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Solubilization of an α-Bungarotoxin-Binding Component from Rat Brain†

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ABSTRACT: Binding of [^{125}I]-α-bungarotoxin to rat brain was investigated. Picomole quantities of specific toxin binding sites per gram of fresh tissue were found in particulate preparations as well as detergent extracts of whole brain. The toxin-binding macromolecules can be solubilized in low concentrations of Triton X-100. Specific binding occurs to a single class of sites with a dissociation constant of 5.6×10^{-11} M. The association rate constant in 10 mM sodium phosphate, pH 7.4, was determined to be 6.8×10^5 M^{-1} s^{-1}; the half-life of the complex was found to be 5.1 h, corresponding to a dissociation rate constant of 3.8×10^{-5} s^{-1}. The binding macromolecules resemble peripheral nicotinic acetylcholine receptors in toxin binding kinetics, solubility, isoelectric point, and hydrodynamic properties.

Considerable information on the biochemistry of nicotinic acetylcholine receptors from peripheral tissues is available at present. Much of the progress in this field has been due to the use of α-bungarotoxin (αBgt[1]) and related neurotoxins which bind with high affinity and selectivity to cholinergic receptors in skeletal muscle and electric organ (Hall, 1972; Changeux, 1975). In contrast, little is known concerning αBgt-binding components of the central nervous system and their relationship with nicotinic receptors.

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[1] Abbreviations used: αBgt, α-bungarotoxin: [^{125}I]-αBgt, [^{125}I]-α-bungarotoxin; CM-cellulose, carboxymethyl-cellulose.

The recent literature contains several reports on the binding of αBgt to particulate brain preparations (Salvaterra and Moore, 1973; Schleifer and Eldefrawi, 1974; Eterović and Bennett, 1974; McQuarrie and Mahler, 1975); while a study of intact membranes is useful and can provide important clues regarding the subcellular localization as well as the drug affinities of toxin binding sites, a further biochemical characterization of the binding substances depends upon their solubilization in an active form. The present communication describes the extraction of toxin binding activity by means of the nonionic detergent Triton X-100. It is shown that binding of αBgt occurs to a specific macromolecule which in several respects resembles a peripheral nicotinic acetylcholine receptor.

Experimental Procedure

Extraction of Rat Brain. Male rats (200–400 g) were decapitated; their brains were removed quickly and homogenized in 10 volumes of 10 mM sodium phosphate, pH 7.4. After centrifugation at 100 000*g* for 30 min, the supernatant was discarded, and the crude particulate fraction was homogenized in 10 volumes (with respect to original tissue weight) of 10 mM sodium phosphate, pH 7.4, 1.0% Triton X-100, and stirred for 1 h at room temperature. Another centrifugation at 100 000*g* for 30 min separated detergent-insoluble matter from the brain extract which contains the toxin binding activity. When necessary the extract was concentrated by ultrafiltration on an XM50 membrane.

Binding Assay. α-Bungarotoxin (αBgt) was purified from *Bungarus multicinctus* venom (Miami Serpentarium) by chromatography on CM-cellulose (Clark et al., 1972), and labeled with ^{125}I using the Chloramine-T procedure (Hunter and Greenwood, 1962). Isolation of [^{125}I]-αBgt of high specific activity is described below.

Binding of [^{125}I]-αBgt to Triton extracts was measured using the DEAE-cellulose disc method (Schmidt and Raftery, 1973). Adsorption of free [^{125}I]-αBgt to discs was of the order of 0.5–2%; occasionally toxin preparations were passed through small columns of DEAE-cellulose prior to use to reduce this background. Linear increase of bound radioactivity was in general seen only up to 10 mg of brain tissue equivalent per disc, possibly because the binding macromolecules are competed off the DEAE-cellulose by excess nonspecific protein. Nonspecific binding was determined by assaying samples previously exposed to native αBgt. Specific binding is defined as binding inhibited by pretreatment with native toxin. Receptor concentrations are given in concentrations of αBgt binding sites.

Protein was assayed by the method of Lowry et al. (1951); precipitates, which form in Triton-containing media upon addition of Folin reagent, are removed by centrifugation.

