constituents in ungerminated seeds was transformed into one smaller arc with about the same electrophoretic mobility as that of the middle constituent (F_2) of the original protein. The most anodic and most cathodic constituents (F_1 and F_3) were catabolized enough to lose their antigenicities. The two components, F_1 and F_3 (represented as two or more bands by Sobolev et al. (1972) for the castor crystalloid protein), were more labile than the protein of intermediate mobility, F_2, which seemed to predominate. This was noted after 6 days' germination or after treatment of the proteins with trypsin. The reasons for this phenomenon are unknown and higher purification of protein F and examination of the structures of its constituents will be required.

In summary, a total protein extract of ungerminated castor seeds contained at least seven antigens of which the CB-1A allergen, the major storage protein, and four cytoplasmic (or subcellular) proteins were identified. Germination of the seeds caused an anodic shift in the CB-1A major antigen and a decrease in the most anodic and cathodic portions of the major storage protein, F. These changes could have been due to a configurational change caused by increased exposure of surface-charged groups on the proteins, but without loss of antigenicity or complete loss of antigenic structure. After germination some proteins were no longer detectable by IEA; but even after 10 days, proteins extracted from the seedlings still contained a significant amount of the major CB-1A antigen.

LITERATURE CITED

"Agricultural Statistics", U.S. Department of Agriculture, U.S. Government Printing Office, Washington, D.C., 1972, p 176.
Altschul, A. M., Ory, R. L., St. Angelo, A. J., *Biochem. Prep.* **10**, 93 (1963).
Berrens, L., *Monogr. Allergy* **7**, 1–298 (1971).
Catsimpoolas, N., Campbell, T. G., Meyer, E. W., *Plant Physiol.* **43**, 799 (1968).
Cherry, J. P., Dechary, J. M., Ory, R. L., *J. Agric. Food Chem.* **21**, 652 (1973).
Coulson, E. J., Spies, J. R., Stevens, H., *J. Am. Oil Chem. Soc.* **37**, 657 (1960).
Coulson, E. J., Spies, J. R., Stevens, H., Shimp, J. H., *J. Allergy* **21**, 33 (1950).
Daussant, J., Neucere, N. J., Conkerton, E. J., *Plant Physiol.* 44, 480 (1969a).
Daussant, J., Neucere, N. J., Yatsu, L. Y., *Plant Physiol.* **44**, 471 (1969b).
Grabar, P., Williams, C. A., *Biochim. Biophys. Acta* **10**, 193 (1953).
Kloz, J., Turkova, V., Klozova, E., *Biol. Plant.* **8**, 164 (1966).
Layton, L. L., Dante, B. T., Moss, L. K., Dye, N. H., DeEds, F., *J. Am. Oil Chem. Soc.* **38**, 405 (1961).
Mottola, A. C., Mackey, B., Walker, H. G., Kohler, G. O., *J. Am. Oil Chem. Soc.* **49**, 662 (1972).
Mourgue, M., Baret, R., Reynaud, J., Bellini, J., *Bull. Soc. Chim. Biol.* **40**, 1453 (1958).
Ory, R. L., Yatsu, L. Y., Kircher, H. W., *Arch. Biochem. Biophys.* **123**, 255 (1968).
Sobolev, A. M., Suvorov, V. I., Safonova, M. P., Prokof'ev, A. A., *Sov. Plant Physiol. (Engl. Transl.)* **19**, 894 (1972).
Spies, J. R., *Ann. Allergy* **25**, 29 (1967).
Spies, J. R., *J. Agric. Food Chem.* **22**, 30 (1974).
Spies, J. R., Barron, J. K., *Ann. Allergy* **24**, 499 (1966).
Spies, J. R., Coulson, E. J., *J. Am. Chem. Soc.* **65**, 1720 (1943).
Spies, J. R., Coulson, E. J., *J. Biol. Chem.* **239**, 1818 (1964).
Waller, J. R., Negi, S. S., *J. Am. Oil Chem. Soc.* **35**, 409 (1958).

