

**Investigations on the Tetrodotoxin Binding  
Component from Electrically Excitable Tissue**

**Thesis by  
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"Words do not express thoughts very well; everything immediately becomes a little different, a little distorted, a little foolish. And yet it also pleases me and seems right that what is of value and wisdom to one man seems nonsense to another."

Herman Hesse

## Abstract

Tetrodotoxin from the Japanese puffer fish was labelled with tritium and purified from the crude mixture obtained. The interaction between the purified [ $^3\text{H}$ ]-tetrodotoxin and membrane suspensions from the olfactory nerve of the long-nosed garfish was investigated using equilibrium dialysis. Tetrodotoxin was shown to bind to membrane suspensions with a dissociation constant  $K_D = 8.3 \text{ nM}$ . The nerve preparation binds 43 picomoles of [ $^3\text{H}$ ]-tetrodotoxin per gm of wet tissue at saturating toxin concentrations. Using various hydrolytic enzymes, the binding component was shown to be a protein embedded in a phospholipid environment. The binding is inhibited below pH 4.0 and is not stable towards heat. Tetrodotoxin binding is not inhibited by the local anaesthetic procaine.

The tetrodotoxin binding component from garfish olfactory nerve membranes was solubilized using the nonionic detergent Triton X-100. Tetrodotoxin binds to the solubilized component with a dissociation constant  $K_D = 2.5 \times 10^{-9} \text{ M}$  and under saturating conditions  $1.95 \times 10^{-12}$  moles of tetrodotoxin are bound per milligram of solubilized protein. Upon solubilization the toxin binding component becomes much less stable towards heat, chemical modification and enzymatic degradation. Sucrose gradient velocity sedimentation

yields an S value of 9.2 for the extracted binding component and from gel filtration data the binding component appears to be slightly larger than  $\beta$ -D-galactosidase.

Tetrodotoxin binding to membrane fragments of the electric organ of Electrophorus electricus was measured and found to be  $25 \times 10^{-12}$  moles of the toxin per gram of wet tissue at saturating conditions with a dissociation constant of  $K_D = 16.7 \times 10^{-9}$  M. Calcium ions at millimolar concentrations were found to inhibit toxin binding to the membrane fragments. The tetrodotoxin binding component was solubilized with the nonionic detergent Lubrol-PX and a convenient assay was developed for measuring the toxin binding to detergent extracts using gel filtration in the centrifuge to separate bound toxin from free toxin. This assay was used to investigate the problem of the stability of the tetrodotoxin binding component in the detergent extract.

Two derivatives of tetrodotoxin were covalently linked to Sepharose-2B in an attempt to synthesize an affinity resin for purification of the tetrodotoxin binding component. It was found that the columns did not display properties which would make them useful since they effected only a minor purification of the binding component with low yields of activity.

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## Abbreviations Used

ATCase	aspartate transcarbamylase
ATPase	adenosine triphosphatase
BTX	batrachotoxin
CSU	catalytic subunit of ATCase
DDT	1, 1, 1-trichloro-2, 2-bis(p-chlorophenyl) ethane
NMR	nuclear magnetic resonance
PALA	N-(phosphonacetyl)-L-aspartate
RSU	regulatory subunit of ATCase
SDS	sodium dodecyl sulfate
STX	saxitoxin
TEA	tetraethylammonium
Tris	tris(hydroxymethyl) aminomethane
TTX	tetrodotoxin

## INTRODUCTION

### Axonal Conduction

When a nerve cell is stimulated in the appropriate fashion, either in vivo or in vitro, a pulse of electrochemical activity is initiated which propagates along the axon. This electrical pulse in turn activates the nerve endings which produce the correct stimulation for exciting or inhibiting the nerve cells adjacent to them. This is the basic unit of activity in the nervous system allowing organisms to perceive and react to stimuli.

It was suggested by Bernstein (1, 2) that the electrical activity of certain cells is due to specific ion fluxes between the inside and the outside of the cell and that some sort of semipermeable membrane separates the inside from the outside of the cell. Later experiments have shown that the above suggestion is indeed the case for nerve axons, and Hodgkin and Huxley in 1952 (3) showed that sodium and potassium ions were the specific ions involved in the permeability mechanism. Hodgkin and Huxley (4-6) went on to develop a precise mathematical model which describes the transient permeability changes towards sodium and potassium arising during an action potential. For the purposes of this discussion a detailed explanation of the Hodgkin and Huxley model is not necessary but the physical



significance suggested by the model is of utmost importance in understanding current theory of axonal conduction.

In describing the events taking place during the propagation of an action potential, it would be very helpful to be able to follow the ion fluxes across the membrane, rather than the potential difference which is usually measured. A method for achieving this has been developed by Cole (7) and Hodgkin et al. (8). The method is called voltage clamp and although it was first used on the giant axon of the squid, it has subsequently been applied to several other preparations including a myelinated nerve. A length of axon is dissected and placed in the apparatus. The interior of the axon is short circuited to abolish the cable properties of the axon by inserting a conducting wire the length of the preparation. This is possible with the squid giant axon because it is approximately 1 mm in diameter. A feedback circuit is set up which supplies any necessary current to maintain the potential difference across the membrane at a set value. This procedure allows one to select a potential difference across the membrane and follow the current necessary to maintain that given potential difference.

Normally the potential difference across the membrane, outside minus inside, is from -70 to -90 mv. This is called the resting potential. If the axon membrane is depolarized to some threshold, approximately -40 to -30 mv, by a post-synaptic potential or

electrical stimulus the axon becomes excited and propagates an action potential. Using voltage clamp one follows the currents produced without having to deal with the explosive nature of the action potential. The axon is held at the resting potential and is step depolarized, let us say, to 0 mv. When this is done it is necessary to apply, first a transient inward current then a steady outward current to maintain the 0 mv potential difference. When the membrane is repolarized to the resting potential the steady outward current, necessary to maintain the 0 mv potential, decays rapidly to zero. Application of this technique to the study of the electrical properties of the axon has led to the following picture of an action potential. In response to a depolarization of the membrane, a transient (about one millisecond) influx of cations generates the action potential which is followed by a transient efflux of cations repolarizing the membrane to the resting potential.

Voltage clamp experiments performed in various external media have led to the conclusion that in squid the early transient influx is carried by sodium and the late steady efflux current by potassium ions. These selective and transient increases in ion permeability have been attributed to the opening and closing of certain ion specific channels located in the nerve membrane. These channels have the property of selectively increasing the permeability of the membrane

towards certain ions. It is the purpose of this report to describe experiments which have helped to elucidate some molecular properties of the sodium ion specific channel in the nerve membrane.

Much confusion has been created as a result of the naming of these ion channels. I will attempt to clarify the confusion, not by renaming the channels, but by indicating the source of confusion. The greatest confusion arises from the fact that, although the ion channels are specific, they are not 100% specific for their respective ions. Thus we find that potassium can pass through the sodium channel since its relative permeability through the sodium channel is  $1/12$  that of sodium (9). Furthermore, it has been found that the ammonium ion can substitute equally as well in the sodium or the potassium channel. Once the realization is made, that the "sodium channel" is just a short-hand name for the "early activated ion channel normally carrying sodium influx," and the "potassium channel" is the "late activated ion channel which normally carries potassium efflux," the confusion disappears.

### The Sodium Channel

Having postulated the existence of the sodium and potassium channels, we can turn our attentions towards their characterization. Are sodium and potassium channels really distinct species, separate

from each other? How does the external medium affect the functioning of the channels? Are the channels actually pores through the membrane, and if so, can they be plugged up? How is the opening and closing of the channels controlled? How many channels are there in a given area of axon membrane? Finally, can the channels or some part of them be isolated as a unique molecular or macromolecular species? In tackling these questions the first approach would be to characterize the channel in situ by monitoring the action of the channel as a function of various external perturbations. By examining the effect of the various perturbations on the channel it might be possible to infer some of the basic characteristics of the channel.

Bertil Hille of the University of Washington has initiated such a study. Hille (10, 11) has concentrated on the sodium channel and has used a myelinated axon from the frog sciatic nerve for his experiments. He has been able to determine the permeability of the channel towards a large number of organic cations. He uses these results to infer a possible structure for the selective ion discriminator in the sodium channel, which lets sodium pass but holds back most other ions. Hille used the voltage clamp method modified for myelinated fibers to determine the flux through the early transient channel, i.e., the sodium channel. He compared these results to those when the node of Ranvier was bathed in a modified Ringers solution in which

the sodium concentration was completely replaced by the given test ion. Hille found that the sodium channel is permeable to a number of small organic cations. These cations are able to substitute for sodium in the following order of effectiveness: sodium<sup>⊕</sup>  $\approx$  hydroxylamine<sup>⊕</sup> > hydrazine<sup>⊕</sup>  $\approx$  ammonium<sup>⊕</sup>  $\approx$  formamidine<sup>⊕</sup>  $\approx$  guanidine<sup>⊕</sup>  $\approx$  hydroxy-guanidine<sup>⊕</sup> > aminoguanidine<sup>⊕</sup>. However methylated derivatives of the above permeant ions are not permeable through the sodium channel, e.g., N-methylhydroxyl amine<sup>⊕</sup>, methyl hydrazine<sup>⊕</sup>, methylamine<sup>⊕</sup>, etc. Larger organic cations such as trimethylamine<sup>⊕</sup>, choline<sup>⊕</sup>, imidazole<sup>⊕</sup>, etc. are also impermeant. From these data Hille proposes a model for the selectivity filter of the sodium channel. His model allows for the sodium ion to remain hydrated with up to three water molecules as it passes through the filter. The filter could then have a three ångstrom by five ångstrom opening which is lined with oxygen atoms. One of the oxygen atoms would be in the ionized state in order to form an electrostatic bond with the positive charge of the permeant cation. The remaining oxygens would be available to form hydrogen bonds with the water molecules, for the inorganic permeants, or the NH or OH functions on the organic cations. Groups with methyl substituents, 3.8 Å across, would not be able to enter the binding site of the filter. Hydroxyl and amino groups, 3.7 Å across, would be able to pass through since the formation of a hydrogen bond

allows for approach between atoms of up to  $0.9 \text{ \AA}$  closer than the Van der Waals contact.

Chandler and Meves (12) measured the permeabilities of inorganic ions through the sodium channel of giant squid axons. The relative permeabilities were as follows:  $\text{Na}^+$  1,  $\text{Li}^+$  1.1,  $\text{K}^+$   $1/20$ ,  $\text{Rb}^+$   $1/40$ ,  $\text{Cs}^+$   $1/61$ . Hille (9) has explored the inorganic cation specificity of the sodium channel in myelinated fibers and confirmed the work of Chandler and Meves (12). The permeability sequence is as follows:  $\text{Na}^+ \approx \text{Li} > \text{Tl}^+ > \text{K}^+$ , where the relative permeability of  $\text{K}^+$  to  $\text{Na}^+$  is 1:12. The permeabilities of  $\text{Rb}^+$ ,  $\text{Cs}^+$ ,  $\text{Ca}^{++}$ , and  $\text{Mg}^{++}$  are too small to measure. The model proposed above accommodates the inorganic ions in the partially hydrated state and therefore does not discriminate between them solely on a basis of size. Hille suggests that the selectivity sequence may be a result of ion-exchange phenomena as the cations interact with the ionized oxygen in his model. Previous work by Hille (13) has lent certain credibility to his assertion that the sodium channel must have an ionized oxygen atom to interact with the positive charge on the permeant ion. He studied the maximal sodium conductance of the sodium channel in a myelinated nerve fiber as a function of pH and found that below pH 6 the sodium conductance decreases rapidly but reversibly. When the maximal sodium conductance is plotted versus pH a titration curve is obtained

which indicates that the protonation of an acidic group with a  $pK_a$  of 5.2 blocks conduction through the sodium channel. It is possible that this group is the ionized oxygen postulated in Hille's model for the selectivity filter. This result further indicated the possibility that the sodium channel is at least partially protein in nature since a  $pK_a$  of 5.2 is quite reasonable for the carboxyl group on a protein.

### Toxins

In attempting to understand the molecular basis of life, scientists in recent years have found that man-made dissecting tools, i.e., dissecting scalpels, microscopes, etc., have not been fine enough to dissect molecular structure. They turned to nature who had already provided them with the tools, genetics and mutations, for fine molecular dissection. More recently this fine scalpel of genetics has been turned towards the dissection of the nervous system, and with some luck should yield some important results. I think, however, that the molecular surgical tools of nature have not yet been used to the fullest and that with the careful exploitation of certain highly neurotoxic agents we will be able to use a very intense molecular "flash light" to explore the molecular biology of the nervous system.

Two figures of voltage clamp data produced by Hille (14, 15) (Figures 1, 2) give a striking visualization of this effective molecular

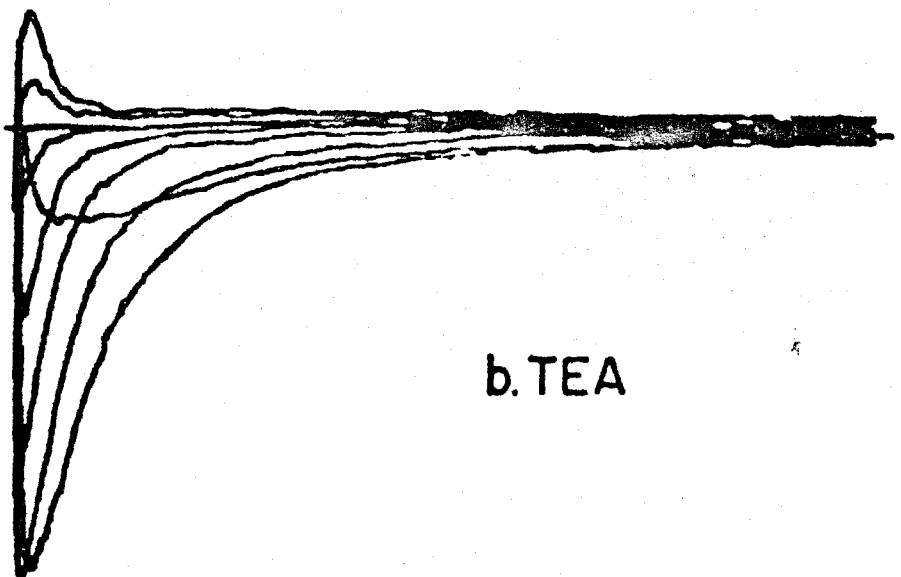
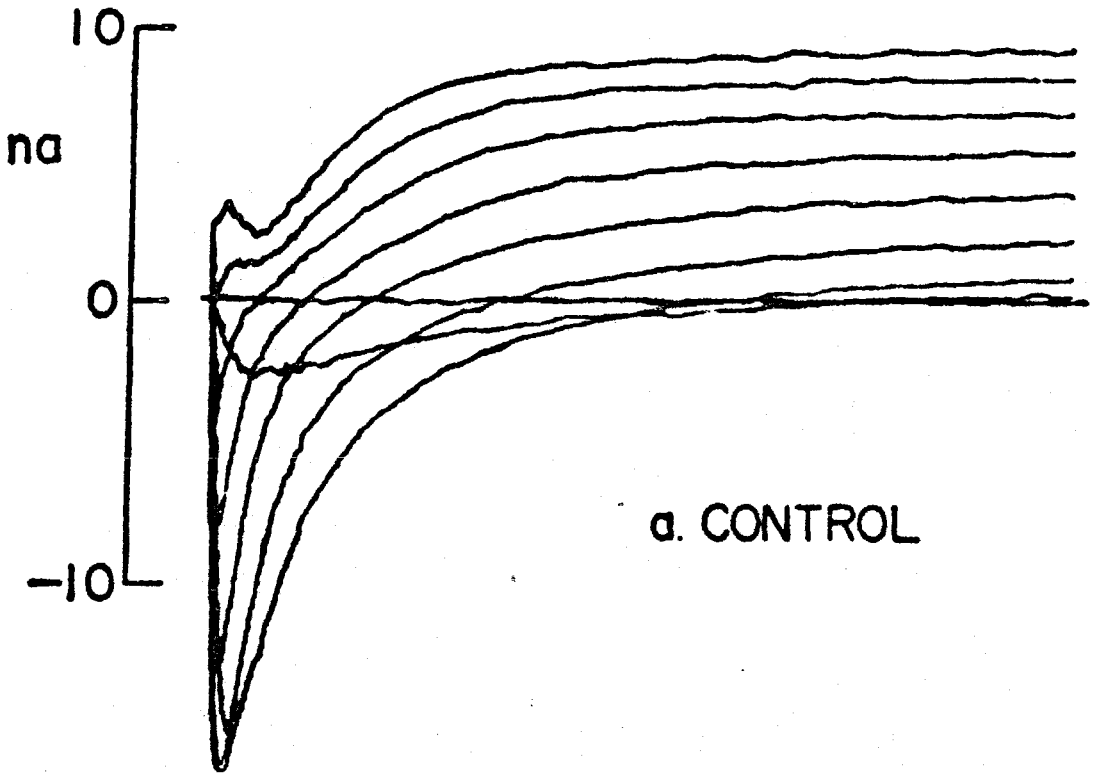
Figure 1. Voltage clamp currents (minus leakage current) in a node of Ranvier in a) Ringer's solution and 6) 6 mM TEA. Hille (15)



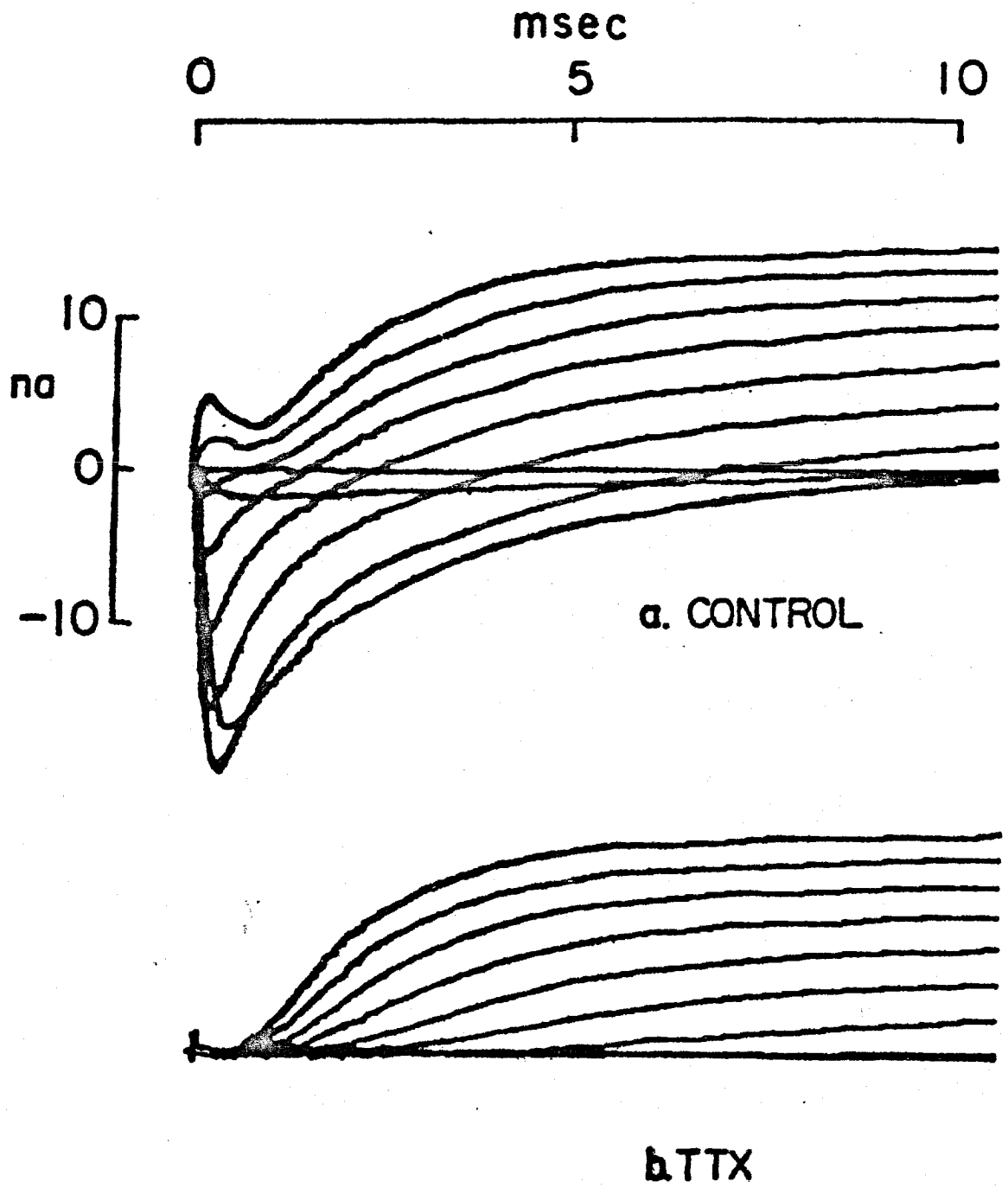
10

msec

0 5 10 15 20



**Figure 2.** Voltage clamp currents (minus leakage currents)  
in a) Ringer's solution and b)  $3 \times 10^{-7}$  M TTX.  
Hille (14)



dissecting tool. In the figures a "family" of voltage clamp traces is shown. They are produced by successive step depolarizations from the resting potential of the nerve membrane to a range of potentials from -60 mv to +75 mv. The figures show the traces obtained in the presence and absence of a drug and a toxin in the perfusion medium. A negative value of current corresponds to an inward flow of cations and a positive current to an efflux of cations from the nerve axon. The traces clearly indicate that tetrodotoxin (TTX) completely abolishes activity in the early transient channel (sodium channel) and that tetraethylammonium (TEA) abolishes current through the late steady channel (potassium channel). These two agents, as well as several other potent neurotoxins, have been well characterized by neurophysiologists as to their specific effect on the conduction of impulses along the axon.

#### Action of the Toxins

The neurotoxins which act specifically on the conduction of impulses along the axon can be separated into a few categories. First, there are those toxins which block the channels, rendering them impermeant to the normal ions. Of these, tetrodotoxin is the most well characterized. The liver and ovaries of the Japanese puffer fish and the eggs of the California newt are the only sources of the

toxin. Presumably this is a good example of convergent evolution, since no other species have been shown to contain TTX. Another sodium channel blocker is saxitoxin (STX) which is the toxin responsible for paralytic shellfish poisoning. This toxin is actually synthesized by a dinoflagellate which is ingested and concentrated by shellfish during certain seasons. Both these toxins are able to block the sodium channel at nanomolar concentrations and with great specificity. Moore et al. (16), Narahashi et al. (17), and Hille (18) have shown that TTX and STX have almost identical actions on myelinated and non-myelinated nerve fibers. The two toxins were able to block the early transient channel even if sodium substitutes, i.e., lithium, were used as the permeant ion. This indicates that the toxins are channel specific rather than sodium ion specific. The potassium channel activation and inactivation remained intact even at 100 times the concentration of the toxins needed to block completely the sodium channel. The toxins act by decreasing the conductance of the membrane towards sodium ions since the kinetics of channel opening and closing remain normal during partial block by the toxins. When the fraction of sodium current remaining in an intoxicated preparation was plotted versus the external toxin concentration, Cuervo and Adelman (19) obtained a dissociation constant for the half maximal block of the squid axon by TTX of  $3.31 \times 10^{-9}$  M. Hille (18) obtained

$1.9 \times 10^{-9}$  M for half maximal block of a myelinated fiber by STX.

The dose response curve in both cases indicates a one to one interaction between the toxin and the sodium channel. The action of the toxins is reversible upon washing with buffer, although Hille (18) and Narahashi et al. (17) find that STX blockage is more readily reversible than TTX blockade. It has been found by Narahashi et al. (20) that the water soluble TTX is not effective in reducing the sodium conductance of the giant squid axon when the toxin is perfused internally even at a concentration of 100 nM which is sufficient for complete blockade when the TTX is perfused externally. This result provides strong evidence that the toxin binding site is located exclusively on the outside face of the nerve membrane and that the TTX molecule is not permeable across the membrane. Besides TTX and STX there are several other drugs which inhibit flux through the sodium channel. These drugs act at concentrations much higher than the nanomolar concentrations necessary for TTX or STX action, and the effects of the drugs are much less specific than the toxins. Hille (14) showed that the effect of the local anaesthetic xylocaine and the tranquilizer, compazine, on myelinated axons is the reduction of the current carried by the sodium channel. The effects are considerably less specific than TTX since at 3.3 mM, xylocaine blocked the potassium channel by 75%. These drugs seem to have no effect upon

the channel's kinetic parameters of opening and closing.

There are no highly toxic compounds which specifically block the potassium channel. However tetraethylammonium acts at millimolar concentrations to block the efflux through the potassium channel. Hille (15) has found a dissociation constant for TEA block of 0.4 mM in myelinated fibers. It was found that the giant squid axon is insensitive to 100 mM TEA in the external perfusion media but that the potassium channel is blocked by 4 mM TEA perfused internally (21, 22).

The second important class of toxins are those which act selectively on the kinetics of the ion channels. Condylactis toxin from the Bermuda anemone, Condylactis gigantea, is a protein of molecular weight 10-15 thousand, which acts to prolong the open state of the sodium channel in the crayfish axon (23), when applied in micromolar concentrations. The effect is not well characterized and in the squid giant axon the toxin has no effect at all. Scorpion venom prolongs the open state of the sodium channel (24), but it has multiple effects such as depressing the conductance of the potassium channel. Finally, there is batrachotoxin (BTX), isolated from the Colombian poison arrow frog, Phyllobates aurotaenia, which is four times as lethal as TTX (25). BTX acts on squid axons by slowly and irreversibly depolarizing the nerve membrane (26). BTX is quite effective

upon both internal and external application. It depolarizes the membrane by irreversibly opening sodium channels. In an experiment with squid axon (26) the effect of TTX upon BTX intoxicated fibers was found to be reversible repolarization of the cell membrane. That is, when TTX was applied to the BTX depolarized cell, the cell repolarized to the resting potential; and when the TTX was washed out the cell was depolarized again. This result suggests that BTX and TTX act at different sites and that the TTX molecule is effective in blocking the sodium channel which the BTX molecule opens up. Albuquerque et al. (27) performed some preliminary experiments, using various sulfhydryl and disulfide reagents, showing that TTX and BTX probably bind to different sites on the membrane. When lobster axons are treated with millimolar concentrations of N-ethylmaleimide or p-chloromercuribenzoate for five minutes the action potential and resting potential are affected only slightly. The axon is now resistant to the application of BTX. The action potential remains and there is no further depolarization of the membrane. On the other hand, pretreatment of axons with these reagents is not able to prevent the effects of TTX applied to the nerves. These results indicate that the BTX binding site is a protein in the nerve membrane and furthermore that certain sulfhydryl groups must remain intact for BTX to have its effect. Indeed, it is not surprising that the specific and complex tasks involved



in the workings of the sodium channel are performed by a protein.

The insecticide DDT and the alkaloidal drugs veratridine and veratrine also act on the kinetic parameters of the channels. Hille (18) explored the action of veratrine and DDT and found that these drugs prolong the open state of the sodium channel when the channels are in the active form. Veratrine causes the channel to remain open about one hundred times as long as the DDT prolonged channel opening. Therefore, at rest the veratrine membrane is depolarized while the DDT treated membrane is not. The effect of veratrine, like that of BTX, can be reversed by TTX.

