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BINDING OF RADIOACTIVE TETRODOTOXIN TO NERVE MEMBRANE PREPARATIONS

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SUMMARY

Tetrodotoxin has been tritiated by the Wilzbach method and purified to 60% radiochemical purity. Tetrodotoxin binds to rabbit brain nerve endings, but not to rabbit liver mitochondria or bovine red blood cell ghosts. Tetrodotoxin also binds to lobster walking leg nerves. The density of tetrodotoxin binding sites on lobster nerve is $22/\mu m^2$. The sites are half saturated at about 25 nM.

INTRODUCTION

Tetrodotoxin was first shown by Narahashi *et al.*[1] to be a specific blocking agent for the Na^+ inflow component of a nerve action potential. Moore *et al.*[2] then showed that tetrodotoxin binds to only a very small number of sites on lobster axon membrane ($13/\mu m^2$). More recently Keynes *et al.*[3] have confirmed this observation ($36/\mu m^2$) and extended it to other species. The low density of tetrodotoxin binding sites is especially interesting in view of the discussion of Hille[4] of the minimum density of Na^+ inflow sites compatible with the known electrical properties of nerves. The most reasonable conclusion from these data is that tetrodotoxin binds only to Na^+ inflow sites on axon membranes. The work of Cuervo and Adelman[5] indicates a one to one stoichiometry for the tetrodotoxin: Na^+ site interaction.

If tetrodotoxin could be prepared in a radioactive form, it would be useful as a tracer for purifying Na^+ inflow sites from nerve tissue for reincorporation into an artifical membrane system. The reversibility of tetrodotoxin binding would complicate the purification process, but would be very advantageous at the final step when the toxin must be removed to restore the function of the Na^+ sites in an artificial membrane system.

The most economical source of large quantities of nervous tissue for biochemical purification is mammalian brain. My colleagues and I have measured the diffusional properties of tetrodotoxin in cat brain[6]. From these data, we have calculated that the concentration of tetrodotoxin binding sites in the lateral geniculate body of the cat brain is comparable with the concentration in lobster leg nerve[7]. Although there may be other tissues morphologically more favorable for the preparation of nerve membrane components[8], the finding of suitable tetrodotoxin binding site concentrations in mammalian brain makes large scale biochemical purification feasible.

METHODS

"Tetrodotoxin free of citric acid" was obtained from Sankyo, Tokyo. Labeiling by the method of Wilzbach[9] was performed by ICN Corp., Irvine, Calif.

Tetrodotoxin concentration was determined by a mouse survival time assay[10]. Mice were injected intraperitoneally with 0.1-ml samples to be assayed, and the elapsed time from injection to death was observed. Each set of assays was done with a large group of 20-g mice of the same strain, age, and sex. First, several mice were injected with known amounts of tetrodotoxin to construct a calibration curve like that in Fig. 1. Then the unknowns were run on mice from the same group. Routine samples such as column fractions were run on only one mouse, but more important determinations were replicated. The reproducibility of replicate determinations was ± 10%.

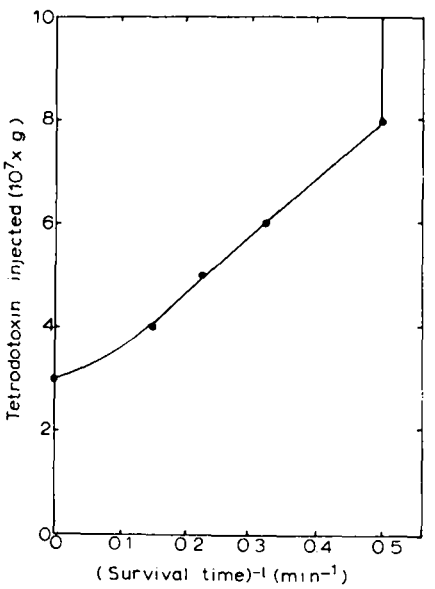


Fig. 1. Calibration of mouse survival time assay for tetrodotoxin. Quantity of tetrodotoxin injected intraperitoneally in 0.1 ml of solution *vs* reciprocal of the survival time. Only the central linear portion was used, corresponding to survival times of 3–20 min.

Bio-Gel P-2 and Bio-Rex 70 were obtained from Bio-Rad Laboratories, Richmond, Calif.

Nerve ending preparations were made by the method of Gray and Whittaker[11], using rabbit brain.

