Equilibrium and Kinetic Properties of the Interaction between Tetrodotoxin and the Excitable Membrane of the Squid Giant Axon

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ABSTRACT Squid giant axons were treated with tetrodotoxin (TTX) in concentrations ranging from 1 nM to 25 nM and the resulting decrease in sodium current was followed in time using the voltage clamp technique. The removal of TTX from the bathing solution produced only partial recovery of the sodium current. This suggests that the over-all interaction is more complex than just a reversible reaction. By correcting for the partial irreversibility of the decrease in sodium current, a dissociation constant of $3.31 \times 10^{-9}$ M was calculated for the reaction between TTX and the reactive site of the membrane. The data obtained fit a dose-response curve modified to incorporate the correction for partial irreversibility when calculated for a one-to-one stoichiometry. The fit disagreed with that calculated for a reaction between two molecules of TTX with a single membrane-reactive site, but neither supported nor disproved the possibility of a complex formed by two reactive sites with one molecule of TTX. Values of the rate constants for the formation and dissociation of the TTX-membrane complex, $k_1$ and $k_2$, respectively, were obtained from the kinetic data. The values are: $k_1 = 0.202 \times 10^8$ M$^{-1}$ min$^{-1}$, and $k_2 = 0.116$ min$^{-1}$. The magnitude of the dissociation constant derived from these values is $5.74 \times 10^{-9}$ M, which has the same order of magnitude as that obtained from equilibrium measurements. Arrhenius plots of the rate constants gave values for the thermodynamic quantities of activation.

INTRODUCTION

The action of tetrodotoxin (TTX) on nerve represents a highly specific membrane affinity (Kao, 1966). It is now well-known that of the two components of the ionic current observed in lobster and squid giant axons under voltage clamp conditions, TTX depresses only the early transient component (Goldman, 1965; Nakamura, Nakajima, and Grundfest, 1965; Narahashi, Moore, and
Scott, 1964; Takata, Moore, Kao, and Fuhrman, 1966). The high degree of TTX specificity in blocking exclusively one of the ionic current components is a rare property reported only for TTX-like compounds (Kao, 1966; Hille, 1968; Narahashi, Moore, and Poston, 1967; Narahashi and Moore, 1968), tetraethylammonium ions (Tasaki and Hagiwara, 1957; Armstrong and Binstock, 1965; Hille, 1967), and urethane (Hagiwara and Saito, 1959).

Both inward and outward sodium ion movements are completely abolished by external TTX in squid giant axons (Moore, 1965), but only the inward sodium current can be totally depressed in lobster axons (Goldman, 1965). When all the sodium in the medium bathing the axon is replaced by other monovalent ions capable of maintaining the excitability of the axon (guanidinium, hydrazinium, hydroxylamine, and lithium), the currents observed under voltage clamp are blocked by TTX (Tasaki and Singer, 1966; Moore, Blaustein, Anderson, and Narahashi, 1967). Thus, TTX inhibits the early phase of the ionic current independent of its direction or of the nature of the carrier. Hence, it has been concluded that TTX acts specifically on the mechanism of the early increase in ionic permeability of the membrane (Moore et al., 1967).

The Hodgkin and Huxley theory (1952) suggests several ways by which the early increase in the permeability of the membrane can be hindered: (a) a decrease in the number of channels capable of becoming permeable \((\Delta g_{Na})\), (b) an increase in sodium inactivation \((\Delta (1-h))\), (c) a decrease in sodium permeability \((\Delta m)\). In experiments in which lobster axons were voltage-clamped by the sucrose gap technique, Takata et al. (1966) obtained evidence that indicates that \(\tau_m\) and \(\tau_h\) are not significantly affected by TTX. This finding implies that \(\alpha_m\), \(\beta_m\), \(\alpha_h\), and \(\beta_h\) are not affected by TTX. Thus TTX does not seem to affect the m and h parameters, and its sole effect seems to be on \(g_{Na}\). Hille (1968) came to the same conclusion for TTX action on the frog node.

It is the express purpose of this paper to determine the kinetics of action of TTX on the membrane conductance of squid giant axons. The extent of the action of TTX was determined by means of the voltage clamp technique. As TTX acts specifically on the sodium channel, attention is focussed on the changes in maximum inward sodium current at a given state of the membrane as a function of [TTX], time, and temperature.

From data gathered in this way, quantitative information about equilibrium and rate constants was derived where possible, and changes in free energy, enthalpy, and entropy were calculated from rate processes. Results are interpreted in terms of several models that have been proposed for the TTX-membrane interaction.

The following assumptions are implicit in this study and will be discussed later: (a) TTX binds to a receptor site on the membrane, forming a complex,
in accord with presently accepted pharmacological theory (Ariens, Simonis, and van Rossum, 1964); (b) a linear relationship exists between the concentration of the drug-receptor complex and the effect produced; (c) the time required by a newly formed drug-receptor site complex to elicit its effect is negligible when compared with the rates at which the complex formation and dissociation proceed, i.e. the change in the site is equivalent to a change in the sodium conductance.

**METHODS**

Most of the procedures and equipment have been described previously (Adelman and Palti, 1969; Frost and Pearson, 1961). Giant axons were obtained from *Loligo pealei* at the Marine Biological Laboratory. The axons were cleaned of all surrounding fibers and most loose connective tissue.

Artificial seawater (ASW) was prepared with deionized water and reagent grade chemicals to the following composition (mm): NaCl, 430, KCl, 10, CaCl₂, 10, MgCl₂, 50, tris (hydroxymethyl)aminomethane, 2, and adjusted to pH 7.40 ± 0.05 at 5°C. TTX was a crystalline preparation obtained from Sankyo Company, Ltd., Tokyo, Japan, in vials containing 100 µg. A stock solution was prepared with a concentration of 10 nanomoles per ml in 0.01 M acetate buffer of pH 4.0 and kept under refrigeration. Under these conditions TTX is stable for several months. When mixing convenient aliquots of this stock solution with ASW to prepare working solutions, the final pH was that of the ASW. Fresh working solution was prepared for each experiment and used without delay to avoid decomposition of the TTX.

The experimental cell and external perfusion system are shown in detail in Adelman and Palti (1969). The TTX-containing solutions were admitted to the cell through the same route as the ASW. Changes were made in solution by switching polyethylene tubes at the entrance to the cell. In this way, the dead space was kept to a minimum. The ASW temperature was monitored with an electronic thermometer connected to a thermistor placed in the inflow close to the center of the cell.