Results

Purification and Properties of Iodinated αBgt. To ensure efficient utilization of radioactive label and to minimize multiple labeling of individual toxin molecules, a 20–50-fold molar excess of toxin over iodine is used for the iodination of αBgt. This results in approximately 90% incorporation of radioactivity into polypeptide as shown by chromatography on Sephadex G-10. Radiotoxin is separated from the excess of unreacted toxin by chromatography on CM-cellulose (Figure 1); following a suggestion by Vogel et al. (1972), bovine serum albumin is included in the chromatography medium to prevent loss of radiotoxin due to adsorption to the column. Several radioactive compounds are obtained, two of which display high binding activity when tested with *Torpedo californica* acetylcholine receptor. The fractions containing the major peak were pooled and used in the binding studies. Since the iodotoxin yield amounts to a few micrograms only, protein measurements and therefore direct determination of specific activity were not feasible. Instead, specific activity was measured by quantitation of binding of iodotoxin to a preparation of *Torpedo* receptor, before and after diluting the radioactive αBgt with a known quantity of native toxin. Specific activity was checked periodically and determined for each day by extrapolation; the radiotoxin was found to retain full binding activity through several ^{125}I half-lives.

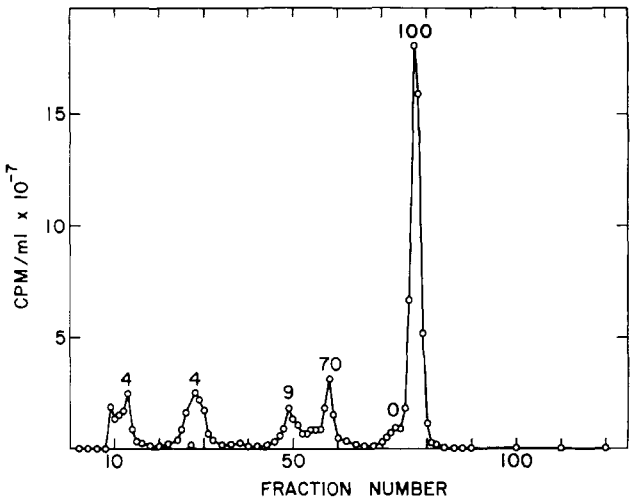

FIGURE 1: Fractionation of iodinated αBgt. Labeling of 0.95 mg of pure αBgt with 10 mCi carrier-free ^{125}I was carried out by the Chloramine-T procedure (Hunter and Greenwood, 1962). The iodinated product was freed of unreacted iodide by chromatography on Sephadex G-10 and applied to a column of CM-cellulose (Whatman CM52) (1.4 × 106 cm) equilibrated in 3 mM sodium phosphate, pH 7.2. Elution was achieved by means of a sodium chloride gradient, started immediately after sample application, with 1.05 l. of 3 mM sodium phosphate pH 7.2, 0.01% sodium azide, and 2 mg per ml of bovine serum albumin in the mixing vessel, and 1.05 l. of the same, including 0.2 M sodium chloride, in the reservoir. Fractions of 10.2 ml were collected and aliquots of 10 μl counted in a dioxane-based scintillator fluid. Numbers indicate relative binding activities of major peaks, measured with purified acetylcholine receptor from *Torpedo californica* electric organ.

Binding to Particulate Fraction. Brain homogenates were incubated with saturating concentrations of [^{125}I]-αBgt. After centrifugation, radioactivity was determined in the supernatant fractions and in pellets washed free of unbound toxin; a value of 0.75 femtomole of specifically bound neurotoxin per mg of original tissue was found.

Binding to Extracts. Binding activity in extracts was determined in a parallel experiment and found to be 0.98 fm (femtomole) per mg, indicating that most, if not all, of the specific toxin binding activity is in fact solubilized by Triton X-100 under the standard conditions (Figure 2). Successful use of the DEAE-cellulose disc assay implies that the neurotoxin receptor from rat brain like its peripheral counterparts is an acidic macromolecule.

To determine optimal extraction conditions, binding activity was measured in extracts obtained with a range of detergent concentrations. As seen in Figure 3 all of the activity was found to be extractable in 0.1% Triton and considerable amounts were solubilized with detergent concentrations as low as 0.02%.

