Properties of the Tetrodotoxin Binding Component in Plasma Membranes Isolated from *Electrophorus electricus*†

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ABSTRACT: The biochemical properties of the electrically excitable sodium channels in the electroplaque of *Electrophorus electricus* were investigated using tritiated tetrodotoxin (TTX) as a specific membrane probe. Membrane fragments from the electroplaque were isolated essentially by differential centrifugation and characterized with respect to the plasma membrane markers acetylcholine receptors, acetylcholinesterase, $(Na^+ + K^+)$ATPase, and [^{3}H]TTX binding. Equilibrium binding studies showed that [^{3}H]TTX bound to a single population of noninteracting receptor sites with an apparent dissociation constant of $6 \pm 1 \times 10^{-9}$ M. The toxin–membrane complex dissociated with a first-order rate constant of 0.012 sec^{-1}. Studies on the pH dependence of complex formation demonstrated the requirement for an ionizable, functional group with a pK of 5.3 and this group has been shown to be a carboxyl. Treatment of the membranes with trimethyloxonium tetrafluoroborate, a carboxyl group modifying reagent, resulted in an irreversible loss in the binding of [^{3}H]TTX, which could be prevented by low concentrations of TTX or saxitoxin. This decrease was due to a reduction in the total number of binding sites and not to a decrease in toxin binding affinities. The relative binding affinities of various monovalent alkali metal and polyatomic cations for the TTX–receptor site showed that this site displayed cation discrimination properties which were similar to those reported previously for the electrically excitable sodium channel in intact nerve fibers. A possible role for this site in the ion selectivity of the sodium channel is proposed.

It is now well established that the propagated action potentials characteristic of electrically excitable nerve and muscle cells result from the transient changes in the permeability of the cell membrane to ions, usually sodium and potassium (Hodgkin and Huxley, 1952a,b). Furthermore, the resulting movement of ions across the membrane in response to a stimulus is believed to occur through highly specialized pores or channels which display marked cation selectivities (see reviews, Hille, 1970; Armstrong, 1975).

The inward movement of sodium ions in the initial phase of the action potential has been shown to be blocked by the puffer fish neurotoxin, tetrodotoxin (TTX)[1] (Moore and Narahashi, 1967; Moore et al., 1967). This poison is thought to bind tightly to the sodium specific channel, thereby preventing the passage of ions through this channel. Because of its high selectivity and binding affinity, TTX has proved to be a valuable tool in studying the properties of the sodium channel. Using radioactive TTX, it has been possible to directly demonstrate the presence of a specific component in various axonal membrane preparations which binds the toxin with affinities comparable to those estimated from electrical measurements using intact nerve preparations (Benzer and Raftery, 1972; Henderson and Wang, 1972; Colquhoun et al., 1972; Hafemann, 1972; Barnola et al., 1973). This toxin binding component has been shown to be a tightly bound membrane protein which can be solubilized by a number of detergents such as Triton X-100 (Henderson and Wang, 1972; Benzer and Raftery, 1973).

In recent years, electrophysiological studies with intact nerves have provided considerable information regarding the functional properties of the sodium channel, particularly with respect to ion selectivity (Chandler and Meves, 1965; Hille, 1972) and gating properties (Armstrong and Bezanilla, 1973; Keynes and Rojas, 1973). On the other hand, the chemical and structural basis for many of these functions still remain obscure.

One approach toward elucidation of some of these biochemical mechanisms is to isolate and characterize the channel components from biological membranes which are electrically excitable. The high specificity of TTX for the sodium channel provides a convenient and sensitive marker for this particular membrane component. Up to the present time, such studies on the biochemical properties of the sodium channels have been restricted mainly to unmyelinated nerve preparations. Isolation procedures using these tissues have not been successful, partly because of the low distribution of sites and the limited quantities of nerve material which are normally available.

One possible tissue source for the sodium channel components is the electric organ of the electric eel, *Electrophorus electricus*. Unlike that of a number of other electric fish such as *Torpedo*, the eel electroplaque is electrically as well as chemically excitable. Nakamura et al. (1965) have shown that in the isolated electroplaque, the inward current during the action potential is carried by sodium ions, and, in addition, is sensitive to TTX. However, the biochemical properties of the TTX-sensitive sodium channel from this tissue have yet to be described.

In this communication, we report the isolation and partial characterization of plasma membranes from the eel

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[1] Abbreviations used are: TTX, tetrodotoxin; STX, saxitoxin; AcChR, acetylcholine receptor; AcChE, acetylcholinesterase; [^{125}I]-α-Bgt, [^{125}I]-α-bungarotoxin; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; TMO, trimethyloxonium ion; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; $(Na^+ + K^+)$ATPase, sodium and potassium ion stimulated adenosine triphosphatase.

electric organ which are enriched in the TTX-binding component. We also describe here some of the biochemical properties of the TTX-binding site, in particular the role of a specific ionizable carboxyl group which appears to be essential in the binding of this toxin.

Experimental Procedure

Preparation of Membrane Fragments. Electrophorus electricus was obtained from Paramount Aquarium, N.Y., and normally ranged in length from 2 to 3 ft. The eels were killed by immersion into ice-water and the main organ was excised and trimmed of extraneous muscle and connective tissue. The organ was used immediately or rapidly frozen and stored at −90°C for later use. No significant differences have been observed between the fresh and frozen samples.

Portions of the electric organ, normally 30–35 g wet weight, were finely minced and suspended in five volumes of ice-cold 0.25 M sucrose. All subsequent steps were carried out at 4°C. The suspension was homogenized with a loose-fitting Teflon-glass homogenizer and centrifuged for 10 min at 600*g*. The supernatant was discarded and the pellet resuspended in 0.25 M sucrose to a final concentration of between 4 and 5 mg of protein/ml. The homogenate was filtered through cheesecloth to remove large aggregates of connective tissue, and sonicated (Branson Sonifier, Model J-22, setting 45) for two bursts of 30 sec each. Much of the remaining connective tissue aggregated into large fibrous clumps, and these were removed by filtration through a fine metal mesh. The opalescent suspension was either applied directly to a discontinuous sucrose density gradient or purified further by conventional differential centrifugation as described below.

Sucrose Gradient Density Centrifugation. The crude membrane suspension (15 ml) was applied to a discontinuous gradient made with nine layers of sucrose, 5 ml each, ranging from 50 to 10% sucrose (w/v). Gradients were centrifuged in an SW-25.2 rotor (Beckman) at 24 000 rpm for at least 10 h. Fractions were collected and analyzed for the various plasma membrane markers as indicated.

