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THE BINDING OF TETRODOTOXIN AND α-BUNGAROTOXIN TO NORMAL AND DENERVATED MAMMALIAN MUSCLE

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SUMMARY

1. The binding of [^{3}H]tetrodotoxin and [^{125}I]iodo-α-bungarotoxin to innervated and denervated rat diaphragm muscle has been measured.

2. A saturable component of tetrodotoxin binding, which was inhibited by saxitoxin, was detected in addition to considerable non-saturable binding. The saturable component had an equilibrium constant of K = 6·1 nM (limits 4·7–7·8 nM) and binding capacity M = 2·5 f-mole/mg wet wt. (limits 2·1–2·8 f-mole/mg).

3. If the saturable component consisted of one-to-one binding of tetrodotoxin to sodium channels, the density of sodium channels would be about 21/μm^2 of surface membrane, a figure similar to that found in other excitable membranes.

4. After denervation the specific tetrodotoxin binding, as measured by the ratio M/K, fell by a factor of 2·8. This change appeared to be due to a fall in binding capacity rather than a decrease in affinity.

5. After denervation the maximum rate of rise of the action potential fell by 27 % and became partially resistant to tetrodotoxin. The maximum rate of rise was at first reduced by tetrodotoxin in similar concentrations to those affecting normal muscle, but even large concentrations which completely blocked normal muscle only reduced the maximum rate of rise by a factor of about 2.

6. Detubulation with glycerol did not appreciably affect the tetrodotoxin sensitivity of normal or denervated muscle.

7. Tetrodotoxin resistance was not observed after denervation of the frog sartorius muscle.

8. [^{125}I]iodo-α-bungarotoxin binding amounted to $3·8 \pm 0·7$ f-mole/mg in innervated muscle and $44·5 \pm 2·1$ f-mole/mg in denervated muscle. Most of the uptake was inhibitable by (+)-tubocurarine.

9. The increase in the labelled bungarotoxin binding is much larger than the specific tetrodotoxin binding of innervated muscle, which renders implausible the possibility that the acetylcholine receptors which appear after denervation are related to the tetrodotoxin resistant-sodium channels.

INTRODUCTION

Tetrodotoxin is known to prevent the generation of action potentials in many excitable tissues by selectively preventing the transient increase in sodium conductance that normally accompanies depolarization of the membrane (Kao, 1966; Hille, 1968; Cuervo & Adelman, 1970).

Various features of its action suggest that the tetrodotoxin molecule may react directly with the sodium channels in the membrane. (1) Extreme selectivity of action. Tetrodotoxin has been shown to affect only one property of the membrane, the maximal increase in sodium conductance attainable by depolarization, $\bar{g}_{Na}$ in the Hodgkin–Huxley notation (Hille, 1968; Cuervo & Adelman, 1970). The sensitivity of the channel to depolarization and the kinetics of its opening and closing are unaltered. (2) The chemical structure of tetrodotoxin. An essential feature of the tetrodotoxin molecule is a positively charged guanidinium group. Since it is known that the guanidinium ion can pass through the sodium channels, it has been suggested that this moiety of the tetrodotoxin molecule interacts directly with the channel (Kao & Nishiyama, 1965; Hille, 1971).

Measurements of the binding of tetrodotoxin to nerve membranes have been made by Moore, Narahashi & Shaw (1967) and Keynes, Ritchie & Rojas (1971) using an indirect method involving the measurement of the depletion of the tetrodotoxin in the medium when a nerve was immersed in it. These results enabled limits to be placed on the number of tetrodotoxin binding sites in the preparation, but did not distinguish between specific and non-specific binding. Colquhoun, Henderson & Ritchie (1972) subsequently measured the binding of tritium-labelled tetrodotoxin by non-myelinated nerves from rabbit, lobster and gar-fish, and with this method were able to distinguish specific and non-specific components of the binding. The dissociation constants estimated from the binding studies agreed very well with estimates made from electrophysiological studies.

In this paper we describe the binding of radioactive tetrodotoxin to rat diaphragm, and compare the dissociation constant so estimated with the concentrations required to alter the electrical properties of those fibres.

We have also measured the binding of radioactive tetrodotoxin to denervated rat diaphragm muscle, following the interesting observation (Redfern & Thesleff, 1971*b*; Harris & Thesleff, 1971; Albuquerque & Warnick,

1972) that denervated muscle becomes relatively resistant to the blocking action of tetrodotoxin. There appears to be a decrease in the binding of tetrodotoxin after denervation, which is quite consistent with the reduction in its physiological effect.

One well-known effect of denervation on muscle fibres is to cause the fibres to become sensitive to acetylcholine at regions away from the motor end-plate (Miledi, 1960), an effect which is well correlated with the appearance of binding sites for α-bungarotoxin at non-junctional regions of the fibre (Miledi & Potter, 1971; Hartzell & Fambrough, 1972; Berg, Kelly, Sargent, Williamson & Hall, 1972; Chang, Chen & Chuang, 1973). In an attempt to see whether these two phenomena – loss of tetrodotoxin sensitivity and development of extrajunctional ACh receptors – might be related, we have measured the change in radioactive iodo-α-bungarotoxin binding after denervation in order to compare it with the change in tetrodotoxin binding.

The results show that the increase in the number of α-bungarotoxin binding sites is very much greater than the decrease in the number of tetrodotoxin binding sites, so it is unlikely that the two phenomena are closely related.

We also report some experiments on frog muscle, which is known to show very marked changes in ACh receptors following denervation (Miledi, 1960). We find that in this species, unlike the rat, denervation does not cause any marked change in susceptibility to the action of tetrodotoxin.

A preliminary report of part of this work has already appeared (Colquhoun, Rang & Ritchie, 1973).

METHODS

Denervation of rat diaphragms

Rats were anaesthetized with halothane or methoxyflurane. The thorax was opened on the left side and the left phrenic nerve cut a few millimetres above the diaphragm. Positive pressure ventilation was used for the short period that the chest was opened.

Binding studies with intact diaphragms

Hemidiaphragms from rats weighing about 250 g were dissected after the animal had been anaesthetized with ether or chloroform and bled out. Two hemidiaphragms with attached costal cartilages were placed in a vial containing 5 ml. Locke solution to which a known concentration of [^{3}H]tetrodotoxin and, in most cases, [^{14}C]inulin, had been added. The vials were closed and shaken gently at room temperature (21 °C) for 4 hr. Because of the small volume of solution (necessary to conserve the [^{3}H]tetrodotoxin) compared with the amount of tissue (1–2 g) in each vial, the medium tended to become acid during the incubation. To counteract this the concentration of Tris buffer (pH 7·2) was increased to 20 mm. The final pH was usually 5·5–6·0.

After 4 hr the muscles were blotted and the bone and tendon were cut away. Each muscle was weighed on a torsion balance and transferred to a counting vial containing 2 ml. 'Protosol' tissue solubilizer (New England Nuclear). The vials were kept at 37° C overnight during which time the muscles dissolved. To each was added 15 ml. 'Aquasol' scintillator (New England Nuclear) containing glacial acetic acid (10 ml./l.) to diminish chemiluminescence.

^{14}C and tritium were counted in separate channels of a liquid scintillation counter, and the counting efficiency determined by internal standards. The amount of [^{14}C]inulin in the incubation medium was adjusted so that the carbon and tritium count rates were roughly equal.

Samples of the incubation medium from each vial were counted in the same way, and an appropriate extracellular space correction was made in calculating the amount of tetrodotoxin bound.

The binding of iodo-α-bungarotoxin by intact muscle was measured by immersing 10–20 hemidiaphragms in a 200 ml. organ bath containing Liley (1956) solution at 20° C bubbled with oxygen. The composition of the Liley solution was (mM): NaCl, 137; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1; $NaHCO_3$, 12; dextrose, 11; sodium phosphate buffer (pH 7), 1. After about 1 hr iodo-α-bungarotoxin was added and the pieces of muscle were removed at fixed intervals. The muscles were washed in eight to ten changes of Locke solution for about 20 hr, then blotted, weighed and transferred to counting pots for counting in a Panax crystal scintillation counter.

Binding studies with homogenized muscle

Because of the variability of results obtained with tetrodotoxin when intact diaphragm was used, a later series of experiments was done on diaphragm homogenates.

