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THE INFLUENCE OF pH ON EQUILIBRIUM EFFECTS OF TETRODOTOXIN ON MYELINATED NERVE FIBRES OF *RANA ESCULENTA*

BY W. ULBRICHT AND H.-H. WAGNER

From the Department of Physiology, University of Kiel, D-23 Kiel, Germany

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SUMMARY

1. The experiments were done on single nodes of Ranvier of *Rana esculenta*. The effects of tetrodotoxin and H ions were determined either by the reduction of the maximum rate of rise, $\dot{V}_A$, of action potentials evoked with threshold stimuli or in the voltage clamp by the decrease of the peak Na permeability, P_{Na}.

2. With the tetrodotoxin sample used throughout the investigation the equilibrium dissociation constant, K_T, of the toxin-receptor reaction at neutral pH was determined to be 2·8 nM. Between 1·55 and 15·5 nM tetrodotoxin the normalized value, A, of $\dot{V}_A$, was found to be related to the normalized toxin concentration $c_T = [TTX]/2{\cdot}8$ nM by the empirical equation $\log [(1-A)/A] = 1{\cdot}22 \log c_T - 0{\cdot}573$.

3. On increasing the pH (up to 8·8) the effect of tetrodotoxin diminished as revealed by an increase in A. The apparent reduction of c_T (as calculated from A) suggests that the toxin is active only in its cationic forms.

4. Weakly acid tetrodotoxin solutions ($7{\cdot}3 < pH \leqslant 5{\cdot}5$) reduced A to a lesser degree than did neutral toxin solutions in spite of the inherent depressing effect of acid pH on A ($A = 0{\cdot}5$ at about pH 5·5). In more acid toxin solutions A decreased again and at pH 4·6 it was about equal to the value in toxin-free solution.

5. When, after equilibration in an acid toxin solution, the perfusate was suddenly changed to neutral Ringer solution A jumped to a higher value A' as measured 1 sec after the switch. Since the blocking effect of hydrogen ions subsided within a fraction of a second while the time constant of the toxin washout is of the order of 1 min, A' reflects the number of Na channels blocked by tetrodotoxin at acid pH.

6. In acid toxin-free solution the peak P_{Na} as obtained in voltage clamp experiments was reduced by a voltage-dependent factor $(c_H + 1)^{-1}$ with

$c_H = [H^+]/K_H(E)$ and $K_H(E) = 2{\cdot}04\,\mu M \exp(0{\cdot}34\,EF/RT)$. Adding tetrodotoxin resulted in another reduction by a constant factor p'_T.

7. Experiments employing various combinations of toxin concentration (3·1–93 nM) and pH values (7·3–5·2) confirm the decreased toxin effect at low pH. Moreover, p'_T was smaller (the additional toxin effect larger) when the membrane had been kept depolarized and thus c_H reduced during equilibration. This suggests that tetrodotoxin cations and H ions compete for the same blocking site. A quantitative fit, however, requires additional assumptions.

INTRODUCTION

Tetrodotoxin reduces the size of the Na permeability of the nerve membrane without altering the rates of permeability change. This fact and the quantitative relation between the degree of inhibition and the toxin concentration suggest that a Na channel is completely blocked when one toxin molecule binds to the channel or to a site in its immediate vicinity (Hille, 1968*a*; Cuervo & Adelman, 1970). This notion is fully compatible with recent data on the rates of the toxin action (Schwarz, Ulbricht & Wagner, 1973). Interestingly an increase in H ion concentration, too, leads to a reversible block of Na channels (Hille, 1968*b*; Drouin & The, 1969; Woodhull, 1973; Drouin & Neumcke, 1974). It is therefore tempting to assume that tetrodotoxin and H ions bind to the same site whose occupation results in the occlusion of a channel. This possibility was implied by Hille (1971) who proposed a 'selectivity filter' of the Na channel that (1) included an oxygen acid group at the proton binding site and that (2) could function as a receptor for binding tetrodotoxin in a blocking position. Incidentally, this kind of proton-toxin interaction at the 'filter' would agree with the idea of Kao & Nishiyama (1965) that the toxin molecule, when bound to its site, reaches into the channel and plugs it. Mullins (1973), in contrast, judged an indirect toxin action more likely.

In the present paper the influence of pH on toxin action is investigated and many features of the decreasing toxin effect indeed point to competition of H ions and toxin molecules for the same site. The results, however, are not unequivocally in favour of straightforward competition. Also, the situation is complicated by the fact that the rates of toxin action are influenced by pH as described in the subsequent paper (Ulbricht & Wagner, 1975*b*). Preliminary accounts of the present results have appeared elsewhere (Wagner & Ulbricht, 1973; 1974; Ulbricht & Wagner, 1975*a*).

METHODS

The measurements were done on single myelinated nerve fibres of the frog, *Rana esculenta*.

Voltage clamp experiments. The nerve fibre was situated in a Plexiglass chamber with four pools separated by two conventional Vaseline seals and one air gap. The node of Ranvier under investigation lay in a pool that was continuously perfused. The feedback amplifier with FET input was used in an arrangement that has been described in detail by Nonner (1969). The normal resting potential was assumed to be -70 mV (inside minus outside; Dodge & Frankenhaeuser, 1958) and it was defined as the holding potential in the voltage clamp for which $h_\infty = 0{\cdot}7$. If necessary, the holding potential was readjusted between runs. The potential values were not corrected for a possible attenuation factor (see Hille, 1971).

In most of the actual measurements hyperpolarizing prepulses of 50 msec duration were employed to remove Na inactivation. The early currents were corrected for leakage and capacity currents to yield I_{Na}. The peak value of this current was converted into the Na permeability, P_{Na}, with the constant field equation using $[Na]_o$ and the experimentally determined equilibrium potential, E_{Na} (see Dodge & Frankenhaeuser, 1959).

The rate of exchange of solutions in the chamber was tested, as before (Schwarz *et al.* 1973), by following I_{Na} when $[Na]_o$ was changed from 118 to 59 mM and back; 89 mM was also tested. One half of the concentration increment was reached, on the average (seventeen measurements on two fibres), after 0·58 sec on increasing and after 0·57 sec on decreasing $[Na]_o$.

Action potential measurements. These measurements were done in the double air gap chamber of Stämpfli (1958) in which the exchange of solutions was much faster (see p. 9). The investigated node was placed in a slit across a thin polyethylene tube through which the test solution flowed continuously. The action potential was electronically differentiated to yield the maximum rate of rise, $\dot{V}_A$. A full description of the method has been published (Vierhaus & Ulbricht, 1971).

Solutions. All solutions were buffered with a mixture (7 mM each) of piperazine dihydrochloride and glycylglycine, the pH being adjusted with NaOH. The total Na concentration was brought to 118 mg ions/l. solution by adding solid NaCl; the solution also contained (in mM) KCl, 2·5; $CaCl_2$, 2·0. The buffer system was chosen since it can be used over a wide range of pH values either side of neutrality; it is non-toxic and does not seem to be different from more conventional buffers (Smith & Smith, 1949; Hille, 1968*b*). All tetrodotoxin solutions were prepared from the same 1 mg sample (Sankyo/Calbiochem). The stock solution contained 0·31 μM toxin and 10 mM acetate buffer (pH 4) and was kept at 4° C to delay deterioration. The working solution was, as a rule, freshly prepared before each experiment.

Relation between maximum rate of rise and toxin concentration. Although it has been shown that the effects of tetrodotoxin on the maximum rate of rise, $\dot{V}_A$, and on P_{Na} are not linearly related (Schwarz *et al.* 1973), an empirical quantitative relation between $\dot{V}_A$ and the toxin concentration, [TTX], appeared desirable. $\dot{V}_A$ was determined with threshold stimuli after the preparation had been equilibrated in a neutral toxin solution; this value was then divided by $\dot{V}_A$ obtained from the same fibre in neutral Ringer solution by the same procedure and the ratio was denoted A. The toxin concentration was also expressed as a dimensionless quantity $c_T = [TTX]/K_T$, where K_T is the equilibrium dissociation constant of the reaction between the toxin and its receptor at the Na channel. K_T was obtained in voltage clamp experiments from toxin-induced reductions of the Na current at neutral pH as described by Schwarz *et al.* (1973). K_T was $2{\cdot}8 \pm 0{\cdot}1$ nM (mean $\pm$ S.E.

of mean, $n = 10$) for the toxin sample used throughout the present investigation. We compiled the mean A for [TTX] between 1·55 and 15·5 nM and obtained the Hill plot of Fig. 1 for which the line of regression was calculated to be $\log [(1-A)/A] = 1{\cdot}22 \log c_T - 0{\cdot}573$ ($r = 0{\cdot}99$). This equation was applied whenever the inherent effect of H ions on the Na channel was negligible as at neutral or alkaline pH. The equation yielded $A = 0{\cdot}5$ for $c_T = 2{\cdot}95$ (8·26 nM tetrodotoxin). This is in excellent agreement with the previous finding of Schwarz *et al.* (1973) that the maximum rate of rise had decreased by 50 % at $c_T = 3{\cdot}03$ which corresponded to 10·9 nM tetrodotoxin when K_T was 3·6 nM.

