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THE BINDING OF LABELLED TETRODOTOXIN TO NON-MYELINATED NERVE FIBRES

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SUMMARY

1. Tritiated tetrodotoxin has been prepared and purified, and its binding to rabbit, lobster, and garfish non-myelinated nerve fibres examined.

2. In each case a component of the binding curve was found that saturated at concentrations of a few nanomolar.

3. In addition, non-specific binding, indicated by a linear dependence of the amount bound on concentration, occurred.

4. The kinetics of wash-in and wash-out of the radioactive toxin were consistent with a model in which all binding was rapid and reversible and in which diffusion into and out of the nerve bundle was rate-limiting. This was shown by numerical solution of the appropriate non-linear diffusion equation. An extension of the limited biophase model that allows for non-specific binding was shown to give good semiquantitative approximations to the proper diffusion equation; and (unlike the latter) the extension was shown to have a relatively simple solution.

5. A number of pharmacological agents were tested for competition with, or perturbation of, tetrodotoxin binding: sodium, calcium and hydrogen ions, lidocaine, batrachotoxin and saxitoxin. Apart from a small calcium effect, only saxitoxin, whose effect on sodium current is similar to that of tetrodotoxin, was found to interfere with binding. Increasing saxitoxin concentrations resulted in reduced amounts of tetrodotoxin binding in a manner consistent with a competition between the two toxins for the same site.

INTRODUCTION

A useful step in any chemical characterization of the sodium channels in nerve membrane would clearly be the identification of some measurable property of the channel that did not depend on the intactness of the tissue. Such a property might be the ability to bind some marker molecule that

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could be suitably tagged. An obvious candidate is tetrodotoxin, which in low concentration specifically reduces the size of the sodium ionic current without altering its kinetics. Colquhoun & Ritchie (1972*a*,*b*) have inferred from electrophysiological experiments that the equilibrium dissociation constant for tetrodotoxin binding in rabbit nerve is small, about 3–4 nM. More direct voltage-clamp experiments on squid axons by Cuervo & Adelman (1970) have demonstrated a reversible component of inhibition of sodium ionic current by tetrodotoxin with an apparent equilibrium constant of 3·3 nM; and, in frog nodes of Ranvier, Hille (1968) showed that the equilibrium dissociation constant for saxitoxin, whose effect is very similar to that of tetrodotoxin, is 1·2 nM. Furthermore, experiments in which the uptake of tetrodotoxin (and saxitoxin) has been measured by bio-assay of the supernatant solution have already demonstrated that a remarkably small amount of binding occurs and have allowed an upper limit to be placed on the density of sodium channels in rabbit (Keynes, Ritchie & Rojas, 1971), lobster (Moore, Narahashi & Shaw, 1967; Keynes *et al.* 1971), and crab (Keynes *et al.* 1971) non-myelinated nerve fibres. Unfortunately, the method used (see Clark, 1933) took too long for more than a few points to be determined, so it was not feasible to determine the time course of adsorption, or to estimate the amount of any non-specific binding.

In the present experiments the interaction between the sodium channels and tetrodotoxin is again examined, but this time by directly measuring the amount of tetrodotoxin bound. Tritium labelled tetrodotoxin has been prepared and its binding to non-myelinated nerve fibres examined as a function of the concentration of tetrodotoxin and under a variety of conditions. As in the earlier experiments (Keynes *et al.* 1971), rabbit and lobster non-myelinated nerves were used because their small average axonal diameter means that they have a relatively large amount of excitable membrane per gram of tissue. In addition, the olfactory nerve fibres of the garfish were used. This latter preparation consists of a more uniform population of non-myelinated fibres with even smaller axonal diameters than the others (Gasser, 1956; Easton, 1965) and in these respects is ideally suitable for measurement of the binding of the toxin to the nerve membrane and for any future studies of membrane constituents.

METHODS

Preparation

Three different preparations of non-myelinated fibres were used in these experiments: *rabbits*, about 10 lb in weight, were killed by injection of air into an ear vein and both cervical vagi, which consist mainly of non-myelinated fibres (Keynes & Ritchie, 1965), were removed and desheathed. A single vagus nerve weighed about 20 mg. *Lobsters*, about 1¼ lb in weight and obtained from the local fishmongers, were stripped of their walking legs and the nerves obtained using the method of Furasawa

(1929). A single lobster yielded about 400 mg of walking leg nerve. *Garfish*, about 2–3 ft long, were obtained from the Gulf Specimen Co., Panacea, Florida. Although the fish did not survive long after arriving from Florida, the olfactory nerves seemed to be in reasonably good physiological condition, judged by their ability to conduct impulses. The dissection procedure has been described by Easton (1965). The two olfactory nerves from a typical garfish were about 150 mm long and yielded about 250 mg of non-myelinated nerve.

Solutions

The normal bathing solutions for the different preparations are shown in Table 1. Modifications of these solutions are described in the text.

TABLE 1. Composition of the bathing solutions used for different species

	Concentration (mM)									
Species	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	SO_4^{2-}	Urea	Dextrose	Sucrose	Tris buffer (pH 7·2)
Rabbit	154	5·6	5	—	154	7·8	—	5	—	2·5 or 10
Lobster	520	13	14	24	609	—	17	—	—	1·0
Garfish	120	2·5	3·5	—	129·5	—	—	24	60	1·0

Design of experiment

In each individual experiment six to fifteen animals were used yielding twelve to one hundred and fifty separate nerves, each of which was sometimes divided in two so as to double the number of individual estimations that could be made. Whenever the effect of a procedure on binding was tested (for example, low sodium) at a given tetrodotoxin concentration, one half of the nerve, or the nerve from the other side of the same animal, was used as a control. Various different pairing and randomization procedures were used. On several occasions a balanced incomplete block design was used (see Colquhoun, 1963). Mostly, however, randomization was achieved simply by pooling all the nerve preparations initially and drawing samples haphazardly from the pool for exposure to the different tetrodotoxin concentrations.

The nerves were soaked for various times in Locke solutions containing the labelled tetrodotoxin. At the end of the soaking-in period they were weighed wet, dried in air at about 60° C for 1–2 hr, weighed dry, and then dissolved in 0·5 ml. of an alkali solubilizer (Protosol, New England Nuclear) to which 0·1 ml. water had been added to facilitate hydrolysis. During this latter procedure the nerves were incubated at about 60° C for 1–3 hr. The radioactivity in the solubilized nerve preparation was determined by adding Bray solution (Bray, 1960) and counting in a liquid scintillation counter. In some experiments, particularly those with lobster nerves, larger volumes of Protosol were required. Furthermore, in these latter experiments considerable chemi-luminescence occurred, when it proved necessary to add sufficient glacial acetic acid to neutralize the solubilizer and to allow the vials to stand for a few days at 4° C until the chemi-luminescence, induced by the digestion, had died out. Uptakes were then expressed as f-mole (i.e. 10^{-15} mole) tetrodotoxin/mg dry of nerve.

In a typical experiment, a vagus nerve (wet weight, 20 mg) that had been exposed to 30 nM tetrodotoxin might contain a total of 50 counts/min (cpm, about 1500 f-mole). At lower concentrations, the amount of bound radioactivity might be as low as 10 cpm. Careful measurement of the background (about 15 cpm) was therefore required; and a number of control experiments were done that demonstrated that

neither the background counts nor the efficiency of the scintillation fluid depended to any important degree on the weight of the particular nerve. Counting each vial for several hours gave a statistical counting error of about 0·5 cpm. Backgrounds from nerves soaked in toxin-free solutions were also reproducible to ± 0·3 cpm.

All experiments were done at room temperature, which was maintained unless otherwise stated at 20° C.

When possible, mean values ± S.E. of mean are given.

Preparation and purification of tritiated tetrodotoxin

Two 5 mg samples of tetrodotoxin, supplied as a freeze-dried powder and free of the citrate buffer that is normally added to stabilize pH, were obtained from Sankyo Chemical Co., Japan. One sample was labelled by the Wilzbach method (Wilzbach, 1957) and the other by a modification of the technique in which a silent electric discharge is used to generate the necessary tritium ions and atoms (Dorfman & Wilzbach, 1959). Both procedures were performed by ICN Corp., Irvine, California, using 20 c of tritium gas in each case. Exchangeable tritium was then removed by dissolving the powder in a small volume of 10^{-3} M acetic acid and freeze-drying. The resulting samples contained 11 and 28 mc total radioactivity respectively for the Wilzbach and gas discharge labelling methods.

Several methods of purification were tried, including thin layer chromatography and gel filtration; but the most successful separations were obtained using high voltage paper electrophoresis at values of pH close to the pK_a of tetrodotoxin, which is 8·5 (Woodward, 1964). The distribution of radioactivity obtained on electrophoresis of the crude material labelled by the gas discharge method is shown in Fig. 1. Essentially, only one peak, II in the diagram, was found that had different electrophoretic mobilities at pH 8·1 and pH 8·5. Peaks I and III represent degradation products not present in the original sample with net charges of 0 and + 1 respectively. Unlabelled tetrodotoxin, which can be detected because it fluoresces after spraying with alcoholic potassium hydroxide and heating (Mosher, Fuhrman, Buchwald & Fischer, 1964), has the same electrophoretic mobility as that of Peak II in Fig. 1 at both values of pH. Accordingly, small batches of tetrodotoxin (0·5 mg) labelled by the gas discharge method were first electrophoresed at pH 8·5 in a Tris-acetate buffer system (0·1 M) as in Fig. 1*a*. After elution and concentration of the peak fractions, they were run again at pH 6·5 in a pyridine:acetic acid:water, 25:1:225, buffer. The resultant material had a radiochemical purity of 85–100 %, as can be seen in Fig. 2, which shows the result of electrophoresis of the final sample at four different pH values bracketing the pK_a. A nominal purity of 90 % was used in the experiments reported here.

The sample that was labelled by the Wilzbach method, when treated identically, did not reveal a clear radioactive peak in the position corresponding to tetrodotoxin, although most of the biological activity (and, therefore, the unlabelled tetrodotoxin) remained. An attempt at partial purification by ion exchange chromatography, followed by gel filtration on Sephadex G-10 (Pharmacia, Sweden), showed that for this method of labelling the main radioactive products were smaller than tetrodotoxin and emerged well after the biological activity on a G-10 column. The radioactivity associated with the tetrodotoxin probably constituted less than 1 part in 50 of the total radioactivity incorporated. By contrast, the gas discharge labelling method, the results of which are described above, gave higher total incorporation and less radioactive impurity, about 1 part in 30 of the total incorporation being present as tetrodotoxin. Conceivably, other methods of labelling may produce better results. For the purposes of this study, however, the material shown in Fig. 2 was considered sufficiently pure and was used throughout after being assayed (see below)

for biological activity against a standard solution of tetrodotoxin of known concentration. The specific activity of the purified tetrodotoxin was approximately 100 mc/m-mole.

