pears primarily to increase the rate of binding since prolonged incubations in the cold will achieve binding levels comparable to short incubations at elevated temperatures. Williams and Gorski (1971) have suggested that the marked temperature dependency of the accumulation of E in uterine nuclei might be totally concerned with the entry of E into the cell. Our data in a cell-free system indicate that equally dramatic effects of temperature can be observed with R–E and C interactions.

It is noteworthy that other investigators studying C–receptor–steroid hormone interactions have not reported on the influence of temperature in these systems and have incubated the components for 1 hr at 4° (Spelsberg *et al.*, 1971; Steggles *et al.*, 1971; Mainwaring and Peterken, 1971). Our data would suggest that 4° for 1 hr would not permit sufficient interaction and would lead to an underestimation of the number of C acceptor sites. This may be important since experiments designed to demonstrate the specificity of receptor–steroid–chromatin binding have largely depended on a comparison of the number of chromatin acceptor sites in various tissues.

Added in Proof

It has been brought to our attention that the EDTA present during the incubation depicted in Figure 1 might inhibit temperature-dependent transformation of receptor in the cytosol which is thought to be required for nuclear binding (Jensen *et al.*, 1971). We have therefore repeated the experiment in the complete absence of EDTA and still obtain the same result.

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Solubilization of a Specific Tetrodotoxin-Binding Component from Garfish Olfactory Nerve Membrane†

Richard Henderson* and Jui H. Wang‡

ABSTRACT: Extraction of the membranes from garfish olfactory nerve cells with detergent has yielded a soluble tetrodotoxin-binding component with an apparent molecular weight of about 500,000. The binding of tetrodotoxin, which is reversible, occurs with a dissociation constant of 6 nM and a dissociation rate of 0.95 min^{-1}. Saxitoxin competes for the same site with an almost identical affinity. The binding of tetrodotoxin is independent both of pH, in the region 6.5–8.5, and of ionic strength, above 0.01 M. It is concluded that there exists in garfish nerve membrane a specific tetrodotoxin binding component, which probably forms part of the sodium ion channel involved in nerve impulse propagation.

Tetrodotoxin and saxitoxin are both potent inhibitors of the regenerative sodium ion conductance change which causes excitability in nerve and muscle membranes. Their action, which is normally reversible, can be explained well on the basis of a one-to-one combination with a specific membrane constituent whose contribution to the sodium ion conductivity is then eliminated (Hille, 1968, 1970; Cuervo and Adelman, 1970). Dissociation constants for binding of toxin ranging between 10^{-8} and 10^{-9} M have been obtained with several nerve preparations, both on the basis of electrical measurements (Hille, 1968; Cuervo and Adelman, 1970; Colquhoun and Ritchie, 1972a,b) and from measurements of the binding of radioactive [^{3}H]tetrodotoxin to intact cells (Colquhoun *et al.*, 1972; Hafemann, 1972). This paper is a report of some of the properties of a soluble tetrodotoxin-binding component obtained from garfish olfactory nerve membrane by extraction with detergents.

The ultimate aim of these experiments is to isolate the sites of toxin binding. Hopefully these will turn out to form at least

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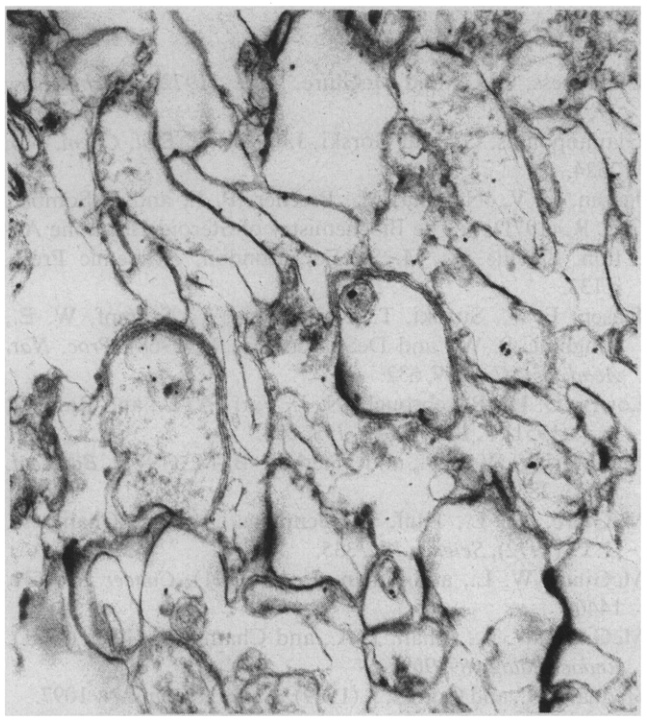