Received for review April 21, 1975. Accepted September 17, 1975.

Isolation of *Gonyaulax tamarensis* Toxins from Soft Shell Clams (*Mya arenaria*) and a Thin-Layer Chromatographic–Fluorometric Method for Their Detection

Lawrence J. Buckley,[1] Miyoshi Ikawa,* and John J. Sasner, Jr.

A thin-layer chromatographic–fluorometric method for the detection and determination of *Gonyaulax tamarensis* toxins in column chromatography fractions is described. Fractions are chromatographed on silica gel plates and the plates sprayed with 1% hydrogen peroxide, heated, and scanned in a fluorometer. By this means 40–400 ng of the various poisons could be determined. Monitoring with this procedure, *G. tamarensis* major toxin was separated into major toxins H and L, with approximate potencies of 1800 and 4200 mouse units per mg, respectively. At pH 4.7 major toxin H was unaffected, even after heating, while L appeared partially converted to H. At pH 8.2 at 16°C major toxin H was unchanged, while L gave H and a small amount of material which corresponded in R_f to saxitoxin. At pH 8.2 heating destroyed both toxins. The fluorescence properties of major toxins H and L and saxitoxin after hydrogen peroxide treatment also suggest similarity in their structures.

Paralytic shellfish poisoning (PSP) is a severe form of food intoxication which occurs in widely scattered areas of the globe (Halstead, 1965). The poison is produced by certain species of marine dinoflagellates which normally make up only a very small portion of the plankton upon which the shellfish feed. Occasionally, however, these toxic dinoflagellates bloom, reaching concentrations as high as 40000 cells/ml. This condition is often called a "red tide". Shellfish, themselves unaffected, filter the dinoflagellates from the water, effectively concentrate the poison, and become toxic to humans and other animals that feed on them.

Shellfish from areas of the United States and Canada affected by outbreaks of PSP are continually monitored for the presence of poison using a modification of the bioassay (mouse test) first employed by Sommer and Meyer in 1937 (see Halstead, 1965). Although chemical (McFarren et al., 1958, 1959) and serological tests (Johnson and Mulberry, 1966) for the estimation of paralytic

Departments of Biochemistry and Zoology, University of New Hampshire, Durham, New Hampshire 03824.
[1]Present address: Department of Pharmacognosy, College of Pharmacy, University of Rhode Island, Kingston, R.I. 02881.

shellfish poison have been developed, they are not widely used since they are complex and time consuming. Recently, Bates and Rapoport (1975) have described a sensitive assay for the shellfish poison saxitoxin (STX) based on a fluorescent derivative formed when STX is treated with alkaline hydrogen peroxide.

Three rather severe outbreaks of PSP along the New England Coast in 1972 and 1974 have generated renewed interest in the chemistry of paralytic shellfish poison. A previous report (Buckley et al., 1975) described the isolation of two toxic components from soft shell clams (*Mya arenaria*) exposed to a bloom of *Gonyaulax tamarensis*. One component (minor toxin) was identified as STX, a potent neurotoxin previously isolated from cultures of *Gonyaulax catenella* (Schantz et al., 1966), from mussels and Alaska butter clams exposed to *G. catenella* (Schantz et al., 1957), and from scallops exposed to *G. tamarensis* (Ghazarossian et al., 1974).

The present report describes the fractionation of the major toxin into two chromatographically pure components, each having potent neurotoxic activity. A new in situ thin-layer chromatographic–fluorometric method for the detection and quantitation of the three neurotoxins is also described.

EXPERIMENTAL SECTION

Mouse Test. The toxicity of aqueous solutions was determined by the intraperitoneal injection of 1 ml of solution into mice (C 57 BL/6J, Jackson Laboratory, Bar Harbor, Maine) weighing approximately 20 g. The death time in minutes was converted to mouse units (MU) using the tables of Sommer (Halstead, 1965), where 1 MU is defined as the amount of toxin required to kill a 20 g mouse in 15 min. Using a U.S. Public Health Service saxitoxin standard the strain of mice used gave a conversion factor of 0.2 μg of saxitoxin per MU.

