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Characterization of Exchange-Labeled Saxitoxin and the Origin of Linear Uptake by Excitable Tissue

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SUMMARY

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Tritium-labeled saxitoxin, which binds saturably to the sodium channels of excitable tissue, also is taken up linearly. Some of the linear uptake seems to represent intracellular uptake of tritiated water. However, the main component of linear uptake is of true toxin and not of radioactive impurity. It results from an accumulation of the divalent toxin in the immediate vicinity of the axonal (and other cell) membranes because of the fixed negative charges on them. A new and general method for determining the radiochemical purity of the exchange-labeled tritiated saxitoxin is described. The temperature dependence of the kinetics of loss of the label from the methylene hydrogens of carbon 12 of labeled saxitoxin is also reported.

INTRODUCTION

Both tetrodotoxin and saxitoxin combine highly specifically and in nanomolar concentrations with some component of the sodium channel in nerve and muscle. With the introduction of tritium-labeled tetrodotoxin by Hafemann (1), and subsequently of tritium-labeled saxitoxin, it became possible to determine readily the density of sodium channels in a variety of excitable tissues (see ref. 2 for references). The present paper deals with three related, largely chemical questions that have arisen in such studies.

First, binding experiments with such labeled toxins have shown a saturable component of uptake corresponding with binding to the sodium channels in excitable tissue. However, there is also a linear, nonsaturable component of uptake. Does this linear component of uptake reflect binding of a labeled impurity (3-6), or is it really nonspecific uptake of true toxin (7, 8)? Second is the question of the radiochemical purity of the labeled toxins. The labeling techniques, particularly the Wilzbach method (9, 10) used for tetrodotoxin labeling, inevitably entail the danger that the final preparation will contain radioactivity not just in the toxin but also in some other compound (perhaps a closely related compound produced in the labeling process) that is difficult to separate by the standard biochemical procedures. How can the radiochemical purity best be assessed? Finally, the most convenient and reliable preparation for such binding experiments is probably the highly specifically labeled, purer, tritiated saxitoxin labeled by a new method described recently (11). This new method of labeling saxitoxin is highly specific because it is based on a temperature-dependent transfer of tritium from triti-

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ated water to slowly exchangeable methylene hydrogens on the toxin. Tritium can thus be exchanged into saxitoxin at high temperature; the labeled toxin can subsequently be stored at low temperatures and binding experiments can be performed at 2–4° with little back-exchange of tritium out of the saxitoxin. No direct determination of the temperature dependence was made in the earlier experiments (11). The present studies were undertaken to characterize precisely the temperature dependence of this reaction and to determine whether the temperature dependence of the amount of radioactivity in the linear component is different from that of the saturably bound radioactivity.

METHODS

Binding experiments were performed lobyter walking leg nerves, desheathed rabbit vagus nerves, and homogenized rabbit brain. The Locke's solution for the rabbit experiments contained NaCl, 15; mM; KCl, 5.6 mM; $CaCl_2$, 2.2 mM; dextrose, 5.0 mM; and morpholinopropanesulfonate buffer, pH 7.2, 10.0 mM. The artificial seawater used in the lobster experiments contained NaCl, 520 mM; KCl, 13 mM; $MgCl_2$, 24 mM; $CaCl_2$, 14 mM; and morpholinopropanesulfonate buffer, pH 7.2, 10 mM. All experiments were conducted at 2–4° to prevent loss of label from the toxin by back-exchange. Whenever possible, means and their standard errors are given.

Homogenized rabbit brain, which was used in the bulk of the experiments, was obtained by homogenizing 5–20 g of brain in about 100 ml of Locke's solution with a tissue homogenizer (Ultra-Turrax) at full speed. The homogenate was then centrifuged for 30 min at 40,000 × g, and the supernatant was discarded. The pellet, except for a small dark brown part that was not easily removed from the wall of the centrifuge tube, was then resuspended in 100–200 ml of Locke's solution. The rabbit vagus and lobster walking leg nerves were dissected as described before (7).

The nerves, or 2–3-ml aliquots of the resuspension of brain homogenate, were then exposed to a variety of concentrations of labeled saxitoxin or of labeled saxitoxin plus unlabeled tetrodotoxin. Pellets of the homogenate (50–200 mg of brain) were then obtained by centrifugation for 30 min at 100,000 × g. The nerves (after being weighed), or the pellets of brain homogenate, were transferred to scintillation vials containing a tissue solubilizer (Protosol, New England Nuclear). After about 3 hr at 50°, scintillation fluid (Aquasol, New England Nuclear) was added, and the radioactivity was determined.

