PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

The Purity of Tritiated Tetrodotoxin as Determined by Bioassay [and Discussion]

S. R. Levinson, D. Colquhon, R. Henderson and J. M. Ritchie

Phil. Trans. R. Soc. Lond. B 1975 **270**, 337-348
doi: 10.1098/rstb.1975.0013

Phil. Trans. R. Soc. Lond. B. **270**, 337–348 (1975) [337]
Printed in Great Britain

The purity of tritiated tetrodotoxin as determined by bioassay

By S. R. Levinson,†
A.R.C. Institute of Animal Physiology, Babraham, Cambridge

The radioactivity binding of a tritiated tetrodotoxin preparation to an electric organ particulate fraction was compared with the binding of biological activity as determined by biological assay. This comparison shows that the chromatographically homogeneous toxin contained a large proportion of radioactive impurities which bind nonspecifically to the electroplax preparation.

Introduction

The interaction of tetrodotoxin with the sodium conductance channel of excitable membranes has been well studied using electrophysiological methods (see the review by Evans 1972). More recently, assays for the binding of tetrodotoxin to various preparations have been used to investigate the occurrence and biochemistry of the sodium channel (Colquhoun, Henderson & Ritchie 1972; Benzer & Raftery 1973; Henderson & Wang 1972; Villegas, Barnola & Camejo 1973; Levinson & Ellory 1973). Most of these studies have used tritiated tetrodotoxin preparations to investigate the concentration dependence of the toxin uptake, and a frequent observation in these studies is the occurrence of non-specific radioactivity uptake in many of the tissues studied, in addition to a saturable specific component thought to be due to interactions of the toxin with the sodium channel. There does not appear to be agreement concerning the nature of this non-specific binding component or even its existence in all tissue preparations. Colquhoun *et al.* (1972) have presented some evidence to support the conclusion that this component is due to non-specific binding of the toxin itself, while Barnola, Villegas & Camejo (1973) have assumed that it is due to binding of radio-impurities present in the toxin solution. Resolution of this point is important, especially in the determination of binding site densities, since these assumptions directly affect calculation of the specific activity of the labelled toxin.

The use of a sensitive bioassay and a high capacity tetrodotoxin binding preparation have enabled a comparison of the uptakes of radioactivity and biological activity to be made in the same preparation (and often in the same samples). This comparison reveals that the non-specific uptake component in the radioassay is not present in the bioassay data, and thus this non-specific component must be due to radioimpurities present in the toxin preparation.

Materials and methods

Bioassay of tetrodotoxin

A freshly excised frog sciatic nerve was desheathed for a distance of 1–2 cm in the region of the thigh. The nerve was then mounted in a three chamber stimulating–bathing–recording apparatus and the bioassay conducted as previously described (Levinson & Ellory 1973). A standard dose–response curve was made by plotting the percentage inhibition of the untreated

† Present address: The Physiological Laboratory, Cambridge.

compound action potential against the concentration of toxin used. The accuracy of the bioassay was mostly limited by changes in the tetrodotoxin sensitivity of the nerve during the course of experiments. These changes were usually predictable as a rapid decrease in sensitivity occurring during the 2 h after mounting of the nerve, followed by a relatively stable period lasting from 3 to 8 h. To minimize the effects of drifting sensitivity, assays were only done during the stable period, and the unknowns were bracketed with standard solutions. Stability of the tetrodotoxin sensitivity was improved by mounting the nerve under slight tension so that replacement of solutions in the bathing chamber would not agitate the nerve and possibly change accessibility of the toxin to the fibre bundle.

The concentration of tetrodotoxin giving 50 % inhibition of the compound action potential (I_{50}) usually ranged from 9 to 15 nM, and appeared to depend on the extent of the desheathing process. The sensitivity of the nerve could be increased irreversibly by treatment of the nerve with phospholipase-C (0.2 mg/ml, for 5 min) in order to measure low concentrations of tetrodotoxin more accurately.

Insensitive nerves (I_{50} approximately 15 nM) had relatively shallow slopes in the low concentration range of the dose–response curve, and thus any error in determining the amount of inhibition of the action potential might translate to a large error in the tetrodotoxin concentration determined. However, most of the insensitive nerves studied gave a slope of about 3 % inhibition per nM tetrodotoxin in the range 3–10 nM. Since the error in determining the inhibition of the action potential is about 2 %, the associated error in toxin concentration in this range should not be greater than 1 nM, and if one restricts the use of insensitive preparations to assay of concentrations over 10 nM, this represents a possible error of about 10 % in the worst circumstances.

