SOME CHEMICAL AND PHYSICAL PROPERTIES OF TWO TOXINS FROM THE RED-TIDE ORGANISM, *GYMNODINIUM BREVE* [1, 2]

BY DEAN F. MARTIN [3] AND ASHIM B. CHATTERJEE [4]

ABSTRACT

A procedure is given for isolating and purifying two toxins (substance I and II) from cultures or blooms of *Gymnodinium breve*. In vitro studies of acetylcholinesterase activity show that the major toxin is not a cholinesterase inhibitor. On the basis of the infrared data the properties of toxin samples isolated from blooms of *G. breve* appear to be identical to those from axenic cultures.

Substance II is a light yellow, low-melting solid. Carbon, hydrogen, and phosphorus are present; sulfur, chlorine, bromine, and nitrogen are absent. The percentage composition is given for carbon, hydrogen, oxygen, and phosphorus; an empirical formula of $C_{55}H_{102}O_{17}P$ is indicated by the analytical data. A molecular weight of 650 was obtained from a sample of substance II.

Substance II was characterized by the absorption spectra (ultraviolet, infrared), by the nuclear magnetic resonance spectrum, and by the specific optical activity. These observations and the mass spectrogram provide useful structural information.

Substance I was characterized by the infrared spectrum which indicated the presence of a carbonyl group. Not enough of substance I was isolated for an elemental analysis.

Evidence for a causal relation between blooms of the Florida red-tide organism, *Gymnodinium breve*, and animal intoxication once rested on seasonal and geographic correlations of these events. The value of the correlation was limited because of the presence of accompanying bacterial species with ichthyotoxic properties (Bein, 1954). The relation was established by Ray and Wilson (1957), who showed that cultures of *G. breve* killed fish, and by McFarren, Tanabe, Silva, Wilson, Campbell, and Lewis (1965), who isolated a ciguateralike poison from oysters and clams (taken from the area in which a bloom occurred), from a bloom of *G. breve*, and from laboratory cultures of *G. breve*.

Before the present study, comparatively little was known about the chemical and biochemical properties of the toxin of *G. breve*. Starr (1958) described attempts to characterize the toxin through bioassays in which mullet and guppies were used to compare the potency of different toxic preparations.

Sasner (1965) reported that the toxin was an endotoxic substance which was soluble in water and ethyl alcohol, insoluble in chloroform, heat labile, acid stable, and slowly dialyzable. The toxic materials in his extracts affected all excitable membranes studied: sciatic nerve, sartorius nerve-muscle preparations, and skin from the leopard frog, *Rana pipiens;* the anterior byssus retractor muscle of Californian mussel, *Mytilus californianus;* and the leg nerves of the spider crab, *Loxyrhynchus* sp. In all cases, the membranes had been rendered unexcitable to electrical stimulation. The toxin caused depolarization and complete loss of the resting potential. According to Sasner (1965), the physiological effects of *G. breve* toxin closely paralleled those of a British gymnodinoid, *Gymnodinium veneficum* (Abbott and Ballantine, 1957). The toxin produced paralysis but was different from a curare-type (synaptic blocking agent) toxin, which is characteristic of *Gonyaulax catenella* (Schantz, Lynch, Vayvada, Matsumoto, and Rapoport, 1966).

A number of toxigenic algae are known; Shilo (1967) included species of dinoflagellates, certain blue-green algae (e.g., *Microcystis aeruginosa*) and several species of Chrysophyta (e.g., *Prymnesium parvum*). Before the present study, however, few of the algal toxins seem to

[1] Contribution No. 19 from the Marine Science Institute, University of South Florida, Tampa, Fla. 33620.

[2] Contribution No. 62 from the Bureau of Commercial Fisheries Biological Laboratory, St. Petersburg Beach, Fla. 33706.

[3] Professor, Department of Chemistry and Marine Science Institute, University of South Florida, Tampa, Fla. 33620.

[4] Visiting Research Assistant Professor, Department of Chemistry, University of South Florida, Tampa, Fla. 33620.

Published November 1970.

have been chemically characterized. Saxitoxin (the toxin of *G. catenella*) has a molecular weight of about 370 and a purine (nitrogenous) base (Schuett and Rapoport, 1962; Schantz et al., 1966). The toxin obtained from *M. aeruginosa* is a cyclic polypeptide composed of 10 amino acid moieties, including D-serine (Bishop, Anet, and Gorham, 1959; Gorham, 1960). Prymnesin, an endotoxin from *P. parvum*, is a glycolipid with a molecular weight of about 23,000 (Paster, 1968). The lipid portion is composed of four long chain acids (myristic, stearic, palmitic, and oleic) and the polysaccharide portion of glucose, mannose, and galactose. The toxin (s) isolated from *G. breve* is thus one of the few algal toxins to be chemically characterized.

MATERIALS AND METHODS

REAGENTS

Most reagents used in this study were analytical grade, and all solvents were Baker [5] analyzed reagents with the following exceptions: Silica gel (grade 62, mesh 60–200) used for chromatography was obtained from Grace Davison Chemical, Baltimore, Md.; bone charcoal was B & A reagent grade; carbon tetrachloride used for spectra was GC-spectrophotometric reagent grade.

SOURCE OF *GYMNODINIUM BREVE*

Organisms were obtained in two ways. Axenic cultures of *G. breve* were obtained from W. B. Wilson (Wilson, 1965) and were subcultured in B-5 medium (Wilson, 1966). Water samples were collected during an outbreak of *G. breve* in the fall of 1967 near Big Pass, off Sarasota, Fla.

