Effects of Deuterium Oxide on the Rate and Dissociation Constants for Saxitoxin and Tetrodotoxin Action

Voltage-Clamp Studies on Frog Myelinated Nerve

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ABSTRACT The actions of tetrodotoxin (TTX) and saxitoxin (STX) in normal water and in deuterium oxide (D$_2$O) have been studied in frog myelinated nerve. Substitution of D$_2$O for H$_2$O in normal Ringer's solution has no effect on the potency of TTX in blocking action potentials but increases the potency of STX by ~50%. Under voltage clamp, the steady-state inhibition of sodium currents by 1 nM STX is doubled in D$_2$O as a result of a halving of the rate of dissociation of STX from the sodium channel; the rate of block by STX is not measurably changed by D$_2$O. Neither steady-state inhibition nor the on- or off-rate constants of TTX are changed by D$_2$O substitution. The isotopic effects on STX binding are observed <10 min after the toxin has been added to D$_2$O, thus eliminating the possibility that slow-exchange ($t_{1/2} > 10$ h) hydrogen-binding sites on STX are involved. The results are consistent with a hypothesis that attributes receptor-toxin stabilization to isotopic changes of hydrogen bonding; this interpretation suggests that hydrogen bonds contribute more to the binding of STX than to that of TTX at the sodium channel.

INTRODUCTION

Early voltage-clamp studies of the action of tetrodotoxin (TTX) and saxitoxin (STX) showed that these toxins block sodium currents with high specificity in many nerve preparations (lobster axon: Takata et al. [1966]; frog node of Ranvier: Hille [1968]; squid axon: Narahashi et al. [1967]), that the blockade was reversible, and that its concentration dependence obeyed the Langmuir isotherm (Langmuir, 1916), (for a review, see Ritchie [1975]). Thus, the binding of toxin to receptor could be described by the reaction:

$$[T] + [R] \xrightarrow{k_1} [T-R],$$

where [T] represents toxin activity and [R] and [T-R] represent receptor and toxin-complexed receptor densities, respectively.
Schwarz et al. (1973) conducted a series of experiments on single myelinated fibers of frog and toad and verified that the above reaction adequately described the kinetics of TTX blockade of sodium currents. Wagner and Ulbricht (1975) extended these kinetic observations using both TTX and STX. They preequilibrated a single node with a known concentration of TTX and subsequently added a known concentration of STX. This procedure produced a nonmonotonic transient blockade of repetitively recorded sodium currents. The characteristics of the transient were predictable if STX and TTX competed for the same receptor sites. They showed the existence of these nonmonotonic transients could be used as a method for identifying competitively acting molecules that block sodium channels with different rate constants.

Despite the wealth of equilibrium and kinetic data, the underlying molecular mechanism of blockade by TTX and STX remains unclear. Kao and Nishiyama (1965) proposed that a guanidinium group, common to TTX and STX, is the blocking moiety that plugs the outer opening of the sodium channel. Camougis et al. (1967) ascribed one of the TTX-Na channel bonds to the formation of an intermolecular hemilactal bond between TTX and the channel. Hille (1975) hypothesized that STX and TTX bind at the "selectivity filter" for the Na channel in the manner proposed by Kao and Nishiyama (1965), where hydrogen bonds, covalent bonds, and electrostatic interactions stabilize the complex.

In many of these proposals, hydrogen would play a role in the binding process, either by engaging in hydrogen bonding or by being transferred from one molecular location to another during the complexation process. In this study, the method of isotopic substitution of hydrogen by deuterium to study the role that hydrogen plays in the binding of STX and TTX to their common receptor is used. It is shown that deuterium replacement for hydrogen slows the dissociation of STX from its receptor, whereas no such effects are seen with TTX. When a solution of STX rapidly replaces one of D2O-STX that had been equilibrated with a nerve fiber, the resulting nonmonotonic transient of repetitively recorded sodium currents suggests that a deuterated STX, with reduced off-rate, competes with STX for common blocking sites on the sodium channel.

METHODS

Compound Action Potential Experiments

The experiments were performed on frog sciatic nerve from either Rana p. pipiens or Rana esculenta. Compound action potentials were recorded using two techniques. A few preliminary experiments were undertaken using a chamber with three pools separated by Vaseline seals. The length of nerve in the center pool, 5-6 mm, was bathed in various test solutions of 150-μl vol. The two side pools, filled with paraffin oil, contained a set of either stimulating or recording electrodes of platinized platinum. The nerve was desheathed and was always excited by supermaximal stimuli.

The majority of the compound action potential experiments were recorded using a single sucrose-gap procedure. A frog sciatic nerve spanned two compartments that
were separated by a block containing a gap through which a sucrose solution flowed, thus electrically isolating the two compartments. One compartment contained the extracellular bath, whereas the other contained the intracellular bath. The action potential was recorded differentially between the two compartments. More than three volumes of the extracellular bath were exchanged when adding new solutions. This procedure was usually sufficient to wash out a test toxin solution so that the action potential was recovered to 95–100% of the control value. Whole nerve experiments were conducted at 20–22°C.

**Preparation of Toxin Solutions**

Reductions in action potential height were measured to assay the relative potency of various toxins. TTX was purchased from Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif., and dissolved in distilled water to yield a citrate-buffered stock solution of 1 mM TTX. STX was provided by the Food and Drug Administration's Microbial Biochemistry Branch, Cincinnati, Ohio, as an acidic 2% ethanol solution of 100 μg ml⁻¹ paralytic shellfish poison (270 μM STX). STX derivatives, neosaxitoxin and gonyautoxin II (GTX II), were kindly supplied by Dr. Y. Shimizu, University of Rhode Island, Kingston, R. I. The toxins were deuterated by dilution of a concentrated stock into 98% D₂O frog Ringer's solution (D-Ringer). The mole fraction of D₂O, the time after dilution (the incubation period), and, in some instances, the temperature of the incubation period were also varied.

The standard control Ringer's solution contained: NaCl (105 mM), KCl (2.5 mM), CaCl₂ (2 mM), and Hepes (2 mM). The pH was adjusted to 7.2 ± 0.05 at 20°C using NaOH. When comparing various drugs or measuring dose response curves, Ringer's solutions containing 12 mM tetraethylammonium chloride (TEA-Ringer) were used to reduce the potassium conductance to negligible values (Koppenhöfer, 1967; Hille, 1967).

**Single Myelinated Nerve Voltage-Clamp Experiments**

Single myelinated nerve fibers were dissected and voltage-clamped using a modified Dodge and Frankenhaeuser (1958) voltage-control method. The chamber in which the fiber was mounted and the voltage-clamp instrumentation were similar to those described by Hille (1971). The linear-leak and linear-capacity currents were electronically subtracted. The fiber was maintained at a temperature of 7–8°C. The holding potential was set so that steady-state inactivation, h₀, was equal to 0.75. In some instances, the axons were held at more negative potentials to further remove inactivation and to reduce the rate of slow inactivation (Fox, 1976). The fiber internodes were cut in 120-mM KCl solution, and 30 min were allowed before any experimental measurements were made.

In some experiments “attenuation artefact” (Dodge and Frankenhaeuser, 1958) was measured by determining the change in reversal potential for the early current when 12 mM TEA-Ringer was substituted by 1:8 normal Na Ringer with 12 mM tetraethylammonium chloride (TEA Cl) (Hille, 1971). The average attenuation was found to be 18%. The potentials reported in this paper were not corrected for attenuation artifact.

The experimental chamber was modified in some experiments to reduce the effective solution-exchange time. At least 10 vol of the nodal pool were exchanged within 2–3 s. The vol of the nodal pool was 150 μl. Usually, 13 2-ml vol of the pool were exchanged when making determinations of time constants. The completeness of the exchange was monitored by observing the appearance of the block of K currents.
by switching from Ringer to 12 mM TEA-Ringer. This procedure also was used to control for temperature transients; repetitively recorded Na currents did not significantly change after the addition of TEA-Ringer, whereas K currents were eliminated. When switching to deuterium-containing toxin solutions, the node was always previously equilibrated with 12 mM TEA D-Ringer.