Apart from the above demonstration that the labelled and unlabelled materials had the same electrophoretic mobility at several pH values, and hence the same

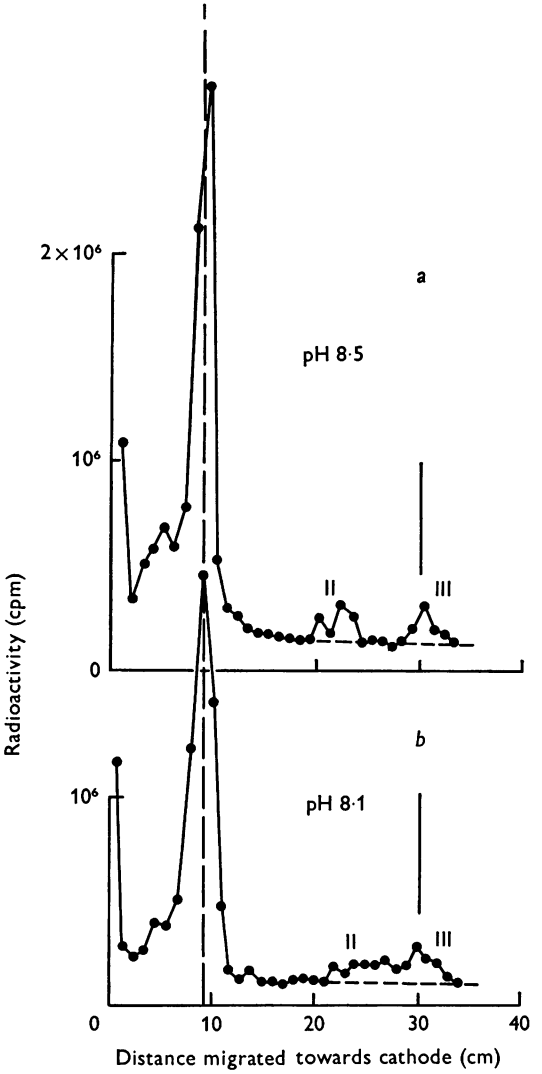

Fig. 1. Distribution of radioactivity obtained on electrophoresis of the crude material tritiated by the gas discharge method: *a*, at pH 8·5 in 0·1 M Tris-acetate buffer; *b*, at pH 8·1 in 0·05 M Tris-acetate buffer. The interrupted vertical line represents the position to which an uncharged marker will migrate during the electrophoresis, which was for 45 min at 100 V/cm. The continuous line is where a singly charged positive particle will migrate.

charge, molecular weight and pK_a, the behaviour of the materials under mild alkaline degradation lends further support to our contention that the labelled and unlabelled compounds were otherwise identical. The half-lives of both labelled and unlabelled tetrodotoxin at 20° C in 0·1 M sodium carbonate buffer pH 9·1 were measured. The disappearance of the radioactive peak on electrophoresis was used to follow the

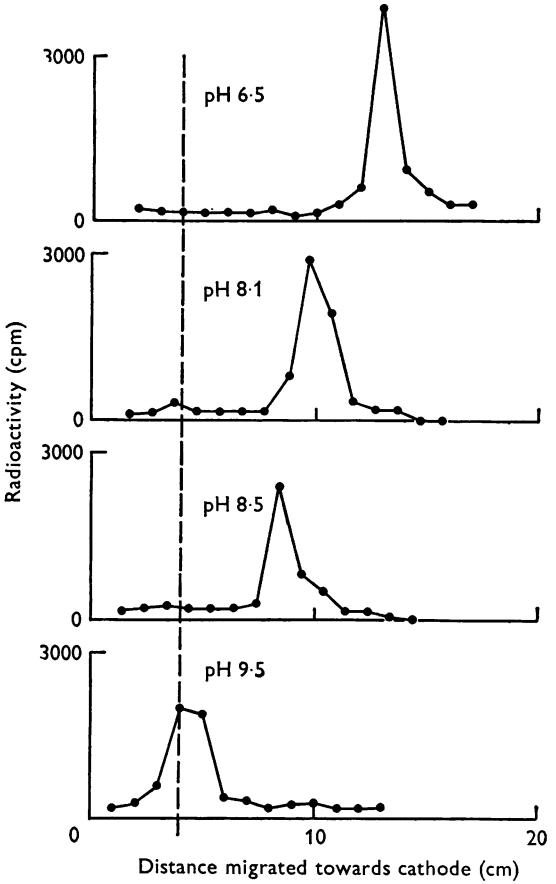

Fig. 2. Electrophoresis of purified tetrodotoxin (TTX) at pH 6·5, 8·1, 8·5 and 9·5. The interrupted vertical line represents the position to which an uncharged marker will migrate during the electrophoresis, which was for 20 min at 100 V/cm.

degradation of the labelled material and the disappearance of a corresponding fluorescent spot produced by spraying with alcoholic potassium hydroxide (Mosher *et al.* 1964) was used to follow the degradation of the unlabelled material. In each case, the half-life was about 2 hr, and the main product was a compound with an electrophoretic mobility at pH 8·1 of approximately zero.

In summary, the crude labelled tetrodotoxin has been purified thirtyfold, to satisfy a number of chemical criteria of purity and homogeneity. Electrophoresis was finally adopted as the simplest method of purification (Figs. 1 and 2). In addition,

preliminary experiments with gel filtration on Sephadex G-10, and thin layer chromatography on carboxymethyl cellulose and silica gel (butanol : acetic acid : water and collidine : water, as used by Mosher *et al.* 1966) also established that the labelled tetrodotoxin had the same mobility as unlabelled material. The fact (Fig. 2) that the purified labelled and unlabelled toxins behaved as though they had the same value of pK_a (8·5) is even stronger evidence of identity, because all known modifications of the toxin invariably perturb the pK_a. For example, O,O′isopropylideneanhydrotetrodotoxin and O-methyl-O′,O″-isopropylidenetetrodotoxin, both derivatives with small modifications some distance from the ionizable hydroxyl group, have pK_a values of 7·5 and 8·3 respectively (Woodward, 1964). Since the purified tetrodotoxin has a specific activity of about 100 mc/mole, only one molecule in 285 actually contains a tritium atom and, therefore, the normal physical methods of analysis such as n.m.r. and infra-red spectroscopy are inappropriate. However, the labelled material used in the following experiments appears homogeneous and identical to the unlabelled material by various reasonable criteria, including one chemical reaction (alkali degradation at pH 9). Finally, several binding experiments similar to those described in the 'Results' section established that an excess of unlabelled tetrodotoxin completely blocks the saturable component of binding of the labelled material, and that dilution of labelled with unlabelled material in the ratio 1 : 5 results in an approximately fivefold reduction in bound radioactivity at both low and high concentrations of tetrodotoxin. The question of the chemical identity of labelled and unlabelled toxin is discussed again later (see pp. 104, 120) where the present binding measurements are compared to those obtained previously with unlabelled material (Keynes *et al.* 1971; Moore *et al.* 1967). Again, the agreement is satisfactory.

Assay of tetrodotoxin activity

During purification, 50 μl. samples were assayed by the method described by Keynes *et al.* (1971, see their Fig. 2). This required very little material but was only moderately accurate. A final assay of the purified material with continuously flowing solutions (as in Fig. 3 of Keynes *et al.* 1971) established the concentration more accurately.

Unlabelled tetrodotoxin, stored at 4° C in citrate buffer, pH 4·8 lost activity with a time constant of roughly 14 months. Corrections were made for this loss of activity; but they were relatively small, since most of the experiments were done within a 3-month period. The absolute concentrations of our solutions is based on the use as a standard of 1·0 mg samples supplied by Sankyo Chemical Co.

RESULTS

The experiments were designed to answer three main questions. (1) In the binding/concentration curve is it possible to distinguish a component associated with the sites responsible for the physiological effect of tetrodotoxin? (2) Are the kinetics of wash-in and of wash-out of the labelled toxin consistent with a model (see, for example, Colquhoun & Ritchie, 1972*b*) in which fast reversible binding is the only cause of uptake? (3) Do any other pharmacological agents alter the observed binding?

Rabbit nerve

Rabbit desheathed cervical vagus nerves were soaked for 2 or 6 hr in various toxin-containing solutions and at the end of this time the total labelled toxin in each nerve was determined. An estimate was then made

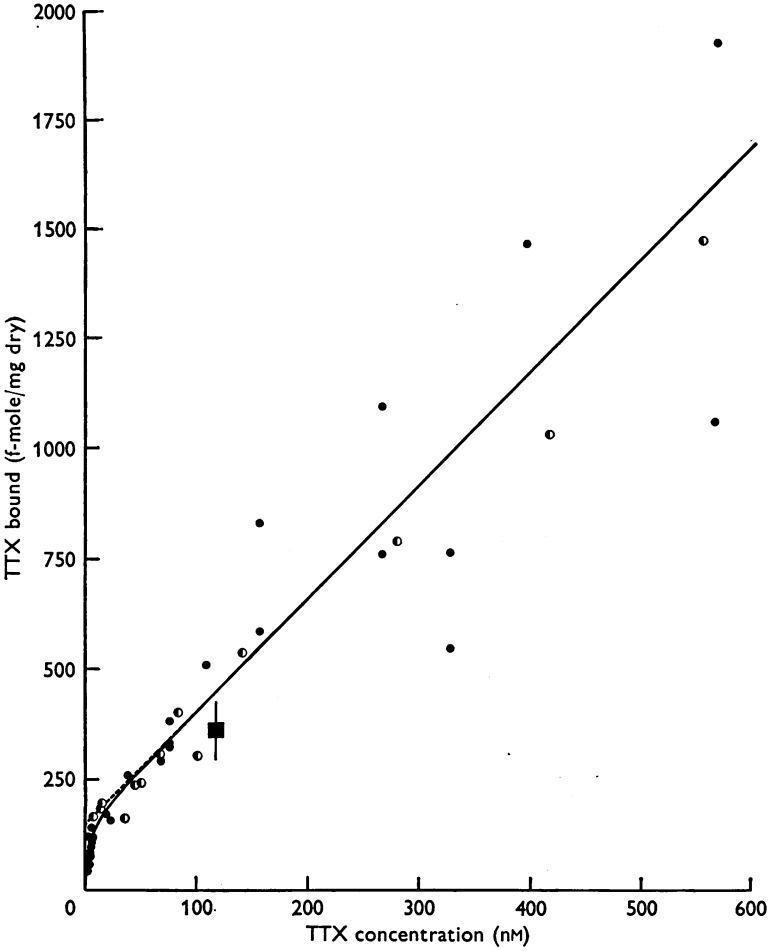

Fig. 3. The uptake of tetrodotoxin (TTX) by rabbit desheathed vagus nerve at different external concentrations of TTX. The nerves were equilibrated for 2 (◐) or 6 (●) hr with the TTX. The symbol ▮ indicates the average value (and twice the S.E.) obtained by Keynes *et al.* (1971). The broken line is the extrapolation of the linear component of the binding curve obtained at high concentration. The continuous line is the relation