### Structure of the Toxins

Considerable effort has been required in order to elucidate the structures of the three potent toxins TTX, STX, and BTX. These three toxins are the most potent nonprotein toxins now known, the toxic protein Botulinum toxin being one of the few molecules which is more lethal. The determination of the structure for each toxin was beset with its own special problems. TTX contains an organic functional group, hemilactal, never before encountered in a natural product. The determination of the structure of BTX was beset with problems of obtaining sufficient amounts of the active venom which can only be obtained in the jungles of Colombia (25). And the determination of

STX had to overcome the problem of its noncrystalline, highly polar and nonvolatile nature, which made the use of X-ray crystallography, and mass spectroscopy impossible (29).

The structure of TTX, determined independently by Woodward (28) and Tsuda et al. (30) was shown to be a water soluble molecule with a  $pK_a$  of 8.5-8.8. This acidic function is due to the dissociation of an OH group in the hemilactal function. At neutral pH the molecule has a positive charge due to a guanidinium group. Furthermore, the hemilactal function was shown to be in equilibrium with a hydroxy-lactone form (see Figure 3). Recently D, L-tetrodotoxin has been synthesized by Kishi et al. (32) confirming the structure proposed by Woodward (28) and Tsuda et al. (30).

BTX was found to be the ester of a steroidal alkaloid and a pyrrole carboxylic acid (31), having a  $pK_a$  of 7.4 (25) and the structure shown in Figure 4.

Only quite recently has a definitive structure for STX been proposed by Wong et al. (29). The OH group was found to have a  $pK_a$  of 8.24 and the guanidinium groups a  $pK_a$  of 11.6 (see Figure 5).

TTX and STX have very similar effects upon the sodium channel, therefore a comparison of their structures should indicate the similarities necessary for effective blockade of the sodium channel. Both have an acidic OH group with a  $pK_a$  of about 8, a guanidinium

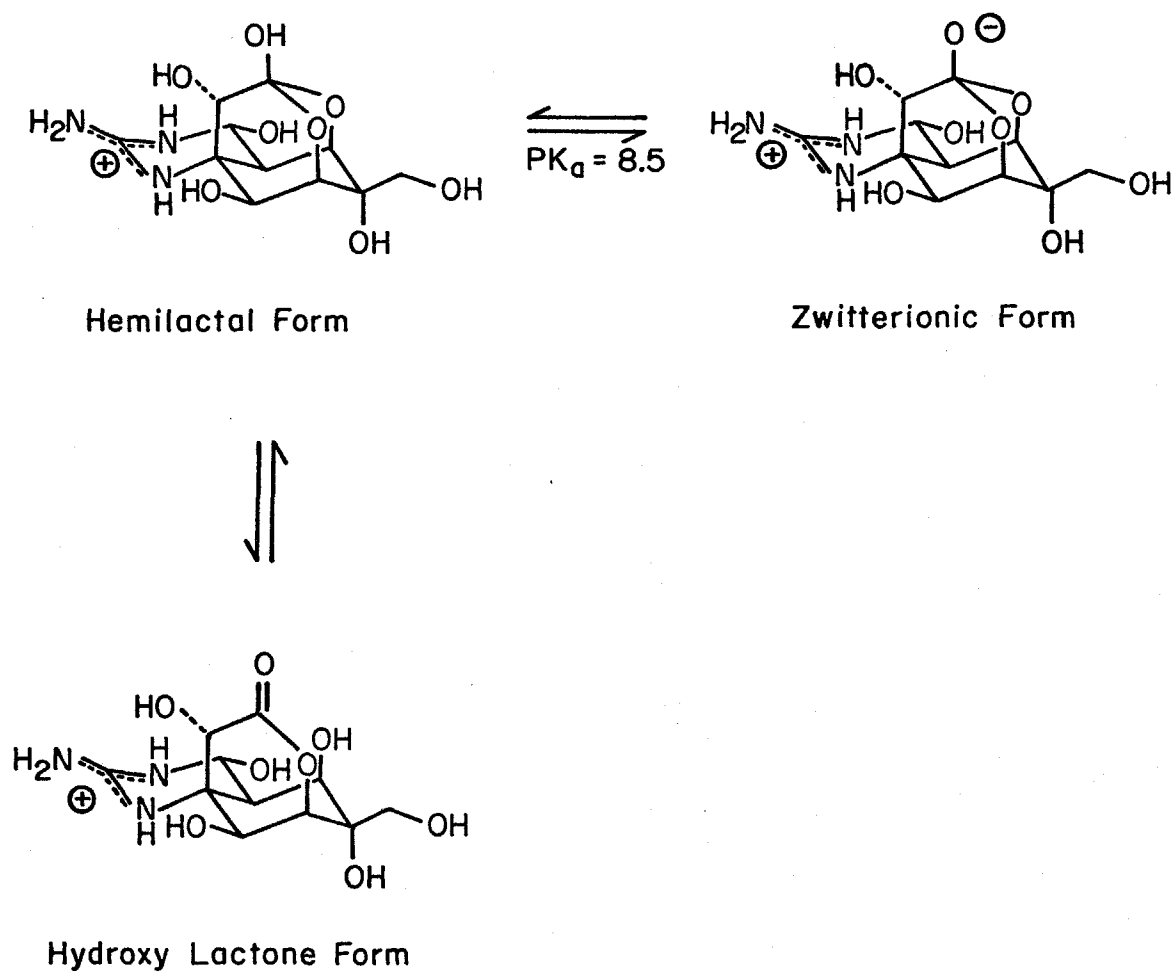


Fig. 3 Tetrodotoxin

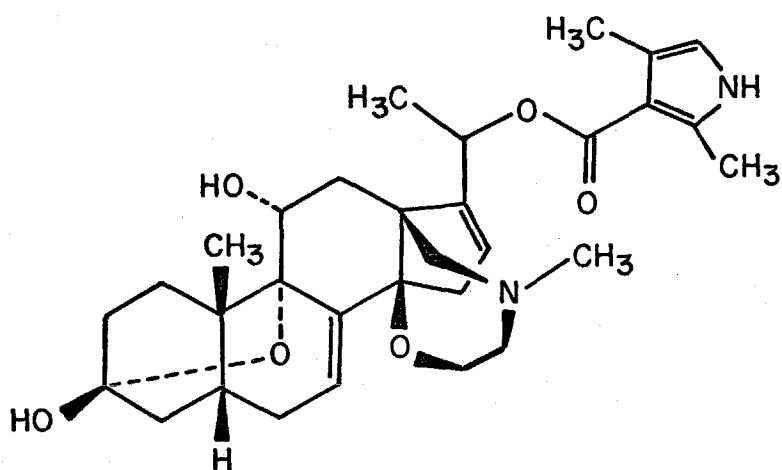


Fig. 4 Batrachotoxin

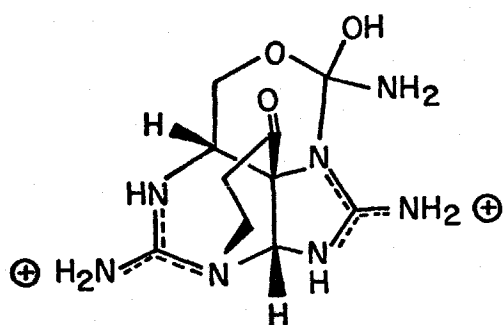


Fig. 5 Saxitoxin

group which is protonated at neutral pH, and various hydrophilic groups which make the compounds water soluble. Both have rigid conformations due to the complex cage work of ring systems. If one makes the assumption that the toxins act by actually plugging up individual sodium channels, and if one accepts Hille's (11) model for the selectivity filter of the channel, then it is not hard to see how a combination of suitably placed hydrogen bonds and ionic interactions between the toxin and the channel could plug the channel by binding tighter to the initial recognition site than any of the possible permeants.

Since the site of action of BTX is not known and no possible models have been proposed, it is impossible to equate its structure to function. One interesting structural note is that the 3,9 hemiketal linkage is very reminiscent of the 4,9 hemiketal linkage in the veratrum alkaloids (33) which have a physiological effect similar to that of BTX.

### Use of Toxins as a Probe

In using the toxins as a molecular dissecting tool of the ion flows in nerve axons, we have encountered two basic groups; toxins which affect the kinetics of opening and closing of the channels, and toxins which affect the actual flow across the channel, presumably by plugging the channel. Further studies in this field which pursue the

actual molecules and mechanisms involved in both these processes should be careful to keep the two processes distinct. The molecule or molecules involved in the actual ion flux may be quite different from those involved in the activation and inactivation of the channels.

TTX can be purchased commercially in its pure form from Sankyo Co. in Japan which obtains large quantities of puffer ovaries from the commercial fisheries in Japan. Since puffer fish is a sought after delicacy in Japan, Sankyo Co. is able to extract tons of the ovaries and obtains about 10 grams of pure TTX per ton of ovary (34). STX is not available commercially at this time and if used as a probe of the sodium channel would have to be isolated, either from cultures of dinoflagellates (35) or from some suitable shellfish. The isolation from shellfish would depend greatly upon the season as they usually contain STX only certain times of the year.

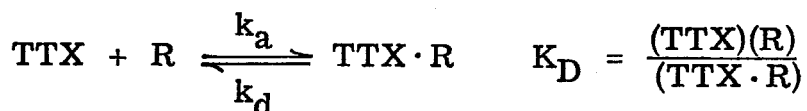
The chemical structure of both toxins is known, as well as many of their physical and chemical characteristics. TTX appears to be chemically unstable at alkaline pH (36), but STX is still active after exposure to a pH 9.7 solution. Therefore in terms of chemical stability it would probably be advantageous to use STX.

The great specificity for the sodium channel blockage has been well demonstrated for TTX and only slightly less so for STX. We have seen already that on axon preparations STX and TTX affect only the

sodium channel during excitation. Furukawa et al. (37) showed that TTX was ineffective in blocking the end plate potential at the neuromuscular junction. Marumo et al. (38) indicate that TTX does not inhibit either active transport of sodium or the sodium-potassium activated ATPase activity in toad bladder. Moore and Narahashi (34) find no effect on active transport in frog skin or red blood cells upon addition of the toxin. Furthermore, neither TTX nor STX affects the presynaptic release of transmitter in nerve muscle preparations. As an interesting note, the nerves of the puffer fish and the newt which produce TTX are highly resistant to blockage by TTX, but still use sodium channels for electrical excitability. These nerves however are quite susceptible to STX poisoning. Evidently the puffer and newt have evolved specialized sodium channels, different enough from normal nerves to be resistant to TTX, but still retaining enough similarity to be poisoned by STX (39). In terms of specificity both STX and TTX would be equally as useful for biochemical studies.

Cuervo and Adelman (19) have done an extensive study on the kinetic and equilibrium interactions between TTX and the giant squid axon. They assumed that the toxin forms a complex with some receptor causing a neurophysiological effect which is quick to manifest itself in comparison to the rate of complex formation. They also assumed that the complex formation was linear with respect to the

effect it elicits. They were able to determine a dissociation constant of  $3.31 \times 10^{-9}$  M for the following reaction:



From independent kinetic data, they obtained an association rate constant  $k_a = 0.202 \times 10^8$  M<sup>-1</sup> min<sup>-1</sup> and a dissociation rate constant  $k_d = .116$  min<sup>-1</sup>. Using these data they calculated a  $K_D = 5.74 \times 10^{-9}$  M which agrees surprisingly well with the equilibrium data.

The above neurophysiological study has not been done for STX, but the results would be interesting to compare to the TTX case. As noted before, Hille (18) reports a value of  $1.2 \times 10^{-9}$  M for half maximal blockage of frog nerve by STX.

The actions of both TTX and STX have been found to be reversible by Narahashi et al. (17). From a physiologist's point of view this means that nerves poisoned with the toxin recover their excitability when washed with a solution containing no toxin. Chemically this probably indicates that the interaction between toxin and sodium channel does not involve covalent bonds. Upon careful analysis Hille (18) found that STX is reversible by 30 sec of washing while TTX poisoning is only partially reversed upon washing for several minutes. This difference between the toxins should be explored thoroughly in



vitro as it may give some information about the requirements for a strong toxin receptor interaction.

The action of these two toxins is also quite sensitive to the hydrogen ion concentration over a pH range where normal nervous activity is constant. It was found by Camougis et al. (36) that TTX is about twice as active at pH 7 as at pH 8.9. Hille (18) showed similarly that STX is relatively inactive at high pH. Furthermore, when the pH of the solution was lowered the STX became active again. Since both the toxins have an OH group with a  $pK_a$  of about 8, these results would indicate that the protonated form of the OH is necessary for activity. This fact also makes sense in terms of Hille's (11) model for the sodium channel selectivity filter. It is entirely possible that the hydroxyl is necessary to form a hydrogen bond with the oxygen functions proposed in Hille's model. Without this hydrogen bond the toxin may not bind tightly enough to produce a block.

Another method for studying the molecular properties of the sodium channel is to modify the toxins chemically and see what effect if any this has on the toxic properties. Tsuda et al. (30) first prepared various derivatives of TTX. Deguchi (40) in Japan, and Narahashi et al. (41) in the U.S. tested the action of the derivatives on various preparations. The derivative deoxytetrodotoxin had about 1/10 the activity of native TTX. The other derivatives appeared to have about

1/100 of the activity or less than the native toxin. All the derivatives have exactly the same physiological effect and only differ in the concentration necessary for activity. This brought up the possibility that the derivatives were contaminated with small amounts of native TTX. The derivatives were analyzed by NMR and deoxytetrodotoxin was found to be contaminated with at least enough pure TTX to account for all the activity that the sample exhibited (34). The limit of resolution of the analysis was about 1% so that the other derivatives could easily be contaminated with enough TTX to account for their toxic properties.

### Channel Counting

A favorite pastime in the game of ion channels is called channel counting. Considerable theoretical effort and some experimental effort has been expended in calculating the number of sodium channels on the membrane surface. Hille (42) presented a complete review of the current status of the theory. He bases his calculations on limiting processes. From a calculation of convergence resistance (called access resistance or spreading resistance) for the mouth of a channel with a radius of  $3A$ , he obtains a maximum conductance for such a pore of 1 nmho in frog Ringers solution and 20 nmho in sea water. Another limiting process is the frequency of ion collisions

with the 3A channel. He obtains a maximum flux of 20 picoamps in frog Ringers and 90 picoamps in sea water. From experimental data, Hille calculates that the conductance of a sodium channel is about 0.1 to 0.2 nmho in frog Ringers and 0.4 to 0.6 nmho in sea water. Using the above he finds that there are 270 sodium channels per square micron in the squid axon and 1200 sodium channels per square micron in the lobster axon. Moore et al. (43) used TTX to estimate an upper limit on the number of sodium channels on the squid axon membrane. They assumed that one toxin molecule bound to one sodium channel. They estimate fewer than 13 channels per square micron. The experiments of Keynes et al. (44) were performed more carefully and they obtained the following estimates: 75/micron<sup>2</sup> for rabbit, 49/micron<sup>2</sup> for crab, and 36/micron<sup>2</sup> for lobster.

The experiments at this stage, although crude, do indicate that the sodium channel or rather TTX binding sites are extremely rare on the axon membrane. Keynes et al. (44) indicate that the ratio of phospholipids to sodium channels in the membrane is 30,000:1. Biochemical investigations on such a minor component will need methods with great sensitivity and tissue with as high a concentration of electrically excitable membrane as possible.

## Choice of Tissue

The experiments described in this report have been carried out on two preparations of electrically excitable tissue: The olfactory nerve from the long-nosed garfish (Lepisosteus osseus) and the electric organ from the electric eel (Electrophorus electricus).

The olfactory nerve from the long-nosed garfish is a fairly large preparation of unmyelinated axons of small diameter (45). It is 1 mm in diameter, approximately 20 cm long, and contains  $10^7$  unmyelinated fibers. The nerve is a homogeneous preparation of axons without large amounts of extraneous Schwann cells. The ratio of axon surface to Schwann cell surface is 30:1. The small diameter of the nerve fibers is also advantageous giving a large surface area of excitable membrane per unit volume of nerve. The ratio of the axon surface area per volume for the garfish to the axon surface area per volume for the squid is 5400:1. The total axon surface area is  $10^3$  cm<sup>2</sup> per cm length of nerve. The fish can be obtained in large numbers from the Midwest where they are frequently caught but rarely eaten, and the nerve can be dissected from the nose without too much trouble after a little practice. The olfactory nerve from the garfish is thus a good source of tissue for preliminary experiments studying the binding of radioactive tetrodotoxin to excitable tissue.

For large scale biochemical studies the garfish olfactory nerve will be of limited usefulness. It is possible, however, to obtain kilogram quantities of the electric organ of the electric eel Electrophorus electricus which will be necessary for the eventual purification of milligram quantities of the components of the sodium channel. The electric organ of the electric eel consists of electrically and chemically (acetylcholine) excitable cells innervated by spinal nerves. Nakamura et al. (46) have studied single cell preparations from the electric organ using a modified voltage clamp method to obtain information about the ionic fluxes of the action potential. The resting potential of the cell is -60 to -90 mv. The voltage clamp data show that upon depolarization there is a transient conductance increase corresponding to the normal sodium activation but this is not followed by the normal steady efflux corresponding to potassium channels. Careful studies show that there is actually potassium inactivation upon depolarization of the electroplax cell. It appears that the normal action potential described by Hodgkin and Huxley (3-6) has been modified by the electric eel so that potassium channels normally open in the resting state close upon depolarization instead of the other way around. The sodium channels appear to be normal since replacement of the sodium in the Ringers with choline abolishes the current. TTX and STX at concentrations of  $3 \times 10^{-7}$  -  $3 \times 10^{-6}$  M block the opening of the channels

rendering the electroplax electrically inexcitable without affecting the potassium inactivation mechanism.

### Properties of the Sodium Channel

The recent studies by neurophysiologists described here have gone quite a way in the elucidation of the mechanisms involved in the production and propagation of action potentials along axons. The use of the highly specific toxins, BTX, STX, and TTX has helped further to understand the workings of the sodium channel. The picture emerging is one in which the sodium channel takes on the properties of a highly complex well-ordered set of processes.

The sodium channel must:

- 1) have an ion discriminator which is able to accept sodium, reject potassium and select among various organic cations.
- 2) have a gating device which is sensitive to the trans-membrane potential. The sodium channels must open when the membrane potential reaches some threshold potential.
- 3) have some ion carrier or pore property to allow the sodium to diffuse freely across the membrane when the channel is in the open state. In this way it may be similar to the antibiotic ionophores which are able to increase the cation permeability in certain natural and artificial membranes.

4) have some time dependent inactivation mechanism. Under voltage clamp conditions the sodium channel is transiently open even when a depolarized voltage is maintained. This indicates that the closing of the sodium channel is some time dependent process and not dependent on membrane potential. The potassium channel on the other hand does not inactivate under the voltage clamp until the potential is again repolarized to the resting potential.

This report describes experiments directed towards the molecular characterization of the part of the sodium channel which interacts with the tetrodotoxin molecule. Of the toxins which interact with components of the sodium channel, tetrodotoxin has been the most extensively studied. Its effect is potent and specific, acting at nanomolar concentrations to block the sodium channel without affecting any other components of the nerve cell. The chemistry of the toxin molecule is well known and it is available commercially in purified form. For these reasons tetrodotoxin was chosen to be labelled with tritium and used to study its interaction with components of the axonal membrane of the garfish olfactory nerve and the electric organ of the electric eel. Through a characterization of the molecule which binds tetrodotoxin it should be possible to learn something about the molecular details of how the sodium channel functions. At the same time, one should be aware that this approach, though an obvious first step,

may not necessarily lead to an understanding of the entire system since the molecules involved in the gating or even the ion carrying mechanism of the sodium channel may be distinct from the molecule which interacts with tetrodotoxin.



## Chapter 1:

### Characterization of the Tetrodotoxin Binding Component from Garfish Nerve Membranes

Recent studies on myelinated fibers from the frog sciatic nerve have determined that several organic cations are able to substitute for sodium in the transient influx of ions (11). From these data Hille proposes a model for the ion selectivity filter of the sodium channel as a small pore  $3 \times 5 \text{ \AA}$  lined with oxygens, capable of forming hydrogen bonds and having one anionic site capable of interacting with cations. The structure of TTX (28, 30), with its positively charged guanidinium group and hydroxyl functions capable of forming hydrogen bonds, is certainly consistent with the hypothesis that blockage of the sodium channel is a result of a specific and tight binding of the toxin to the channel. Some preliminary experiments have been performed in an attempt to measure the binding of TTX to nerves (43, 44, 48). The experiments indicate that TTX does bind to nerves and that binding sites occur at very low concentrations on the nerve membrane.

The specificity of TTX action does not guarantee a specific association of the toxin with the relevant components of the excitable membrane even accepting the quite plausible mechanism of binding proposed by Hille. To insure such interaction in tissue homogenates,

the toxin must be used at concentrations close to those which are active in vivo and under conditions which minimize nonspecific interactions of the cationic TTX molecule with anionic components in the tissue.

Two major problems must be overcome before a biochemical study of the molecules involved in electrical excitability can be begun. Tetrodotoxin must be labelled to a high enough specific activity to be detectable in nanomolar concentrations. This has been accomplished using a modified Wilzbach exchange method (49). Secondly, a preparation of excitable membranes must be found which is suitable for biochemical studies. The olfactory nerve of the long-nosed garfish (Lepisosteus osseus) was chosen as an easily available preparation of unmyelinated fibers having a large surface area of excitable membrane (45). The studies reported here are directed towards characterization of the TTX binding component in excitable tissue using equilibrium dialysis to measure binding; and susceptibility to enzymatic degradation to determine the nature of the membrane components involved.

## Experimental

(1) Materials: Tetrodotoxin (free of citrate) was from Sankyo Co. Ltd. Tokyo. Tritium labelling was performed by International Chemical and Nuclear Corp. Lepisosteus osseus was obtained

live from Minnesota Fish and Game or frozen from Gulf Specimen Co. (Panacea, Florida). Phospholipases, nucleases, hyaluronidase, neuraminidase, and procaine were from Sigma,  $\alpha$ -chymotrypsin from Armour and Co., trypsin from Worthington, and pronase from California Corporation for Biochemical Research.

(ii) Preparation and Purification of [ $^3\text{H}$ ]-Tetrodotoxin. Ten mg of TTX were labelled with tritium using a catalytic exchange method in the presence of 30 curies of tritium. The easily exchanged tritium was removed by solution in dilute acetic acid followed by evaporation. The resulting solid was dissolved in 0.1 M acetic acid and stored at  $-20^\circ\text{C}$ . Purification of [ $^3\text{H}$ ]-TTX was performed on small aliquots of this solution.

Approximately 10 microcuries of the above solution was diluted to 0.4 ml with distilled water titrated to pH 5.0 with acetic acid. The crude material was chromatographed on a  $1.5 \times 55$  cm Biogel P-2 (100-200 mesh) column, equilibrated in distilled water titrated to pH 5.0 with acetic acid. 1.0 ml fractions were collected and the radioactivity of 10  $\mu\text{l}$  aliquots was determined. The fractions which contained nerve blocking activity, as assayed on frog sciatic nerve, were pooled and lyophilized. The material was rechromatographed on the P-2 column and the active fractions lyophilized.

This material was dissolved in 10 mM ammonium acetate pH 7.0 and applied to a 1.0 ml Bio-Rex-70 (200-400 mesh) ( $\text{NH}_4^+$  form) column, equilibrated in 10 mM ammonium acetate pH 7.0. The [ $^3\text{H}$ ]-TTX was eluted using a 100-ml linear salt gradient from 10 mM-200 mM ammonium acetate pH 7.0. 1.0 ml fractions were collected and the radioactivity of 10  $\mu\text{l}$  aliquots was determined. The physiologically active fractions were pooled and lyophilized.

(iii) Analytical Thin-Layer and Ion Exchange Chromatography of [ $^3\text{H}$ ]-TTX. Typically 2-4  $\mu\text{l}$  of solution were spotted on prepared cellulose or silica gel sheets and were developed ~9.0 cm in the solvent tank, cut into 0.5 cm strips, and placed in scintillation vials with 50  $\mu\text{l}$  of water and 1 ml of NCS solubilizer. The radioactivity was determined in a toluene-based scintillator.

For the analytical ion exchange chromatography 10  $\mu\text{l}$  of purified [ $^3\text{H}$ ]-TTX was applied to a Beckman 120-B amino acid analyzer modified for 1 column analyzer and run as usual. The effluent from the column was collected in a fraction collector set to collect 2 min fractions. 100  $\mu\text{l}$  aliquots of the various fractions were counted in a dioxane based scintillator solution. For the degradation studies 25  $\mu\text{l}$  of purified  $^3\text{H}$ -TTX was diluted to 0.2 ml with a solution of  $\text{NH}_4\text{OH}$  at pH 9.0 and incubated for 2 hr at 25°C. The solution was then diluted to 1 ml with the starting buffer and applied to the amino

acid analyzer. Fractions were collected and assayed as above.

(iv) Assay of Nerve Blocking Activity. Frog sciatic nerves, dissected and desheathed, were placed in a Bionix nerve chamber. The diphasic compound action potential was amplified and displayed on a cathode-ray oscilloscope. A 5 mm length of the nerve, between the stimulating and recording electrodes, was immersed in 200  $\mu$ l of frog Ringers solution containing the sample of toxin to be assayed. The percentage decrease in action-potential amplitude after 10 min was taken as a measure of TTX nerve blocking activity. Appropriate controls were performed using Ringers with no toxin added. The concentration of active TTX in a given sample was standardized against known concentrations of unlabelled TTX using the same nerve preparation. The action of TTX was fully reversible upon 1/2 hr of washing with Ringers under these assay conditions.

(v) Preparation of Membranes. Garfish olfactory nerve was dissected according to Easton (45), minced, diluted 1 to 5 with physiological buffer (45), and homogenized in a Virtis "60" homogenizer for 4 min (0°C) at 40,000 rpm. The suspension was either used at this stage (initial binding curve) or centrifuged for 1/2 hr at 45,000 xg. The pellet was resuspended in the appropriate buffer for the enzyme treatments.

(vi) Equilibrium Dialysis Binding Assays. The dialysis tubing was heated for 3 hr in several changes of water over a steam bath and then stored in physiological buffer at 4°C. 0.35 ml of membrane suspension was placed in the tubing and dialyzed against 5.0 ml of physiological solution containing varying amounts of [ $^3\text{H}$ ]-TTX for 12 hr at 4°C with agitation. 0.25 ml aliquots were removed from inside and outside, dissolved in NCS solubilizer, and counted in a toluene-based scintillator using a Packard Tri-Carb Scintillation Spectrometer. It was necessary to determine the efficiency of counting for each sample since the membrane suspension quenched more than the outside solution. For each set of experiments a blank was performed to ensure that the [ $^3\text{H}$ ]-TTX had equilibrated across the dialysis tubing.

(vii) Enzyme Treatments. Nerve membranes were suspended in buffers appropriate for the various enzymes (50), which were used at a concentration of 1 mg/ml except for neuraminidase which was 2.5 mg/ml, and phospholipase A which was 0.2 mg/ml. Incubation was carried out for 1 hr at 25°C. The enzyme-treated membranes were pelleted at 45,000 g for 1/2 hr in a Sorvall centrifuge and resuspended in physiological solution for binding studies. Simultaneous controls were run using the various buffers without addition of the enzymes.

## Results and Discussion

(i) Preparation and Purification of [ $^3\text{H}$ ]-TTX. The crude material (10 mg) labelled by I.C.N. contained 30 millicuries of tritium and approximately 3-4 mg of active tetrodotoxin as measured by the frog sciatic-nerve assay. When extremely small quantities of this crude labelled TTX (approximately 0.4% of the total material) were chromatographed on Biogel P-2 using a buffer of very low ionic strength, the peak of radioactivity containing nerve blocking activity was eluted after the bulk of the inactive radioactive material and with an elution volume greater than the total bed volume of the column (see Figure 6), presumably due to the sorption of the TTX molecule to the resin at very low ionic strength (51). Rechromatography on Biogel P-2 further removed the faster moving inactive radioactive material.