FIGURE 1: Electron micrograph (×20,000) of the preparation of membranes from garfish olfactory nerve obtained by differential centrifugation. Fixed with glutaraldehyde and osmium tetroxide, and stained with uranyl acetate.

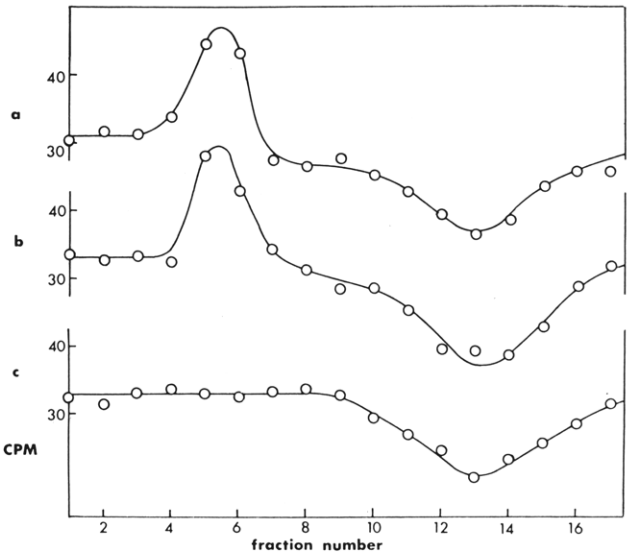

FIGURE 2: Elution profiles obtained by gel filtration of the detergent extract on a small column (0.5 × 10 cm) of Sephadex G-25F. In part a, the column was equilibrated and eluted with a solution of 1 nM labeled-tetrodotoxin in 2% Triton X-100–0.15 M NaCl–0.01 M Tris (pH 7.3). Part b was also eluted with 1 nM labeled tetrodotoxin but in this case the sample was first equilibrated with 50 nM unlabeled tetrodotoxin. In part c, the column was equilibrated and eluted with 1 nM labeled tetrodotoxin and 1000 nM unlabeled tetrodotoxin. The fraction size was 0.17 ml and the applied sample size, 0.25 ml. Flow rate 0.19 ml/min, temperature 20°.

part of the sodium ion channels or carriers which are responsible for the conductance changes. Although this has not yet been achieved, the experiments demonstrate that such a component exists and that its measurable properties, such as those of tetrodotoxin and saxitoxin binding, are almost unchanged compared to the binding properties of intact nerve cells. In addition, several properties not measurable in intact cells, such as the rate of the tetrodotoxin-binding reaction, and some aspects of the structure and stability of the binding component, have been determined.

Materials and Methods

Garfish (heads only) were obtained frozen from the Gulf Specimen Co., Panacea, Fla. Tetrodotoxin was obtained from Sankyo Chemical Co., Japan. Saxitoxin was very kindly supplied by Dr. E. J. Shantz. All other chemicals were reagent grade.

The tetrodotoxin was labeled by exposure to a tritium gas discharge and the radioactive impurities were then removed by high-voltage electrophoresis as described by Colquhoun *et al.* (1972). Approximately 30-fold purification was necessary. Material with a specific activity of 300 Ci/mole was used, slightly higher than previously.