U = TTX bound (in f-mole/mg dry) = 2·6[TTX] + 152[TTX]/(3·0 + [TTX])

where [TTX] is given in nM.

of the amount of toxin in the extracellular space. In most of the experiments the extracellular space was estimated as the wet weight minus the sum of the dry weight and the intra-fibre water, the latter being taken to be

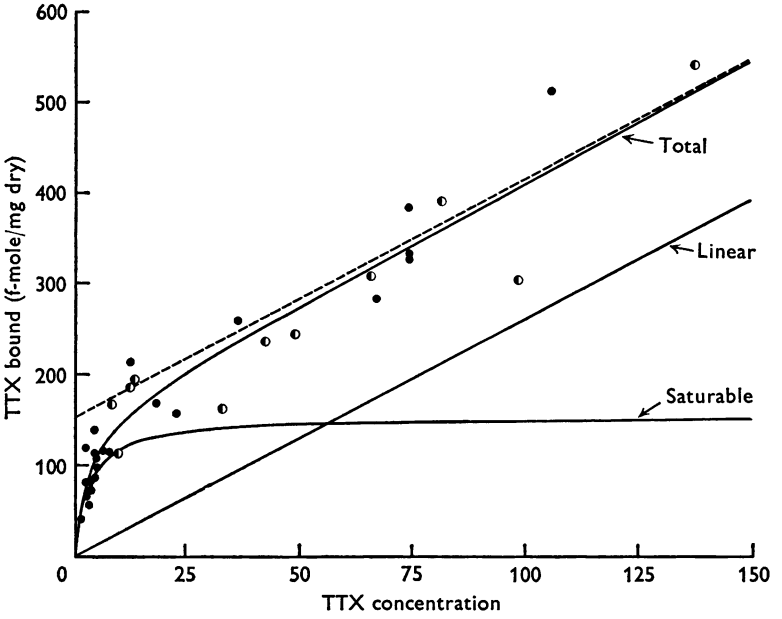

Fig. 4. Same as Fig. 3 on expanded horizontal and vertical scales. The total binding and the two components of binding are also shown.

1·124 × the dry weight (see Rang & Ritchie, 1968). In some experiments, however, the extracellular space was determined directly with [^{14}C]inulin or D-[6-^{14}C]mannitol (Table 2). The intra-fibre water calculated in these experiments agreed quite well with the value obtained by Rang & Ritchie (1968), as shown in Table 2, which gives the intracellular and extracellular spaces for the various nerves used in these experiments. The toxin content of the extracellular space was then subtracted from the total content of the nerve. The remaining uptake, representing all uptake except that in the extracellular space, is shown in Fig. 3 as a function of tetrodotoxin concentration (from 1 to 600 nM); Fig. 4 shows the low concentration end of Fig. 3 on an expanded scale. There are clearly at least two components seen in Figs. 3 and 4; and the results are fitted adequately by a non-saturable component increasing linearly with concentration up to the highest concentrations tested, plus a saturable component that saturates with quite low concentrations of toxin. The kinetics of uptake will be discussed later (see pp. 109–114). However, it is clear from the overlap of the 2 hr (◐) and 6 hr (●) points that uptake has equilibrated in 2 hr at all

concentrations of tetrodotoxin greater than 10 nM. At lower tetrodotoxin concentrations the 2 hr points (see Fig. 10) fell systematically (up to 30–40 %) below the 6 hr points. The large symbol (■) in Fig. 3 represents the average value (with a bar equal to twice the S.E.) obtained by Keynes *et al.* (1971) for the uptake of non-labelled tetrodotoxin at an average concentration of 126 nM (their Table 1). The agreement between the two sets of experiments is clearly satisfactory.

Analysis of the binding curve

The continuous line in Figs. 3 and 4 gives the uptake, U, as

$$U = b[\mathrm{TTX}] + M[\mathrm{TTX}]/(K + [\mathrm{TTX}]), \quad (1)$$

i.e. the sum of a linear component with slope b and a hyperbolic saturable component (Hill, 1909; Langmuir, 1918) with an equilibrium constant K and a binding capacity M. Least squares estimation of the parameters was done using the *Patternsearch* method (Colquhoun, 1971). The results of all 6 hr incubations and of 2 hr incubations with concentrations above 10 nM were included. The points were weighted inversely as their variances (estimated from a smoothed graph made by pooling results from similar concentrations). The interrupted line in Fig. 4, which is the same as the corresponding interrupted line in Fig. 3, shows that visual extrapolation backwards of the binding curve at moderate concentrations of tetrodotoxin (50–150 nM) would almost certainly underestimate M, and hence K. The parameter estimates were: $b = 2{\cdot}6$ f-mole nM^{-1} mg dry^{-1}; $M = 152$ f-mole/mg dry; and $K = 3{\cdot}0$ nM. These values were used to plot the continuous line in Figs. 3 and 4. There is no obvious systematic deviation from the fitted curve at either high or low concentrations, and inclusion of a second saturable component in eqn. (1) did not improve the fit.

The results with rabbit nerve are summarized in Table 3 and indicate a density of saturable sites of $27/\mu\mathrm{m}^2$.

Lobster nerve

Fig. 5 shows the results of an experiment similar to that of Fig. 3 carried out on about 150 walking leg nerves obtained from eighteen lobsters. After dissection, the nerves were pooled; each point on the curve represents a determination made on six to eight nerves (total weight 300–400 mg) selected randomly from the pool. The greater weight of tissue in each sample was particularly advantageous with lobster nerves: whereas in rabbit nerves the extracellular space is only about 3 times the dry weight, in lobster nerve it was found in the present experiments to be 6 times the dry weight (Table 2). The higher value for the extracellular space, together with the value for the specific binding capacity shown in Table 3, meant that in lobster nerve, particularly at high concentrations of tetrodotoxin,

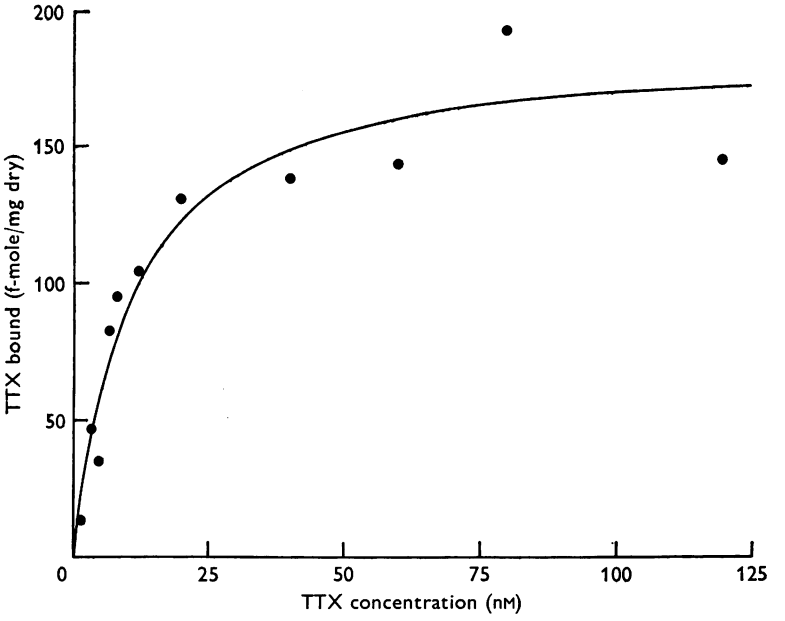

Fig. 5. The uptake of tetrodotoxin (TTX) (after 4 hr) by lobster walking-leg nerves at different external concentrations of TTX. The line is the relation U = TTX bound (in f-mole/mg dry) = 184 [TTX]/(10·2 + [TTX]) where [TTX] is given in nM.

TABLE 2. Extracellular and intracellular spaces of various nerves

Species	No. of expts	Wet wt. / Dry wt. (a)	E.s.* / Dry wt. (b)	E.s.* / Wet wt. (b/a)	I.s.* / Dry wt. ($a-b-1$)
Rabbit	28	4·94 ± 0·09	3·02 ± 0·10	0·613 ± 0·017	0·92 ± 0·08
Lobster	19	10·07 ± 0·12	5·88 ± 0·17	0·546 ± 0·053	3·19 ± 0·22
Gar	16	9·67 ± 0·84	4·09 ± 0·39	0·425 ± 0·013	4·58 ± 0·53

* E.s. = Extracellular space. † I.s. = Intracellular space.

TABLE 3. Specific binding capacities of nerve tissues

Species	K (nM)	Specific binding capacity (f-mole/mg dry)	Non-specific binding (f-mole.nM^{-1}.mg dry^{-1})	Area axonal membrane* (cm^2/mg wet)	Specific binding sites per μm^2
Rabbit	3·0 ± 0·41	152 ± 7·6	2·6 ± 0·09	6	27
Lobster	10·2 ± 3·0	184 ± 26·6	—	7	16
Gar	10·1 ± 1·3	584 ± 41·5	3·4 ± 0·35	143	2·5

* The area of the axonal membranes for rabbit, lobster and garfish are taken respectively, from Keynes & Ritchie (1965), Moore *et al.* (1967), Chacko & Goldman (personal communication). Approximate standard errors were found from the inverse of the matrix of partial second derivatives of the minus log likelihood (not its expectation) with respect to the parameters.

up to 90 % of the toxin in the whole nerve was extracellular; and to obtain a reliable estimate of the bound fraction this extracellular toxin had to be determined accurately. The greater weight of the sample helped in this. But a more accurate knowledge of the extracellular space was still required. The extracellular space was, therefore, determined directly in each sample of lobster nerve by adding D-[1-^{14}C]mannitol as well as [^{3}H]tetrodotoxin to the Ringer solutions. Even with the use of this double labelling procedure the uptake at a high tetrodotoxin concentration was difficult to determine accurately. Nevertheless, it is clear in Fig. 5 that in lobster nerve there is a saturable component that closely obeys the Langmuir relation with an equilibrium dissociation constant of about 10·2 nM and whose maximum binding capacity is 184 f-mole/mg dry. The slope of the linear component was not well determined, and if, as is likely, it was actually somewhat greater than zero, the values for both K and M must be treated as upper bounds for the correct values.