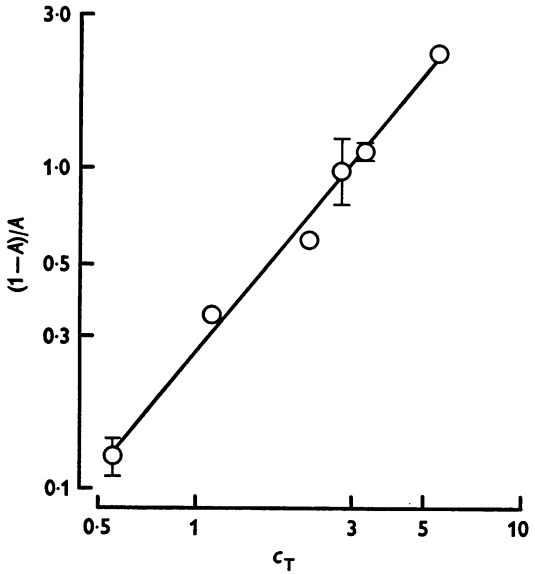

Fig. 1. Empirical relation between normalized maximum rate of rise, A, and tetrodotoxin concentration. Ordinate, $(1-A)/A$; abscissa, c_T = [TTX]/2·8 nM; both on logarithmic scales. The points represent mean values (temperature range 18·5–20·5° C); ± S.E. of mean indicated by vertical bars whenever it exceeded the size of the symbols. Line of regression calculated as $\log [(1-A)/A] = 1{\cdot}22 \log c_T - 0{\cdot}573$.

RESULTS

Effect of pH on maximum rate of rise

The following experiments were done in the double air gap chamber to investigate, in toxin-free solutions, the influence of pH on the maximum rate of rise of the action potential. The standard procedure was to equilibrate the preparation in the test solution and then to apply a train (1 Hz) of threshold stimuli each lasting for several msec; the membrane was kept hyperpolarized by about 30 mV between pulses. The action potentials were electronically differentiated, recorded and the average

TABLE 1. Normalized maximum rate of rise, A, of action potential at different pH. In each fibre A at pH 7·2 was considered to be 1·000. Temperature 18·5–21·0° C

pH	A (mean ± S.E. of mean)	Number of runs	Number of fibres
8·8	1·029 ± 0·011	31	17
8·0	1·014 ± 0·017	5	4
6·5	0·926 ± 0·007	43	9
6·0	0·786 ± 0·007	49	19
5·6	0·636 ± 0·010	27	10
5·3	0·441 ± 0·002	37	15
5·0	0·355 ± 0·023	36	9
4·6	0·126 ± 0·019	8	3

value of the maximum rate of rise of 15–20 consecutive spikes was determined. This value was divided by the control value observed in neutral Ringer solution by the same procedure, the ratio being denoted A, the normalized maximum rate of rise. The results were obtained in the course of toxin experiments or in a separate series in which several pH values were repeatedly tested on the same fibre since reversibility was excellent; they are compiled in Table 1 and one can see that alkaline pH had very little effect on A while increasing [H^+] markedly reduced A. This latter effect was to be expected since earlier voltage clamp studies and the present investigation (see p. 172) revealed a twofold effect of acid pH: reduction of P_{Na} and a shift of the P_{Na}–E relation to more positive membrane potentials (Hille, 1968*b*; Drouin & The, 1969; Woodhull, 1973). Both effects lead to a decrease in the maximum rate of rise but only the reduction of P_{Na} is comparable with the action of tetrodotoxin. Therefore A is not very well suited for a quantitative description of the combined effects of H ions and the toxin, a complication that can be circumvented as shown in the next section.

For the procedure just mentioned it is necessary to assume that the rate of action of hydrogen ions is very fast. To test this point the membrane was stimulated every 20 msec and the changing $\dot{V}_A$ was recorded when the pH was suddenly changed from 7·3 to 5·2 and back by switching perfusates. As in all experiments employing periodic pulses the signals were displayed on the oscilloscope screen as standing pictures and photographed on moving film. Sweep and film speed were chosen as to yield overlapping records. $\dot{V}_A$ obtained in a solution of pH 5·6 served as calibration point since this pH value corresponds to the arithmetic mean of the two [H^+] in the test solutions. It did not occur to us until after the experimental series was finished that what we actually observed was the exchange of two buffer solutions rather than solutions simply

containing hydrogen ions at two different concentrations. Hence the real midpoint for the exchange was given by a mixture, at equal parts, of the two buffer solutions yielding a pH of 6·2. $\dot{V}_A$ for this pH was therefore obtained by interpolation for each experiment and in eighteen runs of six preparations this mid-point was reached after 45 ± 6 msec (mean ± S.E. of mean) on acidification and after 61 ± 7 msec on turning back to neutrality. In these experiments the exchange of Na ions was also tested in an analogous fashion by switching between normal Ringer solution and a solution containing 50% of the normal $[Na]_o$. In three of the six fibres a solution containing 75% of the normal $[Na]_o$ was tested in addition to yield V_A at half the concentration increment; it gave 0·77 of the $\dot{V}_A$ value in normal Ringer solution. The mean time to reach the mid-point was 36 ± 5 msec (± S.E. of mean, $n = 18$) and 27 ± 3 msec (± S.E. of mean, $n = 18$) for decreasing and increasing concentration, respectively. This was slower than the Na exchange in another air gap chamber where the respective half-times were 23 and 19 msec (0·69 τ_c in Vierhaus & Ulbricht, 1971). The fact that in the present investigation the response to a change in pH was slower than to a change in $[Na]_o$ most likely reflects the difference in the diffusion coefficients of the buffer molecules and sodium. At any rate, a new level of $\dot{V}_A$ was clearly reached in much less than 1 sec after changing the pH of the perfusate.

Dependence on pH of tetrodotoxin effect on maximum rate of rise

Fig. 2 illustrates the influence of pH on the effect of 15·5 nM tetrodotoxin as expressed by the mean A, the normalized maximum rate of rise, observed after equilibration in the respective test solutions (open circles). Interestingly the toxin effect was diminished both at alkaline and weakly acid pH as reflected in the increase of A. Only for pH 5·5 did A decrease again. This non-monotonic behaviour of the combined action of H ions and tetrodotoxin is surprising since H ions themselves depress A in a monotonic fashion as outlined in the preceding section and indicated by the crosses in Fig. 2.

Increase of A between pH 7·2 and 5·5 in spite of the marked inhibiting effect of protons *per se* could mean that the toxin effectiveness was progressively diminished. To test this point the perfusate was suddenly switched from the test solution to neutral Ringer solution and within 1 sec a new value of A was determined and denoted A'. A' was assumed to reflect the share of the toxin in the combined action since after 1 sec the inhibiting effect of the H ions should have worn off completely as suggested by the buffer exchange experiments while the offset time

constant of the toxin action at neutral pH is of the order of 1 min (Schwarz *et al.* 1973). The mean values of A' are plotted as filled circles in Fig. 2 and clearly demonstrate that for pH 5·0 very little effect of tetrodotoxin was left.

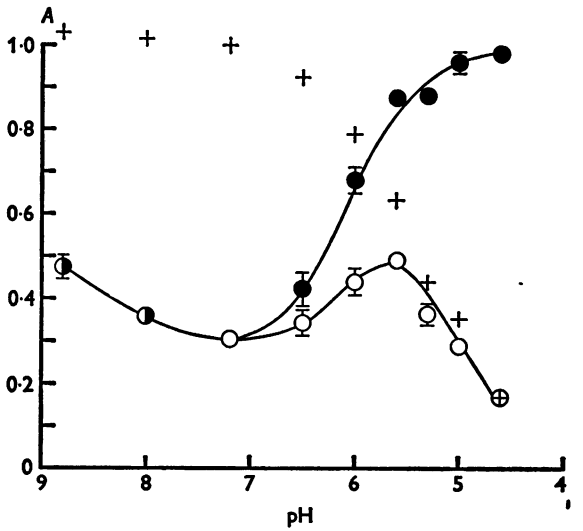

Fig. 2. Modification by pH of the effect of 15·5 nM tetrodotoxin on maximum rate of rise. Ordinate, normalized maximum rate of rise; abscissa, pH of test solution during equilibration with tetrodotoxin. ○, mean equilibrium effect in the toxin solutions, ●, 1 sec after changing to neutral Ringer solution; unchanged values denoted ◑. Vertical bars give ± S.E. of mean. +, mean values in toxin-free solutions. Temperature range 18·5–20·5° C. Curves through points drawn by eye.

