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THE GROWTH AND TOXICITY OF THE FLORIDA RED TIDE ORGANISM, GYMNODINIUM BREVE

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by

Marion Tilton Doig, III

A thesis submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy
in the Department of Chemistry
in
The University of South Florida

June, 1973

Thesis supervisor: Professor Dean F. Martin

Graduate Council
University of South Florida
Tampa, Florida

CERTIFICATE OF APPROVAL

PH.D. THESIS

This is to certify that the Ph.D. thesis of

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with a major in Chemistry
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An Abstract

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ABSTRACT

Mass mortalities of marine organisms in the Gulf of Mexico have been associated with blooms of the unarmored dinoflagellate, Gymnodinium breve. This investigation concerns the factors affecting the growth of G. breve and the properties of a toxin produced by the organism.

A modification of the Flask Test of the Provisional Algal Assay Procedure of the Joint Industry/Government Task Force on Eutrophication was used to determine the response of G. breve to various natural waters and enrichments. The growth-promoting potential of natural waters obtained from areas where outbreaks of G. breve frequently occur was greater than that of waters from other areas, and addition of municipal waste materials to natural waters increased the maximum cell population significantly. Although enrichment, individually, with inorganic nutrients (orthophosphate, nitrate, or ammonia) had relatively little effect on G. breve growth parameters, enrichment with combinations of orthophosphate, nitrate and ammonia equivalent to the concentrations attained by enrichment with municipal waste materials effected an almost identical increase in the maximum population. The enrichment experiments with combinations of nutrients indicated that at concentrations of orthophosphate typical of west Florida coastal waters, the maximum cell population was a linear function of the amount of ammonia added (up to 0.10 ppm NH_3-N).

The addition of orthophosphate or detergent-phosphate to municipal wastes, presumably free of detergent phosphate, did not significantly increase the growth-promoting potential of the waste material. Enrichment of G. breve cultures with nitrilotriacetic acid (NTA) alone or in the presence of sewage had no biostimulatory effect at concentrations of NTA expected if NTA completely replaced phosphates in detergent products.

Soil extract fractions were as effective as sewage (1%, v/v) in increasing the maximum cell population; the substances in the 500-1,000 molecular-weight fraction (iron rich) were the most effective on a mg C-added basis. The growth constant was not affected by enrichment with low molecular-weight compounds but was significantly lowered by the addition of the high molecular-weight substances (>10,000).

The toxin produced by G. breve is an endotoxin which is water insoluble and chloroform soluble. The toxic material was acid stable, but heat and base labile. Photo-inactivation of the toxin occurred during irradiation with ultraviolet, visible, or infrared light.

The crude toxic extract was fractionated by silica gel, LH-20 Sephadex gel and DEAE-cellulose column chromatography, and a 27-fold increase in the specific activity of the toxic material was achieved with recovery of 71% of the total activity. The acidic polysaccharide content of the toxic material increased 5-fold during the purification procedure.

Elemental analysis of the purified toxin indicated an empirical formula of $C_{87}H_{153}O_{18}S$, and the infrared absorption spectrum indicated the presence of one or more carbonyl groups and the absence of

hydroxyl or amine groups. The purified toxin had a maximum absorption at 265 mμ that was optically active. The reduced molar ellipticity at 265 mμ was 3.3×10^3 degrees cm^2/decimole.

The effect of the toxin on the prothrombin time of a standard plasma was determined, and the anticoagulant activity of the toxic material was 1/6 that of carrageenan and 1/70 that of heparin. Available evidence suggests the anticoagulant properties of the toxic material are not responsible for the ichthyotoxicity.

Abstract approved: _____, thesis supervisor

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INTRODUCTION

History

Natural blooms of phytoplankton occur periodically in many locations throughout the world. Under certain conditions, these blooms result in discoloration of large areas of surface water and are commonly referred to as "red water" or "red tide". The terms red water and red tide are somewhat misleading in that the water may appear to be brown, yellow, green, milky, or blue depending upon the concentration, depth, and pigmentation of the causative organisms (Ryther, 1955; Sasner, 1965). In certain bodies of water, the regularity of overblooming has led to colorful names such as the Red Sea or the Vermillion Sea (Gulf of California). Although the discolored water may arise from blooms of diatoms, cocopods, or chromogenic bacteria, the majority of red tides are caused by dinoflagellates and blooms often consist of a single species.

In some areas, red tides are accompanied by mass mortalities of marine organisms. The mortalities may be a result of the liberation of toxic substances by the causative microorganism or such secondary factors as dissolved oxygen depletion or hydrogen sulfide production by decomposing organic matter (Brongersma-Sanders, 1957). Although many dinoflagellates, such as Ceratium fuca, are known to be non-toxic (Dragovich et al., 1965), a number of dinoflagellates have been found to produce toxic substances (cf. Table I).

TABLE I

Distribution of Toxigenic Dinoflagellates

Species	Location	Reference
Gymnodinium breve	Gulf of Mexico	Davis, 1948
Gymnodinium veneficum	English Channel	Abbott and Ballantine, 1957
Peridinium poloicum	Lake Sagami, Japan	Hashimoto et al., 1968
Gonyaulax monilata	Gulf of Mexico	Gates and Wilson, 1960
Gonyaulax catenella	California coast	Sommer et al., 1937
Gonyaulax polyedra	California coast	Schradie and Bliss, 1962
Gonyaulax tamarensis	North Atlantic Coastal Waters	Prakash, 1967

Toxic Florida red tides are most often the result of sporadic blooms of the unarmored dinoflagellate Gymnodinium breve. Large concentrations of this organism cause discoloration of Gulf waters and blooms of G. breve are commonly a fish-killing phenomenon (Davis, 1948). Some twenty toxic red tides (three in Texas, seventeen on the west coast of Florida) were recorded during the period from 1844 to 1960 (Rounsefell and Nelson, 1966). Seven major outbreaks were recorded during the period 1947 to 1960 and associated with G. breve. Typically, the outbreaks along the coast of Florida have occurred in late spring or early fall, most often in the area from Cape Sable to Tarpon Springs (Finucane, 1964).

The mechanism of the development of red tides in estuarine and nearshore waters is uncertain. Dinoflagellates are usually present

throughout the year, however, and it has been proposed that micro-blooms of certain organisms occur at a definite time each year as a part of a regular life cycle (Reijiro, 1967). Whether the micro-bloom spreads and becomes a true bloom seems to depend upon two factors: (1) availability of a suitable environment and/or (2) disappearance of competing or predator organisms (Martin and Martin, 1973). Both factors could be associated with municipal wastes (cf. Harris et al., 1972).

Environment

The environment conducive to red tide outbreaks has been intensively investigated during recent years. A thorough analysis of field data (Rounsefell and Nelson, 1966) indicates the organism thrives from 16 to 27°C, and the reported optimum temperature range is 26 to 28°C (Finucane, 1960). G. breve is a neritic organism with an optimum salinity range of 27 to 37 ppt (Aldrich, 1960). Although G. breve has been shown to be a photoautotrophic organism (Aldrich, 1962), light intensity is probably not a growth-limiting factor (Aldrich, 1960). Other physical factors such as the magnitude and direction of the wind, may be important in the environment of red tide outbreaks. The prevailing winds may produce locally high concentrations of G. breve cells or upwelling of nutrient rich waters (Ryther, 1955). Due to the fragility of the organism, however, blooms may require periods of calm or light winds. There is some evidence that periods of unusually high winds (i.e., hurricanes) may prevent outbreaks under otherwise optimum conditions (Ingle and Martin, 1971).

As long as a suitable environment exists, and in the absence of competing or predator organism, phytoplankton will multiply until the

available supply of some nutrient is exhausted. The nutrient that is exhausted first is said to be "limiting". Although many nutrients are essential for the growth of algae, quantitatively the most important are carbon, nitrogen, and phosphorus, respectivley. There is good evidence that "invasion" of carbon dioxide from the atmosphere supplies sufficient carbon to support algal blooms (Schindler et al., 1972), and much attention, therefore, has been focused on the availability of nitrogen and phosphorus. In certain instances, however, trace elements (iron, cobalt, manganese, etc.) have been shown to be limiting (cf. Doig, 1972).

The nutrient requirements of G. breve have been suggested by two types of studies: (1) analysis of field data and (2) laboratory experiments using G. breve as a bioassay organism. Studies of the first type have indicated that blooms of G. breve may occur in waters that are relatively nutrient-poor (Ryther, 1955; Odum et al., 1955), and the available data suggest that sufficient phosphorus to support red tide outbreaks is always present in the waters along the west coast of Florida (Smith, 1957; Bein, 1957). Dragovich (1960) reported the absence of a relationship between nitrate-nitrite concentrations and the observed incidence of G. breve; and there were no statistically significant correlations of G. breve densities with nitrogen or phosphorus concentrations (Rounsefell and Dragovich, 1966).

In the second type of study, bioassays, inorganic phosphorus, but not inorganic nitrogen, was required in artificial, defined medium; specifically 9 µg P/liter and some form of organic nitrogen was required for significant growth (Wilson, 1966). [Only about 4% of some 6,000 samples obtained from Florida west coast waters had less than 9 µg P/liter,

according to Wilson (1960).] Collier and co-workers (1969) reported, however, "The growth [of G. breve] in natural seawater was most clearly enhanced by the addition of EDTA-Fe, sulfide, N and P."

Although considerable evidence suggests that phosphate and nitrate do not normally restrict the growth of G. breve, some investigators have proposed that the increased intensity of red tides in recent years could be a consequence of increased municipal wastes (cf. Steidinger and Ingle, 1972). An alternative and perhaps more logical explanation is that more red tides are observed because of the presence of more observers.

Other workers have noted that red tide outbreaks are often preceded by periods of heavy rainfall (Slobokin, 1953; Collier, 1954; Aldrich and Wilson, 1960). Presumably, river discharge has some effect other than reducing the salinity of coastal waters because lowered salinity is not biologically essential for G. breve growth (Aldrich and Wilson, 1960). Ingle (1965) suggested that flooded rivers, following periods of heavy rainfall, could leach humic substances from inland bogs and transport these natural chelators and associated metals to potential outbreak areas.

Many investigations have indicated that humic materials stimulate plant growth (Burk et al., 1931; Flaig and Otto, 1951; Dekock, 1955), and the growth rates of cultures of dinoflagellates have been enhanced by the addition of soil extracts (Wilson and Collier, 1955). Prakash and Rashid (1968) have studied the effect of selected soil fractions on the growth of several species of armored dinoflagellates of the genus Gonyaulax. The stimulatory effect of the humic acid fraction (alkali-soluble, acid- and alcohol-insoluble) of marine sediments was

greater than that of the fulvic acid fraction (acid-soluble). The humic acid was further fractionated by gel-filtration chromatography on Sephadex G-10, G-15, G-25, and G-50 gels. The low molecular weight fractions produced the greatest growth enhancement.

Humic substances unquestionably take part in the solubilizing and transport of trace metals (Shapiro, 1957, 1958, 1964; Black and Christman, 1963; Kee and Bloomfield, 1961), and moreover, chelated metals may be required by G. breve. Wilson (1966) reported that the suitability of natural sea water samples (as indicated by the criteria that the G. breve either survived or increased in number) was enhanced in 79% of the samples studied by addition of a chelating agent (EDTA (50 ppm), and iron (0.02 ppm) plus EDTA (50 ppm) enhanced the suitability in 85% of the samples studied. The function of the chelating agent may be two-fold: (1) to detoxify certain metal ions and/or (2) to enhance the availability of other metal ions (Martin and Martin, 1973).

The majority of the soluble humic material in coastal waters is probably from the terrestrial reservoir. Nevertheless, marine sediments may be significant if microblooms arise from excystment of G. breve cysts which are in contact with the sediment. Additional marine humic substances may be generated in situ (Nissenbaum and Kaplan, 1972). Regardless of the source of the humic substances, any study of their effect on G. breve cultures must first overcome a serious problem: the procedures employed in the extraction of humic and fulvic acids from soil humus are rigorous (Felbeck, 1965) and may result in the alteration or complete destruction of compounds of ecological significance. For example, the growth of G. breve may be influenced by the availability of vitamins, auxins, or hormones.

The factors which affect the growth of the Florida red tide organism are a matter of concern because of the dramatic effects that blooms of G. breve have upon the biological community in which they occur. Toxic blooms of G. breve may become serious ecological, economic, and public health problems.

Consequences of Toxicity

The ecological and economic problems associated with blooms of G. breve are a direct result of the toxicity of the organism. Over 100 species of dead animals have been found during red tide outbreaks (cf. Steidinger et al., 1973), and the list includes fish, shellfish, birds, turtles, porpoises, barnacles, and other invertebrates (e.g., sponges). Fish mortalities are particularly extensive and it was estimated that 500 million fish were killed during the 1947 outbreak. The estimate is undoubtedly conservative, and does not include small fish or fish that are consumed by scavangers. The impact of such massive mortalities on sport and commercial fishing is difficult to assess, but analyses of commercial catches following red tide outbreaks indicate that commercial fisheries are not seriously affected (Rounsefell and Nelson, 1966; Torpey and Ingle, 1966). More serious economic consequences result from the large number of dead fish which are washed on shore. These fish pose a serious sanitation and disposal problem and clean-up costs to municipal and county governments are substantial. Although the 1971 outbreak was not as severe as the 1947 outbreak and clean-up efforts were far from adequate, the costs to St. Petersburg alone were $10,000 per day and totaled approximately $500,000. Far greater losses resulted from the fact

that tourists avoided the area during the nationally-publicized outbreak and for several months thereafter. Complete data are not available for the entire area affected by the 1947 red tide, but it has been estimated that the city of Clearwater lost 3.75 million dollars of tourist trade during the outbreak (Martin and Martin, 1973). The economic impact of the 1971 outbreak was also impressive; $12,000,000 for a five-county area, according to some estimates.

Coastal residents may be affected directly, or indirectly, by G. breve blooms. Respiratory and eye irritations from wind-driven aerosols are a direct consequence of the presence of G. breve in off-shore waters (Woodcock, 1948). During the 1971 outbreak, many residents suffered respiratory distress (or other toxic symptoms) whenever the off-shore breeze exceeded about 10 miles per hour, and some were hospitalized. The indirect danger to man is transfer of the toxin through the food chain, particularly through shellfish. Filter-feeding shellfish accumulate dinoflagellate toxins and over 220 human fatalities have been attributed to paralytic shellfish poisoning (Halstead, 1965). Although no deaths have been reported which were a result of ingesting shellfish exposed to blooms of G. breve, mild illnesses caused by ingestion of mussels and clams harvested from Sarasota Bay during a red tide have been reported (McFarren et al., 1965). Also toxic substances have been extracted from oysters, clams, and coquinas collected during the 1967 red tide (Cummins et al., 1971), and oysters exposed to laboratory cultures of G. breve have been shown to be toxic when fed to chicks (Ray and Aldrich, 1965, 1967). Apparently, an incidence of reported shellfish poisoning associated with blooms of G. breve is low for two reasons:

(1) the effects on humans are mild and temporary and (2) salinity often prevents the development of G. breve blooms in areas containing commercially exploitable quantities of shellfish (i.e., estuaries).

