# SOLUBILIZATION AND PARTIAL CHARACTERIZATION OF THE TETRODOTOXIN BINDING COMPONENT FROM ELECTRIC EEL ELECTROPLAQUE

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Frank A. Stackhouse

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## ABSTRACT

The tetrodotoxin (TTX) binding component from the electroplaque of Electrophorus electricus was solubilized and studied using $^{3}$H-TTX purified by a modified procedure using Bio-Rex-70 at pH 8.7. The best extraction yield was obtained from a solubilization in 1-2% Lubrol-PX, at pH 7.6, for 2 hours, at a membrane fragment concentration equivalent to 2.0 - 2.5 g original wet weight tissue/ml. Gel filtration on Sepharose-6B was used to verify that the TTX binding component was solubilized. The molecular weight of the solubilized component was found to be about 200,000 using 10-43% glycerol gradients. A Sephadex G-50 assay was used to study binding. A $K_D$ of 1.2 nM was obtained for $^{3}$H-TTX binding. This binding was affected by cations in a manner similar to that found by Reed and Raftery [Biochemistry 15:944-953 (1975)]. The solubilized material was found to be unstable with respect to TTX binding; this stability was affected by the presence of cations and TTX, but not veratridine or procaine.

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# INTRODUCTION

The transmission of information throughout the nervous system is believed to be accomplished in two ways: (1) chemically, by transmitters such as acetylcholine and glutamate at the neuromuscular junction and most other synapses, and (2) electrically, within nerve and muscle cells and also at some synapses. It is now believed that the propagation of an electrical impulse along a nerve cell axon or muscle fiber is accomplished by sequential changes, in the cell's plasma membrane, of permeability to ions. Under resting conditions the plasma membrane separates concentration gradients of high $Na^+$ outside the cell, low $Na^+$ inside and low $K^+$ outside, high $K^+$ inside. The resting $Na^+$ permeability is very low, while resting $K^+$ permeability is low, but significant. The resting cell, therefore, has a negative potential with respect to the external environment, which is close to that of the $K^+$ battery created by the gradient. Upon stimulation of a portion of a nerve cell, for example, an electrical impulse or action potential propagates along the axon, travelling away from the site of the stimulus. This impulse is generated in two steps: first there is a local depolarization of the membrane at the site of stimulation to a threshold potential which triggers a specific increase in membrane permeability to $Na^+$, allowing the influx of $Na^+$, depolarizing the membrane further and reversing the polarity as the potential approaches that of the $Na^+$ battery. This permeability change is transient, lasting only about one millisecond.

As the $Na^+$ permeability returns to normal, there is triggered a specific increase in $K^+$ permeability, which restores the resting potential, at which point the $K^+$ permeability returns to its original low condition. This local transient depolarization of the membrane serves to bring adjacent sections of the membrane to threshold, which initiates the impulse, stimulating further sections, and propagating the impulse.

## A. Electrophysiological Studies

This general sequence of events, which occurs in almost all nerve and muscle cells, was elucidated over the course of many years. In 1902, Bernstein (1) postulated that nerve impulse might be due to ionic fluxes across the plasma membrane of the nerve cell. He suggested that, at rest, the membrane is permeable to $K^+$ but not to $Na^+$, resulting in a resting potential dependent on the $K^+$ concentration gradient, and that during an action potential the membrane became permeable to $Na^+$, depolarizing the membrane.

This hypothesis was partially confirmed by two major series of experiments. The first was that of Hodgkin, reported in 1937 and 1938 (2-4), which established for the first time the electrical nature of the impulse. Prior to this time, it had not been possible to determine whether the electrical recordings of nerve activity were due directly to the impulse itself or were merely the result of other simultaneous processes. Hodgkin showed that electrical currents flowed across the nerve membrane on the opposite side of a blocked

point from the stimulating electrode, despite the absence of a propagating impulse. This led to his formulation of the local circuit theory, which states that current flows across portions of membrane adjacent to the stimulus in order to complete the circuit begun by the impulse. The magnitude of the potentials created by these currents dropped off exponentially along the length of the nerve, but could be sufficient to excite further portions and propagate the impulse. The all-or-none nature of the impulse resulted from a threshold potential which had to be exceeded in order for the adjacent portions to fire.

The second series of experiments, which elucidated the nature of these currents, were facilitated by two developments: (1) the use of the squid giant axon preparation, and (2) the development of an electrophysiological technique known as the voltage clamp. In 1942, Curtis and Cole (5) used the squid giant axon to accurately record the membrane potential during the transmission of an impulse. They found that the amplitude of the impulse was greater than the resting potential, contrary to Bernstein's proposal. In other words, the membrane did not merely depolarize to zero, but in fact, reversed polarity. In 1949, Hodgkin and Katz (6) postulated that the change in potential during the impulse was due solely to $Na^+$ permeability, while the $K^+$ permeability was low. The potential would then move toward the $Na^+$ battery, unhindered by $K^+$ permeability. The basis for this sodium hypothesis came from their experiments on the effect of sodium on the amplitude of the impulse in squid giant axon. They found that the magnitude of the impulse was proportional to the

concentration of $Na^+$ in the external solutions.

Further progress came about with the development of the voltage clamp technique by Hodgkin, Huxley and Katz (7-11). This technique is necessary due to the voltage dependent nature of the $Na^+$ permeability or conductance. Once the membrane has been depolarized to threshold, the increase in $Na^+$ conductance further depolarizes the membrane, increasing the $Na^+$ conductance in a positive feedback system. In order to study the components of the impulse, without the impulse itself, it is necessary to apply a negative feedback procedure. The voltage clamp does this by applying a current across an isolated piece of membrane just sufficient to maintain a desired voltage across the membrane. Squid giant axon was first used for this technique because it allows the insertion of a fine silver wire down the inside length of the axon in order to eliminate local circuits and keep the entire length of membrane at isopotential. The magnitude and time course of the applied currents are therefore equivalent to the currents which would flow across the membrane at that given voltage under normal conditions. Thus, the investigator can isolate the currents at any membrane potential and relate them to those that constitute the impulse. Further, by replacing the normal external solutions with solutions free of those ions believed to be responsible for the impulse, it is possible to assign the current magnitudes and time constands to the movement of specific ions.

From such data, Hodgkin and Huxley proposed that the initial inward current flow following depolarization to threshold was carried

by $Na^+$ and that in less than 1 millisecond it began to decay to its original low value, despite the maintenance of depolarization. This characteristic of the impulse is known as sodium inactivation. At about the same time that sodium inactivation began, an increase in outward current began, with the two processes combining to return the membrane to resting potential. Hodgkin and Huxley believed this outward current to be carried by $K^+$, but that was not confirmed until their experiments with the flux of radioactive $K^+$ from squid giant axon (12). The time courses of these ionic currents are illustrated in figure 1.

Once it became clear that the properties of the impulse are due to the transient movement of specific ions across the membrane in a defined sequence, questions concerning the mechanism of events in the membrane, on the molecular level, became relevant. One of the first models arose out of Hodgkin and Huxley's mathematical treatment of the impulse (11). They arrived at a theoretical construct for an impulse which closely approximated the actual electrical recordings, and defined three parameters which could be correlated with possible molecular events: $\underline{m}$, related to sodium activation; $\underline{h}$, related to sodium inactivation; and $\underline{n}$ to potassium permeability. In the equations, these parameters are found as $\underline{m^3h}$ and $\underline{n^4}$ for the fractions of the sodium and potassium permeabilities, respectively, that are "turned on and off" as a function of time. The form of these parameters suggested to Hodgkin and Huxley that the ionic currents may result from the distribution of particles in the membrane in

response to voltage -- three m-particles and one h-particle were needed for the sodium pathway and four n-particles were needed for the potassium pathway.

It was later shown that the powers of these parameters are not constant (13), so the particle model becomes less plausible, but at least one important finding came out of the equation -- the parameters for the sodium and potassium permeabilities seem to be mutually independent. This suggests that the pathways for $Na^+$ and $K^+$ are separate and ion-specific. While there are other models which propose convertible sites (14-16), the most attractive model developed for nerve excitability involves the opening and closing of separate sodium and potassium channels located in the membrane.

## B. Ion Channels

Two basic ways that ions may be translocated across the membrane are by carriers and by pores. Armstrong (17) has summarized the evidence against the possibility of carriers, mostly on the basis of kinetics and thermodynamics. The ions are transported during an impulse much faster than is believed possible for known ion carriers, and the energy barrier due to electrostatic repulsion is much higher for a carrier than is actually indicated from the $Q_{10}$ of ion translocation during a nerve impulse. The evidence for the existence of ion pores is now generally considered conclusive.

Even before the question of ion pores had been decided, investigators had been studying the molecular nature of the channels. One

of the first questions to be considered was that of ion specificity. Electrophysiological studies were carried out using solutions in which sodium had been substituted with other ions. It was soon found that $Li^+$ could substitute quite successfully for $Na^+$ and gave voltage clamp data similar to the normal data (13, 18-20). Other ions were also found to pass through the sodium channel with varying degrees of permeability. Chandler and Meves (19) showed the following sequence of ion permeabilities relative to sodium: $Li^+$, 1.1; $K^+$, 1/20; $Rb^+$, 1/40; $Cs^+$, 1/61. The potassium channel also allows the passage of other ions, especially $NH_4^+$ and $Rb^+$; indeed $Rb^+$ has a permeability about equal to that of $K^+$ (21). Although these channels do not discriminate completely against other ions, they are specific enough to merit the names of "sodium channel" and "potassium channel". Moreover, the ability of $K^+$ to substitute to some extent for $Na^+$, does not alone suggest that the channels are the same. The kinetics of the ion fluxes through the channels are different enough to justify the distinction of separate channels.

The ability of other ions to substitute for $Na^+$ can be useful in elucidating a molecular description of the channel. Hille (22, 23) used the myelinated sciatic nerve from frog to determine the permeability of the channel to organic cations. He found the following order of substitution: sodium $\approx$ hydroxylamine $>$ hydrazine $\approx$ ammonium $\approx$ formamide $\approx$ guanidine $\approx$ hydroxyguanidine $>$ aminoguanidine. Methylated derivatives of the above cations, as well as larger cations such as trimethylamine, choline, and imidazole, were not permeable. On

the basis of these data and the data on the alkali metal cation permeabilities, Hille proposed a model for the selectivity of the sodium channel. He suggested the channel was composed of a pore, with minimum dimensions 3 Å × 5 Å, which would allow the passage of a sodium ion hydrated with up to three water molecules. The pore could be lined with oxygen atoms, one of which, in an ionized form, could bond with the permeant cation, while the remainder could form hydrogen bonds with the water molecules or the NH or OH functions on the organic cations. Hille considered the ionized oxygen atom to be quite important in the ion selectivity procedure. He suggested that the anionic character of the pore led to an ion-exchange process which supplemented the size determining factor. Looking more closely at this possibility, Hille determined the maximal sodium conductance of a myelinated nerve fiber as a function of pH. Below pH 6, the conductance was found to decrease rapidly and reversibly. A titration curve of this phenomenon indicated that a protonateable group with a $pK_a$ of about 5.2 was involved in the permeability charge of the membrane (24). This group could contain the ionized oxygen atom proposed to be within the pore. The existence of this group also suggests the possible molecular nature of the sodium channel -- the $pK_a$ of 5.2 is quite reasonable for a carboxyl group of a protein.

## C. Pharmacology of the Channels

Pharmacological studies of nerve and muscle processes have proved extremely useful in probing the molecular nature of the

channels. It has been known for many years that many different compounds, found in various plants and animals, affect the actions of nerve and muscle cells. Quite recently some of these compounds have been isolated, purified and even synthesized for use in neurophysiological and biochemical studies. To this list can be added several synthetically obtained drugs. These compounds have a wide variety of effects and specificities. Some may affect many different types of cells and may affect one cell in many different ways. Others are highly specific, affecting, for example, only a particular kind of synapse. The compounds that will be considered here are those that primarily affect the operation of the ion channels found on the extrasynaptic plasma membrane portions of nerve and muscle cells involved in the conduction of impulses. This class may be divided into two types of agents: (1) channel blockers, which lower the ionic permeability of the channels, and (2) those that affect the kinetics, or time course, of the permeability changes.

The most important of the channel blockers is the highly potent neurotoxin, tetrodotoxin (TTX), found in the pufferfish as well as in a variety of other animals. This toxin had been recognized since early Egyptian and Chinese eras to have physiological effects and has been thoroughly investigated in the last twenty years. In 1960, Narahashi , et al. (25) reported the results of studies on skeletal muscle cells treated with TTX and impaled with microelectrodes. They concluded that TTX blocks the impulse by a selective inhibition of the transient sodium conductance. This was later

confirmed by voltage clamp techniques (26, 27). A set of voltage clamp recordings in normal Ringers solution and in the presence of TTX are shown in figure 2. These represent a family of currents obtained by polarizing the cell at various potentials. Negative readings indicate inward current, while positive tracings indicate outward current. It is clear that TTX abolishes the early influx of current, which is carried by sodium ions. The structure of TTX is shown in figure 3.

A second, well-characterized blocker of the sodium channel is saxitoxin, found primarily in the Alaskan butter clam, but believed to arise from the dinoflagellate, Gonyaulax catanella (28), which the clam ingests. Saxitoxin has been found to have an almost identical action to TTX on nerve membranes (29-31). As with TTX, STX was found to have no effect on the potassium channel. Moreover, the blockade effects of both toxins are specific for the sodium channel molecule, independent of the permeant ion species present. Replacement of the sodium with lithium, for example, did not alter the effects of TTX and STX. The structure of STX is shown in figure 4.

The dose response curves for TTX and STX indicate that these toxins interact with the sodium channel on a one-to-one basis. Half maximal binding of TTX to squid axon was found to be $3.31 \times 10^{-9}$ M (32) and half maximal binding of STX to myelinated fibers was $1.9 \times 10^{-9}$ M (31). TTX is effective only when applied on the outside of the membrane. Internally applied TTX is ineffective even at 100 nM (33). These data indicate that there is a single specific binding site on the

molecule which is responsible for the transient sodium flux.

In addition to these two neurotoxins, there is a group of other compounds which act as channel blockers. These include some local anaesthetics, e. g. xylocaine and procaine, and some tranquilizers, e. g. compazine, which act primarily on the sodium channel (27) and tetraethylammonium (TEA) which acts on the potassium channel. The anaesthetics and tranquilizers act only at significantly higher concentrations than the neurotoxins and are much less specific. TEA is the only major affector of the potassium channel. It blocks the channel at millimolar concentrations with a dissociation constant of 0.4 mM in myelinated fibers (34) and apparently exerts its affect by binding to the inside of the membrane, i. e., when internally perfused (35). Voltage clamp data for TEA are found in figure 6, in similar fashion to figure 2.