Equilibrium binding measurements were made by incubating a nerve ending suspension at 4 °C in 150 mM NaCl buffered to pH 7.5 with 10 mM Tris–HCl buffer, and containing the indicated amounts of tetrodotoxin. The suspension was then centrifuged for 20 min at 25000 × *g* and weighed samples of both supernatant and pellet were prepared for counting by liquid-scintillation spectroscopy. Hyamine hydroxide was used to dissolve the pellets prior to counting.

Lobster walking leg nerves were removed from the anterior two pairs of walking legs of *Homarus americanus*, tied off at both ends, and kept in cold physiological solution (500 mM NaCl, 50 mM $CaCl_2$, 15 mM KCl, 10 mM Tris–HCl buffer (pH 7.4)) until use. Experiments were conducted using physiological solution containing labelled tetrodotoxin and [^{14}C]sucrose. The concentrations of labelled tetrodotoxin and sucrose were constant in all solutions, and the total tetrodotoxin concentration was varied by adding unlabelled tetrodotoxin to some of the solutions. The nerves were exposed to the solutions for 1 h at 4 °C. They were then blotted, the tied ends were removed, and they were weighed and solubilized with Hyamine hydroxide for counting. Wet weights were used throughout.

RESULTS

10 mg of tetrodotoxin was labelled by the method of Wilzbach[9], using 20 Ci of tritium. Exchangeable tritium was removed by dissolving the material in dilute acetic acid and evaporating the liquid. The product was yellow and contained 3.3 mg of tetrodotoxin activity and 441 mCi of tritium.

First stage purification and characterization

The crude radioactive tetrodotoxin was dissolved in 2 ml of water and run on a column of Bio-Gel P-2 with the result shown in Fig. 2. The eluent was water containing about 1 drop of acetic acid per l.

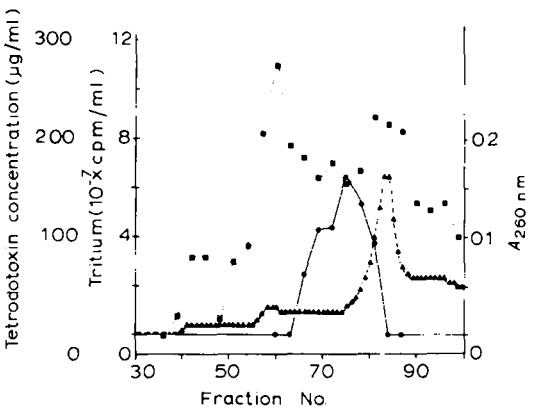


Fig. 2. First stage purification of [^{3}H]tetrodotoxin on a Bio-Gel P-2 column. ●—●, tetrodotoxin bioassay; ▲---▲, absorbance at 260 nm; ■ ··· ■, specific activity of tritium. Tetrodotoxin has no absorbance at 260 nm. Fractions 69–77 were used in subsequent work.

In the preliminary characterization experiment (Table I) a Whittaker nerve ending preparation[11] was made from rabbit brain, using sucrose solutions containing 25 nM [^{3}H]tetrodotoxin from the Bio-Gel P-2 column. Samples were taken from various portions of the gradient, to show the redistribution of [^{3}H]tetrodotoxin by the brain material. The samples containing suspended material were counted as suspensions, and not centrifuged. The radioactivity was concentrated in the nerve ending and mitochondrial regions, but not in the myelin region of the gradient. A control experiment with rabbit liver showed some concentration in the mito-

TABLE I

DISTRIBUTION OF [^{3}H]TETRODOTOXIN IN A WHITTAKER NERVE ENDING PREPARATION, AND IN A CONTROL EXPERIMENT USING LIVER

Initially the three sucrose solutions had the same specific activity, and the brain or liver material was unlabelled.

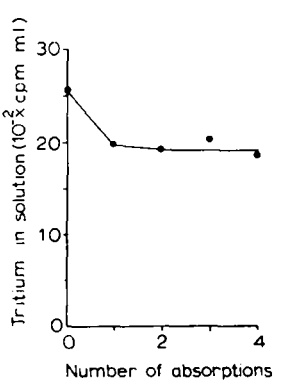
Sample	*Spec. act.* (10^{-3} × *cpm/ml*)	
	Brain	*Liver*
0.32 M sucrose	20.5	20.7
Myelin band	19.0	20.0
0.8 M sucrose	16.7	22.5
Nerve ending band	33.0	22.0
1.2 M sucrose	23.6	22.9
Mitochondrial pellet	33.0	27.0

chondrial pellet only. I interpret this to mean that [^{3}H]tetrodotoxin binds to the nerve ending fraction and to something in the mitochondrial pellet.