The toxicity of blooms of G. breve is also of interest because of the intrinsic pharmacological potential of biotoxins. Baslow (1969, 1971) has noted that many "toxins" ultimately lead to the production of useful drugs, particularly after careful isolation, characterization, and alteration by synthetic means. The term "drug" need not be limited to its strictly medical usage, but should denote any chemical substance that affects a specific physiological function (Fingl and Woodbury, 1965). However, very few marine biotoxins have been chemically characterized to any extent (cf. Doig et al., 1973), and certainly many of the physical, chemical, and physiological properties of the toxin produced by G. breve are as yet undetermined.

Toxin Properties

Subsequent to the development of a suitable media for mass culturing of G. breve (Wilson and Collier, 1955), unialgal and bacteria-free cultures of G. breve were shown to be ichtyocidal (Ray and Wilson, 1957; Starr, 1958), and toxins possessing remarkably different properties have since been partially characterized by a number of workers.

Sasner (1965) isolated a toxic substance that he described as "water and ethyl alcohol soluble, chloroform insoluble, heat labile, acid stable and slowly dialyzable." The toxic material was a general depolarizing agent and rendered all excitable membranes tested inexcitable to electrical stimulation. More recently, Sasner and coworkers (1972) have purified a toxic ether extract by column and thin-layer chromatography on silicic acid. The toxic substance had a

molecular weight of 279, exhibited an anticholinesterase-like activity, and blocked neuromuscular transmission in frog sartorius preparations before rendering the nerve or muscle inexcitable to stimuli. Synaptic effects were blocked by curare, and the end plate appeared to be the primary site of action. No anticholinesterase activity was observed by Siger and co-workers (1972) in similar preparations, and based on the subthreshold actions of the toxin, they concluded that the toxin is an antagonist of the normal function of calcium in the membrane.

Cummins and co-workers (1968) obtained two toxic components after continuous extraction with diethyl ether for 24 hours in a liquid-liquid extractor, elution from a silica gel column using successively increasing quantities of methanol in chloroform, and thin layer chromatography on silica gel (solvent system, $CHCl_3$:CH_3OH:6 $\underline{N}$ NH_4OH, 90:9.5:0.5, v/v). Two toxins, one with a molecular weight of 650 and empirical formula $C_{90}H_{162}O_{17}P$, were also obtained by Martin and Chatterjee (1969, 1970) after extraction with chloroform and silica gel column chromatography. A toxin with a molecular weight and elemental analysis quite similar to the toxin characterized by Martin and Chatterjee has been isolated by extraction with diethyl ether and purification by dry column chromatography on silica gel (Trieff et al., 1972). Finally, three biologically active compounds, one hemolytic and two neurotoxic, were isolated by Spiegelstein, Paster and Abbott (1973); however, there was no evidence that the hemolytic activity is associated with the ichthyotoxicity.

STATEMENT OF THE PROBLEM

The factors affecting the growth of the unarmored dinoflagellate, Gymnodinium breve, have been of considerable interest since G. breve was identified (Davis, 1948) and associated with mass mortalities of marine organisms in the Gulf of Mexico (Davis, 1948; Galtsoff, 1948, 1949; Gunter et al., 1948; Wilson and Ray, 1956). However, the ecological factors which favor the proliferation of a single species to nuisance levels are difficult to determine because blooms are usually short-lived and occur at unpredictable geographic locations and sporadic times. Bioassays with unialgal cultures obviate many of these problems and provide valuable information concerning the relative enrichment characteristics of various waters, wastes, and other nutrient sources.

Circumstantial evidence seems to indicate an association between burgeoning population areas around estuaries with concomitant pollution and red tide outbreaks. Despite the evidence to the contrary, much attention has been focused on the problem of phosphorus control as a means of limiting red tide growth. The simple expedient of removing phosphates from detergent products would eliminate 40 to 70 percent of the phosphorus contained in sewage, and several persons suggested in August 1971 that failure to initiate a ban on phosphorus-containing detergents in Dade County could bring about a red tide outbreak such as was then prevalent in coastal waters from Clearwater to Venice.

The proposed research would determine the response of G. breve cultures to enrichment with inorganic nutrients and domestic wastes, and in addition, the effect of detergent phosphates on the growth of this organism would be evaluated in a realistic manner. The growth-promoting potential of naturally occurring organic substances would be determined for comparison.

The proposed research would also extend our knowledge of the properties of the toxin produced by G. breve; specifically, the extraction and purification procedures described by Martin and Chatterjee (1970) would be expanded, a toxin assay method developed, and the stability, intracellular location, and biological activities of the toxic material determined. The purified toxin would be characterized by chemical and spectroscopic analyses.

The goals and methods of the proposed research are summarized in Fig. 1.

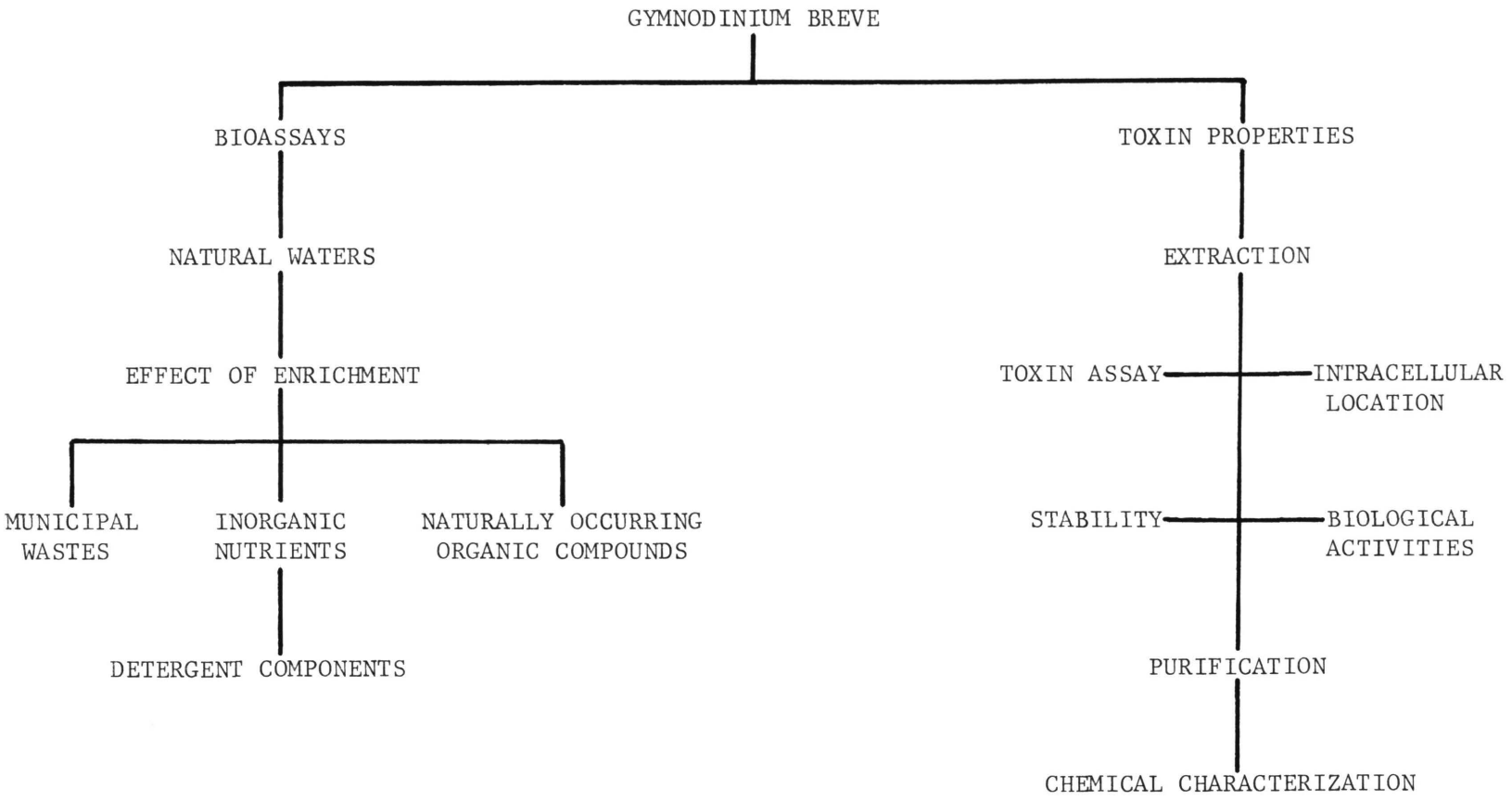

FIG. 1. Goals and methods of the proposed research.

EXPERIMENTAL

Materials

Organisms: Samples of Gymnodinium breve were originally obtained as bacteria-free unialgal cultures from S. M. Ray and W. B. Wilson (Texas A & M Marine Station, Galveston) and were cultured in an enriched sea water medium (B-5, cf. Table II). The sea water used in the preparation of B-5 medium was collected at sampling stations from three to ten miles due west of St. Petersburg in the Gulf of Mexico. Samples were filtered through sand and stored in the dark in five-gallon glass carboys for approximately three months. The "aged" seawater was stirred with activated charcoal (ca. 4 g/5 gal) for 30 min and filtered through 0.45μ and 0.22μ membrane filters (Millipore Corp.). The filtered sea water was then enriched with Fe·EDTA and sulfide solutions and autoclaved at 15 psi for 15 min. The autoclaved medium was stored for at least three days before the vitamin solutions were added aseptically through a disposable Swinnex filter unit (Millipore Corp.) with a 0.22μ membrane filter. Stock cultures were maintained in the enriched sea water medium in 8-liter carboys and aliquots were aseptically removed for bioassays and toxin extractions.

Additional organisms were collected from Old Tampa Bay (for toxin extractions) in July, 1971, during the 1971 red tide outbreak described elsewhere (Simon and Dauer, 1972; Steidinger and Ingle, 1972). There were mass mortalities of fish during the collection period, and G. breve was identified as the major algal species present. Cell counts varied

TABLE II

Chemical Composition of B-5 Culture Medium

(after Brydon et al., 1971)

Component	Amount
Aged sea water	1 liter
Sulfides solution[a]	5.0 ml
Fe·EDTA solution[b]	1.0 ml
Vitamin B_{12}	1.0 µg
Biotin	0.5 µg
Thiamine·HCl	10.0 µg

[a]Sulfides solution (g/liter): NH_4Cl (0.2), K_2HPO_4 (0.1) $NaHCO_3$ (0.2), $MgCl_2 \cdot 6H_2O$ (0.04), $Na_2S \cdot 9H_2O$ (0.15).

[b]Fe·EDTA solution (g/liter): Na_2EDTA (50.0), $FeCl_3 \cdot 6H_2O$ (2.0).

from 250,000 cells per liter to 2,500,000 cells per liter. Approximately 1,000 liters of water were collected.

Water Samples: Surface water samples were collected from an area where red tides frequently occur (Tampa Bay and the Gulf of Mexico one to three miles off St. Petersburg) and from an area where no outbreaks of G. breve had been reported prior to the present study (Biscayne Bay Juno Beach, and the Atlantic Ocean one to three miles off Port Everglades). The nutrient composition of these waters after filtration through 0.22µ membrane filters (Millipore Corp.) and sterilization is summarized in Table III. Two hundred milliter aliquots of these waters

were used in the algal assays, and all enrichments were added aseptically through Swinnex filter units.

TABLE III

Nutrient Composition of Natural Waters Used as Assay Media

Parameter	Juno Beach	Tampa Bay	Biscayne Bay	Gulf of Mexico	Atlantic Ocean
Salinity (ppt)	34.5	32.0	34.0	34.5	34.0
pH	8.20	8.10	8.15	8.20	8.20
Total PO_4-P (ppm)	0.025	1.39	0.125	0.360	0.100
NH_3-N (ppm)	0.021	0.015	0.021	0.001	0.001
NO_3 + NO_2-N (ppm)	0.0174	0.0084	0.0191	0.0075	0.0094
SiO_2-Si (ppm)	2.00	0.56	1.75	0.41	1.24

Enrichments: Enrichment was accomplished by addition of small aliquots of a phosphate solution (0.4894 g KH_2PO_4/liter), a nitrilotriacetic acid solution (10 g NTA/liter), or a detergent solution (0.885 g /liter). The solutions were prepared in triple-distilled water, and equal volumes of triple-distilled water were added to control samples. The detergent was approximately 13% surfactant, 4.3% soap, 5.5% sodium silicate, 45.5% sodium tripolyphosphate and 5% sodium perborate.

Municipal wastes were collected at a small sewage treatment facility (average flow of 2.5 million gallons per day) located in the Miami area where a ban on phosphate detergents had been in effect for six months.

During this period, the influent phosphate concentrations had decreased approximately 40%. The plant is a contact stabilization unit, and the final effluent composition is summarized in Table IV. Enrichments to the assay medium were made in the presence and absence of aliquots of this waste material.

TABLE IV

Composition of the Sewage Treatment Plant Effluent

Parameter	Concentration (ppm)
Five Day BOD	25.5
Chemical Oxygen Demand	80.0
Total Suspended Solids	365
Volatile Suspended Solids	35
Total Organic Carbon	20.1
Total Phosphate	10.0
Kjeldahl Nitrogen	20.5
Ammonia Nitrogen	18.4
Nitrite-Nitrate Nitrogen	0.4
Organic Nitrogen	1.3

Soil Extracts: Soil from a peat bog located in the Tampa area was picked free of macroscopic organisms and large debris and air-dried at room temperature (25 ± 2°C) for 24 hours. The soil sample (200 g) was extracted with 3 liters of distilled water (pH 6.5) for 36 hours with constant mixing. The mixture was filtered through 0.22μ membrane filters (Millipore Corp.) and the characteristics of the filtrate, hereafter referred to as the crude soil extract, were determined (Table V).

TABLE V

Characteristics of the Crude Soil Extract

Characteristic	Value
Final pH	4.5
Total Volume	2500 ml
Total Nonvolatile Material	275 mg/liter
Total Organic Carbon	61 mg/liter
Humic Acid	0.77 mg/liter
Iron	0.096 mg/liter

The crude soil extract was separated into four molecular-weight fractions by means of Amicon Diaflo ultramembranes (UM 05, UM 2, UM 10) using an Amicon filtration cell under a nitrogen pressure of 50 psi. The fraction retained by each membrane was washed and concentrated as described by Gjessing (1970).