The second group of compounds to be discussed is that which affects the kinetics of the permeability changes. One of the most important compounds of this type is the neurotoxin batrachotoxin (BTX), found in the milky skin secretion of the Columbia poison arrow frog Phyllobates aurotaenia. Despite great difficulties in obtaining sizeable quantities of the venom from the frogs which live in remote, dense tropical jungles, a great deal is known about BTX. It is highly toxic, about four times as potent as TTX (36), effectively blocking rat skeletal muscle excitability at concentrations of $0.1\text{-}8.0 \times 10^{-8}$ g/ml (37). Its mode of action, whether internally or externally applied, seems to be to slowly depolarize the membrane by

irreversibly opening sodium channels (38). The effect is antagonized reversibly by TTX (38), which repolarizes the cell back to the original resting potential. The effect of BTX is also antagonized by procaine, but in a different manner from TTX. If both procaine and BTX are applied together and then the nerve preparation is washed with normal solution, no effect of BTX is observed (39). This suggests that procaine blocks the binding of BTX, while the reversible antagonism of TTX indicates that TTX and BTX act at different sites. The structure of BTXis shown in figure 5.

The action of BTX seems to depend on one or more sulfhydryl groups on a membrane bound protein. Treatment of lobster axons with millimolar concentrations of the sulfhydryl modifying agents N-ethylmaleimide or p-chloromercurobenzoate for five minutes to prevent the subsequent action of BTX, while only slightly altering the effect of TTX, again indicating different sites of binding (40). Other evidence which indicates separate sites for BTX and TTX is that Phyllobates is unaffected by BTX, while it retains TTX sensitivity (41). BTX has great potential use as a tool in the study of the sodium channel, limited only by its relative unavailability. Hopefully this problem will be alleviated by its eventual synthesis (42).

A second toxin known to affect the kinetics of the sodium channel is Condylactis toxin (CTX), from the Bermuda sea anemone Condylactis gig ntea. CTX is a protein of molecular weight about 10, 000-15, 000 (43), which prolongs the falling phase of the impulse in crustanean neurons for up to several seconds (44). Voltage clamp studies indicate that

CTX seems to affect the sodium channel by prolonging the early transient sodium current after initial depolarization of the membrane. Its affects seem to be on the sodium inactivation mechanism. It apparently has little or no effect on the potassium current (45). The action of this toxin strongly supports the contention that the sodium and potassium channels are separate, since the total steady state conductance observed during the falling phase in the presence of CTX is far greater than is seen at any point during the normal impulse. Two channels are necessary to carry all of this current simultaneously (46). The main problem with CTX as a biochemical tool is that it is apparently effective only on some invertebrate. It does not affect squid axon (45), and it is claimed that it does not affect vertebrates (47). It apparently has no effect on the electrically excitable Sachs organ of the electric eel, Electrophorus electricus (H. Lester, F. A. Stackhouse and M. A. Raftery, unpublished results).

Another group of toxins which seem to affect the sodium inactivation process comes from the venom of the scorpion. Several species have been investigated, and all seem to have some neurotoxic effect on sodium inactivation. Crude venom from Leiurus quinquestriatus prolonged the impulse of single myelinated nerve fibers from the frog for up to several seconds. The prolonged impulse was shown to be due primarily to a delayed sodium inactivation, and not to a large decrease in potassium permeability (48). Later, voltage clamp studies on frog nodes of Ranvier indicated that there was some simultaneous decrease of potassium current in a scorpion venom

poisoned nerve (49). Studies with the venom from Buthus tamulus on a voltage clamped squid axon defined these effects clearly: (1) the peak sodium current was not affected, (2) the time to peak sodium current was slightly prolonged, (3) the time course of sodium inactivation was greatly prolonged, and (4) the steady state potassium was substantially suppressed (50).

Individual toxins from the venoms of several species have been isolated and characterized. For example, three purified toxins have been isolated from each of the venoms of Androctonus australis Hector and Buthus occitanus tunetunis and five from Leirus quinquestriatus (51). All were identified as basic proteins of molecular weight 6000-10, 000. The animo acid sequences of some of these toxins show them to be relatively homogeneous (52). Toxins from the North American scorpion Centruroides sculpturatus Ewing have been isolated, sequenced, and found to be homologous to the other known toxins (53). Work has also been done with the venoms of the South American scorpion Tityus serrulatus (54-56) and the Asian scorpion Palamneous (Heterometrus) gravimanus (47).

An important aspect of scorpion venom studies on nerve preparations has been the effect of the venom on the gating mechanism of the sodium channel. It has been postulated that the voltage dependent gate is a charged structure able to move in the membrane in response to the applied voltage (17). The movement of this charged structure, which would open and close the sodium pore, constitutes a small "gating current", distinguishable from the ionic current (57, 58).

It is likely that several of the agents which affect the kinetics of the ion flux directly act on this gating structure. Scorpion venom and toxins have been used to study the gating current (59). Two of the purified toxins appear to have slightly different pharmacological effects on sodium inactivation and activation, suggesting that the gating current may have two components. This gating structure is apparently at least partly protein in nature. Internal perfusion of squid axon with the unspecific proteolytic enzymes of pronase results in selective destruction of sodium inactivation without affecting TTX sensitivity or the potassium channel (60).

The venom from the black widow spider, Latrodectus mactans, was also found to affect the kinetics of ion channels. The venom contains a number of components (61), as in scorpion venom, but at least one component in neurotoxic, blocking nerve impulses in a variety of studied systems, such as cockroach neurones (62), crayfish stretch receptors (63), and rat ganglia (64). The effect of this venom may be due in part to a specific presynaptic action involving massive transmitter release (62-65). However, the venom blocks the impulse in squid giant axon (66). This excitability blockade appears to be due to changes in the kinetics of sodium and potassium conductance. Sodium activation, sodium inactivation, and potassium activation were all accelerated, resulting in a reduction of inward current duration, lowering the peak amplitude, and leading to loss of excitability.

A group of toxins from the leaves of plants in the family Ericaceae, the grayonotoxins (GTX) have an action on nerve cells

similar to that of BTX. Studies on squid axon indicate that application of GTX-II, for example, results in a specific increase in resting sodium permeability which is antagonized by TTX and partially antagonized by the anaesthetics procaine and benzocaine (67). The effect differs from BTX in that it is reversible and occurs at a 100-fold higher effective concentration.

A toxin isolated from blue-ringed octopus, Maculotoxin (MTX), has been found to block nerve impulses by interacting with the sodium channel (68). It seems to have several effects on the kinetics: it blocks the transient conductance, slows the turning-on process, and delays the time-to-peak, without affecting sodium inactivation (69).

A number of drugs have been found to affect kinetics. The veratrum alkaloids, which occur in plants of the tribe Veratreae, have a wide variety of effects on ion channels (70). One of these, veratridine, prolongs the open state of the sodium channel, as does the insecticide, DDT (20). Another alkaloid having a similar effect is aconitine, which induces not only the steady state conductance, but also accelerates sodium activation in transient conductance (71).

## D. Biochemical Studies on the Sodium Channel

The prospects for studying the molecular events occurring in the operation of ion channel in nerve membranes seem promising in view of the large number of pharmacological agents available. The logical first choice for study was the sodium channel since TTX has been commercially available for several years. Because it is effective

at very low concentrations, it was expected to be sensitive enough to allow studies on low quantities of sodium channels. Moreover, the TTX binding site appeared to be closely connected with the operation of the pore, if not part of the pore itself. Isolation and purification of the TTX binding component of nerve or muscle membranes would likely lead to the isolation and purification of the pore itself, hopefully in an intact and functional state.

Preliminary biochemical studies on the TTX binding component have been carried out primarily by two groups: Benzer and Raftery at Caltech and Ritchie, Henderson, and coworkers at Yale. Both groups used tritiated TTX to study binding properties and as an assay for activity, and used long-nosed garfish (Lepisosteus osseus) olfactory nerve as the tissue of choice. The garfish was chosen because (1) it is readily available at low cost and (2) it contains easily dissected, unmyelinated nerves having a large surface area of excitable membranes (72).

Benzer and Raftery (73) use TTX tritiated by a catalytic exchange method and purified by chromatography on Biogel P-2 followed by ion-exchange chromatography on Bio-Rex 70 at pH 7.0. Physiological activity was determined by bioassay on frog sciatic nerve. The amount of TTX present in the purified material was determined by correlating blocking activity with a calibration curve for the blocking activity of known concentrations of unlabelled TTX.

The $^{3}$H-TTX was first used to study the binding to homogenized membranes prepared from the garfish nerve. The dissected olfactory

nerve was minced, diluted 1 to 5 with physiological buffer and homogenized at 4°C. Binding to this homogenate was studied by equilibrium dialysis.

Equilibrium dialysis is a method which measures the amount of binding of a small ligand to a protein. A protein solution on one side of a semipermeable membrane (or inside of a bag of such material) is brought to equilibrium with a solution of the ligand in the same solvent on the other side (or outside of the bag). The activity of the ligand is the same on both sides of the membrane, and if the effect of the protein on the activity coefficient is negligible, then the concentrations of the free ligand should also be equal. The difference between the total ligand present per unit volume on the protein side and the free ligand present per unit volume on the other side is equal to the amount of ligand bound per unit volume of protein solution. For a radioactive ligand of known specific activity the amount bound can be assayed by determining the radioactivity of samples in a liquid scintillation counter.

Analysis of the data can be done by considering the theory of reversible ligand binding. For the case of a toxin (T) binding to a protein receptor (R), which has $\underline{n}$ binding sites, the binding equilibria can be represented as:

$$T_{i-1}R + T \rightleftharpoons T_iR \qquad i = 1, 2, 3, \ldots n$$

$$K_i = [T_iR]/[T_{i-1}R][T]$$

For identical, non-interacting sites, these equilibria are characterized by the same intrinsic association constant, $K_A$, and the average number of toxin molecules bound, $\underline{r}$, is given by:

$$r = \frac{\text{moles of T bound}}{\text{mole of R}} = \frac{n K_A [T]}{1 + K_A [L]} \qquad [1]$$

The form of this equation indicates that such binding should follow a rectangular hyperbolic isotherm with an asymptote equal to the maximum theoretical binding, $B_{max}$.

Equation [1] may be rewritten in the form:

$$\frac{1}{r} = \frac{1}{n K_A [T]} + \frac{1}{n} \qquad [2]$$

and a plot of $1/r$ versus $1/[T]$, called a double reciprocal plot, gives a straight line with a slope of $1/n K_A$ and a y-intercept of $1/n$. Another useful value obtained from this plot is the intrinsic dissociation constant, $K_D$, which is equal to the toxin concentration which gives half-maximal binding:

$$K_D = \frac{1}{K_A}$$

At the x-intercept:

$$[T] = -\frac{1}{K_A} \qquad \text{and} \qquad \frac{1}{[T]} = -\frac{1}{K_D}$$

For the case of non-identical, interacting sites, equation [1] may be rewritten to include the Hill coefficient, $\underline{h}$, which is a measure

of the interaction or cooperativity:

$$r = \frac{n [T]^h}{K_A + [T]^h} \qquad [3]$$

Equation [3] may be rewritten as:

$$\log \frac{r}{n-r} = h \log [T] - \log K_A \qquad [4]$$

so that a plot of $\log \frac{r}{n-r}$ versus $\log [T]$, called a Hill plot, gives a straight line with a slope of h and an intercept equal to $\log K_A$. If $h = 1$, there is no cooperativity; if $h > 1$, there is positive cooperativity; and if $h < 1$, there is negative cooperativity.

Benzer's data showed that TTX binding to membrane fragment homogenates showed a characteristic saturation above TTX concentrations of 50 nM. A double reciprocal plot gave a $K_D$ equal to 8.3 nM, which agrees well with in vivo neurophysiological studies (74). It was determined that there were 42 picomoles of saturable binding sites per gram of wet tissue, which corresponds to a calculated density of four binding sites per square micron of membrane surface. A Hill plot of the data gave a Hill coefficient of 1.

In order to probe the nature of the molecule involved in the binding, a portion of the membrane homogenate was centrifuged for 0.5 hours at 45,000 xg, and the pellet was resuspended in buffer containing appropriate enzymes. All of the binding activity sedimented, indicating that it was membrane bound. The only enzymes which

irreversibly decreased toxin binding were phospholipase A and two proteases chymotrypsin and Pronase. Pretreatment with phospholipase A increased the effect of the proteases. These results indicate that the TTX binding component is a protein which is partially buried in the membrane.

The next logical step in the study of a membrane protein is to solubilize the protein intact in order to use the many possible biochemical techniques to purify it. The best solubilizing agents found for membrane proteins are detergents.

Henderson and Wang (75) and Benzer and Raftery (76) have reported successful detergent extraction of the TTX binding component from garfish nerve membranes. Henderson used 1% sodium cholate and 1-2% Triton X-100 to solubilize most of the binding with a specific activity of 1.1 pmoles/mg of protein and a dissociation constant of 6 nM. STX was found to inhibit this binding. Solubilization was accomplished by stirring pelleted membranes with detergent-buffer solutions continuously at 0°C for 4-6 hours and subsequently centrifuging at 50,000-100,000 xg for 1 hour. Any material remaining in the supernatant was defined as solubilized. Henderson found that other detergents, 1.5% Tween-80, 1% digitonin, 1% Brij-35, 1% sodium deoxycholate and 1% sodium dodecyl sulfate were ineffective.

Benzer solubilized membrane fragments by stirring continuously in buffer containing 5% Triton X-100 and 10% glycerol for 10-12 hours at 4°C, and then centrifuged at 100,000 xg for 1 hour. The supernatant contained 1.95 picomoles of activity/mg of protein with a $K_D$

of 2.5 nM. Enzymes treatments on this preparation indicated that the solubilized protein was much more susceptible to proteases. Sensitivity toward phospholipase A indicated that some phospholipid is retained in solubilization. Treatment with disulfide reducing agents indicated that solubilization revealed at least one disulfide necessary for binding activity. Although the extract was very unstable above 10°C, as Henderson had found, Benzer was able to make two molecular weight determinations. Sucrose gradient results showed that the binding component sediments with an S value of 9.2, corresponding to an apparent molecular weight slightly less than 230, 000. On a Sepharose 6B solumn, the binding component eluted with an apparent molecular weight slightly greater than 550, 000. This discrepancy was probably due to the effects of solubilization relating to the shape of the molecule in a complex of associated lipid and detergent.

Benzer then turned his attention to a new source of tissue which could be used for large scale studies (77). He abandoned the use of gar nerve in favor of the electric organ of the electric eel, Electrophorus electricus. The electric organ is an electrically excitable tissue derived from muscle and therefore contains sodium channels and is TTX sensitive. The concentration of sites is close to that of gar nerve while the amount of tissue available per animal is on the order of several hundred grams compared to only a couple grams from gar.