This binding to nerve ending was used to assay the radiochemical purity of the [^{3}H]tetrodotoxin, under the following assumptions:

(i) Tetrodotoxin binds to nerve endings reversibly. (ii) Any material that will not bind to nerve endings is not tetrodotoxin. (iii) Any material that cannot be displaced from nerve endings by excess unlabelled tetrodotoxin is not tetrodotoxin.

The experiment of Fig. 3 shows that only 34 % of the radioactivity in solution can be removed by repeated treatment with excess nerve endings. The mean of three such experiments was 35 %. Excess unlabelled tetrodotoxin displaced 50 % of the bound radioactivity. Therefore I estimate the purity of the [^{3}H]tetrodotoxin at this stage as 17 %.



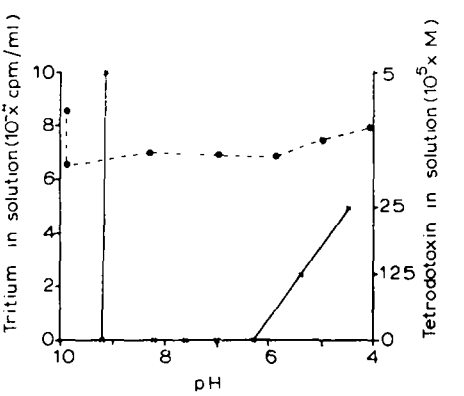
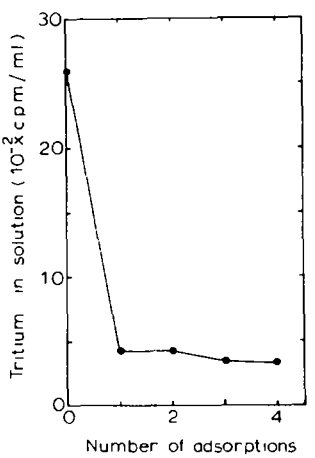


Fig. 3. Adsorption of [^{3}H]tetrodotoxin by successive treatments with excess nerve endings. Only the first treatment brings about a significant decrease in the concentration of tritium in the solution.

Fig. 4. Binding of [^{3}H]tetrodotoxin to Bio-Rex 70 resin. The high pH end of the curves shows the reduction of activity in solution when the resin is added. The rest of the curves show the release of [^{3}H]tetrodotoxin as the pH is lowered. ×—×, tetrodotoxin concentration by bioassay; and ●---●, tritium specific activity, from two separate experiments.

Second stage purification and characterization

Attempts to purify the material by ion-exchange chromatography were unsuccessful, so a batch process was used. Fig. 4 shows two experiments which characterize the binding of tetrodotoxin and [^{3}H]tetrodotoxin to a weakly acidic ion-exchange resin, Bio-Rex 70, initially in the ammonium form. Pure tetrodotoxin binds to the resin at high pH, but is released if the pH is lowered. Correspondingly, some fraction of the radioactivity of [^{3}H]tetrodotoxin is bound at high pH and released when the pH is lowered. Therefore a 1 g sample of Bio-Rex 70 was added to the solution of [^{3}H]tetrodotoxin. This brought the pH to 9.2, causing the tetrodotoxin to bind to the resin. The pH was then adjusted to 8.0 with acetic acid and the resin was collected on a filter. The resin was resuspended in water and brought to pH 4.2 with acetic acid. The resin was then removed by filtration and the solution collected and adjusted to pH 4.85 with NH_4OH. This solution, containing the purified [^{3}H] tetrodotoxin, was frozen and stored for use in the subsequent experiments. Using the same assumptions as before, the adsorption experiment of Fig. 5 shows that 85 % of the radioactivity now binds to nerve endings.



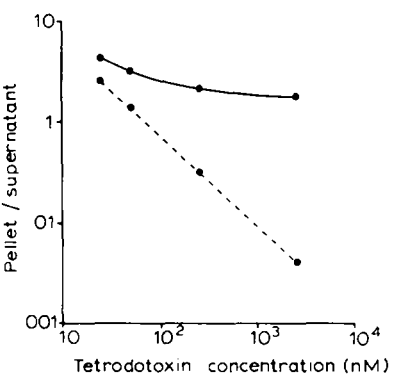


Fig. 5. Adsorption of [^{3}H]tetrodotoxin by successive treatments with excess nerve endings, as in Fig. 3.