Preparation of tritiated tetrodotoxin

This was carried out essentially as described by Colquhoun *et al.* (1972). In the present study 3.5 mg of citrate-free tetrotodoxin (Sankyo Chemical Co.) was tritiated by tritium gas exchange and the unassociated label removed by the Radiochemical Centre, Amersham. This step yielded approximately 3 mg of solid material containing 30 mCi of tritium. The solid was dissolved in 1 ml 10^{-2} M acetic acid (pH about 3.5) and an aliquot removed for purification by high voltage paper electrophoresis. The crude purification was done in 50 mM tris-acetate buffer, pH 7.9, while the final purification was done at pH 6.5 in pyridine-acetate buffer.

The final purification resulted in single coincident peaks of radioactivity and biological activity (as determined by the above bioassay). The total recovered tetrodotoxin was 7 % of that initially submitted to labelling in the original aliquot. Of the total peak of tetrodotoxin recovered, only that section of the electrophoresis paper containing the immediate peak of radioactivity and toxin was used in further studies. In further tests of homogeneity, aliquots of the final purified tetrodotoxin fraction were rechromatographed at pH 6.5 and 8.5, and in both cases single symmetrical peaks of radioactivity resulted. In addition, a sample was chromatographed on the thin layer silica gel system described by Benzer & Raftery (1972), and this too resulted in single coincident peaks of radioactivity and biological activity.

The amount of tetrodotoxin contained in the purified fraction was 22.5 μg, and the purified toxin was stored frozen in 1 ml acetic acid, 10^{-2} M. The overall radioactivity in this fraction was 395 Ci/mol tetrodotoxin. Bioassay of the crude toxin after labelling showed that 88 % of the toxic activity was lost during the labelling process. Thus the tetrodotoxin in the final purified fraction represented a 38-fold purification of the starting labelled material.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Preparation of the electric organ binding fraction

This was done as previously described (Levinson & Ellory 1973), using organ obtained from *Electrophorus electricus* which was frozen for storage. The resultant pellet from the low speed centrifugation steps was diluted with frog Ringer (112 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 10 mM tris pH 7.4) to a final concentration of 1 g wet mass frozen organ per millilitre (about 10–20 mg protein per millilitre). Since binding capacities of material prepared at different times vary in their binding capacities, all of the experiments described were performed on the same preparation.

Bioassay of tetrodotoxin binding in eel particulate material

A reaction mixture was prepared consisting of 0.2–0.4 ml electric organ particulate matter in a total volume of 2 ml tetrodotoxin–Ringer at a known initial concentration of toxin. The mixture was then centrifuged for 1 min in an Eppendorf model 3200 centrifuge, and the resultant supernatant bioassayed for tetrodotoxin as previously described. The amount of toxin bound was taken to be the difference between the initial and final concentrations multiplied by the volume of reaction mixture. The time needed for equilibrium to be established in the reaction mixture must be short as no difference in binding could be detected between samples incubated for 30 s or 15 min (Levinson & Ellory 1973).

Supernatants from samples incubated at high initial tetrodotoxin concentrations were diluted so as to fall within the range of the bioassay sensitivity, since most action potentials were fully inhibited at concentrations above 24 nM. Corrections for the non-hydrated space of the particulate matter were less than 2 % of the total reaction mixture volume, as determined by [^{14}C]inulin space marking.

Radioassay of tetrodotoxin binding in eel particulate material

Reaction mixtures were prepared and processed as above. An aliquot of the initial reaction mixture was assayed for radioactivity by scintillation counting and compared directly with an equal aliquot of the supernatant obtained after centrifugation. The difference in aliquot counts was appropriately scaled to get the total radioactivity bound by the sample.

General conditions

Unless otherwise noted, all studies were done using the titriated tetrodotoxin preparation described above at ambient temperature. Toxin solutions were all made up in the Ringer described above.

Results

Initial observations

The existence of radio-impurities in the labelled tetrodotoxin toxin preparation could most simply be demonstrated by comparison of the fraction of biological activity bound with the fraction of radioactivity bound in the same sample. If the tetrodotoxin was radiochemically pure, these fractions should be equal. In fact, it was found in the range 3–100 nM that the fraction of biological activity bound always exceeded the fraction of radioactivity bound. In a typical example, 0.4 ml of particulate matter bound 11.8 pmol of toxin, reducing the initial

concentration of 2 ml reaction mixture from 12 to 6.1 nM (49 % reduction). In contrast, the amount of radioactivity bound was only 20 % of the initial concentration.

The ratio of fraction radioactivity bound to biological activity bound gives an estimate for the radiochemical purity of the tetrodotoxin, and in this example is 40 %. However, this estimate assumes that the impurities do not bind to the particulate fraction, and in this sense 40 % represents an upper limit to the purity of the toxin. To test this assumption, the concentration dependence of the binding of radioactivity and biological activity was investigated.