A typical preparation of membranes was as follows. Ten frozen garfish heads were quickly thawed in running water and the olfactory nerves were removed and dissected according to the method of Easton (1965). The nerves were immediately placed in a physiological Ringer solution (Easton, 1971) at 0° and washed once or twice until the supernatant was clear. All subsequent operations were carried out at 0–4°. At this stage, the yield from ten medium-sized gar (2.5–3.5 ft long) was approximately 2.5 g of tissue. The nerves were then homogenized in 0.1 M sucrose solution with a Teflon and glass homogenizer using 15 ml of solution. The resulting opaque dispersion was centrifuged at 1000*g* for 5 min and the pellet was washed twice with a total of about 10 ml of 0.1 M sucrose. This removed mainly connective tissue. The combined supernatants were then spun at 50,000*g* for 1 hr. The resultant pellet, containing most of the membrane and almost all of the tetrodotoxin-binding activity, was then used in the experiments described below. The material could be kept frozen at −20° for periods of up to 1 month without loss of tetrodotoxin-binding activity. An electron micrograph of part of such a pellet is shown in Figure 1. Some of the vesicles still contain neurotubules, and mitochondrial fragments are also present. The yield of protein, most of which is membrane protein, by Biuret determination, was from 2.0 to 2.5% of the initial weight of wet tissue, *i.e.*, about 60 mg of membrane protein from ten garfish. This constituted about 20–25% of the dry weight of the whole nerve.

The membranes were extracted with 1 or 2% solutions of various detergents by stirring continuously at 0° for 4–6 hr and, subsequently, centrifuging at 50,000*g* or 100,000*g* for 1 hr. In all cases, at least 2 g of detergent/g of membrane protein was used. The extracting solutions also normally contained 0.15 M NaCl and 0.01 M Tris buffer (pH 7.5). The resultant clear solution often had a slight yellow coloration, but this could be eliminated by washing the membranes in deionized water or 0.15 M NaCl prior to extraction.

Assays of binding of tetrodotoxin to the solubilized membrane preparation were carried out on a small column (0.5 × 10 cm) of Sephadex G-25F (Pharmacia, Sweden), equilibrated, and eluted with a solution containing the desired concentration of radioactive tetrodotoxin. The elution profile (Figure 2a) showed a peak of radioactivity in the void volume where the high molecular weight material from the membrane emerged, followed by a gradual fall in the amount of radioactivity until the back of the column was reached. The radioactivity and hence the tetrodotoxin then rose to the normal

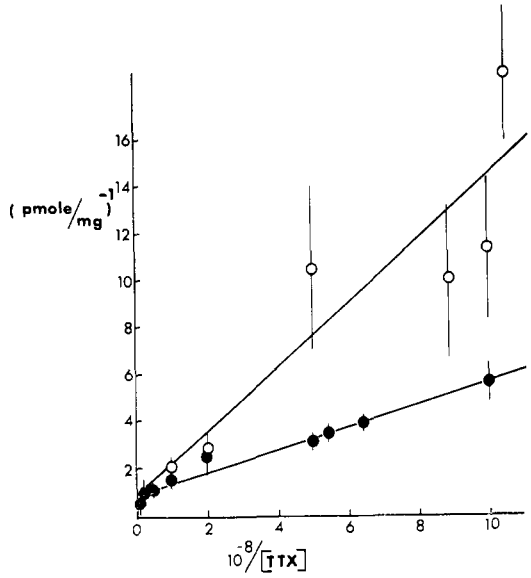

FIGURE 3: Binding of labeled tetrodotoxin to membrane material solubilized in 2% Triton X-100, measured using G-25F column assay at 20°. The open circles are measurements of tetrodotoxin binding in the presence of 15 nM saxitoxin. The solid lines are those predicted theoretically if tetrodotoxin and saxitoxin bind competitively with dissociation constants of 6 and 7 nM, respectively. Errors in these values of ±1.5 and ±2.5 nM are possible.