Extracellular space determination. In early experiments the average extracellular space was determined from parallel experiments on separate batches of the preparation, using [^{14}C]mannitol. Most experiments, however, employed double-labeling techniques using [^{14}C]mannitol together with tritiated saxitoxin. Tritium and ^{14}C efficiencies were calculated from the external standards channel ratio measured on a Beckman LS/3150 scintillation counter.

Bioassay procedures. Samples of the labeled toxin were bioassayed using frog sciatic nerves mounted in a chamber with three pools separated by petroleum jelly. With this method it was possible to determine directly concentrations in 100-μl samples that were of the order of 3–5 nM, or even as low as 0.5 nM under special conditions (for a fuller description, see ref. 11).

Radiochemical purity. The purity of the toxin used was routinely determined (see also RESULTS) by the method described elsewhere (11). Briefly, it was estimated by bioassaying the amount of toxin removed from a bathing solution either by rabbit vagus or by lobster walking leg nerves and comparing it with the amount of radioactivity removed. The apparent specific activity, s_{app}, at the beginning of the series of experiments was 90 dpm/fmole – the biological activity being determined by comparing the concentration of labeled toxin against a standard toxin (standard shellfish poison, serial No. 573, supplied by the Food and Drug Administration). The value of s_{app} was corrected for decay of tritium, falling to about 85 dpm/fmole by the end of the series of

experiments. The radioactivity associated with the toxin proper was determined by equilibrating a small volume (about 1.5 ml, 10–20 nM) with 8–10 desheathed rabbit vagus nerves (about 250 mg). After allowance for activity in the extracellular space, the amounts of radioactivity and of biological activity taken up by the nerves were determined, giving a true specific activity, s_t, of about 66 dpm/fmole. Nine determinations of the purity (s_t/s_{app}) made at the beginning of the series of experiments reported here gave a value of 0.738 ± 0.021. Similar calculations with lobster nerve carried out in the middle of the series (5 months later) and near the end of the series (10 months later) gave purity values of 0.646 ± 0.025 (n = 8) and 0.661 ± 0.011 (n = 8), respectively. The falloff in purity with time probably reflects back-exchange of tritium from toxin during storage.

In all cases the experiment was designed to leave as small as possible a final concentration of toxin in the bath (about 5 nM) to minimize the effect of linear uptake on the calculation of purity. The values of purity given above are based on the assumption that all the radioactivity bound is associated with toxin. However, even in the unlikely event (see RESULTS) that none of the linearly bound radioactivity was associated with toxin, the purity values would be overestimated by only 3–6% because of the relative smallness of the linear component of uptake.

RESULTS

Linear Component

Experiments on the linear component required a tissue in which the linear component was large enough to study, and which was available in large enough amounts so that a binding curve could be determined using aliquots of a single, uniform preparation. Homogenized rabbit brain met these conditions.

Figure 1 shows the uptake of labeled saxitoxin by a homogenized rabbit brain preparation at different external concen-

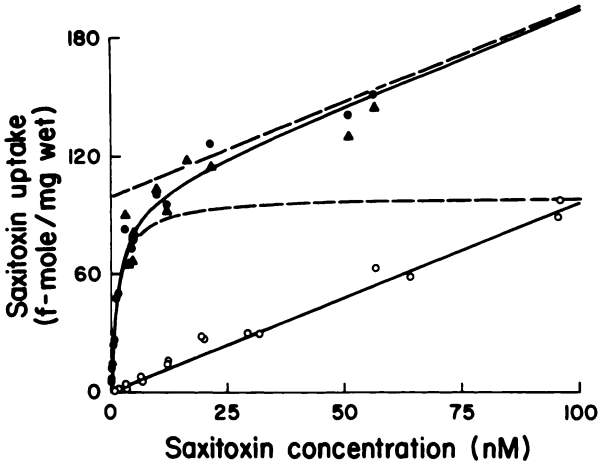

FIG. 1. *Uptake of labeled saxitoxin by homogenized rabbit brain preparation at different external concentrations of toxin determined both by bioassay and by radioactivity measurement*

△, results obtained in bioassay experiments; ○ and ●, in radioactivity experiments. The values for the uptakes of radioactivity were converted to amounts of toxin on the basis of an assumed specific activity of 66 dpm/fmole of saxitoxin. - - -, asymptote of the binding curve. The total binding curve is the relation

$$U = \text{STX bound} = 0.95\,[\text{STX}] + \frac{99.3\,[\text{STX}]}{1.5 + [\text{STX}]}$$

where U is given in femtomoles per milligram, wet weight, and [STX] is the nanomolar concentration of saxitoxin. The linear and saturable components are drawn separately. The binding curves shown are least-squares fits to the points in the presence of varied concentrations of labeled saxitoxin (●) and in the presence of varied concentrations of labeled saxitoxin and 10 μM unlabeled tetrodotoxin (○).