Hemidiaphragms were dissected free of extraneous tissue, blotted, chopped roughly and placed in a weighed flask containing 0·6 M-KCl + 10 mM-MOPS (morpholinopropane sulphonate) buffer pH 7. The volume of medium was 2–3 ml. per hemidiaphragm. KCl was used in order to dissolve as much as possible of the actomyosin after homogenization.

The tissue was cooled on ice and then homogenized for two 30 sec periods with an Ultra-Turrax homogenizer at maximum speed. The homogenate was allowed to stand, on ice, for 30–60 min to dissolve the contractile protein, and then centrifuged at 100,000 *g* for 30 min. The supernatant was poured off and the pellet resuspended in saline solution (140 mM-NaCl) containing 10 mM-MOPS buffer (pH 7). The lower part of the pellet, a brownish mass of mitochondria and connective tissue, was discarded. The final homogenate contained the equivalent of 100–200 mg muscle per ml.

In most experiments 3 ml. homogenate was incubated with 0·5 ml. [^{3}H]tetrodotoxin solution containing D-[^{14}C]mannitol together with about 10 mM non-radioactive mannitol. Replicate 1·5 ml. samples were withdrawn after incubation for 1–4 hr at 20° C with gentle shaking. The samples were centrifuged at 100,000 *g* for 30 min and the supernatant poured off, after removal of a 1 ml. sample for counting.

The inside of the centrifuge tube was wiped dry and the pellet was dissolved by means of a Vortex mixer in 0·5 ml. 10 % sodium dodecyl sulphate solution. The contents of the centrifuge tube were then washed into a counting vial with 15 ml. 'Aquasol' scintillator (see above). To cut down chemiluminescence the scintillator was bubbled with nitrogen before use. Four per cent stannous chloride (0·2 ml.) was added to each vial and the air replaced by nitrogen. With these precautions, chemiluminescence subsided completely within 4–8 hr and the background count rate dropped to 5–7 cpm, which greatly facilitated the counting of low-activity

samples. Counting was continued until at least 5000 tritium counts had been registered. Counting efficiencies were determined by means of internal standards.

The [^{14}C]mannitol counts were taken to represent incubation medium trapped in the pellet, and the total amount of [^{3}H]tetrodotoxin in the pellet was corrected for this to give the amount of bound tetrodotoxin.

For measurements of the binding of iodo-α-bungarotoxin to muscle homogenates, the homogenate was prepared in the same way (except that the extraction of acto-myosin by KCl was usually omitted).

Iodo-α-bungarotoxin was added to 15 ml. homogenate. One ml. samples were removed at intervals and added to 7·0 ml. 140 mM-NaCl buffered to pH 7 with 10 mM phosphate buffer, containing 10^{-6} M non-radioactive bungarotoxin. The tubes were centrifuged at 100,000 *g* for 30 min and the pellets thoroughly washed for 6–8 hr with about four changes of buffer. They were then dissolved in 1 M-NaOH, transferred to counting pots, and counted in a well-type scintillation counter.

Radioactive materials

The tritium-labelled tetrodotoxin was part of the batch prepared by Colquhoun *et al.* (1972). Its specific activity was 250 mc/m-mole. For the later experiments, when the material was more than a year old, it was repurified by high-voltage paper electrophoresis and standardized again by bio-assay (Colquhoun *et al.* 1972). Approximately half of the tetrodotoxin had decomposed (as expected from the time constant for decomposition of unlabelled tetrodotoxin of 14 months found by Colquhoun *et al.* 1972), but the remaining material had the same specific activity as before. When tetrodotoxin concentrations greater than about 50 nM were needed, the specific activity was reduced by adding non-radioactive tetrodotoxin, in order to conserve stocks of [^{3}H]tetrodotoxin.

[^{125}I]α-bungarotoxin was prepared from crude *Bungarus multicinctus* venom (Sigma). The α-toxin was first purified as described by Mebs, Narita, Iwanaga, Samejima & Lee (1972). The venom, 100 mg, was applied to a 75 × 1·5 cm column of Sephadex C50 or CM cellulose (Whatman CM 52), equilibrated with 0·05 M ammonium acetate, pH 5·8. The column was eluted with a 400 ml. ammonium acetate gradient (0·05 M, pH 5·8–0·5 M, pH 7·0) running at 15 ml./hr. The material in the main peak was desalted on a column of Sephadex G-25 and freeze-dried, to give 30·8 mg dry α-bungarotoxin. Assay on the rat diaphragm preparation showed its biological activity to be the same as that obtained by Chang *et al.* (1973).

The toxin was iodinated by the procedure of Vogel, Sytkowski & Nirenberg (1972). In a typical preparation, 2 mg (0·3 μmol) α-bungarotoxin was mixed with 1μ mol ICl, containing about 5 mc ^{125}I (supplied as Na^{125}I by the Radiochemical Centre, Amersham) at 0° C.

Unbound iodine was removed by passing through a 10 ml. column of Sephadex G 25. The [^{125}I]α-bungarotoxin was then separated from unlabelled bungarotoxin by applying the material to a 3 ml. column of Whatman CM 52 and eluting with a NaCl gradient as described by Vogel *et al.* (1972). This procedure completely separated iodo-α-bungarotoxin from α-bungarotoxin. The final yield was 1·1 mg [^{125}I]α-bungarotoxin at approximately 3 c/m-mole.

Analysis of tetrodotoxin binding curves

Least-squares estimation was computed by means of the *Patternsearch* programme (see Colquhoun, 1971). The programme found the parameter values that minimized $S = \Sigma w(y_{obs} - y_{calc})^2$, where y_{obs} is the observed binding, and y_{calc} is the binding calculated, usually from equation (1). The weight, w, for each point was taken as the reciprocal of its variance, which was estimated from a smoothed graph

of S.E. against tetrodotoxin concentration. In the present work the saturable component was much smaller, relative to the linear component, than in earlier work (Colquhoun *et al.* 1972) on small non-myelinated fibres. Therefore it was thought necessary to analyse the experimental errors in more detail than was required in the earlier work. Approximate S.E. for the parameters were found as the square roots of the diagonal elements of the inverse of the $k \times k$ matrix $[-\partial^2 L/\partial\theta_i \partial\theta_j]$, evaluated at the least-square values of the parameters, where the $\theta_i (i = 1, \ldots, k)$ are the parameters, and $L = -S/2$ is the observed log likelihood assuming Gaussian distributions.

The resulting symmetrical error intervals were adequate for precisely determined parameters, but were not very good for the less-accurate parameters, for which the limits of error were considerably non-symmetrical. Therefore estimates of random experimental errors are given as likelihood intervals, which are not in general symmetrical. These are found as follows. If contours of the weighted sum of squared deviations (S defined above) are plotted against the parameters (for a given set of experimental results; see Colquhoun, 1971, pp. 247, 260) the coordinates of the minimum value of S (S_{min} say) are the least squares estimates of the parameters, and the tangents, parallel with the axes, to the extremes of the contour for $S = S_{min} + t^2$, provide upper and lower limits of error (likelihood intervals) for each parameter. If t is taken as 2·0 these will be approximately 95 % confidence intervals.

Electrophysiological experiments

Strips of rat diaphragm muscle 5–10 mm wide were mounted in a Perspex chamber through which flowed oxygenated bathing solution (Liley, 1956).

The temperature was maintained at 30° C. The effect of tetrodotoxin on the rate of rise of the action potential was measured as described by Redfern & Thesleff (1971*a*). A KCl-filled micro-electrode connected to a Medistor capacity-neutralized input stage was used for recording. A second, current-passing electrode filled with potassium citrate was inserted into the same fibre and a hyperpolarizing current sufficient to bring the membrane potential to −95 mV was applied for 30 sec. An action potential was then elicited by a depolarizing pulse lasting 5 msec.

The rate of rise was monitored by an operational amplifier connected as a differentiator. The amplitude and maximum rate of rise of the action potential were read directly from the trace recorded on a storage oscilloscope.

In most cases action potentials were recorded from ten different fibres and average values obtained. The bathing solution was then changed, and the preparation left to equilibrate for 1 hr before repeating the measurements on another group of ten fibres. Preliminary experiments showed that the effect of tetrodotoxin takes up to 60 min to become constant. In a few experiments recovery from the action of tetrodotoxin was followed for 2–3 hr. After 3 hr recovery was 90–95 % complete. In most cases recovery was not tested, the initial control measurements serving as a base line for calculating the effect of two or three concentrations of tetrodotoxin applied in ascending sequence, without intervening recovery periods.