The procedure used for the solubilization of the binding component from eel electroplax (cells of the electric organ) was similar

to that used for gar nerve. Equilibrium dialysis studies of eel membrane fragments indicated that maximal binding was about 25 pmoles/gram of tissue with a $K_D$ of 17 nm. Extracts were obtained which solubilized essentially all of this binding activity with a $K_D$ of 13 nM. The most successful detergent solution consisted of 1% Lubrol-Px, 10% glycerol, 20 mM Tris-HCl (pH 7.4).

A new procedure was developed which allowed completion of a binding assay in a much shorter time than equilibrium dialysis. This method utilizes Sephadex G-50 resin made up of gel beads with pore sizes which allow penetration of small molecules into the beads, while excluding large protein molecules. A column of such resin, subjected to a flow of eluant buffer, retards the passage of free TTX, while permitting the passage of protein and bound TTX. The assay method is as follows: a sample of extract is incubated for 10-15 minutes with a known amount of $^3$H-TTX, and an aliquot is allowed to soak into the top of a small G-50 column set up in a centrifuge tube. The columns were then centrifuged by accelerating to 1500 xg and immediately braked to stop. The free TTX remained in the gel while the band TTX was found in the effluent at the bottom of the tube. The radioactivity of this effluent could be easily determined.

With this reasonably quick assay, a good solubilization procedure, and a good tissue source the prospects for purification and further characterization seem quite good. One major problem remains to be solved, however, and that is the lack of stability of the binding component in extracted form, with at best a half life of about 6 days

at 4°C. In attempts to solve this problem, Benzer added various compounds to the extract after solubilization and centrifugation. None of the compounds added solved the problem which might be due to a missing requirement or the presence of a degrading agent. Compounds which were added included ionic molecules, lysophilic molecules, sodium channel toxins, antioxidants, enzymatic inhibitors, proteins and crosslinking agents.

Despite the presence of this problem, Benzer attempted to purify the TTX binding component by affinity chromatography techniques. He synthesized three different resins which have covalently linked to them a ligand which would bind to the TTX binding component, selectively retarding its passage on a column. Two derivatives of TTX and a derivative of STX were used as these ligands. Briefly, these attempts resulted in only very modest purifications at best.

Along with these solubilization studies, there have been other recent approaches to the characterization of the sodium channel. Using irradiation inactivation, Levinson and Ellory (78) have calculated an approximate molecular weight of 229,000 for the TTX binding component from rat brain and eel electroplax. This agrees well with the value obtained by the sucrose gradient study of Benzer and Raftery (76).

The molecular architecture of the pore has interested several investigators recently. In particular some work has been directed to the study of a possible carboxyl group within

the pore (79). This ionized acid group, with a predicted $pK_a$ of 5-6, could be responsible for the effect of pH on $^3$H-TTX binding observed by Henderson and Wang (75), as well as the effect of pH on intact nerve (23). This group is considered to be the possible binding site for metal cations which inhibit the binding of TTX to intact gar nerve (80,81). Shrager and Profera (82) used a carboxyl modifying reagent, 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide, to reduce the TTX binding to intact carb nerves. However, this reaction is quite drastic and not very specific. Much more conclusive evidence is seen in the work of Reed and Raftery (83) with the treatment of enriched membrane fragments from eel electroplax by the highly specific carboxyl modifying agent trimethyloxonium tetrafluoroborate.

Studies on the molecular mechanism of the ion translocation by sodium channels and its dependency on membrane potential has progressed in recent years, but obviously these studies are still of a preliminary nature. It is hoped that the research directed toward solubilization and subsequent purification will yield material to which more sophisticated biochemical and biophysical techniques may be applied.

# INVESTIGATIONS OF THE TTX BINDING COMPONENT SOLUBILIZED FROM EEL ELECTROPLAX

The work of Benzer (77) on the solubilization of the TTX binding component from eel electroplax laid the foundation for further biochemical characterization of the sodium channel molecule. Several problems remained at the completion of his studies, however: (1) while the solubilization procedure was acceptable, it was inconsistent in producing an extract with a high degree of TTX binding; (2) there was a need for preliminary characterization of the eel TTX binding molecule in a manner similar to that done on the gar nerve TTX binding component; and (3) the lack of stable TTX binding in the extract continued to impede research progress. These are the three areas chosen for this research work, with the hope that success would lead to a broad range of further studies on the sodium channel.

## A. Solubilization Studies

Several areas were investigated in the attempt to find a method of solubilization which optimized the factors of yield, speed, and stability. Several detergents were used for extraction, as well as a series of chaotropic agents. Chaotropic agents, which are molecules that favor the solubility of apolar groups in water, are known to be effective solubilizers of membrane proteins (84, 85). The order of effectiveness of these agents has been found to be:

$SCN^- > ClO_4^- >$ guanidinium$^+$ > urea > $Cl^-$ > $F^-$ (84).

Guanidinium thiocyanate (GdSCN) has been used as a very effective combination (85). Lithium diiodosalicylate (LIS) has also been used for membrane protein solubilization (86).

Lubrol-PX, a non-ionic detergent, was originally used by diluting 25% stock solutions kept on the laboratory bench. Recently, however, it was discovered that such stock solutions contained an impurity or degradation product which lowered the pH of the solution to about 3.5 and was difficult to buffer even in dilute solutions. Removal of this impurity did not solve the problem since more of this material as monitored by pH and conductance appeared within several days. The process is not affected by placing the solution in darkness or by addition of anti-oxidizing agents. It is slowed down by reducing the temperature to 4°C. Standard procedure for the preparation of all Lubrol-PX solutions now used is described in the methods section.

The discovery of the problem with the Lubrol solutions prompted a reappraisal of the efficiency of a Lubrol extraction. The effect of pH on extraction was studied, and subsequent studies were made to determine optimum Lubrol concentration, optimum fragment concentration in the detergent solution, and optimum extraction time.

Materials and Metholds

(i) Purification of $^{3}$H-tetrodotoxin. $^{3}$H-TTX was originally purified by the method described by Benzer and Raftery (73) from crude material tritiated by International Chemical and Nuclear Corp. from unlabelled citrate free TTX obtained from Sankyo Co., Ltd., Tokyo. Only small aliquots of the crude material were purified at a time so it was necessary to repeat the procedure approximately every three months. Briefly, the procedure was as follows: About 10 µCi of the crude material in about 10 µl was loaded onto a 1.5 x 55 cm. Biogel P2 (100-200 mesh) column, equilibrated in very dilute acetic acid (about 2 drops conc. acid/liter). 1.0 ml fractions were collected and the radioactivity of 10 µl aliquots was determined. The peak, which was shown earlier by Benzer to contain physiological activity by its effect on the excitability of frog sciatic nerve, was pooled and lyophilized. This material was dissolved in 10 mM ammonium acetate (pH 6.7) and applied to a 1.0 ml Bio-Rex-70 (200-400 mesh) ($NH_4^+$ form) column equilibrated in 10 mM ammonium acetate (pH 6.7), which was then eluted with a linear gradient of 10-200 mM ammonium acetate (pH 6.7). 1.0 ml fractions were collected and 10 µl aliquots were counted for radioactivity. A peak from about the middle of the gradient was pooled and lyophilized. This purified material was resuspended in dilute acetic acid and used in early studies.

The purity of recent preparations was monitored first by thin layer chromatography with silica gel-G and three different solvent systems, n-butanol:acetic acid:water (8:3:1), t-butanol:acetic acid:water (2:1:1), and 70% syn-collidine (2,4,6-trimethyl pyridine). Unlabelled TTX, STX, tetradonic acid, and tetrodaminotoxin were run as standards. The latter two solvent systems adequately resolved the standards and indicated impurities in the crude labelled material as well as purified material.

Bioassay of the fractions from the Bio-Rex-70 column were performed by Levinson according to a method previously published (87). This also revealed a high degree of impurity in the Bio-Rex-70 peak as indicated by graph 1. Consequently, a modified procedure was developed for the preparation of reasonably purified $^{3}$H-TTX.

The modified procedure utilized only a 1.0 ml Bio-Rex-70 column equilibrated in 10 mM ammonium acetate at pH 8.7 and maintained at 4°C. Crude material was dissolved in 10 mM ammonium acetate (pH 8.7) on ice, and applied to the column. The column was washed with 50 ml of 10 mM ammonium acetate (pH 8.7) and eluted with a 120 ml linear gradient of 10 - 100 mM ammonium acetate (pH 8.7). 1.0 ml fractions were collected in tubes to which had been added sufficient dilute acetic acid in order to weakly acidify the fractions. All the above procedures were carried out at 4°C in order to prevent degradation of the TTX which is rapid at room temperature under

alkaline conditions. Only the acidified fractions were allowed to warm up to room temperature.

Several peaks of radioactivity were eluted with the salt gradient, and the position of the TTX activity was determined by bioassaying, according to the method of Levinson, sufficient fractions to outline a peak. A typical chromatography profile is shown in graph 2. Since the $^{3}$H-TTX was eluted along with some impurities, this peak was usually pooled, lyophilized, and rechromatographed on a new 1.0 ml Bio-Rex-70 column. After the second chromatography, $^{3}$H-TTX was typically obtained with a purity of about 70% and a total yield of about 50%.

Radiopurity was determined by Levinson by correlating the binding of radioactivity and biological activity to eel electric organ particulate material (87). Since the assay indicated that the 70%-pure-$^{3}$H-TTX contained essentially no radioactive impurities which bound non-specifically, these preparations were deemed satifactory for use in this work. The specific activity of the $^{3}$H-TTX could be determined from the purity and the concentration of radioactivity.

(ii) G-50 Assay. The G-50 assay used in this work was similar to that originally developed by Juta Reed (unpublished results) and used by Benzer (77), with some modifications. The early procedure was as follows: Sephadex G-50 (Fine) was swollen in distilled $H_2O$ and then equilibrated in 0.1% Lubrol-PX, 10% glycerol and 20 mM buffer, either Tris-HCl or potassium phosphate, at pH 7.4-7.5 at 4°C. This resin was kept as

a slurry at 4"C until just before needed. Then aliquots of the slurry were pipetted into small, upright syringe barrels, plugged with filter paper, so that the final bed volume was 1.5 ml. The columns were placed in conical centrifuge tubes and kept at 4°C.

The samples were prepared usually in triplicate by pipetting aliquots, from 75 µl to 255 µl, of the material to be assayed into tubes containing the desired amount of $^{3}$H-TTX. The final volume was made up to either 285 µl or 290 µl with the proper detergent buffer. A convenient preparation has been found to be 250 µl of sample and 40 µl of a $^{3}$H-TTX solution which has been diluted to a concentration designed to give a sample concentration of about 150 nM. All tubes were kept on ice throughout the assay. These samples were incubated for 10-15 minutes to allow equilibrium binding.

A 250 1 aliquot was drawn from each sample tube and pipetted onto a G-50 column. The samples were allowed to soak into the columns and the centrifuge tubes with the columns were accelerated in a desk-top centrifuge up to a force necessary to elute the bound TTX but not the free TTX, and then immediately braked to a stop. Benzer had used a force of 1500 x g, but studies on the relation of g-force to eluted material showed that the optimum force was 100 x g. This force gave a low background while allowing essentially all of the bound material to be eluted. The higher force used by Benzer was also acceptable, but it was close to the

threshold for free TTX elution, so that care was needed to prevent high backgrounds and invalid results.

The eluted solutions were caught in the bottom of the centrifuge tubes and were quantitatively transferred, to scintillation vials.

The samples were dried down, and 1.5 ml of NCS solubilizer (Amersham) was added. Radioactivity was determined by counting in a scintillation fluid containing 0.55% Permablend III (Packard) in toluene.

From the known specific activity of the $^{3}$H-TTX and the radioactivity eluted, the bound TTX could be calculated. Benzer had determined that, under these conditions, the bound TTX represented about 60% of the maximum saturated binding.

(iii) Preparation of membrane fragments. The procedure used was similar to that of Benzer (77). A weighed portion of tissue from the electric organ, either fresh or thawed material which had been kept at -80°C, was minced with scissors while dissecting out any large pieces of connective tissue. The minced tissue was homogenized in a cold, glass-Teflon homogenizer with cold distilled $H_2O$ of a volume about four times the tissue volume. All subsequent procedures were carried out at 4°C. This homogenate was centrifuged at 250,000 x g for 1/2 hour. The pellet was resuspended in 1-3 volumes of 20 mM buffer, Tris-HCl or potassium phosphate, pH 7.4-7.5, with a glass-Teflon homogenizer. This homogenate was then sonicated with a Branson sonicator at 45 power for two bursts

of 15 seconds each with at least one minute wait in between to dissipate any heat generated. The sonicated material was filtered through a fine wire mesh to remove coagulated connective tissue fragments. The filtrate was kept at 4°C and was used in that form when reference is made to "membrane fragment homogenate".

(iv) Solubilization procedure. Material for solubilization was obtained by centrifuging the membrane fragment homogenate at 250,000 x g for 1/2 hour at 4°C. For the comparison of solubilizing agents and conditions, the volume of the membrane fragment homogenate was measured and equal aliquots were pipetted into separate centrifuge tubes. It was assumed that the pellets obtained contained approximately equal amounts of membrane material, so that the degree of solubilization could be compared directly.

The pellets were resuspended with a cold glass-Teflon homogenizer in the appropriate extraction solutions. Care was taken to keep all extracts below 10°C at all times. The extraction homogenates were transferred quantitatively to centrifuge tubes. Teflon stir bars were placed inside, and the mixtures were stirred on ice for the appropriate length of time. The extract homogenates were then centrifuged at 250,000 x g for 1 hour. The supernatants were considered "solubilized" material. TTX binding was determined by G-50 assay and/or equilibrium dialysis and protein was determined

by the method of Lowry (87). The binding of TTX in the GdSCN extracts was determined by first reducing the concentration of GdSCN by dialysis because guanidinium is known to inhibit binding (Benzer, unpublished results).

The solubilizing agents used for comparison were of two types, detergents and chaotropic agents. Detergents tested include 1% Lubrol-PX, 1% Lubrol-WX, 1% Triton X-100, 1% Brij 56 and 96, 1% Emulphogene BC-720, 0.5, 1 m and 5% Sarkosyl NL 30, 1% N-Lauroyl Sarcosine, 1% Tween-80, and Lysolecithin (10 mg/ml). All were obtained from Sigma Chemical Co. except Sarkosyl (from Geigy) and Emulphogene (from GAF). Chaotropic agents used include 1.0 M $NaClO_4$, 1.0, 2.0, and 4.0 M NaSCN, 3.0 and 6.0 M Urea, 1.0, 3.0, and 6.0 M GdSCN, and 25, 80, and 300 mM lithium diiodosalicylate (LIS). $NaClO_4$ was obtained from G. Frederich Smith Chemical Co., NaSCN was from Allied Chemical, Urea was ultrapure grade from Schwarz/Mann, GdSCN was from J. T. Baher Chemical Co., and LIS was from Eastman Kodak Co.