PROCEDURE FOR ISOLATION OF TOXINS

We isolated two substances by using the following procedure. Water from the bloom of *Gymnodinium breve* or from laboratory culture media (1×10^6 cells/liter) was adjusted to pH 4.0 by addition of a small quantity of concentrated (12 *M*) hydrochloric acid. Two-liter portions of the solution were then extracted in Fernbach flasks with 30 ml. of chloroform. The mixture was agitated with a mechanical stirrer; the top of the flask was covered with a slotted cardboard to avoid splashing. Each batch was stirred vigorously for 15 minutes and allowed to settle in a modified separatory funnel for 20 minutes. Water from the top layer was discarded by a side tap, and the chloroform extract (substance II) was taken out from the bottom and stored. The combined chloroform extracts were associated with an upper thick interfacial layer consisting of very small bubbles and containing substance I. This layer containing the bubbles was highly toxic to fish. Extracts from a particular batch of water were always kept in separate containers, stored in a refrigerator.

PURIFICATION

The two layers, i.e., the chloroform extract and the interfacial layer, were treated separately.

The clear chloroform layer (substance II) was separated, concentrated, and then purified by column chromatography. We used a separatory funnel to separate the chloroform from the interfacial layer. Generally, an extract containing an appreciable amount of toxin was intensely yellow. The chloroform layer (30 to 40 ml.) was concentrated in a rotary evaporator to one-tenth of its volume, and then passed through a silica-gel column (4 cm. long, 1 cm. diameter) at a rate of 18 to 20 drops per minute. Yellow material, with a greenish-yellow band above it, was adsorbed on top of the column. The colorless chloroform effluent was evaporated (rotary evaporator), and the yellow oily residue was nontoxic to fish. The column was next treated with methylene chloride (four 10-ml. portions), and the yellowish-green band was eluted, leaving the deep brown band on the silica gel. The effluent was evaporated in cold, and the greenish residue obtained was nontoxic to fish. Finally, the brown band was eluted with two 2- to 3-ml. portions of absolute ethanol. The deep yellow effluent was evaporated slowly in a vacuum desiccator. The residue was toxic.

The thick yellow residue (substance II) was dissolved in carbon tetrachloride and purified further. The solution was stirred with bone charcoal and filtered. The colorless carbon tetra-

[5] References to trade names or suppliers in this publication do not imply endorsement of commercial products.

chloride filtrate was evaporated slowly in a desiccator and yielded a light yellow viscous semisolid that was highly toxic.

The interfacial layer (substance I) was separated and the toxin was isolated by removal of solvent. The layer was composed of bubbles trapped in chloroform; normally, a large amount of salt water was associated with it. We washed this layer twice with 250-ml. portions of dilute hydrochloric acid solution (pH 3.5–4.0) in a separatory funnel. The organic layer was collected in long test tubes and allowed to stand over silica gel for drying. Thus purified, substance I was mostly free from sodium chloride. When the organic material was sufficiently dried, flaky dark-green suspended matter was found in the test tubes. Contents of the tubes were transferred to a flask, and the volume was increased 25 percent with absolute ethanol. The mixture was stirred 4 hours, cooled, and filtered. The residue was not toxic. The filtrate was cooled in an ice-water bath and evaporated under reduced pressure.

The residue (substance I) was dissolved in ethanol and purified further. The ethanol solution was cooled in a Dry-Ice bath, stirred with bone charcoal, and filtered. When the filtrate was evaporated, the residue was a toxic, yellowish, oily solid.

THE TOXIN UNIT

One *Gymnodinium breve* fish-kill unit was defined arbitrarily as the amount of toxin per 50 ml. of sea water which under standard conditions killed a 3.0- to 3.5-g. (6- to 7.5-cm.) longnose killifish, *Fundulus similis*, in 7 to 8 minutes. The standard conditions included 50 ml. of test solution in a 400-ml. beaker, controlled temperature (23°±2° C.), and appropriate salinity (32–33 p.p.t.) and pH (8.0–8.2).

EFFECT OF TEMPERATURE

The toxin (substance I in interfacial layer) was maintained at room temperature, 23°±2° C., and periodically tested for potency. The results, summarized in table 1, indicate that a solution of the toxin is stable at room temperature for at least 96 hours.

All toxicity was lost when the sample in chloroform-water solution was heated to 110° C.

TABLE 1.—*Temporal stability of crude toxin (substance I) from* G. breve *at room temperature*

Time	Volume of toxin [1]	Amount of water taken	Length of fish [2]	Approximate weight of fish	Death time	Fish-kill unit
Hours	*Drops*	*Ml.*	*Cm.*	*G.*	*Minutes*	
0	2	50	3.8	1.1	5	1
1	2	50	3.8	1.2	5–6	1
4	2	60	3.8	1.5	5–6	1
8	2	75	3.8	2.0	5–6	1
24	2	75	3.8	2.0	6–7	1
30	2	100	6.4	3.0	6	1
96	2	50	3.5	1.1	6–7	1

[1] Control: two drops of chloroform in same volume of sea water. No effect on fish within 120 minutes.
[2] *Fundulus similis.*

for 5 minutes, and the loss in weight of the sample was 0.3887 g. or 97.5 percent. At this temperature, volatilization of chloroform and water would be expected.

In addition, presumably these data indicate either the volatility or the ease of decomposition of the toxin. Attempts to detect decomposition products with gas chromatography were unsuccessful. An Aerograph vapor phase chromatography unit, equipped with Carbowax 400 column and a thermal detector, was used. Column temperature was maintained at 55° C. The amount of toxin (substance I in ether) may have been insufficient to detect.

Attempts were made to measure the volatility of the sample as follows: One fish was placed in each of two beakers (under the conditions previously defined for the toxin unit). The two beakers were placed in a closed system, and the first beaker was treated with 1 fish kill unit of toxin (substance I). Four minutes later a stream of nitrogen was passed through solution in the first beaker, forcing any evolved gases to pass through the second beaker. The fish in the first beaker died after 6 minutes; the one in the second beaker was unaffected for 12 minutes, after which time the experiment was discontinued. We concluded that the toxin was not volatile at atmospheric pressure at room temperature. However, a loss of toxicity under reduced pressure (when a rotary evaporator is used) was amply indicated.