It should be noted that at alkaline pH the quantities A and A' were virtually identical which is expressed in Fig. 2 by the symbol ◑. This fact may justify a separate description of these experiments in the following section.

Equilibrium effects of alkaline tetrodotoxin solutions

The reduced potency of tetrodotoxin at alkaline pH was studied in two series of experiments. In an early series 15·5 nM toxin was tested only at pH 8·8 yielding a mean A of $0{\cdot}470 \pm 0{\cdot}042$ ($\pm$ S.E. of mean, $n = 9$). This value was confirmed in later, more extensive, experiments in which this toxin concentration was applied at pH 8·7 and 8·0 yielding the respective mean A of $0{\cdot}484 \pm 0{\cdot}011$ and $0{\cdot}360 \pm 0{\cdot}014$ ($n = 4$ in either case). In these experiments measurements were also made 1 sec after changing to neutral Ringer solution to obtain A' whose respective values were $0{\cdot}488 \pm 0{\cdot}013$ and $0{\cdot}360 \pm 0{\cdot}013$.

Decreased toxin activity at high pH has been observed before and generally been attributed to the reversible conversion of the cationic toxin into an inactive zwitterion form (Camougis, Takman & Tasse, 1967; Ogura & Mori, 1958; Hille, 1968*a*; Narahashi, Moore & Frazier, 1969). Before testing this hypothesis we tried to show that whatever happened to the toxin molecule was reversible on re-acidifying. This was done by adding solid buffer (final concentration 13·3 mM) to the alkaline test solution to re-establish neutrality. When, after this procedure, the solution was applied to the same preparation, A was found to decrease again and to assume a value within 15 % of A in the first run in neutral toxin solution indicating that the toxin had indeed regained its potency. When however this procedure was repeated 5 hr after the preparation of the alkaline solution, a test on another fibre revealed a clear loss of potency. Obviously a portion of the toxin had deteriorated at alkaline pH within hours even at room temperature confirming Colquhoun, Henderson & Ritchie (1972) who reported a half-life of 2 hr at pH 9·1 and 20° C. In the second series of experiments we therefore took care to prepare our test solutions immediately before their use.

Two tautomers of the cationic toxin form exist that are in equilibrium with each other, the equilibrium constant, however, being unknown while the apparent pK'_a for the conversion of the cations into the zwitterion form is approximately 8·8 (Tsuda, Ikuma, Kawamura, Tachikawa, Sakai, Tamura & Amakasu, 1964; Goto, Kishi, Takahashi & Hirata, 1965). With this value we calculate for pH 7·2, 8·0 and 8·7 a decreasing cation fraction of 97·5, 86·3 and 55·7 %, respectively. If the toxin action resides only in the cationic configuration and if the two forms are equi-effective relatively little difference between the results at pH 7·2 and 8·0 should be expected. Also, 30·7 nM tetrodotoxin at pH 8·7 should produce a somewhat larger effect than 15·5 nM at pH 7·2 since the 'effective' concentrations are calculated to be $0{\cdot}557 \times 30{\cdot}7$ nM = 17·1 nM and $0{\cdot}975 \times 15{\cdot}5$ nM = 15·1 nM, respectively. These points were indeed confirmed in the series of extended experiments in which 15·5 nM toxin at pH 7·2 yielded a mean A ($\pm$ S.E. of mean) of $0{\cdot}372 \pm 0{\cdot}009$ ($n = 3$) while at pH 8·0 it was 0·360 as mentioned before. It should be noted, however, that in these three experiments A happened to be clearly larger than our over-all mean of $0{\cdot}304 \pm 0{\cdot}008$ ($n = 58$) in 15·5 nM toxin at pH 7·2. Finally, in 30·7 nM tetrodotoxin at pH 8·7 we observed in four fibres $A = 0{\cdot}268 \pm 0{\cdot}023$ and $A' = 0{\cdot}259 \pm 0{\cdot}019$.

These results can be compared to the theoretical predictions in a quantitative way by using our empirical equation (see Methods) to convert e.g. A observed with 15·5 nM toxin at pH 8·0 and 8·7, 0·360 and 0·484, respectively, into c_T values of 4·73 and 3·11. The ratio of these

normalized concentrations, 3·11/4·73 = 0·66, agrees very well with the ratio of the cation fractions, 55·7 %/86·3 % = 0·65. The mean A for 30·7 nM tetrodotoxin at pH 8·7 was 0·268 leading to c_T = 6·72 which is 2·16 times c_T as calculated from A in 15·5 nM at the same pH (3·11). This is in fair agreement with the expected ratio of the nominal concentrations of 30·7/15·5 = 1·98. In summary our results are fully compatible with the idea that the toxin cations are the active forms.

The share of tetrodotoxin in the combined proton-toxin effect on the maximum rate of rise

In the previous Fig. 2 it has been shown for one tetrodotoxin concentration that on decreasing pH the blocking action attributable to the toxin greatly diminished and hence A' approached unity for pH < 5·0. This influence has been studied more extensively employing various combinations of pH and toxin concentration. As a rule two different concentrations were tested at neutral and one acid pH on the same fibre. The mean values of A and A' observed in these experiments are listed in Table 2 which shows, for example, that after equilibration in 6·2 nM tetrodotoxin at pH 6·0 approximately the same fraction of Na

TABLE 2. Maximum rate of rise during (A) and immediately after (A') treatment by acid tetrodotoxin solutions. In 400 nM tetrodotoxin at pH 5·3 the fibres were inexcitable. Temperature 18·0–21·0° C

pH	[TTX] (nM)	$A \pm$ S.E. of mean	(n)	$A' \pm$ S.E. of mean	(n)
6·5	15·5	0·345 ± 0·029	(5)	0·442 ± 0·039	(4)
6·5	31·0	0·197 ± 0·022	(4)	0·274 ± 0·032	(3)
6·0	6·2	0·632 ± 0·019	(6)	0·879 ± 0·013	(6)
6·0	15·5	0·444 ± 0·032	(5)	0·682 ± 0·031	(4)
6·0	50·9	0·196 ± 0·013	(5)	0·366 ± 0·027	(5)
5·6	6·2	0·527 ± 0·023	(3)	0·917 ± 0·026	(3)
5·6	15·5	0·493 ± 0·013	(3)	0·876 ± 0·012	(3)
5·3	6·2	0·419 ± 0·013	(3)	0·973 ± 0·021	(3)
5·3	15·5	0·365 ± 0·024	(7)	0·881 ± 0·014	(7)
5·3	400·0	—	—	0·205 ± 0·021	(4)
5·0	15·5	0·290 ± 0·007	(2)	0·961 ± 0·026	(2)
5·0	31·0	0·246 ± 0·023	(4)	0·898 ± 0·016	(4)

channels was blocked by the toxin as when the preparation had been equilibrated in 15·5 nM at pH 5·3 or 31 nM at pH 5·0 as judged from the almost equal mean A' found in these runs.

For a quantitative description A' was converted into an apparent toxin concentration, c_T^*, with our empirical equation (see Methods) that could be applied here since A' had been determined at neutral pH. The

quantity c_T^* corresponds to the toxin concentration that one would have to apply at neutral pH to achieve an equilibrium effect of the size of A'. At neutral pH the fraction y_T of Na channels blocked by tetrodotoxin is given by $y_T = c_T/(c_T+1)$ where $c_T = [TTX]/K_T$, K_T being the equilibrium dissociation constant of the one-to-one reaction between the toxin and its receptor. By analogy y'_T in the presence of H ions is given as $y'_T = c_T^*/(c_T^*+1)$. When $c_T^* = y'_T/(1-y'_T)$ was plotted against [TTX] in a Hill plot as in Fig. 3, straight lines with slopes of 1·0 could be fitted

