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Contents

Indexed in Current Contents

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The Binding of Saxitoxin and Tetrodotoxin to Excitable Tissue

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Contents

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I. Introduction

The formulation of the ionic hypothesis of nervous conduction by HODGKIN and HUXLEY (1952; see also HODGKIN, 1964), especially with its emphasis on the voltage-clamping technique, made it clear that the action potential in excitable membrane is generated by two ionic currents: an early sodium current and a late potassium current. The nervous membrane, however, is not uniformly permeable to the two cations, sodium and potassium; instead, the currents flow at certain specialized patches or sites on the membrane rather than across all parts of it. Furthermore, pharmacological evidence shows that the two currents flow across the membrane at two different types of sites. For example, the tetraethylammonium ion completely blocks the late potassium current both in the squid giant axon (by internal application, ARMSTRONG and BINSTOCK, 1965) and in frog myelinated nerve fibers (by application, externally, HILLE, 1967; and internally, ARMSTRONG and HILLE, 1972). Similarly — and more germanely for the present review — the early sodium current can be prevented either by saxitoxin or by tetrodotoxin. The toxin has to be applied externally, for example, to lobster giant axons and frog myelinated nerve (NARAHASHI et al., 1964; HILLE, 1968a), for it is quite ineffective when applied internally to the squid giant axon (NARAHASHI et al., 1966). These two toxins are important, not so much because they are active in extremely low concentrations—they are in fact among the most potent nonprotein poisons known; rather it is because their action is so highly specific. Unlike the local anesthetic agents, for example, which block the sodium current in low concentrations but which in higher concentrations also block the potassium current, the two toxins, saxitoxin and tetrodotoxin, even in relatively high concentrations, have only a single known pharmacological action, namely the specific blocking of sodium channels. It is because of this specificity that the extent to which these toxins are bound to conducting tissue can be used as a measure of the number of sodium channels. They are in fact used as specific chemical markers for this important membrane component of excitable tissue (see RITCHIE, 1975a).

The binding of saxitoxin and tetrodotoxin to excitable membrane is characterized by two parameters that provide information about the sodium channel. The first parameter, the maximum saturable uptake of toxin per unit weight of tissue, M, provides a measure of density of sodium channels in a conducting tissue. If the conductance per unit surface area (or the number of ions crossing this unit surface area per action potential) is known, the conductance per sodium channel (or the ionic flux per sodium channel per action potential) can be determined from the M value. The second parameter, the equilibrium dissociation constant of the toxin-sodium channel interaction, K, provides a measure of the affinity of saxitoxin or tetrodotoxin for the sodium channel. By examining the effects of competitive inhibitors of toxin binding, of changes in environment of the sodium channel, and of chemical alteration of the sodium channel, on the apparent value of K_t for saxitoxin or tetrodotoxin binding, one can infer a great deal about the molecular structure and function of the toxin binding site.

The present review will begin by summarizing, in Sections II and III, the structural and electrophysiological basis for the action of saxitoxin and tetrodotoxin. Early binding studies with both unlabeled and labeled toxin will then be discussed in light of their experimental significance (Sect. IV). Studies with unlabeled toxins first indicated the sparsity of sodium channels in nerve membrane. The densities of channels obtained, however, served only as preliminary estimates because of lack of precision of bioassay for measuring toxin uptake. Early studies with radioactive toxins established that the toxins could be labeled without destroying activity. Complete binding curves were obtained, showing that a saturable maximum component of uptake of radioactivity per unit area of membrane surface corresponding to uptake by sodium channels could be determined and that the dissociation constant, K_t, for this binding could also be assessed. However, results obtained by different laboratories on the same preparation for maximum saturable uptake of toxin per unit area varied greatly. It soon became clear that despite the fact that biochemical tests indicated high radiochemical purity, labeled toxin did contain impurities; errors thus arose in the assessment of the amount of toxin uptake. Methods of toxin labeling and determination of purity will be discussed next (Sect. V). In the light of improved methods for determining the purity of labeled toxin, the binding of toxin to nerve and muscle membranes will be discussed next (Sect. VI and VII). Finally, studies characterizing the sodium channel from measurements of toxin channel affinity under a variety of conditions will be discussed (Sect. VIII to XII).