As Table 3 shows, the results with lobster nerve suggest that the density of sites in the saturable component is 16/μm^2. Hafemann (1972) has just published experiments on lobster nerve in which the binding site density for labelled tetrodotoxin was estimated as 22/μm^2 with an equilibrium dissociation constant of 25 nM. These values are somewhat larger than in the present experiments, perhaps because Hafemann's (1972) tetrodotoxin contained more impurities than ours (40 compared with 10 %). The larger value for the equilibrium constant could result from the fact that the time that his nerves were exposed to the labelled tetrodotoxin (1 hr at 4° C in Hafemann's experiments compared with 4 hr at room temperature in ours) was insufficient to allow equilibration.

Keynes *et al.* (1971) found that the uptake of tetrodotoxin by the leg nerves of the lobster *Homarus vulgaris* was 36 f-mole/mg wet. Using the same species as in the present experiment (*Homarus americanus*) Moore *et al.* (1967) estimated the uptake of unlabelled tetrodotoxin by the same nerves to be less than 16 f-mole/mg wet (when exposed to tetrodotoxin in concentrations of 100–300 nM which would be expected to almost completely saturate the sites). This corresponds with an uptake of about 160 f-mole/mg dry (Table 2). The value obtained in the earlier experiments with unlabelled tetrodotoxin in *Homarus americanus* is thus in excellent agreement with the value obtained in the present experiments for the maximum binding capacity of labelled toxin.

Garfish nerve

Uptake of tetrodotoxin by the non-myelinated fibres of the olfactory nerve (Fig. 6) again consists of a saturable and a non-saturable component.

Garfish olfactory nerve, like the lobster nerve, has a large extracellular space, about four times the dry weight (Table 2). However, it was not necessary to determine the extracellular space by double labelling each sample as with the lobster nerve. For although the extracellular correction is nearly as high in the garfish as in the lobster nerve, the specific binding in the garfish nerve turned out to be about four times larger than that in the lobster nerve (Table 3); there was also a larger amount of non-specific binding.

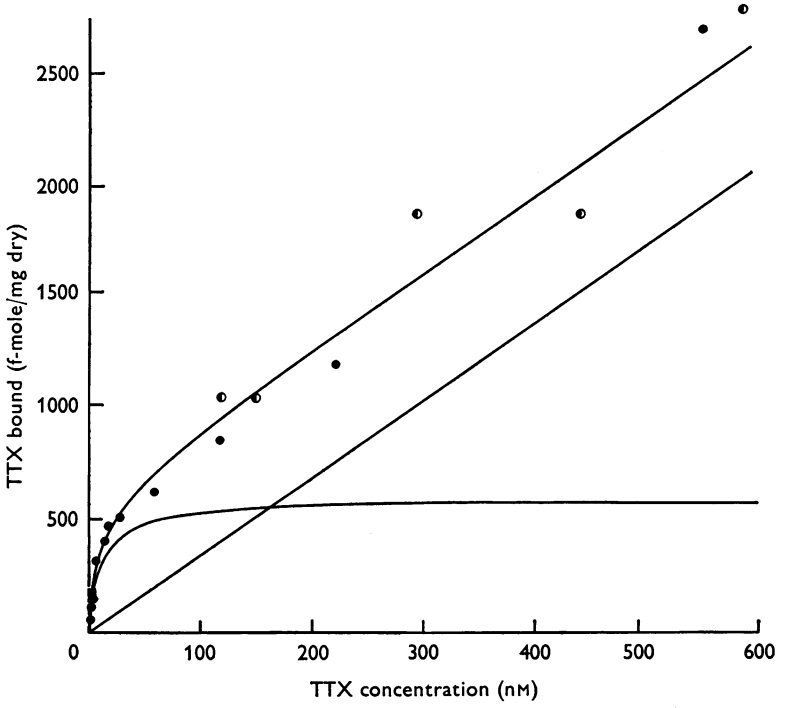

Fig. 6. The uptake of tetrodotoxin (TTX) by the olfactory nerve of the garfish at different external concentrations of TTX. The top line is the total binding drawn to obey the relation

U = TTX bound (in f-mole/mg dry) = 3·4[TTX] + 584[TTX]/(10·1 + [TTX])

where [TTX] is in nM. The two components, linear and saturable, are also shown. The equilibration times were (hr): ◐, 3; ■ (single point), 6; ●, 12.

In the experiment of Fig. 6, uptake of [^{3}H]tetrodotoxin is shown after incubation both for 3 and 12 hr. During the 12 hr incubation, carried out in gar ringer buffered initially to pH 7·5 with 10 mM maleate, the pH fell to 6·5. Longer incubation (up to 24 hr) led to a greater pH change; and the tissue eventually became permeable to mannitol. However, there is very little difference between the amount of uptake at the two times so the

12 hr points are almost certainly close to equilibrium. This conclusion is suggested also by calculations of the sort given on pp. 109–114. The observed mannitol diffusion rate and tetrodotoxin binding capacity predict virtually complete equilibration in 12 hr even if K were as low as 3 nM.

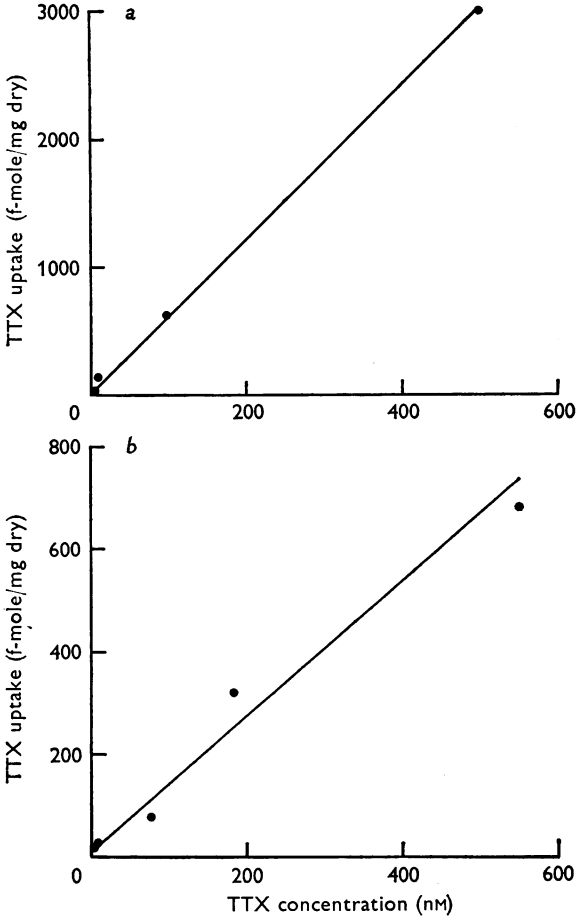

Fig. 7. Binding of radioactive tetrodotoxin (TTX) to *a*, red blood cell ghosts and *b*, the myelinated trigeminal nerve from garfish.

Again the results are fitted well by eqn. (1), with (see Table 3) an equilibrium constant K = 10·1 nM, and binding capacity M = 584 f-mole/mg dry.

The trigeminal nerve that runs with the olfactory nerve in the garfish consists solely of myelinated fibres. The binding curve (Fig. 7*b*) showed mainly non-saturating binding up to 500 nM. Any saturable component also present would have to be small, less than about 15 f-mole/mg dry.

This could represent specific binding at the nodes of Ranvier and, as would be expected, was found to be much smaller than the specific binding (per mg dry of tissue) to the non-myelinated fibres.

Red blood cell ghosts

Red cell ghosts (kindly supplied by Dr J. F. Hoffman) were mixed with solutions containing tritiated tetrodotoxin and ^{14}C-labelled inulin. They were allowed to stand for 30 min, and then spun at 50,000 *g* for 20 min. The pellet and the supernatant were then counted, and the amount of tetrodotoxin bound determined, after a correction was made for the unbound material using the inulin marker. The results are shown in Fig. 7*a*. Only non-specific binding, increasing linearly with concentration of tetrodotoxin, was found. Any saturable binding present was less than 40 f-mole/mg dry weight, representing less than 0·25 sites per square micron of exposed membrane.

Kinetics of wash-in and wash-out of tetrodotoxin

As in all experiments in which the amount of binding to a tissue is measured, it is important to demonstrate that the compound of interest has indeed equilibrated. Furthermore, it must be shown that it has not either irreversibly accumulated in some intracellular compartment or been converted into some entirely different compound. The data in Fig. 3 have already shown that at tetrodotoxin concentrations above 10 nM the difference between the amounts of tetrodotoxin taken up after 2 and after 6 hr is negligible, i.e. equilibration has occurred at least by 2 hr. The following section further demonstrates the agreement between the observed equilibration rates and those predicted on the basis of diffusion into the nerve trunk, assuming that binding equilibrates fairly rapidly (see p. 119) compared with diffusion as proposed by Colquhoun & Ritchie (1972*b*).

The time course of diffusion of mannitol into rabbit vagus nerve is shown in Fig. 8*D*. If the points are taken as lying on a simple exponential curve, the time constant would be about 4·5 min; the line through the points is, however, not an exponential but is a theoretical line determined on the basis of diffusion into a cylinder.

