Pharmacological Modifications of the Sodium Channels of Frog Nerve

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ABSTRACT Voltage clamp measurements on myelinated nerve fibers show that tetrodotoxin, saxitoxin, and DDT specifically affect the sodium channels of the membrane. Tetrodotoxin and saxitoxin render the sodium channels impermeable to Na ions and to Li ions and probably prevent the opening of individual sodium channels when one toxin molecule binds to a channel. The apparent dissociation constant of the inhibitory complex is about 1 nM for the cationic forms of both toxins. The zwitter ionic forms are much less potent. On the other hand, DDT causes a fraction of the sodium channels that open during a depolarization to remain open for a longer time than is normal. The effect cannot be described as a specific change in sodium inactivation or as a specific change in sodium activation, for both processes continue to govern the opening of the sodium channels and neither process is able to close the channels. The effects of DDT are very similar to those of veratrine.

INTRODUCTION

Our understanding of the mechanisms of nervous conduction is based on the lucid description of the ionic permeability changes in the giant axon of the squid given by Hodgkin and Huxley in 1952. Although new techniques have made it possible to refine the observations and to extend them to other axons, few advances concerning the structural or molecular basis of the permeability changes have been made. One structural problem that seems to be answerable at this time is whether the ionic fluxes that underlie the sodium currents, potassium currents, and leakage currents defined in the Hodgkin-Huxley model pass through the same parts of the membrane or through independent specializations: the sodium channels, the potassium channels, and the leakage channels.

In the last few years, pharmacological studies with axons have shown that certain drugs can eliminate one of the components of the ionic current without affecting the other two. The poison of the puffer fish and salamander, tetrodotoxin (TTX), selectively abolishes the sodium currents of the squid giant axon (Nakamura, Nakajima, and Grundfest, 1965 *a*), the lobster giant axon (Narahashi, Moore, and Scott, 1964; Takata et al., 1966), the electric

eel electroplaque (Nakamura et al., 1965 *b*), and the node of Ranvier of frog myelinated nerve (Hille, 1966). Similarly, the paralytic shellfish poison, saxitoxin (STX), eliminates the sodium current of the eel electroplaques (Nakamura et al., 1965 *b*). On the other hand, the tetraethylammonium ion and some related ions selectively abolish the potassium currents of myelinated nerves (Hille, 1967 *a*; Koppenhöfer, 1967). No agents are yet known to reduce the leakage current. These observations form the bulk of the new evidence for independence of the sodium, the potassium, and the leakage channels. These and other arguments have been reviewed by Grundfest (1966) and by Hille (1967 *b*).

This paper further documents some statements made about TTX in an earlier paper (Hille, 1966) and presents some new pharmacological studies with the shellfish poison STX and with the insecticide DDT (1,1,1-trichloro-2,2-*bis* (*p*-chlorophenyl)-ethane). Each of these agents specifically affects the sodium currents, TTX and STX by reducing them and DDT by prolonging them. The results are described with the assumption that the hypothesis of the heterogeneous membrane, the membrane with separate and specific ionic channels, is the correct choice. Indeed, all the observations presented are arguments for this hypothesis.

MATERIALS AND METHODS

Single nodes of Ranvier of large fibers dissected from the sciatic nerve of *Rana pipiens* were studied at low temperatures by the voltage clamp technique of Dodge and Frankenhaeuser (1958). The procedures are discussed in Hille (1967 *a*). Basically the data are recorded with the aid of an on-line digital computer and analyzed in terms of the equations of Hodgkin and Huxley (1952). Except where otherwise noted, the definitions of terms are those of Hodgkin and Huxley (1952).

At almost all times the nerve was clamped at a holding potential between −70 and −90 mv (all potentials are on the absolute or "E" scale of inside potential minus outside). A conditioning *prepulse* lasting 40 msec was followed immediately by the depolarizing *test pulse*. Except where otherwise noted, the prepulse was a 45 mv hyperpolarization from the holding potential.

The Ringer solution had the following composition (mM): NaCl 110, KCl 2.5, $CaCl_2$ 2.0, Tris(hydroxymethyl)aminomethane buffer (pH 7.3) 5.0. Solutions with different values of pH contained instead of the Tris buffer a mixture of 7 mM glycylglycine (Mann Research Labs Inc., N.Y.) and 7 mM piperazine dihydrochloride (K and K Laboratories Inc., Plainview, N. Y.) titrated with NaOH. Other solutions were made by replacing some of the NaCl by LiCl, tetramethylammonium bromide (Eastman), tetraethylammonium chloride (Eastman), tetrodotoxin (Sankyo Company Ltd., Tokyo), and saxitoxin (purified from the dinoflagellate *Gonyaulax catanella*, gift of Dr. E. J. Schantz, Fort Detrick, Maryland). The DDT-containing solutions (*p*,*p*′-DDT 98% pure, gift of Dr. D Woolley, University of California, Davis) were made by adding a few drops of a concentrated solution of the insecticide in 95% ethanol to the Ringer solution. The final concentration of ethanol was less than 0.5%.

As DDT is not soluble in cold water at the concentrations used, the final solution was an opalescent suspension rather than a true solution. In the experiments with DDT, the drug was applied just once for about a minute and then washed away. The effect of the DDT did not diminish over several hours, even through many washings with other solutions.

The "Standard" Node

There are several references in the text to a theoretical "standard" node. This is a complete set of empirical equations for the rate constants (α's and β's) pertaining to the parameters called m, n, and h in the theory of Hodgkin and Huxley, but the equations in this case are derived from voltage clamp experiments with a node of Ran-

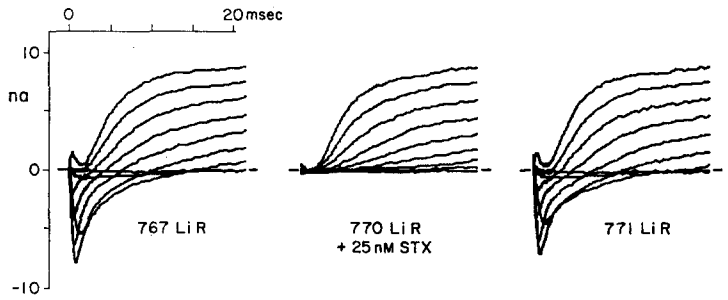

FIGURE 1. Lithium currents and STX. The time courses, drawn by computer, of the voltage clamp currents minus leakage currents in sodium-free lithium Ringer's solution, before, during, and after treatment with 25 nM STX. The curves are recorded at 15 mv intervals from about −60 mv to +60 mv but with a small drift of the amplifiers between the three measurements. T = 6.5°C.

vier. In other words, this is a mathematical model for the responses of a node, comparable to the Hodgkin-Huxley model for the responses of the squid giant axon. The original experimental data for this standard node are taken from "node 7" of Dodge (T = 22°C), which has already been described extensively (Dodge, 1961, 1963). The particular approximating functions used are given in detail in Hille (1967 *b*). They differ only slightly from Dodge's. The standard node is used as a norm for comparison with actual measurements in this paper and in the following paper (Hille, 1968).

