known. Data presented here indicate that there are variations in fungal growth when they are grown on different varieties of maize and groundnut. They also show that a soft endosperm variety (Shakti) supports more fungal growth than did a hard variety (Comp. H-3). This may be due to the fact that a soft endosperm variety permits easier penetration and proliferation of the fungus in the grain. The amount of toxin production, however, among these varieties is not consistent with fungal growth, suggesting that increases or decreases in growth of the fungus do not run parallel to increases or decreases in aflatoxin production. It would thus appear that differences in the amount of toxin produced by the fungus, on different varieties of the same food grain are independent of quantitative differences in growth, but related to qualitative changes characteristic of the genotype. This may be due to the presence of varying amounts of stimulatory and inhibitory factors in the genotype. Further studies are in progress to explore such possibilities.

ACKNOWLEDGMENT

The authors are grateful to S. G. Srikantia, Director, National Institute of Nutrition, Hyderabad, for his keen interest and encouragement, to the Maize-breeder, Maize Research Station, Amberpet, Hyderabad, and All India Coordinator for Oil seeds for providing authentic varieties of maize and groundnuts. We are thankful to R. Bhat and V. Nagarajan for helpful suggestions and to K. Visweswara Rao for statistical analysis.

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Received for review April 21, 1977. Accepted August 15, 1977.

A Chemical Assay for Saxitoxin. Improvements and Modifications

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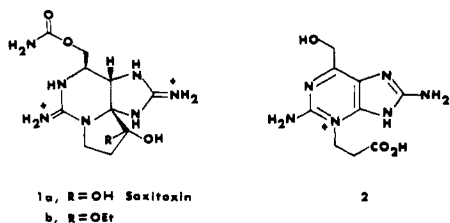
Saxitoxin (**1a**), the paralytic shellfish poison, can be oxidized to a purine (**2**) the concentration of which may be determined by ultraviolet absorbance or fluorescence. This is the basis of a sensitive chemical assay for saxitoxin. Several improvements and modifications are presented, as well as a procedure to check the functioning of each separate operation in the assay. The constant which relates saxitoxin concentration to ultraviolet absorbance after oxidation has been remeasured using pure saxitoxin.

Recently we presented a chemical assay procedure for saxitoxin (**1a**), the paralytic shellfish poison, based on oxidation to a fluorescent purine (**2**) (Bates and Rapoport, 1975). This chemical assay is superior to the previously used mouse bioassay in many respects, and its implementation is being considered for routine analysis of West Coast shellfish samples. The purpose of the present paper is to clarify several procedural details and indicate certain improvements and modifications in the chemical assay which increase its accuracy and reproducibility. Using several samples of purified saxitoxin, we have remeasured the constant which relates saxitoxin concentration to the UV absorbance after oxidation. We also describe a procedure to check the functioning of each separate operation of the assay.



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EXPERIMENTAL SECTION

Materials and Equipment. The concentration of reagent grade hydrogen peroxide (~30%) was determined by titration with potassium permanganate (Welcher, 1963) and a 10% solution was prepared by dilution. When stored at 5 °C, it is stable for months. For routine work, hydrogen peroxide concentrations between 9 and 11% are satisfactory, introducing less than 1% deviation in the amount of **2** produced. Glassware and solvents must be kept free of dust and metallic particles capable of decomposing the hydrogen peroxide. Decomposition will lead to decreased and irreproducible oxidation of saxitoxin.

A stock solution of saxitoxin dihydrochloride monohydrate in water (50–100 μg/mL) is prepared and kept refrigerated in a glass container fitted with a rubber septum to prevent evaporation. Evaporation can be significant when ground glass stoppered flasks are used. After its concentration has been determined, a dilute solution of saxitoxin (~5 μg/mL) is accurately prepared from the original solution and stored in the same way. Volumes of less than 1 mL of either saxitoxin solution must be measured with microliter pipets inserted through the septa. Microliter syringes are totally unsatisfactory as they absorb saxitoxin on their ground glass surfaces.

All solutions should be checked periodically for contamination by fluorescent materials.