Figs. 8*A*, *B* and *C* show the time course of the total (i.e. free + bound) uptake of tetrodotoxin by rabbit vagus nerve for tetrodotoxin concentrations of 3, 12 and 540 nM. The lines in Figs. 8*A*, *B* and *C* are theoretical lines calculated, as described below, on the assumption of diffusion in the presence of rapidly equilibrating binding. The amount of binding was determined at equilibrium (as above), and the rate of diffusion in the absence of binding was estimated from the rate of mannitol equilibration

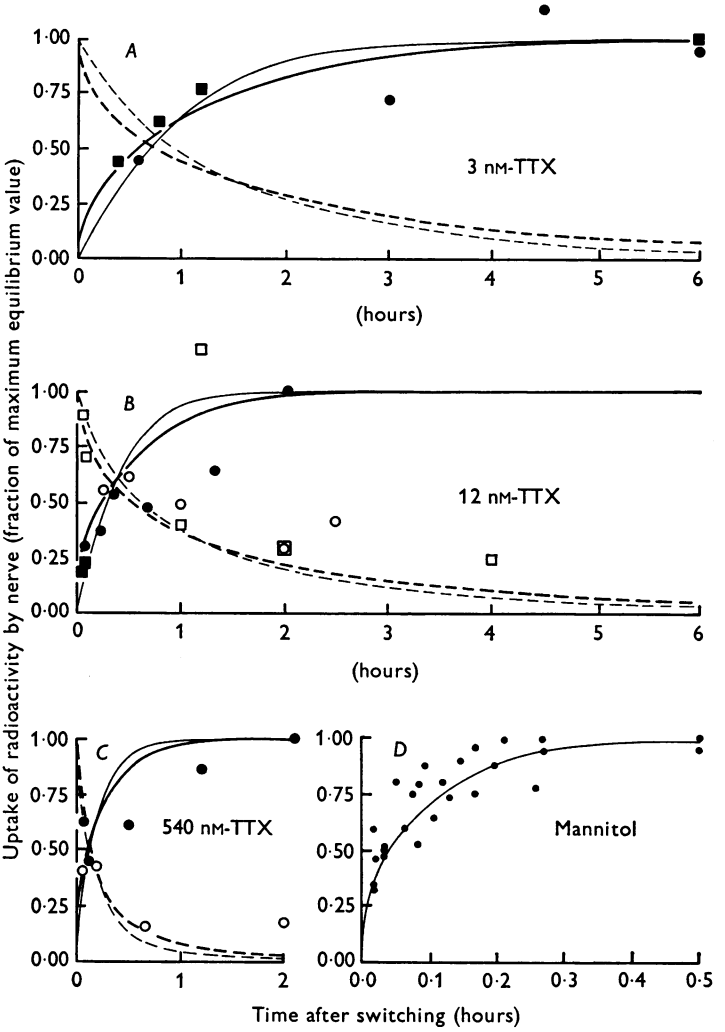

Fig. 8. The time course of the uptake of tetrodotoxin (TTX) (filled symbols) by rabbit desheathed vagus nerve from different external of concentrations of TTX (3, 12, 540 nM) and its subsequent wash-out on being transferred to TTX-free solution (corresponding open symbols). The circles and squares represent separate experiments. The lines are theoretical lines calculated for TTX from the rate of uptake of mannitol (panel *D*) on the basis of: the exact diffusion model (thick lines); and the limited biophase model (thin lines). The continuous lines represent predicted onset curves and the interrupted lines predicted offset curves.

(Fig. 8 *D*). The theoretical curves in Fig. 8 thus involve no arbitrary parameters at all, other than the maximum total uptake in each experiment. The lines fit quite satisfactorily – certainly within the experimental error.

The only exception to this conclusion is that there seems to be a small component (less than 15%) of the total uptake that washes off more slowly than expected.

Prediction of diffusion rate through the tissue

Exact diffusion equation approach. The rate of change of concentration in the absence of binding is assumed to be given by Fick's second law, $\partial c/\partial t = D_{\text{eff}} \nabla^2 c$, where the effective diffusion coefficient perpendicular to the fibre axis, D_{eff}, is assumed constant. Its value was determined for mannitol from Fig. 7*D*, in which the continuous line represents the calculated diffusion taking $a^2/D_{\text{eff}} = 40$ min. (If we take the radius of the nerve, a, as 0·3 mm, this implies D_{eff} is about $3{\cdot}8 \times 10^{-7}$ cm^2 sec^{-1}, i.e. about 1/18 times its value in free solution.) The diffusion coefficient of sucrose (which is similar in size to tetrodotoxin) in solution is about 30% smaller than that of mannitol, so the diffusion coefficient that tetrodotoxin would have if it were not adsorbed will be taken as being smaller in the same proportion, i.e. a^2/D_{eff} will be taken as $40 \times 1{\cdot}3 = 52$ min. Assuming that the interaction with both sorts of binding site is sufficiently rapid for them to remain essentially in equilibrium with the local tetrodotoxin concentration, two terms must be added to the diffusion equation to allow for the reduction in free concentration produced by binding, respectively, to saturable and linear binding sites giving

$$\frac{\partial c}{\partial t} = D_{\text{eff}} \nabla^2 c - m_{\text{sat}} \frac{\partial p}{\partial t} - m_{\text{lin}} \frac{\partial c}{\partial t}, \tag{2}$$

where c is the dimensionless normalized free concentration (free concentration/K), m_{sat} is defined as M/KV (as in Rang, 1966; Colquhoun & Ritchie, 1972*b*); M is the binding capacity, K is the equilibrium constant, and V is the extracellular space (which must be included to make the units of bound and free concentrations the same, so that m_{sat} is dimensionless, as in Colquhoun, 1965), and p is the fraction of saturable sites occupied. The slope of the linear component m_{lin} must also be expressed with bound and free concentrations in the same units, so $m_{\text{lin}} = b/V$ where b is the observed slope. Rearranging eqn. (2), substituting $\partial c/\partial t = (1+c)^2 \partial p/\partial t$ from the Langmuir equation, one gets

$$\frac{\partial c}{\partial t} = \frac{D_{\text{eff}}}{1 + m_{\text{sat}}/(1+c)^2 + m_{\text{lin}}} . \nabla^2 c \tag{3}$$

from which it is clear that diffusion behaves as though the diffusion coefficient were not constant, but varied with concentration, from $D_{\text{eff}}/(1 + m_{\text{sat}} + m_{\text{lin}})$ when few saturable sites are occupied ($c \ll 1$) to $D_{\text{eff}}/(1 + m_{\text{lin}})$ when all saturable sites are occupied ($c \gg 1$). This is a non-linear equation, and it must be solved numerically for the concentration, $c(r, t)$, at distance r from the centre of the nerve at time t. The method used is described in the Appendix. The thick lines in Fig. 8*A*, *B* and *C* are the theoretical total uptake curves calculated from this model, by integration of free and bound concentrations over the fibre radius, taking $M = 150$ f-mole/mg dry, $K = 3{\cdot}0$ nM, $V = 2{\cdot}5$ μl./mg dry, and $b = 2{\cdot}6$ f-mole nM^{-1} mg dry^{-1} so that $m_{\text{sat}} = 20$ and $m_{\text{lin}} = 1$. Clearly, the calculated lines fit the experiments satisfactorily.

The limited biophase model. The fact that the diffusion approach described above can adequately account for the experimental results seems to make it clear that diffusion through the extracellular space is the main rate-controlling factor, rather than diffusion through the Schwann cell layer into the periaxonal space, or the rate of drug-receptor interaction. The two-compartment approach of the limited biophase

model is therefore not physically appropriate; and it may be asked whether the interpretations based on this model by Colquhoun & Ritchie (1972*b*) were justified. It will now be shown that they were; and in this case, at least, the limited biophase model seems to be a reasonable approximation to the diffusion model. In fact, it may be regarded as the approximation, which is much simpler to solve, in which only $N = 1$ annulus is used for integration (see Appendix). It is worth establishing this result in some detail because the relative simplicity of this model should make it widely useful to give at least a preliminary idea of diffusion rate to be expected in the presence of binding, without having to solve a non-linear partial differential equation.

A model of the limited biophase type has already been invoked to explain the fact that diffusion-limited kinetics may closely mimic drug-receptor interaction limited kinetics (Paton & Waud, 1964, 1967; Rang, 1966; Waud, 1967, 1968; Thron & Waud, 1968).

The limited biophase model, which was used by Rang (1966), assumes that the compartment containing the receptors (the biophase) is well stirred and is separated by a diffusion barrier from the drug whose external concentration is constant, and that the receptors equilibrate rapidly with the free ligand in the biophase. The model given by Rang (1966), modified by addition of a term to account for the linear component of adsorption, is

$$\frac{dc}{dT} = (c_0 - c) - m_{sat}\frac{dp}{dT} - m_{lin}\frac{dc}{dT}, \tag{4}$$

where c_0 is the normalized external concentration and $T = t/\tau_0$, τ_0 being the time constant for the exponential equilibration of the biophase concentration, c, that would be seen in the absence of binding. The other definitions are as above. The solution of eqn. (4) is a slight generalization of the solution given by Colquhoun & Ritchie (1972*b*). It is most conveniently written in terms of the fraction of receptors not occupied, $p_f = 1 - p$, and is

$$t/\tau_0 = m_{sat}p_f(\infty)^2\left[\frac{p_f(0) - p_f(t)}{p_f(\infty)} - \log_e p_f^{\dagger}(t)\right] - (1 + m_{lin}).\log_e\left[\frac{p_f(0)}{p_f(t)}.p_f^{\dagger}(t)\right], \tag{5}$$

where

$$p_f^{\dagger}(t) = \frac{p_f(t) - p_f(\infty)}{p_f(0) - p_f(\infty)}. \tag{6}$$

The time corresponding to any specified occupancy can be calculated from this, the total uptake at this time (free + saturable binding + linear binding) being $VKc + Mp + m_{lin}VKc$, where $p(t) = 1 - p_f(t) = c(t)/(c(t) + 1)$. The calculated curves are shown, with the experimental data, as thin lines in Fig. 8*A*, *B* and *C*. These curves assume $m_{sat} = 20$ and $m_{lin} = 1$, as above. For mannitol, the time taken for 63·2 percent equilibration, is about 4·5 min. If it were not bound tetrodotoxin would be expected to be about 30 % slower, as above, so τ_0 has been taken as $4{\cdot}5 \times 1{\cdot}3 = 5{\cdot}85$ min. The calculated lines are in fair agreement with the observations, considering that no arbitrary parameters are involved. It should be noted that the biophase used in the calculation is the whole of the extracellular space of the nerve, and not just the periaxonal space.