trations of saxitoxin. The amounts of toxin bound were determined from the loss of radioactivity from the bathing medium on the basis of a true specific activity of the toxin of 66 dpm/fmole (see METHODS). The total binding curve (solid circles) clearly consists of the sum of a saturable component of binding and a linear component. The points are quite well fitted by the relation

$$\text{Uptake} = b[\text{STX}] + \frac{M[\text{STX}]}{K + [STX]}$$

where [STX] is the saxitoxin concentration, b is a constant, M is the maximum saturable binding capacity, and K is the equilibrium dissociation constant. The values for the parameters (±standard errors) determined by the *Patternsearch* procedure (see ref. 7) were: $b = 0.95 \pm 0.04$ fmol/mg (wet)/nM, $M = 99.3 \pm 3.1$ fmoles/mg (wet), and $K = 1.5 \pm 0.2$ nM. In the presence of a high concentration of unlabeled tetrodotoxin (open circles), the saturable component of uptake is virtually completely inhibited and only the linear component remains. The difference between the two curves (the interrupted line) indicates the saturable component of binding of radioactivity.

The uptake of biological activity was also calculated more directly by bioassay of the initial and final concentrations of toxin in the bathing medium (triangles). The experimental conditions were chosen (small volume, large amount of tissue) so that at all concentrations about half or more of the toxin was removed from the bathing medium by the homogenate (Table 1). If the linear uptake component represents uptake only of impurity and not of true toxin, the triangles ought clearly to fall on, or near, the interrupted line (the saturable component of binding). However, in contrast to the findings in experiments on the uptake of labeled tetrodotoxin by muscle (5), it is clear that the values for uptake of biological activity (triangles) in the present experiments follow the *total* uptake curve much more closely than they do the curve for saturable uptake alone. Nearly all the uptake of radioactivity, therefore, seems to be associated with uptake of toxin.

This conclusion receives additional support from the data in Table 1. If the disappearance of radioactivity is all toxin-associated, estimations of s_t obtained by dividing the radioactivity uptake by the uptake of biological activity would be independent of the concentration in the bathing medium. On the other hand, if only the saturable component of radioactivity were associated with toxin, the estimates of s_t should systematically increase

TABLE 1

Estimation of radiochemical purity of labeled saxitoxin

Final [STX][a]	A. STX added	B. STX uptake	C. Radioactivity added	D. Radioactivity removed	Purity[b]
nM	*pmoles*	*pmoles*	*dpm*	*dpm*	
0.01[c]	21.4	21.4	1.93×10^6	1.29×10^6	0.668
1.31[c]	133.8	130.4	12.0×10^6	8.90×10^6	0.761
1.37[c]	40.1	36.2	3.61×10^6	2.23×10^6	0.684
3.90[c]	40.1	28.8	3.61×10^6	1.76×10^6	0.679
5.84[c]	133.8	118.3	12.0×10^6	8.72×10^6	0.822
8.75	66.9	41.9	6.02×10^6	2.74×10^6	0.727
10.8	267.6	239.9	24.1×10^6	16.1×10^6	0.745
11.7	66.9	32.9	6.02×10^6	2.29×10^6	0.774
24.1	267.6	203.6	24.1×10^6	15.1×10^6	0.824
52.6	401.4	264.6	36.1×10^6	19.3×10^6	0.811
54.6	401.4	256.7	36.1×10^6	18.0×10^6	0.780
					0.752 ± 0.018

[a] STX, saxitoxin.

[b] Calculated as columns (A × D)/(B × C).

[c] Determined by adding 6–9 nM extra saxitoxin before bioassay (11).

with increasing concentration. In practice, as Table 1 shows, the estimated value of s_t is relatively constant ($p < 0.05$), being 0.752 ± 0.018, which is very close to the value of 0.738 independently determined as described under METHODS at about the same time. Hence it appears that the linear uptake of radioactivity by homogenized rabbit brain, at least in experiments on saxitoxin labeled by the tritium exchange method, indeed represents linear, nonspecific uptake of toxin rather than of impurity.