Methods

Cell Enumeration: Cell counts were determined electronically with a Model B Coulter Counter after sterile removal of aliquots from test cultures. The instrument was calibrated with a suspension of polystyrene divinylbenzene spheres (Duke Standard Co.) in filtered sea water, and the calibration factor was 830 μ^3/threshold unit (Aperature Current Setting: 2, Amplification Setting: 4, Aperature Size: 100 μ). The size distribution of an axenic culture of G. breve was determined (cf. Fig. 2) and counts used in determining the growth parameters of test cultures included cells from the entire size range (Lower

Threshold Setting: 8; Upper Threshold Setting: 40). Random variations about some mean value occur at the relatively low particle concentrations of unialgal cultures and the reproducability of a count is a function of the number of particles counted (cf. Table VI).

TABLE VI

Reproducibility of Cell Enumeration with the Coulter Counter (Model B)

Mean Count[a]	Standard Deviation	Rel Std Deviation[b]
20	4	20.0
45	7	16.0
81	6	7.6
143	14	9.8
305	26	8.6
641	35	5.6
1278	47	3.7
2574	81	3.2

[a]Mean (cells/0.5 ml) for 10 replicate determinations.

[b]Relative standard deviation (100 x S.D./Mean).

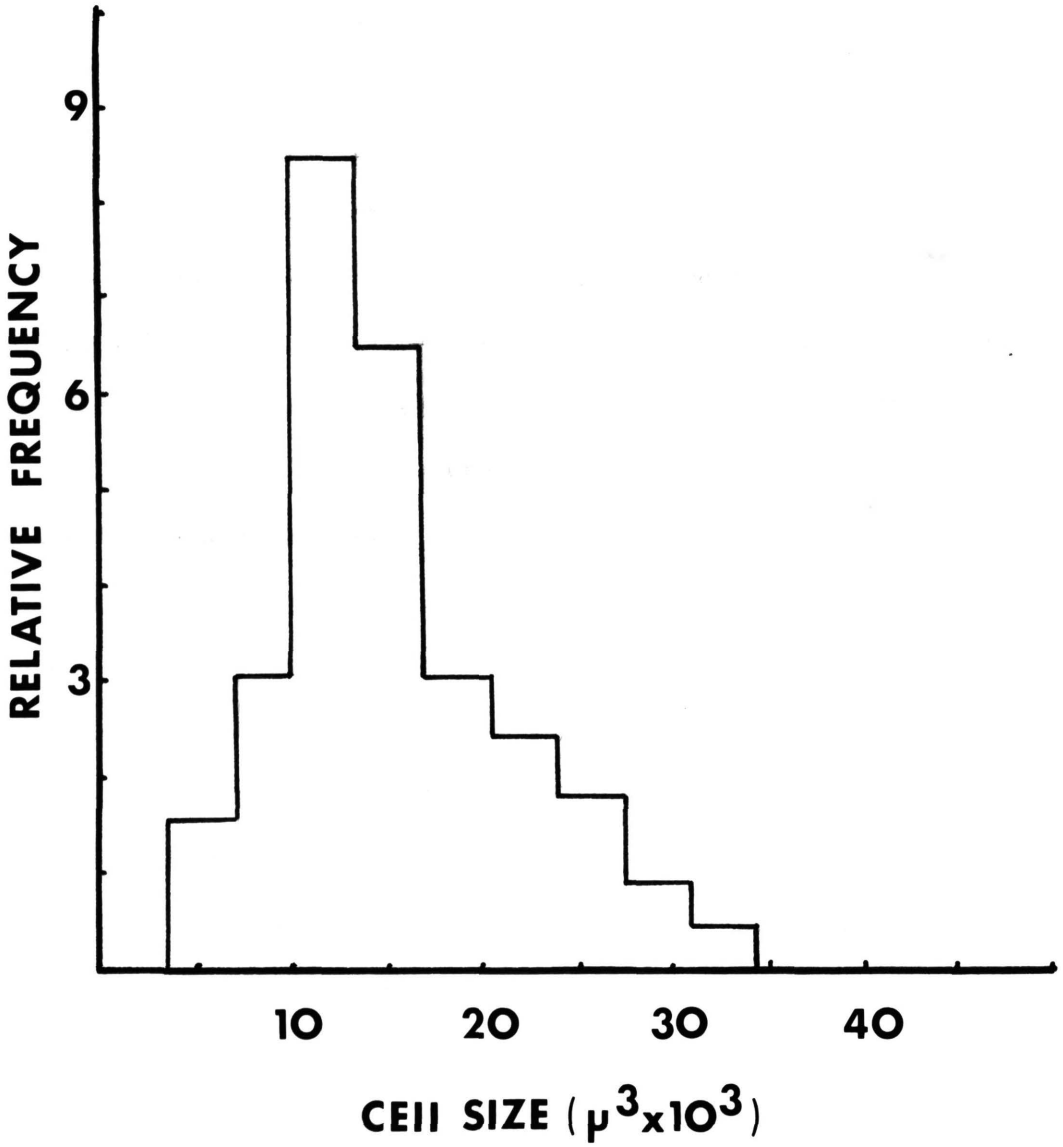

FIG. 2. Size distribution of an axenic culture of Gymnodinium breve.

Bioassays: A modification of the Flask Test of the Provisional Algal Assay Procedure of the Joint Industry/Government Task Force on Eutrophication (1969) was used to determine the response of G. breve to various natural waters and enrichments. Inoculum was aseptically transferred to 200-ml aliquots of test medium in 250-ml Erlenmeyer flasks when the parent (stock) culture had reached the stationary phase of growth. The inoculated flasks (initial count approximately 150 cells/ml) were maintained at 25° ± 2°C under constant illumination of approximately 600 ft-candles, provided by dual banks of 40-watt cool-white fluorescent lamps.

All glassware was rigorously cleaned by washing with detergent (Acationox) and soaking in concentrated hydrochloric acid (12 M). After thorough rinsing with distilled water, the culture flasks were filled with distilled water, stoppered with disposable foam plugs, and autoclaved at 15 psi for 30 min.

Cell counts were determined as previously described (cf. Cell Enumeration) after sterile removal of an aliquot of medium. The aliquots were obtained from triplicate cultures which were counted every two to three days. Aliquots from each culture were counted five to seven times, and all results were used to calculate the mean cell count and the standard deviation of the mean.

Cell viability was determined visually at 100X with a binocular microscope.

Chemical Analyses: Chemical analyses of water and waste samples were performed by the standard procedures described in the EPA methods manual (Environmental Protection Agency, 1971), with several exceptions. Humic acid concentrations were determined spectrophotometrically after extraction (Martin and Pierce, 1971) and trace metal concentrations

were determined by atomic absorption spectroscopy using a Perkin-Elmer Model 403 Atomic Absorption Spectrophotometer equipped with a graphite furnace. Salinities were determined with a hand-held refractometer.

Toxin Extraction: Six/liter aliquots of water were acidified (to pH 4.0) by dropwise addition of concentrated hydrochloric acid (12 M) and extracted with 90 ml of chloroform for 15 minutes. The extractions were carried out in large glass jars with mechanical stirrers.

After extraction, the mixture was allowed to settle for 15 min and the majority of the water was siphoned from the jar. The remainder of the mixture was transferred to a separatory column, and the chloroform layer was removed from the bottom. Also, an interfacial layer was collected and reextracted with 50-ml aliquots of chloroform until the extract was not longer colored (ca. 250 ml). Additional water was removed from the chloroform extract by centrifugation (30,900 x g, 15 min) in a Sorval SRC-2B centrifuge.

Toxin Assay: The toxin activity was characterized by means of ichthyocidal activity toward Poecilia sphenops. The standard assay procedure was to add an aliquot of toxin in methanol to 50 ml of fresh water (pH 7, 23°C) in a 400-ml tall-form beaker, place a 1.0 to 2.5 g fish (P. sphenops 4.0 to 6.0 cm) in the assay mixture, and determine the time (in minutes) to death. Controls with methanol were tested concurrently with each set of assays.

P. sphenops was selected as the assay organism because the fish are commercially available, easily maintained, and very sensitive to G. breve toxin. The first symptom of intoxication was hyperactivity

followed by a period of inactivity during which there was a loss of equilibrium. Death time was defined as the instant at which operculum movement ceased, and the variation of death time with toxin concentration is indicated in Fig. 3. For assay purposes, a toxin unit (TU) was defined as that weight of toxin needed to produce death in 15-30 minutes under standard assay conditions. The limitations of the assay are indicated by the data in Table VII. The death time was recorded for twenty fish of various lengths and weights. The data show no significant correlation of death time with weight, using one toxin unit, though the correlation of weight and length for this fish is significant (even at the $P < 0.001$ level). Effectively, the conditions were so selected that death times corresponding to one toxin unit are reasonably short and still within the concentration-sensitive region of the response curve (Fig. 3).

Toxin Stability: The effects of temperature, pH, light , and storage time on toxicity were determined using 0.200 ml aliquots (0.226 mg of nonvolatile material) of the crude chloroform extract. This aliquot was equivalent to one toxin unit.

The effect of temperature on toxicity was tested by adding an aliquot of toxin (in methanol) to 5.0 ml of distilled water (pH 7.2) in a Pyrex glass test tube (15 x 125 mm). The test tube was stoppered and placed in a constant-temperature bath at the desired temperature for one hour. The rate of inactivation at a fixed temperature was determined under the same conditions except that the tubes were removed at various time intervals. After the tubes were removed from the baths, they were rapidly cooled to room temperature (23°C) and tested for toxicity by the standard assay procedure.

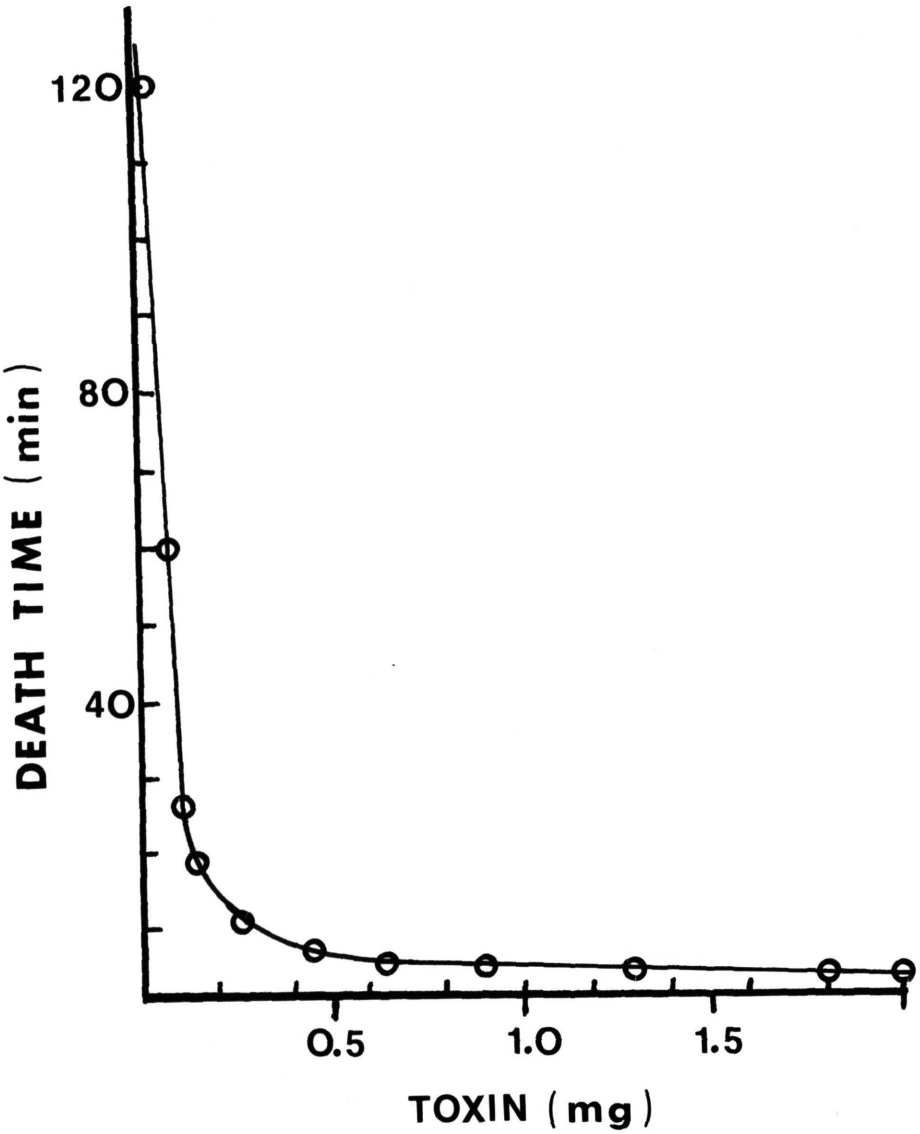

FIG. 3. Response of Poecilia sphenops to Gymnodinium breve toxin.

TABLE 7

Ichthyoxidal Assay of Gymnodinium breve Toxin

Parameter[a]	Mean	Range	Standard Deviation, S	Correlation Coefficient, r
Death Time, min	20.4	15-28	3.8	
Fish Weight, g	1.4	0.7-2.6	0.6	-0.21
Fish Length, cm	4.7	3.9-6.1	0.7	0.90[b]

[a]Based on 20 specimens of Poecilia sphenops.

[b]Significant at $P < 0.001$.

The effect of pH on toxicity was determined by placing the aliquot of toxin (in methanol) in 5.0 ml of distilled water that had been adjusted to pH values ranging from 1 to 13 with hydrochloric acid or sodium hydroxide. This solution was allowed to stand at room temperature (23°C) for 48 hours, and then was diluted to 50 ml, adjusted to pH 7, and assayed.

Aliquots of toxin were placed in 10-ml glass Petri dishes and dried (producing a thin film) at room temperature. The dishes were placed under ultraviolet (9 watt, 5-cm distance), infrared (250 watt, 20-cm distance), or visible (150 watt, 10-cm distance) lamps for 12 hours to determine the effects of each type of radiation on toxicity. Controls wrapped in black paper were placed under each lamp, and an additional control was left in the dark at room temperature. The weight of the toxic residue was determined before and after irradiation. After irradiation, the residues were dissolved in methanol and tested for toxicity in the usual manner.

The toxic solution used in the previously described stability tests was stored in the dark at approximately 10°C, and aliquots were assayed periodically for six months.

Intracellular Location: The intracellular location of the toxin was evaluated by means of continuous centrifugation of lysed and non-lysed samples in a Sorval RC2B centrifuge (SS34 head; 10,000 rpm; 12,100 x g) equipped with a Szent-Gyorgy and Blum continuous-flow system (flow rate: 70 ml/min). The pellets and supernatants from 10-liter samples (10^6 cells/liter) were lysed, extracted, and assayed for ichthyotoxicity as previously described (cf. Methods, Toxin Extraction and Toxicity Assays).

