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Equilibrium Binding of [^{3}H]Tubocurarine and [^{3}H]Acetylcholine by *Torpedo* Postsynaptic Membranes: Stoichiometry and Ligand Interactions†

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ABSTRACT: Studies are presented of the equilibrium binding of [^{3}H]-*d*-tubocurarine (dTC) and [^{3}H]acetylcholine (AcCh) to *Torpedo* postsynaptic membranes. The saturable binding of [^{3}H]dTC is characterized by two affinities: K_{d1} = 33 ± 6 nM and K_{d2} = 7.7 ± 4.6 μM, with equal numbers of binding sites. Both components are completely inhibited by pretreatment with excess α-bungarotoxin or 100 μM nonradioactive dTC and competitively inhibited by carbamylcholine with a K_I = 100 nM, but not affected by the local anesthetics dimethisoquin, proadifen, and meproadifen. The biphasic nature of [^{3}H]dTC binding was unaltered in solutions of low ionic strength and by preparation of *Torpedo* membranes in the presence of *N*-ethylmaleimide, a treatment which yields dimeric AcCh receptors. dTC competitively inhibits the binding of [^{3}H]AcCh and decreases the fluorescence of 1-(5-dimethylaminonaphthalene-1-sulfonamido)ethane-2-trimethylammonium (Dns-Chol) in a manner quantitatively consistent with its directly measured binding properties. It decreases the initial rate of ^{3}H-labeled *Naja nigricollis* α-toxin binding by 50% at 60 nM with an apparent Hill coefficient of 0.58. The stoichiometry of total dTC, AcCh, and α-neurotoxin binding sites in *Torpedo* membranes was determined by radiochemical techniques and by a novel fluorescence assay utilizing Dns-Chol as an indicator, yielding ratios of 0.9 ± 0.1:0.9 ± 0.2:1, respectively. The biphasic equilibrium binding function is not unique to dTC since other ligands inhibited [^{3}H]AcCh binding in a biphasic manner with apparent inhibition constants as follows: gallamine triethiodide (K_{I1} = 2 μM, K_{I2} = 1 mM); Me_2dTC (K_{I1} = 500 nM, K_{I2} = 10 μM); decamethonium (K_{I1} = 100 nM, K_{I2} = 1.6 μM). Carbamylcholine, however, inhibited [^{3}H]AcCh binding with a single K_I = 100 nM. The observed competition between those ligands and [^{3}H]AcCh cannot be completely accounted for by competitive interaction with two different affinities, and the deviations are discussed in terms of the positive cooperativity of the [^{3}H]AcCh binding function itself. It is concluded that dTC binds only to the AcCh sites in *Torpedo* membranes and that those sites display two affinities for dTC but only one for AcCh.

The binding of acetylcholine (AcCh)[1] or other cholinergic agonists by nicotinic cholinergic receptors results in increased permeability of the plasma membrane to cations. Reversible antagonists of this response have been classified as either competitive or noncompetitive on the basis of their alteration of steady-state agonist dose-response relations. *d*-Tubocurarine (dTC) inhibits agonist-induced depolarizations of vertebrate skeletal muscle (Jenkinson, 1960) and of *Electrophorus* (Higman et al., 1963) and *Torpedo* (Moreau & Changeux, 1976) electroplax in a manner consistent with competitive antagonism, while local anesthetic aromatic amines and the piperidine alkaloid histrionicotoxin are examples of noncompetitive antagonists (Bartels & Nachmansohn, 1965; Kato & Changeux, 1976). While the classification of antagonist mechanisms based upon the analyses of depolarization responses may be fortuitous [see, for example, Hubbard et al.

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[1] Abbreviations used: AcCh, acetylcholine; α-BgTx, α-bungarotoxin; DFP, diisopropyl phosphofluoridate; Dns-Chol, 1-(5-dimethylaminonaphthalene-1-sulfonamido)ethane-2-trimethylammonium; dTC, *d*-tubocurarine; meproadifen, 2-(diethylmethylamino)ethyl 2,2-diphenylvalerate; TPS, *Torpedo* physiological saline (250 mM NaCl, 4 mM KCl, 3 mM $CaCl_2$, 2 mM $MgCl_2$, and 5 mM NaP_i, pH 7); TPS–0.1% BSA, TPS supplemented with 0.1% bovine serum albumin.

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(1969) and Ginsborg & Jenkinson (1976)], more recent electrophysiological studies provide further support for the notion that dTC, but not local anesthetics, interacts directly with the AcCh binding site, although an additional noncompetitive mechanism of antagonism can also be identified (Adams, 1975; Manalis, 1977; Katz & Miledi, 1978).

The use of radiolabeled elapid α-neurotoxins provided indirect evidence that agonists and reversible competitive antagonists bind to a common site in vertebrate skeletal muscle (Colquhoun & Rang, 1976) and fish electroplax (Weber & Changeux, 1974b). It is possible to analyze directly the binding of agonists and antagonists to nicotinic postsynaptic membranes isolated (Cohen et al., 1972) from *Torpedo* electric tissue, a unique preparation for the biochemical analysis of the mechanism of permeability control by nicotinic cholinergic receptors [for a review, see Heidmann & Changeux (1978)]. Two distinct classes of ligand binding sites have been characterized in *Torpedo* membranes by the use of radiolabeled cholinergic ligands: the acetylcholine binding site and a binding site for noncompetitive antagonists such as histrionicotoxin and certain local anesthetics (Eldefrawi et al., 1978; Elliott & Raftery, 1979; Krodel et al., 1979). The anesthetic binding site as well as the AcCh binding site is contained within the peptides of the nicotinic receptor itself (Neubig et al., 1979). dTC reduces the equilibrium binding of [^{3}H]AcCh to its binding site (Weber & Changeux, 1974b) but not the binding of [^{14}C]meproadifen to the anesthetic site (Krodel et al., 1979). While it has generally been assumed that cholinergic agonists and competitive antagonists do bind to a common site, it has been reported that dTC reduces the equilibrium binding of [^{3}H]AcCh to both the membrane-bound (Eldefrawi & Eldefrawi, 1977) and detergent-solubilized (Gibson, 1976) *Torpedo* receptor by a noncompetitive or allosteric effect rather than by a competitive interaction at a common binding site. No direct study of the binding of competitive antagonists has allowed a comparison of the number of agonist and reversible antagonist sites or an assessment of the reciprocal effects of these ligands on the other's binding. Such information is necessary to distinguish between possible antagonist mechanisms.

We report here studies of the equilibrium binding of [^{3}H]-dTC to nicotinic postsynaptic membranes isolated from *Torpedo* electric tissue. dTC binds at equilibrium with quite different affinities (K_d = 20 nM and 7 μM) to two sites, each present in a ratio of 1 per 2 α-neurotoxin sites (or per 2 AcCh sites). The heterogeneity of the equilibrium dTC binding function is confirmed by measurement of the effect of dTC on the binding of [^{3}H]AcCh, ^{3}H-labeled *Naja nigricollis* α-neurotoxin, and the fluorescent cholinergic antagonist 1-(5-dimethylaminonaphthalene-1-sulfonamido)ethane-2-trimethylammonium (Dns-Chol). The two affinities for dTC binding are discussed in terms of receptor conformational changes, the organization of subsites on the receptor, and the bidentate nature of the dTC molecule. A preliminary characterization of the binding of [^{14}C]dimethyltubocurarine to *Torpedo* membranes has been reported previously (Cohen, 1978).

Materials and Methods

AcCh Receptor-Rich Membranes. Acetylcholine receptor-rich membrane fragments were prepared from freshly dissected *Torpedo marmorata* electric organs by the method of Cohen et al. (1972), as modified by Krodel et al. (1979), and from *T. californica* and *T. nobiliana* according to Sobel et al. (1977). Fractions from the continuous gradients having the highest specific activity were pooled for binding studies and stored at 4 °C in 37% sucrose–0.02% NaN_3. Specific activities (in micromoles of α-toxin sites per gram of protein) were 1–2 for *T. marmorata* (4 fish) and *T. californica* (10 fish) and 2–3 for *T. nobiliana* (2 fish). Membrane protein concentrations were determined by the method of Lowry et al. (1951) and acetylcholinesterase by the method of Ellman et al. (1961). The binding of ^{3}H-labeled *N. nigricollis* α-toxin to the *Torpedo* membrane was assayed by Millipore filtration (Weber & Changeux, 1974a) with the following modification to reduce nonspecific adherence of [^{3}H]-α-toxin to the filters. The filters were soaked 4 h or more in *Torpedo* physiological saline (TPS: 250 mM NaCl, 4 mM KCl, 3 mM $CaCl_2$, 2 mM $MgCl_2$, and 5 mM NaP_i, pH 7) supplemented with 0.1% bovine serum albumin (TPS–0.1% BSA), and incubation of the membranes with the [^{3}H]-α-toxin was carried out in TPS–0.1% BSA. Because inclusion of BSA reduced by 30–40% the retention of membrane-bound [^{3}H]-α-toxin by HA filters, Millipore GS filters were used which retained over 90% of membrane-bound [^{3}H]-α-toxin in the presence of 0.1% BSA.

