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THE RATE OF ACTION OF TETRODOTOXIN ON MYELINATED NERVE FIBRES OF *XENOPUS LAEVIS* AND *RANA ESCULENTA*

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SUMMARY

1. The experiments were done on single Ranvier nodes of *Xenopus laevis* (voltage clamp) and *Rana esculenta* (action potentials). Rate and size of the effect of tetrodotoxin were determined by the reversible reduction of either the sodium inward current (*Xenopus*) or of $\dot{V}_A$, the maximum rate of rise of the action potential (*Rana*).

2. The results of tetrodotoxin block at equilibrium could be excellently fitted by assuming a one-to-one reaction between toxin molecules and sodium channels of the *Xenopus* membrane with an equilibrium dissociation constant $K = 3{\cdot}60$ nM at room temperature. $\dot{V}_A$ was not linearly related to the fraction of unblocked sodium channels and 10·9 nM tetrodotoxin was necessary on the average to reduce $\dot{V}_A$ to 50 % in *Rana* motor fibres; in sensory fibres a lower concentration sufficed.

3. Onset and offset of the tetrodotoxin effect on *Xenopus* nodes could be quantitatively interpreted as being determined by the rates of the tetrodotoxin channel reaction. Experiments with 3·1 and 15·5 nM tetrodotoxin at room temperature yielded an association rate constant, k_1, of $2{\cdot}94 \times 10^6$ M^{-1} sec^{-1} and a dissociation rate constant, k_2, of $1{\cdot}42 \times 10^{-2}$ sec^{-1}. In these experiments the equilibrium dissociation constant, K, was 3·31 nM. If determined solely from the onset in the two tetrodotoxin concentrations, $k_1 = 3{\cdot}25 \times 10^6$ M^{-1} sec^{-1} and $K = k_2/k_1 = 4{\cdot}08$ nM was calculated.

4. In *Rana* fibres the onset and offset of $\dot{V}_A$ reduction by 15·5 and 31 nM tetrodotoxin was evaluated using the equilibrium effects of intermediary tetrodotoxin concentrations for calibration. The average results at room temperature were $k_1 = 4 \times 10^6$ M^{-1} sec^{-1}, $k_2 = 1{\cdot}4 \times 10^{-2}$ sec^{-1} and $K =$ 3·4 nM.

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5. The very short latency with which $\dot{V}_A$ started to decline when tetrodotoxin was suddenly applied proved that the toxin had ready access to the membrane.

6. The temperature dependence of k_1, k_2 and K in the *Xenopus* experiments could be described by Arrhenius plots yielding activation energies, E_a, of 9·8, 20·5 and 7·0 kcal/mole, respectively, corresponding to Q_{10} values of 1·82, 3·42 and 1·53 (between 12 and 22° C). For k_1, determined from onset alone, E_a = 13·7 kcal/mole (Q_{10} = 2·25) was obtained. Although in *Rana* the temperature dependence of the rate constants could not be determined directly, the Q_{10} for k_2 must have been of the order of 3.

7. The results suggest that the rate of the toxin action on the nodal membrane of *Xenopus* and *Rana* is limited by the tetrodotoxin-sodium site reaction.

INTRODUCTION

In recent years considerable evidence has been found in favour of the idea that during excitation sodium ions move through a finite number of membrane 'channels' which are structurally distinct from the predominant pathways of potassium ions (see review of Hille, 1970). Tetrodotoxin reversibly inhibits this passive movement of Na ions in a way suggesting that a channel is completely blocked when one tetrodotoxin molecule is bound to it or a site in the immediate vicinity (Hille, 1968; Cuervo & Adelman, 1970; Keynes, Ritchie & Rojas, 1971; Colquhoun & Ritchie, 1972*a*, *b*). The affinity of tetrodotoxin for the binding site is very high so that at equilibrium a nanomolar toxin concentration is sufficient to block half of the total channel population. Because of its specificity the tetrodotoxin-site reaction and especially its rates deserve a detailed investigation which may eventually help to elucidate the channel structure. Kinetic studies have so far been done on squid giant axons (Cuervo & Adelman, 1970) and bundles of mammalian C fibres (Colquhoun & Ritchie, 1972*b*). In these two preparations, however, the rate of tetrodotoxin action may be limited by the diffusional access to the membrane which in the squid axon is retarded by the Schwann cell layer (Frankenhaeuser & Hodgkin, 1956) and in the bundle by its extensive extracellular space (Keynes & Ritchie, 1965). In the node of Ranvier, on the other hand, there is free access to the membrane and at its outer surface very fast concentration changes, e.g. of sodium, can be achieved (Vierhaus & Ulbricht, 1971*a*). In the present paper it is shown that tetrodotoxin, too, reaches the membrane with very little delay and on a sudden application the concentration of the toxin should rise almost stepwise if compared to its slow rate of action. Thus the node offers favourable conditions for kinetic studies and the present results most probably represent the true rates of the tetrodotoxin-site reaction,

a notion that is especially supported by the rates' dependence on concentration and temperature. A preliminary account of part of the results has appeared elsewhere (Wagner, Schwarz & Ulbricht, 1972).

METHODS

The measurements were done on single myelinated nerve fibres of the clawed toad, *Xenopus laevis*, and of the frog, *Rana esculenta*. If not otherwise noted motor fibres were investigated.

Setup and measurements. In the voltage clamp experiments on *Xenopus* a plexiglass chamber was used that contained four pools separated by three partitions. The fibre was held in position by Vaseline seals applied to the partitions; the node under investigation lay in a pool that was continuously perfused. The adjacent nodes were kept in isotonic potassium chloride solution. After mounting the fibre one internode was cut to decrease the resistance of the current path. The dimensions of the chamber and the method of recording membrane currents under voltage clamp conditions have been described in detail (Koppenhöfer, 1967); the method is based on that of Dodge & Frankenhaeuser (1958).

The action potentials of *Rana* nerve fibres were recorded in the double air gap chamber of Stämpfli (1958). The investigated node was placed in a slit across a thin polyethylene tube through which the test solution continuously flowed. The solution could be rapidly changed by a stopcock that was situated a few millimetres upstream. To the lever of the stopcock a vane was attached which allowed light to fall on a photocell when, on switching solutions, the lever had completed half of its 30° rotation. The photo current was displayed on the oscilloscope (Tektronix 565) together with the action potential or its time derivative. The latter was obtained by an electronic differentiator with a time constant of 22 μsec. A full description of the method has been published (Vierhaus & Ulbricht, 1971*a*). The action potentials recorded with the air gap technique were 10 to 20% smaller than the 'true' values because the external internodal resistance, although very high, was finite and acted as a shunt; the measured values of $\hat{V}_A$ were diminished in proportion. However, since only relative effects of tetrodotoxin were of interest in the present study, no correction was deemed necessary.

The development or release of the tetrodotoxin block was studied while the preparation was stimulated (*Rana*) or subjected to short depolarizing pulses (*Xenopus*) at a rate of 1/sec unless otherwise noted. The measured signals were displayed on the oscilloscope screen as standing pictures and photographed on moving film. Sweep and film speed were chosen to yield overlapping records.

Potentials are given as deviations, V, from the normal resting potential, depolarizations being positive. In the voltage clamp experiments, the resting potential was defined as the holding potential for which $h = 0{\cdot}7$. If necessary, the holding potential was readjusted between runs. In the *Rana* experiments the resting potential was given by the potential of the neighbouring node which was made inexcitable by cocaine.

Solutions. The Ringer solution used in the *Rana* experiments was based on that of Adrian (1956) and contained (in mM): NaCl, 115; KCl, 2·5; $CaCl_2$, 2·0; Na_2HPO_4, 2·15; NaH_2PO_4, 0·85. The pH was 7·2. For the *Xenopus* experiments 6 mM-TEA chloride was added to this solution to suppress the potassium permeability. All tetrodotoxin experiments on *Xenopus* and *Rana* fibres were done with test solutions derived from one 1 mg sample of tetrodotoxin (Sankyo/Calbiochem). The stock solution contained 3·1 μM tetrodotoxin and 10 mM acetate buffer (pH 4) and was

kept at 4° C to delay deterioration (see Cuervo & Adelman, 1970). The working solutions, freshly prepared before each experiment, had the pH of the Ringer solution (7·2).

Preliminary experiments and analog computations. While the chamber used for *Rana* experiments permitted a change of sodium concentration at the nodal membrane with a time constant of about 50 msec (Vierhaus & Ulbricht, 1971*a*), the situation was less favourable in the *Xenopus* chamber because of its comparatively large dead space. To test the speed of sodium exchange in this chamber the perfusate was switched from a sodium-poor solution (57 mM sodium of the Ringer solution substituted by choline) to a sodium-rich solution (228 mM sodium, obtained by adding crystalline sodium chloride to ordinary Ringer solution) while the membrane was subjected to 1 msec depolarizing pulses at a rate of 20/sec. The pulse strength was adjusted to give maximum sodium currents; between pulses the membrane was kept hyperpolarized by 40 mV. The membrane was periodically depolarized, also after equilibration in the sodium-poor and sodium-rich solution and in two additional solutions containing 90 or 142 mM sodium which were prepared by mixing the two extreme solutions. In each experiment the Na current observed in these four solutions was plotted against sodium concentration to give a calibration curve with which the sodium currents were translated into the momentary sodium concentration at the membrane during the exchange. Between 10 and 90% of the total change, the time course of sodium concentrations could be reasonably fitted by an exponential function. The average time constant in five experiments was 0·65 sec. In order to estimate whether this relatively slow exchange could influence the time course of the tetrodotoxin-induced inhibition of the sodium permeability, calculations were done on an analog computer (Systron-Donner, SD 10/20) following the general procedure of Paton & Waud (1964). The tetrodotoxin-receptor reaction was characterized by the equilibrium dissociation constant, K, and by the dissociation rate constant, k_2, of $1{\cdot}5 \times 10^{-2}$ sec^{-1}, the apparent rate obtained from *Rana* experiments in which an almost stepwise change in drug concentration could be achieved. Ignoring possible complications from diffusion plus binding (see Discussion) it was assumed that at the *Xenopus* membrane the tetrodotoxin concentration rose exponentially in proportion to sodium, i.e. with a time constant of 1 sec. With this value the time course of receptor occupation was computed for the onset in a tetrodotoxin concentration = $5\,K$. Although this was more than the highest concentration used in the *Xenopus* experiments, the onset differed very little from that on a step change in drug concentration. Only during the first 5% of the final receptor occupancy was the onset delayed but for the remaining 95% the two curves ran in parallel with a slight shift of 0·8 sec but with no visible change of slope. The onset curves at lower concentrations and the washout curves were almost indistinguishable from those following a step in tetrodotoxin concentration. Hence the less favourable conditions in the *Xenopus* chamber were of no consequence to the measurement of time constants in experiments with up to 15·5 nM tetrodotoxin.