RESULTS

TTX and STX Block Sodium Channels Specifically

TTX and STX render the sodium channels impermeable to the ions that normally can pass through them; e.g., Na, Li, NH_4, guanidinium, etc. I have studied Na and Li currents. Fig. 1 shows the inhibition of Li currents by STX. Record 767 is a family of voltage clamp currents recorded in a Na-free Li Ringer solution. The early transient (lasting a few milliseconds) inward (negative) currents are Li currents which closely resemble the transient in-

ward Na currents in the standard Ringer solution except that the Li currents in this case are 40% smaller. In the next record the Li currents have been abolished by 25 nM STX, and in the last record they have recovered to full amplitude upon removal of the STX. Experiments like this one but with TTX abolishing Na and Li currents of a node of Ranvier have been reported (Hille, 1966, 1967 *b*). TTX and STX also eliminate the extra component of sodium conductance that is induced by DDT or by veratrine (see below). The depression of the sodium currents by TTX and by STX is complete in 5 sec between 2 and 22°C.

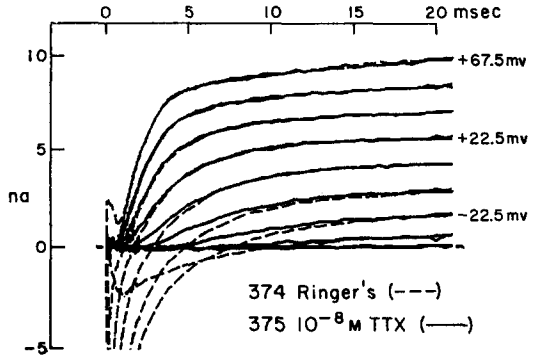

FIGURE 2. Potassium currents in TTX. The superimposed voltage clamp currents minus leakage currents of a node before (dashed curves) and during (solid curves) treatment with 10 nM TTX. The maximum inward sodium current in the Ringer solution is about −17 na, but the records are truncated at −5 na in this figure. The maximum depolarization is to +67.5 mv. Further experiments with this node are shown in Fig. 1 of Hille (1967 *a*). T = 17°C.

TTX and STX do not affect leakage channels. If the normal leakage conductance is called 100, the conductance in 15 measurements on 9 different nodes in TTX was 99.5 ± 5.5 (mean ± SD) and in 6 measurements on 4 different nodes in STX (5 nM or more) 103.2 ± 6.7.

Potassium channels are not affected by STX and TTX. This can be seen qualitatively by inspecting the late time courses of the currents in the three records of Fig. 1 and quantitatively by superimposing records from before and during drug treatment. In Fig. 2 the currents in 10 nM TTX have been drawn in solid lines over the currents in the standard Ringer solution (dashed lines). The records of the inward sodium currents in the Ringer solution have been truncated at −5 na to permit greater enlargement of the rest of the figure. The point to be made is that the dashed lines and the solid lines merge completely after the first few milliseconds demonstrating the insensitivity of potassium currents to the presence of TTX. The same result is obtained with STX. This result can be obtained only if great care is taken to eliminate tem-

perature differences in the solutions and to minimize drift in the amplifiers. In the experiments of Fig. 1 in this paper and of Fig. 1 in Hille (1966) a voltage drift was recorded (about 3 mv in each case), and the measured curves cannot be superimposed satisfactorily.

One Molecule May Block One Channel

At low concentrations TTX and STX reduce the maximum sodium conductance, $\bar{g}_{Na}$, without changing the time course of the diminished sodium currents that remain, or, in terms of the channel hypothesis, the drugs block

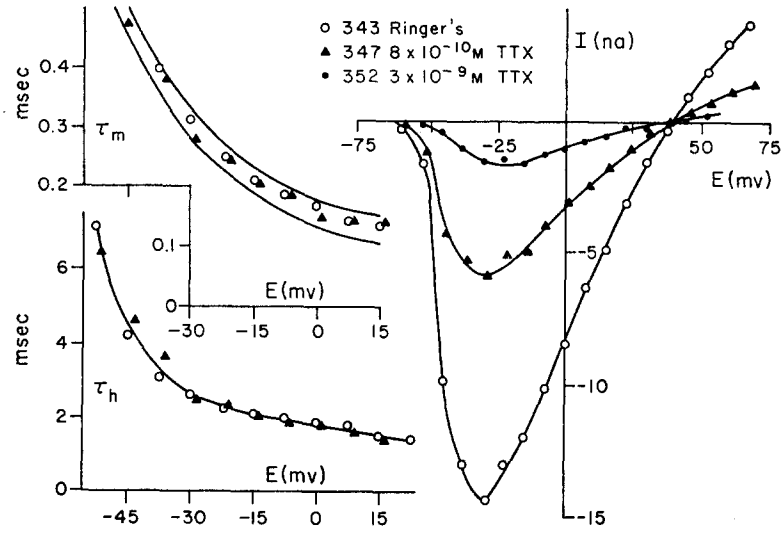

FIGURE 3. Analysis of sodium currents in TTX. The time constants, τ_m and τ_h (left), and the peak current-voltage diagram (right) of the sodium currents of a node before and during treatment with TTX. The two lines drawn on the graph of τ_m indicate the band of uncertainty in the measurement. T = 2.5°C.

some of the sodium channels but in no way affect the opening and closing of those that remain unblocked. Fig. 3 (left) shows the voltage dependence of the time constants, τ_m and τ_h, associated with the rising and falling phases of the sodium current, both in a control solution and in a low concentration of TTX. Within the limits of experimental error there is no change in the time constants attributable to the drug. In the same experiment (Fig. 3, right) the amplitudes of the peak sodium currents are reduced by the same fraction at every voltage, whether the current is inward (negative) or outward. There is a reduction of $\bar{g}_{Na}$ by 60% in record 347 and by 88% in record 352.