Toxin Purification: The toxic material in the crude extract was purified by the procedure summarized in Fig. 4. The column chromatographic procedures were essentially those of Rouser and co-workers (1967). Reagent silica for chromatographic columns (50-200 mesh, G. Frederick Smith Chemical Co.) was activated in an oven at 110°C for 2 hr, and gels were preswelled and columns poured in the first eluting solvent. The column (2.0 cm x 35 cm) was then washed with 10-column volumes of each solvent to be used. Bulk fractions were collected at a flow rate of 3 ml per minute. Sephadex LH-20 gel (25-100 μ) was preswelled in excess chloroform and, after removal of fines, was poured into a 2.6 cm x 30 cm reversed-flow column (Pharmacia, SR 25/45). The column was washed with 10-column volumes of chloroform, the sample (in chloroform) was then placed on the column through a three-way valve (Pharmacia, LV-3), and 10-ml fractions were collected at a flow rate of 1 ml/min with a Gilson Model VL volumetric fractionator. Diethylaminoethyl (DEAE) cellulose (Whatman DE 32, 1.0 meq/g) was washed with one

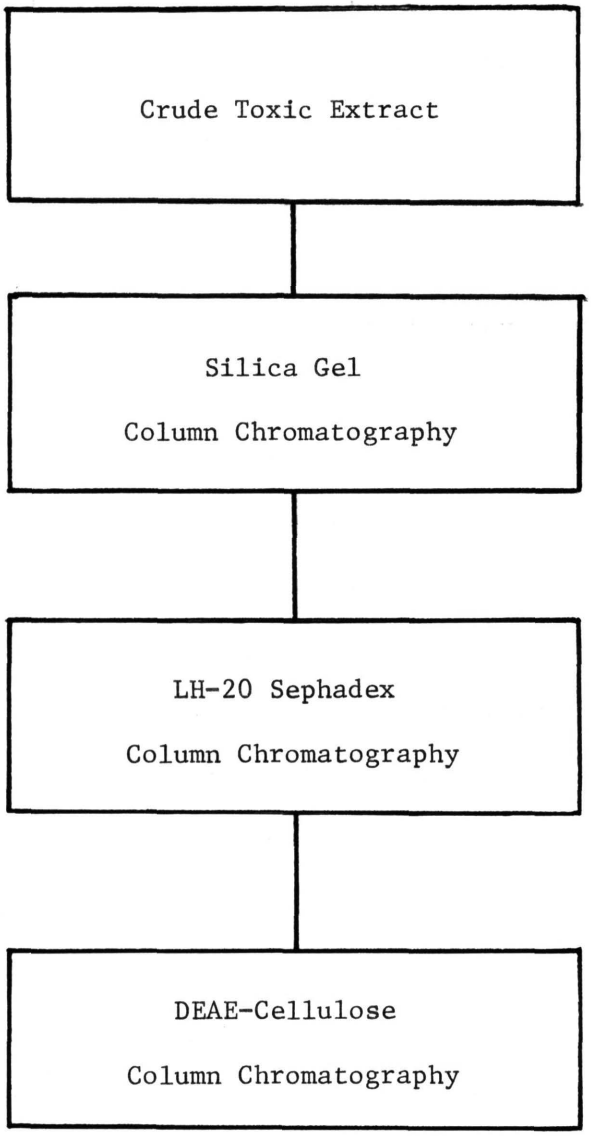

FIG. 4. Summary of Gymnodinium breve toxin purification procedure.

liter of 0.5 N HCl followed by 2 liters of 0.5 N NaOH, preswelled in glacial acetic acid for 12 hr, and poured into a 2.6 cm x 18 cm reversed-flow column after removal of fines. The packed column was washed with 5-column volumes of each solvent to be used as an eluent (in reverse order). Fractions of 12-ml each were collected as previously described at a flow rate of 1 ml/min.

The crude toxic extract and the purified toxin were examined by thin layer chromatography (TLC) on 0.25 mm thick pre-coated TLC plates (Silica Gel F-254, EM Laboratories, Inc.). The plates were activated at 110°C for 30 min prior to use and were developed in chloroform-methanol (90:10, v/v). The chromatographic purity of the purified toxin was determined using a number of additional solvent systems (cf. Table XXI). The dried plates, after chromatography, were examined under long (350) and short (254) wavelength ultraviolet light (Chromato-Vue, Ultraviolet Products Inc.) and were also exposed to iodine vapor for visualization of organic matter.

Acidic Polysaccharide Determination: The acidic polysaccharide contents of the crude toxic extract and the purified toxin were determined by the procedure described by Kim and Martin (1972). The method is based upon the interaction of polysaccharides with a cationic carbocyanine dye in an acidic solution. The dye undergoes a spectral shift to shorter wavelengths in the presence of lipopolysaccharides and a spectral shift to longer wavelengths in the presence of acidic polysaccharides. The amount of acidic polysaccharide present was expressed in terms of heparin equivalents per milligram of material using the relationship: A_{600} = 0.043 μg heparin equivalent.

Anticoagulant Activity: The effect of the toxin (and other test compounds) on prothrombin time was determined with a Fibrometer Precision Coagulation Timer (Becton, Dickinson and Co.) and a standard human plasma (Verify Normal Oxalate). Aliquots (0.050 ml) of test solution (in methanol-water, 50/50, v/v) were added to 0.20 ml of Ortho brain thromboplastin and were placed in a warming well (37°C) of the fibrometer for 5 minutes. A 0.1-ml aliquot of standard plasma was added to the reaction mixture after temperature equilibrium had been established and the time necessary for coagulation was determined.

The addional test compounds included: sodium heparin (147 units/mg, Nutritional Biochemicals), a potent anticoagulant; prymnesin, the toxin isolated from the euryhaline chrysomonad Prymnesium parvum (a gift of Dr. G. M. Padilla, Department of Physiology, Duke University Medical Center); alginic acid (ammonium-calcium salt, type V, Sigma Chemical Co.) from Macrocystis pyrifera, a nonsulfated-polysaccharide with no known anticoagulant activity; and gum carrageenan (potassium salt, Sigma Chemical Co.) from Irish moss, a sulfated-polysaccharide anticoagulant of marine origin.

RESULTS AND DISCUSSION

Bioassays

The response of G. breve to various natural waters and enrichments was evaluated in terms of four growth parameters that were obtained from semi-logarithmic plots of mean cell count (N) as a function of time (t). Typically, the plots consisted of four phases. The lag phase was the period after inoculation during which little or no growth occurred, and often the cell count decreased during this period. Following the lag phase, the cell count increased exponentially (log phase) until the supply of some nutrient was exhausted and a plateau period was then observed. The plateau phase, which was often of long duration, was followed by a decline in the cell population (death phase). Lag times and maximum cell counts were taken directly from the semi-logarithmic plots. The growth constant (K_e) was calculated from data corresponding to the linear portion of the semi-logarithmic plot using the expression:

$$K_e = \frac{1}{(t - t_o)} \ln \frac{N}{N_o}$$

where N and N_o are mean cell counts at times t and t_o. The mean generation time (t_g) was determined from the relationship:

$$t_g = \ln 2/K_e$$

The growth-promoting potential of natural waters from various localities was determined before and after enrichment with iron-EDTA,

sulfides, and vitamins as described by Brydon and coworkers (1971) for B-5 culture medium. The growth parameters for the waters tested are summarized in Table VIII. Enrichment significantly increased the maximum cell concentration in each case, but had little effect on the growth constant. The waters obtained from areas where outbreaks frequently occur seem to have greater potential for supporting the growth of G. breve, but all of the non-enriched cultures supported growth of the organism to cell concentrations greater than the generally accepted toxic level for blooms (ca. 200 cells/ml). These results indicate that although blooms are more likely to occur along the west coast of Florida, they may be sustained for some time in waters from the east coast; and the results are consistent with the fact that a minor red tide outbreak was observed off the coast of southwest Florida during September 1972. Also, the west coast outbreak apparently seeded an unprecedented red tide on the southeast coast of Florida that was heaviest off the coast of Palm Beach during November 1972.

A significant difference in the nutrient composition of the natural waters studied was the phosphate concentration (cf. Table III). The apparent correlation (linear correlation coefficient, $r = 0.990$; probability level, $P = 0.01$) between the phosphate concentrations of these waters and the maximum cell population attained indicated the need for a detailed investigation of the effect of nutrient additions on the growth of G. breve. The rate of photosynthetic production of organic matter in the sea is frequently limited by the availability of inorganic phosphorus or nitrogen; therefore, the effects of additions of phosphate, nitrate, and ammonia to an Atlantic Ocean near-shore sample (Juno Beach) having low nutrient concentrations were determined.

These are summarized in Tables IX, X, and XI and Figure 5. The lag times, growth constants and mean generation times are not significantly affected by nutrient additions except at concentrations of ammonia that may have been high enough to produce an initial toxicity. The maximum cell count is unaffected by nitrate additions and decreased by high concentrations of ammonia. Enrichment with phosphate to 0.075 ppm PO_4-P effects a 30% increase in the maximum cell population; further increases in phosphate concentration have no effect on this growth parameter (cf. Fig. 5). This type of growth, in which the growth rate remains constant but growth continues longer at higher concentrations of the limiting nutrient and thus increases the maximum cell population is often observed in bioassays utilizing batch cultures. O'Brien (1972) has suggested that the rate of growth changes only at very low nutrient concentrations that occur as the maximum population is approached. An alternative explanation is that other rate-limiting factors obscured the effects of added nutrients. In eutrophic waters the limiting nutrient may well be something other than nitrogen or phosphorus.

Eutrophication is most often a consequence of the introduction of domestic wastes into natural waters. The effect of enrichments with municipal wastes on G. breve growth is shown in Table XII. The addition of aliquots of the effluent from a secondary sewage treatment facility to low-nutrient Atlantic Ocean water had a pronounced effect on the growth-promoting properties of the media. The final cell population reached a maximum of approximately three times control values at sewage concentrations of 1%. Thus, enrichment with sewage had a much greater effect on the final yield than equivalent additions of phosphate (or inorganic nitrogen sources) alone. However, addition

Table VIII

Growth of Gymnodinium breve in natural waters

Culture[a]	Lag time (days)	Max. cell count (cells/ml)	K_e ($days^{-1}$)	t_g (days)
AO-NE	2.6	590 ± 40	0.160 ± 0.007	4.3
-E	5.0	3050 ± 250	0.162 ± 0.007	4.3
GM-NE	3.3	800 ± 50	0.180 ± 0.026	3.8
-E	5.0	3500 ± 150	0.197 ± 0.007	3.5
TB-NE	10.0	1400 ± 75	0.145 ± 0.017	4.8
E	7.0	5600 ± 200	0.194 ± 0.013	3.6
BB-NE	4.2	680 ± 40	0.176 ± 0.020	3.9
-E	4.2	2800 ± 150	0.183 ± 0.026	3.8

[a]AO, Atlantic Ocean; GM, Gulf of Mexico; TB, Tampa Bay; BB, Biscayne Bay; NE, non-enriched; E, enriched. (See Materials and Methods for the details of Fe-EDTA + sulfides enrichment.)

Table IX

Effect of phosphate enrichments on G. breve growth parameters

PO_4-P (ppm)*	Lag time (days)	N_{max} (cells/ml)	K_e ($days^{-1}$)	t_g (days)
0.025	2.5	480 ± 30	0.196 ± 0.012	3.5
0.050	2.8	550 ± 40	0.230 ± 0.005	3.0
0.075	2.8	625 ± 55	0.240 ± 0.003	2.9
0.125	2.8	625 ± 60	0.223 ± 0.007	3.1
0.225	2.7	610 ± 35	0.224 ± 0.009	3.1
0.425	2.5	640 ± 45	0.226 ± 0.012	3.1

*Final orthophosphate concentration.

Table X

Effect of nitrate enrichments on G. breve growth parameters in Juno Beach water

NO_3-N (ppm)*	Lag time (days)	N_{max} (cells/ml)	K_e ($days^{-1}$)	t_g (days)
0.017	2.5	480 ± 30	0.196 ± 0.012	3.5
0.042	2.8	470 ± 35	0.256 ± 0.007	2.7
0.067	2.7	480 ± 45	0.257 ± 0.023	2.7
0.117	2.1	530 ± 40	0.215 ± 0.005	3.2
0.217	2.9	460 ± 45	0.225 ± 0.016	3.1
0.417	2.7	480 ± 40	0.218 ± 0.004	3.2

*Final nitrate concentration.

Table XI

Effect of ammonia enrichments on G. breve growth parameters in Juno Beach water

NH_3-N (ppm)*	Lag time (days)	N_{max} (cells/ml)	K_e ($days^{-1}$)	t_g (days)
0.021	2.5	480 ± 30	0.196 ± 0.012	3.5
0.046	2.3	520 ± 35	0.258 ± 0.028	2.7
0.071	2.9	389 ± 65	0.153 ± 0.007	4.5
0.121	2.7	435 ± 40	0.177 ± 0.025	3.9
0.221	3.8	390 ± 45	0.163 ± 0.005	4.3
0.421	3.8	275 ± 35	0.112 ± 0.007	6.2

*Final ammonia concentration.

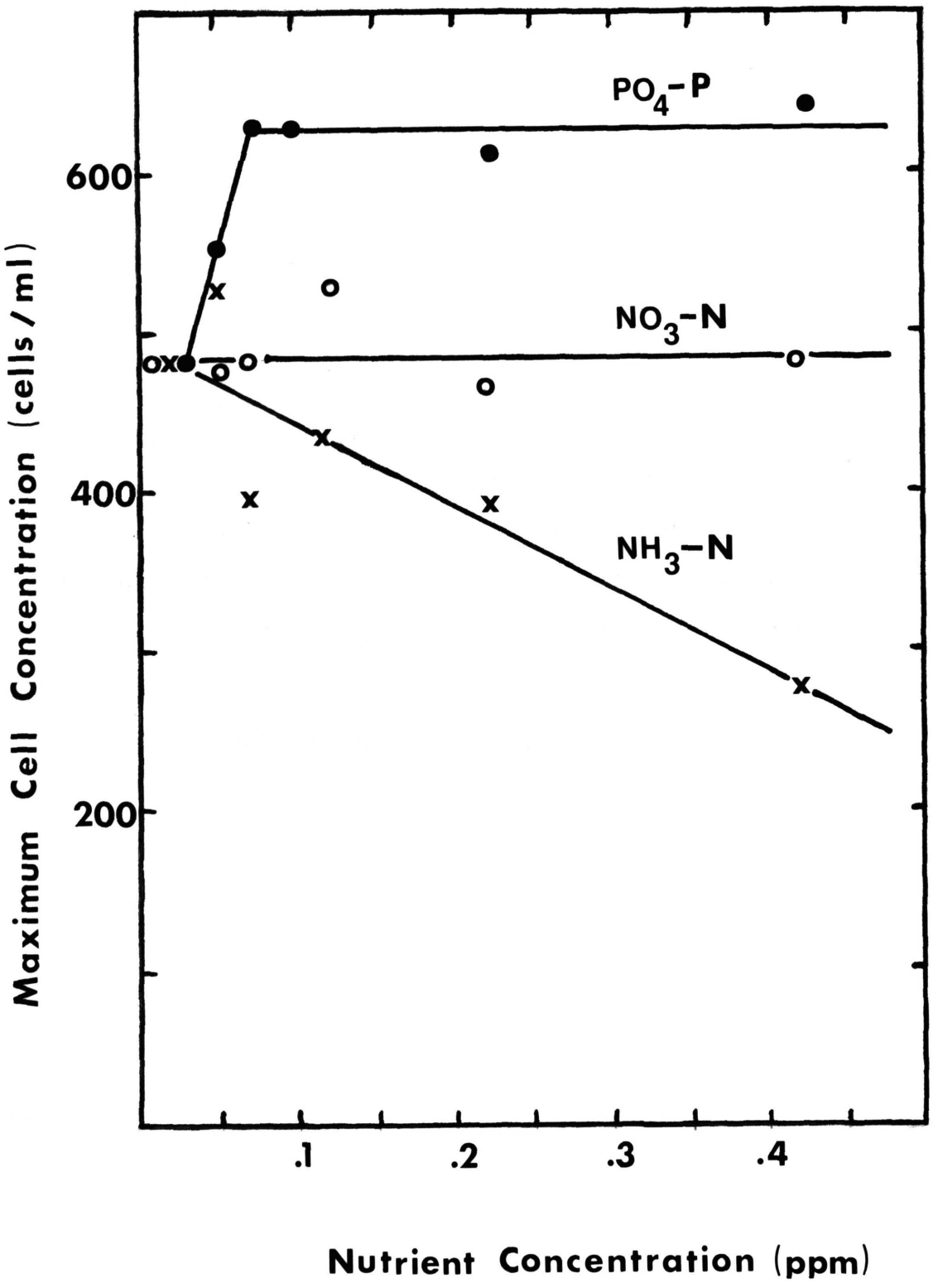

FIG. 5. Effect of inorganic nutrient enrichments on the maximum *G. breve* population.