The Lubrol-Px was prepared as a 5% solution and stirred for several hours at 4°C with Bio-Rad AG501-X8 (20-50 mesh) beads, a mixed-bed ion-exchange resin, designed to remove both anionic and cationic impurities. The treatment was continued until the pH is approximately 7.0 and the conductance was reduced approximately to that of distilled $H_2O$. This stock solution was kept at 4°C in the presence of the beads. Just prior to use, the bottle was removed from the refrigerator, stirred for

short time, and an aliquot was filtered to removed the beads. The filtrate was used to make up fresh solutions.

(v) Determination of optimal pH for extraction. Approximately equal amounts of membrane fragments were solubilized for 10-12 hours in 1% Lubrol-PX, 10% glycerol, and 20 mM buffer at the appropriate pH. Potassium phosphate was used at pH's below 7.6 and Tris-HCl was used at pH's above 7.5. TTX binding activity was determined by G-50 assay at pH 7.5.

(vi) Determination of optimal Lubrol concentration. Approximately equal amounts of membrane fragments were solubilized for 10-12 hours in 10% glycerol, 20 mM potassium phosphate pH 7.5, and concentrations of Lubrol-PX from 0.5% to 4.5%. TTX binding was determined by G-50 assay at pH 7.5.

(vii) Determination of optimal extraction. Approximately equal amounts of membrane fragments were solubilized for times ranging from 1 hour to 25 hours in 1% Lubrol-PX, 10% glycerol and 20 mM potassium phosphate pH 7.5. TTX binding was determined by G-50 assay at pH 7.5.

(viii) Determination of optimal membrane fragment concentration. Approximately equal amounts of membrane fragments were solubilized in varying volumes of 1% Lubrol-PX, 10% glycerol, and 20 mM potassium phosphate pH 7.5. This procedure resulted in concentrations of membrane fragments equivalent to an original tissue weight of 1.3 g/ml to 4.5 g/ml. TTX binding was determined by G-50 assay at pH 7.5.

RESULTS AND DISCUSSION

The results of the various attempts at solubilization are summarized in Table 1. The yields are normalized to the equivalent Lubrol-PX extraction. No solubilizing agent was found to surpass the effectiveness of 1.0% Lubrol-PX in either yield or specific activity. None of the detergents produced an extract with a stability greater than that of Lubrol-PX. GdSCN extracts showed 20-50% better stability, but the yields of these extractions were poor. Attempts to combine the yield of a Lubrol extract with the stability of a GdSCN extract produced acceptable extracts, but no greater stability than Lubrol alone.

The relationship of extractibility to pH is shown in graph 3. Optimal pH appears to be 7.6 to 7.9. From graph 4, the optimal Lubrol concentration is seen to be 1-2% and from graph 5, the optimal extraction time is 2 hrs. Graph 6 shows the relationship of extractibility to membrane fragment concentration. For a 12-14 hr extraction there is little difference between the various concentrations. However, for an extraction time of 5 hrs there is a great disparity between the yield from concentrations of 4.5 g/ml and 2.2 g/ml. This indicates that the extraction time is probably dependent on the total protein=detergent ratio, so that consideration must be made to correlate the proper extraction time for a given homogenate concentration. From these results a fairly consistent extract is obtained with a 1% Lubrol-PX concentration at pH 7.5-7.6 for 2 hrs at a fragment concentration equivalent to 2.0-2.5 net weight tissue/ml.

In the course of these studies it was noted that the quality of the electric organ is a factor in the extractibility. Good extracts were consistently obtained with some organs, while others gave poor and variable yields. Trial and error gives the only good test for acceptable organ.

## B. MOLECULAR WEIGHT STUDIES

Determination of the molecular weight of the solubilized TTX binding component is important for two reasons: (1) it provides a criterion for solubilization, i.e. if the protein is not excluded in the void volume of a Sepharose column, it is considered to be solubilized out of its membrane environment (89) and (2) it provides a correlation of the TTX binding component from electric eel with that of the gar nerve by comparison with the results obtained by Benzer (76). Two methods were used from this determination: (1) gel filtration on a Sepharose 6B column and (2) velocity centrifugation on a glycerol gradient (10-43%).

Materials and Methods.

(i) Solubilization Procedure. The method of solubilization for the molecular weight determinations was as follows: crude membrane fragments were prepared as before and then solubilized in 2% Lubrol PX, 20 mM Na-Hepes, pH 7.6, 1 mM EDTA and in the presence of small amounts of PMSF (phenyl methyl sulfonyl fluoride) and BHMP (2,6-di-tert-butyl-4-hydroxymethylphenyl). All of the above reagents were obtained from Sigma. The membrane

fragment concentration was equivalent to 4 grams original wet weight tissue per ml. This was stirred on ice for about 3 hrs and then centrifuged at 100,000 x g for 1 hr. The supernatant was used for the following determinations.

(ii) Sepharose 6B column. A 1.5 x 40 cm column of Sepharose 6B was packed and equilibrated with 0.1% Lubrol, 20 mM NaHepes (pH 7.6), 50 mM NaCl, and 1 mM EDTA. 1.0 ml of the extract prepared as above was layered on the column and 1.0 ml fractions were collected. 0.25 ml aliquots were assayed for TTX binding activity by G-50 assay. The column was standardized with blue dextra, β-galactosidase, and myoglobin.

iii) Glycerol gradient. 4.5 ml glycerol gradients (10-43%) made with a Beckman density gradient former, contained 0.1% Lubrol, 20 mM NaHepes (pH 7.6), and 1 mM EDTA. 350 µl samples of the above extract were layered on the top, and the gradient were centrifuged for 12 hrs at 60,000 rpm in a SW 65 rotor in a L-2-65B Beckman Ultracentrifuge. 300 µl fractions were collected and 250 µl of each were assayed by G-50 assay. β-glactosidase and catalase were used as standard proteins.

Results and Discussion

The profile of TTX binding activity from the Sepharose 6B column is shown in graph 7. The molecular weight obtained from this profile for the TTX binding component from electric eel is similar to that obtained from the garfish nerve by Benzer (76). The molecular weight appears to be somewhat larger than β-galactosidase, at approximately 600,000.

The profile of TTX binding activity in the glycerol gradient is shown in graph 8. The molecular weight appears to be somewhat lower than catalase, at approximately 200,000. This estimate is again similar to that for the gar nerve protein (76).

The discrepancy between the two molecular weight determinations is probably due to the typical behavior of detergent solubilized membrane proteins on Sepharose. The apparent molecular weight may be quite large due to the presence of a detergent-protein complex, so that the actual molecular weight of the protein would be much lower. The Sepharose column, however, does indicate that the TTX-binding component has been effectively solubilized out of lipid membrane. Very little activity is seen in the void volume where one would expect to find membrane patches.

The molecular weight obtained from the glycerol gradient is probably closer to actual weight of the binding component The estimate of 200,000 is similar to that obtained by Levinson for the TTX binding component from electric eel using irradiation inactivation (78).

## C. BINDING STUDIES

It is important to study the binding of TTX to the extracted protein for at least two reasons: (1) to correlate the characteristics of the extracted binding component with those of the component in its original membrane environment, and (2)

to investigate the nature of the instability in the TTX binding and attempt to correct it. The determination of the $K_D$ of several cations were used as criteria for the correlation with values obtained by Reed and Raftery (83) for electric eel membrane fragments. These binding parameters were studied also with respect to the aging of the extract to determine if the instability was a function of a change in $K_D$ or in the number of sites. The effect of cation binding on the instability was also investigated.

In order to study the binding of TTX to the extract, the G-50 assay was used to provide a quick reliable assay over a wide range of TTX concentrations, unaffected by the varying purity of $^3$H-TTX preparations. Since only bound $^3$H-TTX passes through the G-50 columns and it has been shown that non-specifically binding impurities are negligible, only the original TTX concentration and the intrinsic specific activity of the $^3$H-TTX must be known. In the course of the assay only the radioactivity passing through the column must be measured. In addition the assay required only about 1 hr to complete, making it much better than equilibrium dialysis.

Materials and Methods.

(i) $K_D$ determination: An extract was prepared as described for the molecular weight determinations. 25 µl aliquots were added to tubes containing varying amounts of $^3$H-TTX and brought up to 300 µl volumes with 20 mM NaHepes (pH 7.6). In the course of this study the final TTX concentrations were varied from about 0.5 mM to 100 mM. The tubes were kept on ice for

30 min and then 250 µl aliquots were removed for the G-50 assay. These samples were applied to the G-50 columns, allowed to enter the gel, and quickly centrifuged in order to prevent a change in equilibrium binding. A series of background values were obtained by preparing samples with varying concentrations of $^{3}$H-TTX alone or in combination with heat treated extract (to destroy TTX binding) or with good extract and 2 µM cold TTX.

The eluted samples were transferred quantitatively to scintillation vials. Instead of drying down these samples they were taken up in a scintillation fluid consisting of 5.5 g/l Permablend-III (Packard) in a solution of 25% Triton X-100 (Sigma) and 75% Toluene (90). This scintillation fluid is designed to solubilize up to 1 ml of aqueous sample/ 10 ml scintillator. These samples were counted in a Beckman scintillation counter.

The background values were subtracted from the radioactivity counted, and this value was converted to pico moles $^{3}$H-TTX bound. Since the initial concentration of TTX was known, the free concentration of $^{3}$H-TTX could be calculated.

(ii) Cation binding. The $K_D$ for $^{3}$H-TTX binding to the extract was repeated, as above, with a variety of cations present as the samples were incubated with $^{3}$H-TTX. These cations were $Li^+$, $Na^+$, $K^+$, $Rb^+$, $Ca^+$, $NH_4^+$, and choline$^+$. In all cases $Cl^-$ was the counterion, and the ionic strength was kept constant with choline chloride. It has been reported by Reed and Raftery (83) that there was no appreciable binding of

choline to the TTX site, and this was first confirmed for the solubilized binding component before the rest of the cations were studied.

(iii) Stability studies. The $K_D$ for $^3$H-TTX binding was studied with respect to the aging of the extract and loss of binding activity. An extract was kept on ice for several days, and the $K_D$ determination was repeated at various intervals.

The effect of some of these cations on the loss of binding activity was determined by adding known amounts of the cations to aliquots of an extract and floowing the binding activity over the course of several days. The effects of $^3$H-TTX, veratradine and procaine were also investigated in a similar manner.

Results and Discussion

The $K_D$ for the binding of $^3$H-TTX to solubilized extract was found to be about 1-2 nM, using the G-50 assay as shown in graph 9. This is somewhat lower than the value of 6 nM obtained for $^3$H-TTX binding to membrane fragments (83), but this is probably more reliable due to a more accurate determination of the specific activity of the $^3$H-TTX. The $K_D$ was found to be unaffected by the addition of choline chloride but was altered by the other cations studied. Graphs 10 and 11 show the effect of $NH_4^+$ on the binding of $^3$H-TTX. The apparent $K_D$'s were calculated for $^3$H-TTX binding in the presence of 200, 300, and 400 mM $NH_4Cl$, and these values were replotted with respect to the concentration to give an

apparent $K_i$ for $NH_4^+$. Table 2 shows the values of the apparent $K_i$'s obtained for membrane fragments and for solubilized material. The correlation between the two sets of data are reasonably good. This is a very good indication that the $^3$H-TTX binding component has been successfully solubilized out of its membrane environment without a drastic change in the properties of the protein.

The loss of TTX binding as the extract ages, however, does indicate that the environment of the protein in the solubilized state is not entirely suitable. The study of binding in aging material indicates that the $K_D$ does change significantly with time, and it appears that the loss of TTX binding is due to a decrease in the number of sites. The loss of TTX binding with time is shown in graph 12.

Also in graph 12 is shown the effect on TTX binding of several agents which were added to the extract. It had previously been demonstrated that 5-10 mM $Ca^{2+}$ slowed the loss of TTX binding (John Brabson, unpublished results). In this study it was found that other cations also have this effect. 50 mM $Li^+$, 50 mM $NH_4^+$, and 100 mM $Ca^+$ were at least as successful as 5 mM $Ca^{2+}$. ($Na^+$, $K^+$, and $Rb^+$ were not tested for this effect.) In addition, the presence of $^3$H-TTX in the aging extract clearly slowed the loss of TTX binding, apparently in relation to the free toxin concentration. Veratridine and procaine at the concentrations used failed to have any effect on the TTX binding loss.

These results seem to indicate that binding to the TTX site is important in slowing the loss of activity. All of the cations are presumed to bind to this site (81,83). It is possible that occupancy of this site protects the protein from the slow inactivation process. This finding may be important for the development of a suitable procedure to maintain a high level of TTX binding in the solubilized system.

## D. SUMMARY AND CONCLUSIONS

In the course of this work, optimum conditions were determined for consistent solubilizations in good yield of the TTX binding component from eel. The molecular weight studies indicated that the solubilized eel protein was similar to that solubilized from the gar, and the binding studies indicated that the solubilized protein retained the binding properties found for the protein in intact eel membrane fragments. The instability in TTX binding was found to result from a loss of sites not from a change in $K_D$, and that these sites could be partially protected from inactivation by the binding of cations to those sites.

The work described in this report has resulted in the solubilization and partial characterization of the TTX binding component from electric eel. It is hoped that this work has provided some measure of progress toward the ultimate goal of understanding the mechanisms of nerve function.

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Table 1. Compounds Used in Attempt to Stabilize the TTX Binding Component (77)

Ionic molecules

$MgCl_2$ (2 mM)
$CaCl_2$ (2.2 mM)
EDTA (2 mM, 5 mM)
NaCl (.5 M)
KF (.2 M)

Enzymatic inhibitors

Phenylmethylsulfonylfluoride (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

$^3$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%)

TABLE 2. Comparison of Cation Binding for Intact Membrane Fragments and Solubilized Extract.

| Cations | Apparent Inhibitor Constant $K_i$, $10^{-3}$ M | |
|---|---|---|
| | Membrane Fragments (83) | Extract |
| $Li^+$ | 60 ± 10 | 45 ± 10 |
| $Na^+$ | 71 ± 4 | 70 ± 10 |
| $K^+$ | 135 ± 12 | 120 ± 20 |
| Rb | 207 ± 20 | 180 ± 30 |
| $Cs^+$ | 272 ± 30 | 300 ± 40 |
| $NH_4^+$ | 87 ± 5 | 80 ± 10 |
| $Choline^+$ | >400 | >400 |

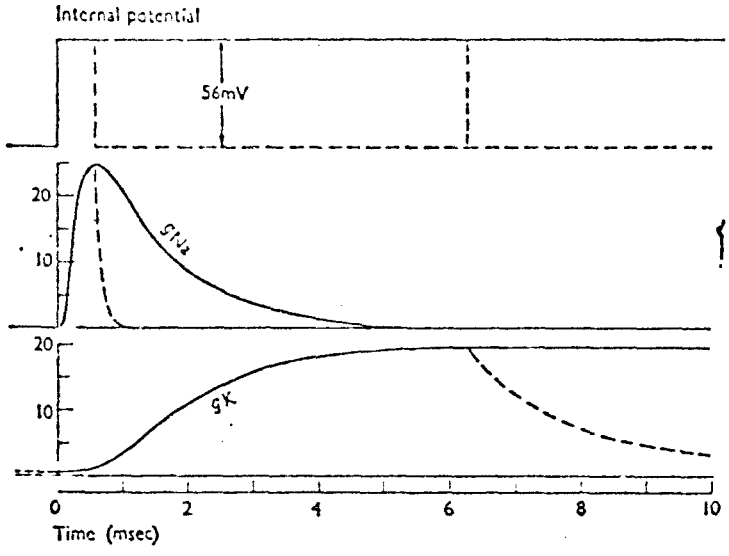



FIG. 1. Time course of Na and K conductance following voltage clamp steps from −65 mV to −9 mV in a squid giant axon. Continuous curves are for maintained depolarization. Broken curves are for a return to the resting potential. Conductance in μmho cm$^{-2}$. Temperature 8.5°C.