The pooled active fractions from the P-2 column, when placed on Bio-Rex 70 and eluted with a linear salt gradient, gave 3 peaks (Figure 7). The radioactive peak containing the physiological activity (fractions 32-56) was sharp and symmetrical suggesting high purity of the labelled toxin.

TLC's on cellulose and silica gel using ethyl acetate:pyridine: water:acetic acid 5:5:3:1 and butanol:acetic acid:water 8:3:9 showed a single peak of radioactivity for the purified [ $^3\text{H}$ ]-TTX. TLC of crude

Figure 6. Chromatography of TTX which had been tritiated by a modified Wilzbach process on a Biogel P-2 column ( $1.5 \times 55$  cm) with water, titrated to pH 5.0 with acetic acid, as the elution buffer. 1.0 ml fractions were collected. 10  $\mu$ l aliquots of appropriate fractions were removed for determination of radioactivity. Fractions No. 100-140 were pooled and contained physiological activity.



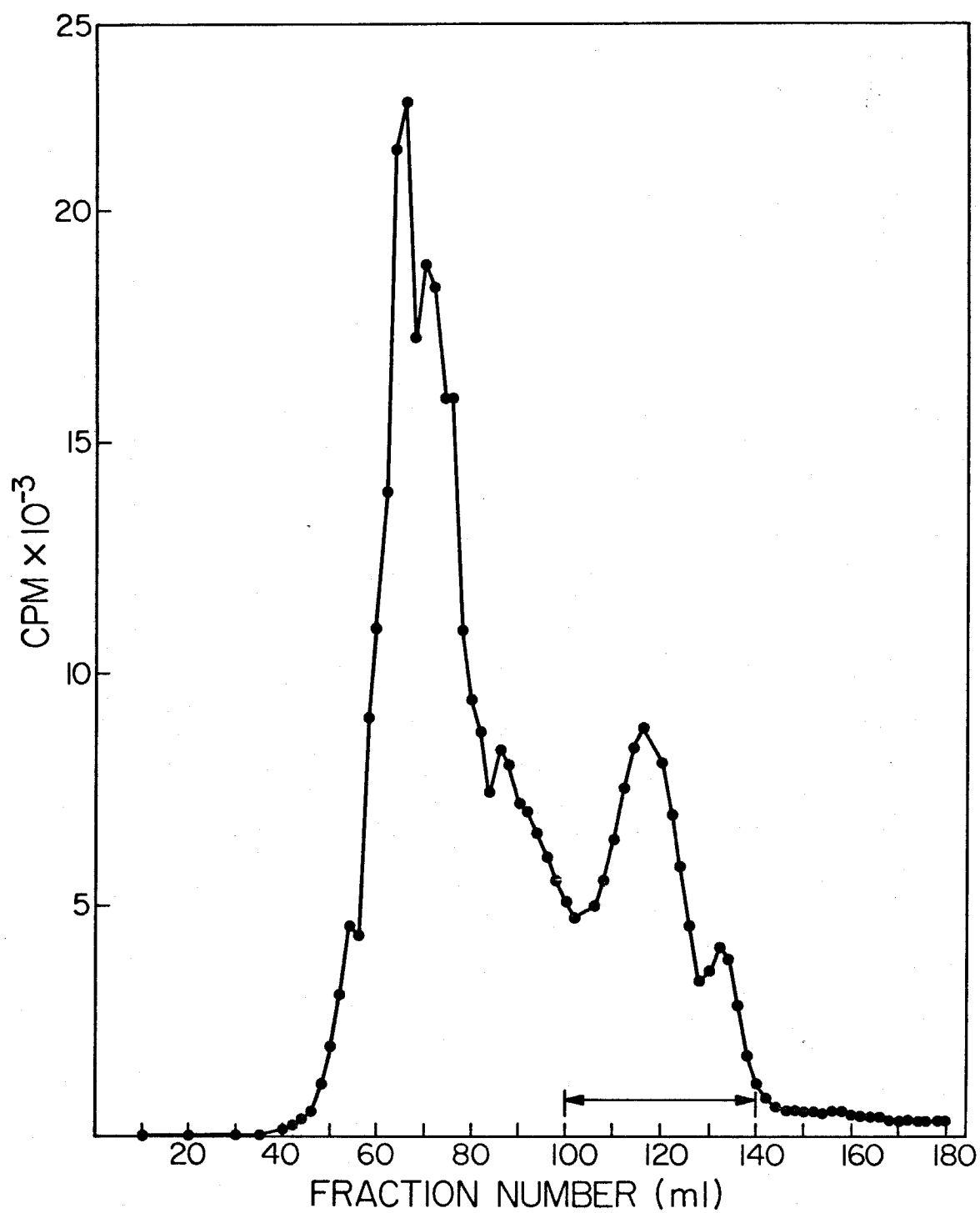
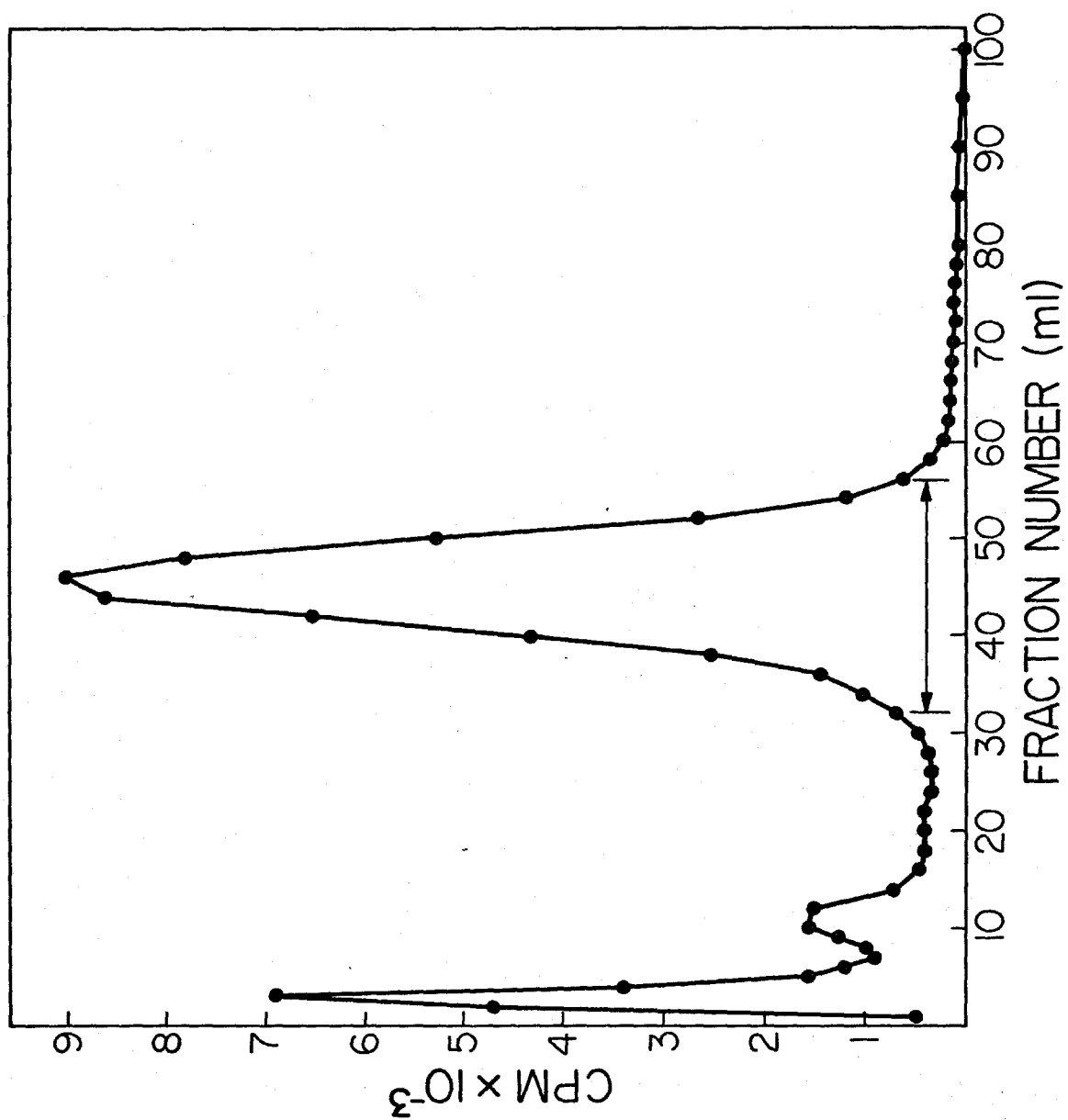


Figure 7. Gradient elution chromatography on Bio-Rex 70 (1 ml bed volume) of the active fractions of [ $^3\text{H}$ ]-TTX twice chromatographed on Biogel P-2. Elution was carried out using a 100 ml ammonium acetate gradient from 10 mM-200 mM at pH 7.0. 1.0 ml fractions were collected and 10  $\mu\text{l}$  aliquots removed for determination of radioactivity. Fractions No. 32-56 were pooled and contained physiological activity.



labelled material showed the majority of the radioactivity was not at the same position as the purified toxin peak (Figure 8). The sharp symmetrical peak on the Bio-Rex 70 column and the single peaks on TLC peaks using two different supports and two different solvent systems was taken as good evidence of the radiochemical purity of the [ $^3\text{H}$ ]-TTX preparation. As a further check of the radiochemical purity of our [ $^3\text{H}$ ]-TTX, a sample of the purified [ $^3\text{H}$ ]-TTX was chromatographed on a Beckman 120-B amino acid analyzer. The column effluent was collected and the fractions assayed for radioactivity. A single sharp peak of radioactive material was eluted slightly after the normal elution position of ammonia, as would be expected for the basic tetrodotoxin molecule (Figure 9). A sample of [ $^3\text{H}$ ]-TTX was incubated at pH 9.0 for 2 hr at 25°C, and then applied to the amino acid analyzer. This alkaline treatment destroys approximately one half of the nerve blocking activity of tetrodotoxin (52). The elution profile of the degraded sample showed 4 major peaks of radioactivity and the peak corresponding to the  $^3\text{H}$ -TTX was greatly reduced in size (Figure 10). The co-chromatography of biological activity and radioactivity in various systems plus the disappearance of the [ $^3\text{H}$ ]-TTX peak on the analyzer simultaneously with the loss of nerve blocking activity indicate that the radioactive material in our sample of [ $^3\text{H}$ ]-TTX does have the properties of native tetrodotoxin.

Figure 8. (a) Thin-layer chromatography on silica gel of purified [ $^3\text{H}$ ]-TTX using butanol:acetic acid:water 8:3:9 as a solvent.

(b) Thin-layer chromatography of crude labelled TTX on silica gel using butanol:acetic acid:water 8:3:9 as a solvent. Note the scale of the ordinate is  $\sim 1/10$  that of Figure (a). Both chromatograms contained approximately equal amounts of active TTX as assayed on frog sciatic nerve.

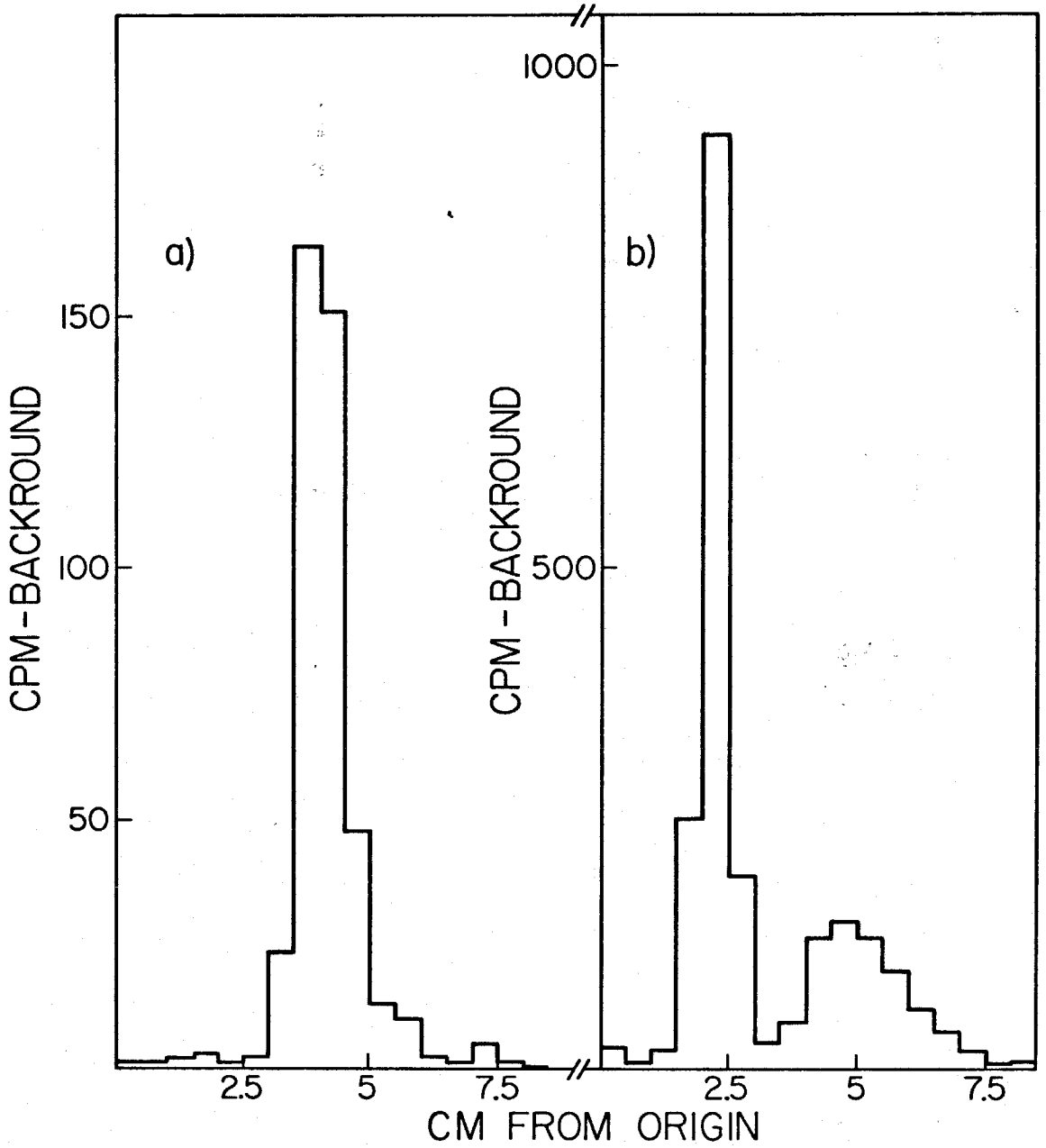


Figure 9. Ion exchange chromatography of purified [ $^3\text{H}$ ]-TTX on a Beckman 120-B amino acid analyzer. Elution was the usual for a one column analysis of amino acids. Two-minute fractions were collected and 100  $\mu\text{l}$  aliquots removed for determination of radioactivity.

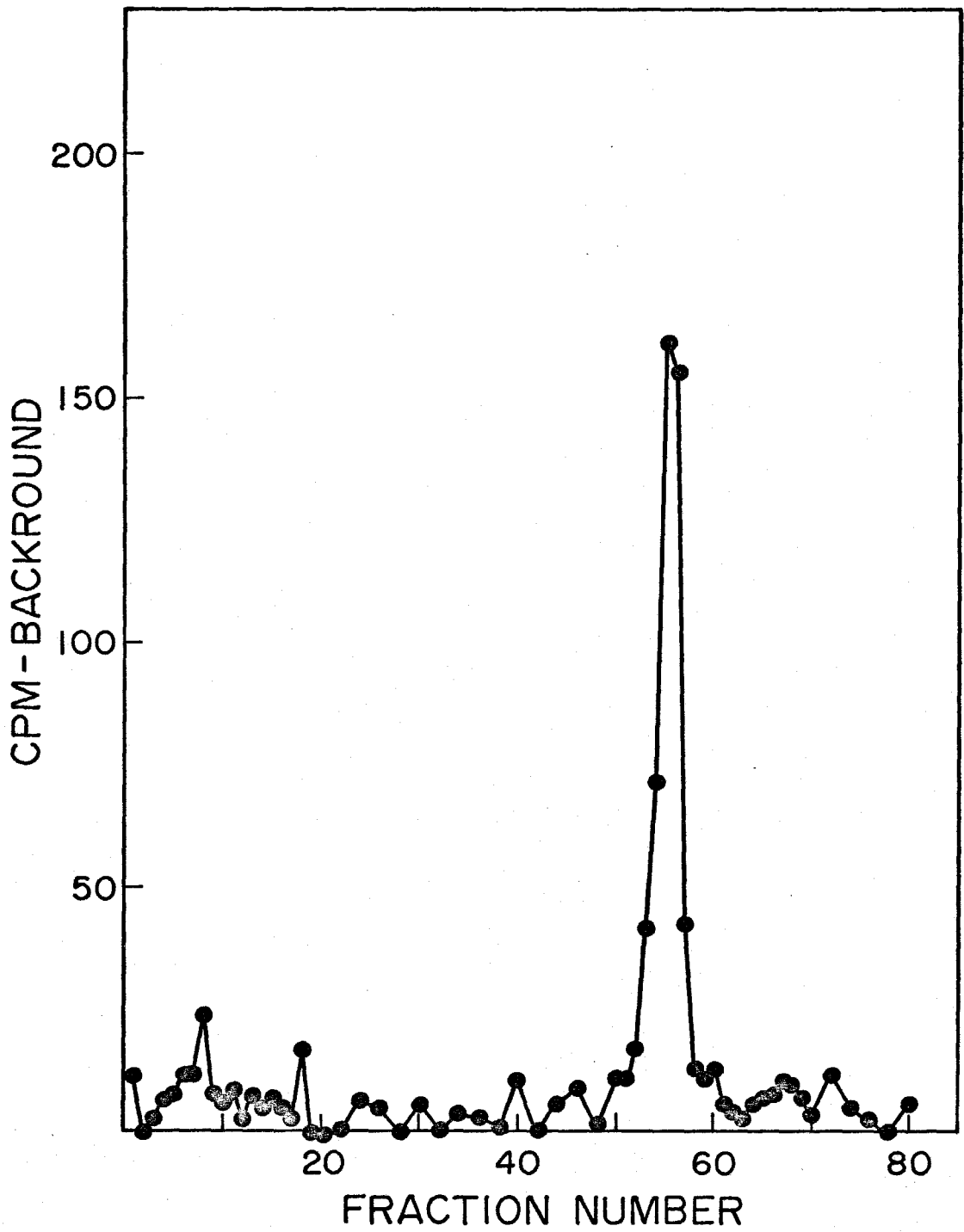
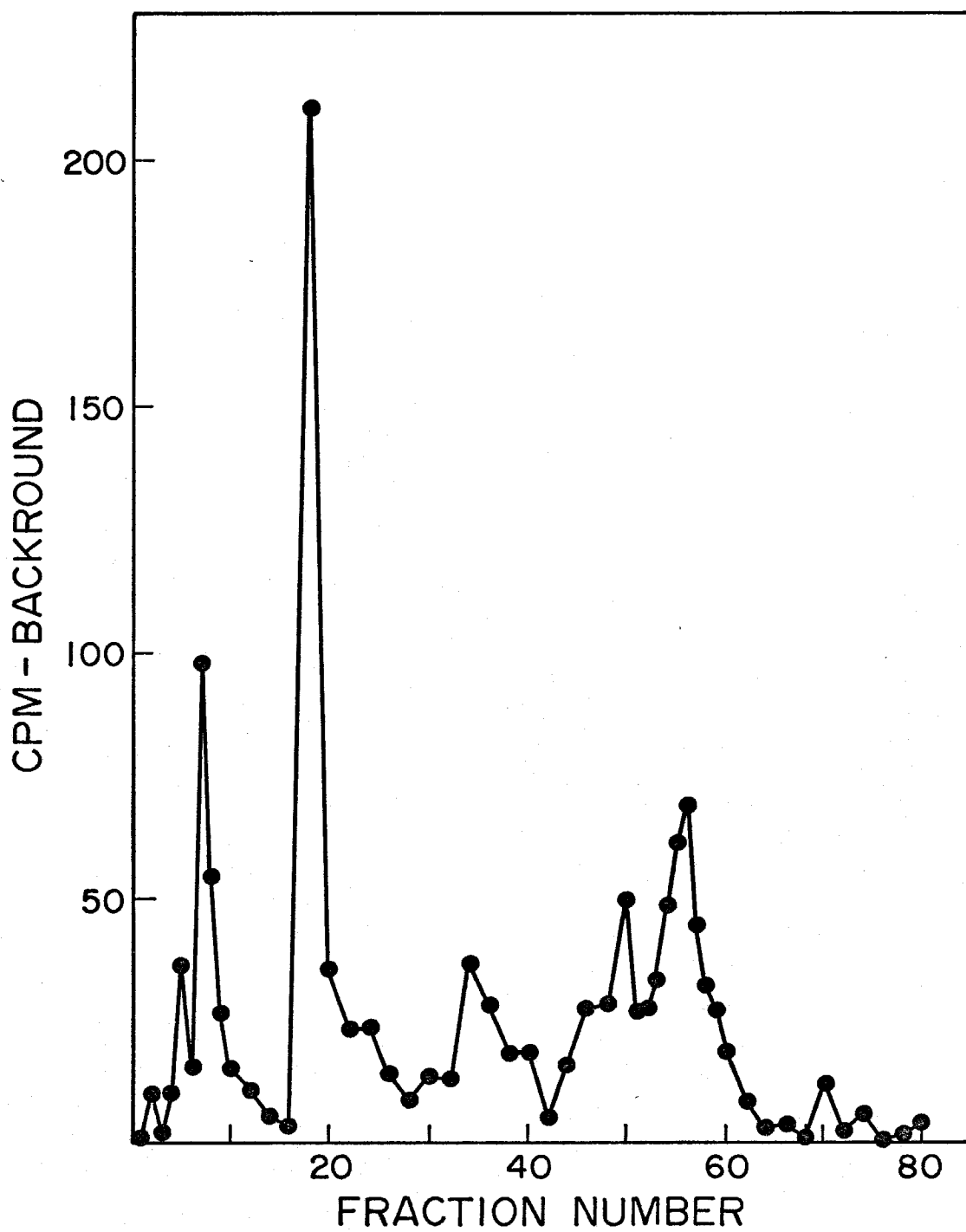




Figure 10. Ion exchange chromatography of purified [ $^3\text{H}$ ]-TTX incubated at pH 9 for 2 hr. The chromatogram was run and analyzed the same way as the chromatogram in Figure 9 except that the input sample contained 2.5 times the amount of radioactivity.



Hafemann (48) has reported difficulty in obtaining radiochemically pure [ $^3\text{H}$ ]-TTX presumably because he has not taken advantage of the sorption properties of Biogel P-2 or the possibility of ionic strength gradient elution on Bio-Rex 70. The purified preparation of [ $^3\text{H}$ ]-TTX obtained here was standardized against known concentrations of unlabelled TTX using the frog sciatic nerve as a bioassay. A specific activity of 0.33 curies/millimole was determined for the purified active  $^3\text{H}$ -TTX.

(ii) Equilibrium Dialysis Binding Studies. This technique has been used to measure the binding of [ $^3\text{H}$ ]-TTX to tissue homogenates of the garfish olfactory nerve. A plot of the amount of [ $^3\text{H}$ ]-TTX bound versus the free [ $^3\text{H}$ ]-TTX concentration (see Figure 11) shows that at nanomolar concentrations [ $^3\text{H}$ ]-TTX binds to nerve homogenates and that the binding sites can be saturated. A double reciprocal plot (Figure 12) yields a straight line and gives a dissociation constant of  $K_D = 8.3 \text{ nM}$  TTX and a maximal binding of 43 picomoles [ $^3\text{H}$ ]-TTX per gram of wet tissue. The reciprocal plot also indicates only one type of high affinity binding site for the toxin. A Hill plot (53) of the data (Figure 13) yields a Hill coefficient of .99 indicating that the binding of TTX is not a cooperative process. Subsequent experiments showed recovery of all the  $^3\text{H}$ -TTX binding in the 45,000 g pellet confirming that the binding component is membrane bound.

**Figure 11.** Binding curve of [ $^3\text{H}$ ]-TTX to olfactory nerve homogenate using equilibrium dialysis. Picomoles of [ $^3\text{H}$ ]-TTX bound per gram of tissue is plotted versus the concentration of free [ $^3\text{H}$ ]-TTX.

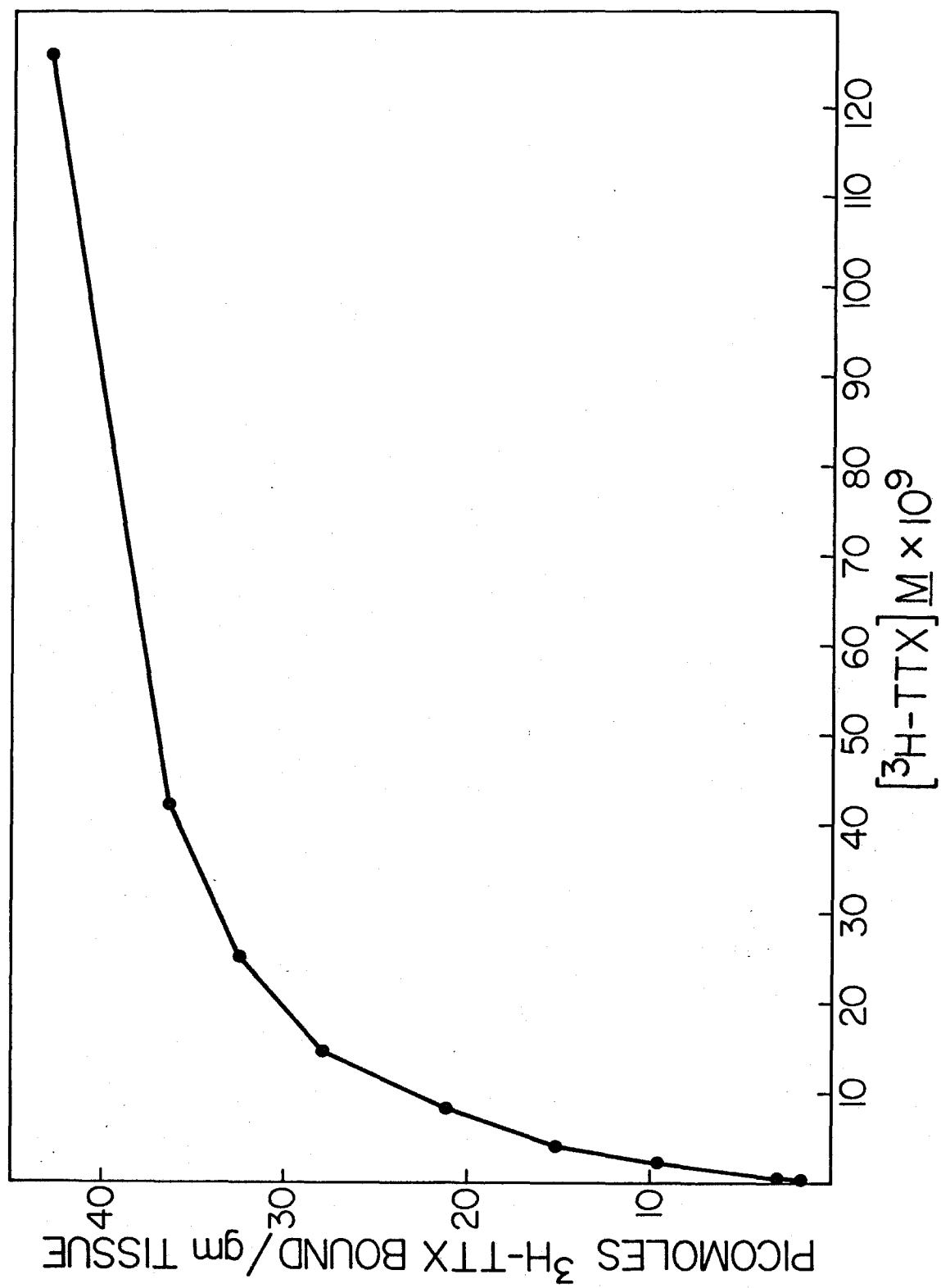


Figure 12. Reciprocal plot of [ $^3\text{H}$ ]-TTX binding to olfactory nerve homogenate. The ordinate shows the reciprocal of the amount of [ $^3\text{H}$ ]-TTX bound in picomoles per gram of wet tissue. The abscissa is the reciprocal of the concentration of free [ $^3\text{H}$ ]-TTX.