Isolation Procedure. Toxic clams (*Mya arenaria*) collected during the 1972 New England Red Tide and kept in the frozen state were used as the source of toxin. The procedure used for the isolation of *G. tamarensis* major and minor toxins from these clams has been previously described (Buckley et al., 1975). This involved the extraction of the toxin from the clams with dilute acidic ethanol and purification by chromatography on weak acid cation exchange resins and gel filtration resins.

The major toxin has been further separated into two components by repeated passes through a 2.6 × 90 cm Bio-Gel P-2 column (200–400 mesh) (Bio-Rad Laboratories, Richmond, Calif.) by the following procedure. Samples of major toxin dissolved in 5 ml of 0.1 *M* acetic acid were applied through a flow adapter, which reduced the dead volume, and eluted with 0.1 *M* acetic acid at a flow rate of 40 ml/hr. Five-milliliter fractions were collected. The effluent from the column was monitored using the mouse test and by spotting 4-μl samples from each fraction onto a 5 × 6 cm piece of silica gel TLC plate. Up to 20 fractions were spotted per plate in several rows, and, without development in any solvent system, the plate was sprayed directly with 1% hydrogen peroxide, heated at 100°C for 30 min, and observed under a long-wave uv lamp (360 nm). Fractions giving fluorescent spots were further evaluated using the TLC–fluorometric procedure described below.

Thin-Layer Chromatography (TLC). TLC was run on precoated silica gel-60 plates (EM Reagent) activated at 110°C for 30 min just prior to use. All samples run in a particular series were applied in the same volume (usually 4 μl with a 2-μl Lang-Levy pipet). A maximum of 12 spots was applied along the origin of a 20 × 20 cm TLC plate. To demonstrate the chromatographic purity of major toxin H and major toxin L, the following solvent systems were used: (B) *tert*-butyl alcohol–acetic acid–water (50:25:25); (C) ethanol–pyridine–water–acetic acid (60:40:20:10); (D) ethanol–water–acetic acid (100:40:25); (E) pyridine–ethyl acetate–water–acetic acid (75:25:30:15). Solvents B, C, and D correspond to those previously similarly designated (Buckley et al., 1975).

In Situ TLC–Fluorometric Assay. Standard solutions containing 100, 80, 60, 40, 20, and 10 μg of toxin/ml were prepared from samples of major toxins H and L, isolated on the Bio-Gel column, and from a saxitoxin standard kindly supplied by Dr. E. J. Schantz (Food Research Institute, Madison, Wis.). Standard solutions of partially purified minor toxin containing 500, 400, 300, 200, 100, and 50 MU/ml were also prepared. If only the relative amounts of the different toxins present in a particular series of samples (e.g. fractions off a column) were to be investigated, no standards were applied to the TLC plates. For the quantitative determination of the concentration of toxin in a sample, standards and test solutions were run on the same plate. The plates were developed in solvent system E for 1.5 hr at room temperature. After air drying for 1 hr, the developed plates were sprayed evenly with 1% hydrogen peroxide for 30 sec. The plates were then heated in an oven at 100°C for 30 min and finally placed in a vacuum desiccator, containing $CaCl_2$, for 1 hr. The fluorescent spots were measured in situ with a Turner Model III fluorometer (Turner Associates, Palo Alto, Calif.) equipped with a TLC Scanner Door. The fluorometer was operated at the 10× setting using the standard (110–850) lamp, a 7–60 narrow pass primary filter (peak 360 nm) and a 47 B narrow pass secondary filter (peak 436 nm). The plates were scanned at a rate of 20 mm/min along the path of sample migration and the fluorescence recorded on a strip chart.

Fluorescence Spectra. Fluorescence spectra were determined in solution with a MK-1 spectrofluorometer using a stabilized xenon lamp (Farrand Optical Co., New York, N.Y.).

