 1.2 to 1.5 liter volume in 2.5 liter Low-form flasks (Corning #4422). They were maintained in an incubator or in an open water tank at 24±1°C and exposed to a 14 hour light period daily using 40 watt cool white fluorescent bulbs (500 foot candles). The culture medium employed was the artificial sea water medium NH15 described by Gates and Wilson (10). New cultures were inoculated with grown cultures to give an initial count of 1000±200 cell per ml, and were allowed to grow undisturbed, with no cell counts taken for approximately two weeks. The growth was observed through the wall of the flask. When dense cell masses could be seen inside the flasks (generally after two weeks), samples of the cultures were counted with a model F Coulter counter. Cell counts usually reached 15-20 x 10^6 cells per liter after two weeks.

Extraction and Purification of Crude Toxin

The entire method of purification of G. breve toxin is depicted in Fig. 1. The pH of the culture medium was adjusted to pH 5.5 with concentrated HCl and was extracted twice with diethyl ether (first with 150 ml, then with 100 ml per liter of medium). Most of the salts in the ether extract were removed by carefully removing the lower aqueous layer which resulted during the concentration of the extract. Additional residues which precipitated from the ether phase

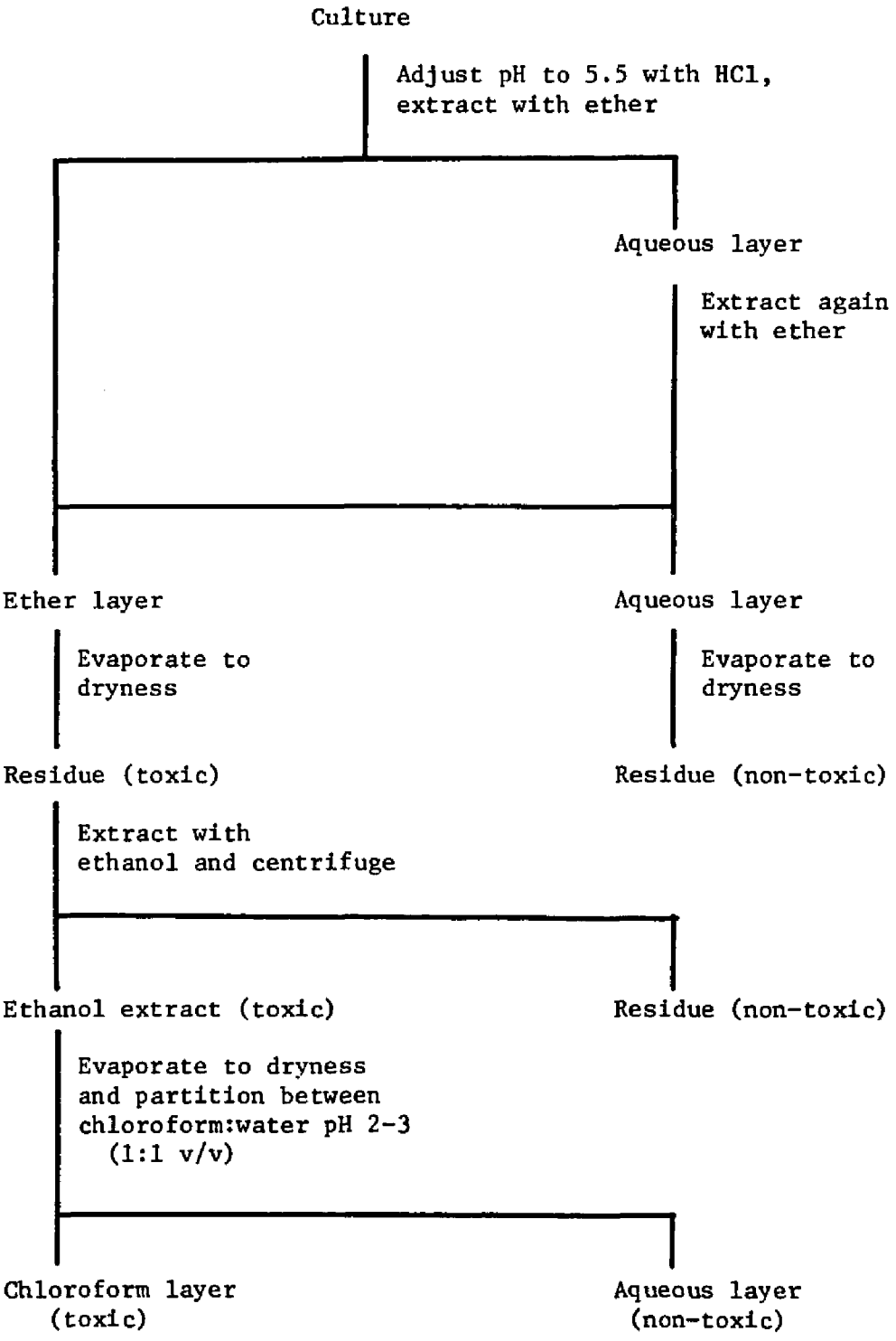


Fig. 1. Scheme of purification of G. breve toxin

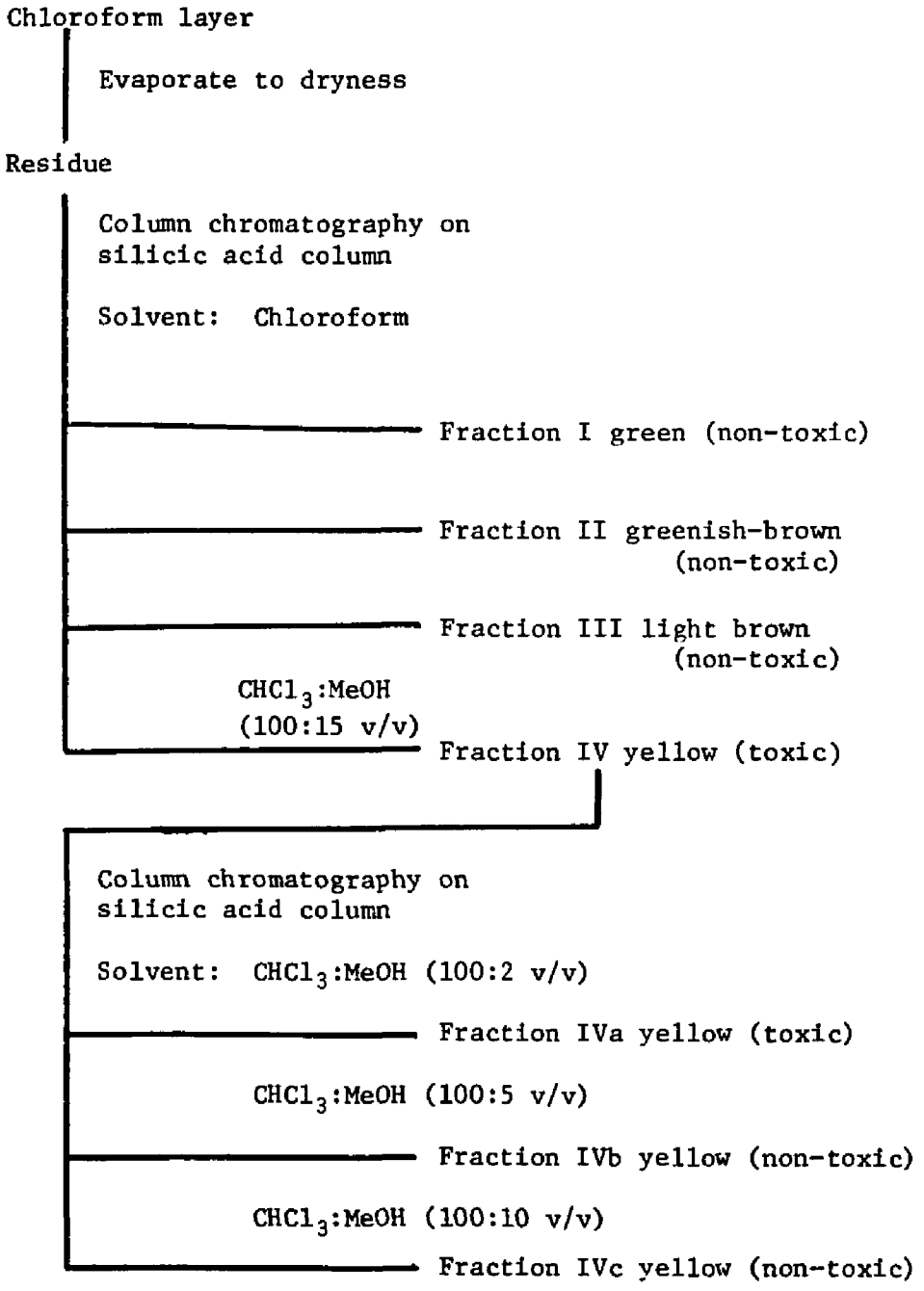


Fig. 1 (Continued)

were removed just prior to taking the sample to complete dryness *in vacuo*. Ethanol (95%) (*ca*. 50 ml) was added to the residue from the ether extract. Upon stirring, a white gelatinous precipitate formed, which was removed from the supernatant solution by centrifugation using a clinical centrifuge. The ethanol solution was evaporated to dryness *in vacuo*. Chloroform (reagent grade) and water (acidified to pH 3.0-3.5 with HCl) were added in equal volume (*ca*. 50 ml each) to the residue and the mixture was shaken in a separatory funnel. The mixture was allowed to stand and the lower chloroform layer was separated and evaporated to dryness *in vacuo* to yield partially purified toxin.

Column Chromatography

Chromatography columns (2.5 x 12.0 cm) were prepared by packing with a slurry of silicic acid (15 g) (SilicAR CC7, 100-200 mesh, Mallinckrodt) in chloroform. The columns were then washed with 250 ml of redistilled chloroform. Partially purified toxin was dissolved in redistilled chloroform (between 45-55 mg in 2 ml) and was placed on the column. The column was eluted with redistilled chloroform (approximately 500 ml) followed by a mixture of chloroform: methanol (100:15 v/v). The yellow band which was eluted with the chloroform:methanol (100:15) mixture was evaporated to dryness *in vacuo* and the residue was dissolved in 2 ml of redistilled chloroform and placed in another column of silicAR of approximately the same dimensions as the first column. The column was eluted with chloroform, chloroform:methanol (100:2), chloroform:methanol (100:5), and chloroform:methanol (100:10) mixtures.

Thin Layer Chromatography

Thin layer chromatography plates were prepared using silica gel G (E. Merck Reagent) containing 13 percent $CaSO_4$ as binder. An approximately 30 percent slurry of silica gel G in water was spread on 20 x 20 cm glass plates using the Brinkmann 0.250 mm TLC spreader. The plates were dried at room temperature for two hours and activated in an oven at 110°C for a minimum of 60 minutes before use. Solutions of partially purified and pure toxin were spotted approximately 2.5 cm from the bottom, using micro sampling pipets (Corning). The plates were developed in chambers containing either chloroform:methanol: acetic acid (100:10:1.0 v/v) or benzene:ethyl acetate:absolute ethanol (80:10:10 v/v) (21). At the end of the run the plates were removed, positioned vertically, and were allowed to dry at room temperature. The spots were located by iodine vapour.

Phosphorus Determination

The amount of organic phosphorus present in pure toxin was determined by a modification of the Bartlett method (22) of phosphorus assay. Twenty five mg of potassium dihydrogen phosphate (KH_2PO_4) was dissolved in 25.0 ml of deionized distilled water to give a stock solution containing 0.228 mg P/ml. The stock solution was further diluted to give a working standard containing 5.69 µg P/ml. Aliquots of 0.05, 0.1, 0.2, 0.25, 0.4, 0.5 and 1.0 ml of working solution were placed in 18 x 150 mm test tubes and the solutions were diluted to 2.0 ml with deionized distilled water. Ten normal sulfuric acid (0.5 ml) was added to each tube, and the tubes were heated in a heating block (Research Specialties Co.) for 30 minutes at 200-210°C. Two drops of 30 percent hydrogen peroxide were added to

each tube and the tubes were heated for an additional 20 minutes. Ammonium molybdate (9.2 ml of a 0.22 percent solution) and Fiske-SubbaRow reagent (0.4 ml) were added to each tube after cooling and the tubes mixed thoroughly and heated for 7 minutes in a boiling water bath. The tubes were allowed to cool, and the optical density was read at 830 mμ on a Bausch and Lomb Spectronic 20. Pure toxin (6.0 mg) was dissolved in 4 ml of redistilled chloroform and aliquots placed in tubes which were run along with the tubes containing standard solutions.

Ultra Violet and Visible Spectrum

The ultra violet and visible spectrum of methanolic solutions of the toxin was recorded on a Cary Model 15 recording spectrophotometer.

Infrared Spectral Measurements

The infra-red spectrum of purified G. breve toxin was recorded on a Perkin-Elmer 337 infra-red spectrophotometer. Spectra were determined on 8-10 percent solutions of toxin in carbon tetrachloride (Fisher spectral grade).

Nuclear Magnetic Resonance Spectrum

The NMR spectra of pure G. breve toxin were recorded on a Varian A60 Analytical NMR spectrometer in conjunction with a C-1024 Time Average Computer (CAT) (Varian Associates, Walnut Creek, California). Pure G. breve toxin (7.913 mg) was dissolved in 0.2 ml of spectral grade carbon tetrachloride and one drop of tetramethylsilane (TMS) was added as an internal reference. At first a spectrum was recorded without using CAT. This was followed by taking the

spectrum using the time average device (CAT). The conditions were as listed below:

	1st spectrum	2nd spectrum	
	0-500 cps	0-300 cps	300-450 cps
Solvent	CCl_4	CCl_4	CCl_4
Concentration (percent)	4	4	4
Scans (No. of times)	-	17	17
Sweep Width	500	150	290 Hz.
Sweep Time	500	25	100 Sec.
Sweep Offset	0.04	450	300 Hz.
Readout time	-	100	250 Sec.
Filter Band	1	1	1
RF Field	0.5	0.5	0.5
Spec. Amp.	100	100	100

Mass Spectral Analysis

The mass spectrum of the pure toxin was recorded by the direct inlet method. The conditions were: ionizing voltage 70 ev, pressure 2.1×10^{-6} and temperature 295 ± 3°C. Another sample of the toxin was examined by high resolution mass spectrometry at the RT Center for Mass Spectrometry, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina, by the direct inlet method at 150°C. The number of carbon atoms was calculated using the formula (23)

$$\frac{[\ (A+1)^+\ ]}{(A^+)} = \underline{ca.}\ 1.1\ \%\ \text{x no. of C atoms} + 0.36\ \%\ \text{x no. of N atoms.}$$

The total number of rings plus double bonds was calculated using the formula (23)

Total rings + double bonds = $X - \frac{1}{2} Y + \frac{1}{2} Z + 1$, where the

empirical formula is $C_x H_y N_z O_n$.

Optical Rotatory Dispersion

The ORD spectrum of the pure toxin was recorded on a Cary Model 60 Recording Spectropolarimeter. The optical rotation was calculated by the formula:

$$\alpha_\lambda = \text{Pen deflection} \times \text{Sensitivity}$$

and specific rotation:

$$[\alpha] = \frac{100}{1 \times C} \times \alpha_\lambda$$

where 1 = length of the path in decimeter

C = concentration in g per 100 ml

α_λ = optical rotation at wave length λ

Toxicity Studies

The toxicity of pure and crude toxin at various stages of purification was monitored by a mouse bio-assay method. A known volume of solution at each stage of purification was evaporated to dryness in a preweighed pear-shaped flask. The weight of residue was determined from the difference in weights before and after evaporating the solution to dryness. The residue was dissolved in a known volume of cottonseed oil, and various aliquots of this solution were injected intraperitoneally into 2-3 mice of 19-20.5 g weight. The mice were watched carefully for 1 hour. If the animal died within one hour the dose was considered lethal. The choice of cottonseed oil was based on the fact that the toxin is soluble in lipid solvents but insoluble in water. Mouse units were calculated using the tables of McFarren *et al*. (15).

Molecular Weight Determination (Osmometric)

A Mechrolab Vapour Pressure Osmometer Model 301A (Mechrolab Inc.,

Mountain View, California) was used for the molecular weight determination. The osmometer was turned on the night before the determination was to be made. Benzil (0.42 g) was dissolved in 2.0 ml of redistilled chloroform, and a series of dilutions was made to give a series of solutions with final concentrations of 0.01-0.10 g mole of solute per liter of solution. One ml of each of these solutions was withdrawn into syringes 1-4 of the instrument. After setting and zeroing the instrument with redistilled chloroform, the sample bead was rinsed with a few drops of standard solution, one concentration at a time, and a drop of solution was suspended from the bead. The zero was checked after about 45 seconds, and the ΔR value was read after exactly 2 minutes. The bead was rinsed off and a new drop of the same solution was suspended, and, after zeroing, ΔR value was again recorded. The process was repeated until a constant ΔR value was obtained for the solution. The other syringes containing increasing concentrations of the solute were read in a similar fashion. Purified G. breve toxin (1.4 mg) was dissolved in 0.25 ml of redistilled chloroform and the solution was withdrawn into syringe 1 of the instrument, and ΔR values were recorded as described above. A calibration curve was drawn by plotting ΔR vs concentration. This permits the g molar concentration of the unknown to be read directly from the graph. The molecular weight of the toxin was calculated using the formula

$$\text{Mol. wt.} = \frac{X}{C}$$

where X = weight of unknown in solution in g per liter.

C = concentration in g mole per liter.

RESULTS

Growth of G. breve Cells

The growth of G. breve cultures is shown in Fig. 2. It is representative of some 150 cultures maintained under the conditions described in MATERIALS AND METHODS. These cultures had a doubling time of approximately 2-3 days. The cultures grown in the open water tank gave the same amount of growth except that it generally took one to two days longer than cultures grown in the incubator to reach peak growth. Starting with mass cultures with initial counts of 1000±200 cells per ml provided a definite advantage when large quantities of cellular material were required. It avoided a lag phase of growth which occurred when cultures were innoculated with fewer number of cells, and permitted harvesting of mature cultures approximately every 14-15 days. If growth was allowed to proceed beyond 18-20 days, cell counts and toxin yields from extraction were erratic. The addition of gibberellic acid did not significantly increase cell numbers at maturity in cultures as suggested by Paster and Abbott (24).

Extraction and Purification of Crude Toxin

The crude toxin yields and toxicity data from representative batches processed as described in MATERIALS AND METHODS is shown in Table 1. An average of 0.961 g of residue was obtained from approximately 12.5 liters of G. breve culture. This residue upon stirring with ethanol gave a white gelatinous precipitate which consisted of salts and proteinacious matter and which gave a positive ninhydrin reaction. The precipitate was insoluble in lipid solvents, slightly soluble in methanol and ethanol, and soluble in water. It was found

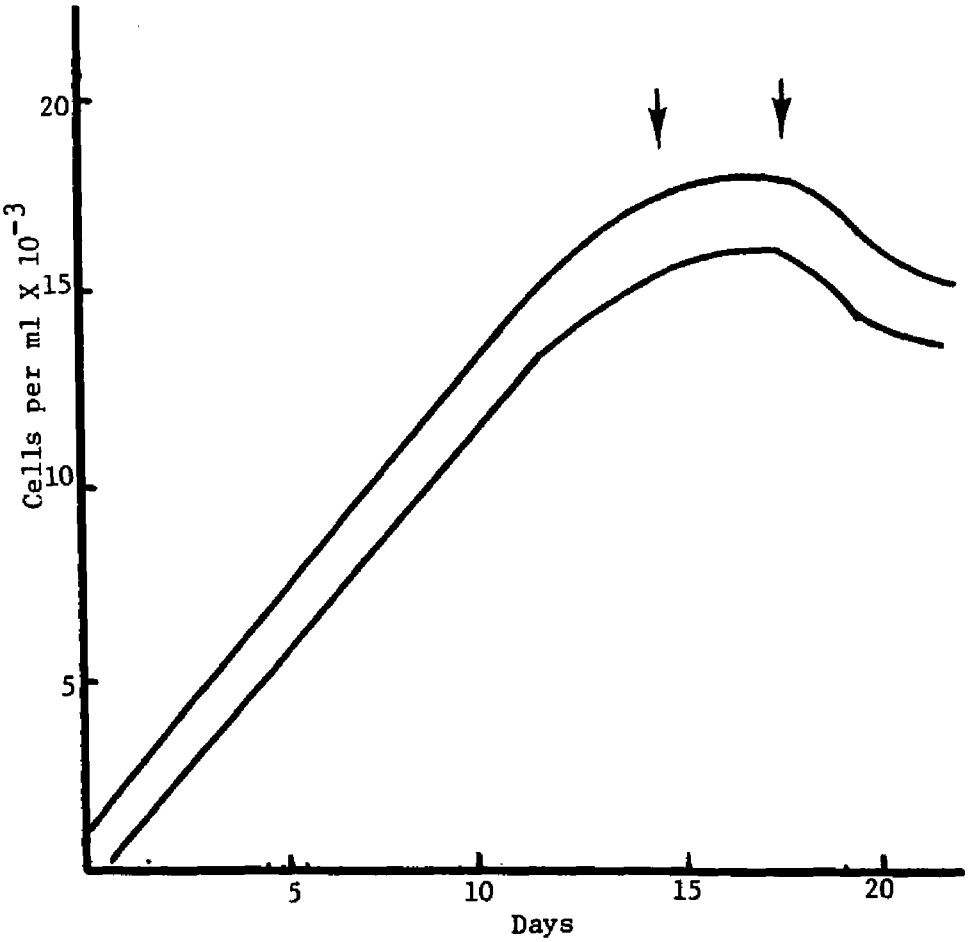


Fig. 2 Growth curve of G. breve cultures. Toxin was was extracted when the cells reached the age indicated between the arrows.