Preparation of Membranes by Differential Centrifugation. The following procedure was routinely used to prepare large quantities of the enriched plasma membranes. The crude membrane suspension was centrifuged at 150 000*g* for 1 h to pellet all particulate material. Pellets were resuspended in 30% sucrose by homogenization, and the suspension was centrifuged at 5000*g* for 20 min. The cloudy supernatant was carefully removed and the viscous pellet discarded. The supernatant was diluted to 15% sucrose by the addition of ice-cold H_2O and centrifuged at 150 000*g* for 1 h. The supernatant was discarded; pellets were resuspended in 0.25 M sucrose and used for further experiments. The membrane fragments were either used immediately or stored at 4°C in 0.02% azide for up to 10 days with little change in any of the TTX-binding properties.

Enzyme and Binding Activities. The $(Na^+ + K^+)$ATPase activity was determined in a medium containing 3 mM ATP, 5 mM $MgCl_2$, 0.5 mM EDTA, 100 mM NaCl, 10 mM KCl, and 0.05 M imidazole-HCl (pH 7.4). Samples were incubated for 10 min at 37° and the phosphate released was determined by the method of Fiske and Subba-Row (1925). Preliminary experiments indicated that approximately 90–95% of the electroplaque membrane ATPase activity was oubain sensitive as has been previously reported (Bauman et al., 1970).

Acetylcholinesterase activity (AcChE) was measured by the method of Ellman et al. (1961) using acetylthiocholine as substrate. The distribution of acetylcholine receptor molecules (AcChR) was monitored by following the binding of [^{125}I]-α-Bgt using the DEAE disc assay described by Schmidt and Raftery (1973). Protein was estimated by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Binding of [^{3}H]TTX. Tetrodotoxin (citrate free) was obtained from Sankyo Ltd., Tokyo, and tritiated using a catalytic exchange reaction (ICN, Tracerlab Inc. Irvine, Calif.). Impurities were removed by chromatography on Bio-Gel P-2 and Bio-Rex 70 (NH_4^+ form) as previously described by Benzer and Raftery (1972). The specific activity of the purified TTX was estimated by the dilution assay as outlined below. Specific activities of approximately 300 Ci/mol were routinely obtained and the radiopurity was estimated to be at least 90% from the chromatographic profile on a Bio-Rad A-6 ion-exchange resin (Benzer and Raftery, 1972).

The procedure for the determination of the specific activity of [^{3}H]TTX involves measuring the fractional reduction in the binding of [^{3}H]TTX to isolated membranes by the addition of known concentrations of unlabeled TTX. From the analysis of the dilution according to the equations described below, the concentration of TTX in the radioactive sample can then be determined. This assay is highly reproducible and has been found to be more reliable than the bioassay which has generally been used in the past.

The reversible binding of TTX to receptor sites follows the Langmuir hyperbolic saturation

$$b = B[\mathrm{TTX}]/([\mathrm{TTX}] + K_d) \quad (1)$$

where b is the binding of toxin at equilibrium, B is the maximum number of binding sites, and K_d is the dissociation constant. The concentration of radioactive and nonradioactive toxin is given by $[TTX_1]$ and $[TTX_2]$, respectively, and b^* is the measured binding of radioactive toxin in dpm. Assuming that TTX_1 and TTX_2 are identical compounds, binding to the same site with equal affinities, then the binding of TTX_2 competitively inhibits the binding of TTX_1 according to the equation

$$b^* = B[\mathrm{TTX_1}]/[[\mathrm{TTX_1}] + K_d(1 + [\mathrm{TTX_2}]/K_d)] \quad (2)$$

Rearranging

$$\frac{1}{b^*} = \frac{1}{B}([\mathrm{TTX_2}] + K_d)\frac{1}{[\mathrm{TTX_1}]} + \frac{1}{B} \quad (3)$$

Experimentally, TTX_1 is varied at fixed levels of TTX_2. Plots of $1/b^*$ vs. $1/TTX_1$, in arbitrary units of radioactivity, will be a series of straight lines, converging at the ordinate at $1/B$. The intercept at the abscissa will be given by $-[TTX_1] = [TTX_2] + K_d$ and the absolute concentration of $[TTX_1]$ can then be determined graphically from the corresponding replot. The specific activities of the toxin obtained using this method were comparable to those estimated by bioassay (Benzer and Raftery, 1972).

The binding of [^{3}H]TTX to membranes was measured by equilibrium dialysis at 10°C. Routinely, the dialysate contained 50 mM NaCl, 10 mM (K) phosphate buffer (pH 7.0), and 0.25 M sucrose. Membranes and dialysate samples were solubilized with NCS (Amersham) and analyzed for radioactivity in a scintillation fluid containing 0.55% Permablend TM-II (Packard) in toluene.

Determination of the Rate of Dissociation of the TTX–

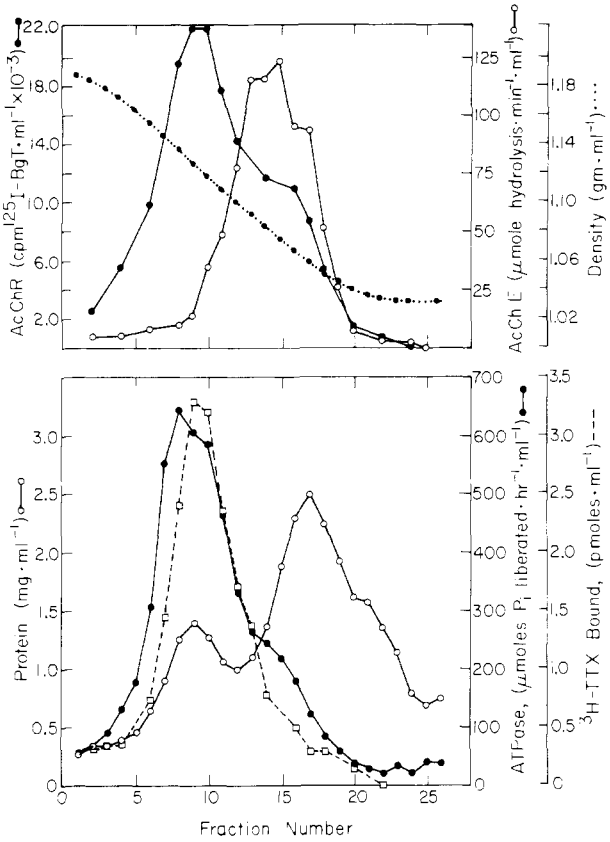

FIGURE 1: Equilibrium sucrose density gradient centrifugation of membrane fragments from *Electrophorus electricus*. Distribution of AcChR, AcChE, $(Na^+ + K^+)$ATPase, [^{3}H]TTX binding, and protein was measured as described in the Experimental Procedure. 2.3-ml fractions were collected for analysis.