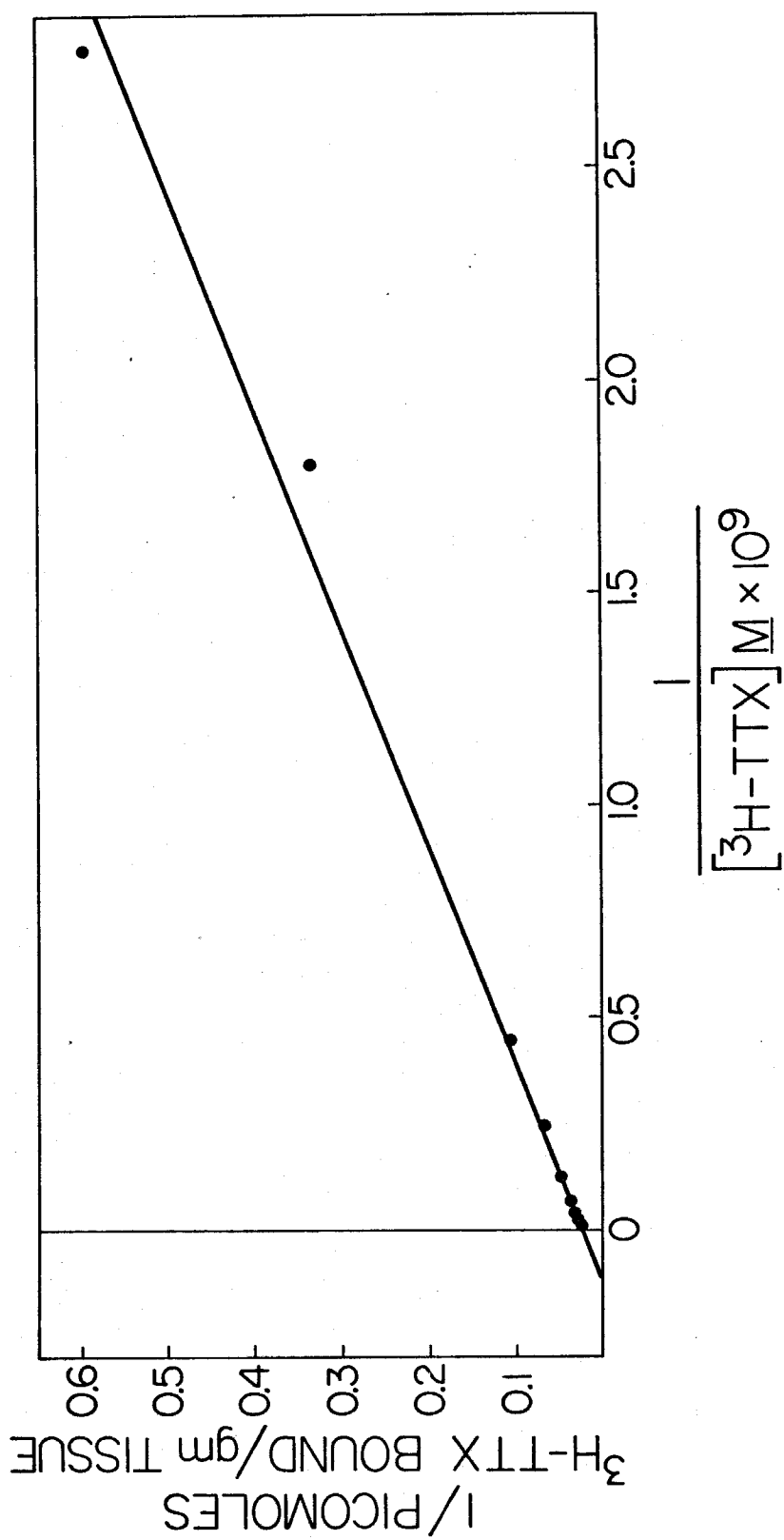
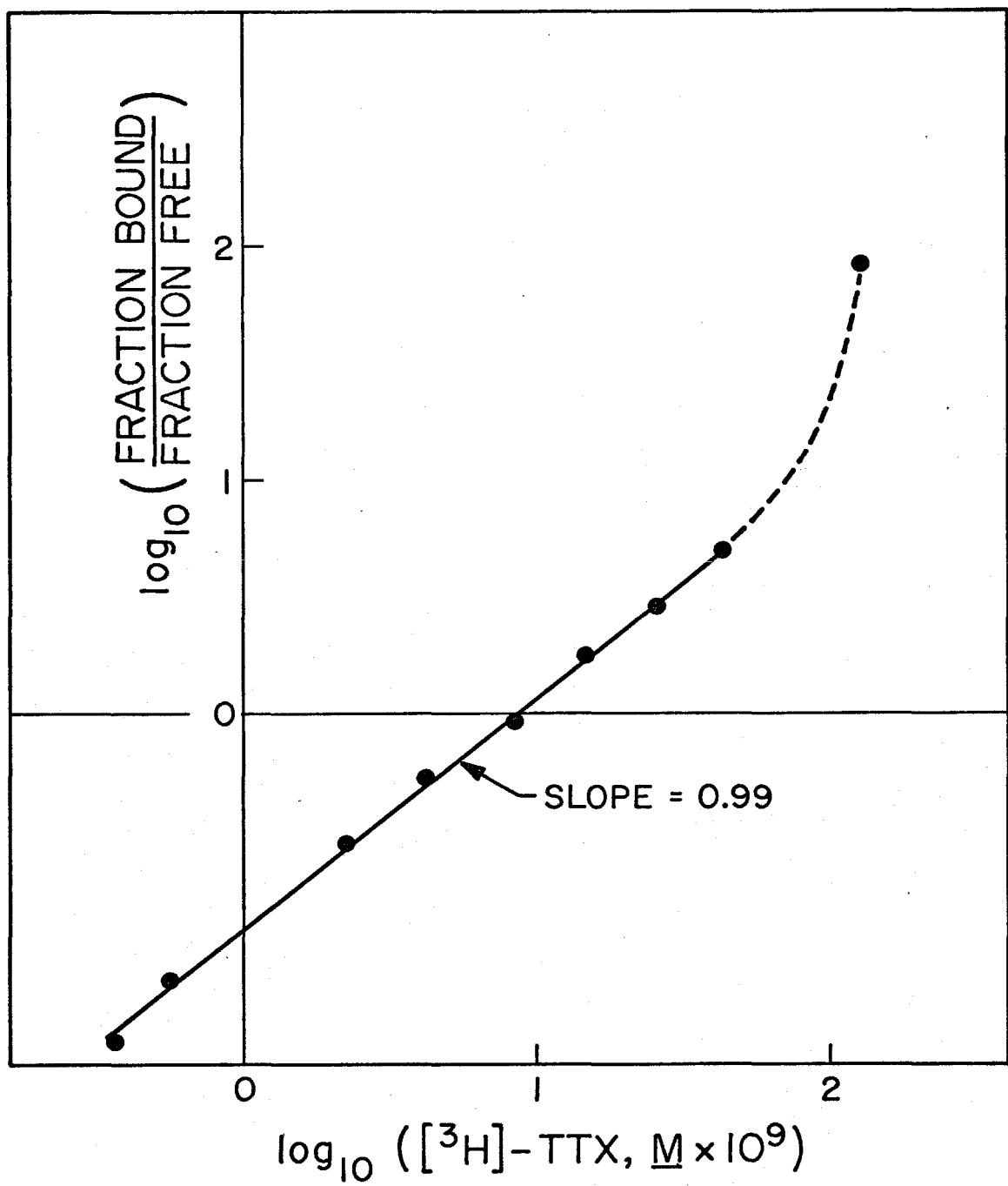


Figure 13. A Hille plot of the binding of [ $^3\text{H}$ ]-TTX to the olfactory nerve homogenate. The  $\log_{10}$  of the free [ $^3\text{H}$ ]-TTX concentration is plotted versus the  $\log_{10}$  of the ratios the amount of component bound to the amount free.

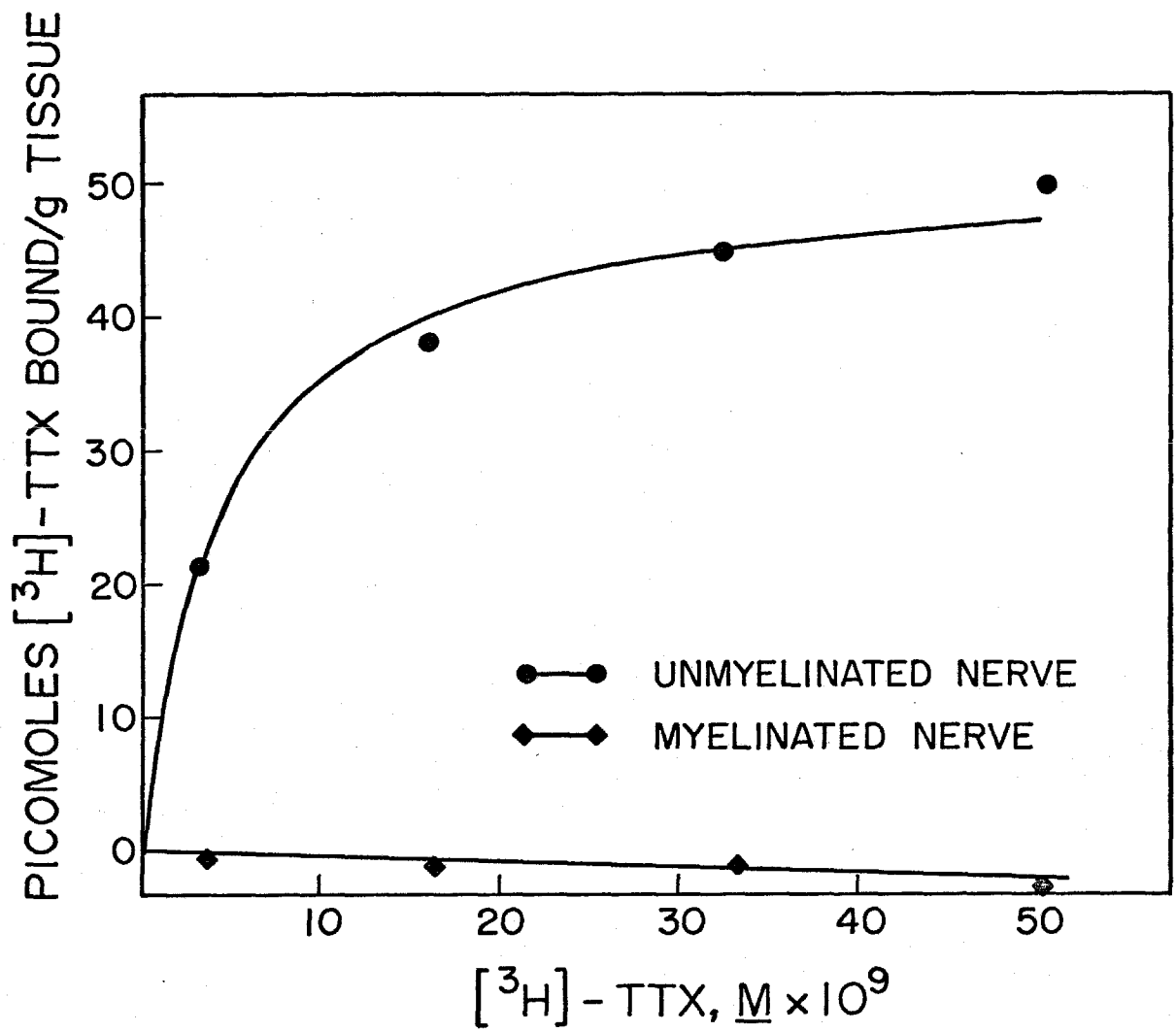




TTX binding studies were performed on tissue homogenates of the branch of the myelinated trigeminal nerve, found adjacent to the olfactory nerve in the gar (45). No [ $^3\text{H}$ ]-TTX binding was detectable over a range of TTX concentrations from 3.5-50 nM (see Figure 14). The limits of sensitivity of the assay indicate a binding of less than 1.5 picomoles per gram of wet tissue. This low value is to be expected for myelinated fibers where the excitable membrane occupies only a small fraction of the membrane surface area.

In order to correlate the number of binding sites with physiological data, it is necessary to determine the number of sites per square micron. Calculations have been made for the olfactory nerve of cross sectional area and surface area of axon per length of nerve (45). Assuming a density of 1 gm/ml for the tissue there are  $6.5 \times 10^{12}$  square microns of axonal surface area per gram of wet tissue. Under saturating concentrations of [ $^3\text{H}$ ]-TTX there are  $2.52 \times 10^{13}$  binding sites per gram of tissue, and therefore 3.9 binding sites per square micron of surface area. This value is lower than values reported for other unmyelinated preparations (43, 44) but agrees well with a recent value obtained for gar olfactory nerve (52). The dissociation constant for TTX binding is also in agreement with physiological studies which determined the concentration of TTX at which the sodium influx was reduced to half its normal value (19).

Figure 14. Binding curve of [ $^3\text{H}$ ]-TTX to olfactory nerve (unmyelinated fibers) homogenates and trigeminal nerve (myelinated fibers) homogenates. Picomoles of [ $^3\text{H}$ ]-TTX bound per gram of tissue is plotted versus the concentration of free [ $^3\text{H}$ ]-TTX.



The agreement of the binding and dissociation data from experiments using whole nerves and the membrane suspension studies reported here encourages the investigation of the biochemical properties of the TTX binding component. Since the TTX binding component retains its binding properties when the axonal membranes are disrupted, the solubilization and isolation of a molecule or molecules involved in electrical excitability becomes possible.

(iii) Enzyme Treatment. The only enzymes found capable of inhibiting toxin binding were phospholipase A and two proteases, chymotrypsin and pronase (Table I). Trypsin treatment had no effect upon binding. Pretreatment of the membranes with phospholipase A for one hr, followed by treatment with various proteolytic enzymes resulted in greater reduction of binding than that found without the pretreatment. This indicates that the protein part of the TTX binding component is embedded in a phospholipid environment which partially protects against attack by proteolytic enzymes. Toxin binding is also inhibited below pH 4 and by heating membrane suspensions to 100°C for 15 min. It is not known whether the inhibition below pH 4 is due to inactivation of the binding protein or merely to protonation of a residue in the active site which must be in the deprotonated state for binding to occur.  $10^{-2}$  M procaine had no effect upon the binding which is in agreement with the notion that such local anaesthetics

Table 1:

<u>Treatment</u>	<u>cpm bound treated material</u> <u>cpm bound control</u>	
Ribonuclease (1 hr)	0.91 ± .05	
Deoxyribonuclease (1 hr)	1.09 ± .05	
Hyaluronidase (1 hr)	1.04 ± .05	
Neuraminidase (1 hr)	1.62 ± .05	
Phospholipase A (1 hr)	0.69* ± .10	
(2 hr)	0.62* ± .10	
Phospholipase C (1 hr)	0.99 ± .05	
Phospholipase D (1 hr)	1.28 ± .05	
		Phospholipase A (1 hr) then proteolytic enzyme (1 hr)
Trypsin (1 hr)	1.10 ± .05	0.10* ± .10
$\alpha$ -Chymotrypsin (1 hr)	0.80 ± .05	0.22* ± .10
Pronase (1 hr)	0.72 ± .05	0.30* ± .10
Dithiothreitol (50 mM)	0.85 ± .05	
Heat 100°C (15 min)	0.18 ± .05	
pH 2	0.09 ± .05	
pH 4	0.22 ± .05	
Procaine 10 <sup>-3</sup> M	1.10 ± .05	
10 <sup>-2</sup> M	0.99 ± .05	

\* Binding measured in supernatant as well as pellet.

block action potentials by some interaction with the lipid phase of the membrane and not at the specific TTX binding site.

Treating the membranes with neuraminidase caused a 62% increase in TTX binding. This same effect has been seen for [ $^{125}\text{I}$ ]- $\alpha$  bungarotoxin binding to membranes of electroplax from electric fish (54). The increase in binding could be due to "unmasking" of binding sites upon cleavage of sialic acid residues from the membrane, or it could simply be due to an increase in nonspecific binding sites on the membrane which could interact with the positive charge on the toxin molecule.

The results of the experiments presented here show that TTX binds to a small number of high affinity sites on the membranes of garfish olfactory nerve and that the membrane binding component appears to be protein or phospholipoprotein in nature. Agreement with previous calculations of the number of sites and the binding affinity suggest that the TTX binding protein may be identical with or related to the molecule or molecules involved in the transient sodium influx of excitable tissue.

These preliminary results encourage the investigation of isolation procedures on the TTX binding protein.

## Chapter 2:

### Characterization of the Detergent-Solubilized Tetrodotoxin

#### Binding Component from Garfish Olfactory Nerve

The experiments in Chapter 1 exploited the great specificity in the binding of TTX to nerve cells by using tritiated TTX as a marker of sodium channel sites in axonal membrane fragments. In order to isolate the molecular component which interacts with tetrodotoxin, the first step would be to extract the component into a soluble phase while at the same time to preserve its tetrodotoxin binding activity. The extraction procedure should be such that there will be a reasonable number of isolation procedures available to use on the extracted preparation. The conformation and the activity of the TTX binding component will certainly change to some extent when it is extracted from the membrane environment, and one should be aware of these possible changes when interpreting results from experiments on solubilized material.

This chapter describes the extraction of the tetrodotoxin binding component from membrane fragments using detergents and describes some biochemical properties of the detergent-solubilized tetrodotoxin binding component including Stokes radius from gel filtration columns,



S value from sucrose gradient velocity sedimentation, and susceptibility towards enzymatic degradation.

## Experimental

(i) Materials. Tritiated tetrodotoxin was prepared as described in Chapter 1. Frozen Lepisosteus osseus heads were obtained from E. Saeugling (Guttenberg, Iowa). Enzymes used were those described in Chapter 1. All chemicals were reagent grade except where specified.

(ii) Preparation of Detergent Extracts. Garfish olfactory nerve was dissected free of blood vessels and myelinated fibers, minced, diluted 1 to 5 with physiological buffer (45) and homogenized in a Virtis "60" homogenizer for 4 min (0°C) at 40,000 rpm. The suspension was centrifuged for 1/2 hr at 45,000 xg and the supernatant discarded. The membrane pellet was suspended in the extraction medium, consisting of garfish physiological solution (45) which had been made 10% in glycerol and the appropriate concentration in detergent. The membranes were extracted with stirring for 10-12 hr at 4°C and centrifuged at 100,00 xg for 1 hr in an L-3-50 Beckman ultracentrifuge. The supernatant was decanted and is subsequently referred to as the detergent extract. It was kept below 10°C at all times.

(iii) Binding Assays. 0.35 ml of detergent extract was placed in the dialysis tubing and dialyzed against 5 ml of the same extraction solution containing various amounts of [ $^3\text{H}$ ]-TTX for 12 hr at 4°C with agitation. 0.25 ml aliquots were removed from inside and outside, dissolved in NCS solubilizer and counted in a toluene-based scintillator solution.

(iv) Enzyme Treatment and Chemical Modifications. For each enzyme treatment, 0.35 ml of Triton extract was incubated with 0.5 mg of the various enzymes (except Phospholipase A which was 0.1 mg per assay) for 10 hr at 4°C. The treated Triton extract was then placed in dialysis tubing and the equilibrium dialysis assay performed.

(v) Molecular Weight Determinations. Sucrose gradient (5-20%) velocity sedimentation was performed according to the method of Martin and Ames (55). The gradients were made up in the Triton extraction solution (5% Triton) with 4.0 nM [ $^3\text{H}$ ] tetrodotoxin distributed uniformly throughout the gradient. A 200  $\mu\text{l}$  sample also containing 4.0 nM [ $^3\text{H}$ ]-TTX was layered on the preformed gradients and centrifuged for 10 hr at 65,000 rpm (420,000 xg) in a Beckman SW 65K rotor. Standard proteins,  $\beta$ -D-galactosidase and catalase were run simultaneously in adjacent tubes. Tubes were pierced at the bottom and 140  $\mu\text{l}$  fractions collected for assay of radioactivity and

protein. Using this method a peak of radioactivity is expected at the position of the tetrodotoxin binding component and a trough at the position of free tetrodotoxin.

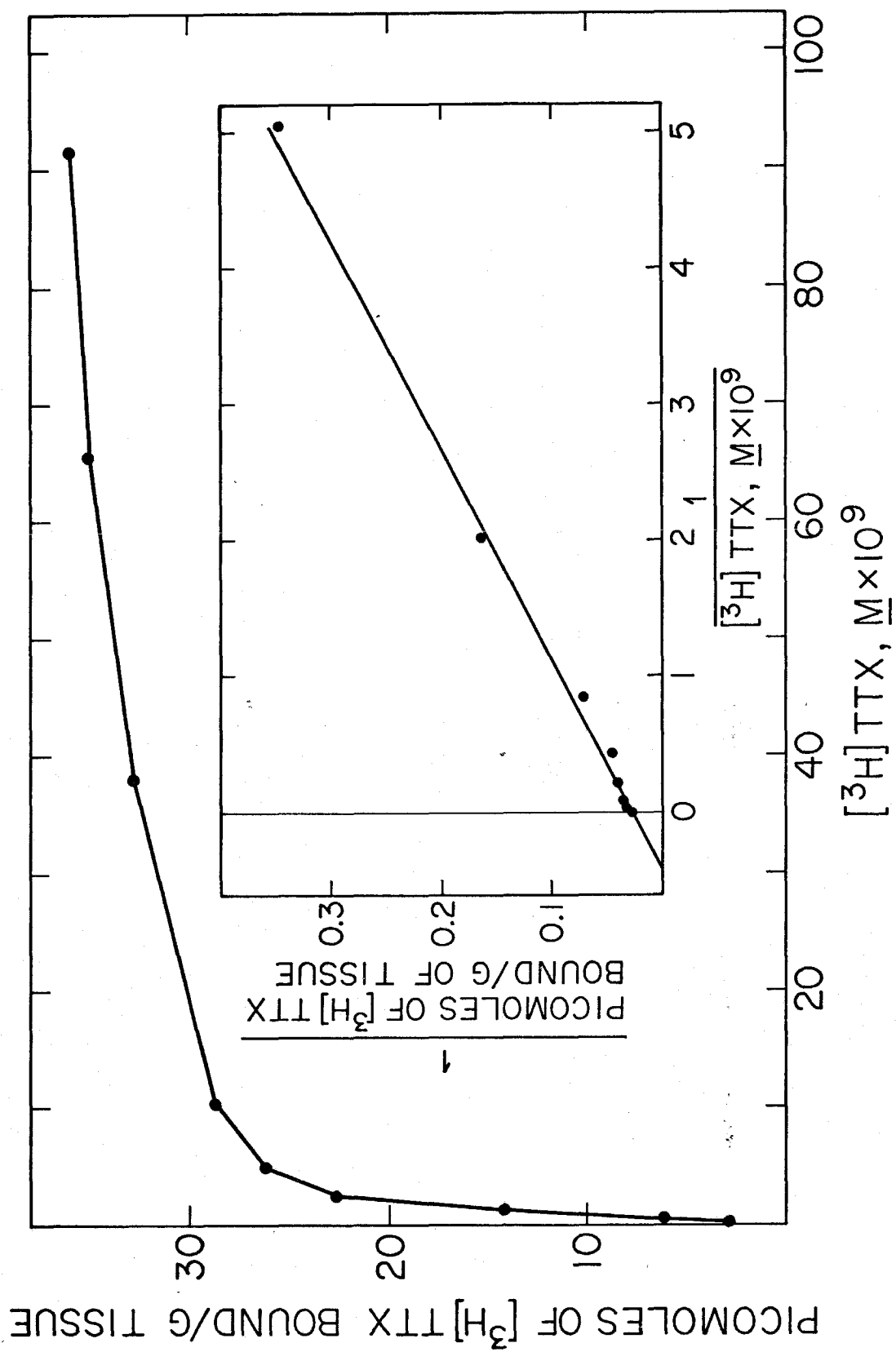
A Sepharose 6B column ( $1.5 \times 98$  cm) was packed and equilibrated in Triton extraction buffer (5% Triton) containing  $0.5 \text{ nM}$  [ $^3\text{H}$ ]-TTX. The column was standardized using blue dextran for the void volume marker and  $\beta$ -D-galactosidase, IgG, and myoglobin as the standard proteins. A 1 ml sample of the Triton extract (5%) containing  $0.5 \text{ nM}$  [ $^3\text{H}$ ]-TTX was applied to the Sepharose column and the amount of radioactivity measured in the effluent. By this method of Hummel and Dreyer (56) a peak of radioactivity corresponds to the elution position of the TTX binding component and the trough to the elution position of free [ $^3\text{H}$ ]-TTX as in the sucrose gradient.

## Results and Discussion

(i) Solubilization. By use of 5% Triton X-100 solutions it was possible to extract greater than 90% of the tetrodotoxin binding activity from membranes. 10% glycerol was used to stabilize the solubilized component.

The binding curve obtained for the Triton-solubilized material (Figure 15) shows a single binding site with a dissociation constant  $K_D = 2.5 \times 10^{-9} \text{ M}$ . Under saturating conditions, there are 39

Figure 15. Binding curve of [ $^3\text{H}$ ]-TTX to the Triton extract (5%) of axon membranes by equilibrium dialysis. Picomoles of [ $^3\text{H}$ ]-TTX bound per gram of tissue is plotted against the concentration of free [ $^3\text{H}$ ]-TTX. The insert shows the reciprocal plot of the binding data.