RESULTS

The H_2O_2–Fluorescence Reaction. Heating the paralytic shellfish poisons in the presence of hydrogen peroxide induced formation of fluorescent derivatives both in solution and on TLC plates. The major toxin, isolated by the procedure previously described (Buckley et al., 1975), showed two distinct fluorescent spots on a TLC plate developed in solvent system E after heating in the presence of hydrogen peroxide (Table I). The top spot, which was much more intense, was called major toxin H, and the lower spot was called major toxin L. Mixtures of STX, major toxin H, and major toxin L gave three distinct fluorescent spots after TLC in solvent system E, treatment with hydrogen peroxide, and heating. The fluorescent derivatives were readily visible under long-wave uv light and showed up as individual peaks when scanned with the Turner Fluorometer (Figure 1). Mixtures of STX and *G. tamarensis* minor toxin gave only a single spot. In actuality, the fluorescence of each toxin was found to be due to two fluorescing materials. This could be demonstrated by H_2O_2 treatment and heating prior to TLC. By this means two fluorescing materials were isolated from each toxin and their fluorescence properties determined (Table I).

A standard mixture of STX and major toxin was used to determine the effect of several variables on the intensity and stability of the fluorescent derivatives. A spraying time of 30 sec with 1% hydrogen peroxide and heating at

Table I. R_f Values of the Toxins and the R_f Values and Fluorescence Properties of Their Derivatives

Compound	R_f[a]	Excitation max, nm	Emission max, nm
Saxitoxin	0.58		
Low R_f fluorescent derivative[b]	0.29	360	415
High R_f fluorescent derivative[b]	0.75	330	383
Minor toxin	0.56		
Low R_f fluorescent derivative	0.28	360	412
High R_f fluorescent derivative	0.74	332	384
Major toxin H	0.74		
Low R_f fluorescent derivative	0.15	360	415
High R_f fluorescent derivative	0.57	333	380
Major toxin L	0.68		
Low R_f fluorescent derivative	0.15	362	417
High R_f fluorescent derivative	0.56	334	386

[a] TLC was run in solvent system E on silica gel plates. [b] The fluorescent derivatives were obtained separately by spotting the toxins onto the plates and spraying with H_2O_2, heating, and desiccating for 1 hr, producing the fluorescent derivatives prior to development in solvent system E. After TLC the fluorescent derivatives were scraped off the plates, the scrapings extracted with 2 ml of water adjusted to pH 4 with HCl, and the extracts spun in a clinical centrifuge. The fluorescence spectra of the supernates were determined.

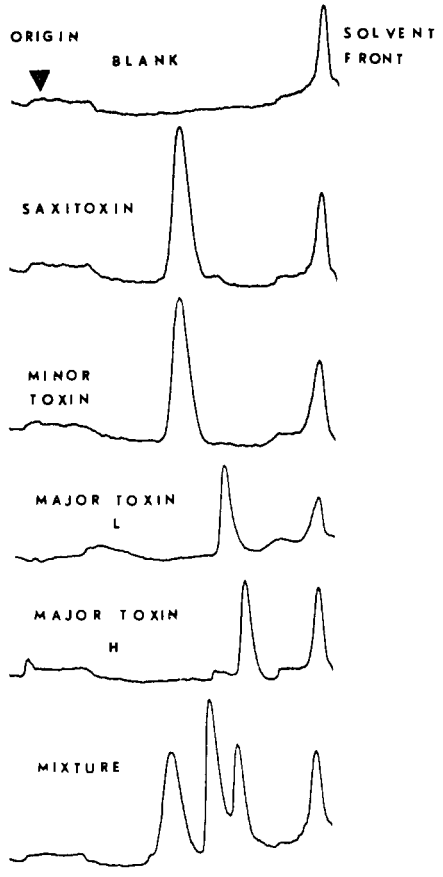

Figure 1. Scans of TLC plates spotted with samples of *G. tamarensis* toxins and developed using the TLC-fluorometric assay procedure.