Prepare Bio-Rex 70 ion-exchange resin, 50–100 mesh

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(200 mL, wet volume) by rinsing with H_2O (3 × 600 mL), 0.5 M H_2SO_4 (3 × 600 mL), H_2O (600 mL), 1 M NaOH (3 × 600 mL), and H_2O (3 × 600 mL). In each case this is done by stirring for 5–10 min and decanting after the resin has settled. Suspend the resin in 0.2 M acetic acid (600 mL) and adjust the pH to 5.0 with H_2SO_4. Rinse with 0.2 M pH 5.0 sodium acetate buffer (2 × 600 mL). This stock of resin, stored in the buffer, may be kept indefinitely and used as needed. Used resin may be recycled by the same process.

A specially designed glass column may be used for ion-exchange chromatography. Our columns consist of a 75 mm length of 6 mm (inner diameter) glass tubing fitted with a medium fritted glass filter on the botton and a 45-mL reservoir of 28 mm tubing on the top. The columns are filled with 2.0 mL of the resin just prior to use.

Ultraviolet spectra were obtained with a Cary 14 spectrophotometer, the accuracy of which was verified using alkaline potassium dichromate (Haupt, 1952).

Fluorescence measurements were obtained with a Perkin-Elmer MPF-2A fluorimeter as previously described, using nonflourescent Pyrex screw-cap vials as cuvettes. Though the fluorescence maximum of **2** is 386 nm (corrected spectrum in quartz cuvette), measuring the fluorescence at 380 nm appears to decrease the flourescence of the unoxidized blank.

Implementation of Chemical Assay. Initially, we recommend checking each of the major operations (oxidation, fluorescence, and ion exchange) before attempting to perform the entire chemical assay. This is illustrated below:

The concentration of a stock solution of saxitoxin is determined by oxidation to the fluorescent purine **2**. Add 2.0 mL of 1.0 M NaOH and 0.05 mL of 10% H_2O_2 to 2.0 mL of saxitoxin stock solution (50–100 μg/mL, withdrawn through septum). After 40 min, measure the absorbance at 335 nm. (This measurement is optional; it is more convenient to measure absorbance at pH 5.) Subtract the absorbance of 10% H_2O_2 (0.05 mL) added to 0.5 M NaOH (4.0 mL) to obtain the net absorbance. A net absorbance of 1.00 will result from oxidation of 225 μg of saxitoxin dihydrochloride (mol wt 372, 4.05 mL × 55.5 μg of saxitoxin/mL). Immediately, adjust the pH to 5.0 with glacial acetic acid (0.16 mL) and measure the absorbance at 333 nm. Subtract the absorbance of 10% H_2O_2 (0.05 mL) added to 0.48 M pH 5 sodium acetate buffer (4.16 mL) to obtain the net absorbance. An absorbance of 1.00 will result from oxidation of 182 μg of saxitoxin dihydrochloride (4.21 mL × 43.2 μg/mL). Calculate the concentration of saxitoxin in the stock solution.

To calibrate the flourescence spectrometer, serially dilute the pH 5 sample of oxidized saxitoxin to 0.1% of its original concentration with 0.12g H_2O_2 in 0.48 M sodium acetate buffer. Measure the fluorescence of this sample, subtracting the fluorescence of the solvent, using excitation at 330 nm and reading the emission at 380 nm. The Raman peak of a separate sample of water (excitation 330, emission 371) is useful as a standard. It may be desirable to prepare a number of dilutions to verify that fluorescence is a linear function of concentration.

Add 2.0 mL of 1.0 M NaOH and 0.05 mL of 10% H_2O_2 to a mixture of 0.020 mL of dilute saxitoxin solution (about 5 μg/mL, withdrawn with a microliter pipet through the septum) and 1.98 mL of water. After 40 min, adjust the pH to 5.0 with glacial acetic acid (0.16 mL) and measure the fluorescence against the Raman peak of water as above. Verify that the fluorescence is equal to that expected from the amount of saxitoxin used. The same procedure may be repeated using different amounts of saxitoxin.