**Series Resistance Artifacts**

In all clamp experiments, precautions were taken to minimize series resistance artifacts. Test pulses employed were in the range from -30 to 20 mV. In experiments designed to measure toxin on- and off-rates, large initial values of the sodium currents were reduced by employing test pulses near $E_{Na}$. Over most of the voltage range used, the current-voltage ($I-V$) relation is relatively linear, simplifying the estimation of series resistance errors, which are produced by shifts in membrane potential attributable to an current-resistance ($I-R$) drop across the series resistance. In some experiments, series resistance estimates were made using a current-clamp method, and the values were similar to other reported values for *R. pipiens* and *R. esculenta*: 0.3 MΩ (Drouin and Neumcke, 1974; Sigworth, 1980).

In most determinations of time constants, the percent block of the currents was kept to <65% to minimize the change in the sodium current, and thus to reduce artifacts from series resistance. To further ensure that the time constants were faithful representations of the kinetic decline or augmentation of sodium current, the following procedure was adopted: time constants were obtained by semilogarithmic plots of the reduction or augmentation of currents from their initial values. Exponentials were fitted to a time range over which currents had decreased to 25% of their initial value or increased to 75% of their final value. With this procedure, the largest voltage error resulting from series resistance was for the axon designated 31 Oct 79: assuming a 0.3 MΩ series resistance, the error was 3.6 mV. This voltage shift produced a 4.5% error in the estimate of the time constants for that axon. Typical values were between 0.5 and 2 mV. This produced errors in time constant determinations attributable to series resistance artifacts that were <3%.

Because isotopic effects on equilibrium dissociation constants agreed well with the corresponding effects on constants obtained from kinetic data, this provided an experimental verification of the nominal error introduced by series resistance into the determinations of the kinetic constants.

Means reported in this paper are given plus or minus the standard error, with the number of observations given in parentheses. Statistical significance was determined by applications of the double-tailed Student's $t$ test, and results were assumed significant if $P < 1%$.

**RESULTS**

**Whole Nerve Experiments**

Fig. 1 represents a typical experiment to obtain dose-response curves for various toxins. Action potentials are recorded every 25 s after the extracellular bathing solutions are changed. The action potential recordings are obtained from a single sucrose-gap recording procedure. In this experiment, three different STX concentrations—4, 10, and 5 nM—are applied to the nerve via the bathing media. In each application of toxin, the peak heights of action potentials monotonically decrease to a new steady state level. Upon readdition
to the control solution, 12 mM TEA-Ringer, the peak heights monotonically increase to the control height. Several replacements of the bathing solution were required before all the toxin was washed out of the nerve.

Fig. 2 represents a dose-response curve obtained using the procedure just described. The plotted points represent the fractional height of a stimulated action potential that remains after the nerve has been equilibrated with a toxin solution. The fraction of the control action potential was computed as the mean fraction of the preceding and subsequent control action potentials. Experiments in which recoveries from a toxin solution were <80% of the original control were discarded.

The triangles represent the experimental results obtained with STX diluted into TEA-Ringer. The potency of the toxin was defined to be the reciprocal of the concentration at which the action potential height had been reduced to one-half the control value. In this instance, the potency of STX was \( \sim 0.4 \text{ nM}^{-1} \).

If the experiments were repeated after preequilibrating the nerve and using toxins diluted into 98% \( \text{D}_2\text{O} \) Ringer solution (TEA D-Ringer), values represented by the squares were obtained. In all of the experiments of this type, the substitution of the TEA D-Ringer alone was sufficient to reduce the action potential height by an average factor of 0.94 ± 0.01 \( (n = 10) \). Thus the reductions in action potential heights produced by toxin addition were always computed using the appropriate control Ringer, (D-Ringer or hydrogen-Ringer \( [\text{H-Ringer}] \)). The dose-response relation (dotted line) in TEA D-Ringer shifts to the left, indicating a relative potency increase at all STX concentrations employed. The solid and dotted curves are drawn only to aid in visualizing the data. The circle represents 2 nM STX in 50% D-Ringer and 50% Ringer. The effects of changes in mole fraction \( \text{D}_2\text{O} \) on degree of block will be discussed later in this report. The potency of the deuterated toxin was \( \sim 0.6 \text{ nM}^{-1} \). Similar results were obtained in three other experiments; the
The average ratio of the protonated to deuterated potencies was 1.45 ± 0.05 (n = 4).

Because the pD (−log10[D⁺]) of a deuterium solution determined by using a glass electrode is underestimated by 0.4 U (Wang and Copeland, 1973), the pD of the 12 mM TEA D-Ringer was adjusted to be equivalent to the pH of 12 mM TEA-Ringer. The results obtained after this correction were not significantly different than the previous results; the ratio of protonated to deuterated STX potency was 1.5.

![Graph showing dose-response curves for STX in two isotopic solvents. The data points show dose-response relationships for STX in normal 12 mM TEA-Ringer (H-Ringer) and 12 mM TEA D-Ringer.](image)

When TTX was used to obtain a dose-response curve, no change in potency in TEA D-Ringer was observed (Fig. 3). Because the change in potency depended on the structure of the toxin molecule, two STX derivatives were tested for potency changes in D₂O: 1-hydroxysaxitoxin (neosaxitoxin [neo STX]) (a singly charged derivative at pH 7.2) and 11-hydroxysaxitoxin sulfate (gonyautoxin II [GTX II]) (Shimizu et al., 1978) (Table I). The results of these experiments, collected in Table II, indicated that substitution at carbon 11 or nitrogen 1 does not substantially affect the increased potency of the deuterated toxins.

The rate at which hydrogen atoms exchange for deuterium could also substantially alter the potency of various toxins in time. The OH and NH
groups of these molecules are rapidly exchangeable (proton-exchange time constants are <1 s). Studies of OH and NH groups in protein side chains have shown that exchange at these sites was close to being diffusion-limited (for review see Englander et al. [1972]). However, protons bound to carbon usually exchange so slowly that they could be neglected. One exception to this is the exchange at carbon 11, a methylene group alpha to a ketone group. In fact, Ritchie et al. (1976) labeled STX with tritium by exchanging the carbon 11 hydrogens by incubating the toxin in tritium oxide (T₂O). Their measured half-time for deuterium exchange into STX at carbon 11 at pH 7, 33°C, using a proton nuclear magnetic resonance assay was 70 min. A relatively

![Graph showing dose-response data for TTX in two isotopic solvents. TEA D-Ringer is the control for TTX (D-Ringer). AP, action potential. T = 20-21°C.](image)

stable labeled STX was obtained because back exchange of the tritium from carbon 11 to the solution had a time constant of 1 wk at 22°C (Ritchie and Rogart, 1977). In the experiments described below, we determined that these slowly exchangeable hydrogens did not contribute to any significant potency shifts.

### STX-D₂O Interactions; Controls and Effect of Mole Fraction

STX was diluted from a stock of 100 µM-13 nM in TEA-Ringer or TEA D-Ringer and incubated along with the two control solution, TEA-Ringer and TEA D-Ringer, at 40°C for ≤400 min. Samples were taken at 160, 275, and 400 min and tested for changes in potency. The full isotopic effect was seen as rapidly as the solutions could be mixed and assayed (10-17 min), and no significant changes in potency were seen thereafter. Therefore, slowly introducing deuterium into the exchange sites at carbon 11 did not significantly change the potency of STX.
When STX in TEA-Ringer was replaced by STX in TEA D-Ringer, a nonmonotonic decrease or increase in action potential height was observed (Fig. 4). The nerve was preequilibrated in 3.8 nM STX in TEA-Ringer, causing the action potential height to monotonically decline to the height shown at the left of Fig. 4. At the first arrow the solution was changed to 3.8 nM STX in 60% D$_2$O TEA D-Ringer. The time-course of decline was nonmonotonic. The steady-state height of the action potential was significantly less than the corresponding one in 3.8 nM STX TEA-Ringer.