100°C for 30 min were found to give optimum results. After desiccation for 1 hr the intensity of the fluorescent spots remained essentially constant for at least 24 hr. For each toxin tested, there appeared to be a linear relationship between the peak height on the recorder chart and the amount of toxin applied in the range of 40 to 400 ng of toxin per spot. Above 400 ng per spot, the fluorescence

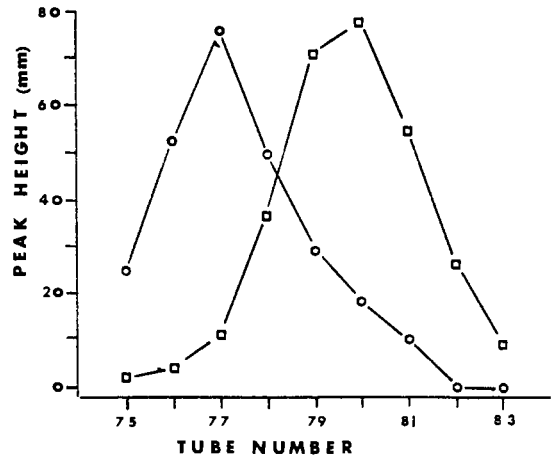

Figure 2. The elution of major toxin H (○) and major toxin L (□) from a Bio-Gel column developed with deionized distilled water followed by 0.1 *M* acetic acid. Peak heights were determined with the TLC-fluorometric assay procedure.

began to diminish and linearity was not maintained, possibly due to quenching.

In order to determine the specificity of the H_2O_2–fluorescence reaction, a number of compounds were tested. Of the compounds tested, only proline, tyrosine, and streptomycin sulfate yielded fluorescent spots. The intensity of these spots, upon scanning with the fluorometer, was less than $^1/_{100}$ of the intensity given by an equal amount of PSP. The following compounds failed to give a fluorescent spot when tested at 5 μg/spot: glutamic acid, aspartic acid, asparagine, alanine, arginine, uracil, guanine, glucose, gluconic acid, acetic acid, acetylcholine, guanidine, sulfaguanidine, creatinine, creatine phosphate, methylguanidine, guanidoacetic acid, aminoguanidine sulfate, creatine hydrate, phosphocreatine, argininosuccinic acid, and canavanine sulfate.

Isolation and Toxicity of Major Toxins H and L. When the major toxin was chromatographed on the Bio-Gel column only one peak of activity was observed with the mouse assay. However, the in situ TLC–fluorometric assay showed that the higher R_f component (major toxin H) was eluted from the column slightly ahead of the lower R_f component (major toxin L) (Figure 2). By pooling and rechromatographing fractions rich in one toxin or the other on Bio-Gel columns, both the major toxin H and major toxin L were isolated. Both purified toxins gave only one spot on TLC plates developed in five different solvent systems followed by spraying with concentrated H_2SO_4 and charring. Major toxin H was found to have a specific toxicity of 1800 MU/mg, and major toxin L had a specific toxicity of 4200 MU/mg.

After major toxin H and major toxin L had been isolated, standard solutions were prepared and utilized to determine the concentrations of all three paralytic shellfish poisons present in a solution using the in situ TLC–fluorometric assay (Table II). Multiplying the concentration in micrograms per milliliter, determined from the graph of peak height vs. micrograms by the specific toxicity in mouse units per microgram (1.8 for major toxin H, 4.2 for major toxin L, and 5.5 for STX) gave the total number of mouse units per milliliter. When this value was compared with the value obtained using the mouse test, good agreement was observed between the two methods for fractions off the Bio-Gel column and for partially purified preparations of *G. tamarensis* minor toxin (Table II).