Fig. 6. Displacement of [^{3}H]tetrodotoxin from nerve endings by unlabelled tetrodotoxin. In all 4 experiments the concentration of [^{3}H]tetrodotoxin was the same. Unlabelled tetrodotoxin was added to bring the total concentration to the value shown on the graph. The ratios of the specific activities of the pellets to the supernatant are plotted on ●—●. Subtraction of a constant (1.89) from each of these numbers is sufficient to arrange them along ●---●.

A displacement experiment is shown in Fig. 6. Several concentrations of unlabelled tetrodotoxin have been used to measure the displacement of [^{3}H]tetrodotoxin. The data plotted are ratios of specific activity of pellets and supernatants. When 1.89 is subtracted from each point, they fall on a straight line on the log–log plot. Following the theoretical treatment developed in Appendix, this 1.89 value corresponds to non-tetrodotoxin radioactive material and solution trapped in the pellet. Depending on how much solution was trapped in the pellet, and on the partition coefficient for the non-tetrodotoxin radioactive material, the purity of the adsorbed

material could be 58–74%. On the basis of these experiments I estimate that the radiochemical purity of the [^{3}H]tetrodotoxin is 60% and the specific activity is 1200 Ci/mole.

Similar binding experiments were performed with rabbit liver mitochondria and bovine red blood cell ghosts, and no specific binding was observed in either case.

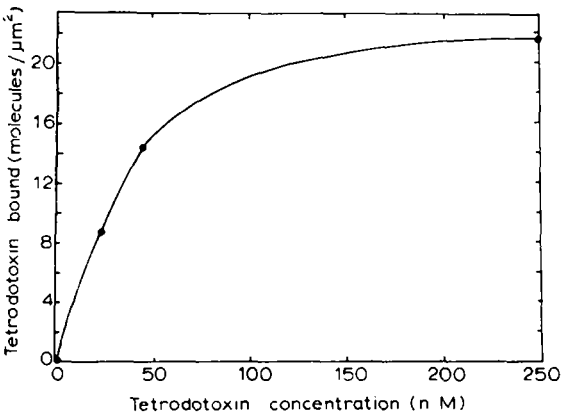


Fig. 7. Number of molecules of [^{3}H]tetrodotoxin bound per μm^2 of axonal area *vs* tetrodotoxin concentration for lobster walking leg nerve.

Experiments with lobster nerves

A double label experiment, with [^{14}C]sucrose as a marker of the extra-cellular space, is shown in Fig. 7. The concentration of [^{3}H]tetrodotoxin was constant at 25 nM in all solutions, and unlabelled tetrodotoxin was added to some solutions to bring the total to 50, 250, and 2500 nM. The concentration of [^{14}C]sucrose was also constant in all solutions. At 25 and 50 nM tetrodotoxin, more ^{3}H than ^{14}C was taken up by the nerves. However, at 250 and 2500 nM, more ^{14}C than ^{3}H was taken up by the nerves. By assuming that the binding sites are completely saturated at 250 and 2500 nM, I calculate that tetrodotoxin reaches 0.85 of the extracellular volume reached by sucrose. Applying this correction factor to all the data leads to the results plotted in Fig. 7. The saturation concentration of bound tetrodotoxin is 2.6 pmoles/g of wet nerve. Using Eisenberg's estimate of 7000 cm^2 of axonal area/g of wet nerve, the final result is 22 ± 4 sites/μm^2 of axonal area. The sites are half saturated at about 25 nM tetrodotoxin.

DISCUSSION

The data confirm that the method of Wilzbach[9] can be used to label tetrodotoxin, although extensive purification is necessary to prepare a useable product. A workable purification scheme has been developed, which is relatively simple while producing [^{3}H]tetrodotoxin of satisfactory purity for use in biochemical preparations.

[^{3}H]Tetrodotoxin binds to nerve endings from rabbit brain, but not to myelin fragments or liver mitochondria. Thus, mammalian brain is a suitable starting material for isolation of tetrodotoxin binding sites. These binding sites might be identical with sodium inflow sites on nerve membranes.

