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Binding of Tetrodotoxin and Saxitoxin to Sodium Channels [and Discussion]

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Binding of tetrodotoxin and saxitoxin to sodium channels

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A useful first step in any chemical characterization of the sodium channels in nerve membrane would clearly be the identification of some measurable property of the channel that does not depend on the intactness of the tissue. To this end, tetrodotoxin and saxitoxin, which bind specifically to sodium channels, have been tritiated and their binding to rabbit, lobster and garfish non-myelinated nerve fibres examined. In each case, a component of the binding curve was found that saturated at concentrations of a few nanomolar. In addition, non-specific binding, indicated by a linear dependence of the amount bound on concentration, occurred. A solubilized membrane preparation from garfish nerve shows the same specific binding component as that of the intact nerve. The saturable component of binding seems to reflect the sodium channel density in nerve, and this is extremely small, being about 27/μm^2 in the rabbit nerve and as small as 6/μm^2 in the garfish nerve.

Introduction

Tetrodotoxin (TTX) and saxitoxin (STX) are the two most potent, non-protein, poisons known. Tetrodotoxin is found in the tissues (particularly the ovaries, muscles, and liver) of various species of the puffer fish *Tetrodon*, i.e. the Japanese *Fugu* fish. Saxitoxin is elaborated by certain species of the dinoflagellate *Gonyaulax*. When conditions of light and temperature are right this organism may multiply so rapidly as to discolour the sea, hence the term 'red tide'. Shellfish feeding on these dinoflagellates concentrate the poison and become poisonous to man, producing paralytic shellfish poisoning.

Although much of the pharmacology of these toxins has long been known (for a review of the earlier literature see Kao (1966)), it was not until comparatively recently that it became clear that the biological effects of these toxins result from a specific inhibition of sodium currents in conducting tissue (for example, lobster giant axon: Takahata, Moore, Kao & Fuhrman (1966); frog nodes of Ranvier: Hille (1968); toad nodes of Ranvier: Schwarz, Ulbricht & Wagner (1973); squid giant axon: Cuervo & Adelman (1970)). The potent and highly specific action of these two toxins has led to an intense study of the molecular mechanism involved, with the hope that some light would be shed on the events occurring in the membrane during excitation (see Hille 1970; Narahashi 1972). These studies have led to the concept that a toxin molecule (T) reacts chemically with a sodium channel (R) according to the relation:

$$T + R \rightleftharpoons TR. \qquad (1)$$

When a channel is combined with a toxin molecule it no longer allows the passage of sodium ions. Voltage-clamp experiments in which the maximum sodium conductance ($\bar{g}_{Na}$) was determined for squid giant axons (Cuervo & Adelman 1970) and for frog nodes of Ranvier (Hille 1970) show that the equilibrium dissociation constant (K) for this reaction is only a few

nanomoles per litre. The experiments reviewed in this paper, which were done to test the validity of this model for small non-myelinated nerve fibres of the rabbit, lobster and garfish, show that there is indeed a saturable component of uptake of these toxins by the non-myelinated nerve fibres that obeys equation (1). Furthermore, the equilibrium dissociation constant for this uptake by the non-myelinated fibres is the same as that determined in electrophysiological experiments. Finally, agents that affect sodium currents in nerve similarly affect binding of tetrodotoxin. It thus seems highly likely that this saturable component of uptake represents binding of toxin to the sodium channels. This being so, the interaction between the channels and the toxin would then provide a means of examining certain important properties of excitable membranes. In the recent work, three questions have been studied.

First, there is the question of the density of sodium channels in excitable membrane. How sparse is their distribution? This question was first examined in lobster nerve by Moore, Narahashi & Shaw (1967), who measured the amount of tetrodotoxin that was removed from a small volume of solution on sequentially bathing in it a series of lobster nerve trunks, each nerve being removed as soon as conduction block occurred. Arguing that the missing toxin had disappeared by being bound to the lobster nerve membrane, thus blocking conduction, they calculated that the density of sodium channels was small indeed, being less than $13/\mu m^2$. Subsequent experiments using a similar bioassay technique, which was modified to improve the reliability of the measurements, confirmed the low density of sodium channels (Keynes, Ritchie & Rojas 1971) in a variety of non-myelinated nerve fibres (rabbit, crab and lobster). However, although in these later experiments the accuracy of the measurements was much improved, no allowance could be made for any non-specific binding of toxin. Making such an allowance requires that the form of the binding curve over a substantial range of concentrations be determined, so that any specific component of binding associated with interaction with the sodium channels can be distinguished from any non-specific binding to the tissues. Unfortunately, determination of this curve with the bioassay technique is impractical because of the long time required to make an individual measurement yielding just a single point on the curve. This difficulty has now been overcome by the use of tritium-labelled tetrodotoxin (Colquhoun, Henderson & Ritchie 1972; Hafemann 1972), use of which allows the binding curve to be determined over a large range of concentrations and the various components of binding to be separated from each other.

A second question concerns the use of these toxins in chemical experiments on the nerve membrane. Clearly, a useful step in any chemical characterization of the sodium channel would be the identification of some measurable property of the channel that did not depend on the intactness of the tissue. Tetrodotoxin and saxitoxin are obvious candidates for marker molecules that could be suitably tagged and followed through the various chemical procedures. A first step in this direction has been the demonstration by Henderson & Wang (1972) that the binding site survives solubilization by the detergent Triton X-100; and this step has already been followed by studies on the stability and biochemical reactivity of the solubilized channel (Benzer & Raferty 1973).

The final question concerns the use of the interaction between tetrodotoxin and the sodium channels to characterize the reaction of the channel with other agents. The principle involved is that another cation or drug, if it binds to the same site as does the toxin, ought to depress toxin binding; and from the nature and extent of the depression of toxin binding, the affinity of the other agent for the binding site can be calculated. For example, Woodhull (1973) has

shown in electrophysiological experiments that hydrogen ions block the sodium currents in frog nodes of Ranvier, apparently by a potential-dependent type of binding to a site in the sodium channel that has a pK_a of 5.5 at zero membrane potential. Furthermore, both calcium ions (Woodhull 1973) and thallous ions (Hille 1972) also reduce the nodal sodium currents. The effect of these cations on toxin binding was, therefore, examined (Colquhoun *et al.* 1972; Henderson, Ritchie & Strichartz 1973). All were found to depress toxin binding, in concentrations in reasonably close agreement with those required to produce corresponding degrees of block in the sodium currents. These three cations thus seem to have a common site of binding and of electrophysiological action. More recent experiments (Henderson, Ritchie & Strichartz 1974) on a variety of other monovalent, divalent and trivalent cations suggest that the site at which the toxins bind is a principal coordination site for cations as they pass through the sodium channel.