Membrane Complex. Membrane samples were equilibrated for 20 min at 4°C with 1.4×10^{-7} M [^{3}H]TTX. Aliquots were transferred to 1.0-ml columns of DE-52 (Whatman) and rapidly washed under vacuum for varying lengths of time with 50 mM NaCl, 10 mM (K) phosphate buffer (pH 7.0), plus 0.25 M sucrose. The membrane particles remained tightly bound to the resin and unbound [^{3}H]TTX was readily washed off. The membranes and bound [^{3}H]TTX were quantitatively eluted with 1% Triton X-100, and the radioactivity was determined. Values are corrected for background [^{3}H]TTX by simultaneously determining the radioactivity bound to the resin in the absence of membranes. This normally amounted to approximately 10% of the total membrane-bound [^{3}H]TTX.

Enzyme Treatment and Chemical Modification. The membrane fragments, between 4 and 5 mg of protein/ml, were incubated for 1 hr at 24°C in the presence of various enzymes at concentrations indicated. All incubations were at pH 7.4, except where neuraminidase was used, and in this case incubations were at pH 6.3. Samples were diluted and the fragments sedimented by centrifugation. Pellets were resuspended, and the binding of [^{3}H]TTX to the final suspension was measured by equilibrium dialysis.

Treatment with Trimethyloxonium Tetrafluoroborate. Trimethyloxonium tetrafluoroborate (TMO), prepared by the method of Curphy (1971), was kindly provided by Dr. Yuan Chao. Inactivation of [^{3}H]TTX binding to isolated membrane fragments was carried out essentially as follows.

To approximately 4 mg of protein/ml of membranes in 0.25 M sucrose and 50 mM (K) phosphate or 50 mM Hepes buffer (pH 7.0) was added solid TMO to final concentrations ranging from 0.5 to 2.0 mg/ml. The pH of the suspension was maintained by the addition of microliter quantities of 2 N NaOH. Samples were incubated on ice for a total of 5 min after the addition of the modifying reagent. Reaction by-products were removed by extensive dialysis, and the binding of [^{3}H]TTX was determined as previously described. Dialysis was normally carried out in solutions containing 0.25 M sucrose, 50 mM NaCl, and 10 mM (K) phosphate (pH 7.0). In toxin protection experiments, the membrane fragments were incubated on ice for at least 30 min with the appropriate toxin or drug, prior to reaction with TMO.

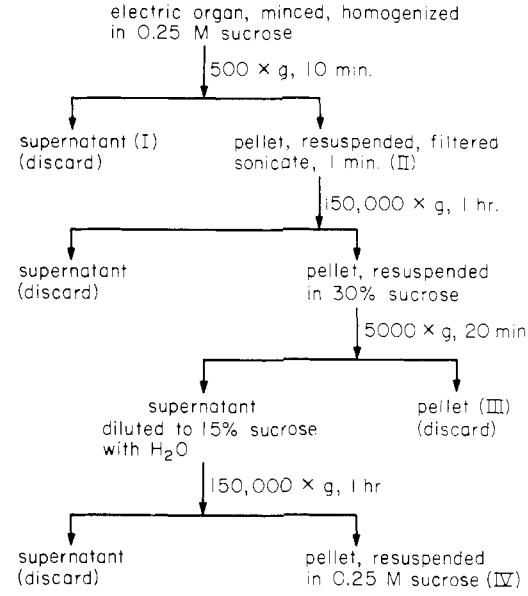

FIGURE 2: Summary of the procedure for isolation of electroplaque membranes enriched in the [^{3}H]TTX-binding component.

Measurement of Exposed Carboxyl Groups. The number of exposed carboxyl groups in the membrane fragments was estimated by following the incorporation of radioactive amine in the presence of water-soluble carbodiimide, as described by Hoare and Koshland (1967). Membrane samples were incubated for various times in 0.1 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 0.5 M glycine ethyl ester plus tracer [^{14}C]glycine ethyl ester. Excess reagents were removed by extensive dialysis and the incorporation of radioactivity into the membranes was estimated after correcting for nonspecific adsorption of the [^{14}C]amine.

Results

Isolation of Electroplaque Plasma Membranes. When the crude electroplaque membrane suspension was applied to a discontinuous sucrose gradient, the membrane fragments banded in two distinct regions of the gradient, d = 1.06 and 1.12 gm/ml as indicated in the profile shown in Figure 1.

The fractions from the gradient were analyzed for the plasma membrane markers $(Na^+ + K^+)$ATPase, AcChR, AcChE, and sodium channel. The results indicated that the heavier band of membranes contained virtually all of the [^{3}H]TTX binding activity, the $(Na^+ + K^+)$ATPase, and approximately 70% of the total AcChR activity as judged by the binding of [^{125}I]-α-Bgt. The lighter membrane fragments were enriched mainly in AcChE activity, but con-

Table I: Analysis of Partially Purified Membrane Fragments.[a]

Fraction	Protein (mg)	AcChR[e]	ATPase (units[b])	[^{3}H]TTX bound[c] (pmol)	AcChE (units)[d]
Homogenization in 0.25 M sucrose, 500*g*					
I supernatant	434	Negl.	10 700	40	9200
II pellet	220	36.1	43 900	303	9910
Homogenization in 30% sucrose, 5000*g*					
III pellet (discard)	11	2.98	1 700	24	241
Supernatant in 15% sucrose, 150 000*g*					
IV pellet	92	27.8	29 900	228	6210
Yield in IV (%)	14	77	55	67	32

[a] Prepared from 23 g wet weight electric organ. [b] Unit = μmol of ATP hydrolyzed/h at 37°C. [c] Binding measured at 17.3×10^{-9} M [^{3}H]TTX at 4°C. [d] Unit = μmol of acetylthiocholine hydrolyzed/min at 24°C. [e] μg of [^{125}I]-α-Bgt bound.

tained about 30% of the total AcChR activity. A similar partial separation of the AcChE and AcChR activities from *Electrophorus electricus* has been reported previously (Duguid and Raftery, 1973). The profile for the AcChR molecules showed a characteristic major peak with a pronounced shoulder at lower densities, although the size of the shoulder varied slightly from one preparation to another. On the other hand, the profile for the [^{3}H]TTX binding was always an essentially symmetrical peak with little or no toxin binding activity associated with the lighter band. Presumably not all of the AcChR molecules are located in membrane fragments derived from the same region of the plasma membrane as are the sodium channels. Such a distribution of AcChR-containing membranes may represent areas of synaptic and extrasynaptic localization.