Purity of Toxin

The argument used in interpreting Fig. 1 is that the bioassayed uptake points (triangles) fit the curve for uptake that is based on the *total* radioactivity and on a value for purity determined as in METHODS better than they do that for saturable radioactivity alone. One drawback in such a calculation of purity is that the procedure necessarily involves relatively small amounts of fluid that can be recovered for bioassay; furthermore, the final concentration is necessarily small (to avoid any large contribution from linear binding) and somewhat variable, depending on the amount of tissue used and its binding parameters. It seemed worthwhile, therefore, to design a method of assessing purity in which a single bioassay of potency could be done under optimal experimental conditions so that the purity could subsequently be estimated solely from radioactivity measurements. This technique provided additional, independent evidence that the linear component involved uptake of true toxin.

The procedure adopted was first to determine the biological activity of the labeled preparation in a separate series of experiments by calibrating it against a standard preparation by bioassay (11). The rest of the experiment, which involved only determinations of radioactivity, started with volumes of labeled and unlabeled saxitoxin at the same concentration. The same amount of tissue (per unit volume) was added to both labeled and unlabeled solutions, and the systems were shaken until equilibration occurred. Because the conditions were identical, the same amount of toxin was necessarily removed from the same unit volume of each solution, and the final concentration of toxin in the unlabeled and labeled equilibrated supernatants must have been the same. The principle of the method is that if the supernatant (obtained by centrifugation) in equilibrium with the unlabeled toxin is added to the equilibrated mixture of labeled toxin and tissue, no change in the concentratiom of toxin in the aqueous phase occurs, so that there can be no change in the amount of toxin bound to the preparation. However, the radioactivity bound to the preparation must change, since the specific activity has been changed by the addition of the unlabeled toxin to the system.

Suppose that a given amount of labeled toxin is equilibrated in an initial volume V: of the total amount originally added to the preparation, an amount b_o will be bound and an amount f_o will be free in the supernatant. The radioactivity in the tissue, U_o, will be $b_o \cdot s_o$, where s_o is the initial specific activity (equal to s_t). On addition of a volume v of the unlabeled supernatant, the amount b_o remains constant, but the specific activity falls, so that the tissue radioactivity U_v also falls. It is easy to show that the ratio U_o/Q_v is given by

$$\frac{U_o}{U_v} = 1 + \frac{v}{V} \cdot \frac{f_o}{f_o + b_o}$$

If U_o/U_v is then plotted against v/V, a straight line is obtained with a slope that gives the unbound toxin as a fraction of the total toxin initially added. The fraction bound, and hence the total amount bound, can thus be determined by subtraction and related to the radioactivity bound.

The experiment was conducted by equilibrating 200 ml of homogenized rabbit brain (diluted to contain 50–100 mg of brain, wet weight, per milliliter) with 50 ml of 200 nM unlabeled saxitoxin. At the same time, 0.5-ml aliquots of 200 nM labeled saxitoxin were equilibrated with 2 ml of the same homogenized rabbit brain preparation in each of the 18 centrifuge tubes. The contents of 16 of the tubes were

then diluted with various volumes (0.5–4.0 ml) of unlabeled supernatant, obtained by centrifuging the equilibrated unlabeled preparation at 40,000 × g for 30 min. Figure 2 (solid circles) shows the plot of U_o/U_v against v/V. The slope of the line, determined by a linear least-squares fit, is 0.53. The amount of toxin bound is thus (1 − 0.53) times the initial amount added, which was 100 pmoles. The bound radioactivity, U_o, was determined to be 2.55 × 10^6 dpm, so that s_t is 54.2 dpm/fmole. With the appropriate value of s_{app} of 86 dpm/fmole, the value for purity, p, becomes 0.63. The experiment of Fig. 2 was done near the end of the series of experiments, and so the value of purity derived from it agrees quite well with the value of 0.65–0.66 determined by other means as described under METHODS.

Linear component. Is the linear uptake of radioactivity associated with toxin or impurity? The calculation above assumes that all the radioactivity taken up by the tissue is associated with saxitoxin. If this assumption were false, and some of the radioactivity were indeed associated with an impurity. the analysis would still be valid, provided that any radioactivity associated with the impurity were first subtracted from the total uptake. This can be done by adding a large amount of unlabeled tetrodotoxin (but no extra volume) to the equilibrating solutions (after the addition of each volume v). All the saxitoxin-associated radioactivity would be displaced from the pellet, leaving behind that associated with the impurity, which could then be used as a correction in the calculation. A separate series of experiments was performed in which enough tetrodotoxin was added to the original 0.5-ml volumes of labeled saxitoxin to bring the final unlabeled tetrodotoxin concentration to 10 μM. Figure 2 (open circles) shows the plot of $(U_o - I_o)/(U_v - I_v)$ at the different fractional dilutions, where I_o and I_v are the tetrodotoxin-insensitive uptakes of radioactivity initially and after addition of volume v, respectively. The slope of this line (0.48) indicates that the bound fraction was 0.52. Since the radioactivity displaceable by the tetrodotoxin (before addition of any unlabeled supernatant) was found to be 1.67 × 10^6 dpm, the apparent value for s_t was estimated as 32.1 dpm/fmole. The value for the purity derived from this, 32.1/86.0, i.e., 0.37, is substantially lower than that determined directly as in METHODS. The premise that the linear component is impurity-associated thus seems to be false.