In some experiments, the muscle was detubulated by treatment with hypertonic glycerol (Eisenberg & Eisenberg, 1968; Eisenberg & Gage, 1969; Howell, 1969) to see whether tetrodotoxin affected the sodium channels in the T-tubules differently from those of the outer membrane. If Liley solution containing 400 mM glycerol was applied for 1 hr at 30° C and then washed away, the fibres mostly had very low membrane potentials (20–30 mV) and would not give proper action potentials. Reducing both the temperature during the glycerol treatment (to 10° C) and the time of exposure (to 40 min) improved the membrane potentials to 60–70 mV but failed to abolish the twitch completely. Washing the glycerol out with Liley solution

diluted to 80 % strength with distilled water resulted in effective abolition of twitches, and the membrane potential usually exceeded 60 mV in about 70 % of the fibres. This procedure was usually used, therefore, and recordings were made only from fibres with resting potentials of at least 60 mV.

Some experiments were done on frog sartorius muscles, bathed in Frog Ringer solution at 20° C. The composition of the Ringer solution was (mM); NaCl, 120; KCl, 2; $CaCl_2$, 2; sodium phosphate buffer (pH7), 2. The frogs were denervated under ether anaesthesia (immersion in water containing 2 % ether) by cutting the sciatic nerve at the hip joint, and the muscles were studied 4–12 weeks later.

RESULTS

The binding of [^3H]tetrodotoxin

Normal innervated muscle

Intact diaphragm. The relationship between the amount of tetrodotoxin bound (corrected for the amount retained in the extracellular space) after 4 hr immersion at room temperature and the final concentration of tetrodotoxin in the medium is shown in Fig. 1. There was a good deal of

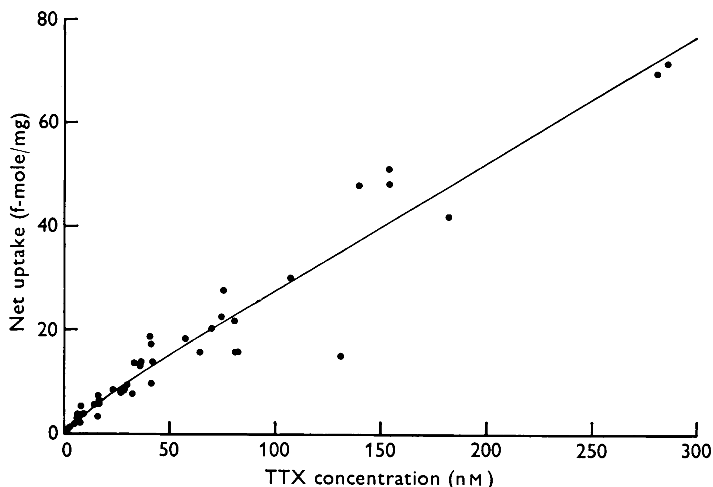

Fig. 1. Binding of tetrodotoxin (TTX) at equilibrium by intact rat diaphragm (innervated) at 20° C in Liley solution, plotted against the tetrodotoxin concentration in the supernatant solution. Points are means of duplicates for the pooled results of experiments 17 May to 13 July 1972. It is not possible to show all the points at low concentrations on this scale (see Fig. 2).

scatter in the measurements, and the relationship did not, at first sight, appear to deviate appreciably from a straight line. It therefore appeared that any component of binding that saturated at a low concentration of tetrodotoxin was small in relation to the experimental error.

However, inspection of the lower end of the binding curve (Fig. 2), and comparison of the slope at very low tetrodotoxin concentrations with that at higher concentrations (Fig. 2, inset), showed clear evidence for the presence of a saturable component.

The results were therefore subjected to a curve-fitting procedure (see Methods) to obtain the best fit to the equation:

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

Approximate tests showed this equation to provide a 'significantly' better fit than a straight line through the origin. It represents the uptake, U, as the sum of a linear component, with slope b, and a hyperbolic saturating component, with binding capacity M and equilibrium constant K.

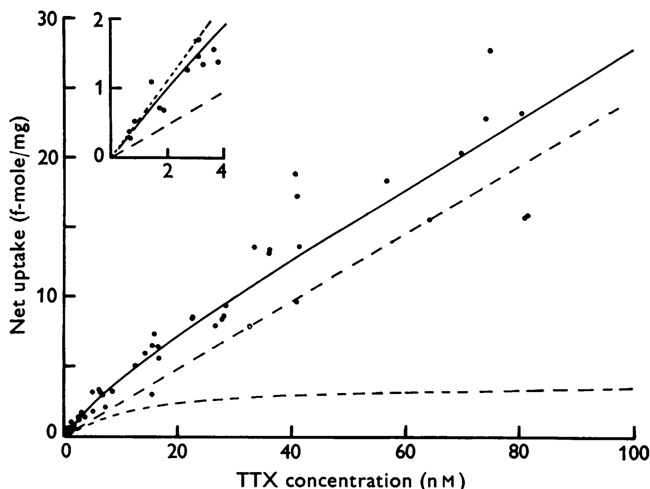

Fig. 2. Binding of tetrodotoxin (TTX) at equilibrium by intact diaphragm. Same results as shown in Fig. 1, but high tetrodotoxin concentrations omitted. The two postulated components of binding, estimated by weighted least-squares, are also shown. The estimates are: $b = 0{\cdot}242$ f-mole mg^{-1}, nM^{-1}, $K = 12{\cdot}7$ nM, $M = 3{\cdot}93$ f-mole/mg. The inset shows the data for very low concentrations only. It shows that the initial slope ($b+M/K$ from equation (1) is clearly greater than the final slope (b). M/K is estimated to be 0·31 f-mole mg^{-1} nM^{-1} in these experiments. If there were no saturable binding it would be zero.

The results are shown in Table 1. The first set of experiments (17 May to 16 June), which were done on normal diaphragms, gives somewhat smaller values of K and M than the second set of experiments (3–13 July), which were done on the normal half of denervated diaphragms. The precision of the results is not great enough to provide convincing evidence for a real difference, especially since M/K (the initial slope of the saturable

TABLE 1. Binding of [^{3}H]tetrodotoxin to normal (innervated) muscle. Weighted least-squares estimates ± approximate S.D. Approximate 95% intervals are given in parentheses (see Methods)

Date	Method	No. of expts. (observations)	b (f-mole mg^{-1} nM^{-1})	K (nM)	M (f-mole/mg)	M/K (f-mole mg^{-1} nM^{-1})
17.v.72 to 16.vi.72	Intact	4 (60)	0·226 ± 0·013	8·52 ± 3·1	2·92 ± 0·8	0·341
3.vii.72 to 13.vii.72	Intact	3 (54)	0·249 ± 0·017	20·7 ± 7·9	6·00 ± 2·0	0·291
All above results	Intact	7 (114)	0·242 ± 0·011	12·7 ± 3·4 (7·11–22·2)	3·93 ± 0·85 (2·50–6·31)	0·31 ± 0·03 (0·26–0·38)
20.vii.73	Homogenized	1 (28)	0·273 ± 0·022	3·15 ± 1·21	2·53 ± 0·81	0·80
22.vii.73	Homogenized	1 (16)	0·67 ± 0·006*	6·18 ± 2·1	2·56 ± 0·43	0·41
24.vii.73	Homogenized	1 (34)	0·102 ± 0·002	8·16 ± 1·9	1·55 ± 0·17	0·19
28.vii.73	Homogenized	1 (22)	0·087 ± 0·004	5·89 ± 1·45	2·19 ± 0·29	0·37
2.viii.73	Homogenized	1 (18)	0·160 ± 0·01	4·75 ± 1·34	2·37 ± 0·44	0·50

* Non-bound tetrodotoxin in pellet not estimated. Estimate of b only should be affected.

component) is similar for both sets. Because of the large positive correlation between estimates of M and K (the correlation coefficient from the approximate covariance matrix was between 0·93 and 0·96 in these experiments; see Colquhoun, 1969, 1971, p. 271), their ratio, M/K, is much more precisely determined than their separate values. Errors for the ratio M/K were found by fitting equation 1 directly to the observations in the form $U = b\,[\mathrm{TTX}] + b'\,[\mathrm{TTX}]/(1 + [\mathrm{TTX}]/K)$, where $b' \equiv M/K$, which gave estimates, and their errors, for b, b' and K.