These nonmonotonic transients were always observed when STX solutions with two different isotopic solvents were exchanged; the magnitude of the effect was dependent on the mole fraction of D$_2$O in the solution. However, the effect was not seen upon changing from TEA-Ringer to TEA D-Ringer or vice versa or with TTX solutions. The effect is consistent with a competitive effect of deuterated and protonated toxin molecules for the same site (Colquhoun, 1968; Wagner and Ulbricht, 1975). The kinetics of the effect are distorted by the increased diffusion time into the bundle of fibers. The actual toxin kinetics for a single fiber were investigated and are discussed later in this paper.

To gain a clearer insight about the STX-D$_2$O interaction, the mole fraction of D$_2$O in the Ringer solution was altered, and the relative effect on the action potential height was monitored. By altering the mole fraction of D$_2$O in the Ringer solution containing STX, the probability of deuterium being present on various exchange sites on the STX molecule can be altered. By changing the mole fraction of D$_2$O in the Ringer solution, the potency of STX will change from that seen in H$_2$O to a value seen in pure D-Ringer. Fig. 5 shows data from such an experiment plotted as the difference of percent block in TEA D-Ringer and TEA-Ringer and normalized. The relationship between percent block and mole fraction (Fig. 5, X) becomes linear at high mole fractions. The relationship deviates significantly from linearity at low mole fractions.

### Table I

| STX AND STX DERIVATIVES |
|--------------------------|
| **STX** | neo-STX | GTX-II |
|----------|---------|--------|
| $R_1$*  | $-$H    | $-$OH  | $-$H   |
| $R_{11}$ | $-$H    | $-$H   | $-$SO$_4$ |

* $R_1$ and $R_{11}$, substituents at nitrogen 1 and carbon 11, respectively.

### Table II

| ISOTOPE EFFECTS ON THE POTENCY OF VARIOUS TOXINS |
|--------------------------------------------------|
| Toxin   | Potency ratio | Number of experiments |
|---------|---------------|-----------------------|
| STX     | $1.4\pm0.05$  | 4                     |
| TTX     | $1.0\pm0.0$   | 2                     |
| GTX II  | 1.5           | 1                     |
| neo-STX | 1.3           | 1                     |
fractions; this effect was seen in Fig. 2 (the 50% TEA D-Ringer point lies near the dose-response curve for STX in TEA-Ringer). In these experiments, the exact relationship between dissociation constant and the mole fraction cannot be determined because action potential height is nonlinearly related to the proportion of unblocked sodium channels.

**Voltage-Clamp Experiments on Single Myelinated Fibers**

To make more-definitive statements about the STX-D$_2$O interaction, experiments were performed on single myelinated axons under voltage-clamp. The shift in the potency implied that the dissociation constant for STX, $K$, would be decreased in the presence of D$_2$O. Therefore, experiments were conducted to determine the basis for this change in the dissociation constant.

**Isotopic Effects on Dissociation Constants**

Nodes of Ranvier were voltage-clamped, and periodic applications of depolarizing test pulses were used to observe the changes in sodium current, $I_{Na}$, before and after solution changes. A 50-ms hyperpolarizing prepulse of 30 or 40 mV always preceded the application of a test pulse. $I-V$ relations were usually taken in all solutions.

To assay for changes in dissociation constant, $K$, the following protocol was
employed: The node was initially exposed to control TEA-Ringer, during which an $I-V$ relation was obtained. The solution was then changed to one containing a specific STX concentration in TEA-Ringer. A test-pulse voltage was randomly chosen in the voltage range of from $-30$ to $20$ mV and used to assay for the decline in $I_{Na}$ after the change to the STX test solution. After steady-state inhibition from the toxin was reached, an $I-V$ relation was obtained and the solution was then replaced by the control, TEA-Ringer. The recovery from toxin block was monitored by periodically observing $I_{Na}$ using the standard test pulse.

This procedure was repeated on the same node using TEA D-Ringer. Solutions were changed only after the node had equilibrated with the exchanged solutions. Data from such experiments were only accepted if recovery of $I_{Na}$ from toxin block in both solvents was $>80\%$ of the control currents measured before toxin application. The degree of block was calculated by dividing the size of the inhibited sodium currents by the means of control $I_{Na}$.

![Figure 5. Effect of mole fraction $D_2O$ on the block of the action potential by STX. The ordinate represents the increase in percent block in $D_2O$ solutions normalized to the maximum increase. Data from three fibers at three STX concentrations, each applied in a separate experiment, are shown. The degree of block increases with increasing mole fraction of $D_2O$ ($X$). (---), a linear relationship between normalized block and mole fraction $D_2O$, and is included for comparison.](image-url)
before and after toxin application. TEA D-Ringer alone reduced $I_{Na}$ by 11% by decreasing sodium permeability $[P_{Na}(D)/P_{Na} = 0.89 \pm 0.07 \ (n = 10); \text{ cf. Bergman (1979); Schauf and Bullock (1979)}]$. The determination of dissociation constants in the two different solvents was made on the same node.

The results of such experiments are contained in Table III. The data were analyzed by assuming that STX reacts reversibly and independently with one receptor (Hille, 1968; Schwarz et al., 1973; Wagner and Ulbricht, 1975).

The experiments for determining the effect of D2O on the dissociation constant were conducted using three different STX concentrations—0.5, 1, and 2 nM (Table III). Also tabulated are the percent of unblocked sodium current, $I_{Na}$, in TEA-Ringer and TEA D-Ringer and their ratio. Dissociation constants, were calculated from the equation:

$$K = [\text{STX}](1 - Y)/Y,$$

where $Y$ represents the fractional receptor occupancy, which is assumed to equal $1 - [I_{Na}(\text{STX})/I_{Na}]$ (Schwarz et al., 1973; Wagner and Ulbricht, 1975).

| Number | a | b | c | d | e | f | g |
|--------|---|---|---|---|---|---|---|
| of fibers | (STX) | $I_{Na}$ | $I_{Na}$ (STX-D2O) | b/c | $K_{H2O}$ | $K_{D2O}$ | $K_{H2O}$ / $K_{D2O}$ |
| nM | | | | | | | |
| 3 | 2.0 | 0.30±0.04 | 0.19±0.03 | 1.6±0.2 | 0.9±0.2 | 0.5±0.2 | 1.8±0.4 |
| 3 | 1.0 | 0.56±0.05 | 0.42±0.01 | 1.4±0.1 | 1.4±0.3 | 0.7±0.02 | 1.9±0.4 |
| 2 | 0.5 | 0.75±0.01 | 0.61±0.02 | 1.23±0.06 | 1.10±0.03 | 0.7±0.02 | 1.5±0.1 |
| Grand mean ± SEM | | | | | 1.2±0.01 | 0.67±0.07 | 1.8±0.2 |
| [TTX] | | | | | | | |
| 1 | 4.0 | 0.42 | 0.43 | 0.98 | 2.90 | 3.01 | 0.96 |
| 1 | 2.4 | 0.54 | 0.51 | 1.06 | 2.81 | 2.50 | 1.12 |
| 1 | 3.0 | 0.57 | 0.53 | 1.07 | 3.98 | 3.38 | 1.18 |
| Mean ± SEM | | | | | 1.09±0.08 | | |

The ratio of the $K$ obtained in H2O compared to D2O was found to be 1.83 ± 0.17 (8). The change in dissociation constant did not depend upon test pulse voltage. This observed ratio can be compared to the potency ratios determined from whole nerve experiments, 1.45 ± 0.05 (n = 4). Because reductions in action potential height because of toxin action are nonlinear measures of reductions in sodium current, the difference in ratios is to be expected.