II. Saxitoxin and Tetrodotoxin

A. Source of the Toxins

To date no satisfactory synthetic substitute for the toxins is available, even though both are rather low molecular weight substances (300–319) of known molecular structure (tetrodotoxin, WOODWARD, 1964; saxitoxin, SCHANTZ et al., 1975). Furthermore, even though the complete synthesis of tetrodotoxin

(KISHI et al., 1972), and of saxitoxin (TANINO et al., 1977) is now possible, synthetic toxin, at the moment, is not generally available to replace the natural product. The toxins currently used are thus of biological origin, and their potency must be verified by bioassay since no chemical tests are available for assaying and identifying them in the concentrations usually used in biological experiments.

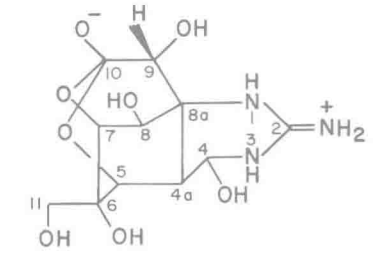
Saxitoxin is produced by a marine dinoflagellate *Gonyaulax catenella* (see SCHANTZ, 1969, 1973; SCHANTZ, et al., 1975). When the conditions of temperature and light are right, this organism reproduces itself (blooms) so rapidly as to discolor the sea, hence the term red tide. Shellfish, especially clams and mussels, feeding on the dinoflagellates at this time become poisonous to man causing paralytic shellfish poisoning. The symptoms of paralytic shellfish poisoning are virtually identical with those of *Fugu* fish poisoning (see below). They are due to the saxitoxin ingested by the shellfish, which is stored for weeks (mussels) or many months (clams), especially in the hepatopancreas or, in the case of clams, also in the siphon. One small mussel may contain 50 human lethal doses.

Tetrodotoxin, the poison classicaly associated with the Japanese *Fugu* fish, occurs in the tissues of at least 40 species of Puffer fish, mostly belonging to the family *Tetraodontidae* (FUHRMAN, 1967). It is most highly concentrated in the ovaries and liver, smaller amounts being found in the intestines and skin. Surprisingly, the identical toxin, originally called tarichatoxin, also occurs in the eggs of various species of Western American newts of the genus *Taricha* (BUCHWALD et al., 1964; MOSHER et al., 1964), in a Pacific goby (NOGUCHI and HASHIMOTO, 1973), and in the skin of some Atelopid Central American frogs (KIM et al., 1975). The biological significance of the toxin in these widely different groups of animals is unknown.

Symptoms of both paralytic shellfish poisoning and *Fugu* fish poisoning reflect the progressive paralysis of the excitability mechanism of nerve and muscle. They begin with numbness in the lips, tongue, and fingertips that may be apparent within a few minutes after eating. This is followed by feelings of numbness and weakness in the legs, arms, and neck, which progress to a general muscular incoordination. In severe poisoning, death occurs from respiratory paralysis. Artificial respiration is the only known antidote. If the patient survives, 24 h prognosis is good with rapid, complete recovery.

B. Structure of the Toxins

Figure 1 shows the structures of saxitoxin (SCHANTZ et al., 1975) and of tetrodotoxin (WOODWARD, 1964). Although the two toxins differ in their chemical structure and one is a monovalent cation while the other is a divalent cation, a certain similarity between the two toxins does exist. For example, tetrodotoxin contains one guanidinium group, and saxitoxin contains two. Guanidinium is an organic cation that is known to be able to pass through the sodium channel, and in sodium-free solution, it can actually substitute for sodium in sustaining excitability (for references, see HILLE, 1971). The presence of the guanidinium moiety seems critical for the pharmacological action of the toxins. In fact, in 1965, KAO and NISHIYAMA were the first to make the now generally accepted suggestion that the