Additional information on the nature of the binding of TTX or STX to the membrane may be obtained from a dose-response curve constructed by plotting $\bar{g}_{Na}$ against the concentration of the drug (Fig. 4). In this graph the

open circles are measurements with STX added to the Ringer solution and the filled circles are measurements with 6 mM TEA in addition to the STX. The line is a simple "rectangular hyperbola" calculated by assuming a dissociation constant of 1.2 nM for a hypothetical inhibitory complex. All the points fall near the line. One of the assumptions used in calculating the line is that a small fraction of the total sodium conductance is eliminated by the formation of a complex between *one* STX molecule and a receptor substance. The simplest interpretation of the result is that individual sodium channels are blocked by single molecules of STX that complex with part of the channel. The graph also shows that the elimination of potassium currents by TEA has no influence on the actions of STX; in other words, TEA and STX do not com-

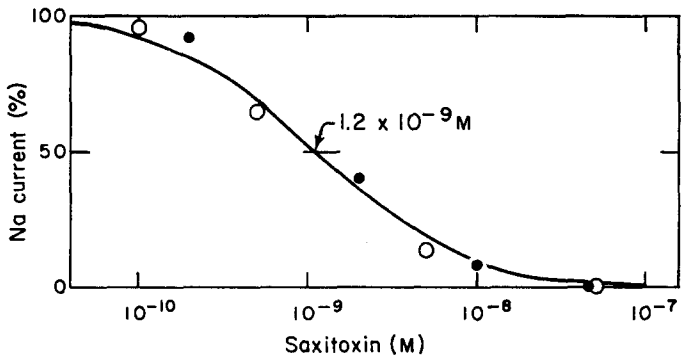

FIGURE 4. Dosage-response to STX. The maximum sodium conductance at various concentrations of STX relative to that in Ringer's solution. The filled circles are from experiments in which the potassium currents have been eliminated by 5 mM TEA. All the measurements are from two nodes. The solid line is the dose-response relation of a system in which one STX molecule binds reversibly to its receptor to produce a fraction of the inhibitory effect. T = 4°C.

pete for the same receptor. Dose-response experiments with TTX have not been as satisfactory as those with STX for reasons given in the next paragraph, but it has been shown that TTX and TEA do not compete for the same receptor by demonstrating that TTX has no influence on the actions of TEA (Hille, 1967 *a*).

Thus far I have suggested that TTX and STX have indistinguishable pharmacological properties, but this is not entirely true. The effects of STX can always be reversed with 30 sec of washing, whereas frequently the effects of TTX are only partly reversed with several minutes of washing. Indeed a treatment with STX can *increase* $\bar{g}_{Na}$ slightly: the recovery after exposures to low concentrations of STX may be to a value of $\bar{g}_{Na}$ 10 or 15% greater than the preceding control. Quick reversibility permits repeated tests with the same nerve, hence experiments with STX are easier. Another factor has compli-

cated experiments with TTX. Different batches from the same supplier have different potencies, even when the samples are kept in a freezer from the time of arrival. One batch (used in experiments 1–500) reduced $\bar{g}_{Na}$ by 50% at about 0.6 nM and another (used subsequently), at about 2 nM. A third batch was unfortunately left at room temperature for 3 yr (dry and in the original ampoule), and 15 nM was required for the same effect. It seems likely that TTX and STX have similar potencies, although further tests should be made with samples of known purity.

The Toxins Act in Their Cationic Forms

The state of ionization of the drug molecule influences its binding to the nerve. A positively charged guanidinium group (pK_a between 11 and 12) makes the TTX and STX molecules cationic at neutral pH. Ionization of the hemi-

TABLE I

THE EFFECT OF pH ON THE ANESTHETIC POTENCY OF TTX AND OF STX

Record No.		664—670			704—707			1020—1041	
Toxin, nM		8.3			12.5		0.4	1.2	1.9
$\bar{g}_{Na}$, nmho		620			1250		540	420	460
pH		$\bar{g}_{Na}$	pH		$\bar{g}_{Na}$	pH	$\bar{g}_{Na}$	$\bar{g}_{Na}$	$\bar{g}_{Na}$
		%			%		%	%	%
8.6	R:	100	7.3	R:	100	7.3 R:	100	100	100
8.6	TTX:	51	10.1	TTX:	85	9.7 R:	96	99	89
5.9	R:	89	7.3	TTX:	16	9.7 STX:	96	99	80
5.9	TTX:	22				7.5 STX:	94	66	25

lactal hydroxyl group (pK_a about 8.8) of TTX converts the cation to the zwitter ion at higher values of pH (Goto et al., 1965; Woodward, 1964). An unidentified group on the STX molecule ionizes in the same range of pH as well (pK_a = 8.1; Schantz, 1960). Using the amplitude of the compound action potential of a desheathed frog nerve as a criterion, Camougis, Takman, and Tasse (1967) have shown that a solution of TTX is approximately twice as potent at pH 7 as at pH 9. They conclude that the cationic form of TTX is more important than the zwitter ion for anesthetic action. I have studied the influence of pH on the depression of $\bar{g}_{Na}$ by TTX and by STX, and I also find that both toxins are relatively ineffective at high values of pH.

The results of experiments on three nodes are given in Table I in terms of the relative values of $\bar{g}_{Na}$ in various test and control solutions. The first experiment (records 664–670) shows that TTX depresses $\bar{g}_{Na}$ more at pH 5.9 than at pH 8.6. The decrease in potency at the high pH is near the factor of two found by Camougis et al. Even in the control Ringer solution, the value

of $\bar{g}_{Na}$ is lower at pH 5.9 than at pH 8.6. This change is attributable to a specific depression of $\bar{g}_{Na}$ in acidic solution that is examined in detail in the following paper (Hille, 1968).

In the next experiment in Table I (records 704–707), the more basic solution has a pH of 10.1, 1.3 pH units above the pK_a of the hydroxyl group of TTX. The concentration is also slightly higher than in the previous experiment. The table shows that 12.5 nM TTX is much less potent at pH 10.1 than 8.3 nM TTX is at pH 8.6, although 12.5 nM is more effective at pH 7.3 than 8.3 nM is at 5.9. The experiment suggests again that the cationic form of TTX is active and that the zwitter ion has much less, if any, activity.