Homogenized diaphragm. The binding curve, after equilibration for at least 1 hr at room temperature, was found to be very similar to that for intact muscle, as exemplified in Fig. 3(*a*). The saturable component is shown more clearly by plotting $U/[\mathrm{TTX}]$ against [TTX], as in Fig. 3(*b*). This converts the linear component to a horizontal line, with ordinate b, and any saturable component is seen as an upward deviation from this line at low concentrations as in Fig. 3(*b*); from equation (1),

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

A second example is shown in Fig. 4*a*, where it is compared with denervated muscle.

The results of analysis of the curves, as described above, are shown in Table 1. The experiments of 20th and 24th July gave rather aberrant values of M/K (possibly because of differences in the yield of membrane particles resulting from minor variations in the centrifugation procedure)

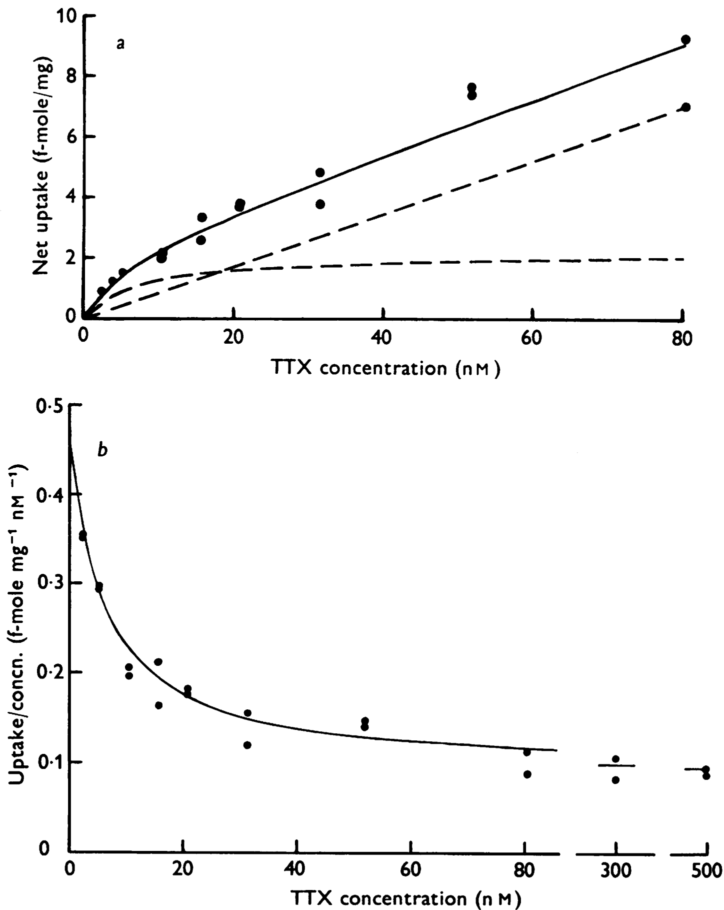

Fig. 3. (*a*) Binding of tetrodotoxin at equilibrium by homogenized rat diaphragm (innervated) at 20° C, plotted against tetrodotoxin concentration. Low concentrations only shown for clarity (see Fig. 3*b* for rest of data). Individual observations for experiment of 28 July 1973. The postulated components of binding are shown. The parameters, estimated as described in Methods, were $b = 0{\cdot}087$ f-mole mg^{-1} nM^{-1}, $K = 5{\cdot}89$ nM, $M = 2{\cdot}19$ f-mole/mg.

(*b*) the same results plotted as uptake/concentration against concentration. The curve through the points is calculated from the parameter estimates used in (*a*). At low tetrodotoxin concentration it deviates upwards from a horizontal line by $M/K = 0{\cdot}37$ f-mole mg^{-1} nM^{-1} (cf. Fig. 4).

so that these experiments could not be pooled with the others for analysis.

Pooled results on innervated muscle. The result of simultaneously fitting all results (except 20. vii. 73 and 24. vii. 73; see above) is shown in Table 3. The equilibrium constant, $K = 6{\cdot}11 \pm 0{\cdot}78$ nM (approximate 95% limits 4·71–7·84 nM), is similar to the value found for non-myelinated nerve fibres (Colquhoun *et al.* 1972). The binding capacity, $2{\cdot}45 \pm 0{\cdot}18$ f-mole/mg (limits 2·11–2·85 f-mole/mg), is a great deal lower than for the small nerve fibres, as expected from the much smaller surface/volume ratio of the muscle fibres.

TABLE 2. Binding of [^{3}H]tetrodotoxin to denervated muscle. Errors are specified as in Table 1

Date	Method	No. of expts. (observations)	b (f-mole mg^{-1} nM^{-1})	K (nM)	M (f-mole/mg)	M/K (f-mole mg^{-1} nM^{-1})
3. vii. 72– 13. vii. 72	Intact	3 (54)	0·239 ± 0·028	–*	–*	0·128 ± 0·028 (0·075–0·355)
30. vii. 73	Homogenized	1 (28)	0·136 ± 0·011	9·90 ± 8·9	1·17 ± 0·84	0·118 ± 0·034
2. viii. 73	Homogenized	1 (18)	0·065 ± 0·007	10·5 ± 3·6	1·65 ± 0·46	0·157
21. viii. 73	Homogenized	1 (18)	0·141 ± 0·009	3·66 ± 4·2	0·470 ± 0·31	0·128

* Insufficient precision to determine separate values of K and M.

Denervated muscle

Intact diaphragm. The analyses of experiments on muscles that had been denervated 5–14 days previously are shown in Table 2. The scatter of results, relative to the size of any saturable component, was too great for M and K to be determined separately. However, M/K was determinate, being $0{\cdot}128 \pm 0{\cdot}028$ f-mole mg^{-1} nM^{-1}. This is lower than for normal muscle, indicating a reduced ability to bind tetrodotoxin after denervation; it is not possible to tell from these experiments whether this is the result of a decrease in binding capacity or an increase in K (i.e. decrease in affinity).

Homogenized diaphragm. The greater precision of experiments on homogenized muscle enabled separate values of K and M to be determined, even though individual experiments had large errors (Table 2). The values of M/K were very similar to those in intact denervated muscle. An example of binding to denervated muscle is shown in Fig. 4, from which it is clear that M/K is smaller in denervated than in innervated muscle.

TABLE 3. Pooled results of experiments on binding of [^{3}H]tetrodotoxin to intact and homogenized muscle. Errors are specified as in Table 1. A least-squares fit was obtained with common values of K and M for all experiments, but separate b values were used for each experiment on homogenized muscle, because b was found to vary from experiment to experiment. The values of b are not given, as they differ little from those given in Tables 1 and 2

	No. of expts. (observations)	K (nM)	M (f-mole/mg)	M/K (f-mole mg^{-1} nM^{-1})
Innervated muscle	10 (170)	6·11 ± 0·78	2·45 ± 0·18	0·401 ± 0·025
		(4·71–7·84)	(2·11–2·85)	(0·355–0·456)
Denervated muscle*	3 (64)	8·49 ± 2·5	1·22 ± 0·29	0·144 ± 0·015
		(4·32–15·3)	(0·76–1·98)	(0·118–0·185)

* Intact muscle experiments, for which errors were large so that M and K were indeterminate, were omitted.

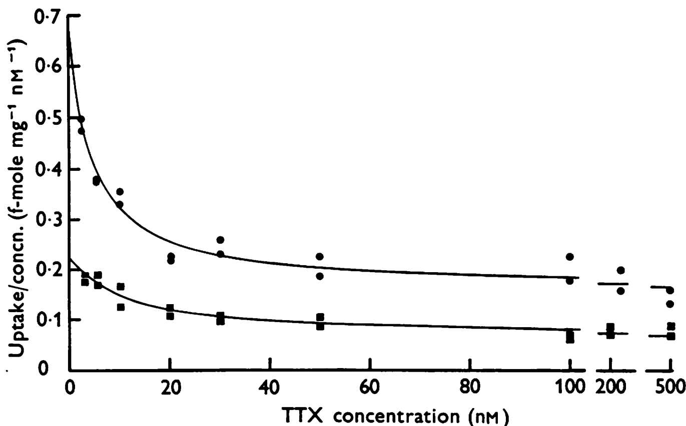

Fig. 4. Binding of TTX at equilibrium to homogenates of the (a) innervated (●), and (b) denervated (■), halves of the same diaphragms (experiment 2, August 1973). Plotted in the form U/[TTX] against [TTX], as in Fig. 3. The lines become horizontal, wth ordinate b, at high concentrations. In both cases an upward deviation, by an amount M/K (because the value of U/[TTX] at [TTX] = 0 is $b + M/K$, from equation (1)), is seen at low concentrations. The size of the upward deviation (and therefore M/K, and the specific binding at low concentrations) is clearly smaller for denervated muscle (M/K = 0·16 in this experiment) than for innervated muscle (M/K = 0·50). The curves drawn through the points are those fitted on the basis of equation (1)).