[^{3}H]Tubocurarine Binding. A Beckman Airfuge microultracentrifugation assay was used to measure the equilibrium binding of [^{3}H]dTC. The protocol has been described in detail by Krodel et al. (1979) to measure the binding of [^{14}C]meproadifen to the *Torpedo* membranes. Bound and free [^{3}H]dTC were determined from the [^{3}H]dTC retained in the pellet and supernatant, respectively, after centrifugation for 15 min at 130000*g*. Nonspecific retention of [^{3}H]dTC by the *Torpedo* membranes was determined by the use of membranes pretreated for 1 h at 23 °C with a 5–10-fold excess of α-bungarotoxin (α-BgTx). Nonspecific binding was linearly proportional to the free [^{3}H]dTC concentration at least to 20 μM. [^{3}H]dTC bound specifically to the *Torpedo* postsynaptic membranes was determined from the difference between the total binding and the nonspecific component determined for that concentration of free [^{3}H]dTC. Except where noted, all binding experiments were done in TPS at 4 °C.

[^{3}H]Acetylcholine Binding. A filtration assay was used to measure the equilibrium binding of [^{3}H]AcCh to membranes in TPS (Neubig et al., 1979; Cohen & Boyd, 1979). Membranes were pretreated for 30 min at 4 °C with 1 mM diisopropyl phosphofluoridate (DFP) to inhibit residual acetylcholinesterase and then diluted to give a final concentration of 50–100 nM α-toxin sites, 0.1 mM DFP. Various amounts of [^{3}H]AcCh and competing drugs were added, and 1.0-mL aliquots were filtered on Whatman GFF filters at 23 °C *without washing.* Filters were dried and counted, and 0.5 mL of the filtrates was counted to determine the concentration of free [^{3}H]AcCh. [^{3}H]AcCh bound specifically to the membrane-bound cholinergic receptor was determined from the difference between the total radioactivity retained on the filter and the [^{3}H]AcCh retained at the same concentration of free [^{3}H]AcCh when membranes had been pretreated for 60 min with a 5–10-fold excess of α-BgTx prior to equilibration with [^{3}H]AcCh. It should be noted that with glass fiber filters the nonspecific binding consists largely of [^{3}H]AcCh trapped in free solution and adsorbed to the filters and not of [^{3}H]AcCh bound to the membranes themselves (N. D. Boyd and J. B. Cohen, unpublished experiments). When competing drugs were used, separate determinations of the nonspecific retention of [^{3}H]AcCh were made for each concentration of added drug. Attempts to use this filtration assay for measuring [^{3}H]dTC binding failed due to the very high nonspecific binding of [^{3}H]dTC to GFF filters.

Liquid Scintillation Counting. All aqueous solutions were counted in a toluene–Triton scintillation solution [T–T–PBD

= 2 parts toluene, 1 part Triton X-100, and 0.4 g/L 2-(4′-*tert*-butylphenyl)-5-(4″-biphenylyl)-1,3,4-oxadiazole]. For experiments with [^{3}H]dTC, the final counting solution contained 0.2 mL of TPS, 0.2 mL of 10% sodium dodecyl sulfate, and 3.5 mL of T–T–PBD. For [^{3}H]AcCh, the final counting solution was 1.0 mL of TPS and 10 mL of T–T–PBD. All filters were counted in 4 mL of toluene containing 3 g/L 2,5-diphenyloxazole and 0.2 g/L 1,4-bis[2-(5-phenyloxazolyl)]benzene. Counting efficiencies were determined by using [^{3}H]H_2O (New England Nuclear) for aqueous containing solutions. Counting efficiency for [^{3}H]AcCh on GFF filters was determined by counting [^{3}H]AcCh spotted on filters and in parallel in the aqueous counting solution. Depending on the scintillation counter used, tritium counting efficiencies ranged from 12 to 30% for 3.5 mL of T–T–PBD, 18 to 30% for 10 mL of T–T–PBD, and 34 to 40% for GFF filters.

Fluorescence Titrations. Membrane binding capacities for AcCh and α-neurotoxins were determined independent of specific activities of radiolabeled compounds by a fluorescence assay. Fluorescence of Dns-Chol excited by energy transfer from receptor tryptophan was measured as described by Cohen & Changeux (1973). Experiments were performed on an Aminco SPF-1000 fluorometer. To ensure maximum stability, we used the ratio fluorescence mode which corrects for fluctuations in lamp output and phototube fatigue. Excitation and emission wavelengths were 287 and 530 nm, respectively, with 10-nm slit widths. A microfluorescence cell (Precision Scientific 52H, 2 × 10 mm path length) was used with 0.3-mL sample volumes. Membrane suspensions (0.2–0.6 μM α-toxin sites in TPS) were equilibrated with 6.6 μM Dns-Chol perchlorate. Aliquots of AcCh or α-neurotoxins were added, and fluorescence was measured with shaking between readings until the signal reached equilibrium (about 1 min for AcCh and 5–30 min for α-neurotoxins); then more was added until no further change occurred. For experiments involving AcCh, membranes were pretreated with DFP as described for [^{3}H]-AcCh binding. The binding capacity of the membranes was determined from a plot of the observed fluorescence intensity as a function of the number of picomoles of ligand added.

α-Neurotoxin concentrations were determined by weight or by absorbance measurements using molar extinction coefficients of 9500 (Bulger et al., 1977), 8300 (Karlsson et al., 1971), and 8600 (Hori & Tamiya, 1976) for α-bungarotoxin, *Naja naja siamensis* α-toxin, and erabutoxin *b*, respectively. The absorbance measurements which were lower, but always within 20% of weight, were used when available.

Radiochemicals. [*N-methyl*-^{3}H]Acetylcholine (batch 8) and [*acetyl*-^{3}H]acetylcholine (batches 18 and 21) were obtained from Amersham with reported specific activities 0.20, 0.25, and 0.25 Ci/mmol, respectively. Radiochemical purity of [*Ac*-^{3}H]AcCh was measured by thin-layer chromatography as described by Lewis & Eldefrawi (1974) and that of [*Ch*-^{3}H]AcCh was determined by high-voltage electrophoresis (Hildebrand et al., 1971). Isotopic dilution studies (see Results) gave a specific activity of 0.42 and 0.22 Ci/mmol for batches 8 and 21, while batch 18 was estimated to be within 10% of the reported value which was then used. Tritiated [13-*N*-^{3}H]-D-tubocurarine (lots 962-165 and 962-258, 20.6 and 18.2 Ci/mmol, respectively) was obtained from New England Nuclear, diluted 30–40-fold with nonradioactive *d*-tubocurarine before use, and stored in H_2O at 4 °C shielded from light. Radiochemical purity was determined by thin-layer chromatography in three systems: (I) ethyl acetate–2-propanol–ammonium hydroxide (9:7:4); (II) methanol–2 M ammonium chloride–ammonium hydroxide (7:3:0.1); (III) butanol–acetic acid–water (25:4:10).

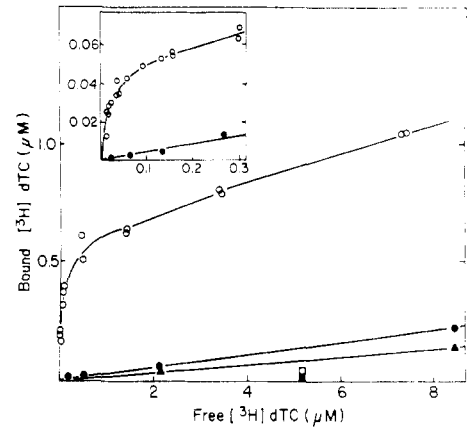

FIGURE 1: Binding of [^{3}H]dTC to *T. marmorata* membranes determined by ultracentrifugation. Membrane suspensions (1.05 μM α-toxin sites in TPS) contained various concentrations of [^{3}H]dTC and either no additions for total binding (○), 5 μM α-BgTx (●), or 100 μM nonradioactive dTC (▲) to measure nonspecific binding. Little or no binding of [^{3}H]dTC in the presence (■) or absence (□) of α-BgTX was detected when membranes were omitted. Inset: high-affinity component of [^{3}H]dTC binding to membranes (150 nM α-toxin sites) in the absence (○) or presence (●) of 1 μM α-BgTx. Lines are smooth curves through the data, and axis labels refer to both the main figure and inset.

Tritiated *N. nigricollis* α-toxin was a gift of Drs. A. Menez, J. L. Morgat, P. Fromageot, and P. Boquet. The relative specific activities of the two lots of [^{3}H]-α-toxin used were determined by the use of nonradioactive α-BgTx. First, the binding of [^{3}H]-α-toxin to the *Torpedo* membranes determined by Millipore filtration was used in conjunction with the concentration of α-neurotoxin sites determined by the fluorescence titration assay to determine a specific activity for lot 1 of 12.8 ± 1.5 Ci/mmol (nine titrations on four membrane preparations) and for lot 2 of 12.2 Ci/mmol (one titration). The specific activity of lot 2 was also determined by preincubating a *Torpedo* membrane suspension with several known substoichiometric amounts of α-BgTx and then assaying the remaining receptor sites with the [^{3}H]-α-toxin by Millipore filtration. The specific activity obtained (13.3 Ci/mmol) was within 10% of that determined by the fluorescence titration.

Chemicals. Acetylcholine (Sigma) and dTC (KK Laboratories) were stored desiccated at −20 °C and room temperature, respectively. Samples of each did not change weight when heated at 50 °C for 1 h under vacuum, so in subsequent experiments they were used without drying. α-Bungarotoxin and *N. n. siamensis* α-toxin were from Miami Serpentarium, and erabutoxin *b* was a gift from Dr. N. Tamiya.