The last experiment in Table I (records 1020–1041) was done in a CO_2-free atmosphere with only a few seconds' delay between the time of mixing of the STX solutions and the time of application. In addition, the solutions that were tested at a high pH were reneutralized and tested again at a low pH. These changes in the procedure were introduced to guard against two possible sources of error in the preceding experiments. The first is that the buffering may be insufficient to prevent a significant lowering of the pH of the alkaline medium when there is a CO_2-containing atmosphere only 100 μ above the nerve. The second is that the low activity of the toxins in the alkaline medium may be due to breakdown of the molecules at high pH. The solutions were allowed to act on the node for 60 sec before the measurements were taken. About 2 min after the STX solution at pH 9.7 had been prepared, the measurement had been completed and enough concentrated pH 4.5 buffer was added to bring the pH down to 7.5. At 0.4 nM concentration the STX is relatively ineffectual at high and at low pH. At 1.2 nM it is again ineffectual at pH 9.7, whereas the reneutralized solution reduces $\bar{g}_{Na}$ by 34%. In this case, after the effects of the reneutralized STX solution had been measured, another solution of 1.2 nM STX (pH 7.3) that had never been made basic was tried. It reduced $\bar{g}_{Na}$ by 43%. This fresh solution was, therefore, only slightly more potent than the one that had been exposed to pH 9.7. The 1.9 nM STX solution had a small effect at pH 9.7 and a profound one at pH 7.5, where $\bar{g}_{Na}$ was reduced to 25%. Immediately after this measurement, another solution of 1.9 nM STX was buffered at pH 9.7, tested, rebuffered at pH 7.5, and retested. The value of $\bar{g}_{Na}$ rose again to 64% at pH 9.7 and fell to 19% at pH 7.5. Thus the block by STX may be reversed by raising the pH without removing the toxin.

All the experiments of Table I demonstrate that in basic solutions STX and TTX are ineffectual. The experiments in the following paper (Hille, 1968) show that in the absence of toxins, pH changes in the range from pH 6.5 to pH 10.1 have only small effects on the voltage clamp responses of the node of Ranvier. Thus the change in potency of the toxins in this range is probably attributable to the change in the ionization of the toxins themselves. The bind-

ing of STX and of TTX to the sodium channel probably requires the cationic form of the molecule.

DDT Retards the Closing of Sodium Channels

The primary action of DDT is to prolong the transient increase of sodium permeability elicited by a depolarization of the node. This effect is easily seen in the voltage clamp currents following a short depolarizing test pulse. If a normal node is repolarized when the sodium conductance is high (in the first few milliseconds of a test pulse), the sodium conductance falls rapidly and exponentially from the high value it had at the moment of repolariza-

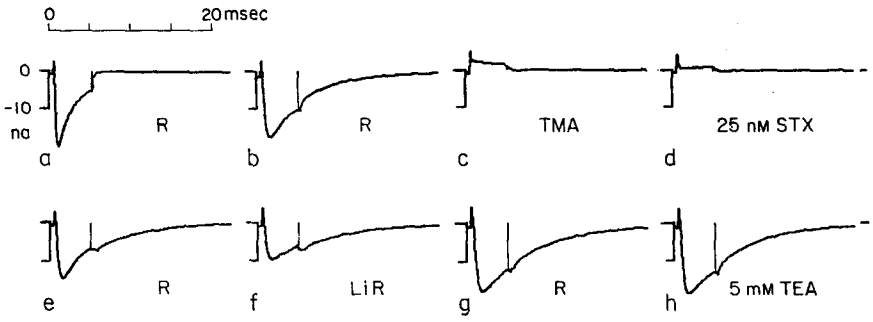

FIGURE 5. The principal action of DDT. The time course, drawn by computer with no subtractions, of the voltage clamp current of a node before (*a*) and after (*b–h*) treatment with 140 μM DDT. The records include the last 0.5 msec of the 40 msec hyperpolarizing prepulse, the 4 msec depolarizing test pulse, and the first 17 msec at the holding potential after the test pulse. The voltage of the test pulse was initially − 15 mv but changed slowly as the holding potential drifted several millivolts during the 60 min period of the measurements. A vertical line extending to the level of zero current in each record signifies the end of the test pulse. The solutions are Ringer's solution (*a*, *b*, *e*, *g*), and sodium-free tetramethylammonium Ringer's (*c*), sodium-free lithium Ringer's (*f*), and 25 nM STX (*d*) or 5 mM TEA (*h*) in Ringer's. Node records 775–777. T = 6.5°C.

tion to a very low steady-state value. Fig. 5 *a* shows such an experiment with a node before it is treated with DDT. Following the 4 msec test pulse, the sodium current turns off in a fraction of a millisecond and the current returns to the steady base line level typical of a resting node. For the theoretical standard node, the steady-state sodium conductance at −75 mv (the resting potential) is 0.01% of $\bar{g}_{Na}$. Unfortunately when the experiment of Fig. 5 was recorded, a low digital time resolution (0.5 msec) was employed for all parts of the record after the first 3 msec (see Hille, 1967 *a*), so the fast "sodium tails" were not faithfully recorded.

After the nerve is exposed to DDT (Fig. 5 *b*), a prominent "tail" of inward current appears that persists for more than 10 msec. The following observations show that the tail is due to a persistent sodium conductance. Like the

transient inward sodium current in the test pulse, the persistent tail of current disappears in a sodium-free medium made with tetramethylammonium chloride (Fig. 5 *c*) and is reversibly abolished by 25 nM STX (Fig. 5 *d*) and by 25 nM TTX (not illustrated). Lithium substituted for sodium gives a 40% smaller transient current and a 40% smaller persistent tail current (Fig. 5 *f*). Neither the transient sodium current nor the tail current is affected by 5 mm TEA (Fig. 5 *h*). Furthermore, although the action potential of a node treated with DDT is initiated at an approximately normal threshold voltage and rises normally, it may take 6–15 msec to fall halfway back to the base line. Sometimes the prolongation of the action potential can be more appropriately described as a long, large, negative afterpotential. Thus the persistent tail of current has the ionic and the pharmacological (but not the kinetic) properties that define the sodium current of normal nerves, and, therefore, DDT acts on the sodium channels of the node.

