MOLECULAR PHARMACOLOGY, 8, 285–292

The Kinetics of the Interaction between Tetrodotoxin and Mammalian Nonmyelinated Nerve Fibers

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(Received November 24, 1971)

SUMMARY

Kinetic measurements were made of the blockade by tetrodotoxin (TTX) of sodium channels in the nonmyelinated fibers of the desheathed rabbit vagus nerve. The onset rate was found to increase with TTX concentration, whereas the offset rate after removal of TTX was found to be relatively independent of the TTX concentration to which the preparation had been exposed. It has been shown that the rate of action of tetrodotoxin may well be controlled by diffusion (slowed by simultaneous adsorption) rather than by the rate of the drug-receptor interaction. Nevertheless, the equilibrium constant calculated independently from kinetic measurements, on the wrong assumption that the rates are controlled by the drug-receptor interaction, should be roughly correct. The equilibrium constant so calculated was 3.18 nM, confirming the previous independent estimate made from equilibrium measurements of TTX blockade.

INTRODUCTION

Cuervo and Adelman (1) investigated, in voltage-clamp experiments, the rate at which tetrodotoxin acts on the squid axon, by following the tetrodotoxin-induced reduction in sodium current. These authors assumed, as we shall, that the interaction between the tetrodotoxin molecule[1] and its membrane receptor (R), insofar as it is reversible (see ref. 1 and DISCUSSION below), can be represented by the reaction

$$\text{TTX} + R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{TTX-}R \qquad (1)$$

where the receptors are supposed to be identical and independent. This mechanism implies the Langmuir binding equation (2), which was used by Cuervo and Adelman (1) and by Colquhoun and Ritchie (3). The values for the law of mass action rate constants in squid axon were found (1) to be $k_1 = 0.202 \times 10^8$ M^{-1} min^{-1} and $k_2 = 0.116$ min^{-1}. The ratio of these, i.e., the dissociation equilibrium constant, $K = k_2/k_1$, was 5.74 nM, in fair agreement with the value obtained in the same preparation from equilibrium measurements, which was 3.31 nM (1). In experiments on the desheathed rabbit cervical vagus nerve, Colquhoun and Ritchie (3) estimated, from equilibrium measurements, a similar value of about 3–5 nM for the equilibrium constant of tetrodotoxin acting on the small nonmyelinated C fibers. We have now used this information, together with new evidence on the kinetics of action of tetrodotoxin on mammalian C fibers, as a basis for discussing the general question, often raised when the interaction of a drug with a receptor is being examined: namely, whether the observed rates of onset

This work was supported in part by Grant NS-08304 from the United States Public Health Service.

[1] The abbreviation used is: TTX, tetrodotoxin.

and offset of action of a drug really represent the rates of drug-receptor interaction or whether they reflect the rates of diffusion into, and out of, some compartment containing the receptors. The work of Rang (4), Waud (5), and Thron and Waud (6) has already indicated that in some circumstances diffusion-controlled rates may mimic drug-receptor interaction-controlled rates. We have extended quantitatively the treatment of the model used by these authors into the range required by our experimental results. We conclude that the ratio of the apparent forward and backward rate constants may still give a fairly good estimate of the equilibrium constant, even when the rates are diffusion-controlled, if the drug concentration is not too high.

METHODS

Adult rabbits were killed by injection of air into an ear vein, and a cervical vagus was removed, desheathed, and mounted in a sucrose gap for electrical recording of the compound action potential of the nonmyelinated fibers (7). The part of the nerve from which the action potential was recorded was continuously perfused with Locke's solution, or with one of a series of modified Locke's solutions that could be selected by means of a tap. Switching from one solution to another was complete within a few seconds, and produced little or no electrical artifact (7). The nerve was stimulated with single maximal shocks (1 msec duration; 5–10 times threshold) every 40 sec throughout the experiment. The distance between the stimulating cathode and the recording Locke-sucrose interface was usually about 3 mm.

Solutions. Normal Locke's solution contained NaCl, 154 mM; KCl, 5.6 mM; $CaSO_4$, 5 mM; dextrose, 5 mM; and Tris buffer (pH 7.2), 2.5 mM. Low-sodium Locke's solutions were made by replacing sodium chloride with an equimolar amount of choline chloride. The choline (Eastman Kodak) was recrystallized from ethanol.

All experiments were done at room temperature, about 20°.

Whenever possible, means and standard errors of the results are given.