RESULTS

Dose–response relation at room temperature

Voltage clamp experiments. In a first series of experiments on *Xenopus* fibres the tetrodotoxin-induced inhibition of sodium currents was studied at equilibrium. The experiments were started in Ringer solution by determining the leakage conductance with hyperpolarizing impulses. Then the peak inward current–voltage relation was obtained with depolarizing

pulses each being preceded by a 50 msec hyperpolarization by 40 mV to remove any inactivation of the sodium permeability. The tetrodotoxin was applied for at least 6 min (which was more than sufficient for reaching a steady effect) and the procedure was repeated. The preparation was then washed for at least 10 min after which leakage conductance and current–voltage curve were again determined in Ringer solution. As a rule, three concentrations of tetrodotoxin were tested in each fibre, the toxin treatment alternating with periods of thorough washing.

Peak sodium currents, I_{Na}, were obtained from the observed peak inward currents after correction for leakage currents. The leakage conductance was unaffected by tetrodotoxin. The I_{Na}–V curves of the tetrodotoxin-treated membrane and of the control intersected the abscissa ($I = 0$) at approximately the same potential. The potential at which a maximum sodium current was observed varied somewhat although not systematically. For any larger depolarization I_{Na} of the tetrodotoxin-treated membrane was a constant fraction of I_{Na} in Ringer solution. This justified using an appropriate depolarizing pulse of constant amplitude to follow the onset and offset of the tetrodotoxin-induced inhibition of I_{Na} as described in a later section. This simplified method was also employed in part of the steady-state experiments.

After washing, I_{Na} recovered to between 80 and 100% of the control value, complete recovery being the exception. However, there was no clear indication that tetrodotoxin bound irreversibly to a fraction of membrane sites as reported for the squid giant axon by Cuervo & Adelman (1970). In particular, we could not confirm their observation that once irreversible binding had taken place, recovery after a second exposure was to the same level as the first recovery. In our experiments the loss of I_{Na} after repeated applications of the toxin was progressive and little dependent on tetrodotoxin concentration. The decrease was more likely of the slow unspecific kind that is unavoidable in excised fibres for it was also observed at a comparable rate when the preparation was left in Ringer and subjected to periodic depolarizing pulses. Therefore, the I_{Na} values before tetrodotoxin application and after washout were averaged and the value observed in the toxin solution was expressed as percentage of this average. In eight fibres we found the relative I_{Na} to be (mean ± S.E. of mean): $90{\cdot}7 \pm 1{\cdot}6$% in 0·31 nM ($n = 3$), $70{\cdot}5 \pm 1{\cdot}8$% in 1·55 nM ($n = 3$), $53{\cdot}1 \pm 1{\cdot}6$% in 3·1 nM ($n = 8$), $17{\cdot}6 \pm 1{\cdot}6$% in 15·5 nM ($n = 5$) and $12{\cdot}2 \pm 1{\cdot}7$% in 31 nM ($n = 3$). In five further experiments, made 3 months later with 3·1 and 15·5 nM tetrodotoxin from the same stock solution, the respective mean values were $51{\cdot}1 \pm 1{\cdot}2$ and $16{\cdot}6 \pm 1{\cdot}7$%.

The individual data of the former series of experiments are given in Table 1 together with quantities calculated from these data. For the calcu-

lation a first order reaction was assumed whereby each sodium site (receptor; R) reacts independently with a single tetrodotoxin molecule (Hille, 1968; Cuervo & Adelmann, 1970; Keynes *et al.* 1971; Colquhoun & Ritchie, 1972 *a*, *b*):

$$TTX + R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} TTX.R,$$

TABLE 1. Equilibrium inhibition of I_{Na} by TTX

Axon	[TTX] (nM)	I_{Na} in % of control	y_∞	$\frac{1-y_\infty}{y_\infty}$	K (nM)
a	0·31	88·0	0·120	7·33	2·27
b	0·31	90·5	0·095	9·53	2·95
c	0·31	93·5	0·069	14·39	4·46
a	1·55	67·0	0·330	2·03	3·15
b	1·55	73·0	0·270	2·70	4·19
c	1·55	71·5	0·285	2·51	3·89
a	3·1	60·0	0·400	1·50	4·65
b	3·1	52·0	0·480	1·08	3·36
c	3·1	57·5	0·425	1·35	4·19
d	3·1	51·5	0·485	1·06	3·29
e	3·1	53·0	0·470	1·23	3·50
f	3·1	50·0	0·500	1·00	3·10
g	3·1	55·0	0·450	1·22	3·79
h	3·1	46·0	0·540	0·85	2·64
d	15·5	16·0	0·840	0·19	2·95
e	15·5	18·5	0·815	0·23	3·52
f	15·5	12·5	0·875	0·14	2·21
g	15·5	19·5	0·805	0·24	3·76
h	15·5	21·5	0·785	0·27	4·26
a	31·0	10·5	0·895	0·12	3·64
e	31·0	10·5	0·895	0·12	3·64
g	31·0	15·5	0·845	0·18	5·69

Mean K ($\pm$ S.E. of mean) $3{\cdot}60 \pm 0{\cdot}17$ nM

where k_1 and k_2 are the association and dissociation rate constants, respectively. It was further assumed that I_{Na} in the presence of tetrodotoxin is proportional to the fraction of unoccupied sites, $1-y_\infty$, where y_∞ is the fractional receptor occupancy at equilibrium as defined by

$$y_\infty = \frac{[TTX.R]}{[R]+[TTX.R]}. \quad (1)$$

Table 1 contains y_∞ and $(1-y_\infty)/y_\infty$ i.e. the ratio of free sites to occupied sites. From this ratio the equilibrium dissociation constant, $K = k_2/k_1$, was calculated according to

$$K = \frac{1-y_\infty}{y_\infty}[TTX] \quad (2)$$

and also listed in Table 1. The mean value was 3·6 nM. Finally, in a Hill plot of these data the logarithm of the ratio $y_\infty/(1-y_\infty)$ was plotted against log [TTX] and the regression line was calculated. Its slope was 0·97 (correlation coefficient $r = 0{\cdot}99$) and thus agreed well with the theoretical slope of 1·0 for the assumed type of reaction.

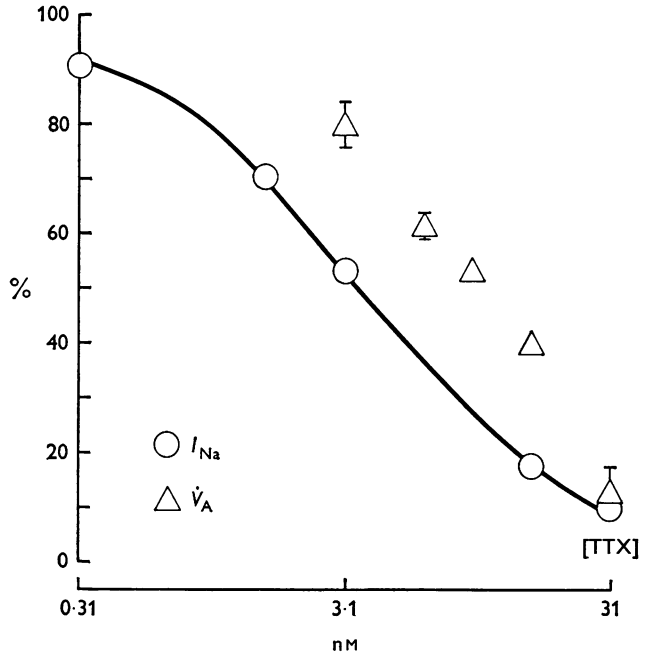

Fig. 1. Steady-state effect of TTX on I_{Na} (○) and on $\dot{V}_A$ (△), the maximum rate of rise of the action potential, at room temperature; Both I_{Na} and $\dot{V}_A$ are given in % of the respective values in Ringer solution, [TTX] is plotted on a logarithmic scale. The points are mean values obtained from eight experiments on *Xenopus* fibres (○) and as many experiments on *Rana* fibres (△). Where the S.E. of mean exceeds the symbol size it is given by the vertical bars. The curve through the circles was calculated assuming the relative I_{Na} to be proportional to $K/([TTX]+K)$ with $K = 3{\cdot}6$ nM.

Maximum rate of rise. The stationary effect of tetrodotoxin on the maximum rate of rise, $\dot{V}_A$, of the action potentials was investigated in *Rana* fibres. Throughout the experiments the nodal membrane was hyperpolarized by 10–20 mV and stimulated with 30 μsec cathodal impulses of threshold strength.