Temperature Dependence of Loss of Tritium from Labeled Toxin

The method of labeling of the toxin used in the present and previous experiments (11–13) relies on the fact that at a rela-

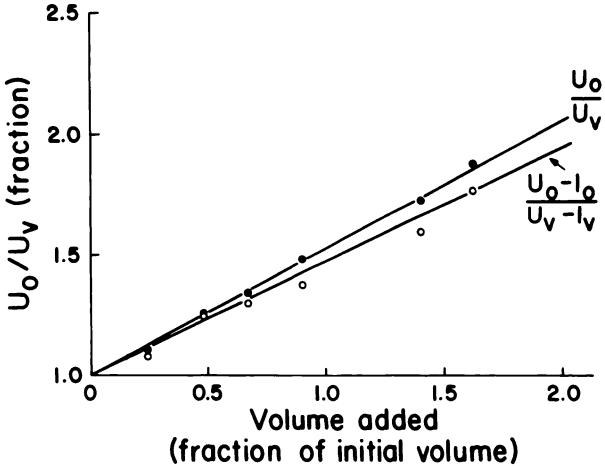

FIG. 2. *Determination of purity of labeled saxitoxin from measurements of radioactivity uptake by homogenized rabbit brain*

For a full description, see the text.

tively high temperature (50°) tritium readily exchanges with protons attached to carbon 12 of the saxitoxin molecule (14), whereas at low temperatures loss of the tritium from this position is extremely slow.

The precise temperature dependence of the loss of tritium from position 12 was studied in the present experiments by exposing samples of the toxin for various times at different temperatures (from 3–12 hr at 50° to 40–120 hr at 22–24°). Small volumes (usually 3 ml) of brain homogenate (each equivalent to about 100–200 mg of brain, wet weight) were then exposed at 2–4° to the same concentration of saxitoxin (final concentration, about 20 nM) made up from the various temperature-treated samples. Saxitoxin is known to be extremely heat-stable (15). It was assumed, therefore, that any decrease in radioactivity taken up by the tissue must reflect a decrease in the specific activity of the toxin rather than a decrease in total toxin bound.

In all the tests at any given temperature the uptake of radioactivity by the pellet was found to fall exponentially with time of exposure to the increased temperature (Fig. 3A). The higher the temperature, the greater was the rate of loss of radioactivity. At 50° the time constant of decay was about 5 hr; at room temperature (22–23.5°) it was about 1 week. In an Arrhenius plot (Fig. 3B) the natural logarithm of the rate constant (k_t) was found to be a linear function of the reciprocal of absolute temperature (T). The slope of the line indicated an activation energy of about 24 kcal/mole. Extrapolation of this Arrhenius plot to temperatures lower than those studied indicated that the time constant of decay at 0° was about 6 months, while the time constant of decay at −70°, the usual storage temperature of our toxin, would be about 2 million years (although the phase change in the water on freezing almost certainly invalidates this latter calculation).

Linear component. Prior exposure of the saxitoxin to high temperatures not only reduced the amount of radioactivity that was bound saturably (i.e., the tetrodotoxin-sensitive component) but also reduced the uptake of linear, tetrodotoxin-insensitive radioactivity. This therefore excludes the possibility that the linear uptake component represents mainly radioactivity in a heat-stable impurity. The fractional decrease in tetrodotoxin-insensitive uptake fell linearly with the fractional decrease in saturable uptake, as might be expected if the linear component of radioactive uptake reflected uptake of toxin. The line did not, however, pass through the origin. Rather, extrapolation showed that 0.38 of the initial linear radioactivity remained when the saturable radioactivity would have been zero. A likely explanation is that the decrease in the