Although sucrose gradients are useful for qualitative analysis, they are time consuming and tedious for any routine, large-scale preparation of membrane fragments, as needed for our studies. A differential centrifugation procedure was therefore developed, using varying densities of sucrose to selectively isolate only those membranes banding at approximately d = 1.12 g/ml, which would presumably contain the TTX-binding components. This procedure is summarized in Figure 2. The initial homogenization serves to disrupt the cells, releasing intra- and extracellular contents, but tends to keep the membranes as rather large intact sheets which could then be collected by low-speed centrifugation. More disruptive homogenization procedures, such as with the VirTis homogenizer generally used to disrupt the electric organ for isolating the AcChR, were unsuitable for our studies. Under such circumstances, considerable variability and loss of recovery of the TTX-binding components were generally found.

The sonicated crude membrane suspension at stage IV (Figure 2) was resuspended in 30% sucrose. The membranes with [^{3}H]TTX-binding activity remained in the supernatant after centrifugation, and the pellets contained any remaining connective tissue fragments as well as other cell debris.

Various fractions in the purification procedure were analyzed for the plasma membrane markers, and the results are shown in Table I. The corresponding numbers indicate the stage of purification according to the scheme outlined in Figure 2. The final membrane suspension normally contained approximately 65–75% of the total [^{3}H]TTX-binding activity and the AcChR molecules, but only 10–15% of the total protein and represents a five- to sevenfold enrichment of the TTX-binding components on a protein basis.

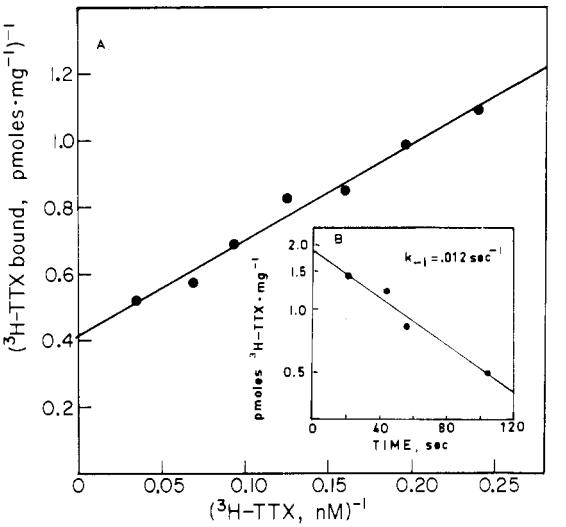

FIGURE 3: (A) Double reciprocal plot of the binding of [^{3}H]TTX to isolated electroplaque membranes. Binding was measured under equilibrium conditions at 4°C in the presence of 50 mM NaCl, 10 mM (K) phosphate buffer (pH 7.0), and 0.25 M sucrose. (B) Dissociation of the [^{3}H]TTX membrane complex using the DE-52 filtration method described in text.

Binding of [^{3}*H*]*TTX.* The binding of [^{3}H]TTX to the membrane fragments followed normal hyperbolic saturation, characteristic of a single type of noninteracting binding site. The corresponding double reciprocal plot, shown in Figure 3A, was linear; there was no evidence of significant nonspecific binding over the concentration range studied. The apparent dissociation constant was $6 \pm 1 \times 10^{-9}$ M and the maximum number of binding sites normally ranged from 2 to 3 pmol/mg of protein. The total number of TTX-binding sites in the electric organ varied from 15 to 25 pmol/g wet tissue weight. In general, lower yields were obtained from larger eels. Table II lists the various membrane preparations used previously by other laboratories to study the binding properties of [^{3}H]TTX. For comparison, the apparent dissociation constants, as well as the number of binding sites, is included for each tissue.

The formation of the [^{3}H]TTX–membrane complex is reversible and can be represented by the simple equilibrium

Table II: Binding of [^{3}H]TTX to Intact Excitable Membrane.

Tissue	Apparent Dissociation Constant (M)	Binding Capacity (pmol/gm wet weight)	Ref
Lobster nerve	25×10^{-9}	2.6	Hafemann, 1972
	4×10^{-9}		Barnola et al., 1973
	10.2×10^{-9}	18	Colquhoun et al., 1972
Garfish olfactory nerve	8.3×10^{-9}	42	Benzer and Raftery, 1972
	10.1×10^{-9}	60	Colquhoun et al., 1972
Rabbit vagus nerve	3×10^{-9}	31	Colquhoun et al., 1972
Rat diaphragm	12.7×10^{-9}	3.9	Colquhoun et al., 1973
Electroplaque	6×10^{-9}	15–25	This study

$$R + TTX \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} R{\cdot}TTX \qquad (4)$$

where R is the toxin binding component in the membrane. In addition, $K_d = k_{-1}/k_{+1}$ where k_{-1} is the first-order rate constant for the dissociation of the complex, and K_d is the equilibrium dissociation constant. This rate constant was determined as described in the Experimental Procedure, and the results are shown in Figure 3B. From the slope, k_{-1} was estimated to be 0.012 sec^{-1}, the complex dissociating with a half-time of approximately 57 sec. Using $K_d = 6 \times 10^{-9}$ M, the association rate constant can be estimated to be 2×10^6 M^{-1} sec^{-1}, considerably slower than expected for a diffusion-limited reaction in a homogeneous system.

Enzyme Treatment and Chemical Modification. Treatment of the membrane fragments with the proteolytic enzymes, trypsin, chymotrypsin, and Pronase, resulted in a substantial loss of the binding of [^{3}H]TTX, as shown in Table III. Although treatment with phospholipase C resulted in some inactivation, almost total loss of binding was observed with phospholipase A at considerably lower concentrations. The TTX-binding component in the electroplaque membranes, like that in the nerve membrane (Benzer and Raftery, 1972), appears to be a protein which is probably stabilized in the membrane by specific phospholipid interactions. Other enzymes such as neuraminidase, hyaluronidase, and collagenase had no effect on [^{3}H]TTX binding. In addition, lectins such as concanavalin A, wheat germ agglutinin, and castor bean agglutinin at concentrations up to 1 mg/ml were ineffective, although some aggregation of membranes occurred with concanavalin A and castor bean agglutinin. Sulfhydryl reagents, such as dithiothreitol and iodoacetamide, were also without significant effect.