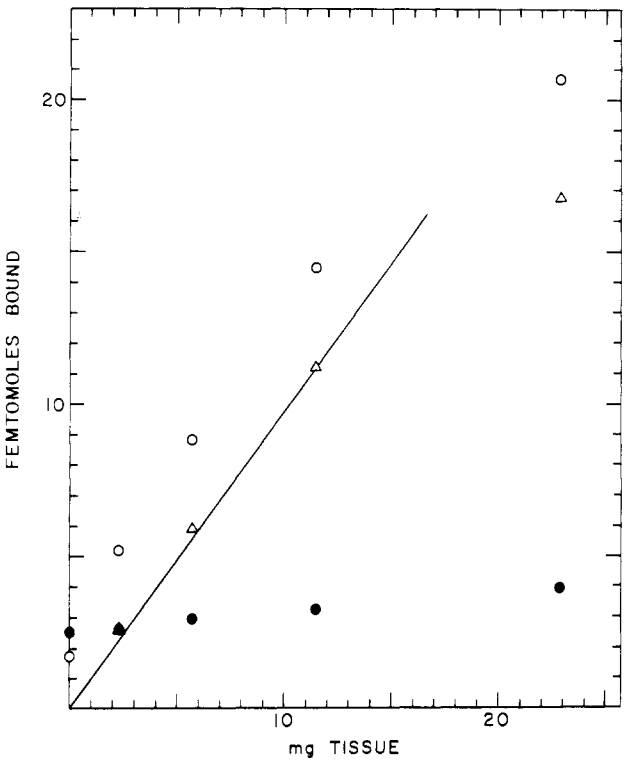

FIGURE 2: Binding of [^{125}I]-αBgt to brain extract. A brain extract was prepared as described in Methods, with 1 g of original tissue corresponding to 3.5 ml of extract. Experimental (○) and control samples (●) were pretreated with 1/200 volume of buffer and 10^{-4} M αBgt per ml, respectively; 0.1-ml aliquots of these extracts and several dilutions thereof were incubated with 0.025 ml each of 7×10^{-8} M [^{125}I]-αBgt for 1.5 h at room temperature. Aliquots of 0.1 ml were then pipetted onto DEAE-cellulose discs, washed, and counted. Bound radioactivities are plotted as a function of tissue weight per disc. Triangles represent data corrected for nonspecific binding, most of which occurs to DEAE-cellulose rather than to brain extract.

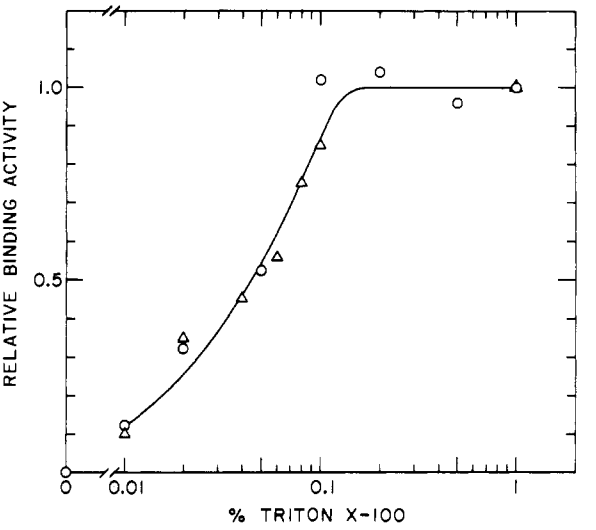

FIGURE 3: Extraction of toxin binding activity as a function of Triton concentration. Brain tissue was homogenized in approximately 30 volumes of 10 mM sodium phosphate, pH 7.4. After stirring at room temperature for 1 h with the indicated concentrations of Triton X-100, insoluble material was removed by centrifugation, and the supernatant solutions assayed for αBgt binding. Values are normalized with respect to binding activity extracted by 1% Triton. Different symbols (○, △) refer to separate experiments.