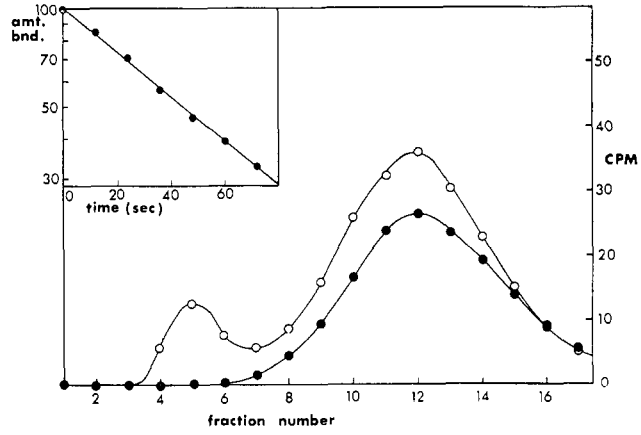

FIGURE 4: Gel filtration (○) as in Figure 2 of a sample of the detergent extract equilibrated with an initial concentration of 5 nM labeled tetrodotoxin and run under a small hydrostatic pressure. The control experiment (●) was run under identical conditions but with a sample containing only labeled tetrodotoxin. The column was previously equilibrated and eluted with a solution of 1000 nM unlabeled tetrodotoxin in 2% Triton X-100–0.15 M NaCl–0.01 M Tris (pH 7.3). The fraction size was 0.17 ml, and the sample size 0.25 ml. Flow rate 0.85 ml/min, temperature 20°. The difference between the two profiles represents tetrodotoxin which was initially bound to membrane material, some of which became dissociated during the run. The difference between the profiles has been analyzed (see text) to give the amount of toxin which remained bound at different times after the start of the run (insert). The straight line indicates an exponential decay with a rate constant of 0.95 min^{-1}.

level again. The whole assay took about 5 min. The radioactive peak emerging in the void volume was taken to represent bound tetrodotoxin and was integrated after subtraction of the background level of free tetrodotoxin. Inclusion of an excess of unlabeled tetrodotoxin or saxitoxin during the assay resulted in the complete abolition of the peak, as would be expected and as Figure 2c shows. The samples which were applied to the column contained no radioactive tetrodotoxin, so that the elution profile shows an additional dip at the back of the column, seen most clearly in Figure 2c, but also in Figure 2a,b where it is superimposed on the gradual fall due to binding of the toxin to the material in the void volume peak.

Results

Solubilization and Binding. Using the Sephadex column described above to assay soluble fractions for their ability to bind tetrodotoxin, several methods of solubilization were tried in an attempt to extract from the membranes the specific tetrodotoxin-binding component. It was found that neither deionized water nor 1 M NaCl would remove the tetrodotoxin binding activity from the pellet.

Solutions of 1.5% Tween-80, 1% digitonin, and 1% Brij-35, all nonionic detergents, also failed to solubilize the binding activity, and left most of the protein still in the membrane pellet. Sodium deoxycholate (1%) was ineffective. Sodium dodecyl sulfate (1%) dissolved the whole membrane immediately, but no binding activity was present in the resultant solution. Only sodium cholate (1%) or Triton X-100[1] (1 or 2%) successfully solubilized most of the membrane proteins, including the tetrodotoxin-binding activity, leaving a small insoluble residue which was removed during the centrifugation. Presumably these latter two are sufficiently disruptive of the lipid structure of the membrane under the conditions used, to remove most of the proteins, but are mild enough that the tertiary structures of the proteins are maintained intact.

The binding of tetrodotoxin to the material solubilized in 2% Triton is shown as a function of the concentration of tetrodotoxin in Figure 3. The amount bound follows closely that expected for binding to a single component with a dissociation constant, K = 6 nM, present at a concentration, N = 1.1 pmoles/mg of membrane protein (both ±25%). These numbers should be compared to those determined by Colquhoun *et al.* (1972), in intact nerve with K = 10 nM and N = 2.5 pmoles/mg of membrane protein, using the data given in the Methods section of this paper to make their results comparable. The agreement is reasonable considering that the really accurate measurement in both cases is the ratio K/N. The values of K/N are consistent with retention in the detergent solubilized preparation of 70–80% of the initial tetrodotoxin-binding activity. The values of the dissociation constant before and after solubilization agree within experimental error. Figure 3 also shows the binding of labeled tetrodotoxin in the presence of 15 nM saxitoxin. The binding appears to be competitive and is consistent with an almost identical affinity of saxitoxin for the same site.