Effect of pH on [^{3}H]TTX Binding. As previously shown, the binding of [^{3}H]TTX to electroplaque membrane fragments follows normal hyperbolic saturation, with an apparent dissociation constant of 6×10^{-9} M. Recently, evidence has been reported to suggest that the binding of TTX, at least to axonal membranes, is pH dependent and involves an anionic species at the binding site (Henderson et al., 1974). The possibility of a similar functional group in the electroplaque membranes participating in toxin binding was therefore investigated here by studying the effect of pH on the binding of [^{3}H]TTX to the electroplaque plasma membranes. The dissociation constant for the toxin–membrane complex was measured at varying pH. In each case, the apparent K_d' was determined from a double reciprocal plot, following the saturation of binding sites with increasing concentrations of [^{3}H]TTX. The buffers used in the dialysis were either 10 mM phosphate or 10 mM cacodylate. At all pH values tested, ranging from 5.3 to 6.4, no appreciable difference was found in the total number of binding sites, indicating that there were no irreversible effects of pH on the binding. The apparent dissociation constant, K_d', was unchanged between pH 6.9 and 7.7. An increase in the apparent dissociation constant with decreasing pH would be expected if H^+ and TTX compete for the same group at the binding site on the membrane.

Table III: Effect of Enzyme Treatment on the Binding of [^{3}H]TTX to Electroplaque Plasma Membranes.

Conditions[a]	Fraction of Control
Trypsin	
1 mg/ml	0.37
0.5 mg/ml	0.49
Chymotrypsin	
1 mg/ml	0.39
0.5 mg/ml	0.32
Pronase, 1 mg/ml	0.38
Phospholipase A	
0.11 mg/ml	0.08
0.05 mg/ml	0.12
Phospholipase C, 1 mg/ml	0.64
Neuramindase, 1 mg/ml	0.90
Hyaluronidase, 1 mg/ml	1.01
Collagenase, 1 mg/ml	1.01

[a] Including electroplaque membranes, 4.5 mg/ml of protein. Incubations were for 1 h at 24°C.

Assuming that the binding of H^+ to the site has a dissociation constant of K_H, it can be shown that

$$K_d' = K_d + K_d([H^+]/K_H) \qquad (5)$$

where K_d' is the apparent dissociation constant for the binding of toxin at any $[H^+]$, K_d is the dissociation constant where $[H^+] \ll K_H$. Taking the logarithms of both sides of eq 5 and rearranging give

$$\log (K_d' - K_d) - \log K_d = pK - pH \qquad (6)$$

For a single ionizable group involved in binding, a plot of $\log (K_d' - K_d) - \log K_d$ vs. pH will be a straight line with a slope of -1. From such a plot, as shown in Figure 4, the apparent pK_H can be estimated to be 5.3. Presumably the protonated form of this group substantially reduces the affinity of TTX for the receptor site.

Effects of Carboxyl Group modifying Reagents. The nature of the ionizable group involved in TTX binding was further investigated. The effects of carboxyl group modifying reagents were first studied since previous reports suggested that a specific carboxyl group was possibly involved in toxin binding.

Treatment of the electroplaque membrane fragments with water-soluble carbodiimide (EDC) in the presence of excess amine, using the method described by Hoare and Koshland (1967), resulted in such extensive modification of surface carboxyl groups that the membranes aggregated. Binding of [^{3}H]TTX was irreversibly and rapidly lost, but it was unclear whether this resulted from a specific modification of the toxin-binding region or from secondary effects involving gross changes in the charge distribution on the

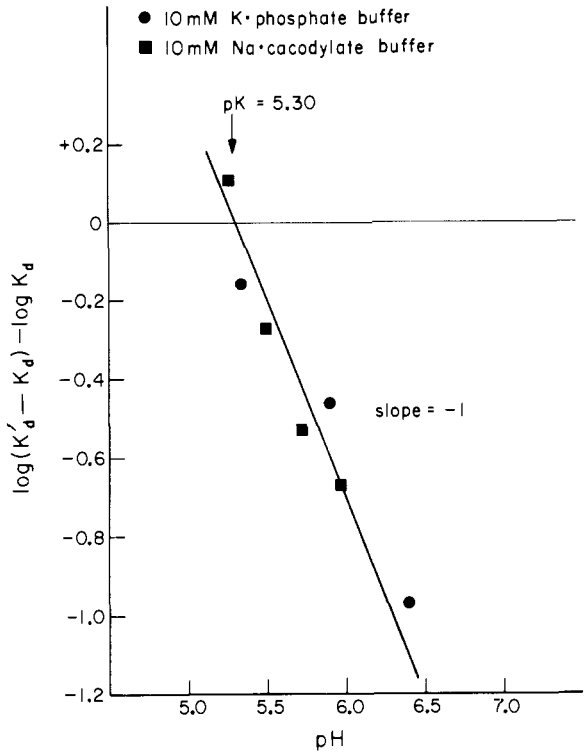

FIGURE 4: Effect of pH on the binding of [^{3}H]TTX to the isolated electroplaque membranes. The apparent dissociation constant (K_d') for the [^{3}H]TTX membrane complex was determined at various pH's from a double reciprocal plot as shown in Figure 3A. Details of the analysis of the data are given in the text.

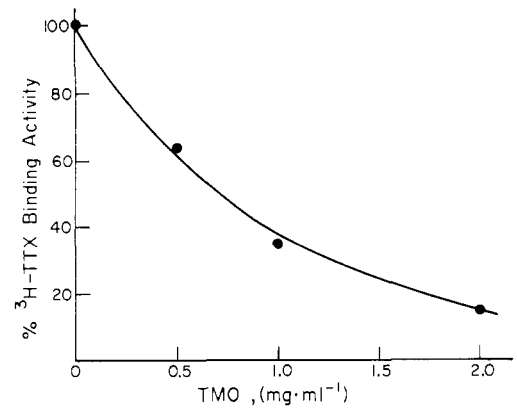

FIGURE 5: Inactivation of [^{3}H]TTX binding to the electroplaque membranes after treatment with increasing concentrations of trimethyloxonium tetrafluoroborate (TMO). Membranes were treated with TMO for 5 min at 4°C and [^{3}H]TTX binding determined as described in the Experimental Procedure.

Table IV: Esterfication of Carboxyl Groups of Membrane Fragments.

Conditions[a]	μmol of [^{14}C]Glycine Ethyl Ester Incorporated/mg of Protein: No Treatment	TMO Treatment[b]
Expt I		
pH 7.0; 18-h incubation	1.20	1.24
Expt II		
pH 6.0; 4-h incubation	0.78	0.74
6-h incubation	1.06	1.07

[a] Incubations were carried out in the presence of 0.1 M carbodiimide (EDC) plus 0.5 M glycine ethyl ester (plus trace [^{14}C]-glycine ethyl ester), 4–5 mg of membrane protein/ml. [b] Membranes were treated with 2 mg/ml of TMO for 5 min at 4°C.

surface of the membrane. Furthermore, the inactivation by carbodiimide treatment could not be prevented even by excess concentrations of TTX.