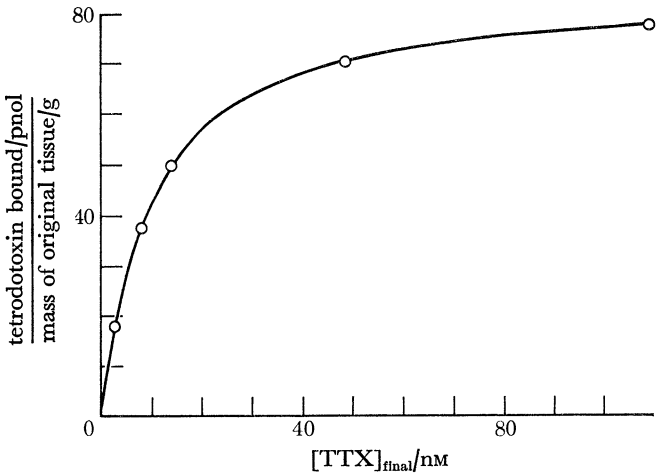

FIGURE 1. Binding curve of tritiated tetrodotoxin to electric organ particulate matter as determined by bioassay. Points are from a single experiment.

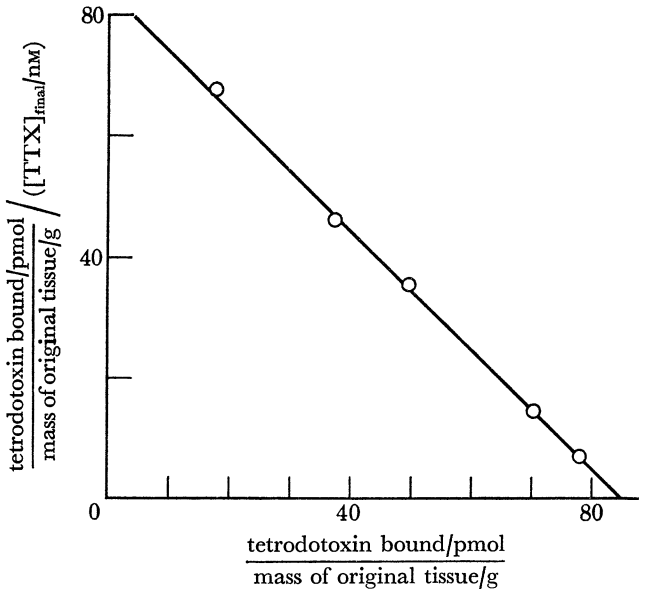

FIGURE 2. Data of figure 1 on a Scatchard plot. The points are well fitted by a straight line. Its slope and its intercept with the abscissa give an affinity constant of 10.5 nM and a maximum binding of 87.5 pmol/g.

Uptake of biological activity

The uptake of biological activity as a function of tetrodotoxin concentration is shown in figure 1. This plot indicates a single saturable component occurring in the range studied (2.4–110 nM), and this indication is confirmed by graphing the data on a Scatchard plot

(figure 2) in which saturable Langmuir binding would give a straight line (Scatchard, 1949). The bioassay data do give an excellent straight line, with an affinity constant K_D of 10.5 nM and a maximum binding of 85.7 pmol tetrodotoxin/g frozen electric organ.

Uptake of radioactivity

Figure 3 shows the binding of radioactivity as a function of tetrodotoxin concentration. Since the experimental conditions were the same in the bioassay experiments, the bioassay data were used to calculate the final toxin concentration. The equation used was

$$[\mathrm{TTX}]_f = \tfrac{1}{2}\{([\mathrm{TTX}]_i - 8.85) + ([\mathrm{TTX}]_i^2 - 3.6[\mathrm{TTX}]_i + 78.3)^{\frac{1}{2}}\},$$

where $[\mathrm{TTX}]_f$ and $[\mathrm{TTX}]_i$ are the final and initial concentrations of tetrodotoxin in the sample in μg/l. This equation was derived from the mass action law and the stoicheiometry of the binding. The points in figure 3 suggest that binding is made up of two components, a saturable component and a linear component; the linear binding at high tetrodotoxon concentrations is indicated by the dotted line. This observation is again confirmed by a Scatchard plot of the data (figure 4), in which the radioactive binding is seen to consist of a linear portion for the specific component at low binding levels followed by a curvilinear tail, as might be expected if a linear, nonspecific component was superimposed on a specific component.