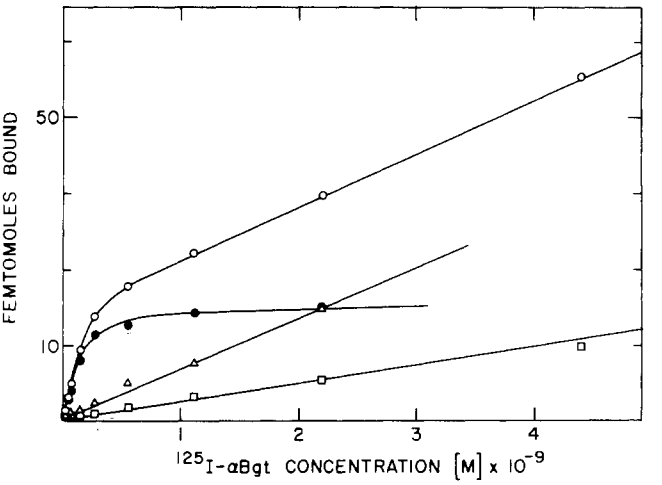

FIGURE 4: Demonstration of a saturable binding component. Brain extract, representing 7.8 mg of original tissue, was incubated with the indicated concentrations of [^{125}I]-αBgt in a total volume of 0.125 ml of 10 mM sodium phosphate, pH 7.4, 0.1% Triton X-100. After overnight incubation at room temperature, 0.1-ml aliquots were assayed for bound radioactivity, using the DEAE-cellulose disk assay (○). A parallel series differed only by treatment of extract with 6×10^{-8} M αBgt prior to addition of [^{125}I]-αBgt (△); in another control, extract was replaced by buffer (□). Data corrected for nonspecific binding are also shown (●).

The existence of a saturable binding component can be demonstrated by measuring bound radioactivity as a function of [^{125}I]-αBgt concentration (Figure 4). After corrections for nonspecific binding of toxin to DEAE-cellulose as well as to brain extract, a high-affinity saturable component is seen amounting to approximately 2.2 fm per mg of tissue. In 20 experiments carried out over a period of 1 year, this value ranged from 0.7 to 2.4, with an average of 1.4.

To obtain a quantitative measure for toxin affinity, the binding data in Figure 4 can be replotted as a function of the concentration of unbound toxin, by subtracting bound toxin from total toxin; a half-saturation value of approximately 4×10^{-11} M is thus obtained.

The dissociation constant for the interaction of toxin and toxin binding site can also be determined by separately measuring rates of toxin binding and toxin release. Experiments of this kind are described in Figures 5 and 6. The data suggest a single on-rate constant, 6.8×10^5 M^{-1} s^{-1}. The half-life of the receptor-[^{125}I]-αBgt complex is found to be 5.1 h, corresponding to an off-rate constant of 3.8×10^{-5} s^{-1}. Thus a dissociation constant of 5.6×10^{-11} M is calculated, in good agreement with the equilibrium data in Figure 4.

Results of gel permeation and sedimentation velocity analysis are shown in Figures 7 and 8. It is apparent that [^{125}I]-αBgt binds to a macromolecule similar to the acetylcholine receptor from *Torpedo californica* electric organ with respect to its radius of gyration; the bulk of the detergent-solubilized central receptor sediments with catalase (sedimentation coefficient 11.4). Its isoelectric point is 4.9, as seen in Figure 9.

Discussion

The concentration of toxin binding sites described in the present report is in good agreement with values previously published by Salvaterra and Moore (1973), Eterović and Bennett (1974), and McQuarrie and Mahler (1975); it is comparable to receptor densities found in skeletal muscle (Berg et al., 1972; Alper et al., 1974). What concentration