The voltage clamp system was a modification of the point-control system of Cole and Moore (1960) and the circuit is shown in Adelman and Palti (1969).

After the axon was in place in the cell and the axial wire inserted, a short depolarizing pulse was applied to the axon through the axial wire and the resulting membrane action potential measured. The axon was discarded if the action potential was lower than 100 mv. The micropipette electrode was then inserted through the membrane and the reference electrode was placed in position. A set of current records at different command potentials was obtained to define the normal current-voltage relationship of the axon, and immediately the bathing solution was changed from ASW to TTX-containing ASW. Then, a series of three pulses, separated by intervals of about 10 sec, was applied every 1 or 2 min until the magnitude of the peak inward current reached a new steady value. The amplitude of the pulses was slightly changed in the course of the experiment in order to define the current-voltage curve in the range that contained the maximum peak sodium current.

In order to increase the efficiency of determining the range of maximum sodium
current, double pulse experiments were performed in which two different pulses were applied with a temporal separation of 50 msec for each sweep of the oscilloscope. The resolution in the time scale of the records was necessarily reduced, but the peak sodium currents could be easily measured. In this manner, it was possible to define the desired range of the current-voltage curve in only 10 sec.

**TABLE I**

**EFFECT OF TTX ON EQUILIBRIUM VALUES OF THE MAXIMUM PEAK SODIUM CURRENT**

<table>
<thead>
<tr>
<th>Axon</th>
<th>[TTX]</th>
<th>Temperature</th>
<th>Type of clamp</th>
<th>( I_{\text{Pmax}} ) values</th>
<th>( I_{\text{max}} ) recovery</th>
<th>( \Delta I_{\text{in}} = (I_{\text{INa}} - I_{\text{IK}}) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-65</td>
<td>25.0</td>
<td>15.0</td>
<td>Triangular</td>
<td>3.00</td>
<td>0</td>
<td>0.13</td>
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<tr>
<td>S-59</td>
<td>25.0</td>
<td>15.0</td>
<td>Sine</td>
<td>2.60</td>
<td>0.15</td>
<td>1.70</td>
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<td>S-52</td>
<td>15.0</td>
<td>7.1</td>
<td>Pulse</td>
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<td>1.37</td>
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<td>S-66</td>
<td>15.0</td>
<td>7.0</td>
<td>Sine</td>
<td>3.55</td>
<td>0.40</td>
<td>3.00</td>
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<tr>
<td>S-40</td>
<td>10.0</td>
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<td>2.36</td>
<td>0.42</td>
<td>2.00</td>
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<td>4.5</td>
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<td>1.90</td>
<td>0.38</td>
<td>1.42</td>
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<td>2.64</td>
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<td>3.38</td>
<td>0.92</td>
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<td>S-51</td>
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<td>Pulse</td>
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<td>0.85</td>
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<td>1.95</td>
<td>0.50</td>
<td>1.27</td>
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<tr>
<td>S-62</td>
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<td>Triangular</td>
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<td>3.50</td>
</tr>
<tr>
<td>S-63</td>
<td>2.5</td>
<td>7.0</td>
<td>Pulse</td>
<td>3.00</td>
<td>1.15</td>
<td>1.90</td>
</tr>
<tr>
<td>S-64</td>
<td>2.5</td>
<td>7.0</td>
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<td>2.10</td>
</tr>
<tr>
<td>S-49†</td>
<td>1.0</td>
<td>10.0</td>
<td>Pulse</td>
<td>3.08</td>
<td>1.95</td>
<td>2.34</td>
</tr>
<tr>
<td>S-61‡</td>
<td>1.0</td>
<td>5.0</td>
<td>Triangular</td>
<td>4.20</td>
<td>2.85</td>
<td>3.45</td>
</tr>
</tbody>
</table>

\* Initial current value before TTX exposure; \( I_{\text{INa}} \) = equilibrium Na current value after TTX exposure; \( I_{\text{IK}} \) = equilibrium current value in recovery plateau.

† Kinetic data from these axons showed too much scatter to be analyzed accurately; these axons do not appear in Table III.

In a few experiments, a varying potential control voltage clamp was used in order to determine the entire sodium current-voltage relationship in a single oscilloscope sweep. In this method, the usual step command voltages were replaced with triangular or sinewave potentials. As slowly varying potential waves (sine or triangular) give practically pure \( I_{\text{INa}} \) and rapidly varying potential waves give practically pure \( I_{\text{IK}} \), direct on-line displays of the sodium and potassium I-V characteristics were obtained within milliseconds. For example, it has been shown by Palti and Adelman

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on theoretical and experimental grounds that at frequencies between 500 and 700 Hz, the total membrane current for depolarizing clamped wave forms is within a few per cent of the separated $I_{Na}$ and that an entire $I_{Na}$ vs. $E_m$ relationship can be obtained in a single oscilloscope sweep for a potential swing of ± 150 mV from the resting or holding potential. The type of voltage clamp system used is specified for each experimental axon in Table I.

After the maximum peak sodium current had reached a steady value, the bathing solution was changed back to ASW and the maximum peak sodium current was followed until a new steady value was reached.

RESULTS

A. General Characteristics of the Action of TTX

1. EFFECT OF TTX ON THE IONIC CURRENTS

Previous studies, using concentrations ranging from 15 nM to 100 nM TTX, have shown a very rapid onset of the effect of TTX in blocking the excitability of axons (Takata et al., 1966). In order to study the kinetics of inactivation by TTX, it was decided to employ a lower TTX concentration range in order to obtain enough precision in the measurements to assure a satisfactory temporal definition of the variations in the membrane sodium current-voltage relation in the region of maximum current density. This definition took about 20 sec with the single pulse experiments and about 10 sec with the double pulse experiments. The concentrations of TTX chosen were 1, 2.5, 5, 10, 15, and 25 nM. As these concentrations did not completely abolish the sodium current (with the possible exception of 25 nM), determination of the equilibrium blocking point became possible.

The membrane currents obtained with single pulse and double pulse experiments are shown in Fig. 1. Fig. 1A illustrates a series of currents at constant pulse potential at 1 min intervals after changing the external perfusion solution from ASW to TTX-containing ASW. It is evident that the delayed outward current is not affected by TTX, while the early inward transient current carried by sodium ions is greatly decreased.