Prediction of the occupancy rate: relation between diffusion and biophase models. Appealing features of the limited biophase approximation are that lengthy numerical calculations are unnecessary, and that the question of what response will be observed in a tissue in which the drug concentration is non-uniform is side-stepped. Fig. 9 (right-hand series) shows predictions of the occupancy rate of the sort made by Colquhoun & Ritchie (1972*b*) for large m_{sat}, but using eqn. (4) with $m_{sat} = 20$ and $m_{lin} = 1$. The results predict a close approximation to exponential onset and offset of

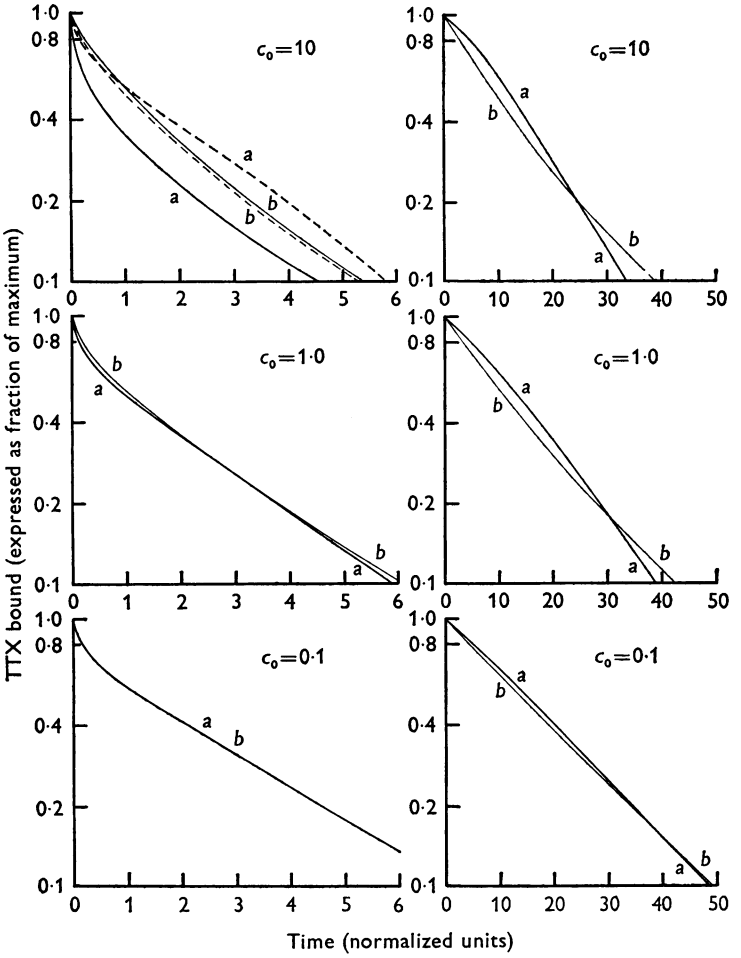

Fig. 9. Theoretical predictions of the kinetics of onset (*a*, thick lines) and offset (*b*, thin lines) of the occupancy of saturable sites for different external normalized concentrations (c_0) of drug. In the bottom left-hand panel the onset and offset curves nearly superimpose, so only the former is drawn. For all curves m_{sat} = 20 and m_{lin} = 1, as found for the rabbit desheathed vagus nerve.

The left-hand panels are predicted on the basis of the exact diffusion model. The ordinate is the apparent occupancy, p_{app} (see text p. 114). The right-hand panels are predicted by the limited biophase model. The ordinate is the occupancy, p. The ordinates are all expressed as $[p(t)-p(\infty)]/[p(0)-p(\infty)]$. The abscissae are normalized time, T, for the offset curves, and $T(c_0+1)$ for the onset curves (T is defined differently for the two models, see pp. 111, 112 and Appendix).

The interrupted lines in the top left-hand panel show the mean occupancy (i.e. $p(r,t)$ averaged over the radius, r, see p. 124). This is the only case for which it differs substantially from the apparent occupancy, and even in this case it implies essentially the same conclusions, which shows that the conclusions do not depend critically on the method used to average the occupancy over the nerve trunk.

receptor occupancy. The rate constant for offset is predicted to be very nearly independent of c_0, and the onset rate constant is very close to $(c_0 + 1)$ times the offset rate constant. In other words, the predictions mimic closely drug-receptor interaction kinetics (see Hill, 1909; Rang, 1966; Colquhoun & Ritchie, 1972*b*) for which the plots would be straight and superimposed for all c_0. In fact, Colquhoun & Ritchie (1972*b*) observed approximately exponential onset and offset rates of occupancy, and, by assuming that the ratio of onset to offset rate constants was $(c_0 + 1)$ as for drug-receptor interaction kinetics, they found $K = 3{\cdot}2$ nM, close to the values found both from equilibrium physiological measurements (Colquhoun & Ritchie, 1972*a*), and from binding in the present work. The actual value of the offset time constant in Fig. 9 is about 80 min over the first part of the offset. This is somewhat larger than the value (46 min) inferred from physiological measurements by Colquhoun & Ritchie (1972*b*) (see Discussion).

The left-hand series in Fig. 9 show similar predictions based on the proper diffusion model. Again, an onset rate constant $(c_0 + 1)$ times the offset rate constant is predicted. In order to get an idea of how the predictions of the proper diffusion model agree with those above, the occupancy at each depth in the nerve trunk (see Appendix) was converted into a conduction velocity for fibres at that depth, using, as an approximation, the relation determined at equilibrium for the whole trunk in Fig. 6 and eqn. (6) of Colquhoun & Ritchie (1972*a*). The response observed in conduction velocity experiments was then predicted as the mean conduction velocity, found by averaging over the trunk by numerical integration (see Appendix). (This is not strictly correct if measurements are made to the peak of the compound action potential.) The mean conduction velocity was converted to an apparent occupancy, p_{app}, as described by Colquhoun & Ritchie (1972*b*). This is the occupancy which, if it were uniform, would produce the given conduction velocity. The results of these calculations are shown in the left-hand panels of Fig. 9. It is seen that the semilogarithmic plots have similar slopes and are roughly straight and parallel over most of their length, again indicating that onset and offset are approximately exponential, with the offset rate constant independent of c_0, and the onset rate constant $(c_0 + 1)$ times the offset rate constant, just as expected for drug-receptor interaction kinetics. The offset rate constant over the first half of the offset is about 88 min (see Discussion). Again, it is predicted that the correct equilibrium constant will be found from measurement of onset and offset rates, as found experimentally by Colquhoun & Ritchie (1972*b*). But the same result would have been obtained if one had used the simpler, limited biophase model, which, in this case at least, is seen to be a very reasonable approximation to the proper diffusion model.

These calculations thus suggest that the slowness of tetrodotoxin action is largely attributable to diffusion time through the extracellular space; and once it arrives at the binding sites the tetrodotoxin taken up by the rabbit vagus nerve then equilibrates with these sites fairly rapidly.

The kinetic arguments just presented would have been vitiated if the nerves were actively converting the radioactive tetrodotoxin into some non-toxic compound. A check was therefore made to ensure that this was not the case. Six nerves were incubated in a small volume of 540 nM tetrodotoxin for 20 hr, and the biological activity of the supernatant solution then assayed against a control solution not exposed to any nerve. The final tetrodotoxin concentrations of the two solutions were within 4% of one another.

Effect of various procedures on the tetrodotoxin uptake

The results obtained so far indicate that binding to the non-myelinated fibres is made up of two components, one saturable and the other, in the range tested, not saturable. In an attempt to learn something about the nature of the binding, experiments were performed on rabbit vagus nerves to see how the uptake is affected by other agents that are thought to act on or near the sodium channel: saxitoxin, whose electrophysiological effect is identical with that of tetrodotoxin in that it reduces the sodium current; the local anaesthetic, lidocaine; batrachotoxin, the effect of which on squid

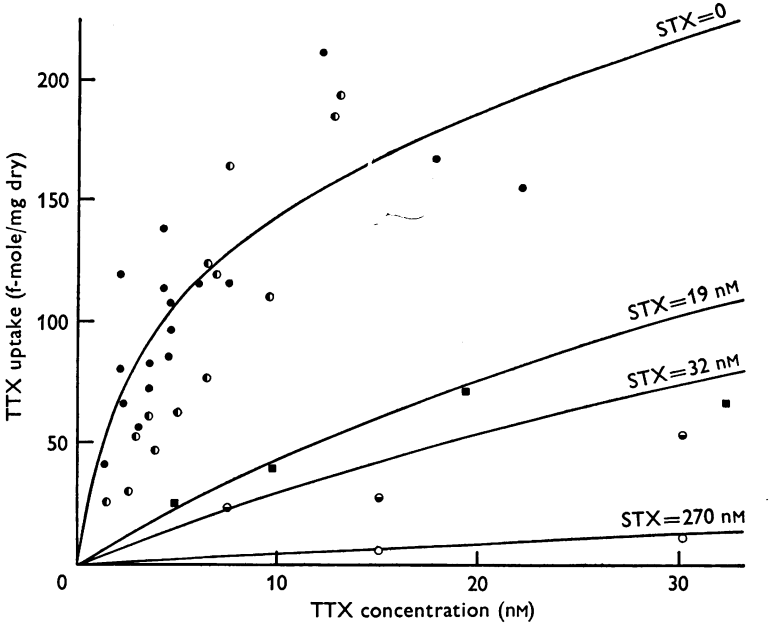

Fig. 10. The effect of saxitoxin (STX) on the uptake of tetrodotoxin (TTX) by rabbit desheathed vagus nerves at different concentrations of TTX. The top line through the control points is the same as appears in Figs. 3 and 4. The STX concentration was (nM): ●, 0 (6-hr equilibration); ◐, 0 (2-hr equilibration), 2 hr points below 10 nM not used in fitting control curve; ■, 19; ◒, 32; ○, 270. The lines are calculated (see text).

axon is antagonized by tetrodotoxin; sodium ions, which pass through this channel, calcium, whose presence is in some way related to the function of the sodium channels; low temperature, which according to Colquhoun & Ritchie (1972*b*) affects the equilibrium of the interaction between tetrodotoxin and the channel; and finally, the effect of pH, which by altering the net charge of either the toxin or the receptor site may alter the intensity of the toxin/receptor interaction (Camougis, Takman & Tasse, 1967).

Saxitoxin. Saxitoxin, kindly supplied by Dr E. J. Schantz, was found to depress the uptake of labelled tetrodotoxin. Although the experimental points are not very precise, the effect is clearly large (Fig. 10). In fact, the tetrodotoxin binding was reduced by large saxitoxin concentrations to below that expected if only the specific component were completely abolished leaving the non-specific component unaffected. The lines drawn through the experimental points were, in fact, calculated on the assumption that the linear component of tetrodotoxin binding is the bottom end of a hyperbola of low affinity and high capacity for tetrodotoxin, and that saxitoxin could also compete with tetrodotoxin for these non-specific sites. Least squares estimates were found as above. They gave equilibrium constants for saxitoxin of 1·6 nM for the specific (sodium channel) sites, and 14·5 nM for the non-specific sites.

The experiments with saxitoxin were mostly done separately from those in which the tetrodotoxin binding curve was determined, so it is possible that the low tetrodotoxin uptake at high saxitoxin concentrations merely reflects random variability of the slope of the linear component of tetrodotoxin binding. On this basis, the experimental results for only those experiments in which saxitoxin was used were fitted on the assumption that tetrodotoxin had the equilibrium constant and binding capacity determined from the experiments of Fig. 3, and that saxitoxin competed only for specific binding sites. In this case, the equilibrium constant for saxitoxin was estimated to be 1·0 nM and the slope, b, was 1·6, rather than 2·6 previously, a difference attributed, on this model, to inter-experiment variation.

