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Phil. Trans. R. Soc. Lond. B 1975 **270**, 365-375
doi: 10.1098/rstb.1975.0016

Phil Trans. R. Soc. Lond. B. **270**, 365–375 (1975) [365]
Printed in Great Britain

The rate of action of tetrodotoxin on sodium conductance in the squid giant axon

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When tetrodotoxin is applied to or washed away from the squid giant axon, the rates at which the sodium conductance is blocked and unblocked are an order of magnitude smaller than those reported for the isolated node of Ranvier. This slowing is to be expected if in squid the tetrodotoxin binding sites act as a saturable sink in series with the barrier to free diffusion imposed by the presence of the Schwann cell. A comparison has been made between the rates observed experimentally and those calculated for a computer model of the system, in order to estimate the apparent density in the membrane of both specific and non-specific tetrodotoxin binding sites. The figure thus obtained for the number of sodium channels in the squid giant axon, several hundred per square micrometre, agrees well with those derived from other lines of argument.

It was reported in 1970 by Cuervo & Adelman that the reaction between tetrodotoxin and the sodium channels in the squid giant axon, which may be represented as

$$\mathrm{TTX} + \mathrm{Ch} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{TTX.Ch_{blocked}}, \tag{1}$$

was a rather slow one, the value of the rate constant for association, k_1, being 0.2×10^8 M^{-1} min^{-1}, while that for dissociation, k_2, was 0.1 min^{-1}. At the isolated node of Ranvier, on the other hand, the reaction is apparently about ten times faster, since Schwarz, Ulbricht & Wagner (1973) found that k_1 was 1.8×10^8 M^{-1} min^{-1} and k_2 was 0.85 min^{-1}.

In discussions on the implications of Cuervo & Adelman's results, it was pointed out to us by Sir Alan Hodgkin that if in the case of the squid axon the tetrodotoxin binding sites acted as a saturable sink in series with the barrier to free diffusion between the surface of the membrane and the bathing solution imposed by the Schwann cell, a considerable slowing might be expected of the blocking action of tetrodotoxin on the sodium conductance. His argument ran as follows. The observations of Frankenhaeuser & Hodgkin (1956) on the accumulation of potassium immediately outside the membrane during repetitive stimulation of a squid axon were well fitted by a model in which there is a space of thickness s between the membrane and the Schwann cell, and the Schwann cell is regarded as a layer of negligible thickness with a permeability coefficient P_K. The time constant τ_{FH} for the change in external potassium concentration during a train of impulses is then given by

$$\tau_{FH} = s/P_K. \tag{2}$$

As we have confirmed, τ_{FH} is generally of the order of 50 ms at about 12 °C, so that if s is taken as 3×10^{-6} cm, P_K will be 6×10^{-5} cm/s. Now, writing the quantity of tetrodotoxin binding sites in moles per unit area as N, the equilibrium dissociation constant for the binding

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as $K(= k_2/k_1)$, and the fraction of channels that remain unblocked by tetrodotoxin as z, it can be shown that

$$z_\infty(z_0 - z) + \left(z_\infty^2 + \frac{Ks}{N}\right) \ln \frac{z_\infty - z_0}{z_\infty - z} + \frac{Ks}{N} \ln \frac{z}{z_0} = \frac{P_{\mathrm{TTX}} K}{N} t, \tag{3}$$

where z_0 and z_∞ are respectively the initial and final values of z. Since Ks/N is very much smaller than 1, it follows that towards the end of a washing out experiment in a tetrodotoxin-free solution where $z_\infty = 1$, z recovers exponentially with a time constant given by

$$\tau_z = \frac{N}{P_{\mathrm{TTX}} K}. \tag{4}$$

So that from (2) and (4)

$$\tau_z = \frac{P_{\mathrm{K}}}{P_{\mathrm{TTX}}} \frac{N}{Ks} \tau_{\mathrm{FH}}. \tag{5}$$

If there are 200 binding sites in each square micrometre of membrane, the effective concentration of sites in the Schwann cell space ($= N/s$) is just over 10 μM. The value of K is around 3 nM and $P_{\mathrm{K}}/P_{\mathrm{TTX}}$ can be taken as 3. Equation (5) therefore predicts that even if the binding reaction proceeds quite rapidly, τ_z will be about 500 s, which is close to what Cuervo & Adelman (1970) actually observed.

We were at that time (November 1971) anxious to be able to count the sodium channels in the squid giant axon, but the bioassay method of Keynes, Ritchie & Rojas (1971) for determining tetrodotoxin binding was not readily applicable in this instance, and a supply of suitably labelled tetrodotoxin was not yet available. We therefore set out to measure the apparent rate of action of tetrodotoxin and saxitoxin on the sodium conductance of the squid axon as a means, albeit indirect, of estimating the number of binding sites. A preliminary account of our findings has been given to the Society of General Physiologists (Keynes, Rojas & Taylor 1973).