Fig. 1B shows a set of two voltage-clamped pulses with the associated membrane currents recorded on a single CRO trace. The two pulses were applied to increase the rate of data collection. In order to insure that the time between these pulses was sufficient to remove most of the sodium inactivation, the interval between pulses was ≥ 50 msec. Current-voltage relations derived from the records by plotting peak inward and steady-state outward currents vs. the applied potential (see Fig. 2) further demonstrate the drastic and specific action of TTX on the sodium current. In Fig. 2, the time in minutes after application of TTX appears adjacent to each curve. These current-voltage relations were used in estimating the maximum values of the peak sodium currents. These maxima show a slight shift on the voltage axis...
with time of exposure to TTX. The magnitude of this shift was of the order of 10 mV and was reversed when TTX was removed from the bathing solution. The significance of this phenomenon will not be discussed in this paper. According to Takata et al. (1966), any small changes in sodium conductance parameters should be unimportant when compared with changes ensuing from the effect of TTX on the maximum sodium conductance, $g_{Na}$. Hille (1968) concluded that the TTX effect on frog nodes was on $g_{Na}$ exclusively.

Figure 1. Membrane currents (above) as a function of time in response to voltage clamp pulses (below). A, successive records obtained upon the application of 10 nm TTX to axon S-41. Upper current record was obtained in TTX-free ASW, and successively smaller records were obtained at approximately 1 min intervals after the application of TTX. Membrane potentials are labeled on the voltage record. Compare with Fig. 2. B, record illustrating membrane current responses to two 10 msec voltage clamp pulses separated by 50 msec. Axon S-57 in ASW. As indicated on the voltage record, the second pulse was smaller in amplitude than the first. See text.

2. TIME COURSE OF THE CHANGE IN MAXIMUM PEAK SODIUM CURRENT When the maximum values of the peak sodium currents, taken from the current-voltage diagrams, are plotted against time, curves of the type shown in Fig. 3 (A and B) are obtained. The salient features of these curves are the two plateaus that appear during the exposure to TTX and after the removal of TTX. Under the assumption that the first of these plateaus represents a state of equilibrium, the effect of TTX on the sodium current will be treated as the consequence of a reversible reaction, following the usual interpretation of drug-receptor interactions. This assumption is supported by the fact that the
normalized points representing the first plateau, obtained with various concentrations of TTX acting on axons that had different initial sodium currents, fit fairly well a dose-response curve, as will be seen later. Isolated squid axons usually show some progressive decrease in current values as a function of time. This progressive deterioration of the preparations was much slower than the effect of TTX.

In all experiments performed, the magnitude of the maximum peak sodium currents at zero time was greater than after recovery from TTX exposure.

The difference was larger than could be expected from the progressive decrease in currents as estimated from the decline in the steady-state values of $I_{K}$ (see Table I and Fig. 3 A). In Fig. 3 A, maximum peak sodium current ($I_{Pmax}$) values, potassium current ($I_{K}$) values obtained at $E_{m} = +106$ mv, and the resting potential ($E_{RP}$) obtained between clamping periods, are plotted as a function of time. The axon was voltage-clamped to two triangular wave forms having frequencies of 550 and 55 Hz. At 550 Hz, the sodium current-voltage relation was determined, and at 55 Hz, the potassium current-voltage relation.

It is concluded that, in addition to a reversible TTX membrane interaction, some other phenomenon occurs. Thus, in Fig. 3 B, three regions are defined: A is proportional to the extent of the process that confers some degree of
FIGURE 3. A, values of maximum peak sodium current, $I_{P_{\text{max}}}$ (filled circles); potassium current, $I_K$ (open circles) at $E_m = +106$ mV; and the resting potential, $E_{RP}$ (continuous line) between brief triangular wave voltage-clamping periods. Axon exposed to 2.5 nM TTX and subsequently to ASW. The holding potential was set equal to the initial resting potential of $-60.5$ mV. $I_{P_{\text{max}}}$ and $I_K$ were obtained using frequencies of 550 Hz and 55 Hz, respectively. B, depression of the maximum peak sodium current upon exposure to 15 nM TTX and its recovery in ASW. Ordinate, $I/I_0$ in per cent of $I_0$, where $I$ is the maximum peak sodium current during the exposure and recovery and $I_0$ is the maximum peak sodium current initially in ASW. Abscissa, time in minutes where $t_0 = time$ at which TTX exposure begins, $t_1 = time$ at which TTX exposure ends, and $t_2 = time$ at which maximum peak current reaches a recovery plateau. For definition and significance of areas A, B, and C, see text. Axon S-52. Temperature = 7°C.
irreversibility on the action of TTX; B, proportional to the surface density of still excitable sites; and C, proportional to the extent of the reversible reaction. As it appears necessary to take A into account for the study of the equilibrium constant of the main reaction, a detailed consideration will be made later. For the present, it is sufficient to say that the amplitude of the maximum peak sodium current, $I_{p_{\text{max}}}$, will be taken as a measure of the surface density of TTX-free sites available for sodium transport, i.e.:

$$B = P(I_{p_{\text{max}}})$$

where $P$ is a constant proportionality factor, and $B$ is the fraction of sites not found by TTX.

B. Equilibrium

Table I summarizes the effect of TTX on the values of $I_{p_{\text{max}}}$ by comparing the relative levels of the steady initial, test, and recovery states of the sodium current. In Table I, $I_0$ is the $I_{p_{\text{max}}}$ value before the application of TTX (at $t = 0$), $I_t$ is the $I_{p_{\text{max}}}$ value achieved during the plateau of TTX effect (at $t = t_1$), $I_s$ is the $I_{p_{\text{max}}}$ value once recovery from TTX had reached a steady state (at $t = t_2$), $\Delta I_K$ is the proportional decrease in potassium steady-state current at the end of the recovery period as compared with the initial $I_K$ value before addition of TTX, and $I_s/I_0$ is the percentage recovery of $I_{p_{\text{max}}}$ in ASW at $t = t_2$.

Fig. 4 A illustrates a control experiment showing the relationship between $I_{p_{\text{max}}}$ and $I$ values obtained from a typical axon externally perfused with ASW for 125 min. The axon was voltage-clamped to two different triangular wave forms. With the method described by Palti and Adelman, the sodium current-voltage relation was obtained at 657 Hz and the potassium current-voltage relation at 65.7 Hz. The $I_K$ values plotted corresponded to a membrane potential of +60 mV. It can be seen in Fig. 4 A that the general declines of $I_{p_{\text{max}}}$ and $I$ values with time parallel each other.