ELEMENTAL QUALITATIVE ANALYSIS

Purified samples of toxin (substance I and substance II) were fused with sodium and decomposed with water, and the solution was analyzed for nitrogen, sulfur, halogen (chlorine,

bromine, or iodine), and phosphorus by the procedures of Vogel (1956). Positive tests were obtained only for phosphorus. In all cases, the results were compared with known compounds to verify the test.

ELEMENTAL QUANTITATIVE ANALYSIS

Semimicro quantitative analyses for carbon, hydrogen, and nitrogen were performed by Peninsular Chem Research, Inc., Gainesville, Fla. (table 2).

Organic phosphorus was determined by a conventional method (Martin, 1968). The calibration curve (fig. 1) was prepared with a

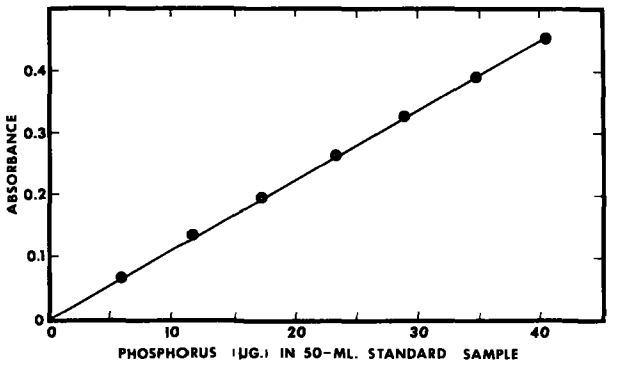

FIGURE 1.—Calibration plot for organic phosphorus determination. Standard sample prepared by decomposition of 9.8 mg. of disodium *p*-nitrophenylphosphate, followed by dilution to 50 ml. with triple-distilled water at 705 mμ.

phosphorus standard (disodium *p*-nitrophenylphosphate). Exactly 0.89 mg. of substance II was decomposed and diluted to 50 ml.; two 25-ml. portions had absorbances of 0.058 and 0.055, which corresponded to 1.41 percent P. A second analysis was performed similarly with 0.99 mg. of substance II, except that 6.968 μg. of phosphorus (as phosphorus standard) was added; total absorbances of 0.115 and 0.118 were obtained, which gave a corrected value of 1.35 percent P.

Total oxygen was determined by Schwarzkopf Microanalytical Laboratory, Woodside, N.Y. Exactly 0.200 mg. of toxin sample produced 0.115 mg. of carbon dioxide.

TABLE 2.—*Elemental quantitative analysis of substance II (bloom sample)*

Sample	Carbon	Hydrogen	Nitrogen	Phosphorus	Oxygen
			Percent		
1.....	47.05	6.94	0.00	0.00	0.00
2.....	49.60	7.60	[1](0.6)	1.41	[2] 41.4
3.....	0.00	0.00	0.00	0.00	41.8

[1] Not analytically significant.
[2] Calculated by difference from 100 percent total.

MOLECULAR WEIGHT

The molecular weight of substance II (bloom sample), determined by Galbraith Laboratories, Inc., Knoxville, Tenn., by vapor-pressure osmometry, was 650 (chloroform solvent, 0.03 *M*).

ABSORPTION SPECTRA

The carbon tetrachloride solution of substance II from the bloom sample had no absorption in the visible region of the spectrum; its absorption maximum was at 269.5 to 270 mμ (2.5 mg./3 ml. in CCl_4) in the ultraviolet region. When the solution was diluted, the absorption maximum broadened and was 265 to 270 mμ for the most dilute solution (table 3).

The prominent absorption bands of the infrared absorption spectra of various toxin fractions (as carbon tetrachloride solutions) were determined (table 4). Tentative assignments are also indicated and are compared with standard values given by Bellamy (1958).

TABLE 3.—*Ultraviolet absorption of carbon tetrachloride solutions of substance II at 269 mμ*

Concentration	Maximum absorbance
(Mg./1 ml. CCl_4)	
0.833	1.020
.556	0.730
.371	0.490
.247	0.340

The similarity of substance II samples isolated from bloom water and from culture medium is indicated by the infrared data obtained with a Perkin-Elmer 337 infracord. Substance I could not be isolated in sufficient quantity from culture medium for analysis. The bloom sample of substance I seems to be different from the bloom sample of substance II; the infrared spectrum of the former indicates the presence of a carbonyl group, whereas the spectrum of the latter does not.

TABLE 4.—*Characteristic infrared absorption frequencies of three toxin samples dissolved in CCl_4*

Frequency [1]	Possible assignment	Literature value [1]
	Substance II (bloom sample)	
3,000 (vs)	C-H (paraffinic) (unsaturated)	3,000 (s)
2,400 (s)	P-H	PH_3, 2,327–2,421
1,422 (br,s)	PC_6H_5	1,450 (s), 1,000 (m)
1,266 (s)	$+P(OR)_2$	1,265 (s)
1,217 (vs)	$P-OC_6H_5$	1,218 (P-O-aromatic) (s)
920 (m)	P-O-P	970–940 (m)
667 (vs)	P-C (aliphatic)	750–650 (s)
	Substance I (bloom sample)	
2,940 (s), 2,865 (s)	$-CH_3$	2,962, 2,872 ± 10 (s)
2,905 (s)	$-CH_2-$	2,926, 2,553 ± 10 (s)
1,730 (m)	$-CO-$	1,700–1,750 (s)
1,460 (m)	$-C-CH_3$	1,450 ± 20 (m)
1,265 (s), 1,010 (s)	$+P(OR)_2$	1,265 (s), 1,050–1,000 (s)
1,210 (s)	$(CH_3)_2-C-R$	1,250–1,200 (s)
1,080 (s)	$-CH_2-O-CH_2$	1,150–1,160 (vs)
1,010 (s)	cyclopropyl	1,020–1,000 (m)
	Substance II (culture medium sample)	
2,950 (s)	$-CH_3$	2,962, 2,872 ± 10 (s)
2,900 (s)	$-CH_2-$	2,926, 2,553 ± 10 (s)
1,263 (s)	$+P(OR)_2$	1,265
1,215 (vs)	POC_6H_5	1,218 (P-O-aromatic) (s)
667 (vs)	P-C (aliphatic)	750–650 (s)

[1] Intensity abbreviations: (vs) very strong; (s) strong; (m) medium; (br) broad.