Well cleaned axons were mounted intact in a perspex chamber of the type described by Rojas, Taylor, Atwater & Bezanilla (1969) for voltage-clamp measurements. The voltage-clamp system of Bezanilla, Rojas & Taylor (1970) was used to control the potential of the central 2.5 mm of the axon, with positive feedback to compensate as completely as possible for the Schwann cell series resistance. During the experiments, artificial seawater containing various amounts of tetrodotoxin flowed into the chamber at one end and was removed by suction at the other, the rate of flow being such that the solution in the chamber was changed 1.5 times per second. The temperature of the chamber and of the inflowing solution was thermostatically controlled. The measurements of sodium conductance while the tetrodotoxin concentration was changing were made from photographic records of the peak inward current for single voltage-clamp pulses 1–2 ms in duration, which took the membrane potential to +10 mV from a holding level of −70 mV maintained for the preceeding 600 ms. Accurate timing of the records was ensured by operating the oscilloscope camera with a relay driven from a Devices Digitimer which provided pulses at exact intervals between 1 and 60 s. Before and after each kinetic run, the steady-state sodium conductance was measured by recording a complete I–V curve. The artificial sea water contained 460 mM NaCl, 10 mM KCl, 10 mM $CaCl_2$, 50 mM $MgCl_2$, and 5 mM tris Cl at pH 7.5; the KCl was sometimes omitted to keep the resting potential as high as possible.

The fraction z of the sodium channels not occupied by tetrodotoxin was obtained by dividing the peak inward current in any given record by its control value for the same voltage-clamp

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pulse in the absence of tetrodotoxin. The size of the pulses was chosen to bring the membrane potential to the linear part of the I–V curve, at a point where the current was still reasonably large. The instantaneous values of z measured in this way usually agreed well at the beginning and end of each run with those derived from the full I–V curves, although they were unavoidably susceptible to error from leakage currents when the sodium current was close to being completely blocked. Figure 1 shows how z varied with time for an axon to which tetrodotoxin was applied and then washed away, first at a concentration of 3 nM and then at 300 nM. It will be noted that in each case the time course was approximately exponential, as could be verified by plotting the quantity $(z_\infty - z)/(z_\infty - z_0)$ logarithmically against time. As may be seen in figure 2, after a small initial time lag the points fitted well on straight lines over the rest of the range covered. This was a rather consistent finding in virtually all the experiments, which meant that the result of any given run could be expressed with reasonable accuracy as a single time constant τ_z. For the removal of tetrodotoxin, τ_z was almost the same for an initial concentration of 3 nM, 30.2 min, as it was for 300 nM, 29.7 min. For the application of 3 nM tetrodotoxin, τ_z was 16.7 min, while for 300 nM it fell to 0.57 min.

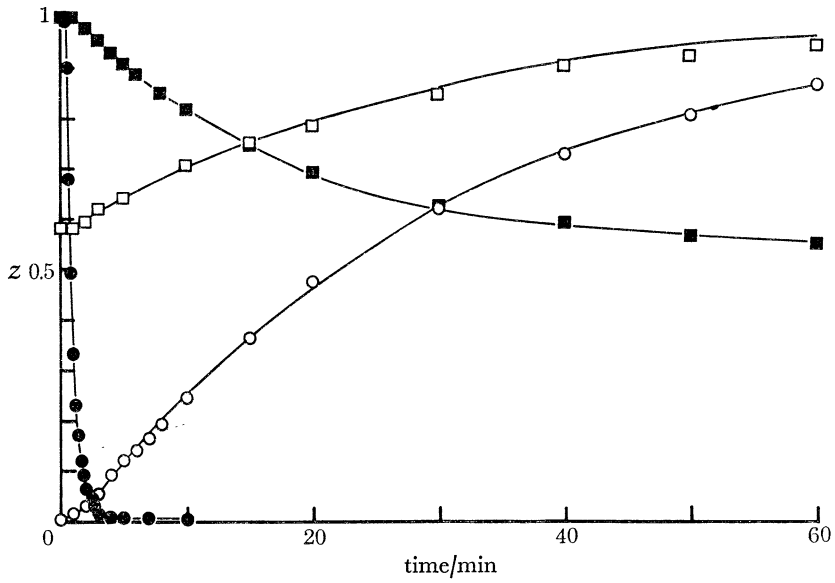

FIGURE 1. The time course of the change in the fraction of sites unoccupied by TTX, z, on application (filled symbols) and removal (open symbols) of 3 nM (squares) and 300 nM (circles) TTX. z was calculated as the ratio of the peak inward current during a test voltage-clamp pulse of constant size to the current at the start (for 'on' curves) or finish (for 'off' curves) of the run. Axon no. 6D1. Temperature about 5 °C. The equilibrium dissociation constant K was determined from I–V curves recorded at the end of each run as 4.5 nM with 3 nM TTX, and as 1.0 nM with 300 nM TTX.