In three similar experiments that tested for isotopic changes in the dissociation constant for TTX, there were no significant differences when TEA-Ringer was substituted by TEA D-Ringer (Table III). The mean ratio of dissociation constants was found to be 1.09 ± 0.08 (n = 3). It thus appeared that the changes in dissociation constant because isotopic substitution were indeed molecule dependent, substantiating similar results in whole nerve experiments.
Isotope Effects on Rate Constants

A series of experiments was conducted to determine the effects of deuterium substitution on the rates of onset and offset of toxin action. In these experiments the experimental system and chamber were modified to ensure a rapid and complete interchange of solutions.

The protocols for these experiments were quite similar to those used to test dissociation constants, as just described. Test pulses were applied repetitively, and the corresponding sodium currents were displayed with an increasing trigger delay on a storage oscilloscope. The completeness of the solution change was always tested initially by observing the block of potassium current after the solution change from Ringer solution to 12 mM TEA-Ringer. The first record after solution interchange (1–3 s) always had negligible potassium currents.

Fig. 6A and B illustrates one such experiment, where the rates of onset and offset of STX block were determined in TEA-Ringer and TEA D-Ringer. In the first series of records, the solution was switched from TEA-Ringer to 0.5 nM STX TEA-Ringer (top panel, at first arrow). The first record was the current obtained in TEA-Ringer, and the second, delayed record was recorded 7 s after the solution change had been initiated. For experiments of this type, all solution changes occurred in <4 s, with most occurring between 2 and 3 s. The second trace was usually recorded within 7–9 s after solution change initiation. Thereafter, traces were recorded at regular intervals. In the experiment of Fig. 6, records were taken every 19 s. The envelope of the sodium currents represents the kinetic of blockade of sodium channels by STX. The entire sequence of currents to the steady state was measured, but is not shown. Fig. 6 B shows the recovery from STX block after replacement of TEA-Ringer in the A pool. The steady-state recovery value of $I_{Na}$ is not shown, but it does not differ substantially from the last peak $I_{Na}$ value shown (last record on right-hand side). The lower panels illustrate an identical sequence of events, except that the fiber and the toxin were preequilibrated with TEA D-Ringer. (In this particular experiment, application of STX in TEA D-Ringer actually preceded the application of TEA Ringer.) Recovery from toxin solutions was >80% for both cases. Experiments were included in the data analysis if recovery from toxin block was ≥80% of the control sodium currents before toxin addition.

Time constants for onset and offset were obtained from semilogarithmic plots of the decline or recovery of the peak sodium currents in time. The rate constants for the reaction

$$\text{STX} + R \xrightarrow{k_1} \text{STX-R} \xrightarrow{k_2}$$

were then obtained from the expression (Schwarz et al., 1973):

$$k_2 = 1/\tau_{\text{off}} \quad (3)$$

$$k_1 = (1/\tau_{\text{on}} - 1/\tau_{\text{off}})/[\text{STX}] \quad (4)$$
Most experiments were performed in either TEA-Ringer or TEA D-Ringer. It was most desirable to measure the STX dissociation and rate constants for the two solvents on the same node, to minimize variation from node to node. This was achieved for all parameters of blocks in four experiments (paired experiments). Fig. 7 illustrates the analysis of toxin-blocking kinetics for one such experiment, that of Fig. 6. The rate of blockade in the TEA D-Ringer is almost identical to the rate found for STX in TEA-Ringer. The differences are statistically insignificant.

In contrast, when the rates of dissociation are compared, they are found to be substantially different (Fig. 8). The rate of dissociation in the TEA D-
Ringer is almost one-half that found for normal TEA-Ringer. Similar results were found in three other paired experiments. The means of the kinetic and equilibrium constants for the paired experiments were: $k_1$: $4.6 \pm 1.3 \times 10^6$ M$^{-1}$s$^{-1}$; $k_2$: $0.82 \pm 0.11 \times 10^{-2}$s$^{-1}$; $K$: $1.4 \pm 0.2$ nM in TEA-Ringer, and $k_1$: $6.4 \pm 1.4 \times 10^6$ M$^{-1}$s$^{-1}$; $k_2$: $0.46 \pm 0.03 \times 10^{-2}$s$^{-1}$; $K$: $0.6 \pm 0.1$ nM in TEA D-Ringer.

The differences in $k_1$ are not significant, however the differences in $k_2$ and $K$ are significant. The ratios $k_{2H}/k_{2D}$ and $K_{H}/K_{D}$ are found to be 1.78 and 2.3, respectively. If other paired experimental determinations of $K_{H}/K_{D}$ are combined with these, the overall ratio of $K_{H}/K_{D}$ was found to be $1.82 \times 0.17$. 

Table 7. Onset of block by STX (data from Fig. 6A and C). Open circles represent the mean of three determinations on the same fiber. The filled triangles represent a single determination of the on-rate using STX in TEA D-Ringer. Currents are recorded at various times [$I_{Na}(t)$] after addition an STX solution; the current decrements from an initial value [$I_{Na}(0)$] to a steady-state value [$I_{Na}($∞$)$].
The combined paired and unpaired experimental determinations of rate constants are summarized in Table IV. Three different concentrations of STX were used—0.5, 1, and 2 nM STX. Tabulated are the percent block of $I_{Na}$, and the time constants for onset and offset of STX in the two different isotopic media. The forward and reverse rate constants are also shown and have been calculated from Eqs. 3 and 4. The ratio of $k_2$ to $k_1$ is also shown and can be compared with $K$, the equilibrium dissociation constant computed from the values of percent block that have also been tabulated. The values agree reasonably well, and the difference between the two means is not statistically significant.

**Figure 8.** Reversal of block shows STX-receptor dissociation. $\circ$, $\square$, Two different determinations of the off-rate of STX. The line is drawn to fit the $\circ$. $\triangle$, A single determination of the off-rate using STX in TEA D-Ringer. Currents are recorded in time $[I_{Na}(t)]$ after wash out of an STX solution; the current increments from an initial value $[I_{Na}(0)_{STX}]$ to a steady-state value $[I_{Na}(\infty)]$. 
The time constant for dissociation of the STX-channel complex is significantly increased in D-Ringer, and this is reflected by the approximate halving of the off-rate constant in D-Ringer compared with the value obtained in TEA-Ringer. This result was found to be independent of the concentration of STX employed. The rate constant for onset, $k_1$, was unaffected by the presence of deuterium.

The paired values for the ratios of the kinetic and equilibrium constants are not statistically different from the mean values for the same quantities obtained using the compilation of both paired and unpaired data.

The results of these experiments indicate that $D_2O$ affects the on-rate of STX action nominally, if at all, and reduces the off-rate of STX by a factor of about one-half. The isotope effects on the equilibrium constants agree well with the effects obtained for the rate constants.