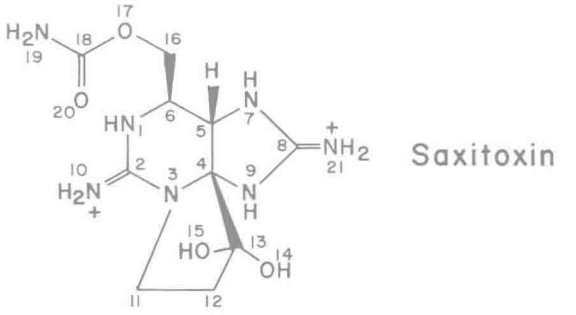


Fig. 1. The structure of saxitoxin (SCHANTZ et al., 1975) and of tetrodotoxin (WOODWARD, 1964)

guanidinium group found in both toxin molecules enters the sodium channel, where it then becomes stuck because the rest of the molecule is too bulky to pass. SMYTHIES et al. (1971) have suggested that tetrodotoxin ties together in a highly efficient manner various parts of the sodium channel. As a result, the passage of sodium ions through the channel is physically prevented and the consequence is a block in conduction. From an analysis of the permeability of various organic cations that pass through the sodium channel, HILLE (1971) proposed that the part of the channel where the toxins finally stick is near a narrow ionic selectivity filter that is approached through a pore 3 Å × 5 Å. About one-quarter of the way into the pore is an anionic group, probably a carboxyl group, that is responsible both for binding the cationic toxins and for binding a variety of metal cations, including sodium. The pore is lined by six oxygen atoms, which hydrogen bond to the toxin molecule stabilizing it in position. According to this model, the sodium channel would function by first binding one of a variety of inorganic or organic cations to the same cationic binding site, each with a different affinity. The selectivity filter then presents another energy barrier, through which the cations pass with a rate determined by the energy requirement for each particular ion. Using this model and studying the permeability of a variety of organic and inorganic cations with voltage-clamp measurements, HILLE (1975a) was able to determine approximate dissociation constants for the cationic binding site and heights of energy barriers for the selectivity filter.

The recent determination of the structure of saxitoxin, first by SCHANTZ et al. (1975) and subsequently by BORDNER et al. (1975), has allowed HILLE (1975b)

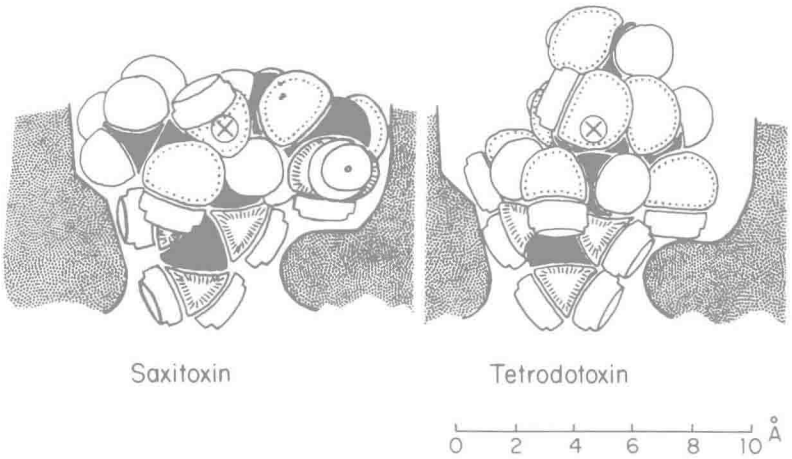


Fig. 2. Saxitoxin and tetrodotoxin on their receptors. The shading on the atoms of toxin represent: carbon, black; hydrogen, white; oxygens, dotted margins; nitrogen, dashed margins. The stippled areas represent the receptor in the sagittal section with the narrow selectivity filter below. Most of the receptor is hydrogen bond accepting, and there is a negative charge associated with the selectivity filter. A circled X has been drawn in the same position with respect to the receptor in two cases. The X falls on a hydroxyl group attached to an unusually electropositive carbon

to refine further the structure of the channels. According to this refinement, the 3 Å × 5 Å opening to the selectivity filter is approached through an antechamber of larger molecular dimensions, about 9 Å × 10 Å. This structure allows the guanidinium moiety of tetrodotoxin or the guanidinium moiety of saxitoxin around the 8-carbon atom to be inserted into the selectivity filter of the pore; in the case of saxitoxin, the second, planar guanidinium group around the 2-carbon remains resting against the pore (Fig. 2, taken from HILLE, 1975b). Such a model helps to explain why even minor changes in the structure of either saxitoxin or tetrodotoxin lead to almost complete loss of biological activity; for example, the low biological activity of alkyl, deoxy, and anhydro derivatives of tetrodotoxin are readily accounted for (HILLE, 1975b) by the loss of hydrogen bonds between modified toxin and sodium channels.

