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HPLC SEPARATION AND COMPARATIVE TOXICITY OF SAXITOXIN AND ITS REACTION PRODUCTS

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A chromatographic method was developed that was used to purify saxitoxin and separate it from its chemically modified products and the reagents used in the reactions. The separation time is about 10 minutes. Using differential-refractive-index detection, quantitation of the products (±10%) can be done on 30–100 µg of toxin, A simple bioassay with crab leg nerves in vitro was used in conjunction with the chromatography to determine, within a factor of two, the inhibition binding constants of saxitoxin and its products. The binding constant for saxitoxin at ambient temperature, 18–21°C, is K_i ~80 nM. The acid-hydrolysis product has K_i ~8 µM under the same conditions. The chemistry of saxitoxin was investigated using the chemical and bioassays.

Saxitoxin, the toxic component of the red tide, is one of the most potent nerve toxins known. Its structure is shown in Fig. 1. The toxins acts at nanomolar concentrations by binding to nerve and blocking the voltage-dependent sodium currents [1]. As such, it is a useful tool in characterizing the sites of the sodium-current regulators. Also, a better understanding of the toxin binding site itself may follow from measuring differences in toxicity with known chemical changes in this local anesthetic.

Schantz and his co-workers suggested that hydrolysis of saxitoxin (3 h, 7.5 M HCl, 100°C) produces a cleavage and loss of urethane at C(13) leaving an alcohol group [2]. He named the product decarbamylsaxitoxin. Using an assay on rats, the toxicity of decarbamylsaxitoxin was found to be close to that of saxitoxin itself. Kishi and his associates described a method that would reverse this process by reforming the urethane using excess chlorosulfonylisocyanate in formic acid followed by hydrolysis in warm water [3]. Neither group was able to separate saxitoxin and decarbamylsaxitoxin.

We have found a method for separating saxitoxin from the acid-hydrolysis product using ion-pairing reversed-phase chromatography. With it we can show that the reaction of the acid-hydrolysis product with chlorosulfonylisocyanate does not

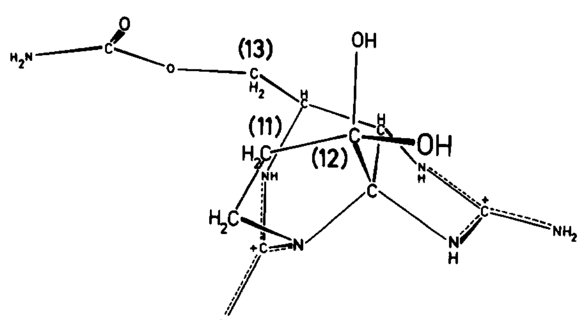

Fig. 1. Structure of the saxitoxin molecule with germane atom numbering.

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lead to reformation of saxitoxin. Further, in assays using crab nerve in vitro, the acid-hydrolysis product is seen to be at least 100-times less effective than saxitoxin in blocking the propagated action potential. In addition, we show that the acid-hydrolysis product may not be, in fact, decarbamylsaxitoxin, or the chlorosulfonylisocyanate reaction does not proceed as believed.

Experimental

The saxitoxin used was received from the FDA Cincinnati Food Research Laboratory. The chromatography was done using a reverse-phase method utilizing the ion-pairing reagent pentanesulfonic acid. This acid is prepared by oxidation of pentane thiol with hydrogen peroxide in acetic acid with rapid stirring under reflux until cool [4]. The opalescent solution is then warmed for a few hours until clear, exhaustively evaporated under vacuum at ambient temperature, and then quantitated by titration with KOH.

The chromatography column used was a 25 cm long Waters μ-Bondapak/C18 which, after extensive use, had a plate count of only 500. This was determined as suggested in the manufacturers manual (Waters No. CU84588) using the standard tangent method (see, for example, Ref. 5) on a sample of acenaphthene with a mobile phase of CH_3CN/H_2O (60:40, v/v).

The mobile phase used for the saxitoxin chromatography was water with 25 mM formic acid (1 ml/l) and 3 to 4 mM pentanesulfonic acid. With a flow rate of 1.5 ml/min (Waters model 6000 pump) the separation can be completed in about 8 min. See Fig. 3.

