

Density and Distribution of Tetrodotoxin Receptors in Normal and Detubulated Frog Sartorius Muscle

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ABSTRACT Tetrodotoxin (TTX) binding was measured in muscles which were either in normal condition or which had been "detubulated" by glycerol-induced osmotic shock. In both cases the binding of TTX was found to saturate at high TTX concentrations. Maximum binding in normal fibers was 35 pmol/g wet weight, and that figure was reduced to 16 pmol/g after glycerol treatment. The dissociation constant for binding to the surface membrane was 3 nM, which is in the range of values obtained by electrophysiological measurements of the effect of TTX on the maximum rate of rise of the action potential. The results suggest that the dissociation constant in the transverse tubules may be higher than that in the surface. Control experiments indicate that the effects of glycerol treatment are limited to the accessibility of the receptors to the toxin and result in no alteration of the affinity of the binding site. TTX receptors in the transverse tubules may be recovered after glycerol treatment by homogenization of the fibers. The measurements suggest that the density of sodium channels in the surface membrane is about $175/\mu\text{m}^2$ and that in the transverse tubular membrane is $41\text{--}52/\mu\text{m}^2$.

INTRODUCTION

The surface membrane of frog skeletal muscle is capable of conducting action potentials through the activation of tetrodotoxin-sensitive sodium channels. It has been suggested that the inward spread of excitation along the transverse tubules (T system) may be mediated by a similar system (Costantin, 1970; Costantin and Taylor, 1971; González-Serratos, 1971; Bezanilla et al., 1972; Caputo and Dipolo, 1973; Bastian and Nakajima, 1974). Tetrodotoxin (TTX) is a highly selective and potent inhibitor of sodium channels in excitable membranes (Narahashi, 1972). The binding of this toxin to cell surfaces has been utilized as a measure of the density of sodium channels in a number of preparations (Moore et al., 1967; Keynes et al., 1971; Colquhoun et al., 1972, 1974; Almers and Levinson, 1975). Frog sartorius muscle is potentially valuable in these studies because it is composed of a relatively homogeneous population of fibers, is susceptible to electrophysiological measurements via intracellular recording, and allows a disconnection of the T system from the cell surface. This latter decoupling may be accomplished in *Rana pipiens* by the use of glycerol-induced

osmotic shock (Howell and Jenden, 1967; Eisenberg and Gage, 1967). After this treatment, fibers retain the ability to conduct action potentials in the surface membrane, but fail to contract. Morphological studies have shown that the T system is no longer accessible to extracellular solutes (Eisenberg and Eisenberg, 1968; Franzini-Armstrong et al., 1973). Provided that glycerol treatment does not alter the toxin-receptor interaction, this procedure may be useful in determining the distribution of sodium channels between surface and transverse tubular membranes. We have studied the binding of TTX to normal and "detubulated" muscles using a bioassay technique. Our results suggest that about one-half of the sodium channels in frog sartorius muscle are located in the transverse tubular system. A preliminary report of this work has been presented (Jaimovich et al., 1975).

METHODS

Freshly isolated sartorius muscles from healthy frogs, *R. pipiens*, were used throughout this investigation. Their mean wet weight was 75 ± 18 mg (1 SD).

Solutions and Reagents

The composition of the standard Ringer's fluid was the following (in mM): NaCl, 115; KCl, 2.5; CaCl₂, 1.8; Na₂HPO₄, 2.15; NaH₂PO₄, 0.85, pH, 7.2. Low [Na⁺]_o solutions were made by partial replacement of Na⁺ by choline⁺ in the above solution on a 1:1 basis. In the experiments where low [Na⁺]_o solutions were used D-tubocurarine (10⁻⁵ g/ml) was also added to all solutions. The hypertonic solution (glycerol-Ringer's fluid) used for "detubulation" was prepared by adding 400 mM glycerol to standard Ringer's fluid. The solution with high [Ca⁺⁺]_o and [Mg⁺⁺]_o contained (in mM): NaCl, 115; KCl, 2.5; CaCl₂, 5.0; MgCl₂, 5.0; Na₂HPO₄, 1.1; NaH₂PO₄, 0.4, pH, 7.2. TTX was added to solutions daily from a refrigerated stock solution of 2×10^{-5} M.

TTX was obtained from Sankyo Co., Tokyo, Japan. D-tubocurarine chloride was purchased from Squibb and Sons, Inc., New York. [¹⁴C]sucrose and [¹⁴C]inulin were obtained from New England Nuclear, Boston, Mass. All other compounds were reagent grade.

Glycerol Treatment

In some muscles the transverse tubular system was disrupted by bathing in glycerol-Ringer's fluid for 75 min followed by a 75-min exposure to high [Ca⁺⁺]_o and [Mg⁺⁺]_o solution (Howell and Jenden, 1967; Eisenberg et al., 1971). Usually at the end of this period no twitch can be recorded, while 90% of the transverse tubules are disconnected from the surface membrane and are inaccessible to extracellular markers (Franzini-Armstrong et al., 1973). After the exposure to high [Ca⁺⁺]_o and [Mg⁺⁺]_o the muscles were washed for about 1 h in normal Ringer's fluid before measuring TTX binding. This protocol seems to slow the depolarization usually seen in glycerol-treated muscles (Eisenberg et al., 1971; Venosa and Horowicz, 1973).

Electrical and Mechanical Measurements

Resting and action potentials were recorded using the method recently described by Stefani and Schmidt (1972) for pyriformis and iliofibularis muscles. This method allows recording in isotonic media without motion artifacts. In the present experiments a sartorius muscle was stretched to 1.2–1.3 times its body length, rolled around a Plexiglas rod and placed in a suitable chamber. In this condition, movement associated with a

twitch was considerably reduced so that single and, in many instances, several action potentials from a single fiber in normal Ringer's fluid could be easily obtained with no apparent damage to either electrode or cell. Conventional glass microelectrodes filled with 3 M KCl (6–20-M Ω resistance) were used. They were connected to the input of a cathode follower with negative capacitance compensation. The output of the cathode follower was connected to one channel of a storage oscilloscope. This signal was also electronically differentiated so that both the action potential and its time derivative (\dot{V}) were displayed on the oscilloscope and photographed. The magnitude of the peak twitch tension from muscles in the presence of different solutions was recorded using a different arrangement which has been described in a previous paper (Venosa, 1974).