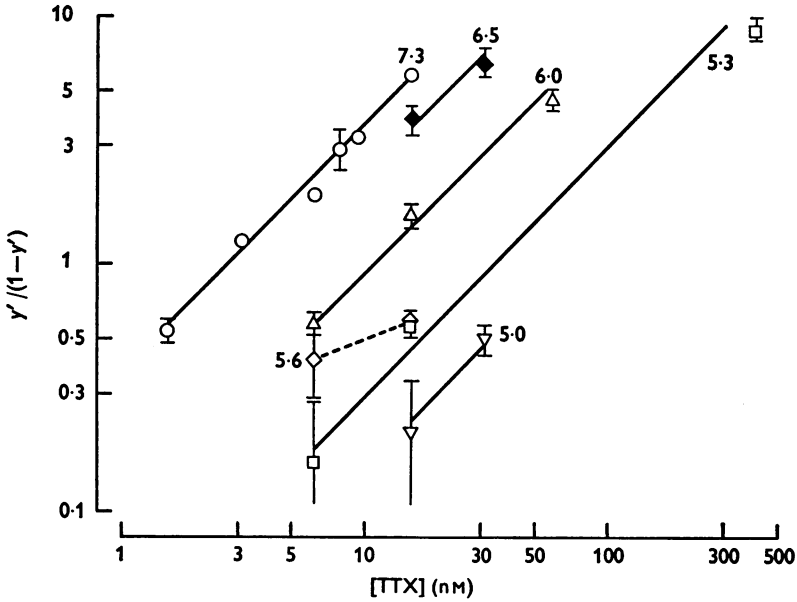

Fig. 3. Hill plot of the fraction of Na channels blocked by tetrodotoxin at several pH values. Ordinate, $y'_T/(1-y'_T)$, where y'_T, the partial receptor occupancy by the toxin, is calculated from A' as described in the text. Abscissa, tetrodotoxin concentration in nM. Both quantities on a logarithmic scale. The points and bars are calculated from the mean A' ± one S.E. of mean; each pH value being given a separate symbol. To the points, with the exception of ◇, straight lines with a slope of 1·0 have been fitted by eye and the numbers next to them denote the pH. Temperature range 18·5–20·5° C.

to the points belonging to one pH value with the exception of the two points determined for pH 5·6. The lines were shifted to higher toxin concentrations as the pH decreased. Thus at pH 7·3 only 1·55 nM tetrodotoxin was necessary to block one third of the Na channels so that $y'_T/(1-y'_T) = 0{\cdot}5$, while at pH 5·0, 31 nM had to be applied to achieve the same degree of block.

In our attempt to find an explanation for the reduced toxin action

it appeared necessary to study quantitatively the toxin-like action of H ions in the presence and absence of tetrodotoxin. As has already been pointed out, *A* is not suited for this purpose. Therefore voltage clamp experiments had to be done in which we could distinguish between the relevant action on P_{Na} and the additional shifting effect of H ions.

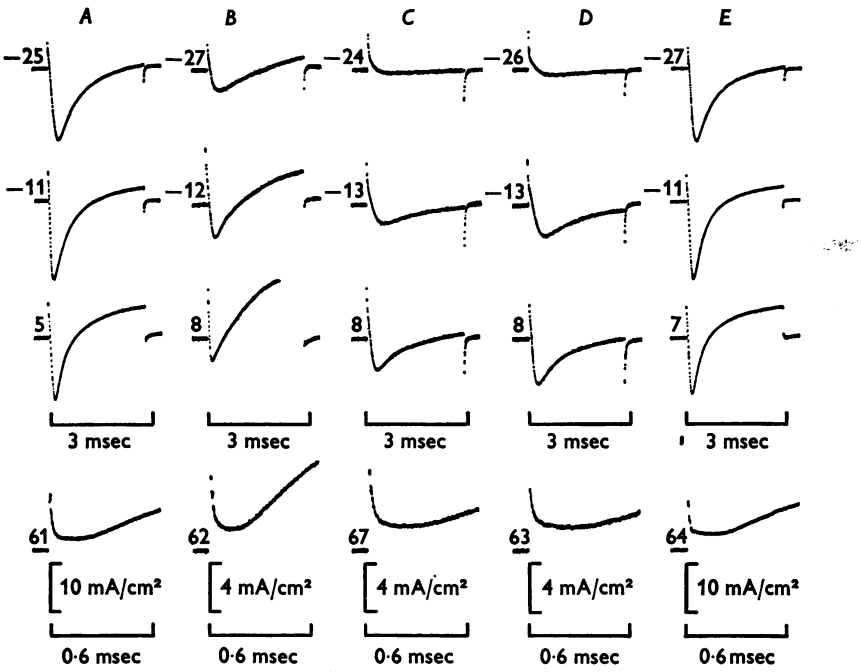

Fig. 4. Original voltage clamp records. The calibration of current density in mA/cm² is given at the bottom of each column. The time scale is 3 msec per horizontal bar for all records except for those in the bottom row where it is expanded 5 times. Columns *A* and *E* were obtained in neutral Ringer solution (pH 7·2), *B* in 9·3 nM tetrodotoxin at pH 7·2, *C* in 9·3 nM tetrodotoxin at pH 5·6 and *D* in toxin-free solution of pH 5·6. The numbers at the left margin of each record give the absolute membrane potential during the test pulse. 18·5° C.

Inhibition of Na current by tetrodotoxin and H ions

In this series of voltage clamp experiments the preparation was successively equilibrated in the following solutions: (1) neutral Ringer solution, (2) neutral toxin solution, (3) acid toxin solution, (4) neutral Ringer solution, (5) acid Ringer solution and (6) neutral Ringer solution. In each solution a complete current–voltage curve was determined extending, as a rule, to E = 130 mV. In the experiments employing

pH 5·3, however, the neutral toxin solution (run 2) was not applied until after run 6 and this last run consisted of only a few test impulses. Original records from such an experiment are shown in Fig. 4. Here the first and second column give the results of runs 1 and 2, illustrating the fact that in either solution the maximum inward current, with which

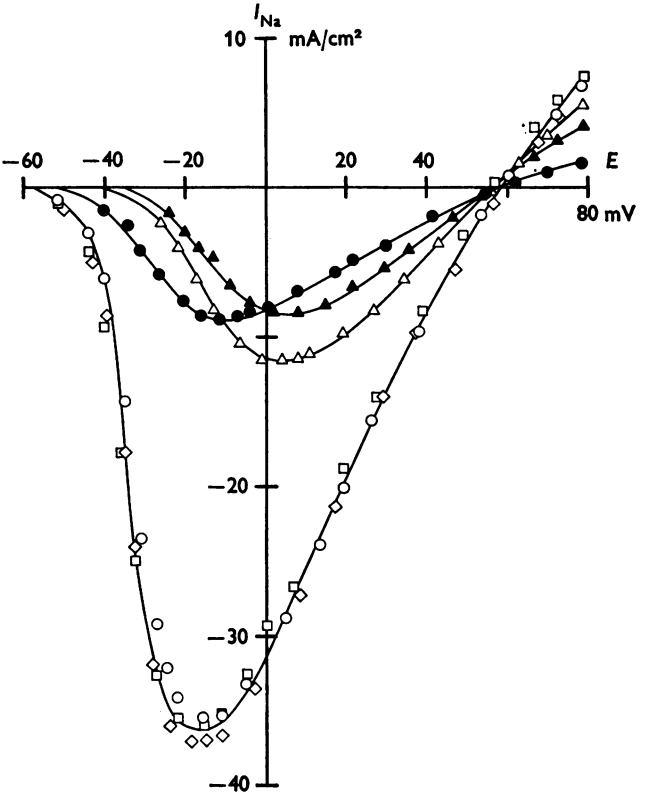

Fig. 5. Sodium current–voltage curves in neutral and acid solutions with and without tetrodotoxin. Ordinate, I_{Na}, current density in mA/cm², inward current negative. Abscissa, absolute membrane potential during test pulses in mV. ○, ◇, and □, denote runs 1, 4, and 6, respectively in Ringer solution of pH 7·2; ●, run 2 in 9·3 nM tetrodotoxin at pH 7·2; ▲, run 3 in 9·3 nM toxin at pH 5·6; △, run 4 in Ringer solution of pH 5·6. The same fibre as in Fig. 4.

we are concerned here exclusively, is observed at about $E = -12$ mV while the absolute amplitudes are, of course, greatly reduced in 9·3 nM tetrodotoxin. The third and fourth column shows records at pH 5·6 (runs 3 and 5) where the maximum inward current did not occur until E was about $+8$ mV. At this pH the relative reduction by the toxin

was clearly less than at neutral pH. The last column was obtained at the end of the experiment (run 6) and it demonstrates the excellent reversibility of the effects. The traces in the bottom row were recorded, at increased sweep speed, near E_{Na}, the equilibrium potential for Na ions at which the early Na current vanishes. Thus these records illustrate the constancy of E_{Na} in the various test solutions.