C. Structure and Activity Relationships in Saxitoxin and Tetrodotoxin Derivatives

1. The Effect of pH on Saxitoxin and Tetrodotoxin

Tetrodotoxin can exist in solution either as a zwitterion or as a cation in two forms, all three being in equilibrium with each other (WOODWARD, 1964). The pKa for the equilibrium between the cationic and zwitterionic forms is about 8.8. Similarly, the 2-nitrogen of saxitoxin will be in its cationic form at acidic values of pH. Studies on the effect of varying the pH of the bathing solution on the action of

both saxitoxin and tetrodotoxin have indicated that the cationic form is the one that seems to be important in producing nerve block (CAMOUGIS et al., 1967; OGURA and MORI, 1968; HILLE, 1968a; NARAHASHI et al., 1969).

2. Relatively Inactive Saxitoxin and Tetrodotoxin Derivatives

Most modifications of saxitoxin and tetrodotoxin, except those described in Section B. III. 3 below, are relatively inactive and so only a few examples will be mentioned, rather than discussing the toxin derivatives comprehensively. For instance, in the dihydro derivative of saxitoxin formed by reduction of saxitoxin with hydrogen at 1 atmosphere pressure over Adams platinum catalyst, the two hydroxyl groups at the 13-carbon atom are replaced by hydrogens: dihydrosaxitoxin is not toxic (MOLD et al., 1957). Furthermore, eight derivatives of tetrodotoxin, prepared by degradation of the parent toxin, have been studied both chemically (TSUDA et al., 1964) and pharmacologically (DEGUCHI, 1967; NARAHASHI et al., 1967; OGURA and MORI, 1968). The least active of these degradation products is tetrodonic acid. The most active, deoxytetrodotoxin, is one to five orders of magnitude less potent than tetrodotoxin. Even then, it is not clear how active these derivatives truly are, because although the purity of the crystalline samples of the compounds was verified by infrared spectra, NMR, and mass spectra, etc., the possibility of contamination with trace amounts of tetrodotoxin, which would not be detected by any chemical or physical means, could hardly be eliminated.

3. Pharmacological Active Derivatives of Saxitoxin and Tetrodotoxin

Until recently, all attempts to make structural alterations to either saxitoxin or tetrodotoxin that retained biological activity have been unsuccessful. Such active derivatives would seem to be essential to provide a functional organic group on the molecule that would allow preparation of radioactively or fluorescently labeled toxins. Despite the widespread use of both toxins to block sodium currents in excitable membrane, this lack of active derivatives has impeded progress in characterizing and isolating the sodium channel. For example, the activities of the tetrodotoxin derivatives referred to in Section II. C. 2. were so low that they might well have been due to traces of unreacted tetrodotoxin. Within the last 2 years, however, two extremely exciting findings have been made of active derivatives both of tetrodotoxin (TSIEN et al., 1975) and of saxitoxin (GHAZAROSSIAN et al., 1976). Furthermore, GUILLORY et al. (1977) have just reported the first covalent binding of a tetrodotoxin analogue to sodium channels using an aryl azido photoaffinity label.

TSIEN et al. (1975) have modified tetrodotoxin to give two derivatives, one of which retains considerable pharmacological activity. The first compound, which is virtually devoid of biological activity, is nortetrodotoxin in which the hydroxy and methoxy groups attached to the 6-carbon atom (Fig. 1) are replaced by a ketone group. Activity is, however, restored when the nortetrodotoxin is treated with methoxamine. The resultant compound, the methoxamine of nortetrodotoxin or a tetrahydryl intermediate, is about one-third as potent as tetrodotoxin

itself; such potency cannot be explained by contamination with unreacted tetrodotoxin because the product is formed through the intermediate nortetrodotoxin that contains the biological activity of not more than 0.5% tetrodotoxin. This finding of Tsien et al. (1975), that an active methoxamine product can be formed from a relatively inactive compound suggests that the 11-end of the tetrodotoxin molecule (Fig. 1) has a significant role to play in the binding to the membrane receptor. Previous speculation had attached all the importance to the guanidinium moiety and to the cationic head at the 2-position. Other carbonyl group reactions based on nortetrodotoxin might enable a more detailed mapping of the structure activity of relation of this portion of the molecule. The six position would thus seem to be a reasonable starting point for attaching ligands irreversibly with retention of reasonable pharmacological activity.