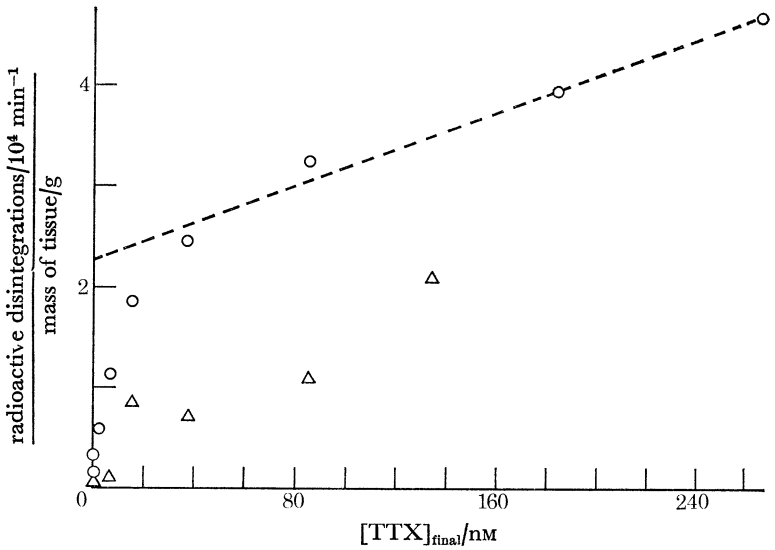

FIGURE 3. Binding of radioactivity to the same preparation as used in figure 1. Radioactivity bound per gram of tissue is plotted against the final concentration of tritiated tetrodotoxin in the sample. Points are averages of two determinations on the same preparation. The dotted line represents the linear binding visible at high tetrodotoxin concentrations. The triangles show binding of radioactivity in the presence of 6 μM unlabelled tetrodotoxin.

From these plots, the amount of radioactivity bound due to specific binding may be determined in two ways. First, the dotted line in figure 3 may be extrapolated back to the ordinate to give as its intercept the amount of specific binding at saturation. In a similar way the linear specific part of the Scatchard plot may be extrapolated to the abscissa (after taking the non-specific component into account) to obtain an independent value for the bound radioactivity. The agreement is good, giving 2.25×10^4 and 2.20×10^4 disintegrations min^{-1} g^{-1} of frozen organ respectively.

Calculation of the actual specific activity and purity of the tetrodotoxin

The specific activity is obtained from the ratio of saturable radioactivity binding

$$(2.225 \times 10^4 \text{ d. min}^{-1} \text{ g}^{-1} = 1.01 \times 10^{-8} \text{ Ci/g})$$

to saturable biological activity bound (85.7 pmol/g) at full saturation. The value thus obtained is 117.4 Ci/mol. The actual purity of the tetrodotoxin relative to the general activity (395.0 Ci/mol) is therefore 30 %.

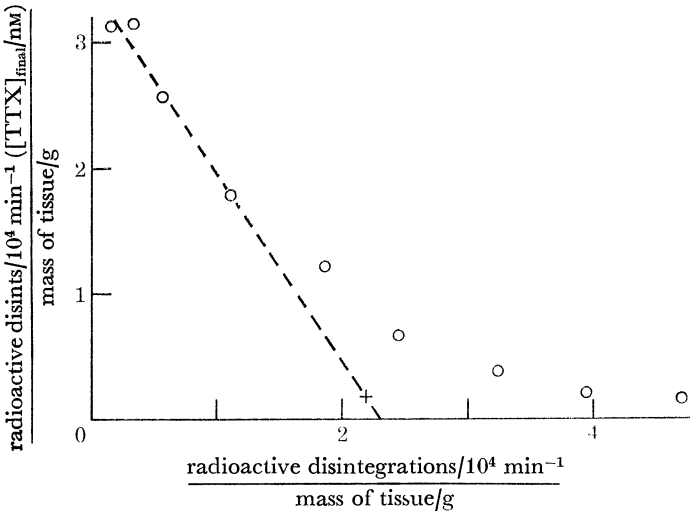

FIGURE 4. Data of figure 3, on a Scatchard plot. Ordinate: radioactivity bound per gram of tissue divided by the final concentration of tritiated tetrodotoxin in the sample. Abscissa: radioactivity bound per gram of tissue. The plot indicates a linear portion followed by a curvilinear tail. The linear component has been extrapolated to an abscissa intercept corrected for the contribution of the curvilinear tail (cross). Maximum binding thus obtained: 2.20×10^4 disintegrations min^{-1} g^{-1}.

Preliminary observations on the nature of the binding components

With tritiated compounds the most usual source of non-specific uptake is the binding of tritiated water which might be formed in the solution by exchange of label from the compound. However, this could not be the case in the present study since removal of water by lyophilization resulted in less than a 2 % loss in radioactivity. Thus the radio-impurity would appear to be non-volatile.