TABLE I

Yields and Toxicity of G. breve Toxin at Various Stages of Purification

	Residue from Ether Extract					Residue from Ethanol Extract				Residue from Chloroform Layer			
	Volume of Cult. (L)	Wt. of residue (g)	Toxicity			Wt. of residue (g)	Toxicity			Wt. of residue (g)	Toxicity		
			Dose (mg)	S.T.[a] (min)	MU[b]		Dose (mg)	S.T.[a] (min)	MU[b]		Dose (mg)	S.T.[a] (min)	MU[b]
1	15	1.28	19.6	21.5	4.5	0.799	18.0	15.0	6.5	0.0822	--	--	--
2	15	--	--	--	--	0.612	10.6	21.3	4.4	0.0529	--	--	--
3	--	0.326	16.2	20.0	4.5	0.081	8.27	26.0	4.2	0.0500	--	--	--
4	10	0.580	20.9	18.0	5.0	0.431	--	--	--	0.1080	2.1	22.0	4.5
5	--	--	--	--	--	0.618	--	--	--	0.2030	--	--	--
6	8	0.610	--	--	--	0.195	12.7	35.0	3.9	0.0280	3.2	17.0	5.5
7	15	1.113	18.2	16.0	6.0	0.829	--	--	--	0.0516	2.4	14.5	7.0
8	--	--	--	--	--	--	--	--	--	0.0785	1.8	20.0	4.5
9	--	1.24	--	--	--	0.970	7.5	22.5	4.4	0.1150	1.8	20.0	4.5
10	10	0.765	18.0	12.0	8.0	0.294	8.5	21.6	4.5	0.1090	1.5	18.0	5.0
11	16	1.360	17.4	23.0	4.2	0.792	--	--	--	0.0858	1.35	26.7	4.3
12	--	1.290	--	--	--	0.521	--	--	--	0.0691	--	--	--
Ave	12.7	0.961	18.4	18.4	5.37	0.558	10.9	23.6	4.65	0.0819	2.00	19.7	5.04

[a] S.T. - Survival Time.

[b] Mouse Units present in lethal dose as calculated from the tables of McFarren et al (15).

TABLE I (Continued)

	1st Chromatography				2nd Chromatography						Remarks
								Toxicity			
	Fr.I (mg)	Fr.II (mg)	Fr.III (mg)	Fr.IV (mg)	Fr.IVa (mg)	Fr.IVb (mg)	Fr.IVc (mg)	Dose (mg)	S.T.[a] (min)	MU[b]	
1	--	--	--	--	--	--	--	--	--	--	
2	--	--	--	11.0	3.00	0.7	--	0.15	6.0	11	
3	23.0	1.7	3.4	16.0	--	--	--	--	--	--	
4	120.0	26.3	13.5	28.4	6.70	3.45	0.40	0.23	15.0	6.5	Residues from 4 and 5 were combined and 311.0 mg was placed on the 1st column.
5											
6	--	--	--	--	--	--	--	--	--	--	
7	8.3	5.9	3.1	9.5	4.48	3.5	0.61	0.112	15.0	6.5	32 mg was placed on 1st col.
8	22.5	2.1	3.5	25.2	12.90	5.7	3.0	--	--	--	
9	13.4	24.0	9.5	39.4	10.70	4.3	1.4	0.114	18.0	5.0	110 mg was placed on 1st col.
10	25.8	9.1	3.6	43.2	9.2	3.2	1.8	0.113	19.0	5.0	
11	22.6	4.6	6.9	35.2	8.5	4.4	2.1	0.105	16.7	6.5	
12	--	--	--	18.8	4.2	--	--	--	--	--	
Ave.	33.66	10.5	6.2	25.1	7.4	3.6	1.85	0.137	13.2	7.5	

to be non-toxic to mice when injected intraperitoneally. The percent recovery of soluble toxic material from ethanol extraction was 58.1 percent. The next step in purification involved the partitioning of this residue between chloroform and water. For the partitioning the pH of the water was adjusted to pH 2-3. This was based on observations that the toxin was more completely extracted at a lower pH than at the usual water pH of 6-6.5. An average of 81.9 mg of crude toxin per 12.5 liters of culture was obtained from the chloroform extract. A recovery of 14.68 percent of the residue from the ethanol extract was obtained. The variation from batch to batch in the percent recovery of crude toxin at various stages of purification may be due to differences in the composition of the ether extract from various batches of G. breve cultures.

Column Chromatography

The data from column chromatographic purification are given in Table 1. In each run approximately 50 mg of residue from the chloroform extract was placed on the column. During elution three bands were seen moving down, the first (Fraction I) was green in colour, the second (Fraction II) had a brown tinge and the third (Fraction III) was yellowish. The fractions were cut on the basis of these coloured bands. The variation in the amount of residue obtained from various fractions as percent of partially purified toxin can be safely attributed to differences in the composition of chloroform extracts from various G. breve culture lots. In some cases, where the culture medium had a light green colour, an additional band of greenish-blue colour was seen moving just behind the first green band (Fraction I). After collecting the third band (Fraction III)

from the column, the column was eluted with a solution containing 15 percent methanol in chloroform. Only one band (Fraction IV) was seen moving down with this solvent. Fraction IV was evaporated to dryness and was placed on a second column. The second column was eluted with increasing concentrations of methanol in chloroform. Three yellow bands were seen moving down (as shown in Table I). The first yellow band (Fraction IVa) was eluted with solvent containing 2 percent methanol in chloroform and was found to be toxic when tested as described in MATERIALS AND METHODS. The latter two bands (Fractions IVb and IVc) which were eluted with chloroform:methanol (100:5 v/v) and (100:10 v/v) respectively, were non-toxic when tested by the mouse bio-assay method.

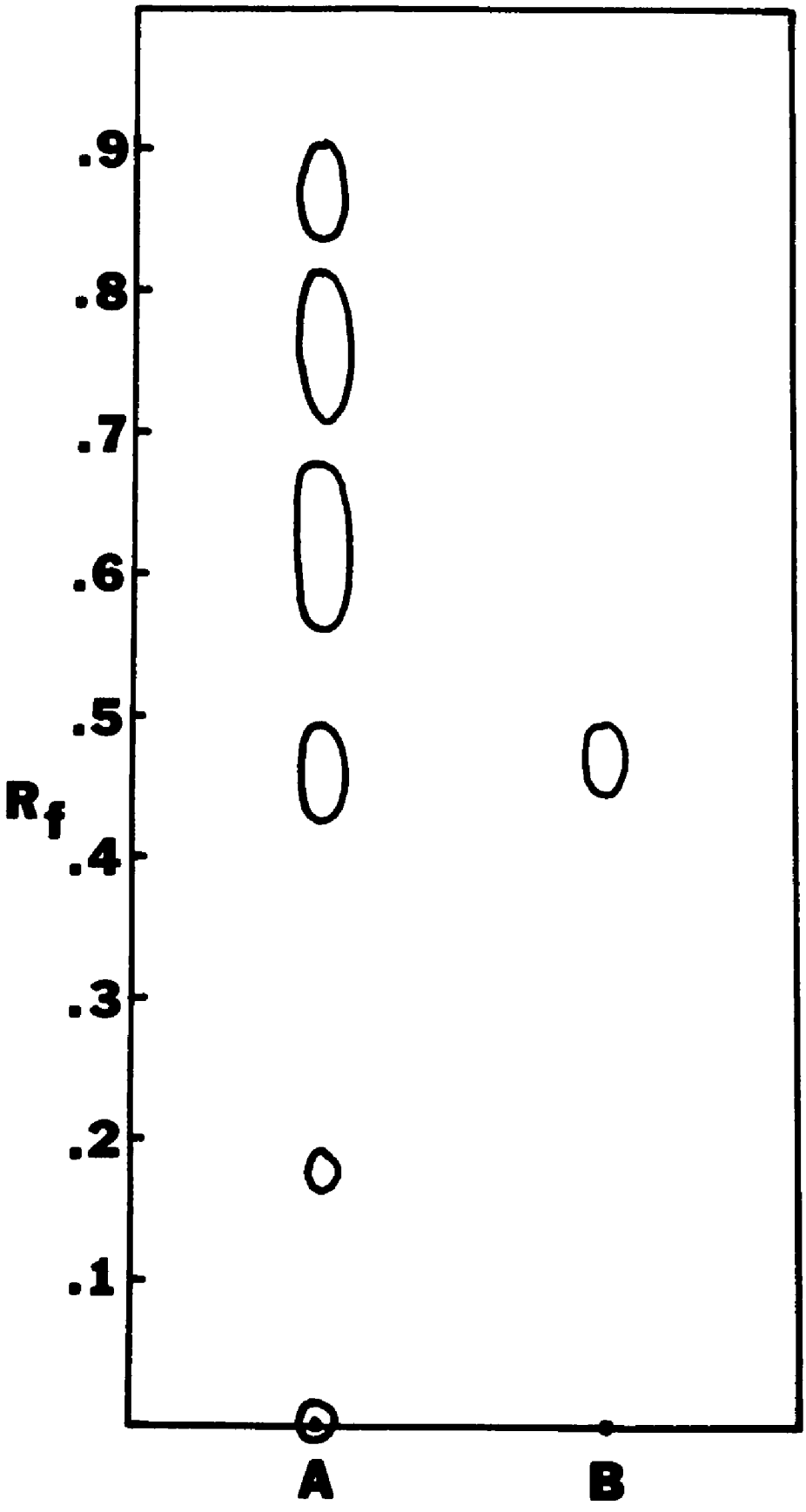
Thin Layer Chromatography

The thin layer chromatographic pattern of pure and crude (chloroform extract) G. breve toxin is shown in Fig. 3. The crude toxin showed the presence of six spots with Rf values of 0.88, 0.78, 0.62, 0.48, 0.19, and one which stayed at the point of application of the sample. The pure G. breve toxin (Fraction IVa) had a Rf value of 0.48. The Rf value of pure toxin was nearly the same in both chloroform:methanol:acetic acid (100:10:1.0 v/v) and benzene:ethyl acetate:absolute ethanol (80:10:10 v/v).

Toxicity Studies

The average fold increase in toxicity during the purification process as a function of stage of purification is depicted in Fig. 4. The residue from the ether extract (18± 2 mg) gave a survival time of about 18±2 minutes, which represents 5-6 mouse units when calculated from the tables of McFarren et al.(15). Thus about 3.5 ± 1 mg of residue

Fig. 3 Thin layer chromatography of G. breve toxin in chloroform: methanol: acetic acid (100:10:1 v/v). (A) Chloroform extract (B) Pure toxin (fraction IVa).


.9
.8
.7
.6
.5
R_f
.4
.3
.2
.1
A
B

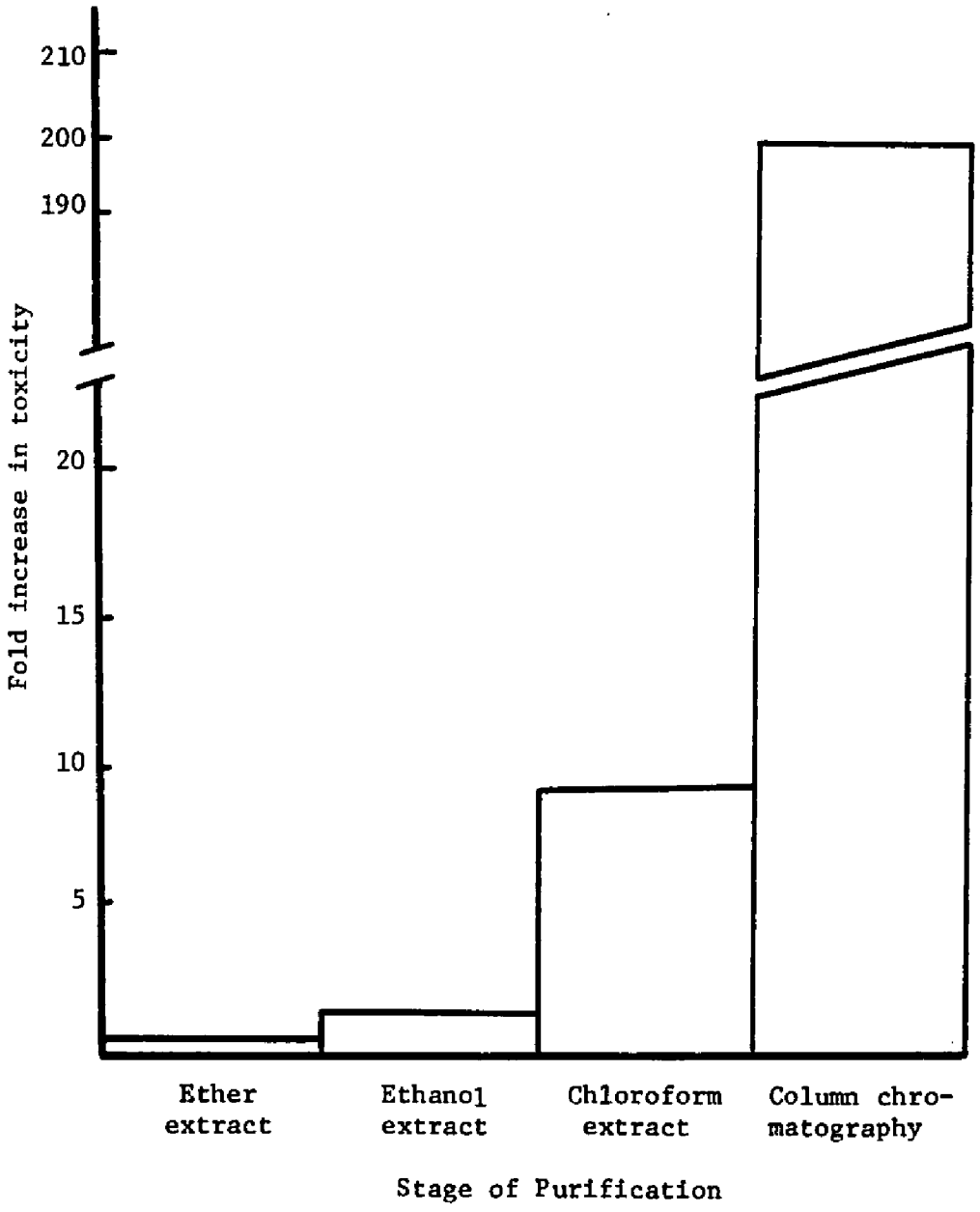


Fig. 4 Fold increase in toxicity at various stage of purification.

from the ether extract is equivalent to one mouse unit or the crude toxin had an activity of *ca.* 0.29 MU per mg. The minimum lethal dose in mg of toxin at various stages of purification is shown in Fig. 5. The pure *G. breve* toxin at a 0.113±0.03 mg level gave an average survival time of 14.9 minutes (Table I) (omitting run 4) representing about 6.5 MU according to McFarren *et al.* (15). Thus an average of 0.017 mg of pure toxin constituted one mouse unit or the toxin had an activity of about 59 MU per mg of solid. There was a significant batch to batch variation (Table I) in the potency of the crude extract but variation decreased as the purification proceeded. No detailed study was made to this batch to batch variation.

The reaction of the mice to the toxic effects of *G. breve* toxin were similar to those recorded by Starr (13) and McFarren *et al.* (15). The characteristic symptoms produced by *G. breve* included respiratory irregularity, violent twitching, hind limb paralysis, and death by respiratory failure.

Phosphorus Determination

The calibration curve for the phosphorus determination, prepared with potassium dihydrogen phosphate, is shown in Fig. 6. The optical density of solutions containing various concentrations of phosphorus, and the unknown is shown in Table II. Pure *G. breve* toxin, 3.0 mg, had 0.15 µg of phosphorus as determined from the calibration curve, and the percent phosphorus was found to be 0.005 percent. This value is far less than that reported by Martin and Chatterjee (18) and Trieff *et al.*(20, 21).

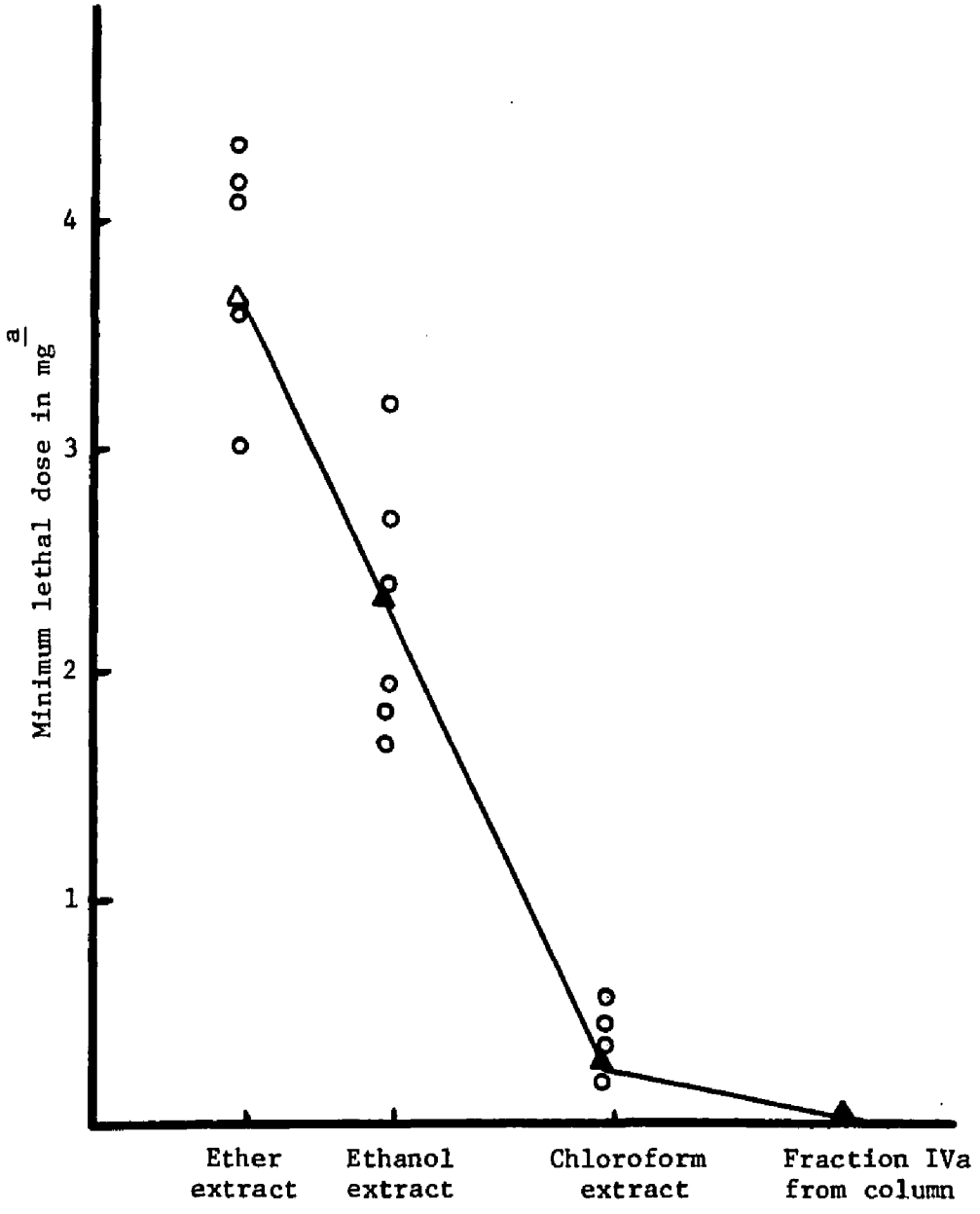


Fig. 5 Minimum lethal dose of G. breve toxin at various stage of purification
(a) Calculated from the table of McFarren et al.(15)

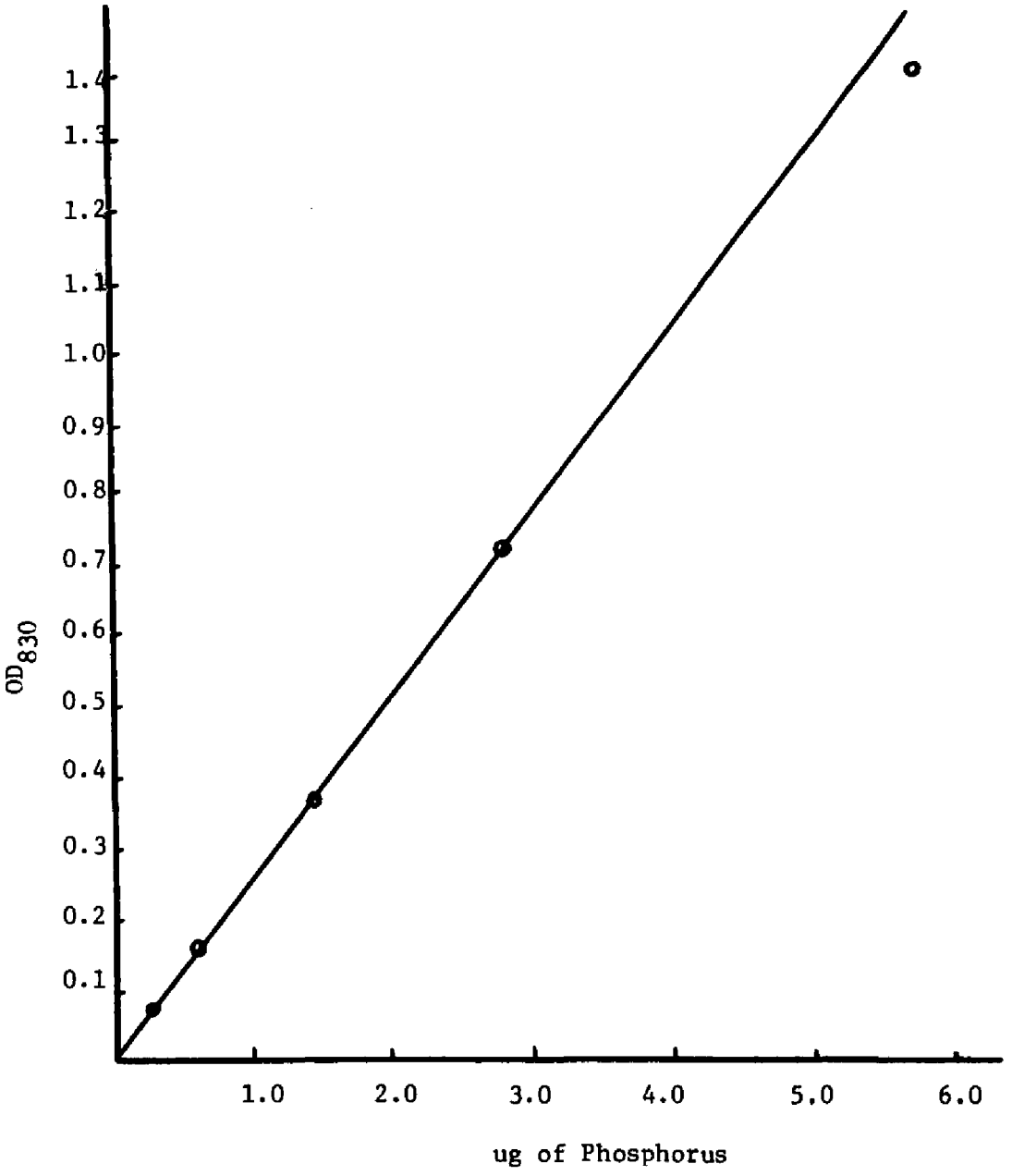


Fig. 6 Calibration plot for organic phosphorus determination.