Stability and Interconversion of the Toxins. Major

Table II. Estimation of Toxin Concentration and Activity Using the TLC-Fluorometric Assay and Comparison with the Mouse Test

Sample no.[a]	TLC-fluorometric assay: Concn, μg/ml Ma H	Ma L	STX	Total,[b] MU/ml	Mouse assay, MU/ml
1	0	0	0	0	0
2	8	0	0	14	24
3	65	0	0	117	109
4	113	0	0	203	192
5	126	0	0	226	194
6	70	4	0	143	198
7	62	8	0	146	184
8	11	8	0	100	127
9	0	24	0	101	102
10	0	0	98	543	500
11	0	0	18	100	100

[a] Samples 1-9 were consecutive fractions from a typical run of major toxin on a Bio-Gel column. Samples 10 and 11 were partially purified preparations of minor toxin. [b] Calculated by multiplying μg of Ma H/ml by 1.8, μg of Ma L/ml by 4.2, and μg of STX/ml by 5.5 (see text).

toxins H and L were each diluted to a concentration of 2 μg/ml with either 10 m*M* NaH_2PO_4 or $NaHCO_3$. These dilutions, which gave a final pH of about 4.7 and 8.2, respectively, were placed in a water bath at 16°C for 30 hr or 93°C for 30 min. After the incubation period, the samples at pH 8.2 were adjusted to pH 4-5 with HCl. One-half the sample was used directly for the mouse assay, the other half was lyophilized, and the residue taken up in distilled water to give a final concentration of 40 μg/ml ($^1/_{20}$ the volume before lyophilization). These preparations were then evaluated using the TLC-fluorometric assay (Table III). At pH 4.7, major toxin H appeared to be unaffected even after heating at 93°C for 30 min, while major toxin L appeared to be partially converted to major toxin H at both 16 and 93°C. At pH 8.2, major toxin H appeared to be unchanged after 30 hr at 16°C, while major toxin L appeared to give major toxin H and a small amount of material corresponding in R_f to STX. At pH 8.2 both major toxins H and L were destroyed by heating.

DISCUSSION

Unlike shellfish exposed to blooms of *G. catenella* which appear to contain only STX, extracts of clams exposed to *G. tamarensis* contain a mixture of at least three toxins. The in situ TLC-fluorometric assay provides a means of distinguishing between these three toxins, quantitating each toxin individually, and estimating their combined potency. This technique was utilized in the isolation of major toxin H and major toxin L. Shimizu et al. (1975) have demonstrated that clams exposed to *G. tamarensis* contain at least three toxins. How major toxins H and L correlate with the toxins reported by these authors has not been determined.

The identical R_f values of the fluorescent derivatives of major toxin H and major toxin L in several solvent systems and the similar fluorescence properties of the derivatives from the H_2O_2 treatment of these toxins and STX would indicate that major toxin H and major toxin L are chemically similar and that they are also probably related to STX. The chemical similarity is also borne out by the pH-temperature stability studies in which major toxin L appeared to undergo a partial conversion to major toxin H at pH 4.7 and where major toxin L at pH 8.2 broke down and appeared to yield a small peak corresponding to STX. The presence of only STX in 10-year old extracts of scallops which had been exposed to *G. tamarensis* and the weakly basic nature of the *G. tamarensis* toxins as

Table III. Stability of Major Toxins H and L

Conditions: pH	Temp, °C	Time, hr	TLC-fluorometric assay: Final concn, μg/ml[a] Ma H	Ma L	STX	Total act.,[b] MU/ml	Mouse test, MU/ml
Major Toxin H							
4.7	16	30	34	4	0	3.9	3.6
4.7	93	0.5	36	4	0	4.0	4.6
8.2	16	30	40	0	0	3.6	4.0
8.2	93	0.5	0	0	0[c]	0	0
Major Toxin L							
4.7	16	30	17	18	0	5.3	7.6
4.7	93	0.5	28	15	0	5.6	7.1
8.2	16	30	14	8	9	5.4	7.1
8.2	93	0.5	0	0	0[c]	0	0

[a] TLC-fluorometric assay done on solutions concentrated to give an initial H or L level of 40 μg/ml (see text). [b] Calculated by multiplying μg of H/ml by 1.8, μg of L/ml by 4.2, and μg of STX/ml by 5.5 and dividing the sum by 20 to correspond to the initial concentration of 2 μg/ml used in the mouse assay. [c] Although a small peak was discernible, its value was negligible.

compared to the strongly basic nature of STX has led Ghazarossian et al. (1974) to postulate that, on aging, *G. tamarensis* toxins break down to give STX and speculate that perhaps an amine oxide is breaking down to give an amine STX.