One carboxyl group modifying reagent which has been shown to display some selectivity is trimethyloxonium tetrafluoroborate (TMO) or Meerwein's reagent. TMO and the triethyl derivative have been used to modify specific carboxyl groups in a number of proteins such as lysozyme (Parsons et al., 1969), trypsin (Nakayama et al., 1970), acetylcholinesterase (Rawn and Leinhard, 1974), and more recently an anionic group of the AcChR molecule involved in ligand binding (Chao et al., 1975). The reaction with carboxyl groups occurs rapidly at neutral pH, with the formation of the methyl ester. Other amino acid side chains such as those of methionine and histidine will also react with TMO (Yonemitsu et al., 1969); however, these reactions only appear to occur with the free amino acids and have not been observed with any proteins.

Treatment of the electroplaque plasma membranes with concentrations of TMO ranging from 0.5 to 2 mg/ml resulted in a progressive loss in the binding of [^{3}H]TTX, as shown in Figure 5. The inactivation was irreversible and no recovery was observed even after extensive dialysis at pH 9.0, conditions which might be expected to cause hydrolysis of a particularly labile methyl ester. At the concentrations of the reagent used in these experiments, no aggregation of membranes was observed.

The modification of membrane carboxyl groups by TMO appeared to occur with some specificity, since only a small percentage of the total available carboxyl groups was methylated under the conditions described here. The exposed carboxyl groups were determined by measuring the incorporation of radioactive amine in the presence of water-soluble carbodiimide. Prior treatment of the membranes with TMO at concentrations sufficient to result in at least 80% inactivation of [^{3}H]TTX binding did not appear to significantly reduce the total number of exposed carboxyl groups, as shown in Table IV. Although there is some variation in the total modifiable carboxyl groups from one membrane preparation to another, pretreatment with TMO resulted in no reduction in the relative incorporation of [^{14}C]glycine ethyl ester within a single preparation over the same time period. These results suggest that probably less than 5–8% of the total carboxyl groups which can be modified by carbodiimide treatment are in fact methylated by TMO.

The inactivation effects of TMO on [^{3}H]TTX binding could result from either a direct effect at the binding site, or from secondary effects due to modification of other carboxyl groups near but not at this site. These alternatives were tested in the protection experiments illustrated in Figure 6. In these studies electroplaque membranes were treated with TMO (2 mg/ml) in the presence of increasing concentrations of unlabeled TTX. After extensive dialysis to remove reaction products and TTX, the number of toxin binding sites remaining was determined using [^{3}H]TTX. Even at concentrations of TTX not far from the apparent dissociation constant a substantial protection from TMO inactivation was observed. At 5×10^{-8} M TTX and higher, almost 90% of the original binding activity was retained after treatment. The effect of TMO appeared to be a direct one in-

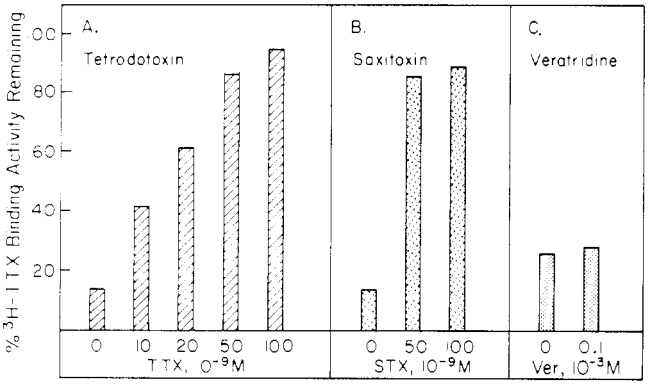

FIGURE 6: Effect of tetrodotoxin, saxitoxin, and veratridine on the inactivation of [^{3}H]TTX binding by TMO treatment. Membranes were treated with 2 mg/ml of TMO at 4°C in the presence of toxins at concentrations indicated. The [^{3}H]TTX binding remaining after treatment was determined by equilibrium dialysis.

Table V: Binding of Monovalent Cations to the Tetrodotoxin Site.

Cation	Apparent Inhibition Constant K_i, 10^{-3} M
A. Metal cations	
Li^+	60 ± 10
Na^+	71 ± 4
K^+	135 ± 12
Rb^+	207 ± 20
Cs^+	272 ± 30
B. Polyatomic cations	
Hydrazine	56 ± 6
Ammonium	87 ± 5
Choline	>400[a]
Tetramethylammonium	>400[a]
Tetraethylammonium	>400[a]

[a] Limits of detection.

volving modification of at least one carboxyl group at the toxin-binding site.

The effect of saxitoxin (STX) was also investigated. Saxitoxin, the paralytic shell-fish poison, has been shown to be pharmacologically very similar to TTX (Kao and Nishiyama, 1965; Narahashi et al., 1967). Both toxins contain guanidinium groups, which are presumably protonated at neutral pH, and both are believed to bind at the same site on the sodium channel, as indicated by competition studies (Colquhoun et al., 1972). As expected, STX, also at relatively low concentrations, was as effective as TTX in protecting the TTX-binding site, as shown in Figure 6.

Veratridine, the plant alkaloid shown to increase the sodium conductance, presumably by allowing the sodium channels to remain open (Ulbricht, 1969), was found to give no protection against TMO inactivation, even at concentrations as high as 1×10^{-4} M. Although the effects of both TTX and veratridine are directed to the sodium channels, these compounds do not apparently interact with the membrane at the same carboxyl-containing site.

The decrease in toxin binding appeared to result from a reduction in the number of binding sites and not from a decrease in binding affinity. Equilibrium dialysis studies using TMO-treated membranes (1 mg of TMO/mg of protein) clearly showed that while there was no change in the measured dissociation constant as compared to control untreated membranes, the total number of binding sites in the treated membranes was reduced by nearly 50% (J. K. Reed and M. A. Raftery, unpublished observations). The effect of TMO, then, appeared to be an all-or-none phenomenon, methylation of the active site carboxyl group resulting in a total block in the binding of [^{3}H]TTX to the membrane.

Effect of Cations on [^{3}H]TTX Binding. There is considerable evidence to suggest that in isolated nerve fibers, the sodium channels allow passage of other cations in addition to Na^+ (Chandler and Meves, 1965; Hille, 1971, 1972). For example, Li^+ appears to be at least as permeable as Na^+, whereas K^+, Rb^+, and Cs^+ have only limited permeabilities. Hille (1972) has suggested that these discrimination properties may be related not only to such geometric properties as pore size, but possibly also to other molecular or electrostatic determinants in the channel. In particular, a role for a single ionizable carboxyl group in sodium conductance has been postulated (Hille, 1968; Woodhull, 1973). Since the binding of TTX to the receptor site at the sodium channel also appears to involve an ionizable carboxyl group, it was of interest to learn if this functional group displayed any of the cation selectivity properties previously observed for the sodium channel from electrical measurements.