Table I. Oxidation of Known Concentrations of Saxitoxin to Purine 2 to Determine UV Standard

Sample	Concn yielding 1.00 absorbance unit, μg/mL[a]
Saxitoxin·2HCl·H_2O	55.5
Saxitoxin·2HCl·H_2O	56.6
Saxitoxin·2HOAc·H_2O	56.8[b]
Saxitoxin·2HCl·H_2O·EtOH (**1b**)	58.8[b]

[a] Concentration of saxitoxin·2HCl·H_2O yielding an absorbance of 1.00 after oxidation as described in the Experimental Section. [b] Concentration recalculated as described in the Experimental Section.

Apply 0.020 mL of the dilute saxitoxin solution (about 5 μg/mL) to a column of previously prepared Bio-Rex 70 resin, elute the column and oxidize half the eluent, as described in the next section, omitting centrifugation. Measure the fluorescence of the oxidized portion and subtract that of the unoxidized blank. Verify that the fluorescence is as expected.

Shellfish Assay. Extract the shellfish with aqueous trichloroacetic acetic acid, neutralize, centrifuge, and apply to Bio-Rex 70 column as originally described (Bates and Rapoport, 1975). Elute the column with 30 mL of 0.2 M pH 5.0 sodium acetate buffer, 25 mL of H_2O, and 1.0 mL of 0.25 M H_2SO_4, and discard the eluents. Elute with 3.9 mL of 0.25 M H_2SO_4 and collect eluent in a centrifuge tube. Mix, then divide into two equal volumes in centrifuge tubes. Add 2.0 mL of 1.3 M NaOH and 0.05 mL of 10% H_2O_2 to one portion and mix. Substitute H_2O for H_2O_2 in the other. Centrifuge at 1000*g* for 1 min and transfer supernatants into cuvettes. Forty minutes after H_2O_2 was added, neutralize to pH 5 with ca. 0.16 mL of glacial acetic acid. Measure the fluorescence of the oxidized portion and subtract that of the unoxidized blank as above. The Raman peak of a separate sample of water (excitation 330 nm, emission 371 nm) is useful as a standard and corresponds to approximately 0.024 μg of saxitoxin (mol wt 372)/gram of shellfish.

Determination of UV Standard Using Known Concentrations of Saxitoxin. The values displayed in Table I, relating saxitoxin concentration to UV absorbance after oxidation, were obtained as follows: Saxitoxin dihydrochloride monohydrate (Bates and Rapoport, 1975) and crystalline saxitoxin dihydrochloride ethyl hemiketal monohydrate (**1b**) (Bordner et al., 1975) were purified as before. The hemiketal (**1b**) was quantitatively hydrolyzed to saxitoxin in aqueous solution at 5 °C. Removal of the ethanol formed did not influence the oxidation to **2**. Saxitoxin diacetate monohydrate was prepared by elution from Bio-Rex 70 resin with 0.05 M acetic acid (Bates and Rapoport, 1975).

All samples gave satisfactory elemental analysis. The purest sample of saxitoxin dihydrochloride monohydrate (first sample in Table I) gave acceptable bioassay (4560 MU/mg, Swiss-Webster CD-1 LBL strain; 4500 MU/mg, Swiss-Webster Fairfield Strain), optical rotation ($[\alpha]_D^{H_2O}$ = +128 ± 2°), and electrophoresis (Gelman cellulose polyacetate, 200 V, 0.25 M pH 8.7 tris buffer; visualization with 0.5 M NaOH + 0.1% H_2O_2, Weber, Jaffe, and Benedict-Bahre sprays). No impurities were seen in the ^{13}C NMR spectrum of purified saxitoxin dihydrochloride.

Each sample was dried and carefully weighed without reabsorption of water. Several aqueous solutions with concentrations between 65 and 85 μg of saxitoxin/mL were

prepared from each sample. Identical values were obtained when solutions prepared from the same sample were oxidized. Solutions were kept refrigerated in flasks fitted with septum caps, and no evaporation occurred.