TABLE II

Optical density at 830 nm of standard phosphorus solutions and of the purified G. breve toxin

	Phosphorus ug	OD_{830}
1	0.000	0.00
2	0.284	0.08 (0.08)[a]
3	0.569	0.16 (0.16)
4	1.422	0.36 (0.37)
5	2.845	0.72 (0.72)
6	5.690	1.40 (1.50)
7	Toxin solution	0.04 (0.04)

[a] Results of duplicate run in parentheses.

Ultraviolet and Visible Spectrum

The ultraviolet and visible absorption spectrum of the pure G. breve toxin is shown in Fig. 7. Aside from strong absorption at wavelengths below 250 nm, a maximum was observed at 410 nm with shoulders at 433 and 465 nm. The yellow colour of the toxin and its absorption maximum at 410 nm would indicate that the toxin might be contaminated with a small amount of pigments giving it a yellow colour. The reason for this assumption is discussed later.

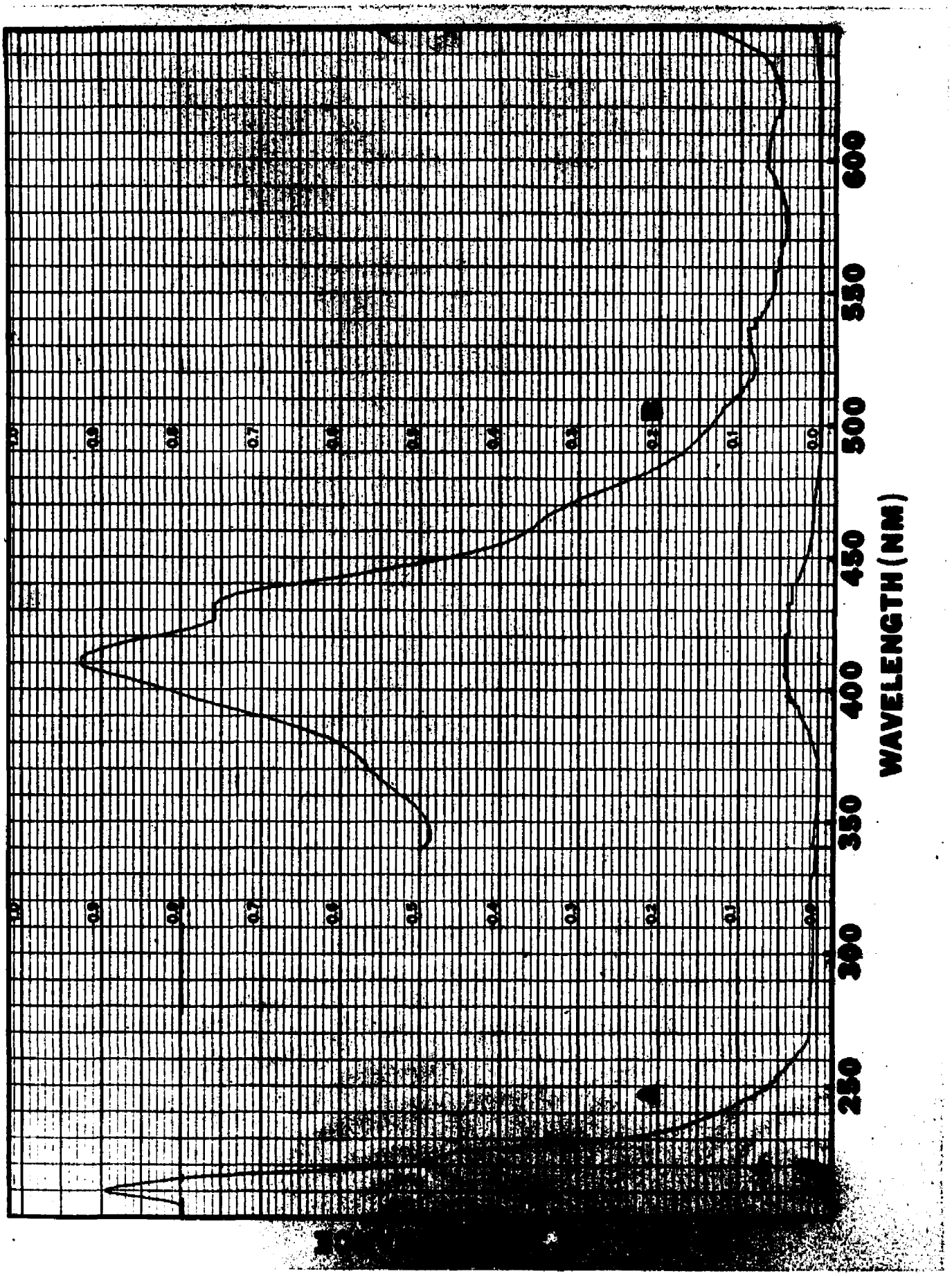
Infra-red Spectrum

The infra-red spectrum of pure G. breve toxin is shown in Fig. 8. The characteristic infra-red absorption frequencies with tentative assignments obtained by comparison with standard values given by Bellamy (25, 26) is given in Table III. The spectrum indicates the absence of hydroxyl, amino, amide, and aromatic residues and the presence of a strong carbonyl absorption. A strong band in the region of 1100-1050 cm^{-1} indicates the presence of either an ester, carboxylic, aldehydic, or alcoholic group.

Nuclear Magnetic Resonance Spectrum

The NMR spectrum of the pure toxin is shown in Fig. 9. The spectrum had a very high noise level and only with the help of the C-1024 Time Average Computer was a spectrum recorded which could be integrated for the relative number of protons. Two strong signals at 1.17δ and 1.27δ showed six and eight protons respectively, which definitely belonged to methylene groups which could be present in or on a strained ring system. Another absorption between 1.7δ to 1.9δ, consisting of six protons, could be ascribed to methylene protons in the proximity of an electronegative atom or group. A very weak

Fig. 7 Ultraviolet and Visible Spectrum of G. breve toxin in methanol. Curve B was taken with a more concentrated solution than curve A to show the detail in the visible region.


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
250
300
350
400
450
500
550
600
WAVELENGTH(NM)

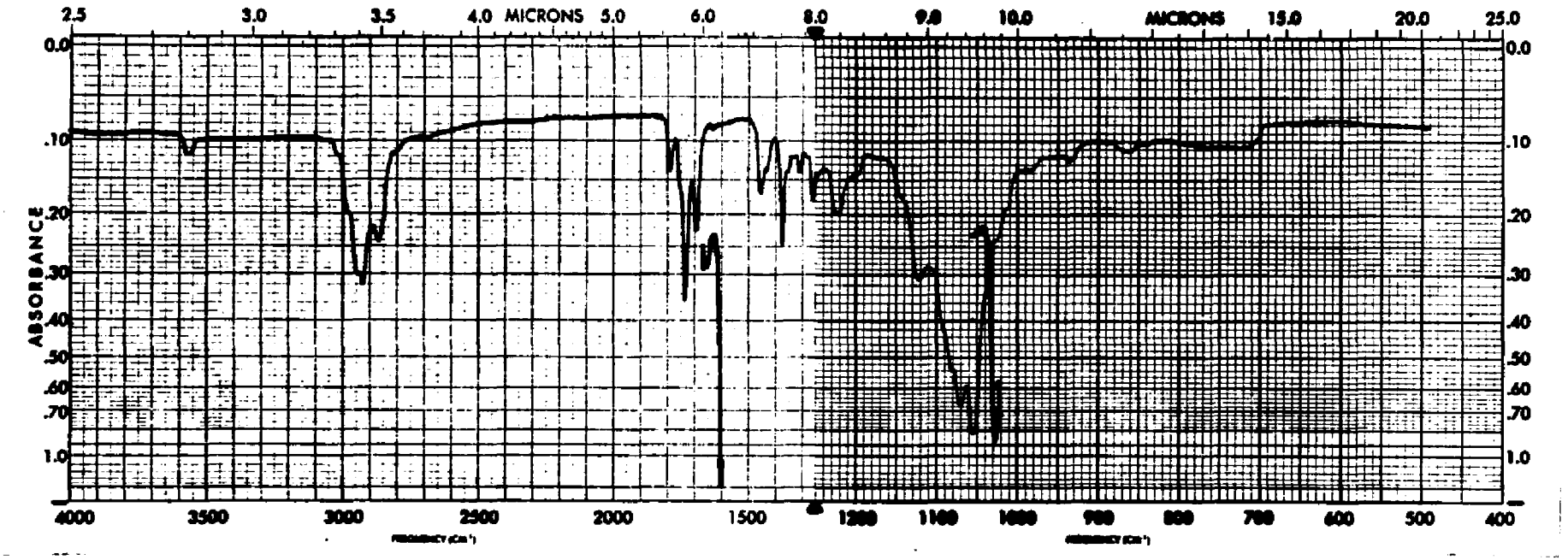


Fig. 8 Infra-red Spectrum of G. breve toxin.

TABLE III

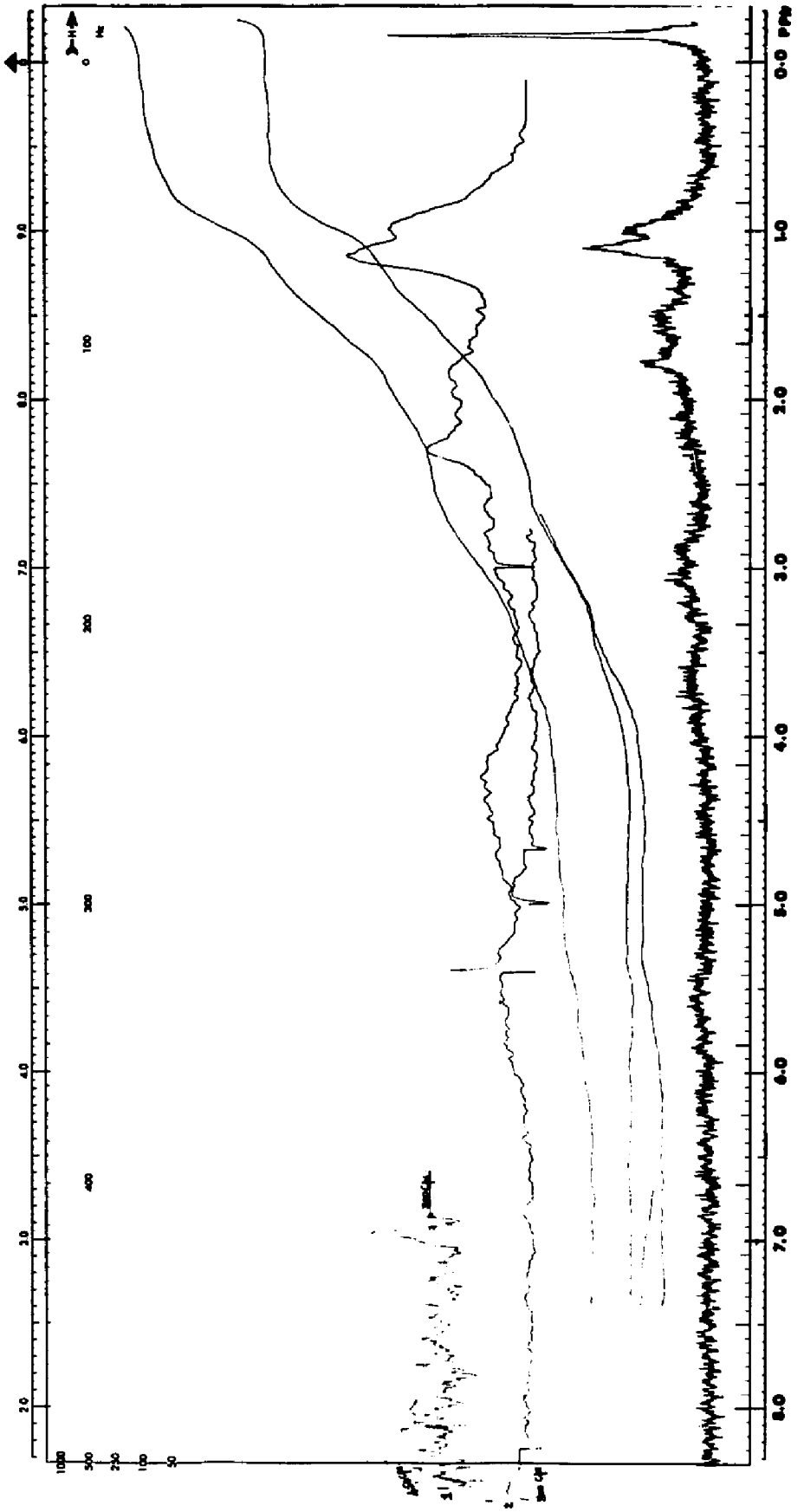
Characteristic Infra-red Absorption Frequencies of

G. breve Toxin Dissolved in CCl_4.

Frequency[a] cm^{-1}	Possible	Literature value cm^{-1}
3570-3560	overtone or N-H	3500-3100 (s,m)
3025 (sh)	C-H Olefinic	3040-3010 (s,w)
2980 (sh)	C-H Paraffinic	
2925 (s)	C-H Paraffinic	2962-2853 (s,M)
2860 (s)	C-H Paraffinic	
1795 (w)	C=O lactone, anhydride	near 1800 (m) near 1800 (m)
1740 (s)	C=O ester, lactone	1770-1735 (s)
1700 (m)	C=O ketone	1710-1690 (s)
1450 (m)		
1390 (m)	C-H Paraffinic	1465-1359 (s,m)
1380 (m)		
100-1050 (s)	C-O- ester, acid aldehyde, alcohol	1100-1000 (s,m)

[a] Intensity abbreviations: strong (s), shoulder (sh), medium (m), and weak (w).

Fig. 9. 60 MHz. NMR spectrum of G. breve toxin in CCl_4.



absorption was recorded at a further down field region (5.7δ) which had only one proton and could be assigned to a proton present on an unsaturated system. No absorption was observed in the region between 7δ and 12δ indicating the absence of aldehydic, carboxylic, and aromatic protons.

Optical Rotatory Dispersion Spectrum

The ORD spectrum of a 0.983 mg per ml solution, in methanol, of pure G. breve toxin is shown in Table IV and in Fig. 10. The toxin had a maximum at 260 nm, a minimum at 235 nm and cross overs at 246 and 223 nm. The specific rotation was found to be + 264°at 260 nm and -244.15° at 235 nm.

Mass Spectrum

The ionization pattern of the high resolution mass spectrum of G. breve toxin is shown in Fig. 11. The mass-charge ratios (m/e) and the relative abundances from mass spectral analysis are shown in Table V. The probable molecular ion had a m/e of 279.1627, which demands that it should have an odd number of nitrogen atoms to qualify as a molecular ion (27). The probable molecular formulae with molecular weight between 279.159 and 279.16 are listed in Table IX. On the basis of reasons discussed elswhere only formulae $C_{16}H_{23}O_4$ and $C_{19}H_{21}NO$ are possible. The (A + 1) to (A) ratio gives a value of 18.7 carbon atoms for the compound. The base peak had a m/e value of 149 and the maximum relative abundance at m/e 149 was set at 100 percent. The tentative formula for the base peak can be either $C_{10}H_{13}O$ or $C_{10}H_{15}N$.

TABLE IV

Optical and Specific Rotation of G. breve Toxin Solution (in methanol) at Various Wave-lengths.

	Wave length mμ	α (degrees)	Specific rotation (degrees)
1	290	+ 0.0130	+ 132.248
2	280	+ 0.0190	+ 193.285
3	270	+ 0.0250	+ 254.323
4	265	+ 0.0255	+ 259.409
5	260	+ 0.026	+ 264.496
6	255	+ 0.020	+ 203.458
7	250	+ 0.011	+ 111.902
8	246	0.000	000.000
9	240	- 0.016	- 162.767
10	235	- 0.024	- 244.150
11	230	- 0.022	- 223.804
12	225	- 0.012	- 122.075
13	223	0.000	000.000

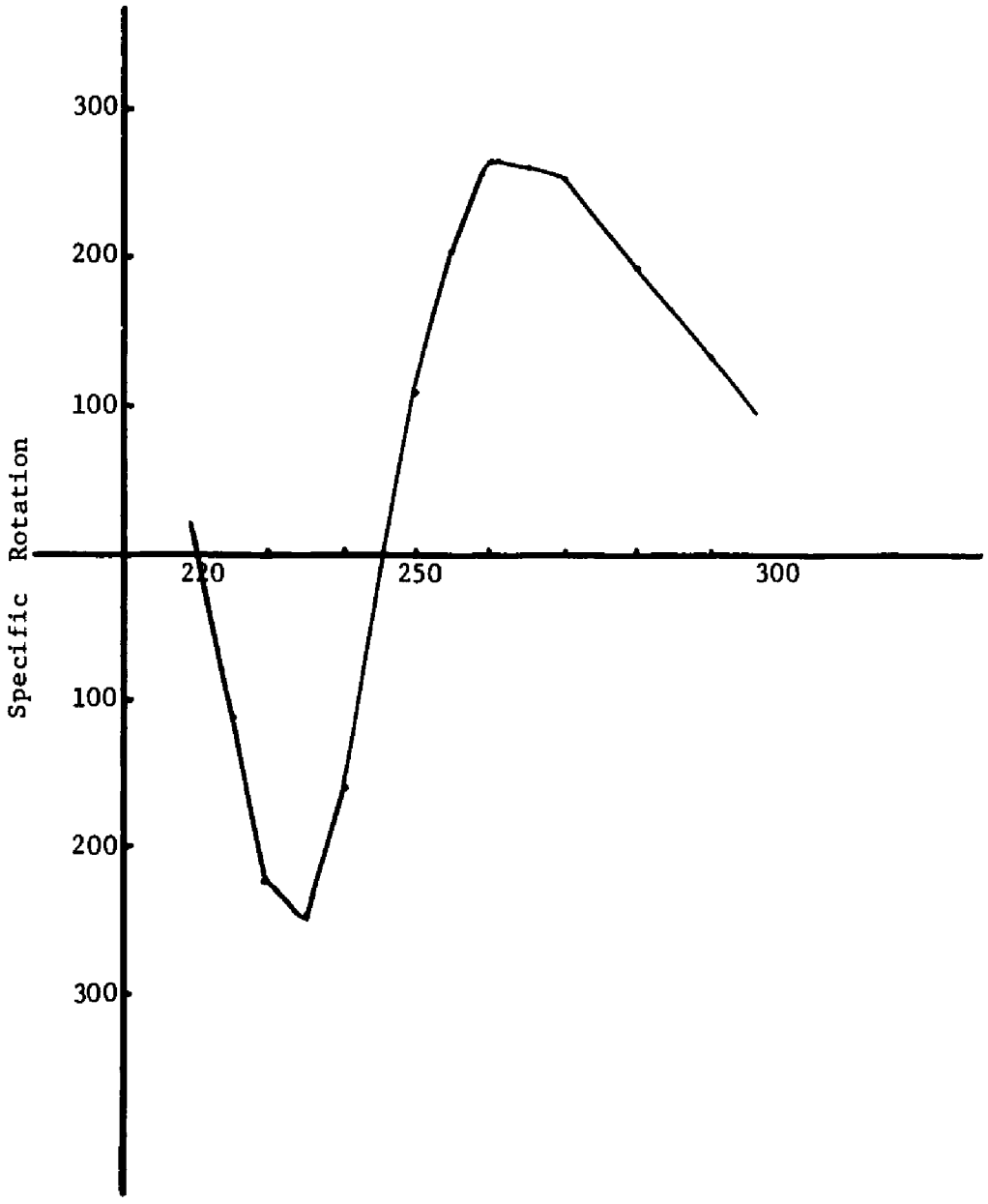


Fig. 10 Optical Rotatory Dispersion Spectrum of G. breve toxin.