Bioassay of Tetrodotoxin

The bioassay for TTX (Moore et al., 1967; Keynes et al., 1971) was performed using a method similar to that described by Shrager and Profera (1973), but utilizing a frog sciatic nerve. The nerve was desheathed over a 10–15-mm segment and was placed in a Plexiglas chamber designed to isolate a 2-mm portion of the desheathed region between two air gaps. The nerve was sealed in place with Vaseline and stimulation and recording of compound action potentials were by means of external platinum electrodes. One recording electrode was placed at the center of the 2-mm test region. A dose-response curve (Fig. 1) was measured by applying successive 20- μ l aliquots of Ringer's fluid containing known TTX concentrations to the test segment, washing with Ringer's fluid between applications. Equilibrium was usually reached within 15 min and reversibility on washing was consistently close to 100%. 20- μ l aliquots of Ringer's fluid containing unknown concentrations of TTX were applied in similar manner and the TTX concentration

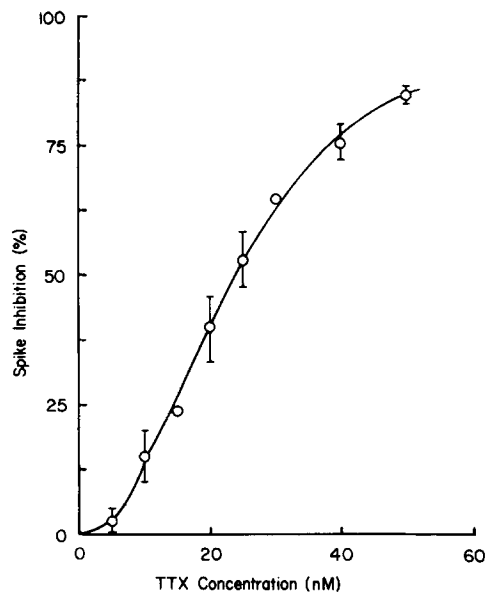


FIGURE 1. Dose-response curve for TTX bioassay. Percent inhibition of the amplitude of the compound action potential of desheathed frog sciatic nerve is shown as a function of TTX concentration. Each point represents the mean of 4–17 measurements and the vertical bars show the SD.

determined from the resulting equilibrium height of the compound action potential and the dose response curve (measured for each assay nerve). The bioassay procedure was carried out at room temperature.

TTX Binding Procedure

From two to four isolated muscles were drained by blotting the tendons with filter paper, and their wet weight determined. They were then incubated in 0.2–1.0 ml of Ringer's fluid containing 10–200 nM TTX and with [¹⁴C]sucrose (1 mM carrier, 5×10^5 cpm/ml) or [¹⁴C]inulin (carrier-free, 5×10^5 cpm/ml) added as a marker for the extracellular space. The system was kept at room temperature (21–23°C) with stirring, and after 30–180 min, samples were taken for the bioassay and radioactive counting. Muscles to be glycerol treated were weighed before placing them in the glycerol-Ringer's fluid. In some experiments, noted below, muscles were incubated in 100 ml of Ringer's fluid containing TTX, for periods of up to 6 h and then removed, drained as above, and incubated in 0.2–0.3 ml of Ringer's fluid containing [¹⁴C]sucrose for 40–60 min. Samples were taken from this solution for bioassay and ¹⁴C counting. The extracellular fluid volume was determined from the dilution of radioactivity after incubation. In some experiments, groups of four muscles were either cut with scissors or were homogenized in several 10-s periods with a cell disrupter (Tekmar Co., Cincinnati, Ohio, model SDT 182) in 5-ml Ringer's fluid. In order to measure the TTX binding, the homogenate was centrifuged for 30 min at 17,000 g and the pellet resuspended by homogenization in 1 ml of Ringer's fluid containing TTX and [¹⁴C]sucrose. After incubation for at least 30 min at room temperature, the suspension was centrifuged again and the supernatant assayed for TTX and ¹⁴C. Binding was referred to the original intact wet weight of the muscles.

As a control, e.g., for K⁺ leakage, normal and glycerol-treated muscles were incubated in 0.2–1.0 ml of Ringer's fluid in the absence of TTX for the same period as in the TTX binding experiments. Aliquots from these solutions similar in quantity to those used in binding studies had no effect on the compound action potentials of sciatic nerves prepared for assay.

RESULTS

Internal Potential Measurements

Before embarking upon a study of the binding of tetrodotoxin to sartorius muscles, electrical responses in the presence of different concentrations of the drug were measured. The results from these studies provided indications as to the concentration range to be studied for binding and also provided a physiological response curve to be compared to the binding data. Fig. 2 *a* illustrates an action potential and its time derivative in the presence of normal Ringer's fluid. Fig. 2 *b* shows similar records obtained from the same muscle about 20 min after addition of 12 nM TTX to the external fluid. A steady state was generally reached within 15 min. The overshoot and the maximum rate of rise of the action potential, \dot{V}_{\max} , are reduced and time to \dot{V}_{\max} is increased after exposure to TTX. Fig. 2 *c* and *d* show the same sequence in glycerol-treated fibers. In agreement with the results of Eisenberg and Gage (1967) the negative afterpotential seen in normal muscle is absent after detubulation. The action of TTX on the spike in glycerol-treated fibers, however, as seen in Fig. 2 *d*, is similar to that in normal muscle.

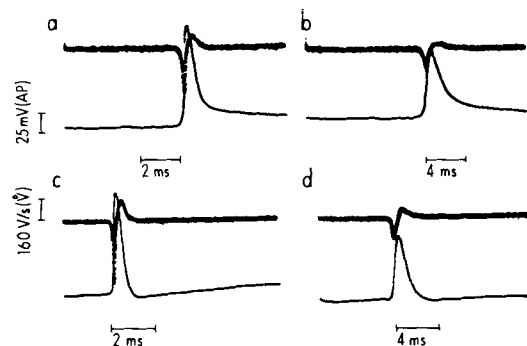


FIGURE 2. Records of intracellular action potentials (AP) and their time derivatives (\dot{V}) from different muscle fibers under the following conditions: (a) normal muscle in the presence of normal Ringer's fluid, (b) same muscle as in a 20 min after addition of 12 nM TTX to the bathing medium, (c) glycerol-treated muscle in normal Ringer's fluid, and (d) same muscle as in c 20 min after adding 12 nM TTX externally. The base line for \dot{V} corresponds to $V = 0$ for the action potential.