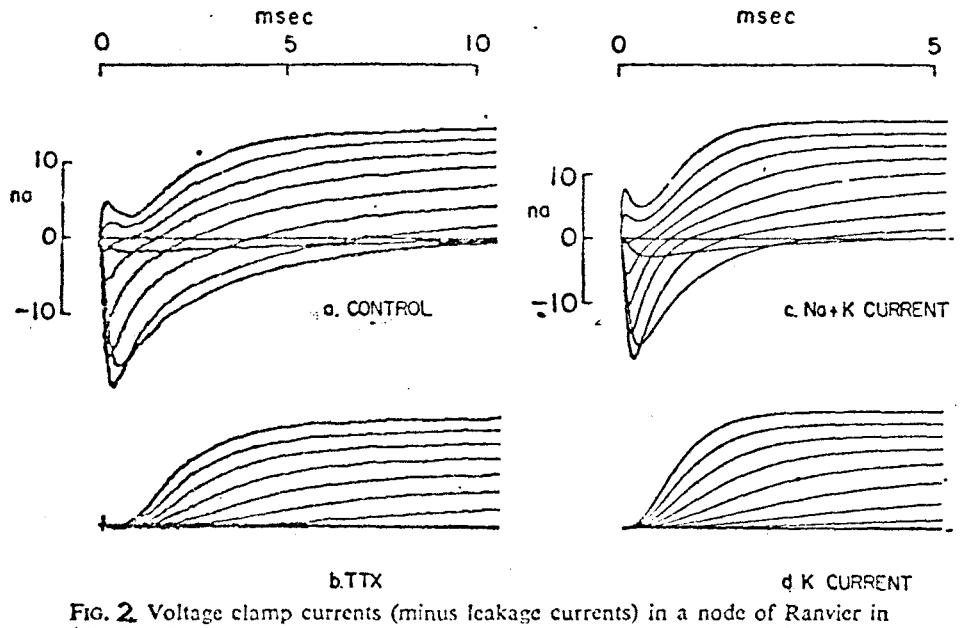



FIG. 2. Voltage clamp currents (minus leakage currents) in a node of Ranvier in (a) Ringer's solution and (b) 300 nM TTX compared with (c) the sum of Na and K currents and (d) K current alone computed from a model node. The currents correspond to potentials spanning the range from −60 mV to +75 mV in 15 mV steps. Outward current is positive. Temperature (a, b) 13°C; (c, d) 22°C.

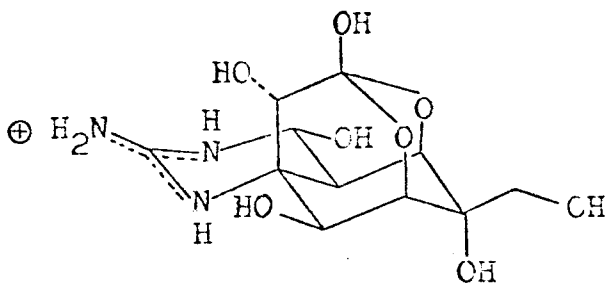


Fig. 3 Tetrodotoxin

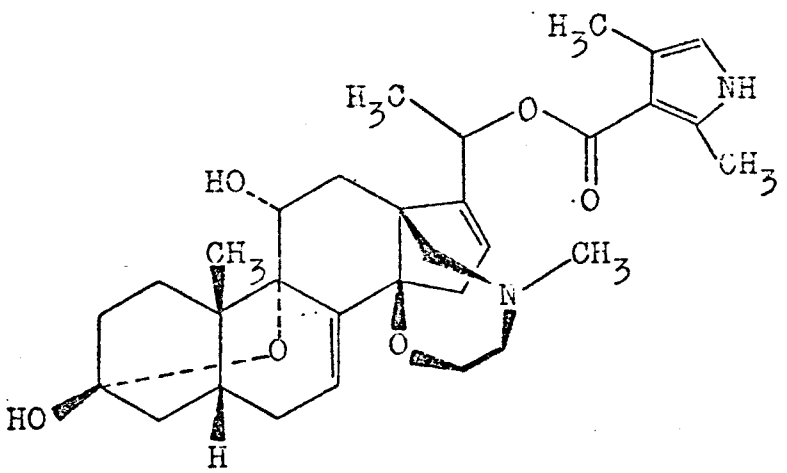


Fig. 4 Batrachotoxin

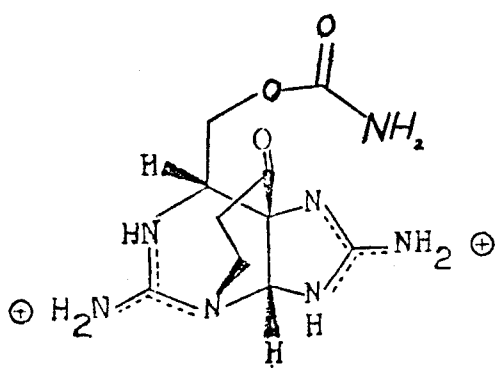


Fig. 5 Saxitoxin

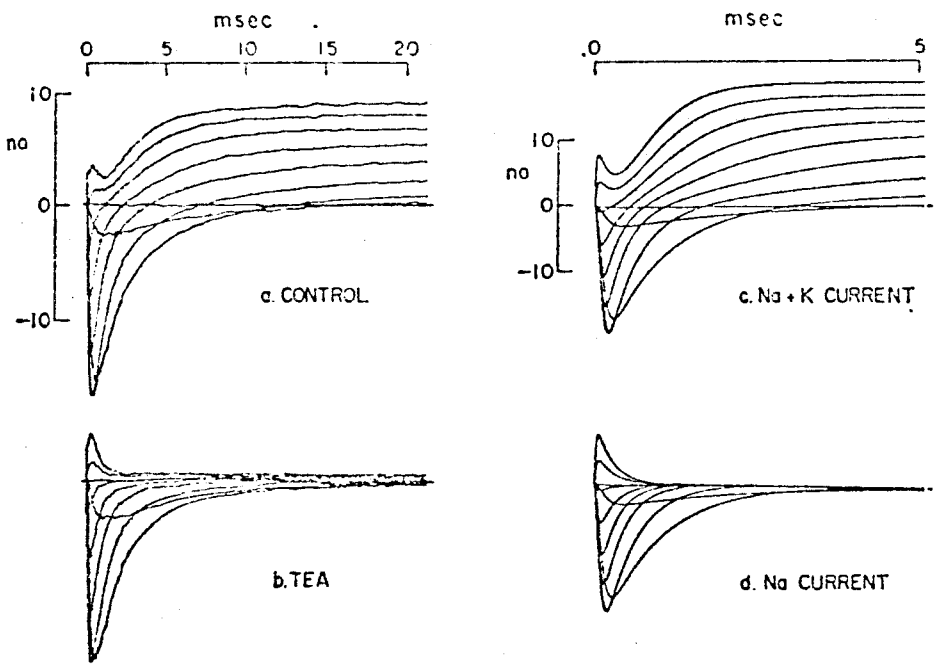
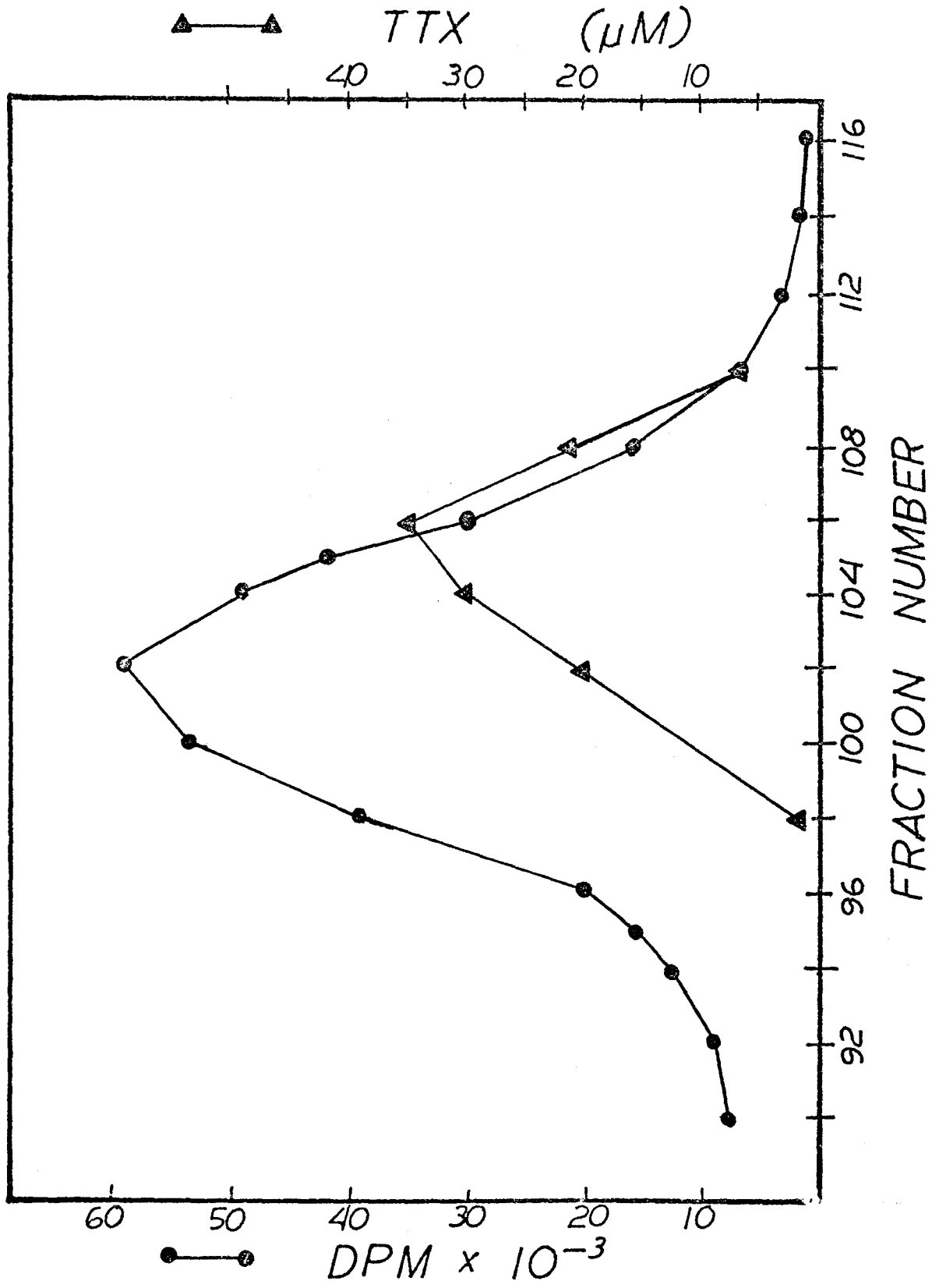
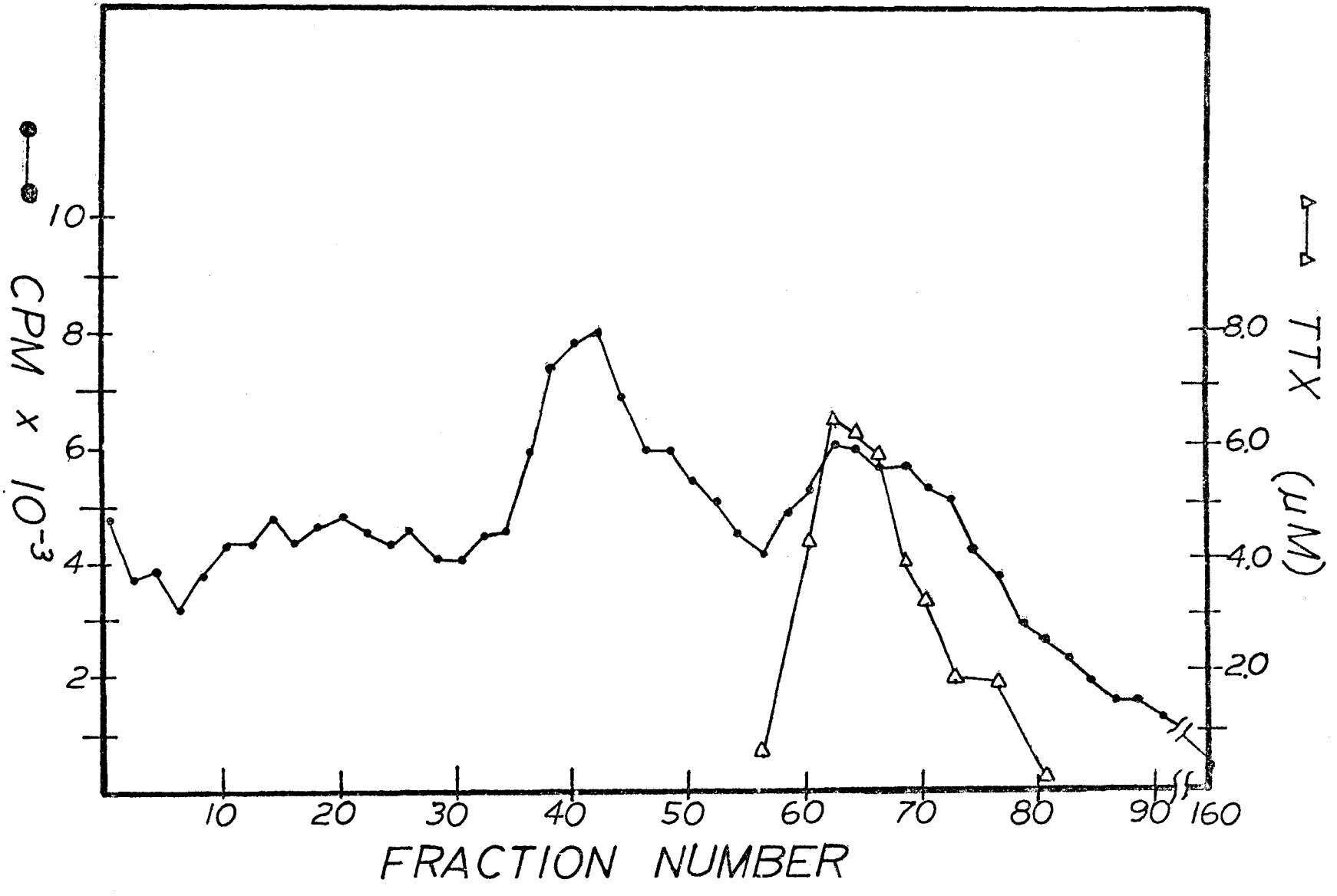



FIG. 6. Voltage clamp currents (minus leakage currents) in a node of Ranvier in (a) Ringer's solution and (b) 6 mM TEA compared with (c) the sum of Na and K currents and (d) Na currents alone computed from a model node Temperature (a, b) 11°C; (c, d) 22°C.