THEORETICAL CONSIDERATIONS

It will help in the interpretation of the observed rate of action of tetrodotoxin to make some quantitative predictions about the expected rate of diffusion of tetrodotoxin into the tissue when the drug is simultaneously adsorbing to saturable sites. The simplest model appropriate to this situation is a modification of the biophase model of Furchgott (8) and of Frankenhaeuser and Hodgkin (9). This model postulates a diffusion barrier separating the receptors from the external fluid, the compartment containing the receptors being referred to as the biophase, for example, the periaxonal space: the sites themselves thus form part of the biophase. This model has been modified to allow for the adsorption of drug by Rang (4), whose "limited biophase model" will now be discussed.

If it is assumed that the biophase can be treated as well mixed, we obtain

$$\frac{dc_i}{dT} = (c_o - c_i) - \frac{M}{KV} \cdot \frac{dp_b}{dT} \qquad (2)$$

The terms used in this and subsequent equations are defined as follows:

K = equilibrium constant (moles/liter)

c = normalized (dimensionless) concentration unit ≡ drug concentration/K

c_i = normalized concentration of free (unbound) drug in biophase at time t

c_o = normalized concentration in external solution, assumed constant

V = volume of biophase per unit weight of tissue

k_p = permeability constant for diffusion barrier; it is assumed to be the same in both directions, so at equilibrium $c_i(\infty) = c_o$

τ_0 = V/k_p = time constant for exponential change of concentration in the biophase in the absence of adsorption

t = time

T = normalized (dimensionless) unit of time, defined as t/τ_0

M = capacity of binding sites, in moles per unit weight of tissue
p_b = fraction of binding sites blocked at time t
M/KV = ratio of binding capacity to amount of drug unbound in biophase when drug concentration = K, i.e., $c_i = 1$

If we assume that the binding sites can be identified with the receptors, and that they equilibrate rapidly so that at all times we have the Langmuir equation $p_b = c_i/(1 + c_i)$, then we can substitute $dc_i/dT = (c_i + 1)^2\, dp_b/dT$ into Eq. 2. The resulting equation was given by Rang (4), who presented some analogue computer solutions of it for values of M/KV up to 10. In the present case results for much larger M/KV are needed, and it will be convenient to use the analytical solution. Integration of Rang's equation gives[2]

$$\frac{t}{\tau_0} = \frac{M}{KV}\, p_f(\infty)^2 \left[\frac{p_f(0) - p_f(t)}{p_f(\infty)} - \ln p_f^{\dagger}(t)\right] - \ln\left(\frac{p_f(0)}{p_f(t)}\right) - \ln p_f^{\dagger}(t) \qquad (3)$$

where $p_f(t) = 1 - p_b(t) = 1/(c_i + 1) =$ fraction of receptors *not* occupied at time t, and

$$p_f^{\dagger}(t) \equiv \frac{p_f(t) - p_f(\infty)}{p_f(0) - p_f(\infty)}$$

When the binding is large relative to the amount of unbound drug in the biophase, M/KV being at least 100–200, the terms other than the first in Eq. 3 become negligible at most t and c, so that the plots of occupancy against time will have the same appearance for a given drug concentration, whatever the value of M/KV. In this case occupancy can be plotted against t/τ, where $\tau \equiv \tau_0 M/KV$, and this is done in Fig. 1 for drug concentrations $c_o = 0.1$, 1.0, and 10, corresponding (3) to TTX concentrations of about 0.3, 3, and 30 nM. Numerical calculations show that the curves in Fig. 1 are an excellent approximation to the exact result (Eq. 3) when M/KV is at least a few hundred, but in case of doubt the full Eq. 3 should be used. The curves for offset show log $p_b(t)$ plotted against t/τ. For onset the plots show log $[p_b(\infty) - p_b(t)]$ against $t(c_o + 1)/\tau$. The reason for plotting in this way is to facilitate comparison with the alternative model in which the rates of onset and offset are dependent only on the rate of interaction of drug with receptor, with the rate constants k_1 and k_2 (see Eq. 1). In the latter case offset and onset are exponential with rate constants k_2 and $k_2(c_o + 1)$, respectively (2). Therefore, if results conforming to the receptor interaction model were plotted as in Fig. 1 (with $\tau = k_2$), both onset and offset would fall on a single straight line with slope = -1. This sort of plot was used by Rang (4), who shows some solutions for smaller M/KV values. It may be noted that in the caption of Rang's Fig. 3 the words "solid" and "interrupted" should be interchanged.[3] Over a wide range of concentrations, and for M/KV values not less than 4 or so (see Fig. 1 and ref. 4), it is seen that onset and offset are slowed, by adsorption, by a factor approaching M/KV.