Assuming, as seems to be roughly the case, that the rate of equilibration of the tetrodotoxin in the Schwann cell space with the binding sites is fast compared with the rate of equilibration with the concentration in the bathing solution, a qualitative impression of the time course of the change in tetrodotoxin concentration y in this space can be obtained by calculating instantaneous values of y from the steady-state relation that follows from equation (1),

$$\frac{1-z}{z} = \frac{y}{K}. \tag{6}$$

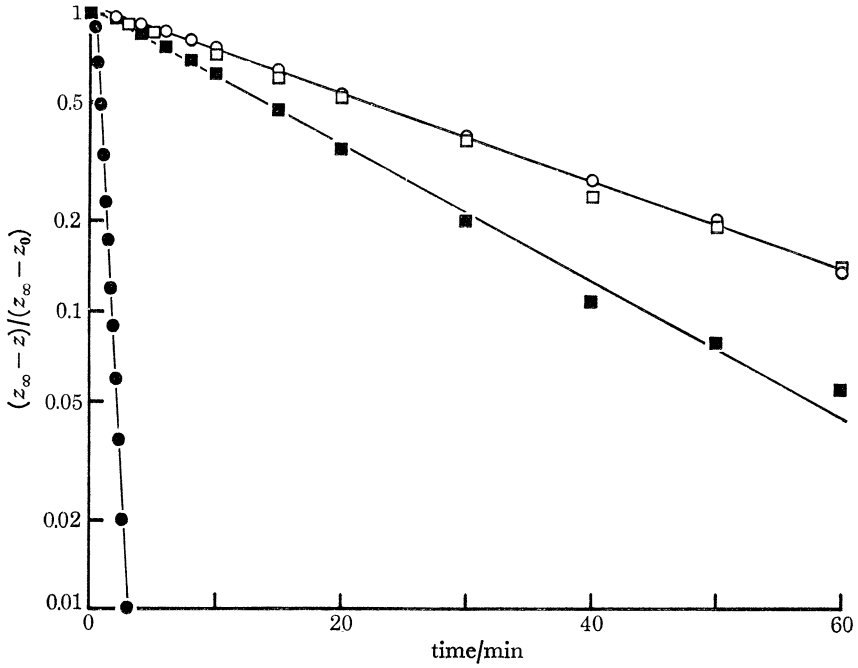

FIGURE 2. The results of figure 1 replotted in a standard reduced form, using the same symbols.

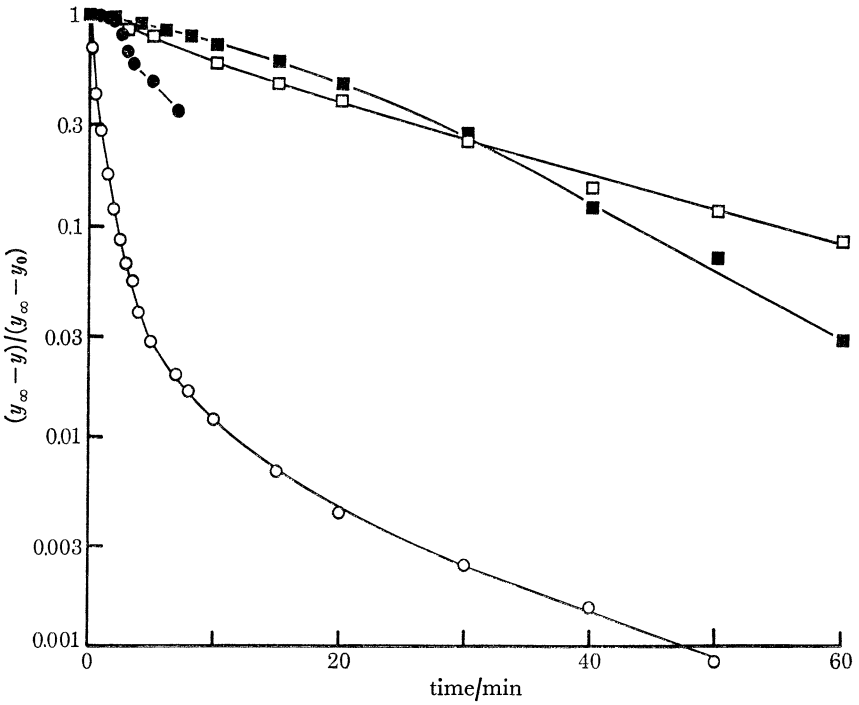

FIGURE 3. The changes in the concentration of TTX at the membrane surface, y, plotted in the reduced form used for figure 2. Experiment of figures 1 and 2, same symbols.

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Figure 3 shows the result of conducting this operation for the experiment of figures 1 and 2, and plotting $(y_\infty - y)/(y_\infty - y_0)$ logarithmically against time. It is seen that for the application and removal of 3 nM tetrodotoxin, y follows a roughly exponential time course with a large time constant. For the application of 300 nM, however, the calculation suggests that during the first 2 min the surface concentration changes much more slowly than it does later on, in contrast to what happens on returning to a tetrodotoxin-free solution, when y initially displays a time constant approaching that for changes in the external sodium concentration, but after about 10 min slows down to the rate observed in the low concentration range. This type of behaviour was an invariable feature of all the experiments, and is precisely what would be expected for a system incorporating a saturable sink in the membrane, since the lag caused by the sink in the attainment of diffusion equilibrium would be apparent only over the range of concentrations at which the binding sites were taking up or releasing tetrodotoxin. Once all the sites were occupied, there would be no appreciable slowing effect.