The solutes in the effluent were detected using a Waters model R401 to measure differential refractive index. Occasionally ultraviolet light absorption at 253 nm with a Cecil model CE212 was used in addition. The refractive index response was linear with concentration and was calibrated using aliquots of milligram samples of saxitoxin weighed after drying in vacuo ($< 10^{-3}$ torr) for a day. The partial molar refractive indices were assumed to be the same for saxitoxin and its various products. The accuracy of this assumption depends on two characteristics of the species involved. First, the molecules' masses and atomic constituents are the same, such as if only a bond is broken. Second, the molecular charge remains the same, since this affects the molecule-solvent interactions.

Qualitative peak measurements could be made with 10 μg of toxin and quantitative ones ($\pm$ 10% area) with 30 to 100 μg samples. Thus beginning with 100 μg of toxin, numerous reactions could be run sequentially on the sample. Also, reaction rates could be followed as long as the products were stable on the columm.

The bioassay was carried out using the apparatus as indicated in Fig. 2. This method for measuring nerve conduction originated in the work of Hodgkin [6]. All assays were conducted at ambient temperature, 18–21°C. To a solution of artificial seawater (mM; 460 NaCl, 50 $MgCl_2$, 10 KCl, 10 $CaCl_2$, 2.5 HCO_3^-, pH 7.8) were added aliquots of a quantitated fraction from the HPLC until conduction block occurred. That is, the nerve exhibited a block of the conduction of the action potential which could be reversed by washing

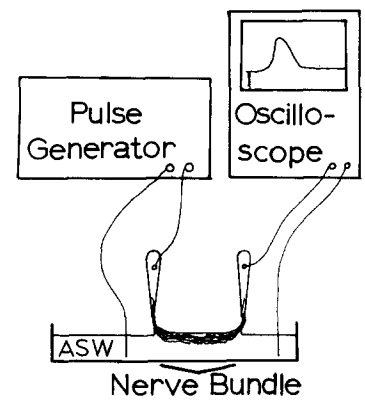

Fig. 2. Diagrammatic representation of the bioassay apparatus. The pulse generator is a simple, standard physiological excitation apparatus. Pulses needed are approximately a millisecond long with potentials between 0.1 and 10 V. The oscilloscope has at least a 1 mV/cm ordinate. The figure shows a typical pulse with 1 ms/cm scale on the abscissa. The ends of the nerves in the bundle must be above the surface of the artificial seawater (ASW) bath. The supports for the nerve bundle and the cotton thread ties can be tweezers or simply conducting wires. The nerves will stay on the wires without clamping if kept wet. The nerves must not be allowed to dry out and so usually are immersed completely while equilibrating with a sample. They may be reproducibly positioned for the measurement either by positioning the reservoir on a laboratory jack or raising and lowering the nerve on a ring-stand support.

thoroughly with artificial seawater. The block usually occurred within 3 min equilibration time after the final aliquot of the toxic fraction was added.

The assay was done using the excised walking-leg nerves from *Carcinas maenas* or *Maia squinado* (Marine Biological Laboratory, Plymouth, U.K.). The nerves from the two were used interchangably. The nerves were obtained by the pulling out method of Furusawa [7] but clipping and pulling off only one leg segment at a time. If the bundle was long enough to use (3 to 5 cm) it was clipped off onto a glass plate wetted with artificial seawater. The bundle was tied at both ends with cotton thread and stored in artificial seawater until used. If a nerve was not long enough after removing one leg segment, a second joint was clipped. In general, 18–20 nerve bundles can be obtained from a *Maia squinado* and 10 from the smaller *Carcinas maenas*. A single nerve bundle can be used for up to a few hours, measuring sequential fractions with no problem, especially if the fractions are not toxic. In general, for pure saxitoxin, the conduction is not fully reversible and, for greatest accuracy, a fresh nerve should be used for each toxic sample tested. Large excesses of sodium pentanesulfonate had no effect on the toxicities.