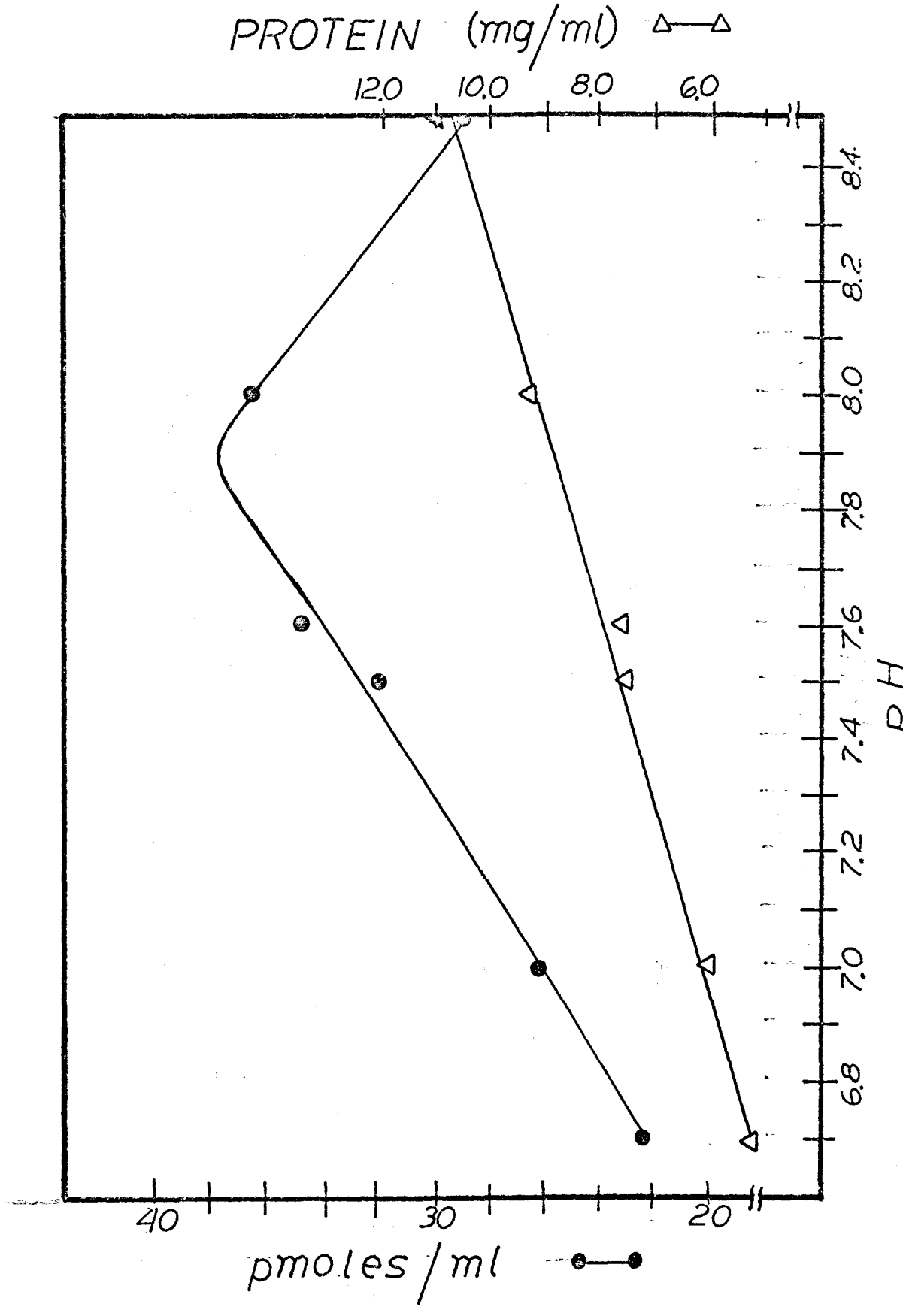
GRAPH 1. A portion of the gradient elution chromatography on Bio-Rex-70 for the purification of $^{3}$H-TTX as described by Benzer (73). 1.0 ml fractions were collected and 10 µl aliquots were removed for determination of radioactivity. The concentrations of TTX were determined by bioassay.


TTX (μM)
40
30
20
10
116
112
108
104
100
96
92
FRACTION NUMBER
60
50
40
30
20
10
DPM x 10⁻³

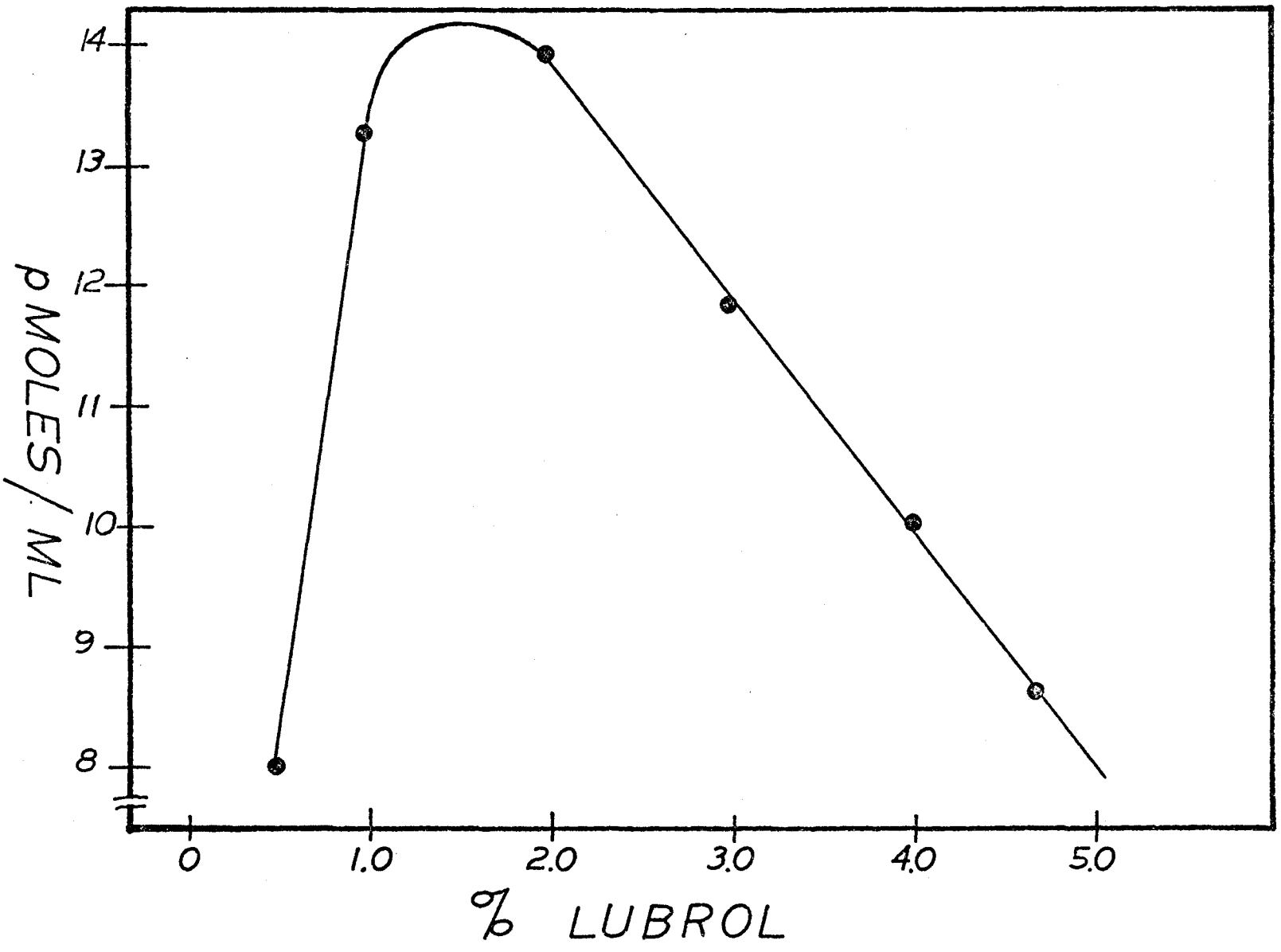
GRAPH 2. Gradient elution chromatography on Bio-Rex-70 at pH 8.7 and 4°C. of the crude $^{3}$H-TTX. A preliminary wash with 10 mM ammonium acetate of the 1.0 ml column removed a large amount of radioactivity, and the 10-100 mM ammonium acetate gradient was begun at fraction 1. 1.0 ml fractions were collected and 10 µl were removed for determination of radioactivity. The concentrations of TTX were determined by bioassay.


CPM x 10-3
10
8
6
4
2
TTX (μM)
8.0
6.0
4.0
2.0
10
20
30
40
50
60
70
80
90
160
FRACTION NUMBER

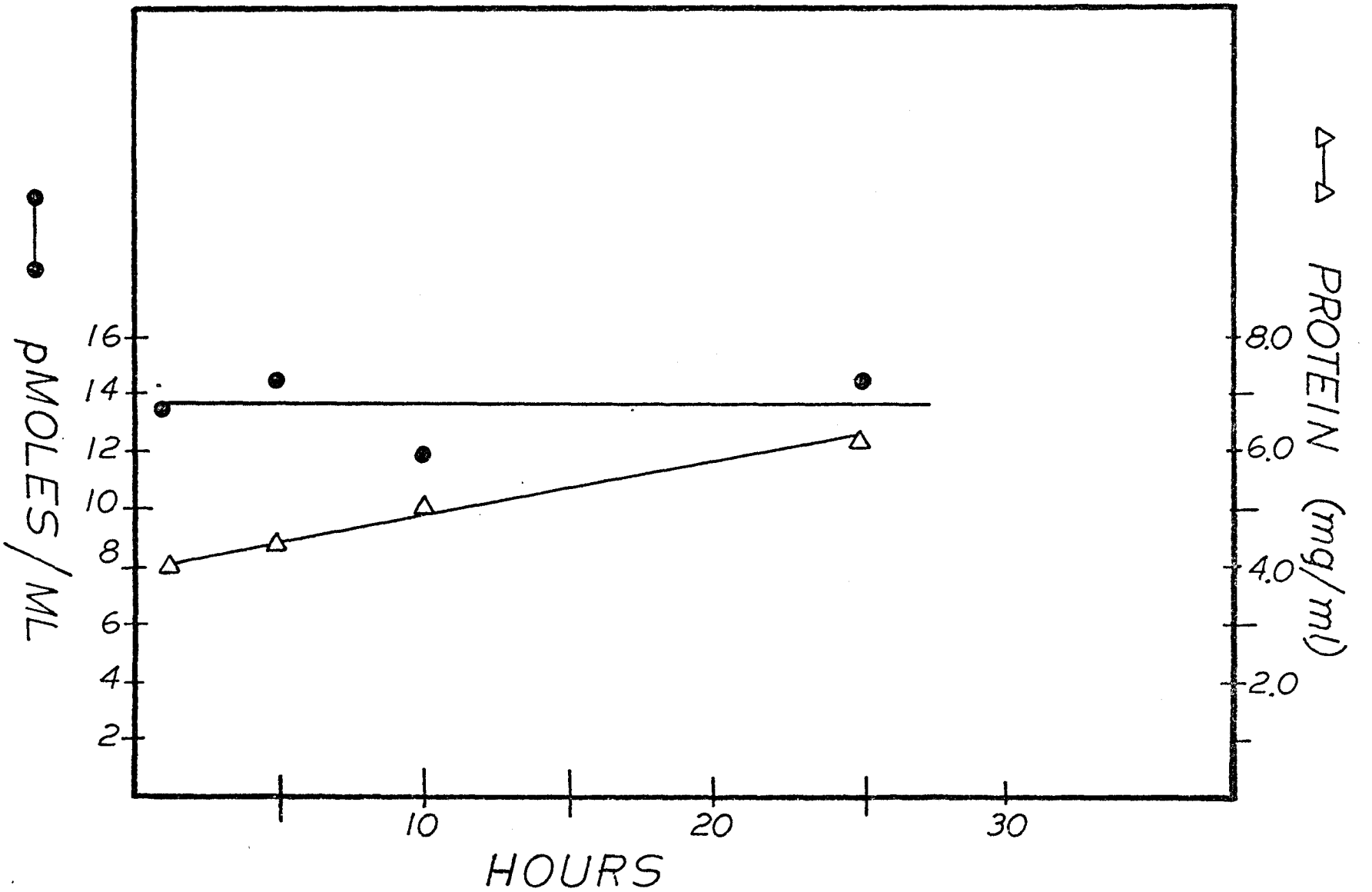
GRAPH 3. Effect of pH on extraction yield. TTX binding (pmoles/ml) was determined by G-50 assay; protein was determined by the method of Lowry.