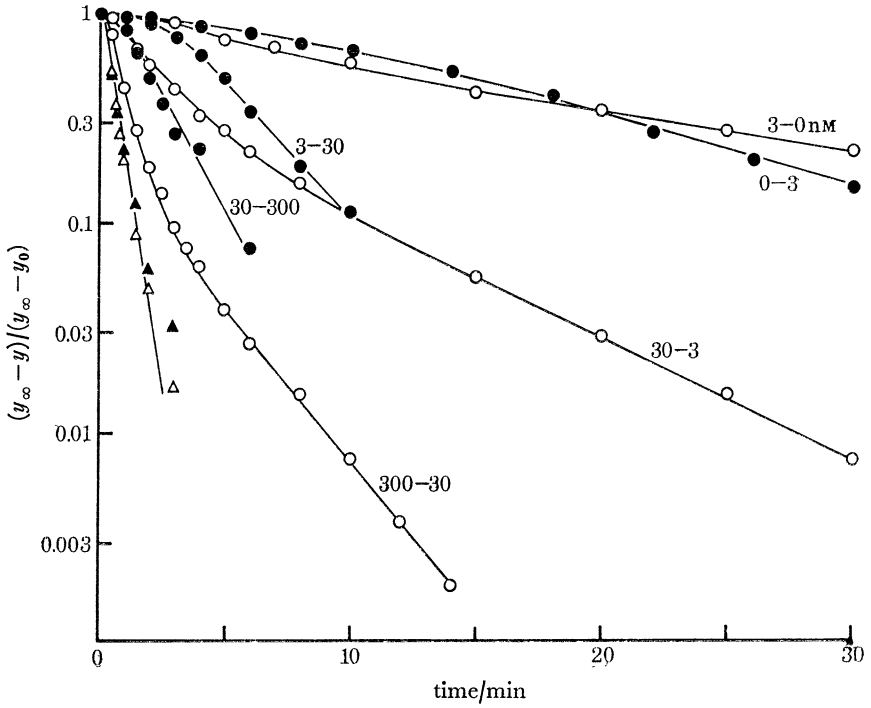

FIGURE 4. The changes in surface concentration of TTX plotted in reduced form for an experiment in which the bathing solution contained successively 3, 30, 300, 30, 3 and 0 nM TTX. Filled circles: [TTX] stepped up. Open circles: [TTX] stepped down. Triangles: time course of change in external [Na] on halving the concentration in the bathing solution (△), and on restoring it to normal (▲). Axon no. 7D1. Temperature 7 °C. The steady-state value of K determined from I–V curves was 2.6, 2.7 and 2.4 nM at 3, 30 and 300 nM TTX respectively.

In order to underline the point still further, we have plotted in a similar fashion in figure 4 the results of an experiment in which the concentration of tetrodotoxin was raised in succession to 3, 30 and 300 nM, and then lowered back to zero in the same steps. Only between 30 and 300 nM tetrodotoxin did the time constant approach that for changes in external sodium concentration measured in the same axon.

In order to treat the data quantitatively, it was first necessary to decide on a realistic model for which the time course of the changes in sodium conductance or z could be calculated by means of standard computer programs. Derivation of an analytical solution was out of the question except for the crude model considered by Frankenhaeuser & Hodgkin (1956), since incorporation of a saturable sink constituted a nonlinear boundary condition for the diffusion equation. The model finally chosen contained the following elements:

(1) A membrane containing N moles of tetrodotoxin binding sites per unit area, for which the fractional occupancy $1-z$ follows the equation

$$dz/dt = -zy_s k_1 + (1-z)\, Kk_1, \tag{7}$$

where K is the equilibrium dissociation constant for the binding reaction, and k_1 is its association rate constant. The system also comprises a quantity B per unit area of non-specific binding sites, whose occupancy is directly proportional to the tetrodotoxin concentration in the immediate vicinity, and does not exhibit saturation.

(2) A space between the membrane and the Schwann cell of thickness s, in which the concentration of tetrodotoxin is y at time t.

(3) A Schwann cell which behaves as a very thin diffusion barrier with a permeability coefficient P_{TTX}.

(4) An unstirred external layer of thickness l across which tetrodotoxin diffuses with a diffusion constant D_{TTX}, in contact with the well stirred bathing solution.

The respect in which this model seemed least likely to prove satisfactory was the assumption that the Schwann cell layer could be regarded as a uniform barrier of negligible thickness. As was pointed out by Frankenhaeuser & Hodgkin (1956), its low permeability actually results from the fact that extracellular ions are only able to reach the membrane via the tortuous gaps between the cells in the mosaic enveloping the giant axon. Recent measurements of electron micrographs of the greater part of the periphery of one axon, for which we are greatly indebted to Dr F. B. P. Wooding, have given the average intervals between gaps as about 13 μm, and the average diffusion distance along the gaps to the loose connective tissue outside as about 10 μm. These figures agree reasonably well with experimental measurements of P_K and of the electrical resistance of the Schwann cell layer. Using them as a basis, the behaviour of an anatomically more realistic model has been computed, and it turns out that representing the Schwann cell layer as a thin uniform barrier does not in fact make a great difference to the results. Both models agree in predicting that for the release of a pulse of potassium at the membrane, τ_{FH} is determined by s and P_K, and is relatively little affected by the characteristics of the unstirred layer, while the time constant τ_{Na} for the change in [Na] at the membrane when the concentration in the bathing solution is stepped up or down depends mainly on D and l. This fits with experimental observations on the effect of incomplete removal of the small fibres and connective tissue when dissecting the axon.