The HPLC fractions were dried before adding to a nerve bath since they were strongly acidic and the toxin was relatively too dilute for immediate use. The solvent eluting the samples from the column was either evaporated off at ambient temperature in an air stream or by freeze drying in vacuo. Results were the same in both cases. No differences in effect were seen in toxicities of samples tested before and after chromatography. This was true for both saxitoxin and acid-hydrolysis product (when the latter is corrected for the quantities and toxicities of impurities in the unpurified sample).

The hydrolysis of saxitoxin was done using the method of Schantz and co-workers [2] except on a smaller scale. Usually 1 mg of saxitoxin was heated in 0.5 ml of 7.5 M HCl for 3 h at 100°C. The mixture was cooled, frozen, and evaporated under vacuum. The resulting products were then dissolved in distilled water to 10 μg original saxitoxin per μl. This solution was directly chromatographed or stored at liquid nitrogen temperature.

Reactions with chlorosulfonylisocyanate were carried out in formic acid for 15 min at 5°C followed by hydrolysis in warm water (Kishi, Y., personal communication). Typically, 500 μg of acid-hydrolysis product was placed in 2 drops of AR formic acid and cooled in ice. To this, 10 μl of chlorosulfonylisocyanate was added by microsyringe, and the reaction followed by HPLC. It was complete in 15 min. The solution was frozen and taken to dryness under vacuum. Three drops of cold, distilled water were added and then heated to 60°C for 15 min. The sample was the lyophilized, a known volume of water added by microsyringe, and the solution aliquots injected directly into the HPLC. Any storage was done at liquid nitrogen temperature.

Low level tritium labeling was done using Ritchie's method [8] with 5 mCi/ml tritiated water. After reacting, the solution was frozen, lyophilized, and a known volume of water added. This solution was injected directly for chromatography. The specific activity could be determined directly from the radioactivity and the concentration as determined from the differential refractive index peak height of the saxitoxin fraction.

Results

Chromatography

As a measure of the efficacy of the chromatographic method, in Fig. 3a we see the material received from the FDA has at least two impurities totaling approx. 5% of the area detected by differential refractive index. These are the low peaks immediately before and after the main saxitoxin peak.

As seen in Fig. 3 a–c, the product of acid hydrolysis of saxitoxin can be separated from saxitoxin using this method. The two have not previously been separated. After the 3-h hydrolysis, usually about 4% of the original saxitoxin remained. This was determined by measuring the difference in toxicity between the whole peak including the tail and a partial fraction from the middle of the peak (and see below).

Following chemical transformations of saxitoxin and its products is also simplified by the method. For example, the speed of the HPLC method allows us to corroborate and enlarge on Kishi's observation concerning the extreme lability

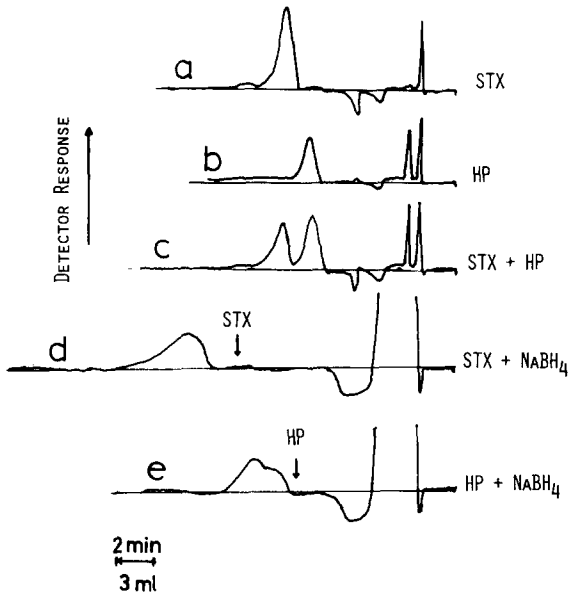

Fig. 3. Chromatograms with differential refractive index detector response on the abscissa versus volume on the ordinate. See the text for description of the conditions. Arrows mark the expected peak positions of saxitoxin (STX) and the acid-hydrolysis product (HP).

of the *gem*-diol at C(12) in saxitoxin [3]. Following the reaction over time, the condensation product was seen to be hydrolyzed in acid in less than 15 min at 60°C. Methanol yields a far less stable ketal and/or hemiacetals. Presumably they decompose under the conditions present in the column since only a small amount of product was ever observed. Additionally, attempts to acetylate using acetylchloride or acetic anhydride in acetic acid as solvent followed by mild hydrolysis (60°C, 15 min) yielded only saxitoxin. All reactions were thus run without protecting groups for the *gem*-diol.