After a repolarization from the test pulse, the sodium conductance of the DDT-treated node falls in two phases. In the first phase it falls very rapidly, as in the normal node, until it is reduced to 10–30% of its initial value. At this point, the second persistent component remains. In the following, the word tail is used exclusively to denote the *persistent* component of conductance of the poisoned nerve. Following depolarizations to less than 0 mv and lasting less than 5 msec the decay of the tail is invariably exponential. The time constant of decay shortens as the voltage following the test pulse is made more negative. For example the time constant at 6°C in one case was more than 70 msec at −5 mv, 9 msec at −80 mv, and 3 msec at −125 mv. Although the time constant of decay does not depend on the prepulse or on the test pulse, the shape of the early part of the tail does depend on these factors. Following test pulses longer than 5 msec or to a voltage greater than 0 mv, the tail does not begin to decay, and may even increase slightly, for 2–6 msec after the repolarization, and then the typical exponential decay begins. All measurements of the tail amplitude in the next sections were made at a time during the exponentially falling part of the tail. In one case the measurements have been extrapolated back to the moment of repolarization assuming a continuous exponential decay (Fig. 6). This method of extrapolation cannot be fully justified since a several milliseconds' delay in the decay was apparent in the current records with the longer test pulses.

DDT-Treated Nodes Have Two Components of Sodium Conductance

Comparison of records *a* and *b* in Fig. 5 shows that DDT prolongs the sodium current *during* the test pulse as well as *after* the repolarization. When does this prolongation develop? The kinetic factors that control the generation of the tail conductance can be studied in experiments with test pulses of constant voltage and variable duration. The open circles of Fig. 6 show the sodium

conductance during the tail *following* test pulses of the indicated durations. After test pulses shorter than 2 msec there is almost no tail. A test pulse 12 msec long elicits the largest tail, and even after a 32 msec test pulse the tail is large. Fig 6 also shows the time course of the sodium conductance *during* the test pulse (solid line, derived by subtracting the current in TTX from the total current). It rises to a peak at 3.5 msec in a normal manner but does not fall exponentially and does not return to the base line. This observation shows that the persistent conductance associated with the tail is superimposed on the transient conductance during the test pulse. Indeed by subtracting the component in the tail (open circles) from the total sodium conductance (solid line) one obtains a transient component (filled circles) that rises and

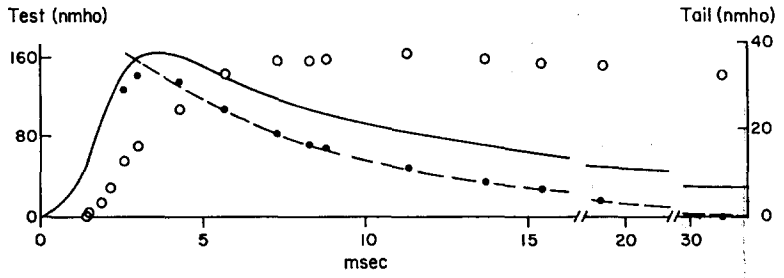

FIGURE 6. The two components of sodium conductance in a node treated with DDT. The time course of the sodium conductance (solid line) during a long test pulse to −5 mv and the magnitude of the tail conductance (open circles) after repolarization from test pulses of the indicated durations. The tail conductance is an extrapolation from 10 msec after the repolarization back to the moment of repolarization assuming an exponential decay of the tail with a time constant of 13.7 msec. The filled circles show the calculated transient component of the conductance during the test pulse, the difference between the values of the open circles and the solid line. An exponentially decaying curve with a time constant of 7.2 msec (dashed line) shows the expected fall of the transient conductance. The node has been treated with 70 μM DDT. Node record 1754. T = 2.5°C.

falls with the same wave form *and* time constants as the transient sodium conductance of a normal node. The dashed line is an exponential curve falling with a time constant of 7.2 msec. This is within 10% of the time constant τ_h for this node with corresponding conditions before the treatment with DDT. Thus the DDT-treated node behaves as though some of its sodium channels open and close normally while others close only slowly once they have opened.

In Fig. 6 and in the two other cases I have studied, the persistent component of conductance (open circles) develops with some milliseconds of delay after the transient component (filled circles) appears. This delay suggests a possible precursor-product relationship between the two components that is examined further in the next section.

The DDT Tail is Proportional to the Transient Sodium Conductance

If the transient component of the sodium conductance is actually a precursor of the persistent component, then the conductance during the tail should be quantitatively dependent on the peak conductance during the transient. I have tested for such a dependence in experiments in which the transient component is systematically varied through changes in the parameter h (variations of the prepulse) and in the parameter m (variations of the test pulse).

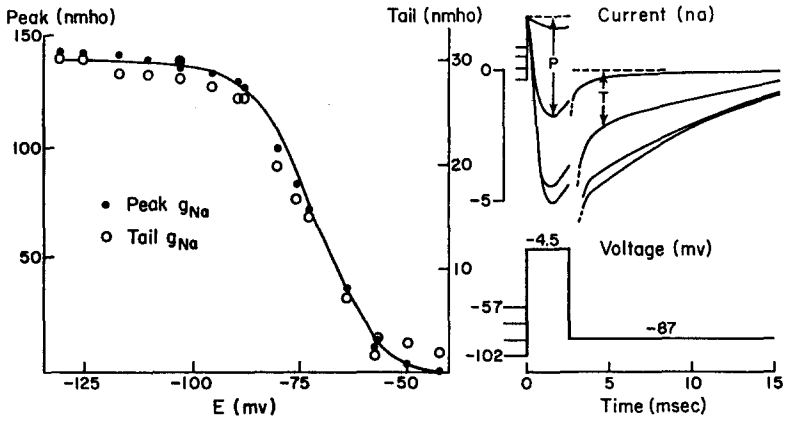

FIGURE 7. Sodium inactivation and DDT. The sodium conductance at the peak (P, filled circles) of the sodium current elicited by the test pulse and during the tail of current (T, open circles) 2 msec after repolarization of a node treated with 140 μM DDT. The conductances are plotted against the voltage (E) of the conditioning prepulse. The solid line is taken from the theoretical standard node but assuming $\bar{g}_{Na}$ = 470 nmho. The insets at the right show the method of measurement with four tracings from the original records of current minus the capacity current. Node records 605–607. T = 6.5°C.