Spraying TLC plates with H_2O_2 and heating appears to be a sensitive method for the detection of STX and the other *G. tamarensis* toxins, quantities of the order of 0.04 μg being detectable. A sensitive method for the detection of STX was recently described (Proctor et al., 1975) in which 0.01 μg could be detected on TLC plates by spraying with Fast Blue B Salt followed by NaOH. These authors report that this reagent was nearly two orders of magnitude more sensitive than Jaffe or Weber Reagents, sprays used previously for the detection of STX on paper chromatograms. The H_2O_2-fluorescence reaction appears to be rather specific for the *G. tamarensis* toxins since a variety of amino acids, purines, carbohydrates, and choline and guanidine compounds failed to show this reaction at 5-μg levels.

Although the TLC-fluorometric method described in this paper is sensitive and selective and works well on chromatographic fractions, where considerable concentration has been achieved, it cannot be used in its present form for the estimation of the individual toxins in crude shellfish extracts. The standard mouse assay for STX can detect ca. 0.2 μg of the poison per ml (volume injected per mouse) or 40 μg of STX per 100 g of shellfish meat in a standard extract of 2 ml per g of shellfish meat. The Bates and Rapoport (1975) alkaline H_2O_2-fluorometric method is about 100 times more sensitive and can detect ca. 0.003 μg of STX per 2 ml (volume used in the assay) or 0.3 μg of STX per 100 g of shellfish meat in an extract of 2 ml per g of shellfish meat. In absolute quantity the amount detectable by the present method (0.04 μg) lies between the value for the mouse assay and the Bates and Rapoport method. However, since only 4 μl of solution is used, the applied concentration would need to be ca. 10 μg/ml. The maximum safe level of PSP (as STX) has been set at 80 μg of STX per 100 g of shellfish meat. At 2 ml of extract per g of shellfish this level of STX would yield a solution containing 0.4 μg per ml. Therefore, what is needed to adapt this method for the estimation of the individual toxins in shellfish exposed to *G. tamarensis* is a simple rapid method for extracting the toxins which will leave

behind the major portion of material which interferes with the TLC separation and which effects approximately a tenfold increase in concentration.

LITERATURE CITED

Bates, H. A., Rapoport, H., *J. Agric. Food Chem.* **23**, 237 (1975).
Buckley, L. J., Ikawa, M., Sasner, J. J., Jr., in "Proceedings of the First International Conference on Toxic Dinoflagellate Blooms", Massachusetts Science and Technology Foundation, Wakefield, Mass., 1975, pp 423–431.
Ghazarossian, V. E., Schantz, E. J., Schnoes, H. K., Strong, F. M., *Biochem. Biophys. Res. Commun.* **59**, 1219 (1974).
Halstead, B. W., "Poisonous and Venomous Marine Animals of the World", Vol. 1, U.S. Government Printing Office, Washington, D.C., 1965, pp 157–226.
Johnson, H. M., Mulberry, G., *Nature (London)* **211**, 747 (1966).
McFarren, E. F., Schantz, E. J., Campbell, J. E., Lewis, K. H., *J. Assoc. Off. Agric. Chem.* **41**, 168 (1958).
McFarren, E. F., Schantz, E. J., Campbell, J. E., Lewis, K. H., *J. Assoc. Off. Agric. Chem.* **42**, 399 (1959).
Proctor, N. H., Chan, S. L., Trevor, A. J., *Toxicon* **13**, 1 (1975).
Schantz, E. J., Lynch, J. M., Vayvada, G., Matsumoto, K., Rapoport, H., *Biochemistry* **5**, 1191 (1966).
Schantz, E. J., Mold, J. D., Stanger, D. W., Shavel, J., Riel, F. J., Bowden, J. P., Lynch, J. M., Wyler, R. S., Riegel, B., Sommer, H., *J. Am. Chem. Soc.* **79**, 5230 (1957).
Shimizu, Y., Alam, M., Fallon, W. E., in "Proceedings of the First International Conference on Toxic Dinoflagellate Blooms", Massachusetts Science and Technology Foundation, Wakefield, Mass., 1975, pp 275–285.