PROTEIN (mg/ml) △—△
12.0
10.0
8.0
6.0
8.4
8.2
8.0
7.8
7.6
7.4
7.2
7.0
6.8
pH
40
30
20
pmoles/ml ●—●

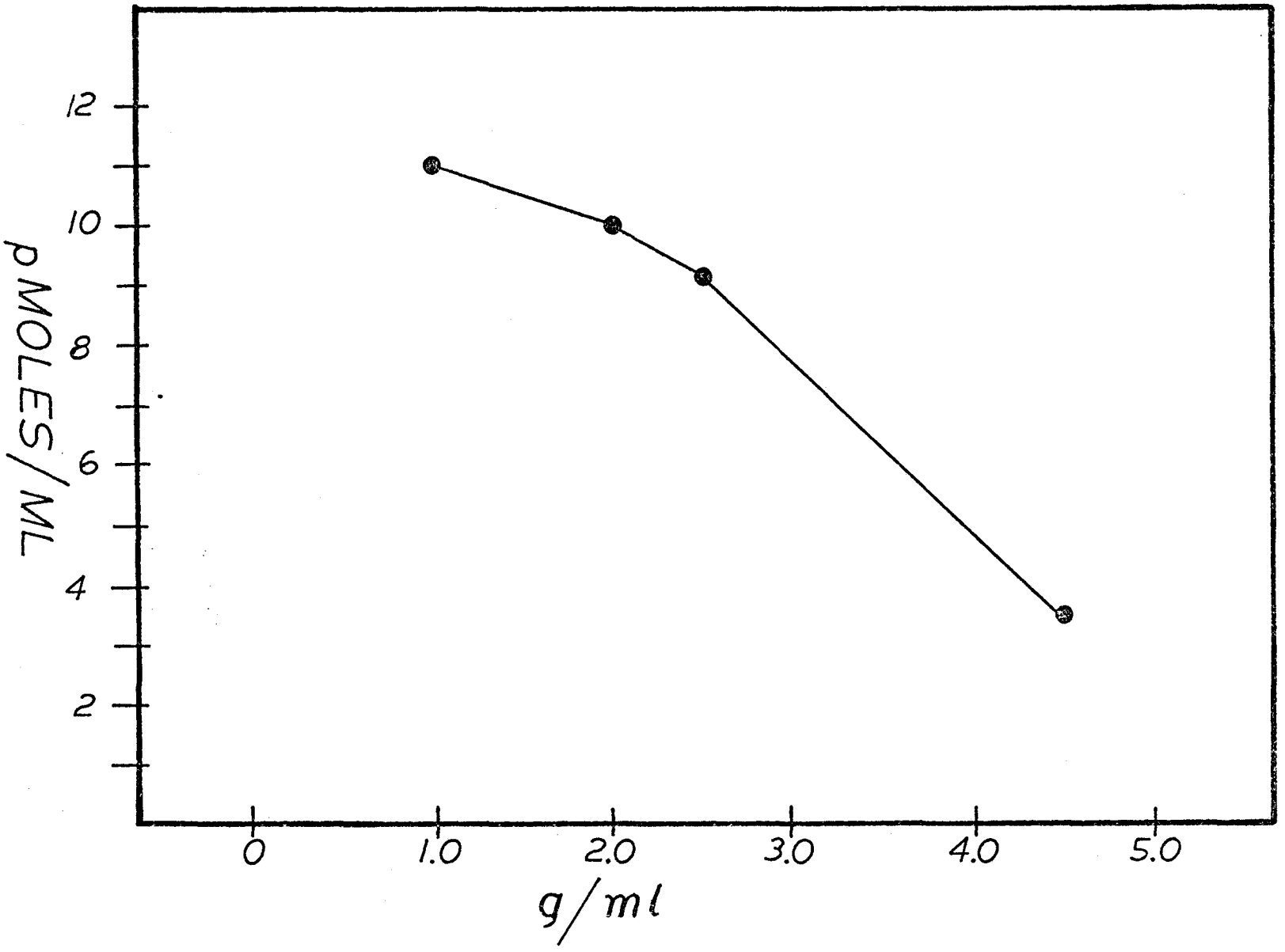
GRAPH 4. Effect of Lubrol-PX concentration on extraction yield. TTX binding (pmoles/ml) was determined by G-50 assay.


14
13
12
11
10
9
8
p MOLES / ML
0
1.0
2.0
3.0
4.0
5.0
% LUBROL

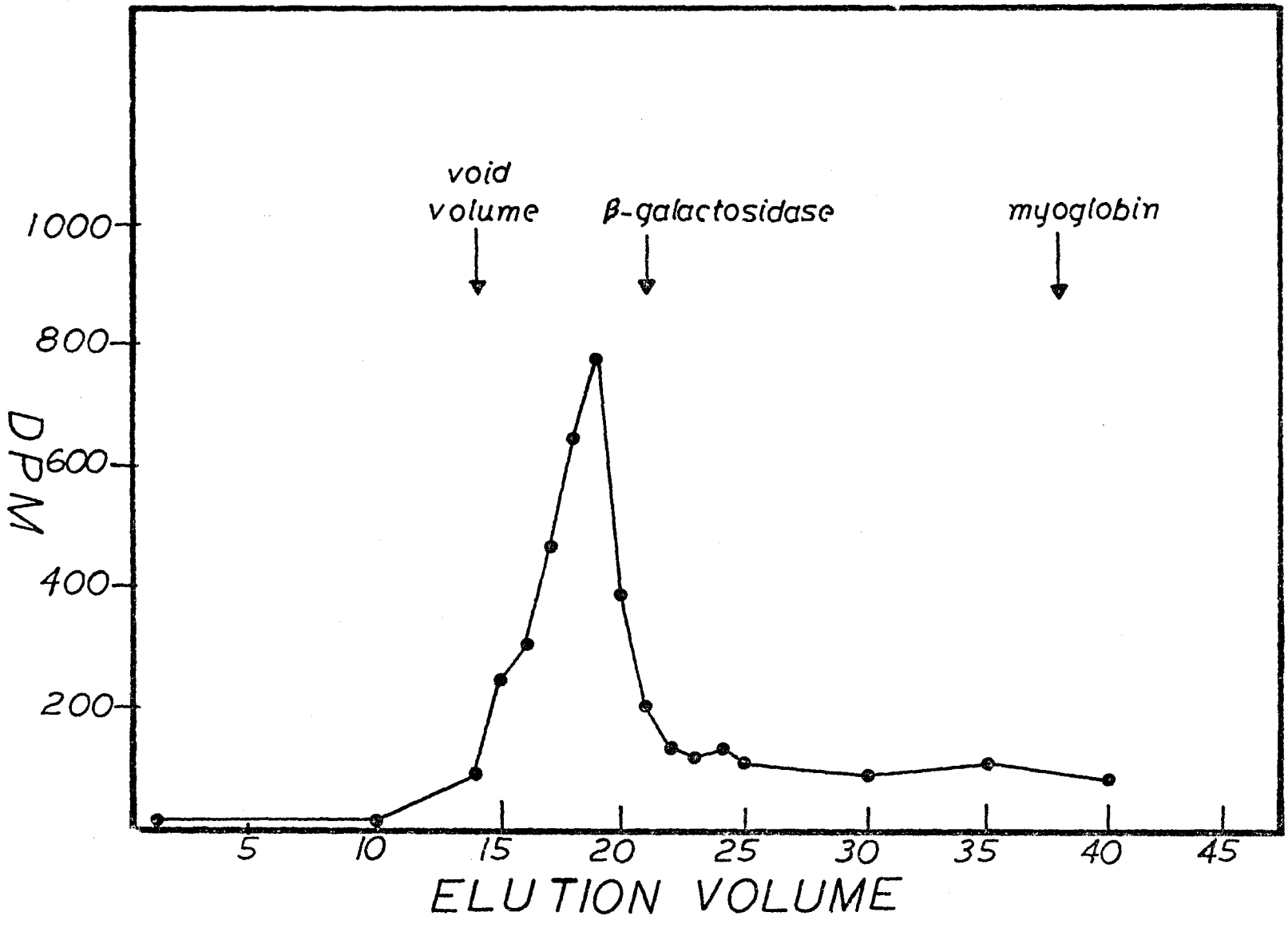
GRAPH 5. Effect of extraction time on extraction yield. TTX binding (pmoles/ml) was determined by G-50 assay and protein was determined by the method of Lowry.


pMOLES / ML
PROTEIN (mg/ml)
16
14
12
10
8
6
4
2
8.0
6.0
4.0
2.0
10
20
30
HOURS

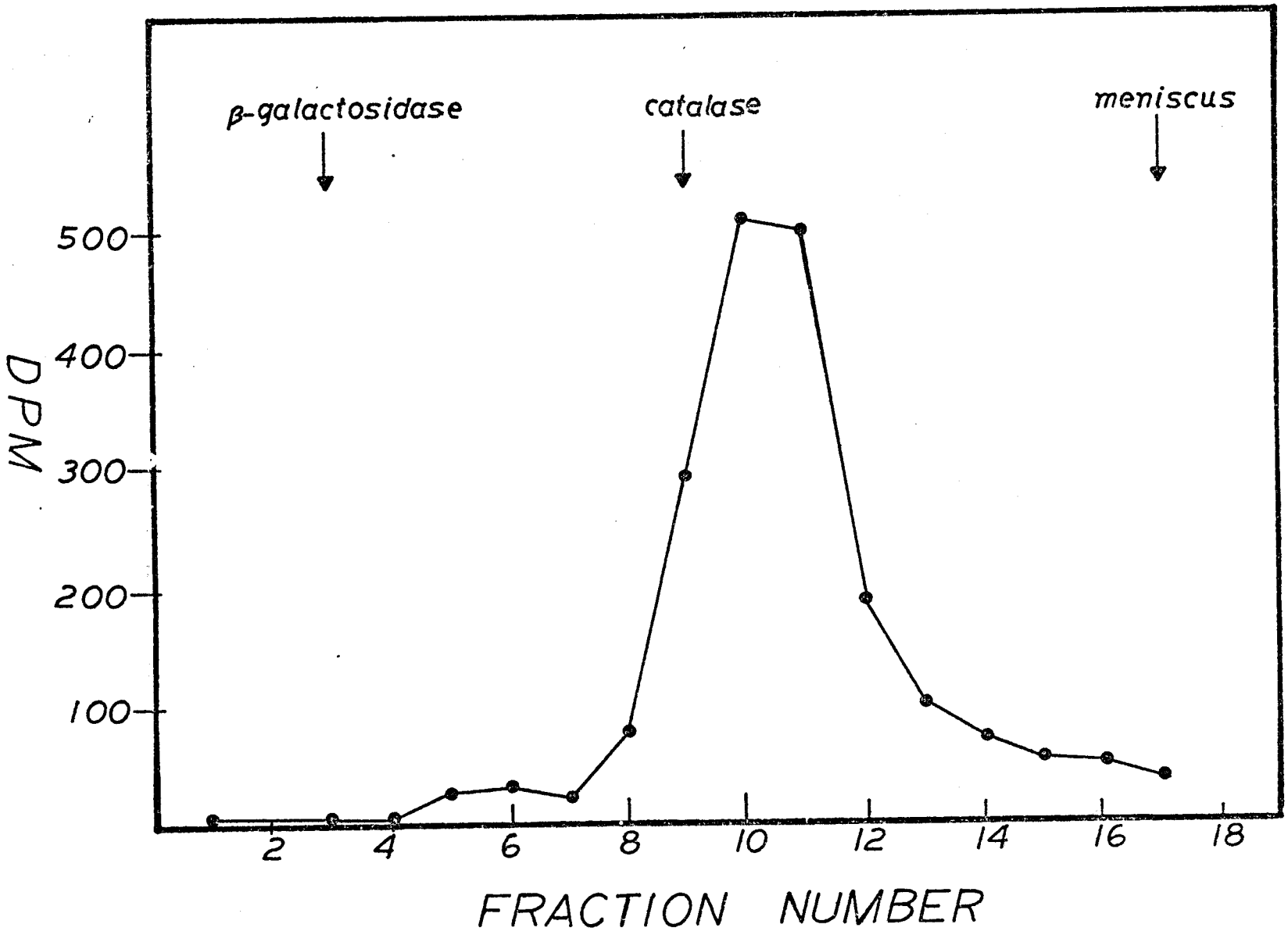
GRAPH 6. Effect of membrane fragment concentration on extraction yield. The concentration of membrane fragments was equivalent to that obtained from the original wet weight tissue (g/ml). TTX binding (pmoles/ml) was determined by G-50 assay.


12
10
8
6
4
2
p MOLES/ML
0
1.0
2.0
3.0
4.0
5.0
g/ml

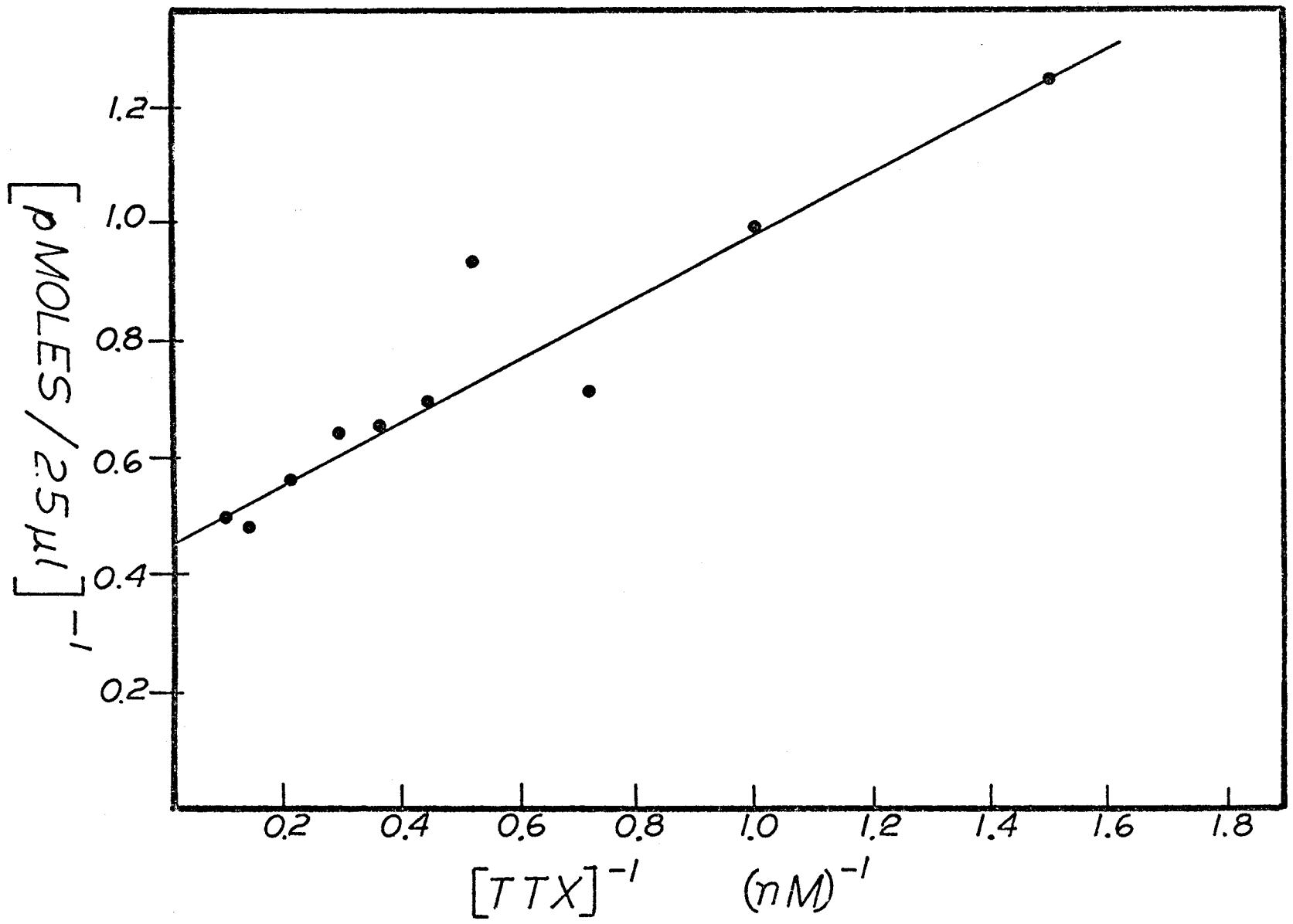
GRAPH 7. Gel filtration on Sepharose 6B of the solubilized material. TTX binding (DPM) was determined for a 250 µl aliquot by G-50 assay.