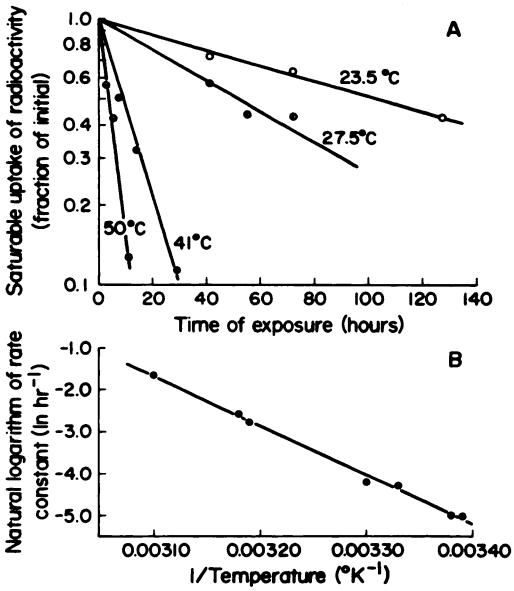

FIG. 3. *Temperature dependence of exchange labeling of saxitoxin*

A. Decay of the specific activity of tritium-labeled saxitoxin with time of exposure to different temperatures. The exposure temperature is noted beside each curve.

B. Arrhenius plot (of two experiments, that in Fig. 3A and another) showing temperature dependence of the loss of tritium from exchange-labeled saxitoxin. The natural logarithm of the rate constant of loss (k_T) of tritium is plotted against the reciprocal of absolute temperature (T). The line, drawn by eye, is the equation

$$\ln k_T = 1.20 \times 10^4 \left(\frac{1}{T}\right) + 36.02$$

specific activity of labeled toxin during heat treatment inevitably entails an increase in the concentration of tritiated water. Furthermore, tritiated water is probably present in the original labeled toxin. If this tritiated water equilibrates with the water in any cells left intact after the homogenization procedure, or in any intravesicular compartment, because of penetration of the cell membrane by water or by tritium, it would not be allowed for in the extracellular space correction. The result would be an apparent tetrodotoxin-insensitive, heat-stable, linear component of uptake. Direct support for this suggestion that tritiated water has access to some space denied to [^{14}C]mannitol was obtained in double-labeling experiments in which the tritiated water and [^{14}C]mannitol spaces were determined simultaneously. In 12 such tests, the tritiated water space was always greater, being 1.196 ± 0.003 times that of the [^{14}C]mannitol space.

In the experiment of Fig. 3, extrapolation showed that at the time when all the label would have exchanged from the toxin into water the tissue would still have an amount of radioactivity in it that was 0.38 of the total linear uptake, seemingly associated with tritiated water. Under normal experimental conditions, however, the contribution of this component would be relatively small. For example, in the experiments on homogenized rabbit brain, in which the radiochemical purity was 0.65–0.74, the contribution of radioactivity from this compartmentalized tritiated water would represent a fairly small fraction of the total linear uptake of radioactivity (being at most 0.26–0.35 × 0.38, i.e., 0.10–0.13). Nor would it be expected to contribute largely to the linear component in *intact* preparations. In garfish olfactory nerve, where the purity of the toxin used was about 0.74 (11), the intracellular fiber water is about 0.5 μl/mg (wet) (Table 1) (11). Therefore, even with complete equilibration of the water in the extracellular and intracellular spaces, there would be an apparent linear uptake of toxin of only 0.13 fmole/mg (wet)/nM, which is less than a quarter of the observed apparent linear uptake of 0.57 fmole/mg (wet)/nM (11). The bulk of the linear uptake thus seems to reflect true uptake of toxin.

Measurements of purity as in Fig. 2, which were made at about the same time as the temperature experiments just described that suggested that 0.38 of the linear uptake of radioactivity by rabbit brain homogenate was in tritiated water, indicated that at that time about 0.36 of the radioactivity in the preparation of labeled toxin was not in saxitoxin (and was presumably in tritiated water). Under the conditions of Fig. 1, therefore, where the impurity was only 0.26, a smaller fraction 0.10 of the linear component of radioactive uptake would be expected to be associated with tritiated water. Indeed, only 0.90 of the linear uptake would be toxin-associated, so that the points for bioassayed toxin uptake (triangles) would be expected to fit a curve whose asymptote would intercept the *y* axis at the same point as in Fig. 1 but whose slope would be less. Furthermore, the purity determined when this factor is not taken into account (Table 1) would be too high. Both expectations are fulfilled (Fig. 1 and Table 1).

Effect of pH. Some labeled toxin was incubated at 40° for 4–12 hr, but at pH 4. The specific activity of this toxin also fell exponentially with time of exposure to the high temperature. But the rate constant of decay was only about $^{6}/_{10}$ the corresponding rate constant at pH 7.2, suggesting that acid solutions slow the exchange reaction.