Kinetics of Binding. If the above binding assay was carried out after equilibration of the solubilized material with an excess of unlabeled tetrodotoxin, the same elution profile was obtained (Figure 2a,b). This already shows that, within the period of the assay, approximately 5 min, sufficient time was available for dissociation of the unlabeled tetrodotoxin and subsequent binding of the labeled material. By running the same Sephadex G-25F column five or ten times faster under a larger hydrostatic pressure, it proved possible to measure the rate of dissociation.

[1] Triton X-100 is a (9–10) poly(oxyethylene ether) of *p-tert*-octylphenol and has an average molecular weight of 620.

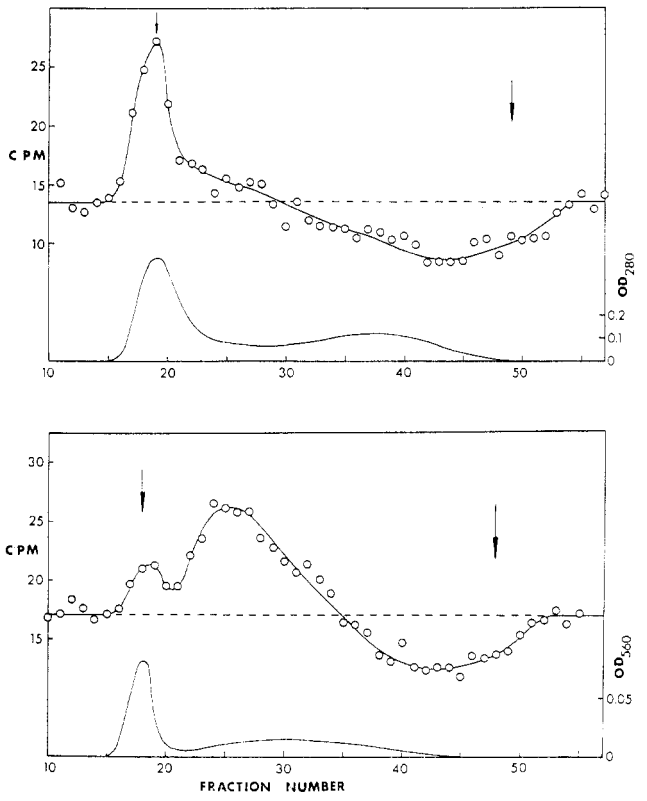

FIGURE 5: Gel filtration on a column (0.9 × 45 cm) of Sepharose 6B, of nerve membrane solubilized in 1% sodium cholate (a) and 2% Triton X-100 solutions (b) containing 0.15 M NaCl–0.01 M Tris (pH 7.3). The fraction size was 0.65 ml and flow rate 10 ml/hr, temperature 0°. The lower curves represent protein concentrations by Biuret determination or OD_{280}, and the two arrows mark the positions of the void volume and total volume of the column.

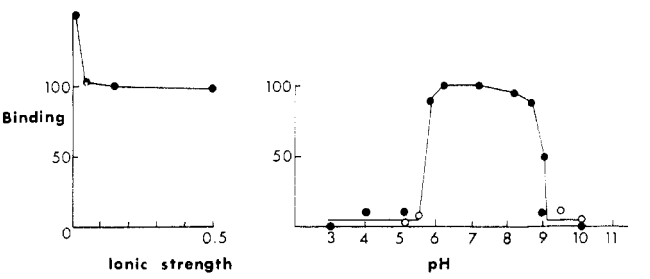

FIGURE 6: Dependence of binding of 1 nM tetrodotoxin to solubilized nerve membrane on ionic strength of NaCl (a) and pH (b). The open circles in part b represent measurements at pH 7.2 after exposure to the indicated pH for 15 min at 0°.