Effect of TTX Concentration on the Maximum Rate of Rise of Action Potentials and Twitch Tension

In general, the average resting potential of glycerol-treated fibers was about 10 mV more positive than that of normal fibers (see Venosa and Horowicz, 1973). In order to compare electrical parameters before and after treatment, in a given muscle, only fibers with internal potentials more negative than -85 mV were analyzed. In all experiments, glycerol-treated muscles failed to twitch upon supramaximal stimulation. Furthermore, it has been shown that after treatment using the procedures described, 90% of the transverse tubules are disrupted (Franzini-Armstrong et al., 1973).

The effects of different TTX concentrations on \dot{V}_{\max} for both normal and glycerol-treated fibers are summarized in Fig. 3. The points represent means \pm 1 SD recorded from between 4 and 62 fibers for each TTX concentration. The overlap of the data suggests that there is no significant change in the effects of TTX on \dot{V}_{\max} after detubulation. The TTX concentration causing a 50% reduction of \dot{V}_{\max} is in the range of 5–7 nM in both cases. At concentrations of 20 nM and above, no action potentials could be elicited by stimulation.

The magnitude of the peak twitch tension in whole muscles decreased with increasing TTX concentrations in a manner similar to that observed for \dot{V}_{\max} . The twitch is reduced to one-half its normal value by 8 nM TTX or by reducing $[\text{Na}^+]_o$ to 40% of its original value. The twitch fails either in TTX concentrations above 20 nM or in external media with less than 25 mM $[\text{Na}^+]_o$.

Tetrodotoxin Binding

Since measurements of binding were to be made at equilibrium, we first investigated the dependence of TTX binding on incubation time, for our experimental conditions of temperature and stirring. The results are shown in Fig. 4, which

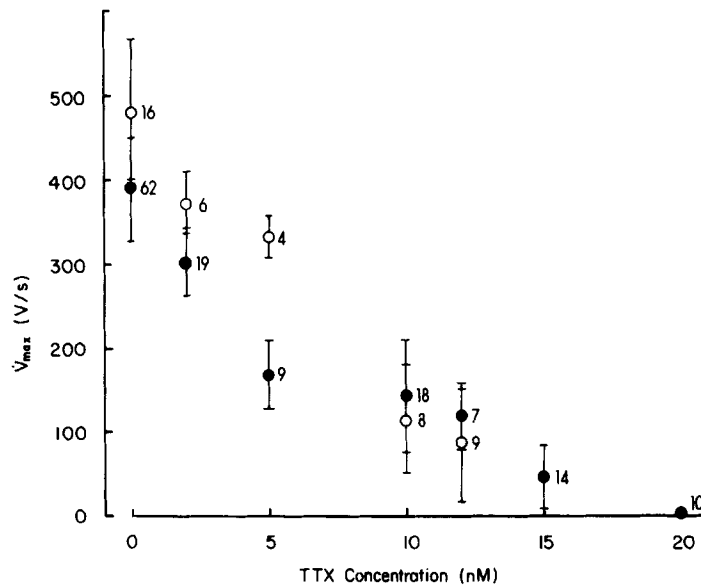


FIGURE 3. Maximum rate of rise of the action potential in normal (●) and glycerol-treated (○) muscles as a function of TTX concentration. The vertical bars represent 1 SD and the numbers next to the points indicate the number of fibers studied.

includes data at several different TTX concentrations. In all experiments maximal binding was reached in less than 30 min and this time was therefore taken as the minimum incubation time.

Tetrodotoxin binding was measured in both normal and glycerol-treated muscles. In each experiment two to four isolated muscles were incubated in a known initial concentration of TTX for from 30 to 60 min. The equilibrium concentration of TTX was then measured via the bioassay technique. Toxin binding (b) was calculated according to Eq. 1.

$$b = \frac{[\text{TTX}]_i V - [\text{TTX}]_f V'}{w}, \quad (1)$$

where $[\text{TTX}]_i$ is the known initial concentration of TTX, $[\text{TTX}]_f$ is the final concentration of TTX, V is the initial volume of the incubation medium containing TTX before exposure to the muscle, V' is obtained by addition of the extracellular fluid of the muscles measured with ^{14}C inulin or ^{14}C sucrose (see Methods) to V , and w is the wet weight of the muscles. Fig. 5 illustrates binding as a function of the equilibrium TTX concentration. In both normal and detubulated muscles the binding saturates as the toxin concentration is increased. There seems to be no significant linear, nonsaturable component as has been found in other preparations using different assay techniques (Colquhoun et al., 1972, 1974). Maximum TTX binding in normal muscle is on the order of 35 pmol/g wet weight, and this is reduced by about one-half after glycerol treatment. This difference might represent binding to TTX receptors in the transverse tubules.

In order to test whether the reduction in TTX binding after glycerol treatment was due to an effect other than detubulation, several control experiments were carried out. As mentioned above, glycerol-treated fibers had lower resting potentials than normal fibers. We tested for the possibility that TTX binding may be affected by depolarization or by damage to the surface membrane. Paired muscles were dissected from four frogs. Transverse cuts were made in one member of each pair with scissors so that all fibers were sectioned in at least 10 places. All muscles were soaked in normal Ringer's fluid for 1 h and were then tested for binding. The results are given in Table I, row *b*, and show no significant difference between untreated and cut muscles.

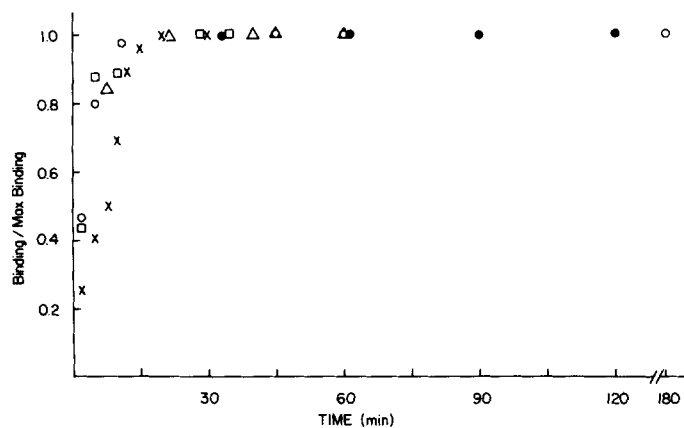


FIGURE 4. Kinetics of TTX binding. Each symbol represents a different experiment done with four muscles in which aliquots were taken at different times from the incubation medium and TTX concentration was measured. The maximum binding for each experiment was used for normalization. The final TTX equilibrium concentrations were, for normal muscle, (\circ , \bullet) 7 nM, (Δ) 25 nM, (\times) 30 nM; and for glycerol-treated muscle (\square) 30 nM.