If the biophase can be identified with the periaxonal space, then $M/KV \cong 3500$ for the rabbit vagus preparation (see DISCUSSION), and therefore the graphs in Fig. 1 can be used. At low concentrations (relative to K) it is clear that onset and offset of occupancy (and concentration) become simple exponential processes with rate constant $\tau_0 M/KV$. At high concentrations, such as those used in the present experiments, the occupancy no longer changes in a precisely exponential way. But at $c_o = 1$ the deviation is small and even at $c_o = 10$ it is not huge, and the rates of onset and offset are slowed, by adsorption, by a factor approaching M/KV. Unless the drug concentration is very high, the onset starts with rate constant $\tau_0 M/KV$ and then speeds up as the occupancy increases, whereas offset is initially fast and then settles down to this rate, as might be expected.

Over the 100-fold range of concentration shown in Fig. 1, the offset rate varies relatively little, in rough agreement with the

[2] A. L. Hodgkin, personal communication; H. P. Rang and D. Colquhoun, unpublished results; D. Thron, personal communication.

[3] H. P. Rang, personal communication.

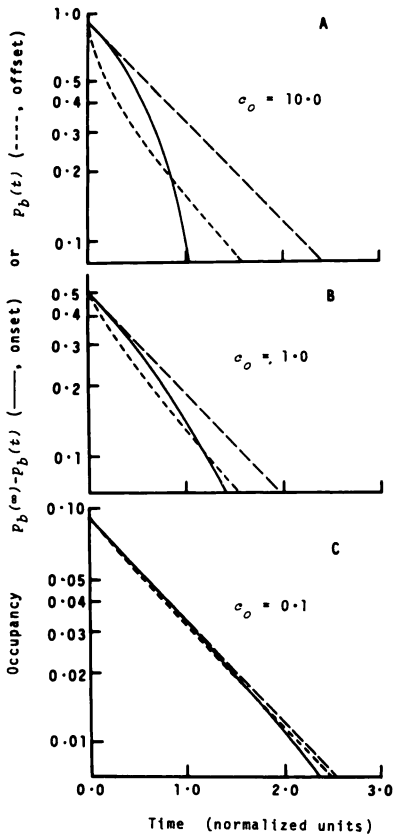

FIG. 1. *Calculated time course of occupancy according to limited biophase model, where M/KV is large (at least 100–200)*

For offset (-----) $p_b(t)$ is plotted (on a logarithmic scale) against t/τ, where $\tau = \tau_0 M/KV$ and $\tau_0 = V/k_p$ = time constant in the absence of adsorption; for onset (——) $p_b(\infty) - p_b(t)$ is plotted (logarithmically) against $t(c_o + 1)/\tau$ as explained in the text. For reference, straight lines (– – –) with slope corresponding to a time constant of τ are shown. The values (c_o) of the concentration of the applied drug (in multiples of the equilibrium dissociation constant K) were: A, 10; B, 1.0; C, 0.1.

simple Langmuir model, according to which it should be constant (k_2). Over the range of concentrations used in the experiments, corresponding to $c_o \cong$ 3–30, the offset rate curves on the limited biophase model are virtually identical (like that shown for c_o = 10). Furthermore, the onset and offset curves in Fig. 1A, B, and C have similar slopes, at least in the central range of occupancy (around 30–70 %, and the onset curves are very similar over the range c_o = 3–30 (like that shown for c_o = 10). Thus the onset rate constant is predicted to be faster than offset rate by a factor similar to that predicted by the simple Langmuir model, viz. (c_o + 1), as was observed (see EXPERIMENTAL RESULTS). Consequently in both models the ratio of the onset (κ_{on}) and offset (κ_{off}) rate constants is about the same. Approximately the same value of K is obtained from the relationship $K = \kappa_{off}[TTX]/(\kappa_{on} - \kappa_{off})$ whether the rate constants are determined by the limited biophase model or by the simple Langmuir model. This means that the estimate of K from the observed onset and offset rates (Table 1) may be roughly correct, even though the observed rates give completely wrong estimates of k_1 and k_2.