Nature of Linear Uptake Component

The experiments on the temperature dependence of the specific activity of exchange-labeled toxin suggest that any impurity present (other than tritiated water) has a temperature stability of labeling that is the same as that of labeled saxitoxin. Indeed, the experiments of Figs. 1 and 2 suggest that the main contribution of uptake of radioactivity to the linear component is associated with true toxin. A likely explanation for the component of linear uptake of toxin is that it represents the increased concentration of toxin in the Debye-Hückel layer in the immediate vi-

cinity of the membrane resulting from the presence of fixed negative charges on it. Many nerve membranes contain fixed charges, whose magnitude is of the order of 10^6–$10^7/\mu m^2$ (see ref. 16). To maintain electrical neutrality, therefore, there must be an excess of cations in the fluid adjacent to the negatively charged membrane, divalent cations being preferred over monovalent cations. This excess—for a compound present in very small amounts—would be proportional to the concentration of the drug, and there would be a linear component of uptake as a result.

The idea that the fixed charges on the membrane are responsible for the linear component of uptake can be tested qualitatively. If the monovalent sodium chloride (154 mM) of the Locke's solution is replaced by an isosmotic amount of divalent calcium chloride (117 mM), the amount of saxitoxin attracted to, and held by, the fixed charges should be reduced. On the other hand, if the sodium chloride is replaced by a nonionic molecule such as sucrose (270 mM), the amount of saxitoxin so held should be much increased. Figure 4 shows that this is indeed the case. Thus, in normal Locke's, sucrose-Locke's, and calcium-Locke's solution, there is little change in the maximum binding capacity. The linear uptake coefficient, however, more than doubles in sucrose-Locke's solution and falls to about one-third in calcium-Locke's solution.

In calcium-Locke's solution the saxitoxin and calcium ions, being both divalent, would contribute roughly equally to the screening effect of the fixed surface charges. The membrane area in the homogenized brain preparation is not known. However, it is known for garfish olfactory nerve, where the nonspecific binding capacity is about 0.6 fmole/mg (wet)/nM (11). Assuming that this too is reduced to one-third in calcium-Locke's solution, one calculates that the amount linearly bound in calcium-Locke's solution at an external saxitoxin concentration of 1 nM would be 0.2 fmole/mg (wet). With an axonal area of 65 cm^2/mg (wet) (17), and an equal amount of Schwann cell membrane bounding the periaxonal space, there would thus be 1 positive charge/50 μm^2 of membrane associated with this saxitoxin. But for a 1 nM saxitoxin concentration (and a calcium concentration of 117 mM) this would amount to only a fraction (10^{-9}/0.117) of the total extra cationic charge associated with the fixed negative charges. This total would thus amount to about 1 charge/50 A^2, which is very close to the values reported by Schauf and others for nerve (see ref. 16). The charge density would certainly be lower than 1/50 A^2, because no allowance has been made for other, nonaxonal, membranes such as that of the nonperiaxonal surface of the Schwann cells and of connective tissue. Nor has any allowance been made for the component of linear uptake of radioactivity that is associated with intracellular tritiated water.

Equilibrium Dissociation Constant

The experiments of Fig. 4 were designed mainly to reveal effects on the linear component of binding. However, the affinity of the toxin for the binding site was also determined, although less accurately, and found to be affected in a way consistent with the above explanation. The presence of fixed negative charges on the membrane would increase the local concentration of the toxin near the binding site relative to that in the bulk of the solution; screening of these charges, by reducing the field associated with them, would reduce this local concentration. The hypothesis would thus require that the value of K decrease in sucrose-Locke's solution and increase in calcium-Locke's solution. Figure 4 shows that experimentally this is indeed the case: the value of K is more than halved in sucrose-Locke's and increased 70% in calcium-Locke's solution.

DISCUSSION

Consideration of fixed charges on the nerve membrane, and of their screening by ions in the bathing medium, leads to the theoretical expectation of apparent linear binding of any cationic drug, particularly a divalent compound such as saxitoxin. This theoretical expectation is indeed confirmed by the present experi-