The next step was to choose plausible values for all the parameters except the numbers of specific and non-specific binding sites. We hoped originally that from measurements of τ_{FH}, τ_{Na} and R_s, the Schwann cell series resistance, we could derive an independent set of parameters for each individual axon, but this involved many more computations than could easily be handled, and instead we have to reply on standard values based on averages. For the reasons discussed by Frankenhaeuser & Hodgkin (1956), s has been taken throughout as 3×10^{-6} cm. Their value for P_K at room temperature was 6×10^{-5} cm/s, but our average for

R_s of a little over $3\Omega\,cm^2$ corresponds to about 10×10^{-5} cm/s. Taking 8×10^{-5} cm/s as a compromise, and allowing for the fact that in free solution the diffusion constant for sucrose, whose molecular mass is midway between those of tetrodotoxin and saxitoxin, is 0.28 times that for KCl, P_{TTX} would be 2.2×10^{-5} cm/s, or say 1.4×10^{-5} cm/s at the temperature at which the experiments of figures 7 and 8 were conducted. If the diffusion constant for Na^+ ions at room temperature is 1×10^{-5} cm^2/s, then D_{TTX} would be 0.5×10^{-5} cm^2/s, or 0.33×10^{-5} cm^2/s at the lower temperature, since the Q_{10} for τ_{Na} was found to be 1.42. From the standard analytical solution for diffusion in an unstirred layer

$$\tau_{Na} = 4l^2/D_{Na}\pi^2 \qquad (8)$$

so that since τ_{Na} averaged 27 s, l may be taken as 0.0210 cm. From the experiments of Schwarz *et al.* (1973) on tetrodotoxin, k_1 was 3×10^{-6} M^{-1} s^{-1} at 20 °C with a Q_{10} of 1.8; at 6–7 °C it may therefore be taken as 1.4×10^{-6} M^{-1} s^{-1}. For saxitoxin, Henderson, Ritchie & Strichartz (1973) found rates of association almost 4 times higher than for tetrodotoxin, so that at the low temperature k_1 would be 5.6×10^{-6} M^{-1} s^{-1}.

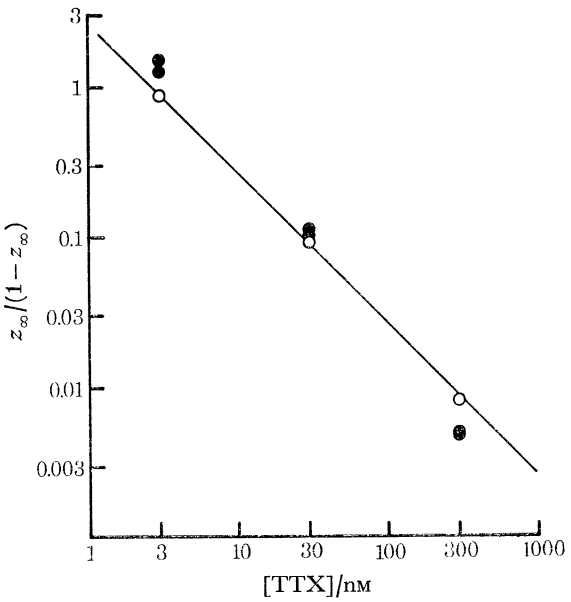

FIGURE 5. $z_\infty/(1-z_\infty)$ plotted against [TTX] on logarithmic scales for the experiment of figure 4. Open circles: values obtained from *I–V* curves. Filled circles: values obtained from single pulse measurements at the end of each kinetic run. The slope of the line is 1.0.

In seeking a computed solution to match any given experimental run, the parameters that remained to be chosen were the equilibrium constant K and the numbers of specific and non-specific binding sites. The choice of K was complicated by an internal contradiction in the experimental evidence that we have so far been unable to resolve to our complete satisfaction. As may be seen in figure 5, a test of equation (6) conducted by plotting $\lg\{z_\infty/(1-z_\infty)\}$ against lg [TTX] for an experiment in which an axon was exposed in turn to 3, 30 and 300 nM TTX gave points (the open circles) that lay on a straight line with a slope of -1.0 if z_∞ was calculated from the slope conductance of complete *I–V* curves recorded after a steady state of blocking had been reached. A similar experiment with saxitoxin gave the same result, as did the assembled values for a large number of other axons treated with different concentrations of TTX

and STX, though the scatter was then much greater because of individual variation. When, however, z_∞ was calculated from the measurements of I_{Na} for single pulses of fixed size made during the kinetic runs, the points (filled circles in figure 5) consistently lay on a line with a slope of 1.2–1.3. In other words, although the steady state determinations indicated that K was satisfactorily constant over a 100-fold range of concentrations, the single pulse determinations gave values about one third as great at 300 nM as they were at 3 nM. The most likely explanation for the discrepancy is that the single pulse measurements were subject to error from the addition of a leakage current pulse of constant amplitude, which became ever more important as I_{Na} fell; a leakage pulse whose size was less than 1 % of that for I_{Na} in the unblocked condition would suffice. The only argument against this suggestion is that on one occasion, the experiment of figures 1, 2 and 3, the steady state determination of K showed the same apparent reduction at 300 nM TTX as that for the single pulses; and since the final recovery of the sodium conductance in a TTX-free solution after several marathon runs was as usual – and contrary to the reports of Cuervo & Adelman (1970) – close to 100 %, the results were hard to reject. But the fact that the washing-out curves for 3 and 300 nM TTX were almost perfectly superimposable when plotted as in figures 2 and 3 argues rather strongly that K must actually have been the same for two runs.

Since the occurrence of appreciable non-specific or linear binding of TTX in squid axons has been questioned by Levinson & Meves (1975) some comment is necessary on the inclusion of such binding in our computer model. In preliminary computer runs in which the parameter B was omitted, we found that the solutions failed to resemble the experimental runs in that plots of $\ln\{(z_\infty - z)/(z_\infty - z_0)\}$ invariably displayed a slope that increased continuously with time instead of remaining roughly constant as in figure 2. Only by incorporating a linear component could we obtain solutions with the single time constant that we always observed experimentally. It may be noted that precisely the same is true of the analytical solution for the simplified model given in equation (3). If it is modified by incorporating a linear binding component B it becomes

$$z_\infty(z_0 - z) + \left(z_\infty^2 + \left(1 + \frac{B}{s}\right)\frac{Ks}{N}\right)\ln\frac{z_\infty - z_0}{z_\infty - z} + \left(1 + \frac{B}{s}\right)\frac{Ks}{N}\ln\frac{z}{z_0} = \frac{P_{TTX}\,K}{N}\,t, \qquad (9)$$

which with a suitable choice of the magnitude of B does approximate to a single exponential. Equation (9) is formally identical with equation (5) of Colquhoun, Henderson & Ritchie (1972). A further point is that the values of B chosen to fit our data are such that in the majority of Levinson & Meves's (1975) experiments the non-specific binding would have been much smaller than the specific binding.

Having settled the fixed parameters, and knowing the values of K and the initial and final concentrations of TTX to be taken for any given run, we are now in a position to see how large the specific binding N and the unspecific binding B have to be in order to obtain a respectable match between the experimental and computed time courses for z. The fitting had to be done by trial and error, aided by the observation, which seemed intuitively reasonable, that the time constant for a change in z reduced to the standard form $(z_\infty - z)/(z_\infty - z_0)$ was more sensitive to N than to B for the washing away of TTX, but was mainly determined by B when the external [TTX] was raised, especially at high concentrations where the specific binding sites were saturated within the first few minutes. This point is illustrated by figure 6, which shows the dependence of the time constant on N and B for the family of computer runs described in the legend.

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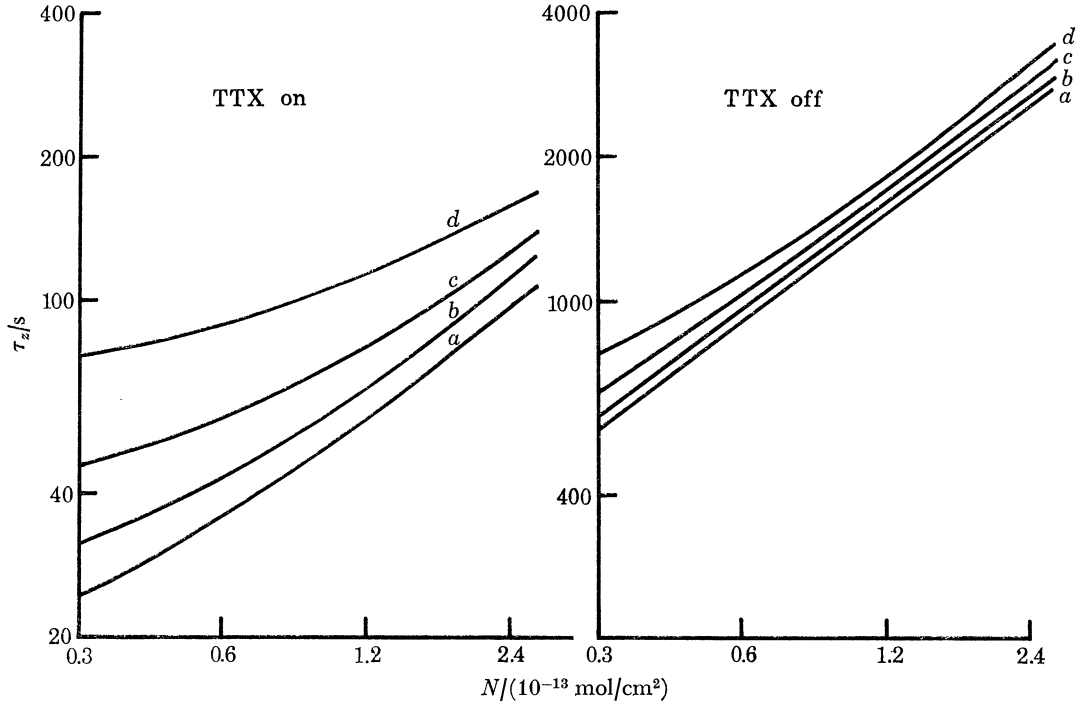

FIGURE 6. The dependence of the computed time constant τ_z on N and B for the application and removal of 25 nM TTX. The parameters used in the calculation were: K, 6 nM; k_1, 2.4×10^{6} M^{-1} s^{-1}; s, 3×10^{-6} cm; P_{TTX}, 1.4×10^{-5} cm/s; D_{TTX}, 0.5×10^{-5} cm^2/s; l, 0.0215 cm. Values of B were: a, 3×10^{-16}; b, 6×10^{-16}; c, 12×10^{-16}; d, 24×10^{-16} mol/cm^2 per nM TTX.

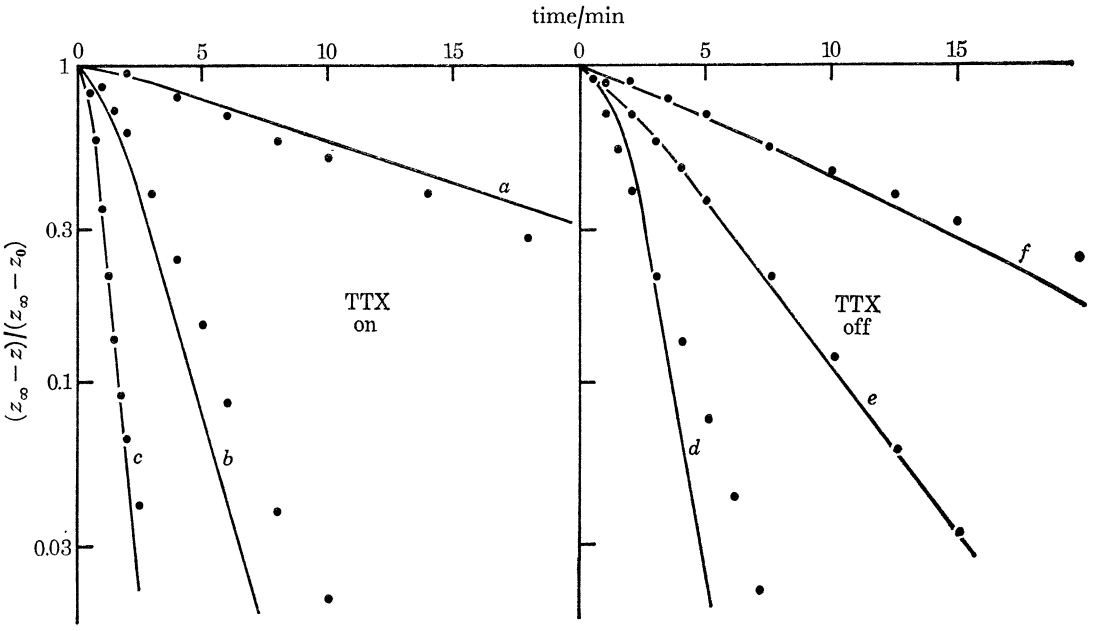

FIGURE 7. Comparison between observed and computed time course of z for experiment of figure 4. Filled circles are the experimental points, lines are the computer solutions. Parameters common to all runs were: k_1, 1.4×10^{6} M^{-1} s^{-1}; s, 3×10^{-6} cm; P_{TTX}, 1.4×10^{-5} cm/s; D_{TTX}, 0.5×10^{-5} cm^2/s; l, 0.0215 cm; N, 0.87×10^{-13} mol/cm^2; B, 0.99×10^{-15} mol/cm^2 per nM TTX. Values of K were: a, 0–3 nM TTX, 3.7 nM; b, 3–30 nM TTX, 2.9 nM; c, 30–300 nM TTX, 1.3 nM; d, 300–30 nM TTX, 1.6 nM; e, 30–3 nM TTX, 3.5 nM; f, 3–0 nM TTX, 4.5 nM.

The experiments that provided the best test of the fitting procedure were two in which the concentrations of TTX and STX respectively were altered stepwise up and down the sequence 3, 30, 300, 30, 3 and 0 nM. Figure 7 shows a comparison between the experimental and computed time courses of $(z_\infty - z)/(z_\infty - z_0)$ when N was taken as 0.87×10^{-13} mol/cm² membrane = 522 sites/μm² membrane, and B was taken as 0.99×10^{-15} mol/cm² membrane per nM TTX. The agreement is by no means perfect, but any other choice for N would have worsened the fit for 3 nM on and off, and any other for B would have spoiled it for 300 nM on and off. The chosen compromise distributes the lack of agreement more or less evenly.

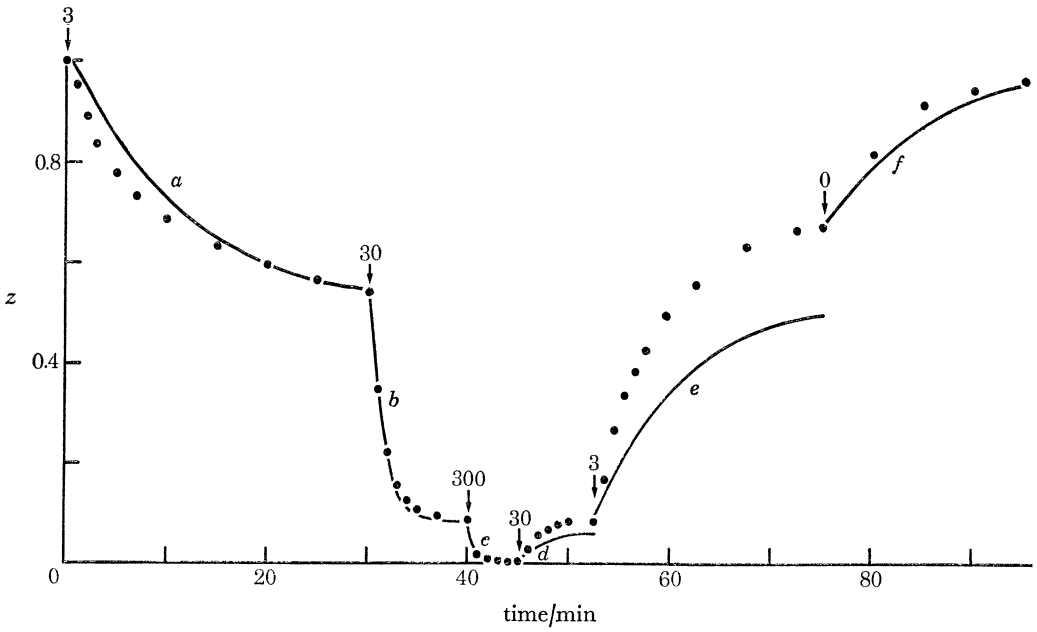

FIGURE 8. Comparison between observed and computed time course of z for an experiment similar to that of figure 4, using STX instead of TTX. Filled circles are the experimental points, lines are the computer solutions. Axon no. 8D1. Temperature 7 °C. Parameters common to all runs were: k_1, 4×10^6 M^{-1} s^{-1}; s, 3×10^{-6} cm; P_{STX}, 1.4×10^{-5} cm/s; D_{STX}, 0.5×10^{-5} cm²/s; l, 0.0215 cm; N, 0.54×10^{-13} mol/cm²; B, 0.9×10^{-15} mol/cm² per nM STX. Values of K were: a, 0–3 nM STX, 3.5 nM; b, 3–30 nM STX, 2.7 nM; c, 30–300 nM STX, 0.9 nM; d, 300–30 nM STX, 1.0 nM; e, 30–3 nM STX, 2.7 nM; f, 3–0 nM STX, 6.6 nM. Mean value of K determined from I–V curves was 3.4 nM.

Figure 8 shows the results of a similar experiment with STX, this time plotting z itself on a linear scale. Again the selection of N as 0.54×10^{-13} mol/cm² membrane = 324 sites/μm² membrane, and of B as 0.90×10^{-15} mol/cm² membrane per nM STX spreads the misfit impartially. In no individual run was there more than 30 % disagreement between the observed and computed time constants, and the average discrepancy was zero. Although the two sets of runs may appear to agree worse in figure 8 than in figure 7, this is a false impression arising from the different manner in which they have been presented. Employment of the reduced form of z as in figure 7 glosses over the problem arising from the apparent decrease in K in the kinetic runs at the higher concentrations of TTX or STX. Although this can partially be mimicked by appropriate changes in K in the computations, it is impossible to make the observed and computed values of z agree both at the start and at the finish of each run. Probably the most satisfactory way of circumventing the difficulty would be to use for the computations for each axon only the mean value of K obtained from the steady-state I–V curves,

and having calculated the size of the leakage pulse that would yield the same final value of K in the kinetic runs, to apply it as a constant correction to all the pulse measurements before working out the time constants for the changes in z.

Since this suggestion has not yet been put into effect, we are still some way from achieving our original objective of calculating values of N and B to fit all our data. However, the experiments of figures 7 and 8 are fully representative of many others as far as the sizes of τ_z are concerned, and we are therefore confident that our final estimate for the number of high-affinity specific TTX or STX binding sites will be in the range of 300–600/μm^2 membrane. Our faith in this conclusion is, of course, reinforced by the fact that studies with tritiated tetrodotoxin reported at this Meeting (Levinson & Meves 1975) have now given values of the same order of magnitude, as have measurements of gating currents (Keynes & Rojas 1974; Rojas & Keynes 1975; Armstrong & Bezanilla 1974).

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