The ability of large excesses of unlabelled toxin to compete with both radioactive binding components was tested by measuring the binding in the presence of 6 μM unlabelled tetrodotoxin (triangles, figures 3) or 2 μM saxitoxin. Both conditions abolished all observable saturable binding, but did not appear to affect the linear non-specific component. The failure of the unlabelled toxin to compete for the non-specific component might be explained by binding impurities present only in the radiolabelled preparation. However, an equally likely explanation is that the nonspecific component represents binding to a very large 'sink' and, in the conditions of this experiment, the occupancy of the sink would be so small that competition by the unlabelled toxin preparation would not be seen.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Discussion

The results demonstrate the existence of substantial amounts of binding radioimpurities in chromatographically homogeneous tritiated tetrodotoxin prepared in this laboratory. The fact that the bioassay failed to show any significant non-specific binding over the range studied might call into question the interpretation of Colquhoun *et al.* (1972) that the substantial non-specific binding observed in their preparations was due to non-specific binding of the toxin itself. The alternative interpretation that non-specific binding is caused by radioimpurities in the toxin solution is consistent with the conclusions of Barnola *et al.* (1973); it is also consistent with the work of Benzer & Raftery (1972), who failed to find any non-specific binding to gar olfactory nerve with their tritiated tetrodotoxin which was purified by ion exchange chromatography. In this laboratory, comparisons have also been made of the radioactive and bioactive uptakes in piglet grey matter homogenates (Levinson & Ellory 1973; Levinson, unpublished results) and frog sartorius muscle (Almers & Levinson 1975). In the ranges studied (0–180 nM tetrodotoxin) radioassays all showed substantial non-specific binding in addition to a specific component, while bioassay only showed a saturable uptake.

Correct interpretation of the non-specific binding component is necessary in calculation of the binding site density. If one assumed in this study that all of the radioactivity in the solution was bound to tetrodotoxin, one would arrive at a specific activity that is about 3 times greater than the actual specific activity determined here, and thus the calculation of the density of binding sites would be a factor of three too low. For instance, the number of binding sites in the squid giant axon which was obtained as 553 sites/μm^2 (see the following communication by Levinson & Meves), using the specific activity obtained in the present study as 117.4 Ci/mol, would only be 164 sites/μm^2 if the general activity of 395.0 Ci/mol was used.

Preliminary experiments indicate only that the radioimpurities are non-volatile and formed during the labelling or purification process. One might speculate, on the basis of the chromatographic data, that the impurity is in equilibrium with the tetrodotoxin and has roughly the same pK_A. In fact, anhydroepitetrodotoxin has been shown to be in equilibrium with tetrodotoxin under certain conditions, and has a roughly similar pK_A (Goto, Kishi, Takahashi & Hirata 1965). However, the equilibrium found between anhydroepitetrodotoxin and tetrodotoxin rarely gives concentrations of the epimer higher than that of tetrodotoxin, which would be required if one is to account for the large amount of impurities observed in the present study. Also, it would be difficult to explain why anhydroepitetrodotoxin should bind non-specifically to tissues at low concentrations when tetrodotoxin apparently does not.

Finally, it should be emphasized that the findings of this study do not invalidate the use of tritiated tetrodotoxin in studies of the sodium channel, as long as the nature and degree of possible non-specific interactions are taken into account. In a future paper I will try to show that tritiated tetrodotoxin can be an invaluable tool in studies of preparations that have too little binding to be studied by bioassay techniques.

The author would like to thank Dr W. Almers, Dr M. H. Evans and Dr H. Meves for helpful criticism of the results of this study. Financial support from the Wellcome Trust is gratefully acknowledged.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

References (Levinson)

Almers, W. & Levinson, S. R. 1975 *J. Physiol., Lond.* (In Press.)
Barnola, F. V., Villegas, R., Camejo, G. 1973 *Biochim. biophys Acta* **298**, 84.
Benzer, T. I. & Raftery, M. 1972 *Proc. natn Acad. Sci. U.S.A.* **69**, 3634.
Benzer, T. I. & Raftery, M. 1973 *Biochem. Biophys. Res. Comm.* **51**, 1622.
Colquhoun, D., Henderson, R. & Ritchie, J. M. 1972 *J. Physiol., Lond.* **227**, 95.
Evans, M. H. 1972 *Int. Rev. Neurobiol.* **15**, 83.
Goto, T., Kishi, Y., Takahashi, S. & Hirata, Y. 1965 *Tetrahedon* **21**, 2059.
Henderson, R., & Wang, J. 1972 *Biochemistry* **11**, 4565.
Levinson, S. R. & Ellory, J. C. 1973 *Nature New Biol.* **245** (**143**), 122.
Scatchard, G. 1949 *Ann. N.Y. Acad. Sci.* **51**, 660.
Villegas, R., Barnola, F. V. & Camejo, G. 1973 *Biochim. biophys Acta* **318**, 61.