The chromatographic method's sensitivity to changes in chemical structure can be seen when sodium borohydride reacts with saxitoxin, specifically and rapidly reducing the *gem*-diol [9]. The shift in the elution peak is seen in Fig. 3d. The major peak is a combination of two components, as seen from the simultaneous ultraviolet light detection (not shown) of the effluent. Assuming the fractions contain no saxitoxin, their $K_i \sim 10^{-4}$ M (vide infra). The effects of partial blocking (lengthening and broadening of the propagated action potential pulse on the oscilloscope screen) were seen. However, comparison with saxitoxin showed this could result from about one part per thousand saxitoxin impurity. The result seen in Fig. 3e suggests that the acid hydrolysis of saxitoxin does not effect the C(12) position since the borohydride reaction occurs there and shifts the peak position.

Toxicities of the fractions

Given a simple Langmuir absorption * for the binding and toxic action [10] and that the conduction ceases when about 80% of the channels are inhibited [11,12], the concentration at which conduction ceases is about four times the inhibition constant *. This simple assay then allows the approximate inhibition constant, K_i, leading to an action potential block to be determined within a factor of two and the relative toxicity values of various products with the same order of error. Values reported here are K_i = (concentration for block/4).

From nerve conduction blocking experiments, $K_i \sim 8\,\mu$M for a homogeneous fraction of the hydrolysis product; block of conduction occurs at about 30 μM (3 separate hydrolyses and quantitations). On the other hand, the K_i for saxitoxin was found to be approx. 80 nM under the same conditions. This is at least 100-times more effective than the hydrolysis product. This result suggests that

* A Langmuir isotherm, originally used to describe the adsorption of gases on solids, can simply be expressed as $n = a/(1 + a)$, where n is the fraction of available sites that are bound. In this case, the parameter a is directly proportional to the concentration of saxitoxin. The calculation made is arithmetically equivalent to the calculation of the fraction of enzyme-substrate complex in the first step of a Michaelis-Menten kinetics calculation with the rate constant $k_2 = 0$ (see, for example, Ref. 17). The binding constant for the reaction STX + site $\rightleftharpoons$ STX · site is the point where site = STX · site and $n = 0.5$. This is the point where the binding constant equals the toxin concentration. At this point, $a = 1$. When 80% of the sites are bound with the toxin, $n = 0.8$ and $a = 4$. That is, the concentration of toxin is four times that at the half-bound situation. Similarly for $n = 0.7$, $a = 2.3$, and for $n = 0.9$, $a = 9$. Since the nerve ceases to conduct when between 70% and 90% of the channels are blocked ($0.7 < n < 0.9$), we can determine the binding constant within a factor of about two simply by knowing the concentration of toxin (or reaction product) at which the conduction is blocked. We then calculate the inhibition binding constant to be (concentration at conduction block/4). STX, saxitoxin.

the time-dependent assay using rat mortality may reflect additional factors beyond the affinity of the various molecular species for the nerve site.

Using $\Delta H_d^\circ = 8.6$ kcal/mol, the value of $K_i \sim 35$ nM corrected to 4°C. The value of ΔH_d° was calculated using the data of Weigele and Barchi [13] for rat synaptosomes. With the correction for the temperature difference, the K_i for saxitoxin agrees with Strichartz and Hansen Bay [14] within the error limit. They used the nerves of *Homarus americus*. The inhibition binding constant found here is high compared to the values found by Ritchie et al. [8]. The difference could arise due to the 10-min equilibration time used here as opposed to 6 to 8 h used in their radiochemical studies. Alternately, at least part of the disagreement may be due to differences in ionic strength and pH.