**Table IV**

| Number of fibers | Percent block | [STX] nM | $\tau_{on}$ s | $\tau_{off}$ s | $k_1 \times 10^{-6}$ nM s | $k_2 \times 10^2$ s$^{-1}$ | $k_2/k_1$ | $K$ (nM) |
|------------------|---------------|----------|--------------|--------------|------------------------|------------------------|-----------|---------|
| 5                | 0.5           | 32±2     | 10±6         | 140±7        | 5.0±0.8                | 0.74±0.04              | 2.0±0.3   | 1.1±0.09 |
| 3                | 0.5           | 43±6     | 135±28       | 238±21       | 7±2                    | 0.42±0.04              | 0.7±0.3   | 0.7±0.2  |
| 2                | 1.0           | 39±2     | 84±33        | 116±23       | 8±2                    | 0.8±0.2                | 1.1±0.4   | 1.4±0.4  |
| 2                | 1.0           | 64±6     | 77±1         | 219±49       | 8.0±0.9                | 0.5±0.1                | 0.6±0.2   | 0.6±0.1  |
| 3                | 2.0           | 71±2     | 46±3         | 125±16       | 7±1                    | 0.8±0.1                | 1.2±0.2   | 0.8±0.07 |
| 2                | 2.0           | 82±2     | 63±4         | 247±3        | 6.0±0.6                | 0.4±0.01               | 0.7±0.1   | 0.4±0.06 |
| Grand means ± SEM|               | 7.1±0.9  | 7.1±0.9      | 1.6±0.3      | 1.0±0.1                | 0.57±0.06              |           |         |

Table V summarizes data from a similar set of experiments designed to detect any isotopic changes in rate constants for TTX action. The tabulated quantities are identical to those for STX action. These experiments indicate that there is no significant effect of deuterium on the rate of onset or offset of TTX. These results are consistent with the previous findings, which indicated that there was no isotopic effect on the dissociation constant for TTX.

An experiment was performed to test whether rapidly changing the nodal bathing solution preequilibrated with STX in TEA D-Ringer to one containing STX in TEA-Ringer would produce a nonmonotonic transient decay of sodium currents. Such nonmonotonic transients can be predicted to occur in certain instances when one competitive drug is substituted for another (Colquhoun, 1968). The magnitude and time-course of the transient depends upon the initial conditions and the ratio of off-rates (Schwarz et al., 1973). Similar nonmonotonic transients have been observed when STX was added to a node that had been preequilibrated with TTX (Schwarz et al., 1975).

The results of such an experiment are shown in Fig. 9. The node is initially equilibrated with 1 nM STX in TEA D-Ringer, and then the extracellular solution was rapidly changed (3 s) to one containing 1 nM STX in TEA-
Ringer. The sequence of clamp currents obtained before (A) and (B–D) the isotope solution change is shown. The envelope of the peak currents describes the nonmonotonic transient change in $I_{Na}$.

If the peak heights of $I_{Na}$ are normalized to the steady-state $I_{Na}$, found in TEA-Ringer after the recovery from toxin addition and plotted versus time, a nonmonotonic transient of the form shown in Fig. 10 results. The sodium current initially decreases, and then increases in magnitude. The steady-state sodium current is greater in 1 nM STX in TEA-Ringer than in 1 nM STX in TEA D-Ringer. This reflects the steady-state potency increase in D-Ringer. The presence of the nonmonotonic transients is consistent with the idea that the STX molecule is competing with a deuterated STX for common sites. When this experiment was repeated, a similar nonmonotonic transient decay of $I_{Na}$ was seen (data not shown).

### Table V

**ISOTOPE EFFECTS ON TIME AND RATE CONSTANTS FOR TTX ACTION**

| Fibers | Percent block | $\tau_{on}$ | $\tau_{off}$ | $k_1 \times 10^4$ | $k_2 \times 10^2$ | $k_s/k_1$ | $K$ | Ringer's solution (H$_2$O or D$_2$O) |
|--------|---------------|------------|-------------|-----------------|-----------------|------------|-----|--------------------------------------|
| 19 Dec 79 | 4.0 | 64 | 155 | 318 | 0.827 | 0.314 | 3.8 | 2.3 | H$_2$O |
| 13 Jan 80 | 3.0 | 43 | 225 | 375 | 0.592 | 0.267 | 4.5 | 4.0 | H$_2$O |
| 14 Dec 79 | 4.0 | 55 | 195 | 420 | 0.667 | 0.238 | 3.5 | 3.3 | D$_2$O |
| 15 Jan 80 | 3.0 | 47 | 228 | 360 | 0.585 | 0.263 | 4.5 | 3.4 | D$_2$O |
| **Means ± SEM** | | | | | 0.71±0.17 | 0.29±0.03 | 4.1±0.5 | 3.1±1.2 | H$_2$O |
| | | | | | 0.64±0.07 | 0.25±0.02 | 4.0±0.7 | 3.3±0.1 | D$_2$O |

Two exponentials, fitting the initial reduction and the subsequent recovery of $I_{Na}$, could be extracted, and their values corresponded closely with the time constant for onset of STX and the time constant for removal of deuterated STX, respectively. The mean value of these time constants from the two experiments were: $\tau_{on} = 55$ s and $\tau_{off} = 330$ s. Estimates of the time constants are subject to a number of experimental errors. Changing the solvent from D$_2$O to H$_2$O or vice versa usually alters the leak-current contribution to the total current, and this alteration grows in time and may interfere with the analysis of the kinetics of the transient. Solvent changes also alter the series-resistance contribution to the measured membrane potential and affect the time-course and amplitude of the sodium-current kinetics. Some of these effects were minimized by using axons that exhibited only slight reductions in $P_{Na}$ when TEA D-Ringer was substituted for TEA-Ringer, but the other effects contributed errors to the time-constant determinations. In Fig. 9, TEA D-Ringer substitution reduced peak $I_{Na}$ to 0.93 of that in TEA-Ringer. The most significant error in this experiment was the change in the leak current, and this could be corrected if the time-course of the leak change was rapid compared with the fastest exponential. For these two experiments, the leak change had completely subsided within 3–5 s after the solution change, so that the great proportion of the time of block was unaffected by the error.
introduced by the leak change. Therefore, by a careful choice of experimental conditions, most of the errors could be reduced to small levels.

The observed nonmonotonic decay of \( I_{\text{Na}} \) (Fig. 10) can be described by a set of four simultaneous first-order reactions: both deuterated STX and STX compete for receptors which themselves may be either deuterated or hydro-

\[
y = y_\infty + c_1 e^{-(k_{11}\text{STX}+k_{12})t} + c_2 e^{-(k_{22}\text{STX}+k_{23})t} + c_3 e^{-(k_{33})t} + c_4 e^{-(k_{44})t},
\]

Figure 9. Nonmonotonic transient recovery from STX (D\(_2\)O) blockade. A single nerve fiber was equilibrated in TEA D-Ringer. The first recorded sodium current (A) is the current obtained from a test pulse (\(-5\) mV). At the arrow, the solution bathing the node was quickly changed (2 s) to 1 nM STX in TEA D-Ringer. The second record was obtained in that solution 3 s after the solution interchange. Thereafter, each record (in A) was observed every 10 s. After equilibration, the solution was again switched to 1 nM STX in TEA-Ringer (first arrow in B). The first recorded trace was obtained 3 s after solution exchange. Thereafter, each recorded trace was obtained every 10 s (continued through C and D). The envelope of peak \( I_{\text{Na}} \) produced a nonmonotonic recovery transient. The holding potential was \(-95\) mV. \( T = 8\) °C. Axon: 4 Feb 80.

genated. The exchange and back-exchange of deuterium into or out of sites on the toxins is assumed to be much faster than the toxin-binding kinetics. This model of the receptor-binding process leads to a set of simultaneous coupled first-order differential equations which can be adequately described by:
where \( y \) represents the fraction of receptors bound and the \( k \)'s the rate constants for the various binding reactions. The \( c \)'s depend upon the time constants and the initial conditions, \( y^{(i)}(0) \), (see Colquhoun [1968]). If the rate of exchange and back-exchange of deuterium into receptor sites approximates the binding rates, the equation must be modified by adding a particular solution. However, adequate fits are obtained without introducing this additional assumption.