Fig. 4 B plots additional values of $I_{p_{\text{max}}}$ and $I_K$ obtained from the same axon after the above control period. $I_K$ values corresponded to the same membrane potential as in Fig. 4 A. Each point represents immediately successive values of $I_P$ and $I_K$ obtained with the wave forms used in Fig. 4 A. The theoretical curve drawn through the points represents the relation when the per cent change in $I_{p_{\text{max}}}$ is equal to the per cent change in the test $I_K$. $\Delta I_P$ and $\Delta I_K$ over a given time interval were equal throughout such control experiments. $\Delta I_K$ was used to estimate the reduction in $I_P$ values that would have occurred if no TTX had been used. $(I_0-I_s)/I_0$ was used to estimate the extent of the irrecoverable effect of TTX on the sodium system. Note in Table I that $\Delta I_K$ was always less than the irrecoverable fraction.

If we temporarily assume, for a reversible reaction at the membrane surface...
between TTX and a receptor on the axon membrane, a mechanism of the type

$$\text{TTX} + \beta \xrightarrow{k_1/k_2} \gamma$$
where $\beta$ and $\gamma$ represent the unbound and bound receptors, respectively, whose concentrations are represented by $B$ and $C$ in Fig. 3 B, then the following equilibrium relationship obtains between the fraction of membrane sites blocked and the concentration of TTX:

$$\frac{B[TTX]}{C} = \frac{k_2}{k_1} = K.$$  \hspace{1cm} (2)

$C$ is the fraction of sites reversibly bound by TTX, $B$ is the fraction of sodium-conducting sites not bound to TTX, and $K$ is the dissociation constant of the complex. Equation 2 may be recast in the form:

$$\frac{C}{B_o} = \frac{1}{1 + \frac{K}{[TTX]}}$$  \hspace{1cm} (3)

where $B_o$ is proportional to the total number of reversible sites per unit area of membrane, and $C = B_o - B$.

Equation 3 may be expressed by plotting $C/B_o$ vs. log [TTX] which gives a sigmoid dose-response curve. Equation 3 is analogous to the Langmuir (1916) adsorption isotherm for adsorption of gaseous molecules by solid surfaces.

As Table I indicates that the initial $I_{\text{rmax}}$ values are not recoverable after TTX application, the value of $B$ related to the reversible reaction ($B_o$) may be estimated by assuming that the value of $A$ and $I$ equals the value of $A$ at $t_t$. Thus, $A_1 = A_2$. Furthermore, as $B$ is considered to be represented by $I_{\text{rmax}}$ (equation 1), then

$$\frac{A_1}{B} = \frac{A_2}{B} = \frac{I_0 - I_t}{I_0}$$  \hspace{1cm} (4)

The above assumptions were tested on two axons by using successive exposures to TTX. Once an equilibrium $I_{\text{rmax}}$ value was reached in TTX, the axon was returned to ASW and a recovery equilibrium was then established. TTX was applied at the same concentration again until a new equilibrium $I_{\text{rmax}}$ value was reached in TTX. ASW was then reapplied and the $I_{\text{rmax}}$ values were allowed to reach a new recovery equilibrium. In both axons, the second recovery was to the same level as the first recovery. These experiments are taken as evidence that once TTX is applied to an axon a fraction of sodium sites is irreversibly blocked, and that successive exposures to TTX do not increase this fraction. Examination of Table I indicates that this irrecoverable fraction is not a function of the [TTX] over the range from 1 nM to 25 nM. Thus it appears that TTX concentrations from 1 nM to 25 nM saturate the irreversible component and that for our purposes the $A_1$ fraction...
may be taken as a constant estimated by \((I_0 - I_2)/I_0\). Thus

\[ B_1 = \frac{I_1}{I_2} \quad \text{and} \quad C_1 = \frac{I_2 - I_1}{I_1} \]

where \(B_1\) and \(C_1\) are the estimated values of unbound and bound sites in the

**TABLE II**

VALUES OF THE DISSOCIATION CONSTANT \(K\), FOR THE REACTION BETWEEN TTX AND THE SODIUM CHANNEL AT 7°C

<table>
<thead>
<tr>
<th>Axon</th>
<th>[TTX]</th>
<th>(h^*)</th>
<th>(h^*)</th>
<th>(B_1 = \frac{h}{h_1})</th>
<th>(C_1 = \frac{h - h_1}{h_1})</th>
<th>(2B_1)</th>
<th>(2C_1)</th>
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<tbody>
<tr>
<td></td>
<td>(\text{nm})</td>
<td>(\text{ms/cm}^2)</td>
<td>(10^{-9} \text{ M}^*)</td>
<td>(10^{-9} \text{ M}^*)</td>
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<tr>
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<td>0.773</td>
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<td>3.770</td>
<td>0.775</td>
<td>0.225</td>
<td>3.44</td>
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</tr>
</tbody>
</table>

Average \(K = 3.31 \times 10^{-9} \text{ M}\)

* Corrected for expected progressive decline in \(I_N\) as estimated from decline in \(I_K\).

**TABLE II**

VALUES OF THE DISSOCIATION CONSTANT \(K\), FOR THE REACTION BETWEEN TTX AND THE SODIUM CHANNEL AT 7°C

The steady state \((t = t_4)\) for a given concentration of TTX. Table II gives values for \(B_1\) and \(C_1\) for different concentrations of TTX. These values were derived from the data in Table I by correcting the appropriate values of \(I_{P_{max}}\) for a linear spontaneous decline as estimated from the \(\Delta I_K\) values scaled in time, and by normalization to 7°C.
The value of $K$ may be determined from equation 2 as follows:

$$K = \frac{B_i[TTX]}{C_1}.$$  \hspace{1cm} (5)

Values of $K$ are given in Table II. The average $K$ value was $3.31 \times 10^{-9} \text{ M}$.

With the use of equation 3, it is possible to calculate $C_1/B_0$ from the value of $K$ and $[TTX]$. In Fig. 5, $C_1$ is plotted against the log $[TTX]$. The points are from Table II and the curves are theoretical. Curve I in Fig. 5 is the graph of equation 3 for the average value of $K$ from Table II. The fit is fairly good, which shows that, except for some possible intrinsic errors in determining the points, a value of $3.31 \times 10^{-9} \text{ M}$ is quite reasonable for the dissociation constant. As previously mentioned, the agreement between the calculated curve and the experimental points is indicative of a reversible reaction.