Discussion

D. Colquhoun, R. Henderson & J. M. Ritchie

In studies of the uptake of a drug by tissue a specific, saturable component is usually found reflecting the binding of the drug to its receptor. In addition, a non-specific component, characterized by an uptake that increases linearly with increasing concentration in the bathing medium, is often found – for example, the uptake of atropine by guinea pig ileum (Paton & Rang 1965); ouabain by heart (Godfraind & Lesne 1972); insulin by fat cells (Cuatrecasas 1971). Such linear, non-saturable uptake may reflect the lower end of a hyperbolic component of uptake into a pool of sites such as connective tissue, or tissue proteins generally, with a high capacity and low affinity for the drug. But it could also reflect accumulation of the drug in some tissue compartment such as the extracellular or intracellular spaces, or, in the case of charged compounds, accumulation in the double layer surrounding a charged membrane. Finally, the drug may also be metabolized into other compounds that are concentrated by the tissue. Colquhoun, Henderson & Ritchie (1972) found that labelled tetrodotoxin (TTX) was taken up both specifically by its presumed receptor, and non-specifically. Colquhoun *et al.* (1972) discussed in detail the reservations they had about their findings. These reservations still apply to their studies. They also apply to the findings of Levinson (1974).

Levinson (1974) has prepared a tritium-labelled tetrodotoxin and has added small amounts of a particulate fraction from eel electric organ to small volumes of solution containing the labelled toxin. By using both bioassay and scintillation counter techniques he has shown that the extent of disappearance of biological activity from the supernatant was about three times that of the radioactive label. On this basis he concluded that his preparation was only about 30 % pure. In principle, this test does show that the labelled and unlabelled compounds are different. However, as a quantitative determination of 'the purity of tritiated tetrodotoxin' it cannot be relied upon, for if the labelled and unlabelled compounds are different (as the test shows) and the experimenter has no idea of what (or how many) different labelled compounds there might be in his solution, then he is in no position to pick one binding component from each of his binding curves and to say: this component from the unlabelled TTX binding curve corresponds to this other component from the labelled TTX binding curve. Even if the binding constants from the saturable components of both curves are equal – and those of Levinson (1974) are within a factor of 2 (10.0 nM and 6.7 nM) – it does not help. The situation is best illustrated by the example from Colquhoun *et al.* (1972), who considered what would happen if a compound that was very closely related to tetrodotoxin were labelled inadvertently.

The physical and chemical properties (charge, molecular mass, pK_a, etc.) of the labelled compound might prevent its separation from the toxin by most normal methods, and as a result of its slightly changed structure, its binding to the sodium channels might be increased or reduced but not eliminated. The results of any binding study would then be similar to those obtained by Levinson (1974), in that the binding of labelled and unlabelled compounds would differ. Of course, if no account was taken of the altered binding of the radioactive compound, an increased or reduced estimate of the number of binding sites would result. If, in addition, the specific activity of the labelled compound in an impure mixture were unknown, then no determination of the purity or homegeneity of the preparation could be made by this method.

The above is a brief summary of the ambiguities that are almost always present when the Wilzbach method of labelling is used. The ambiguities are not removed even when the binding of labelled and unlabelled compounds agree as we shall show it did in the studies of Colquhoun *et al.* (1972). What Levinson (1974) has done is to determine the number of binding sites for tetrodotoxin in the electric organ of *Electrophorus electricus* by *bioassay methods*, and to show that a labelled preparation has a component of binding with a similar affinity. *If the behaviour of all the impurities is the same* in other tissues, then the preparation can be used to estimate the number of binding sites in, for instance, squid giant axons (Levinson & Meves 1974). It is in these other studies, where binding curves using the bioassay method are not possible, that the ambiguities arise; and they arise to a greater extent with preparations containing demonstrable impurities.

For this reason it is imperative that Wilzbach labelled tetrodotoxin should be purified to the greatest extent. To this end, we devised a simple and direct purification procedure using only two steps and producing very high resolution (Colquhoun *et al.* 1972). Although only a 30-fold purification was necessary to produce a homogeneous sample (by several criteria) from our crude, labelled material, the method would, if required, produce much greater purifications. The data of Levinson (1974) show that the amount of tetrodotoxin in his preparation was reduced by degradation during the labelling process to 22 % , a much more drastic reduction than that obtained in our case (to 60 %), and one which would result in the presence of a correspondingly larger proportion of impurities. The fact that Levinson did not obtain a correspondingly larger degree of purification suggests that the purification of his tetrodotoxin was inadequate (unless a much cleaner labelling method was used, which seems unlikely). Taken together with his estimate of 30 % purity, it would seem to justify the estimates by Colquhoun *et al.* (1972) that their samples were close to being radiochemically pure and discount the suggestion that their binding site densities were underestimated.