Ghazarossian et al. (1976) have similarly been successful in their work with saxitoxin. They have now prepared and described the biological properties of an acid hydrolysis product of saxitoxin that retains biological activity. In this compound, the carbamyl group attached at the 17-oxygen of the saxitoxin molecule (Fig. 1) has been replaced by hydrogen, yielding decarbamyl saxitoxin. This has about the same order of potency as saxitoxin itself — which is perhaps not unexpected since the guanidinium moiety that is thought to insert into the selectivity filter is intact, as also are 12-carbon atom and its environment which remain available for the proposed nucleophilic attack by the receptor. The importance of this first biologically active hydrolysis product of saxitoxin, as with the active derivative of tetrodotoxin of Tsien et al. (1975), is that it opens up the possibility of developing a radioimmunoassay for the toxins as well as affinity chromatographic and affinity labeling techniques for isolating the toxin receptor.

III. Electrophysiological Properties of Saxitoxin and Tetrodotoxin

It has been known for many years that both toxins exert powerful effects on the excitable membranes of nerve and muscle (for reviews see: Kao, 1966; Evans, 1972; Blankenship, 1976). In the mid-1960's voltage-clamp experiments made it clear that these toxins selectively abolish the sodium currents of excitable membrane (squid giant axon, Nakamura et al., 1965a; Moore et al., 1967a; lobster giant axon, Narahashi et al., 1964; Takata et al., 1966; electric eel electroplaque, Nakamura et al., 1965b; and the node of Ranvier myelinated nerve, Hille, 1966, 1968a). Figure 3, which is taken from Hille (1968a), illustrates the fundamental electrophysiological action of the two toxins. Figure 3 shows the voltage-clamp currents in a single myelinated nerve fiber. In the absence of toxin (left-hand record), two components are seen: an early downward deflection due to current in the sodium channels, and a later upward deflection due to current in the potassium channels. In the presence of saxitoxin, the early current is completely abolished while the late current remains unaffected (right-hand record). The effect is rapidly reversed on removing the toxin. This figure also illustrates another important point, namely that the action of the toxin depends on an action on the channel, and does not represent an interaction

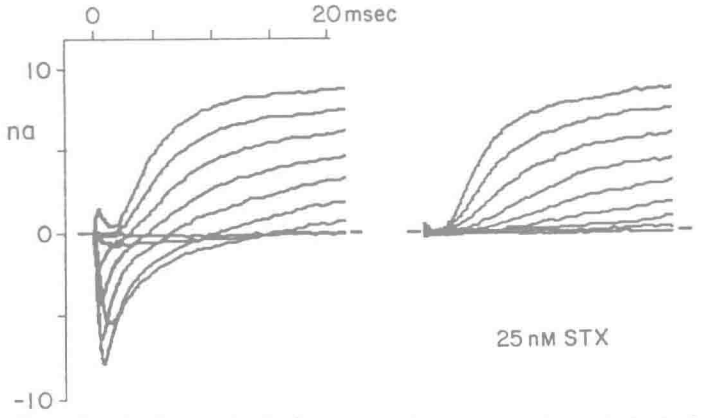


Fig. 3. The effect of saxitoxin on the ionic currents in a voltage-clamped single frog node of Ranvier. A downward deflection indicates an inward current. The records, which were drawn by computer, represent the voltage-clamped currents minus the leakage currents in sodium-free lithium Ringer solution before (left-hand records) and during (right-hand records) treatment with 25 nM saxitoxin. The temperature was 6.5° C. (Taken from HILLE, 1968)

specifically with the sodium ion; for in this experiment the sodium of the Ringer solution had been replaced by lithium. The lithium ion, since it is able to replace the sodium ion as far as the conduction of the spike is concerned, is, like sodium, unable to pass through the channel that has been blocked by the toxin. Other experiments (for references, see HILLE, 1970) again emphasize that it is the blockage of the channel itself that is involved, and neither the particular ion nor its direction of flow through the channel is important. For example, under the appropriate conditions, the sodium currents flowing during a voltage-clamp experiment may be entirely outward rather than inward as normally occurs during an action potential. Even these outward currents, however, are blocked by tetrodotoxin.