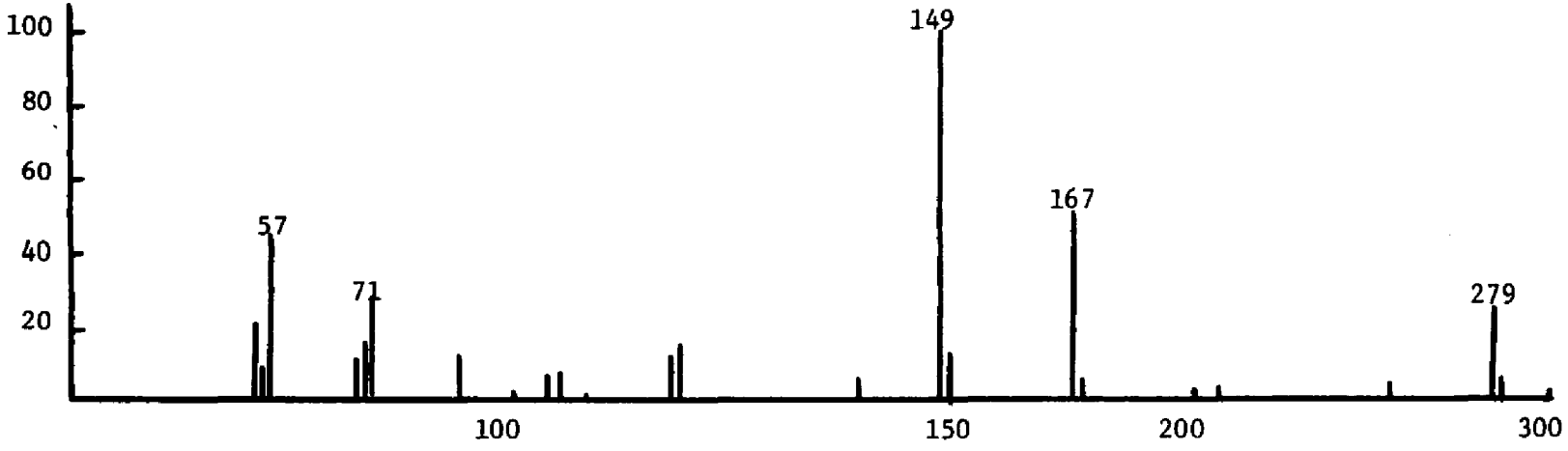


Fig. 11. High Resolution Mass Spectrum of G. breve toxin

TABLE V

Mass-charge Ratio (m/e) and Relative Abundances and Tentative Formulae from the Mass Spectral Analysis of G. breve Toxin.

m/e	Relative abundance percent	Possible formula
55	22	-
56	10	-
57	24	-
69	12	-
70	20	C_5H_{10}, C_4H_8N, C_4H_6O
71	28	C_5H_{11}, C_4H_9N, C_4H_7O
95	6	-
97	8	-
112	11	$C_7H_{12}O$, $C_7H_{14}N$
113	16	C_8H_{17}, $C_6H_{11}NO$
149	100	$C_{10}H_{15}N$, $C_{10}H_{13}O$, $C_9H_{11}NO$
167	30	$C_{12}H_9N$, $C_{10}H_{17}NO$
205	4	-
251	6	$C_{17}H_{17}NO$
279	24	$C_{19}H_{21}NO$, $C_{10}H_{23}O_4$
280	4.5	-

Molecular Weight Determination (Osmometric Method)

The calibration curve for the molecular weight determination, prepared using benzil, is shown in Table VI and Fig. 12. The Δ R values for the chloroform solution of benzil and of G. breve toxin (5.6 g/liter) is given in Table VI . The molecular weight was found to be 288 by this method.

Acid Base Stability of Toxin

The stability of toxin towards acid and base was determined by dividing the ethanol extract from 50 liters of G. breve culture into three equal portions. One was used to determine the toxicity, and the other two were refluxed separately with either 1.0N HCl or 1.0N NaOH for 12 hours. After refluxing and cooling the mixtures were extracted with equal volumes of diethyl ether. The pH of the ether extracted aqueous phase from the acid hydrolysis was adjusted to pH 11-12, and the pH of the ether extracted aqueous phase from the basic hydrolysis was adjusted to pH 1-2. These were then extracted with equal volumes of diethyl ether. The ether layer, separated in each extraction was evaporated to dryness in vacuo and was tested for toxicity by the mouse bio-assay method. The toxicity was lost in both cases after refluxing indicating that the toxin is unstable under both acidic and basic hydrolytic conditions.

Nature of Toxin

In order to determine the acidic, basic, or neutral nature of the toxin, residue from ethanol extract was partitioned between equal volumes of 1.0N NaOH and chloroform. The two layers were separated. The aqueous layer was acidified to pH 2-3 and re-extracted with an equal volume of chloroform. The two layers were separated,

Table VI

ΔR Values for the Standard Benzil Solution in Chloroform.

	g moles of benzil per liter of $CHCl_3$	ΔR
1	0.01	4
2	0.02	8
3	0.03	13
4	0.04	15
5	0.05	22
6	0.06	26
7	0.07	30
8	0.08	36
9	G. breve toxin sol.	7.8

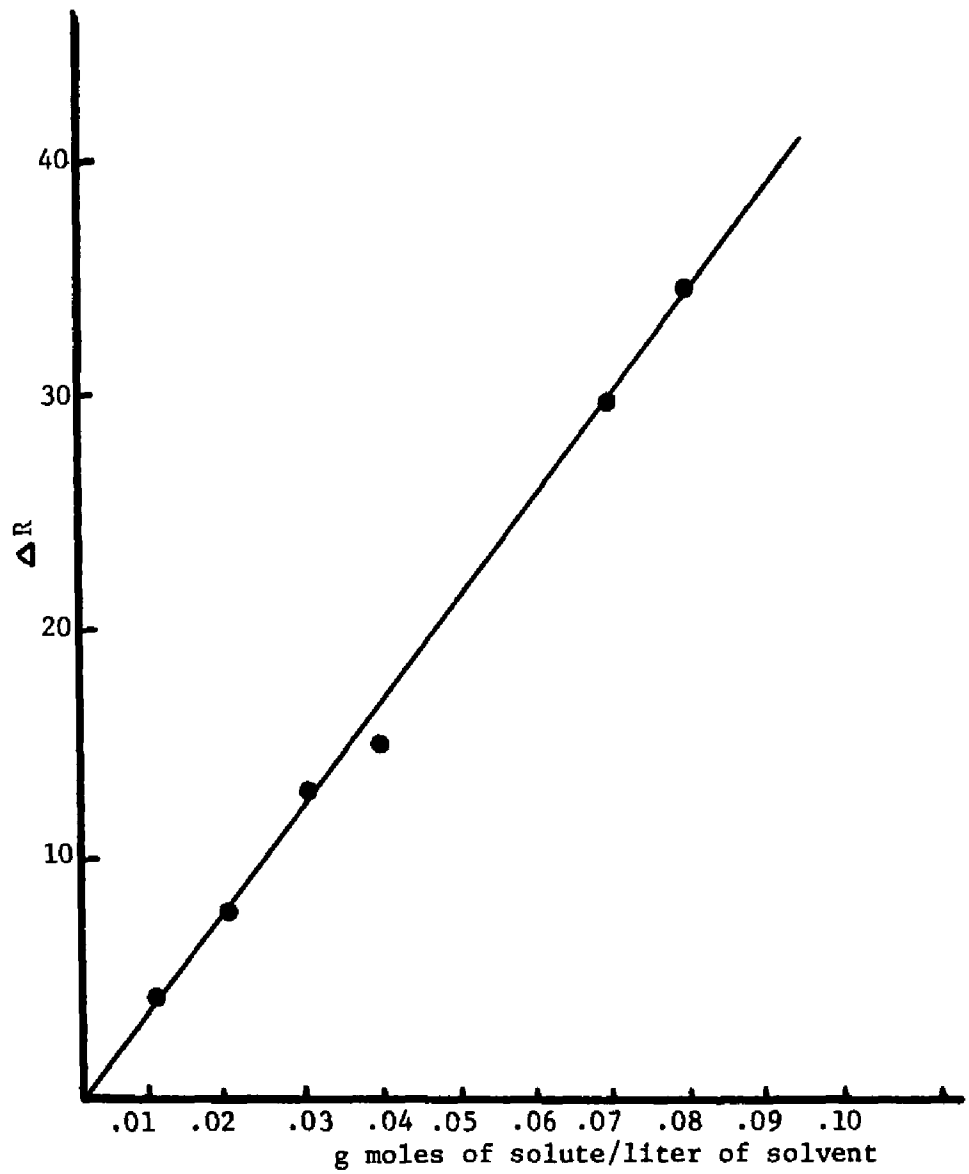


Fig. 12 Calibration curve for the molecular weight determination of G. breve toxin by osmometric method.

both taken to dryness in vacuo and found to be non-toxic when tested on mice. The chloroform layer from the first partitioning was re-partitioned with an equal volume of water (pH 2-3). Both chloroform and aqueous layer were separated brought to dryness in vacuo, and were tested for toxicity by the mouse bio-assay method, only the chloroform layer was found to be toxic to mice indicating that the toxin is neutral in nature. In case of an acidic toxin the toxicity should have been present in the chloroform layer from the repartitioning of the original basic aqueous phase after acidification. In the case of a basic toxin the toxicity should have been present in the aqueous phase resulting from repartitioning the original chloroform phase between dilute acid. The entire experiment is depicted schematically in Fig. 13.

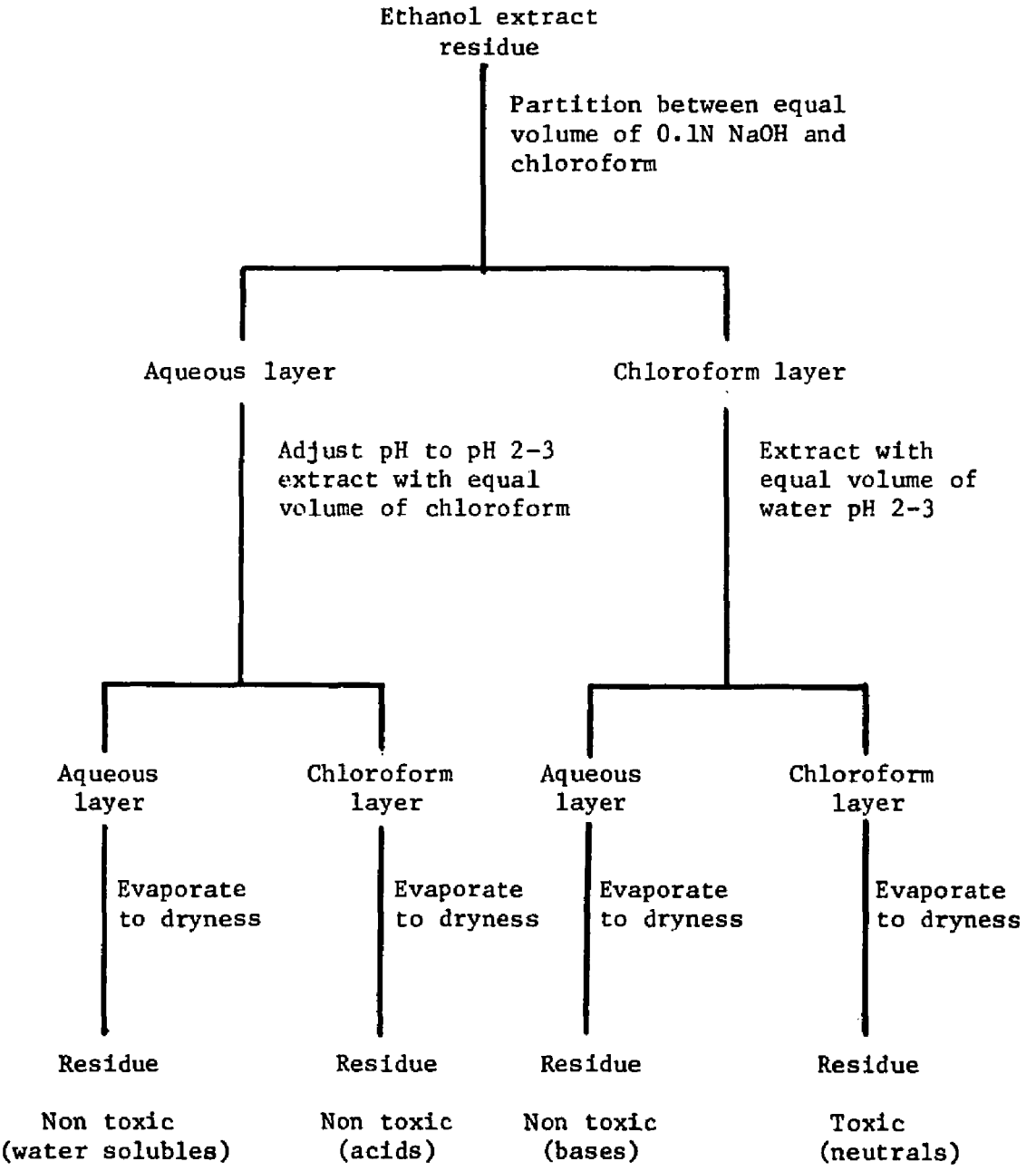


Fig. 13: Flow diagram for the determination of the acidic, basic, or neutral nature of G. breve toxin.

DISCUSSION

In comparing the results with those of other workers in the field, a number of factors must be taken into consideration, such as, growth conditions, methods of isolation of the toxin, and methods for testing for toxicity. These have varied widely and therefore differences in results obtained by various groups of investigators might be explained on these terms.

Although not always reported, incubation times have varied somewhat. Cummins and Stevens (19) report using an incubation period of 3-6 weeks. It has been our observation that after 14-18 days, growth and toxicity began to decline. The population densities of our G.breve cultures ranged from 15-18 x 10^3 cells per ml. This is slightly less than that reported by Paster and Abbott (24) when gibberellic acid was added to the growth medium. However, it is about ten times more than that reported by Cummins and Stevens (19). Both Trieff et al. (20, 21) and Martin and Chatterjee (18, 28) did not mention the peak densities of their cultures.

Comparisons of toxicity can be only approximated because no standard method has been adopted for testing G. breve toxin preparations. From Table VII it is noteworthy that the LD_{50} dose of toxin isolated by Trieff et al, (20, 21) is approximately 25 times less than the fast acting toxin isolated by Cummins and Stevens (19). Our G. breve had a LD_{100} of 0.85 mg/kg body weight, which is nearly twice the LD_{50} dose of the toxin isolated by Trieff et al. (20, 21). It is however, difficult to compare the data of Trieff et al. (20,21) who used polysorbate-80 as the vehicle and our results using cottonseed oil. The former, being an emulsifier, has been suggested (20) to

give a faster rate of absorption of toxin than cottonseed oil. In comparison to the toxin isolated by Cummins and Stevens (19), our G. breve toxin is about 15 times more toxic. Sasner et al. (29) reported that our pure G. breve toxin (fraction IVa) is an inhibitor of cholinesterase activity. However, both Trieff et al. (20) and Martin and Chatterjee (28) have failed to detect any anti-cholinesterase activity in their pure toxin. The reason for this discrepancy is not known unless we surmise that different toxic fractions of G. breve have been isolated. The multiplicity of the toxic fractions produced by this organism have been referred to in the INTRODUCTION.

The toxicological and some physico-chemical data obtained in our laboratory along with those reported by Cummins and Stevens (19), Martin and Chatterjee (28) and Trieff et al. (20, 21) are given in Table VII. Although it is difficult to conclude much from some of this data, there is general agreement that the toxin is soluble in organic solvents. Table VIII gives the elemental composition and molecular weight of our G. breve toxin and of toxin reported by other workers. Wide variations are apparent in the percentages of C, H, N, O, and P. It is noted that Trieff et al. (21) reported different elemental analysis data for the pure G. breve toxin isolated by TLC, dry-column chromatography (DCC), and TLC and DCC combined. It should also be mentioned that the correct elemental formula, calculated from the elemental analysis data for the pure G. breve toxin isolated by Martin and Chatterjee (18, 28) should be $C_{90}H_{161}O_{57}P$ and not $C_{90}H_{161}O_{17}P$ as reported in their communications (18, 28). Trieff et al. (20,21) and Martin and Chatterjee (18, 28) reported the presence of a small percentage of phosphorus in their purified toxin, while our G. breve toxin had only a negligible (0.005%) amount of phosphorus. It would

TABLE VII

G. breve Toxin: Some Physico-Chemical and Toxicological Properties.

Properties	Trieff *et al* (20, 21)	Martin and Chatterjee (18, 28)	Cummins and Stevens (19)	Our laboratory
Color and State	Yellow glassy substance	Light yellow solid	a) Slow acting: gray powder b) Fast acting: light yellow oil	Light yellow glassy substance
Nature	No data	No data	a) Slow acting: neutral b) Fast acting: acidic	Neutral
Solubility	$(C_2H_5)_2O$, $CHCl_3$ C_2H_5OH	$CHCl_3$, CCl_4 C_2H_5OH	$CHCl_3$, CCl_4 $(C_2H_5)_2O$, C_2H_5OH	$CHCl_3$, CCl_4, $(C_2H_5)_2O$, CH_3OH, $(CH_3)_2CO$, Pet. ether, C_2H_5OH

TABLE VII(Continued)

Properties	Trieff et al (20, 21)	Martin and Chatterjee (18, 28)	Cummins and Stevens (19)	Our laboratory
Stability				
Acidic hydrolytic conditions.	Stable	No data	No data	Unstable
Basic hydrolytic conditions	Unstable	No data	No data	Unstable
Melting point	No data	Low melting,	Decomposes in presence of air; 140-150°C in absence of air	Decomposes above 250°C
Cholinesterase activity	Non-inhibitor	Non-inhibitor	No data	Inhibitor
LD_{50} (I.P. in mouse)	0.5 mg/kg in 0.5% polysorbate -80 in saline	No data	a) Slow acting: 26mg/kg 240 min. b) Fast acting: 13 mg/kg 40 min.	No data
LD_{100} (I.P. in mouse)	No data	No data	No data	0.85 mg/kg in cotton-seed oil.

TABLE VIII

G. breve Toxin: Elemental Analysis, Emperical and Molecular Formulae and Molecular Weight.

	Trieff *et al* (20, 21)			Martin and Chatterjee	Cummins and Stevens[a]	Our laboratory
	TLC	DCC	TLC,DCC	(18, 28)	(19)	
Elemental analysis						
Carbon	63.15	61.91	70.92	49.60	63.27	63.9
Hydrogen	8.99	9.75	10.10	7.6	8.08	10.1
Nitrogen	0.00	0.00	0.00	0.0	0.70	0.0
Oxygen	26.36	20.32	17.5	14.4[b]	no data	26.0[b]
Phosphorus	1.22	2.66	1.90	1.41	no data	0.005
Calculated Emperical formula[c]	$C_{134}H_{226}O_{49}P$	--	--	$C_{90}H_{161}O_{57}P$	$C_{102}H_{157}N$	--
Calculated Emp. Wt.[d]	2538	1164	1630	1545	--	--
Expt. Mol. Wt. Osmometry	468	520	694	650	--	288
Mass spectrum	--	--	--	--	--	279

[a] Data per slow acting gray powder.

[b] Calculated by difference from 100% total.

[c] Calculated Empirical formulas on the basis of one P/molecule or one N/molecule.

[d] Based on one P or one N atom per molecule.

appear that phosphorus is an impurity rather than a part of the toxin molecule, because the inclusion of one atom of phosphorus results in molecular weights much in excess of those found by osmometry. It is believed also that the calculation of empirical formulas based on elemental analysis is as yet very premature, because the preparations can still be grossly contaminated even though they are chromatographically pure. However, these analysis are useful criteria for comparing various toxin preparations.

The G. breve toxin isolated by this author had a visible absorption spectrum characteristic of pigments, and is believed to be due to pigment contaminants. This assumption is supported by the fact that the toxin showed a weak absorption due to unsaturation in its IR and NMR spectra. Trieff et al. (20) reported that their toxin had absorption maxima at 256 mμ and 273 mμ, while Martin and Chatterjee (28) reported that their toxin had an absorption band between 265-270 mμ.

The infra-red spectrum of G. breve toxin indicated the presence of a carbonyl functional group. Trieff et al. (20, 21) reported the presence of a definite carbonyl absorption (1725 cm^{-1}) in the IR spectrum of their pure toxin, and suspected the presence of an ester group, which was supported by the fact that the toxin was unstable under alkaline hydrolytic conditions.