To further investigate this matter, binding experiments were carried out with homogenates (see Methods) from normal and glycerol-treated muscles. The results of those experiments are shown in Table 1, rows *c*, *d*₁, and *d*₂ and in Fig. 6. As is seen, this procedure does not measurably alter the maximum binding observed in normal muscles. On the other hand, glycerol-treated muscles exhibited a dramatic increase in binding capacity after homogenization with the maximum binding being about equal to that found in normal muscles. This strongly suggests that the only effect of glycerol treatment on binding is the disconnection of the T system from the external medium with no apparent effect on the TTX receptors.

These experiments also suggest that the TTX-receptor interaction is independent of the integrity of the surface and tubular membranes. Furthermore, it seems that intracellular membranes (sarcoplasmic reticulum, mitochondria, etc.) are apparently free of any significant TTX binding.

In another control experiment we tested for a possible effect of glycerol on the

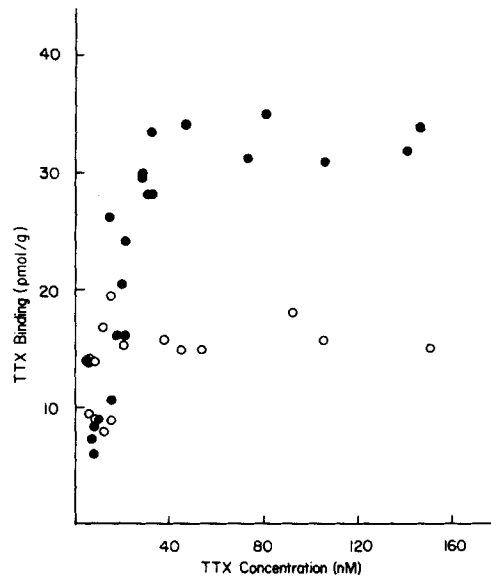


FIGURE 5. TTX binding per unit weight as a function of TTX concentration. Each point was calculated from the binding to three to four muscles incubated in 0.2-1.0 ml of Ringer's fluid containing TTX. ● Normal muscles. ○ Glycerol-treated muscles.

TABLE I
CONTROL EXPERIMENTS FOR GLYCEROL TREATMENT

Preparation	Treatment	Row refer- ence	TTX concen- tration	TTX binding	
				Control	Treated
			nM	pmol/g	pmol/g
Frog muscle	Glycerol incubation without osmotic shock	a_1	41	31	30
		a_2	21	24	27
Frog muscle	Cutting of all fibers	b	30	30	32
Frog muscle	Homogenization	c		35*	37*
Glycerol-treated frog muscle	Homogenization	d_1	57‡	16*	38
		d_2	65‡	16*	39
Crayfish claw nerves	Glycerol osmotic shock	e_1	80	295	320
		e_2	7	63	63

* These values correspond to the maximum binding, obtained by linear regression analysis from data at several TTX concentrations (see Fig. 6).

‡ Concentrations refer to treated muscles only and are in the range where binding to glycerol-treated muscles is maximal.

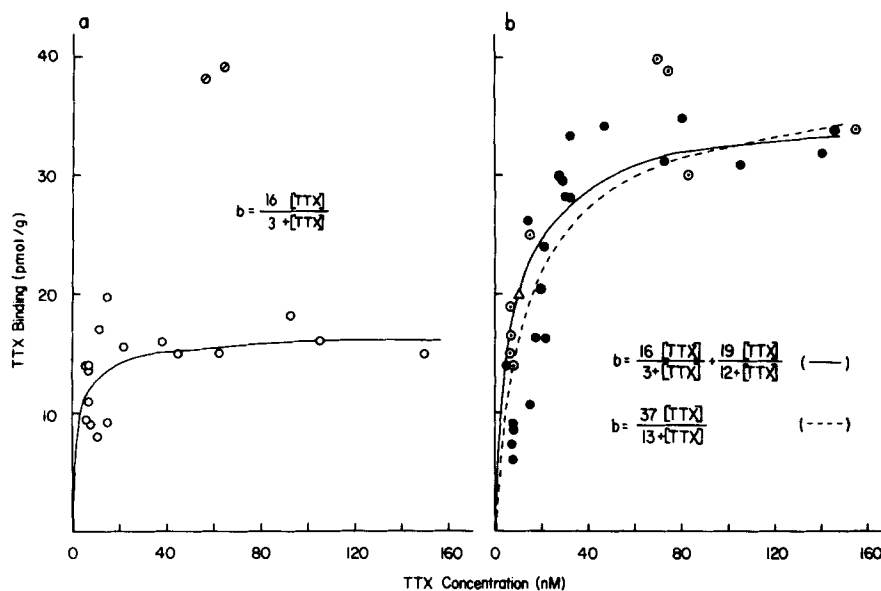
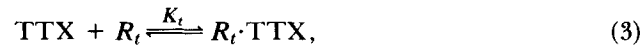
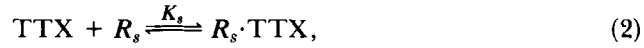


FIGURE 6. Fit of the binding data to equations shown: (a) glycerol-treated muscles: (○) intact, (⊙) homogenate; (b) normal muscles: (●) intact, (⊙) homogenate, (△) value estimated from the long-term experiment (Table II). The curves are drawn according to the equations. Parameters in *a* were calculated by linearizing the general form of the binding equation, using the Hanes-Woolf procedure (Hanes, 1932; Endrenyi and Kwong, 1973). Parameters in the equations in *b* were derived by the procedure described in the text.

number of TTX binding sites, independent of detubulation. Several muscles were placed in Ringer's fluid containing 400 mM glycerol in the usual way. The return to normal Ringer's fluid was made in four steps, decreasing the concentration of glycerol by 100 mM in each step. The peak twitch tension was monitored during this procedure and was not detectably different from that of untreated muscles. Tetrodotoxin binding to these muscles at two concentrations is given in Table I, rows a_1 and a_2 , along with data for paired, untreated muscles. Again, no significant difference was detected. Finally, we tested for the possible reduction of binding sites in surface membranes in a preparation containing no transverse tubules after glycerol-induced osmotic shock. Claw nerves from 20 crayfish (*Procambarus clarkii*) were dissected free by pulling out. Half the nerves were placed in normal Van Harreveld's (1936) saline containing 666 mM glycerol, for 70 min. They were then transferred back to normal Van Harreveld's saline and allowed to stand for 90 min. This treatment represents an osmotic shock roughly equivalent to that applied to frog muscle in these studies. After this treatment crayfish nerves remain excitable, although the externally recorded compound action potentials were somewhat reduced. TTX binding in these nerves was identical to that in untreated, paired controls (Table I, rows e_1 and e_2).