Table XII

Effect of enrichment with municipal wastes on the growth of *G. breve*[*]

Sewage (%)	PO_4-P (ppm)	NO_3-N (ppm)	NO_3-N (ppm)	Lag time (days)	N_{max} (cells/ml)	K_e ($days^{-1}$)	t_g (days)
0.00	0.025	0.0174	0.021	2.5	480 ± 30	0.196 ± 0.012	3.5
0.10	0.035	0.0178	0.039	3.0	590 ± 35	0.238 ± 0.016	2.9
0.25	0.050	0.0184	0.067	2.9	740 ± 50	0.232 ± 0.026	3.0
0.50	0.075	0.0194	0.113	2.9	900 ± 75	0.229 ± 0.020	2.9
1.00	0.125	0.0214	0.205	3.7	1225 ± 75	0.251 ± 0.009	2.8
2.00	0.225	0.0254	0.389	3.8	1250 ± 95	0.198 ± 0.014	3.5

[*]Total nutrient concentrations are given (Juno Beach water).

of a combination of phosphate, nitrate and ammonia equivalent to the concentrations of these nutrients in sewage produced an almost equivalent effect on the final yield of cells (cf. Table XIII). Apparently, at a concentration of 0.10 ppm PO_4-P, the maximum cell concentration is a linear function of the amount of ammonia nitrogen added. Such a phosphate concentration is typical for west Florida coastal waters in which red tide outbreaks generally occur. Although it is difficult to extrapolate from laboratory studies in axenic cultures to natural situations, the results of the present study indicate that sources of inorganic nitrogen may control the development of G. breve blooms in many instances, and this conclusion is consistent with the results of field studies (Florida Board of Conservation, 1966; Wilson, 1966).

The waste material used in the previous enrichment studies was collected at a treatment plant located in the Miami area where a ban on phosphate detergents was in effect. During the six-month period that the ban had been in effect, influent phosphate concentrations had decreased approximately 40 percent. In order to evaluate the effect of detergent phosphate on the growth of G. breve in a realistic manner, enrichments with phosphate and detergent phosphate were made in the presence of various amounts of this municipal waste material. The effect of phosphate enrichment in the presence of sewage is summarized in Table XIV. A comparison of Tables XII and XIV indicates that the additional phosphate has a slight stimulatory effect at low sewage concentrations which is not evident at higher sewage levels. Evaluation of the effect of equivalent additions of detergent phosphates was complicated by the fact that some component of the detergent (perhaps the surfactant) was often fatal to the organism.

Table XIII

Effect of enrichment with nutrient combinations on

G. breve growth in Juno Beach water

Culture	Lag time (days)	N_{max} (cells/ml)	K_e ($days^{-1}$)	t_g (days)
Control-No Additions	2.6	470 ± 25	0.266 ± 0.026	2.6
Added PO_4-P (0.10 ppm) NO_3-N (0.01 ppm) NH_3-N (0.10 ppm)	2.3	1090 ± 45	0.262 ± 0.011	2.6
Added PO_4-P (0.10 ppm) NO_3-N (0.01 ppm) NH_3-N (0.05 ppm)	2.2	875 ± 40	0.252 ± 0.005	2.7
Added PO_4-P (0.10 ppm) NO_3-N (0.01 ppm)	2.1	665 ± 30	0.240 ± 0.008	2.9
Added Sewage (1%)	2.7	1240 ± 55	0.268 ± 0.012	2.6

Table XIV

Effect of phosphorus enrichments in the presence of sewage on the growth of G. breve in Juno Beach water[a]

Sewage	Lag time (days)			N_{max}			K_e			t_g		
(%)	A	B	C	A	B	C	A	B	C	A	B	C
0.00	2.8	2.7	2.8	625 ± 60	585 ± 55	440 ± 45	0.223 ± 0.007	0.187 ± 0.004	0.218 ± 0.002	3.1	3.7	3.2
0.10	2.5	5.5	4.7	795 ± 75	580 ± 60	520 ± 40	0.220 ± 0.029	0.123 ± 0.016	0.147 ± 0.006	3.2	5.6	4.7
0.25	2.9	4.2	4.4	915 ± 45	775 ± 35	720 ± 50	0.239 ± 0.016	0.165 ± 0.006	0.158 ± 0.005	2.9	4.2	4.4
0.50	2.7	5.1	NG	900 ± 40	885 ± 45	NG ±	0.200 ± 0.032	0.166 ± 0.013	NG ±	3.5	4.2	NG

[a] A = + 0.100 ppm PO_4, B = + 0.025 ppm Detergent-P, C = + 0.050 ppm Detergent-P, NG = no growth.

The effect of nitrioltriacetic acid (NTA) on the growth of G. breve was also determined. NTA is a strong chelating agent and has been a leading candidate for use as a phosphate substitute in detergent products, the function of the sodium tripolyphosphates in detergent formulations being to chelate calcium and magnesium in hard waters. Stock solutions of NTA were added to cultures of G. breve in the presence and in the absence of added sewage because sewage would be the major pathway of NTA into receiving waters. Expected concentrations of NTA in municipal sewage influents range from 15 to 20 ppm (Thom, 1971; Hamilton, 1972). However, degradation to carbon dioxide, water and inorganic nitrogen is essentially complete in biological sewage treatment processes (Swisher et al., 1967), and the average concentration of NTA in surface waters would be much less than 5 ppm. Although Christie (1970) has reported that NTA was able to serve as the sole source of nitrogen in cultures of Chlorella pyrenoidosa, the addition of NTA (at 5 and 10 ppm concentrations) either alone or in the presence of sewage was not stimulatory to G. breve (Table XV). These results are in agreement with those of Swedish investigators who found the influence of NTA on algal growth to be species dependent and to vary from stimulatory to inhibitory (Thom, 1971). The present studies in axenic cultures give no information concerning the possible mobilization of metals from bottom deposits by NTA and their subsequent availability for phytoplankton nutrition. Sediment-NTA interactions should be investigated because certain metal-NTA complexes are resistant to biological degradation and may persist in the environment (Hamilton, 1972). Also, previous studies (Martin et al., 1971) have indicated that chelated metals may be significant factors in red tide blooms.

Table XV

Effect of nitrioltriacetic acid (NTA)

on G. breve growth

Culture[a]	Lag time (days)	Max. Cell Count (cells/ml)	K_e ($days^{-1}$)	t_g (days)
Control	1.6	500 ± 60	0.150 ± 0.010	4.6
5 ppm NTA	1.5	365 ± 140	0.116 ± 0.012	6.0
10 ppm NTA	1.6	520 ± 80	0.167 ± 0.044	4.1
2% Sewage	3.5	5000 ± 410	0.151 ± 0.010	4.6
2% Sewage + 5 ppm NTA	3.8	4750 ± 190	0.182 ± 0.006	3.8

[a]In the presence of sewage, effects of NTA on growth may be due to chelation of matals rather than NTA serving as a source of nitrogen. Chelation may detoxify or increase the availability of metals.

Typically, soil extracts are excellent sources of chelated metals (e.g., iron), and the addition of soil extracts to cultures of dinoflagellates is a common practice (Wilson, 1966). The effect of several molecular-weight fractions of a soil extract on the growth of G. breve was determined and compared to the effects of various other naturally-occurring organic substances on the growth parameters (cf. Table XVI). In order to evaluate better the results of the study, an examination of the characteristics of the soil extract fractions (cf. Table XVII) is necessary.

A large percentage (46%) of the organic substances present in the crude extract were in the 1,000-10,000 molecular-weight range; however, the substances in the 500-1,000 molecular-weight range are associated with more iron (on a μg Fe/mg C basis). The percentages given (cf. Table XVII) are valid for the recovered material, but may not be valid for the initial crude extract. Although 85.5% of the total organic carbon (and 78.0% of the total iron) in the crude extract was present in the combined fractions, the recovery of different components was probably variable. Losses of organic material are due mainly to adsorption onto the filters, and the recovery of high molecular-weight material should be less efficient than that of low molecular-weight substances because the high molecular-weight fractions come into contact with more filters.

Each of the soil extract fractions was as effective as sewage (1% v/v) in increasing the maximum cell population (cf. Table XVI); however, the substances in the 500-1,000 molecular-weight (iron rich) fraction were the most effective on a mg C-added basis. The growth constant was not affected by enrichment with low molecular-weight

Table XVI

Effect of naturally-occurring organic substances on the growth of G. breve in Gulf of Mexico water

Culture[b]	Lag time (days)	Max. Cell Count (cells/ml)	K_e ($days^{-1}$)	t_g (days)
Control	2.3	1020±50	0.230±.006	3.0
F1 (0.5 ml)	2.5	1375±80	0.223±.010	3.1
F1 (1.0 ml)	2.8	1860±85	0.231±.003	3.0
F2 (0.5 ml)	2.0	1490±90	0.224±.003	3.1
F2 (1.0 ml)	1.8	2125±105	0.241±.011	2.9
F3 (0.5 ml)	2.3	1200±60	0.202±.014	3.4
F3 (1.0 ml)	2.5	1600±75	0.179±.016	3.9
GA (10^{-8} M)	1.8	1100±60	0.201±.025	3.4
GA (10^{-7} M)	1.7	1090±55	0.194±.009	3.6
GA (10^{-6} M)	0.9	1030±75	0.173±.002	4.0
Sewage (1%)	2.8	1975±75	0.226±.004	3.1

[a] F1, F2, and F3 refer to molecular weight fractions of 500-1,000, 1,000-10,000, and >10,000, respectively. GA, Gibberellic acid. (cf. Table XVII.)

Table XVII

Characteristics of the soil extract fractions

Molecular Weight Range	Volume (ml)	Organic Carbon (ppm)	Organic Carbon (mg)	% of Total Organic Carbon	Iron (ppm)	Iron (µg)	% of Total Iron	µg Fe/mg C
0-500	3000	8	24	18.4	0.005	15.0	8.0	0.62
500-1,000	25	660	16.5	12.6	2.040	51.0	27.3	3.09
1,000-10,000	25	2400	60	46.0	2.460	61.6	32.9	1.03
>10,000	25	1200	30	23.0	2.380	59.6	31.8	1.98

compounds but was significantly lowered by the addition of the high molecular-weight substances.

Responses of G. breve to gibberellic acid (a plant growth hormone) enrichment were similar to those observed by Paster and Abbott (1970) with respect to the lag time (which decreased); however, the maximum cell population was not increased, and the growth constant decreased. In enriched media (cf. Table XVIII), the addition of gibberellic or tannic acid (1 ppm) did not significantly affect the growth parameters. Higher concentrations of tannic acid (5 and 10 ppm) caused the cells to die within a day. The final pH of the media was unaffected by the addition of tannic acid (up to 10 ppm), and,therefore, the demise of the G. breve cells must be attributable to some other phenomenon such as precipitation of the tannic acid at the salinity of the media (35 ppt).

Toxin Stability

Using one toxin unit, the thermal-, pH-, and photo-stability of the toxin was determined after incubation at defined temperature and pH conditions.

Thermal inactivation of the toxin during a one-hour incubation period was not detected over a temperature range of 15-70°C; above the last temperature inactivation occurred (cf. Fig. 6). The toxin activity was also determined as a function of length of incubation at 80°C, and loss of activity followed a first-order rate pattern with a half life of 0.5 hr., and inactivation constant of 0.023 min^{-1}.

The activity-pH profile shows a dramatic change at pH 9-10 (cf. Fig. 7). The toxin (one toxin unit) is stable to incubation

Table XVIII

Effect of selected organic substances on the growth of G. breve in enriched media[a]

Culture[b]	Lag time (days)	Max. Cell Count (cells/ml)	K_e ($days^{-1}$)	t_g (days)
Control	3.4	2595±125	0.156±.003	4.4
GA (10^{-8} M)	3.3	2500±160	0.158±.009	4.4
GA (10^{-7} M)	3.8	2465±115	0.158±.006	4.4
GA (10^{-6} M)	3.1	2425±120	0.156±.003	4.4
TA (1 ppm)	5.0	2455±145	0.168±.002	4.1
Sewage (1%)	8.0	4200±200	0.141±.009	4.9

[a]Gulf of Mexico water with B-5 enrichments.

[b]GA, Gibberellic acid; TA, tannic acid.