Assuming the stoichiometry:

$$2 \text{TTX} + \beta \rightleftharpoons \gamma$$

equation 2 becomes

$$\bar{K} = \frac{B_i[TTX]^2}{C}.$$  \hspace{1cm} (6)
and equation 3 becomes

$$C_1 = \frac{1}{B_0} \frac{1}{1 + \frac{[\text{TTX}]}{K}}.$$  \hspace{1cm} (7)

It is possible to calculate a dissociation constant from equation 6 by following the same procedure as before. The value obtained is $2.84 \times 10^{-17}$ M. Curve II in Fig. 5, calculated from equation 7 with this constant, does not fit the data, indicating that this stoichiometry is not correct.

For the stoichiometry

$$\gamma \frac{k_3}{k_1} \beta + \text{TTX}$$

to be possible, it would be necessary that the $\beta$-molecules be located near each other at the surface because, compared with the mobility of the free TTX molecules, they can be considered as fixed in space. The degree of proximity would have to be such that one molecule of TTX could react simultaneously with two of $\beta$. Then, the kinetic behavior of two $\beta$-molecules would be identical to that of a single reactive site and the equilibrium equation would not contain a squared term. The effects of the disappearance of two molecules of $\beta$ for each effective collision with one of TTX and the appearance of two molecules of $\beta$ for each dissociated molecule of $\gamma$ would be reflected in the value of the rate constants, which would be twice the value they have in the simpler case of a one to one stoichiometry. Since $\beta$ is being measured through a property without any knowledge of the magnitude of the factor relating $\beta$ and $\gamma$, this case might go completely undetected.

C. Rate Measurements

1. THE RATE CONSTANTS OF THE REVERSIBLE REACTION  The reversible interaction between TTX and membrane may be represented by the reaction represented by equation 2. The initial conditions for all the experiments are $C = 0$, $B = B_0$, while the concentration of TTX is maintained constant in time, except for the moments of transition during changes of the solution bathing the axon. For these initial conditions the rate of the reverse reaction can be neglected during the first stages of the process, when the amount of $\gamma$ formed is still small. Then

$$-\frac{dB}{dt} = k_3[\text{TTX}]B.$$  \hspace{1cm} (8)
which upon integration yields

$$\ln \frac{B}{B_0} = -k_1[TX]t$$  \hspace{1cm} (9)$$

Hence, a plot of $\log I/I_0$ against $t$ will be a curve practically linear near $t = 0$. The slope of this portion will be equal to $-k_1[TX]/2.303$.

**Figure 6.** Curves illustrating the washout of the experimental cell with a dye upon changing from one experimental solution to another. The effect of perfusion flow is illustrated. Ordinate, logarithm of the normalized change in the response of a photocell to transmitted light through the axon cell upon changing from a clear solution to a solution containing dye. $V$ is proportional to the transmitted light during the solution change, $V_0$ is proportional to the transmitted light in the clear solution, and $V_00$ is proportional to the transmitted light in the solution containing dye at equilibrium. Abscissa, time in seconds after changing perfusion from clear to a solution with dye.

When applying this method of calculating $k_1$ it is essential to make sure that the washout time of the cell does not introduce a sizable error. For that purpose the time course of the change in solutions was followed by switching, during normal perfusion flow, to a colored solution and measuring the light transmitted through the axon cell with an International Rectifier silicon solar cell of 1 cm² in area, placed directly above the portion of the axon cell where the membrane currents are measured. The photocell generates a voltage which is a linear function of the intensity of the incident light, within the range of
intensities used. This voltage was fed directly into the oscilloscope and followed in time with a slow sweep. Photographic records were obtained in the usual manner.

Fig. 6 shows several of the curves obtained at different flow rates covering a range wider than the one used in the experiments. The ordinates represent on a logarithmic scale the fraction of the original solution still remaining in the cell at a given time. Since most of the curves deviate from exponential behavior after some time, a useful index of the speed of change of solutions is the time at which this is 90% complete. For the slowest flow rate tested this was 15 sec.

![Figure 7. Curves illustrating the development in time of TTX blockade, as a function of time and [TTX]. $I/I_0$ is proportional to the fraction of membrane sites remaining unblocked by TTX. Time zero is the onset of exposure to the [TTX] given at the right of each curve. Axons S-51, S-47, and S-53 in 5 nM, 10 nM, and 15 nM TTX, respectively.](image)

The first current measurements were never made less than 1 min after switching to another solution, and these were well within the linear portion of the plot suggested by equation 9. It can be said with confidence, then, that the effect of the lag in the concentration of TTX on the values obtained for $k_1$ is insignificant.

In Fig. 7, a group of curves plotting semilogarithmically $I/I_0$ vs. $t$ for different concentrations of TTX is shown and all the values of $k_1$ [TTX] obtained from the slopes of the linear portions of this type of curve appear in Table III.

For the second phase of the experiments, when the concentration of TTX is made zero in order to induce recovery, the reaction becomes a simple decomposition of $\gamma$ into $\beta$ and TTX with rate constant $k_2$. The initial conditions are: $C = C_1$ and $B = B_1$ and the final conditions: $C = C_2$ and $B = B_2$. Then,

$$\frac{dB}{dt} = (k_2)C.$$
Taking

\[ C = (B_2 - B) \]  \hspace{1cm} (10)

\[ \frac{dB}{(B_2 - B)} = (k_2) \, dt \]  \hspace{1cm} (11)

**Table III**

<table>
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<tr>
<th>Axon</th>
<th>[TX]</th>
<th>T</th>
<th>1/T</th>
<th>k(TX)</th>
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<th>k2</th>
</tr>
</thead>
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<tr>
<td></td>
<td>min</td>
<td>°C</td>
<td>m⁻¹</td>
<td>min⁻¹</td>
<td>min⁻¹</td>
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* Only equilibrium values at t2 were obtained. Kinetic data for k1 determination were not sufficient.
‡ Kinetic data for k1 too scattered to be useful. Equilibrium data in Table II.
§ Axon was killed in clamp during recovery, and no k1 data and no recovery equilibrium data were obtained. Axon does not appear in Tables I and II.

which becomes, upon integration between B₁ and B,

\[ \ln \frac{B_2 - B}{B_2 - B_1} = -k_3(t - t_1). \]  \hspace{1cm} (12)

Hence, a plot of log \((I_2 - I)/(I_3 - I_1)\) vs. \((t - t_1)\) will be a straight line with slope equal to \(-k_3/2.303\). The k3 values obtained in this way also will not be affected by the lag in changing solutions. Fig. 8 illustrates the curves that result from plotting \((I_2 - I)/(I_3 - I_1)\) vs. t and Table III contains the values of k3 obtained in all the experiments in which the axons did not show evidence of spontaneous rapidly deteriorating currents up to the completion of the exper-
iment. The values of $k_2$ should be independent of the concentration of TTX and therefore the variations observed are due to differences in temperature and to scatter.

The values for $k_1$ and $k_2$ determined experimentally are fairly constant, indicating that any important error in them should be relatively constant. The work of Camougis, Takman, and Tasse (1967), showing that the three structures of TTX that coexist in solution do not have the same blocking ability, points out one of these errors. It will affect not only $k_1$ and, consequently, the dissociation constant derived kinetically, but also the dissociation constant obtained from equilibirium measurements. The equilibrium constant between the two cationic forms, and which species is the reactive one, are not known. Hence, the magnitude of this error cannot be estimated. Since the effective

\begin{figure}
\centering
\includegraphics[width=0.5\textwidth]{figure8}
\caption{Curves illustrating the time course of recovery in ASW from TTX blockade. The ordinate is proportional to the fraction of membrane sites remaining blocked by TTX. Time zero is the time at which TTX perfusion was changed to perfusion with ASW. Axons S-53 and S-55 after 15 nM and 10 nM TTX, respectively. See text.}
\end{figure}

concentration of TTX is lower than its molar concentration by a factor $q$, it is seen that

$$k'_1 q = k_1$$

where $k_1$ is the experimentally determined constant and $k'_1$ is the actual value of the rate constant. Then, the actual value of the dissociation constant,

$$K' = \frac{k_2}{k'_1} = q \frac{k_2}{k_1}.$$ 

Also,

$$K = \frac{B_1}{C_1} [TTX], \text{ and so, } K' = q \left(\frac{B_1}{C_1}\right)[TTX]$$
which shows that both values of the dissociation constant are equally affected by this factor.

2. THE ENERGIES OF ACTIVATION OF THE FORWARD AND REVERSE REACTIONS  Kinetic data over a range of temperatures usually follow an empirical equation proposed by Arrhenius which is, in logarithmic form,

\[ \log k = \frac{-E_a}{2.303R} + \log A \]  \hspace{1cm} (13)

where \( E_a \) is the Arrhenius activation energy; \( T \) the absolute temperature; and \( A \) the Arrhenius factor, which has the dimensions of frequency for first-order reactions. From the slope of this curve it is possible to calculate \( E_a \).

The information about the dependence of the rate constants on temperature gathered in this investigation, permits the calculation of the energies of activation of the reactions in both directions (Daniels and Alberti, 1961). In Fig. 9, values of \( k_1 \) and \( k_2 \) are plotted, semilogarithmically, against the reciprocal of the absolute temperature. Fig. 9 contains the information necessary to perform the calculation given by equation 13 for the forward and reverse reaction. The lines in Fig. 9 are best fits to the data and determine the following equations:

\[ \log k_1 = -1129 \frac{1}{T} + 11.34 \]

\[ \log k_2 = -2593 \frac{1}{T} + 8.32. \]

The absolute reaction rate theory of Eyring (Glasstone, Laidler, and Eyring, 1941) predicts the following relationship:

\[ \log k = \log \frac{tRT}{N \hbar} + \frac{\Delta S^f}{2.303R} - \frac{\Delta H^f}{2.303R} \cdot \frac{1}{T} \]  \hspace{1cm} (14)

where \( t \) is the transmission coefficient, the fraction of the times that an activated molecule proceeds to products instead of reverting to reactants, \( \Delta S^f \) is the entropy of activation, \( \Delta H^f \) is the enthalpy of activation, \( N \) is Avogadro’s number, and \( \hbar \), Planck’s constant.

When equations 13 and 14 are compared, it can be shown that

\[ E_a = \Delta H^f + RT \]  \hspace{1cm} (15)

and, taking \( t \) equal to one, as is usually done,

\[ A = e^{\frac{RT}{N \hbar}} \exp \left( \frac{\Delta S^f}{R} \right) \]
which makes it possible to calculate $\Delta H^f$ and $\Delta S^f$ with the information obtained from a plot of $\log k$ vs. $1/T$.

When this information is obtained for the two rate constants, $k_1$ and $k_2$, of a reversible reaction, we have, at a given temperature, the free energy of activation:

$$\Delta F^o = \Delta H^o - T(\Delta S^o),$$  \hspace{1cm} (16)

$$\Delta F^o = \Delta F^1_2 - \Delta F^1_1$$ \hspace{1cm} (17)

$$\Delta H^o = \Delta H^2_2 - \Delta H^2_1$$ \hspace{1cm} (18)

$$\Delta S^o = \Delta S^2_2 - \Delta S^2_1$$ \hspace{1cm} (19)
where the standard changes in the thermodynamic quantities are those of the reverse reaction.

The following equations are obtained from the slopes of the lines in Fig. 9 and equation 13:

\[
\frac{-E_{a_1}}{2.303R} = -1129 \quad \text{and} \quad \frac{-E_{a_2}}{2.303R} = -2593.
\]

Then

\[
E_{a_1} = 5168 \text{ cal mole}^{-1}
\]
\[
E_{a_2} = 11,865 \text{ cal mole}^{-1}
\]

and using equation 15:

\[
\Delta H_1^\circ = E_{a_1} - RT = 4611 \text{ cal mole}^{-1}
\]
\[
\Delta H_2^\circ = E_{a_2} - RT = 11,308 \text{ cal mole}^{-1}
\]

at 7°C. Thus,

\[
\Delta H^\circ = E_{a_2} - E_{a_1} = 6697 \text{ cal mole}^{-1}.
\]

From the calculations using equations 14 and 16 through 19, at 7°C:

\[
\Delta S_1^\circ = -9.2 \text{ cal deg}^{-1} \text{ mole}^{-1},
\]
\[
\Delta S_2^\circ = -22.1 \text{ cal deg}^{-1} \text{ mole}^{-1},
\]

and

\[
\Delta S^\circ = \Delta S_2^\circ - \Delta S_1^\circ = -12.9 \text{ cal deg}^{-1} \text{ mole}^{-1}.
\]

Thus,

\[
\Delta F_1^\circ = \Delta H_1^\circ - T(\Delta S_1^\circ) = 7179 \text{ cal mole}^{-1},
\]
\[
\Delta F_2^\circ = \Delta H_2^\circ - T(\Delta S_2^\circ) = 17,499 \text{ cal mole}^{-1},
\]

and

\[
\Delta F^\circ = \Delta F_2^\circ - \Delta F_1^\circ = 10,320 \text{ cal mole}^{-1}.
\]

3. COMPARISON OF DISSOCIATION CONSTANTS DERIVED FROM KINETIC STUDIES WITH THOSE FROM EQUILIBRIUM DATA

From Fig. 9, the values of \( k_1 \) and \( k_2 \) at 7°C may be readily obtained from the best fit lines. \( k_1 = 0.202 \times 10^8 \text{ M}^{-1} \)
min⁻¹, and \( k_2 = 0.116 \text{ min}^{-1} \). Since the dissociation constant,

\[
K = \frac{k_2}{k_1}
\]

the value for \( K \) from the kinetic data is \( 5.74 \times 10^{-9} \text{ M} \). As the value of \( K \) at 7°C obtained from the equilibrium data is \( 3.31 \times 10^{-9} \text{ M} \), these two values are in reasonable agreement.

**DISCUSSION**

In the course of this work, axons were exposed to different concentrations of TTX and, after the current had reached a steady value, the toxin was removed and recovery of the current followed until another steady value was attained. This behavior is interpreted as mainly the consequence of a reversible process.

One result of this investigation has been the resolution of the interaction of TTX with the axon membrane into two separate processes of which the reversible one has been quantitatively characterized. The other process, whose effect is not reversed by removing TTX, cannot be measured separately, thus presenting a serious difficulty in elucidating its possible complex mechanism.

In interpreting the experimental results, several important assumptions have been made. At the root of the problem lies the question of whether a linear dependence exists between the extent of the reaction and the reduction of the maximum peak sodium current. This question can be discussed in two parts: the relationships between the extent of reaction and effect, and between the effect and maximum peak sodium current. Ariens et al. (1964) state that, in its most general way, the interaction between a drug and a reactive site in a biological structure will elicit a stimulus that is transmitted to the effector site. The magnitude of the stimulus is thought to be proportional to the extent of the reaction, but the relationship between stimulus and effect may have any form. An inquiry about this form cannot be attempted until a great deal is known about the detailed chemistry of the phenomenon. In the present case, the effect consists of a hindrance of the mechanism by which the sodium conductance of the membrane is increased upon depolarization. This hindrance conceivably may occur in many ways, from a complex process consisting of a reaction of TTX with molecules distant from the effector sites, having a stimulus transmission that decays with distance and including the possibility of a superposition of fields of influence of different reactive sites, to a simple plugging action on pore apertures. If the only important manifestation of the effect is a reduction of \( g_{Na} \), as the results of Takata et al. (1966) and Hille (1968) indicate, it would be tempting to favor an almost direct action of TTX on the gate. Based on a one to one TTX-site reaction, Moore, Narahashi, and Shaw (1967) have estimated that there are 13 sites per \( \mu^2 \) of axon surface in lobster nerves. Based on the following: (a) the similarity of the action of TTX and
saxitoxin, \( h \) the fact that both have guanidinium groups in the toxin molecules, and \( c \) the reported ability of guanidinium ions to support excitability in the absence of sodium (Tasaki, Singer, and Watanabe, 1965), Kao and Nishiyama (1965) have suggested that the guanidinium moiety enters the sodium pathway and is held there by reactions between other parts of the molecule and the cell membrane surface. This type of interaction would completely block the affected gates, appearing as a reduction of \( g_{Na} \) with no effect on the kinetics of \( h \) or \( m \), and is compatible with a linear relationship between the extent of the reaction and its effect. Nevertheless, since this matter is still in a speculative stage, this linearity remains an assumption.

The strict validity of the maximum peak sodium current as a linear measure of the action of TTX on the sodium channel depends on the exclusiveness of the TTX effect on \( g_{Na} \) and on the constancy of the voltage at which the maximum peak sodium current is obtained. Within the Hodgkin and Huxley (1952) equations, of those parameters that TTX could be expected to alter \((g_{Na}, m, h)\), only a decrease of \( g_{Na} \) would result in a linear change of \( I_{Na} \). The results of Takata et al. (1966) suggest that the first condition is met. With respect to the second, the present work has shown a slight shift of the maximum peak inward current along the voltage axis (Fig. 3). Nevertheless, the resultant relationship does not deviate from linearity to any significant extent because, as the peak currents decrease, the I/V curve flattens and the current values for slightly different voltages become practically the same. Although the relationship between extent of the reaction and intensity of the effect is not known, it is justifiable to assume linearity and the available evidence supports the use of maximum peak \( I_{Na} \) as a measure of this effect. As a result, it is reasonable to consider that the extent of the reaction is being assessed by the electrical measurements used.

The incompleteness of recovery of the sodium current, previously discussed, has been reported by other authors (Narahashi et al., 1964; Takata et al., 1966) and found to be diminished by high Ca\(^{++}\) concentrations. These authors, working with high concentrations of TTX on lobster axons, found that recovery was poor after strong depression of the sodium current, while a large degree of reversibility was observed for applications of very short duration (2 or 3 min) that produced about 50% reduction. In the experiments reported here, the treatment with lower concentrations of TTX (1 to 5 nM) for longer periods of time (not less than 20 min) always allowed recovery to the same level as that obtained after higher [TTX] was applied. These recoveries were obtained even after 80% reduction of the currents. The disagreement between these findings may be due to a species difference, or to the use of the sucrose gap method with lobster axons which leads to early deterioration of the axon segment in the gap (Julian, Moore, and Goldman, 1962), but most probably reflects the complexity of the whole interaction.
Correction for leakage current was not made. The error thus introduced is not considered important as \( I_L \) (leakage current) should not be influenced by TTX, and at the potential values used to measure \( I_{\text{meas}} \), the value of \( I_L \) should be small. Furthermore, values used in calculations were ratios, and \( I_L \) values should cancel. The principal source of error in the values of \( k_1 \) is that the slopes from which they are calculated are based on only a few experimental points. The error from secondary processes or from deterioration of the preparation should be small for \( k_1 \). For \( k_2 \), on the other hand, secondary processes and deterioration are the weakest points. However, each of the \( k_2 \) values was based on many experimental points, and the axon deterioration was corrected for using the decline in \( I_L \) as an index. While these possible errors should be borne in mind, none is considered as seriously influencing the values given in this paper.

Two values of the dissociation constant \( K \) of the drug-receptor complex have been obtained from two sets of independent measurements. At 7°C, the ratio of the rate constants is \( 5.74 \times 10^{-9} \) M, while the equilibrium measurements and the dose-response curve suggest a value of \( 3.31 \times 10^{-9} \) M. As the equilibrium data are not affected at all by rates of penetration through external layers, and as the TTX concentration close to the membrane should represent, at equilibrium, the [TTX] in the perfusion solution, one may consider that the fair agreement between these \( K \) values indicates that TTX is able to get to the membrane and be washed off with only minimal interference from external layers. In frog node, where there is no real external barrier (Hille, 1967) the dissociation constant for STX reaction with the sodium channel (Hille, 1968) is \( 1.2 \times 10^{-9} \) M, which is of the same order of magnitude as the TTX value of \( 3.31 \times 10^{-9} \) M.

The value of \( k_1 \) probably represents the onset of both the reversible and the irreversible processes as no simple way of subtracting the \( A \) process from the \( C \) process could be achieved for the onset of the reactions. The lack of knowledge about the concentration of the active form of TTX adds a systematic error to the equilibrium value of \( K \), as shown in the results. Woodward (1964) reported a value of 9 for the equilibrium constant between the two cationic forms of Gougoutas' hydrochloride, a TTX derivative. If this value is applied to TTX, together with a pK\(_a\) of 8.8, at pH 7.4 the concentration of the lactone form is about 9% of the total TTX concentration and that of the cationic hemilactal form is approximately 80% (Camougis et al., 1967). This calculation suggests that the values reported here for \( K \) could be in error by a large factor. Even in the worst cases, though, the standard thermodynamic quantities for activation would be affected to a much lesser extent.

In view of the previous analysis of assumptions and errors and of the uncertainty regarding the effective concentration of TTX, it is clear that the results of this work can be regarded only as first approximations. In addition,
the interpretation of thermodynamic data in terms of actual physicochemical processes taking place at the surface of a membrane is seriously limited by our ignorance of the structure and distribution of its reactive groups and of the extent to which a change in one molecule may induce alterations in neighboring structures. Nevertheless, several interesting conclusions may be derived from such data.

Since a molecule of TTX contains an ionized guanidinium group in one end, its interaction with the membrane could be conceived as purely electrostatic between this positive ion and negative fixed charges at the external end of the sodium channels. The free energy of dissociation of such a complex is of the order of 4,000 cal mole\(^{-1}\) (Frost and Pearson, 1961), while the \(\Delta F^\circ\) calculated is about 10,000 cal mole\(^{-1}\), suggesting a more stable binding. This agrees with the reported lack of blocking activity of some TTX derivatives that have the guanidinium groups in their molecules (Narahashi, Moore, and Poston, 1966, 1967). Further support for this view comes from the value of the energy of activation and the entropy of activation for the formation of the complex \((E_a\) and \(\Delta S^\circ\), respectively). The first has a value of about 5,000 cal mole\(^{-1}\) while that for the formation of a salt bond between two oppositely charged ions would be much smaller. When covalent bonds or electron transfers are not involved, the entropy of activation should have a positive value because the most important effect in connection with the change in entropy is the release of the water of solvation from its relatively rigid structure as the ions approach (Frost and Pearson, 1961). The value obtained for \(\Delta S^\circ\) is \(-9.2\) cal deg\(^{-1}\) mole\(^{-1}\), which is typical of more complex reactions in which the activated complex has less degrees of freedom than the molecules of the reactants. These results do not necessarily imply the nonparticipation in the reaction of the positive guanidinium group, but they suggest that an energetically and statistically more important process takes place possibly in conjunction with the ion-ion interaction. This combination of the two types of processes is in agreement with the value of \(-12.93\) cal deg\(^{-1}\) mole\(^{-1}\) for the standard entropy change of the reaction, \(\Delta S^\circ\). A relatively large positive value would be expected for the dissociation of a molecule from the membrane brought about without separation of charges. The small negative value found is probably the result of this effect plus the loss of entropy associated with the solvation of two oppositely charged ions that break apart. Additional evidence, necessary to establish without doubt the role of the guanidinium group, should be obtainable from studies on the effect of changes in ionic strength on the kinetics of the reaction.

Camougis et al. (1967) proposed two other models for the reaction in which the group occupying position 10 in the molecule of TTX plays very important roles, either by hydrogen bonding to the membrane or by forming an intermolecular hemilactal with the receptor. In order to account for the \(\Delta H^\circ\) of
about 7,000 cal mole\(^{-1}\) found, several hydrogen bonds would be necessary in addition to the ion-ion interaction. Of the other OH groups of the molecule of TTX, the only one known to be important for biological action is the one in position 4. Yet, its significance seems not to be strictly associated with hydrogen bonding, since its substitution with a hydrogen atom decreases the biological activity only 10 times while replacing it with an NH\(_2\) group produces a 100-fold reduction (Tsuda, Ikuma, Kawamura, Tachikawa, Sakai, Tamura, and Amakasu, 1964).

In general, the presently available evidence indicates that there are at least three parts of the TTX molecule of great importance for biological action: the guanidinium moiety, suggested by its presence in the similar poison, saxitoxin, and by the ability of guanidinium ions to enter the sodium channel, is the most likely part of the molecule to effect a direct block of the sodium currents by reducing \(g_{\text{Na}}\) only; the OH in position 4 (Narahashi et al., 1967; Deguchi, 1967), since its substitution results in decreased activity; and the group in position 10, either OH or carbonyl, as indicated by the effect of pH on the potency of TTX (Hille, 1968). It is unjustifiable to assign an essential role to the oxygen bridge between C\(_{10}\) and C\(_{4}\) on the basis of the inactivity of tetrodonic acid as has been done (Kao, 1966), because this compound shares with the similarly inactive anhydrotetrodotoxin the oxygen bridge between C\(_{4}\) and C\(_{6}\) and hence the absence of the OH group in position 4.

The results of this investigation are compatible with the role suggested for the guanidinium group and indicate the existence of at least one additional binding site. This supplementary link provides the stability that the TTX-membrane complex has. Its nature has not been established, but it is clear that a single H bond will not account for the energy changes involved.

The authors wish to thank Drs. Y. Palti and D. G. Davis for their helpful suggestions and criticisms and Mrs. Nancy Laue for her technical assistance.

This work was supported by United States Public Health Service research grant No. NB 04601 and by National Science Foundation research grant No. GB 15588.

Received for publication 5 August 1969.

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