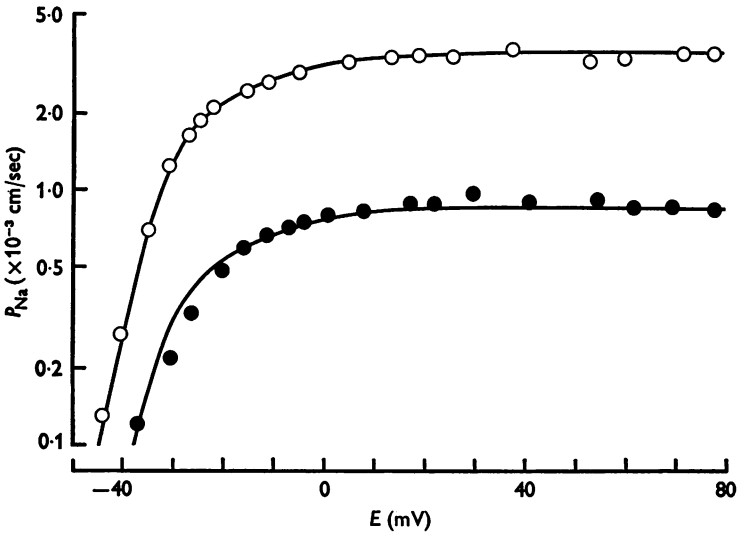

Fig. 6. Equilibrium effect of neutral toxin solution on peak sodium permeability, P_{Na}, as a function of membrane potential. Ordinate, P_{Na} in cm/sec as calculated from peak I_{Na}, on a logarithmic scale. Abscissa, absolute membrane potential, in mV, during test pulses. ○, points in neutral Ringer solution (run 1 of Fig. 5); ●, in neutral toxin solution (9·3 nM, run 2 in Fig. 5). Curve through ○ drawn by eye, curve through ● from former curve multiplied by 0·245. The same preparation as in Figs. 4 and 5.

The current records were later corrected for leakage and capacity currents to determine I_{Na} and Fig. 5 shows the I_{Na}–E curves thus obtained. This Figure nicely summarizes the main observations: shift of the curves to more positive potentials at low pH, reduced I_{Na} in the toxin solutions and unchanged E_{Na}. For the quantitative evaluation, however, it was preferable to calculate P_{Na}, the peak sodium permeability, as described in the Methods. Fig. 6 gives an example of P_{Na}, plotted on a logarithmic scale, as a function of the potential, E, during the test pulse; the open circles refer to neutral Ringer solution (run 1) while filled circles represent measurements in neutral toxin solution (9·3 nM; run 2). The curve through the open circles was drawn by eye; it saturates for $E > 10$ mV. From

this curve the curve connecting the filled circles was obtained by multiplying by 0·245. This illustrates that the toxin simply reduces the number of operating channels (Hille, 1968*a*; Schwarz *et al.* 1973), in this case to about one quarter as one would expect for [TTX] $\approx 3\,K_T$.

The results in the other test solutions (including the first run in neutral Ringer solution) are shown as semilogarithmic P_{Na}–E plots in Fig. 7 in which the good reproducibility justified fitting a common curve to the points from three runs (1, 4, and 6) in Ringer solution of pH 7·2. The points observed in the toxin-free solution of pH 5·6 (open triangles), however, were shifted to the right and did not saturate. Additional treatment with 9·3 nM tetrodotoxin (filled triangles) led to a further reduction of P_{Na} by a constant factor, p'_T, which in the semilogarithmic plot resulted in a parallel downward shift of the curve.

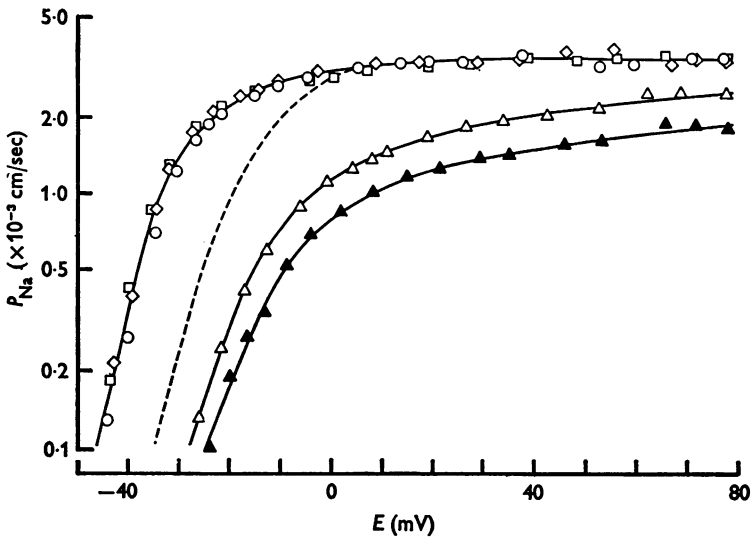

Fig. 7. Equilibrium effect of acid toxin and toxin-free solution on peak Na permeability, P_{Na}. Ordinate, P_{Na} in cm/sec, on a logarithmic scale. Abscissa, membrane potential, E, during test pulses in mV. ○, ◇, and □, in Ringer solution of pH 7·2 (runs 1, 4, and 6 of Fig. 5); △, in Ringer solution of pH 5·6 (run 5); ▲, in 9·3 nM tetrodotoxin at pH 5·6 (run 3). Interrupted curve obtained from curve in acid toxin-free solution after correction for voltage-dependent proton binding; it was shifted by 13·5 mV to more positive potentials.

The characteristic changes of P_{Na} in the acid toxin-free solutions have been studied in detail by Woodhull (1973) who ascribed them to a combination of shift to more positive potentials and of voltage-dependent block of Na channels by hydrogen ions. This latter effect can be formally

treated like the tetrodotoxin-induced block so that at equilibrium the fraction of channels blocked by protons, y_H, is given by

$$y_H = \frac{c_H}{c_H + 1}, \tag{1}$$

where $c_H = [H^+]/K_H$. K_H, in contrast to K_T, is dependent on membrane potential with the idea that an equilibrium is attained before, on a sudden depolarization, I_{Na} has reached its maximum. Woodhull (1973) derived the following relation between K_H and E

$$K_H = b_{-1}/b_1 \exp(\delta EF/RT), \tag{2}$$

where b_{-1}/b_1 is K_H for $E = 0$, thus having the dimension of a concentration and δ is a dimensionless quantity ($\delta < 1$); E is the absolute membrane potential and F, R, and T have their usual meaning.

In our experiments $K_H(E)$ was obtained from ratios of P_{Na} in acid and neutral Ringer solution (runs 4 and 5) at various values of $E > 10$ mV, i.e. beyond the region complicated by the shift. These ratios correspond to $1 - y_H = (c_H + 1)^{-1}$, from which K_H can be easily computed. As can be seen from eqn. (2), a plot of ln K_H *vs.* E gives a straight line whose slope is proportional to δ. Extrapolation to $E = 0$ then yields b_{-1}/b_1. According to Woodhull's theory these two parameters should be independent of pH and the mean values obtained in our experiments at pH 6·0, 5·6, 5·3 and 5·2 indeed were not significantly different as shown in Table 3. We therefore computed over-all means that were $\delta = 0{\cdot}34$ and $b_{-1}/b_1 = 2{\cdot}04$ μM. These values were in the range of Woodhull's (1973) data that yielded a lower mean δ of 0·26. This difference, however, may only be apparent since Woodhull based her calculations on data that had been corrected for a mean attenuation artifact of 24%. Without this correction her mean value would have been $0{\cdot}26 \times 1{\cdot}24 = 0{\cdot}32$ i.e. very close to our mean value derived from uncorrected data.

Once $K_H(E)$ is determined for a given preparation the P_{Na}–E curve in acid Ringer solution can be corrected for the voltage-dependent block by H ions so that for $E > 0$ the corrected curve is identical with that observed at neutral pH. For $E < 0$, however, the correction yields deviating P_{Na} values which in Fig. 7 are represented by the interrupted curve that is shifted parallel to more positive potentials by the 'true' amount, ΔE; it was 13·5 mV in this particular case. The P_{Na}–E curves in acid *toxin* solution can also be corrected for $K_H(E)$ if allowance is made for the constant reducing factor p'_T. The shift is then determined in the same way and in Table 3 it is denoted $(\Delta E)_{TTX}$ to distinguish it from $(\Delta E)_{RI}$ as observed in acid Ringer solution. As can be seen in Table 3 the two (ΔE) values did not significantly differ for a given pH value so that one may conclude that tetrodotoxin does not interfere with the mechanism by which protons produce this shift. Table 3 nicely illustrates the decreasing toxin effect at low pH. Thus at pH 5·6, 9·3 nM tetrodotoxin had to be applied to achieve the same additional reduction in P_{Na}, reflected

in p'_T, as 3·1 nM did at pH 6·0; for approximately the same reduction at pH 5·3, 15·5 nM toxin was required. Before attempting a quantitative explanation of these effects it appears worth discussing, in the following section, a few mechanisms by which hydrogen ions may affect the toxin-induced block.