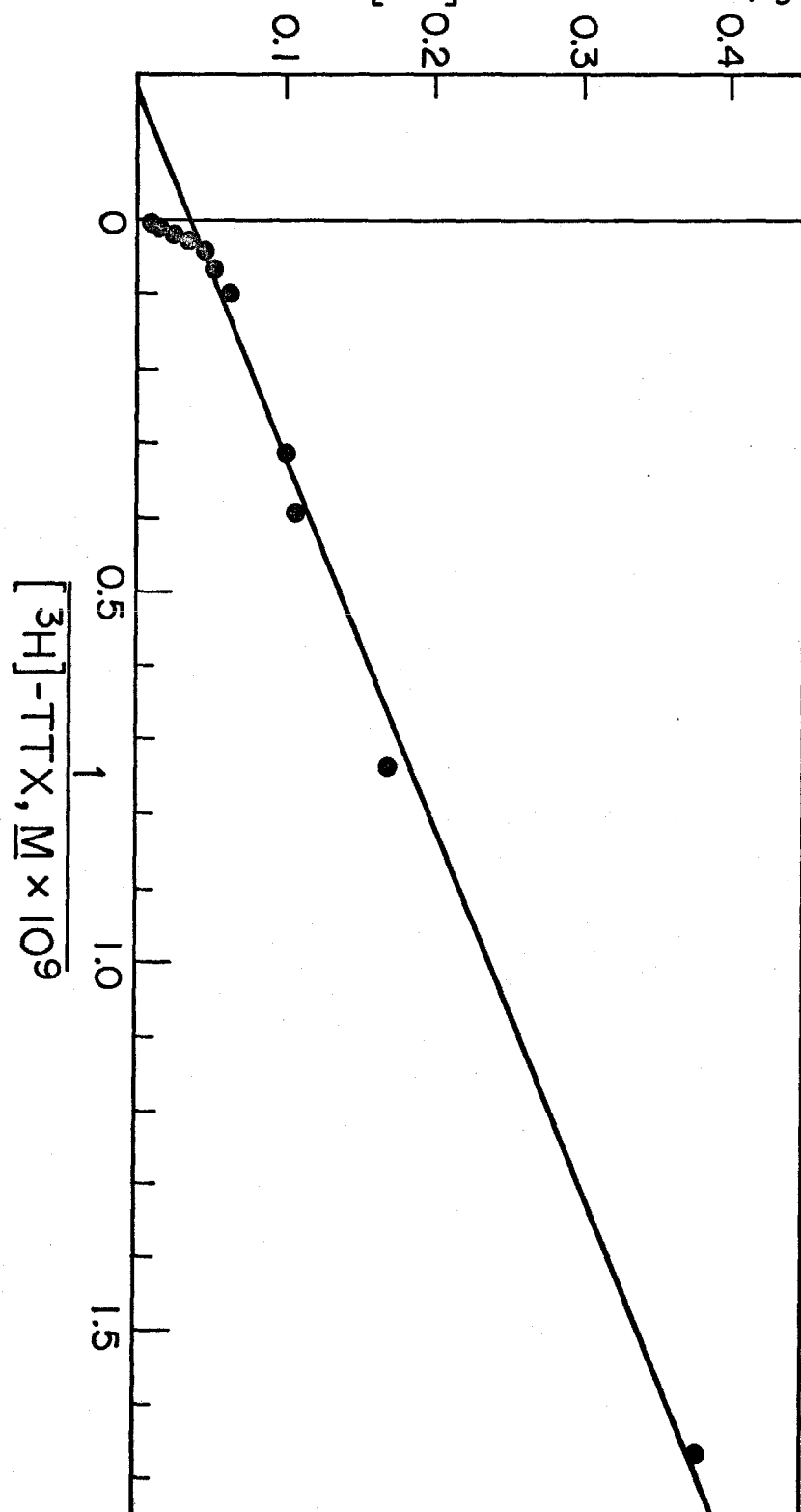


picomoles of toxin bound per gram of wet tissue or 1.95 picomoles of toxin per milligram of solubilized protein as measured by the Lowry method (57). It is interesting to note that the affinity of tetrodotoxin for the binding component is increased upon solubilization from  $K_D = 8.3 \times 10^{-9}$  M obtained from the membrane fragments. This phenomenon is possibly due to increased accessibility of the toxin binding site in the solubilized material. Several nonionic detergents with structures similar to that of Triton-X-100 such as Lubrol-PX and Emulphogene were also effective in solubilizing the TTX binding component.

When the garfish nerve membranes are extracted with 1% sodium cholate and the cholate extract analyzed by equilibrium dialysis the double reciprocal plot (Figure 16) gives a set of points which can be fitted well to two straight lines. At low concentrations of [ $^3\text{H}$ ]-TTX there appears to be a small number (29 picomoles/gm tissue) of high affinity sites ( $K_D = 5.8 \times 10^{-9}$  M). These numbers are similar to those obtained with the 5% Triton extract, and the membrane fragments and therefore probably represent the specific TTX binding. At higher concentrations of [ $^3\text{H}$ ]-TTX the binding does not saturate and the reciprocal plot indicates a large number of low-affinity binding sites. These sites do not correspond to any data obtained previously and are presumably an artifact of the sodium cholate extraction. It is possible

Figure 16. Reciprocal plot of the binding of [ $^3\text{H}$ ]-TTX to the 1% sodium cholate extract using equilibrium dialysis. The reciprocal of the picomoles of [ $^3\text{H}$ ]-TTX bound per gram of original wet tissue is plotted versus the reciprocal of the free [ $^3\text{H}$ ]-TTX concentration.

1  
PICOMOLES OF  $[^3\text{H}]$ -TTX BOUND/g TISSUE





that the anionic cholate molecule binds to the surface of the extracted molecules and creates "nonspecific" binding sites for the cationic [ $^3\text{H}$ ]-TTX.

(ii) Enzyme Treatments and Chemical Modifications. The most striking finding (Table 2) is the decreased stability of the tetrodotoxin binding component when it is extracted from the membrane. The Triton extract is quite sensitive to trypsin, chymotrypsin and pronase, whereas the binding component is relatively insensitive towards proteolytic digestion in the membranes (Table 1). The solubilized binding component is unstable at room temperature for any length of time so that all procedures must be performed below 10°C. At 0-4°C, however, it is stable for several days. Apparently the binding component contains at least one disulfide bond necessary for binding activity which is partially protected against reduction when the component is in the membrane (Table 1). The solubilized binding component retains a phospholipid requirement as seen by its sensitivity towards inactivation by Phospholipase A. It is possible that the solubilized binding component retains a certain amount of associated phospholipid which is necessary for binding activity, even in the presence of Triton X-100.

Table 2: Effects of Enzyme Treatment and Chemical Modification on Tetrodotoxin Binding in Triton Extracts

Treatment	Fraction of binding activity remaining after treatment
Ribonuclease	0.87
Deoxyribonuclease	0.87
Hyaluronidase	0.92
Neuraminidase	1.02
Phospholipase A	0.23
Phospholipase C	0.96
Trypsin	0.07
$\alpha$ -Chymotrypsin	0.25
Pronase	0.11
Heat 25°C (1 hour)	0.15
DTT 5 <u>mM</u>	0.12
ICH <sub>2</sub> CONH <sub>2</sub> 5 <u>mM</u>	0.97

(iii) Molecular Weight Determinations. The sucrose gradient and the Sepharose 6B column show that the [ $^3\text{H}$ ]-TTX binds to a component in the Triton extract which is distinct in its size properties from the bulk of the extracted protein. The sucrose gradient results show that the binding component sediments slightly slower than catalase but faster than the majority of the proteins in the 5% Triton extract with an apparent S value of 9.2 (Figure 17). There is also a fast sedimenting peak at the bottom of the centrifuge tube which is presumably due to aggregation or incomplete solubilization of the binding component. In contrast to its behavior on sucrose gradients, the binding component appears slightly larger than  $\beta$ -D-galactosidase in the Sepharose 6B elution profile (Figure 18). Similar results have also been reported by Henderson and Wang (58). The discrepancy in size estimation between sucrose gradients and gel filtration could be due to many factors including the shape, amount of associated lipid, and amount of bound detergent. Similar discrepancies in size between sucrose gradient and gel filtration data have been observed for detergent extracts of the acetylcholine receptor from electric fish (59), and may reflect an artifact due to the presence of the Triton detergent.

Figure 17. Sucrose gradient sedimentation of the Triton extract.  $\beta$ -D-galactosidase ( $S_{20, w} = 16$ ) and catalase ( $S_{20, w} = 11.4$ ) as marker proteins and the sample were run simultaneously. 140  $\mu$ l fractions were collected and 100  $\mu$ l aliquots were assayed for radioactivity and protein (57). The radioactivity measurements were corrected for the low level of [ $^3$ H]-TTX (269 dpm/100  $\mu$ l) found throughout the preformed gradient.

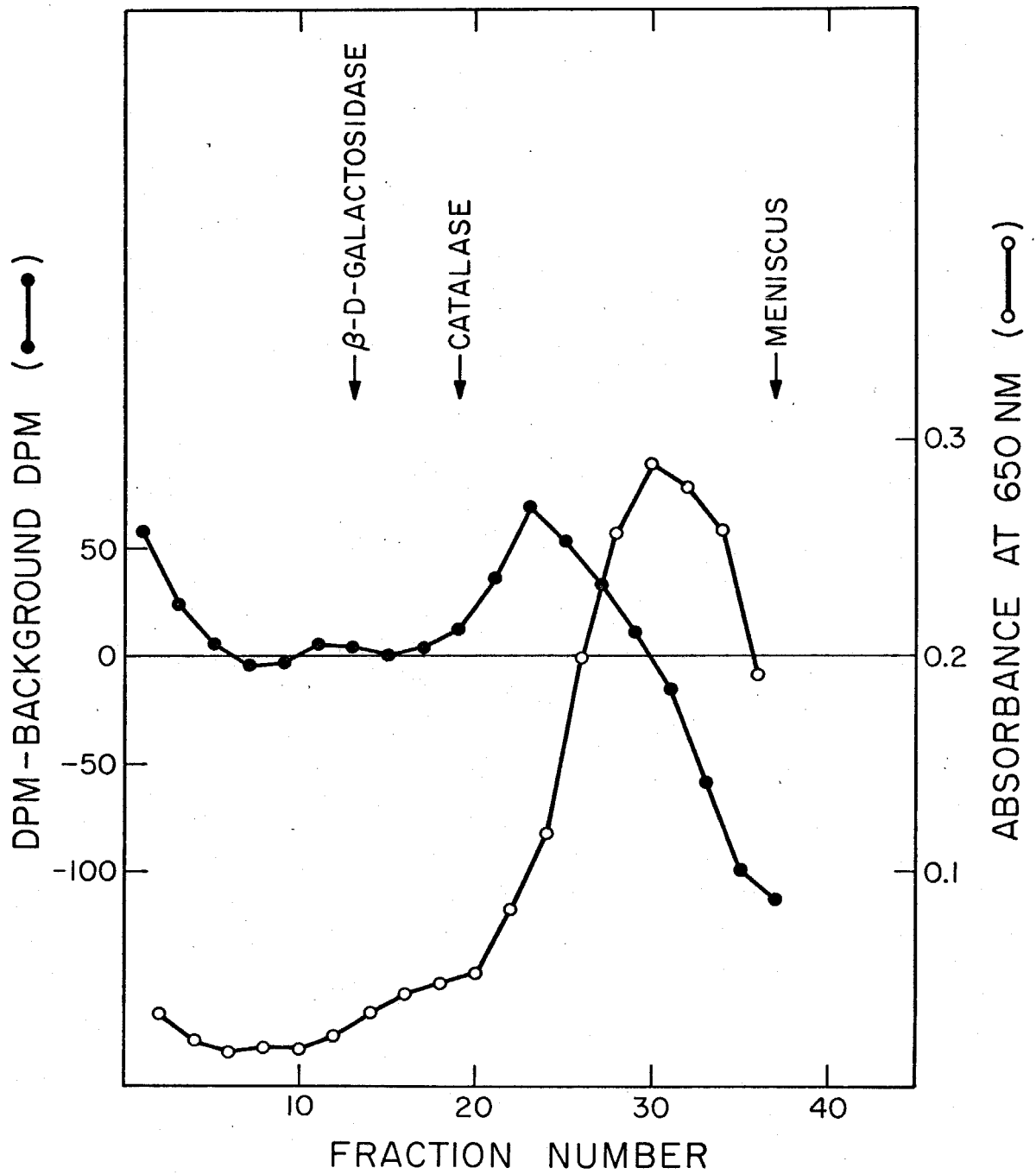
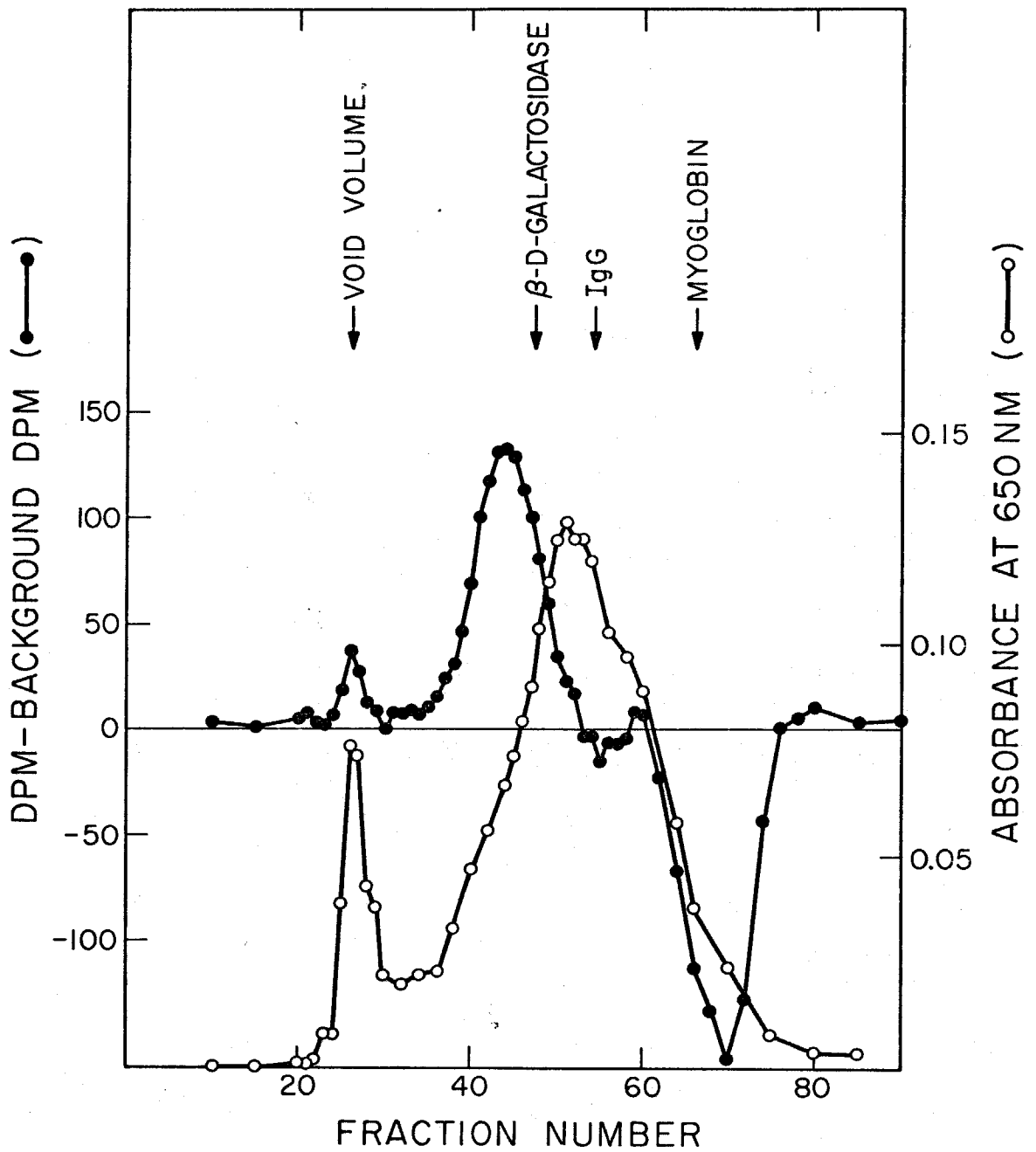


Figure 18. Sepharose 6B chromatography of 1 ml of Triton extract. The column was calibrated using  $\beta$ -D-galactosidase, IgG and myoglobin. The fraction size was 2.0 ml. 1.0 ml aliquots were assayed for radioactivity and 100  $\mu$ l for protein determination. Radioactivity was corrected for the constant background of [ $^3$ H]-TTX (345 dpm/ml) in the elution buffer.



### Chapter 3:

#### Preliminary Characterization of the Tetrodotoxin Binding

##### Component from Electric Eel

The preliminary results in the characterization of the tetrodotoxin binding component from garfish olfactory nerve have been promising. However, in order to isolate the binding component in sizeable quantities (milligrams) it will be necessary to use a tissue source which is available in kilogram quantities and has a concentration of binding sites approximately equivalent to that of the garfish. The electric organ from the electric eel is an electrically excitable system which has tetrodotoxin-sensitive sodium channels (see Introduction). The electric eel is an appropriate choice of tissue because the other components of the organ related to its electrical properties such as the  $\text{Na}^+ - \text{K}^+$  stimulated ATPase (60) the acetylcholine esterase (61) and the acetylcholine receptor (62) are under intensive study. In this chapter, I will describe preliminary studies on the binding of TTX to eel membrane fragments and the solubilization of the binding component using nonionic detergents.

In order to effect a purification of the TTX binding component, it would be quite advantageous to have an assay for TTX binding which is fast and accurate, using small amounts of material. The standard



equilibrium dialysis assay is too tedious and time consuming to be useful for the purification of the binding component. An assay for tetrodotoxin binding which uses gel filtration in the centrifuge to separate free [ $^3\text{H}$ ]-TTX from the [ $^3\text{H}$ ]-TTX bound to the detergent extract will be described which is based on that developed by Lefkowitz et al. (63) for the binding of norepinephrine to the  $\beta$ -adrenergic receptor protein. The application of the assay to problems of the stability of the TTX binding component in detergent solutions will also be discussed.

## Experimental

(i) Materials. Purified [ $^3\text{H}$ ]-tetrodotoxin was prepared as described in Chapter 1. Electrophorus electricus were from Paramount Aquariums, Ardsley, New York. Eels were either kept alive until use or the excised electric organ was kept frozen at  $-90^\circ\text{C}$  until use. Detergents and lipids were from Sigma Inc. All procedures were performed at  $0-4^\circ\text{C}$  unless specified.

(ii) Preparation of Membrane Fragments and Detergent Extracts from Eel Electric Organ. The main electric organ from Electrophorus electricus was excised and dissected free of connective tissue and large nerve fibers. Approximately 20 g of the organ was minced with scissors and homogenized in 2-4 volumes of cold

distilled water in a teflon-glass homogenizer for approximately 2 min. The resulting suspension was centrifuged at 50,000 rpm (250,000 xg max) for 1/2 hr in a Beckman 60-Ti rotor. The supernatant was discarded and the pellet suspended in 30-40 ml of 20 mM Tris-HCl pH 7.4 at 4°C using a teflon-glass homogenizer. The suspension was sonicated for two 15 second intervals at 45 power in a Branson J-22 sonicator, and then filtered through cheese cloth to remove large clumps of connective tissue. The suspension was centrifuged at 50,000 rpm (250,000 xg max) for 1/2 hr and the supernatant discarded. The pellet was either suspended in eel Ringers solution (64) or 0.1 M NaCl, 0.01 M potassium phosphate buffer, pH 7.0 for the membrane fragment binding studies. For detergent extraction, the pellet was suspended a solution containing the appropriate concentration of detergent, 10% glycerol and 20 mM Tris-HCl pH 7.4 at 4°C. The membranes were extracted for 12 hr at 0°C with stirring. After 12 hr the extracted membranes were centrifuged at 50,000 rpm (250,000 xg max) for 1 hr, and the extract was carefully decanted. The extract was stored at 0°C and never subjected to temperatures higher than 4°C.

(iii) Equilibrium Dialysis Binding Assays. Thin dialysis tubing (Union Carbide) was boiled for 3 hr with 3 changes of water. 0.75 ml of membrane suspension or detergent extract was placed in

the dialysis tubing and dialyzed against 5 ml of solution of the same salt and buffer composition containing various concentrations of [ $^3\text{H}$ ]-TTX. After 12 hr at 4°C, 0.5 ml aliquots were removed from inside and outside, dissolved in NCS solubilizer and counted for radioactivity in a toluene based scintillator solution. The ionic strength in the equilibrium dialysis experiments was always kept above 0.1 to eliminate artifacts due to the Donnon equilibrium across the dialysis membrane.

(iv) Sephadex G-50 Assay of [ $^3\text{H}$ ]-TTX Binding. Sephadex G-50 fine was swollen and equilibrated in 0.1% Lubrol-PX, 10% glycerol and 20 mM Tris-HCl pH 7.4 at 4°C. Small columns of the G-50 with a 1.5 ml bed volume were packed in 2.5 ml plastic syringes. The columns were prepared just prior to their use. The sample to be assayed was always made up to 285  $\mu\text{l}$  of which 250  $\mu\text{l}$  was loaded on the gel. The sample contained from 25-280  $\mu\text{l}$  of extract and 5-40  $\mu\text{l}$  of [ $^3\text{H}$ ]-TTX solution, the remaining volume up to 285  $\mu\text{l}$  was extraction buffer. Typically the sample and [ $^3\text{H}$ ]-TTX were incubated for 10 min at which point the sample was loaded carefully on to the top of the syringe column. The sample was allowed to soak into the gel and then centrifuged in an MSE desk top centrifuge by accelerating to 3000 rpm (1500 xg max) and immediately braking to stop. The effluent (approximately 1 ml) was collected and transferred quantitatively

to a plastic scintillation vial. Samples were evaporated under a stream of air to remove excess water. The sample was then dissolved in NCS tissue solubilizer (Amersham/Searle Inc.) and the radioactivity determined in a toluene based scintillator solution. A control sample containing no protein was always run to determine the background. For the initial characterization of the assay the following parameters were varied: the amount of protein assayed, the concentration of [ $^3\text{H}$ ]-TTX added, the preliminary equilibration time of the sample with [ $^3\text{H}$ ]-TTX, and the incubation time of the sample on the G-50 column before centrifuging.

(v) Extract Stability Studies. In order to determine the effect of various reagents on the time stability of the TTX binding component, the given reagent was added to the sample either just before extraction or right after the extract had been centrifuged. The extract was stored at 0°C and assayed using the G-50 assay along with the control sample after several days. These experiments were performed with both eel and garfish extracts.

## Results and Discussion

(i) [<sup>3</sup>H]-TTX Binding to Eel Membrane Fragments. Equilibrium dialysis binding studies of [<sup>3</sup>H]-TTX to eel electric organ membrane fragments suspended in Ringers solution shows binding to sites with a single dissociation constant  $K_D = 13.9 \times 10^{-9} \text{ M}$  and a maximal binding of 14.6 picomoles per gram of wet tissue weight (Figure 19). This value, although only 35% of the amount of TTX binding in the garfish membranes, is quite encouraging for eventual purification. The amount is approximately one-half the number of  $\alpha$ -Bungarotoxin binding sites reported in the eel (59), and it has been possible to purify the acetylcholine receptor from this tissue (62). When the equilibrium dialysis binding studies are performed in a buffer lacking divalent cations, 0.1 M NaCl, 0.01 M K/K<sub>2</sub> PO<sub>4</sub> pH 7.0, it is found that the maximal TTX binding is increased to 25 picomoles per gram of tissue with a dissociation constant  $K_D = 16.7 \times 10^{-9} \text{ M}$  (Figure 20). Subsequent experiments have shown that the binding is inhibited in the presence of millimolar concentrations of calcium ions. Magnesium ions in the same concentration range do not affect the binding of [<sup>3</sup>H]-TTX (J. Reed, unpublished data). Similar results have been obtained by Henderson et al. (65) with the binding of [<sup>3</sup>H]-TTX and [<sup>3</sup>H]-saxitoxin to intact rabbit vagus nerves and solubilized garfish olfactory nerve membranes. In their preparations the

Figure 19. Double reciprocal plot of equilibrium dialysis [ $^3\text{H}$ ]-TTX binding to eel membrane fragments in eel Ringers solution. The reciprocal of the picomoles of [ $^3\text{H}$ ]-TTX bound per gram of wet tissue weight is plotted versus the reciprocal of the free [ $^3\text{H}$ ]-TTX concentration. The maximum amount bound is 14.6 picomoles/gm tissue with a dissociation constant  $K_D = 13.9 \times 10^{-9} \text{ M}$ .

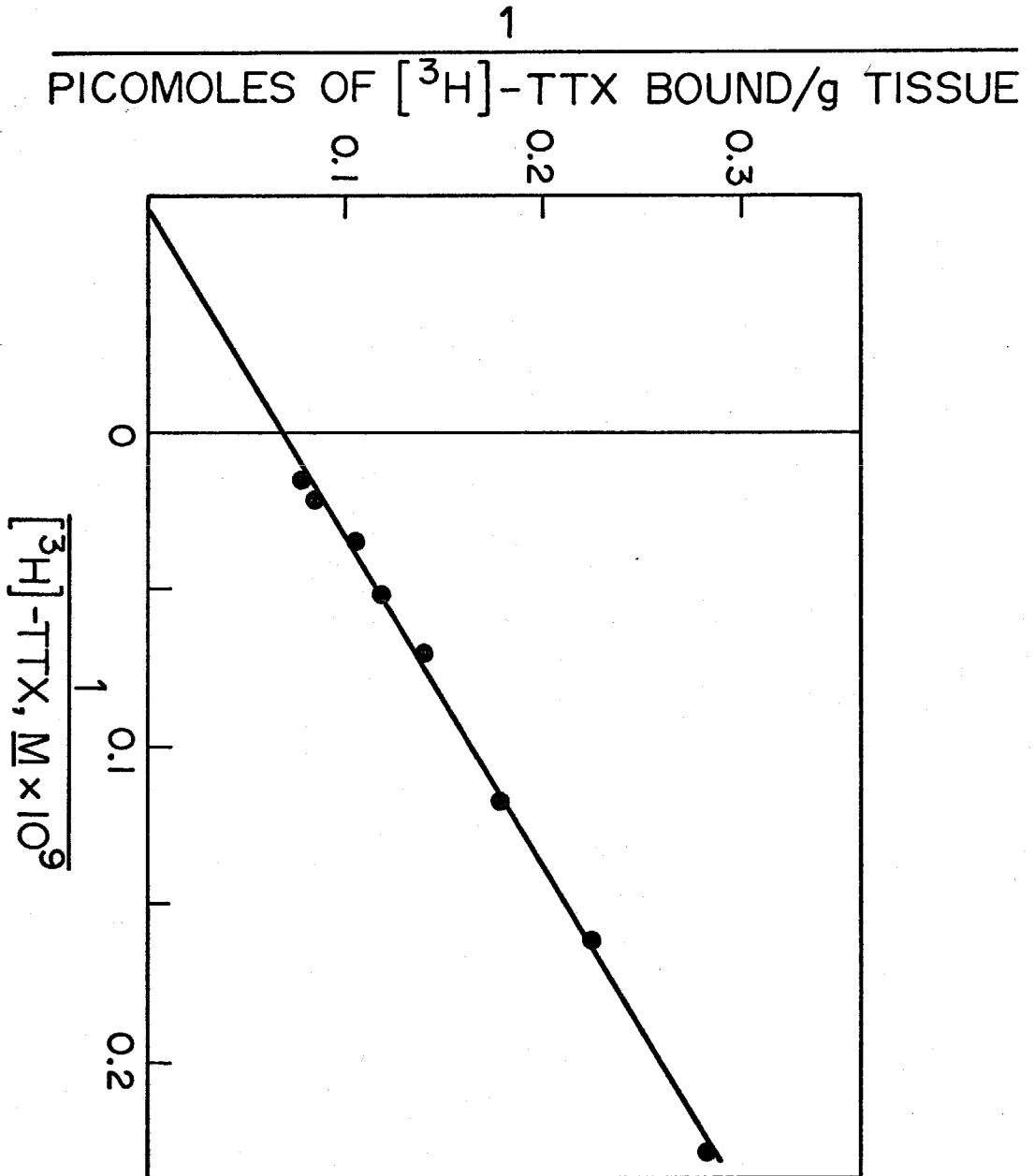
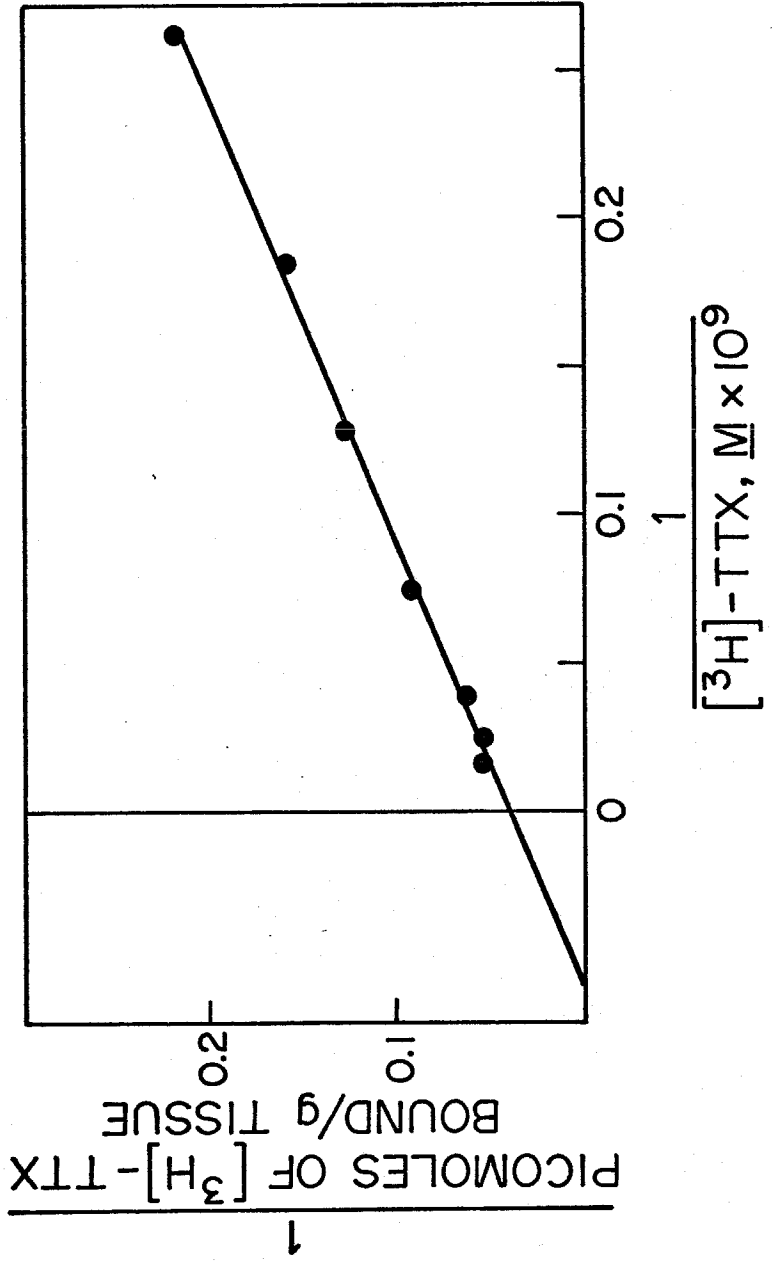


Figure 20. Double reciprocal plot of [ $^3\text{H}$ ]-TTX binding to eel membrane fragments in 0.1 M NaCl and 0.01 M potassium phosphate pH 7.0. The reciprocal of the picomoles of [ $^3\text{H}$ ]-TTX bound per gram of tissue is plotted against the reciprocal of the free [ $^3\text{H}$ ]-TTX concentration. The maximum amount bound is 25 picomoles per gram of tissue with a dissociation constant  $K_D = 16.7 \times 10^{-9}$  M.