As indicated in the RESULTS, pure G. breve toxin had certain centers or units of asymmetry and had a positive specific rotation of 264.4 degrees and a negative specific rotation of 244.1 degrees. The G. breve toxin isolated by other workers did not have any optical rotation at these wave lengths. However, Martin and Chatterjee (18) reported that their pure toxin had an optical rotation of +68 degrees

at 589 mμ.

The NMR data of our pure G. breve toxin is similar to that reported by Martin and Chatterjee (28), but not identical. Similar to Martin and Chatterjee (28) our toxin had two signals at unusually low values (1.17δ and 1.27δ). The signal intensities suggest that the absorptions belong to either methyl or methylene protons present on or in a strained ring or a straight chain aliphatic hydrocarbon (methylene groups in an aliphatic hydrocarbon would absorb at 1.25δ). The NMR pattern is very complex and very little information could be obtained from a 60 MHz spectrum such as this. The absence of absorption signals in the regions of 7δ, 11δ, and 12δ supports the assumption that G. breve toxin does not contain aromatic, aldehydic, or carboxylic groups in the molecule. The possibility of phosphate ester groups in the molecule, as suggested by Trieff et al.(21) was ruled out on the basis that our G. breve toxin had a negligible amount of phosphorus.

The possible molecular formulae, with a molecular weight between 279.158-279.168, with their theoretical elemental composition, along with the data from the elemental analysis of our G. breve toxin are given in Table IX . The most probable molecular formula for the m/e 279.1627 would be $C_{19}H_{21}NO$ (279.1623). This formula is supported by the percent abundance of the P + 1 peak, which indicates the presence of 19 carbon atoms in the molecule. The presence of an odd number of nitrogen atoms in the formula would indicate that this is the molecular ion. The difference in the percentage of carbon and hydrogen in the theoretical and experimental values might be due to the presence of a small amount of contaminants such as silicic acid (the presence of a very small amount of inorganic salts and silica

TABLE IX

G. breve Toxin: Possible Molecular Formulae and Theoretical and Experimental Elemental Composition

Molecular Formula [a]	Molecular Weight	Elemental Composition			
		Carbon	Hydrogen	Nitrogen	Oxygen
$C_{12}H_{21}N_6O_2$	279.1569	51.6	6.8	20.1	8.96
$C_{12}H_{25}NO_6$	279.1681	51.6	8.96	5.01	34.4
$C_{13}H_{21}N_5O_2$	279.1695	55.9	7.52	25.0	11.46
$C_{14}H_{21}N_3O_3$	279.1582	59.5	7.44	15.9	17.0
$C_{16}H_{23}O_4$	279.1596	69.3	8.3	00.0	23.1
$C_{17}H_{19}N_4$	279.1609	73.1	6.81	20.0	00.0
$C_{19}H_{21}NO$	279.1627	81.3	7.52	5.0	5.3
Experimental	279.1623	63.9	10.1	00.0	26.0 [b]

[a] All possible molecular formulae with molecular weight between 279.1582 - 279.1695.

[b] Calculated by difference from 100 percent total.

was detected during the mass spectral analysis of the pure G. breve toxin). The formula $C_{19}H_{21}NO$ is also supported by the relative number of protons present in the molecule when calculated by integrating the NMR signals. The reasons for the discrepancy between the high degree of unsaturation indicated by the mass spectral formula and the low degree of unsaturation indicated by the NMR spectrum is not known.

SUMMARY AND CONCLUSIONS

A new toxic fraction from the dinoflagellate Gymnodinium breve has been isolated by the method of column chromatography, and has been partially characterized by physico-chemical methods. The crude toxin has a LD_{100} of about 200 mg/kg, while the pure toxin had a LD_{100} of 0.85 mg/kg, showing roughly a 203 fold purification. The toxin was neutral in nature and was found to be unstable under both acidic and basic hydrolytic conditions. The toxic fraction has a molecular weight of 279.1627 by mass spectroscopy, and the most probable molecular formula was found to be $C_{19}H_{21}NO$. The IR spectrum indicated the absence of aromatic, carboxylic, and aldehydic groups in the molecule. Strong absorptions in the region of 1790-1700 cm^{-1} indicated the presence of a carbonyl group(s). The NMR spectrum showed that the toxin could be a long chain aliphatic molecule containing at least two methyl groups. On the basis of present information it is not possible to assign a specific formula to the toxin, but this investigation has certainly opened a channel for the further investigation of the chemical nature of the toxin of the dinoflagellate Gymnodinium breve.

BIBLIOGRAPHY

1. Ballantine, D., and B.C. Abbott. Toxic marine flagellates: their occurrence and physiological effects on animals. J. Gen. Microbiol. 16: 274-281 (1957).

2. Sommer, H., and K.F. Meyer. Paralytic shellfish poisoning. Arch. Path. (Lab. Med.) 24: 560-598 (1937).

3. Schantz, E.J., J.M. Lynch, G. Vayvada, K Matsumoto, and H. Rapoport. The purification and the characterization of the poison produced by Gonyaulax catenella in axenic culture. Biochemistry 5: 1191-1195 (1966).

4. Rapoport, H., M.S. Brown, R. Oesterlin, and W. Schuett. Saxitoxin. 147th National Meeting, American Chemical Society, Philadelphia, Penn. 1964, pp. 3N.

5. Schantz, E.J. Algal Toxin. In: Properties and Products of Algae, Zazjic, J.E. (ed.), Plenum Press, New York, 1970 pp. 83-96.

6. Evans, M.H. Two toxins from a poisonous sample of mussels Mytilus edulis. Br. J. Pharmacol. 40: 847-865 (1971).

7. Prakash, A., and F.J.R. Taylor. A red tide bloom of Gonyaulax acatenella in the strait of Georgia and its relation to paralytic shellfish toxicity. J. Fish. Res. Board Can. 23: 1265-1270 (1966).

8. Schradie, J., and C.A. Bliss. The cultivation and toxicity of Gonyaulax polyedra. Lloydia 25: 214-221 (1962).

9. Connell, C.H., and J.H. Cross. Mass mortality of fish associated with the protozoan Gonyaulax in the Gulf of Mexico, Science 112: 359-363 (1950).

10. Gates, J., and W.B. Wilson. The toxicity of Gonyaulax monilata to Mugil cephalus. Limnol. Oceangr. 5: 171-174 (1960).

11. Ray, S.M., and D.V. Aldrich. Ecological interactions of toxic dinoflagellates and molluscs in the Gulf of Mexico. In: Animal Toxins, Ressell, F.E., and P.R. Saunder (eds.), Pergamon Press, Oxford and New York, 1967, pp. 75-83.

12. Abbott, B.C., and D. Ballantine. The toxin from Gymnodinium veneficum Ballantine. J. Marine Biol. Assoc. U.K. 36: 169-189 (1957).

13. Starr, T.J. Toxin from Gymnodinium brevis. Texas Repts. Biol. and Med. 16: 500-507 (1958).

14. Ray, S.M., and D.V. Aldrich. Gymnodinium breve: Induction of shellfish poisoning in chicks. Science 148: 1748-1749 (1965).

15. McFarren, E.F., H. Tanabe, F.J. Silva, W.B. Wilson, J.E. Campbell, and K.H. Lewis. The occurrence of ciguatera-like poison in oyster, clam, and Gymnodinium breve cultures. Toxicon 3: 111-113 (1965).

16. Cummins, J.M., A.A. Stevens, B.E. Huntley, W.F. Hill, Jr., and J.E. Higgins. Some properties of Gymnodinium breve toxin(s) determined biologically in mice. In: Drugs from the Sea, Transactions of the Drugs from the Sea Symposium, Univ. of Rhode Island, 27-28 August, 1967, Frendenthal H.D. (ed.), Marine Technology Socity, Washington, D.C., 1968, p. 213.

17. Spikes, J.J., S.M. Ray, D.V. Aldrich, and J.B. Nash. Toxicity variation of Gymnodinium breve cultures. Toxicon 5: 171-174 (1968).

18. Martin, D.F., and A.B. Chatterjee. Isolation and characterization of toxin from Florida red tide organism. Nature 221: 59-60 (1969).

19. Cummins, J.M., and A.A. Stevens. Investigation on Gymnodinium breve toxin in shellfish. Report of U.S. Dept. of Health, Education, and Welfare, Public Health Service, Gulf Coast Water Hygiene Lab., Daulphin Island, Alabama, 1970, p. 76.

20. Trieff, N.M., J.J. Spikes, S.M. Ray, and J.B. Nash. Isolation and purification of Gymnodinium breve toxin. In: Toxins of plant and animal origin, Proceedings of 2nd International Symposium on Animal and Plant Toxins, Tel Aviv, Israel, February 1970, DeVaries and E. Kochva (eds.), Gorden and Breach, London, 1971, pp. 557-577 (in press).

21. Trieff, N.M., M.N. Venkatasubramanian, and S.M. Ray. Purification of Gymnodinium breve - Dry column chromatography technique. Texas Repts. Biol. and Med., (in press).

22. Bartlett, G.R. Phosphorus assay in column chromatography. J. Biol. Chem. 234(3): 466-468 (1959).

23. McLafferty, F.W. Interpretation of mass spectra. W.A. Benjamin, Inc., New York, 1967, pp. 210-211.

24. Paster, Z., and B.C. Abbott, Gibberellic acid: A growth factor in the unicellular alga Gymnodinium breve. Science 169: 600-601 (1970).

25. Bellamy, L.J. Infra spectra of complex molecules. Methuen and Co. Ltd., London, 1956, p. 323.

26. Bellamy, L.J. Advances in infra-red group frequencies. Methuen and Co. Ltd., London, 1968, p. 304.

27. McLafferty, F.W. Interpretation of mass spectra. W.A. Benjamin, Inc., New York, 1967, p. 32.

28. Martin, D.F., and A.B. Chatterjee. Some chemical and physical properties of two toxins from the red tide organism Gymno-

dinium breve. Fishery Bull 68(3): 433-443 (1970).

29. Sasner, J.J., M. Ikawa, F. Thurberg, and M. Alam. Physiological and chemical studies on Gymnodinium breve Davis. Toxicon (in press).

PART II

APHANIZOMENON FLOS-AQUAE

INTRODUCTION

Poisoning by waterblooms of blue-green algae is virtually world-wide in occurrence. They have been most frequently reported in the central part of North America, especially from the states of North Dakota, South Dakota, Minnesota, Iowa, Wisconsin, Illinois, and Michigan and from the provinces of Alberta, Manitoba, Saskatchwan, and Ontario. Six species of blue-green algae have been reported for their toxic effects. These are Nodularia spumigena, Microcystis aeruginosa, Anabaena flos-aquae, Aphanizomenon flos-aquae, Gloetrichia echinulata, and Coelosphaerium kutzingianum. Of these six species it has been predominantly Microcystis, Anabaena, and Aphanizomenon that have been to blame for the most dramatic and serious poisoning outbreaks. Rose (1) reported a severe outbreak of poisoning by waterblooms of blue-green algae in Storm Lake, Iowa, in which thousands of gulls and ducks were killed. The alga responsible for the poisoning was later found to be Anabaena flos-aquae. Similarly in South Africa in 1943 thousands of cattle, sheep, and many other animals were killed behind the Vaal Dam in the Transvaal when the reservoir developed a poisonous bloom of blue-green algae. The alga responsible for the poisoning was later identified as Microcystis aeruginosa (2, 3). Poisoning of fish by blue-green algae has been reported frequently (4, 5, 6, 7). In many cases of fish kills however, death has been caused by anoxia resulting from the decomposition of large masses of algae under shallow water resulting in oxygen depletion. In other cases, however, fish have been killed while there was still an adequate oxygen supply. In these cases poisonous substances have been implicated. Sawyer et al (8) reported a serious outbreak of poisoning by waterblooms of cyano-

phyceae in Kezar Lake, North Sutton, New Hampshire, in 1966, which killed more than six tons of fish. The predominant algal species in the bloom was identified as Aphanizomenon flos-aquae (L) Ralfs. Similar algal populations in Lake Winnesquam and in Lake Skatutakee in New Hampshire also resulted in mass mortality of fish in 1966. Kezar Lake contained almost unialgal toxic blooms of A. flos-aquae during the summers of 1967 through 1970. The exact conditions contributing to the massive local growth of these organisms are unknown. However, scientists of the United States Fish and Wild Life Service, as well as several independent investigators, have contributed to a realization that the conditions necessary for the blooms of these blue-green algae are complex.

The toxin from M. aeruginosa has been purified and studied extensively by Bishop and Gorham (9). They showed that the toxin (named microcystin) is a cyclic peptide consisting of ten amino acid units with a molecular weight of 1200. It is apparently insensitive to many proteolytic enzymes and upon acid hydrolysis gives L-valine, L-leucine, L-alanine, L-ornithine, L-aspartic acid, L-glutamic acid, and D-serine. Murthy and Capindale (10) reported the isolation of a new toxic peptide from this alga which has arginine, tyrosine, and proline in addition to the amino acids reported by Bishop and Gorham (9).

Another important toxin from a blue-green alga is that from Anabaena flos-aquae which is extremely toxic to many farm animals and which kills very rapidly. This toxin was described by Gorham (11) as a very-fast death factor. This toxin has not been isolated in pure state.

Jackim and Gentile (12), using a method similar to that described for the isolation of saxitoxin, reported the isolation and characterization of a toxin from laboratory cultures of Aphanizomenon flos-aquae. They reported that the Aphanizomenon toxin is similar to saxitoxin in its chemical and physiological properties.

Another toxin of great interest is one produced by a yellow-brown alga Prymnesium parvum. This alga grows in brackish water ponds and estuary waters and produces a toxin that is lethal to fish and gill-breathing animals. The toxin produced by this organism inhibits the transport of oxygen across the gill membranes and has been a serious problem in the commercial carp tanks in Israel. Several investigators in Israel have isolated and studied the toxin extensively (13, 14, 15, 16). It is a glycolipid with a molecular weight of about 2300 (17). The lipid portion is composed of four long chain fatty acids (myristic, oleic, stearic, and palmitic) and the polysaccharide portion of glucose, mannose, and galactose. Very recently Martin and Pedilla (18) reported the characterization of Prymnesium parvum toxin by means of hemolytic kinetics.

MATERIALS AND METHODS

Concentration and Collection of Aphanizomenon

Thick concentrates of Aphanizomenon flos-aquae were collected from Kezar Lake during the height of the bloom, during summers of 1968, 1969, and 1970 by continuous flow centrifugation. De Laval continuous flow centrifuges were set up at lakeside and the lake water was centrifuged at the rate of 12 liters per hour. Thick algal concentrates so obtained were taken to the laboratory at the end of the day's run in quart plastic containers, where they were kept in a deep freezer until ready to be processed.

Isolation and Purification of Crude Toxin

The frozen concentrate (about 500-1000 ml) of A. flos-aquae cells was thawed and diluted 2-3 times with water, if the concentrate was too thick. The mixture was acidified to pH 3 with 6 N HCl and heated to 80-90°C. The heated mixture was chilled immediately in an ice bath and centrifuged at 10,000 x g for 15 minutes. The supernatant was decanted off and saved, and the residue was extracted an additional two times by repeating the above procedure. The supernatants were combined and evaporated to dryness in vacuo at 30°C. Any excess of HCl was removed by leaving the flask (containing the residue) overnight in a vacuum desiccator containing sodium hydroxide pellets. The residue was extracted three times with hot ethanol, using about 150 ml each time. The ethanol extract was evaporated to dryness in vacuo. The residue so obtained was dissolved in about 100 ml of water which had been acidified to pH 3 with HCl and the solution was shaken with 150 ml of chloroform in a separatory funnel. The two layers were allowed to separate in the cold room. The aqueous layer was separated

from the chloroform layer and was evaporated to dryness *in vacuo* at 25-30°C.

High Voltage Electrophoresis

The residue from the aqueous extract was dissolved in 5 ml of distilled water and 0.5 ml of this solution was streaked onto 3 MM Whatman paper near what will be the anode region. The buffer used in high voltage electrophoresis was prepared by mixing pyridine, glacial acetic acid, and water (133:4.6:1876 v/v) (19) and had a pH of 6.41. Direct current at 45 volts per cm was passed for 1.5 hours. After the run was over, the paper was dried in a hood, and the entire paper between the origin and the cathode end was cut into small pieces and eluted with water. The eluate was evaporated to dryness *in vacuo*. The residue was dissolved in less than 2 ml of water and the solution was centrifuged in a clinical centrifuge to remove insoluble material. The supernatant was applied to Whatman 3 MM paper and rerun on high voltage electrophoresis as described above. After electrophoresis two test strips were cut off from the paper and one was sprayed with ninhydrin and the other with Weber reagent. Most of the toxicity was associated with ninhydrin- and Weber-positive region. The ninhydrin- and Weber-positive region was cut out from the remainder of the sheet and eluted with water (pH 4-5) and the eluate was evaporated to dryness *in vacuo*.

Column Chromatography

A chromatography column (2.5 x 15 cm) was prepared by packing with a slurry of hydrogen form of a weak cation exchange resin Amberlite IRC-50 (20-50 mesh size, Mallinckrodt Chemical Works). The hydrogen form of the resin was prepared by stirring the resin in 1M HCl, followed by washing with distilled water till the pH of the solution rose to

pH 6.5. The column was washed with an additional 400 ml of water, and 1.0 ml of an aqueous solution of the residue from the high voltage electrophoresis was placed on the column. The column was first eluted with water and then with 1.0 M acetic acid and 0.1 N HCl, respectively. Fractions of 20 ml volume were collected. In preliminary runs in order to locate the toxic material, each fraction was evaporated to dryness and tested for toxicity by the mouse bio-assay method. In subsequent runs fractions were combined and tested for toxicity. The toxic fractions were further combined and concentrated to dryness in vacuo. Any excess of acid was removed by leaving the flasks overnight in a vacuum desiccator containing sodium hydroxide pellets.

Thin Layer Chromatography

Preparative thin layer chromatography plates were prepared by spreading a 20 percent slurry of Cellulose NM (Macherey, Nagel and Co.) on 20 x 20 cm glass plates with a Desaga Thin Layer Spreader (Brinkmann Instruments, Inc.) set at 0.5 mm. The plates were dried at room temperature and stored in a glass chamber over calcium chloride. Just before use the plates were activated for 10 minutes at 110°C. The residue from the toxic fractions from column chromatography was dissolved in about 0.5 ml of water and spotted at about 1 cm intervals on the TLC plates. The plates were developed in a t-butanol; acetic acid: and water (2:1:1, v/v) system (20) for about 4 hours. At the end of the run the plates were dried in air and an end portion was sprayed with ninhydrin and Weber reagents. The region reacting positive to both ninhydrin and Weber reagents was scraped off and the toxin was eluted from the cellulose with water (pH 4-5). The eluate was evaporated to dryness in vacuo. The residue was dissolved in

0.5 ml of water and the insoluble particles of cellulose were removed by centrifugation and the supernatant was lyophilized. For qualitative thin layer chromatography 0.25 mm thick cellulose NM plates were used, which were prepared as described above except the thickness of the spreader was set at 0.25 mm. After thin layer chromatography the plates were air dried in a hood and then sprayed with various location reagents to detect the toxin. These location reagents included Jaffe reagent (21), Alpha-naphthol-diacetyl reagent (22), Weber reagent (22), Benedict-Behr reagent (23), Sakaguchi reagent (24), and ninhydrin. These reagents give specific color reactions with guanidine, mono- and N,N'-disubstituted guanidines, creatinine, lactams of α-guanidino-acids, N-substituted-3-nitroguanidines, etc.

Cellulose Column Chromatography

A cellulose column (3.5 x 15 cm) was prepared by packing with a slurry of cellulose (Whatman CC41, microgranular form for TLC) in t-butanol:acetic acid: and water (2:1:1 v/v) solution. The cellulose was allowed to equilibrate in the solution for 6 hours before the column was packed. The column was washed with another 250 ml of solvent system. About 250 mg of crude toxin was dissolved in 2 ml of solvent system and was placed on the column, which was then eluted with the t-butanol, acetic acid, and water (2:1:1 v/v) system. Fractions of 5 ml volume were collected.

Hydrogenation of Crude Toxin

Crude toxin (33.75 mg) from IRC-50 column chromatography was dissolved in 10 ml of distilled water and 250 mg of platinum dioxide was added. Hydrogenation was carried out at room temperature and pressure for 4 hours. After hydrogenation the mixture was filtered through Whatman No. 1 filter paper, and the filtrate was lyophilized.

The residue was tested for toxicity by dissolving it in known volume of distilled water and injecting various aliquots of this solution into mice.

Toxicity Studies

Sommer and Meyer (25) published the data showing the relationship of mouse units to survival time for paralytic shellfish poison, and defined a mouse unit as the amount of poison that would kill a 20 g mouse in 15 minutes. The most constant results were obtained when the dilution was adjusted so that the time of death was in the neighbourhood of 11-12 minutes. Small aliquots of toxin solution were removed at each stage of purification, and each was lyophilized in a preweighed serum bottle. After lyophilization each bottle was weighed again and the weight of residue calculated by the difference in the weights. The residue was dissolved in a known volume of distilled water. Various aliquots of the solution were injected intraperitoneally into 2-3 mice of 19-20.5 g in weight, and the survival times were recorded. Mice which survived over 20 minutes were considered to have received a sublethal dose. Because of the similarities in chemical and physiological properties between Aphanazomenon toxin and paralytic shellfish poison, the mouse units per mg of solid toxin was calculated from the data of Sommer and Meyer (25). The fold increase in toxicity at various stages of purification was determined by:

$$\text{Fold increase in toxicity} = \frac{\text{min. LD of dried cells}}{\text{min. LD at stage n}}$$

Infra-red Spectrum

The infrared spectrum of Aphanazomenon toxin was recorded on a Perkin-Elmer 337 Infra-red Spectrophotometer. Pure toxin (4.5 mg)

was mixed with 70 mg of KBr (spectral grade, E. Merck reagents) and 10 ml of water was added. The solution was lyophilized for about 24 hours to remove water. The residue was pressed in a Mini-press (Wilks Scientific Corporation, South Norwalk, Conn.) to form a pellet. As a control 75 mg of KBr was dissolved in 10 ml of water and the solution was lyophilized for 24 hours. The residue was pressed in the Mini-press to form a pellet.

RESULTS

Blooms of Aphanizomenon flos-aquae occurred almost regularly during the summers of 1966-1970 in certain New Hampshire lakes. Blooms in Kezar Lake were monitored by the Water Resources Research Center, University of New Hampshire, Durham. Just before collection of the blooms, the toxicity of the bloom water was tested by the mouse bio-assay method as described in MATERIALS AND METHODS. Generally four quarts of concentrate were collected at the end of a day's run, which represented about 150 liters of centrifuged lake water. The blooms of A. flos-aquae lasted almost a week each year.

Isolation and Purification of the Toxin

The weights of the residues and toxicity data, recorded at each stage of purification, from several representative batches are shown in Table I. An average of 2.7 g of residue was obtained from the supernatant after extraction and centrifugation of 0.86 liters of concentrate. The variation in the amount of residue obtained and toxicity at each stage of purification could be due to the condition of the bloom on the day of collection. The residue obtained from the subsequent ethanol extract was about 1.65 g per batch, which represented an average recovery of 61.4 percent. The recovery of solids in the toxic aqueous phase from the subsequent chloroform-water partitioning step was 58.7 percent.

High Voltage Electrophoresis

At the end of the first high voltage electrophoresis the buffer in the buffer chamber serving as anode was found to have a yellowish tinge. The anode side buffer was evaporated to dryness in vacuo and the

TABLE I

A. flos-aquae Toxin: Yield and Toxicity at Various Stages of Purification

	Cell suspension					Supernatant[a]				Ethanol extract				Aqueous layer			
	Vol. ml	Wt. g[b]	Toxicity ug	S.T.[c]	MU[d]	Wt. g	Toxicity ug	S.T.[c]	MU[d]	Wt. g	Toxicity ug	S.T.[c]	MU[d]	Wt. g	Toxicity ug	S.T.[c]	MU[d]
1	600	3.94	400	12.0	1.0	1.22	115.0	7.0	1.4	0.530	62.5	6.0	1.6	0.461	31.2	15.5	1.0
2	1000	5.87	350	9.0	1.2	3.01	100.0	3.5	4.0	2.090	75.0	4.5	2.3	1.200	49.4	3.5	4.0
3	1000	6.60	350	8.0	1.3	1.65	230.0	3.5	4.0	0.836	180.0	7.5	1.3	0.561	120.0	2.5	6.0
4	900	5.94	400	5.5	1.7	3.81	120.0	4.5	2.5	2.500	140.0	6.0	1.6	1.620	87.5	3.0	5.0
5	800	4.10	350	6.0	1.6	1.95	170.0	4.7	2.5	1.050	92.0	3.5	4.0	0.871	80.0	3.5	4.0
6	1100	8.20	850	7.0	1.4	3.90	210.0	6.5	1.7	1.950	150.0	7.0	1.4	0.960	100.0	4.0	2.5
7	650	8.11	1040	12.0	1.0	3.20	250.0	6.5	1.7	2.600	166.5	5.0	1.9	1.100	107.0	5.0	1.9
Av.	864	6.10	484	8.5	1.3	2.67	170.7	5.1	2.5	1.650	123.7	5.7	1.7	0.968	82.1	5.2	1.9

[a] Fron centrifugation.

[b] Dry weight of cells determined by lyophilizing a small aliquot of the cell suspension.

[c] Survival time in minutes.

[d] Mouse units calculated from the data of Sommer and Meyer based on paralytic shellfish poison.

TABLE I (Continued)

	1st High voltage electrophoresis				2nd High voltage electrophoresis				IRC-50 column chromatography				Thin layer chromatography				Remark
	Wt.	Toxicity			Wt.	Toxicity			Wt.	Toxicity			Wt.	Toxicity			
	mg	ug	S.T.[c]	MU[d]	mg	ug	S.T.[c]	MU[d]	mg	ug	S.T.[c]	MU[d]	mg	ug	S.T.[c]	MU[d]	
1	373.0	-	-	-	44.6	22.1	3.5	4.0	15.5	20.1	3.4	4.5	1.5	2.5	5.0	1.9	40 mg was placed on IRC-50 column.
2	475.0	33.4	6.0	1.7	100.0	25.0	3.2	4.5	41.4	22.0	3.2	4.5	4.0	3.5	4.0	2.5	70 mg was placed on IRC-50 column.
3	195.0	35.0	5.5	1.7	42.9	27.5	2.5	6.0									
4	429.1	-	-	-	106.5	25.0	4.0	2.5	47.5	-	-	-	12.0	2.5	5.0	1.9	140 mg was placed on IRC-50 column.
5	492.0	-	-	-	89.5	25.0	4.2	2.5	18.4	-	-	-	6.2	2.5	5.1	1.9	45 mg was placed on IRC-50 column.
6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	used for cellulose column chromatography.
7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Av	392.8	34.2	5.7	1.7	76.7	24.9	3.4	3.9	30.7	21.0	3.3	4.5	5.9	2.7	4.9	2.0	

residue was found to be non-toxic when tested by the mouse bio-assay method. When the material eluted from the origin to cathode region of the paper was subjected again to paper electrophoresis, the paper from the second high voltage electrophoresis had three ninhydrin-positive bands, but only the fastest of these bands gave a Weber-positive reaction (Fig. 1). This Weber-positive region of the paper was cut into small pieces and was eluted with acidified water (pH 4). The residue from the eluate was toxic to mice when injected intraperitoneally (approximately 3.9 mouse units per 25 ug). On a cellulose MN TLC plate the residue showed the presence of 5 ninhydrin-positive spots (Fig. 2). Only the Rf 0.31 spot gave a Weber-positive reaction

IRC-50 Column Chromatography

The elution pattern of A. flos-aquae toxin from the second high voltage electrophoresis on an IRC-50 column is shown in Fig. 3. The column was first eluted with water followed by 1.0 M acetic acid and 0.1 M HCl, respectively. The first fraction from the column was light yellow in color which, when evaporated to dryness, gave a yellow residue. The residue was dissolved in water and was found to be non-toxic when tested by the mouse bio-assay method. Of all the collected fractions only fractions 13-19, which represented the start of the 1.0 M acetic acid eluant, were toxic when injected intraperitoneally into mice. The toxic fractions were combined, evaporated to dryness and dissolved in 0.5 ml of water (pH 4).

Thin Layer Chromatography

The thin layer chromatographic patterns of residues obtained from the aqueous layer after chloroform partitioning, second high voltage electrophoresis, and combined fractions 13-19 from the IRC-50

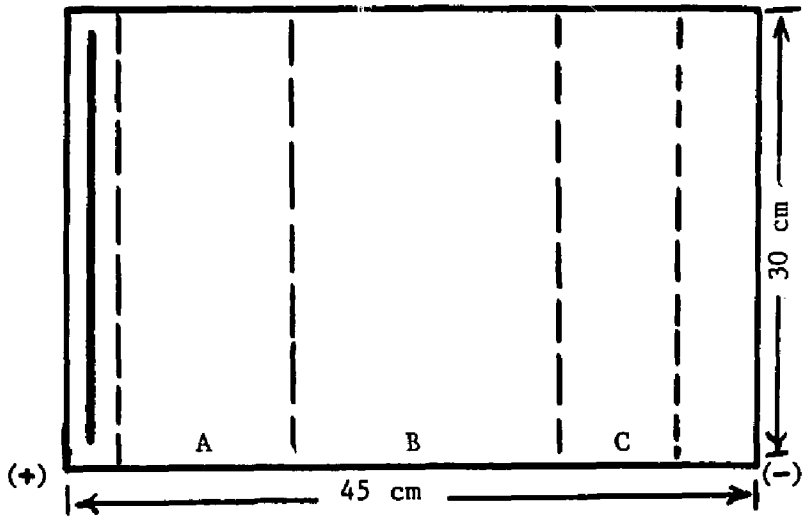


Fig. 1. Second high voltage electrophoresis of A. flos-aquae toxin. A, B, and C indicate the regions which gave ninhydrin positive bands. Toxicity and a Weber-positive test was found associated with region C only.

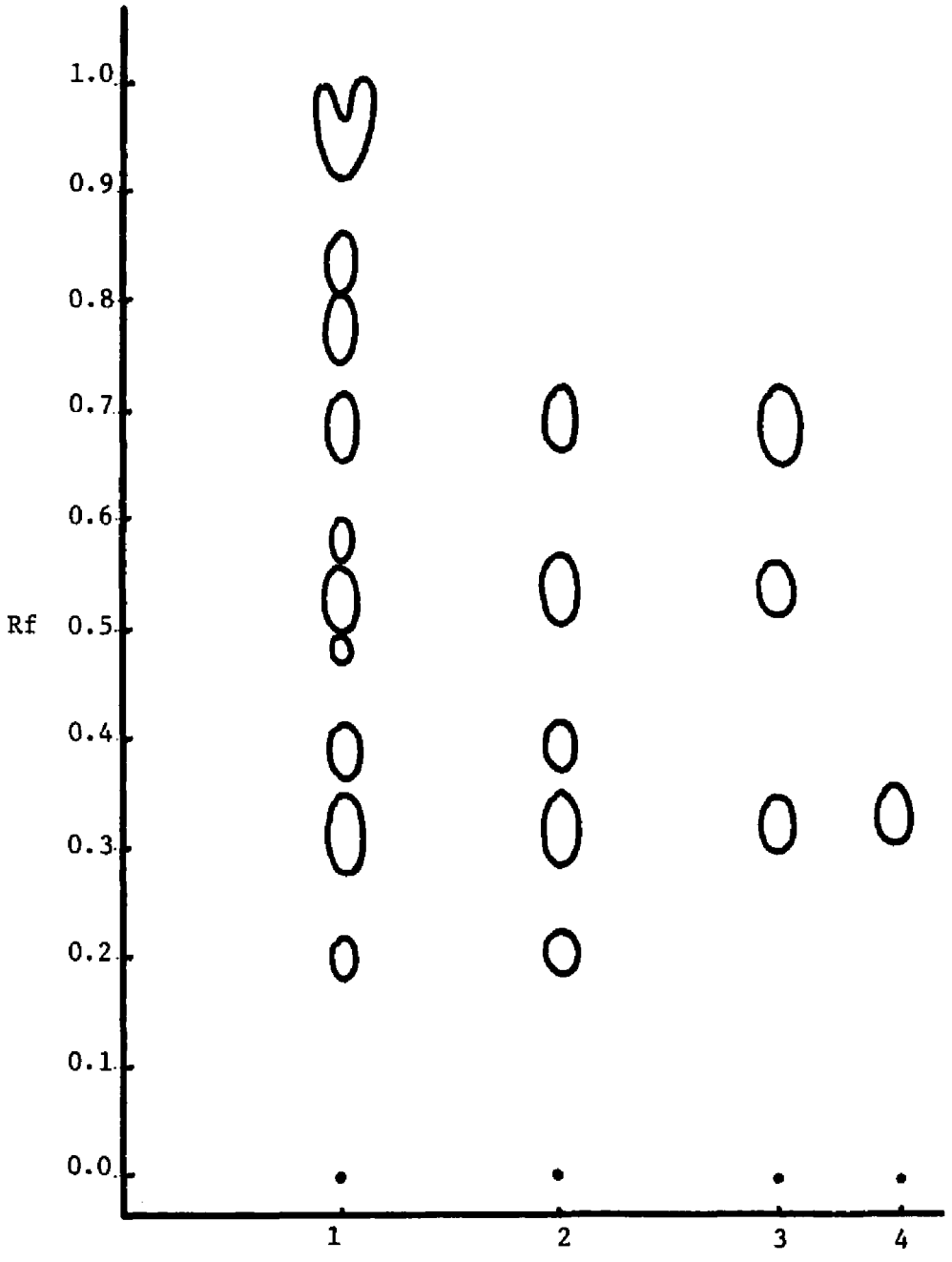


Fig. 2. Thin layer chromatography of A. flos-aquae toxin on cellulose MN. (1) Aqueous extract; (2) residue from 2nd high voltage electrophoresis; (3) fractions 13-19 from IRC-50 column chromatography; (4) pure A. flos aquae toxin from preparative TLC. Ninhydrin positive spots indicated. Only the Rf 0.31 spot was Weber positive. Solvent system, t-butanol, acetic acid, water (2:1:1 v/v).

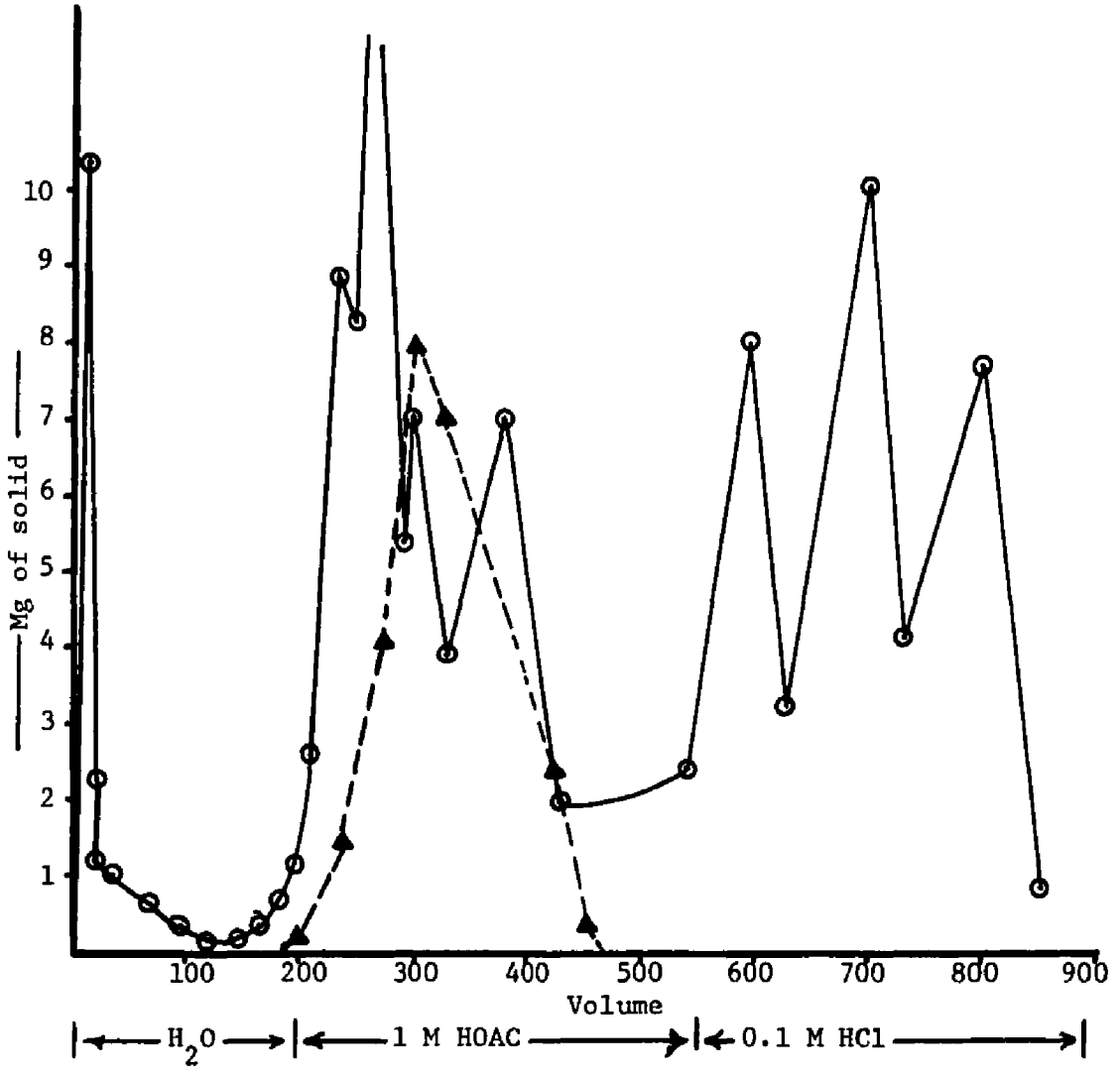


Fig. 3 IRC-50 column chromatography of A. flos aquae toxin. Mg of residue (o——o), toxicity (▲---▲).

column chromatography are shown in Fig. 2. The residue from the column chromatography gave three spots on preparative thin layer plates when the plate was sprayed with ninhydrin reagent. The spots had Rf values of 0.68, 0.54, and 0.31. The spot with Rf 0.31 also gave a Weber-positive test. The area corresponding to the ninhydrin- and Weber-positive spot was scraped off as described in MATERIALS AND METHODS to recover the pure toxin.

Experiments with Cellulose Column Chromatography

As an alternate method of purification not involving high voltage electrophoresis, toxin from the aqueous layer after chloroform partitioning was placed on a cellulose CC41 column. The elution pattern of A. flos-aquae toxin is shown in Fig. 4. Fractions of 5 ml were collected and were combined, on the basis of TLC, to give 5 combined fractions of different compositions. The TLC patterns of these combined fractions are shown in Fig. 5. Both combined fractions 3 and 4 were found equally toxic (49 and 51 mouse units per mg of solids, respectively), when tested by the mouse bio-assay method. The total recovery of combined solids from the cellulose column chromatography was 84.19 percent, and fractions 3 and 4 together gave 57.3 mg of partially purified toxin.

Toxicity Studies

The average fold increase in toxicity during the purification process as a function of stage of purification is shown in Fig. 6. The mouse units per mg of solids at various stages of purification, calculated from the data of Sommer and Meyer (25), is shown in Fig. 7. The pure A. flos-aquae toxin at an average level of 2.75 ug gave an average survival time of 4.8 minutes (Table I) representing about

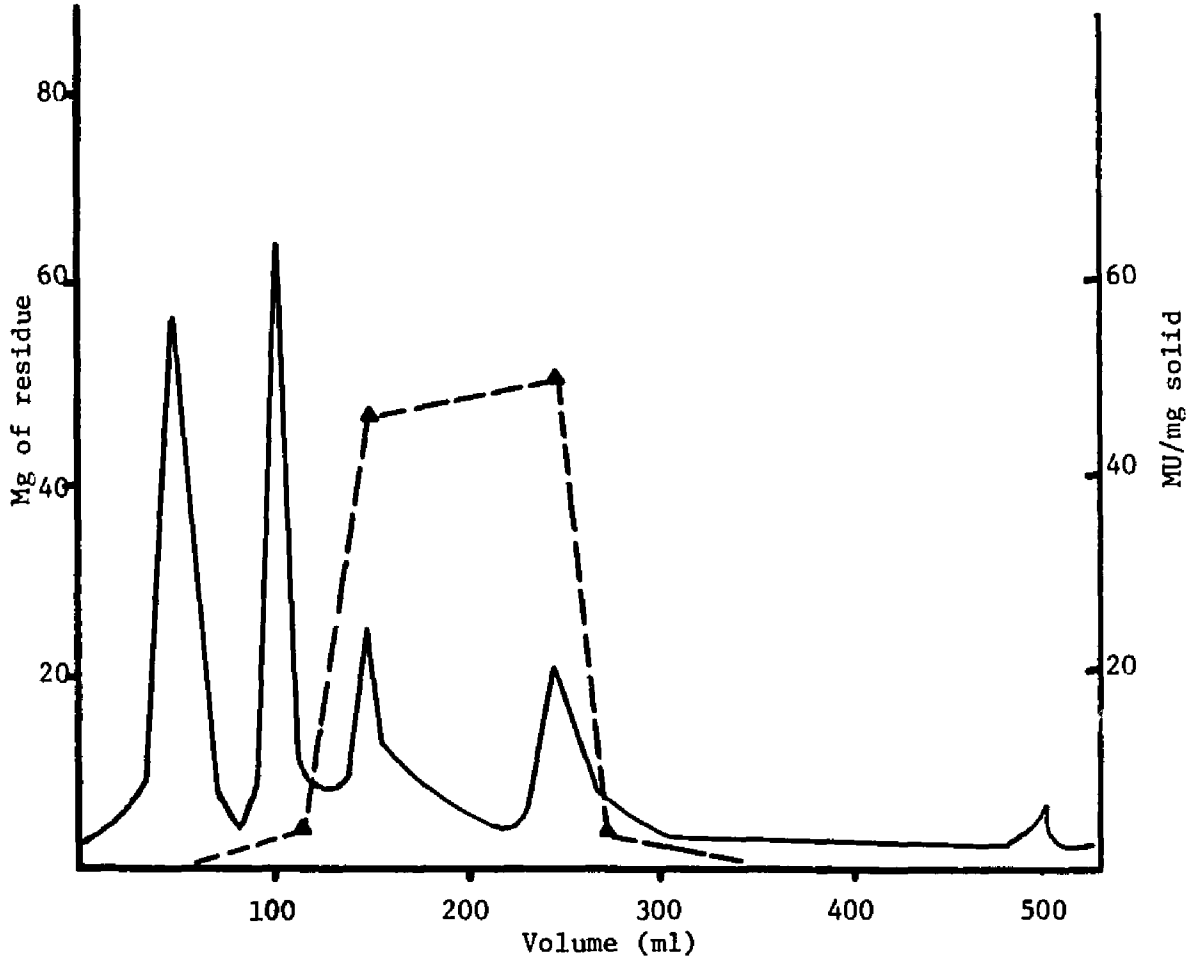


Fig. 4. Cellulose CC41 column chromatography (3 x 15 cm) of A. flos-aquae toxin. Mg of residue (o——o); MU/mg solid (▲) calculated from the data of Sommer and Meyer (25).