We have attempted to fit the experimental data to the simplest binding model by assuming that the reaction of TTX with its receptors is of the first order in both surface and tubular membranes, i.e.,



where R_s represents the receptors in surface membranes, R_t represents the receptors in tubular membranes, and K_s and K_t denote the equilibrium dissociation constants of the surface and tubular membrane binding sites, respectively.

Then, the total binding is given by the equation:

$$b = \frac{T_s[\text{TTX}]}{K_s + [\text{TTX}]} + \frac{T_t[\text{TTX}]}{K_t + [\text{TTX}]}, \quad (4)$$

where $[\text{TTX}]$ is the equilibrium concentration of TTX and T_s and T_t are the maximal binding capacities of the surface and tubules, respectively.

If we assume that in glycerol-treated muscles the tubular membranes are virtually inaccessible, the experimental data can be described by the equation:

$$b_s = \frac{T_s[\text{TTX}]}{K_s + [\text{TTX}]}, \quad (5)$$

where b_s is the binding in the surface membrane. Fig. 6 *a* shows the curve fitted by linear regression analysis of the data to a rearranged form of Eq. 5, i.e.,

$$\frac{[\text{TTX}]}{b_s} = \frac{K_s}{T_s} + \frac{1}{T_s} [\text{TTX}]. \quad (5a)$$

This procedure yields a K_s of 3 ± 2 nM (mean ± 1 SD) and a surface membrane binding capacity, T_s , of 16 ± 1 pmol/g wet weight of muscle.

From the difference between the binding in normal muscles for TTX concentrations greater than 8 nM (see below) and the values of b_s given by Eq. 5 a T_t of 19 ± 1 pmol/g wet weight and K_t of 12 ± 5 nM were estimated. In this case the linear regression was less satisfactory ($r = 0.97$) than that found for fitting Eq. 5 *a* to all the data for glycerol-treated muscles ($r = 0.99$). The solid line in Fig. 6 *b* illustrates the curve obtained by using Eq. 4 and the derived parameters as described above from the glycerol and normal muscle binding data.

We have also attempted to fit the experimental data for normal muscles to a single hyperbolic function of the form:

$$b_n = \frac{T_n[\text{TTX}]}{K_n + [\text{TTX}]}, \quad (6)$$

where the n subscript refers to normal muscle, and all binding sites are taken to be equivalent. Linear regression analysis of all points at toxin concentrations greater than 8 nM yields values of T_n and K_n of 37 ± 2 pmol/g wet weight and 13 ± 4 nM, respectively. The dashed curve in Fig. 6 *b* is drawn according to Eq. 6, using these parameters. In this case the fit ($r = 0.97$) was not significantly different from the fit obtained using Eq. 4. However, the equilibrium constant

calculated for the single-hyperbola model in normal muscle (13 ± 4 nM) is significantly different ($P < 0.01$) from that obtained in glycerol-treated fibers (3 ± 2 nM). This result would imply that there is a change in the binding characteristics of the TTX receptor in the surface after glycerol treatment. However, the electrophysiological data (Fig. 3) suggest no significant alteration of surface receptor sites after detubulation.

In both the double- and single-hyperbola models the derived curves provide fits to the data that are better at high TTX concentrations than at low concentrations. The data from homogenates at these concentrations, however, fall more closely to the curve. One possible explanation for the finding that at low TTX concentrations the binding in intact muscles is lower than the predicted value, is that the reaction may not have achieved equilibrium at the time of the measurements.

In order to evaluate this possibility an additional experiment was performed, designed to test for a very slowly equilibrating component of TTX binding in normal muscle at low toxin concentrations. This experiment is illustrated by the data in Table II. For the long incubation time required (6 h), exposure to TTX was carried out in a large volume and binding measured after release of the toxin from the muscle in a small volume of Ringer's fluid (row I). This procedure was followed in order to avoid possible contamination of the incubation medium with K^+ , etc., and possible deterioration of the muscle due to anoxia. As a control for the release procedure an experiment was carried out with an initial incubation for a short time, at a high TTX concentration, followed by a release phase similar to that used above (row II). Four normal muscles were incubated at room temperature for 6 h in a large volume (100 ml) of Ringer's fluid containing 10 nM TTX (uptake phase). The muscles were then drained and transferred to a small volume (0.2 ml) of TTX-free Ringer's fluid containing [^{14}C]sucrose, and incubated for 60 min with stirring (release phase). At the end of the release phase a new equilibrium was established between bound and unbound TTX. Aliquots of 20- μ l bathing fluid were then assayed. Controls for this long-term, large volume uptake and subsequent release procedure consisted of incubation

TABLE II
LONG-TERM BINDING EXPERIMENT ON NORMAL MUSCLE

Row reference	Incubation time	Equilibrium TTX concentration, uptake phase	TTX bound	Equilibrium TTX concentration, release phase	TTX released
	<i>min</i>	<i>nM</i>	<i>pmol/g</i>	<i>nM</i>	<i>pmol/g</i>
I*	360	10	—	7	10.5
II‡	30	40	30	7.5	21

* Muscles were incubated in 100 ml of Ringer's fluid containing 10 nM TTX for 6 h, then transferred to 0.2 ml of TTX-free Ringer's fluid for 60 min. Aliquots of the final incubation medium were assayed.