Electrophysiological determination of equilibrium dissociation constant for TTX interaction with sodium channels

The nature of the interaction between toxin molecules and the sodium channels depicted in equation (1) is fully characterized in terms of an equilibrium dissociation constant (K) given by $K = [T]\,[R]/[TR]$. An alternative expression for this constant, which is the concentration required to half saturate the pool of sodium channels, can be obtained by rearrangement of the above relation giving:

$$K = [T]/(1/p_f - 1), \tag{2}$$

where p_f is the fraction of sodium channels not combined with toxin. The uptake (U) of toxin is given by the Langmuir, saturable, hyperbolic, relation:

$$U = M[T]/([T] + K), \tag{3}$$

where M is the maximum binding capacity. The surest identification of sodium channels in binding experiments would, therefore, be the demonstration of a hyperbolic component of binding with an equilibrium dissociation constant (K_{bind}) equal to the corresponding constant (K_{ep}) determined in electrophysiological experiments. For giant non-myelinated axons (Cuervo & Adelman 1970) and for nodes of Ranvier of myelinated fibres (Hille 1970; Schwartz *et al.* 1973) the value of K_{ep} is of the order of a few nanomoles per litre. This value was calculated on the basis of equation (2) with p_f being taken to be proportional to the maximal sodium conductance $\bar{g}_{Na}$, which was determined in voltage-clamp experiments. There are, however, no comparable estimates for small non-myelinated fibres because they are too small for voltage-clamping, which is unfortunate since the binding studies are necessarily done on small fibres because of the requirement for a large surface/volume ratio. The value K_{ep} in the larger nerves cannot just be assumed to apply to the small fibres. For even if the binding sites *per se* were identical in the various membranes, structural differences, or differences in surface charge, would be expected to alter the apparent value of K_{ep}. Fortunately, although K_{ep} cannot be determined directly, an indirect approach allows it to be determined in small fibres (Colquhoun & Ritchie 1972*a*).

The method assumes that the small nonmyelinated fibres obey the Hodgkin–Huxley equations, and is based on the independence principle. A computer program, kindly lent by Professor W. K. Chandler, was used to predict how the conduction velocity (or size) of an

externally recorded action potential of a nerve would be expected to vary when the sodium concentration in the bathing medium was reduced. In order to do this, the full Hodgkin–Huxley equations were solved for conduction velocity: the rate constants, conductances, etc., for squid axon at 6.3 °C given by Huxley (1959) were used, the internal sodium concentration being kept constant (or partially reduced). The computation was carried out for a series of values for the maximum sodium conductance, and the family of curves so obtained, relating

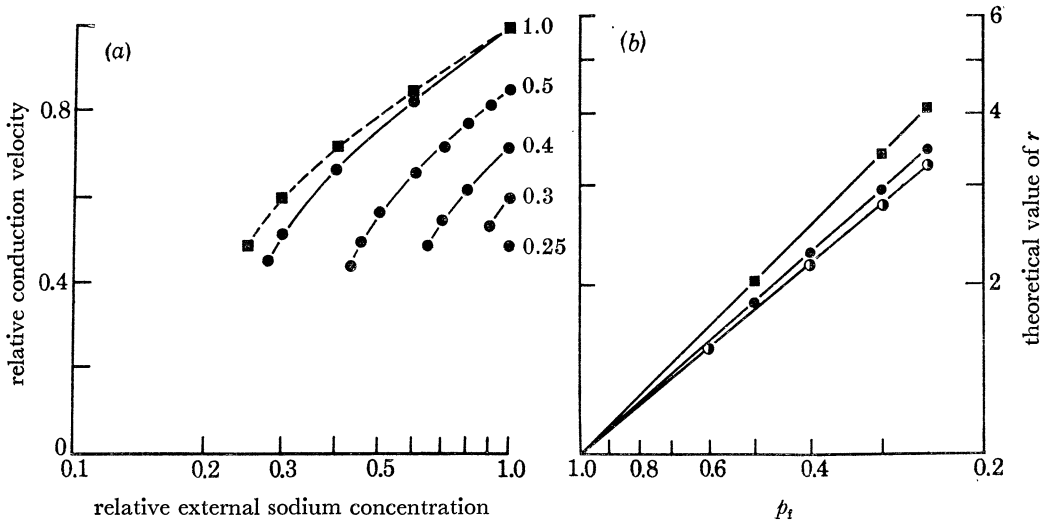

FIGURE 1. Theoretical model based on Hodgkin–Huxley equations.

(*a*) Theoretical relation between relative conduction velocity and relative external sodium concentration. It is usually assumed that p_f is proportional to $\bar{g}_{Na}$. The input values given by Huxley (1959) for squid axon at 6.3 °C were used. ■, $[Na]_i/[Na]_o$ and, hence E_{Na}, assumed constant; ●, internal sodium concentration constant. The fraction of sodium channels not blocked, p_f, is marked at the right of the curves.

(*b*) Theoretical prediction. The value of lg *r* is the horizontal distance between the conduction velocity/lg $[Na]_o$ curves before and after adding TTX so as to leave a fraction p_f of the sodium channels not blocked. ■, $[Na]_i/[Na]_o$ constant. Squid axon at 6.3 °C. Slope corresponds to $n = 1$ (see text). ●, $[Na]_i$ constant. Squid axon at 6.3 °C. Values of lg *r* from figure 1*a*. Slope corresponds to $n = 1.14$. ◑, $[Na]_i$ constant. Squid axon at 6.3 °C except that $\bar{g}_{Na}$, $\bar{g}_K$ and $\bar{g}_{Li}$ all increased threefold from values (Huxley 1959) used for other curves. Slope corresponds to $n = 1.20$. Taken from Colquhoun & Ritchie (1972*a*).

the conduction velocity and the logarithm of the external sodium concentration (expressed as a fraction of its usual value), is shown in figure 1*a* (for $\bar{g}_{Na} = 1.0$, 0.5, 0.4, 0.3 and 0.25 of its normal value). As might have been expected, as the relative external sodium concentration was reduced, the predicted conduction velocity fell. The important point for the present analysis is that the different curves obtained with each different value of $\bar{g}_{Na}$ were all parallel to one another, each curve simply being shifted to the right by a progressively increasing amount (equal to lg *r*) as $\bar{g}_{Na}$ progressively was decreased. For a variety of boundary conditions a plot of lg *r* against lg $\bar{g}_{Na}$ (the latter corresponding with lg p_f) gave a straight line passing through the point (1, 1) in figure 1*b*, which means that:

$$r^n = 1/p_f, \tag{4}$$

where *n* is some constant. A similar analysis, with the height of the external recorded action potential used instead of conduction velocity, gave a similar result (Colquhoun & Ritchie 1972*a*). In theory, therefore, if *n* were known, the fraction of free (unpoisoned) channels can be determined without directly evaluating $\bar{g}_{Na}$ by determining *r* experimentally.