TABLE 3. Mean values ± S.E. of mean of quantities that characterize the results at low pH in the presence and absence of tetrodotoxin. Equilibration in acid toxin solution took place at a holding potential of −70 mV. Temperature 16·0–18·3° C

pH	[TTX] (nM)	$(\Delta E)_{Bi}$ (mV)	$(\Delta E)_{TTX}$ (mV)	p'_T	δ	b_{-1}/b_1 (μM)	n
6·0	3·1	8·7 ± 0·7	8·7 ± 0·4	0·76 ± 0·02	0·33 ± 0·09	1·88 ± 0·11	3
5·6	9·3	14·8 ± 1·6	15·8 ± 0·7	0·76 ± 0·02	0·44 ± 0·08	1·84 ± 0·58	3
5·6	15·5	—*	—*	0·57 ± 0·01	—*	—*	3
5·6	31·0	—*	—*	0·41 ± 0·01	—*	—*	4
5·3	15·5	18·3 ± 1·5	16·3 ± 0·7	0·72 ± 0·04	0·33 ± 0·04	2·05 ± 0·15	5
5·2	15·5	—*	—*	—†	0·30 ± 0·03	2·29 ± 0·33	4
				Over-all mean	0·34 ± 0·03	2·04 ± 0·14	15

* Parameter not evaluated.
† p'_T determined for $E_{HP} \neq -70$ mV, see Table 4.

Models of proton-toxin interaction

(1) It is now generally believed that the shift of the P_{Na}–E curve at low pH (see Fig. 7) is due to the reduction of a negative potential at the outer surface of the membrane. This in turn reduces the concentration of cations, including toxin cations, in the double layer. For a given nominal (bulk) toxin concentration the effective concentration immediately outside the membrane would thus be lower at acid pH, the reducing factor being exp $(-\Delta EF/RT)$ where ΔE is the observed shift of the P_{Na}–E curve (see Table 3). A mechanism of this kind has been assumed by Mozhaeva & Naumov (1972) to explain the effect of pH on the block of K channels by tetraethylammonium ions.

(2) Another possibility is that protons and toxin cations compete for the same receptor whose occupation by either agent results in the occlusion of the Na channel. At equilibrium with an acid toxin solution, one fraction of channels, y'_T, will be blocked by tetrodotoxin and another fraction, y'_H, by protons, the prime denoting the presence of the other blocking agent. These fractions are given by

$$y'_T = \frac{c_T}{c_T + c_H + 1} \tag{3}$$

and

$$y'_H = \frac{c_H}{c_T + c_H + 1}, \tag{4}$$

where c_T and c_H are the normalized concentrations of tetrodotoxin and hydrogen ions as defined on pp. 168 and 173, respectively. From eqn. (3) it is clear that y'_T and its complement p'_T also depend on c_H. Since c_H is a function both of $[H^+]$ and membrane potential, p'_T depends on the potential during equilibration, i.e. the holding potential, E_{HP}. An extensive derivation of p'_T (E_{HP}) is given in the Appendix. Here it may suffice to present the result

$$p'_T = \frac{c_H(E_{HP})}{c_T + c_H(E_{HP}) + 1}, \tag{5}$$

where c_T is assumed to be insensitive to E_{HP}. This point has been confirmed in the experiments of the following section. Eqn. (5) then predicts an increase of p'_T for more negative values of E_{HP} since on hyperpolarization $K_H(E)$ decreases (see eqn. (2)) and c_H therefore increases for a given pH value.

(3) There is also the possibility that H^+ and tetrodotoxin bind to separate (and independent) sites, one each per channel, and that the occupation of one site suffices to block the channel. In this case the observed decreasing toxin action at low pH, as described by p'_T, would have to be attributed solely to a diminished 'affinity' of the toxin site.

It is clear that mechanism (1) could be combined with (2) or (3) but that only (2) suggests right away that the toxin effect at acid pH should also depend on the potential at which the membrane is being held during equilibration. Experiments to test this point are described in the following section.

Effect of holding potential on toxin-induced block

In the following experiments the nerve fibres were equilibrated at two holding potentials, -50 and -90 mV. With $E_{HP} = -90$ mV no prepulses were necessary to obtain maximum Na currents; for $E_{HP} = -50$ mV the hyperpolarizing prepulses were adjusted for maximum currents. These currents, however, were clearly smaller than those observed with $E_{HP} = -90$ mV. The depressing effect of sustained depolarization to -50 mV developed but slowly and thus clearly differed from the common h-type inactivation of P_{Na}. Therefore, measurements were not started until up to 10 min had passed after a change in E_{HP} and care was taken that the measurements in the toxin solutions and in the control were done under exactly the same conditions.

These slow changes have been observed in lobster (Narahashi, 1964), squid (Adelman & Palti, 1969) and frog axons (Koppenhöfer & Vogel, 1969; Fox, 1975). In our experiments this phenomenon was somewhat variable but it appeared to be more pronounced at neutral pH. Thus in toxin-free solution of pH 7·2, I_{Na} at a test pulse potential, E_t, of $+10$ mV

and for $E_{HP} = -50$ mV (employing hyperpolarizing prepulses) was on the average $45 \pm 9\%$ (mean $\pm$ S.E., $n = 3$) of I_{Na} at the same pulse potential but for $E_{HP} = -90$ mV (no prepulses). In toxin-free solutions of pH 5·3 this fraction was $63 \pm 5\%$ ($n = 7$).

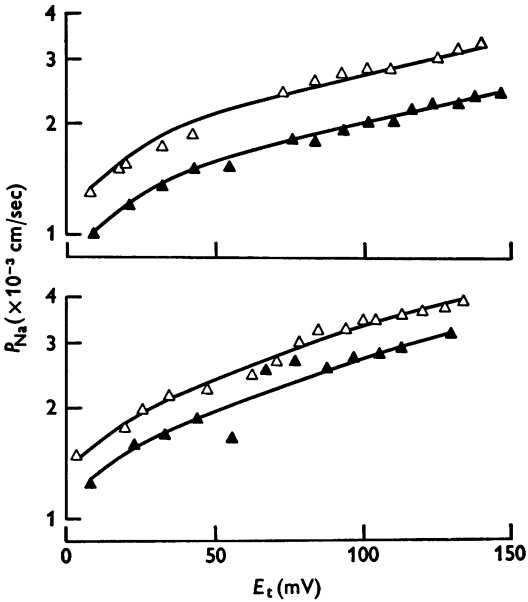

Fig. 8. Dependence of additional toxin effect at low pH on holding potential during equilibrium. Ordinates, peak P_{Na} in cm/sec, logarithmic scales. Abscissae, membrane potential, E_t, during test pulses in mV. For the upper diagram the membrane was equilibrated at a holding potential, E_{HP} of -50 mV, for the lower diagram at $E_{HP} = -90$ mV. In either diagram △ refers to points in Ringer solution of pH 5·2, ▲ to 15·5 nM tetrodotoxin at pH 5·2. The curves through △ were drawn by eye, those through ▲ by multiplication by $p'_T = 0{\cdot}74$ for $E_{HP} = -50$ mV and by $p'_T = 0{\cdot}82$ for $E_{HP} = -90$ mV. Note the depressing effect of a steady depolarization (upper diagram) on P_{Na} in toxin-free solution. 16·2° C.