NUCLEAR MAGNETIC RESONANCE SPECTRA

The background of nuclear magnetic resonance spectroscopy was given by Pople, Schneider, and Bernstein (1959). The spectrum was obtained for a 5-percent (w/v) solution of purified substance II (bloom sample) in $CDCl_3$. The spectrum had two signals: one at 6.31τ and the other at 9.38τ. Tetramethylsilane (TMS) was used as an external, not an internal, standard to avoid contaminating the sample.

A spectrum was also obtained of a 1-percent (w/v) carbon tetrachloride solution (2.5 mg. of substance II in 0.3 ml. of solvent). Again, an external standard TMS was used. Signals were obtained at 6.4 (singlet) and 9.45τ (singlet, triplet).

A signal at 6.3τ may be ascribed to a methylene group attached to an electronegative atom or moiety, e.g., oxygen. The signal at 9.38τ represents an unusually high tau value. One possibility is contamination from silicone grease, though this seems unlikely in view of the precautions taken. The signal intensity suggests a second possibility—that the signal can be ascribed to a methylene in a strained ring. For example, cyclopropane $(CH_2)_3$ has a signal at 9.778τ. The presence of a cyclopropyl moiety seems unlikely because of the absence of prominent absorption peaks in the 1,000 to 1,200 cm.$^{-1}$ region of the infrared region (Yukawa, 1965). For comparison, a methylene group in an aliphatic hydrocarbon would produce a signal at 8.75τ.

OPTICAL ACTIVITY

A Bendix Corporation NPL automatic polarimeter, Type 143A, fitted with a mercury-green lamp (546.1 mμ), was used for all optical activity measurements. The precision of this instrument is ±0.0002°.

The instrument was calibrated with sucrose solution (table 5), by the following technique. Exactly 100 divisions (1 complete rotation) on the fine adjustment zero-control knob corresponds to 10 m.° (=0.01 degree) of rotation of the plane of plane-polarized light. The reading for pure solvent (for a given measurement) was adjusted to zero by using the fine adjustment knob, and the reading on the knob was recorded. Next, the test solution was placed in the cell and the galvanometer pointer was returned to zero. The reading on the knob was recorded. The difference between the two readings (for the solvent and that for the test solution) was converted to millidegrees of observed rotation, α (100 divisions=10 m.°).

For all test solutions, the specific rotation $[\alpha]^{25°}_{546m\mu}$ was calculated from the usual relationship (Vogel, 1956): $[\alpha] = 100\,M/(\ell pd)$, where α is the observed rotation, ℓ is the length of the light path, (0.1 dm), p is the concentration

TABLE 5.—*Specific optical activity of toxin samples at 25° C. and 546 mμ*

Sample	Sign of rotation	Observed rotation	Specific activity
		α, m.°	$[\alpha]^{25°}_{546m\mu}$
1. Sucrose solution, (3.062%)	+	241.0	78.38
2. Substance II in absolute ethanol			
a. After column chromatography	+	2.25	68.2
b. After charcoal treatment	+	3.5	47.0
3. Sample 2b (above) after evaporation of solvent in vacuum desiccator and resolution in absolute ethanol	−	0.3	4.0