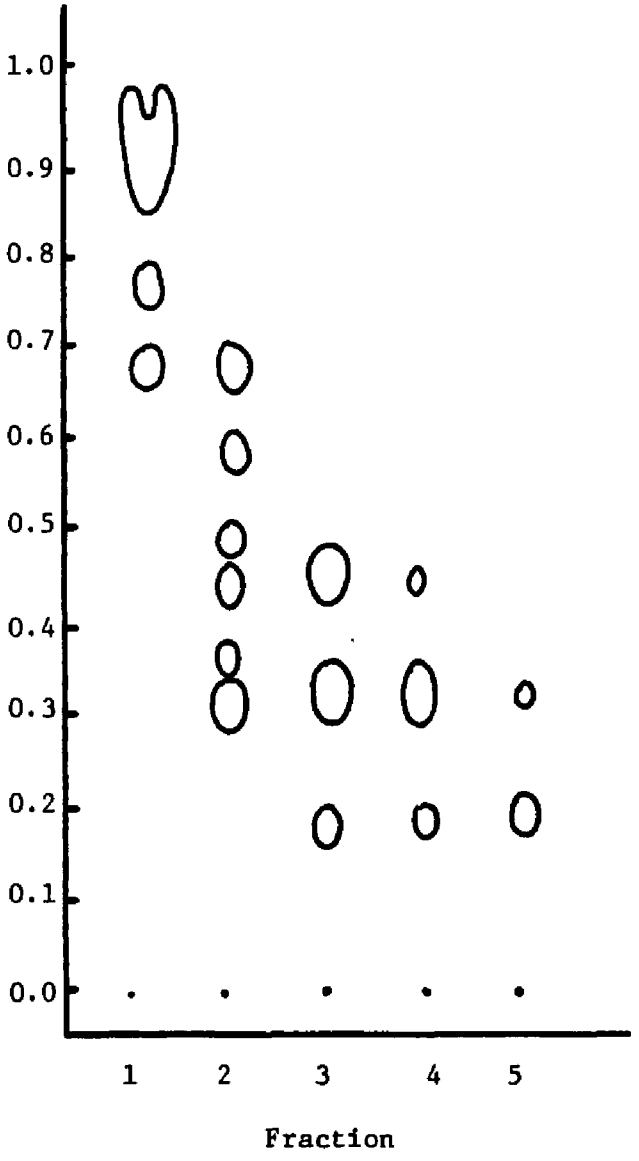


Fig. 5. Thin layer chromatography of combined fractions from cellulose CC41 column. Solvent system: t-butanol: acetic acid: water (2:1:1 v/v).

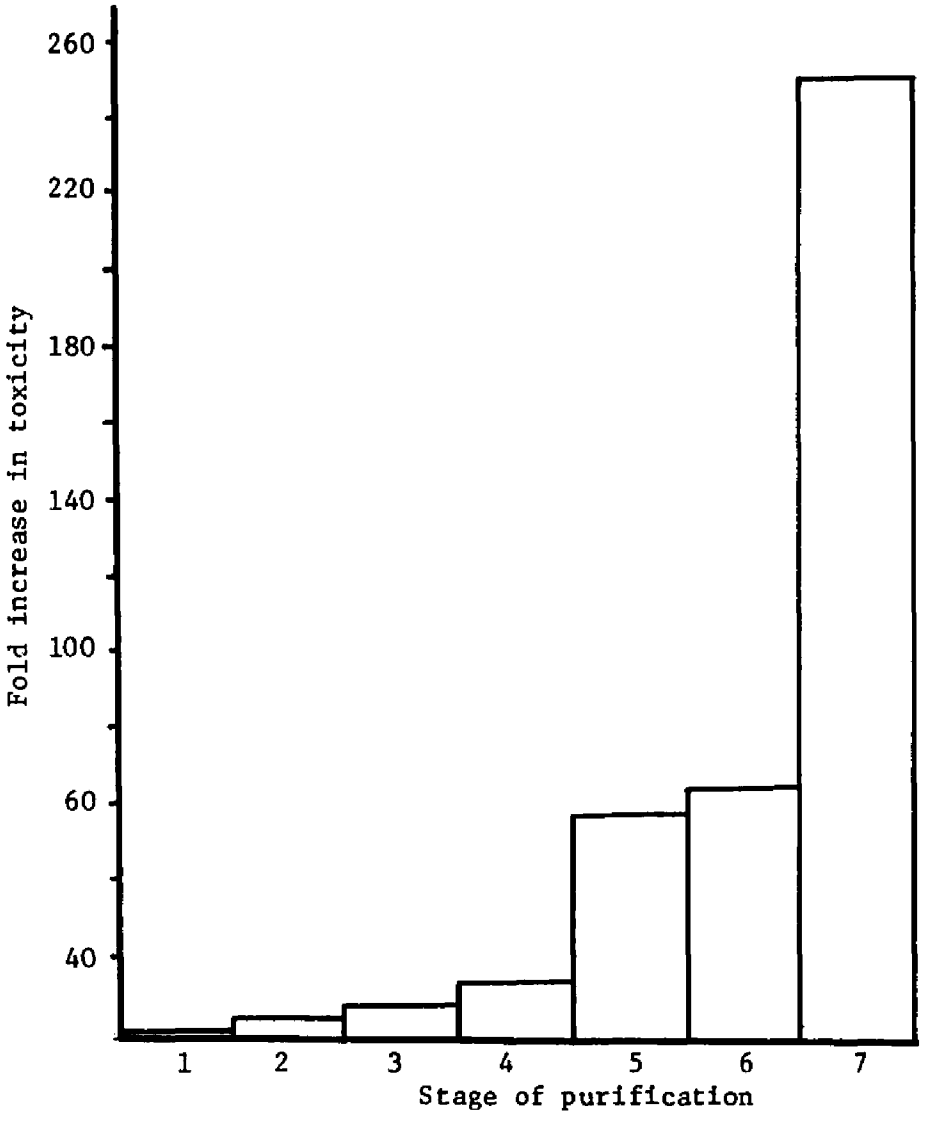


Fig. 6. Fold increase in toxicity of A. flos aquae toxin as function of stage of purification. (1) Cell suspension, (2) supernatant from centrifugation, (3) ethanol extract, (4) aqueous extract, (5) 2nd high voltage electrophoresis, (6) IRC-50 column chromatography and (7) thin layer chromatography.

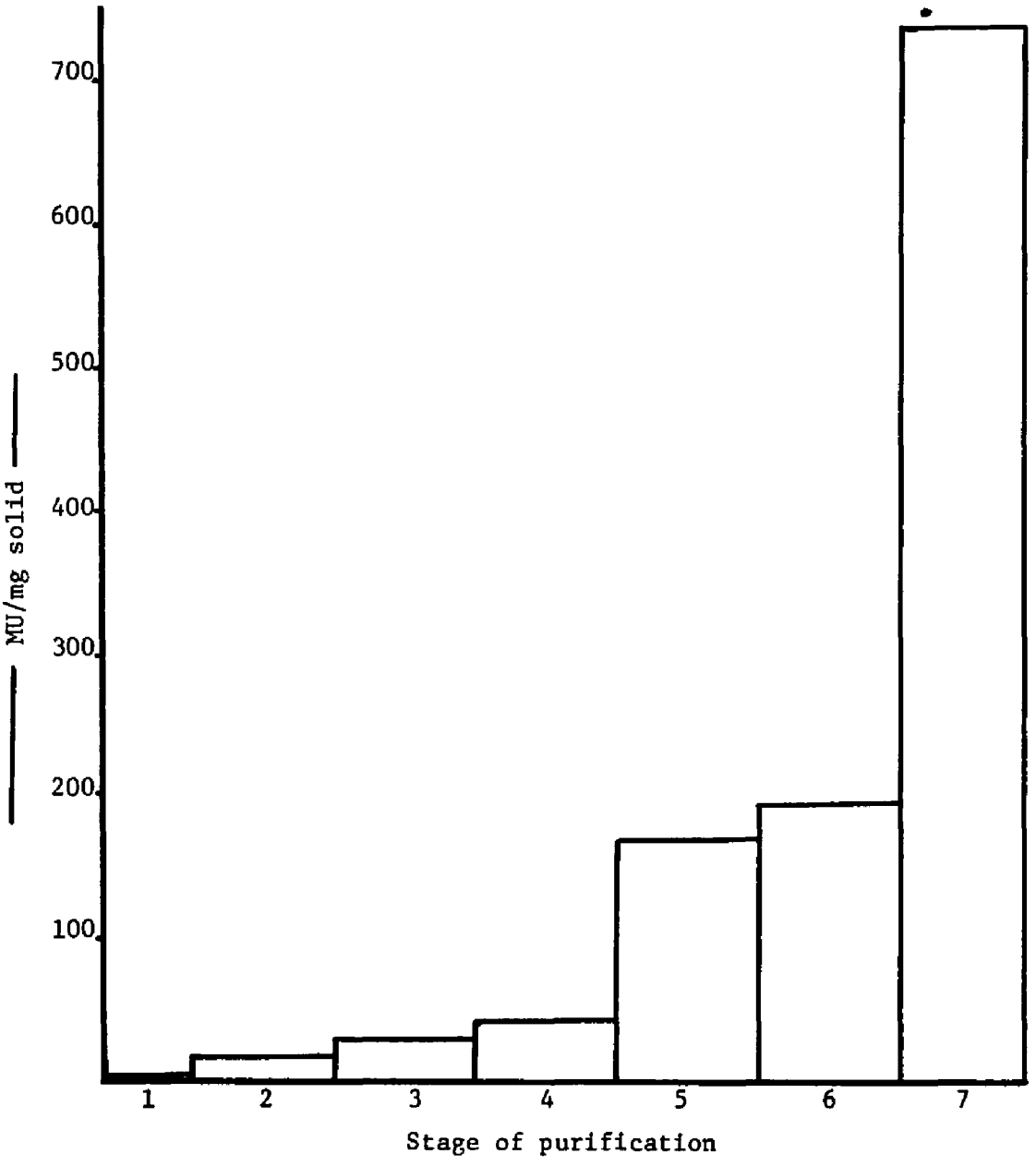


Fig. 7 Toxicity of A. flos-aquae toxin (MU/mg) at various stages of purification. (1) Cell suspension, (2) supernatant from centrifugation, (3) ethanol extract, (4) high voltage electrophoresis, (6) IRC-50 column chromatography, and (7) thin layer chromatography.

2.0 mouse units according to Sommer and Meyer (25). Although there was significant batch to batch variation (Table I) in the potency of crude and partially purified toxin, the pure toxin had a fairly constant activity.

Physical and Chemical Properties

The A. flos-aquae toxin was a very pale yellow glassy substance of hygroscopic nature. It absorbed moisture within a fraction of a minute. No ultraviolet or visible absorption spectrum above 220 mμ was observed for the A. flos-aquae toxin. Due to the hygroscopic nature of the toxin it was difficult to record an infra-red spectrum which was known to be free of water. The infra-red spectrum of the toxin in KBr pellet along with the spectrum of a KBr pellet prepared under the same conditions but using water instead of toxin solution is shown in Fig. 8. The pure toxin was non-volatile and did not ionize even at 310°C in mass spectrometer. Similarly all attempts to record a 60 MHz. NMR spectrum in D_2O were unsuccessful. The latter was hampered by the very limited quantity of the toxin available. The Rf values and TLC patterns of A. flos-aquae toxin and saxitoxin on cellulose MN plates in several solvent systems are given in Table II and Figs. 9, 10 and 11. The colour reactions of A. flos-aquae toxin in comparison to saxitoxin are shown in Table III.

The migration characteristics of A. flos-aquae toxin and that of saxitoxin were investigated by high voltage electrophoresis using pyridine, acetic acid and water (133:4.6:1863 v/v) buffer as described in MATERIALS AND METHODS. The toxin were located with Weber and diacetyl reagents. The migration pattern of A. flos-aquae toxin and saxitoxin are shown in Fig. 12. Our A. flos-aquae toxin showed a slower rate of migration than saxitoxin under these conditions.

Fig. 8. Infra-red spectrum of A. flos-aquae toxin in KBr pellet. (A) A. flos-aquae in KBr, (B) KBr pellet (CA) Calibration for curve A (CB) Calibration for curve B.

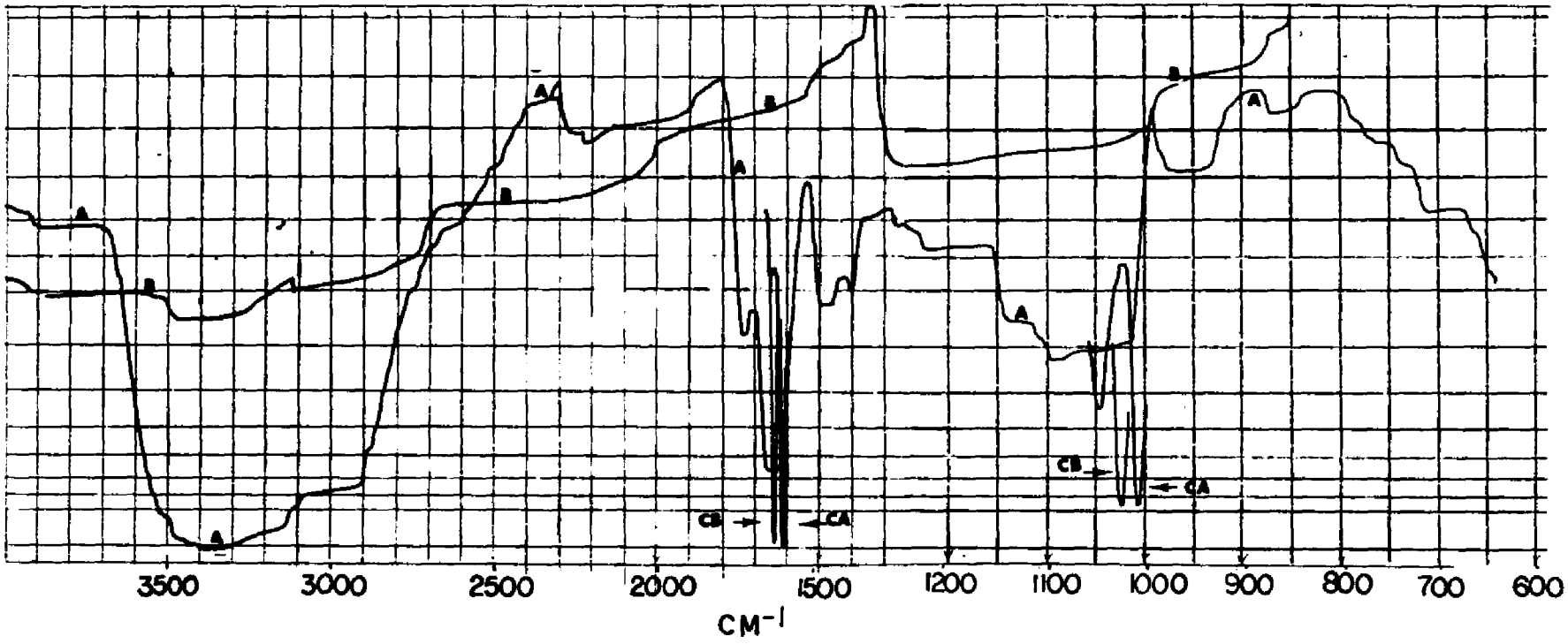

A
B
A
B
A
B
A
B
A
CB
CA
CB
CA
3500
3000
2500
2000
1500
1200
1100
1000
900
800
700
600
CM⁻¹

Table II

Rf Values of A. flos-aquae Toxin and Saxitoxin in Various Solvent Systems.

Solvent	Composition	R_f	
		Saxitoxin	A. flos-aquae toxin
a	t-Butanol: acetic acid: water (2:1:1 v/v) (20).	0.39	0.31
b	n-Butanol: pyridine: water (1:1:1 v/v) (29).	0.31 0.17	0.10
c	n-Butanol: acetic acid: water (120:30:50 v/v) (29).	0.32 tailing	0.15
d	t-Amyl alcohol: pyridine: water (7:7:6 v/v) (20).	0.38	0.57
e	Phenol: water (80:20 v/v) (20).	0.35	0.40
f	n-Propanol: acetic acid: water (60:15:15 v/v) (29).	0.56 0.36	0.26
g	Isopropanol: conc. ammonium hydroxide: water (100:5:10 v/v) (29).	0.07	0.01

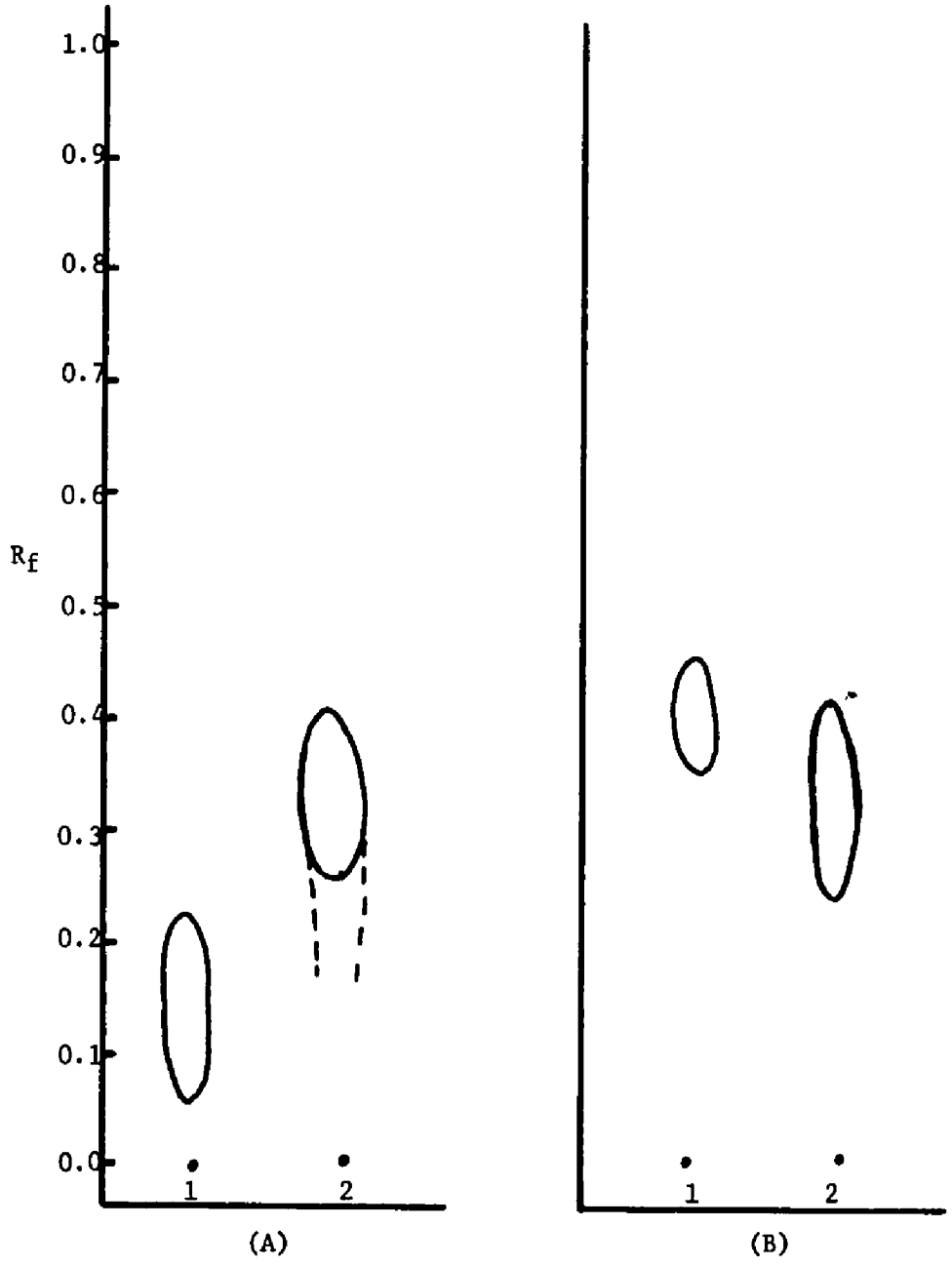


Fig. 9. Thin layer chromatography of A. flos-aquae toxin (1) and saxitoxin (2) on cellulose MN plates. Solvent systems: (A) n-butanol: acetic acid: water (120: 30: 50 v/v); (B) phenol: water (80: 20 v/v).