‡ Muscles were incubated in 0.3 ml of Ringer's fluid containing 50 nM TTX for 30 min and aliquots were removed and assayed. The muscles were then transferred to 0.3 ml of TTX-free Ringer's fluid for 60 min and aliquots again removed for assay.

of normal muscles at high TTX concentrations for shorter times, in small volumes. Binding at this stage was measured using an aliquot of the incubation medium. The muscles were then transferred to a small volume of TTX-free Ringer's fluid containing [¹⁴C]sucrose, and at the end of the release phase, aliquots of the bathing medium were assayed for TTX. These control experiments were designed to result in a final TTX equilibrium concentration after release (ca. 7 nM) similar to that obtained in the long-term experiment with the lower concentration of TTX. The binding expected after a short-term incubation with an equilibrium concentration of 7 nM TTX is 9 ± 3 pmol/g (Fig. 5). This value of binding agrees well with that found by taking the difference between the amount of TTX bound after short-term incubation (30 pmol/g; Table II, row 2) and that released when the muscles were subsequently reincubated in a TTX-free medium (21 pmol/g). This suggests that binding after 6 h in 10 nM TTX was on the order of 20 pmol/g ($10.5 + 9$ pmol/g; Table II, row 1), which is not far from the value of 20.5 pmol/g predicted by the equation given in Fig. 6*b* for a TTX concentration of 10 nM, from the short-term experiments.

It should be noted, however, that the curve which has been fit to the data from intact muscles for short-term incubations at high TTX concentrations predicts a higher TTX binding than that actually measured at low concentrations of TTX. This implies that the binding measured at low TTX concentrations has not reached equilibrium values. This is also suggested by the fact that the binding obtained with homogenized muscles at low TTX concentrations was higher than that of intact muscles and comes closer to the curve (Fig. 6*b*). On the other hand, the kinetic experiments illustrated in Fig. 4 seem to indicate that equilibrium is apparently reached within 30 min at both high and low concentrations. The probable explanation for this discrepancy is that the scatter in the measurements precludes an accurate measurement of a small, slow component in the binding at low TTX concentrations using intact muscles.

DISCUSSION

Effect of Glycerol Treatment on TTX Binding

The results of these experiments indicate that 55% of the TTX binding sites in frog sartorius muscle is eliminated after disconnection of the T system by glycerol osmotic shock. The glycerol treatment employed here is known to uncouple contraction from excitation by interrupting the continuity between surface and transverse tubular membranes (Howell and Jenden, 1967; Gage and Eisenberg, 1969). It has been shown that in glycerol-treated muscles the T tubules which remain accessible to lanthanum (an extracellular marker) represent 10% of the amount found in normal fibers (Franzini-Armstrong et al., 1973). We have considered the possibility that some of the reduction in binding after glycerol treatment might be due to effects other than detubulation. Muscles exposed to identical glycerol concentrations, but returned to normal Ringer's fluid in a manner designed to avoid strong osmotic shock retained the ability to contract and also retained full TTX binding capacity. Homogenization of detubulated fibers allows the recovery of the fraction of TTX binding lost

after glycerol treatment, suggesting that this latter procedure preserves the integrity of the TTX receptors, while rendering some of them inaccessible to the toxin. This conclusion is further supported by the control experiments on nonmyelinated nerve, which resulted in no loss of TTX binding after an equivalent glycerol-induced osmotic shock. Also, the dissociation constant after detubulation is close to the electrophysiologically derived value, and is of the same order as that found in nerve fibers (Cuervo and Adelman, 1970; Hille, 1968; Schwarz et al., 1973). Glycerol treatment is known to result in partial depolarization of some fibers. However, depolarization itself, produced either by high external potassium concentrations (Almers and Levinson, 1975) or by cutting or by homogenization failed to reduce toxin binding. The results of these control experiments therefore suggest that the difference in TTX binding between normal and glycerol-treated muscle is a measure of the binding capacity of the transverse tubular membrane and that the TTX binding measured in detubulated muscles mainly represents binding to the surface membrane. Further, these data suggest that the physical integrity of the surface and T-system membranes is not essential for toxin binding, and that other cellular components, such as the sarcoplasmic reticulum and mitochondria, lack receptors for TTX.

Specificity and Stoichiometry of TTX Binding

DETUBULATED FIBERS The binding of TTX to glycerol-treated muscle saturates at a TTX concentration within one order of magnitude of that required for 50% reduction of \dot{V}_{\max} . These data can be fitted by a single hyperbolic function with a dissociation constant close to that predicted by electrophysiological measurements. This result agrees well with the data obtained in nerve fibers (Hille, 1968; Cuervo and Adelman, 1970; Schwarz et al., 1973) that suggest a 1:1 interaction between TTX and its receptor. Within the range of toxin concentrations used we find no indication of a linear, nonsaturable component of binding, such as that described in other preparations, using [^3H]TTX (Colquhoun et al., 1972, 1974). For a discussion of this linear component see Almers and Levinson (1975), Levinson (1975), and Colquhoun et al. (1975).

NORMAL FIBERS In normal muscles TTX binding is also found to saturate at concentrations within one order of magnitude of the physiologically active range. The data could be fit about equally well by either a single- or double-hyperbola model. However the former case, in which tubule and surface receptor sites are taken to be identical yields a value for the equilibrium constant in normal muscle significantly different from that in detubulated fibers. Since the electrophysiological data suggest that surface receptors are unaltered by glycerol treatment, we consider the binding model with different apparent equilibrium constants for surface and tubule sites to be more probable. Owing to the scatter in the data at low TTX concentrations and the uncertainty of having achieved equilibrium at these concentrations, it is not possible to reach a definite conclusion concerning the stoichiometry of TTX binding in the T system. The simplest assumption is to consider the stoichiometry to be the same as that for the surface membrane, i.e., 1:1. However, we are then led to the conclusion that the

apparent dissociation constant in the transverse tubules is different from that in the surface. This does not necessarily imply that the receptor for TTX is a different chemical entity in the two cases. The local environment surrounding the receptor may vary due to different densities of fixed surface charge, the presence of adjacent membranes (e.g., terminal cisternae), or variation in surface forces due to differing geometry.

Density and Distribution of TTX Receptors

We measure a maximum TTX binding in normal muscle of 35 pmol/g wet weight (Table III). This value may be compared with that recently reported by Almers and Levinson (1975) of 22 pmol/g. The difference in these results may be accounted for by the different surface-to-volume ratios of the muscle fibers

TABLE III
DENSITY AND AFFINITY OF TTX RECEPTORS IN SURFACE
AND TRANSVERSE TUBULAR MEMBRANES

	TTX binding	[TTX] for half saturation	Density of binding sites
	pmol/g	nM	molecules/ μm^2
Normal muscle	35 \pm 2	—	—
Glycerol-treated muscle	16 \pm 1	3 \pm 2	175 \pm 11*
Normal minus glycerol-treated muscle	19 \pm 1	12 \pm 5	41–52‡

All values are obtained from equations used to fit the data of Fig. 6.

* Surface membrane area = 552 cm²/g of drained muscle (Venosa, 1974).