The parameter h reaches different steady-state values, h_∞, during prepulses of differing voltage. The amplitude of the peak of the transient increase of sodium conductance during a test pulse is always proportional to the value of h established during the preceding prepulse. If in this kind of experiment the conductance during the tail is also recorded, there will be enough information to show whether the tail (T) and the peak (P) are proportional. Fig. 7 shows that they are. Other similar experiments also show that the voltage dependence of h_∞ is not affected by the treatment with DDT.

The magnitude of the peak sodium conductance depends also on the parameter m which reaches different steady-state values, m_∞, during test pulses of differing voltage. A comparison of the peak conductances and the tail conductances (Fig. 8) shows that both conductances depend on the volt-

age of the test pulse in the same manner. Other similar experiments show that DDT does not appreciably affect the voltage dependence of m_∞.

The experiments of Figs. 7 and 8 demonstrate that the amplitude of the tail induced by DDT is proportional to the peak of the transient conductance increase during the test pulse under a wide range of conditions. Therefore the number of sodium channels that remain open during the tail is proportional to the number that open during the test pulse. The experiments that I have presented suggest that DDT has no effect on sodium channels that are not open; however, when a channel opens, it may (with a certain

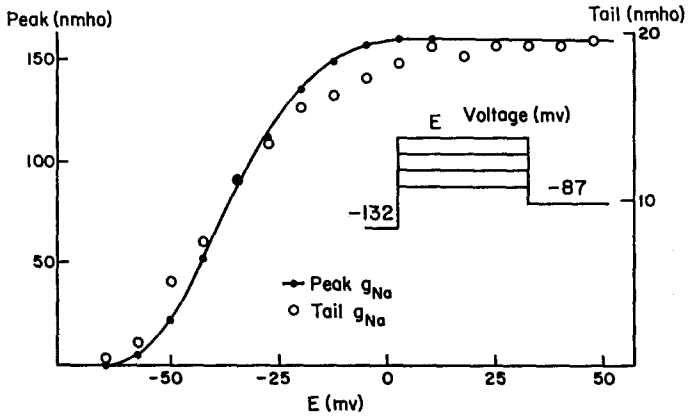

FIGURE 8. Sodium activation and DDT. The sodium conductance at the peak (filled circles) and during the tail (open circles) of the sodium current elicited by 13 msec test pulses of variable voltage (E). The solid line is a smooth curve drawn to pass through the filled circles. The tail conductance is measured 7 msec after the repolarization. The measurements are taken from the same node as the experiment in Fig. 7. Node record 603. T = 6.5°C.

probability) be held open for an unusual length of time or, on the other hand, it may close normally.

This concludes the major findings with DDT. In other experiments I have found that the amplitudes and time courses of potassium currents are not affected by the drug. Over a period of several hours the maximum sodium conductance, $\bar{g}_{Na}$, decreases more rapidly than it would in an untreated nerve, but this effect is temporally quite distinct from the appearance within a minute of the persistent tail phenomenon. It should be mentioned again that in all my experiments the DDT is applied only for a minute at the beginning, so that when the persistent tail reappears after STX or TTX is removed from the nerve, it is not because I have supplied new molecules in the bathing medium. However, the very great lipid solubility of DDT may well cause it to accumulate in excess in all the membranes associated with the axon and the myelin sheath.

DISCUSSION

The Actions of the Drugs

The experiments show that TTX, STX, and DDT act specifically on sodium channels either to close them or to hold them open. These actions abolish or prolong action potentials and probably lead to the death by muscular weakness and axonal block (TTX and STX) or by excitation and convulsion (DDT) that follows an overdose of one of the poisons.

My observations with the marine toxins agree entirely with those of other authors who have used different preparations. With the lobster giant axon, 15–100 nM TTX selectively reduces $\bar{g}_{Na}$ without changing the kinetics of the remaining sodium currents or the voltage dependence of the peak sodium conductance and of the steady-state sodium inactivation (Narahashi et al., 1964; Takata et al., 1966). With the squid giant axon, 150 nM TTX blocks sodium currents (Nakamura et al., 1965 *a*) and lithium currents (Moore, Blaustein, Anderson, and Narahashi, 1967). The sodium currents of electroplaques of *Electrophorus electricus* are abolished by 150 nM TTX and by similar concentrations of STX (Nakamura et al., 1965 *b*). The action potentials of myelinated fibers isolated from frog nerve fail rapidly and without accompanying depolarization in 3 nM TTX and in 3 nM STX (Kao and Fuhrman, 1963; Dettbarn et al., 1965; Kao and Nishiyama, 1965). Although the block with either drug is considered reversible, Kao (1966) describes the reversal from high concentrations (300 nM) of TTX as much slower than from STX. A transient enhancement of the action potential is produced in the first few seconds after STX is applied (Kao and Nishiyama, 1965; Dettbarn et al., 1965), a phenomenon that is probably related to the supernormal recovery of $\bar{g}_{Na}$ from low concentrations of STX.

The mode of action of TTX and of STX remains unknown. One plausible possibility is that the cationic form of the molecule binds to complementary groups, including a negative charge, at the sodium channel, thus blocking the passage of ions by obstructing their diffusion path through the membrane. The pH dependence of the blocking action shows that the cation is the active form, and the pH dependence of the responses of the nerve in the absence of toxin suggests that a negative charge is found at the sodium channel (Hille, 1968). This simple binding picture is especially well supported for STX by its very quick action, its ready reversibility, and its simple dose-response behavior.

The electrophysiological literature on the cellular actions of DDT is not large. Shanes (1949 *a* and *b*, 1951) found that it enhanced oscillations and induced repetitive firing following a single shock to frog sciatic nerve or to crab leg nerve (but not to squid giant axon). He did not observe the large

negative afterpotentials I have reported, perhaps because his preparations were not desheathed and possibly also because he may not have used an organic solvent to assist in suspending the DDT in the bathing medium. A single shock to a cockroach giant axon bathed for several hours in 100 μM DDT produces repetitive firing and a long negative afterpotential that Narahashi and Yamasaki (1960) originally attributed to a reduction in the potassium currents. Their test of this hypothesis was indirect and open to the alternative interpretation of a prolongation of sodium currents. Narahashi has recently observed a specific prolongation of sodium currents in poisoned lobster giant axon (personal communication), and undoubtedly the effects on the cockroach axon are the same.