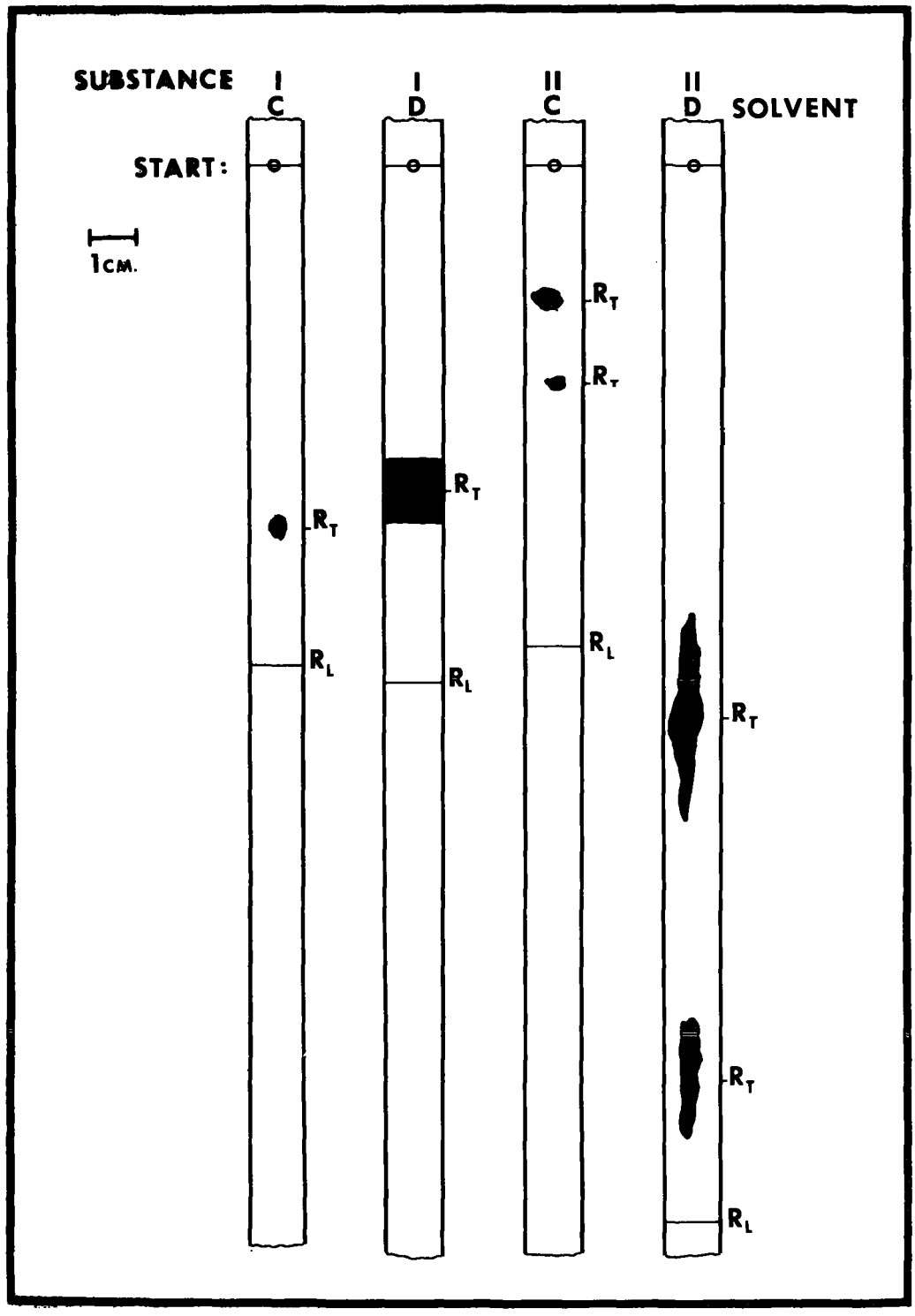

FIGURE 2.—Descending paper chromatograms of toxins (see table 6).

(g. solute/100 g. solution), and d is the density (g./ml.) of the solution (table 5).

The optical activity measurements have several points of interest. First, the sample of substance II is optically active and is dextrorotatory. Some loss of optical activity seemed to occur during the purification scheme (sample 2a versus 2b, table 5). This loss may be due to the removal of a volatile component, as indicated by the further loss of optical activity accompanying evaporation (sample 2b versus 3). It appeared, qualitatively, that there was no loss of toxicity associated with the loss of optical activity.

CHROMATOGRAPHY

TLC (thin-layer chromatography) and paper chromatography (Heftmann, 1967) were used to characterize the toxin and to determine if the purified toxin contained one component or two, as suggested by molecular weight versus weight data from the empirical formula.

When an Eastman chromatogram (for TLC) with silica-gel absorbent was used, only one spot was located (with ultraviolet light) with two different solvent mixtures. The R_f values [6] indicate that the effect of change of solvent polarity was small. The behavior of the toxin with TLC (silica gel) was consistent with column chromatography, i.e., only one fraction was obtained.

TABLE 6.—*R_f values for toxin chromatograms* [1]

Chromatogram	Toxin sample	Solvent mixture	R_f [2]
TLC (silica gel)	Substance II (bloom)	A	0.89
Do	do	B	.90
Paper	Substance I (bloom)	C	.72
Do	do	D	.63
Do	Substance II (bloom)	C	.83, .38
Do	do	D	.85, .52

[1] Solvent mixture A, 10 percent (v/v) chloroform-absolute ethanol; solvent mixture B, 10 percent (v/v) chloroform-isoamyl alcohol; C, absolute ethanol; D, isoamyl alcohol.

[2] The R_f value is defined as the ratio of the distance traveled by a substance (R_T) to the distance traveled by the solvent (R_L).

Another system, paper chromatography, was selected to see if more than one component was present in the fraction. Descending paper chromatography was effected with different solvents (table 6). The chromatograms were run for 5 hours, after which the paper strips were dried, developed with a mixture of minhydrinphenol methyl cellosolve mixture, and washed with dilute sodium hydroxide solutions. Two components were indicated (fig. 2, table 6). R_f values for one component seemed independent of solvent polarity; the R_f values for a second were affected by change in solvent polarity.

[6] The R_f value is defined as the ratio of the distance traveled by a substance (R_T) to the distance traveled by the solvent (R_L).

ACETYLCHOLINESTERASE ACTIVITY

A potentiometric determination (Jensen-Holm, Lausen, Milthers, and Moller, 1959) was used to measure enzyme activity. The brains of five sheepshead minnows, *Cyprinodon variegatus*, were placed in 12 ml. of distilled water, cooled in an ice-water bath, and homogenized for 40 seconds with an ultrasonic macerator operated at full power. The test solution was prepared by diluting 2.0 ml. of the homogenate to 4.5 ml. with distilled water. The pH was adjusted to 7.8 with 0.02 N sodium hydroxide solution, and 0.05 ml. of substrate (0.1 M acetylcholine iodide) was added. The cholinesterase activity was determined poten-

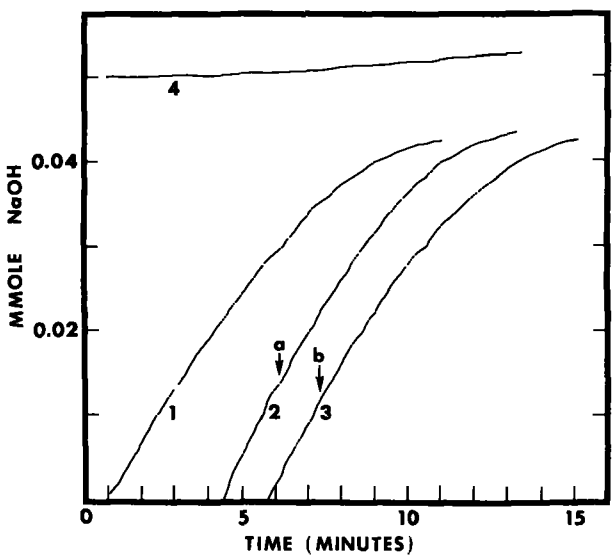

FIGURE 3.—Potentiometric determination of cholinesterase activity, micromoles of sodium hydroxide added as a function of time (minutes). Curve 1, standard run; curve 2, 0.02 ml. of toxin solution added at point a; curve 3, 0.4 ml. of toxin solution added at point b; curve 4, spontaneous acid liberation in absence of homogenate.

tiometrically at 38° C. with an automatic titrator operated as a pH-stat unit. Enzyme activity was expressed as micromoles of sodium hydroxide added per minute (fig. 3) to a pH 7.5 end-point.