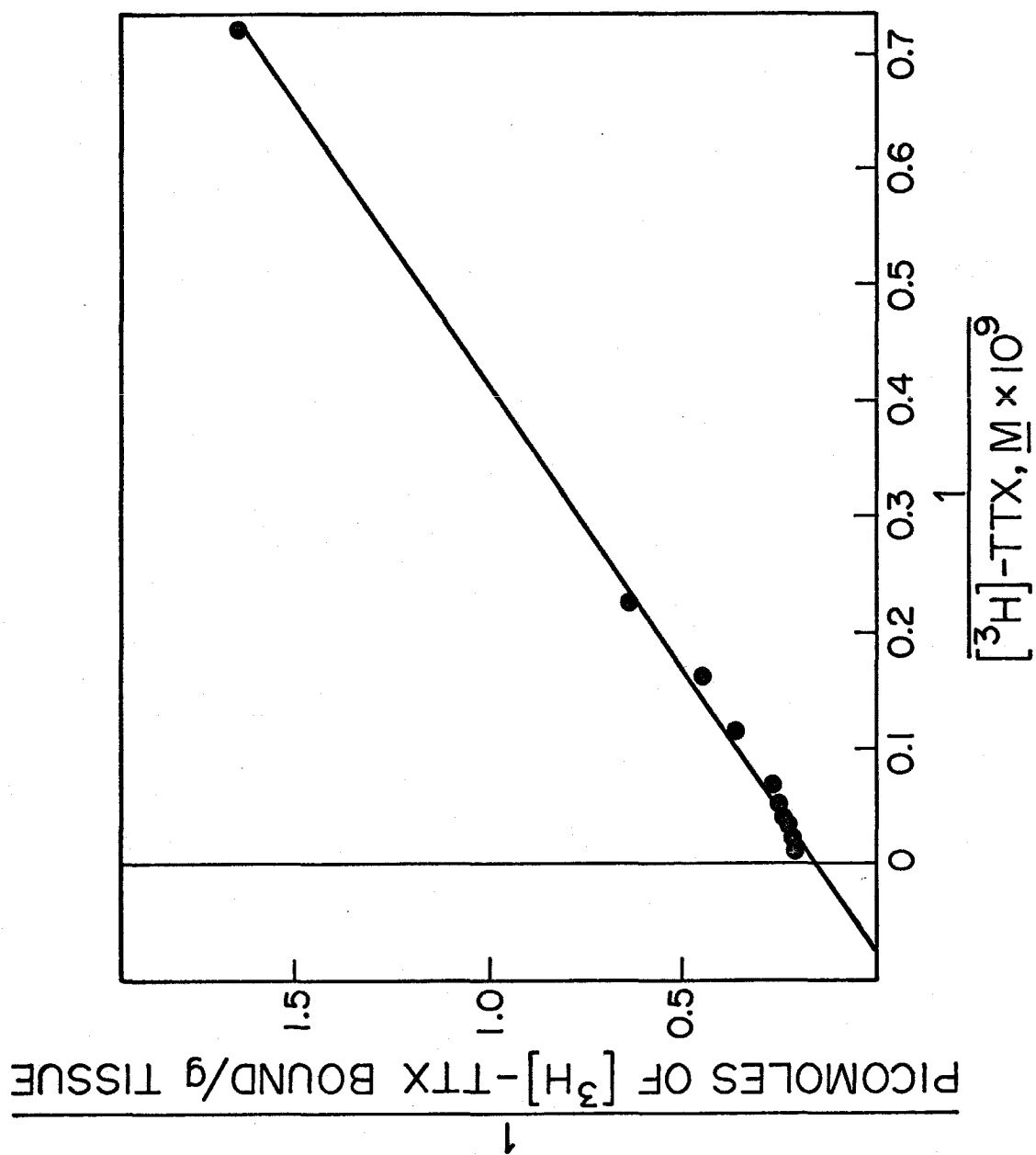


equilibrium dissociation constants for  $\text{Ca}^{++}$  displacing bound toxin is approximately 20-30 millimolar. High calcium concentrations have been found to increase the threshold potential for nerve stimulation (66), and Woodhull (67) finds that calcium ions block current through the sodium channel with a dissociation constant of 23 millimolar. Therefore, the antagonism between toxin binding and calcium ions that I have observed may not be surprising.

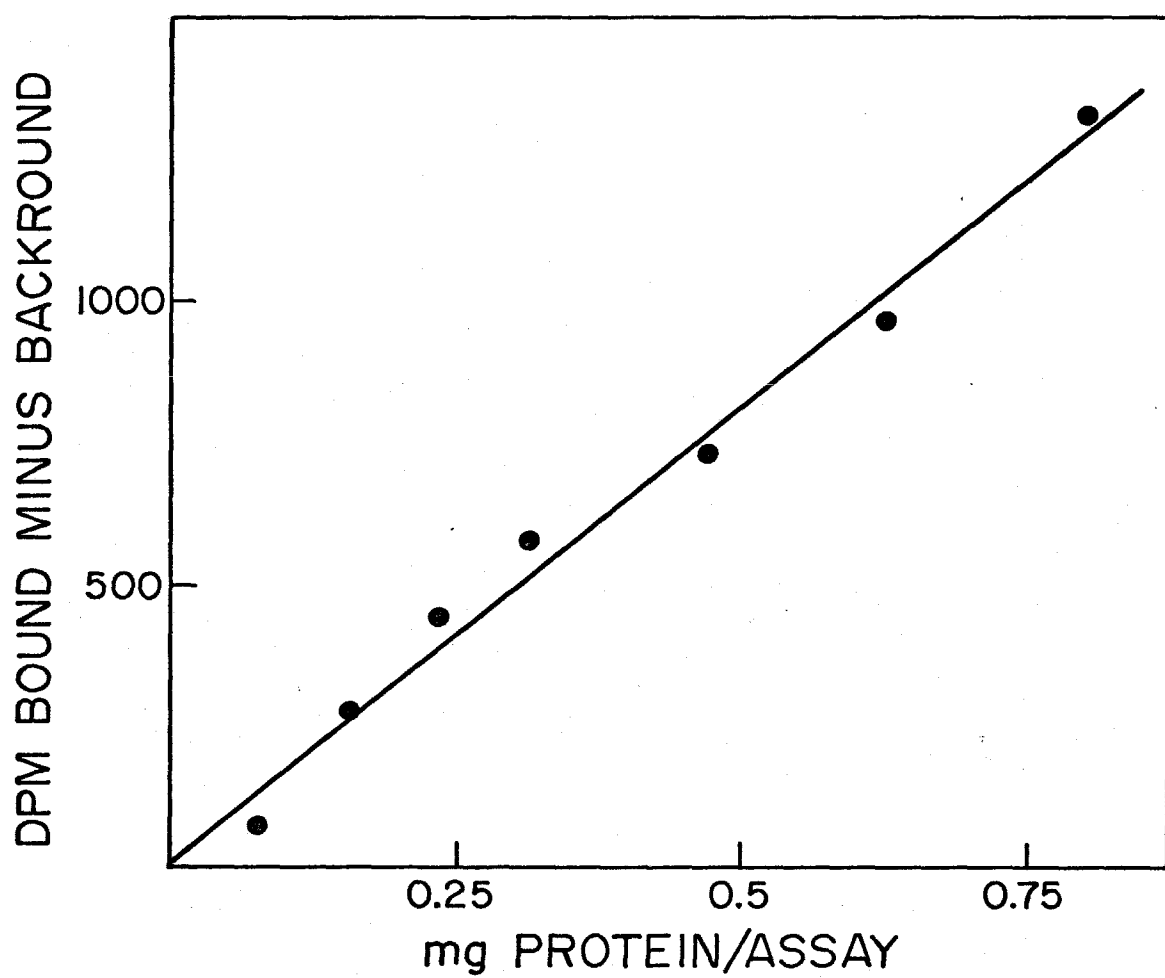
(ii) Solubilization of the TTX Binding Component. Using the G-50 binding assay described in this chapter it was found that detergent extracts of eel membrane fragments could be prepared having an  $[^3\text{H}]$ -TTX binding capacity of up to 25 picomoles per gram of wet weight tissue although this value varied somewhat from preparation to preparation. A medium containing 1% Lubrol-PX, 10% glycerol, 20 mM Tris-HCl pH 7.4 at 4°C was found to be the most efficient solubilizer, although Triton-X-102 and Brij-96 could also be used to solubilize the binding component. From equilibrium dialysis binding studies of the solubilized binding component (Figure 21) a dissociation constant  $K_D = 13.3 \times 10^{-9} \text{ M}$  was obtained which is not significantly different from the value obtained for the membrane fragments.

(iii) Sephadex G-50 Assay of  $[^3\text{H}]$ -TTX Binding. In order for an assay to be useful during the purification of the TTX binding component, it should be reproducible, fast, and linear over a

Figure 21. Double reciprocal plot of equilibrium dialysis binding of [ $^3\text{H}$ ]-TTX to a 1% Lubrol extract of eel membrane fragments. The reciprocal of the picomoles bound per gram of tissue is plotted versus the reciprocal of the free [ $^3\text{H}$ ]-TTX concentration.



**Figure 22.** The plot shows the linear relationship between the amount of 1% Lubrolextract assayed by the Sephadex G-50 assay and the amount of [ $^3\text{H}$ ]-TTX bound. Various amounts of extract made up to 285  $\mu\text{l}$  and containing  $\approx 200 \text{ nM}$  [ $^3\text{H}$ ]-TTX were incubated for 10 min at  $0^\circ\text{C}$  then 250  $\mu\text{l}$  aliquots were removed and assayed on small G-50 columns as described in the methods section of this chapter.



reasonable protein concentration range. Using equilibrium dialysis it takes approximately 24 hr before usable data can be obtained; but with the Sephadex G-50 assay results are available within two hr after the start of the assay. The assay gives a linear relationship between bound counts and the amount of extract assayed (Figure 22). The background radioactivity obtained in the absence of protein was approximately 150 dpm. All points on the curve except the lowest one are at least twice background. Experiments were also carried out to determine the extent of [ $^3\text{H}$ ]-TTX binding after various equilibration times with the 1% Lubrol extract. Figure 23A shows that the binding reaction is >90% complete after 2.5 min incubation of 200 nM [ $^3\text{H}$ ]-TTX with the 1% Lubrol extract. Typically several assays were run at the same time and therefore it was necessary to allow the samples to incubate on the gel for up to several minutes before centrifuging. Figure 23B shows that the sample can be incubated on the gel for up to fifteen minutes without significantly affecting the binding of [ $^3\text{H}$ ]-TTX and furthermore, that the assay is reproducible to  $\pm 3\%$  of the mean for identical samples.

In order to choose the appropriate concentration of [ $^3\text{H}$ ]-TTX to use in the Sephadex G-50 assay, the same amount of 1% Lubrol extract was assayed in the presence of several concentrations of [ $^3\text{H}$ ]-TTX. Figure 24 is a double reciprocal plot of the results

Figure 23. (A) Identical amounts of 1% Lubrol extract were equilibrated with 200 nM [ $^3\text{H}$ ]-TTX for various times ranging from 2-30 min, prior to the G-50 assay. The plot shows that after a 10 min equilibration time the binding of [ $^3\text{H}$ ]-TTX to the binding component is essentially complete.

(B) The samples containing a given amount of 1% Lubrol extract and 200 nM [ $^3\text{H}$ ]-TTX were applied to the Sephadex G-50 gel and allowed to incubate for various times ranging from 0.5-15 min before centrifugation. The plot shows no decrease in binding over this time interval.



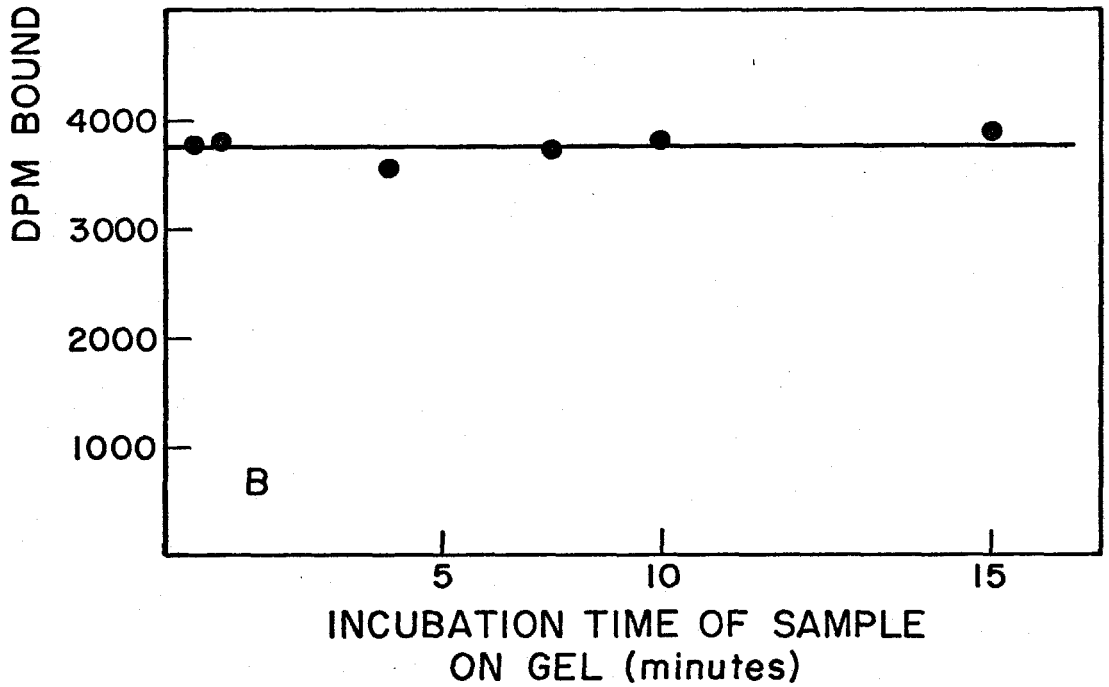
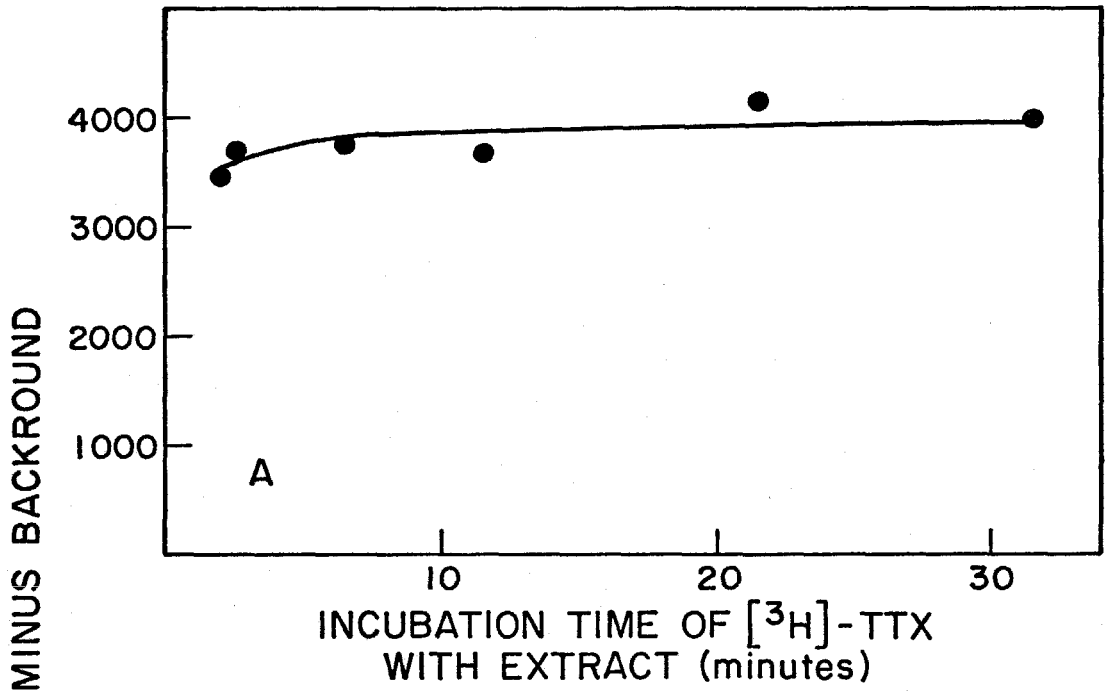
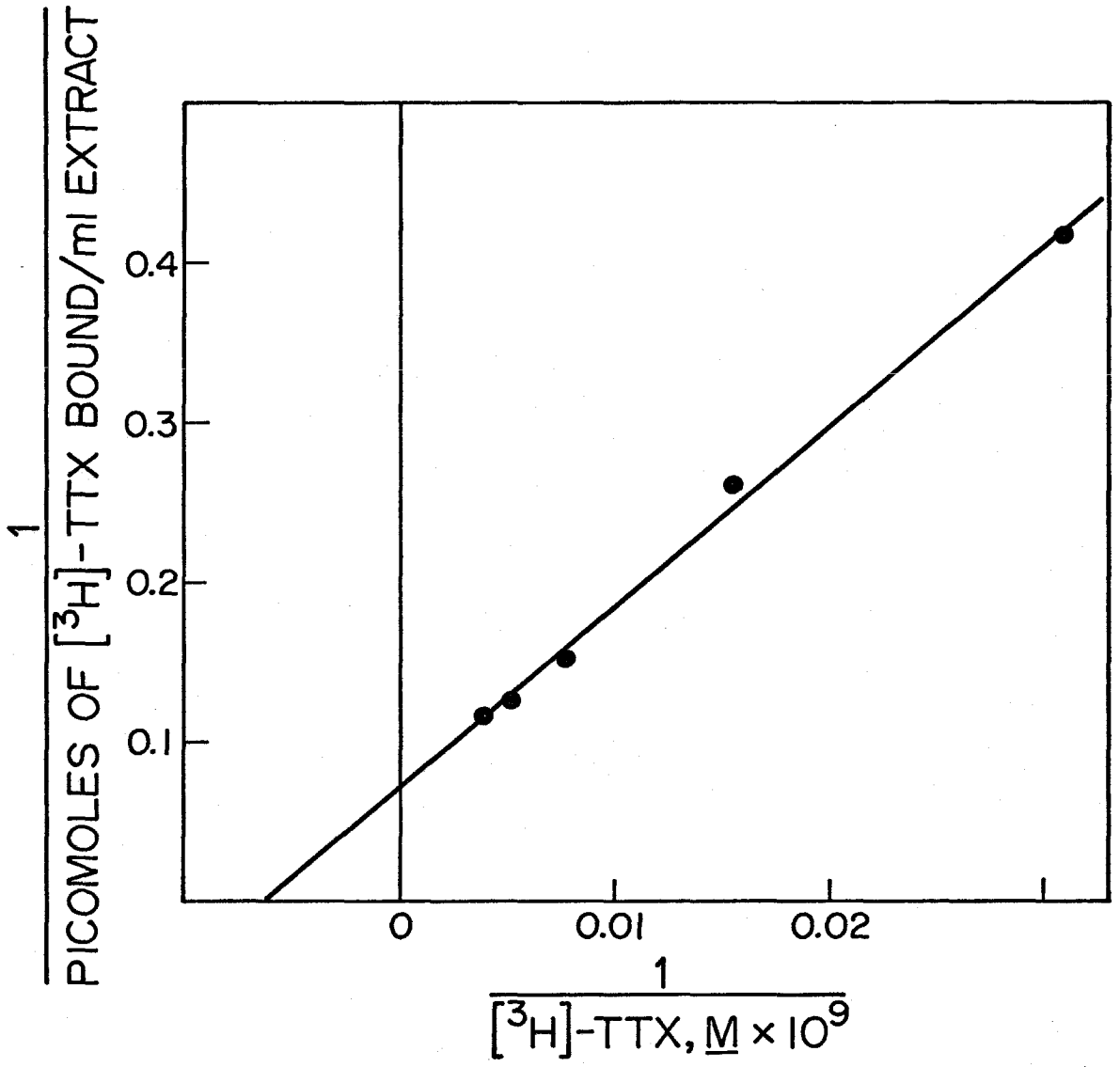


Figure 24. Double reciprocal plot of [ $^3\text{H}$ ]-TTX binding to 1% Lubrol extract using the Sephadex G-50 assay. The reciprocal of the picomoles of [ $^3\text{H}$ ]-TTX bound per ml of extract is plotted against the reciprocal of the concentration of [ $^3\text{H}$ ]-TTX used in the assay. Half-maximal binding is obtained at 160 nM [ $^3\text{H}$ ]-TTX.



obtained. The binding is saturable and half-maximal binding occurs at about 160 nM [ $^3\text{H}$ ]-TTX. Routine assays were carried out in 200 nM [ $^3\text{H}$ ]-TTX. With this concentration one obtains approximately 60% of the maximal binding with a background radioactivity of about 150 dpm.

Using the Sephadex G-50 assay, one can measure the [ $^3\text{H}$ ]-TTX binding in detergent solubilized samples reproducibly and quickly. The maximal amount of binding can be determined by doing the assay at several [ $^3\text{H}$ ]-TTX concentrations and extrapolating the data to saturating conditions. The assay cannot be used to determine the [ $^3\text{H}$ ]-TTX binding in particulate samples because the particles are too large to pass through the Sephadex gel.

(ii) Extract Stability Studies. When the detergent extract of the garfish nerve or electroplax is stored at 0°C in the presence of 1% detergent the half-life of the TTX binding component is approximately six days. When the extract is incubated at 20°C the half-life is less than one hr. A stability problem of this magnitude can be dealt with if the preparation contains a high concentration of the component being studied. When detergent extract, which is 90% inactivated, is assayed for TTX binding one obtains values which are not significantly above the background radioactivity of the assay. For this reason, considerable effort was expended trying to prolong the

lifetime of the detergent extracted TTX binding component. Because the reason for the instability is unknown the approach was fairly random. Compounds were added to the detergent extract in hopes that they were a necessary factor for stabilization. Five classes of compounds were investigated including inorganic ions, hydrolytic enzyme inhibitors, toxins active on the sodium channel, lipids, and several antioxidants. None of the compounds tried had a significant effect on the stability of the binding component. Table 3 lists the compounds and concentrations used. The only procedure which was found to affect the stability of the binding component significantly was a change in detergent concentration. The garfish extract, stored in 5% Triton-X-100 for 6 days retained 3% of the TTX-binding activity while an extract stored in 1% Triton retained approximately 50% of the binding activity. Further progress on the biochemical characterization of the detergent solubilized binding component will depend on whether the stability problem can be solved. The extremely low concentration of the TTX binding component in detergent extracts and the problems encountered in preliminary attempts at purification (see Chapter 4) make it imperative that the problem of stability be solved.

Table 3: Compounds Used in Attempt to Stabilize the  
TTX Binding Component

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Ionic molecules

MgCl<sub>2</sub> (2 mM)

CaCl<sub>2</sub> (2.2 mM)

EDTA (2 mM, 5 mM)

NaCl (.5 M)

KF (.2 M)

Enzymatic inhibitors

Phenylmethanesulfonyl fluoride  
(sat. sol'n)

Sodium azide (.02%)

Anti-oxidants

D- $\alpha$ -tocopherol (.01%)

Butylated-hydroxytoluene (.01%)

2, 6-di-tert-butyl-4-hydroxy methyl phenol (.01%)

Nordihydroguaiaretic acid (.01%)

Lipophilic molecules

Cholesterol (2 mg/ml)

Lysolecithin (2 mg/ml)

Lecithin (2.5 mg/ml)

Dimethyl formamide (2 mg/ml)

Sodium channel toxins

<sup>3</sup>H-tetrodotoxin (50 nM)

Veratridine (0.1 mM)

Others (proteins and crosslinking  
reagents)

Bovine serum albumin (1%)

Dimethylsuberimidate (0.1%, 0.059%,  
0.01%)

Glutaraldehyde (0.1%, 0.05%, 0.01%)

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## Chapter 4

### Synthesis and Preliminary Trials of Affinity Resins for the Tetrodotoxin Binding Component

A specific tetrodotoxin binding component which is presumably an integral part of the sodium channel has been identified in several preparations of electrically excitable tissue (52, 68, 69, Chapters 1 and 3). The binding component has been solubilized with nonionic detergents and characterized using gel filtration and sucrose density gradient centrifugation (58, Chapters 2 and 3). Using the electroplax from the electric eel it should be possible to purify quantities of the TTX binding component sufficient for biochemical characterization. Due to the low concentration of binding component in the electroplax and the instability of the detergent solubilized binding component, it will be necessary to have a purification method which is both fast and very specific.