On either model, the equilibrium constant for the interaction of saxitoxin with the specific binding sites (1·6 or 1·0 nM) was close to the value (1·2 nM) found by Hille (1968) using the voltage-clamped frog node.

Fig. 10 also illustrates that at concentrations less than about 10 nM the uptake during a 2-hr soak (◐) was significantly less than during a 6-hr soak, i.e. equilibration had not occurred. Only a 2-hr soak was used in the saxitoxin experiments; but because of the high concentrations of saxitoxin used, and because the equilibrium constant for saxitoxin is about the same as that for tetrodotoxin, the 2-hr soaking period was almost certainly adequate for equilibration.

Lidocaine in a concentration of 1 mM was found to block the non-myelinated fibres of the desheathed rabbit vagus nerve almost completely in less than 1 min. Exposure to this concentration of the local anaesthetic for 6 hr, however, had no effect on the uptake of tetrodotoxin (Table 4).

Batrachotoxin, a toxin isolated from the Brazilian frog, depolarizes nerve membranes (Narahashi, Deguchi & Albuquerque, 1971). This action is antagonized by tetrodotoxin. Experiments were therefore done to see

whether or not batrachotoxin interfered with the binding of tetrodotoxin. The results (in Table 4) indicated that batrachotoxin in a concentration of 500 nM had no effect on the uptake of tetrodotoxin by rabbit nerve.

External sodium concentration. Several experiments were done to test the effect of decreasing the external sodium concentration (by replacing it with choline) on the uptake of tetrodotoxin (Table 4). The uptake of tetrodotoxin from low external concentrations of toxin is largely specific; and as Table 4 shows in the reduced sodium bathing medium containing only 7 % sodium, this uptake was increased by 21 %. The effect is, therefore, small, and is barely significant ($P \simeq 0{\cdot}1$), i.e. no large effect of sodium on binding appears to be present. This was important to determine because the method used by Colquhoun & Ritchie (1972*a*) to determine the equilibrium dissociation constant for binding necessarily involved changing the external sodium concentration. A large effect of sodium on binding would have complicated their method.

Calcium. When the calcium concentration was raised to 22 mM (by adding chloride to calcium-free Locke solution), or reduced to zero (together with addition of 1 mM-EDTA), the tetrodotoxin uptake was reduced to about 70 % of its initial value (Table 4).

Low temperature. On the basis of electrophysiological experiments on rabbit vagus nerve Colquhoun & Ritchie (1972*a*) had suggested that the equilibrium dissociation constant for tetrodotoxin decreases with decreasing temperature. It seemed worth while, there fore, to test this suggestion more directly in the present experiments. However, as can be seen in Table 4, little or no effect of temperature on binding was obtained.

Dependence on pH. Nerves were exposed to a concentration of about 5 nM tetrodotoxin for 2 hr at several values of pH between 5·7 and 10·1. The buffer used in these experiments was that described by Ritchie & Greengard (1961) whose buffering capacity is reasonably constant over this wide range. As seen in Fig. 11, pH had relatively little effect on the uptake except at very high values (above 9·0) where the main effect is due to degradation of the tetrodotoxin. At pH 9, for example, the half life of tetrodotoxin is about 2 hr (see Methods). The reduced binding above pH 9 is, therefore, almost certainly caused by the reduced tetrodotoxin concentration resulting from its degradation. This is further substantiated by the observation that uptake from a solution of tetrodotoxin previously exposed to pH 10·0 for 2 hr and then brought back to pH 7·7 was much reduced. Unfortunately, the lifetime of tetrodotoxin at high pH is too short to allow sufficient time for equilibration of the inward diffusion. It was, therefore, not possible to say whether or not the zwitterionic form of tetrodotoxin existing above pH 8·5 had a different affinity for nerve membranes.

TABLE 4. Each number is the mean value of two or three determinations of uptake at a given concentration of TTX by nerves under normal conditions (*a*) and by paired nerves exposed to the testing procedure (*b*)

[TTX]	Condition tested	Control uptake (f-mole/mg) *a*	Test uptake (f-mole/mg) *b*	Ratio (*b*/*a*)
8	500 nM batrachotoxin	66	64	0·97
8		77	66	0·86
23		211	212	1·00
23		136	145	1·07
				Mean 0·98 ± 0·04
8	1 mM lidocaine	164	162	0·99
26·0		239	270	1·13
76·4		499	494	0·97
				Mean 1·03 ± 0·05
4	10 mM-Na	139	155	1·12
4		121	159	1·31
7·2		86	143	1·66
4·8		54	78	1·44
4		149	120	0·81
4		220	175	0·80
8		250	275	1·10
8		210	307	1·46
283†		1522	2340	(1·54)
283†		1074	829	(0·77)
660†		2691	2210	(0·82)
18†		203	321	(1·58)
				Mean 1·21 ± 0·11
12†	22 mM-Ca*	220	90	(0·41)
5·5		134	95	0·71
13·7		243	180	0·74
27·4		374	217	0·58
1037†		4610	1230	(0·27)
				Mean 0·68 ± 0·07
4	0-Ca and EDTA	139	78	0·56
4		121	97	0·80
2		36	28	0·78
				Mean 0·71 ± 0·08
4	Low temp. (10° C)	139	112	0·81
4		121	117	0·97
7		100	108	1·08
				Mean 0·95 ± 0·08

* High Ca solution hypertonic.
† Not included in average.

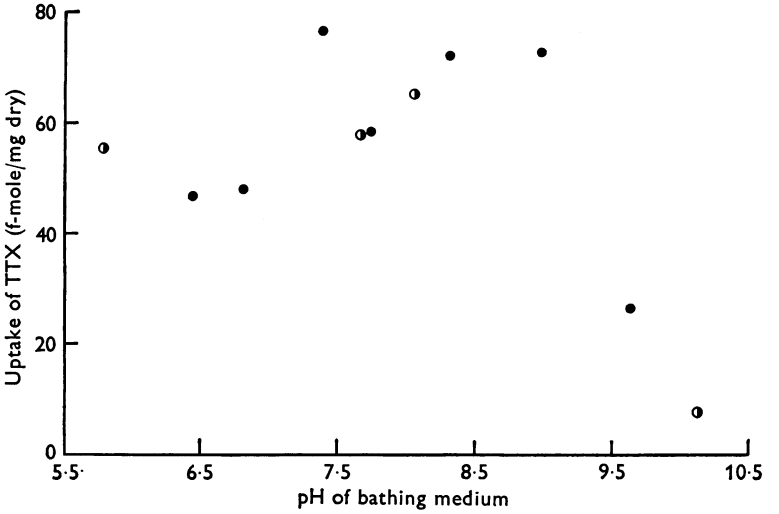

Fig. 11. The dependence on external pH of the uptake of tetrodotoxin (TTX) by the rabbit desheathed vagus nerve. The external concentration in the two experiments illustrated was 4·8 (◑) and 5·8 (●) nM respectively.

DISCUSSION

We have demonstrated the existence of a saturable binding component for tetrodotoxin to bundles of small non-myelinated nerves. Non-specific binding to the nerve also occurs. However, the membranes from red blood cells and the Schwann cells of myelin also bind tetrodotoxin non-specifically; indeed, non-specific binding of drugs to tissue seems to be a general phenomenon (e.g. Paton & Rang, 1965). The kinetics of binding and release of tetrodotoxin are described by a model in which diffusion into and out of the tissue is rate-limiting, the actual equilibration of the toxin at the receptor sites being relatively rapid. Wagner, Schwarz & Ulbricht (1972) estimate that the dissociation rate constant for tetrodotoxin in the frog node of Ranvier is about $1{\cdot}5 \times 10^{-2}$ sec^{-1}. This, if it were correct for vagal C fibres, is sufficiently fast relative to diffusion that one would expect allowance for the rate of drug-receptor interaction would make little difference to the kinetic predictions. In the case of rabbit vagus nerve, Colquhoun & Ritchie (1972*a*,*b*) have estimated the equilibrium constant for binding of tetrodotoxin to sodium channels as 3–4 nM, based on equilibrium and kinetic experiments in which the effects of tetrodotoxin were balanced by increasing the external sodium concentration. Our finding of a saturable component with a 3·0 nM dissociation constant is in good agreement with this value. The offset rate for receptor occupancy estimated from physiological measurements (Colquhoun & Ritchie, 1972*b*) was

almost twice as fast as that predicted (p. 114) in the present work. In view of the combined experimental errors, and of the indirectness of the methods by which both estimates were obtained from the experimental observations, this agreement is reasonably good. Furthermore, it is quite possible that some, or all of the difference is real because the experimental conditions for the physiological experiments in a sucrose-gap apparatus are somewhat different from those in binding experiments: for example, the flow rate over the nerve trunk surface is much faster in the physiological experiments, so presumably the unstirred layer is thinner.

A small word of caution is necessary, however. It is possible that what we have isolated from a mixture of tetrodotoxin degradation products is not tetrodotoxin but some very closely related compound for which the charge, pK_a, molecular weight, and chemical behaviour under mild alkaline conditions are almost identical. The compound might still be biologically active, but have, perhaps, a reduced binding constant compared to tetrodotoxin itself. The results of any binding study would then be almost identical with those obtained using pure labelled toxin, except that the number of binding sites would be underestimated by the factor by which the affinity was reduced. However, the close agreement between the uptake observed by Keynes *et al.* (1971) for rabbit nerve (and between the uptake by lobster nerve by Moore *et al.* 1967) using unlabelled tetrodotoxin and our binding curves makes this occurrence, although still possible, unlikely.

The presence of a small number of high affinity binding sites for tetrodotoxin is, nevertheless, established beyond doubt; and the evidence linking these sites to those responsible for the inhibition of the sodium currents, although indirect, is compelling. First, the idea that tetrodotoxin acts by binding to a specific ionic channel or carrier in the axonal membrane is supported by two findings: (1) the kinetics of the sodium transient under voltage clamp are not affected by the extent of tetrodotoxin block, only $\bar{g}_{Na}$; and (2) the relation between tetrodotoxin concentration and peak sodium current under voltage clamp is consistent with a model in which the binding of one tetrodotoxin molecule completely blocks one channel (Hille, 1970; Cuervo & Adelman, 1970). Secondly, the coincidence between the dissociation constant obtained electrophysiologically (although indirectly) for rabbit nerve and that obtained from the binding studies is clearly satisfactory. And lastly, the number of specific binding sites observed is consistent with other estimates of the number of ionic conducting channels based on the assumption that the channels are discrete structures with an opening little larger than the sodium ions that pass through them (Hille, 1970).