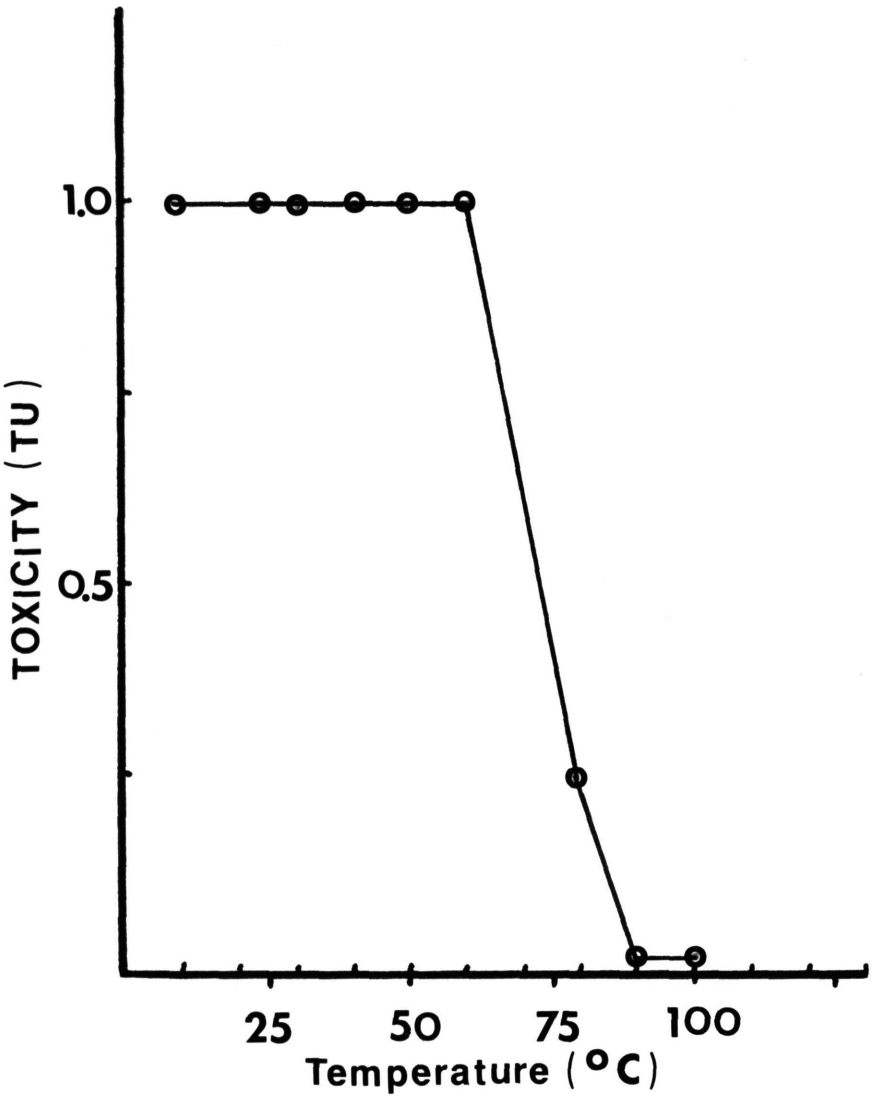

FIG. 6. Effect of incubation (1 hr) at elevated temperature on toxicity (1 toxin unit).

(48 hours, 23°C) at pH values of 1-9, but at pH 10, 75% of the activity is lost and at pH 11 and 12 all activity was lost under these conditions.

The temporal stability was also evaluated. Samples (one toxin unit) were tested at various times during a 200-day period after storage in the dark at 10°C. The initial death time was 25 min, the final was 26 min, with a mean and standard deviation (22 ± 3) that is within the experimental error indicated in Table VII.

The observations of temporal-, pH-, and thermal stability indicated a toxin that should persist in sea water and be mitigated only by dilution. The photo-stability, however, when measured gave a significantly different ecological view (cf. Table XIX). The controls were designed to indicate any thermal effects and inactivation occurred with each type of radiation. The order of inactivation appeared to be uv < vis < ir, though strict comparison is not valid because of the difference in intensity of incident radiation.

Intracellular Location

Two different methods of extraction yielded useful information concerning the intracellular location of the toxin.

First, extraction of cultures of G. breve that were near the end of log-phase growth yielded approximately one toxin unit (ca. 0.3 mg of crude toxic material) per 10^6 cells. After continuous-flow centrifugation of 10 liters of culture media (5 x 10^6 cells/liter), the pellet and supernatant were extracted and 80% of the toxin (40 toxin units) was found associated with the pellet. Therefore, the toxin is an endotoxic substance as was the chloroform-insoluble toxin described by Sasner (1965). The toxic material in the supernatant (less than 20% of

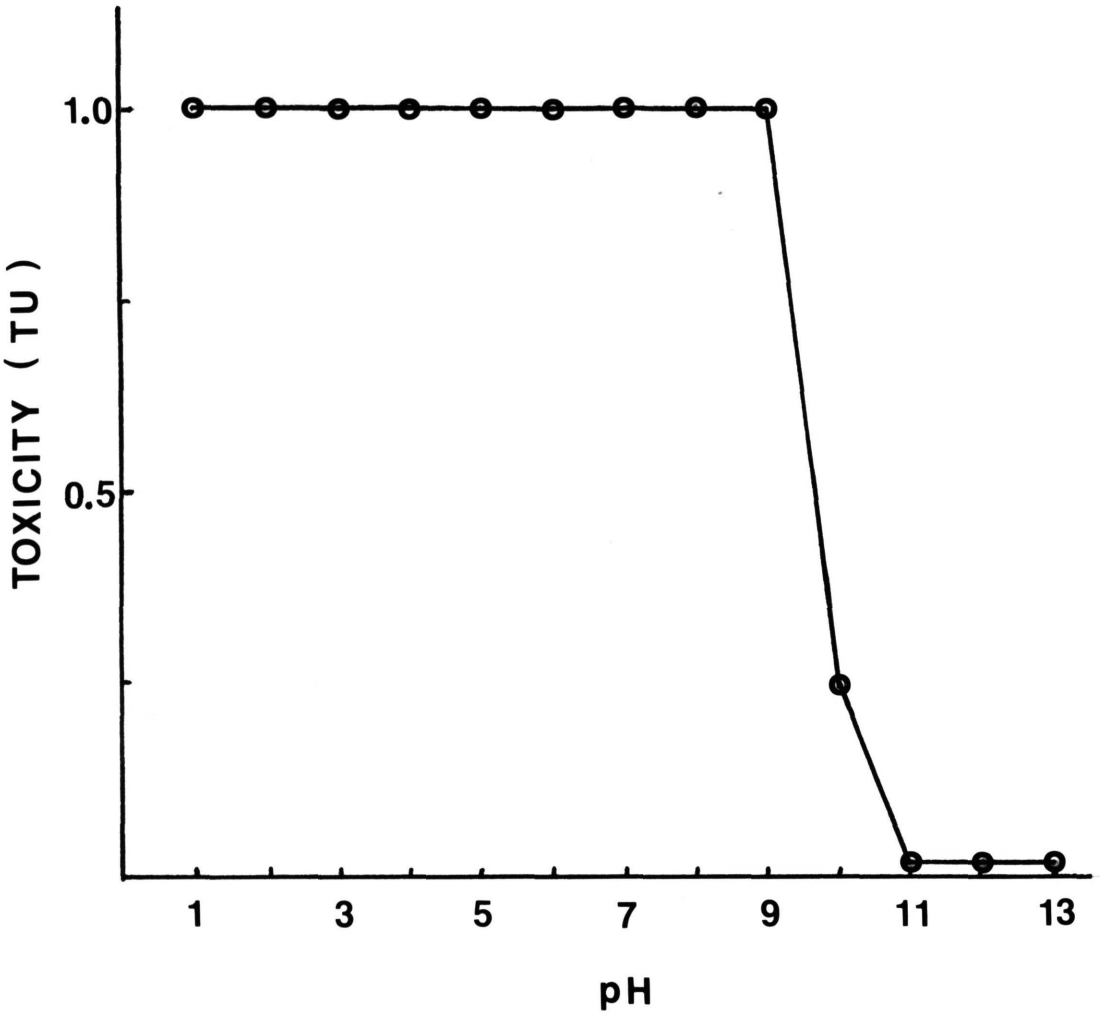

FIG. 7. Effect of incubation (48 hr) at various pH values on toxicity (1 toxin unit).

Table XIX

Photoinactivation of *Gymnodinium breve* toxin[a]

Radiation	Test (T) or Control (C)	Death Time (min)	Toxin Units
None	C	30	1
Ultraviolet	C	15	1
Ultraviolet	T	90	1/2
Visible	C	19	1
Visible	T	360	<1/4
Infrared	C	35	*ca.* 1
Infrared	T	>720	*ca.* 0

[a]One toxin unit.

of the initial material) may have been released during centrifugation (G. breve is an unarmored dinoflagellate and is, therefore, somewhat fragile) or upon the decomposition of dead G. breve during the lag and log phases of growth.

In a second procedure, cells within cultures of G. breve were lysed (by acidification to pH 4 and mechanical stirring for 30 min) prior to centrifugation. Extraction of pellet and supernatant indicated the toxic material was not associated with the pellet. Approximately 100% of the toxin (ca. 50 toxin units) was recovered in the extract of the supernatant, and no toxin was detected in the extract of the pellet. These results are subject to three interpretations: (1) the toxin is present in the cytoplasmic fluid, (2) the toxin is released from the particulate cell matter by the lysing procedure, or (3) the toxin is associated with particulate cell material which is not sedimented at 12,500 x g.

Column Chromatographic Fractionation: The crude toxic extract was fractionated on the basis of adsorption characteristics, size, and polarity by column chromatography on silica gel, LH-20 Sephadex gel, and DEAE-cellulose, respectively. The crude toxic extract was placed on a silica-gel column and non-toxic materials were eluted with 500 ml of chloroform followed by 250 ml of methylene chloride. The toxin was eluted from the silica gel column with 250 ml of methanol. Re-chromatography of the methanol eluent on silica gel (eluting with benzene, acetone, carbon tetrachloride, or mixtures of chloroform and methanol) did not effect a further purification of the toxic material.

The methanol eluent was lyophilized, and the yellow toxic material was dissolved in 10 ml of chloroform and placed on a LH-20 Sephadex

column. The toxin was eluted with chloroform-methanol (19:1, v/v), and 10-ml fractions were collected. Fractions 4-7 were toxic (cf. Fig. 8) and were pooled for chromatography on DEAE-cellulose. The elutant for the first 30 fractions (12-ml each) was chloroform and the next 35 fractions were eluted with glacial acetic acid. Fractions 3-9 were toxic (cf. Fig. 9), and the pooled fractions constitute the purified toxin.

The results of the purification procedure are summarized in Table XX. A 27-fold increase in the specific activity (toxin units/mg) was achieved with recovery of 71% of the total activity (toxin units).

Thin-Layer Chromatography: The crude toxic extract and the purified toxin were examined by thin-layer chromatography (TLC) on silica gel, and the results are shown in Fig. 10. The crude toxic material was separated into six components when the plates were developed in chloroform-methanol (90:10, v/v) and the spot corresponding to an R_f range (ratio of distance traveled by a compound to the distance traveled by the solvent) of 0.61 to 0.68 contained the only toxic material. Simultaneous chromatography of the purified toxin yielded a single component with an R_f range of 0.58 to 0.70. The chromatographic homogeneity of the purified toxin was established by TLC in the solvent systems listed in Table XXI. In each solvent system, the purified toxin migrated as a single spot. The toxic material was blue when examined under long or short wavelength ultraviolet light, and gave brown spots upon exposure to iodine vapor.

FIG. 8. Column chromatography of Gymnodinium breve toxin on Sephadex LH-20 gel.

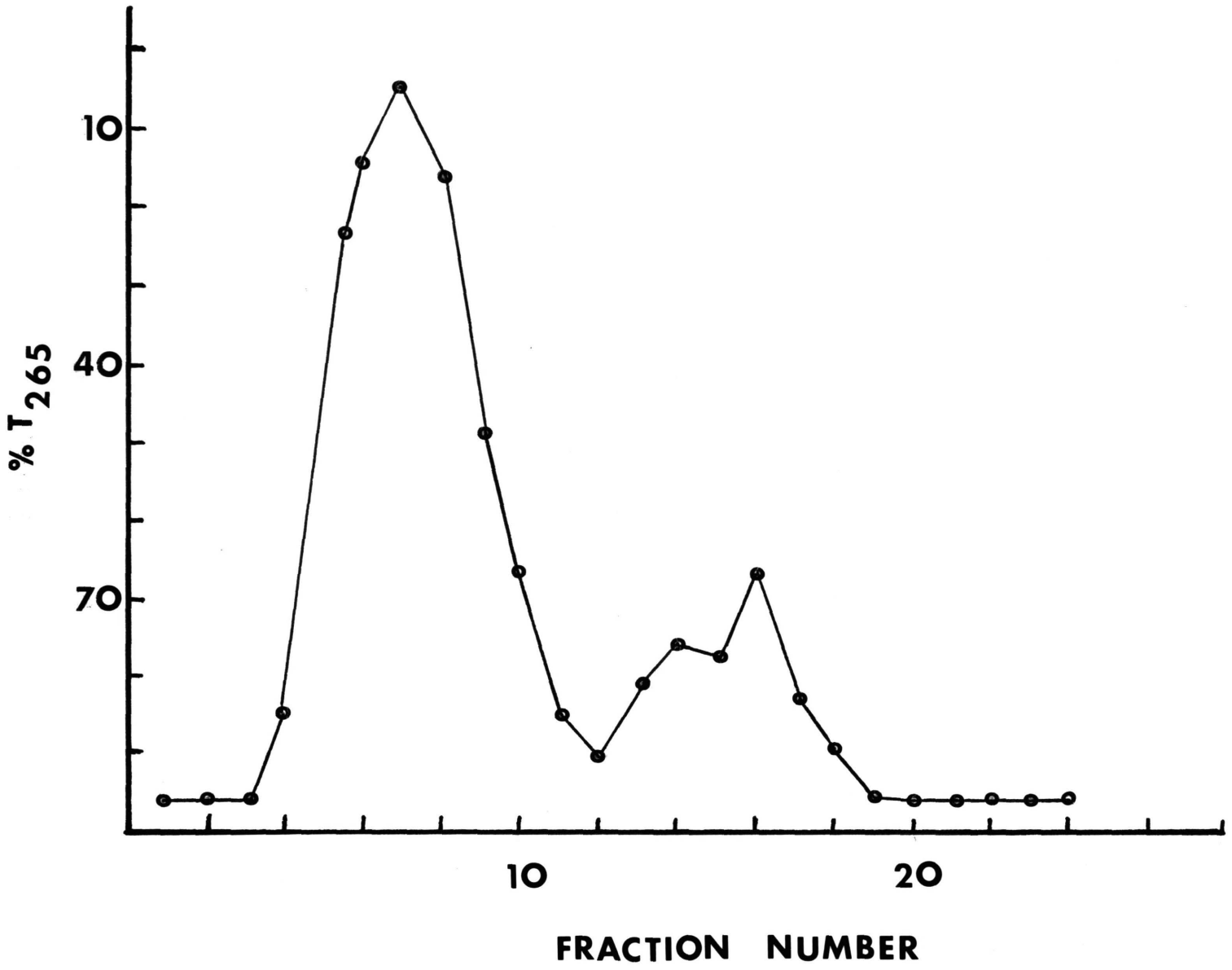
10
40
70
% T265
10
20
FRACTION NUMBER

FIG. 9. Column chromatography of Gymnodinium breve toxin on DEAE-cellulose.