Since Levinson (1974) carried out his purification 'essentially' as described by Colquhoun *et al.* (1972), it is worth while asking whether his modifications might not be expected to give a less pure product. First, in using an initial electrophoretic step at pH 7.9 the maximum degree of separation of tetrodotoxin from possible impurities was not obtained. For maximum resolution, the electrophoresis should be carried out at pH 8.5, the pK_a of the toxin (Colquhoun *et al.* 1972). At this pH the toxin has a charge of 0.5 and a mobility of 0.5 compared to a fully protonated molecule. An impurity with a pK_a of 8.8, say, would then have a mobility of 0.67, and would be clearly separated from the tetrodotoxin. By contrast, at pH 7.9, the mobilities would be 0.80 and 0.87, for tetrodotoxin and the impurity respectively, and the separation would be less than half. Secondly, the separations should be performed by allowing the toxin

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

to move as far as possible from the origin (20–30 cm), thereby obtaining the greatest resolution of the peaks. Levinson (personal communication) ran his samples over shorter distances (8 cm). The combination of these two modifications would certainly result in a product of lower purity.

Whatever impurities there are remaining in Levinson's preparation, they cannot of course be volatile since the tritium, which is exchangeable with water, is removed in the first instance by repeated evaporation. Neither can anhydroepitetrodotoxin be involved. For, if anhydroepitetrodotoxin were in equilibrium with labelled tetrodotoxin, it would be in equilibrium equally with unlabelled tetrodotoxin and precisely the same types of binding would occur. Only if a distinctly different compound was present as a radioactive impurity would the binding results differ.

Clearly, the ultimate answer to possible criticisms of results obtained using compounds labelled by procedures like the Wilzbach method is to develop a simple, clean method of labelling that anyone can use without difficulty. Where this is not yet possible, however, such methods as the Wilzbach are very useful, but *must* be accompanied by detailed and painstaking purification, and even then reservations remain.

A second point concerns the conclusion by Levinson that his radioactive impurities give rise to additional non-specific binding. It concerns on the one hand whether a substantial linear component is indeed *present* in the labelled study and, on the other, whether a linear component is indeed *absent* in the bioassay.

In Levinson's radioactive experiments the presence of a linear component depends nearly entirely on two measurements, the uptakes at two final concentrations, of 180 and 260 nM, that are high enough to saturate the specific component virtually completely. There are two ways of determining uptake of labelled drug by tissue. The determination that is subject least to experimental error is the method (used by Colquhoun *et al.* 1972) in which the actual *uptake* of radioactivity into the tissue is determined directly. The second method, which is used by Levenson, determines the *disappearance* of labelled drug from the bathing medium. Unfortunately, at high concentrations this latter method becomes intrinsically unreliable. Thus, at 180 and 260 nM there are decreases of about 5% and 4% in the radioactivity of the solution respectively. While a determination of such small decreases is not entirely outside the bounds of experimental possibility, it seems unlikely, especially in view of internal evidence that there is indeed a great experimental variability in Levinson's results. This variability is clearly seen in experiments (Levinson's figure 3) in which a large amount of unlabelled toxin was added to the bathing medium to inhibit almost completely the saturable binding of labelled TTX. The remaining uptake, which consisted only of the linear component, showed a considerable scatter in the points about a straight line fitting them. This scatter must also have been present in the original uptake curve; and its presence necessarily casts doubt on the reliability of a claim for a linear component based on the two measurements at high concentration. Furthermore, these latter data show, seemingly, an internal inconsistency. For the slopes of the two straight lines (one based on high concentrations of labelled TTX and the other based on experiments with low concentrations of labelled TTX in the presence of high concentrations of unlabelled toxin), which ought to be equal, in fact differ by a factor of about 1.8.

Just as the existence of a linear component in the labelled experiments seems to require further statistical justification, so there seems to be little evidence, at the moment, for the absence of a linear component in the bioassay experiments. The highest concentration studied

is 110 nM. The experiments with labelled toxin, which used concentrations up to 260 nM, suggest that 110 nM is not high enough for the presence or absence of a linear component to be established. But, as discussed earlier, the technique of measuring the disappearance of activity from the supernatant becomes extremely unreliable at high concentrations. For example, from the data on Levinson's figure 1 at the highest concentration used, 110 nM, the amount of toxin in the supernatant would drop by 30.4 pmol from an initial value of 250.4 pmol (220 + 30.4 pmol), i.e. by 12 %. At 200 and 300 nM final concentrations the corresponding falls would be 7 % and 5 % respectively. These are small differences to detect in any pharmacological bioassay experiment (Levinson's own estimate of possible error is 10 %) – so small that some evidence has to be presented that the conclusion (that there is no linear component) is statistically sound.