Pooled results on denervated muscle. Table 3 shows the combined analysis of the results of all three experiments on homogenized muscle.

The tetrodotoxin binding capacity (M) is seen to be reduced after denervation. There may also be a small decrease in affinity (increase in K), but this does not approach statistical significance.

Effect of saxitoxin on TTX binding

In two experiments on homogenized diaphragm (innervated) it was found that the addition of saxitoxin (100 nM) virtually abolished the saturable component of binding, as shown, for example, by the abolition of the upward deviation at low tetrodotoxin concentration when the binding curve was plotted in the form uptake/concentration against concentration (cf. Fig. 3*b*). This agrees well with the finding in nerve that saxitoxin and tetrodotoxin appear to compete for the same binding site (Colquhoun *et al.* 1972; Henderson, Ritchie & Strichartz, 1973).

Binding of iodinated α-bungarotoxin

The aim of these experiments was to test whether the increase in ACh receptors that occurs after denervation was similar to, or grossly different from, the decrease in tetrodotoxin binding sites. To measure the number of receptors by means of binding studies with α-bungarotoxin, it was necessary to obtain evidence; (*a*) that all of the receptors were labelled under the conditions of the experiment; and (*b*) that all of the labelled sites were receptors. The first point was checked by determining whether the binding had reached saturation and the second by measuring the inhibitory effect of (+)-tubocurarine on iodo-α-bungarotoxin binding.

Preliminary studies on intact muscle showed that saturation was not readily achieved (Table 4). Thus at a fixed iodo-α-bungarotoxin concentration (125 nM) the binding increased markedly between 4 and 8 hr. Also, the binding measured after 3 hr was much greater at an iodo-α-bungarotoxin concentration of 625 nM than at 125 nM. It seemed likely that the slow equilibration with iodo-α-bungarotoxin was the result of slow diffusion into the muscle, so we did some experiments on homogenized muscle, in which diffusion delays should be much reduced. These experiments generally gave much more satisfactory saturation. Thus, in the experiment in Fig. 5*b* the binding to denervated muscle in the presence of 12·5 nM bungarotoxin had reached equilibrium after 2 hr. In other experiments a small increase in binding (10–15%) occurred between 2 and 3 hr, and we have used measurements made at 3 hr for the purpose of quantitative comparison. Normal muscle, even though the total binding was much less than that of denervated muscle, also showed a slight increase between 2 and 3 hr incubation (Fig. 5*a*).

TABLE 4. Binding of iodo-α-bungarotoxin to normal and denervated rat diaphragm muscle (intact hemidiaphragm)

I. Effect of incubation time (125 nM I-BuTX at 37° C)

Time (h)	I-BuTX bound (f-mole/mg) Normal	Denervated
2	3·8	40·8
4	6·0	52·8
8	12·5	87·0

II. Effect of I-BuTX concentration on uptake measured after 3 hr incubation.

Concn. (nM)	I-BuTX bound (f-mole/mg) Normal	Denervated
25	2·6	19·0
125	4·8	38·5
625	6·3	69·1

Tubocurarine inhibited the binding of iodo-α-bungarotoxin (Fig. 5). In the experiment shown in Fig. 5*b*, 10^{-6} M(+)-tubocurarine reduced the rate constant for iodo-α-bungarotoxin binding by a factor of 3·5. In other muscles the dissociation constant (K_{TC}) for tubocurarine blocking ACh receptors is close to 4×10^{-7} M (Jenkinson, 1960; Rang & Ritter, 1969). If the binding of tubocurarine to a receptor prevents the attachment of iodo-α-bungarotoxin (Lester, 1972), and if tubocurarine equilibrates rapidly with the receptors (Waud, 1967), then the factor by which the binding of iodo-α-bungarotoxin should be slowed down is $([TC]/K_{TC}) + 1$, which is equal to 3·5, as found experimentally, for 10^{-6} M tubocurarine.

In intact muscle, tubocurarine slows down iodo-α-bungarotoxin binding by much less than the theoretical factor, probably because diffusion rather than association with receptor is the rate limiting step in iodo-α-bungarotoxin binding under these conditions (Porter, Chiu, Wieckowski & Barnard, 1973).

The amount of iodo-α-bungarotoxin bound to homogenates after incubation for 3 hr in the presence of 12·5 nM iodo-α-bungarotoxin was $3·8 \pm 0·7$ f-mole/mg with normal muscle (mean of six experiments) and $44·5 \pm 2·1$ f-mole/mg with muscle denervated 14–18 days previously (mean of nine experiments).

Electrophysiological experiments

The maximum rate of rise of the action potential ($\dot{V}_{max}$), the amplitude of the action potential, and the resting membrane potential were all

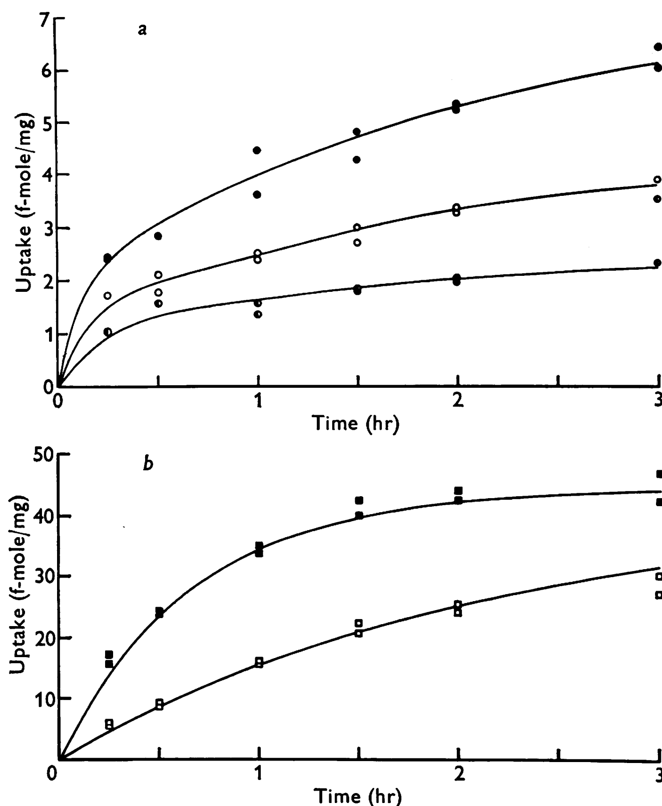

Fig. 5. Binding of iodo-α-bungarotoxin (12·5 nM) to rat diaphragm homogenate at 20° C.

(*a*) Innervated half of diaphragm from operated rats, in phosphate buffered saline (●), and in the presence of 1·0 μM (○) and 10·0 μM (◐) (+)-tubocurarine. The lines are drawn by eye through the observation.

(*b*) Denervated halves of the same diaphragms in phosphate buffered saline (■), and in the presence of 1·0 μM (+)-tubocurarine (□). Note different scale for the ordinate. The control curve is a single exponential with asymptote 44·8 f-mole/mg and rate constant 0·0247 min^{-1} (i.e. half-time 28 min). The curve through the results in the presence of tubocurarine is a single exponential with the same asymptote, but a rate constant 3·5-fold less than the control value, i.e. 0·00797 min^{-1} (half-time 98 min).

reduced 5–10 days after denervation (Table 5), in agreement with the findings of Redfern & Thesleff (1971*a*, *b*); see also Albuquerque, Schuh & Kaufmann, 1971; Albuquerque & McIsaac, 1969.