Affinity chromatography is a method in which solid supports, having specific ligands covalently linked to them, are used to adsorb selectively the macromolecules to be purified. The adsorbed macromolecule is then eluted by incubating the resin with a sufficient concentration of the ligand in solution. A large purification of the specific

macromolecule can often be obtained in one step from starting material containing very low concentrations of the macromolecule (70). This chapter describes the synthesis of and preliminary experiments on two possible affinity resins which may be useful for the purification of the TTX binding component.

Two chemical derivatives of tetrodotoxin were used for synthesis of the affinity resins, one having a free amino group (tetrodamino-toxin) and the other a free carboxyl group (tetrodonic acid). The derivatives appear have an identical physiological action to that of unmodified tetrodotoxin except that they are active at higher concentrations. Tetrodaminotoxin is active at concentrations approximately two orders of magnitude higher than TTX and tetrodonic acid is at least four orders of magnitude less potent (40, 41). These values may be underestimates of the true active concentration range of the derivatives due to the presence of an unknown quantity of native TTX. By use of ligands which are active at concentrations between  $10^{-6}$  M and  $10^{-4}$  M it may be possible to obtain affinity resins which can be eluted under relatively mild conditions, in contrast to resins synthesized from ligands active at  $10^{-9}$  M or less which may require strong denaturing conditions for elution.

Following the suggestions of Cuatrecasas (70, 71) a large pore agarose gel was used in the synthesis of the resin; this allows the



TTX binding component to diffuse into the matrix of the gel. The ligands were attached to the end of a long flexible "arm" which allowed a greater accessibility to the binding component in solution. The chemical structure of the arm was chosen to contain a limited number of charged groups and care was taken to limit the concentration of the charged ligand itself. Schmidt and Raftery (72) have shown that with increasing concentrations of a charged ligand on an affinity resin the behavior of the resin can change from that of highly selective affinity resin to that of a nonspecific ion-exchange resin.

## Experimental

(i) Materials. Sepharose 2-B was from Pharmacia, cyanogen bromide and succinic anhydride from Matheson Coleman and Bell, hexamethylenediamine from Eastman Organic Chemicals and the water-soluble carbodiimide, 1 ethyl-3-(3-dimethylaminopropyl)-carbodiimide from Cyclo Chemicals. Tetradaminotoxin and tetrodonic acid were kindly supplied by Dr. Yoshito Kishi of Nagoya University. Other reagents used were of reagent grade unless specified.

(ii) Synthesis and Coupling of the Flexible "Arm" to Agarose Beads for the Affinity Resins. The cyanogen bromide activation and coupling of hexamethylenediamine to the agarose was performed according to Porath et al. (73). Subsequent reactions followed the

methods of Cuatrecasas (71). To 200 ml of washed Sepharose 2-B in 100 ml of ice cold .24 M potassium phosphate buffer pH 12 was added with stirring, 0.2 g (1.9 mmoles) of cyanogen bromide in dioxane over a two minute interval. The reaction mixture was stirred for ten minutes after which the activated resin was filtered and washed quickly with four liters of cold 0.1 M sodium bicarbonate buffer pH 10. The washed resin was added to a solution containing 23.2 mg (0.2 mmole) of hexamethylenediamine in 200 ml of cold distilled water and titrated to pH 10 with HCl. The reaction mixture was agitated at 4°C for 24 hr, after which it was filtered and washed with water.

To 200 ml of the aminoalkyl sepharose in 200 ml of cold water was added, 0.4 g (4 mmole) of succinic anhydride and stirred at 4°C. The pH was raised to 6.0 and maintained at that pH with 2 N NaOH. After the reaction had stopped taking up base, the suspension was agitated for 5 hr at 4°C, filtered and washed with water.

200 ml of the succinylaminoalkyl sepharose, in 200 ml of distilled water was mixed with 0.464 g (4 mmole) of hexamethylenediamine and titrated to pH 4.7. A solution of 6.2 g (40 mmole) of the water soluble carbodiimide in 20 ml of water was added over a period of 5 min. The pH was maintained at 4.7 for 1 hr. The reaction mixture was then agitated for 24 hr at room temperature after which the resin was filtered and washed with water.

(iii) Synthesis of the Tetrodonic Acid Affinity Resin. To 10 ml of aminoalkylaminosuccinylaminoalkyl sepharose in 5 ml distilled water containing 1 mg (3  $\mu$ mole) of tetrodonic acid and titrated to pH 4.7 was added, 0.31 g (2 mmole) of the water soluble carbodiimide in 2 ml of water over 5 min. The reaction mixture was titrated at pH 4.7 for 1 hr and then agitated for 48 hr at room temperature. The tetrodonic acid affinity resin was filtered, washed with water, and stored at 4°C in the presence of 0.02% sodium azide.

(iv) Synthesis of Tetrodaminotoxin-Sepharese 2-B Resin. To 100 ml of the aminoalkylsuccinylaminoalkyl sepharose, synthesized as described previously, was added, 100 ml of cold distilled water and 0.4 g (4 mmole) of succinic anhydride. The pH was raised and maintained at 6.0 with 2 N NaOH for approximately 1/2 hr. The reaction mixture was then agitated for 12 hr at 4°C after which the resin was filtered and washed with water. A suspension of 10 ml of the above resin, 5 ml of distilled water and 1 mg (3.2 mmole) of tetrodaminotoxin was titrated to pH 4.7. To this mixture, 0.31 g (2 mmole) of the water soluble carbodiimide in 2 ml of water was added over 5 min and the reaction was titrated at pH 4.7 for 1 hr. After agitation at room temperature for 48 hr, the tetrodaminotoxin affinity resin was filtered, washed with water and stored in 0.02% sodium azide at 4°C.

(v) Preliminary Experiments with the Tetrodaminotoxin and Tetrodonic Acid Resins. The affinity resins were packed into columns and equilibrated in a solution containing 0.1% Lubrol-PX, 10% glycerol, and 10 mM Tris pH 7.4 (L-G-T buffer). The columns were always prewashed with the L-G-T buffer containing 1 M NaCl and 0.1 M  $\text{CaCl}_2$ . Detergent extracts from electroplax were applied to the columns which were then washed and eluted with various concentrations of NaCl,  $\text{CaCl}_2$  and [ $^3\text{H}$ ]-TTX in L-G-T buffer. The eluants were assayed for protein by the method of Lowry *et al.* (57) and for TTX binding by the Sephadex G-50 assay. Equilibrium dialysis binding studies were performed on aliquots of the tetrodaminotoxin resin immediately after a typical run to determine if any active binding component had remained bound to the resin.

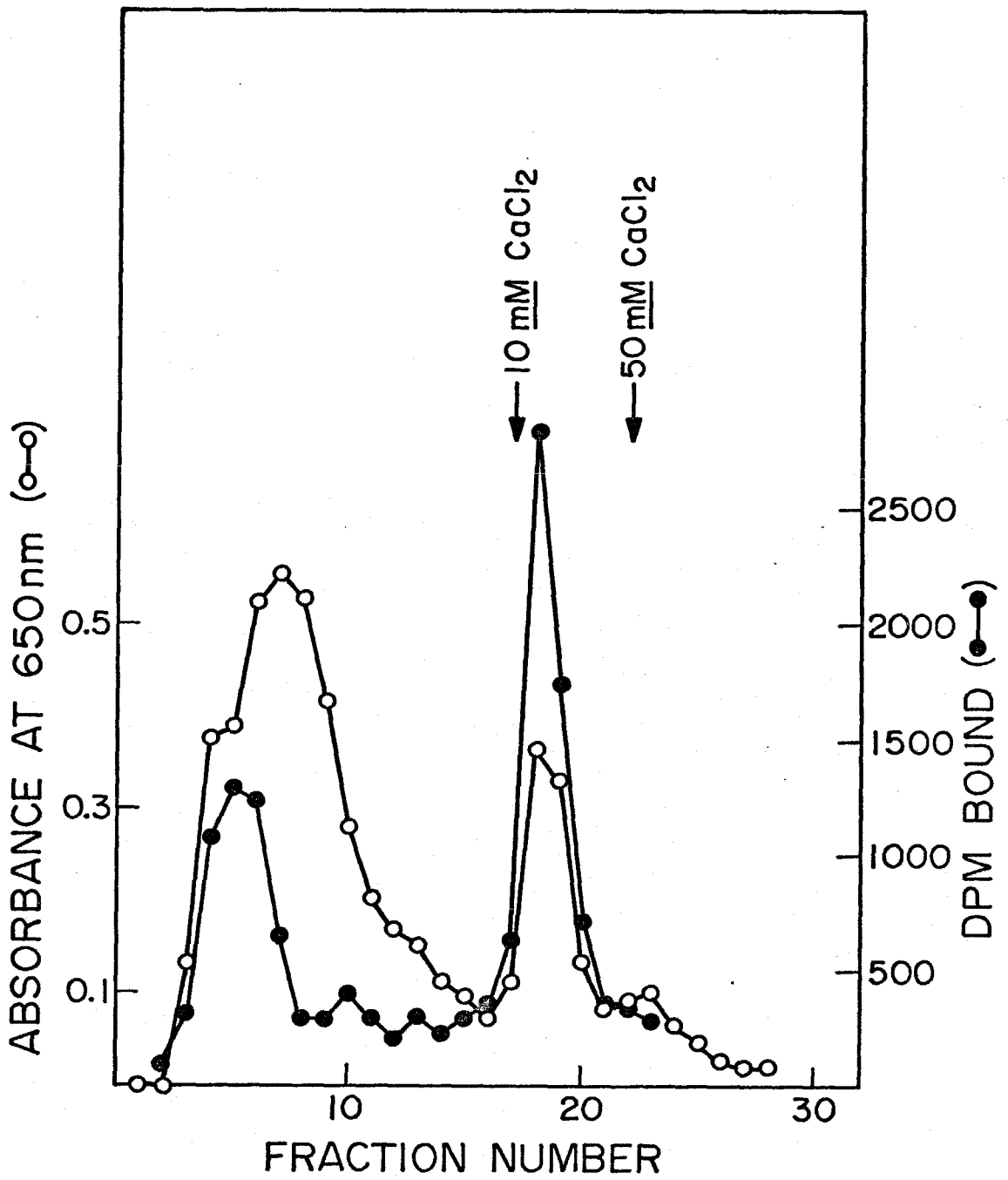
Control experiments were carried out using unmodified Sepharose 2-B in place of the affinity resin. Detergent extract samples were applied to the control columns which were washed and eluted the same way as the affinity columns. Controls were also carried out using DEAE cellulose, guanidoethylcellulose and guanidosepharose, in place of the affinity resin, to monitor the behavior of the TTX binding component when adsorbed to a solid support. The detergent extract was applied to the columns at neutral pH and eluted with NaCl under a variety of conditions including: 1% detergent, 0.1% detergent, linear

salt gradients and step gradients.

## Results

A sample of 8 ml of 1% Lubrol-PX extract of electroplax was applied to the tetrodaminotoxin resin equilibrated in L-G-T buffer. The column was washed with 10 ml of L-G-T buffer and eluted with 10 ml of L-G-T buffer containing 10 mM  $\text{CaCl}_2$  followed by 20 ml of L-G-T buffer containing 50 mM  $\text{CaCl}_2$ . Figure 25 shows the elution profile obtained. Experiments using the tetrodonic acid column gave similar results. It is important to note that although 100% of the applied protein was recovered in the total effluent, only 33% of the applied TTX binding activity was recovered. Furthermore, the TTX binding activity which is eluted with the buffer containing 10 mM  $\text{CaCl}_2$  is associated with a substantial amount of protein. Subsequent experiments with the tetrodaminotoxin resin showed that the TTX binding could be eluted with NaCl in L-G-T buffer at the same ionic strength as the elution using  $\text{CaCl}_2$  even though calcium ions inhibit TTX binding at much lower concentrations than sodium ions. Elution with L-G-T buffer containing up to 0.1 M  $\text{CaCl}_2$  did not increase the yield of binding activity in the effluent. When an attempt was made to elute the adsorbed TTX binding activity from the tetrodaminotoxin column with 150 nM [ $^3\text{H}$ ]-TTX, no detectable binding

Figure 25. Chromatography of 8 ml of a 1% Lubrol-PX extract of eel electroplax on the tetrodamino-toxin resin. Sample application and elution were as described in the results. Fractions of 2.0 ml were collected, 100  $\mu$ l were assayed for protein and 224  $\mu$ l assayed for TTX binding. The sample contained 35.2 mg of protein and bound 320 picomoles of [ $^3$ H]-TTX. A total of 35.3 mg of protein and 104 picomoles of [ $^3$ H]-TTX binding activity were recovered in the effluent.



activity or protein was observed in the effluent. Equilibrium dialysis binding studies were carried out on aliquots of the resin after an experiment and no binding of [ $^3\text{H}$ ]-TTX to the resin was observed over a range of concentrations from 1.5 to 70 nM [ $^3\text{H}$ ]-TTX.

Columns of unmodified Sepharose 2-B eluted with the same buffers as the tetrodaminotoxin columns gave yields of 80 to 100% of the applied TTX binding component in the void volume.

The detergent extract was chromatographed on a number of anion exchange resin to investigate the apparent loss of TTX binding activity when the extract is adsorbed to the tetrodaminotoxin resin. At neutral pH and low ionic strength, no TTX binding activity is eluted in the void volume when the extract is applied to the resins. No binding activity can be recovered at all in the eluent if the elution buffer is made 1% in a nonionic detergent or if elution is attempted with a linear salt gradient. However, up to 60% of the TTX binding activity applied to the column can be recovered if the elution buffer contains only 0.1% detergent and elution is carried out with a step gradient of salt. This behavior indicates that the solubilized binding component may be even more unstable when adsorbed to a resin than it is in solution.



## Discussion

Preliminary experiments using the two tetrodotoxin derivative resins show that these resins are of limited use for a large scale purification of the tetrodotoxin binding component from electroplax. The purification factors obtained are modest, the yields of purified material are extremely low and elution with specific ligands has not been possible.

It is conceivable that the 70% of the TTX binding not recovered during elution of the resin remained tightly bound to it. However, no TTX binding was observed on the resin after a typical run. Under the conditions of this assay, TTX binding would be measurable if the binding component were bound to the resin through the active site with an apparent dissociation constant of less than  $10^{-5}$  M. The possibility that the TTX binding component is tightly bound to the resin cannot be completely excluded. If this were the case, then it would be of interest to synthesize a resin with a much lower concentration of ligand sites. The elution of the TTX binding component from such a resin would be easier due to the reduced ligand concentration; but synthesis of the resin would be difficult because of the slow reaction rate of the carbodiimide coupling at low concentration.

The other possibility, that the TTX binding component is inactivated when adsorbed to the affinity resin, is more likely.

Because tetrodaminotoxin is so much less potent than unmodified TTX it may not be reasonable to assume that the TTX binding component binds to the affinity resin that tightly unless more than one ligand can interact with a single TTX binding component. This possibility seems remote due to the low concentration of ligand sites on the resin (less than  $5 \times 10^{-4}$  moles per liter of resin). Schmidt and Raftery (72) have calculated that a ligand substitution of  $10^{-3}$  moles per liter of resin would correspond to a nearest neighbor distance of 100 Å, assuming ligands distributed evenly through the resin and positioned at the intersections of a cubic lattice. The apparent size of the TTX binding component would preclude interaction with more than one ligand on the resin.

The instability of the TTX binding component on anion exchange resins lends support to the hypothesis that the binding component is also being inactivated on the tetrodaminotoxin resin rather than binding to it very tightly.

The tetrodotoxin-derivative resins are of little use for purification methods at the present time. Furthermore, it seems apparent that the problem of the instability of the detergent solubilized binding component will have to be solved before much progress can be made in the purification and further characterization of the binding component.

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### Abstracts of the Propositions

**Proposition 1:** Action potentials in denervated and embryonic muscle tissue are resistant to the effects of tetrodotoxin. In order to understand the differences between these sodium channels and the normal ones, it is proposed to study the [ $^3\text{H}$ ]-TTX binding to innervated, denervated and embryonic muscle tissue.

**Proposition 2:** Ionic currents, different from the currents observed during an action potential but induced by depolarization, have been observed in nerves and are thought to arise from movements of molecules in the membrane associated with the opening of sodium channel gates. Black widow spider venom has been shown to increase the kinetics of opening and closing of the sodium channel. It is proposed to apply the venom to nerves and measure the effect on these presumed "gating currents". If the time course of the currents are altered, it would be strong evidence that the observed currents are indeed related to the opening and closing of sodium channels.

**Proposition 3:** Electrically excitable calcium channels have been found in paramecia which control movement of the cilia. There are drugs which at very low concentrations are capable of blocking calcium action potentials in certain muscles. It is proposed to study the effects of these



calcium channel antagonists on the electrical activity of paramecia, and if appropriate, to study the binding of radioactively labeled antagonists to normal and mutant paramecia in an attempt to characterize the molecular properties of their calcium channels.

Proposition 4: Intracellular particles containing what are thought to be newly synthesized acetylcholine receptors have been identified in cultures of chick muscle. It is proposed to study, in vitro, the mechanism of incorporation of these new receptor molecules into the plasma membrane.

Proposition 5: It is proposed to react aspartate transcarbamylase with specific reagents containing  $^{19}\text{F}$  in an attempt to label the protein at a small number of well-defined sites and use  $^{19}\text{F}$ -nuclear magnetic resonance to study the tertiary and quaternary structural changes occurring during catalysis.

## Proposition 1

### A Study of the Binding of Tetrodotoxin to Innervated, Denervated, and Embryonic Muscle Membranes

#### Introduction

Adult skeletal muscle exhibits two forms of electrical activity. The muscle cell can be depolarized by the application of acetylcholine, and the muscle membrane can conduct an action potential similar to that observed in nerve fibers. This can be induced by electrical stimulation of either the muscle fiber itself or the innervating nerve. Del Castillo and Katz (1) have shown, using iontophoretic application of acetylcholine, that the acetylcholine sensitivity of innervated muscle fibers is localized primarily at the end-plate region. Miledi (2) has shown that the sensitivity decreases with increasing distance from the end-plate. The action potential of the muscle, like that of nerves, can be blocked using low concentrations of tetrodotoxin without affecting the cholinergic response of the muscle fiber (3).

When the nerve innervating a muscle is denervated, the muscle becomes hypersensitive to the application of acetylcholine (4). It was found that one to two weeks after denervation the entire muscle membrane becomes as sensitive to acetylcholine as the original end-plate region (5). The depolarization produced by the extra-junctional receptors and the

pharmacology of the response are similar to those of the junctional receptors. Recently it has been shown that the distribution of and the increase in acetylcholine sensitivity upon denervation can be correlated with the distribution and density of acetylcholine receptor molecules as measured by  $^{125}\text{I}$ - $\alpha$ -bungarotoxin binding (6). The denervated muscle, though hypersensitive to acetylcholine, does remain electrically excitable (5).

Upon denervation of mammalian skeletal muscle, there is a change in the sensitivity of the action potential to inhibition by tetrodotoxin. The spike generating mechanism becomes resistant to blockage by high concentrations of TTX (7). A solution of  $10^{-5}$  M TTX only reduces the amplitude of the action potential in denervated fibers, whereas  $10^{-6}$  M TTX completely abolishes the action potential in innervated muscle. In rat muscle the development of TTX resistant spikes is maximal five days after denervation and the time course is coincident with that of the spread of extrajunctional acetylcholine sensitivity (8). Denervated muscles of cat and mouse also exhibit this increased resistance to tetrodotoxin. It is interesting to note that denervated muscles do not gain an appreciable increased resistance to saxitoxin over innervated controls, even though the cellular action of saxitoxin is almost identical to that of tetrodotoxin (9). The depolarizing effect of batrachotoxin on the resting potential of the muscle membrane is unaffected by denervation (10), and TTX was

only partially able to repolarize the batrachotoxin depolarized muscle. Reduction in external sodium ions does antagonize the batrachotoxin induced depolarization indicating that in denervated muscle batrachotoxin still acts by opening sodium channels in the membrane.

The TTX resistant action potential was measured in external media of various ionic compositions to determine those ions whose flux is responsible for the spike (11). When the sodium in the external medium was replaced partially with sucrose the amplitude of the spike was reduced, while in the absence of sodium no spike was observed. Increasing the calcium ion concentration in the sodium-free medium did not restore the action potential. The TTX resistant action potential, therefore, has the same ionic specificity as the normal muscle action potential. The increased acetylcholine response of denervated muscle can be blocked by various cholinergic drugs without affecting the TTX resistant spike; and the spike can be blocked by  $10^{-5}$  M saxitoxin without affecting the acetylcholine response (8). These results imply that the ion fluxes induced by acetylcholine and the TTX resistant spike in denervated muscle are carried by two distinct molecules, although it is possible that the extrajunctional receptors are interacting in some way with the sodium channels to make them resistant to TTX.

The effects of several inhibitors of RNA and protein synthesis on skeletal muscle of mice in vivo were studied in order to determine if these

processes are required for the development of extrajunctional receptors and TTX resistant spikes after denervation (12). Injected one day after denervation, actinomycin D, which inhibits RNA synthesis without affecting protein synthesis, inhibited the spread of extrajunctional receptors and the appearance of TTX resistant spikes for four days. The drug failed to inhibit these changes when administered later than two days after denervation. Chloramphenicol and cycloheximide, which inhibit protein synthesis, also blocked the changes brought about by denervation. It was concluded that synthesis of new proteins is required for the denervation induced changes in the muscle and that the motor-nerves themselves may exert some influence on the expression of certain genes in the muscle.

Fetal rat diaphragm is also sensitive to acetylcholine over the entire muscle surface (13). Muscle fibers from one day old rats could generate action potentials in the presence of  $10^{-6}$  M TTX. After 10 days the TTX resistant spikes could be elicited only in the end-plate regions and after 20 days there were no TTX resistant spikes. The adult end-plate innervation is established at approximately the same time that the spikes become totally sensitive to TTX. The establishment of innervation is also correlated with a restriction of the acetylcholine sensitivity to the end-plate regions (14).

A similar phenomenon is observed in vitro using cultured chick embryonic heart cells. During the normal development of chick hearts the action potentials and beating activity are initially insensitive to TTX, but become sensitive after approximately one week of incubation (15, 16). Isolated embryonic chick heart cells in culture are insensitive to  $3 \times 10^{-7}$  M TTX whereas aggregates of these cells are sensitive to this concentration (17). Exposure of the aggregates to trypsin renders them insensitive to TTX, although the beating of the aggregate continues. The resensitization process requires protein synthesis since the aggregates remain resistant to TTX even in the presence of cycloheximide.

The in vitro embryonic heart muscle system also exhibits the interesting property of TTX desensitization (18).  $3 \times 10^{-5}$  M TTX blocks spontaneous beating in heart cell aggregates from four-day old embryos, but after 2-3 hr of incubation with TTX the fraction of beating aggregates increases from 20% to 75%. Moreover, the beating aggregates were resistant to an additional dose of TTX. Desensitization cannot be seen in aggregates from chicks older than 5 days and fails to occur in the presence of cycloheximide. Desensitization is reversible after 2-3 hr of wash. The process presumably involves a change in the mode of spike generation from a sodium to a calcium flux, since the desensitized spike is blocked by the calcium channel antagonist D-600 (19) and is augmented by an increased calcium ion concentration in the external medium.

## Proposed Experiments

It is proposed to study directly the binding of tetrodotoxin to various preparations of innervated, denervated and embryonic muscle tissue in order to gain some information on the physiological changes which occur during the transition of the muscle from a TTX sensitive to TTX insensitive state. The techniques used would be those developed for measuring binding of TTX to nervous tissue (20, 21), either in the intact cell or in tissue homogenates. A preliminary report has appeared (22) showing that it is possible to measure the TTX binding in intact rat diaphragm. The binding studies would show if the sodium channels in denervated muscle had an altered dissociation constant for TTX binding or merely an increase in the density of channels on the membrane. An increase in the density of sites would be similar to that seen for the acetylcholine receptor upon denervation, while a change in TTX binding affinity could reflect synthesis of new altered sodium channels or a change in the membrane environment which affects the binding of TTX to the endogenous sodium channels. These two alternatives can be distinguished by carrying out binding studies using nonionic detergent extracts of the muscle membranes where the constraints of the original membrane structure on the sodium channels are minimal (23).

The conversion from sodium spikes to calcium spikes during the process of TTX desensitization using the in vitro chick heart system

would also be interesting to study. Using a radioactive calcium channel antagonist (see Proposition 3) and [ $^3\text{H}$ ]-tetrodotoxin one could quantitate both calcium channel and sodium channel sites in the muscle. It would then be possible to distinguish between the following mechanisms for desensitization. It may be possible that sodium channels in the membrane are being modified in some way to change their ion specificity to calcium. Alternatively there may be a de novo synthesis of calcium channels without an effect on either the concentration or properties of the existent sodium channels. Using the same in vitro system one would like to know the ionic specificity of the spikes controlling the TTX-resistant beating activity in the embryonic muscle fibers. It may be possible that during normal development the heart muscle changes the specificity of its spike generating mechanism from calcium to sodium.

The results of these experiments may provide some insight into mechanisms of development and plastic change in electrically excitable systems.



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## Proposition 2

### Gating Currents in Squid Axons

#### Introduction

Recently Armstrong and Bezanilla (1) and Keynes and Rojas (2) have observed ionic currents in the giant axon of squid, distinct from the action potential currents, which they feel are related to the gating currents predicted by Hodgkin and Huxley (3). These currents would be associated with rearrangements of molecules in the membrane which open and close the sodium channels. The gating current should be an efflux of positive ions or influx of negative ones for opening of the sodium channel induced by depolarization and the opposite for closing of the channels. It is hypothesized that the gating current arises from a conformational change of the voltage sensitive membrane protein responsible for the opening of the sodium channel gates. The current may be caused by the redistribution of charged groups on the molecule itself or an effect on the inorganic ion distribution near the membrane due to a conformational change.

The putative gating currents are very small in comparison to the current through the sodium channels; thus special experimental protocol and signal averaging techniques are necessary to measure them (1). The squid giant axon was used because of the convenience in

manipulation of the internal and external solutions. The axon was perfused internally with a solution in which the impermeant ion, cesium, replaced the potassium. In the external medium, tris(hydroxymethyl)methylammonium replaced the permeant sodium ions of the artificial sea water. In some experiments  $2 \times 10^{-7}$  M TTX was added to reduce further any possible currents through the sodium channel. The remaining leakage current was subtracted electronically. A large tail of capacitative current was eliminated by algebraically summing the current obtained from exactly matched pulses of opposite sign. An electronic window was also used which shuts out the first 10-30  $\mu$ sec of signal following the onset of the pulse and thereby eliminates the remaining capacitative transient. The currents obtained are summed and averaged for 2,000 matched sets of pulses. The analyzed data reveals a transient outward current at the onset of the pulse which rises for 80  $\mu$ sec to a peak of 13 pA/ $\mu^2$  when the axon is held at -70 mv and pulses of  $\pm 70$  mv are used. The peak current through the sodium channel under these conditions would be 300 times as large. When the current is observed at the end of the pulse, an inward current is seen which is equal in area but opposite in sign to the current observed at the beginning of the pulse.

As evidence that the observed currents are really gating currents Armstrong and Bezanilla (1) show that the patterns are only obtained

from functional nerves. The time course of the current is rapid enough to correspond to activation of the sodium channel gates and the net charge movement during the current,  $300 \text{ changes}/\mu^2$ , is in agreement with the low density of sodium channels on the squid axon (4). Using similar procedures Keynes and Rojas(2) have observed the same gating currents and find that these currents are reduced reversibly by procaine. Tetrodotoxin does not affect the gating currents.