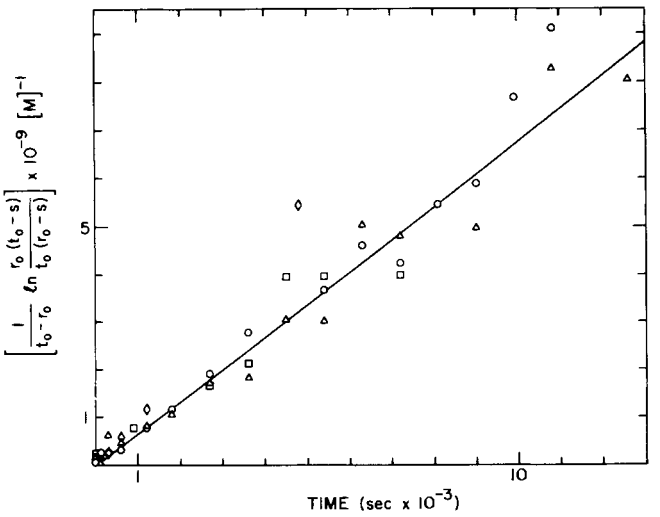

FIGURE 5: Determination of on-rate constant. Brain extract containing "r_0" M toxin binding sites was incubated with "t_0" M of free [^{125}I]-αBgt at room temperature in a total volume of 5 ml of 10 mM sodium phosphate, pH 7.4, 0.1% Triton X-100. Reaction was started by adding radioactive toxin. Aliquots of 0.1 ml were withdrawn at the times indicated and pipetted onto DEAE-cellulose discs which were processed as described in Methods. A control experiment was carried out using brain extract pretreated with native αBgt, to determine the extent of nonspecific binding at any time point; the concentration of specifically bound toxin is designated "s". Different symbols (○, □, △) refer to independently conducted experiments, "r_0" varied from 0.3 to 1.3 × 10^{-11} M, "t_0" from 1.8 to 3.9 × 10^{-10} M.

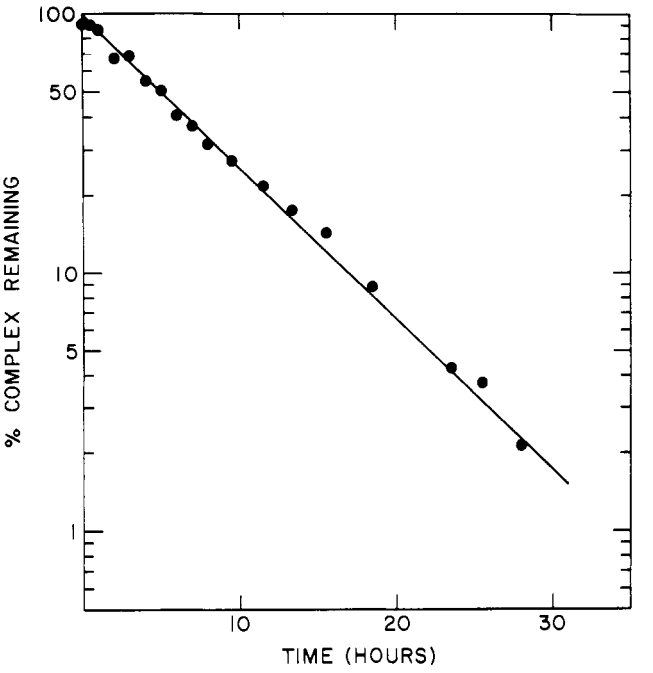

FIGURE 6: Rate of release of bound [^{125}I]-αBgt from binding macromolecules. Brain extract, 1.2 × 10^{-10} M in toxin binding sites, was incubated with 1.0 × 10^{-10} M [^{125}I]-αBgt, in a total volume of 4.5 ml of 10 mM sodium phosphate pH 7.4, 0.1% Triton X-100. After 15 h, 0.5 ml of 5.5 × 10^{-6} M αBgt was added. At the times indicated, 0.1-ml aliquots of the mixture were pipetted onto DEAE-cellulose discs, and the amount of bound [^{125}I]-αBgt was determined. Values are normalized with respect to a control experiment in which buffer was added instead of native toxin, to correct for possible receptor inactivation during the course of the experiment.

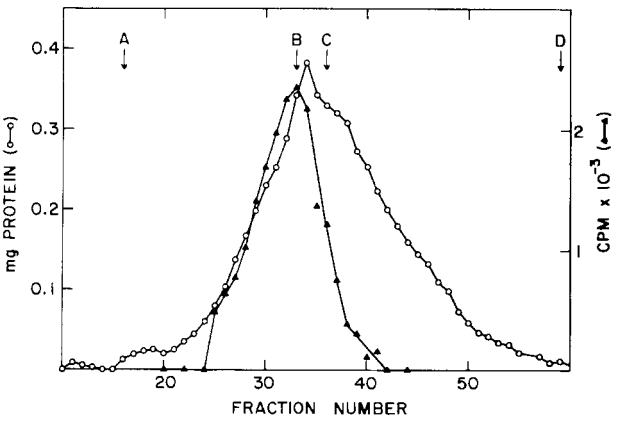

FIGURE 7: Gel permeation chromatography. Brain extract (2.9 ml, corresponding to 7 g of fresh tissue) was chromatographed on a column of Sepharose 4B (2.5 × 79 cm) equilibrated in 10 mM sodium phosphate, pH 7.4, 0.02% sodium azide, 0.1% Triton X-100. Elution was carried out in the same buffer. Fractions of 8.7 ml were collected. Protein assays were done on 0.5-ml aliquots. Aliquots of 0.1 ml were incubated with 0.025 ml of 1.4 × 10^{-9} M [^{125}I]-αBgt for overnight at room temperature, and bound toxin was determined in 0.1 ml of the incubation mixture by the DEAE-cellulose disc assay. Elution positions of markers (indicated by arrows) were determined in separate runs: (A) blue dextran; (B) *Torpedo californica* acetylcholine receptor; (C) catalase; (D) αBgt.

of toxin binding sites should have been expected in the central nervous system? In view of the enormous synapse density of the brain, one might have predicted higher values, possibly approaching the 1000 fm per mg found in *Torpedo* electric tissue (Miledi et al., 1971; Raftery et al., 1972). However, it is important to keep in mind that nicotinic cholinergic synapses constitute only a fraction of all central synapses; furthermore, autoradiographic (Polz-Tejera et al., 1975) and biochemical (J. Schmidt, unpublished) studies indicate that considerable regional differences do exist; certain areas of the central nervous system, such as the retina and the optic tectum of the chick, display receptor levels of up to 50 fm per mg of tissue, comparable to those found by Changeux et al. (1970) and Raftery et al. (1971) in the electric organ of *Electrophorus electricus*. Interestingly, estimates of the concentration of muscarinic binding sites in whole brain exceed the number of αBgt binding sites by one to two orders of magnitude (Burgen et al., 1974; Yamamura and Snyder, 1974).

Eterović and Bennett have reported that [^{125}I]-αBgt prepared by the Chloramine-T procedure does not give reproducible results when used with brain preparations and suggest that oxidation damage of the neurotoxin molecule may occur during iodination. There is no doubt that the Chloramine-T procedure yields a fairly large number of iodinated products when used with pure αBgt as a substrate; some of these are biologically active while others are not. It is conceivable that use of the iodinated product without further purification may give rise to extensive nonspecific binding. At any rate, in our hands purified iodotoxin binds to peripheral and central receptors with comparable specificity (if saturability is accepted as a criterion of specificity). This is of importance since binding levels are low in the central nervous system and iodination with ^{125}I yields a product that is almost two orders of magnitude more radioactive than αBgt containing an equal number of tritium atoms.

Is there a unique species of macromolecule in the brain which binds [^{125}I]-αBgt? Evidence in favor of this assumption is provided by the kinetic analysis of receptor–toxin complex formation which proceeds like a simple bimolecu-