Results

Binding of [^{3}H]dTC at Equilibrium. The equilibrium binding of [^{3}H]-*d*-tubocurarine to *Torpedo* postsynaptic membranes was determined for [^{3}H]dTC concentrations varying between 10 and 10000 nM (Figure 1). Qualitative examination of the observed total binding indicates that it must be characterized by multiple saturable binding components as well as a nonsaturable component. Furthermore, most of the binding was receptor specific in the sense that it was displaced by α-BgTx. The binding of [^{3}H]dTC in the presence of α-BgTx was linearly dependent on [^{3}H]dTC concentration up to 20 μM, and this nonspecific binding comprised less than 15% of the total binding at 10 μM dTC. One hundred micromolar dTC did displace slightly more [^{3}H]dTC from *Torpedo* membranes than did excess α-BgTx, but this increment

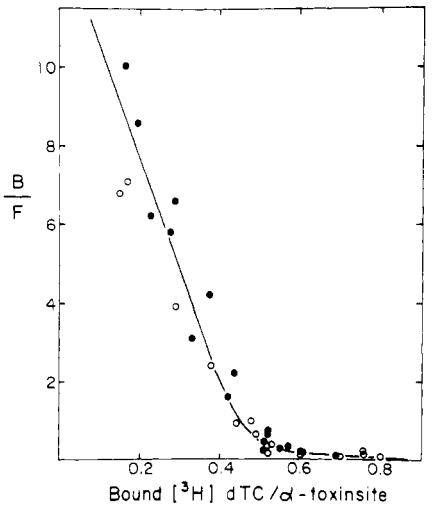

FIGURE 2: Scatchard plot showing two components of specific [^{3}H]-dTC binding to *T. marmorata* membranes. Experimental procedures were as described under Materials and Methods. Pooled data from two preparations (○ and ●) of membranes represent five experiments conducted at receptor (α-toxin site) concentrations of 1.05 and 1.91 μM (○) and 0.15, 0.75, and 0.75 μM (●). A nonlinear least-squares fit of bound and free dTC to a two-site binding model, $B = B_1/(1 + K_{d1}/L) + B_2/(1 + K_{d2}/L)$, gave the following parameters (±SD): $B_1 = 0.45 \pm 0.03$ site/α-toxin site, $K_{d1} = 33 \pm 6$ nM, $B_2 = 0.49 \pm 0.12$ site/α-toxin site, $K_{d2} = 7.7 \pm 4.6$ μM. Data points are means of duplicate determinations, and the line was calculated according to the least squares fitted parameters.

was only 5% of the total binding at 10 μM and was much less than the total number of receptor sites. Due to the greater binding specificity of α-BgTx than dTC, the former ligand was used to define receptor-specific binding.

The specifically bound [^{3}H]dTC cannot be characterized by a single class of binding sites but is consistent with two sites, one of high and one of low affinity. The high-affinity component of the [^{3}H]dTC binding function can be readily characterized by measuring the binding of [^{3}H]dTC at low concentrations of receptor and of [^{3}H]dTC (Figure 1 inset). This high-affinity binding is present and exhibits comparable binding parameters in all three species of *Torpedo* studied. The dissociation constants are 31 ± 15, 16 ± 6, and 7 nM for *T. marmorata* (five determinations), *T. californica* (two determinations), and *T. nobiliana* (one determination), respectively, with roughly half as many high-affinity dTC sites as α-neurotoxin sites in all species (see below for details on site stoichiometry). The low-affinity component, also displaced by α-BgTx, is best seen in the Scatchard plot in Figure 2 which contains pooled data from five experiments in two preparations of *T. marmorata* postsynaptic membranes. No estimate of the K_d for the low-affinity binding can be made directly from such a plot because the observed binding at high dTC concentrations always includes the high- as well as the low-affinity binding. The low-affinity K_d was estimated from the data of Figure 2 by fitting the data to a model consisting of two binding sites (a high-affinity binding site at concentration B_1 with dissociation constant K_{d1} and a low-affinity site at a concentration of B_2 with dissociation constant K_{d2}). According to such a model, for any concentration, L, of free dTC the total bound dTC is $B = B_1/(1 + K_{d1}/L) + B_2/(1 + K_{d2}/L)$. A nonlinear least-squares program (Bard, 1967) was used to identify the best parameters. The solid curve in Figure 2 was calculated according to the parameters obtained: $B_1 = 0.45 \pm 0.03$ site/α-toxin site and $B_2 = 0.49 \pm 0.12$ site/α-toxin site; $K_{d1} = 33 \pm 6$ nM and $K_{d2} = 7.7 \pm 4.6$ μM. Low-affinity, α-toxin displaceable [^{3}H]dTC binding is also present in post-

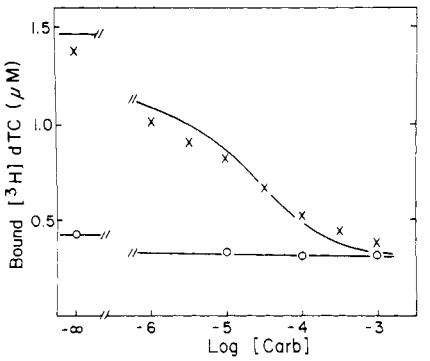

FIGURE 3: Displacement of [^{3}H]dTC binding by carbamylcholine. The binding of 10 μM [^{3}H]dTC to *T. californica* membranes (1.6 μM toxin sites) was measured in the presence of different concentrations of carbamylcholine by using the microultracentrifugation assay (see Materials and Methods). Binding to normal membranes (×) and to α-BgTx-treated membranes (○) is shown. Data points are means of duplicate determinations, and the lines are theoretical curves for dTC binding to two sites ($B_1 = B_2 = 0.72$ μM, $K_{d1} = 35$ nM, $K_{d2} = 8$ μM) which bind carbamylcholine with a single affinity ($K_d = 0.1$ μM).

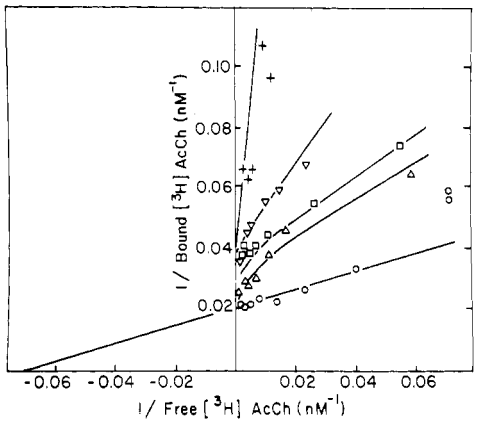

FIGURE 4: dTC inhibition of [^{3}H]AcCh binding to *T. marmorata* membranes at equilibrium. Suspensions of DFP-treated membranes containing 50 nM toxin sites were equilibrated for 30 min at 22 °C with various concentrations of [^{3}H]AcCh and dTC: (○) 0; (△) 0.3; (□) 1; (▽) 10; (+) 100 μM. 1-mL aliquots were filtered on GFF filters; counting of filters and filtrates and determination of specific α-BgTx displaceable binding were as described under Materials and Methods. The lines are drawn according to the two-site inhibition model (see text) with a $K_d = 14$ nM for AcCh and $K_{I1} = 15$ nM and $K_{I2} = 7$ μM for dTC with equal numbers of low- and high-affinity sites.

synaptic membranes isolated from *T. californica* (Figure 8).

Carbamylcholine Inhibition of [^{3}H]dTC Binding. α-Bungarotoxin (M_r 8000) is considerably larger than tubocurarine or acetylcholine, and it was conceivable that α-BgTx could prevent the binding of [^{3}H]dTC while a small cholinergic agonist might not displace dTC from either its high- or low-affinity binding site. For example, the local anesthetic meproadifen was bound with high affinity in the presence of dTC but not in the presence of α-BgTx (Krodel et al., 1979). When the binding of [^{3}H]dTC was measured in the presence of different concentrations of carbamylcholine, it was found that carbamylcholine displaced all of the specifically bound [^{3}H]-dTC (Figure 3). Furthermore, the concentration dependence of the effect of carbamylcholine was consistent with a competitive interaction between carbamylcholine which binds with $K_d = 100$ nM (J. B. Cohen and N. D. Boyd, unpublished experiments) to each of two independent sites and dTC binding to those sites with $K_{d1} = 35$ nM and $K_{d2} = 8$ μM.

dTC Inhibition of [^{3}H]AcCh Binding. Additional information can be obtained about dTC binding by measuring its

displacement of other ligands from the cholinergic receptor. We studied the effect of dTC at concentrations between 0.2 and 100 μM on the equilibrium binding of [^{3}H]AcCh to *Torpedo* postsynaptic membranes. Figure 4 is a double-reciprocal plot of AcCh binding in the presence of different concentrations of dTC. These data are not compatible with either competitive or noncompetitive inhibition at a *single* homogeneous site, but they can be fit by assuming a competitive interaction at two different sites.

The AcCh binding function in such a model can be written as $B = B_1/[1 + (K_{d1}/A)(1 + I/K_{I1})] + B_2/[1 + (K_{d2}/A)(1 + I/K_{I2})]$, where B, A, and I are the concentrations of bound and free AcCh and free dTC, respectively, B_1 and B_2 are the concentrations of AcCh binding sites, and K_{d1}, K_{d2}, K_{I1}, and K_{I2} are the dissociation constants for AcCh and dTC binding to those sites. The solid lines in Figure 4 are theoretical calculations of the binding of one ligand (AcCh) to a single class of homogeneous sites ($K_d = K_{d1} = K_{d2} = 14$ nM) in the presence of another ligand (dTC) which binds to half of those sites with a $K_{I1} = 15$ nM and to the other half with a $K_{I2} = 7$ μM. These values agree very well with the directly determined binding of [^{3}H]dTC and suggest that the interaction of dTC and AcCh is simply competitive, once the two dTC affinities are taken into account. It should also be noted that dTC inhibited over 80% of the binding of AcCh, but the low-affinity component of its binding necessitates very high concentrations of dTC (100 μM) to produce that degree of inhibition.

dTC Inhibition of [^{3}H]-α-Toxin Binding. The interaction of dTC with its binding sites in the *Torpedo* membranes was also characterized by its capacity to modify the initial binding kinetics of the ^{3}H-labeled *N. nigricollis* α-toxin. Assays of this sort have been used to characterize both the equilibrium binding and the kinetics of binding of nonradioactive cholinergic ligands [reviewed in Heidmann & Changeux (1978)]. In the absence of dTC the kinetics of binding of ^{3}H-labeled *N. nigricollis* α-toxin to membrane suspensions prepared from *T. californica* electric tissue were characterized by a bimolecular association rate constant, $k_T = (1.2\text{–}1.8) \times 10^7$ M^{-1} min^{-1} (at 23 °C in TPS–0.1% BSA), which is similar to values reported for the binding of the same α-neurotoxin to *T. marmorata* membranes in TPS (Weber & Changeux, 1974a). The k_T characterizing the interaction of ^{125}I-labeled *N. n. siamensis* α-neurotoxin with *T. californica* membranes has been reported to be 3×10^7 M^{-1} min^{-1} (Weiland et al., 1976) while that of [^{125}I]-α-BgTx has been reported to be 6×10^5 M^{-1} min^{-1} in TPS (Blanchard et al., 1979). When the *Torpedo* membranes were preincubated with dTC prior to exposure to the [^{3}H]-α-toxin, dTC reduced the initial rate of binding of the [^{3}H]-α-toxin in a concentration-dependent manner (Figure 5). C_{50}, the concentration of dTC reducing the initial rate of binding by 50%, was equal to 60 nM, a value similar to the $C_{50} = 170$ nM characterizing the effect of dTC on the binding of the same [^{3}H]-α-toxin to *T. marmorata* membranes (Weber & Changeux, 1974b). The biphasic character of the dTC binding function is reflected in the full concentration dependence of its inhibition of [^{3}H]-α-toxin binding which cannot be characterized by simple hyperbolic binding (solid line in Figure 5), but it exhibits a Hill coefficient of 0.58, suggesting multiple sites or negative cooperativity [see also Weiland & Taylor (1979)]. However, the observed inhibition of [^{3}H]-α-toxin binding kinetics is not fit by the two-site model using parameters that successfully reproduced the direct binding of [^{3}H]dTC and its inhibition of [^{3}H]AcCh binding (dashed line), despite the fact that those parameters generate a similar Hill coefficient of 0.53. This points out the difficulty of using the α-neurotoxins as quantitative probes of small ligand binding to the cholinergic receptor. Qualitatively, nevertheless, the deviation from a hyperbolic inhibition curve is in the direction expected for multiple dTC binding affinities.

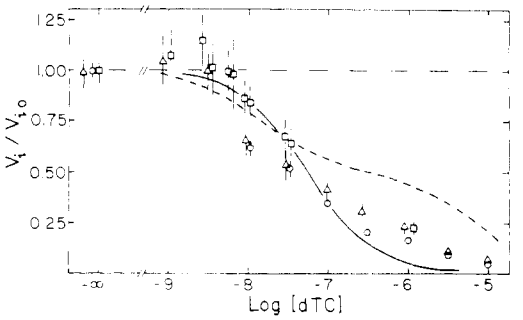

FIGURE 5: dTC inhibition of the initial rate of binding of ^{3}H-labeled *N. nigricollis* α-toxin to *T. californica* membranes. Equal (4 mL) volumes of TPS–0.1% BSA containing 2 nM α-toxin sites and 12 nM ^{3}H-labeled *N. nigricollis* α-toxin were preincubated for 30 min at 22 °C with various concentrations of dTC. The binding reaction was initiated by mixing the two solutions, and then 1-mL aliquots were filtered on Millipore GS filters at 20–120-s intervals. Filtration intervals were selected for each dTC concentration so that seven data points could be obtained during the linear phase of the binding reaction, and the seven values of counts per minute bound vs. time were fitted by a linear least-squares program to determine the slope ±SD. Error bars for control values (no drug) are the standard deviation of triplicate determinations, and those for experimental points are the combined error from control values and the SD of the least squares fitted slope. Data are from experiments in three preparations (△, ○, □) of membranes; control values of initial rate (V_{io}) correspond to bimolecular association constants of $(1.2\text{–}1.8) \times 10^7$ M^{-1} min^{-1}. The solid line is the expected inhibition pattern for a single hyperbolic binding site with $K_D = 56$ nM, and the dashed line is for two sites present in equal amounts with $K_{d1} = 15$ nM and $K_{d2} = 7$ μM.

Stoichiometry of α-Toxin, AcCh, and dTC Binding. The initial observations that there were approximately half as many high-affinity dTC sites as toxin sites and that dTC inhibited about half of the AcCh binding with high affinity prompted us to undertake a study of the stoichiometry of dTC, AcCh, and α-neurotoxin binding to *Torpedo* membranes. There is considerable confusion in the literature concerning the stoichiometry of α-toxin and reversible ligand binding to the cholinergic receptor [for a review, see Heidmann & Changeux (1978)]. We have taken two independent approaches to determine site stoichiometries. First, a titration assay was utilized which does not depend on the determination of the specific activities of radiolabeled compounds, and, secondly, radioactive ligand binding assays were done with specific activities, radiochemical purity, and counting efficiencies monitored as described under Materials and Methods.

In Figure 6 are shown titrations of the AcCh and α-neurotoxin binding capacities of *T. californica* membranes using Dns-Chol fluorescence (Cohen & Changeux, 1973) as an indicator. Addition of either AcCh or α-neurotoxins resulted in a decreased fluorescence intensity linearly proportional to the amount of ligand added for at least 80% of the titration curve. The observed linear titration of the Dns-Chol fluorescence is to be expected for AcCh as well as the α-neurotoxins because the dissociation constants characterizing the binding of AcCh ($K_d = 10$ nM) as well as the α-neurotoxins ($K_d \leq 0.1$ nM) are at least a factor of 20 less than the concentration of binding sites, and the concentration of Dns-Chol is less than its dissociation constant ($K_d = 20$ μM). The residual fluorescence detected in the presence of saturating concentrations of AcCh or α-neurotoxin was similar for both ligands and is due to Dns-Chol free in solution and bound to other

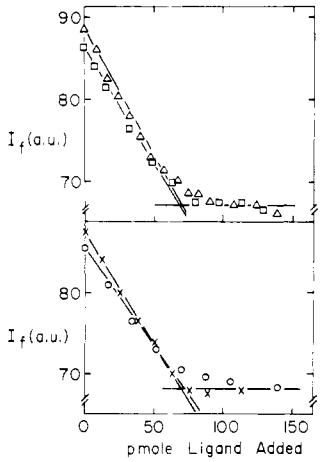

FIGURE 6: Titration of AcCh and α-neurotoxin binding sites using Dns-Chol as a fluorescence indicator. Membranes from *T. californica* preincubated with DFP were diluted with TPS at 22 °C to a final concentration of ~0.2 μM toxin sites. Aliquots (0.3 mL) were made 6.6 μM in Dns-Chol (K_D = 20 μM), and fluorescence was measured before and after addition of small aliquots of AcCh (△, □), *N. n. siamensis* α-toxin (×), or erabutoxin *b* (○). Ordinate: fluorescence intensity at 550 nm, expressed in arbitrary units. The intersection of the slanted and horizontal lines indicates the number of AcCh and α-toxin binding sites in the cuvette, and these values are 64 and 65 pmol (213 and 217 nM) for AcCh and 67 and 68 pmol (223 and 227 nM) for *N. n. siamensis* α-toxin and erabutoxin *b*, respectively.

Table I: AcCh and α-Neurotoxin Binding Capacities of *Torpedo* Membranes Determined by Fluorescence Titrations[a]

species	prepn no.	titrating ligand (site concn, μM) α-BgTx	erabutoxin *b*	*N. n. siamensis*	AcCh
T. marmorata	1	5.0	5.2	5.1	
			4.4		
T. marmorata	2		5.6		5.1
			5.0		
T. marmorata	4		3.8		4.4
T. californica	8		2.3	2.2	2.1, 2.2

[a] The concentration of binding sites for various ligands was determined by titrating the fluorescence of Dns-Chol (6.6 μM) with those ligands as described under Materials and Methods. Membranes were diluted 10-fold with TPS for the assays, and values on the same horizontal line were determined on the same day.

sites in the *Torpedo* membranes. The assay provides no direct determination of the amount of Dns-Chol displaced from the receptor, but it does permit the determination of the concentration of AcCh high-affinity or α-neurotoxin binding sites in the *Torpedo* membranes. Extrapolation of the linear portion of the fluorescence titration assays shown in Figure 6 of AcCh and two different α-neurotoxins indicated the presence of 64 and 65 pmol of AcCh binding sites and 67 and 68 pmol of binding sites for *N. n. siamensis* α-toxin and erabutoxin *b*, respectively. Titration of several membrane preparations (Table I) gave nearly identical numbers of sites for three different α-toxins and for AcCh. Consistent with the results of the previous sections, displacement of Dns-Chol by dTC gave a biphasic curve (Figure 7). Low concentrations of dTC resulted in a reduction of Dns-Chol fluorescence that paralleled the effects of AcCh and α-toxin until ~50% of maximum displacement. High concentrations of dTC were necessary to displace the remaining Dns-Chol, and even 3 μM dTC resulted only in a 75% displacement of Dns-Chol. Higher concentrations of dTC were not used because at 100 μM dTC photolysis of dTC generated a fluorescent species, an effect not seen below 10 μM.

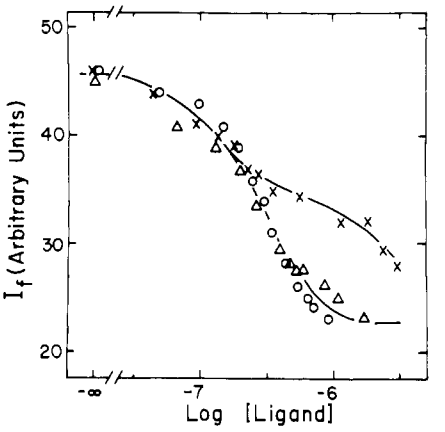

FIGURE 7: Displacement of Dns-Chol by dTC, AcCh, and erabutoxin *b*. *T. marmorata* membranes at a final concentration of 0.55 μM toxin sites in TPS were titrated as described in Figure 6 with dTC (×), AcCh (△), and erabutoxin *b* (○).

Quantification of the number of binding sites for [^{3}H]AcCh in the *Torpedo* membranes depends upon a knowledge of the specific activity and radiochemical purity of the [^{3}H]AcCh. The specific activity of the [^{3}H]AcCh was determined by isotope dilution in an assay utilizing the [^{3}H]AcCh binding capacity of the *Torpedo* membranes. In a typical experiment, the specific binding of [^{3}H]AcCh (2.00×10^5 dpm/mL, nominally 360 nM) to a suspension of membranes containing 95 nM α-toxin sites was determined in the presence of 0–1000 nM added nonradioactive AcCh. When the data were plotted as 1/(counts per minute bound) vs. the concentration of added nonradioactive AcCh, the x intercept was −415 ± 30 nM, which is equal to $-A^*$ where A^* is the total concentration of radioactive AcCh (nM) in the [^{3}H]AcCh added to the reaction mixture. This result can be derived as follows. Under the conditions of this assay, the free [^{3}H]AcCh is much greater than K_d and the receptor sites are fully occupied. Thus, the number of counts per minute bound equals SR_0, where S is the specific activity of the [^{3}H]AcCh and R_0 is the concentration of binding sites. Adding an amount A of nonradioactive AcCh decreases the specific activity of the AcCh to $S[A^*/(A^* + A)]$ and the counts per minute bound to $S[A^*/(A^* + A)]R_0$. Taking a reciprocal yields

$$1/(\text{cpm bound}) = \frac{1}{SR_0} + \frac{1}{SR_0}\frac{A}{A^*} \qquad (1)$$

which when plotted as mentioned above gives a line with a slope of $(SR_0A^*)^{-1}$, a y intercept of $(SR_0)^{-1}$, and an x intercept of $-A^*$. The radiochemical purity of the [^{3}H]AcCh used in this experiment was determined by thin-layer chromatography to be 96% AcCh both before and after incubation with DFP-treated membranes for 5 h. From these results, the specific activity of this batch of AcCh can be calculated to be 0.96(2.00 $\times 10^5$ dpm)/415 pmol or 0.21 ± 0.02 Ci/mmol. For a lot of [*N-methyl*-^{3}H]acetylcholine stated by the manufacturer to be of 0.2 Ci/mmol, we determined a specific activity of 0.42 Ci/mmol and that the stock solution contained 33% of its counts as choline after routine storage in ethanol at −20 °C for 7 months. The specific activities of ^{3}H-labeled *N. nigricollis* toxin and [^{3}H]dTC were determined by comparison with fluorescence titrations and 30–40-fold isotopic dilution, respectively, as described under Materials and Methods (see also Discussion for the assumption involved).

Pooling the data from 20 [^{3}H]-α-toxin assays, 11 fluorescence titrations with α-toxins, 24 [^{3}H]AcCh binding curves, and 4 AcCh fluorescence titrations on 12 preparations of *Torpedo* membranes, we find a stoichiometry of 1.11 ± 0.18

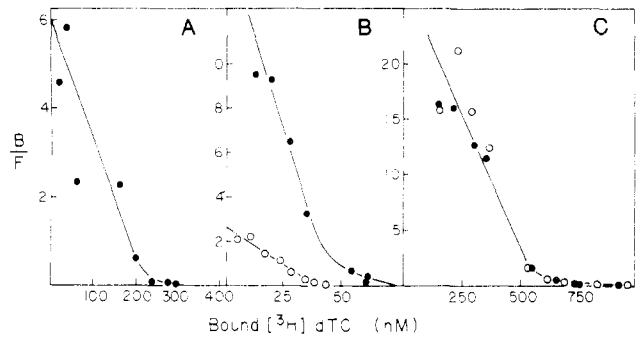

FIGURE 8: Effect of *N*-ethylmaleimide, low salt, and local anesthetics on specific [^{3}H]dTC binding. (A) *N*-Ethylmaleimide (10 mM) was added to the initial homogenization of *T. californica* tissue, and membranes were prepared as usual. Equilibrium binding of [^{3}H]dTC to these membranes (350 nM α-toxin sites) is shown. (B) Binding of [^{3}H]dTC to *T. californica* membranes (100 nM α-toxin sites) was determined in TPS (○) or in a low-salt buffer (20 mM NaCl and 5 mM NaP_i, pH 7) (●). (C) Binding of [^{3}H]dTC to *T. californica* membranes (1000 nM α-toxin sites in TPS) was measured in the presence (●) and absence (○) of 10 μM dimethisoquin.

α-toxin sites/AcCh site. Also, 7 determinations of the number of high-affinity dTC sites gave 0.41 ± 0.01 high-affinity dTC site/α-toxin site, which is 0.46 ± 0.07 high-affinity dTC site/AcCh site by comparison to the α-toxin/AcCh stoichiometry. The number of low-affinity sites determined by a nonlinear least-squares fit of the data in Figure 2 gave 0.49 ± 0.12 low-affinity dTC site/α-toxin site and 1.10 ± 0.17 α-toxin sites/total dTC sites. It is difficult to obtain precise estimates of the number of low-affinity dTC sites due to the large amount of nonspecific binding observed with high free [^{3}H]-dTC concentrations.

Examination of Conditions That Might Modify the dTC Equilibrium Binding Function. We wished to determine whether either the high- or low-affinity dTC binding components could be altered by factors known to alter the structural and functional properties of the membrane-bound *Torpedo* receptor. First, we examined the effects of sulfhydryl reagents that are known to interconvert monomer (9 S) and dimer (12 S) forms of the *Torpedo* receptor (Chang & Bock, 1977; Hamilton et al., 1977; Sobel et al., 1977). *Torpedo* postsynaptic membranes prepared under our standard conditions yield upon detergent solubilization a mixture of 9S and 12S forms of the *Torpedo* receptor (Sobel et al., 1977; R. Neubig, unpublished experiments). Membranes prepared by our standard procedure with the additional inclusion of 10 mM *N*-ethylmaleimide in the homogenization medium yield upon detergent solubilization over 90% of the receptor in the dimeric (12 S) form (Chang & Bock, 1977; R. Neubig, unpublished experiments). Although we did not characterize extensively the binding of [^{3}H]dTC to these membranes, the number of high-affinity binding sites was about half the number of α-toxin sites and a low-affinity binding component was present (Figure 8A). These results indicate that the biphasic nature of the dTC binding function is not sensitive to the conditions that alter the equilibrium between monomeric and dimeric forms of the receptor.

When the equilibrium binding of [^{3}H]dTC was measured at low ionic strength (20 mM NaCl and 5 mM P_i, pH 7.0), the number of high-affinity sites was found to be unaltered, although the affinity for dTC was increased (Figure 8B). At low ionic strength K_{d1} = 2.6 nM, while K_{d1} = 13 nM for the same *Torpedo* membranes in TPS. Because the nonspecific interaction of the [^{3}H]dTC with the *Torpedo* membranes was increased by a factor of 5.5 at low ionic strength, it was not possible to quantify the low-affinity component of the dTC binding function with any greater precision than was possible for *Torpedo* membranes in TPS.

The equilibrium binding of [^{3}H]dTC to *Torpedo* membranes in TPS was measured in the presence of aromatic amine noncompetitive antagonists. Ligands such as dimethisoquin and prilocaine actually increase by a factor of 2 or 3 the affinity with which [^{3}H]AcCh is bound at equilibrium to the *Torpedo* membranes and convert the positively cooperative [^{3}H]AcCh binding function into a hyperbolic one (Cohen et al., 1974). It was of interest to determine whether these ligands would alter the apparent negative cooperativity of [^{3}H]dTC binding and produce hyperbolic binding. The presence of 10 μM dimethisoquin did not result in hyperbolic binding of [^{3}H]dTC (Figure 8C), nor did it have any effect on the low-affinity component of [^{3}H]dTC binding. Small changes in the dissociation constant for high-affinity [^{3}H]dTC binding, such as those observed for [^{14}C]Me_2dTC (Cohen, 1978; Krodel et al., 1979), would not have been detected under these conditions since the concentration of high-affinity binding sites (500 nM) was far in excess of the dTC binding constant (K_{d1} = 20 nM). In other experiments, we established that 10 μM meproadifen or 50 μM proadifen did not displace any of the low-affinity dTC binding. Since these concentrations are sufficient to displace [^{14}C]meproadifen from the local anesthetic binding site in the *Torpedo* membranes (Krodel et al., 1979), we conclude that the low-affinity dTC binding is not to the anesthetic binding site.

Competition between Cholinergic Ligands and [^{3}H]AcCh. In order to identify structural features of dTC related to the biphasic nature of its binding function, we measured the equilibrium binding of [^{3}H]AcCh in the presence of other cholinergic ligands. Both flexible and rigid polyfunctional antagonists were found to exhibit biphasic competition with [^{3}H]AcCh. Dimethyltubocurarine [or more properly named *O*,*O*',*N*-trimethyltubocurarine (Everett et al., 1970)] was characterized by inhibition constants K_{I1} = 0.5 μM and K_{I2} = 10 μM. The inhibition by decamethonium, which is an antagonist of the carbamylcholine-stimulated efflux of $^{22}Na^+$ from *Torpedo* vesicles (Popot et al., 1976), was fit by constants K_{I1} = 0.1 μM and K_{I2} = 1.6 μM. Gallamine triethiodide, a trifunctional antagonist, was characterized by K_{I1} = 2 μM and K_{I2} = 1 mM (Figure 9A). However, the two-site competitive inhibition model does not fit the gallamine data completely (see Discussion).

The effect of mono- and bisquaternary ammonium agonists on the equilibrium binding of [^{3}H]AcCh was also analyzed. Carbamylcholine inhibited the binding of [^{3}H]AcCh in a competitive manner with a single K_I = 100 nM (Figure 9B). It can also be seen in Figure 9 that in the absence of competing ligands, the equilibrium binding of [^{3}H]AcCh cannot be characterized by a single high-affinity binding constant but by a positively cooperative binding function. However, it is clear that the effect of carbamylcholine on the [^{3}H]AcCh binding is quite different from that of gallamine: gallamine, like dTC, results in a biphasic [^{3}H]AcCh binding function while carbamylcholine does not. The observed positive cooperativity of the [^{3}H]AcCh binding function is in distinct contrast with the apparent negative cooperativity of the [^{3}H]dTC binding function.

Another agonist that was characterized was suberyldicholine [$(CH_3)_3N^+CH_2CH_2OOC(CH_2)_6COOCH_2CH_2N^+(CH_3)_3$]. This potent bifunctional agonist stimulates the efflux of $^{22}Na^+$ from *Torpedo* vesicles with a half-maximal effect at a concentration of 0.3 μM (R. Neubig, unpublished experiments), and it reduces the initial rate of binding of ^{3}H-labeled *Naja*

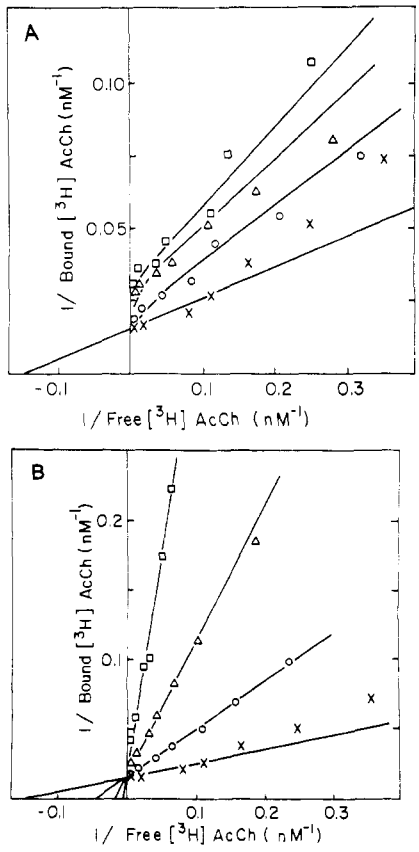

FIGURE 9: Effect of gallamine and carbamylcholine on binding of [^{3}H]AcCh at equilibrium. Suspensions of DFP-treated *T. californica* membranes containing 50 nM α-toxin sites were incubated for 30 min at 22 °C with various concentrations of [^{3}H]AcCh and (A) gallamine triethiodide [(×) 0; (○) 10; (△) 100; (□) 300 μM] or (B) carbamylcholine [(×) 0; (○) 0.2; (△) 0.6; (□) 2 μM]. Specific AcCh binding was determined by filtration as described under Materials and Methods. (A) Lines are calculated curves for the two-site competition model (see Results) with K_D = 7 nM for AcCh and K_{I1} = 2 μM and K_{I2} = 1 mM for gallamine, and (B) straight lines are drawn through the data points for carbamylcholine.

α-neurotoxin at a concentration of ~10 nM (Barrantes, 1978). The inhibition of the equilibrium binding of [^{3}H]AcCh indicated that suberyldicholine was bound with a slightly higher affinity than AcCh. The competition data could be reasonably well fit by a single binding constant, K_I = 10 nM, but they could be better fit by two distinct binding constants differing by a factor of 3–5 (K_{I1} = 5 nM and K_{I2} = 25 nM). The available data are not of sufficient precision to conclude that this bifunctional agonist does interact with two distinct binding constants with the cholinergic binding site, and further studies of other bifunctional agonists will be necessary to clarify this matter.

Discussion

This paper is the first study of the direct binding of dTC to the nicotinic cholinergic receptor. The equilibrium binding of [^{3}H]dTC by the *Torpedo* postsynaptic membranes in TPS is characterized by three components: by a nonspecific interaction with the membranes characterized by a partition coefficient, P = 3%/(mg of protein mL), and by two sites, each present in equal amounts but binding dTC with a high (K_d = 35 nM) and low (K_d = 7 μM) affinity. Both the low- and high-affinity binding of [^{3}H]dTC are reversible and are displaced by the cholinergic agonist carbamylcholine (Figure 3) and by α-BgTx (Figure 1), but not by local anesthetic noncompetitive antagonists (Figure 9). Comparison of the residual binding of [^{3}H]dTC to *Torpedo* membranes in the presence of α-BgTx or high concentrations of nonradioactive dTC reveals that dTC displaces ~5% more [^{3}H]dTC than does α-BgTx (Figure 1). This small amount of binding is not displaced by concentrations of local anesthetics sufficient to displace [^{14}C]meproadifen from the local anesthetic and histrionicotoxin binding site in the *Torpedo* membranes. Although it is possible that this small amount of binding "displaceable" by dTC but not by α-BgTx is associated with a low-affinity specific binding site in the membranes, we feel it is more likely that the high dTC concentrations reduce the nonspecific partitioning of dTC into the membranes and in the following discussion we focus on the saturable binding of [^{3}H]dTC that is displaceable by α-BgTx.

We will discuss later the fact that the observed dTC binding function does not in itself provide evidence for physically distinct sites binding dTC with high and low affinity. However, in the first part of the discussion we will refer to sites binding dTC with high or low affinity.

The inhibition of [^{3}H]AcCh binding by dTC (Figure 4) can be explained over a 300-fold range of dTC concentrations by a competitive model which takes into account the two binding constants for dTC. Furthermore, the binding constants estimated from the competition experiment (15 nM and 7 μM) agree very well with the values from the direct binding experiments. The fact that the dTC binding function must be characterized by both high- and low-affinity components explains the paradoxical observations that very high concentrations of dTC (100 μM in Figure 4) are necessary to completely displace AcCh from the site binding dTC with low affinity while at an intermediate concentration of dTC (1 μM) its interaction with its high-affinity site is such that it appears to displace AcCh noncompetitively. This effect of dTC is not a result of noncompetitive inhibition, and, in fact, the concept of noncompetitive inhibition used by enzymologists is not applicable to ligand binding studies. Noncompetitive inhibition of enzymes is due to the formation of nonproductive ternary complexes of enzyme, substrate, and inhibitor in which substrate binding is not affected [see, for example, Segel (1975)].

The observed competition between AcCh and dTC can be accounted for by a simple model that assumes that dTC and AcCh bind to a common site. This model predicts that the total number of high- and low-affinity dTC sites should be equal to the number of high-affinity AcCh binding sites present in the isolated *Torpedo* membranes. We determined by the use of radiochemical methods and by the use of an independent fluorescence assay that this prediction is correct. The combined results of these two assays lead to the conclusion that the number of AcCh binding sites is equal to 0.93 ± 0.15/α-neurotoxin site and that the total number of dTC binding sites is equal to 0.93 ± 0.12/α-toxin site.

In determining the number of sites of [^{3}H]AcCh and [^{3}H]-dTC, we took particular care in the determination of the radiochemical purity and in the specific activities of the ligand. The specific activity of the [^{3}H]dTC depended on the purity of the nonradioactive dTC used to dilute the radiochemical tracer (90% by TLC on system III) and the radiochemical purity of the tracer itself (93% by TLC on system II). The number of high-affinity dTC sites was half the number of AcCh sites when determined radiochemically. The number of binding sites for the α-neurotoxins as well as for AcCh were determined by the use of a fluorescence titration assay.

The fluorescence titration assay described here is a rapid and reproducible means to determine receptor site concentrations without many of the difficulties associated with the use

of radiochemicals. The accuracy of the results depends only on the use of ligands having a high affinity for the receptor and on the careful determination of the chemical purity and quantities used. It is possible to estimate the error in the determination of the AcCh site concentration when the fluorescence assay is used with a ligand such as AcCh that is bound with a dissociation constant of ~15 nM. At a receptor site concentration of 0.2 μM, over 84% of the added AcCh will be bound at half-occupancy, resulting in less than a 20% overestimation of the number of AcCh sites. Most titrations were done at site concentrations of 0.4–0.6 μM, where the overestimation is less than 5–10%. The acetylcholine used was pure as determined by high-voltage electrophoresis and dry as described under Materials and Methods. The concentration of α-neurotoxin binding sites was determined by the use of three different α-neurotoxins. All α-toxins were greater than 98% pure as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. A potential source of error in the assay was that the different α-toxins bind at different rates. α-BgTx, in particular, binds relatively slowly, and it was necessary to allow times as long as 30 min for the fluorescence signal to reach equilibrium. For the three different α-neurotoxins, the concentration of binding sites in the *Torpedo* membranes differed by less than 10% and also differed from the concentration of AcCh sites by less than 15%. Furthermore, the fluorescence titration with dTC gave results indicating that dTC interacts with high affinity with only half the AcCh or α-BgTx binding sites.

The ratio of AcCh to α-neurotoxin binding sites in the *Torpedo* nicotinic receptor has been a source of considerable disagreement. Determination of site stoichiometries for the receptor isolated in detergent solution is particularly difficult because the presence of detergents dramatically reduces the affinity with which reversible agonists are bound (Sugiyama & Changeux, 1975; Chang & Bock, 1979). However, there is also no agreement concerning the ratio of AcCh to α-neurotoxin sites in the *Torpedo* postsynaptic membranes, with reports of either 0.5 (Raftery et al., 1975; Schimerlik et al., 1979) or 1 (Weber & Changeux, 1974b; Sugiyama & Changeux, 1975; Damle et al., 1976) AcCh bound per α-toxin. It has recently been reported (Weiland et al., 1979) that one molecule of *p*-(trimethylammonium)benzenediazonium fluoroborate, an alkylating analogue of phenyltrimethylammonium, is bound per α-neurotoxin site. One possible source of the uncertainties concerning the relative stoichiometry of ligand binding sites is the inaccurate specific activities reported by radiochemical companies [see also Chang & Bock (1977)] as well as the lack of monitoring of radiochemical purity during routine use. However, it is also possible that the disagreements reflect differences in the ligand binding properties of the membrane-bound *Torpedo* receptor prepared in different laboratories.

The simplest interpretation of the 1:1 stoichiometry of AcCh and dTC binding and their mutual inhibition is that they bind competitively to the same sites. An allosteric inhibition model could also generate these results, but such an explanation seems unnecessary in terms of the data presented here and unwarranted in terms of the following observations. Interaction of both dTC and AcCh with *Torpedo* membranes results in a common conformation of the AcCh receptor, as indicated by its high-affinity equilibrium binding of [^{14}C]meproadifen (Krodel et al., 1979) and the kinetics of [^{3}H]AcCh binding (Cohen, 1978; N. Boyd, unpublished experiments). The allosteric mechanism predicts that dTC and AcCh should result in different receptor conformations while the competitive model is consistent with the two ligands producing a common equilibrium receptor conformation [see also Grunhagen & Changeux (1976)].

The existence of a ligand binding function consistent with multiple sites (or negative cooperativity) is not unique to dTC. Gallamine triethiodide exhibits a biphasic inhibition of [^{3}H]-AcCh binding (Figure 9A), and Eldefrawi & Eldefrawi (1977) reported apparently noncompetitive inhibition of AcCh binding by 10 μM gallamine triethiodide. Also, its inhibition of the binding of [^{125}I]-α-toxin was characterized by a Hill coefficient of 0.6 (Weiland & Taylor, 1979). A fluorescence study with 1,10-bis(3-aminopyridino)decane suggests multiple affinities for this ligand's interaction with *Torpedo* membranes (Bode et al., 1979). Decamethonium displaces [^{3}H]AcCh with two affinities differing by a factor of 20 [see also Gibson (1976)], and this ligand and an extensive series of methylene-linked bisquaternary ammonium compounds inhibited the binding of [^{125}I]-α-toxin as if they bind to the receptor with negative cooperativity (Weiland & Taylor, 1979). Although decamethonium is often a nicotinic partial agonist, it is an antagonist of the cholinergic permeability response of the isolated *Torpedo* vesicles (Popot et al., 1976). The inhibition of [^{3}H]-AcCh binding by suberyldicholine, an agonist on the *Torpedo* vesicle preparation, could be accounted for by binding constants differing by less than a factor of 5. While the bisquaternary ammonium compounds generally interact with the receptor in a manner consistent with two binding constants, ligands such as carbamylcholine (Figure 9B; Eldefrawi & Eldefrawi, 1977) and nicotine (Gibson, 1976; Eldefrawi & Eldefrawi, 1977) do displace [^{3}H]AcCh with a single affinity. Experiments with monoquaternary ammonium antagonists or other bisquaternary ammonium agonists will be necessary to determine whether the two apparent affinities are related to the bifunctional nature of the ligands or to the fact that they are antagonists.[2]

A more detailed analysis of the inhibition of [^{3}H]AcCh binding by dTC (Figure 4) and by gallamine (Figure 9A) indicates a lack of fit by the simple two-site competitive model at low AcCh–low inhibitor concentrations and at high AcCh–high inhibitor concentrations, particularly for gallamine. Much of this discrepancy is due to the fact that the equilibrium binding of [^{3}H]AcCh in the absence of inhibitors in those experiments is not hyperbolic but rather positively cooperative, characterized by Hill coefficients of 1.3 ± 0.1. Such a binding function has been reported by various research groups [for example, Weber & Changeux (1974b), Cohen et al. (1974), Schiebler et al. (1977), and Eldefrawi et al. (1978)] but not by all (Raftery et al., 1975). Systematic errors such as those caused by radiochemical impurities can cause the appearance of positive cooperativity even when the ligand binding function is hyperbolic (Builder & Segel, 1978). We have observed cooperative binding of [^{3}H]AcCh for experiments where the radiochemical purity of the [^{3}H]AcCh has been monitored and where impurities due to [^{3}H]AcCh hydrolysis during the course of the experiment cannot account for the nonhyperbolic binding function. The inclusion of positive cooperativity in the AcCh binding in a cooperative two-conformation–two-site competition model [model II in Gibson (1976)] can give a better fit of the data, but this is to be expected since it has

[2] Recent experiments with two monoquaternary antagonists, dihydro-β-erythroidine and triethyl(4-phenylbutyl)ammonium iodide, showed two K_I values (1 μM and 40 μM) for the former and one K_I (50 μM) for the latter. These results indicate that (1) multiple affinities are not a general characteristic of antagonists and (2) two positive charges are not necessary to generate multiple affinities.

four additional adjustable parameters. Minimally, the model introduces two new parameters if the receptor conformational isomerization constant and the dissociation constants for the binding of AcCh to each conformation are determined independently from the kinetics of binding of [^{3}H]AcCh (Cohen & Boyd, 1979; N. D. Boyd and J. B. Cohen, unpublished experiments). Full analysis of the interaction of antagonists with the AcCh binding sites will be most effectively completed when direct measurement of ligand binding kinetics permits a definition of their interactions with different receptor conformations. At this time it is more useful to discuss the possible origins of the biphasic dTC binding function.

There are several physical situations that can lead to the two affinities observed for dTC binding: (1) two populations of receptors, (2) steric effects causing a half-sites phenomenon, (3) allosteric negative cooperativity of binding, or (4) two distinct binding sites on each receptor molecule.

Although there is little information concerning possible sources of receptor heterogeneity such as junctional and extrajunctional receptors in *Torpedo*, the nicotinic receptor extracted by detergents from *Torpedo* electric tissue does appear heterogeneous in the sense that it exists in monomeric and dimeric forms (Chang & Bock, 1977; Hamilton et al., 1977). However, *Torpedo* membranes prepared in *N*-ethylmaleimide yield only the dimeric form of the receptor, but dTC is still bound with high affinity to only half the α-toxin sites in those membranes (Figure 8B). Thus, this heterogeneity cannot explain the biphasic dTC binding function. A second apparent heterogeneity in the *Torpedo* membranes is found in the fact the membrane-bound receptor exists in conformations with different affinities for AcCh (Weber et al., 1975). However, even in the absence of cholinergic ligands, the two conformations are in equilibrium with each other with about 20% of the sites binding AcCh with high affinity and 80% with low affinity (Heidmann & Changeux, 1979; Cohen & Boyd, 1979). This ratio does not agree with the equal numbers of high- and low-affinity dTC sites, and because the conformations are interconvertible, the equilibrium binding of dTC should be characterized by a single apparent K_d just as is seen for AcCh. Thus, interconvertible conformations cannot explain the two affinities observed for the equilibrium binding of [^{3}H]dTC.

A theoretical description of the binding of multivalent ligands (DeLean et al., 1979) points out that apparently heterogeneous binding curves can result from steric effects when multivalent ligands interact with a homogeneous population of multivalent binding sites. For example, if two AcCh binding sites are close together, two bulky dTC molecules might not fit together without interfering with each other (Figure 10A), while two AcCh or an AcCh and a dTC would not interfere. An alternative model could be where two AcCh sites share a single accessory site (Figure 10B). Both of these models are compatible with two apparent K_d values and the equal number of total dTC sites and AcCh and toxin sites, but they also make the testable prediction that the AcCh sites are very close together ($\sim$2–3 nm), which is surprising since two α-toxin molecules can bind to them without much interference.

The other possible factors contributing to the heterogeneous dTC binding function can be discussed with reference to the known structure of the *Torpedo* receptor. The monomeric form of the *Torpedo* nicotinic receptor, characterized by a molecular weight of $\sim$250 000 under nondenaturing conditions, is made up of four peptides [α, M_r 41 000; β, M_r 50 000; γ, M_r 60 000; δ, M_r 65 000 [reviewed in Heidmann & Changeux (1978)]]. The α subunit is associated with the AcCh

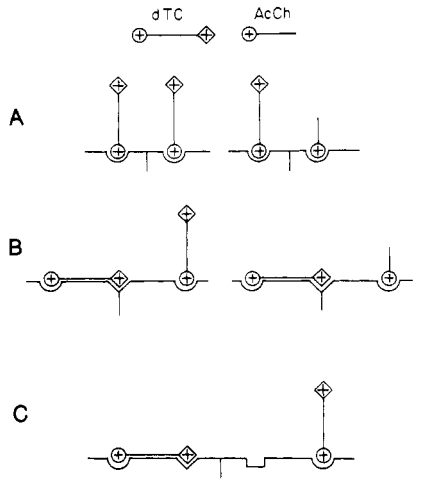

FIGURE 10: Diagrams of possible models explaining the biphasic dTC binding. (A) The repulsive interaction between the two "free" positive charges of adjacently bound dTC molecules increases the energy of binding of the second dTC. An AcCh molecule lacking a second positive charge does not interfere with the binding of a second AcCh or a dTC at the adjacent site. (B) The high-affinity dTC binding is due to the interaction of both positive charges of the first dTC molecule bound to the receptor, while the second molecule can only interact with one positive charge since the "accessory site" is already occupied. The accessory site is *not* the local anesthetic binding site (see text). (C) This model represents two distinct binding sites on one receptor molecule and thus is different from models (A) and (B), which can be classed as steric inhibition models. Identical AcCh sites (semicircles) with different accessory sites suggest a mechanism whereby AcCh binds to the receptor with a single affinity but dTC binds with two very different affinities.

binding site since it is labeled by the covalent affinity labels (maleimidobenzyl)trimethylammonium (Weill et al., 1974) and (trimethylammonium)benzenediazonium (Weiland et al., 1979). Since there is one α-neurotoxin site or (trimethylammonium)benzenediazonium bound per 125 000 daltons, a proposed subunit stoichiometry is $\alpha_2\beta\gamma\delta$ (Reynolds & Karlin, 1978). Damle & Karlin (1978) have shown that (maleimidobenzyl)trimethylammonium is bound by half as many sites as α-BgTx and that reduced binding is due to two classes of sites and not to negative cooperativity. Hence, although negative cooperativity is a possible explanation for the biphasic dTC binding function, it seems more likely to be due to the existence of cholinergic binding sites that are differentiated by their capacity to bind dTC but not AcCh. The distinction between ligands containing one and more than one charged nitrogen could be due to differences in an accessory site (Figure 10C) adjacent to the AcCh binding site (Chao et al., 1975). Two different sites could occur on one receptor molecule if they were on different subunits or on the same subunit in different conformations (Figure 10C). It should be noted that the two α subunits of a receptor of composition $\alpha_2\beta\gamma\delta$ (or $\alpha_2\beta$ or $\alpha_2\beta\delta$) can never be in exactly the same environment. Further studies will be necessary to determine unambiguously the origin of the biphasic dTC binding function.

Perhaps the most intriguing question raised by the observed dTC equilibrium binding function is the relationship between occupancy of the receptor by dTC and blockage of the cholinergic permeability response. It has been reported (Popot et al., 1976) that dTC inhibits competitively the carbamylcholine-stimulated efflux of $^{22}Na^+$ from the *Torpedo* vesicles with an inhibition constant (K_{ap}) determined by the dose-ratio method of 2.3 μM. Such a result appears to suggest that occupancy of half the receptor sites by dTC bound with high affinity is without effect on function. However, the analysis of the permeability response of the *Torpedo* vesicles should

be performed on the millisecond rather than the second–minute time scale because at those longer times the flux response is not a direct measure of channel activation. The activation of only a fraction of the receptors in each vesicle results in rapid equilibration of $^{22}Na^+$ between intravesicular and extravesicular space (Neubig et al., 1979; R. R. Neubig and J. B. Cohen, unpublished experiments). In the presence of spare receptors it is difficult to distinguish between competitive and noncompetitive antagonism. For example, on the 10-s time scale, dTC appears to inhibit the carbamylcholine-stimulated efflux competitively with a K_{ap} of 2 μM, but it inhibits the response associated with the partial agonist phenyltrimethylammonium noncompetitively with a K_{ap} equal to 0.2 μM. dTC inhibits noncompetitively the agonist-stimulated efflux of $^{22}Na^+$ from cholinergic vesicles isolated from *Electrophorus* (Hess et al., 1976) and from chick skeletal muscle in culture (T. Gibbs, personal communication). At least in the *Torpedo* vesicles the apparent noncompetitive antagonism is probably a reflection of the complicated interaction between dTC and the cholinergic binding site. It is striking that we have found no evidence that dTC interacts at equilibrium with the local anesthetic binding site in the *Torpedo* membranes: 10 μM dimethisoquin does not inhibit the low-affinity [^{3}H]dTC binding (Figure 8C), and 100 μM dTC does not inhibit the binding of [^{14}C]meproadifen (N. S. Shera and J. B. Cohen, unpublished experiments). The fact that equilibrium binding of dTC by the cholinergic receptor is characterized by two affinities differing by almost a factor of 500 explains some of the anomalies reported previously in ligand binding studies and provides a basis for the analysis of the functional consequences of receptor occupancy by this classic nicotinic antagonist.

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Hybrid Glycosaminoglycans Synthesized by Monolayers of Chick Embryo Arterial Fibroblasts†

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ABSTRACT: Monolayer cultures of arterial fibroblasts from 13-day chick embryonic aorta incorporated $^{35}SO_4^{2-}$ into glycosaminoglycans containing both glucuronic and iduronic acids. Bacterial chondroitinase ABC converted more than 98% of the $^{35}SO_4$-labeled polymer to mono- or disaccharides, including (1) *N*-acetyl-D-galactosamine 4-sulfate, (2) $\Delta^{4,5}$-glucuronic acid 2- or 3-sulfate→*N*-acetylgalactosamine 6-sulfate, and (3) the unsaturated disaccharides normally obtained from chondroitin 4-sulfate and chondroitin 6-sulfate sequences. Chondroitinase AC converted only 77% of the $^{35}SO_4$-labeled polymer to the same mono- and disaccharides and yielded, in addition, the following oligosaccharide products: (1) $\Delta^{4,5}$-glucuronic acid→*N*-acetylgalactosamine 4- or 6-sulfate→iduronic acid→*N*-acetylgalactosamine 6- or 4-sulfate; (2) *N*-acetylgalactosamine 4-sulfate→iduronic acid 2- or 3-sulfate→*N*-acetylgalactosamine 6-sulfate; (3) $\Delta^{4,5}$-glucuronic acid→*N*-acetylgalactosamine 4-sulfate→(iduronic acid→*N*-acetylgalactosamine 4-sulfate)$_2$; (4) $\Delta^{4,5}$-glucuronic acid→*N*-acetylgalactosamine 4- or 6-sulfate→(iduronic acid→*N*-acetylgalactosamine 6- or 4-sulfate)$_2$; (5) higher oligosaccharides containing iduronic acid and *N*-acetylgalactosamine 4-sulfate.

Three types of polymeric structures were distinguished in early studies on the sulfated glycosaminoglycans of connective tissues: chondroitin 4-sulfate, composed of repeating GlcUA→GalNAc-4-SO_4 disaccharides (A units),[1] chondroitin 6-sulfate, composed of repeating GlcUA→GalNAc-6-SO_4 disaccharides (C units), and dermatan sulfate, composed of repeating IdUA→GalNAc-4-SO_4 disaccharides (B units) (Jeanloz, 1970; Mathews, 1975). More recently, evidence has been presented suggesting that chondroitin sulfate is a copolymer of A and C disaccharide units (Habuchi et al., 1973; Kimata et al., 1974; Seno et al., 1974, 1975), and it has also been recognized that dermatan sulfate is a hybrid structure composed predominantly of B units but also containing A and C units (Fransson & Malmström, 1971; Habuchi et al., 1973; Cöster et al., 1975; Malmström et al., 1975a). All of these polymers occur as protein–polysaccharides in which the repeating disaccharide units are linked to a core protein through a unique galactosyl–galactosyl–xylose linkage region (Rodén & Horowitz, 1978).

Present evidence indicates a close biosynthetic relationship among the three disaccharide types found in these connective tissue polysaccharides (Rodén & Horowitz, 1978). Chondroitin, the initial biosynthetic product, is made up of repeating GlcUA→GalNAc disaccharide units and is attached to the core protein through the usual linkage region. It serves as the unsulfated polymeric precursor for copolymers of A, B, and C units. Specific sulfotransferases catalyze the transfer of

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[1] Abbreviations used: GlcUA, D-glucuronic acid; IdUA, L-iduronic acid; GalNAc, *N*-acetyl-D-galactosamine; unit A, GlcUA→GalNAc-4-SO_4; unit B, IdUA→GalNAc-4-SO_4; unit C, GlcUA→GalNAc-6-SO_4; ΔDi-4S, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-β-D-*gluco*-hex-4-enopyranosyluronic acid)-4-*O*-sulfo-D-galactose; ΔDi-6S, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-β-D-*gluco*-hex-4-enopyranosyluronic acid)-6-*O*-sulfo-D-galactose.

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