void volume
β-galactosidase
myoglobin
1000
800
600
400
200
DPM
5
10
15
20
25
30
35
40
45
ELUTION VOLUME

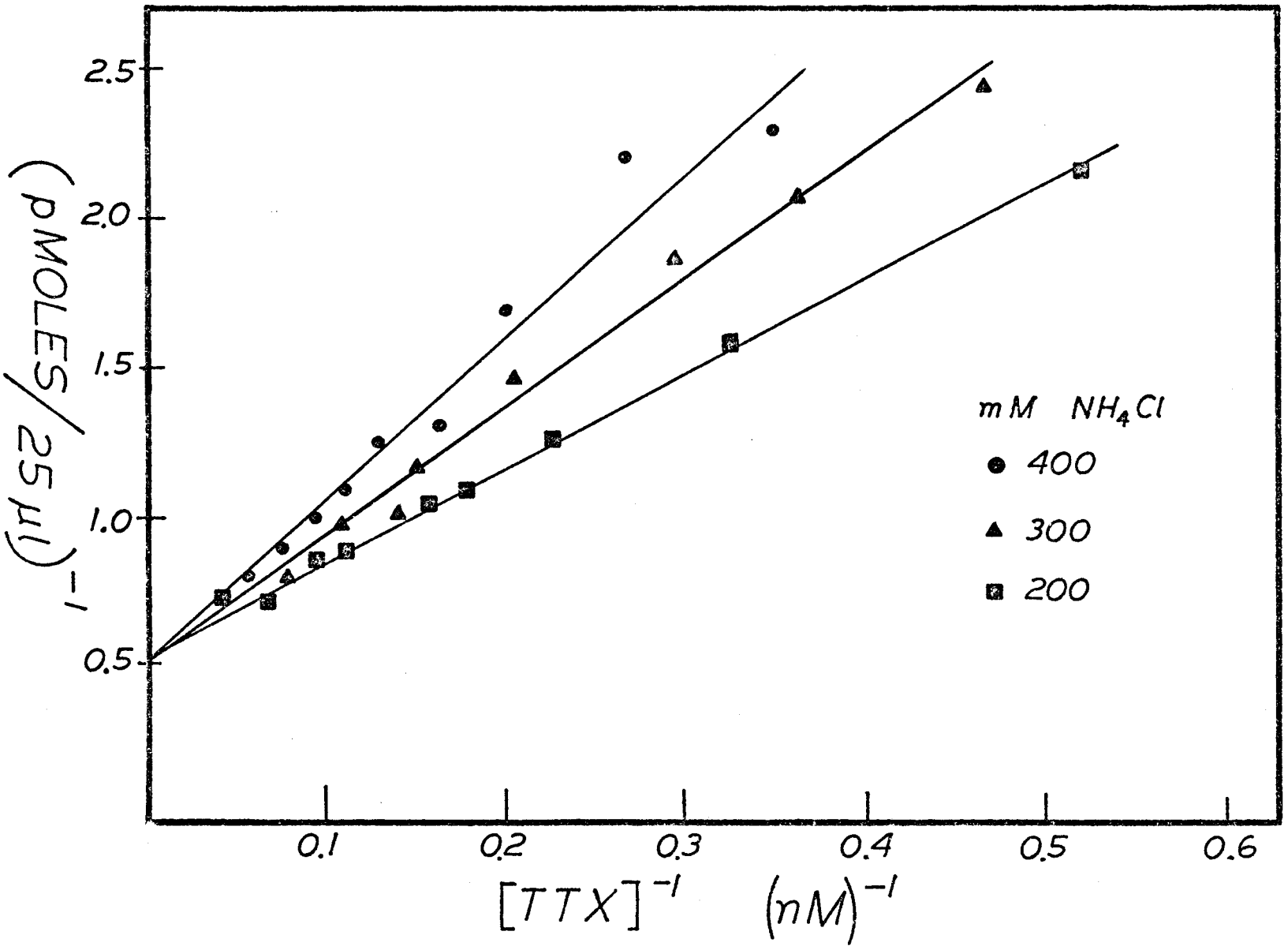
GRAPH 8. 10-43% glycerol gradient of solubilized material The 5 ml gradient was centrifuged in a SW 65 rotor at 60,000 RPM, for 12 hrs. 300 µl fractions were collected, and 250 µl aliquots were assayed for TTX binding (DPM) by G-50 assay.


β-galactosidase
catalase
meniscus
500
400
300
200
100
DPM
2
4
6
8
10
12
14
16
18
FRACTION NUMBER

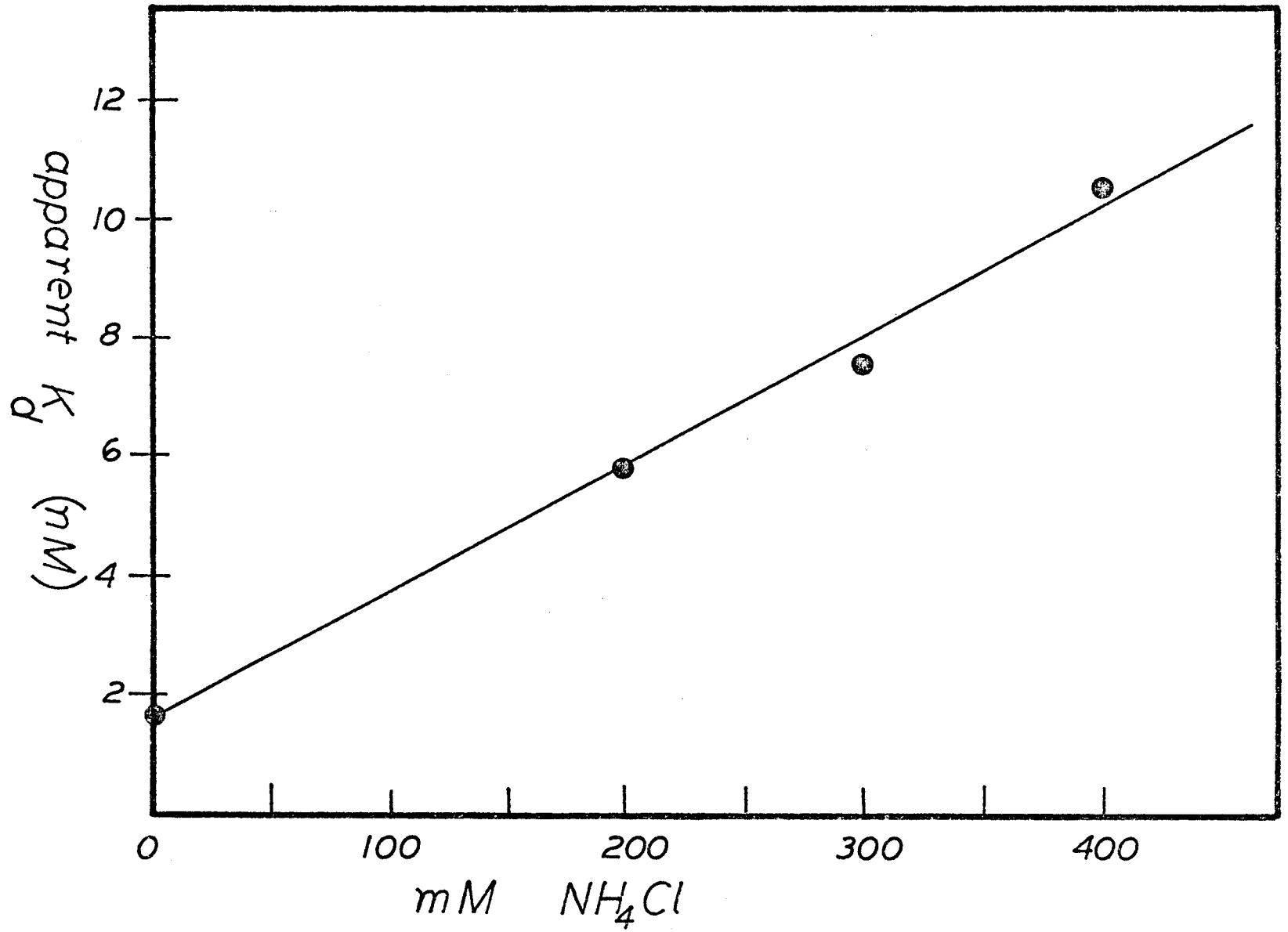
GRAPH 9. Reciprocal plot of the binding of $^{3}$H-TTX to the Lubrol extract using G-50 assay. The $K_D$ of 1.2 nM for binding was obtained from the negative reciprical of the intersection of line with the abscissa.


1.2
1.0
0.8
0.6
0.4
0.2
[pMOLES / 2.5 µl]$^{-1}$
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
[TTX]$^{-1}$ (nM)$^{-1}$

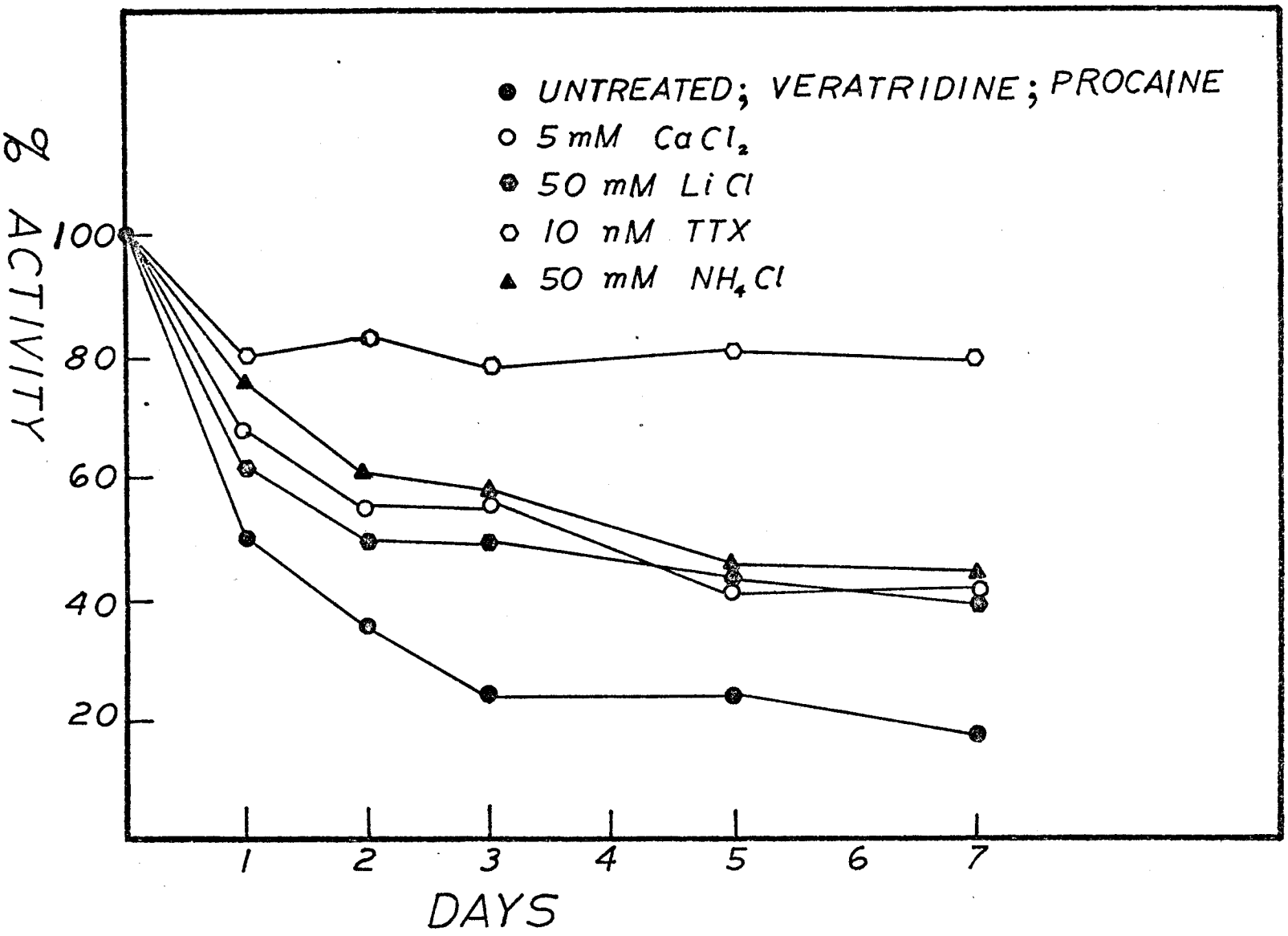
GRAPH 10. Reciprical plot of $^{3}H$-TTX binding to the Lubrol extract in the presence of $NH_4CL$. $K_D$ 's for binding were determined as in graph 9.


2.5
2.0
1.5
1.0
0.5
$(pMOLES/25\ \mu l)^{-1}$
0.1
0.2
0.3
0.4
0.5
0.6
$[TTX]^{-1}$ $(nM)^{-1}$
mM $NH_4Cl$
400
300
200

GRAPH 11. Replot of the apparent $K_D$'s obtained from graph 10 versus the concentration of $NH_4Cl$. The $k_i$, apparent inhibition constant for $NH_4^+$, was estimated as the concentration of $NH_4CL$ which would result in an apparent $K_D$ equal to $2(K_D)$.


apparent $K_d$ (mM)
12
10
8
6
4
2
0
100
200
300
400
mM $NH_4Cl$

GRAPH 12. The loss of TTX binding activity in the Lubrol extract with time. The extract was either left untreated or one of the reagents was added.


UNTREATED; VERATRIDINE; PROCAINE
5 mM CaCl2
50 mM LiCl
10 nM TTX
50 mM NH4Cl
% ACTIVITY
100
80
60
40
20
1
2
3
4
5
6
7
DAYS