The mode of action of DDT and of the alkaloids in veratrine seems to be very similar. Veratridine-treated nodes of Ranvier have a persistent component of sodium conductance that appears rapidly (within milliseconds) on depolarization and that decays slowly (time constants 0.1–1 sec) on repolarization (Ulbricht, 1965). I have used veratrine in a few voltage clamp experiments (Hille, 1967 *b*) and find that the persistent component of sodium conductance induced by veratrine is blocked by TTX and by Xylocaine but not by TEA. As with DDT, the magnitude of the persistent conductance depends on the magnitude of the transient sodium conductance increase elicited by a test pulse, but with veratrine there is always a small inward sodium current even when the potential is clamped at the standard resting potential −75 mv; i.e., veratrine is a depolarizing agent whereas DDT is not. The effects of veratrine can be at least partly reversed by washing. I think that, like DDT, veratrine acts primarily by holding sodium channels open once they have opened, and that because veratrine holds them open for up to 100 times longer, it depolarizes the nerve more effectively.

One might imagine that an alternative description to "holding open sodium channels" is "prevention of sodium inactivation," but this, I believe, would be wrong. In the theory of Hodgkin and Huxley (1952), two time-variant parameters, m and h, govern the changes of the sodium conductance. In a normal nerve the turning off of the sodium conductance occurs because of a decrease in h if the nerve is much depolarized (typical sodium inactivation during a test pulse) or because of a decrease in m if the nerve is repolarized or hyperpolarized (typical sodium tail experiment). In the DDT- or veratrine-treated nerve, the persistent conductance does not turn off at the normal rate either in the depolarized node (Fig. 6) or in the hyperpolarized node (Figs. 5 and 7). Thus the processes described by the m parameter and by the h parameter both appear to be overridden when DDT or veratrine holds sodium channels open. With veratridine there is evidence that the steady-state value (after several seconds) of the persistent sodium conductance *is*

determined by the value of m_∞ (see Fig. 2 in Ulbricht, 1965). This has not been tested quantitatively.

The Separation of the Currents

One of the principal problems that faces the investigator who uses the voltage clamp technique is to break down the record of total ionic current into its three components, the sodium, potassium, and leakage currents. This paper shows that the sodium currents may be eliminated by TTX or STX. The potassium currents that remain after treatment with TTX or STX are totally abolished by 60 mM tetraethylammonium ion (Hille, 1967 *a*) and have a time course that closely fits the formulae for the potassium current involving the parameter n raised to the fourth power given by Hodgkin and Huxley (1952, equations 6 and 7). Thus, there are now four practical and equivalent procedures for resolving the potassium component of the ionic current in the frog node of Ranvier: the sodium substitution method (as first applied to the squid giant axon by Hodgkin and Huxley), the mathematical method of fitting the formula for the potassium current to the late part of the current record (Dodge, 1963), the criterion of sensitivity to tetraethylammonium ion, and the criterion of insensitivity to TTX or to STX. Although TTX is now in wider use, I recommend STX as a better agent for the separation of currents because of its more rapid reversibility. Once the time course of the potassium current is known, the sodium current may be calculated by a single subtraction.

The equivalence of these four very different methods for separating the components of current is the strongest argument for the hypothesis of heterogeneity. The ionic permeability mechanisms therefore seem to comprise three chemically and spatially distinct entities, the sodium channels, the potassium channels, and the leakage channels. Much as the names of enzymes often specify the principal or normal substrate of the enzymes without including the many minor or artificial substrates, so the names *sodium* and *potassium* applied to the channels designate the ions whose permeation is essential to normal nervous signalling without including the long list of other cations that are not of such direct physiological importance.

The Number of Ionic Channels

The discovery of lipid-insoluble agents that bind selectively to sodium channels has introduced a new method to estimate the number of sodium channels. Moore, Narahashi, and Shaw (1966) calculate that approximately 13 TTX molecules or fewer are removed from solution per square micron of nerves surface when a nerve trunk from the lobster walking leg in the poison is blocked. On the very plausible assumption that one TTX molecule is needed to block one channel, Moore et al. conclude that there are at most 13 sodium

channels per square micron. The conductance of a single channel could be calculated from this estimate if the maximum sodium conductance $\bar{g}_{Na}$ of the axons in this nerve were known. Let us assume that $\bar{g}_{Na}$ of these axons and of a giant axon from the circumesophageal nerve of the lobster are the same: 1.2 mho cm^{-2} or 12 nmho μ^{-2} (unpublished analysis by C. D. Hopkins and B. Hille of records kindly provided by J. P. Pooler, J. W. Moore, and T. Narahashi). Then the conductance of one channel is about 0.9 nmho, or for the same channel bathed in Ringer's solution, which has less than one-quarter of the sodium in seawater, the conductance would be about 0.2 nmho.

Del Castillo and Suckling (1957) studied fluctuations of the subthreshold response of nodes of Ranvier of nerve fibers from *Rana temporaria* and suggested that "the subthreshold response is made up by the addition of a variable number of unit potential changes (equal to $\frac{1}{100}$–$\frac{1}{200}$ of the action potential)." In their preparation (described by del Castillo and Stark, 1952) the average threshold depolarization is one-quarter of the action potential height, and so the subthreshold response seems to be composed of 25 to 50 unitary events. The sodium conductance of the theoretical "standard node" (see Methods) at the firing threshold is 5 nmho, so del Castillo and Suckling may have observed fluctuations of conductance of the order of 0.1–0.2 nmho, very close to the conductance of a single sodium channel estimated from the adsorption of TTX.

There are two theoretical methods for calculating limiting properties of sodium channels. They are presented in the Appendix. One is to calculate the conductance associated with a hypothetical pore through a membrane from Ohm's law, and the other is to determine the maximum rate of arrival of new ions at the mouth of a hypothetical pore using Fick's law. With the assumptions given in the Appendix and for Ringer's solution, the results are that a channel cannot have a conductance greater than 0.3 nmho and cannot pass more than 40 pa of steady current. It may be noted parenthetically that the calculations in the Appendix are general and might apply to other ionic channels such as those at the motor end plate or those in receptor structures.