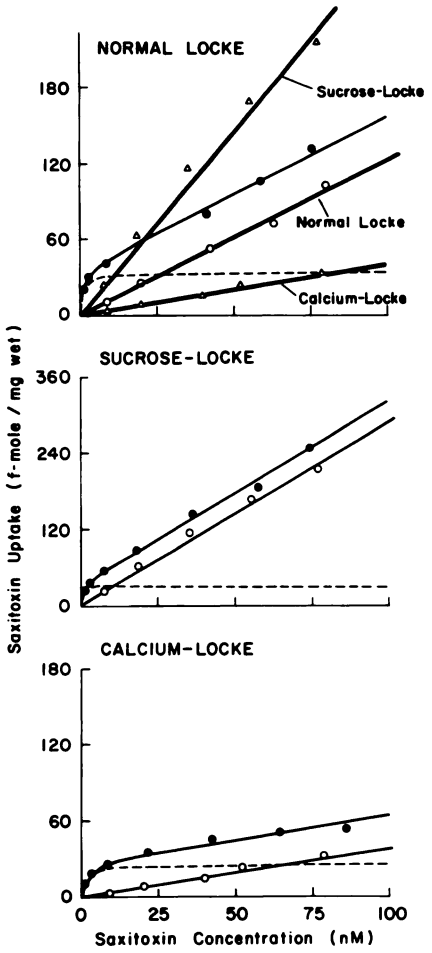

FIG. 4. *Effect of ionic composition of bathing medium on uptake of labeled saxitoxin by homogenized rabbit brain at different external concentrations of toxin*

The preparations (a total of 12 for each curve) were equilibrated for about 1 hr at 2–4° with the saxitoxin, and a pellet was obtained by centrifugation. The binding curves shown are least-squares fits to the points: ●, in the presence of varied concentrations of labeled saxitoxin; ○, in the presence of varied concentrations of labeled saxitoxin and 10 μM unlabeled tetrodotoxin. The total binding curves (●) are the following relations. For normal Locke's:

$$U = \text{STX bound} = 1.2\,[\text{STX}] + \frac{33.8\,[\text{STX}]}{1.0 + [\text{STX}]}$$

For sucrose-Locke's:

$$U = \text{STX bound} = 2.9\,[\text{STX}] + \frac{31.9\,[\text{STX}]}{0.4 + [\text{STX}]}$$

For calcium-Locke's:

$$U = \text{STX bound} = 0.39\,[\text{STX}] + \frac{26.4\,[\text{STX}]}{1.7 + [\text{STX}]}$$

where U is given in femtomoles per milligram, wet weight, and [STX] is the nanomolar concentration of saxitoxin. The linear and saturable components are drawn separately. In each panel the interrupted line indicates the saturable component of binding. The thick lines in the top panel show the curves for the linear component in each solution, plotted on the same scale for comparison. (Note that the scale for the experiment in sucrose-Locke's solution is different.)

ments on the uptake of tritium-labeled saxitoxin by rabbit brain. Calculation of the purity of such preparations gives results that are consistent only on the supposition that the bulk of linear uptake in this system is of true toxin. Indeed, a linear component of uptake of labeled markers for membrane proteins is found in many systems (see ref. 18 for review). Therefore it is surprising that in bioassay experiments on muscle neither Almers and Levinson (5) nor Jaimovich *et al.* (6) found a linear component of uptake of tetrodotoxin.

This conclusion does not, of course, exclude the possibility that in other systems linear uptake of impurity also occurs. For example, the linear component of binding with tetrodotoxin, being monovalent, should be much less than that of saxitoxin. In practice, however, it is of the same order, or even greater (7, 19). It seems likely, therefore, as Almers and Levinson (5) had already suggested, that with tetrodotoxin a substantial amount of uptake is associated with a radioactive impurity, which is perhaps not surprising in view of the drastic nature of the Wilzbach process of labeling (see ref. 11).

The presence of radiochemical impurities in labeled membrane markers that otherwise seem to be pure by standard biochemical tests is probably not unique to saxitoxin and tetrodotoxin. The new method described, based on dilution of labeled toxin with unlabeled toxin, requires that the labeled and unlabeled marker concentrations be matched only once and under optimal conditions. This can be done at very high concentrations,

perhaps chemically if tests are available (for instance, see ref. 20 for a fluorometric assay of tetrodotoxin). The method described is therefore generally applicable.

The measurement of the temperature dependence of the rate constant of loss of label from saxitoxin confirms that the toxin can be stored safely for long periods of time at freezer temperatures of −70°. It also accounts for the efficiency of the labeling process, which is carried out at 50°. At 50° the time constant of loss of tritium from the isotope is about 5 hr. However, labeling is even faster than this time constant suggests. Because of kinetic isotope effects (e.g., ref. 21), the rate of loss of hydrogen from carbon 12 in saxitoxin, which would be expected to be the rate-limiting step in the labeling process, is considerably greater than the rate of loss of tritium measured in the present experiments. For example, at 25° the rate of loss of hydrogen molecules is 16 times faster than the rate of loss of tritium (Table 3-1 of ref. 21). The insertion of tritium into the molecule would thus be expected to proceed with a time constant that is a good deal smaller than the 3 hr allowed in the exposure.

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