Each sample (2.0 mL) was oxidized with 1.00 M NaOH (2.0 mL) and 10% H_2O_2 (0.05 mL). After 40 min, the absorbance in 0.5 M NaOH at 335 nm was measured, subtracting the absorbance due to the alkaline hydrogen peroxide. Table I shows values obtained by dividing the concentration of saxitoxin dihydrochloride monohydrate (mol wt 372.1) originally present (in 4.05 mL at time = 0) by the absorbance after oxidation. Concentrations of the diacetate monohydrate and ethyl hemiketal dihydrochloride monohydrate were recalculated to reflect the ratio of their molecular weights to that of the dihydrochloride monohydrate.

RESULTS AND DISCUSSION

Several modifications have been made in the saxitoxin chemical assay procedure which increase its accuracy and reproducibility: Saxitoxin is now eluted from the ion-exchange column with 3.9 mL of 0.25 M H_2SO_4 rather than 4.0 mL of 0.5 M HCl. The presence of chloride ion in the subsequent oxidation of saxitoxin to **2** appears to decrease the fluorescence, whereas sulfate has no effect. Since the resin contracts slightly in acid, application of 3.9 mL affords 4.0 mL of eluent. The concentration of NaOH added in the oxidation step has been increased from 1.2 to 1.3 M in order to afford the 0.5 M final concentration of base after dilution and neutralization of the acidic eluent. Higher concentrations of NaOH will decrease the yield of **2**.

It has always been difficult to establish the absolute concentration of a saxitoxin solution. The only primary standard is accurate preparation of a solution from a carefully weighed sample of totally pure saxitoxin. In practice, this is not trivial. Minute quantities of highly hygroscopic, amorphous saxitoxin, purified from natural sources must be utilized. The mouse bioassay has been employed as a secondary standard, relying on a reference standard of known concentration, but it suffers from differences in the susceptibility of various mouse strains and moderate reproducibility (Schantz et al., 1958). When no interfering compounds are present, optical rotation is a relatively accurate measure of saxitoxin concentration; however, relatively large quantities of saxitoxin are required.

Oxidation of saxitoxin to purine **2**, the concentration of which can be measured spectrophotometrically, provides a simple, specific, more precise secondary standard, eliminating the necessity for recourse to the primary standard, once an accurate value relating the ultraviolet absorbance to saxitoxin concentration is determined. To obtain such a value, four samples of saxitoxin were used: two samples of saxitoxin hydrochloride, one sample of saxitoxin diacetate, and one sample of saxitoxin dihydrochloride produced by aqueous hydrolysis of the crystalline ethyl hemiketal of saxitoxin (**1b**). Accurately prepared solutions of each sample were oxidized in 0.5 M NaOH with 0.125% hydrogen peroxide. Table I shows the concentrations of saxitoxin dihydrochloride monohydrate which, when originally present, produced an absorbance of 1.00 after oxidation. Reasonable agreement is observed among the four samples; however, the first sample in Table I is clearly the purest. Consideration of possible uncertainties in measurements gives a value of 55.5 ± 1 μg/mL-absorbance unit for the conversion factor. The error in our previously reported value of 39.7 (Bates and Rapoport, 1975) has been traced to slow evaporation of water from the saxitoxin solution.

Except for the modifications noted above, the chemical assay for saxitoxin in shellfish is performed as originally described. For routine work, some time may be saved by extracting the shellfish with 0.2 M pH 5 sodium acetate buffer, rather than trichloroacetic acid, eliminating the need for neutralizing the extract before applying it to the ion-exchange column. The acetate buffer extracts 90% of the saxitoxin extractable by the trichloroacetic acid.

The experimental section describes a procedure for checking the functioning of each separate operation in the chemical assay. The detailed procedure, with improvements and modifications as noted here, should facilitate implementation of the chemical assay for routine analysis of shellfish samples.

ACKNOWLEDGMENT

We are grateful to Scott Rogers for the purification of saxitoxin and the ^{13}C NMR spectra, and to Paul Baba, California State Department of Health, for the Fairfield strain of Swiss-Webster mice.

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Received for review August 8, 1977. Accepted October 17, 1977.