[^{3}H]Tetrodotoxin also binds to lobster nerves, and the observed density of 22 ± 4 sites/μm^2 falls between the earlier results of 13/μm^2 and 36/μm^2. The dissociation constant is 25 nM. This is in reasonable agreement with the work of Keynes *et al.*[3] for lobster nerve. However, the results of Hille[12] for frog nerve and Cuervo and Adelman[5] for squid nerve are both an order of magnitude lower.

APPENDIX

Mathematical description of the distribution of an impure radioactive tracer between supernatant and a pellet to which it binds reversibly

Assume a first-order reaction between tetrodotoxin and a binding site S,

$$ST \rightleftharpoons S + T, \qquad K = \frac{[S]T}{[ST]} \tag{1}$$

where T is the total concentration of unbound tetrodotoxin, including both radioactive tetrodotoxin (T^*) and non-radioactive tetrodotoxin (T°),

$$T = T^* + T^\circ \tag{2}$$

If $\bar{S}$ is the total of $[S]$ and $[ST]$, then we may write

$$[ST] = \frac{\bar{S} \cdot T}{K + T}$$

Now consider the total specific activity C_1 of the supernatant phase,

$$C_1 = \alpha T_1^* + B_1^* + N_1^* + \alpha(ST^*)_1 \tag{3}$$

where α is the molar specific activity of [^{3}H]tetrodotoxin, and subscripts 1 and 2 refer to supernatant and pellet, respectively. N^* represents the specific activity of those radioactive impurities which do not bind to the pellet, and B^* represents the specific activity of the remaining radioactive impurities, which distribute themselves between supernatant and pellet according to the equilibrium

$$\frac{B^*_2}{B^*_1} = K_B \tag{4}$$

The total specific activity C_2 of the pellet will be

$$C_2 = fC_1 + B_2^* + \alpha(ST^*)_2 \tag{5}$$

where f is a number less than 1 describing what fraction of the pellet is merely trapped supernatant. The ratio of the specific activities of pellet/supernatant is

$$\frac{C_2}{C_1} = \frac{fC_1 + B_1^* K_B + \alpha T_1^* \dfrac{\bar{S}_2}{K + T_1}}{N_1^* + B_1^* + \alpha T_1^* \left(1 + \dfrac{\bar{S}_1}{K + T_1}\right)} \tag{6}$$

For high tetrodotoxin concentrations, $T \gg K$, this can be simplified to

$$\frac{C_2}{C_1} = \frac{fC_1 + B_1^* K_B + \alpha T_1^* \frac{\bar{S}_2}{T_1}}{N_1^* + B_1^* + \alpha T_1^*} \tag{7}$$

Subtracting a constant from both sides of the equation, we arrive at

$$\frac{C_2}{C_1} - \left(f + \frac{B_1^* K_B}{C_1}\right) = \frac{\alpha T_1^* \frac{\bar{S}_2}{T_1}}{N_1^* + B_1^* + \alpha T_1^*} \tag{8}$$

If we now differentiate the logarithm of both sides of the equation,

$$\frac{\partial \ln \left[\frac{C_2}{C_1} - \left(f + \frac{B_1^* K_B}{C_1}\right)\right]}{\partial \ln T_1} = -1 \tag{9}$$

This is the origin of the straight line in Fig. 6. This equation shows that the constant which must be substracted from all the data points to bring them into a straight line is simply

$$f + \frac{B_1^* K_B}{C_1}$$

The properties of these equations suggest a simple experimental method for using impure [^{3}H]tetrodotoxin to measure the concentration of tetrodotoxin binding sites in insoluble material, like nerve membrane preparations. If one experiment is run using only [^{3}H]tetrodotoxin and a parallel experiment is run using the same concentration of [^{3}H]tetrodotoxin *plus* a large excess of unlabelled tetrodotoxin, then the difference between the pellet/supernatant ratios is

$$\left(\frac{C_2}{C_1}\right)_{T_1 = T_1^*} - \left(\frac{C_2}{C_1}\right)_{T_1 \gg T_1^*} = \frac{\alpha T_1^*}{C_1(K + T_1^*)} \bar{S}_2 = \beta \bar{S}_2 \tag{10}$$

where β is a constant, dependent on T_1^*. So if a series of different nerve membrane preparations are run at the same [^{3}H]tetrodotoxin concentration with and without excess unlabelled tetrodotoxin, this is a simple way of measuring relative tetrodotoxin binding site concentrations.

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