In seven experiments on motor nerve fibres at 20–22° C the node of Ranvier was successively exposed to five concentrations of tetrodotoxin for about 5 min, each application except the last being followed by a washing period of about equal duration. Recovery of $\dot{V}_A$ was excellent and

the Ringer value at the end of the fourth wash was, with one exception, more than 95 % of the starting value of $\dot{V}_A$. This nearly complete recovery may be deceptive since for small decreases in $\bar{P}_{Na}$, the theoretical maximum sodium permeability, the relative decrease in $\dot{V}_A$ is clearly smaller. Thus Frankenhaeuser & Huxley (1964) calculated a reduction to 66·4 % when $\bar{P}_{Na}$ was halved. As a consequence small decreases in $\bar{P}_{Na}$ may have gone undetected. This view is supported by the observation that 0·31 nM tetrodotoxin, which in *Xenopus* reduced I_{Na} to 90·7 %, had no clear effect on $\dot{V}_A$ in *Rana*. Therefore, a tetrodotoxin concentration below 3·1 nM was not systematically tested. In the seven experiments and in one additional preparation in which only 6·2 and 15·5 nM tetrodotoxin was applied the following mean values (± S.E. of mean) of $\dot{V}_A$ relative to the preceding value in Ringer solution were obtained: 81·1 ± 3·0 % in 3·1 nM, 64·7 ± 1·9 % in 6·2 nM, 54·7 ± 1·5 % in 9·3 nM, 40·3 ± 1·3 % in 15·5 nM and 12·9 ± 2·6 % in 31 nM. These mean values are represented as triangles in Fig. 1 together with the mean values of I_{Na} observed in equivalent *Xenopus* experiments. Since, as will later be shown, K of the tetrodotoxin-site reaction in *Rana* was equal to that in *Xenopus*, Fig. 1 illustrates the non-linear relation between I_{Na} and $\dot{V}_A$.

Interestingly, in sensory fibres of *Rana* a stronger effect of tetrodotoxin on $\dot{V}_A$ was observed. These fibres were diagnosed by their repetitive response to long cathodal impulses. Only two preparations were tested but the results were unequivocal: $\dot{V}_A$ was 67·5 and 67·8 % in 3·1 nM, 47·2 and 48·0 % in 6·2 nM, 33·9 and 35·2 % in 9·3 nM, and 17·8 % in 15·5 nM. In 31 nM no action potential could be elicited.

Onset and offset of tetrodotoxin effect on I_{Na} at room temperature

In these voltage clamp experiments on *Xenopus* fibres the membrane was depolarized every second by a 1 msec impulse that was slightly stronger than necessary for obtaining a maximum inward current. During the train of impulses the perfusate was changed from Ringer solution to a 3·1 nM tetrodotoxin solution which was kept on until no further reduction of the peak inward current could be detected. The solution was then washed out and in the later phase of the washout the pulse rate was decreased to 0·5 or 0·2 Hz. Once a stationary value had been obtained in Ringer solution the procedure was repeated with 15·5 nM tetrodotoxin. After correction for the leakage current the peak values of I_{Na} were plotted against the time after the change of solutions as shown in Fig. 2. In this Figure the sodium current is expressed relative to its initial value in Ringer solution before each application of the toxin; open symbols refer to 3·1 nM, filled symbols to 15·5 nM tetrodotoxin. Fig. 2 illustrates three important points. 1. The action of tetrodotoxin was rather slow considering the speed with which

the sodium concentration could be changed at the membrane (see Methods). 2. The onset rate of action clearly increased with increasing tetrodotoxin concentration while the offset rate was virtually constant. 3. Both onset and offset were exponential functions of time as revealed by the calculated curves. Hence when the decrement or increment of I_{Na} was plotted on a semilogarithmic scale, straight lines could be fitted to the points. From the slope of these lines the time constants of onset and offset, τ_{on} and τ_{off}, were calculated; these are listed in Table 2.

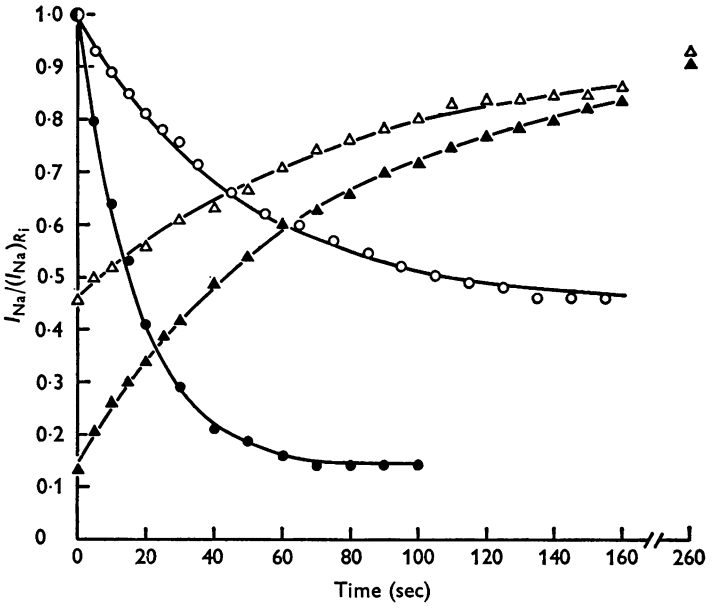

Fig. 2. Development of and recovery from TTX-induced inhibition of I_{Na} of a *Xenopus* node at room temperature. Ordinate, I_{Na} relative to its value before the application of 3·1 nM-TTX (open symbols) or 15·5 nM-TTX (filled symbols). Abscissa, time after the change of solutions. Circles and triangles refer to measurements during onset and offset, respectively. The curves were calculated from eqns. (3) to (6) with y_∞ and the time constants listed in Table 2 for axon *k*.

A simple explanation of these findings is to assume that onset and offset are determined by the rate of the tetrodotoxin reaction with the membrane receptors. In that case receptor occupancy, y, rises as follows on a step change in tetrodotoxin concentration (Hill, 1909)

$$y = y_\infty (1 - e^{-(k_1[TTX]+k_2)t}), \tag{3}$$

where y_∞, k_1 and k_2 have the meaning given on p. 169. Eqn. (3) clearly shows that the onset rate increases as the concentration of tetrodotoxin is

raised. When tetrodotoxin is rapidly washed away, occupancy declines from its initial value, y_0, at a rate that is determined by k_2 only,

$$y = y_0 \, e^{-k_2 t}. \tag{4}$$

From eqn. (3) and (4) it follows directly that

$$k_1[\mathrm{TTX}] + k_2 = 1/\tau_{on} \tag{5}$$

and

$$k_2 = 1/\tau_{off} \tag{6}$$

so that

$$k_1 = \frac{1/\tau_{on} - 1/\tau_{off}}{[\mathrm{TTX}]}. \tag{7}$$

TABLE 2. Rates of TTX action on I_{Na} at room temperature

Axon	τ_{on} (sec)	τ_{off} (sec)	k_1 (10^6 M^{-1} sec^{-1})	k_2 (10^{-2} sec^{-1})	y_∞	K (nM)	k_2/k_1 (nM)
			(1) Action of 3·1 nM-TTX				
g	41·0	54·2	1·92	1·85	0·450	3·79	9·64
h	37·4	63·7	3·56	1·57	0·540	2·64	4·41
i	55·4	82·0	1·89	1·22	0·478	3·39	6·46
j	48·0	77·0	2·53	1·30	0·524	2·82	5·14
k	45·0	76·5	2·95	1·31	0·507	3·01	4·44
l	50·0	90·0	2·87	1·11	0·468	3·52	3·87
m	40·5	86·0	4·21	1·16	0·465	3·57	2·76
	Mean		2·85	1·36		3·25	5·25
	± S.E. of mean		± 0·32	± 0·10		± 0·16	± 0·85
			(2) Action of 15·5 nM-TTX				
g	13·5	49·4	3·47	2·02	0·805	3·76	5·82
h	12·3	49·0	3·93	2·04	0·785	4·26	5·19
i	23·4	81·5	1·97	1·23	0·877	2·17	6·24
j	17·2	69·0	2·82	1·45	0·852	2·69	5·14
k	17·4	78·0	2·88	1·28	0·853	2·67	4·44
l	15·7	72·0	3·21	1·39	0·800	3·86	4·33
m	16·2	87·5	3·25	1·14	0·790	4·12	3·51
	Mean		3·08	1·51		3·36	4·95
	± S.E. of mean		± 0·23	± 0·14		± 0·31	± 0·35

Eqn. (6) and (7) were used to calculate the rate constants in Table 2. The last three columns of the Table give the equilibrium occupancy, y_∞, the equilibrium dissociation constant, $K(y_\infty)$ calculated from y_∞ (eqn. (2)) and the ratio $k_2/k_1 = K$. With the exception of axon *m*, this ratio was consistently larger than $K(y_\infty)$. In Table 2 the results with 3·1 and 15·5 nM tetrodotoxin are listed separately; the slight differences in the mean constants, however, were not statistically significant. Therefore, the results obtained in the two concentrations of tetrodotoxin were lumped

leading to the following over-all means ($\pm$ S.E. of mean; $n = 14$): $k_1 = 2{\cdot}96 \pm 0{\cdot}19$ (10^6 M^{-1} sec^{-1}), $k_2 = 1{\cdot}42 \pm 0{\cdot}09$ (10^{-2} sec^{-1}), $K = 3{\cdot}31 \pm 0{\cdot}17$ nM, $k_2/k_1 = 5{\cdot}10 \pm 0{\cdot}44$ nM.

The constants k_1 and K can also be obtained from the onset in two different tetrodotoxin concentrations. Let τ_{on} and τ'_{on} be the onset time constants in [TTX] and [TTX]', respectively. Eqn. (5) then yields, by eliminating k_2,

$$k_1 = \frac{1/\tau'_{on} - 1/\tau_{on}}{[\text{TTX}]' - [\text{TTX}]}. \tag{8}$$

Also, since $k_2/k_1 = K$, eqn. (5) can be written

$$1/(k_1 \tau_{on}) = [\text{TTX}] + K \tag{9}$$

so that

$$\frac{\tau_{on}}{\tau'_{on}} = \frac{[\text{TTX}]' + K}{[\text{TTX}] + K} \tag{10}$$

from which we obtain

$$K = \frac{\tau'_{on}[\text{TTX}]' - \tau_{on}[\text{TTX}]}{\tau_{on} - \tau'_{on}}. \tag{11}$$

In Table 3 the values of k_1 and K were calculated with eqns. (8) and (11) and it is interesting to note that K derived from onset alone (4·0 nM) agreed better with K obtained at equilibrium (3·3 nM) than did k_2/k_1 (5·1 nM) when computed from onset and offset time constants.

TABLE 3. Constants calculated exclusively from the onset in 3·1 nM (τ_{on}) and in 15·5 nM-TTX (τ'_{on}). K was obtained with eqn. (11), i.e. from kinetic data

Axon	$1/\tau_{on}$ (sec^{-1})	$1/\tau'_{on}$ (sec^{-1})	k_1 (10^6 M^{-1} sec^{-1})	K (nM)
g	0·0244	0·0741	4·01	2·99
h	0·0267	0·0813	4·40	2·98
i	0·0181	0·0427	1·98	5·97
j	0·0208	0·0581	3·01	3·82
k	0·0222	0·0575	2·85	4·72
l	0·0200	0·0637	3·52	2·58
m	0·0247	0·0617	2·98	5·17
	Mean		3·25	4·03
	$\pm$ S.E. of mean		$\pm$ 0·30	$\pm$ 0·48

Onset and offset of tetrodotoxin effect on maximum rate of rise

In this series of experiments on *Rana* fibres the maximum rate of rise, $\dot{V}_A$, of the action potential was recorded while the perfusate was suddenly changed from Ringer to a tetrodotoxin solution. Immediately after the change, $\dot{V}_A$ began to decrease at a rate that depended on the concentration of tetrodotoxin as illustrated by Fig. 3. In this Figure the steady-state

reduction of $\dot{V}_A$ by the higher concentration was less than expected from the mean values in Fig. 1. This difference was caused by the 3 msec super-threshold stimuli of constant amplitude that were employed in the kinetic experiments to prevent spike failure when the threshold rose under the influence of the toxin. As will later be shown, the effect of stimulus strength on $\dot{V}_A$ did not impair the kinetic results.

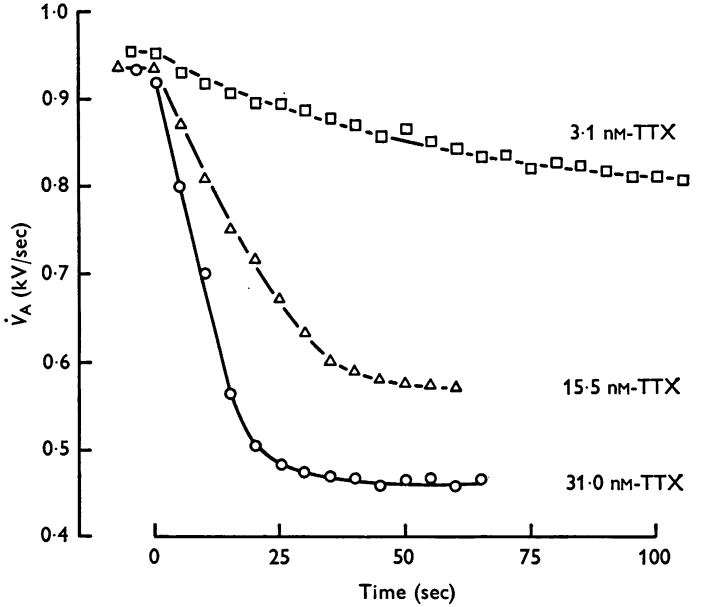

Fig. 3. Onset of the TTX effect on $\dot{V}_A$, the maximum rate of rise of the action potential. Ordinate, $\dot{V}_A$ in 10^3 V/sec; abscissa, time after change from Ringer solution to either 3·1 nM (□), 15·5 nM (△) or 31 nM-TTX (○). The curves were drawn by eye. Experiment on a *Rana* fibre at 22° C.

Since $\dot{V}_A$ is not linearly related to $\bar{P}_{Na}$ or the fraction of free sodium sites, plots of the kind shown in Fig. 3 and the pertinent washout curves do not directly yield the rates of receptor occupation. To arrive at these rates a more indirect approach had to be chosen which rested on the following assumptions. 1. The reaction of tetrodotoxin with the membrane sites is of the same type as in *Xenopus* so that the changes of receptor occupancy, $y(t)$, are given by eqns. (3) and (4). 2. The relation between $\dot{V}_A$ and y, although non-linear, is instantaneous and monotonic and is reproducible for a given preparation. The validity of these assumptions can be tested with the aid of washout curves whose underlying process, the exponential decline of y, is independent of tetrodotoxin concentration according to eqn. (4). From this equation it follows that an offset curve,

$y(t)$, starting with y_0 must be part of another offset curve whose initial value is y_0', provided $y_0' > y_0$. Hence, if the assumed relation between $\dot{V}_A$ and y is correct, one should be able to superimpose the curve along which $\dot{V}_A$ recovers during washout. This criterion was tested in six experiments yielding very satisfactory results. An example is given in Fig. 4 which shows the superposition of curves recorded during the washout of three different concentrations of tetrodotoxin.

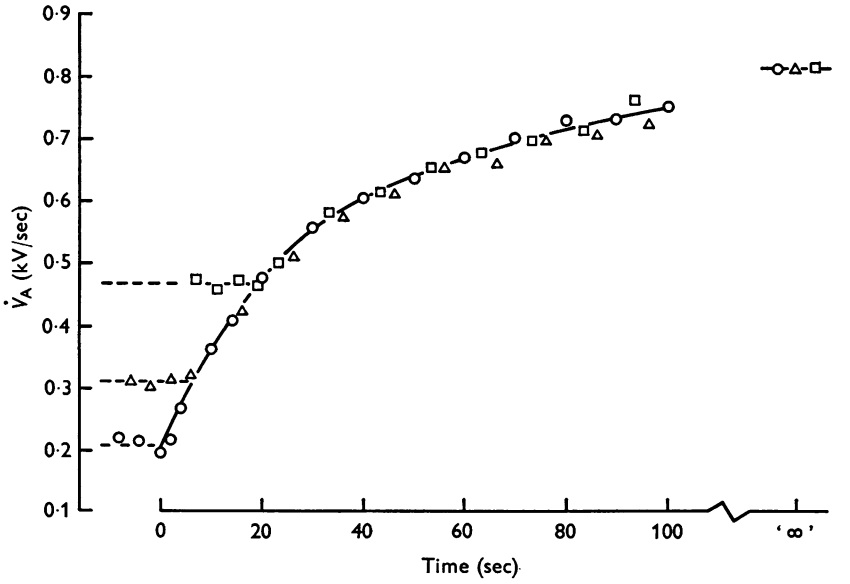

Fig. 4. Recovery of the maximum rate of rise, $\dot{V}_A$, following exposures to 6·2 nM (□), 15·5 nM (△) and 31 nM-TTX (○). Ordinate, $\dot{V}_A$ in 10^3 V/sec; abscissa, time from the washout of the highest [TTX]. Common curve drawn by eye. Experiment on a *Rana* fibre at 19·5° C.

The time course of $\dot{V}_A$ during onset and offset was characterized by time intervals from which the rate constants of the tetrodotoxin-site reaction were eventually calculated as described in the Appendix. In each experiment the node was first equilibrated in 3·1 nM tetrodotoxin and $\dot{V}_1$, the stationary value of $\dot{V}_A$, was determined at a rate of stimulation of 1/sec. After the toxin had been completely washed away, 15·5 nM tetrodotoxin was applied under continuous stimulation until $\dot{V}_A$ had reached a steady value, $\dot{V}_2$, and subsequently the complete washout was recorded. This procedure was finally repeated with a 31 nM concentration. Throughout the experiment the duration and strength of the stimuli were kept constant. The values of $\dot{V}_A$ during the kinetic runs were plotted as a function of time and smooth curves were drawn through the points. From

the onset curve in 31 nM tetrodotoxin, t_1 and t_2 were determined as the time at which $\dot{V}_A$ had declined to $\dot{V}_1$ and $\dot{V}_2$, respectively. During washout, t_{II} and t_I denoted the respective times after the change of solution at which $\dot{V}_A$ had recovered to $\dot{V}_2$ and $\dot{V}_1$. The curves obtained with 15·5 nM tetrodotoxin were analysed accordingly and the time to reach $\dot{V}_1$ (the only point of calibration) during onset and offset was denoted t_3 and t_{III}, respectively.

On three fibres the entire procedure was repeated with threshold stimuli which required continuous readjustment of stimulus strength during onset and offset. As a consequence not every stimulus was successful. Nevertheless, when the results were analysed using values of $\dot{V}_1$ and $\dot{V}_2$ that were also obtained with threshold stimuli, the characteristic times (t_1, t_2, etc.) did not significantly differ from those determined with constant superthreshold impulses in the same preparation. Hence the use of superthreshold impulses in the kinetic experiments appeared to be justified.

The mean results (± S.E. of means) from all kinetic experiments at 20–22° C were: $t_1 = 5{\cdot}7 \pm 0{\cdot}5$ sec ($n = 10$), $t_2 = 18{\cdot}5 \pm 1{\cdot}1$ sec ($n = 11$), $t_3 = 10{\cdot}8 \pm 0{\cdot}6$ sec ($n = 10$), $t_I = 44{\cdot}1 \pm 3{\cdot}5$ sec ($n = 9$), $t_{II} = 5{\cdot}3 \pm 0{\cdot}6$ sec ($n = 11$) and $t_{III} = 38{\cdot}6 \pm 3{\cdot}0$ sec ($n = 10$). It should be noted here that eqn. (4) and our assumptions require t_{III} to be equal to $t_I - t_{II}$, which clearly was the case. The mean time values were used for the calculations that are given in detail in the Appendix with the following results: $k_1 = 4 \times 10^6$ M^{-1} sec^{-1}, $k_2 = 1{\cdot}4 \times 10^{-2}$ sec^{-1} and $K = 3{\cdot}4$ nM. The constants compare favourably with those found in the kinetic experiments on *Xenopus* fibres (see p. 177): $k_1 = 3 \times 10^6$ M^{-1} sec^{-1}, $k_2 = 1{\cdot}4 \times 10^{-2}$ sec^{-1} and $K = 3{\cdot}3$ nM.

In another three fibres at 23–24° C the effect of 155 nM tetrodotoxin was compared with that of 31 nM. The procedure was somewhat different since $\dot{V}_1$ was obtained after equilibration in 6·2 nM (instead of 3·1 nM) so that t_1 and t_I had a different meaning in these experiments. Also, the toxin was applied only until $\dot{V}_A$ had decreased below $\dot{V}_2$ which, as usual, corresponded to the steady effect in 15·5 nM tetrodotoxin. Since the washout was started before a stationary effect had been attained, only $\Delta t_{off} = t_I - t_{II}$ could be determined. The mean Δt_{off} (± S.E. of mean) observed after tetrodotoxin applications of 31 and 155 nM was $12{\cdot}7 \pm 0{\cdot}7$ and $12{\cdot}7 \pm 0{\cdot}9$ sec, respectively. This is in good agreement with eqn. (4) which predicts the rate of offset to be independent of concentration. The onset in 155 nM tetrodotoxin was rather fast so that a more reliable measure of it was obtained from $\Delta t_{on} = t_2 - t_1$; its mean value (± S.E. of mean) was $1{\cdot}2 \pm 0{\cdot}1$ sec. This agreed well with $\Delta t_{on} = 1{\cdot}3$ sec as calculated with eqn. (3) using the mean k_2 and K determined for a slightly lower temperature (see preceding section). The calculation of Δt_{on} for 31 nM tetrodotoxin yielded 8·1 sec, the observed value was $7{\cdot}7 \pm 0{\cdot}2$ sec (± S.E. of mean). Only for Δt_{off} did

the calculated value, 17·1 sec, clearly differ from the observed value (12·7 sec).

Finally, in four experiments at 21° C the onset in 31 nM tetrodotoxin was tested at different rates of stimulation, 0·2, 2, and 20 Hz. The observed onset curves were practically identical; slight deviations that were occasionally found with 0·2 Hz were most likely the result of the poor temporal resolution at this low frequency. When the frequency was increased from 0·2 to 20 Hz during the onset the transition was smooth and the curve could fully be superimposed on one determined at a single frequency.

Latency of tetrodotoxin effect on $\dot{V}_A$ in Rana fibres

The relatively fast onset in 155 nM tetrodotoxin which, incidentally, rendered the fibre inexcitable within a few seconds, permitted an estimate of the latency with which the first sign of the toxin action on $\dot{V}_A$ appeared after application. In these experiments the node was stimulated at a rate of 50 stimuli/sec to improve the temporal resolution; the film speed was increased accordingly. As in all previous experiments on *Rana* fibres, $t = 0$ was defined by the step in photo current when the stopcock was turned (see Method). About 40–50 msec passed after this event before the new solution had reached the node. This short period could previously be neglected; in the latency experiments, however, some correction was attempted by recording, under identical circumstances, the delay with which $\dot{V}_A$ responded to an abrupt change in sodium concentration. An example is given in Fig. 5 where the triangles denote $\dot{V}_A$ when sodium was suddenly changed from 59 mM (choline substituting for sodium) to 118 mM (Ringer solution) in the absence of tetrodotoxin. In a second run the solution was changed from 59 mM sodium to Ringer solution that contained 155 nM tetrodotoxin. As a result (circles) $\dot{V}_A$ first increased, due to the rising concentration of sodium, but soon decreased again as the toxin started to act. The difference between the two curves, representing the tetrodotoxin effect, was drawn as an interrupted line in Fig. 5 and it is obvious that there was very little delay in the onset of the toxin action.

In five tests of the kind illustrated by Fig. 5, done on two fibres, $\dot{V}_A$ started to increase 58 ± 5 msec (mean $\pm$ S.E. of mean; $n = 6$) and the tetrodotoxin action set in 136 ± 6 msec ($n = 5$) after the photoelectric signal. Comparable results were obtained on another two fibres to which the same concentration of tetrodotoxin was applied while the sodium concentration was kept at 118 mM. In these experiments the stimulus frequency was raised to 80 Hz and the mean latency of the tetrodotoxin action was 121 ± 7 and 194 ± 17 msec (eight runs in each fibre). In these two fibres a sudden change from 59 to 118 mM sodium (without tetrodotoxin) was also tested and the mean latency of the increase in $\dot{V}_A$ was 70 ± 2 and

97 ± 3 msec ($n = 8$ and 9). Considering these results and those of the experiments illustrated by Fig. 5, the first sign of tetrodotoxin action was observed, on the average, about 75 msec after the start of the pure sodium effect. This interval consisted of the time that tetrodotoxin required, in excess of that for sodium, to diffuse through the perinodal space and of the reaction time until a minimum fraction of sites was occupied to give the first detectable effect on $\dot{V}_A$. This fraction was not exactly known but since

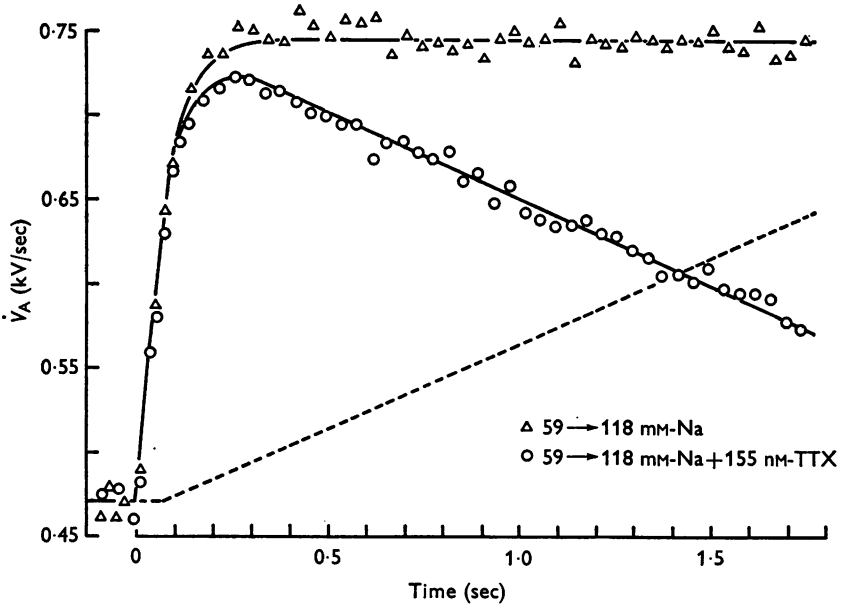

Fig. 5. Latency of the effect of 155 nM-TTX on $\dot{V}_A$ of action potentials elicited every 20 msec. Ordinate, $\dot{V}_A$ in 10^3 V/sec; abscissa, time after the start of the sodium effect. Triangles refer to a change from 59 to 118 mM-Na without TTX, circles refer to a change from 59 to 118 mM-Na + 155 nM-TTX. The smooth curves were drawn through the points by eye and the difference between the two curves, the TTX effect, was given as an interrupted line. *Rana* experiment at 20·8° C.

at equilibrium with 0·31 nM tetrodotoxin ($y_\infty = 0{\cdot}08$) hardly any effect was observed we shall assume $y = 0{\cdot}04$ to be the minimum. With 155 nM tetrodotoxin ($y_\infty = 0{\cdot}98$), $\tau_{on} = 1{\cdot}6$ sec can be calculated from our earlier data and $y = 0{\cdot}04$ would be attained after 65 msec leaving a smaller fraction of the interval to be accounted for by the excess diffusion time due to the expected lower diffusion coefficient of tetrodotoxin. In spite of the rather arbitrary assumptions, our latency experiments thus strongly suggest that the toxin had ready access to the sodium sites of the nodal membrane.

Influence of temperature on the tetrodotoxin action on Xenopus nodes

In this series of voltage clamp experiments the membrane was depolarized every 2 sec by impulses (V = 50–60 mV) whose duration was adjusted at each temperature to last well beyond the peak of the inward current. The hyperpolarizing prepulse (V = −40 mV) was increased in duration to 150 msec. Only one concentration of tetrodotoxin, 3·1 or 15·5 nM, was

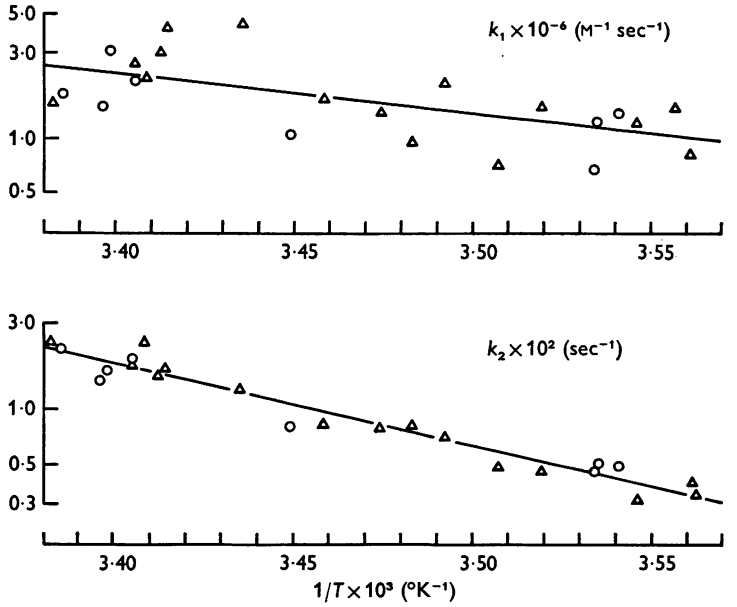

Fig. 6. Dependence of the rate constants k_1 and k_2 on temperature. Arrhenius plot of data from 10 *Xenopus* experiments with either 3·1 nM (○) or 15·5 nM-TTX (△). Logarithmic ordinates of the upper and lower diagram for k_1 and k_2, respectively. The abscissae give the reciprocal of the absolute temperature. The lines of regression were calculated as log $k_1 = 13{\cdot}6737 - 2151(1/T)$, k_1 being expressed in M^{-1} sec^{-1}, and log $k_2 = 13{\cdot}4892 - 4482(1/T)$, k_2 being expressed in sec^{-1}. The respective correlation coefficients were $r = 0{\cdot}62$ and $r = 0{\cdot}97$. The correlation was based on $23 - 2 = 21$ pairs of observations being significant to the levels $P < 0{\cdot}005$ and $P < 0{\cdot}001$, respectively.

tested in each preparation. The sequence of the two or three temperatures was varied to avoid possible systematic errors. At each temperature the onset and offset of the I_{Na} inhibition were recorded and the toxin was completely washed out before another run at a different temperature was started. The results were analysed as described before. The rate constants obtained from ten fibres are shown in the two Arrhenius plots of Fig. 6,

i.e. the constants were plotted on a logarithmic scale versus the reciprocals of the absolute temperature. In both the upper (k_1) and lower (k_2) plot, circles and triangles refer to experiments with 3·1 and 15·5 nM tetrodotoxin, respectively. The lines through the points were calculated lines of regression which yielded the following values for 22° C ($1/T = 3{\cdot}388 \times 10^{-3}$ $°K^{-1}$): $k_1 = 2{\cdot}44 \times 10^6$ M^{-1} sec^{-1} and $k_2 = 2{\cdot}02 \times 10^{-2}$ sec^{-1}. For 12° C ($1/T = 3{\cdot}507 \times 10^{-3}$ $°K^{-1}$), k_1 was $1{\cdot}35 \times 10^6$ M^{-1} sec^{-1} and k_2 was $0{\cdot}59 \times 10^{-2}$ sec^{-1}. Hence the Q_{10} for this range of temperature was 1·81 and 3·42, respectively. The Arrhenius energy of activation, E_a, was obtained from the slopes of the regression lines which are equivalent to $-E_a/2{\cdot}303R$, where R is the universal gas constant. The calculation of E_a gave 9·84 kcal/mole (k_1) and 20·5 kcal/mole (k_2).

The mean k_2 in the earlier series of experiments at room temperature was $1{\cdot}4 \times 10^{-2}$ sec^{-1} which corresponds with the value for 19° C in Fig. 6. The agreement was less satisfactory for k_1 which, when calculated for this temperature, was $2{\cdot}05 \times 10^6$ M^{-1} sec^{-1} as compared to $2{\cdot}96 \times 10^6$ M^{-1} sec^{-1} in the previous experiments. Although this discrepancy may reflect the larger scatter of the k_1 values, another possible explanation is that the tetrodotoxin stock solution had lost some of its potency in the 3–4 months that had passed between the two series of experiments. While k_2 would be unaffected, the value of k_1 as calculated with eqn. (7) on p. 176 would be too small if the actual tetrodotoxin concentration were smaller than the nominal one used in the calculation. The following results also point to a partial inactivation of the tetrodotoxin stock.

To assess the temperature dependence of K, the equilibrium dissociation constant, all temperature experiments with 3·1 nM tetrodotoxin were gathered, i.e. the four experiments contained in Fig. 6 and another six incomplete experiments. Each preparation was tested at two different temperatures and the equilibrium occupancy, y_∞, was determined as in the early series at room temperature; between the two runs the toxin was completely washed out. K was calculated from y_∞ with eqn. (2) and the results, again presented in an Arrhenius plot, are shown in Fig. 7. From the line of regression $E_a = 7{\cdot}0$ kcal/mole is calculated and $K = 4{\cdot}0$ nM is obtained for 19° C which is to be compared with the mean K of 3·6 nM as observed in the earlier experiments at room temperature (see Table 1). Again, a slight inactivation of the tetrodotoxin stock solution could account for the difference. The values of K calculated for 22 and 12° C were 4·59 and 2·99 nM, respectively, corresponding to a Q_{10} of 1·53 for this range of temperature. Compared with this the ratio of the Q_{10} values for the rate constants, $3{\cdot}42/1{\cdot}81 = 1{\cdot}89$, suggested a higher Q_{10} for K.

An attempt was also made to assess the temperature dependence of k_1 as determined from the onset alone with the aid of eqn. (8). Therefore,

$1/\tau_{on}$ (3·1 nM tetrodotoxin) and $1/\tau'_{on}$ (15·5 nM tetrodotoxin) were correlated with $1/T$ and the respective lines of regression were found to be $\log(1/\tau_{on}) = 9{\cdot}8545 - 3385\ (1/T)$ and $\log(1/\tau'_{on}) = 9{\cdot}4467 - 3134\ (1/T)$ with $r = 0{\cdot}94$ and 0·82. From these equations we calculated $1/\tau_{on} = 0{\cdot}02424\ \text{sec}^{-1}$ and $1/\tau'_{on} = 0{\cdot}06720\ \text{sec}^{-1}$ for 22° C and $1/\tau_{on} = 0{\cdot}00958\ \text{sec}^{-1}$ and $1/\tau'_{on} = 0{\cdot}02850\ \text{sec}^{-1}$ for 12° C. Inserting these values into eqn. (8) yielded $k_1 = 3{\cdot}47 \times 10^6\ \text{M}^{-1}\ \text{sec}^{-1}$ for 22° C and $1{\cdot}54 \times 10^6\ \text{M}^{-1}\ \text{sec}^{-1}$ for 12° C which corresponds to a Q_{10} of 2·25. With this value the ratio of

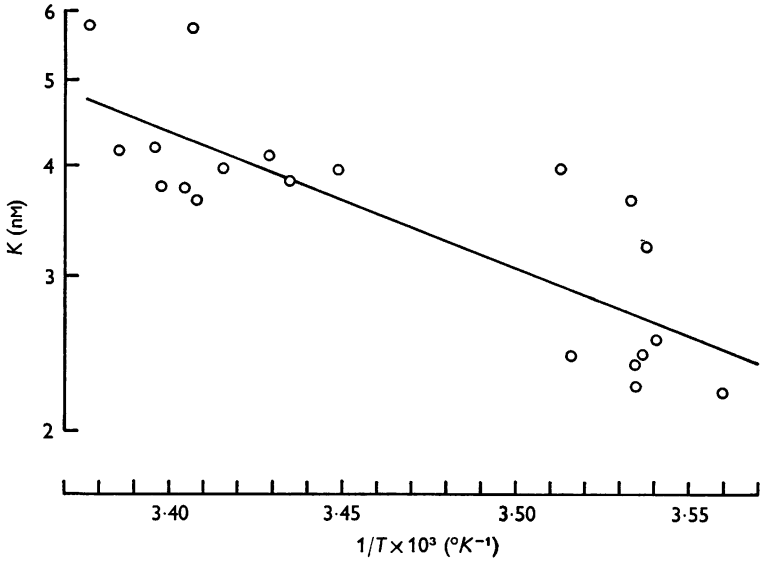

Fig. 7. Temperature dependence of K, the equilibrium dissociation constant. Arrhenius plot of data from ten equilibrium experiments with 3·1 nM-TTX on *Xenopus* fibres. Ordinate, K plotted on a logarithmic nM scale. Abscissa, reciprocal of the absolute temperature. If K is expressed in nM, the line of regression is given by $\log K = 5{\cdot}8553 - 1534(1/T)$ with a correlation coefficient $r = 0{\cdot}82$. The correlation was based on $20 - 2 = 18$ pairs of observations with $P < 0{\cdot}001$.

the Q_{10} for k_2/k_1 becomes $3{\cdot}42/2{\cdot}25 = 1{\cdot}52$ which is equal to the Q_{10} for K as observed in the equilibrium experiments. In an Arrhenius plot of k_1 determined solely from the onset, a slope of -3000 is obtained which is equivalent to an activation energy of 13·7 kcal/mole. It should be noted that a decrease of the effective tetrodotoxin concentration of the stock would affect only the absolute values of k_1 but not their change with temperature.

Temperature effect on the rate of tetrodotoxin action on $\dot{V}_A$

In these experiments on *Rana* fibres the rate of action of 31 nM tetrodotoxin was investigated. The procedure was of the modified kind described on p. 180, i.e. $\dot{V}_1$ and $\dot{V}_2$ were the stationary $\dot{V}_A$ in 6·2 and 15·5 nM tetrodotoxin, respectively. Also, to keep the duration of an experiment at a reasonable length, the toxin was washed out before $\dot{V}_A$ had reached its steady-state value in 31 nM so that only $\Delta t_{off} = t_I - t_{II}$ could be measured

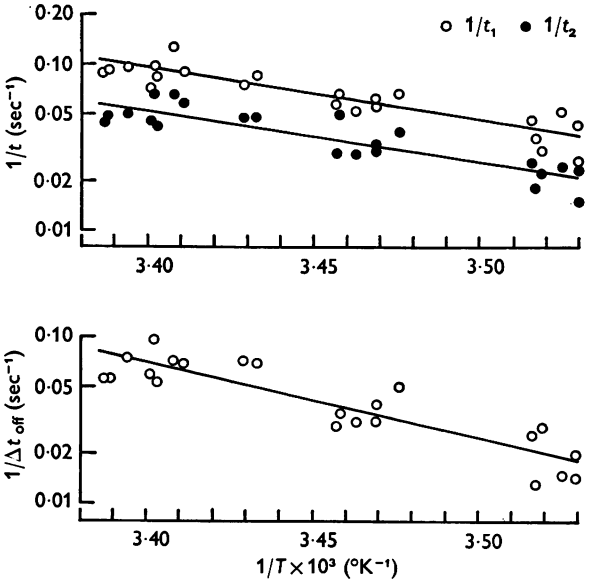

Fig. 8. Temperature dependence of the times characterizing the onset and offset of the effect of 31 nM-TTX in ten *Rana* fibres. Ordinates, reciprocals of the time values in sec^{-1}; abscissae, reciprocal absolute temperature. The upper Arrhenius plot shows $1/t_1$ (○) and $1/t_2$ (●), the lower plot shows $1/\Delta t_{off}$. The calculated lines of regression were: log $(1/t_1) = 9{\cdot}6691 - 3149\,(1/T)$, log $(1/t_2) = 9{\cdot}4486 - 3160\,(1/T)$ and log $(1/\Delta t_{off}) = 13{\cdot}7595 - 4390\,(1/T)$. The respective correlation coefficients, r, were 0·90, 0·90 and 0·89. Each of the correlations was based on $22 - 2 = 20$ pairs of observations; for each correlation $P < 0{\cdot}001$.

to characterize the offset. The entire procedure of calibrating and of recording the onset and offset was repeated after each step in temperature because $\dot{V}_A$ itself depends on temperature (see e.g. Hodgkin & Katz, 1949). The sequence of the steps was varied and at each temperature the characteristic times t_1, t_2 and Δt_{off} were determined. Fig. 8 summarizes the results of ten experiments in the form of Arrhenius plots with the reciprocals of the times as the dependent variables; $1/t_1$ and $1/t_2$ are shown in the upper plot,

$1/\Delta t_{off}$ in the lower plot. In the upper plot the lines of regression ran practically in parallel so that the Q_{10} for $1/t_1$ and $1/t_2$, 2·37 and 2·33 (between 12 and 22° C), were almost identical. The Q_{10} for $1/\Delta t_{off}$ was 3·33 in this range of temperature. Of these data only $1/t_2$ could be compared with the results of the earlier experiments at room temperature since only $\dot{V}_2$ had a common definition for the two series. In the early experiments the mean t_2 was 18·5 sec so that $1/t_2 = 0{\cdot}054$ sec^{-1} which in Fig. 8 is the mean value at 21·7° C ($1/T = 3{\cdot}392 \times 10^{-3}$ °K^{-1}), thus demonstrating the good agreement between the two series.

The difference in Q_{10} values for the quantities characterizing onset and offset is to be expected if, as in *Xenopus*, k_2 has a higher Q_{10} than k_1 since the offset is determined by k_2 alone while the onset rate depends on both k_1 and k_2. However, the precise relation between the Q_{10} for the reciprocals of the time values and the Q_{10} for the rate constants is more complicated because of the implication that K decreases in the cold. Consequently, the equilibrium receptor occupancy in 6·2 and 15·5 nM tetrodotoxin increases whereby that in 6·2 nM is more affected than that in the higher concentration.

This complication and, possibly, the larger scatter of the data in the temperature experiments led to inconsistent results when the original method of the Appendix was used to calculate k_2 and K. Therefore, definite assumptions about the Q_{10} for K were made on which, however, the values of k_2 in the cold depended. For a Q_{10} of 1·53 as determined in the equilibrium experiments on *Xenopus* (Fig. 7) and with the average *Rana* data at room temperature ($k_2 = 1{\cdot}4 \times 10^{-2}$ sec^{-1} and $K = 3{\cdot}4$ nM), $k_2 = 0{\cdot}41 \times 10^{-2}$ sec^{-1} was calculated for 11·7° C as described in the later section of the Appendix. Hence a drop in temperature by 10 degrees reduced k_2 by a factor of 1·4/0·41 = 3·42 which is identical with the Q_{10} for k_2 determined in the *Xenopus* experiments. This agreement is clearly fortuitous since assuming a higher Q_{10} for K would also increase the Q_{10} for k_2.

While only a rough estimate of the temperature dependence of k_2 can be made, a lower limit can be obtained by considering the extreme case that K is independent of temperature. The equation of the Appendix (A5) and (A6) then predict that in Fig. 8 the lines of regression for both onset and offset should have the same slope which clearly was not the case. On the other hand, a Q_{10} for K as low as 1·3 still yields a $Q_{10} = 3{\cdot}1$ for k_2 so that one may conclude that the real Q_{10} for the dissociation rate constant is at least 3 and that there is no basic difference in the processes underlying the tetrodotoxin action on *Rana* and *Xenopus* nodes.

DISCUSSION

The present experiments show that, within the precision of the measurements, the reaction of tetrodotoxin with the nodal membranes of *Xenopus* and *Rana* appears to be identical in size, rate and temperature dependence. The equilibrium dissociation constant, K, of this reaction of about 3·5 nM at room temperature agrees well with the earlier observations in amphibian nerve that 3 and 31 nM tetrodotoxin, respectively, reduce the peak I_{Na} to about 50 % (Hille, 1966) and 10 % (Koppenhöfer & Vogel, 1969). Quite similar results were obtained with giant axons of squid (Cuervo & Adelman, 1970) and lobster (Takata, Moore, Kao & Fuhrman, 1966) as well as in non-myelinated rabbit fibres (Colquhoun & Ritchie, 1972*a*) in which binding of tritium-labelled tetrodotoxin has been found to contain a Langmuir component with K of about 3 nM (Colquhoun, Henderson & Ritchie, 1972). Exact comparison of different preparations, however, has to consider temperature (Colquhoun & Ritchie, 1972*a* and this paper) and pH (Narahashi, Moore & Frazier, 1969) and it is limited by possible variations in the potency of different tetrodotoxin samples. This was pointed out by Hille who, in a later publication (1968), reported K to be as low as 0·6 nM, however, at low temperature. Finally, it should be noted that a considerably higher concentration of tetrodotoxin is needed to halve the compound action potential (Keynes *et al.* 1971) or the conduction velocity (Colquhoun & Ritchie, 1972*a*). Similarly, Fig. 1 of our paper shows that slightly more than 10 nM tetrodotoxin was necessary for a 50 % reduction of $\dot{V}_A$.

While there is surprisingly good agreement of the equilibrium experiments, the kinetic data of different preparations vary considerably as can be illustrated by the onset in 15 nM tetrodotoxin. In our experiments the mean τ'_{on} at 20° C was 17·5 sec (see line of regression on p. 185). The equivalent time constant calculated from the mean data of the rabbit vagus (Colquhoun & Ritchie, 1972*b*) is 5·4 min. At 7° C the mean τ'_{on} of our experiments is 55·3 sec while the time constant calculated from the squid data is 2·4 min (Cuervo & Adelman, 1970). Finally, the onset time constant is about 6 min in lobster axons at 7° C (estimated from Fig. 3*a* in Takata *et al.* 1966). Thus the fastest onset of tetrodotoxin action is found in amphibian nodes of Ranvier, most probably because of the easy access to its membrane as suggested by our latency experiments. However, compared to the rate of action of tetraethylammonium ions on the nodal potassium permeability (Vierhaus & Ulbricht, 1971*b*) the tetrodotoxin effect develops rather slowly. This is confirmed in a short communication of Stämpfli (1968) who mentions that a concentration of 30 nM produces 50 % of its effect within 8 sec. Hille's (1968) earlier, considerably higher

rates of action have turned out to be erroneous; in a recent clarifying experiment he found rates that were within 40% of ours (B. Hille, personal communication). The important question then is whether the rates observed in our experiments are the true rates of the tetrodotoxin-receptor reaction as has tacitly been assumed in the treatment of our data.

The answer to this question is not as obvious as it may seem. Of course, straightforward diffusion limitation of the reaction can be ruled out because of the short latency, the exponential recovery of I_{Na} during washout which was independent of tetrodotoxin concentration and because τ_{off}/τ_{on} was equal to $([TTX]+K)/K$. This relation is predicted by eqns. (5) and (6) for the case of a rate-limiting tetrodotoxin-receptor reaction. Unfortunately, this is not an unequivocal criterion since an approximately 'correct' ratio of time constants can be expected in the case of a more complicated access limitation introduced by Rang (1966; see also Thron & Waud, 1968; Colquhoun & Ritchie, 1972*b*). The original concept is based on a space that contains the receptors and to which access of the drug is limited by a diffusion barrier. If the receptor concentration, i.e. the site density in relation to the volume of the space, is high, rapid binding of tetrodotoxin to the sites can markedly retard the rise in tetrodotoxin concentration in this compartment and in turn slow the rate of receptor occupancy. The original equation of Rang (1966) has recently been presented in an integrated form by Colquhoun & Ritchie (1972*b*) for the case that the drug concentration in the receptor-containing compartment rises exponentially in the absence of binding. Let τ_0 be the time constant of this concentration increase and let the binding be instantaneous and to the receptors only, then τ_0' during the initial onset approaches $\tau_0 M/KV$ for values of M/KV of several hundred, where M = concentration of binding sites, K = equilibrium dissociation constant of the drug-site reaction and V = volume of the compartment.

We have done a few calculations on a desk calculator using the complete equation. Our object was not so much to attempt a quantitative fit of our observed τ_{on}' as to test the model on changing the temperature. Hence $M/KV = 100$ at room temperature was rather arbitrarily chosen. The decline of y/y_0 during the washout of 15·5 nM tetrodotoxin was calculated and plotted against t/τ_0, whereby $y_0 = 0{\cdot}812$ since $K = 3{\cdot}6$ nM. To simulate a drop in temperature by 10° C, M/KV was raised to 150 to allow for the decrease of K (see Fig. 7); y_0 was increased accordingly to 0·866. These were the only changes to be made since binding was assumed to be so rapid that it should still be 'instantaneous' in the cold. The resulting offset curves were intermediate between the curves in Fig. 1 *a* and *b* of Colquhoun & Ritchie (1972*b*), i.e. exponential only in their later parts. The dimensionless time value $t_{0{\cdot}5}/\tau_0$, at which y had declined to $0{\cdot}5\,y_0$ was in-

creased by a factor of 1·36 on cooling. Considering that τ_0 is determined by diffusion with a relatively low Q_{10} of, say, 1·3, $t_{0.5}$ should not increase by more than a factor of 1·8 ($= 1{\cdot}36 \times 1{\cdot}3$) when the temperature drops by 10° C. This is in clear contrast to the observed Q_{10} of 3·42 for $1/\tau_{off} = k_2$. The corresponding calculation for the onset revealed hardly any change of $t_{0.5}/\tau_0$ so that $t_{0.5}$ was solely determined by τ_0. Again, a higher Q_{10} of 2·4 for $1/\tau'_{on}$ was found in the experiments with 15·5 nM tetrodotoxin (see p. 185). Hence neither the temperature dependence nor the shape of the experimental offset (and onset) curves agreed with the original model of instantaneous binding to receptors within a compartment to which access was limited. For a more realistic description a finite reaction speed must probably be incorporated into this model. This would complicate the calculation to the extent that it appears justified only if such a compartment could be precisely defined for the node. In contrast to the squid giant axon where the functional Frankenhaeuser–Hodgkin space seems to have its clear anatomical substrate (Frankenhaeuser & Hodgkin, 1956), no indication of a compartment has so far been found in the node (Meves, 1961; Ulbricht, 1963; Vierhaus & Ulbricht, 1971*a*).

In their squid experiments Cuervo & Adelman (1970) found k_2/k_1 ($= K$) to be 1·7 times K obtained from equilibrium data. In the present study a similar though less severe discrepancy was observed: the average k_2/k_1 of the data in Table 2 (5·1 nM) was 1·5 times K from equilibrium results (3·3 nM). The agreement was better with the kinetic data determined from onset alone (4·0 nM; last column in Table 3) which were only 1·2 times K. The significance of these differences and of comparable inconsistencies in the respective Q_{10} values remained unclear.

The squid experiments (Cuervo & Adelman, 1970) yielded, at 7° C, $k_1 = 0{\cdot}202 \times 10^8$ M^{-1} min^{-1} ($= 0{\cdot}34 \times 10^6$ M^{-1} sec^{-1}) and $k_2 = 0{\cdot}116$ min^{-1} ($= 0{\cdot}19 \times 10^{-2}$ sec^{-1}) while in our experiments k_1 was $1{\cdot}0 \times 10^6$ M^{-1} sec^{-1} and k_2 was $0{\cdot}31 \times 10^{-2}$ sec^{-1} at this temperature (see Fig. 6). Incidentally, k_1 calculated solely from the onset at 7° C was also $1{\cdot}0 \times 10^6$ M^{-1} sec^{-1}. The lower values in squid may reflect some diffusion-controlled process, especially since the rates were less dependent on temperature than in amphibian nerve. From the squid data Q_{10} values of 1·38 and 2·10 can be extracted for k_1 and k_2, respectively (between 7 and 17° C). These are to be compared with the respective Q_{10} values of 1·81 (or 2·25 if derived from the onset alone) and 3·42 in our experiments. If the temperature dependence can be taken as an indication of the rate-controlling process, access limitation appears to play a role in the squid giant axon rather than in the node of Ranvier. Hence the rates determined in our experiments should not differ very much, if at all, from the true rates of the tetrodotoxin receptor reaction.

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APPENDIX

Calculation of rate constants of the drug-receptor reaction from temporal changes in the maximum rate of rise

The following procedure was used to derive the rate constants of the tetrodotoxin site reaction from the time values determined in the kinetic experiments on *Rana* fibres. We introduce an analytical unit of concentration $c = [\mathrm{TTX}]/K = k_1(\mathrm{TTX}]/k_2$ (Waud, 1968) so that eqn. (1) becomes

$$y_\infty = \frac{c}{c+1} \tag{A1}$$

and eqn. (3)

$$y = y_\infty(1 - \mathrm{e}^{-(c+1)k_2 t}) \tag{A2a}$$

or

$$1 - \frac{y}{y_\infty} = \mathrm{e}^{-(c+1)k_2 t}. \tag{A2b}$$

At $t = t_a$ during the onset, y assumes the value y_a which is equivalent to the steady-state occupancy when the receptors are at equilibrium with some lower concentration $c_a = ac$, where $a < 1$. Hence y_a can be related to c_a according to eqn. (A1) so that

$$\frac{y_a}{y_\infty} = \frac{ac + a}{ac + 1}. \tag{A3}$$

Introducing eqn. (A3) into (A2b) yields

$$\frac{1-a}{ac+1} = \mathrm{e}^{-(c+1)k_2 t_a}. \tag{A4}$$

so that

$$1/t_a = k_2(c+1)\Big/\ln\frac{ac+1}{1-a} = k_2 A. \tag{A5}$$

When a is known, A is a function of c only.

In our experimental situation the values of y were unknown but it was postulated that $\dot{V}_1$ and $\dot{V}_2$ corresponded to two definite values, y_1 and y_2. Hence in the experiments with 31 nM tetrodotoxin, y_1 was equivalent to y_a with $a = 0{\cdot}1$ and y_2 to y_a with $a = 0{\cdot}5$. According to eqn. (A5) we have $1/t_1 = k_2 A_1$ and $1/t_2 = k_2 A_2$ so that $t_2/t_1 = A_1/A_2$ which is solely determined by c. The ratio A_1/A_2 was computed and plotted against c, being 3·61 for $c = 6$ and 2·77 for $c = 15$. The ratio of the observed mean values was $t_2/t_1 = 18{\cdot}6/5{\cdot}7 = 3{\cdot}26$ which corresponded to $c = 8{\cdot}6$ from which $K = [\mathrm{TTX}]/c = 31$ nM$/8{\cdot}6 = 3{\cdot}61$ nM was obtained. Since $A_1 = 13{\cdot}2$ for

$c = 8{\cdot}6$, k_2 was calculated from t_1 to be $1{\cdot}33 \times 10^{-2}$ sec^{-1} and, since $k_1 = k_2/K$, $k_1 = 3{\cdot}68 \times 10^6$ M^{-1} sec^{-1} was obtained.

The offset was analysed accordingly by introducing eqn. (A3) into (4) resulting in a relation analogous to eqn. (A5)

$$1/t'_a = k_2 \Big/ \ln \frac{ac+1}{ac+a} = k_2 A', \tag{A6}$$

where t'_a is the general expression of t_{I}, t_{II}, etc. and A' depends only on c since a is known. The actual calculation for the washout of 31 nM tetrodotoxin was done with $\Delta t_{\mathrm{off}} = t_{\mathrm{I}} - t_{\mathrm{II}}$ leading to a similar relation $1/\Delta t_{\mathrm{off}} = k_2 A'_{1-2}$. To eliminate k_2, the ratio $\Delta t_{\mathrm{off}}/t_1 = A_1/A'_{1-2}$ was computed as a function of c and plotted. The observed mean Δt_{off} was $44{\cdot}1 - 5{\cdot}3 = 38{\cdot}8$ sec so that $\Delta t_{\mathrm{off}}/t_1 = 38{\cdot}8/5{\cdot}7 = 6{\cdot}81$ which corresponds to A_1/A'_{1-2} for $c = 10{\cdot}87$ yielding $K = 2{\cdot}85$ nM. Since A'_{1-2} was 2·07 for this value of c, $k_2 = 1/(\Delta t_{\mathrm{off}} \times 2{\cdot}07) = 1{\cdot}25 \times 10^{-2}$ sec^{-1} was obtained; $k_1 = 4{\cdot}38 \times 10^6$ M^{-1} sec^{-1} was calculated as a consequence of $k_1 = k_2/K$. Finally, from the experiments with 15·5 nM tetrodotoxin, $c = 8{\cdot}35$ was obtained from the ratio t_{III}/t_3 which gave $K = 3{\cdot}71$ nM. The rate constant k_2 was computed from $1/t_3$ and $1/t_{\mathrm{III}}$ yielding an identical value of $1{\cdot}49 \times 10^{-2}$ sec^{-1} so that $k_1 = 4{\cdot}02 \times 10^6$ M^{-1} sec^{-1}. The three sets of values derived from the onset and offset in the experiments with 15·5 and 31 nM tetrodotoxin agreed surprisingly well and their average was $k_1 = 4 \times 10^6$ M^{-1} sec^{-1}, $k_2 = 1{\cdot}4 \times 10^{-2}$ sec^{-1} and $K = 3{\cdot}4$ nM.

A change in temperature affects eqn. (A5) and (A6) doubly since both k_2 and c change. To simplify the evaluation of k_2 from the observed characteristic times t_1, t_2 and Δt_{off}, the Q_{10} for K (= 1·53) as determined in the *Xenopus* experiments was introduced into the calculation using the average *Rana* data given above. From these data $c = 31$ nM/3·4 nM = 9·1 is obtained at room temperature (21·7° C, see Results) so that at 10 degrees below, c increases to $9{\cdot}1 \times 1{\cdot}53 = 13{\cdot}9$. For this value of c, $A_1 = 9{\cdot}60$, $A_2 = 5{\cdot}40$ and $A'_{1-2} = 5{\cdot}79$ whereby in this series $y_1 = y_a$ with $a = 0{\cdot}2$ so that the definition of t_1 and $\Delta t_{\mathrm{off}} = t_{\mathrm{I}} - t_{\mathrm{II}}$ differed from that in the preceding section. At 11·7° C the mean values (Fig. 8) of $1/t_1$, $1/t_2$ and $1/\Delta t_{\mathrm{off}}$ were $4{\cdot}08 \times 10^{-2}$ sec^{-1}, $2{\cdot}27 \times 10^{-2}$ sec^{-1} and $2{\cdot}22 \times 10^{-2}$ sec^{-1}, respectively, so that the following k_2 values were calculated with eqns. (A5) and (A6): $0{\cdot}43 \times 10^{-2}$ sec^{-1}, $0{\cdot}42 \times 10^{-2}$ sec^{-1} and $0{\cdot}38 \times 10^{-2}$ sec^{-1} with an average of $0{\cdot}41 \times 10^{-2}$ sec^{-1}. Since at 21·7° C the average k_2 was $1{\cdot}4 \times 10^{-2}$ sec^{-1}, a Q_{10} of 3·42 is obtained for this rate constant.

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