Thus the only distinction that can be used in the present experiments between the simple Langmuir kinetics and limited biophase kinetics is the curvature of the semilogarithmic plots in Fig. 1A. However, moderate curvature is difficult to detect in semilogarithmic plots unless the results are very precise, especially when, as in the present experiments, the asymptote in the onset curves is estimated so as to make the line as straight as possible. In addition, the limited biophase model is probably a fairly crude approximation to the actual physical situation, so that the exact shape of the curves cannot be taken too literally. A more realistic model for diffusion into the nerve trunk would use the diffusion equation [as used, for example, by Ruspini and Hafemann (10) as a model for diffusion of TTX in the brain]. However, the difficulty of relating the measured response to p_f when p_f varies with distance as well as time has discouraged us from attempting to use this model.

Thus, under a wide range of conditions, the limited biophase model predicts results that are at least qualitatively, and often quantitatively, similar to those expected with the simple Langmuir model.

EXPERIMENTAL RESULTS

Kinetics of tetrodotoxin effect. When tetrodotoxin was applied to the desheathed

rabbit vagus nerve, the amplitude of the compound action potential of its nonmyelinated fibers progressively declined with time as more and more of the sodium channels were blocked by the toxin. The time course of fall of the compound action potential height after application of tetrodotoxin in normal Locke's solution was not usually exponential (Fig. 2, ◐). This confirms that the physiological response is not linearly related to occupancy (see also ref. 3). However, when the occupancy (fraction of receptors occupied; see below) by tetrodotoxin was calculated, it was usually found that the onset and offset of occupancy were approximately exponential, as shown in Fig. 2 (●). Occupancy was estimated, as described elsewhere (3), by first measuring at various t the ratio, r, of the concentrations of sodium that result in equal responses

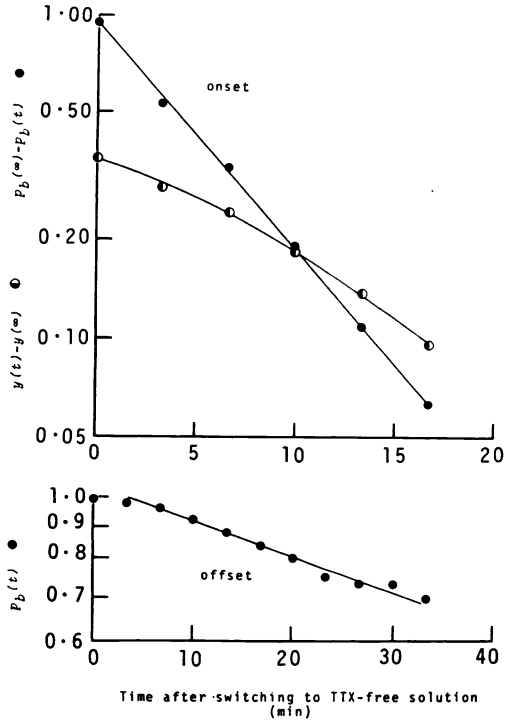

FIG. 2. *Kinetics of onset (upper graph) and of offset (lower graph) of action of TTX on mammalian nonmyelinated nerve fibers at about 20°*

The ordinates are plotted on a logarithmic scale. Upper curve, onset; lower curve, offset. The height of the compound action potential at time t is denoted $y(t)$ where $y(0) = 1$ and the occupancy is denoted $p_b(t)$.

(compound action potential heights) in the presence (for time t) and absence of tetrodotoxin. The tetrodotoxin was applied in normal Locke's solution, so that the numerator of r was always the normal external sodium concentration (154 mM), the denominator being found by interpolation on the (compound action potential height)/log (external sodium concentration) curve determined in the absence of tetrodotoxin. The occupancy was then found from the relation $p_f = 1/r^n$, given by Colquhoun and Ritchie (3), the coefficient n being estimated separately in each experiment.

The experimentally observed onset rate constants (κ_{on}) and offset rate constants (κ_{off}) for occupancy are collected in Table 1. In each experiment the offset rate constant was relatively independent of the concentration of toxin to which the preparation had been exposed; the interpretation of these results is considered under DISCUSSION. On the other hand, the onset rate constant increased roughly in proportion to the concentration of tetrodotoxin used. At all concentrations, and particularly at lower ones, the onset rate constant was considerably less than the rate constant for exchange of cations like sodium and potassium, which is about 1.0 min (11). Even allowing for the larger size of tetrodotoxin, the slowing in the rate of onset (and in the rate of offset) of action thus seems to be determined largely by some factor in addition to diffusion to the site of action in the membrane.