Since the action of the toxins is on the time- and voltage-dependent sodium channels, it is not surprising that the toxins have no marked effect on resting potential. For in normal resting preparations these channels are almost entirely closed, $\bar{g}_{Na}$ being close to zero at rest. However, the sodium channels are not *entirely* closed at rest. For example, HODGKIN and KATZ (1947) showed that the resting sodium permeability accounts for 2–4 mV of the resting potential, and FREEMAN (1971) has demonstrated that application of 300 nM tetrodotoxin to the bathing medium hyperpolarizes squid axons by 5 mV. These observations can be readily accounted for if, because of the presence of a drug like veratridine (see below) or for some other reason, some fraction of the channels remains open at rest. In that case, blocking the channels by saxitoxin or tetrodotoxin will lead to a hyperpolarization.

One of the more important findings in HILLE's (1968a) study of the two toxins is illustrated in Figure 4 which shows the dose-response curve of saxitoxin. As HILLE pointed out, this curve is quite consistent with the idea that it is the combination of a single toxin molecule with a single sodium channel that results in the blocking action. Had, for example, two toxin molecules been required

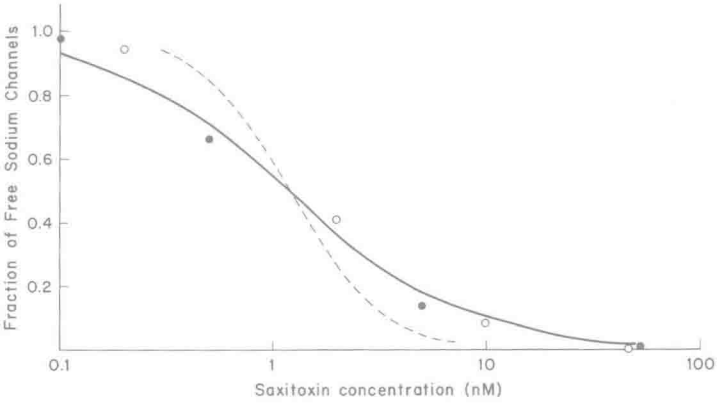


Fig. 4. The dose-response relationship for saxitoxin in frog single myelinated nodes of Ranvier. The ordinate is the maximum sodium conductance at various concentrations of saxitoxin relative to that in normal Ringer solution. The full circles are from experiments in which the potassium currents have been eliminated by 5 mM TEA. The solid line is the theoretical dose-response relationship of a system in which one saxitoxin molecule binds reversibly to its receptor to produce its effect; the broken line shows a similar theoretical relationship if two saxitoxin molecules are required

for each channel, the slope of the dose-response curve (interrupted line, Fig. 4) would have been much steeper at its midpoint than found by HILLE (1968a). Furthermore, in squid giant axons, CUERVO and ADELMAN (1970) showed that the dose-response curve for tetrodotoxin is again fitted quite well by a Langmuir-type dissociation curve.

Subsequent experiments with other tissues have in general confirmed this notion. For example, in systems of small nonmyelinated fibers voltage-clamping is difficult, if not impossible, and so it is difficult to determine how the sodium conductance, $\bar{g}_{Na}$, is progressively decreased by increasing concentrations of toxins. Yet it is important to estimate K_t electrophysiologically in these tissue since many of the chemical studies of toxin-channel interaction depend on the use of small diameter fibers because they contain a huge area of membrane surface. For this reason, COLQUHOUN and RITCHIE (1972a) used a model, based on arguments involving the independence principle and numerical solutions of the Hodgkin-Huxley equations, to show that the effects of various concentrations of tetrodotoxin on the curves relating the height of the action potential or the conduction velocity of the compound action potential to the logarithm of the external sodium concentration are consistent with the idea that one tetrodotoxin molecule binds to, and blocks, a single sodium channel.