‡ The range in density values reflects the possible range of tubular membrane area for fibers with an average diameter equal to 55 μm (2,200–2,800 cm²/g of drained muscle) (Peachey, 1965; Hodgkin and Nakajima, 1972).

employed. The average fiber diameter in the sartorii used in these experiments was approximately 55 μm , while that in the work of Almers and Levinson (1975) was 80 μm . If binding is referred to surface area, assuming cylindrical geometry (and neglecting the T system) rather than wet weight, the data for normal muscle are identical in the two cases (ca. 380 sites/ μm^2). Colquhoun et al. (1974) have reported [³H]TTX binding to rat diaphragm muscle with an estimated specific component of 2.5 pmol/g. The average fiber diameter in this preparation was of the order of 40 μm . Aside from the species difference, we have no explanation for the rather large difference in the apparent receptor density in rat diaphragm as compared with that in frog sartorius muscle.

There have been a number of estimates of the density of TTX receptors in nerve membranes. Of particular interest here are those made on relatively homogeneous preparations. In the very small fibers of garfish olfactory nerve (diameter, 0.25 μm) values of 3.9 sites/ μm^2 (Benzer and Raftery, 1972) to 5.6 sites/ μm^2 (Colquhoun et al., 1972, 1974) have been reported. In squid giant axons (diameter 400–600 μm) TTX binding has been measured at 553 sites/ μm^2 (Levinson and Meves, 1975). Our measurements suggest a value of 175 sites/ μm^2 (Table III) for the surface membrane of frog muscle (diameter 55 μm). This estimate,

uncorrected for the small fraction of transverse tubules remaining after glycerol treatment, thus falls between values for smaller and larger nerve fibers. There have also been many estimates of dissociation constants in nerve both from TTX binding and from electrophysiological studies, and almost all fall in the range 3–10 nM (Hille, 1968; Cuervo and Adelman, 1970; Colquhoun and Ritchie, 1972; Schwarz et al., 1973). Our measured value of 3 nM for the surface membrane thus agrees well with those studies.

Values of 41–52 sites/ μm^2 can be calculated for the binding density in tubular membranes from the difference in TTX binding between normal and glycerol-treated fibers, and from the area of tubular membrane for the average fiber diameter (Table III). If one assumes that each TTX binding site is associated with one sodium channel and that the electrical characteristics of the channels are the same for tubular and surface locations, then the density of sodium channels and the limiting sodium conductance in the tubular walls is about one-quarter that in the fiber surface. From the numerical construction of action potentials in frog sartorius muscle Adrian and Peachey (1973) concluded that the ratio of sodium channel density in the tubules to that in the surface is 1:20. Although this ratio is smaller by a factor of 5 than that based on the TTX binding figures in this report, numerical reconstruction of action potentials permits a wide choice of parameters for any given model network. In addition, the model network chosen for numerical analysis influences the choice of a parameter such as the limiting sodium conductance in the transverse tubules. It is likely, therefore, that a ratio of 1:4 for the density of sodium channels in the tubules to that in the surface can be accommodated in theoretical and numerical reconstruction of action potentials.

The time-course of TTX binding in our experiments was significantly faster than that reported by Almers and Levinson (1975). From their Fig. 3 the uptake of the toxin by muscles exposed to 10 nM TTX at 4°C has a half time of about 17 min. Our experiments, on the other hand, carried out at 21–23°C, have a mean half time of approximately 5 min (Fig. 4), which is close to the half time for the exchange of Tris⁺ for Na⁺ (2.2 min) by diffusion in the same preparation, and in the same temperature range (Venosa, 1974). In two control experiments, with the incubation step carried out at 2°C, the rate of binding was considerably slowed, with an estimated half time of 15 min, in reasonable agreement with the results of Almers and Levinson (1975). Since changes in temperature should have only a small effect on the diffusion constant of the drug this suggests that the rate of reaction between the toxin and its binding site may be a significant factor in determining the kinetics of binding at low temperatures.

One final point worth considering is the maximum conductance of a single sodium channel. Almers and Levinson (1975) using all the measured TTX binding sites, without allowance for sites in the T system, and the maximum sodium conductance measurements by Adrian et al. (1970) and Ildefonse and Roy (1972) calculate the limiting sodium conductance to be 0.6–1.4 pmho. These values will be larger if tubular sites are to a degree electrically isolated and therefore do not contribute their full share to the peak sodium conductance of muscle membrane. In the limit where tubular sites are completely isolated our

results suggest that these values could be multiplied by a factor of about 2. This brings the calculated single-channel conductance in muscle closer to the more recent estimates for this parameter in nerve (Keynes and Rojas, 1974; Levinson and Meves, 1975).

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REFERENCES

- ADRIAN, R. H., W. K. CHANDLER, and A. L. HODGKIN. 1970. Voltage clamp experiments in striated muscle fibres. *J. Physiol. (Lond.)*. **208**:607-644.
- ADRIAN, R. H., and L. D. PEACHEY. 1973. Reconstruction of the action potential of frog sartorius muscle. *J. Physiol. (Lond.)*. **235**:103-131.
- ALMERS, W., and S. R. LEVINSON. 1975. Tetrodotoxin binding to normal and depolarized frog muscle and the conductance of a single sodium channel. *J. Physiol. (Lond.)*. **247**:483-509.
- BASTIAN, J., and S. NAKAJIMA. 1974. Action potential in the transverse tubules and its role in the activation of skeletal muscle. *J. Gen. Physiol.* **63**:257-278.
- BENZER, T. I., and M. A. RAFTERY. 1972. Partial characterization of a tetrodotoxin binding component from nerve membrane. *Proc. Natl. Acad. Sci. U.S.A.* **69**:3634-3637.
- BEZANILLA, F., C. CAPUTO, H. GONZALEZ-SERRATOS, and R. A. VENOSA. 1972. Sodium dependence of the inward spread of activation in isolated twitch muscle fibres of the frog. *J. Physiol. (Lond.)*. **223**:507-523.
- CAPUTO, C., and R. DiPOLO. 1973. Ionic diffusion delays in the transverse tubules of frog twitch muscle fibres. *J. Physiol. (Lond.)*. **229**:547-557.
- COLQUHOUN, D., R. HENDERSON, and J. M. RITCHIE. 1972. The binding of labelled tetrodotoxin to non-myelinated nerve fibres. *J. Physiol. (Lond.)*. **227**:95-126.
- COLQUHOUN, D., R. HENDERSON, and J. M. RITCHIE. 1975. The purity of tritiated tetrodotoxin as determined by bioassay. Discussion of S. R. Levinson. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **270**:337-348.
- COLQUHOUN, D., H. P. RANG, and J. M. RITCHIE. 1974. The binding of tetrodotoxin and α -bungarotoxin to normal and denervated mammalian muscle. *J. Physiol. (Lond.)*. **240**:199-226.
- COLQUHOUN, D., and J. M. RITCHIE. 1972. The kinetics of the interaction between tetrodotoxin and mammalian non-myelinated nerve fibers. *Mol. Pharmacol.* **8**:285-292.
- COSTANTIN, L. L. 1970. The role of sodium current in the radial spread of contraction in frog muscle fibers. *J. Gen. Physiol.* **55**:703-715.
- COSTANTIN, L. L., and S. R. TAYLOR. 1971. Active and passive shortening in voltage-clamped frog muscle fibres. *J. Physiol. (Lond.)*. **218**:13-15P.
- CUERVO, L. A., and W. J. ADELMAN, JR. 1970. Equilibrium and kinetic properties of the interaction between tetrodotoxin and the excitable membrane of the squid giant axon. *J. Gen. Physiol.* **55**:309-335.
- EISENBERG, B., and R. S. EISENBERG. 1968. Selective disruption of the sarcotubular system in frog sartorius muscle. *J. Cell. Biol.* **39**:451-467.
- EISENBERG, R. S., and P. W. GAGE. 1967. Frog skeletal muscle fibers: Changes in electrical