Three types of experiments were performed: (1) testing 15·5 nM tetrodotoxin at pH 5·2; (2) testing 31 and 93 nM tetrodotoxin, successively, at pH 5·3; (3) testing 3·1 nM tetrodotoxin at pH 7·2. Within each series the sequence of E_{HP} was varied to avoid possible systematic errors. In the first series p'_T was determined as illustrated by Fig. 8. This Figure contains two pairs of semilogarithmic $P_{Na} - E$ plots ($E > 0$) as obtained at pH 5·2 in toxin-free solution (open triangles) and 15·5 nM tetrodotoxin (filled triangles). For the upper pair the membrane was held at -50 mV so that hyperpolarizing prepulses by about 30 mV had to be applied for 50 msec to obtain maximum currents during the test pulses. When the

points were measured in successive runs in the two relevant solutions the curves drawn through them were separated by a constant vertical distance corresponding to a constant factor $p'_T = 0{\cdot}74$. After the toxin had been washed out at pH 5·2 and after another run at pH 7·2, the holding potential was changed to -90 mV (no prepulses) and the procedure was repeated. The results are shown in the lower panel of Fig. 8. In spite of the scatter in the points near E_{Na} where I_{Na} is very small and P_{Na} thus hard to determine, the two curves again ran in parallel but the

TABLE 4. Dependence of p'_T on holding potential, E_{HP}, during equilibration in neutral and acid toxin solution; mean values ± S.E. of mean. Temperature 15·5–18·1° C

pH	[TTX] (nM)	p'_T for $E_{HP} = -50$ mV	n	p'_T for $E_{HP} = -90$ mV	n
7·2	3·1	0·52 ± 0·03	3	0·50 ± 0·01	3
5·2	15·5	0·60 ± 0·05	4	0·80 ± 0·02	4
5·3	31·0	0·53 ± 0·02	4	0·74 ± 0·02	3
5·3	93·0	0·24 ± 0·02	4	0·48 ± 0·02	4

distance was clearly smaller and hence p'_T larger, viz. 0·82. In the two other series a similar experimental procedure was adopted but p'_T was determined more simply as the ratio of the maxima of $I_{Na}(E_t)$ in the respective solutions. Table 4 summarizes the results of all our experiments in which E_{HP} was changed. As mentioned before, p'_T was insensitive to E_{HP} at neutral pH ($c_H \approx 0$) and according to eqn. (5) this should be the case if c_T did not depend on the holding potential. At acid pH, however, the influence of E_{HP} is marked and as predicted by eqn. (5): when the membrane is equilibrated in the acid toxin solution during a steady hyperpolarization ($E_{HP} = -90$ mV) more channels are blocked by protons and consequently fewer by tetrodotoxin so that the additional inhibiting effect of the latter is diminished and hence p'_T increased as observable on testing. It may be worth pointing out here that the successful extrapolation of $K_H(E)$ to potentials near the resting level would extend Woodhull's (1973) original interpretation of voltage-dependent block of channels with open gates also to closed ones, which is not at all obvious.

While the results agree qualitatively with the implications of eqn. (5), the quantitative test reveals systematic deviations. Thus when $c_H(E_{HP})$ is calculated with the empirical $K_H(E)$ as obtained from eqn. (2) with our data given on Table 3 and introduced in eqn. (5) to solve for c_T, $K_T = [TTX]/c_T$ turns out to depend on pH and E_{HP} for finite values of c_H which is clearly at variance with simple competition. This is shown in Table 5 in which the mean results of various combinations of [TTX] and pH are listed in the order of increasing c_H and in which K_T has

been calculated under three different assumptions. The first assumption, denoted (1), is straightforward competition showing the tendency of K_T to increase with c_H. If we correct these values for the (hypothetical) change in effective toxin concentration due to changes in the surface potential as outlined in the preceding section, the second column of K_T

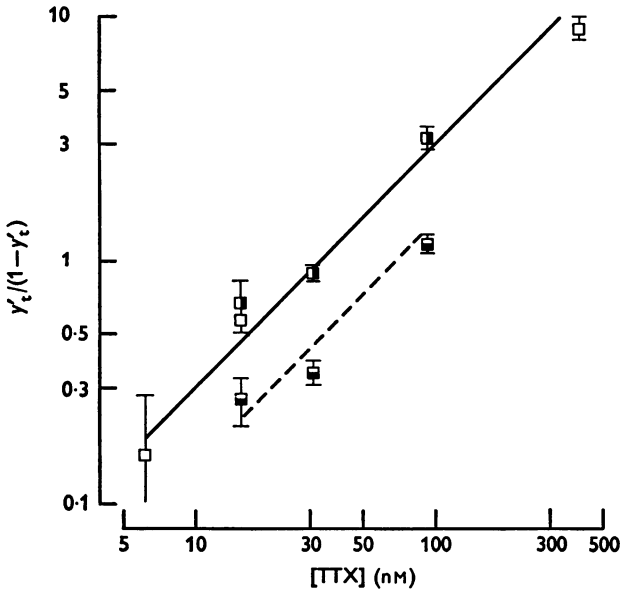

Fig. 9. Hill plot of mean voltage and current clamp results at pH 5·3 or 5·2. Ordinate, $y'_T/(1-y'_T)$ with y'_T = fraction of Na channels blocked at equilibrium by tetrodotoxin; logarithmic scale. Abscissa, tetrodotoxin concentration in nM, logarithmic scale. □, from maximum rate of rise (A'; see Fig. 3); ◨, from voltage clamp results at a holding potential $E_{HP} = -50$ mV; ⬓, at $E_{HP} = -90$ mV. The bars denote the range of ± S.E. of mean from the mean p'_T or A'. The continuous line (slope of 1·0) is that of Fig. 3 for pH 5·3; the interrupted line is drawn in parallel through ⬓.

is calculated and values become more uniform. Finally, column (3) presents K_T values necessary to describe the toxin effects in the case of two independent sites per channel, i.e. where an enormous loss of 'affinity', expressed by the large increase in K_T, has to be assumed. Of course, a similar correction as for column (2) could be applied but this would at best halve the K_T values. The comparative constancy of K_T calculated for the case of competition plus surface charge effects appears to be a point in favour of this explanation. However, there is no *a priori* reason why K_T should *not* change.

The mean values compiled in Table 5 stem from voltage as well as current clamp experiments and the two series at pH 5·2 that yielded

deviating (too low) K_T values were obtained with the latter method. This raises the question to what extent these results can be compared. Fig. 9 may give an answer since here the voltage clamp results of Table 4 for pH 5·3 are presented in a Hill plot together with the points (open squares) for this pH as derived from the maximum rate of rise. It may be fitting here to note that $y'_T/(1-y'_T) = (1-p'_T)/p'_T$. The continuous straight line in Fig. 9 is taken from the relevant plot of Fig. 3 and one can see that it also fits the points obtained in voltage clamp runs after equilibration at $E_{HP} = -50$ mV. Although the membrane was definitely not depolarized between stimuli when A' was determined, the agreement with the pertinent p'_T data appears to be quite good considering the completely different methods. The values obtained at $E_{HP} = -90$ mV, however, are fitted by another (interrupted) line that is shifted parallel as if it belonged to points observed at a lower pH. Thus both voltage and current clamp results show the same general dependence on pH as revealed by the Hill plots although the absolute values of p'_T may depend somewhat on the type of results used for their calculation. This probably contributes to the scatter of K_T values in Table 5.

TABLE 5. Apparent equilibrium dissociation constant, K_T, of toxin-site reaction as calculated for the case of (1) competition, (2) competition and surface potential-induced change in effective toxin concentration and (3) exclusive change of 'affinity' of the toxin receptor in a two-site situation

					K_T calculated for assumption			
pH	E_{HP} (mV)	c_H^*	[TTX] (nM)	Mean p'_T	(1)† (nM)	(2)‡ (nM)	(3)§ (nM)	n
7·2	−50	0·06	3·1	0·52	3·2	3·2	3·4	3
7·2	−90	0·10	3·1	0·50	3·1	3·1	3·4	3
6·0	−70	1·27	3·1	0·76	4·3	3·0	9·8	3
5·6	−70	3·18	9·3	0·76	7·1	3·9	29·7	3
5·6	−70	3·18	15·5	0·57	4·9	2·7	10·0	3
5·6	−70	3·18	31·0	0·41	5·2	2·8	21·7	4
5·3	−50	4·86	31·0	0·53	6·0	3·0	35·2	4
5·3	−50	4·86	93·0	0·24	5·0	2·5	29·3	4
5·2	−50	6·13	15·5	0·60	3·3	1·5	23·5	4
5·3	−70	6·34	15·5	0·72	5·4	2·7	39·6	5
5·3	−90	8·35	31·0	0·74	9·4	4·7	87·9	3
5·3	−90	8·35	93·0	0·48	9·2	4·6	86·0	4
5·2	−90	10·52	15·5	0·80	5·4	2·4	62·2	4

* $c_H = [H^+]/K_H(E_{HP})$; $K_H = 2{\cdot}04 \exp(0{\cdot}34\, E_{HP}F/RT)$ [μM].

† $K_T = [TTX]/c_T$; $c_T = (1/p'_T - 1)(c_H + 1)$.

‡ $K_T = \exp(-\Delta EF/RT)\,[TTX]/c_T$; c_T obtained as for †; mean ΔE from Table 3: 8·7, 15·3 and 17·4 mV for pH 6·0, 5·6 and 5·3. Value for pH 5·2 estimated to be 20 mV.

§ $K_T = [TTX]/c_T^*$; $c_T^* = (1/p'_T - 1)$.

DISCUSSION

The present equilibrium experiments show that the effect of tetrodotoxin on the nodal membrane has an optimum near pH 7·2 since both at more alkaline and more acid pH the fraction of Na channels blocked by a given toxin concentration is smaller than at neutral pH.