In summary, four points must be made. First, the presence of a non-specific component of uptake of tetrodotoxin does not in itself indicate the presence of an impurity. Many other explanations are possible (see above). Secondly, there are good reasons for believing that Levinson's purification of labelled TTX made inadequate use of the methods we devised originally, and could be easily improved. His suggestion that other TTX samples are as impure as his own must therefore be doubted. Indeed, Levinson's value of 30 % purity comes from the observation of a threefold discrepancy between the uptake calculated from labelled experiments and the corresponding value determined in bioassay experiments. However, using essentially the same test, namely comparing the results of a radioactive assay and those of a bioassay, Colquhoun *et al.* (1972) showed that *no such discrepancy* exists in their experiments. Thus, a value of 450 fmol/mg dry tissue is obtained for uptake of labelled TTX from a least squares fit to the data in the radioactive experiments of Colquhoun *et al.* (1972). This value does not differ significantly from the corresponding value of 360 ± 65 (s.e.) fmol/mg (10 experiments) obtained by Keynes, Ritchie & Rojas (1971) for unlabelled TTX. Similarly, the maximum uptake by lobster nerve (*Homarus americanus*) in the labelled experiments was 184 ± 27 fmol/mg dry tissue (Colquhoun *et al.* 1972), which again does not differ significantly from the corresponding value of 160 fmol/mg dry tissue for uptake of unlabelled tetrodotoxin by the nerves of the same species found by Moore, Narahashi & Shaw (1967). If anything, in both the rabbit and lobster nerves, the labelled uptakes are higher than those of unlabelled toxin. This agreement, found by us but not by Levinson, is consistent with the purity of our TTX; but measurements at a single concentration do not conclusively demonstrate purity. Independent, and more compelling, evidence comes from the observed agreement between the values of uptake for both rabbit and garfish nerve using labelled saxitoxin and those using labelled tetrodotoxin (Henderson, Ritchie & Strichartz 1973): since the chemical structure of saxitoxin is quite different from the puffer fish poison, it is unlikely that the same type of impurity (with the same scaling factor) would be produced by the labelling procedure and remain undetected during purification. Finally, the agreement between labelled and unlabelled uptake by the rabbit vagus nerve also holds for saxitoxon, though the reliability of the comparison is not as great as with tetrodotoxin because fewer measurements were made. For the three experiments on table 3 of Keynes, *et al.* (1971) at an average concentration of 180 nM (145–240 nM) the uptake of labelled STX was 450 ± 50 (s.e.) fmol/mg dry tissue. This agrees reasonably well with the value of 340 fmol/mg predicted from the least squares analysis of the data of Henderson *et al.* (1973).

The general conclusion seems inescapable. From the arguments given above, there seems

no reason whatsoever at the moment 'to call into question the interpretation of Colquhoun *et al.* (1972)', or those of Henderson *et al.* (1973), and of Ritchie (1974). The binding site densities obtained in these studies remain the best estimates available.

References

Colquhoun, D., Henderson, R. & Ritchie, J. M. 1972 The binding of labelled tetrodotoxin to non-myelinated nerve fibres. *J. Physiol., Lond.* **227**, 95–126.

Cuatrecasas, P. 1971 Insulin-receptor interactions in adipose tissue cells: Direct measurement and properties. *Proc. natn. Acad. Sci., U.S.A.* **68**, 1264–1268.

Dorfman, L. M. & Wilzbach, K. E. 1959 Tritium labelling of organic compounds by means of electric discharge. *J. phys. Chem.* **43**, 799–801.

Godfraind, T. & Lesne, M. 1972 The uptake of cardiac glycosides in relation to their actions in isolated cardiac muscle. *Br. J. Pharm.* **46**, 488–497.

Henderson, R., Ritchie, J. M. & Strichartz, G. R. 1973 The binding of labelled saxitoxin to the sodium channels in nerve membranes. *J. Physiol., Lond.* **235**, 783–804.

Keynes, R. D., Ritchie, M. J. & Rojas, E. 1971 The binding of tetrodotoxin to nerve membranes. *J. Physiol., Lond.* **213**, 235–254.

Levinson, S. R. 1974 The purity of tritiated tetrodotoxin as determined by bioassay. *Phil. Trans. R. Soc. Lond.* B **270**, 337–348 (this volume).

Levinson, S. R. & Meves, H. 1974 The binding of tritiated tetrodotoxin to squid giant axons. *Phil. Trans. R. Soc. Lond.* B **270**, 349–352 (this volume).

Moore, J. W., Narahashi, T. & Shaw, T. I. 1967 An upper limit to the number of sodium channels in nerve membrane? *J. Physiol., Lond.* **188**, 99–105.

Paton, W. D. M. & Rang, H. P. 1965 The uptake of atropine and related drugs by intestinal smooth muscle of the guinea-pig in relation to acetylcholine receptors. *Proc. R. Soc. Lond.* B **163**, 1–44.

Ritchie, J. M. 1974 Binding of tetrodotoxin and saxitoxin to sodium channels. *Phil. Trans. R. Soc. Lond.* B **270**, 319–336 (this volume).

Wilzbach, K. E. 1957 Tritium-labelling by exposure of organic compounds to tritium gas. *J. Am. chem. Soc.* **79**, 1013.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES