# Affinity Labeling of One of Two α-Neurotoxin Binding Sites in Acetylcholine Receptor from *Torpedo californica*†

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ABSTRACT: Various theoretical models are proposed to account for the observed fact that the nicotinic acetylcholine receptors isolated from the electric tissues of *Electrophorus* and *Torpedo* bind twice as much α-neurotoxin (toxin) as the affinity label, 4-(*N*-maleimido)benzyltrimethylammonium iodide (MBTA). When the degree of MBTA labeling is considered as a function of the degree of toxin occupancy for the receptor that is partially bound with toxin, however, different curves are predicted by different models for various possible kinetics of toxin binding to receptor. For the *Torpedo* receptor, both in solution and in membrane fractions, it was found experimentally that the extent of [$^3$H]MBTA labeling decreases linearly as the degree of toxin occupancy increases. Taken together with the known kinetics of binding of toxin to *Torpedo* receptor, these data are shown to be consistent only with the model that assumes that the two toxin binding sites on the receptor are not identical; i.e., only one of them possesses the structure necessary for the reaction with MBTA at an enhanced rate, and this labeling by MBTA is blocked by toxin only when toxin is bound to the receptor at the labeling site. Furthermore, it is shown that the data are consistent only with a model in which the two sites are initially kinetically identical with respect to toxin binding. There may, however, be negative cooperativity in the binding of toxin to the two sites.

Acetylcholine receptors, which transduce the binding of the neurotransmitter, acetylcholine, into ion permeability changes of the postsynaptic membrane, have been isolated and purified from the electric tissue of the electric eel, *Electrophorus electricus,* and the marine rays, *Torpedo* species (reviewed in Karlin, 1977a; Changeux, 1975), and recently from rat muscle (Froehner et al., 1977b). In general, three different types of reactions have been used to assay the binding sites of these receptors, namely, rapidly reversible binding of acetylcholine and congeners, very slowly reversible binding of α-neurotoxins, and covalent reaction with affinity labels. In the present work, we examine the interaction of the latter two of these reactions.

An affinity alkylating agent, 4-(*N*-maleimido)benzyltri[$^3$H]methylammonium iodide ([$^3$H]MBTA) has been shown to alkylate specifically a sulfhydryl group near an acetylcholine (and toxin) binding site of the dithiothreitol-reduced receptor. [$^3$H]MBTA covalently labels a single subunit in the receptor from both *E. electricus* and *T. californica* (Reiter et al., 1972; Karlin & Cowburn, 1973; Weill et al., 1974; Karlin et al., 1975). This labeling is completely blocked by prior saturation of the receptors with α-neurotoxin.

Both forms of the receptor from *T. californica,* which is a mixture of monomer of molecular weight 250 000 and of dimer (Reynolds & Karlin, 1978), and the single form of receptor from *E. electricus,* bind one α-neurotoxin molecule per 125 000 daltons of protein. These receptors are labeled, however, to the extent of one MBTA per 250 000 daltons (McNamee et al., 1975; Karlin et al., 1975). Thus, there are two α-neurotoxin sites per receptor monomer, but only one site per monomer is labeled by MBTA. There are two possible explanations for this. Either there is only one site per monomer susceptible to reduction by dithiothreitol and affinity labeling by MBTA, or there are two such sites; the reaction at one of the sites, however, renders the second unreactive.

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We shall first describe models incorporating the two possibilities for MBTA labeling and show that these models predict different relationships between the degree of occupation of receptor sites by α-neurotoxin and the extent of subsequent labeling by MBTA. In addition, we shall show that these relationships depend on the kinetics of α-neurotoxin binding. Experimental determination of the dependence of MBTA labeling on the degree of occupancy by α-neurotoxin permits us to choose between the alternative explanations of MBTA labeling and to draw conclusions about the kinetics of α-neurotoxin binding. Similar models have been considered in connection with the half-of-the-sites reactivity shown by certain oligomeric enzymes (Bernhard & MacQuarrie, 1973).

## Theory

Receptor has two distinct sites for the binding of toxin per 250 000 daltons; i.e., two sites per receptor monomer. We call these sites arbitrarily A and B (Figure 1). It is assumed that each receptor monomer is an independent unit even when part of a receptor dimer; i.e., the sites on different receptor monomers are independent of one another. There may, however, be interactions between the two sites on the same monomer.

We consider the occupation of the sites, A and B, by toxin. Four different species or states of occupation are possible (Figure 1). If $y$ is the degree of occupancy of all sites, then

$$y = \frac{n_{1A} + n_{1B} + 2n_2}{2} \quad (1)$$

where the $n$'s are the relative concentrations or mole fractions of the various species (Figure 1).

We now consider the reaction of MBTA with receptor in the four states of toxin occupation, under conditions ensuring complete reaction with available sites. We consider three models (Figure 1) of availability of sites for reaction with MBTA consonant with the observation that MBTA, in the absence of toxin, labels one-half of the number of sites that bind toxin.

*Model I.* MBTA labels only one of the two toxin sites, say site A only, regardless of whether site B is occupied by toxin

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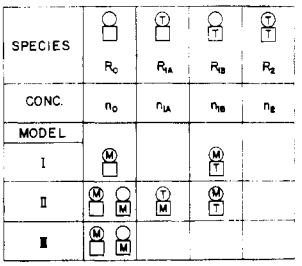


FIGURE 1: Upper part depicts schematically the four species that may exist in a mixture of receptor and less than saturating amount of toxin. (O) Site A; (□) site B. T denotes that the site is occupied by toxin; $R_0$, receptor with no toxin bound; $R_{1A}$, toxin bound at site A; $R_{1B}$, toxin bound at site B; $R_2$, toxin bound at both sites A and B. In the lower part are shown schematically the models of MBTA labeling and its blocking by toxin. Only the species which may be labeled by MBTA are shown for each model. M denotes the site that may be labeled by MBTA. In models II and III, the two sites designated as O and □ are actually indistinguishable.

or not. MBTA labeling is blocked by toxin only when it is bound at site A. The species that will be labeled by MBTA are $R_0$ and $R_{1B}$.

*Model II.* MBTA labels either of the two sites, which are equivalent, but only one per receptor monomer; further, if both are vacant either one will be labeled, if one is occupied by toxin the other will be labeled. The species that will be labeled are $R_0$, $R_{1A}$, and $R_{1B}$.

*Model III.* MBTA labels only receptor with no bound toxin. Just one toxin molecule bound at either site completely blocks MBTA labeling. For this model it makes no difference whether MBTA actually labels a particular one of the two toxin sites or either of them, as long as it labels only one site per receptor monomer, because the only species that will be labeled is $R_0$.

The number of sites available for MBTA labeling depends on the model. If $m$ is the fraction of the total toxin sites that can be labeled by MBTA, we have for the three models:

$$\text{I:} \quad m = \frac{n_0 + n_{1B}}{2} \tag{2}$$

$$\text{II:} \quad m = \frac{n_0 + n_{1A} + n_{1B}}{2} \tag{3}$$

$$\text{III:} \quad m = \frac{n_0}{2} \tag{4}$$

Equations 1-4 define implicit relationships between $m$ and $y$ for these models. In general, it will be necessary to know the $n$'s to determine $m$ as a function of $y$. For all models, $m = 0.5$ when $y = 0$ and $m = 0$ when $y = 1$.

The values of the individual $n$'s for any value of $y$ depend on the kinetics of toxin binding. On the time scale of our experiments, the dissociation of the receptor-toxin complexes is negligible; hence, we need consider only the association rates. We consider three cases: (i) there is one rate constant; (ii) the sites bind toxin independently with different rate constants; (iii) the sites bind toxin with either positive or negative cooperativity.

The first case (i) is treated most simply by a probability argument. The degree of saturation, $y$, is also the probability that any site will be occupied by toxin. The probabilities, and hence the relative concentrations, of each of the toxin occupation states are given in Table I. Combining these relative concentrations and eq 2-4, we obtained $m$ as a function of $y$ for the three models (Table I). For model I, $m$ is a linear function of $y$ and, for II and III, $m$ is a parabolic function of $y$ (Figure 2a).

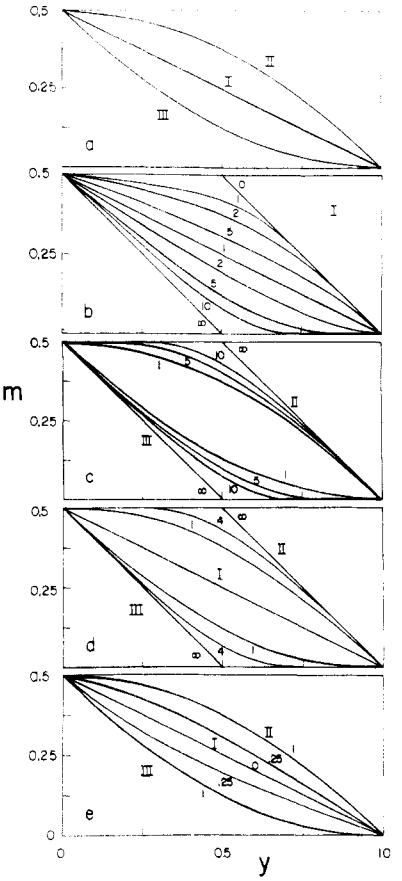


FIGURE 2: Theoretical curves for the three models showing $m$, the degree of MBTA labeling, as a function of $y$, the degree of toxin occupancy, for various cases of toxin binding kinetics. (a) Toxin binds to receptor consisting of a single homogeneous class of sites (case i). (b and c) Two classes of sites with two independent rate constants $k_A$ and $k_B$ (case ii). Curves are shown for various $k_A/k_B$; (b) model I; (c) models II and III. (d and e) Cooperativity in toxin binding (case iii). The rate constants $k_1$ and $k_2$ refer to first and second toxin molecules that bind to a receptor monomer. Curves are shown for various $k_1/k_2$: (d) negative cooperativity, $k_1 > k_2$; (e) positive cooperativity, $k_1 < k_2$.

Case ii of toxin binding kinetics may also be treated by a probability argument, taking $y_A$ and $y_B$ as the probabilities of occupation of sites A and B, respectively (Table I). Since $y = (y_A + y_B)/2$, whenever $y_A$ is not equal to $y_B$, $1 - y_A$ will not be equal to $1 - y$, and, hence, $m$ will no longer be a linear function of $y$ in model I. Moreover, $y^2$ is greater than $y_A y_B$, and $1 - y_A y_B$ will be greater than $1 - y^2$ and $(1 - y_A)(1 - y_B)$ will be less than $(1 - y)^2$, and hence $m$ as a function of $y$ will deviate even further from linearity in models II and III, case ii compared with case i (Figures 2b and 2c). The exact relationships between $m$ and $y$ in these cases are obtained by solving the kinetic equations governing the population of the various toxin occupation states (see Appendix).

In case iii of cooperativity in toxin binding, it is assumed that the sites are initially identical with respect to toxin binding. The first toxin molecule binds to either site of a receptor monomer with an association rate constant $k_1$. The second toxin molecule binds to the other site of the same receptor monomer with a different rate constant $k_2$.

We have negative, zero, or positive cooperativity depending upon whether $k_1$ is greater than, equal to or less than $k_2$. Curves for zero cooperativity are of course identical with those in Figure 2a.

Since the probability of occupancy of a specific site is not defined, we use the probabilities of singly and doubly occupied

TABLE I: Expressions for the Mole Fractions of the Four Species, $y$, and $m$'s for the Three Models (Figure 1), in Terms of Probabilities (of Site Occupancies or of Receptor–Toxin Complexes) for Various Cases of Toxin Binding Kinetics.[a]

| | (i) Single class of sites $k_{1A} = k_{1B} = k_{2A} = k_{2B}$[b] | (ii) Two classes of sites $k_{1A} = k_{2A} = k_A$ $k_{1B} = k_{2B} = k_B$[b] | (iii) Cooperativity in binding $k_{1A} = k_{1B} = k_1$ $k_{2A} = k_{2B} = k_2$[b] |
|---|---|---|---|
| $n_0$ | $(1-y)^2$ | $(1-y_A)(1-y_B)$ | $1-2y_1-y_2$ |
| $n_{1A}$ | $y(1-y)$ | $y_A(1-y_B)$ | $y_1$ |
| $n_{1B}$ | $y(1-y)$ | $y_B(1-y_A)$ | $y_1$ |
| $n_2$ | $y^2$ | $y_A y_B$ | $y_2$ |
| $y$ | $y$ | $\frac{y_A+y_B}{2}$ | $y_1+y_2$ |
| $m$ (model I) | $\frac{1-y}{2}$ | $\frac{1-y_A}{2}$ | $\frac{1-y}{2}$ |
| $m$ (model II) | $\frac{1-y^2}{2} = \frac{1-y}{2}+\frac{y(1-y)}{2}$ | $\frac{1-y_Ay_B}{2}$ | $\frac{1-y}{2}+\frac{y_1}{2}$ |
| $m$ (model III) | $\frac{(1-y)^2}{2} = \frac{1-y}{2}-\frac{y(1-y)}{2}$ | $\frac{(1-y_A)(1-y_B)}{2}$ | $\frac{1-y}{2}-\frac{y_1}{2}$ |

[a] $y$ = fraction of total sites occupied by toxin. $y_A$ = fraction of A sites occupied = $n_{1A} + n_2$. $y_B$ = fraction of B sites occupied = $n_{1B} + n_2$. $y_1$ = fraction of total sites occupied by toxin in singly occupied receptor monomers = $(n_{1A} + n_{1B})/2$. $y_2$ = fraction of total sites occupied in doubly occupied monomers = $n_2$. [b] See Figure 5 for the explanation of rate constants.

receptor monomers to express the mole fractions and $m$'s for various models (Table I). Model I predicts a straight line for all $k_1$, $k_2$ (Figures 2d and 2e). This is obvious since toxin shows no preference toward a site, i.e., whether it is A or B, $y_A = y_B = y$ for all $y$'s.

For models II and III, $m$–$y$ curves must be computed as described in the Appendix. Deviations from the curves for zero cooperativity now depend upon whether $k_1 > k_2$, or $k_1 < k_2$ (Figures 2d and 2e). If $k_1 > k_2$, because of negative cooperative interactions, bound toxins would prefer to be on different receptor monomers. Hence, $n_{1A}$ and $n_{1B}$ would increase at the expense of $n_0$ and $n_2$. Thus, $y_1 > y(1-y)$, and hence $m$–$y$ curves become even more nonlinear (Figure 2d).

On the other hand, if $k_1 < k_2$, toxin molecules prefer to be on the same receptor monomer because of positive cooperative interactions. Thus, $R_{1A}$ and $R_{1B}$ tend to disappear, $y_1 < y(1-y)$, and $m$–$y$ curves for models II and III now approach the straight line predicted by model I (Figure 2e). In the case of positive cooperativity, it becomes impossible to distinguish between the three models for MBTA labeling. Fortunately, there is no evidence for positive cooperativity in the kinetics of toxin binding.

## Materials and Methods

Membrane fractions were prepared from *T. californica* electric tissue by a modification of the method for preparing "microsacs" (Hazelbauer & Changeux, 1974). Two hundred grams of either fresh or frozen (at −196 °C) tissue was thawed, minced with scissors, and homogenized in 2 vol of either 10 mM $CaCl_2$ (unbuffered) or 10 mM Mops,[1] 1 mM EDTA, 0.5 mM $PhCH_2SO_2F$, pH 7.4, at "high" speed for 2 min in a 1-gallon capacity Waring Blendor. The homogenate was filtered through a double layer of gauze and centrifuged at 6000 rpm for 20 min in a Beckman 19 rotor. The supernatant was again filtered through two layers of gauze, layered over 60 mL of 35% (w/v) sucrose in 10 mM Mops, 1 mM EDTA, 0.1 mM $PhCH_2SO_2F$, pH 7.4, and centrifuged at 19 000 rpm for 18–20 h. The pellet was resuspended in 10 mM Mops, 1 mM EDTA, 0.1 mM $PhCH_2SO_2F$, pH 7.4, and stored in liquid nitrogen until use. Typically, such suspensions contained 10–20% sucrose, 2–6 mg of protein per mL of suspension and 0.6 to 1.4 nmol of toxin binding sites per mg of protein.

Purified receptor from *T. californica* electric tissue was prepared essentially as described before (Karlin et al., 1976b) except that the affinity gel was derivatized with bromoacetylcholine. [$^3$H]MBTA was prepared as described before (Karlin, 1977b) and stored as acetonitrile solution under liquid nitrogen.

Crude, lyophilized venom from *Naja naja siamensis* was obtained from Miami Serpentarium. Its principal $\alpha$-neurotoxin was purified according to Karlsson et al. (1971) and stored at −20 °C as a concentrated solution (~6 mg/mL) in 50 mM sodium phosphate buffer (pH 7.0), 3 mM $NaN_3$. [$^3$H]-Methyl-$\alpha$-neurotoxin was prepared by reaction of the $\alpha$-neurotoxin with formaldehyde and $NaB^3H_4$ (Rice & Means, 1971; Biesecker, 1973; Ong & Brady, 1974; Karlin et al., 1976b; cf. Klett et al., 1973). The reactions were carried out in a well ventilated hood at 0 °C as follows: To 2 mg of $\alpha$-neurotoxin in 333 $\mu$L of 50 mM $NaPO_4$ (pH 7.0 at 25 °C) were added 500 $\mu$L of 0.2 M sodium borate (pH 9.0 at 25 °C) and 100 $\mu$L of 46 mM formaldehyde in the same borate buffer. After 90 s, 8.4 $\mu$mol of $NaB^3H_4$ (6 Ci per mmol, from Amersham/Searle Corp.) in 100 $\mu$L of dimethylformamide was added gradually over 1 min. The mixture was stirred for 30 min, 20 $\mu$L of 3.5 M acetic acid was added, and stirring was continued for 5 min at 0 °C and 40 min at room temperature to release tritium from unreacted $NaB^3H_4$. The reaction mixture was passed through a column (26 × 0.9 cm) of Bio-Gel P6 (Bio-Rad) in 90 mM ammonium acetate (pH 6.5) at 0.4 mL per min. The toxin emerged at the column void volume and was collected in a total volume of about 5 mL. This solution was lyophilized. The residue was dissolved in 1 mL of 50 mM $NaPO_4$ (pH 7.0), 3

[1] Abbreviations used: MBTA, 4-($N$-maleimido)benzyltrimethylammonium iodide; Mops, 2-($N$-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride.

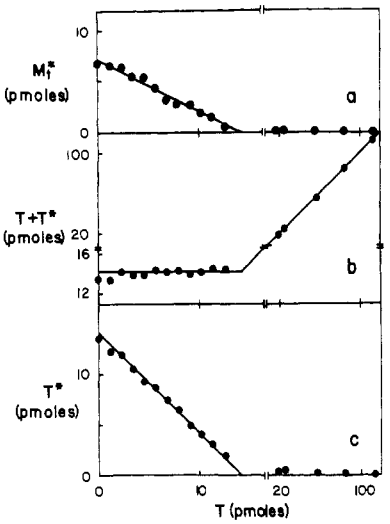


FIGURE 3: Experimental data on [$^3$H]MBTA and [$^3$H]methyl-toxin assays of purified *Torpedo* receptor–native toxin mixtures. All mixtures contained the same number of receptor sites, $R$, but varying amounts of added native toxin, $T$; and all data refer to the same total number of receptor sites. Each point is the mean of duplicate determinations. (a) $M^*_t$, total [$^3$H]MBTA labeling vs. $T$. (b) $T + T^*$ vs. $T$, where $T^*$ is the amount of [$^3$H]methyl-toxin bound. The horizontal line indicates the mean value of $R$. (c) $T^*$ vs. $T$. A straight line with a slope = −1 and both intercepts = $R$ is shown for comparison.

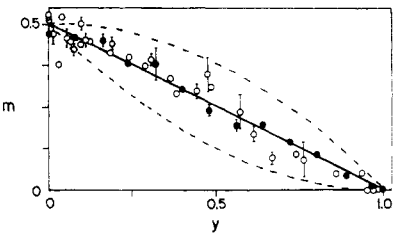


FIGURE 4: The degree of MBTA labeling, $m$, vs. the degree of toxin occupancy, $y$, for *Torpedo* receptor in solution: (●) purified receptor; (○) Triton X-100 solubilized membrane. Data are from four independent experiments; each point is the mean of duplicate determinations. The curves are theoretical predictions for the three models, for a single rate constant for binding of toxin (as in Figure 2a).

mM $NaN_3$ and passed through a column (56 × 0.9 cm) of Bio-Gel P30 in the same buffer at 0.2 mL per min. Single coinciding peaks of protein and of radioactivity were eluted at 1.5 void volumes. The concentration of α-neurotoxin in the pooled peak fractions was determined by absorbance at 279 nm and an extinction coefficient of 1.06 per mg per mL (Karlsson et al., 1971). About 1.6 mg of [$^3$H]methyl-α-neurotoxin was recovered in 8 mL. The specific activity was about 10 Ci per mmol. the labeled toxin was stored at −196 °C.

Since toxin has a tendency to stick to glass or plastic, all dilute solutions of toxin were freshly made from frozen stock. It was found that the presence of BSA (bovine serum albumin) at a concentration of 100 μg/mL prevents toxin from sticking to containers; hence all dilute solutions of toxin were prepared in corresponding buffers containing 100 μg/mL BSA (see also Colquhoun & Rang, 1976).

Affinity labeling assay of the receptor, both in solution and in membrane fractions, with [$^3$H]MBTA was performed as described previously (Karlin et al., 1976a,b; Karlin, 1977b).

For [$^3$H]methyl-toxin binding assay, the DE81 filter disc method of Klett et al. (1973) was modified as follows: 50 μL of a solution of receptor containing 0.2–3 pmol of toxin sites in buffer A (100 mM NaCl, 10 mM Mops, 0.2% Triton X-100, pH 7.4) was mixed with 50 μL of a solution containing about 7 pmol of [$^3$H]methyl toxin in the same buffer (A) and incubated for 15–20 min at room temperature. The incubation mixture was then diluted with 5 mL of buffer B (10 mM NaCl, 10 mM Mops, 0.2% Triton X-100, pH 7.4) and filtered under low vacuum (~50 mmHg) through two discs of DE81 filters (Whatman) which were previously washed with 4 mL of B buffer. The tube which contained the incubation mixture was washed twice with 5 mL of B buffer and washings were transferred to the filters. The filters were washed with 15 mL of B buffer and partially dried under vacuum. They were then transferred to a counting vial, 0.5 mL of 1 M HCl was added, and, after 10 min, 10 mL of Scintisol Complete (Isolab) was added. After 20 min, the $^3$H radioactivity was counted in a scintillation counter. To determine the nonspecific binding of excess free [$^3$H]methyl-toxin by the filters, a similar filtration was performed in which either the receptor was omitted from the incubation mixture, or a large excess of native toxin was added to the mixture. This background correction was always very low, about 1% of the total radioactivity present in the incubation mixture. To convert the observed radioactivity to pmol of [$^3$H]methyl-toxin bound, an aliquot of [$^3$H]methyl-toxin solution was spotted on washed and dried filters and counted under identical conditions to the assay samples.

The assay was found to be very reproducible and the recovery of the receptor–toxin complex by the filters was generally above 90%. It was linear at least up to 4 pmol of toxin sites in 100 μL of incubation mixture.

Experiments to study the degree of [$^3$H]MBTA labeling of *Torpedo* receptor as a function of the degree of occupancy by toxin were performed with membrane, membrane solubilized in Triton X-100 solution, and purified receptor in Triton solution. *Torpedo* membrane was either solubilized in TNP50 (0.2% Triton X-100, 50 mM NaCl, 10 mM $NaPO_4$, 1 mM EDTA, 3 mM $NaN_3$, pH 7.0) for studies in solution, or diluted in np50 (same solution as TNP50 but without Triton X-100) for studies in membrane. Purified receptor was diluted in TNP50.

Typically 0.1-mL diluted solutions (or membrane suspensions) containing 60–90 pmol of toxin binding sites were mixed with 0.2-mL solution of native toxin in TNP50 (or NP50 as the case may be) containing 100 μg/mL BSA. For each experiment a number of such mixtures were made and incubated for 30 min at room temperature to ensure complete reaction. (Under the conditions used, the association reaction is complete within a few minutes.) These mixtures contained the same number of toxin sites but different amounts of native toxin so as to cover the whole range of toxin occupancy, 0–100%. Duplicate aliquots were assayed with [$^3$H]methyl-toxin to determine the number of sites unoccupied by native toxin and, hence, by subtraction from the total number of sites, the number of sites occupied by native toxin. Duplicate aliquots were also assayed for sites available for affinity labeling by [$^3$H]MBTA.

Two blank mixtures without receptor, one without and the other with added native toxin, were also made and assayed for [$^3$H]MBTA labeling and [$^3$H]methyl-toxin binding. These provided the background corrections, i.e., nonspecific binding of [$^3$H]MBTA and of [$^3$H]methyl-toxin to filters. The nonspecific labeling by [$^3$H]MBTA was determined as the extent of labeling in the presence of native toxin in large excess over receptor and was subtracted from the total labeling to obtain the extent of specific labeling.

## Results and Discussion

We now determine the number of sites which can be spe-

TABLE II: Coefficients of Linear Regression Equation, $m = a + by$, Fitted to Experimental Data.

| Coefficient | Receptor preparation: Purified | Solubilized membrane | Membrane |
|---|---|---|---|
| $a$ | 0.50 ± 0.01 | 0.51 ± 0.01 | 0.48 ± 0.02 |
| $b$ | −0.51 ± 0.02 | −0.52 ± 0.02 | −0.45 ± 0.04 |

TABLE III: Statistical Deviation of the Data from the Curves Predicted by Each of the Models as Measured by $s_2^2/s_1^2$.[a]

| Model | Receptor preparation: Purified | Solubilized membrane | Membrane |
|---|---|---|---|
| I | 1.5 | 1.7 | 2.8 |
| II[b] | 31 | 8.8 | 6.6 |
| III[b] | 34 | 11.4 | 6.8 |

[a] $s_2^2/s_1^2$ is the $F$ ratio, the mean square deviation of the mean data points from the theoretical curve divided by the variance of the data points. It tests the hypothesis that the data fit the curve predicted by a particular model; the smaller the value of $F$, the better is the fit. [b] For these models, we consider case i of toxin binding kinetics since these curves are closest to the straight line of model I.

cifically labeled with [$^3$H]MBTA as a function of the extent of occupancy of sites by native toxin added previously. The extent of occupancy by native toxin in each case is determined by assaying the unoccupied sites with [$^3$H]methyl-toxin.

It is assumed in the theoretical section that $\alpha$-neurotoxin binds irreversibly and completely to receptor. If this were true under the conditions of our experiments, all of the native toxin added would be bound by the receptor sites or if toxin is in excess over the sites the latter would be saturated. Labeled toxin added subsequently should bind only to unoccupied sites, if any, and should not displace bound native toxin. This implies that as the amount of native toxin added ($T$) increases, the amount of [$^3$H]methyl-toxin bound subsequently ($T^*$) should decrease in a linear fashion, as long as $T < R$, where $R$ is the total number of toxin binding sites. When $T > R$, $T^*$ should be zero. Moreover, if all of the added native toxin is completely bound by the receptor, the sum $T + T^*$ should remain constant and equal to $R$ so long as $T < R$.

These expectations are fulfilled, as can be seen from the data on purified *Torpedo* receptor shown in Figure 3 (b and c). Thus, there is neither dissociation nor displacement nor redistribution of bound native toxin within 30–60 min that the toxin assay and MBTA labeling experiments take. Similar results were also obtained with crude solubilized receptor (Triton X-100 solubilized membrane) and with receptor in membrane. Hence, we can obtain from experimental data both $m$, the degree of MBTA labeling, and $y$, the fraction of toxin sites occupied by native toxin, with the assurance that both refer to the same mixture of receptor–toxin complexes. The value of $R$ is obtained from data as in Figure 3b as the mean number of total toxin binding sites. $y$ is calculated as

$$y = T/R$$

In Figure 3a, $M^*_t$, the total [$^3$H]MBTA labeling of receptor partially occupied by native toxin, is plotted vs. $T$ for purified receptor. $M^*_t$ is seen to decrease linearly with increasing $T$ as long as the latter is less than $R$. Similar results were also obtained for solubilized membrane and for receptor in membrane. For higher values of $T$ ($>R$), the receptor sites are saturated, and the residual reaction with [$^3$H]MBTA is by definition, nonspecific. The nonspecific labeling by [$^3$H]MBTA, $M^*_{ns}$, is practically zero for the purified receptor (Figure 3a). For membrane and for solubilized membrane, however, $M^*_{ns}$ is found to be significant, and $M^*_s$, the specific labeling of the receptor by [$^3$H]MBTA, is obtained as the difference, $M^*_s = M^*_t - M^*_{ns}$. The degree of specific [$^3$H]MBTA labeling is calculated as

$$m = M^*_s/R$$

We now compare the experimental results with the theoretical predictions. In Figure 4, the $m$–$y$ data on purified receptor and on solubilized membrane are shown superimposed on the theoretical $m$–$y$ curves of Figure 2a. All of the data are seen to follow the straight line predicted by model I and are so far removed from the parabolic curves predicted by models II and III that the latter two can definitely be ruled out. The least-squares lines fitting the data closely approximate in slope and intercept the theoretical line predicted by model I, case i or iii; i.e. slope = −0.5 and intercept = 0.5 (Table II). The $F$ test for the deviations of the data from the theoretical curves indicates that model I alone is consistent with the data (Table III).

Data on receptor in membrane fractions (no detergent) showed considerable scatter; however, statistical analysis favors model I over II and III for these data too (Tables II and III).

We, therefore, conclude that of the two toxin sites on the *Torpedo* receptor monomer, only one is affinity labeled by MBTA, and this labeling is blocked by $\alpha$-neurotoxin only when it binds at the MBTA labeling site. We also conclude that the two sites behave identically with respect to binding of toxin, at least initially, and that the sites either bind toxin independently with the same rate constant or show negative cooperativity.

Our experimentally observed relationship between $m$ and $y$ could result from there being two types of receptor molecules in the preparation, only one of which reacts with MBTA and both of which bind toxin. Although the receptor preparations contain monomer and dimer, these two forms have identical specific activities with respect to MBTA labeling and toxin binding, and the ratio of toxin binding to MBTA labeling is 2 (McNamee et al., 1975). In any case, if two forms of receptor molecule exist which differ in susceptibility to MBTA labeling, we have not been able to separate them during purification since the ratio of toxin binding to MBTA labeling is 2 for membrane, crude extract, receptor purified by affinity chromatography, and separated monomer and dimer forms (Damle et al., 1976). We conclude that the heterogeneity in binding sites with respect to reaction with MBTA is in all likelihood *intra*molecular rather than *inter*molecular.

We have observed two apparent rates of [$^3$H]methyl-toxin association with *Torpedo* receptor in solution (unpublished), as have Raftery et al. (1975) using iodinated $\alpha$-bungarotoxin. Others have observed only one rate of toxin association with *Torpedo* receptor (Weber & Changeux, 1974) and with *Electrophorus* receptor (Klett et al., 1973; Weber & Changeux, 1974; Maelicke et al., 1977). For muscle receptor one (Colquhoun & Rang, 1976) and two (Brockes & Hall, 1975) association rates have been reported.

The observation of two apparent rates could be due to either negative cooperativity between initially identical sites, or heterogeneity in labeled toxin, or heterogeneity of receptor molecules. We have ruled out the possibility of independent sites on the same monomer unit of receptor binding toxin at different rates. (Comparison of Figures 2b and 4 shows that

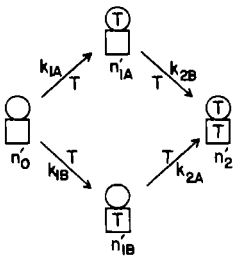


FIGURE 5: General scheme for the kinetics of toxin binding to receptor. (○) Site A; (□) site B. T denotes that the site is occupied by toxin. $n'$ represents the relative concentration of a species at any time $t$.

the experimental data are inconsistent with two classes of sites binding toxin independently at rates which differ even by a factor of as small as two, while the observed apparent rates differ by a factor of 4 to 7.) It is possible, however, that monomer and dimer, for example, could bind toxin at different rates. As long as the sites on any one monomeric unit of receptor bind toxin at the same rate, intermolecular heterogeneity will still fall under case i above.

Our results on *Torpedo* receptor differ from those of Maelicke et al. (1977) on *Electrophorus* receptor. They infer that there are pairs of close, interacting toxin binding sites, and that two toxin molecules can be displaced by one small, reversibly binding ligand. We find that labeling one site of two on *Torpedo* receptor in solution with MBTA prevents the binding of only one of two toxin molecules (Karlin et al., 1978); i.e., one site of two is still free to bind toxin. This result is more consistent with the toxin sites on *Torpedo* receptor being separate and distinct.

We conclude that there are two types of toxin sites that have the properties that they bind toxin at identical rates and yet differ in reactivity toward MBTA. The absence of a disulfide bridge near one of the binding sites, or its decreased susceptibility to reduction, or to affinity alkylation after reduction could account for the lack of reaction with MBTA. The two sites, though differing in structure at some level, could bind toxin at the same rate. Such nonequivalence of sites has also been demonstrated in glyceraldehyde-3-phosphate dehydrogenase, an enzyme with otherwise identical subunits, that exhibits half-of-the-sites reactivity (Bernhard & MacQuarrie, 1973).

In receptor purified from rat muscle, the ratio of toxin binding to MBTA labeling is one, and MBTA labels two different polypeptide chains (Froehner et al., 1977a) compared with one chain in electric tissue receptors (Karlin et al., 1975). If in fact toxin sites overlap MBTA sites, as has been inferred (Prives et al., 1972), then in muscle receptor, two different toxin sites have been identified, both of which, however, in this case react with MBTA. In the cases of *Torpedo* and *Electrophorus* receptors, we have yet to locate the inferred second type of toxin binding site, which does not react with MBTA.

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## Appendix

*General Scheme for the Kinetics of Association of Toxin with Receptor.* We assume that all added toxin binds irreversibly and completely with available sites. Since there are two toxin binding sites on a receptor monomer, the general association reaction scheme between toxin and the receptor monomer will have four association rate constants as shown in Figure 5. The rate equations can be written in a straightforward manner. No explicit general solution exists; however, by eliminating time as an independent variable, we can obtain relationships between the concentrations of various species valid at all times including $t = \infty$, when the binding reaction is complete (see, e.g., Benson, 1960). These relationships are

$$n_{1A} = \frac{n_0 p_4}{p_4 - 1}(1 - n_0^{p_4 - 1}) \quad \text{(A1)}$$

$$n_{1B} = \frac{n_0 p_2}{p_3 - 1}(1 - n_0^{p_3 - 1}) \quad \text{(A2)}$$

where

$$p_1 = k_{1A}/(k_{1A} + k_{1B})$$

$$p_2 = k_{1B}/(k_{1A} + k_{1B})$$

$$p_3 = k_{2A}/(k_{1A} + k_{1B}) \neq 1 \quad \text{(A3)}$$

$$p_4 = k_{2B}/(k_{1A} + k_{1B}) \neq 1$$

For the special case of $p_4 = 1$ or $p_3 = 1$, we have

$$n_{1A} = -p_1 n_0 \ln n_0 \quad \text{(A4)}$$

$$n_{1B} = -p_2 n_0 \ln n_0 \quad \text{(A5)}$$

By difference, we obtain $n_2$ as

$$n_2 = 1 - n_0 - n_{1A} - n_{1B} \quad \text{(A6)}$$

Equations A1–A6 yield $n_{1A}$, $n_{1B}$, and $n_2$ as functions of $n_0$. By eq 1, $y$, and by eq 2–4, $m$, are thus also expressed as functions of $n_0$. Therefore, as we let $n_0$ decrease from 1 to 0, $y$ increases from 0 to 1, and we can calculate for each model, the value of $m$ corresponding to each value of $y$.

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# Two-Step Purification of the Major Phosphorylated Protein in Reticulocyte 40S Ribosomal Subunits†

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ABSTRACT: In reticulocytes, a single ribosomal protein, S13, has been shown to be phosphorylated by the cAMP-regulated protein kinases. The 40S ribosomal subunits were phosphorylated in vitro with [$\gamma$-$^{32}$P]ATP to facilitate the identification of S13 during the two-step purification procedure. Total ribosomal protein from the 40S subunit was fractionated by phosphocellulose chromatography in urea, and S13 was purified to homogeneity by gel filtration on Sephadex G-100. The protein was identified by the radioactive phosphate, by molecular weight, and by the migration characteristics in a two-dimensional polyacrylamide gel electrophoresis system. Thin-layer electrophoresis of partial acid hydrolysates of S13 showed that more than one phosphorylated residue was present in the same oligopeptide, indicating at least some of the phosphoryl groups were clustered in the protein molecule.

Studies on the incorporation of radioactive inorganic phosphate into ribosomal proteins in mammalian cells have indicated the major site of phosphorylation was a single 40S ribosomal protein. This protein has been designated S6 in rat liver (Gressner & Wool, 1974a) and S13 in rabbit reticulocytes (Traugh & Porter, 1976), and has been shown to contain multiple phosphoryl groups (Gressner & Wool, 1974a; Traugh & Porter, 1976; Lastick et al., 1977; Treloar et al., 1977; Roberts & Ashby, 1978). In rat liver, phosphate incorporation into S6 was stimulated by glucagon (Blat & Loeb, 1971; Gressner & Wool, 1976a) dibutyryl cAMP (Gressner & Wool, 1976a), cyclohexamide, puromycin (Gressner & Wool, 1976b), ethionine (Treloar et al., 1977), and under conditions of regeneration (Gressner & Wool, 1974a). Stimulation has also been shown in rabbit reticulocytes (Cawthon et al., 1974), pituitary slices (Barden & Labrie, 1973), hamster islet cell tumor (Schubart et al., 1977), and rat cerebral cortex (Roberts & Ashby, 1978) with cAMP, and in baby hamster kidney fibroblasts (Leader et al., 1976), and HeLa cells (Lastick et al., 1977) by addition of serum.

The protein, S13, which was phosphorylated intracellularly by incubating intact reticulocytes in a nutritional medium with radioactive inorganic phosphate, was modified in vitro by cAMP-regulated protein kinases isolated from rabbit reticulocytes (Traugh & Porter, 1976). The incorporation of radioactive phosphate into S13 has been used as a means of rapidly identifying the protein during this two-step purification to homogeneity. The purification of S6 from rat liver has been described previously by Wool & co-workers (Collatz et al., 1976).

## Experimental Procedures

*Materials.* Urea (Mallinckrodt) was purified and used fresh, or stored in aliquots and frozen. Sodium dodecyl sulfate and pyronine G were obtained from BDH Chemicals Ltd. The [$\gamma$-$^{32}$P]ATP was prepared as described previously (Hathaway et al., 1978).

*Preparation of Ribosomal Subunits.* Reticulocytes were prepared from New Zealand doe rabbits, and ribosomal subunits were purified by zonal centrifugation as described previously (Traugh & Traut, 1974) and stored in small aliquots at −70 °C.

*Phosphorylation of 40S Ribosomal Subunits.* The phosphorylation mixture contained in a reaction volume of 25 mL: 50 mM Tris-HCl, pH 7.5; 3 mM $MgCl_2$; 0.0014 mM cAMP; 0.14 mM [$\gamma$-$^{32}$P]ATP; 1.22 mg/mL of 40S subunits; protein kinase. Phosphorylation was started by addition of 1000 enzyme units of type I cAMP-regulated protein kinase per mg of 40S subunits and incubated at 30 °C for 100 min. The

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