Received for review June 26, 1975. Accepted October 13, 1975. Supported in part by a Dissertation Year Fellowship to the senior author from the Graduate School, University of New Hampshire, and grants from the Department of Commerce, NOAA, Office of Sea Grant Programs to the University of New Hampshire, and from the New England Regional Commission. Published with the approval of the Director of the New Hampshire Agricultural Experiment Station as Scientific Contribution No. 782.

Conversion of 1,1,1-Trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) to Water-Soluble Products by Microorganisms

Fredrick W. Juengst, Jr.,[1] and Martin Alexander*

Forty-three percent of the bacteria isolated from sea water and marine sediments converted between 5 and 10% of the DDT supplied in vitro to water-soluble products, and 35% transformed less than 5% of the insecticide to water-soluble metabolites. Several water-soluble compounds generated from DDT by *Mucor alternans* were partially characterized and were found to be different from known products of DDT metabolism. However, the insecticide was not converted to water-soluble products in model marine ecosystems supplemented with a large number of organic compounds and incubated aerobically, anaerobically, and with supplemental inorganic nutrients.

DDD (1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane) and DDE (1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene) are formed from DDT (1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane) in soil, water, plants, and animals. Although these compounds can be generated by photochemical reactions (Miller and Narang, 1970; Plimmer et al., 1970), microorganisms are apparently important in their formation in certain ecosystems. In addition, 1-chloro-2,2-bis(*p*-chlorophenyl)ethylene, 2,2-bis(*p*-chlorophenyl)ethylene, 1-chloro-2,2-bis(*p*-chlorophenyl)ethane, bis(*p*-chlorophenyl)methane, 4,4'-dichlorobenzophenone (DBP), 4,4'-dichlorobenzhydrol (DBH), and 2,2-bis(*p*-chlorophenyl)acetic acid (DDA) are produced in model freshwater and sewage ecosystems under controlled laboratory conditions (Pfaender and Alexander, 1972). Most of these metabolites have not been isolated from marine waters, but this may result from the minute amounts of the intermediates that accumulate.

DDD and DDE have been reported to accumulate in marine environments (Butler, 1969). Whether these compounds are generated by the activity of microorganisms or by photochemical reactions is not known, but Patil et al. (1972) showed that such products appeared in cultures of marine microorganisms. Some of the isolates were also noted to synthesize 2,2-bis(*p*-chlorophenyl)-ethanol and 2,2-bis(*p*-chlorophenyl)ethane. Products of more extensive degradation of the insecticide have not been reported in marine waters. Considering the environmental problems associated with the use of this insecticide, it is surprising that products representing the removal of only one of the 14 carbon atoms in the DDT molecule have been described. This may partly result from the widespread use of hexane extraction procedures so that only hexane-soluble metabolites are characterized. By contrast, Anderson et al. (1970) observed that *Mucor alternans* converted DDT to water-soluble compounds as well as hexane-soluble products. Miyazaki and Thorsteinson (1972) subsequently reported that *Nitzschia* sp. and an unnamed diatom converted DDT to an unidentified water-soluble product.

Because the water-soluble compounds may represent new groups of DDT metabolites generated by pathways of degradation not heretofore characterized, a study was initiated of the possible synthesis of water-soluble products from DDT by marine microorganisms, and an attempt was made to characterize the water-soluble metabolites formed from the insecticide by *M. alternans*.

MATERIALS AND METHODS

Marine bacteria were isolated and maintained either on a medium containing 2.0 g of yeast extract, 0.05 g of K_2HPO_4, 15 g of agar, and 1000 ml of sea water that was allowed to stand for at least 30 days before use or on a medium described by ZoBell (1946) and containing 2.0 g

Laboratory of Soil Microbiology, Department of Agronomy, Cornell University, Ithaca, New York 14853.
[1]Present address: Calor Agriculture Research, Okemos, Michigan 48864.