From the previously described experiments, we have determined \( k_{+1}, k_{-1}, \) and \( k_{-4} \), which represent onset and offset rate constants for STX and the offset...
DISCUSSION

Comparison of Results

The experimental results compare favorably with previously reported kinetic and equilibrium constants for STX and TTX obtained from amplitude changes of sodium currents in voltage-clamped nodes of Ranvier. Table VI compares our values obtained using TEA-Ringer solution with previously reported values for TTX (Schwarz et al., 1973) and STX (Wagner and Ulbricht, 1975). Schwarz et al. (1973) obtained $Q_{10}$'s for their equilibrium and kinetic data, and these are tabulated for comparison. Also tabulated are estimates of $Q_{10}$'s for these same constants using the present data at 7-8°C.

| Preparation | Temperature | $K$ | $Q_{10}$ | $k_1 \times 10^{-7}$ | $Q_{10}$ | $k_2 \times 10^{-7}$ | $Q_{10}$ | Reference |
|-------------|-------------|-----|----------|---------------------|----------|---------------------|----------|-----------|
| STX         |             |     |          |                     |          |                     |          |           |
| Rana esculenta | 16 | 1.38 | 1.01±0.23* | 1.76±0.14* | Wagner and Ulbricht, 1975 |
| Rana pipiens | 8  | 1.04±0.10 (1.42) | 0.60±0.09 (1.82) | 0.79±0.02 (2.72) | This paper |
| TTX         |             |     |          |                     |          |                     |          |           |
| Xenopus laevis | 22 | 3.25±0.16 | 1.53 | 2.85±0.32 | 1.82 | 1.96±0.10 | 3.42 | Schwarz et al., 1973 |
| Rana pipiens | 8  | 3.1±1.2 (1.03) | 0.71±0.17 (2.73) | 0.29±0.03 (3.06) | This paper |

* Obtained from standard deviation of five means presented in Schwarz et al. (1973).
‡ Figures in parentheses were calculated using data of this paper for lower temperature values and Wagner and Ulbricht (1975) or Schwarz et al. (1973) for TTX and STX data at higher temperatures. $Q_{10}$ values not in parentheses were calculated from data presented in single references.
§ Calculated using onset data alone.

and the previously obtained data of Schwarz et al. (1973) at 22°C and that of Wagner and Ulbricht (1975) at 16°C. The TTX data of Schwarz et al. was obtained using fibers from *Xenopus laevis*, whereas Wagner and Ulbricht used those from *R. esculenta*.

Wagner and Ulbricht (1975) did not obtain $Q_{10}$ values for STX rate and equilibrium constants; however, estimates can be made from their results at 16°C and the present results obtained at 8°C. The $Q_{10}$'s derived using this procedure are: for $K$, 1.42; for $k_1$, 1.82; and for $k_2$, 2.72. The magnitudes of these $Q_{10}$'s are very similar to those reported for the rate and equilibrium constants for TTX action. It seems reasonable to assume that the differences between the reported rate and equilibrium constants of Wagner and Ulbricht and the present results can be attributed solely to the temperature dependence of these parameters.

Nonmonotonic Exponential Transients

Wagner and Ulbricht (1975) have observed nonmonotonic decays and recoveries of Na currents when a competitively acting toxin was added or removed.
from a medium previously equilibrated with another toxin. They described the importance of the ratio of off-rates of the two competitively acting toxins in producing the nonexponential process. In their experiments, TTX and STX were employed and shown to be competitive blockers. They used computer simulations to show that ratios of off-rates from \( \sim 1.5 \) to \( 10 \) produce observable nonmonotonic transients. At the temperature they employed (16°C), the ratio of off-rate constants was approximately \( \frac{k^{\text{STX}}_2}{k^{\text{TTX}}_2} = 1.7 \) (obtained from data of Schwarz et al. [1973]).

Wagner and Ulbricht (1975) emphasized that the presence of the nonmonotonic decays demonstrated competitive blockade at a single site and noted its utility as an indicator of competitive action. In the present experiments, similar nonmonotonic transients have also been observed. In sucrose-gap recordings (data not shown), transient overblock or transient underblock of successively recorded action potentials was seen upon substitution of equipotent concentrations of STX for TTX or vice versa, respectively.

Both recording techniques revealed nonmonotonic transients when deuterated STX solutions were replaced by STX solutions. The transients observed in the sucrose gap were complicated because they arose from two sources, time-dependent changes in action potential kinetics, primarily a result of the slowing effect of deuterium on the Na current kinetics, and nonmonotonic transients as a result of competitively acting toxins, and were further complicated by diffusion of toxins into and out of the nerve bundle. The kinetics of the transients observed in the single-node experiments suggest that the off-rate of the deuterated toxin relative to the protonated toxin is reduced by a factor \( <1/1.5 \). This conclusion agrees with the direct measurements of the ratio of dissociation constants and off-rates on single node experiments: \( \frac{K^{\text{STX}}_D}{K^{\text{STX}}_H} = \frac{k^{\text{STX}}_2}{k^{\text{STX}}_2} = 0.5 \) (Tables III and IV). Thus, the experimental results are consistent with the theory that a deuterated STX molecule can compete with hydrogenated STX for common sites.

Because STX has different classes of exchangeable hydrogens, we tested whether the reduced rate of toxin-receptor dissociation arose from deuteration at rapid- or slow-exchange sites. The rapid-exchange sites are exchanged within \( <1 \) min, whereas the slow-exchange sites exchange over many hours. There are two slow-exchange sites on the STX molecule, which presumably could be involved in the isotopic effects. Insertion of deuterium into these slow-exchange sites at the \(-\text{OH}\) groups on carbon 12 produced no significant change in the potency of the STX molecule. This negative result is gratifying, for the same site has been used extensively to label STX with tritium. Presumably, insertion of tritium at this site also has negligible effects on the kinetic parameters of STX action.

When the solvent of an STX solution bathing the node is rapidly changed from \( \text{D}_2\text{O} \) to \( \text{H}_2\text{O} \), the sodium currents undergo a nonmonotonic transient overblock, the kinetics of which can be described by the reactions of hydrogenated STX blocking unoccupied receptors and deuterated STX dissociating from blocked receptors (Fig. 10). The slow dissociation of deuterated STX from receptors was followed for \( >10 \) min, during which the nodal membrane was completely bathed by STX in TEA-Ringer. Because this observed relief
of block is adequately described by the kinetics of dissociation of deuterated STX from the receptor, we conclude that protons affecting the isotopic potency on STX do not exchange with solvent protons when the toxin is bound.

**Theoretical Considerations**

Because STX and TTX are cations that bind to charged membrane receptors in an aqueous solution, the role of water must be considered in their reaction mechanisms. Both toxins will interact with water molecules, forming hydrogen bonds between water and \(-\text{OH}\) and \(-\text{NH}_2\) groups on the toxins and ordering water molecules in a hydration shell around their charged guanidinium groups. Opposing these stabilizing contributions from bond formations between toxins and solvent molecules is the disorganizing effect of the toxin molecules on the structure of water; to describe completely the thermodynamics of the binding reactions, we must account for all of the enthalpic and entropic changes in the solvents as well as those of the toxins and the receptors. The available data are insufficient for such an accounting, but we can make some hypotheses about the reactions based on the measurements reported here and elsewhere and on thermodynamic values from simpler systems.

Our observations show that the isotopic effect of D$_2$O on STX affinity exclusively results from a slowing of the dissociation of the toxin-receptor complex; no effect of D$_2$O on the kinetics of TTX action is observed. Both toxins contain guanidino groups (one in monovalent TTX, two in divalent STX) and have several \(-\text{OH}\) and \(-\text{NH}_2\) groups, and it has been hypothesized that both form ionic and hydrogen bonds with their common receptor. In addition, a weak, covalent, hemilactal bond between TTX (Camougis et al., 1967) or STX (Hille, 1975) and the receptor has been postulated. However, such a hemilactal bond is unlikely for STX because it would require the presence of a carbonyl group at the carbon 12 position. At pH 7.0, STX is almost exclusively in the dihydroxy form; <1% of the molecules exist in the keto form and raising the pH to 7.5, which favors the presence of the keto form (Shimizu et al., 1981) has no effect on the potency of the toxin (G. R. Strichartz, unpublished observation). The gem-diol groups are not weakly acidic—the pK$_a$ of 8.2 reported for STX is a result of the ionization of the guanidinium moiety around nitrogen 8 (Shimizu et al., 1981), not to the \(-\text{OH}\) groups on carbon 12—thus, the formation of a covalent bond between STX and receptor is unlikely.