The activity of the toxin was tested as follows. In a succeeding trial, the enzyme activity was measured again, and after 2 minutes an aqueous solution of the toxin (substance II dispersed in water by means of the ultrasonic macerator) was added. The determination curves (fig. 3) indicate the addition of 0.2 ml. or 0.4 ml. of toxin solution has no effect on homogenate activity. The activity of the toxin solution was verified: 0.2 ml. of solution (*ca.* 0.15 mg. substance II) added to 30 ml. of sea water caused death of sheepshead minnows (5.5 cm., 4.1 g.) within 3 minutes.

MASS SPECTRAL ANALYSIS

The mass spectrum of substance II (bloom sample) was obtained by using the following conditions: ionizing voltage 70 e.v. pressure 1.5 $\times 10^{-6}$ mm. Hg., and temperature 50° C. (table 7).

TABLE 7.—*Mass-charge ratios (m/e) and relative abundances from mass spectral analysis of substance II.*

m/e	Relative abundance	m/e	Relative abundance
27	11.0	151	37.0
31	30.9	153	13.5
42	54.1	155	13.5
45	80.0	157	13.5
57	62.7	160	30.8
59	77.5	240	14.9
63	19.6	258	8.6
67	22.1	266	6.3
69	54.0	290	9.0
72	60.6	301	6.3
83	60.1	309	5.0
86	56.5	310	5.0
100	45.5	311	5.0
102	24.7	319	5.0
107	16.1	320	5.0
121	17.2	321	5.0
124	18.1	333	4.8
128	24.8	334	4.8
131	15.4	335	4.8
138	15.1	348	4.8
141	15.1	349	4.8
143	15.1	[1] 350	4.8

[1] The amplified scale spectrum (100 times regular intensity) indicates stable species (with relative abundance of less than 1) at m/e of 375, 390, 450, 418, 440, 477, 508. Maximum relative abundance is 80.

SOME CHEMICAL CHARACTERISTICS AND STRUCTURAL FEATURES OF THE TOXIN

The purpose of the present study was to isolate the toxins of *Gymnodinium breve* blooms and cultures and to determine the chemical and physical properties of these toxins. During the course of the study, sufficient information was obtained from these properties to indicate some structural features of the toxins. The topics will be considered in order; a review of the chemical characteristics of the toxin is necessary to understand the structural characteristics. Unfortunately, not enough substance I was isolated to evaluate its chemical characteristics, and most of the discussion must be confined to substance II.

CHEMICAL CHARACTERISTICS

Several results of the present study are consistent with those of previous investigators.

First, several workers (McFarren et al., 1965; Sasner, 1965; Cummins, Stevens, Huntley, Hill, and Higgins, 1968) have also found two (or more) toxins which differ in solubility and other physical properties. In the present study, two toxins—substances I and II—were separated from *Gymnodinium breve* bloom water. Both have been separated from cultures of *G. breve*, although substance I was isolated only in trace quantities and could easily have been missed.

Secondly, the major toxin (substance II) is essentially an endotoxin. This determination was made in the present study by dividing a 1–1. sample of *G. breve* culture into two portions and extracting the medium before (portion A) and after (portion B) the cells had been removed by gravity filtration. Detectable amounts of toxin could be isolated from portion A but not from portion B. An alternative possibility, complete adsorption of the toxin in the filter paper, has not been observed.

Thirdly, the major toxin of *G. breve* may be a neurotoxin, but it evidently does not inhibit cholinesterase activity in vitro.

Fourthly, the toxins (substance I or II) seem to volatilize under reduced pressure, although the toxin is not readily volatilized from aqueous solution by a stream of nitrogen.

The study provided the following results that apparently have not been reported previously.

1. The major toxin (substance II) isolated from a bloom of *G. breve* appears to be similar to that isolated from cultures of *G. breve*. This