The effect of tetrodotoxin in normal and denervated muscle is shown in Fig. 6. Although the denervated muscle starts off with a lower rate of rise, it is much less sensitive to tetrodotoxin. Thus in normal muscle, $\dot{V}_{max}$

TABLE 5. Comparison of resting and action potentials in normal and denervated rat muscle at 30° C

	No. of fibres	Resting potential (mV)	Maximum rate of rise (V/sec)	Amplitude of action potential (mV)
Normal	110	79·9 ± 1·3	520 ± 23	108·7 ± 2·3
Denervated 6–19 days	130	67·0 ± 1·3	377 ± 18	88·7 ± 1·7

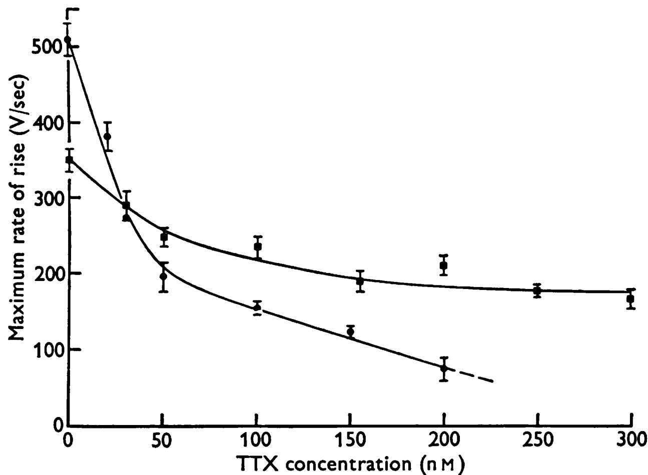

Fig. 6. Maximum rate of rise ($\dot{V}_{max}$, V/sec) of the action potential as a function of tetrodotoxin concentration, in innervated (●) and denervated (■) rat diaphragm muscle fibres, after holding membrane potential at −95 mV for 30 sec. Liley solution at 30° C.

is roughly halved at 40 nM tetrodotoxin, whereas in denervated muscle this effect was achieved only by the highest tetrodotoxin concentrations tested (250–300 nM), concentrations that completely blocked action potential generation in normal muscle. In normal muscle, at tetrodotoxin concentrations of 150 nM or more, a proportion of the ten fibres tested usually failed to respond with clear-cut action potentials. These were recorded as showing zero rate of rise, a procedure that may have biased slightly the calculation of the mean rate of rise.

We were interested in the correlation between the blocking action and the binding of tetrodotoxin. It was notable that the binding studies suggested that the dissociation constant was approximately 6 nM, whereas six times this concentration was required to diminish the rate of rise by 50%. The simplest assumption, therefore, that the maximum rate of rise decreases in proportion to $\bar{g}_{Na}$ (which is anyway unlikely on theoretical

grounds), seems to be untrue, and we must postulate a markedly non-linear relationship between $\dot{V}_{max}$ and $\bar{g}_{Na}$, such that 50 % reduction of $\bar{g}_{Na}$ causes only about 10 % reduction of $\dot{V}_{max}$ and about 85 % reduction of $\bar{g}_{Na}$ is required to halve $\dot{V}_{max}$.

A non-linearity of this kind has been demonstrated for the node of Ranvier of frog nerve fibres (Schwarz, Ulbricht & Wagner, 1973). In this preparation $\bar{g}_{Na}$ is reduced to 50 % by 3·6 nM tetrodotoxin whereas a 50 % reduction of $\dot{V}_{max}$ requires 10·9 nM tetrodotoxin, a threefold difference. In rat muscle there is about a sixfold difference between the dissociation constant (4·7–7·8 nM) estimated from tetrodotoxin binding, and the concentration that reduces $\dot{V}_{max}$ by 50 % (40 nM).

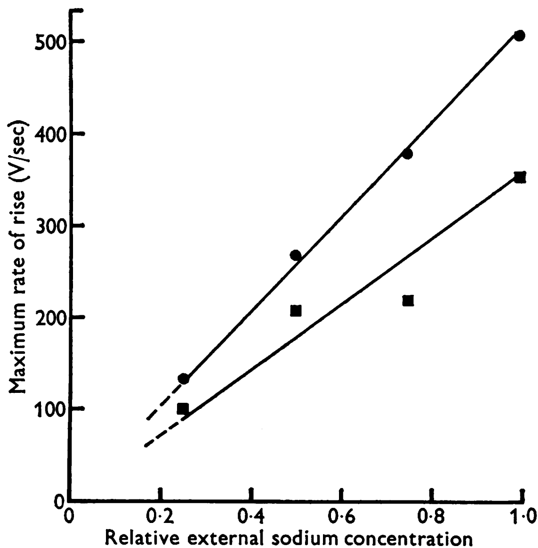

Fig. 7. Maximum rate of rise of the action potential as a function of external sodium concentration (relative to value in normal Liley) in innervated (●) and denervated (■) muscle fibres. Conditions as for Fig. 6.

Colquhoun & Ritchie (1972*a*, *b*) calculated the dissociation constant for tetrodotoxin in mammalian non-myelinated nerve fibres (in which the measurable electrophysiological quantities, conduction velocity and amplitude of the compound action potential are even less direct as measures of $\bar{g}_{Na}$ than the maximum rate of rise used in these experiments). Their approach was to use a null method based on matching the effect of a given concentration of tetrodotoxin against the effect of reducing the external sodium concentration by a factor, *r*. The apparent dissociation constant for tetrodotoxin, K_{app} was calculated from

$$K_{app} = [\mathrm{TTX}]/(r-1).$$

This value, as they had predicted, tended to increase at increasing tetrotodoxin concentrations, and a correction based on the independence principle (see Colquhoun

& Ritchie, 1972*a*; equation (8)) was used to provide a more reliable estimate of the dissociation constant.

Similar experiments on the rat diaphragm showed that, with both normal and denervated muscle, the relationship between $\dot{V}_{max}$ and $[Na]_o$ was nearly linear (Fig. 7). K_{app}, calculated as above to be about 40 nM, did not increase with tetrodotoxin concentration in the manner expected from the analysis of Colquhoun & Ritchie (1972*a*), indicating that it is inappropriate to use this approach here.

It has therefore not proved possible to get a valid estimate of the dissociation constant from these electrophysiological measurements, because the model previously employed does not describe the results with muscle fibre.

One reason for this could be that the independence principle is not applicable. Another is that the sodium conductance at any moment depends not only on $\bar{g}_{Na}$, but also on the degree of activation of the channels ($m^3 h$), which is highly sensitive to the shape of the early part of the action potential, and could easily be different for action potentials of equal maximum rate of rise produced on the one hand by adding tetrodotoxin and on the other by reducing $[Na]_o$, thus invalidating the analysis.

Because of this we attempted to compute the effect of reducing $\bar{g}_{Na}$ or $[Na]_o$ on the muscle action potential, using a programme for frog muscle (Adrian, Chandler & Hodgkin, 1970), modified as in Colquhoun & Ritchie (1972*a*, p. 537). However, the computations predicted failure of propagation when either $[Na]_o$ or $\bar{g}_{Na}$ was reduced by 50 %. Since action potentials were maintained at sodium concentrations lower than this (Fig. 7), it did not seem a satisfactory basis on which to calculate the reduction of $\bar{g}_{Na}$ by tetrodotoxin. The computed model treated the tubular membranes as passive elements. It is possible that a more elaborate programme, incorporating regenerative properties in the tubular membrane (Adrian & Peachey, 1973) might resolve the discrepancy, but we have not tried this.

Fig. 6 suggests that the main difference between normal and denervated muscle is that the denervated fibres are blocked to a maximum of about 50 % by tetrodotoxin at a concentration of 150 nM. Increasing the concentration to 300 nM has little or no further effect. The concentration range (0–150 nM) over which tetrodotoxin exerts a graded blocking effect is about the same in normal and denervated muscle. A tentative conclusion is that a denervated muscle fibre has, in addition to a population of sodium channels with the normal sensitivity to tetrodotoxin (responsible for the blocking effect in the range 0–150 nM), a resistant population sufficient in number to enable the fibre to propagate action potentials with a maximum rate of rise of about 200 V/sec. The resistance of these channels to tetrodotoxin is probably not absolute since Harris & Thesleff (1971) found that $\dot{V}_{max}$ in denervated fibres declined appreciably at much higher tetrodotoxin concentrations, up to 10^{-5} M. The change that follows denervation is thus more likely to be the appearance of a population of tetrodotoxin-resistant sodium channels, and the loss of some of the normal tetrodotoxin-sensitive channels, than a change in the affinity of all of the channels for tetrodotoxin (see Discussion).