This leaves ionic bonds and hydrogen bonds for consideration. The evidence for ionic bonding is indirect. Metal and organic cations and protons compete with both STX and TTX for the receptor (Henderson et al., 1974; Reed and Raftery, 1976; Weigele and Barchi, 1979; Reed and Trzos, 1979), and alkylation of a putative carboxyl group near the outer opening of the sodium channel eliminates both toxin binding (Reed and Raftery, 1976) and toxin sensitivity (Spalding, 1980; Sigworth and Spalding, 1980). The ion-competition results have been cited as evidence for a cation-binding acidic group being physically part of the toxin receptor (Henderson et al., 1974; Hille,
However, binding of cations at such groups could exert a profound effect on toxin action through mechanisms other than a direct steric competition. For example, specific charges located near the toxin receptors in lobster walking-leg nerves can account for significant differences in dissociation constants (Strichartz and Hansen Bay, 1981). Cation competition per se is not evidence that the acidic group of the receptor must be juxtaposed with a bound toxin molecule. We do believe that ionic bonds are important for STX action; neutralization of the guanidino charge at nitrogen 8 significantly lowers the potency of the toxin. Ionic interactions can occur over a substantial distance compared with the dimensions of the toxin molecule.

Hydrogen bonds also contribute to the free-energy change of binding. The studies in D₂O support this idea, although the observed isotope effects can arise from a number of mechanisms (Schowen, 1977). Of the various sources of the isotopic effects, the most likely is an exchange labeling by deuterium of the reaction center of STX that slows the dissociation of STX from its receptor.

In theory, the isotope effects could arise from other sources: (a) Water may be a reactant. (b) The reactant structure may be indirectly altered by the deuterated solvent. (c) The solvent may cause bonding changes in the solvation shell of the toxin. (d) Differences in viscosity, dielectric constant, or other properties of the solvent may produce rate changes. The reaction kinetics of STX and TTX are pseudo-first order in the forward direction and first order in the reverse direction, and, as yet, there is no evidence that water is a product or reactant in the binding. These experiments suggest that the fourth source is unlikely; if the rate-limiting step of onset of block by either STX or TTX were diffusion, the rates for both toxins should have been measurably slowed by the 25% increase in viscosity of the solvent when D₂O was used. Similarly, other solvent effects are unlikely to affect the off-rate of STX alone. With regard to the second source, most enzyme studies have indicated only small structural changes of proteins in a D₂O solvent; x-ray crystallography studies of ribonuclease show no structural changes when exchange with deuterium (Bellow and Harker, 1961). Bonding changes in the solvation shell (source 3) may play a role; however, our demonstration in sucrose-gap experiments of isotopic effects of very similar size for doubly charged STX and zwitterionic, singly charged GTX and neo-STX reduces the likelihood that this source measurably contributes to the observed isotopic effect. Thus, deuteration of the reaction center is the most likely mechanism for kinetic isotope effects.

The kinetic isotope effects observed in these experiments may be primary or secondary. A primary effect arises when the hydrogen or deuterium is involved in a bond that is broken in the rate-limiting step. \( k_H/k_D \) ratios for such reactions are generally between 2 and 10 (Kirsch, 1977). Secondary effects are usually observed when the hydrogen or deuterium is attached to a chemical group that participates in the reaction. Such secondary isotopic effects are generally found to have \( k_H/k_D \) ratios of 1.02–1.4 (Kirsch, 1977).

The data obtained do not allow an unequivocal determination of the nature
of the isotopic effect. A number of rapid-exchange sites are known to exist on
the STX and TTX molecules, and others may exist on their receptor, and
exchange at each of these sites could potentially contribute to an overall
isotopic effect. Because this is the case, we prefer to interpret the data as a
sum of isotopic effects. Within this framework, the data are consistent with a
model in which the isotopic effect arises from changes in hydrogen bonding of
specific functional groups of the STX molecule with its receptor. The amino
and diol groups of STX are the likely candidates, because each of these groups
exchanges rapidly with deuterium.

Experimental studies and quantum-mechanics calculations have been em-
ployed to estimate the effect of deuterium substitution on hydrogen bonding
(Nemethy and Scherega, 1964; Rabinovich, 1970). The studies were conducted
on deuteroalcohols and deuteroamines. Experimental determinations of
changes in vapor pressure or osmotic coefficients were utilized to obtain
estimates of the increase in the degree of association when deuterium was
substituted for hydrogen molecules containing hydroxyl or amino groups.
Deuterium substitution acts to increase the energy of association of a single
hydrogen bond (O—H—O) by ~100 cal/mol in alcohols and by 240 cal/
mol in water at 25°C. The increase in bond energy is generally larger for
hydrogen bonds made by amino groups.

Our results show that D_2O solvent substitution reduces the K_D for STX
from 1.2 to 0.67 nM at 8°C (Table III). This increased affinity corresponds to
an increase in the free energy of STX binding of 0.4 kcal/mol, which can be
accounted for by two to four hydrogen bonds becoming deuterium bonds.

The isotopic effects on rate and equilibrium constants can be described by
an equation which is a simplified expression of a more general statistical
mechanical expression (see Bigeleisen and Mayer [1947]; Wolfsberg and Stern,
[1964]; and Cleland et al., [1976]):

$$\frac{K_1}{K_2} \sim \exp\left[\frac{h(Y_1 - P_1)}{2kT}\right],$$

where 1 and 2 refer to the light and heavy species, respectively, R and P refer
to reactants and products, respectively, h is Plank's constant, and k is Boltz-
mann's constant, and the y's represents the real normal mode frequencies of
vibration, expressed in reciprocal centimeters. A similar expression is obtain-
able for the rate constants, except that the product state is replaced by the
transition state of the molecule. Our data on the rate and equilibrium
constants put restraints upon the vibrations and rotations of the reacting
groups in the transition and product state. Thus, estimates of the vibrational
spectra of the various reacting groups could be obtained spectroscopically,
and the groups contributing to the isotope-sensitive bonding identified on this
basis.

If STX or TTX engages in hydrogen bonding to the receptor, deuterium
replacement at these sites should stabilize the bonds. Because no significant
changes in the on-rate constants were detected for either STX or TTX upon
deuteration, this restrains the sum of the various vibration frequencies in the
transition state to be equivalent to the identical sum for the reactants. Because
the off-rate constant for STX is halved upon deuteration, the sum of energy of the isotopically sensitive vibrations is reduced when STX is bound. No such effect is seen when TTX binds to its receptor, indicating that either the isotopically sensitive vibrational changes of the reactants are compensated by equal and opposite changes in the product state, or that hydrogen bonding plays a minimal role in TTX binding.

If the isotopic effects arise from exchange of deuterium at a number of sites in the STX molecule, the method of fractionation factors (Buddenbaum and Shiner, 1976) could be used to obtain specific information about the reaction center. Coupled with studies of STX derivatives, this may elucidate the method of STX blockage of Na channels.

In conclusion, the off-rate of the STX dissociation reaction is sensitive to the presence of deuterium in the Ringer's solution, and can be attributed to primary or secondary isotopic effects. In either case, the reduction in the off-rate arises from isotopically sensitive changes in zero-point energy contributions of the various real normal-mode vibrations of the molecule. The results are consistent with deuterium substitution acting to strengthen hydrogen bonds between STX and its receptor.

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REFERENCES

BELLO, J., and D. HARKER. 1961. Crystalization of deuterated ribonuclease. Nature (Lond.). 192:756.

BERGMAN, C. 1977. Sodium conductance and gating currents in myelinated axons. Proceedings of the International Union of Physiological Sciences. (Paris). 12:193. (Abstr.).