In addition, Bezanilla and Armstrong (5) report three procedures which block both sodium channel currents and the presumed gating currents. The procedures are reversible, with both the sodium channel currents and gating currents recovering together. Internal perfusion with  $10 \text{ mM ZnCl}_2$  suppresses current through the sodium channels and potassium channels (6). The gating currents, both at the beginning and at the end of test pulses, are also blocked by  $10 \text{ mM ZnCl}_2$  and recover after washing. Bezanilla and Armstrong (5) show that the sodium channels and gating currents are blocked by a 5 millisecond depolarizing pulse to  $+10 \text{ mv}$ . The current through the sodium channel and the gating current recover after 25 msec. Finally, both currents can be blocked by a prolonged depolarization to  $+56 \text{ mv}$  for 2 min. The recovery of the currents has a half-time of 50 sec. The authors state that the simultaneous blockage of both the sodium currents and the putative gating currents is good evidence that the currents observed are indeed

the gating currents. Even stronger evidence that the currents are gating currents would be obtained by showing that some procedure affected both the kinetics of sodium channel activation and the kinetics of the gating current in the same way.

The action potential of a squid giant axon is reversibly blocked by application of  $10^{-6}$  grams per ml of black widow spider venom in the external medium (7). Prior to blockage the rate of depolarization of the spike is increased slightly and the rate of repolarization is increased by a factor of two. In voltage clamp experiments the time to the peak inward current is decreased to 56% of the control and the time to the steady state current is decreased to 42% of control. There is little effect on the peak inward current but the steady state current is increased slightly over control. These effects are reversible. By analyzing the tail currents obtained upon repolarization after various durations of a depolarizing pulse, it was found that the sodium channel inactivation in the venom-treated axon is also significantly faster than in the untreated axon.

### Proposed Experiments

It is proposed to apply black widow spider venom to the squid giant axon and measure both the presumed gating currents and the sodium channel currents using the method of Bezanilla and Armstrong

(1). If it is found that the kinetics of both of the currents are increased, it would be strong evidence that the two currents are functionally linked. If the kinetics of the currents do not change in a similar manner, one should question the claims that these small outward currents are related to opening of sodium channel gates. Except for the short pulse depolarizations to +10 mv for 5 msec, all the other procedures which block both sodium channels and gating currents are the result of fairly gross modifications of the nerve. It is estimated that the lipid soluble procaine reaches high concentrations within the membrane during nerve blockade and has many pharmacological effects. High concentrations of  $\text{Zn}^{++}$  and sustained depolarizations certainly affect the ionic balance surrounding the membrane. Being able to show an effect of low concentrations of a specific pharmacological agent, such as black widow spider venom, on the gating currents would certainly strengthen the case that the observed currents are not an artifact. Therefore, it would be advantageous to use a purified fraction of the black widow spider venom, containing the axonal toxin, but not the synaptic toxins which are known to be present (8, 9).

Assuming the experiments show an effect of the venom on the gating currents, it would be interesting to study the component in the axonal membrane which interacts with the black widow spider venom.

One would like to know the relationship of this component to the tetrodotoxin binding component which has been identified in axonal membranes (4, 10).



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### Proposition 3

#### Electrically Excitable Calcium Channels in Paramecium

##### Introduction

Recent electrophysiological and behavioral studies have shown that the unicellular ciliate *Paramecium* is a good model for the study of integrated responses of organisms to their environment. Eckert (1) has presented a review of the mechanisms of the paramecium's behavior. The responses of paramecia to stimuli are limited. The most common one is the avoidance response to mechanical stimuli, ultraviolet irradiation, and certain chemical stimuli. Mechanical stimuli directed at the anterior end of the paramecium causes a temporary reversal of the direction of ciliary power stroke which accelerates the animal in the reverse direction. The cilia then resume their normal stroke and the paramecium travels off in another direction (1). Mechanical stimulation of the posterior end of the animal causes the cilia to beat more rapidly and the animal is accelerated in its forward motion (2). One can study the chemical requirements of the avoidance response by using paramecia which have been treated with the nonionic detergent triton-X-100 (3). The cell interior is freely exchangeable with the exterior, but the paramecia are still able to swim. They swim forward in a medium composed of 4 mM ATP, 4 mM MgCl, 50 mM KCl, 10 mM Tris and

a  $\text{Ca}^{++}$  concentration between  $10^{-8}$  and  $10^{-7}$  M. With increasing concentrations of  $\text{Ca}^{++}$ , the cilia shift the plane of their power stroke and above  $10^{-6}$  M  $\text{Ca}^{++}$  the paramecium swims backward. In an electrical field paramecia orient their anterior end towards the cathode and swim towards the cathode (1). These results indicate that there is some relationship between calcium, electric currents and locomotor activity in paramecia.

Paramecia are large enough to be impaled with microelectrodes and studied using conventional electrophysiological techniques. When the anterior side of a paramecium is stimulated mechanically its membrane is transiently depolarized from the normal resting potential, which is about -30 mv in 1 mM  $\text{K}^{+}$  and is dependent on the external potassium concentration (4, 5). This mechanoreceptor potential is graded in response to the stimulus intensity and the external calcium concentration and it spreads passively throughout the cell. This depolarization causes a regenerative depolarization or spike which is also graded in response to stimuli and carried by calcium ions. The increase in intracellular calcium concentration due to the spike activates the reversal of the power stroke of the cilia and the animal swims backward. Forward motion is then restored when the intracellular calcium concentration returns to the resting level either by diffusion, active extrusion or sequestering of the ions.

In response to mechanical stimuli at the posterior end of the paramecium, the membrane is transiently hyperpolarized reaching a maximum 50 msec after the stimulus and decaying slowly to the resting potential. This response is also graded according to the stimulus intensity, but does not exhibit the regenerative properties of the depolarizing response. The conductance of the hyperpolarized membrane is increased sixfold over the resting conductance and is carried by an efflux of potassium ions (2, 4, 5).

The cell membrane is also sensitive to electrical stimuli. A depolarizing pulse of current produces a regenerative depolarization similar to the regenerative response to anterior mechanical stimulation. The depolarization is graded in amplitude and rate of rise according to the stimulus intensity. The overshoot is insensitive to lithium, sodium, chloride and tetrodotoxin but increases with increasing concentrations of calcium, barium or strontium ions in the external medium. Adding barium or strontium to the external medium converts the graded response to an all or none spike. This behavior is similar to that seen in crustacean muscle (6) where graded calcium spikes are also converted to an all or none response in the presence of barium or strontium.

The paramecium can respond to external mechanical or depolarizing chemical and electrical stimuli with a regenerative

calcium influx. It has also been shown that an increase in intracellular calcium causes a reversal in the direction of swimming. Finally, it has been shown, using high speed cinematography and simultaneous electrophysiological recording, that reversal of ciliary beating was produced during the calcium spike but never during a purely electronic depolarization of the membrane (1). It is apparent that the behavioral responses of paramecium are coordinated by systems which are similar to the nervous systems of multicellular animals. One might expect to gain information pertinent to the understanding of complex nervous systems from a study of the simple "nervous system" in paramecium.

A beginning in this regard has been made by Kung who has initiated a genetic approach to the study of membrane excitation. Changes in behavior due to defects in membrane activity were used to isolate mutants (7, 8). Paramecium aurelia is well suited for this investigation since pure clones can be grown axenically (9) and a large fraction of the cell membrane can be isolated easily (10). Three interesting behavioral mutants have been identified in paramecium (11, 12). The first, called 'Pawn', can only swim forward. They are unable to respond to cation or mechanical stimuli. "Pawn" does not respond to electrical depolarization with a regenerative calcium spike, although the passive responses of the membrane are the same as the wild type paramecium. "Pawns" do respond to the presence of sodium

ions with a depolarization but there is no evidence of the calcium spikes seen with wild type. The mutational block is "Pawn" probably occurs in the mechanism responsible for the upstroke of the action potential.

The mutant "Fast-2" has an accelerated forward motion and is not sensitive to sodium. In the presence of sodium there is no depolarization, but normal action potentials can be elicited in the presence of barium or by electrical stimulation. The mutational block is presumably in the preliminary depolarizing response to sodium but not in the regenerative spike mechanism.

Finally there is "Paranoic" which has a severe overreaction to sodium but not other stimuli. In response to sodium, the membrane undergoes prolonged depolarizations up to 20 seconds in length. The blockage in this mutant is presumably in the repolarization mechanism of the membrane.

In a study of the mechanisms of electrical excitability in the paramecium, it would be quite helpful to have some pharmacological agents capable of interfering with the normal response. The use of tetrodotoxin, batrachotoxin, tetraethylammonium and other related compounds have proved useful in work with the electrical responses of nerves and muscle. Recently, a group of compounds has been found to block the excitation-contraction coupling in mammalian heart and rat uterine muscle (13, 14).  $4.4 \times 10^{-6}$  M verapamil ( $\alpha$ -isopropyl- $\alpha$ -

[(N-methyl-N-homoveratryl)- $\gamma$ -aminopropyl]-3,4-dimethoxyphenyl-acetonitrile  $\cdot$  HCl or  $1 \times 10^{-6}$  M D-600, a methoxy derivative of Verapamil, decrease transmembrane calcium spikes in cat myocardial fibers to 30% of control (15,16). The calcium currents were studied under voltage clamp and the sodium spike was inactivated with a conditioning depolarization. Increasing the calcium concentration in the external medium antagonizes the drug effect. The sodium spike in heart is unaffected by these drugs in contrast to the effect of the local anaesthetic, Procaine, which blocks both the sodium and calcium spikes. These calcium channel antagonists have been used to study independently the two spike mechanisms in the heart one consisting of a sodium flux and the other a calcium flux. 1 mM Prenylamine lactate, N-(3,3-diphenylpropyl)- $\alpha$ -methyl phenethylamine lactate, which has the same effect on heart muscle as Verapamil and D-600 (17), also blocks the action potential in crayfish muscle (18) showing that excitable calcium channels from widely divergent species are sensitive to these drugs. Another drug, Bay-a-1040, whose formula has not been disclosed, is the most active calcium channel antagonist so far described and is several fold more potent than D-600 (17).

### Proposed Experiments

It is proposed to use calcium channel antagonists to study the mechanism of electrical excitability in paramecium. At first, the

physiological effect of the drugs on the paramecium's behavior and electrical responses would be studied. Although the drugs are active on mammalian heart and smooth muscle as well as on crustacean muscle, it is not clear that they will act on the calcium spikes in paramecium. Assuming these drugs affect paramecium calcium channels, it also must be shown that the drugs have a negligible effect on processes of the paramecium unrelated to the calcium action potential.

Specifically it would be important to know if the drugs affect the non-regenerative receptor response to mechanical and chemical stimuli, the active transport of calcium and the response of the cilia to calcium ions. This last effect can be studied using Triton-X-100 extracted paramecium (3) and different concentrations of calcium in the presence of calcium channel antagonists.

If these drugs are found to be potent and specific inhibitors of the regenerative calcium spike in paramecium, then it would be possible to study the molecular properties of the spike generating mechanism using radioactive drug molecules as a probe. The drugs could be labelled with tritium either by specific synthesis or random exchange (21). The binding of the drugs to the cytoplasmic membrane of paramecium would be measured directly using equilibrium dialysis and characterized, as have other membrane proteins involved in excitable systems, using hydrolytic enzymes, gel filtration, density gradient



centrifugation, etc. (22, 23). The excitable membrane of paramecium is a convenient preparation since the cilia, with attached cytoplasmic membrane, can be isolated from the whole paramecium and further separated into interior contents and exterior membrane fractions by established methods (10).

After the properties of the excitable channel in wild-type paramecium have been established, one could study the various behavioral mutants isolated by Kung. By analyzing the specific biochemical changes correlated with a behavioral mutation in paramecium one can begin to understand the mechanisms producing the wild-type behavior. The possibilities for mutant excitable channels are numerous. They may have altered thresholds for excitation, creating hypo- or hyper-excitable mutants, they may be altered in their ionic specificity, their numbers may be altered, or they may become temperature sensitive.

It is possible that the drugs will have no effect on the spike generating mechanism in paramecium. In this case the drugs may still be useful in characterizing the excitable calcium channels in mammalian heart and smooth muscle and crustacean muscle, although problems obtaining a purified concentrated membrane preparation may be great and the interesting genetic experiments could not be carried out.

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## Proposition 4

### The Incorporation of Newly Synthesized Membrane

#### Proteins into Membranes

#### Introduction

Recently a mechanism has been identified which facilitates transport of phospholipids between membrane structures and is thought to function in the distribution of newly synthesized lipids between membrane organelles (1, 2). Phosphatidylcholine was found to exchange between rat liver mitochondria and microsomes (3). This exchange was stimulated by a 100,000 xg supernatant from the rat liver. This stimulation of lipid exchange has also been observed in beef heart (4) and guinea-pig brain (5), where exchange is stimulated by a soluble non-dialyzable component. The supernatant component from rat liver stimulates phospholipid exchange not only between natural membrane structures but also between artificial liposomes and mitochondria and between two different artificial phospholipid structures. Using the exchange of radioactively labelled phosphatidylcholine between artificial liposomes and mitochondria as an assay, Ehnholm and Zilversnit (6) have purified two active proteins from beef heart. The proteins stimulate the exchange of phosphatidylcholine and sphingomyelin but showed little or no activity towards phosphatidylethanolamine or

cholesterol. The isoelectric points of the two proteins were 4.7 and 5.5 with molecular weights of 21,000 and 29,500, respectively, as measured by S.D.S. gel electrophoresis.

Very little is known about the insertion of proteins into membranes. A considerable portion of the ribosomes in eukaryotic cells is associated with membranous structures in the form of the rough endoplasmic reticulum (7), but it is not clear that these ribosomes are responsible for synthesis of membrane proteins. If they are, the mechanism for transport of newly synthesized proteins into correct membrane organelles is unknown. In E. coli and B. megaterium membrane-bound ribosomes account for 30% of the total polyribosomes but their function is unknown. Osborn (8) suggests that they may represent the site of synthesis of membrane proteins.

Fox (9) finds no evidence for localized membrane synthesis at a fixed focus in E. coli but does show that newly synthesized membrane proteins are preferentially associated with newly synthesized lipids. Using fatty acid auxotrophs of the bacteria and studying inducible  $\beta$ -galactoside transport, he finds that the characteristics of galactoside transport are dependent on the type of fatty acid in the bacterial growth medium. A study of the  $\beta$ -galactoside transport in bacteria, which have been induced for transport and shifted to a new fatty acid in the growth medium simultaneously, shows that the characteristics of the induced

galactoside transport are the same as those observed when the bacteria have been grown entirely on the new fatty acid. These results suggest that newly synthesized membrane proteins may be inserted into the membrane as a lipid-protein complex, but the mechanism of this insertion is unclear.

In experiments designed to investigate the synthesis of acetylcholine receptor molecules in primary cultures of chick muscle, Fambrough (10) finds that the number of  $\alpha$ -bungarotoxin binding sites increases about 20% per hour in rapidly dividing cells. The synthesis of new receptors can be blocked by cycloheximide. When he blocks all of the surface receptors with unlabelled  $\alpha$ -bungarotoxin and then measures [ $^{125}\text{I}$ ]- $\alpha$ -bungarotoxin binding to cell homogenates, he finds a set of internal toxin binding sites which sediment like small mitochondria. During rapid growth the number of internal receptors may be as high as 25% of the total number of receptor molecules. A three hr exposure of cells to cycloheximide causes a 30% reduction in the number of internal receptors which is balanced by an equivalent increase in the number of external receptors. These results suggest that the internal pool of receptors are newly synthesized proteins waiting to be inserted in the membrane.

## Proposed Experiments

It is proposed to use the in vitro muscle system described by Fambrough (10) to study the incorporation of newly synthesized membrane proteins into the plasma membrane. The intracellular particles containing the newly synthesized acetylcholine receptor would be isolated from the plasma membranes. This would be accomplished using a combination of standard differential and density gradient centrifugation techniques. The best approach to the cell disruption would be to use a mild homogenization procedure which leaves the plasma membranes as large sheet-like structures easily separable from the small intracellular receptor particles. The incorporation of the newly synthesized receptors into the plasma membranes would be measured as follows: The receptors on the plasma membrane would be blocked by unlabelled  $\alpha$ -bungarotoxin and washed free of unbound toxin. The intracellular particles would then be incubated with the plasma membranes under a variety of conditions after which the particles would be separated. The plasma membrane fraction would be assayed for appearance of new receptors by [ $^{125}\text{I}$ ]- $\alpha$ -bungarotoxin binding. To measure the possible exchange of lipids between the intracellular particles and plasma membranes one would isolate the intracellular receptor particles from muscle cells grown on radioactive lipid precursors and measure the radioactivity in unlabelled plasma membranes after incubation with the labelled particles.

Finally, the incorporation of receptors into unrelated membranes such as liver cell plasma membranes or artificial lipid membranes would be measured.

Three possible mechanisms for incorporation of new receptor molecules into the plasma membrane can be distinguished with these experiments. It is possible that there is a soluble protein similar in function to the phospholipid exchange protein isolated by Zilversmit which catalyzes the exchange of membrane proteins between membrane structures. In this case, one would find that some supernatant fraction of the muscle cell would stimulate incorporation of newly synthesized receptor molecules into the plasma membrane. Depending on the specificity of this protein, it might also be possible to stimulate incorporation of new receptor into artificial lipid systems. Alternatively, the new receptors may be incorporated into the plasma membrane by fusion with the membrane at certain specific sites on the plasma membrane. Transfer of lipids from the intracellular receptor particles to the plasma membrane would accompany the incorporation. In this case one would not expect the receptor particles to fuse with artificial systems lacking the specific recognition site. However, by mixing various plasma membrane components with the artificial systems it might be possible to isolate the specific recognition site and obtain fusion of the receptor particles with the artificial membranes.



Finally, it is a distinct possibility that the intracellular receptor particles incorporate randomly into the plasma membrane whenever the two species collide with sufficient force. Under these conditions, one would expect that the particles would fuse with artificial systems as efficiently as with the plasma membrane and that there would be no specific factor to stimulate the fusion.

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## Proposition 5

### A Study of Aspartate Transcarbamylase Using Specific NMR Probes

#### Introduction

Aspartate transcarbamylase (ATCase) from E. coli was first isolated by Gerhart and Pardee. It catalyzes the first reaction in the pathway for the biosynthesis of pyrimidines. Since it is the first enzyme in a biosynthetic pathway, it is subject to regulation by substrates as well as by the end products of the pathway. It is a fairly large enzyme with a molecular weight of 310,000 daltons (1). The enzyme is composed of two types of subunits which can be separated from each other by heating (2) or with p-mercuribenzoate. One subunit, designated the catalytic subunit (CSU), carries out the catalysis but is no longer subject to allosteric control. The other, called the regulatory subunit (RSU), does not catalyze the reaction but does interact with the allosteric effectors. Catalytic and regulatory subunits can be mixed together to form the native enzyme. Certain modified subunits can also be mixed with native subunits to form hybrids (3). There are two catalytic subunits per enzyme consisting of three polypeptide chains each, and there are three regulatory subunits per enzyme containing two polypeptide chains each (3). The catalytic trimer has a molecular weight of 100,000. The regulatory dimer a molecular weight of

30,000 (4, 5) and contains two zinc atoms (6).

X-Ray crystallographic studies which have progressed to 5.5 Å resolution show that native ATCase is  $90 \times 110 \times 110$  Å in its largest dimensions and has an interior aqueous space of  $25 \times 50 \times 50$  Å (7). The two catalytic trimers are located above and below a belt of the three regulatory dimers (8). These results have confirmed earlier electron-microscopic data (9). Each catalytic chain has one sulfhydryl near the active site, which is probably accessible from the interior cavity and appears to communicate with the exterior through six 15 Å diameter channels in the vicinity of the regulatory subunits (8). Warren et al. speculate that the allosteric behavior may be controlled by regulation of access of substrates through these channels. They also find that the zinc binding site in the RSU is 24 Å away from the nearest CSU thiol.

ATCase is activated by substrates, aspartate and carbamyl phosphate (2, 10). CTP inhibits and ATP activates catalysis. The activation by substrates (homotropic effects) is similar to the effects observed in the binding of oxygen to deoxy-hemoglobin, in which the binding of one oxygen increases the affinity of the other subunits for oxygen.

The postulated mechanism for the heterotropic and homotropic effects is that the allosteric regulator forces the enzyme to change its

conformation between at least two states, one with high affinity and one with low affinity for substrate (11). There is, in fact, evidence that the conformation of the enzyme does change upon binding of the allosteric effectors. The reactivity of the thiols and peptide bonds in the enzyme change upon binding (11), the sedimentation rate shifts (12), the optical rotation of the molecule at 233 nm changes (13), and the ultraviolet spectrum of the enzyme changes in the presence of carbamyl phosphate and carbamyl phosphate plus succinate (14).

Gregory and Wilson (15) have studied the effects of bromosuccinic acid on the native ATCase. They found that in the absence of substrate, alkylation with bromosuccinic acid inactivates the enzyme. Using  $^{14}\text{C}$ -bromosuccinate, they found 0.8 residues react per catalytic polypeptide chain and 1.1 residues per regulatory chain. However in the presence of carbamyl phosphate and a competitive inhibitor, maleate, the enzyme is activated by treatment with bromosuccinate. The activation results in about one modified residue per catalytic chain and 2 residues per regulatory chain. With both the activation and inactivation there is a loss of the interaction between subunits which gives rise to the allosteric properties. However, the catalytic and regulatory subunits are not dissociated by the treatment. The inactivation of the enzyme is likely due to the reaction of the halo acid with the enzyme at the active site, while in the case of activation by the halo acid, the active site is

protected by the presence of substrate.

There is one sulfhydryl group in each catalytic polypeptide chain situated near the active site (16). In the catalytic subunit the sulfhydryl groups do not react with alkylating agents, even at high concentrations, but they do react slowly with 5, 5' -dithiobis(2-nitrobenzoate) and p-hydroxymecuribenzoate to form inactive derivatives. In the presence of the enzymatic inhibitors succinate and inorganic phosphate the CSU does not react with the two sulfhydryl reagents. This result is strong evidence that the sulfhydryl is near the active site. The CSU-thio-nitrobenzoate derivative can react with cyanide to give the CSU-S cyano derivative which regains full enzymatic activity. The enzymatic activity of this derivative indicates that although located near the active site the free sulfhydryl is not required for activity. The S-methyl derivative of the CSU can be synthesized by reacting denatured CSU in concentrated guanidinium chloride with methyl iodide, followed by renaturation of the CSU according to Rosenbusch and Weber (6). The S-methyl derivative had no free sulfhydryl groups, 0.8 to 0.9 S-methyl cysteine groups per catalytic polypeptide chain, and 73% of the initial enzymatic activity (17). The S-methyl and S-cyano derivatives can be recombined with the zinc regulatory subunits to form active derivatives of native ATCase which then respond normally to both homotropic and heterotropic effectors. The sulfhydryl at the active

site is therefore not essential for allosteric behavior either. Modifications of this sulfhydryl group leading to anionic derivatives inactivate the enzyme due to reduced binding of the anionic substrate, aspartate. The anionic derivative formed by potassium permanganate oxidation binds carbamyl phosphate well but binds succinate only weakly (17).

In an attempt to mimic the transition state for the ATCase reaction, Collins and Stark (18) synthesized N-(phosphonacetyl)-L-aspartate (PALA) which combines features of both the substrates in one molecule. It binds to the enzyme about 1000 times more tightly than the most tightly bound substrate, carbamyl phosphate. The inhibition of carbamyl phosphate binding by PALA is competitive but inhibition with aspartate is not, indicating an obligatory order in the binding for catalysis, first carbamyl phosphate then aspartate.

### Proposed Experiments

Recent studies with hemoglobin (19, 20) have shown that [ $^{19}\text{F}$ ]-NMR labels suitably placed on the molecule can be used to study the allosteric mechanisms of this protein. I propose the specific labelling of ATCase with a [ $^{19}\text{F}$ ]-NMR label, using the techniques and reagents mentioned in the introduction, to obtain a suitable probe with which to study the behavior of the enzyme. Both  $\text{CF}_3\text{CH}_2\text{CH}_2\text{CO}_2\text{H}$  and  $\text{HO}_2\text{CCF}_2\text{CH}_2\text{CO}_2\text{H}$  can be obtained commercially and presumably

brominated in the alpha position by the Hell-Volhard Zelinsky reaction. Either of these reagents could be reacted with the enzyme in the absence of substrate, using the conditions of Gregory and Wilson (15). The subunits could then be separated using the heat treatment of Gerhart and Pardee (2) and regulatory subunits isolated with one modified residue per chain. This labelling reaction would also be carried out in the presence of PALA the transition state analogue of Collins and Stark (18) which protects the active site. The subunits would then be dissociated and the catalytic subunit with one fluorine label per chain could be isolated. Having obtained these selectively labelled subunits, it would be necessary to characterize their behavior. The catalytic properties of the modified catalytic subunit and the binding properties of the allosteric effectors to the modified regulatory subunit would be compared to the behavior of the native subunits.

Hybrids using modified subunits and native subunits would be made (3, 17). The reannealed enzyme would be checked for catalytic and allosteric behavior. Presumably the modified catalytic-native regulatory hybrid would have a greater chance of regaining its allosteric properties, because according to Markus et al. (11) most modifications of the regulatory subunit lead to loss of allosteric properties. This hybrid enzyme could then be studied by NMR using the  $\text{CF}_3$  or  $\text{CF}_2$  group as a probe of the local environment of the



catalytic subunit. With this probe it would be possible to study the details of the conformational changes exhibited by the enzyme in response to the addition of various of the allosteric regulators.

In another approach to obtaining a specific [ $^{19}\text{F}$ ]-NMR probe on ATCase, one could use the method of Jacobson and Stark (17) to label the sulfhydryl in the CSU with bromo-trifluoroacetone. The reaction would have to be carried out on denatured CSU in guanidinium chloride due to the unreactivity of the sulfhydryl in the native CSU. The derivative would be renatured then reannealed with the RSU to form a derivative of ATCase with a  $\text{CF}_3$  group near the active site. There is a good chance that this derivative would have full enzymatic and allosteric activity since the derivative is small and uncharged, like the S-methyl and S-cyano derivatives which are fully active (17). This derivative would be useful in monitoring tertiary changes in the active site upon binding substrate. It also may be possible to monitor quaternary structural changes in ATCase using this derivative if the zinc ions in the regulatory subunits can be replaced by paramagnetic ions. Recently cadmium ions have been substituted for the zinc ions with only a slight perturbation of the enzymatic activity (21), showing that zinc is not obligatory for activity. There is some evidence that a derivative containing the paramagnetic ion, cobalt(II) has been made (22), although no evidence of enzymatic activity was presented. If an

active paramagnetic derivative could be made using the trifluoroacetylated ATCase, one could study the paramagnetic broadening of the fluorine resonance. Since the inorganic ion binding site is on the regulatory subunit and the broadening of the [ $^{19}\text{F}$ ] resonance is strongly dependent on distance, any change in the  $\text{CF}_3$  to  $\text{Co(II)}$  distance would be visible as a change in the NMR spectrum and would reflect quaternary structural changes. Experiments using this system would be helpful in proving or disproving the model Jacobson and Stark (22) have presented to explain homotropic cooperativity. They suggest that in the native enzyme three carbamyl phosphate molecules can bind without a major quaternary change. When the first succinate binds this triggers a major conformational change to the high affinity state to which the last three carbamyl phosphate molecules to bind readily. The proposed experiments should allow simultaneous observation of tertiary and quaternary changes produced by binding of less than saturating concentrations of the various substrates.

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