Studies on glycerol-treated muscle

Because the results suggested that there might be two types of sodium channel in denervated muscle, differing in their susceptibility to tetrodotoxin, it seemed possible that they might correspond to the channels in the transverse tubular system (Adrian, Costantin & Peachey, 1969; Costantin, 1970; Gonzalez-Serratos, 1971; Bezanilla, Caputo, Gonzalez-Serratos & Venosa, 1972; Adrian & Peachey, 1973), and those of the outer membrane. If this were the case, elimination of the T-tubules should cause either complete resistance to tetrodotoxin, or restore the normal sensitivity.

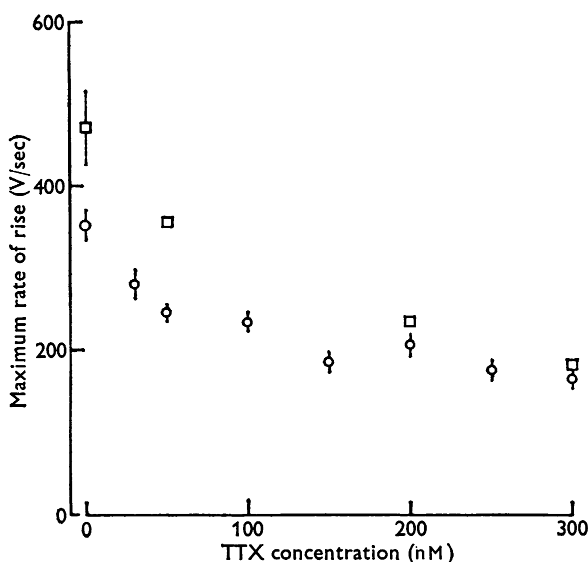

Fig. 8. The maximum rate of rise of the action potential in denervated rat diaphragm in Liley solution at 30° C. ○, control observations; □, after glycerol treatment (see Methods).

The results showed that detubulation by treatment with glycerol made no appreciable difference to the tetrodotoxin sensitivity of either denervated muscle (Fig. 8), or of innervated muscle, so it does not appear that differences between the sodium channels of the surface membrane and those of the tubules can account for the effects seen.

Studies on frog muscle

In order to test whether the effect of denervation seen in mammalian muscle also occurs in other species, we did some experiments on denervated frog muscle. Interestingly, in this species neither the rate of rise of the

action potential nor its sensitivity to tetrodotoxin is altered after denervation (Fig. 9). Since this work was done it has been reported that neither embryonic nor adult denervated chick muscle is resistant to tetrodotoxin (Harris, Marshall & Ward, 1973; Harris, Marshall & Wilson, 1973). It therefore appears that it is by no means a universal phenomenon, and, since the development of extrajunctional acetylcholine sensitivity is well documented for frog muscle (Miledi, 1960), this finding makes it unlikely that the two processes are closely related.

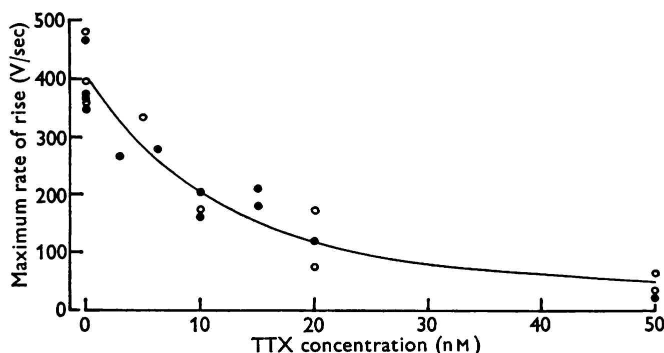

Fig. 9. The maximum rate of rise of the action potential of frog sartorius muscle in frog Ringer at 20° C. ●, normal innervated muscle; ○, denervated muscle.

DISCUSSION

Tetrodotoxin binding in innervated muscle

Intact and homogenized diaphragm both bind tetrodotoxin to similar extent. Despite a considerable amount of non-specific binding it is possible to distinguish a saturable component of binding of the same sort as found in nerve (Colquhoun *et al.* 1972). In normal (innervated) muscle the equilibrium constant for the saturable component of 6 nM (Tables 1 and 3), is similar to the values found in other tissues, which are collected in Table 6. The binding capacity is about 2·5 f-mole/mg wet wt. As expected from the smaller surface/volume ratio of muscle fibres relative to C fibres, this value is much smaller than found in nerve (Colquhoun *et al.* 1972), but it suggests a rather similar density of binding sites to that found in nerve. Thus if it were assumed that 75% of the tissue weight consisted of 40 μm diameter fibres of density 1·07, the superficial membrane area would be 0·7 cm^2 mg. The density of tetrodotoxin binding sites would therefore be about twenty-one sites/μm^2 if all sites were on the superficial muscle fibre membrane (cf. 27 sites/μm^2 in rabbit vagus C fibres; Colquhoun *et al.* 1972). However, there is reason to believe that

TABLE 6. Tetrodotoxin binding and sodium channel density in different preparations

Tissue	Binding capacity	Site density (sites/ μm^2)	Dissociation constant (nM)	Method	Reference
Rabbit vagus nerve	152 f-mole/mg dry	27	3·0	TTX binding	Colquhoun *et al.* (1972)
	—	—	4·0	Measurement of action potential	Colquhoun & Ritchie (1972*a*, *b*)
Gar-fish olfactory nerve					
Intact	584 f-mole/mg dry	5·6‡	10·1	TTX binding	Colquhoun *et al.* (1972)
Membrane fraction	42 f-mole/mg wet nerve	3·9	8·3	TTX binding	Benzer & Raftery (1972)
Solubilized membranes	*ca.* 250 f-mole/mg dry	—	6	TTX binding	Henderson & Wang (1972)
	—	—	12	TTX interaction with saxitoxin binding	Henderson *et al.* (1973)
Lobster leg nerve					
Intact	260 f-mole/mg dry	22	25	TTX binding	Hafemann (1972)
	184 f-mole/mg dry	16	10·2	TTX binding	Colquhoun *et al.* (1972)
Membrane fraction	—	28	4·0	TTX binding	Barnola, Villegas & Camejo (1973)
Squid giant axon	—	—	3·3	Voltage clamp	Cuervo & Adelman (1970)
	—	50–400	—	Measurements of gating currents	Armstrong & Bezanilla (1973); Keynes & Rojas (1973)
Frog myelinated nerve	—	—	3·4	Voltage clamp	Schwarz *et al.* (1973)
Toad myelinated nerve	—	—	3·6	Voltage clamp	Schwarz *et al.* (1973)
Rat diaphragm muscle					
Normal	2·5 f-mole/mg wet	21*	6·1	TTX binding	Present study
Denervated	1·2 f-mole/mg wet or 0·9 f-mole/mg wet†	10* 7·5*†	8·5	TTX binding	

* This value might be reduced if there were tetrodotoxin-binding sites in the transverse tubules (see text, p. 220).

† Value assuming that K does not change after denervation.

‡ This is larger than the value given by Colquhoun *et al.* (1972) because it is based on a better estimate of axon area, viz. 65 cm^2/mg wet (Easton, 1971).

there are sodium channels in the transverse tubular system also (Adrian *et al.* 1969; Costantin, 1970; Gonzalez-Serratos, 1971; Bezanilla *et al.* 1972), and a molecule as small as tetrodotoxin should have easy access to these channels. If we assume, as Adrian & Peachey (1973) estimate for frog muscle, that the maximum sodium conductance of the tubular system would be about 0·15 times that of the associated surface membrane for a 40 μm diameter fibre, and that each tetrodotoxin binding site corresponds with a sodium channel (see below), this would imply a density of around 18 channels/μm^2 of surface membrane (and about 1 channel/μm^2 of tubular membrane).