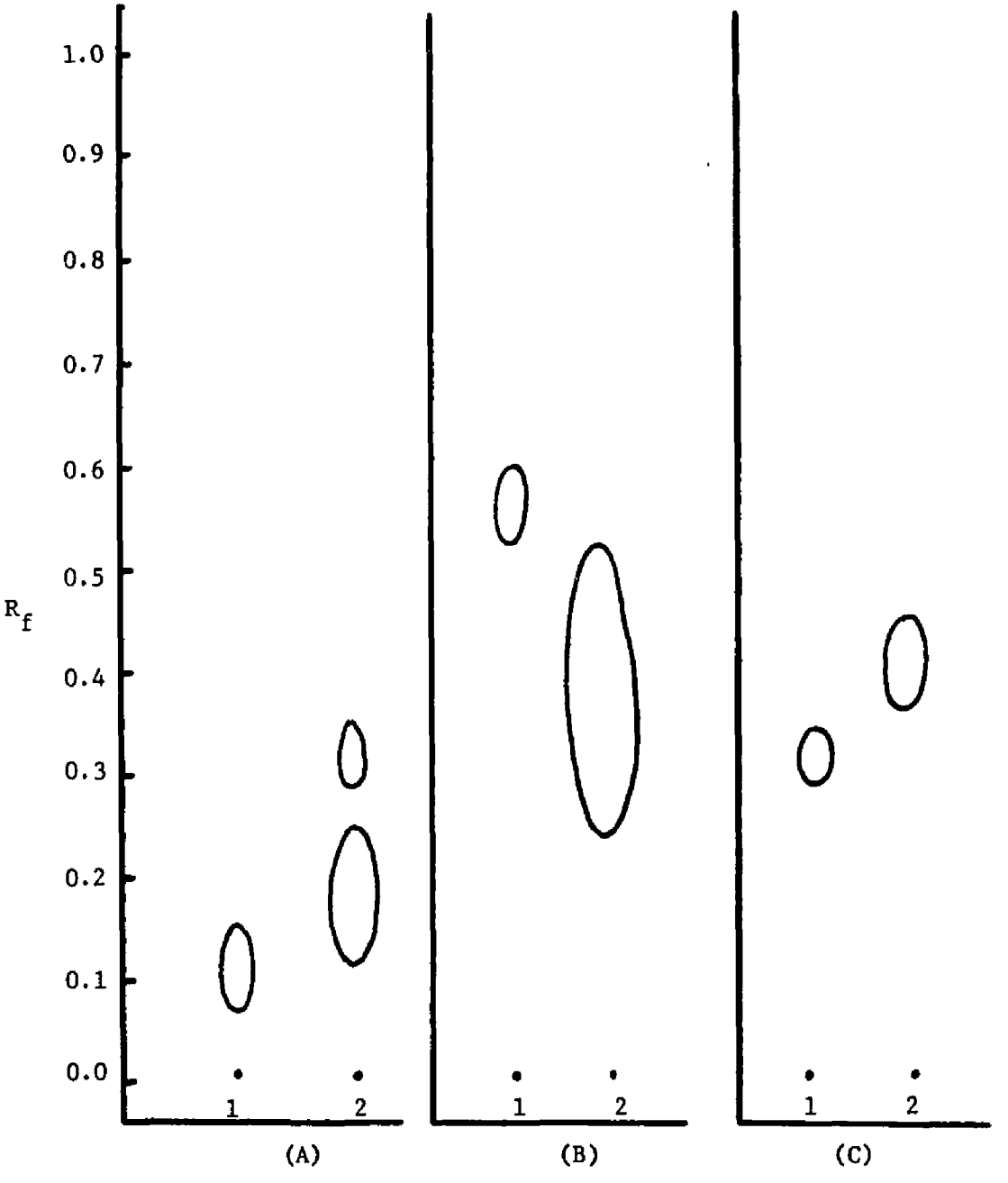


Fig. 10. Thin layer chromatography of A. flos-aquae toxin (1) and saxitoxin (2) on cellulose MN plates. Solvent systems: (A) n-butanol: pyridine: water (65: 65: 65 v/v); (B) t-amyl alcohol: pyridine: water (35: 35: 30 v/v); (C) t-butyl alcohol: acetic acid: water (2: 1: 1 v/v).

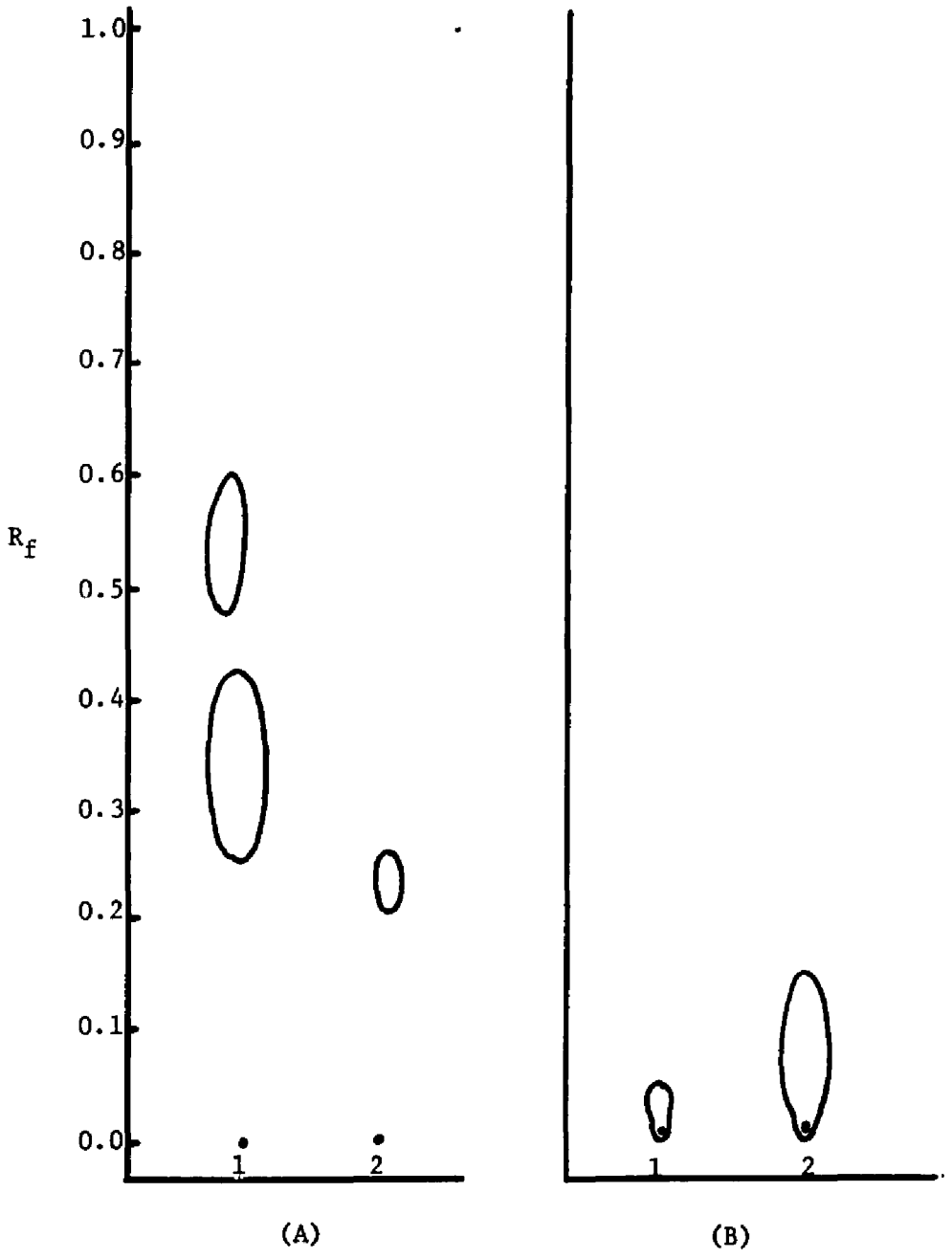


Fig. 11. Thin layer chromatography of A. flos-aquae toxin (1) and saxitoxin (2) on cellulose MN plates. Solvent systems: (A) n-propanol: acetic acid: water (60: 15: 15 v/v) (B) isopropanol: conc. ammonium hydroxide: water (100: 5: 10 v/v).

Table III

Color Reactions of A. flos-aquae Toxin and Saxitoxin.

	Reagent	Saxitoxin[a]	A. flos-aquae toxin
1	Ninhydrin	Negative Yellow[b]	Purple
2	Jaffe reagent (21)	Red	Negative
3	Weber reagent (22)	Deep red	Deep red
4	Diacetyl reagent (22)	Purple red	Purple red
5	Sakaguchi reagent(24)	Orange	Orange
6	Benedict-Behre reagent (23)	Purplish red	Red
7	Bromocresol green (26)	Basic	Basic

a Generously supplied by Dr. E. J. Schantz.

b Yellow according to Jackim and Gentile (12).

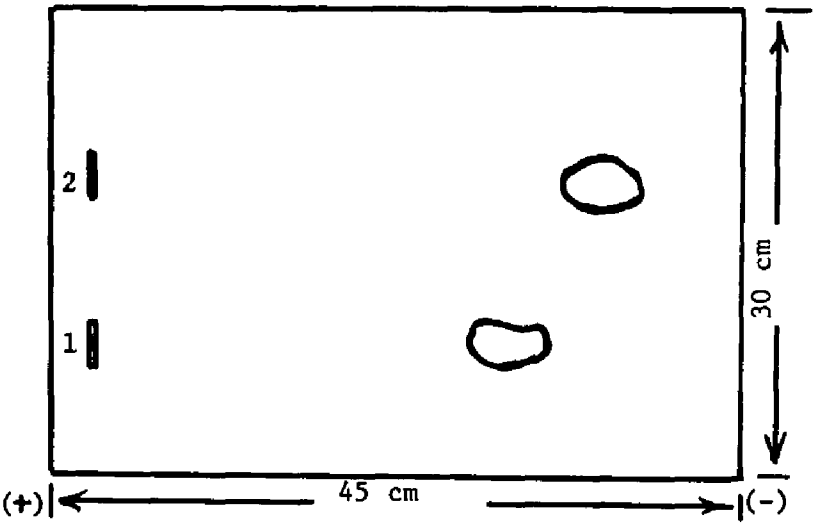


Fig. 12. High voltage electrophoresis of A. flos-toxin (1) and saxitoxin (2). Buffer: pyridine: acetic acid: water (133:4.6:1863 v/v) pH 6.41. Location reagent : Weber and diacetyl reagents

slower rate of migration than saxitoxin under these conditions.

DISCUSSION

The results of the toxicity studies indicate the presence of a powerful toxin in the natural blooms of A. flos-aquae collected from Kezar Lake. Sawyer et al. (8) reported that the toxin from A. flos-aquae had potent nerve and muscle-blocking activity, destroying conduction in these tissue without apparently affecting the transmembrane resting potential.

Purification of A. flos-aquae toxin from the blooms resulted in a component which was responsible for most of the activity. In contrast to saxitoxin it did not bind well to the sodium form of carboxylate Amberlite resin, and eluted with acetic acid. All attempts to purify A. flos-aquae toxin by the methods described by Jackim and Gentile (12) were unsuccessful. These involved the use of carboxylate ion-exchange resin or the use of preparative paper chromatography. Nor was fractionation of toxin achieved when the partially purified toxin from IRC-50 column chromatography was passed through acid washed alumina, as described by Schantz et al. (26). These experiments support this author's assumption that the toxin from A. flos-aquae natural blooms is chemically different from that described by Jackim and Gentile (12) from the laboratory cultures of A. flos-aquae. In chromatographic behavior on carboxylate resin our A. flos-aquae toxin resembled the major toxin isolated by Evans (27) from a poisonous sample of mussels Mytilus edulis.

Difference in the chemical nature of our A. flos-aquae toxin and saxitoxin is also supported by the fact that the A. flos-aquae toxin was insensitive to hydrogenation, whereas saxitoxin has been reported (20) to lose its activity on hydrogenation at room temperature.

The hygroscopic nature of our A. flos-aquae toxin made it very difficult to record an IR spectrum of the toxin for a comparison with that of saxitoxin. An IR spectrum of partially hydrated A. flos-aquae toxin in a KBr pellet showed the presence of a hydroxyl or amino group as well as the presence of a carbonyl group. The exact position of the absorption band could not be determined from the spectrum. Due to the limited supply of the pure toxin the degradative comparison of our toxin with that of saxitoxin, reported by Schuett and Rapoport (28) and Wong et al. (29) was not possible. However both TLC and high voltage electrophoretic comparisons of A. flos-aquae toxin and saxitoxin were made. As shown in Fig. 9-11 and Table II our A. flos-aquae toxin had different Rf values than saxitoxin in several solvent systems. The migration characteristics of A. flos-aquae toxin and saxitoxin under the influence of an electrical field of 2000 volts was also different. As shown in Fig. 12 our toxin has a slightly slower rate of migration than does saxitoxin. The slow rate of migration of our toxin indicate that it is less basic than saxitoxin which could be due to the presence of a fewer number of tertiary nitrogen atoms than saxitoxin.

As shown in Table III, A. flos-aquae toxin and saxitoxin showed differences in their reactions to various location reagents. A. flos-aquae toxin gave a purple color with ninhydrin and a negative Jaffe test, whereas saxitoxin is ninhydrin negative or is reported to give a yellow color (12) and is Jaffe-positive. Positive reactions with Weber, diacetyl, and Sakaguchi reagents indicated the presence of a guanidine group(s) in A. flos-aquae toxin (30). A red color with Benedict-Behr reagent (instead of purplish-red given by creatine and

saxitoxin) indicate the presence of a substituted creatine or an active methylene or methyl groups in A. flos-aquae toxin molecule.

The toxicity data indicated that our toxin had a toxicity of 745 Mouse units per mg of solid which is about one-seventh that of saxitoxin as reported by Schantz et al. (26).

SUMMARY AND CONCLUSION

A new toxic fraction from the natural blooms of blue-green alga Aphanizomenon flos-aquae from Kezar Lake, North Sutton, New Hampshire, has been isolated and purified by the method of high voltage electrophoresis, column chromatography and preparative thin layer chromatography. The toxin had a LD_{100} of 60.5 µg per kg.. It was found to be basic in nature and had no ultraviolet and visible absorption spectrum above 220 mµ. Due to its hygroscopic nature, attempts to record an IR spectrum of the toxin, free of water, were not totally successful. The results however, did indicate the presence of hydroxyl or amino groups and of carbonyl group. The migration of the toxin in several solvent systems on cellulose MN plates showed that A. flos-aquae toxin was different from saxitoxin. Similarly in high voltage electrophoresis the A. flos-aquae toxin had a slower rate of cathodic migration than saxitoxin. A. flos-aquae toxin, in contrast to saxitoxin, gave a negative Jaffe test which is reported to be specific for creatinine residues in a molecule. The colour reactions of A. flos-aquae toxin suggested the presence of substituted guanidine group(s) in its molecule.

The behavior of A. flos-aquae toxin on carboxylate resin Amberlite IRC-50, and acid washed alumina as well as on cellulose MN plates in various solvent systems suggests that the toxin is not similar to saxitoxin but may be more similar to another toxin isolated from poisonous samples of the mussel Mytilus edulis.

BIBLIOGRAPHY

1. Rose, E.T. Toxic algae in Iowa lakes. Proc. Iowa Acad. Sci. 60: 738-745 (1953).
2. Styne, D.G. Poisoning of animals by algae (scum or waterbloom) in dams and ponds. Union of South Africa, Dept. of Agr. and Forestry. Govt. Printer, Pretoria, 1945.
3. Stephens, E.L. Microcystis toxica sp. n.: A poisonous alga from the Transvaal and Orange Free State, Trans. Roy. Soc. So. Africa 32: 105-112 (1945).
4. Carl, G.C. Flora and fauna of brackish water. Ecol. 18: 446-453 (1937).
5. Prescott, G.W. Objectionable algae with reference to killing of fish and other animals. Hydrobiol. 1: 1-13 (1948).
6. Mackenthum, K.M., E.F. Herman, and A.F. Bartsch. A heavy mortality of fishes resulting from the decomposition of algae in Yahara River, Wisconsin. Trans. Am. Fisheries Soc. (for 1945) 75: 175-180 (1948).
7. Astakhova, T.M., M.S. Kun, and D.L. Teplyi. Cause of carp disease in lower Volga. Dokl Akad. Nauk. S. S. S. R. 133: 1205-1208 (1960) (A. I. B. S. Transl. Doklady) (Biol. Sci. Sect.) 133: 579-581 (1961).
8. Sawyer, P.J., J. H. Gentile, and J.J. Sasner. Demonstration of a toxin from Aphanizomenon flos-aquae (L) Ralfs. Can. J. Microbiol. 14: 1199-1203 (1968).
9. Bishop, C.T., E.F.L.J. Anet, and P.R. Gorham. Isolation and identification of the fast-death factor in Microcystis aeruginosa NCR-I Can. J. Biochem. Physiol. 37: 435-471 (1959).

10. Murthy, J.M. and J.B. Capindale. A new isolation and structure for the endotoxin from Microcystis aeruginosa NCR-I. Can. J. Biochem. 48: 508-510 (1970).

11. Gorham, P.R. Toxic waterblooms of blue-green algae. Can. Vet. J. 1: 235-245 (1960).

12. Jackim, E. and J. Gentile. Toxins cf a blue-green alga: Similarity to saxitoxin. Science 162: 915-916 (1968).

13. Otterstrom, C.V. and E. Steeman-Nielsen. Two cases of extensive mortality in fishes caused by the flagellate Prymnesium parvum carter. Rept. Danish Biol. Sta. 44: 5-24 (1939).

14. Reich, K., F. Bergmann, and M. Kidron. Studies on the homogeneity of Prymnesin, the toxin isolated from Prymnesium parvum carter. Toxicon 3: 33-39 (1965).

15. Parnas, I. The toxicity of Prymnesium parvum (a review). Israel J. Zool. 12: 15-23 (1963).

16. Shilo, M. and R.F. Rosenberger. Studies on the toxic principles formed by the chrysomonad Prymnesium parvum carter. Ann. N.Y. Acad. Sci. 90: 866-876 (1960).

17. Paster, Z. Prymnesin: The toxin from Prymnesium parvum carter. Rev. Intl. Oceanogr. Med. 10: 247-257 (1968) (through Oceanog. Abstr. 15: A 582).

18. Martin, D.F. and G.M. Pedilla. Characterization of Prymnesium parvum toxins by means of hemolytic kinetics. Environmental lett 1: 199-203 (1971).

19. Groskopf, W.R., J.W. Holleman, E. Margoliash, and I.M. Klotz. Amino acid sequences of peptide from tryptic digest of Golfingia gouldii hemerythrin. Biochemistry 5: 3783-3796 (1966).

20. Mold, J.D., J.P. Bowden, D.W. Stanger, J.E. Maurer, J.M. Lynch, R.S. Wyler, E.J. Schantz, and B. Riegel. Paralytic shellfish poison VII. Evidence for purity of the poison isolated from the toxic clams and mussels. J. Am. Chem. Soc. 79: 5235-5238 (1957).

21. Ames, S.R. and H.A. Risley, Aminoaciduria in progressive muscular dystrophy. Proc. Soc. Expt. Biol. Med. 68: 131-135 (1948).

22. Roche, J., N.V. Thoai, and J.L. Hait, Metabolism of guanidyl derivatives III: Chromatographic analysis of guanidyl derivatives. Biochem. Biophys. Acta 14: 71-75 (1954).

23. Carr, J.J. Reactions of aromatic nitro compounds with active methyl, methylene, methine groups in presence of base. Anal. Chem. 25: 1859-1863 (1953).

24. Smith, I. Color reactions on paper chromatograms by dipping technique. Nature 171: 43-44 (1953).

25. Sommer, H. and K.F. Meyer. Paralytic shellfish poisoning. Arch. Pathol. (Lab. Med.) 24: 560-598 (1937).

26. Schantz, E.J., J.D. Mold, D.W. Stanger, J. Shavel, F.J. Riel, J.P. Bowden, J.M. Lynch, R.S. Wyler, B. Riegel, and H. Sommer. Paralytic shellfish poison VI. A procedure for the isolation and purification of the poison from toxic clam and mussel tissue. J. Am. Chem. Soc 79: 5230-5235 (1957).

27. Evans, M.H. Two toxins from a poisonous sample of mussels Mytilus edulis. Br. J. Pharmacol. 40: 847-865 (1971).

28. Schuett, W. and H. Rapoport. Saxitoxin, the paralytic shellfish poison. Degradation to a pyrrolopyrimidine. J. Am. Chem. Soc. 84: 2266 (1962).

29. Wong, J.L., M.S. Brown, K. Matsumoto, R. Oestertin, and H. Rapoport. Degradation of saxitoxin to a pyrimido [2, 1-b] purine. J. Am. Chem. Soc. 93: 4633-4634 (1971).

30. Smith, I. and H.G. Horsewell, In: Chromatographic and Electrophoretic Techniques, Vol. I. Chromatography, p. 286-294 (Smith, I. ed.). Interscience Publishers, New York, 1969.