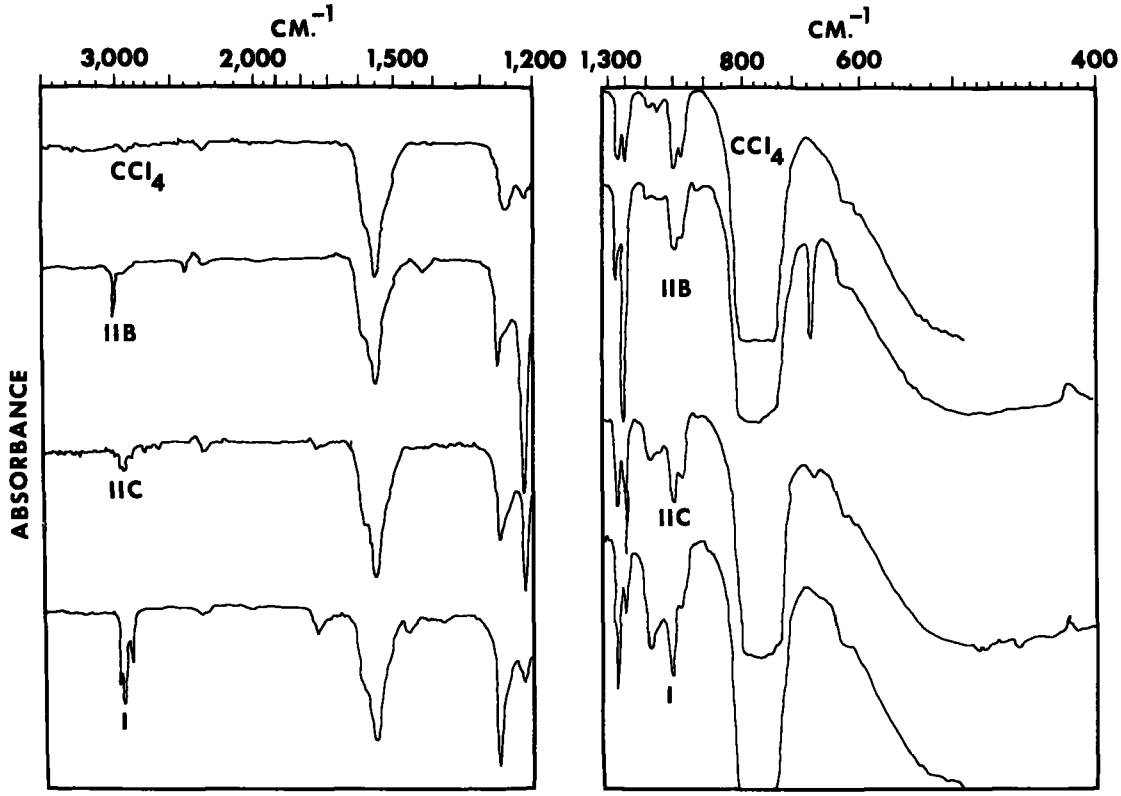

FIGURE 4.—Infrared absorption spectra of toxins in CCl_4. The instrument used provides spectra in two regions, low and high wavelength, as indicated. (Reference cell contained air.) Key to spectra; CCl_4, pure solvent; IIB, substance II (bloom sample); IIC, substance II (culture sample); I, substance I (bloom sample).

observation is based on a study of infrared spectral data (table 4). These data also indicate that substances I and II differ; notably a carbonyl group is indicated by the spectrum of substance I (fig. 4) but not by the spectrum of substance II.

2. The major toxin (substance II) does not contain nitrogen according to qualitative and quantitative analysis (table 2). This appears to be the first report of a phosphorus-containing toxin isolated from dinoflagellate blooms, a fact that is of considerable interest because organophosphorus compounds have been used as model compounds in the study of factors controlling nerve activity (Nachmansohn, 1961).

3. An empirical formula of $C_{90}H_{162}O_{17}P$ was calculated from the mean values of the quantitative elemental analytical data of substance II (table 2). Substance I could not be isolated in the quantity required for elemental analysis. As usual, the validity of the empirical formula is governed by the validity of the elemental analysis. In this instance, the reliability is indicated by replication of carbon and hydrogen analysis for different samples. The phosphorus analysis is the limiting factor in the accuracy of the formula. The results with a "spiked" sample suggest decomposition is complete; the values are within the experimental error.

4. A molecular weight of 650 was determined for substance II. This is about one-half the value of the empirical formula weight (1,545) and indicates each unit of substance II exists as two particles in chloroform (and presumably in other polar solvents). The observation gives no information about the relative sizes of the two particles. The two particles must be similar in properties because only one band is obtained with chromatography (thin-layer or column) using silica gel. Also, two bands indicative of two components were obtained when substance

II was subjected to descending paper chromatography (table 6).

5. Three methods have been devised for detecting the toxin. These are: a toxicity test under a standard set of conditions; a spectroscopic test using the absorbance of a CCl_4 solution at 270 mμ (ε obs.=790, ε calc.=1900)[7]; and the optical activity test that detects certain centers or units of asymmetry.

6. Substance II is optically active, which indicates a center or molecular unit of asymmetry (Morrison and Boyd, 1967). An example of a center of asymmetry would be an asymmetric carbon atom as in 1-alanine; an example of a molecular unit of asymmetry would be tris (ethylenediamine) chromium (III) ion. The specific optical rotation of substance II is +68 (maximum, table 6) which may be compared with +128 for the poison of *Gymnodinium catenella* (Schantz et al., 1966). The former value was obtained at 546 mμ, and the latter was probably obtained at 589 mμ.

STRUCTURAL FEATURES

A complete structural determination of the toxin(s) was beyond the scope of the present study, but several structural features became apparent. These are valid, of course, for the toxin at the described state of purification.

First, the spectrum of substance I evidently contains a carbonyl group, =C=O; that of substance II does not (fig. 3). Both substances have infrared spectra that are consistent with organophosphates R-PO(OR)$_2$, where R is an oxygenated hydrocarbon moiety.

Second, the toxin molecule contains some asymmetric feature, either an asymmetric carbon atom(s) or a larger asymmetric unit.

Third, some structural features are indicated by the mass spectrum (Beynon, 1960; Biemann, 1962), though the spectrum must be interpreted with some care because of the volatility of substance II. For example, the maximum formula weight of a stable fragment is 508. Any fragments having greater formula weights are too volatile or too short-lived to be detected. It is also possible that the substance readily decomposes into three fragments with similar formulas; thus, the value of 508 corresponds to about one-third of the calculated empirical formula weight.

Finally, it may be hypothesized that a toxin portion of molecular weight 650 decomposes into two fragments with m/e values of about 508 and 147. The latter fragment might be a phosphate ester OP(OR)OR.

Peak assignments, which appear to be reasonable on the basis of available data (table 7), include the following:

1. The peak at m/e=31 probably corresponds to phosphorus-31.
2. The peak at m/e=63 is attributable to a PO_2 unit, which should come from the R'-PO(OR)$_2$ structure.
3. The P(OR)(OR') fragment might be assigned to the peaks at m/e=138–143. If R and R' is CH_2CH_3, a m/e value of 137 would be expected; if R is $CH=CH_2$ and R' is $CH_2CH=CH_2$ m/e value of 142 would be expected. The second possibility is consistent with the ultraviolet absorption spectrum. Cleavage of the vinyl group would account for peaks at about 27 ($CH=CH_2$) and 121 [PO(OCH=CH_2)OCH$_2$]. Further cleavage of a methylene fragment would account for a peak at about 107.