Discussion

The results show that a conflict arises in the known chemistry of saxitoxin. The hydrolysis product may not be decarbamylsaxitoxin or the reaction with chlorosulfonylisocyanate in formic acid may not reform the urethane yielding saxitoxin. This conclusion derives from the following observations*. The reaction of chlorosulfonylisocyanate with the hydrolysis product was followed by taking aliquots over time and was seen to be complete after 15 min at 0–5°C. At least two unresolvable products were seen chromatographically. From the peak positions (elution times) and low toxicity (approximately that of the HP) only an insignificant part of the products could be saxitoxin. To test this further, the products were rehydrolyzed (3 h, 100°C, 7.5 M HCl) which should have yielded 'decarbamylsaxitoxin' again. But none was found, using the HPLC method. A quantity corresponding to 5% of the original material could have been detected.

In addition, chlorosulfonylisocyanate reacting with saxitoxin alone (formic acid, 0°C, 15 min) yielded a product with toxicity less than 10^{-4} of that of saxitoxin. The product was not identified but may be the allophanic acid ester [15,16]. (Reaction with urethane under the same conditions yielded a stable reaction product as seen by thin-layer chromatography on silica with diethyl ether development and iodine visualization.) Since the acid-hydrolysis product reacted with chlorosulfonylisocyanate yielding a more toxic product than the reaction of chlorosulfonylisocyanate with saxitoxin, it suggests that no modification occurs at C(12). Possible explanations for the seeming paradox are that perhaps some further decomposition beyond the putative loss of urethane [2] occurs upon hydrolysis of saxitoxin, or the hydrolytic cleavage itself does not occur at C(13). On the other hand, it may be that chlorosulfonylisocyanate reacts with decarbamylsaxitoxin to form a sulfamide [15,16] or other possible products arising from use of a bifunctional reagent.

In any case, the suggestion that saxitoxin might be a useful reagent for affinity chromatography of the binding site or as a site specific fluorescent label appears difficult to reconcile with the data presented here. It appears extremely difficult to modify the structure without significantly decreasing the affinity for the toxin binding site as measured by the equilibrium assay. Of course, the extraordinary sensitivity of the binding strength to the structure makes the toxin and its products excellent probes of the site.

Note added in proof: (Received March 26th, 1982)

A paper on the chemistry of saxitoxin has appeared recently [18].

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References

1 Evans, M.H. (1972) Int. Rev. Neurobiol. 15, 83–166
2 Ghazarossian, V.E., Schantz, E.J., Schnoes, H.K. and Strong, F.M. (1976) Biochem. Biophys. Res. Commun. 68, 776–780

* Authentic decarbamylsaxitoxin was unavailable for testing.

3 Tanino, H., Nakata, T., Kaneko, T. and Kishi, Y. (1977) J. Am. Chem. Soc. 99, 2818–2819
4 Backer, H.J. (1935) Rec. Trav. Chim. 54, 205–207
5 Willard, H.H., Merritt, L.L. Jr. and Dean, J.A. (1974) Instrumental Methods of Analysis, 5th edn., pp. 536–538, Van Nostrand, New York
6 Hodgkin, A.L. (1937) J. Physiol. 90, 183–210
7 Furusawa, A. (1929) J. Physiol. 67, 325–342
8 Ritchie, J.M., Rogart, R.B. and Strichartz, G.R. (1976) J. Physiol. 261, 477–494
9 Chaikin, S.W. and Brown, W.G. (1949) J. Am. Chem. Soc. 71, 122–125
10 Henderson, R., Ritchie, J.M. and Strichartz. G. (1973) J. Physiol. 235, 783–804
11 Colquhoun, D. and Ritchie, J.M. (1972) J. Physiol. 221, 533–553
12 Baker, P.F. and Rubinson, K.A. (1975) Nature 257, 412–414
13 Weigele, J.B. and Barchi, R.L. (1978) FEBS Lett. 91, 310–314
14 Strichartz, G.R. and Hansen Bay, C.M. (1981) J. Gen. Physiol. 77, 205–221
15 Graf, R. (1968) Angew. Chem. Int. Ed. Engl. 7, 172–182
16 Hagemann, H. (1977) Angew. Chem. Int. Ed. Engl. 16, 743–750
17 Moore, W.J. (1972) Physical Chemistry, 3rd Edn. Chap. 8, Prentice-Hall, Englewood Cliffs, NJ
18 Koehn, F.E. et al. (1981) Bioorg. Chem. 10, 412–428