The binding of a number of alkali metal cations and small polyatomic cations to the toxin receptor site was determined from equilibrium dialysis studies, measuring the apparent inhibition constant K_i, from [^{3}H]TTX competition experiments. The effect of each cation on the dissociation constant for the toxin–membrane complex was estimated from a plot, as shown in Figure 7A for Na^+. The binding of [^{3}H]TTX was measured at varying concentrations of the cation, and the observed data were found to fit well the equation

$$\frac{1}{b^*} = \frac{K_d}{B}\left\{1 + \frac{[I]}{K_i}\right\}\frac{1}{[TTX]} + \frac{1}{B} \qquad (7)$$

for competitive binding of an inhibitor [I] with an inhibition constant, K_i. K_i was estimated from a replot (Figure 7B) of the "apparent K_d" or $K_d\{1 + ([I]/K_i)\}$ as a function of [I].

In all cases, chloride was the counterion and the ionic strength was kept constant by the addition of choline chloride; preliminary studies indicated that there was no appreciable binding of choline to the TTX site. For all the cations studied, inhibition of [^{3}H]TTX binding appeared to be strictly competitive.

Table V lists the various cations studied in these experiments, in order of decreasing binding affinities. For the alkali metal cations, the binding affinity followed the sequence $Li^+ \approx Na^+ > K^+ > Rb^+ > Cs^+$ and for the polyatomic cations, the sequence was hydrazine > ammonium $\gg$ choline $\approx$ tetramethylammonium $\approx$ tetraethylammonium.

Discussion

In recent years there has been considerable interest in the molecular basis for the membrane cation permeability changes which characterize chemically and electrically excitable cells. This is particularly exemplified by the current studies in this and other laboratories on the chemical and functional characteristics of the purified AcChR (Schmidt and Raftery, 1972; Meunier et al., 1972; Klett et al., 1973; Karlin, 1973) which is involved in postsynaptic membrane depolarization. Similar studies on the ion channels responsible for the generation of action potentials in nerve and muscle membranes have not yet been possible, since attempts to

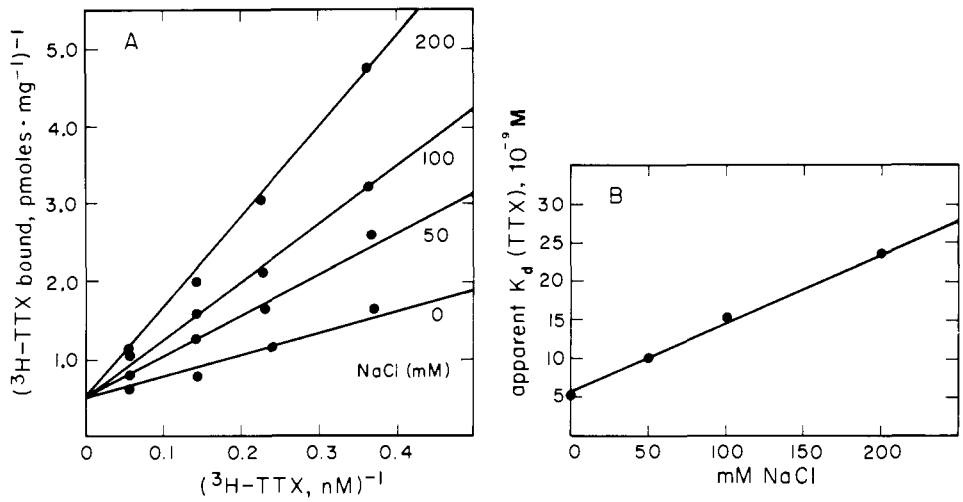

FIGURE 7: Inhibition of [^{3}H]TTX binding to electroplaque membranes by NaCl. The reduction in the binding of toxin was measured at increasing concentrations of NaCl as indicated. The apparent inhibition (or dissociation) constant, K_i, for the cation–receptor complex was estimated from the replot (B) of the double reciprocal plot as shown in (A).

isolate these components have been unsuccessful to date. Nevertheless, it has been possible to indirectly study some of the biochemical features of the sodium-specific channel by using radioactive TTX as a probe for this system.

In the studies reported here, we have used the electroplaque of *Electrophorus electricus* as a tissue source for electrically excitable membranes. Pharmacologically, the electric organ is very similar to skeletal muscle (Keynes and Martins-Ferreira, 1953; Nakamura et al., 1965) and thus represents a unique model system in studies of not only electrical but also chemical excitability functions of biological membranes. The individual cells or electroplaques of the electric organ are asymmetric, only the posterior surface is innervated and excitable and presumably contains the AcChR molecules and sodium channel components involved in the generation of action potentials. The opposite face is inexcitable and contains the cation pump mechanisms, the ($Na^+ + K^+$)ATPase, which functions in maintaining the cellular cation concentration gradients (Schoffeniels, 1959).

The electroplaque membrane fragments used in our studies are apparently derived from both the innervated and noninnervated regions of the cell, as judged by the recovery of the plasma membrane markers, ($Na^+ + K^+$)ATPase, AcChR, and [^{3}H]TTX binding. Recently, we have been able to separate these two populations by repeated sucrose density gradient centrifugation and preliminary studies indicate that these two fractions are not only functionally but also chemically distinct (J. K. Reed, C. D. Linden, and M. A. Raftery, unpublished observations).

Studies on the [^{3}H]TTX binding properties of electrically excitable membranes have in the past been restricted mainly to unmyelinated nerve preparations (see Table II). Although these tissues generally contain more toxin binding sites than the electroplaque, measured on a wet weight basis, the quantities of electric organ readily available for such studies far exceeds that of any nerve or muscle preparation yet described. Furthermore, the density of [^{3}H]TTX binding sites in the isolated electroplaque membranes is sufficiently high, between 2 and 3 pmol per mg of protein, such that many of the chemical properties of this component in the native membrane environment can now be studied using conventional methods such as equilibrium dialysis.

The experiments described here show that [^{3}H]TTX binds tightly, with an apparent dissociation constant of 6 ± 1×10^{-9} M to a homogeneous population of noninteracting receptor sites. The toxin–membrane complex dissociates with a first-order rate constant, k_{-1} of 0.012 sec^{-1}, very similar to that reported by Henderson and Wang (1972) for a detergent-solubilized axonal membrane preparation. More recently, Schwarz et al. (1973) have reported studies on the kinetics of TTX inhibition of sodium currents in isolated nerves. The rate constants obtained, $k_{+1} = 3.3 \times 10^6$ M^{-1} s^{-1} and $k_{-1} = 0.014$ s^{-1}, agree remarkably well with those found here, and may suggest that the TTX-binding site in these different tissues may be very similar if not identical. Disruption of the electroplaque and subsequent fractionation of the membrane have not apparently altered the toxin-binding properties.