- properties after disruption of transverse tubular system. *Science (Wash. D.C.)*. **158**:1700-1701.
- EISENBERG, R. S., J. N. HOWELL, and P. C. VAUGHAN. 1971. The maintenance of resting potentials in glycerol-treated muscle fibres. *J. Physiol. (Lond.)*. **215**:95-102.
- ENDRENYI, L., and F. H. F. KWONG. 1973. Design and analysis of hyperbolic kinetic and binding experiments. In *Analysis and Simulation of Biochemical Systems*. H. C. Hemper and B. Hess, editors. North Holland, Amsterdam. 219-237.
- FRANZINI-ARMSTRONG, C., R. A. VENOSA, and P. HOROWICZ. 1973. Morphology and accessibility of the transverse tubular system in frog sartorius muscle after glycerol treatment. *J. Membr. Biol.* **14**:197-212.
- GAGE, P. W., and R. S. EISENBERG. 1969. Capacitance of the surface and transverse tubular membrane of frog sartorius muscle fibers. *J. Gen. Physiol.* **53**:265-278.
- GONZÁLEZ-SERRATOS, H. 1971. Inward spread of activation in vertebrate muscle fibers. *J. Physiol. (Lond.)*. **212**:777-799.
- HANES, C. S. 1932. The effect of starch concentration upon the velocity of hydrolysis by the amylase of germinated barley. *Biochem. J.* **26**:1406-1421.
- HILLE, B. 1968. Pharmacological modifications of the sodium channels of frog nerve. *J. Gen. Physiol.* **51**:199-219.
- HODGKIN, A. I., and S. NAKAJIMA. 1972. Analysis of membrane capacity in frog muscle. *J. Physiol. (Lond.)*. **221**:121-136.
- HOWELL, J. N., and D. J. JENDEN. 1967. T-tubules of skeletal muscle: Morphological alterations which interrupt excitation contraction coupling. *Fed. Proc.* **26**:553. (Abstr.).
- ILDEFONSE, M., and G. ROY. 1972. Kinetic properties of the sodium current in striated muscle fibres on the basis of the Hodgkin-Huxley theory. *J. Physiol. (Lond.)*. **227**:419-431.
- JAIMOVICH, E., R. A. VENOSA, P. SHRAGER, and P. HOROWICZ. 1975. Tetrodotoxin (TTX) binding in normal and "detubulated" frog sartorius muscle. *Biophys. J.* **15**:255a. (Abstr.).
- KEYNES, R. D., J. M. RITCHIE, and E. ROJAS. 1971. The binding of tetrodotoxin to nerve membranes. *J. Physiol. (Lond.)*. **213**:235-254.
- KEYNES, R. D., and E. ROJAS. 1974. Kinetics and steady-state properties of the charged system controlling sodium conductance in the squid giant axon. *J. Physiol. (Lond.)*. **239**:393-434.
- LEVINSON, S. R. 1975. The purity of tritiated tetrodotoxin as determined by bioassay. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **270**:337-348.
- LEVINSON, S. R., and H. MEVES. 1975. The binding of tritiated tetrodotoxin to squid giant axons. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **270**:349-352.
- MOORE, J. W., T. NARAHASHI, and T. I. SHAW. 1967. An upper limit to the number of sodium channels in nerve membrane? *J. Physiol. (Lond.)*. **188**:99-105.
- NARAHASHI, T. 1972. Mechanism of action of tetrodotoxin and saxitoxin on excitable membranes. *Fed. Proc.* **31**:1124-1132.
- PEACHEY, L. D. 1965. The sarcoplasmic reticulum and transverse tubules of the frog's sartorius. *J. Cell Biol.* **25**:209-232.
- SHRAGER, P., and C. PROFERA. 1973. Inhibition of the receptor for tetrodotoxin in nerve membranes by reagents modifying carboxyl groups. *Biochim. Biophys. Acta.* **318**:141-146.
- SCHWARZ, J. R., W. ULBRICHT, and H. H. WAGNER. 1973. The rate of action of tetrodotoxin on myelinated nerve fibres of *Xenopus laevis* and *Rana esculenta*. *J. Physiol. (Lond.)*. **233**:167-194.
- STEFANI, E., and H. SCHMIDT. 1972. A convenient method for repeated intracellular

recording of action potentials from the same muscle fibre without membrane damage. *Pfluegers Arch. Eur. J. Physiol.* **334**:276-278.

VAN HARREVELD, A. 1936. Physiological saline for crayfish. *Proc. Soc. Exp. Biol. Med.* **34**:428-432.

VENOSA, R. A. 1974. Inward movement of sodium ions in resting and stimulated frog's sartorius muscle. *J. Physiol. (Lond.)* **241**:155-173.

VENOSA, R. A., and P. HOROWICZ. 1973. Effects on sodium efflux of treating frog sartorius muscle with hypertonic glycerol solutions. *J. Membr. Biol.* **14**:33-56.