All the electrophysiological experiments, therefore, are consistent with the idea that binding or uptake of toxin is Langmuir, hyperbolic, in character. The uptake, U_t, from a given bathing concentration of toxin, $[T]$, is thus given by:

$$U_t = M/(1 + K_t/[T]), \tag{1}$$

where M is a constant equal to the maximum binding capacity of the tissue and K_t is the equilibrium dissociation constant. The value of K_t for the various tissues,

i.e., the external concentration at which half the sites are saturated, is very small, being 0.1–10 nM.

Whether or not binding is Langmuir or whether more than one toxin molecule binds to a single channel are critical questions deserving future study. For all estimates of channel density made so far rely on the assumption of a one-to-one correspondence; and although the evidence for this is reasonable (HILLE, 1968a; CUERVO and ADELMAN, 1970; COLQUHOUN and RITCHIE, 1972a), it is certainly not absolute.

IV. Early Binding Studies

A. Unlabeled Toxin

The earliest attempt to determine the sodium channel density in nerve membrane was made by MOORE et al. (1967b). They used an elegant technique, first used about 50 years earlier to determine the density of glycoside binding sites in heart muscle (see CLARK, 1933). They estimated the amount of tetrodotoxin taken up by a nerve membrane at the time of block and hence determined the sodium channel density. Up to seven lobster nerve trunks were dipped successively in a small volume of artificial sea water containing 300 nM tetrodotoxin. Each nerve trunk bound some of the toxin in the solution, and because of cumulative uptake by the successive nerves dipped into it, the solution was eventually left with an insufficient concentration of toxin to block conduction. After allowing for dilution of the initial toxin-containing solution by the extracellular spaces of the lobster nerve trunks, MOORE et al. (1967b) argued that there were probably fewer than 13 sodium channels per μm^2 of axon in lobster nerve, assuming that each adsorbed tetrodotoxin molecule blocks no more than one sodium channel. Subsequently, using a similar bioassay procedure, KEYNES et al. (1971) confirmed the sparseness of tetrodotoxin binding sites (i.e., sodium channels) in the nonmyelinated nerves of lobster and also of crab and rabbit. For the walking leg nerves of lobster (a different species from that studied by MOORE et al., 1967b) KEYNES et al. (1971) found a channel density of $36/\mu m^2$. For crab it was $49/\mu m^2$ and for the rabbit vagus nerve $75/\mu m^2$. Later studies (see Sect. VI.A.1.) on the density of sodium channels in these nonmyelinated preparations using saxitoxin that had been highly specifically labeled suggests that the true density is higher than that obtained either by MOORE et al. (1967b) or by KEYNES et al. (1971).

Although more reliable numbers for the channel densities in these and other tissues are now available from the later work, these earlier studies and the techniques involved should not be ignored. For it is important to realize that although radioactive studies have largely replaced, because of convenience, the earlier studies based solely on bioassay, all such radioactive studies ultimately depend on bioassay for their reliability. In the absence of any unique chemical identification of either saxitoxin or tetrodotoxin, the radioactive experiments necessarily depend on a bioassay uptake experiment of the kind just described so that the amount

of toxin bound to the preparation can be related to the amount of radioactivity bound, hence yielding the specific radioactivity and radiochemical purity of the toxin.

B. Labeled Toxin

To be quite sure that the toxin taken up by the preparation is bound to the sodium channels and not to some other component requires that the character of such uptake be identical with that of the known electrophysiological interaction between the toxin and the sodium channel derived from voltage-clamp experiments. Thus, one should be able to demonstrate that the binding is Langmuir in type, i.e., the uptake is a hyperbolic function of the bathing concentration of toxin, and the concentration at which sites (M) become half saturated (K_t) should be of the order of a few nanomolar. The uptake, thus, is given by Equation 1. Such complete binding curves are difficult to construct in bioassay experiments of the type described above because each individual determination takes too long for more than a few points to be determined on a single preparation. Furthermore, the bioassay method is limited at both extremes of concentration. On the one hand, at low concentrations, it is difficult to determine the amount of toxin removed by the preparation because low concentrations of toxin (less than a few nanomolar) are extremely difficult to bioassay accurately. And on the other hand, at high concentrations, the bioassay again becomes inaccurate because the limited amount of toxin removed results in very little difference between the final and initial bathing concentrations (which is difficult to determine given the errors inherent in any bioassay). A major advance, therefore, was made when HAFEMANN (1972) showed that it was possible to use the WILZBACH method to label the toxins radioactively. Following this demonstration, COLQUHOUN et al. (1972) determined the complete binding curves of such a tritium-labeled tetrodotoxin to a variety of nonmyelinated nerve fibers (rabbit vagus, lobster walking leg, garfish olfactory nerves). Two components of uptake of labeled compound were always seen: (1) there was a linear nonspecific component of binding, and (2) there was a hyperbolic saturable component of binding that was half-saturated at concentrations of toxin of a few nanomolar, i.e., the same order as the corresponding value determined directly by voltage-clamp experiments in squid giant axons (CUERVO and ADELMAN, 1970) and in myelinated nerve fibers (HILLE, 1968a), and indirectly in small nonmyelinated nerve fibers (COLQUHOUN and RITCHIE, 1972a). Subsequent experiments (see Sect. VI.A.1.) have shown that the absolute sodium channel densities obtained in these initial experiments were too low — presumably because of undetected radioactive impurity in the preparation (see Sect. V.B.). Nevertheless, the fact that a component of the binding curve that saturated at concentrations of a few nanomolar was found strongly suggested that the component being studied was indeed the sodium channels. It should be noted that in subsequent experiments where the labeled toxin was used solely as an indicator of the interaction of various other drugs and cations with the sodium channel (as, for example, in HENDERSON et al., 1974), the fact that the preparation may have been impure does not affect the validity of the conclusions reached

that the toxins seem to act at a metal cation binding site in the sodium channel, nor does it affect the correctness of the calculations of the dissociation constants for the competing cations.

V. Methods of Labeling

A. The Wilzbach Method

In the Wilzbach method (WILZBACH, 1957) of labeling, the toxin is exposed to a highly radioactive tritium gaseous atmosphere for several weeks (HAFEMANN, 1972); or in the modified WILZBACH method (DORFMAN and WILZBACH, 1959), it is exposed for only a few hours but in the presence of an electric discharge (COLQUHOUN et al., 1972). The labeling produced is fairly nonspecific, and considerable breakdown of the irradiated toxin is likely, so that extensive subsequent purification is necessary. COLQUHOUN et al. (1972, 1975) and HENDERSON et al. (1973) pointed out the danger of either the WILZBACH or the modified Wilzbach method of labeling, namely, the possibility that the final preparation may contain radioactivity not just in the toxin but in some other closely related compound produced in the labeling process. These impurities may be difficult to separate by the standard biochemical procedures available.

B. Errors Due to Impurity, Their Assessment, and Their Elimination

The errors that may arise as the result of impurities may perhaps be best seen by considering a hypothetical example. Imagine, for example, that a binding curve is obtained for a milligram of nerve bathed in a variety of concentrations of a labeled, impure toxin solution. Suppose biochemical tests fail to detect any impurity and so the solution is taken to be radiochemically pure; and suppose, further, that bioassay and scintillation counting of this toxin solution show that the radioactivity of the bathing solution corresponds with 90 dpm/f-mol. The preparation shows a maximum saturable uptake of radioactivity of 9000 dpm/mg. If all the radioacitivity is truly associated with toxin, this would correspond with a toxin uptake (hence channel density) of 100 f-mol/mg wet tissue.

However, suppose that two-thirds of the radioactivity in the bathing solution is not in the toxin but in a closely related impurity not detected by biochemical tests (so that the true specific activity of the tetrodotoxin is only 30 dpm/f-mol and not the apparent 90 dpm/f-mol); an uptake of 6000 dpm/mg would response with a channel density of 300 f-mol/mg, i.e., three times the first estimate. Clearly, the first calculation would represent a drastic underestimation of binding sites. It is important to assess directly the fraction of the radioactivity actually associated with the toxin rather than relying on indirect biochemical means to assure the quantitative accuracy of binding experiments.

LEVINSON (1975) first pointed out the problem in assessing the purity of WILZBACH-labeled tetrodotoxin, even when it seems to be radiochemically pure