ACKNOWLEDGMENTS

J. R. Linton provided helpful discussion and assistance in the study of acetylcholinesterase activity; J. A. Zoltewicz, University of Florida, helped us obtain the mass spectrogram of substance II; S. M. Ray and W. B. Wilson, Texas A&M University, provided initial cultures and advice; the BCF (Bureau of Commercial Fisheries) gave financial support; and the Public Health Service gave a Research Career Award (to DFM, 1 KO 4 GM 425691) from the National Institute of General Medical Sciences. We were helped by personnel of the BCF Biological Laboratory, St. Petersburg Beach, including James E. Sykes, Director, who with J. K. McNulty contributed helpful advice and criticisms; J. H. Finucane, who helped collect bloom samples; and Dorothy Hartswick, who helped prepare this manuscript for publication. Personnel

[7] The observed value is based on a molecular weight of 650; the calculated value is based on an empirical formula weight of 1545.

of the Gulf Coast Marine Health Sciences Laboratory, Dauphin Island, reviewed this manuscript.

LITERATURE CITED

ABBOTT, B. C., and DOROTHY BALLANTINE.
1957. The toxin from *Gymnodinium veneficum*. J. Mar. Biol. Ass. U.K. 36: 169–189.

BEIN, SELWYN JACK.
1954. A study of certain chromogenic bacteria isolated from "red-tide" water, with a description of a new species. Bull. Mar. Sci. Gulf Carib. 4: 110–119.

BELLAMY, L. J.
1958. The infra-red spectra of complex molecules. 2d ed. John Wiley & Sons, New York, 425 pp.

BEYNON, J. H.
1960. Mass spectrometry and its applications to organic chemistry. Elsevier, Amsterdam, 640 pp.

BIEMANN, KLAUS.
1962. Mass spectrometry organic chemical applications. McGraw-Hill Book Co., New York, 370 pp.

BISHOP, C. T., E. F. L. J. ANET, and P. R. GORHAM.
1959. Isolation and identification of the fast death factor in *Microcystis aeruginosa* NRC-1. Can. J. Biochem. Physiol. 37: 453–471.

CUMMINS, JOSEPH M., ALAN A. STEVENS, BOB E. HUNTLEY, WILLIAM F. HILL, JR., and JAMES E. HIGGINS.
1968. Some properties of *Gymnodinium breve* toxin(s) determined bioanalytically in mice. *In* H. D. Freudenthal (editor), Proceedings, Drugs from the Sea Conference, Marine Technological Society (Washington, D.C.), pp. 213–228.

GORHAM, PAUL R.
1960. Toxic waterblooms of blue-green algae. Can. Vet. J. 1: 235–245.

HEFTMANN, ERICH.
1967. Chromatography. 2d ed. Reinhold Publishing Corp., New York, 896 pp.

JENSEN-HOLM, J., H. H. LAUSEN, K. MILTHERS, and KNUD O. MOLLER.
1959. Determination of the cholinesterase activity in blood and organs by automatic titration. Acta Pharmacol. Toxicol. 15: 384–394.

MARTIN, DEAN F.
1968. Analytical methods: marine chemistry 1. Marcel Dekker, Inc., New York, 280 pp.

MCFARREN, E. F., H. TANABE, F. J. SILVA, W. B. WILSON, J. E. CAMPBELL, and K. H. LEWIS.
1965. The occurrence of a ciguatera-like poison in oysters, clams, and *Gymnodinium breve* cultures. Toxicon 3: 111–123.

MORRISON, ROBERT THORNTON, and ROBERT NEILSON BOYD.
1967. Organic chemistry. 2d ed. Allyn and Bacon, Inc., Boston, Mass., 1204 pp.

NACHMANSOHN, DAVID.
1961. Chemical factors controlling nerve activity. Science 134: 1962–1968.

PASTER, Z.
1968. Prymnesin: the toxin from *Prymnesium parvum* Carter. Rev. Int. Oceanogr. Med. 10: 249–257. *Through* Oceanogr. Abstr. 15: A582.

POPLE, J. A., W. G. SCHNEIDER, and H. J. BERNSTEIN.
1959. High-resolution nuclear magnetic resonance. McGraw-Hill Book Co., New York, 501 pp.

RAY, SAMMY M., and WILLIAM B. WILSON.
1957. Effects of unialgal and bacteria-free cultures of *Gymnodinium breve* on fish, and notes on related studies with bacteria. U.S. Fish Wildl. Serv., Fish. Bull. 57: 469–496.

SASNER, JOHN JOSEPH, JR.
1965. A study of the effects of a toxin produced by the Florida red tide dinoflagellate, *Gymnodinium breve* Davis. Ph. D. thesis, Univ. Calif., Los Angeles, 84 pp.

SCHANTZ, EDWARD J., JOSEPH M. LYNCH, GEORGE VAYVADA, KEN MATSUMOTO, and HENRY RAPOPORT.
1966. The purification and characterization of the poison produced by *Gonyaulax catenella* in axenic culture. Biochemistry 5: 1191–1195.

SCHUETT, WOLFGANG, and HENRY RAPOPORT.
1962. Saxitoxin, the paralytic shellfish poison: degradation to a pyrrolopyrimidine. J. Amer. Chem. Soc. 84: 2266–2267.

SHILO, MOSHE.
1967. Formation and mode of algal toxins. Bacteriol. Rev. 31: 180–193.

STARR, THEODORE J.
1958. Notes on a toxin from *Gymnodinium breve*. Tex. Rep. Biol. Med. 16: 500–507.

VOGEL, ARTHUR I.
1956. A textbook of practical organic chemistry. 3d ed. Longmans Green & Co., London, Ch. XI, 1188 pp.

WILSON, WILLIAM B.
1965. The suitability of sea-water for the survival and growth of *Gymnodinium breve* Davis; and some effects of phosphorus and nitrogen on its growth. Ph. D. thesis, Texas A&M Univ., 103 pp.
1966. The suitability of sea-water for the survival and growth of *Gymnodinium breve* Davis; and some effect of phosphorus and nitrogen on its growth. Fla. State Bd. Conserv. Mar. Lab., Prof. Pap. Ser. 7, 42 pp.

YUKAWA, YASHUHIDE (EDITOR).
1965. Handbook of structural organic analysis. W. A. Benjamin, Inc., New York, 779 pp.