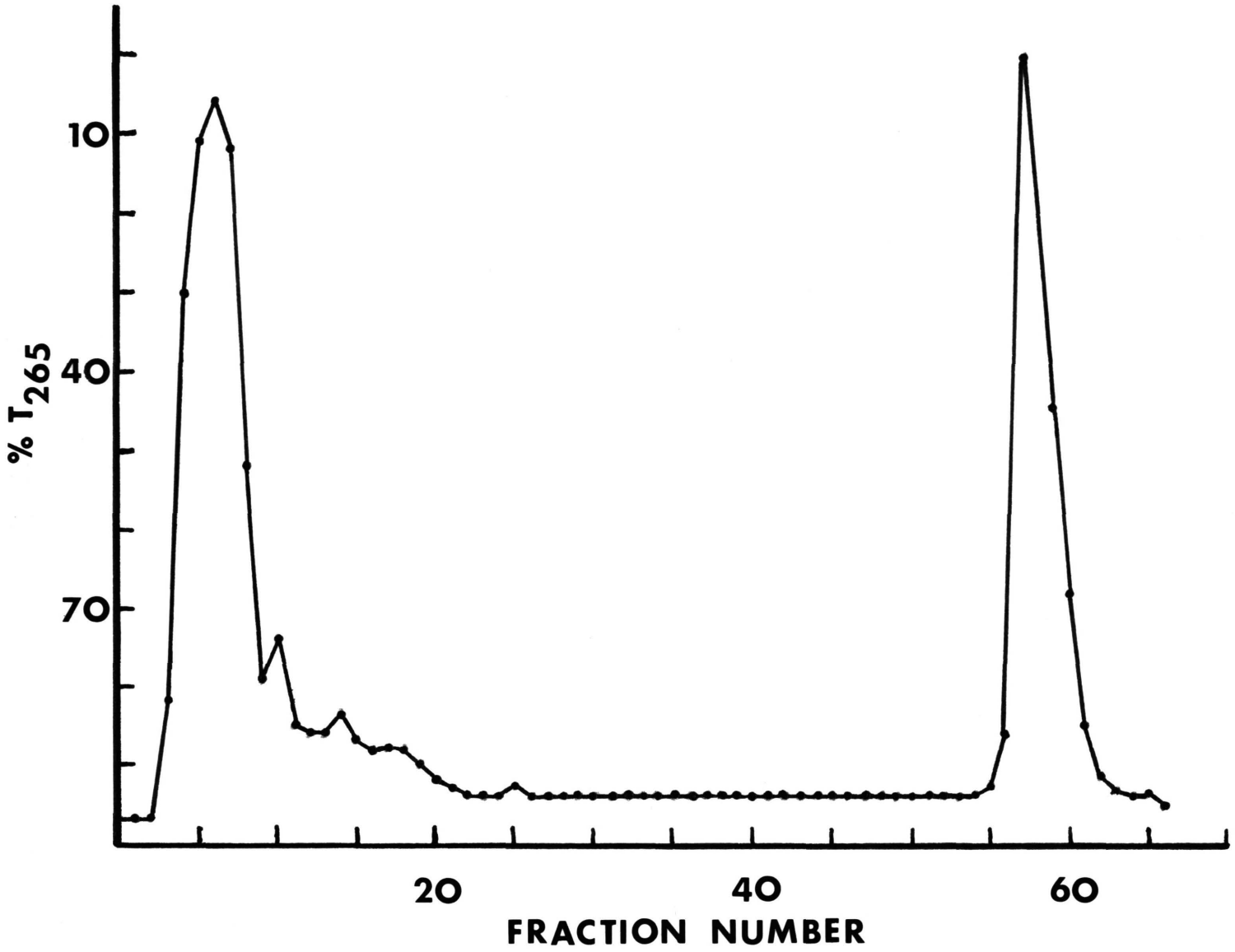
10
40
70
% T_{265}
20
40
60
FRACTION NUMBER

Table XX

Summary of the purification of a toxin from *Gymnodinium breve*

Fraction	Yield (mg)	Specific Activity (TU/mg)	Total Activity (TU)	Reative Activity	Recovered Activity (%)
Crude Toxic Extract	270	4.43	1200	1.0	100
Methanol Eluent from Silica Gel	112	9.76	1090	2.2	91
LH-20 Fractions (4-7)	51	17.3	882	3.9	74
DEAE-Cellulose Fractions (3-9)	7	121.8	853	27.3	71

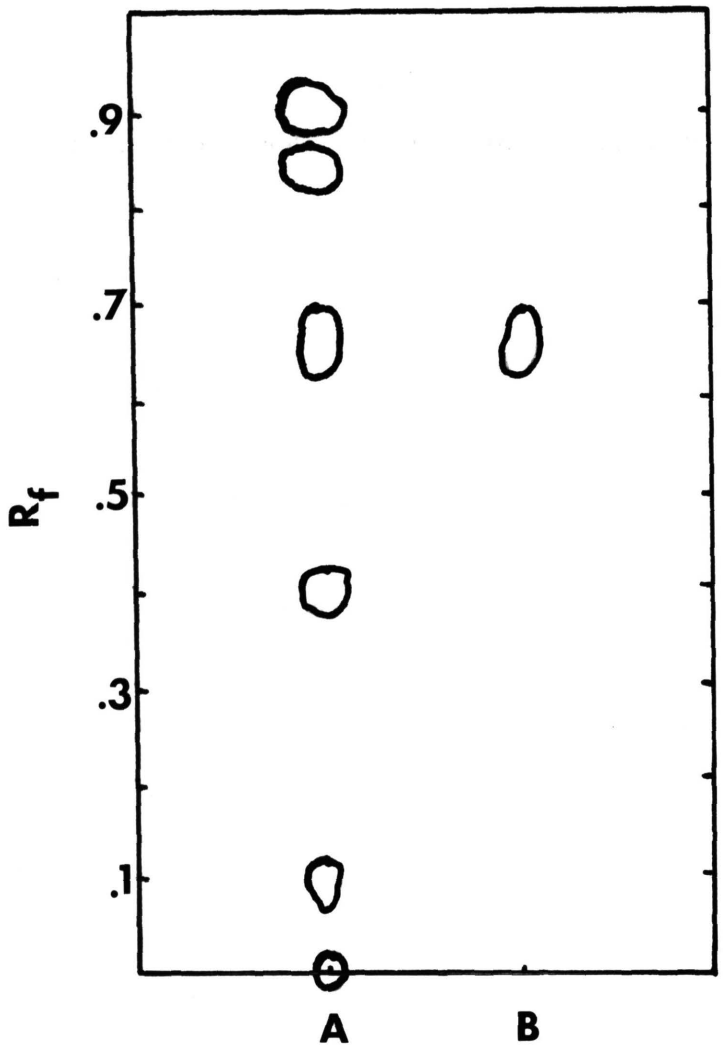

FIG. 10. Thin-layer chromatography of the crude toxic extract (A) and the purified toxin (B) on silica gel in chloroform-methanol (90:10, v/v).

Table XXI

Thin-layer chromatography of purified Gymnodinium breve toxin

Solvent System (v/v)	R_f Range
Chloroform:Methanol (90:10)	0.58-0.70 (0.64)
Chloroform:Methanol:Acetic Acid (100:20:1)	0.85-0.89 (0.87)
Methanol:conc. NH_4OH (100:1.5)	0.64-0.74 (0.69)
Ethyl Acetate:n-Propanol:conc. NH_4OH (40:30:3)	0.64-0.76 (0.70)
Benzene:Ethyl Acetate:Ethanol:Acetic Acid (79:10:10:1)	0.57-0.64 (0.61)
Chloroform:Methanol:Acetic Acid (60:20:20)[a]	0.89-0.95 (0.93)

[a]Chromatography on DEAE-Cellulose (Whatman DE81) sheets.

Characterization: Semi-micro quantitative analyses for carbon, hydrogen, phosphorus, nitrogen, and sulfur were performed by Chemalytics, Inc., Tempe, Arizona (cf. Table XXII), and oxygen was calculated by difference from 100 percent. Assuming the traces of nitrogen and phosphorus are not associated with the toxin, the remainder of the analytical data indicate an empirical formula of $C_{87}H_{153}O_{18}S$ which corresponds to a minimum molecular weight of 1517. However, the mass spectrum (obtained at Florida State Univ.) of the purified toxin had a parent peak (maximum m/e) of 619 which agrees reasonably well with a molecular weight of 650 determined by vapor-pressure osmometry (Martin and Chatterjee, 1970). It is evident from the analytical data that the purified toxin is not an unique isolated compound.

The purified toxin had a maximum absorption at 265 mμ and no absorption in the visible region of the spectrum. The observed infrared absorption frequencies indicated the presence of one or more carbonyl groups (1720 and 1780 cm^{-1}) and the absence of N-H or O-H stretching absorptions (3100-3500 cm^{-1}) indicates the absence of hydroxyl or amino groups in the purified toxin (cf. Appendix A).

The absorption in the ultraviolet region (265 mμ) corresponds to an optically active absorption band. The reduced molar ellipticity at 265 mμ was calculated from the expression:

$$[\theta'] = \frac{3}{n_\lambda^2 + 2} \; 100 \; \frac{\Psi}{lm}$$

where Ψ is the measured ellipticity in degrees, l is the light path in cm, m is the concentration in moles/liter (assuming a molecular weight of 619), and n_λ is the refractive index at wavelength λ. The calculated value was 3.3×10^3 degrees cm^2/decimole.

TABLE XXII

Elemental analyses of purified *Gymnodinium breve* toxin(s)

Element	Present Study	Percent Martin and Chatterjee (1970)	Trieff et al., (1972)
G	67.95	49.60	70.92
H	9.94	7.60	10.10
N	0.50	0.60	-
P	0.20	1.41	1.99
S	2.08	-	-
O	19.33	41.4	17.54

Acidic Polysaccharide Content

The acidic polysaccharide contents (in μg heparin equivalents/mg of sample) of the crude toxic extract and the purified toxin were 3.72 and 20.5, respectively (cf. Table XXIII). This represents a 5.5-fold increase in the polysaccharide content during the purification procedure. The 27.3-fold increase in toxicity during the same procedure indicates the toxic properties of the extract are probably not associated with the anionic polysaccharide present in the purified toxin. Also, the solubility properties of the polysaccharide indicate the material must be associated with a lipophillic substance.

Table XXIII

Acidic polysaccharide determination data

Sample	Weight (μg)	A_{600}	H. Eqv.	H. Eqv./mg	Conc. Factor
Heparin	0.5	0.022	-	-	-
Heparin	1.0	0.043	-	-	-
Heparin	3.0	0.128	-	-	-
Heparin	5.0	0.231	-	-	-
Crude Toxin	330	0.051	1.19	3.72	-
Purified Toxin	220	0.194	4.51	20.5	5.5

Anticoagulant Activity

The variation of clotting time with concentration of G. breve toxin is given in Fig. 11, and heparin and carageenan are included for comparison. Two other compounds tested had no discernable effect on the prothrombin time for human blood plasma. Alginic acid tends to form gels that limited solubility to 0.50 mg/0.05 ml aliquot, and at this concentration no anticoagulant activity was evident. Prymnesin was also inactive, even at a concentration of 4.0 mg/0.05 ml aliquot, which is hemolytically active.

The order of effectiveness of the anticoagulants is evident from inspection of Fig. 11; however, a more quantitative comparison of activities can be made in terms of the amounts of anticoagulant required to double the prothrombin time. The values are given for the three anticoagulants in Table XXIV. On this basis, G. breve toxin had 1/6 the activity of carrageenan and 1/70 the activity of heparin. Values reported for heparin and carrageenan differ somewhat from those reported previously. The additional heparin needed to double the prothrombin time, as noted in the present study, may be due to the use of oxalated blood plasma instead of citrated plasma (cf. Lenahan et al., 1966). The use of oxalated plasma may also be responsible for the additional carrageenan required in the present study; however, variations in the amount of carrageenan required to double the pro thrombin time are probably inevitable because the activity depends upon the relative proportion of different isomers. The carrageenan used in this study, for example, may be relatively rich in the less active kappa fraction (cf. Percival and McDowell, 1967).

FIG. 11. Comparison of the effects of Gymnodinium breve toxin, carrageenan, and heparin on prothrombin time (G, C, and H, respectively).

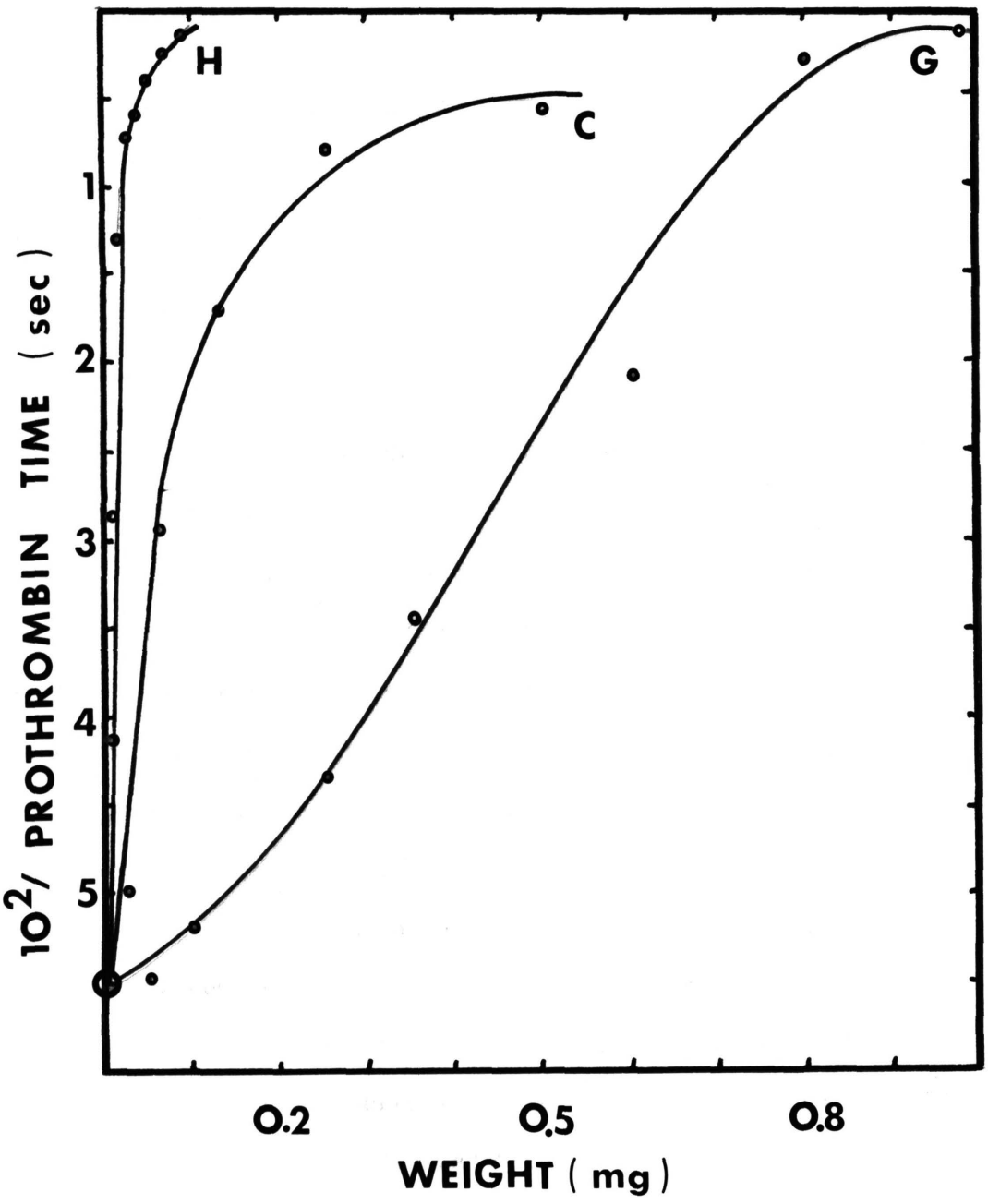
H
C
G
1
2
3
4
5
10^2/ PROTHROMBIN TIME (sec)
0.2
0.5
0.8
WEIGHT (mg)

Table XXIV

Comparison of the amounts of anticoagulant required to double the prothrombin time

Anticoagulant	Amount Required (μg) Present Study	Hawkins and Leonard (1963)
Heparin	5	3
Carrageenan	60	40
G. breve Toxin	350	-

Although G. breve toxin inhibited the clotting process at concentrations corresponding to one toxin unit, the toxic symptoms and relatively fast death times for intoxicated fish indicate the anticoagulant properties of the toxin are not responsible for the ichthytoxicity. Martin (1966) reached a similar conclusion concerning the anticoagulant activity observed in extracts from the sea anemone Rhodactis howesii. Based on differential inactivation of the various activities by heat, he concluded that the high molecular-weight agent responsible for the anticoagulant properties was not responsible for the hemolytic or neurotoxic effects of the anemone extract.

Prior to the present study, the anticoagulant properties of toxins from red tide organisms were unknown; however, a number of substances exhibiting anticoagulant activity had been isolated from marine organisms (cf. Table XXV). All of the known anticoagulants isolated from marine species are sulfated-polysaccharides, and the anticoagulant activity is a function of the extent of sulfation (Baslow, 1969).

Table XXV

Substances with anticoagulant activity isolated from marine organisms

Source[a]	Substance
Chlorophyta, Rhodophyceae, many species (algae)	Carrageenan
Chlorophyta, Phaeophyceae, many species (algae)	Laminarin
Mollusca, Pelecypoda, Spisula solidissima (clam)	Mactin A
Echinodermata, Ophiuroidea, Ophiocomina nigra (brittle star0	Sulfated polysaccharide
Cnidaria, Anthozoa, Rhodactis howesii (sea anemone)	Heat stable, high mol. wt. substance
Chordata, Mammalia, Balaenoptera physalus (whale)	ω- Heparin

[a]Ref. Baslow, 1969.

The sulfur (2.08%) and anionic polysaccharide (20.5 heparin equivalents/mg) content of the purified toxin suggested that the anticoagulant activity may be associated with a sulfated polysaccharide present in the purified toxin; however, the presence of other types of anticoagulants in the purified toxin cannot be ruled out. For example, phosphatidylserines have anticoagulant activities (Turner et al., 1972), and the purified toxin also contained traces of both nitrogen (0.50%) and phosphorus (0.2%). In either case, the specific activity of the

anticoagulant must be substantial if it is present as a trace contaminant of the toxin, and the presence of such a potent anticoagulant in G. breve should be investigated in future studies.

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APPENDIX A

Spectra

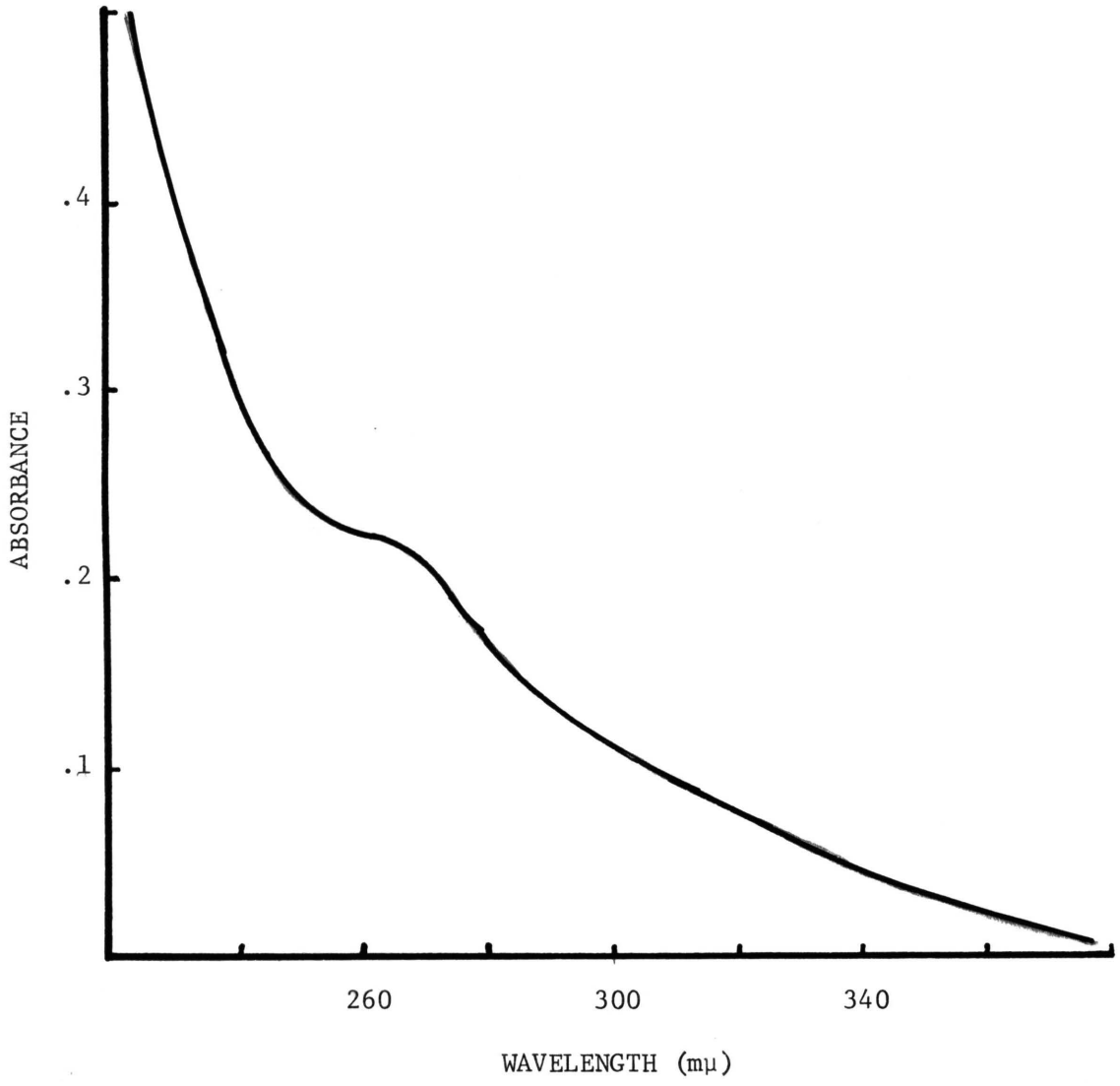

FIG. 12. Ultraviolet spectrum of purified Gymnodinium breve toxin (in methanol).

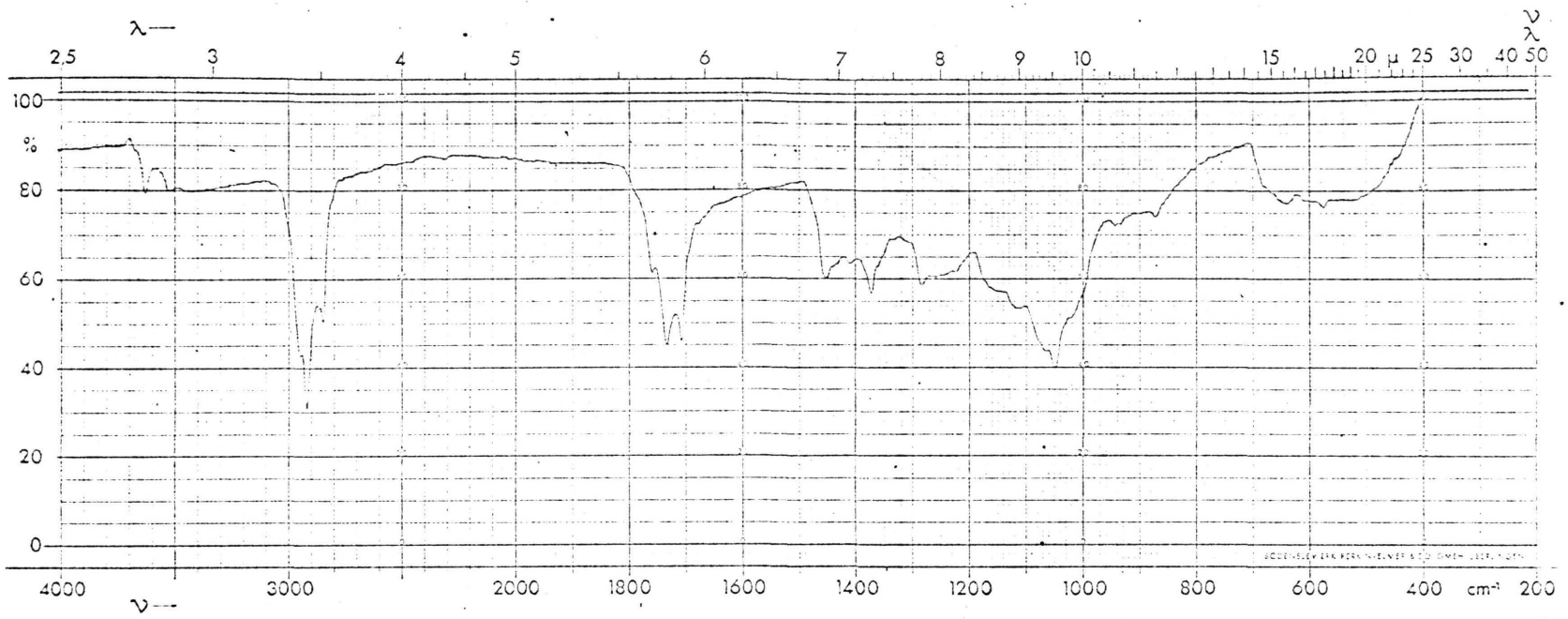

FIG. 13. Infrared spectrum of purified Gymnodinium breve toxin (in carbon tetrachloride).

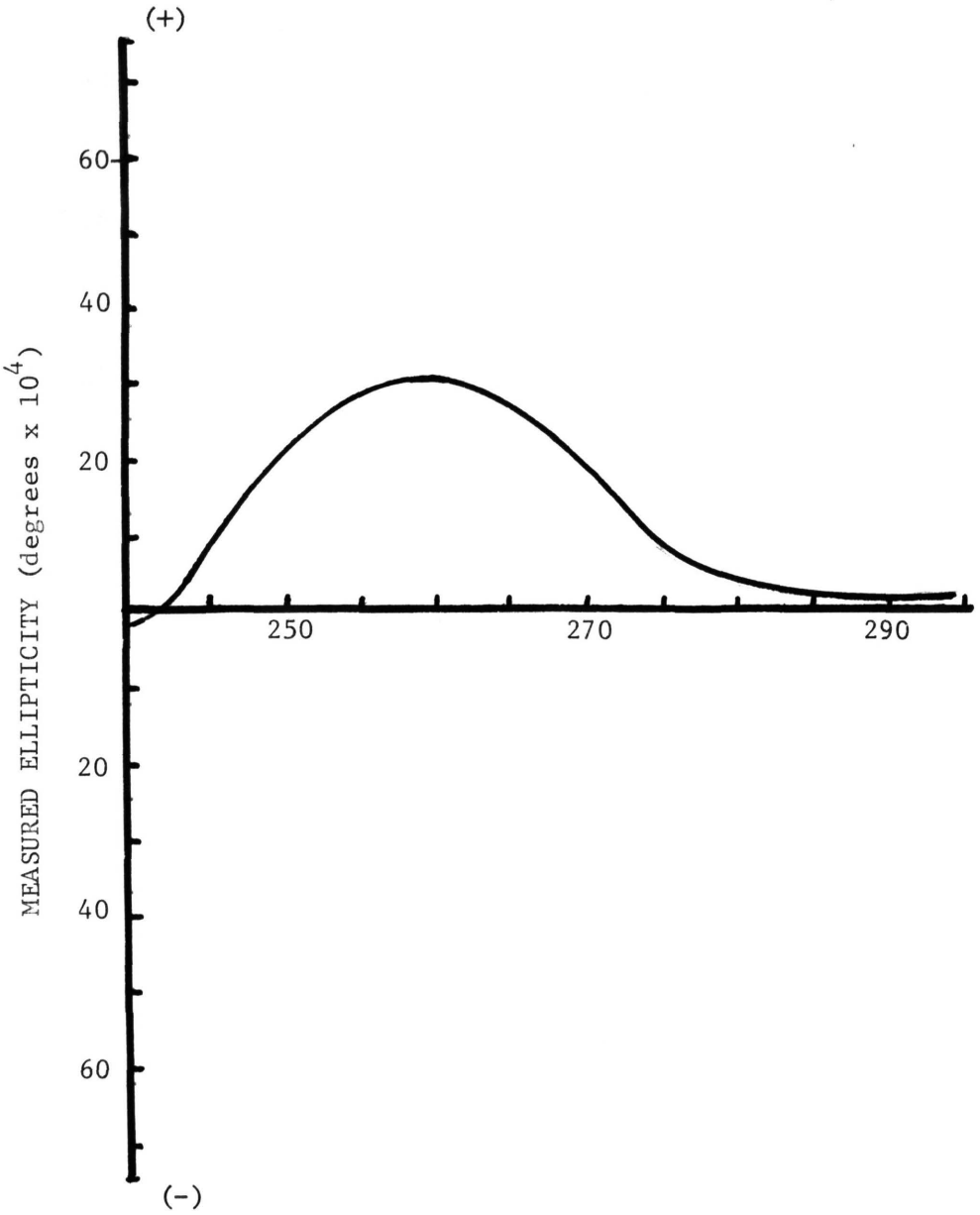

FIG. 14. Circular Dichroism spectra of purified Gymnodinium breve toxin (in methanol).