The results of the two experimental measurements and of the two theoretical calculations agree well. Disregarding for the moment the problem of individual and species differences, I think that one can now assume that the conductance of a single sodium channel bathed in 0.1 M NaCl is roughly 0.1 or 0.2 nmho and in 0.5 M NaCl, roughly 0.5 or 1.0 nmho, and that a channel is not capable of passing more than 40 pa and 200 pa of sodium current under these conditions. The standard frog node of Ranvier, with a maximum sodium conductance of 750 nmho, would then have roughly 5000 sodium channels and could pass no more then 200 na of inward sodium current from 0.1 M NaCl. For a node with 30 μ^2 of membrane the channels would

occupy less than 10^{-4} of the total area and the distance between individual channels might be as great as 800 A (almost 0.1 μ). Most other axons have a smaller sodium conductance per unit area, so the channels would occupy a correspondingly smaller fraction of their area and would lie further apart. These calculations suggest that the ionic channels of excitable tissues constitute such an exceedingly minor component of the cell membrane that experiments to look for them by physical or chemical methods will have to employ very great sensitivity.

APPENDIX

The purpose of this appendix is to present calculations based on simple physical chemical principles that suggest a lower limit to the number of channels. In treating structures with dimensions of the order of atomic diameters, macroscopic phenomenological laws that are based on the properties of a continuum may have little meaning. Nevertheless, it is instructive to carry through the calculations from our macroscopic point of view to get a sense of the order of magnitude of the events invoved. In the remote future, it may become possible to take explicit account in these calculations of the effects of water structure and of the structure of the ionic channels.

In the calculations I assume an ionic channel to be a cylindrical pore 3 A in radius (r) and 5 A in length (L) that penetrates a dielectric membrane. The radius is chosen to be about as large as the hydrated radius of the ions and the length is assumed to be only a few atomic diameters, with the rest of the membrane thickness containing a much wider opening that connects the short pore to the surrounding fluids. The resistivity (ρ) of the aqueous medium is 100 ohm cm, and the concentration (n) and diffusion constant (D) of the electrolyte are 10^{-4} moles cm^{-3} and 10^{-5} cm^{-2} sec^{-1}. These values are appropriate for Ringer's solution.

1. *The Resistance of the Pore* The resistance of a conducting structure is equal to the integral of the resistance along the paths of current flow. For the cylindrical pore the result is

$$R_{\text{pore}} = \rho \frac{L}{\pi r^2} = \frac{100 \times 5 \times 10^{-8}}{\pi \times (3 \times 10^{-8})^2} = 2 \times 10^9 \text{ ohms}$$

One could easily argue that this calculated resistance should be doubled because all the anions should be excluded from the pore. In addition to resistance within the pore, any measurement would also include the access resistance on both sides; i.e., the resistance along the convergent current paths from the bulk medium to the narrow pore. This is approximately equal to twice the integral resistance from infinity to a hemispherical shell of radius 3 A or

$$R_{\text{access}} = 2 \times \frac{\rho}{2\pi r} = \frac{100}{\pi \times 3 \times 10^{-8}} = 10^9 \text{ ohms}$$

The access resistance sets an absolute upper limit to the conductance of an ionic channel (1.0 nmho). Thus, on the basis of access resistance alone, the theoretical standard

node of Ranvier with values of $\bar{g}_{Na}$, $\bar{g}_K$, and $\bar{g}_L$ of 750, 130, and 25 nmho must have at least 750, 130, and 25 channels of the three types if the assumptions made in the model are appropriate. The total resistance associated with any real pore would be the sum of the resistance in the pore and the access resistance. For the conditions assumed above, the total resistance is three times the access resistance (equal to a conductance of about 0.3 nmho) and, therefore, the estimates of the numbers of channels would be three times the minimum numbers given above.

2. *The Diffusion-Limited Velocity* It is possible to estimate the rate of arrival of new ions at the mouth of a pore from Fick's law of diffusion. The problem is exactly analogous to the problem of calculating the rate of encounter-controlled (diffusion-limited) chemical reactions for which a method was first proposed by Smoluchowski (1918). The diffusion equation can be solved for a spherical sink of radius r in an infinite medium of molecules at a concentration n, using the boundary condition $n = 0$ on the surface of the sink. The desired quantity is the flux ϕ of molecules into the sink. The time-dependent solution starting from a uniform distribution of the molecules is (Frost and Pearson, 1961)

$$\phi_{sink} = 4\pi rDn\,[1 + r(\pi Dt)^{-1/2}]$$

After about 10^{-7} sec the molecules near a sink of radius 3 A have entered the sink, and the flux is within 1 % of the steady-state value:

$$\phi_{sink} = 4\pi rDn$$

The derivation and limitations of this equation have been discussed in many places in the literature of chemical kinetics. Some of these papers consider the effects of water structure, of crowding of the sinks, and of attractive and repulsive forces between the particles (see, for example, Noyes, 1960).

The steady-state solution can be used for the pore model if we assume that there is hemispherical sink at one end of the pore:

$$\begin{aligned}\phi \text{ pore} &= 2\pi rDn = 2\pi \times 3 \times 10^{-8} \times 10^{-5} \times 10^{-4} \\ &= 2 \times 10^{-16} \text{ moles sec}^{-1} \\ &= 1.3 \times 10^{8} \text{ ions sec}^{-1}\end{aligned}$$

If the sink has a single negative charge, the limiting flux of univalent positive ions is approximately doubled (Frost and Pearson, 1961). Thus, if the chosen conditions imitate those of an ionic channel, I estimate that each channel could support a maximum flux of 2.6×10^8 ions sec^{-1} which corresponds to an ionic current of 40 pa. In a good experiment, a node of Ranvier may have sodium, potassium, and leakage currents of 25, 20, and 4 na, corresponding to at least 625, 500, and 100 of the three kinds of ionic channels. The lower limit on the number of sodium channels must be doubled because only about one-half of the sodium channels are open when the maximum sodium current occurs; i.e., g_{Na} does not exceed 0.5 times $\bar{g}_{Na}$ during this measurement. Furthermore, one does not generally arrange experimental conditions to obtain

the maximum possible currents, and, undoubtedly, larger potassium and leakage currents could be obtained by using larger depolarizations. The estimated lower limit on the number of channels would have to be increased accordingly.

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Added Note Experiments with DDT, TTX, and STX similar to some given here, but with the lobster giant axon, have been published since my paper was submitted (Narahashi, T., and H. G. Haas. 1967. *Science.* **157**:1438; Narahashi, T., H. G. Haas, and E. F. Therrien. 1967. *Science.* **175**:1441).

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