The electroplaque membrane fragments are vesicular with an internal volume, determined by loading with $^{22}NaCl$, of between 0.2 and 0.6 μl/mg of protein. From preliminary studies on the efflux of $^{22}Na^+$ from preloaded vesicles, using the procedure described by Kasai and Changeux (1971) we have been unable to show any substantial effect on the flux rate with either TTX or veratridine (J. K. Reed and M. A. Raftery, unpublished observations). Whether this implies any functional perturbations of the sodium channel during the isolation of these membranes remains to be determined.

In recent years electrophysiological studies by Hille and his colleagues using intact nerves have provided convincing evidence for a role of an ionizable group in the membrane which is essential in the in vivo function of the sodium channel during excitation (Hille, 1968; Woodhull, 1973). Because of its low pK, around 5.2, this group is generally thought to be a single ionizable carboxyl, possibly located at the opening of the sodium channel. The recent studies of Henderson et al. (1974) on the binding of [^{3}H]TTX to isolated nerve fibers indicated that a group with a pK of between 5 and 6 is involved in the binding of the toxin to the membrane site and it has been speculated that these two groups may be identical (Hille, 1975).

Our studies on the effect of pH on the binding of [^{3}H]TTX to the electroplaque membranes show that an ionizable group with a pK of 5.3 is also involved in toxin binding in this system and presumably protons and TTX compete for this binding site.

The chemical nature of this functional group has now

been clearly established by the modification experiments with trimethyloxonium tetrafluoroborate, an alkylating reagent which has been shown to specifically modify carboxyl groups in a number of proteins. The modification of the sodium channel carboxyl by this reagent occurred preferentially since only a small percentage of the total exposed carboxyl groups in the membrane were esterified under these conditions. Possibly, the small positively charged oxonium ion resembles the protonated guanidinium groups of the toxin molecule sufficiently to act as an affinity reagent.

Because of this apparent selectivity, TMO may prove to be a valuable tool to study further some of the chemical and physical properties of the sodium channel. Using tritiated TMO, with a high specific activity, it may be possible to introduce a covalent radioactive label directly at the TTX binding site and thus provide a convenient irreversible marker to identify the membrane constituent(s) involved. Such a probe would also greatly facilitate the further purification and chemical characterization of the sodium channel from detergent-solubilized membranes. These studies are currently in progress in our laboratory.

The cation selectivity properties of the sodium channel in intact nerves have been investigated in a number of laboratories. Using squid giant axons Chandler and Meves (1965) found that the relative permeability of the channel to alkali metal cations follows the sequence $Li^+ > Na^+ > K^+ > Rb^+ > Cs^+$. Since this is also the selectivity sequence for cation binding to a high field strength anion, according to the theories developed by Eisenman (1962), it was proposed that the sodium channel contains at least one negatively charged binding site which favors interaction with cations of smaller crystal radius such as Li^+ and Na^+. Similar cation selectivities were reported by Hille (1972), who also extended this model further in suggesting that this high field strength anion is actually the ionizable group with a pK of 5.2, possibly a carboxyl, which is essential in the channel permeability during excitation.

The binding of [^{3}H]TTX to the sodium channel in the electroplaque membranes also requires the presence of an ionizable carboxyl group with a pK of 5.3. Furthermore, the site also binds a number of monovalent metal and polyatomic cations with varying degrees of affinity as shown in Table V. Interestingly, the sequence of binding affinities for the alkali metal cations is identical with the sequence for the relative permeability of the sodium channel determined from electrical measurements on intact nerves; the cations which have the highest affinity also appear to be the most permeant.

Henderson et al. (1974) have recently reported studies on the effects of a variety of monovalent, divalent, and trivalent cations on the binding of [^{3}H]TTX to intact nerves and solubilized nerve membranes. Estimates of the dissociation constant for Na^+ ranged from approximately 0.6 M in the solubilized preparation to greater than 1.3 M in the intact nerve. Similar variabilities were found for Li^+ and K^+, and it was unclear whether there was any discernible order for cation selectivity from these experiments which was consistent for both the intact and solubilized preparations.

In the studies reported here, the binding of monovalent cations to the electroplaque membranes was measured under equilibrium conditions, varying both cation and toxin over a range of concentrations, and the apparent dissociation constants, obtained from these experiments, were highly reproducible from one membrane preparation to another. The cation binding constants for the electroplaque preparations determined from these studies do not agree with those reported by Henderson et al. (1974). The sodium channel site in the electroplaque appears to bind all the metal cations with affinities almost an order of magnitude greater than those found for the nerve preparations.

If this anionic site involved in [^{3}H]TTX binding functions in some capacity as an ion selectivity filter, as has recently been suggested (Hille, 1975), then presumably it may also display similar cation selectivities with respect to binding affinities. The interaction of Na^+ with this site is obviously sufficiently weak to pose no appreciable barrier to the passage of the ion, and yet it is nevertheless strong enough to provide at least some measure of ion selectivity for the channel. The binding of the various polyatomic cations also agrees with this pattern. The channel is considerably more permeable to hydrazine than to ammonium (Hille, 1971); hydrazine also binds significantly tighter to the TTX site. The larger organic cations, which are apparently impermeant (Hille, 1971), do not appear to bind to any measurable extent.

Although the sequence for relative permeabilities agrees well with the sequence for binding to the TTX site, the absolute magnitude of the selectivities do not. While Na^+ binds to the site with an affinity twice that of K^+, the permeability of the channel to Na^+ is nearly 12 times that of K^+ (Chandler and Meves, 1965; Hille, 1972). The significance of this discrepancy is not entirely clear at the present time; however, it should be pointed out that the dissociation constants are determined under equilibrium conditions, while the cation permeabilities are measured under nonequilibrium conditions and probably depend on a number of kinetic factors, including, for example, ion mobilities, as has been pointed out by Eisenman and Krasne (1973).

Recently, Armstrong (1975) has suggested that the sodium channel functions not by preferentially binding cations, as we suggest here, but rather by excluding nonpermeant ions, and that the rate of transport through the membrane depends only on the cation-site association rate constants. Our studies on the electroplaque membranes indicate that the sodium channel site can distinguish between cations in terms of equilibrium binding affinities and while this gives no information as to the individual rate constants for complex formation, it nevertheless describes at least some of the possible geometric or electrostatic determinants of the channel. Although other properties of the channel may be more important in determining ion permeabilities under physiological conditions, it is possible that the interaction of the cation to the TTX-binding site, however weak, may be an obligatory step in the permeation.

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