If the saturable binding we observe is not to the sites responsible for

inhibition of sodium currents, then either the physiological sites are present in even smaller concentrations than we observe, or the action of tetrodotoxin is not mediated through a small number of high affinity sites. Neither of these possibilities can be excluded; but if either applies, we are left with no explanation for the saturable sites that we do observe. The identification of sodium channels with tetrodotoxin binding sites is, therefore, entirely reasonable; and future use of tetrodotoxin binding as an assay in the isolation of the membrane components responsible for sodium ion conductivity would seem promising.

That lidocaine and batrachotoxin have no effect on tetrodotoxin binding is consistent with the expectation that their action on sodium channels is from the lipid phase inside the membrane, whereas tetrodotoxin acts from the aqueous phase, being insoluble in lipid. Saxitoxin, having an identical action to tetrodotoxin, appears to act competitively at the same site, again as expected. The notable absence of significant effects of sodium or calcium on tetrodotoxin uptake implies that specific binding sites for these ions, if they exist, are at points separate from the tetrodotoxin binding sites.

We must finally, as others have done before us (Moore *et al.* 1967; Keynes *et al.* 1971), record our amazement at the sparsity of sites. For the rabbit vagus, 152 f-mole/mg dry corresponds to about twenty-seven sites/μm^2, or, for a square array, about 0·2 μm between sites. If one binding site corresponds with one sodium channel (see Cuervo & Adelman, 1970) and if the conductance of one channel were about 0·1 nmho, as postulated by Hille (1970), this density would correspond to $\bar{g}_{Na} = 0{\cdot}27$ mho/cm^2, similar to the values already obtained experimentally for squid (0·12 mho/cm^2) and lobster (0·53 mho/cm^2) giant axons. The density is even lower in the garfish olfactory nerve in which 584 f-mole/mg dry corresponds to less than three sites/μm^2 – quite close to the value estimated for squid axon by Hille (1970) on the basis of indirect estimates of the conductance per channel. This would be equivalent, if the sites were in a square array, to a distance of 0·6 μm between sites, i.e., for an average 0·24 μm diameter axon, about $2\frac{1}{2}$ fibre diameters. Perhaps this explains why the olfactory nerve is more sensitive to block by tetrodotoxin (personal observation) than is the rabbit vagus nerve where, although the binding is three times stronger, 10 times more channels per unit area are present. It may also explain why the conduction velocity in garfish non-myelinated fibres is lower than might have been expected from the velocity in rabbit non-myelinated nerve fibres on the basis of a simple change of diameter. Thus, the ratio of the actual average conduction velocities of rabbit and garfish fibres is 5, whereas that predicted on the basis of the ratio of the square roots of their mean diameters (Rushton, 1951) is only 1·7.

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APPENDIX

Solution of the equation for diffusion in the presence of instantaneous saturable adsorption

The nerve trunk is treated as an infinite cylinder. Substituting the appropriate definition of ∇^2, and using the normalized time $T \equiv tD_{\text{eff}}/a^2$, and also normalizing the distance, r, from the centre of the cylinder as $R \equiv r/a$, eqn. (3) becomes

$$f(c).\frac{\partial c}{\partial T} = \frac{\partial^2 c}{\partial R^2} + \frac{1}{R}\frac{\partial c}{\partial R}, \tag{A 1}$$

where $f(c) \equiv 1 + m_{\text{lin}} + m_{\text{sat}}/(1+c)^2$ represents the factor by which the diffusion coefficient is reduced.

A predictor-corrector method (see, for example, Ames, 1969) was used to solve eqn. (A 1). This method is convenient because only linear equations need be solved. The cylinder is divided into N annuli of width (in terms of R) $h = 1/N$, and time (T) is divided into steps of length k. The normalized concentration at $R = ih$ and $T = jk$ is denoted $c_{i,j}$ $(0 \leqslant i \leqslant N,\ 0 \leqslant j)$.

The $N+1$ concentrations at each i (radius) value at zero time ($j = 0$) are given by the initial conditions. The method allows one to calculate the $N+1$ concentrations at $j+1$ from those at j. The calculation proceeds in two steps. First, the *predictor* calculates concentrations at the intermediate value of $j+\frac{1}{2}$; then, using these values to estimate the non-linearity, $f(c)$, the *corrector* gives the concentrations at $j+1$. A finite difference approximation to the right-hand side of eqn. (A 1) may be defined as

$$L(c_{ij}) \equiv \frac{c_{i-1,j} - 2c_{i,j} + c_{i+1,j}}{h^2} + \frac{1}{ih}.\frac{c_{i+1,j} - c_{i-1,j}}{2h}. \tag{A 2}$$

The concentration at the edge of the cylinder ($i = N$) is set to the value in the external solution. The boundary condition at the centre of the cylinder ($i = 0$) is given by replacing the right-hand side of eqn. (A 1) by its limiting value as $R \to 0$, $2\partial^2 c/\partial R^2$, i.e. eqn. (A 2) is replaced by

$$L(c_{0,j}) = \frac{4(c_{1,j} - c_{0,j})}{h^2}. \tag{A 3}$$

Using these definitions, the predictor analogue of eqn. (A 1) is

$$f(c_{i,j}).\frac{(c_{i,j+\frac{1}{2}} - c_{i,j})}{\frac{1}{2}k} = L(c_{i,j+\frac{1}{2}}) \tag{A 4}$$

for $0 \leqslant i \leqslant N-1$. Rearrangement of these equations gives a set of N *linear*

equations of the form $\mathbf{Ac} = \mathbf{b}$ where $\mathbf{A}$ is a known $N \times N$ tridiagonal matrix, $\mathbf{b}$ is an $N \times 1$ vector of known quantities. This was solved for $\mathbf{c}$, the $N \times 1$ vector of values of $c_{i,\,j+\frac{1}{2}}$ ($0 \leqslant i \leqslant N-1$, the Nth value being given by the boundary conditions), using the algorithm given by Carnahan, Luther & Wilkes (1969, pp. 441, 446). These values are then used in the corrector analogue of eqn. (A 1) viz.*

$$f(c_{i,\,j+\frac{1}{2}}) \cdot \frac{c_{i,\,j+1} - c_{i,\,j}}{k} = \frac{[L(c_{i,\,j+1}) + L(c_{i,\,j})]}{2}. \qquad \text{(A 5)}$$

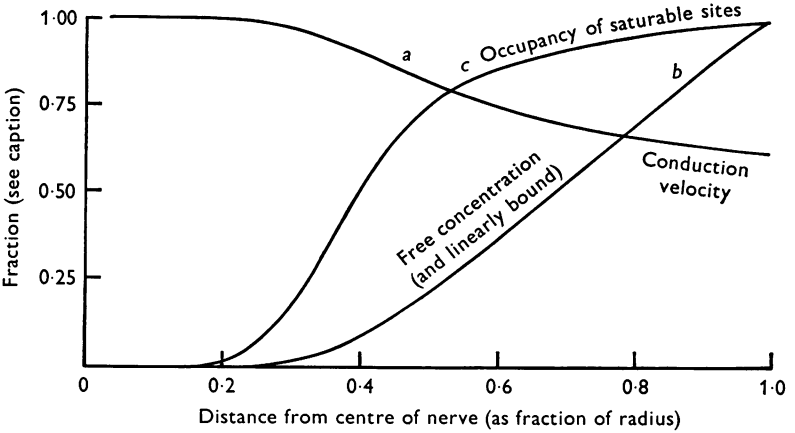

Fig. 12. The solution of the diffusion equation for the onset of a drug present in a normalized concentration of 10 (e.g. about 30 nM TTX since K is 3 nM). The solution takes $m_{\text{sat}} = 20$, $m_{\text{lin}} = 1$, as found for the rabbit desheathed vagus, and uses $N = 50$ and $k = 0{\cdot}0001$. The solution shown is for $T = 0{\cdot}35$ (i.e. $t \simeq 18$ min for TTX on the vagus, for which $a^2/D \simeq 52$ min). At this time the total quantity of free (and linearly bound) TTX in the nerve trunk have reached about half (50·9 %) of their equilibrium values, whereas the saturable component is 79·1 % equilibrated so the mean occupancy is 0·72. The apparent occupancy (see p. 114 and Fig. 9) was 0·88.

The three lines show the variation with radial distance from the centre of the cylinder of the computed values of a, conduction velocity as a fraction of its value before TTX b, concentrations of free, and linearly bound, TTX as fractions of their equilibrium values and c, occupancy of the saturable sites as a fraction of its equilibrium value.

Again, using eqns. (A 2) and (A 3), and the results of the predictor stage, this rearranges to a set of N linear tridiagonal equations which can be solved, as above, for the concentrations at the next time step, $c_{i,\,j+1}$, for $0 \leqslant i \leqslant N-1$.

* Many of the calculations were done using

$$f(c_{i,\,j+\frac{1}{2}}) \cdot (c_{i,\,j+1} - c_{i,\,j+\frac{1}{2}})/(\tfrac{1}{2}k)$$

on the left-hand side of eqn. (A 5). This gives results of comparable accuracy.

The accuracy of solutions obtained was judged by comparison with the known solutions in the linear case, and by their constancy when h and k were reduced. For longer times, sufficient accuracy was obtained using $N = 20$, $k = 0{\cdot}001$. For short times $N = 50$, $k = 0{\cdot}0001$ or $N = 100$, $k = 0{\cdot}000025$ were used. The external concentration is supposed to undergo a step change at $t = 0$, so the point $c_{N,0}$ is singular, but, as long as the earliest time steps are not used, it makes no difference whether this point is set to the initial external concentration or to the initial internal concentration. A typical solution is shown in Fig. 12, which also shows the conduction velocity at different depths, estimated as on p. 114.

The uptake, at time t, of free material, and of material bound to saturable and linear sites, was found by numerical integration (using Simpson's rule) of each of these components over the radius of the nerve trunk. For example, the occupancy at R, T is $p(R,T) = c(R,T)/[1+c(R,T)]$ so the amount of drug bound to the saturable component at time t is

$$2M\int_{R=0}^{R=1} p(R,T).R.\mathrm{d}R,$$

which has the same dimensions as M. The mean occupancy is simply this quantity divided by M. The free and linearly bound uptakes are calculated similarly, and the total of the three components is plotted, with the experimental observations, in Fig. 8.

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