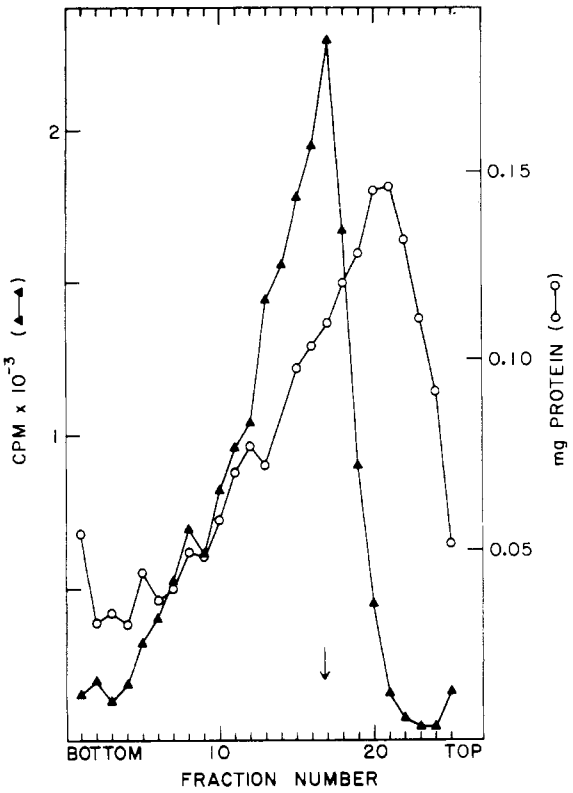

FIGURE 8: Sucrose density gradient centrifugation of brain extract. Brain extract, 0.5 ml, corresponding to 0.65 g of tissue, was layered on a linear gradient of sucrose (20–5%) in 10 mM sodium phosphate, pH 7.4, 0.1% Triton X-100, and centrifuged in a Beckman Spinco SW 27 rotor at 27 000 rpm for 20 h. The gradient was collected in 1.56-ml fractions. Aliquots of 0.1 ml each were assayed for [^{125}I]-αBgt binding as described in Methods. Aliquots of 0.1 ml each were assayed for protein. The position of catalase (arrow) was determined in a parallel run.

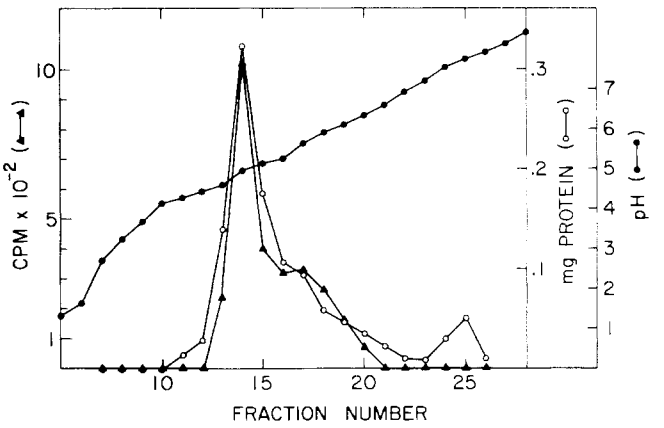

FIGURE 9: Isoelectric focusing of brain extract. Brain extract, corresponding to approximately 0.9 g of tissue, was subjected to isoelectric focusing in a 110-ml LKB column. The sucrose gradient (50/5%, w/v) contained 2.5 ml of ampholine (pH 3.5–10), 0.1% Triton X-100. The experiment was carried out at 5 °C overnight. Fractions of approximately 3.5 ml were collected and their pH's were determined. Portions of 1 ml were dialyzed against 10 mM sodium phosphate, pH 7.4, 0.1% Triton X-100; aggregated material redissolved during dialysis except for some precipitate in fraction 14. Aliquots of 0.5 and 0.1 ml of the dialyzed samples were assayed for protein and [^{125}I]-αBgt binding, respectively.

lar reaction. The on-rate constant is comparable to that determined for the system *Torpedo californica* receptor–αBgt (1.5×10^6 M^{-1} s^{-1} in 20 mM sodium chloride (Schmidt and Raftery, 1974)). Likewise, dissociation of the complex proceeds with a unique rate constant. Chromatographic and sedimentation data are of relatively minor significance in deciding whether a unique toxin receptor exists in brain because the hydrodynamic behavior of the binding activity is expected to primarily reflect the homogeneity of receptor-carrying micelles rather than that of the receptors themselves. Nevertheless, a comparison of toxin binding and protein profiles indicates that activity resides with a select group of macromolecules. While the toxin binding activity from brain co-chromatographs with *Torpedo californica* receptor on Sepharose 4B, it sediments considerably faster on sucrose gradients than the 9.5S species predominant in *Torpedo* extracts. In isoelectric focusing, toxin binding activity peaks at a pH of 4.9, an isoelectric point very close to those determined for electroplax receptors (Raftery et al., 1971, 1972).

Based on the present data no solid claim can be made concerning the identification of the toxin binding molecule as the central nicotinic receptor. Yet this notion is supported by: (a) the consensus that αBgt and related neurotoxins are specific probes for the nicotinic acetylcholine receptor in peripheral synapses; (b) the membrane location of the central toxin binding macromolecule, as shown by its water insolubility and Triton solubility; (c) the similarity of several biochemical properties of central and peripheral toxin receptors.