The results are shown in Figure 4, where material incubated for 10 min with labeled tetrodotoxin has been applied to the top of a column equilibrated with an excess of unlabeled tetrodotoxin. During the run, which from the time that the flow was started until the emergence of the void volume peak was 72 sec, about 65% of the initially bound toxin became dissociated and, because of the excess of unlabeled material present, did not become rebound. The labeled toxin that had initially been bound to the solubilized component was separated from the initially unbound toxin by running a blank containing only free toxin and scaling the magnitude of the two elution profiles so that the trailing edge of the profiles coincided. The difference between the two profiles then represents tetrodotoxin which was initially bound to the membrane material. The void volume peak represents tetrodotoxin remaining bound after 72 sec. By integrating the difference in profiles from the front to the back of the column, the amount bound at intermediate times during the run was also obtained, and, when plotted as shown in Figure 4 (insert) gives an exponential decay of the amount of toxin bound with a rate constant of 0.95 min^{-1}, corresponding to a half-life of 44 sec.

As a check on this scaling procedure, the amount of bound and unbound material in the initial sample was calculated independently using the known dissociation constant and capacity, obtained from the data in Figures 2 and 3, together with the known amount of labeled tetrodotoxin added (5-nM initial concentration). The fraction of the tetrodotoxin which was thereby calculated to bind was 31% of the total added, compared to the amount measured in the experiment, which was 32%. The fact that these numbers agree is not only satisfactory, but, since the incubation was for 10 min during which time the labeled tetrodotoxin would be bound and released an average of three times, it demonstrates that the toxin is not degraded during binding, but is released in a form which retains its ability to bind. If the tetrodotoxin had been either hydrolyzed or degraded in some other manner to an inactive compound as a result of any special chemistry which occurred during the binding interaction, only 10% of the toxin would be left after 10 min and very little difference between the two profiles in Figure 4 would have been obtained. This therefore shows that binding of tetrodotoxin is *rapid* and *reversible* and results in *no destruction* of the toxin.

Gel Filtration. Application of samples of the Triton or cholate solubilized material to a Sepharose-6B column which was previously equilibrated and eluted with 1 nM labeled tetrodotoxin yielded the results shown in Figure 5. In Figure 5a, with 1% sodium cholate, all the tetrodotoxin-binding activity emerged in the void volume, and therefore represented material with an apparent molecular weight of several million, probably an aggregate. In Figure 5b, with 2% Triton X-100, most of the binding activity emerged in a broad peak which covered the range of apparent molecular weight of 500,000 to 1,000,000. A small amount of material, perhaps 10%, was also present in the void volume peak. The aggregation state of the tetrodotoxin-binding material therefore seems to vary depending on the detergent that is used to solubilize, and even with Triton X-100, some material remains in a very high molecular weight complex. Denatured protein (2 hr at room temperature, see next section) gives no binding in the void volume or elsewhere with the same column, so presumably both peaks in the elution profile of Figure 5b represent specific binding.

Stability. Because of the difficulty experienced in attempts to purify further the binding component, mainly due to loss of activity, the effects of pH and ionic strength on the binding of tetrodotoxin were investigated. First, some mention of stability under normal conditions (pH 7, $I = 0.15$) should be made. If the solubilized material was kept at 0–4°, very little drop in ability to bind tetrodotoxin was observed over a period of several days. However, even after 1 hr at room temperature (20°), the binding activity had completely disappeared. The half-life was about 30 min at 20°, sufficiently long to assay, but not to carry out any purification. This lack of stability of the solubilized material is in contrast to its relatively stable behavior while still in the membrane and presumably reflects the degree to which the environment inside a detergent micelle differs from that within the lipid bilayer.