With this theoretical background, experiments using the sucrose gap method (Armett & Ritchie 1960) were carried out on the non-myelinated fibres on the desheathed rabbit vagus nerve to determine how the conduction velocity (or height) of the compound action potential did in practice vary when the external sodium in the bathing medium was progressively decreased from its normal value in Locke solution (154 mM) to nearly zero by being replaced by choline. The relation between conduction velocity (or action potential height) and the logarithm of the external sodium concentration was first determined for the normal nerve.

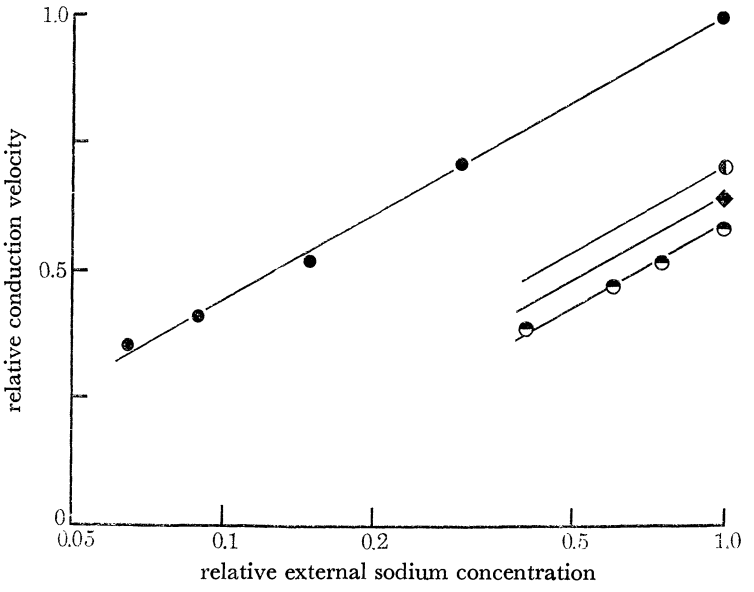

FIGURE 2. The conduction velocity of the peak of the compound action potential of a rabbit desheathed vagus nerve at 31 °C with different external sodium concentrations and in the presence of the following concentrations of tetrodotoxin (nM): ●, 0; ◐, 8; ◆, 18; and ◓, 24. The lines are theoretical lines calculated from equations (4) and (6) (i.e. Langmuir binding of TTX has been assumed) giving the best least-squares fit. Taken from Colquhoun & Ritchie (1972*a*).

It was then redetermined after the nerve had been exposed to one or more concentrations of tetrodotoxin – analagous to decreasing $\bar{g}_{Na}$ in the computer studies. This second relationship was identical to the first except that it was shifted to the right by tetrodotoxin (figure 2), a series of toxin concentrations yielding a family of parallel curves displaced progressively to the right. The experimental results in figure 2 are thus fully consistent with the theoretical model shown in figure 1*b*, which was then used to calculate the fraction of free sodium channels from the relation $1/p_f = r^n$ (Colquhoun & Ritchie 1972*a*). From equations (2) and (4), $K_{ep} = [T]/(r^n - 1)$. The values of r were therefore determined for at least two different concentrations of tetrodotoxin: the latter equation was then solved numerically for both n and K_{ep}.

On this basis the equilibrium dissociation constant for the interaction of tetrodotoxin and the specific receptors associated with the sodium channels in these small mammalian non-myelinated fibres was estimated to be quite similar to the values found in the squid and frog by the more direct methods, being about 3–5 nM at 20 °C. Subsequent experiments on the kinetics of the onset and offset of block in these mammalian fibres by tetrodotoxin fully confirmed this finding (Colquhoun & Ritchie 1972*b*).

The above analysis has only been carried out for the non-myelinated fibres of the rabbit

vagus nerve. However, independent estimates of the value of K, for subsequent comparison with the values obtained in binding experiments, have also been made for garfish olfactory nerve fibres. Recently, Henderson & Strichartz (1974) have measured the efflux of ^{22}Na from garfish non-myelinated nerve fibres. This flux is inhibited by both tetrodotoxin and saxitoxin, being halved by concentrations of 12 nM (tetrodotoxin) and 6 nM (saxitoxin). These results give values for the equilibrium dissociation constant that can then be compared with the corresponding values obtained in binding experiments with garfish nerve (table 1).

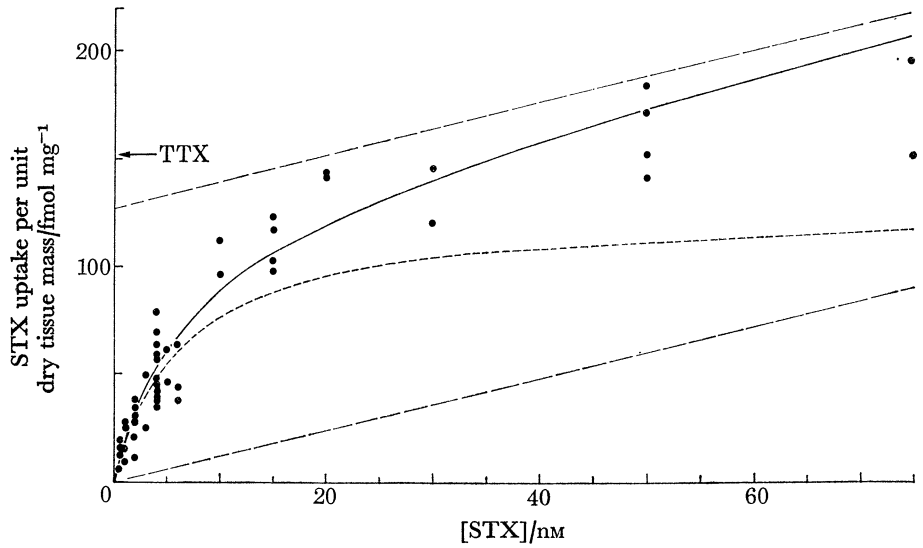

FIGURE 3. The uptake of labelled saxitoxin by rabbit desheathed vagus nerve at different external concentrations of STX. The nerves were equilibrated for 6 h with the STX. The broken line is the asymptote of the binding curve. The total binding curve is the relation:

$$U = \mathrm{STX}_{\mathrm{bound}} = 1.2[\mathrm{STX}] + 127[\mathrm{STX}]/(6.7 + [\mathrm{STX}]),$$

where U is given in fmol/mg dry tissue and [STX] is given in nM. The linear and saturable components are drawn separately. The total binding curve is a least squares fit to the points that are shown, together with others (not shown) at higher concentrations up to 600 nM. Taken from Henderson *et al.* (1973).

THE BINDING OF LABELLED TETRODOTOXIN AND SAXITOXIN TO EXCITABLE MEMBRANES

Preparation and purification of tetrodotoxin

Tetrodotoxin supplied as a freeze-dried powder and free of the citrate buffer that is normally added to stabilize pH, and saxitoxin, generously supplied by Dr E. J. Schantz, were labelled by exposure to a silent electric discharge (Dorfman & Wilzbach 1959). Exchangeable tritium was then removed by dissolving the powder in a small volume of 10^{-3} M acetic acid and freeze drying. It was then purified by electrophoresis (Colquhoun *et al.* 1972; Henderson *et al.* 1973). After purification the final sample was assayed for toxin activity by the bio-assay method described by Keynes *et al.* (1971). The final sample had a specific activity of 100–400 mCi/mmol and appeared identical with the parent toxin by a variety of chemical criteria of purity and homogeneity.

Binding to nerve

Rabbit vagus

The uptake of radioactive saxitoxin by desheathed rabbit vagus nerve during a 6 h soak is shown in figure 3. The continuous line is a least squares fit to the experimental points (weighted as in Colquhoun *et al.* 1972) of the equation

$$U = b[\mathrm{STX}] + M[\mathrm{STX}]/(K + [\mathrm{STX}]). \quad (5)$$

This represents the sum of a linear, non-saturable, component and a component whose maximum binding capacity is M and whose equilibrium dissociation constant is K. For rabbit vagus nerve the value of M for saxitoxin is about 127 fmol/mg dry tissue, similar to the value of 154 fmol/mg dry tissue obtained by Colquhoun *et al.* (1972) for the same nerve using radioactive tetrodotoxin. The dissociation constant is 6.7 ± 3.0 nM. A similar value, of 3 nM, was obtained by Colquhoun *et al.* (1972) for tetrodotoxin.

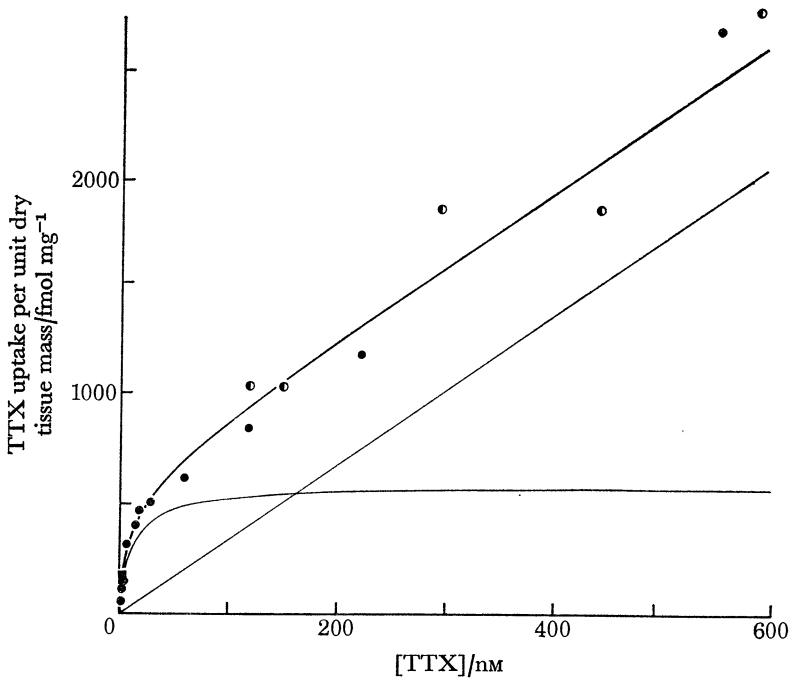

FIGURE 4. The uptake of tetrodotoxin by the olfactory nerve of the garfish at different external concentrations of TTX. The top line is the total binding drawn to obey the relation:

$$U = \mathrm{TTX}_{\mathrm{bound}} = 3.4[\mathrm{TTX}] + 584[\mathrm{TTX}]/(10.1 + [\mathrm{TTX}]),$$

where U is given in fmol/mg dry tissue and [TTX] is in nM. The two components, linear and saturable, are also shown. The equilibration times were (h): ◐, 3; ■ (single point), 6; ●; 12. Taken from Henderson *et al.* (1973).

Lobster and garfish

Similar experiments were carried out on the walking nerve of the lobster, which had been used in previous studies (Moore *et al.* 1967; Keynes *et al.* 1971), and on the non-myelinated fibres of the garfish olfactory nerve (Colquhoun *et al.* 1972; Henderson *et al.* 1973). This latter nerve has the great advantage of being composed of extremely small non-myelinated fibres,

with no myelinated fibres accompanying them and little connective tissue. A single fish can yield about 10–100 times as much tissue as the rabbit and the nerve has about ten times the membrane area per gram of tissue as does the vagus nerve (Easton 1971).

In both nerves a saturable Langmuir-type hyperbolic component of binding was clearly distinguished (figure 4; table 1) with an equilibrium dissociation constant of a few nanomoles/litre.

TABLE 1. SPECIFIC BINDING PROPERTIES OF VARIOUS TISSUES FOR TETRODOTOXIN (VALUES IN PARENTHESES ARE FOR SAXITOXIN)

	non-myelinated nerve fibres			solubilized membrane garfish	muscle, rat diaphragm
	rabbit	lobster	garfish		
equilibrium dissociation constant/nM	3.0 (6.7)	10.2	10.1	6–12† (6–7)	6.1
maximal binding capacity of wet tissue/fmol mg^{-1}	31 (26)	18	60	—	2.5
maximal binding capacity of dry tissue/fmol mg^{-1}	152 (127)	184	584	1100 (1400)‡	—
axonal area per unit mass/cm^2 mg^{-1}	34	71	630§	—	0.7
number of binding sites/μm^2	27 (22)	16	6	—	18

† Various estimates (see Table 1 of Henderson *et al.* 1973).
‡ Per mg membrane protein.
§ Based on a wet/dry mass ratio of 9.67 (Colquhoun *et al.* 1973) and an axonal area of 65 cm^2/mg wet tissue (Easton 1971).

Binding to normal and denervated muscle

Normal muscle

Binding experiments with tetrodotoxin have also been carried out for normal and denervated rat diaphragm (Colquhoun, Rang & Ritchie 1973, 1974), initially on intact hemidiaphragms but later on muscle homogenates to improve reliability. Apart from the great increase in the accuracy of the measurements with the homogenates, there appeared to be no essential difference in the results of the two preparations. In muscle, as in nerve, uptake again consists of the sum of a saturable and a non-saturable component, although the latter is relatively very much larger than in nerve (figure 5*a*). The saturable component is shown more clearly by plotting $U/[\mathrm{TTX}]$ against [TTX] as in figure 5*b*; from equation (5),

$$U/[\mathrm{TTX}] = b + M/(K + [\mathrm{TTX}]).$$

This procedure converts the linear component to a horizontal line with ordinate b; and any saturable component is seen as an upward deviation from this line at low concentrations. Such an analysis suggests that the saturable component has an equilibrium constant (K) of 6.1 nM and a binding capacity of 2.5 fmol/mg wet tissue.

Denervated muscle

One well-known effect of denervation on muscle fibres is to cause them to become sensitive to acetylcholine at regions away from the motor endplate, an effect that is well correlated with the appearance of binding sites for α-bungarotoxin at non-junctional regions of the fibre (Miledi & Potter 1971). Such denervated fibres also become relatively resistant to the blocking

action of tetrodotoxin (Harris & Thesleff 1971; Redfern & Thesleff 1971*a*, *b*), and there is a decrease in the binding capacity of the toxin (Colquhoun *et al.* 1974). The question whether these two phenomena are directly related has been examined by measuring the number of α-bungarotoxin binding sites in this preparation and comparing this with the number of tetrodotoxin binding sites. Such experiments (Colquhoun *et al.* 1973; Colquhoun *et al.* 1974) show that on denervation the number of α-bungarotoxin binding sites increases from a normal value of about 3 fmol/mg wet tissue to about 44.5 fmol/mg wet tissue. The increase in α-bungarotoxin binding, therefore, is much larger than the specific tetrodotoxin binding in normal muscle, which is only 2.5 fmol/mg wet tissue and which is reduced to about half by denervation (Colquhoun *et al.* 1974). These findings thus render unlikely the possibility that the acetylcholine-sensitive receptors that appear after denervation are obtained by conversion of the tetrodotoxin-sensitive channels of normal muscle.

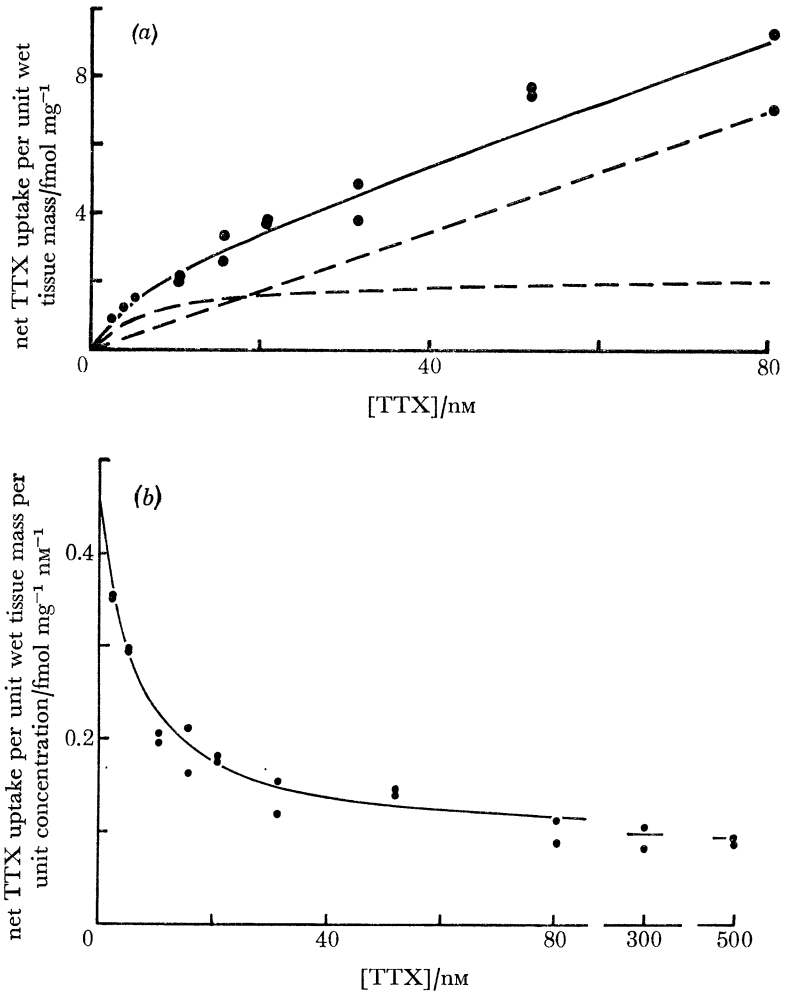

FIGURE 5. (*a*) Binding of tetrodotoxin at equilibrium by homogenized rat diaphragm (innervated) at 20 °C, plotted against TTX concentration. For clarity, only low TTX concentrations are shown. The postulated components of binding are: $b = 0.087$ fmol/mg^{-1} nM^{-1}, $K = 5.89$ nM, $M = 2.19$ fmol/mg.

(*b*) The same results plotted as uptake/concentration against concentration. The curve through the points is calculated from the parameter estimates used in (*a*). At low TTX concentration it deviates upwards from a horizontal line by $M/K = 0.37$ fmol mg^{-1} nM^{-1}. Taken from Colquhoun *et al.* (1974).

The density of sodium channels

Nerve

The uptake of both tetrodotoxin and saxitoxin by the rabbit vagus nerve consists of a saturable component superimposed on a gradual linear uptake that does not saturate. The dissociation constant for saxitoxin is about 7 nM and that for tetrodotoxin about 3 nM. These are similar to the values determined indirectly for tetrodotoxin on rabbit non-myelinated fibres and for the values obtained in voltage-clamp experiments on larger fibres (Hille 1970; Cuervo & Adelman 1970). Although saxitoxin and tetrodotoxin do have some structural similarities, they are entirely different molecules. However, they have the same site of physiological action; and the finding that the binding capacity for the two molecules is similar seems to provide additional evidence that the toxin binding sites are indeed the sodium channels or an integral part of them. Using the value, therefore, of 6000 cm^2/g wet tissue for the axonal area of the non-myelinated fibres of the rabbit vagus nerve obtained by Keynes & Ritchie (1965), one obtains a channel density in this nerve of about 22–27/μm^2. In lobster nerve, where the area of axonal membrane is about 7000 cm^2/g (Moore *et al.* 1967), a channel density of 16/μm^2 is obtained, close to that originally obtained by Moore *et al.* (1967). The largest specific binding capacity per unit mass of tissue was obtained with the garfish olfactory nerve, being 584 fmol/mg dry tissue or 60 fmol/mg wet tissue. Since there is 65 000 cm^2 axonal membrane per gram of nerve (Easton 1971), the channel density must be about 6/μm^2. The sodium site density on all the nerve fibres examined is thus extraordinarily low. For the rabbit vagus the value obtained (22–27/μm^2) corresponds, for a square array, with about 0.2 μm between sites. For garfish olfactory nerve the corresponding value is 0.6 μm between sites, i.e. about $2\frac{1}{2}$ fibres diameters. This might explain why the conduction velocity in garfish non-myelinated fibres is lower than might have been expected from the velocity in rabbit non-myelinated fibres on the basis of a simple change in diameter. According to Rushton's (1951) theory, in which the channel density would be presumably independent of fibre size, one would expect the ratio of the average conduction velocities of rabbit and garfish fibres to depend on the ratio of the square roots of the mean diameters, namely 1.7: in actual fact the ratio is found to be 5.

Comparison of the density of sodium channels with the density of sodium pumping sites in nerve membranes

The evidence is strong that the sodium pump in excitable membranes (or an important component of it) is a sodium/potassium dependent ATPase first described for crab nerve by Skou in 1957 (see Skou 1971 for review). The most decisive single piece of evidence for this is that both the ATPase and the sodium pump are specifically inhibited by cardiac glycosides such as ouabain. This fact has been made use of to determine the density of sodium pumps in a variety of tissues (Baker & Willis 1969; Hoffman 1969), including nerve (Landowne & Ritchie 1970; Baker & Willis 1972; Ritchie & Straub 1975). If it is assumed that a single pumping site is blocked when it combines with a single molecule of glycoside, the amount of drug bound to the tissue when pumping is just completely inhibited must indicate the density of pumping sites. As figure 6 shows, uptake of ouabain by garfish olfactory nerve consists of a linear, non-saturable, component together with a saturable component of binding with an equilibrium dissociation constant of about 0.5 μM and a maximum binding capacity of about 22 pmol/mg dry tissue, i.e. about 300 sites/μm^2. By comparison, the tetrodotoxin binding in

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this same nerve, shown in figure 4, is 584 fmol/mg dry tissue. Thus, in garfish olfactory nerve, as in the rabbit vagus nerve (Landowne & Ritchie 1970), there are about 30 times as many sodium pumps as sodium channels. Similarly, for the squid giant axon the pumps greatly outnumber the channels, by a factor of 10–100 (Baker & Willis 1972).

Muscle

In normal muscle the maximal binding capacity is 2.5 fmol/mg wet tissue. If it is assumed that 75 % of the tissue mass consists of 40 μm diameter fibres of density 1.06 g/cm³, the superficial membrane area would be 0.7 cm²/mg. The density of tetrodotoxin binding sites should, therefore, be about 21/μm² if all the sites were on this superficial muscle fibre membrane. If, however, there are sodium channels in the transverse tubular system, and if we assume that its maximum sodium conductance is about 0.15 times that of the associated surface membrane (Adrian & Peachey 1973), the true density would be about 18 channels/μm² of surface membrane (and about 1 channel/μm² of tubular membrane). The value for the superficial muscle fibre membrane is thus extraordinarily close to that obtained in the mammalian non-myelinated fibres.

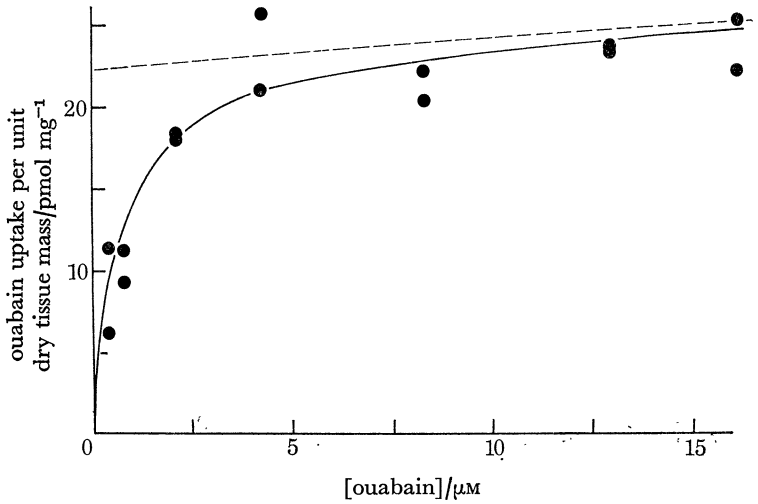

FIGURE 6. The uptake of labelled ouabain by garfish olfactory nerve at different external concentrations of ouabain (Ou). The nerves were equilibrated for 6 h with the ouabain. The broken line is the asymptote of the binding curve. The total binding curve is the relation:

$$U = \mathrm{Ou}_{\mathrm{bound}} = 0.191[\mathrm{Ou}] + 22.25[\mathrm{Ou}]/(0.51 + [\mathrm{Ou}]),$$

where U is given in pmol/mg dry tissue and [Ou] is given in μM. The total binding curve is a least squares fit to the points that are shown, together with several others (not shown) at higher concentrations up to 300 μM. Taken from Ritchie & Straub (1975).

If the maximum sodium conductance for the mammalian muscle is similar to that for frog skeletal muscle, being about 180 mS/cm² at 20 °C (Adrian, Chandler & Hodgkin 1970; Ildefonse & Roy 1972; Adrian & Peachey 1973), the conductance for an open channel must thus be of the order of 10^{-10} S (Hille 1970; Colquhoun *et al.* 1972). This is close to the conductance measured for the ionic channel opened by acetylcholine at the frog neuromuscular junction, which is about 10^{-10} S (Katz & Miledi 1972) or $0.2–0.3 \times 10^{-10}$ S (Anderson & Stevens 1973). It is, however, much greater than the corresponding value for the sodium channels of the squid giant axon, which is about 2.5×10^{-12} S (Keynes & Rojas 1974).

Biochemical studies on the sodium channel

A significant advance in the chemical study of sodium channels was made in 1972, when Henderson & Wang showed that it was possible to solubilize the sodium channels of nerve (by Triton X-100). The full biochemical potential of this work is still to be realized. The studies done so far show that most of the sodium channels originally present in garfish nerve membrane can be recovered in the solubilized preparation. Interestingly, only the specific saturable component of binding remains: the linear component is absent. Thus, without any correction for a linear component, a plot of the reciprocal of the uptake against the reciprocal of the bathing concentration yields a straight line (figure 7). This might mean that the phospholipid, much of which is left behind in the extraction procedure, is involved in non-specific binding. Unfortunately, further steps to purify the extract have so far failed.

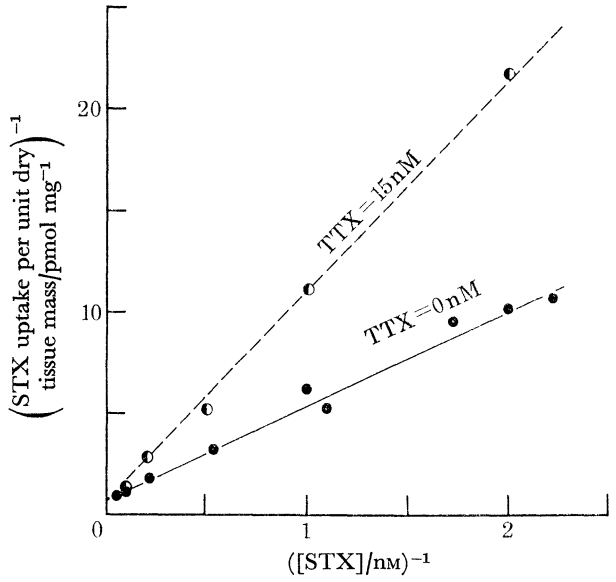

Figure 7. The uptake of labelled saxitoxin by solubilized garfish nerve membranes. Note the double reciprocal plot. The lines are least squares fit to the points in the absence (●) and presence (◐) of 15 nM tetrodotoxin. Taken from Henderson *et al.* (1973).

Using essentially the same technique described by Henderson & Wang (1972) to obtain solubilized sodium channels, Benzer & Raftery (1973) have studied the effect of a variety of agents on the ability of the preparation to bind tetrodotoxin. Upon solubilization the binding component, the putative sodium channel, becomes less stable toward heat and chemical modification and enzymatic degradation. Whereas ribonuclease, deoxyribonuclease, hyaluronidase and neuraminidase have relatively little effect on binding ability, phospholipase C, trypsin, chymotrypsin and pronase markedly reduce binding.

In a related study, Benzer & Raftery (1972) have prepared a homogenate of garfish nerve and obtained a preparation of membranes in which the sodium channels, or at least the ability to bind tetrodotoxin, have remained intact. The various enzymes tested on the solubilized preparation (see last paragraph) were also examined in this preparation of membrane

fragments. Again only phospholipase A and the two proteases, trypsin and pronase, appear to be capable of inhibiting toxin binding. This suggests that the site to which tetrodotoxin binds in the nerve membrane is a protein, or a phospholipoprotein, in nature.

Characterization of the sodium channel

Drugs affecting conduction and the binding site

Equation (1) predicts that the uptake of toxin by the sodium channels is given by

$$U = [T]/([T]+K).$$

If some other agent I also reacts with the sodium channel so that one has

$$I + R \rightleftharpoons IR, \tag{6}$$

the uptake of toxin by the preparation will be reduced to a new value given by

$$U_I = [T]/\{[T]+K(1+[I]/K_I)\}, \tag{7}$$

where [I] is the concentration of this agent (regarded as an inhibitor) and K_I the equilibrium dissociation constant of the reaction in equation (6).

Table 2. The ratio of the total uptake of labelled toxin by rabbit vagus nerve in the presence and absence of various drugs and at low temperature

	toxin concentration nM		no. of expts, n	ratio, δ
lidocaine (1 mM)	TTX	8–76	3	1.03
lidocaine (1 mM)	STX	4	3	1.21
veratrine (50 g/ml)	STX	7–10	6	0.83
batrachotoxin (500 nM)	TTX	8–23	4	0.98
low temperature (10 °C)	TTX	4–7	3	0.95
TTX (500 nM)	STX	4	3	0.14

There is no reason to believe that local anaesthetics or veratrine act at the same site as does tetrodotoxin. It is not surprising, therefore, that neither local anaesthetics nor the veratrine alkaloid affects toxin binding either in intact nerve ($\delta = U_I/U \approx 1$, table 2) or in the solubilized preparation. Batrachotoxin does not affect tetrodotoxin binding; nor does lowering the temperature by 10 °C. However, both tetrodotoxin and saxitoxin would be expected to reduce the binding of the labelled toxin. This expectation is confirmed by experiment (table 2), and so provides an alternative method for evaluating the equilibrium dissociation constants for the toxins by regarding the added toxin as an inhibitor of labelled toxin uptake. Calculations on this basis (equation (7)) yield values that are comparable with those determined directly (Henderson *et al.* 1973).

Cations and the binding site

The fact that the affinity of tetrodotoxin for its binding site is the same as its affinity for the sodium channel determined in electrophysiological experiments strongly suggests that the binding site in the uptake experiments is indeed identical to the site in the sodium channel studied electrophysiologically. Further evidence for this comes from an examination of the effect of various cations on the binding of toxin. In electrophysiological experiments Woodhull

(1973) has shown that hydrogen ions block sodium currents at frog nodes of Ranvier, apparently by a potential-dependent type of binding to a site in the sodium channel that has pK_a of 5.5 at zero membrane potential: the value becomes lower at positive membrane potentials and higher at negative membrane potentials, being about 5.9 at -40 mV, which is thought to be the value for the resting potential in mammalian non-myelinated nerve fibres (Keynes & Ritchie 1965). In good agreement with this, the uptake of toxin is found to be markedly dependent on external pH. As figure 8 shows, binding is much reduced in acidic solutions, being virtually abolished at about pH 4.5. An analysis of this curve (Henderson *et al.* 1973) indicates that protons compete with the toxins for binding to a site whose pK_a is about 5.9.

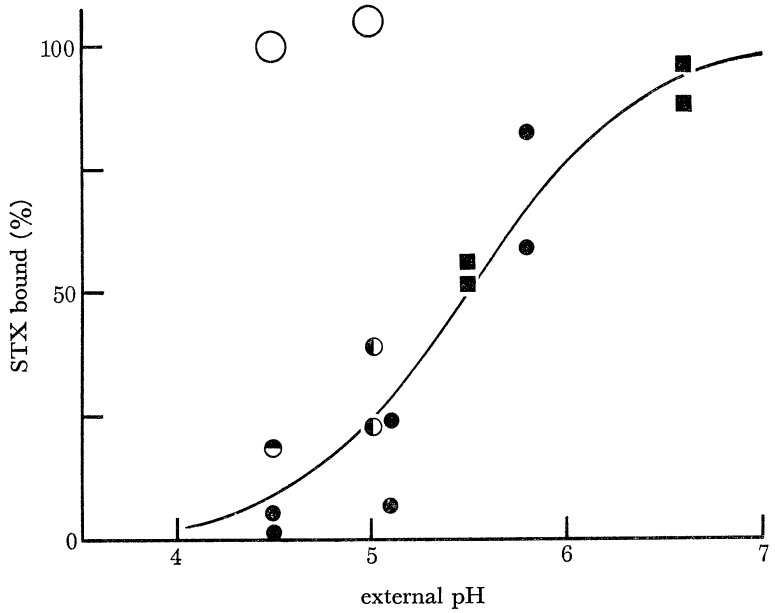

FIGURE 8. The dependence on external pH of the saturable uptake of saxitoxin by the desheathed rabbit vagus nerve. The uptakes at the different values of pH are expressed relative to the uptakes by paired nerves from solutions of the same STX concentration but at pH 7.0. Uptake was measured after 6 h exposure time in solutions containing STX at 6 nM (●), 7 nM (■), 8 nM (◐) and 200 nM (◓) concentrations. The open circles (○) represent the uptakes at pH 7.0 by nerves that had previously been exposed for 3 h in saxitoxin-free solutions at the pH indicated. Taken from Henderson *et al.* (1973).