Neurophysiological evidence and additional experiments on subcellular location, ligand binding properties, and regional distribution of toxin binding sites are necessary to clarify this point. Such experiments are presently being conducted; studies on autoradiographic localization of αBgt in rat and chick brain (Polz-Tejera et al., 1975), in conjunction with some previous observations concerning the sensitivity of the toxin receptor to certain nicotinic drugs as well as the association of binding activity with isolated nerve endings (Salvaterra and Moore, 1973; Eterović and Bennett, 1974; McQuarrie and Mahler, 1975), make it increasingly likely that αBgt and related toxins are highly specific ligands of central nicotinic receptors. If this is in fact the case, it may be of interest to recall that complete solubilization of αBgt binding activity can be effected under conditions used to trim down synaptosomes to "synaptic complexes". Such complexes are prepared by treating nerve endings with 0.1% Triton X-100 and consist of pre- and postsynaptic membrane patches still adhering to each other; they can be purified and have been regarded as highly concentrated preparations of synaptic constituents (De Robertis, 1971). However, in view of the ease with which the nicotinic acetylcholine receptor is solubilized, the possibility has to be considered that neurotransmitter receptors and other important constituents may get lost during detergent maceration of synaptic membranes.

Note Added in Proof

After submitting this manuscript we learned of an additional study of [^{125}I]-αBgt binding to rat brain particulate preparations. The observations reported by P. M. Salvaterra, H. R. Mahler, and W. J. Moore ((1975), *J. Biol. Chem. 250,* 6469–6475) indicate that toxin binding activity is highest in synaptosomal fractions, and thus provide further evidence for the identity of α-bungarotoxin binding sites and nicotinic acetylcholine receptors in the central nervous system.

Acknowledgment

We thank Ms. Mercedes Diaz for skillful technical assis-

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The Subcellular Distribution of Adenylate and Guanylate Cyclases in Murine Lymphoid Cells†

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ABSTRACT: Membrane vesicles can be prepared from murine lymphoid cells by nitrogen cavitation and fractionated by sedimentation through nonlinear sucrose density gradients. Two subpopulations of membrane vesicles, PMI and PMII, can be distinguished on the basis of sedimentation rate. The subcellular distribution of adenylate and guanylate cyclases in these membrane subpopulations have been compared with the distribution of a number of marker enzymes. Approximately 20–30% of the total adenylate and guanylate cyclase activity is located at the top of the sucrose gradient (soluble enzyme), the remainder of the activity being distributed in the PMI and PMII fractions (membrane-bound enzyme). More than 90% of the 5′-nucleotidase and NADH oxidase activities detected in lymphoid cell homogenates are located in PMI and PMII fractions, whereas succinate cytochrome *c* reductase activity is detected only in the PMII fractions. In addition, β-galactosidase activity is distributed in the soluble and PMII fractions of the sucrose density gradients. On the basis of the fractionation patterns of these various enzyme activities, it appears that PMI fractions contain vesicles of plasma membrane and endoplasmic reticulum, whereas PMII fractions contain mitochondria, lysosomes, and plasma membrane vesicles. Approximately 30–40% of the adenylate and guanylate cyclase activities in PMII can be converted to a PMI-like form following dialysis and resedimentation through a second nonlinear sucrose gradient. Adenylate and guanylate cyclases can be distinguished on the basis of sensitivity to nonionic detergents.

The experiments described here are preliminary to developing methods to study how lymphocyte mitogens interact with cell surface membranes and initiate the intracellular biochemical changes that lead to DNA synthesis and cell division. A large number of agents have now been shown to exert selective mitogenic activity on murine bone-marrow-derived (B[1]) (Andersson et al., 1972; Coutinho et al., 1974;

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[1] Abbreviations used: B and T lymphocytes, bone-marrow-derived and thymus-derived lymphocytes, respectively; LPS, bacterial lipopolysaccharide; cyclic GMP, guanosine 3′:5′-monophosphate; PBS, phosphate buffered saline; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; PMI and PMII, the first and second plasma membrane fractions, respectively; NADH, nicotinamide adenine dinucleotide reduced form.