The maximum sodium conductance, $\bar{g}_{Na}$, for frog skeletal muscle has been found to be about 180 mmho/cm^2 at 20° C (Adrian *et al.* 1970; Ildefonse & Roy, 1972; Adrian & Peachey, 1973). This value would be expected to be a measure mainly of superficial membrane sodium channels because of the resistance in series with any tubular channels. If we assume a similar value for rat muscle, it would suggest that the conductance of an open channel is of the order of $1{\cdot}0 \times 10^{-10}$ mho. It is interesting that this is close to the values suggested for the sodium channel in nerve, about 10^{-10} mho (Hille, 1970; Colquhoun *et al.* 1972), and also close to the conductance measured for the ionic channel opened by acetylcholine at the frog neuromuscular junction which is about 1×10^{-10} mho (Katz & Miledi, 1972) or $0{\cdot}2$–$0{\cdot}3 \times 10^{-10}$ mho (Anderson & Stevens, 1973). Measurements of a membrane current attributed to movement of the sodium channel gates (see Table 6) in the squid axon have suggested considerably greater sodium channel density than has been found from tetrodotoxin binding in other tissues. It is not yet known whether or not this reflects a real difference in channel density between squid axon and other tissues. If the conductance of the channels were proportional to the sodium concentration (which is greater for the squid than for muscle), and if the channels had similar permeability in squid and muscle, the similar $\bar{g}_{Na}$ values for these tissues would have led one to expect a greater rather than a lesser density of channels in muscle relative to the density in squid axon.

The considerations just discussed support the proposition that the observed saturable component of tetrodotoxin binding is due to sodium channels. Further support comes from the observation that the saturable component of binding is virtually abolished in the presence of 100 nM saxitoxin, which, like tetrodotoxin, blocks the sodium current in skeletal muscle (Kao, 1966; Evans, 1972). In the case of nerve it was possible to provide additional evidence by showing that the affinity of tetrodotoxin for the sodium channels measured by an indirect electrophysiological method, agreed with affinity found for the saturable binding component (Colquhoun & Ritchie, 1972*a*, *b*; Colquhoun *et al.* 1972). Unfortunately the

theory used for nerve does not give satisfactory results with muscle (see Results), possibly because of the considerable extra complication offered by the transverse tubular system in muscle.

It is nevertheless of interest that in experiments on frog nerve (Schwarz *et al.* 1973) found that the concentration of tetrodotoxin required to reduce the rate of rise of the action potential by 50 % (10·9 nM) was three times as great as the concentration required to halve $\bar{g}_{Na}$ (3·6 nM). If a similar effect occurs in rat muscle, the electrophysiological results would suggest that $\bar{g}_{Na}$ might be halved at approximately 13 nM tetrodotoxin, a value similar to the equilibrium constant estimated from the binding studies.

Tetrodotoxin binding in denervated muscle

After denervation we find a 27·5 % reduction of $\dot{V}_{max}$ (Table 5) and partial resistance to the action of tetrodotoxin, confirming the results of Redfern & Thesleff (1971*a*, *b*) and Harris & Thesleff (1971). We also find that in denervated fibres $\dot{V}_{max}$ falls initially over the same range of tetrodotoxin concentrations as in innervated fibres, but then becomes more or less independent of tetrodotoxin concentration at about half its initial value (i.e. about one third of the value in innervated muscle). A simple interpretation of this observation would be that roughly one third of sodium channels remain unchanged after denervation (the relation between $\dot{V}_{max}$ and sodium conductance being unknown in denervated fibres, a precise statement cannot be made). There is a clear reduction in the saturable component of tetrodotoxin binding after denervation. This is shown most precisely by the reduction of M/K by a factor of 2·8 in denervated muscle (see Table 3). The ratio M/K is the initial slope of the saturable component, in the low concentration region where binding is directly proportional to concentration. It is not possible to be quite as certain about changes in the individual values of K and M following denervation. It is an unfortunate statistical fact that, whatever method is used to estimate K and M, there is a strong positive correlation between their estimates ($r > 0{\cdot}9$ from the approximate covariance matrix in the present experiments), and there is also a considerable negative correlation ($r = -0{\cdot}5$ to $-0{\cdot}9$ in the present experiments) between the estimate of b and those of both M and K (see Colquhoun 1969; Oliver, 1970). This means that experiments in which errors lead to an overestimate of M will almost certainly over-estimate K also (and underestimate b). Consequently M/K is more precisely estimated than either parameter alone (see Tables 1–3 and pp. 207–209). The results in Table 3 nevertheless suggest that the fall in M/K is mostly due to a fall in binding capacity rather than an increase in K (decrease in affinity for tetrodotoxin), a conclusion which would be compatible with the electrophysiological results.

It seems likely therefore that denervation brings about a reduction in the number of normal, tetrodotoxin-sensitive sodium channels, together with the appearance of tetrodotoxin-resistant channels. It might be simplest to suppose that a fraction of the normal channels becomes transformed into tetrodotoxin-resistant channels, but we have no direct evidence for this.

It is interesting that the change in the sodium channels following denervation appears to be incomplete, and it seemed possible that this could result from the channels of the surface membrane and those of the T-tubules responding differently. This does not seem to be the case, however, since we found no marked effect of detubulation by means of glycerol on the sensitivity of denervated muscle to tetrodotoxin. We conclude either that the surface and tubular sodium channels respond to denervation in the same way, or that only the behaviour of the surface channels influences the rate of rise of the action potential appreciably.

Binding of iodo-α-bungarotoxin

The estimate of binding of iodo-α-bungarotoxin in homogenized innervated muscle after 3 hr was $3{\cdot}8 \pm 0{\cdot}7$ f-mole/mg, which is somewhat higher than values found by others, e.g. 1·7 f-mole/mg (Chang *et al.* 1973), 1·25 f-mole/mg (Berg *et al.* 1972), 3·2 f-mole/mg (Porter *et al.* 1973), 2·4 f-mole/mg (Hartzell & Fambrough, 1972). In calculating these values from the published figures, values of 10^4 end-plates per hemidiaphragm, and a weight of 200 mg have been assumed to make units comparable. After denervation an increase in binding (at 3 hr) to $44{\cdot}5 \pm 2{\cdot}1$ f-mole/mg is seen. This increase of 41 f-mole/mg is similar to the values found by others in the rat diaphragm, e.g. 49 f-mole/mg (Chang *et al.* 1973), 59 f-mole/mg (Miledi & Potter, 1971). In most of our experiments the binding was still increasing slowly at 3 hr, as was the difference in binding between innervated and denervated muscle. Therefore our value for the difference in binding capacity between denervated and normal muscle is probably a slight underestimate.

It is considerably larger than the specific tetrodotoxin binding capacity of innervated muscle (about 2·5 f-mole/mg), which renders implausible the possibility that the change following denervation consists of a conversion of the normal sodium channels from a tetrodotoxin (but not ACh) sensitive ion channel into an acetylcholine (but not tetrodotoxin) sensitive channel. This conclusion is supported by the failure of denervation to affect the tetrodotoxin sensitivity of frog muscle, even though it is known to cause extrajunctional ACh receptors to appear (Miledi, 1960; Miledi & Potter, 1971).

Effect of (+)-tubocurarine on iodo-α-bungarotoxin binding

The rate of iodo-α-bungarotoxin binding to denervated muscle homogenate (Fig. 5*b*) is slowed to about the extent predicted theoretically, a factor of $1+([TC]/K_{TC})$, on simple suppositions, namely (*a*) tubocurarine and iodo-α-bungarotoxin bind in a mutually exclusive fashion to the receptor; (*b*) iodo-α-bungarotoxin binds irreversibly; (*c*) tubocurarine equilibrates rapidly with the receptors not occupied by iodo-α-bungarotoxin; (*d*) diffusion of iodo-α-bungarotoxin is not rate-limiting. The good agreement between this theory and the experimental result (Fig. 5*a*) suggests that most of the iodo-α-bungarotoxin in denervated muscle is bound to ACh receptors. Subsequent experiments have shown that a small fraction (about 10%) of this binding is resistant even to very high concentrations of tubocurarine, and presumably represents binding to non-receptor sites.

It is interesting that the binding studies on intact, as opposed to homogenized muscle, showed much slower binding of iodo-α-bungarotoxin and also much less inhibition by tubocurarine, in agreement with the results of Miledi & Potter (1971) and Berg *et al.* (1972). Presumably in this situation diffusion of iodo-α-bungarotoxin into the muscle rather than the intrinsic rate of association becomes rate-limiting. The slower its diffusion, the more nearly will local equilibrium, at each depth from the surface of the tissue, be approached. And, because iodo-α-bungarotoxin combines virtually irreversibly, the *equilibrium* state would be complete occupancy of receptors by iodo-α-bungarotoxin, whatever the tubocurarine concentration. Therefore, when diffusion is rate-limiting, tubocurarine would be expected to reduce the rate of iodo-α-bungarotoxin binding by considerably less than the 'theoretical' factor.

We wish to thank Dr R. H. Adrian and Professor W. K. Chandler for providing the computer programs for the muscle action potential, and for useful discussions.

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