Furthermore, both calcium ions (Woodhull 1973) and thallous ions (Hille 1972) cause a blockage of the channels in voltage-clamp experiments, the concentrations required to reduce the sodium currents to half being about 20–30 mM. Again, in agreement with these electrophysiological results, calcium and thallous ions compete with the toxins for their binding sites (table 3), the equilibrium dissociation constant for these two cations being 20–30 mM (Henderson *et al.* 1973). Although the values of K obtained were somewhat scattered, they did not seem to depend in any systematic way on either the concentration of labelled toxin, or of the inhibitory cation. There is thus reasonably close agreement between the concentrations of Ca^{2+}, Tl^{+} and H^{+} required to block sodium currents of frog nodes, and the concentrations required to produce corresponding decreases in the binding of the toxins. It is tempting, therefore, to conclude that one is dealing with the same site in all cases; namely a binding site that may be the principal coordination site for monovalent cations as they pass through the sodium channels.

Both tetrodotoxin and saxitoxin would thus seem to act by binding in the channel at this site (see Kao & Nishiyama 1965).

Further demonstration that factors that affect the sodium currents in the channels similarly affect toxin binding must necessarily await further electrophysiological experiments. Binding experiments, however, can continue to be done to characterize the putative sodium channel.

TABLE 3. INHIBITION OF THE SATURABLE COMPONENT OF SAXITOXIN BINDING BY THALLOUS ION THAT REPLACED EQUIMOLAR AMOUNTS OF SODIUM ION IN THE LOCKE SOLUTION

(The ratio of the saxitoxin uptakes in thallous-Locke and sodium-Locke, which is the value of δ_{spec}, was obtained by making the relatively small ($< 10\%$) correction for the linear uptake to the total uptake. K for saxitoxin binding was taken as 6.7 nM. The thallous ion concentrations noted are solution concentrations calculated by accounting for incomplete dissociation of the added thallous nitrate. The inhibitory equilibrium dissociation constant for thallous ion is:

$$K_{Tl+} = [Tl^+]\, K\delta_{spec}/(K+[STX])\,(1-\delta_{spec}).$$

Taken from Henderson *et al.* (1973).)

$[Tl^{+1}]$ nM	[STX] nM	δ_{spec}	K_{Tl} mM
121	5	0.141	11.37
121	3	0.089	8.17
121	4	0.230	22.64
121	4	0.247	24.82
84	10	0.571	44.86
84	10	0.424	24.81
54	10	0.723	(56.55)†
54	10	0.345	11.41
28	3	0.337	9.83
28	3	0.447	15.63
10	3	0.690	15.06
10	3	0.655	12.85
		mean ± s.e.	18.31 ± 3.20

† Value 12 s.e. from mean and therefore excluded.

Recent experiments (Henderson *et al.* 1974) suggest that the same single site in the nerve membrane that binds the toxin is also able to bind at least eight divalent and trivalent cations as well as the monovalent cations, lithium, thallium and hydrogen ions. All the previous electrophysiological observations on blockage of sodium currents by Ca^{2+}, H^+, Tl^+ and Li^+ show concentration dependences that are consistent with cation binding at this same site. In general the affinity of the trivalent cations for the site is very high ($K < 1$ mM), that of the divalent ions moderate ($K \approx 20$–30 mM) whereas the monovalent cations have a relatively low affinity. In the light of these considerations, Henderson *et al.* (1974) have suggested that the site of toxin binding is near the metal binding cation binding site that is the main coordination site for sodium ions as they pass through the channel.

FUTURE EXPERIMENTS

The binding of tetrodotoxin and saxitoxin provide a promising tool for examining the nature of the sodium channel in excitable membrane. It is unfortunate that the electrophysiological determination of the dissociation constant of the small non-myelinated fibres used to compare with the corresponding value in binding experiments was necessarily obtained

indirectly. A direct determination of K electrophysiologically in small C fibres seems unlikely at the moment. However, the recent gating-current measurements in squid (Armstrong & Bezanilla 1973; Keynes & Rojas 1973) indicate a much larger density of sodium channels (several hundred/μm^2) than in the small non-myelinated fibres. It might be possible, in that case even with the presently available labelled toxins, to discern a saturable component of binding to the giant axon of the squid and to obtain an equilibrium dissociation constant, and a maximum binding capacity, that could be compared directly with the corresponding values obtained electrophysiologically. Current attempts to improve the specific activity of the labelled toxins would, if successful, greatly aid such a comparison. Furthermore, a labelled preparation of high specific radioactivity would certainly be useful in any attempt to examine the number of binding sites at nodes of Ranvier, by autoradiographic techniques.

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Discussion

L. G. M. Gordon (*Physiological Laboratory, Downing Street, Cambridge*)

If the blocking position of tetrodotoxin in the sodium channel has been correctly described by Hille then the binding constant which you have measured should be dependent on the membrane potential. Did you investigate the effect of the field on the binding of the poisons, TTX and STX?

[A negative reply followed from J. M. Ritchie.]

There are two possible ways in which the effect of the electrostatic field on the binding

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constants of TTX can be measured. The first method involves pronase which eliminates the inactivation of the sodium channels. In the presence of a sufficiently small concentration of TTX which partially blocks the non-inactivating sodium conductance and with the potassium pathway blocked, the steady-state current–voltage relations of the sodium conductance will contain the essential information. If the TTX binding constant is independent of the electrostatic field then the conductances measured in the presence and absence of TTX will be linearly related but a non-linear relation will give a direct measure of the field-dependent nature of the binding.

The second method utilizes the difference in the time constants for the TTX uptake and the relaxation from a polarized state to the resting state. The former time constant is much longer than the latter so that with the condition of blocked potassium channels, the time course for the sodium conductance after a voltage step from the resting state will depend on the amount of TTX bound and this in turn will be dependent on the pre-pulsed conditioning of the axon (assuming that the TTX binding is dependent on the field). The axon is initially clamped at a potential away from the resting state for a sufficient time to equilibrate the TTX. The clamp is then removed for a short time to allow the axon to regain its activity and a depolarizing voltage step made before the TTX can again equilibrate. The time course of the sodium conductance will be dependent on the prepulsed potential, if, and only if the binding of TTX is independent of the field.

J. F. Lamb (*Physiology Department, Bute Medical Buildings, St Andrews, Scotland*)

I wish to raise a rather different point which might be considered as a fourth part of your discussion. There is evidence that transport systems in other kinds of cells are genetically controlled. I have in mind that the uridine uptake in the amino acid transport system and perhaps density of sodium pumps can be controlled by the cell. Is there any evidence of this kind of control system for sodium channel density? For example, are there any experiments on the effect of protein synthesis inhibitors on the number of binding sites?

[A negative reply followed from J. M. Ritchie].

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