BIGLEISEN, J., and M. G. MAYER. 1947. Calculations of equilibrium constants for isotope exchange reactions. J. Chem. Phys. 15:261-267.

BUDDENBAUM, W. E., and V. J. SHINER, JR. 1976. ¹³C kinetic isotope effects and reaction coordinate motions in transition states for SN₂ displacement reactions. Can. J. Chem. 54:1146-1161.

CAMOUGIS, G., B. H. TAKMAN, and J. R. P. Tasse. 1967. Potency difference between the zwitterion form and the cation forms of tetrodotoxin. Science (Wash. D. C.). 156:1625-1627.

CLELAND, W., M. O'Leary, and D. Northrop, editors. 1976. Isotope Effects on Enzyme-catalyzed Reactions. University Park Press, Baltimore.

COLQUHOUN, D. 1968. The rate of equilibration in a competitive n drug system and the auto-inhibitory equations of enzyme kinetics: some properties of simple models for passive sensitization. Proc. Roy. Soc. Lond. B. Biol. Sci. 170:135-154.

DODGE, F. A., and B. FRANKENHAEUSER. 1958. Membrane currents in isolated frog nerve fiber under voltage clamp conditions. J. Physiol. (Lond.). 143:76-90.

DROUIN, H., and B. Neumcke. 1974. Specific and unspecific charges at the sodium channels of the nerve membrane. Pfluegers Arch. Eur. J. Physiol. 351:207-229.

ENGLANDER, W. S., N. W. DOWNER, and H. Teitelbaum. 1972. Hydrogen exchange. Annu. Rev. Biochem. 41:903-924.
Fox, J. M. 1976. Ultra-slow inactivation of the ionic currents through the membrane of myelinated nerve. Biochim. Biophys. Acta. 426:232–244.

Henderson, R., J. M. Ritchie, and G. R. Strichartz. 1974. Evidence that tetrodotoxin and saxitoxin act at a metal cation binding site in the sodium channels of nerve membrane. Proc. Natl. Acad. Sci. U. S. A. 71:3936–3940.

Hille, B. 1967. The selective inhibition of delayed potassium currents in nerve by tetraethylammonium ion. J. Gen. Physiol. 50:1287–1302.

Hille, B. 1968. Pharmacological modifications of the sodium channels of frog nerve. J. Gen. Physiol. 51:1199–219.

Hille, B. 1971. The permeability of the sodium channel to metal cations in myelinated nerve. J. Gen. Physiol. 59:637–658.

Hille, B. 1973. The receptor for tetrodotoxin and saxitoxin. A structural hypothesis. Biophys. J. 15:615–619.

Kao, C. Y., and A. Nishiyama. 1965. Actions of saxitoxin on peripheral neuromuscular systems. J. Physiol. (Lond.). 180:50–66.

Kirsch, J. F. 1977. Secondary kinetic isotope effects. In Isotope Effects on Enzyme-Catalyzed Reactions. W. Cleland, M. O'Leary, and D. Northrop, editors. University Park Press, Baltimore. 100–121.

Koppenhöffer, E. 1967. Die Wirkung von Tetraäthylammoniumchlorid auf die Membranströme Ranvierscher Schnürringe von Xenopus laevis. Pfluegers Arch. Eur. J. Physiol. 293:34–55.

Langmuir, I. 1916. The constitution and fundamental properties of solids and liquids. J. Am. Chem. Soc. 38:2221–2295.

Narahashi, T., H. G. Haas, and E. F. Therrien. 1967. Saxitoxin and tetrodotoxin: comparison of nerve blocking mechanism. Science (Wash. D. C.). 157:1441–1442.

Nemethy, G., and H. A. Scheraga. 1964. Structure of water and hydrophobic bonding in proteins. IV. The thermodynamic properties of liquid deuterium oxide. J. Chem. Phys. 41:680–689.

Rabinovich, I. B. 1970. Influences of Isotopy on the Physicochemical Properties of Liquids. Consultants Bureau, New York.

Reed, J. K., and M. A. Raftery. 1976. Properties of the TTX binding component in plasma membranes isolated from Electrophorus electricus. Biochemistry. 15:944–953.

Reed, J. K., and W. Trzos. 1979. Interaction of substituted guanidines with the tetrodotoxin-binding component in Electrophorus electricus. Arch. Biochem. Biophys. 195:414–422.

Ritchie, J. M. 1975. Binding of tetrodotoxin and saxitoxin to sodium channels. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 270:319–336.

Ritchie, J. M., and R. B. Rogart. 1977. Characterization of exchange-labeled saxitoxin and the origin of linear uptake by excitable tissue. Mol. Pharmacol. 13:1136–1146.

Ritchie, J. M., R. B. Rogart, and G. R. Strichartz. 1976. A new method for labelling saxitoxin and its binding to non-myelinated fibres of the rabbit vagus, lobster walking leg, and garfish olfactory nerves. J Physiol. (Lond.). 261:477–494.

Schäuf, C. L., and J. O. Bullock. 1979. Modifications of sodium channel gating in Myxicola giant axons by deuterium oxide, temperature, and internal cations. Biophys. J. 27:193–208.

Schöwen, R. L. 1977. Solvent isotope effects on enzyme reactions. In Isotope Effects on Enzyme-Catalyzed Reactions. W. Cleland, M. O’Leary, and D. Northrop, editors. University Park Press, Baltimore. 64–99.

Schwarz, J. R., W. Ulbricht, and H. H. Wagner. 1973. The rate of action of tetrodotoxin on myelinated nerve fibers of Xenopus Laevis and Rana Esculenta. J. Physiol. (Lond.). 233:167–194.

Shimizu, Y., C. P. Hsu, W. E. Fallon, Y. Oshima, and I. Miura. 1978. Structure of neosaxitoxin.
SHIMIZU, Y., C. P. Hsu, and A. GENENAH. 1981. Structure of saxitoxin in solutions and steriochemistry of dihydrsasxitoxins. J. Amer. Chem. Soc. 103:605-609.

SIGWORTH, F. J. The variance of sodium current fluctuation at the node of Ranvier. 1980. J. Physiol. (Lond.). 307:97-129.

SIGWORTH, F. J., and B. C. SPALDING. 1980. Chemical modification reduces the conductance of sodium channels in nerver. Nature (Lond.). 283:293-295.

SPALDING, B. C. 1980. Properties of toxin-resistant sodium channels produced by chemical modification in frog skeletal muscle. J. Physiol. (Lond.). 305:485-500.

STRICHARTZ, G. R., and C. M. HANSEN BAY. 1981. Saxitoxin binding in nerves from walking legs of the lobster Homarus americanus. Two classes of receptors. J. Gen. Physiol. 77:205-221.

TAKATA, M., J. W. MOORE, C. Y. KAO, and F. A. FUHRMAN. 1966. Blockage of sodium conductance increase in lobster giant axon by tarichatoxin (tetrodotoxin). J. Gen. Physiol. 49:977-988.

WAGNER, H. H., and W. ULBRICHT. 1975. The rates of saxitoxin action and of saxitoxin-tetrotoxion interaction at the node of Ranvier. Pfluegers Arch. Eur. J. Physiol. 359:297-315.

WANG, J. H., and E. COPELAND. 1973. Equilibrium potentials of membrane electrodes. Proc. Natl. Acad. Sci. U. S. A. 70:1909-1911.

WEIGELE, J. B., and R. L. BARCHI. 1979. Saxitoxin binding to the mammalian sodium channel. Competition by monovalent and divalent cations. FEBS (Fed. Eur. Biochem. Soc.) Lett. 95:49-53.

WOLFSBERG, M., and M. J. STERN. 1964. Validity of some approximation procedures used in the theoretical calculation of isotope effects. Pure Appl. Chem. 8:225-242.