The dependence of binding of tetrodotoxin on pH and ionic strength (NaCl) is shown in Figure 6. In Figure 6a, binding is constant from 0.05 to 0.5 M NaCl and only a small increase is present at very low (0.01 M) ionic strength. In particular, no specific reduction in tetrodotoxin binding with increasing sodium ion concentration is seen. In Figure 6b, the binding is constant between pH 6.5 and 8.5. The falloff above and below this range of pH is due to irreversible denaturation, as shown by the binding experiments carried out at pH 7.2 after incubation at different pH's for 10–15 min at 0°. The falloff at high pH seems to occur somewhat more slowly than would be expected if the zwitterionic form of tetrodotoxin which exists at high pH (pK_a = 8.5–8.8) did not bind. The effect, however, is only just significant, and in view of the experiments (Camougis *et al.*, 1967; Hille, 1968) which suggest that the cationic form may be more active, any definitive statement should await further experiments with a more stable preparation. It should be emphasised that the experiments at high pH shown in Figure 6b were all carried out within 5 min of preparation of the tetrodotoxin solutions, so that even at pH 10 where the half-life of the tetrodotoxin, due to alkaline degradation, is about 15 min (Colquhoun *et al.*, 1972), significant degradation would not have occurred. At pH 9 and below, with a half-life of 2 hr or more, ample time was available to carry out the assays.

Discussion

The above results have demonstrated that it is possible to extract a specific tetrodotoxin-binding component from garfish olfactory nerve membranes in good yield. Once extracted, the dissociation constant for binding of tetrodotoxin is 6 nM and for saxitoxin, by competition, 7 nM. The binding is reversible with a dissociation rate of 0.95 min^{-1}.

One test of whether this component is the one which mediates the inhibition of sodium ion permeability (and is therefore the receptor for tetrodotoxin) is whether the binding and rate constants for the toxin interaction are the same as those which govern the sodium currents. Although the effect of different concentrations of the toxins on the membrane sodium ion currents has not been measured on the garfish nerve for technical reasons, results with other species are available and provide encouragingly similar values. For instance, with the squid giant axon (Cuervo and Adelman, 1970), there is a reversible component of inhibition of sodium current by tetrodotoxin with a dissociation constant of 3.3 nM, and with frog myelinated nerves (Hille, 1968), the peak sodium current in single nodes of Ranvier is inhibited by saxitoxin with a dissociation constant of 1.2 nM. In the single case of frog myelinated nerve where the rate of inhibition of the sodium currents by tetrodotoxin has been tentatively estimated (Wagner *et al.*, 1972) the offset rate was 0.90 min^{-1}, very close to the dissociation rate of 0.95 min^{-1} measured here with the garfish preparation. It therefore appears, as Wagner *et al.* suggested that the rate of reaction of tetrodotoxin with single nodes of Ranvier is indeed limited by the intrinsic rate of reaction with the receptor and not by diffusion, as is normally the case with whole tissue (Colquhoun *et al.*, 1972). Again, the agreement is satisfactory.

The apparent molecular weight of the tetrodotoxin-binding component of 500,000 or more obtained by gel filtration on Sepharose 6B may seem rather large for a membrane protein. However, it is typical of many other membrane proteins which have been solubilized in nonionic detergents like Triton X-100. For instance, rhodopsin, a polypeptide of 40,000 molecular weight forms micelles in digitonin solutions of about 300,000 molecular weight (Hubbard, 1954); the ($Na^+ + K^+$)-activated ATPase from kidney or brain, containing a phosphorylatable peptide of 90,000 molecular weight, behaves as a particle of 700,000 molecular weight in several detergents (Kyte, 1971; Kahlenberg *et al.*, 1969; Uesugi *et al.*, 1971); and the cholinergic receptor of electric eel appears in Triton X-100 solutions with an apparent molecular weight of about 500,000 (Meunier *et al.*, 1971; Raftery *et al.*, 1971). Indeed micelles of Triton X-100 detergent alone in water have an average molecular weight by light scattering of 70,000–100,000 (Shick, 1967; Kushner and Hubbard, 1954). More useful structural information therefore must await complete purification. The best material so far obtained, after gel filtration, is at most 1% pure, representing about a 20-fold purification relative to the total membrane protein.

In summary, the tetrodotoxin-binding component obtained in the above experiments from garfish olfactory nerve membrane is probably the same component through which the physiological effects on the sodium currents are mediated (the receptor) and, as such, is most likely to be part of the sodium channel itself. Further purification, characterization and reconstitution should clarify this point.

Acknowledgments

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