The diminished blocking action at increased pH is most probably caused by a reversible transformation of the active cationic forms of the toxin into an inactive zwitterion configuration. This interpretation has been proposed by previous authors who, however, have presented only few data that can directly be compared with our results. Thus Camougis *et al.* (1967) found in desheathed frog nerves that 12·8 nM tetrodotoxin at pH 7·05 was about as effective as 22·4 nM at pH 8·8. With $pK'_a = 8·8$ we calculate cation fractions of 98·3 and 44·3 %, respectively at the two pH values. Hence the cation concentrations are $0·983 \times 12·8$ nM $= 12·6$ nM and $0·443 \times 22·4$ nM $= 9·9$ nM and their Fig. 2 indeed suggests that the neutral toxin solution was slightly more effective. Hille (1968*a*) documents only one complete experiment on a frog nerve fibre in which 12·5 nM tetrodotoxin at pH 10·1 reduced $\bar{g}_{Na}$ to 85 % of the control value in Ringer solution while the same toxin concentration at pH 7·3 led to a reduction of $\bar{g}_{Na}$ to 16 %. Solved for (apparent) c_T values we obtain 0·18 and 5·25, respectively. The ratio $0·18/5·25 = 0·034$ is comparable to the ratio of cation fractions that one calculates with $pK'_a = 8·8$, i.e. $4·8\%/96·9\% = 0·050$. Narahashi *et al.* (1969) report that 30 nM tetrodotoxin at pH 7 and 9 reduced $\bar{g}_{Na}$ of the squid giant axon to 27 and 65 %, respectively, which corresponds to apparent c_T values of 2·70 and 0·54. Their ratio, $0·54/2·70 = 0·20$, however, is smaller than the ratio of cations calculated for the two pH values, $38·7\%/98·4\% = 0·39$ and the discrepancy may well have been caused by some irreversible degradation of the toxin in the alkaline solution.

The reduced toxin effect in acid solutions appears to have different reasons but the situation is complicated by the inherent blocking actions of protons. The present results on functioning membranes are, at least in part, confirmed by binding studies employing tritiated tetrodotoxin ([^{3}H]TTX) and saxitoxin ([^{3}H]STX). Thus binding of [^{3}H]STX is reduced at acid pH in a way compatible with competition as described by our eqns. (3) and (4), yielding $K_H = 1·4$ and 2·3 μM for rabbit vagus and garfish olfactory, respectively (Henderson, Ritchie & Strichartz, 1973). In an earlier study the [^{3}H]TTX uptake by rabbit vagus at pH 5·7 was also less than at neutral pH (see Fig. 11 of Colquhoun *et al.* 1972). Binding of [^{3}H]TTX to nerve membrane homogenates at pH 4·0 was reduced to 22 % of the control binding at neutral pH (Benzer & Raftery, 1972)

while binding to a solubilized membrane preparation was unaffected by pH between 8·5 and 6·5 but was drastically reduced below, reportedly because of irreversible denaturation of the binding protein (Henderson & Wang, 1972).

The mechanism of the diminished toxin action at low pH cannot uniquivocally be determined from the present results. Therefore three values of K_T for each combination of tetrodotoxin concentration and pH have been presented in Table 5 reflecting three different approaches. One of them, assuming competition of protons and toxin cations for the same site plus surface potential-induced changes in the effective toxin concentration immediately outside the membrane, led to K_T values that were relatively little dependent on pH or c_H. Actually, in simple competition K_T should be independent of c_H which could very well have been the case considering the possible errors in our computations. Thus $c_H(E_{HP})$ had to be obtained by extrapolating an average empirical equation of $K_H(E)$ to potentials (-50 to -90 mV) where the proton-induced block of Na channels could not directly be measured. Similarly, only an average correction for changes in the effective toxin concentration could be applied since individual ΔE data were not available for most preparations. In this connexion it should be noted that no independent evidence exists for the applicability of this correction to the present results although it has been helpful in explaining the pH dependence of the TEA-induced block of K channels (Mozhaeva & Naumov, 1972). Clearly, the possible changes in the effective toxin concentration would be quite insufficient as the *sole* explanation of the apparent increase of K_T by a factor of over 80 (see third K_T column on Table 5) since just doubling K_T already requires a shift by 17·5 mV.

Although the modified competition model appears to be the most plausible explanation of our results it must be considered with reservation for the following reasons. First, toxin binding, in contrast to that of protons, is independent of the holding potential as shown in Table 4. This is at variance with Woodhull's (1973) hypothesis of voltage-dependent binding which should apply equally to all agents bound to the same site. This contradiction could be avoided by a modified interpretation of proton binding as shall be discussed in the following paper (Ulbricht & Wagner, 1975*b*). Secondly, the rate of dissociation of toxin depends on c_H, which shows that H ions do not just compete for the blocking site but are more intricately involved in toxin binding. The experiments establishing this important fact are presented in the subsequent paper.

We should like to thank Miss E. Dieter for unfailing technical help and Drs B. Hille (Seattle, U.S.A.) and W. Vogel for commenting on the manuscript. The support of this work by the Deutsche Forschungsgemeinschaft is gratefully acknowledged.

APPENDIX

Derivation of $p'_T(E_{HP})$

If tetrodotoxin cations and H ions compete for the same site the fraction of Na channels blocked at equilibrium is given by

$$y'_\infty = y'_T + y'_H = \frac{c_T}{c_T + c_H + 1} + \frac{c_H}{c_T + c_H + 1}, \tag{A 1}$$

where y'_T and y'_H are the partial receptor occupancies by the toxin molecules and protons and c_T and c_H are the respective normalized concentrations as defined on pp. 168 and 173. Since c_H is a function of membrane potential both y'_T and y'_H are influenced by $c_H(E_{HP})$ and hence by the holding potential. Therefore the adequate description of the total receptor occupancy after equilibration at $E = E_{HP}$ is

$$y'_\infty(E_{HP}) = y'_T(E_{HP}) + y'_H(E_{HP}). \tag{A 2}$$

The validity of eqn. (A 2) cannot directly be tested since at $E = E_{HP}$ the gates of the Na channels are closed. To open them a cathodal test pulse of amplitude E_t has to be applied during which a few channels that previously had been blocked by protons become unblocked since a new proton-site equilibrium is reached soon after the start of the impulse (see Woodhull, 1973). While $y'_H(E_{HP})$ thus reduces to $y'_H(E_t)$, the toxin-site reaction is much slower (Schwarz *et al.* 1973) so that channels blocked by tetrodotoxin at E_{HP} remain so during the test pulse, and so does $y'_T(E_{HP})$. At $E = E_t$ the total receptor occupancy, $y'(E_t)$, is then

$$y'(E_t) = y(E_{HP}) + y'_H(E_t). \tag{A 3}$$

The term $y'_H(E_t)$ can be derived from the basic rule which is also inherent in eqn. (A 1) that each blocking agent equilibrates with the fraction of receptors left free by the other agent, i.e. $1 - y'_T(E_{HP}) = p'_T(E_{HP})$ in this case. Hence we obtain

$$y'_H(E_t) = \frac{c_H(E_t)}{c_H(E_t) + 1} p'_T(E_{HP}) \tag{A 4}$$

and since $c_H(E_t)/[c_H(E_t) + 1] = y_H(E_t)$, i.e. corresponding to the occupancy by protons in the absence of tetrodotoxin, we can write

$$y'_H(E_t) = y_H(E_t) \times p'_T(E_{HP}). \tag{A 5}$$

Introducing (A 5) into (A 3) and denoting $p'(E_t) = 1 - y'(E_t)$ leads to

$$p'(E_t) = p'_T(E_{HP}) - [y_H(E_t) \times p'_T(E_{HP})]. \tag{A 6}$$

Finally, since $1 - y_H(E_t) = p_H(E_t)$, we obtain

$$p'(E_t) = p'_T(E_{HP}) \times p_H(E_t). \tag{A 7}$$

The p's denote the fractions of channels not occupied under the respective experimental circumstances and are hence proportional to P_{Na}. Therefore eqn. (A 7) expresses the fact illustrated by Fig. 7 that for any value of E_t, P_{Na} observed in acid toxin solution ($\propto p'(E_t)$) equals P_{Na} in acid Ringer solution ($\propto p_H(E_t)$) *times* a constant factor $p'_T(E_{HP})$. This factor is complementary of $y'_T(E_{HP})$ so that from the first right hand expression of eqn. (A 1) we obtain

$$p'_T(E_{HP}) = \frac{c_H(E_{HP})+1}{c_T+c_H(E_{HP})+1}, \qquad \text{(A 8)}$$

i.e. the eqn. (5) on p. 21.

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