Reversibility. Little or no evidence was obtained in the present experiments for an irreversible component of action of TTX. Thus, in 10 experiments at 20°, preparations were exposed to TTX (5–80 nM; mean, 36 nM) until equilibrium was attained (1–2 hr). The relationship between the conduction velocity and the logarithm of the external sodium concentration was then determined, to permit the measurement of r for the subsequent estimation of occupancy (3). The conduction velocity rather than the height of the compound action potential was used as an index of TTX action in this set of experiments, because the possible irreversible changes sought were likely to be small and the conduction velocity measurement is

TABLE 1

Onset (κ_{on}) and offset (κ_{off}) rate constants for TTX action

Expt.	[TTX]	κ_{on}	κ_{off}	$(\kappa_{on} - \kappa_{off})/[TTX]$	$\kappa_{off} \cdot [TTX]/(\kappa_{on} - \kappa_{off})$
	nM	min^{-1}	min^{-1}	$nM^{-1}\ min^{-1}$	*nM*
1	25	0.783	0.0264	0.0303	0.87
	50	1.203	0.0196	0.0201	0.98
2[a]	50	0.239	(0.0647)	(0.0035)	(18.49)
	100	0.538	(0.0647)	(0.0047)	(13.77)
3	15	0.390	0.0196	0.0247	0.79
	25	0.450	0.0196	0.0172	1.14
4	30	0.171	0.0118	0.0053	2.23
5	10	0.088	0.0358	0.0052	6.88
	30	0.218	0.0358	0.0061	5.87
6	10	0.073	0.0169	0.0056	3.02
7	10	0.139	0.0295	0.0110	2.68
	25	0.187	0.0181	0.0068	2.66
8	25	0.170	0.0385	0.0053	7.26
	50	0.470	0.0352	0.0087	4.05
9	25	0.110	0.0156	0.0038	4.11
10	20	0.099	0.0149	0.0042	3.55
11	20	0.153	0.0117	0.0071	1.65
Mean ± SE			0.0233 ± 0.0024	0.0108 ± 0.0020	3.18 ± 0.55

[a] Not included in means.

less affected by the recording conditions than the action potential height (3). Washing for 1–2 hr in a toxin-free solution led to recovery of the conduction velocity to 92.3% of its original value. However, after a simple determination of the conduction velocity/log (sodium concentration) relationship in the *absence* of TTX in the same experiments, the conduction velocity recovered to only 95.6% of its original value. The difference in the recovery after determining the conduction velocity/log (sodium concentration) relationship in the presence and absence of TTX thus seems slight (3.3% ± 1.4). However, a recovery of conduction velocity to within 3.3% of its control value corresponds with a recovery of p_f only to 85.6% of its original value (based on a value of q of 0.49; see Eq. 6 and p. 549 of ref. 3). But even this much larger lack of recovery is not good evidence for irreversibility; Table 1 shows that recovery of occupancy for TTX occurs with a time constant of about 46 min, so that recovery to 85.6% would take about 90 min, which might well account for much of the observed apparent irreversibility.

DISCUSSION

It was shown by Hill in 1909 (2) that the model specified in the INTRODUCTION (Langmuir adsorption) predicts that the offset and onset of occupancy $p_b(t)$, after a step change in drug concentration, would be exponential with rate constants k_2 and $(k_1 \cdot [TTX] + k_2)$, respectively. If the observed rates of onset and offset of occupancy (rate constants κ_{on} and κ_{off}) in Table 1 were controlled by the rate of reaction between drug and receptor, it follows that k_2 should be estimated by κ_{off}, and k_1 by $(\kappa_{on} - \kappa_{off})/[TTX]$. The ratio of these estimates, $k_2/k_1 = K$, should provide an estimate of the equilibrium constant from kinetic measurements. This estimate can then be compared with that obtained independently from equilibrium measurements. Fifteen such estimates on 10 different preparations gave a value of K of 3.18 ± 0.55 nM (see Table 1), which agrees reasonably well with the estimates of 2.38 and 3.05 nM made by Colquhoun and Ritchie (3) from equilibrium measurements of action potential and conduction velocity, respectively. Unfortunately, this agreement does not im-

ply that the estimates of k_1 and k_2 from kinetic measurements are even roughly right. It is shown under THEORETICAL CONSIDERATIONS that even if k_1 and k_2 were nearly infinite, results quite similar to those observed, including an approximately correct value for K, are predicted by a simple diffusion model. The model postulates that the interaction with the receptor is rapid, and that the rate of action of the drug is controlled by a diffusion barrier separating the compartment containing the receptors (the biophase; e.g., the periaxonal space) from the external solution. When a substantial proportion of drug is bound, the drug concentration in the biophase changes much more slowly than if the drug were not bound. A suitable measure of binding (4) is M/KV (see also THEORETICAL CONSIDERATIONS), where M = binding capacity per unit amount of tissue, K = equilibrium constant, and V = volume of biophase per unit amount of tissue; thus M/KV is the ratio of the binding capacity to the amount of unbound drug present in the biophase when the drug concentration is K, i.e., about 3 nM. The following calculation suggests that this factor may be important in mammalian C fibers. If the width of the periaxonal space is about 100 A, the volume of the periaxonal space would be given approximately by $V \times 34 \times 100 \times 10^{-6} \times 10^{-3} = 3.4 \times 10^{-2}$ μl/mg, dry weight, there being 34 cm^2 of membrane per milligram, dry weight (11). The tetrodotoxin uptake, M, is 3.6×10^{-13} mole/mg, dry weight (12). Taking $K \cong 3$ nM, we obtain $M/KV \cong 3500$ (this is an upper bound for the correct value because the value of M used is an upper bound for the specific uptake). The amount bound is very much larger than the amount free in the extracellular space, and it was shown under THEORETICAL CONSIDERATIONS that this would be expected to make the approach to equilibrium a few thousand times slower than for a substance that was not bound. It is difficult to make further quantitative predictions, because of uncertainty about what the rate of diffusion would be in the absence of binding. For example, the theoretical analysis by Greengard and Straub (13) suggests that diffusion of small molecules out of the periaxonal space is rapid, in the absence of adsorption, and is measured in milliseconds, so that even when multiplied by a factor of a few thousand the slow onset of action of the tetrodotoxin could barely be accounted for, and the slowness would probably have to be ascribed to the receptor-drug interaction. However, Keynes and Ritchie (11) have shown experimentally that the sodium and potassium in the periaxonal space equilibrate with the sodium and potassium in the bulk of the solution with a much larger time constant, of about 1 min; and Colquhoun, Henderson, and Ritchie[4] have found a time constant of 4.5 minutes for the equilibration of the extracellular space with [1-^{14}C]D-mannitol. At the moment it is not clear which is the relevant time constant for the purposes of calculation. If the value of Keynes and Ritchie (11) is the appropriate one, it is possible that the barrier to diffusion encloses a much larger volume than just the periaxonal space, so that the effect of adsorption on equilibration time would not be so pronounced. At most, however, this volume (V) could not exceed the extracellular space, which is about 2.4 μl/mg, dry weight (14), and so M/KV could still be about 50. There would still be much more bound drug than unbound drug in the biophase. The time constant of 1 min for sodium equilibration might well be reflected as a time constant (κ_{off}) in the region of 40 min, as observed experimentally. Certainly, the findings by Hille (15) that TTX produces a steady level of inhibition within 5 sec of its application to frog nodes and that complete recovery may occur within 15 sec support the idea that the toxin-receptor interaction is indeed very fast.

Further experiments are clearly necessary to resolve this critical question. It is probably better at the moment to regard the rate constants that have been obtained merely as providing an empirical description of the kinetics of onset and offset, without ascribing to them any particular theoretical meaning. However, as the THEORETICAL CONSIDERATIONS show, even if the values of the apparent rate constants k_1 and k_2 are in error, their ratio, k_2/k_1, may give an approximately correct estimate of the equilibrium dissociation constant. In fact, the value of K derived

[4] Unpublished observations.

from the kinetic measurements in this paper agrees well with that previously determined from the equilibrium measurements in the same preparation (3).

Similar remarks probably apply to the work of Cuervo and Adelman (1) on the rate of TTX action on squid axon. If the density of binding sites on the squid axon is similar to that on C fibers, calculations of the sort given above suggest that the presence of the binding sites should reduce the rate of TTX equilibration by a factor of a few thousand in the squid axon[5] and in any other axons with a periaxonal space of similar thickness, so that in these axons, too, the rate of equilibration may well not reflect the rate of drug-receptor interaction.

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