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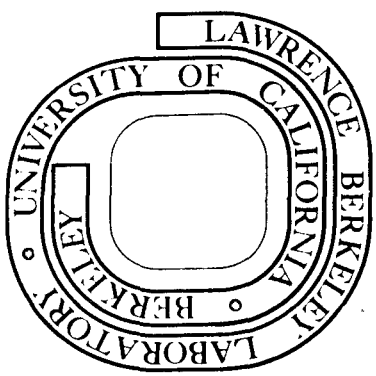

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EFFECTS OF THIO-GROUP MODIFICATION AND CA^{++} ON AGONIST-SPECIFIC STATE TRANSITIONS OF A CENTRAL NICOTINIC ACETYLCHOLINE RECEPTOR[†]

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[1]Abbreviations used: nAChR, nicotinic acetylcholine receptor(s); CNS, central nervous system; DTT, dithiothreitol; ACh, acetylcholine; NEM, N-ethylmaleimide; DTNB, 5,5'-dithio-bis-(2-nitrobenzoic acid); α-Bgt, α-bungarotoxin; [^{3}H]α-Bgt, [^{3}H] labeled α-bungarotoxin; EGTA, ethyleneglycol-bis-(β-amino ethyl ether) N,N'-tetraacetic acid; IC_{50}, concentration of competitor required to block 50% specific [^{3}H] α-Bgt binding; K_D^{app}, apparent dissociation constant for [^{3}H]α-Bgt binding; n_H, Hill number for competitor binding to nAChR; mAChR, muscarinic acetylcholine receptor.

[2]The term 'thio-groups' is used to designate organic sulfur-containing residues, specifically, disulfides and sulfhydryls.

ABSTRACT

Agonist-binding affinities of central nervous system nicotinic acetylcholine receptors (nAChR) are sensitive to the duration of exposure to agonist. These agonist-induced changes in receptor state may be mimicked by appropriate modification of receptor thio-groups and/or by manipulation of solvent ionic composition. In the absence of Ca^{++}, the concentration of acetylcholine (ACh) necessary to prevent half of specific [^{3}H]-labeled α-bungarotoxin binding is ∿1mM for nAChR treated with dithiothreitol (DTT) or N-ethylmaleimide (NEM) (low affinity states), and ∿40 μM for nAChR treated with dithio-bis-nitrobenzoic acid (DTNB) or for native nAChR pretreated with ACh (high-affinity states). Addition of Ca^{++} results in an increase in effectiveness of ACh toward blocking toxin binding. None of these treatments alter toxin or antagonist binding, nor are there observed differences in Hill numbers for agonist binding. Agonists competitively inhibit toxin binding to low-affinity states, but non-competitive inhibition is observed for binding to high-affinity states. Values of ACh dissociation constants determined from these data fall within the range of values determined physiologically with nAChR from other systems. The data indicate that the redox state of brain nAChR thio-groups and Ca^{++} may mediate physiologically important changes in receptor state during activation and desensitization.

INTRODUCTION

A fundamental property of neurotransmitter receptors in their physiological role of regulating ion translocation and nerve impulse initiation is their selective response to agonists. Recently, reports have appeared describing agonist-specific changes in receptor affinity for cholinergic agonists. These changes have been detected by inhibition of curaremimetic neurotoxin binding to peripheral (Weiland et al., 1976., 1977; Weber et al., 1975., Colquhoun & Rang, 1976; Barrantes, 1976; Lee et al., 1977) and central (Łukasiewicz & Bennett, 1978a; 1978b) nAChR[1]. These results suggest that nAChR selectively and actively responds to agonists, and make possible speculation that the observed affinity states of nAChR correlate with the different physiological states of the receptor. In these studies, it was shown that the low-affinity state of nAChR is transient. That is, application of cholinergic drug to nAChR causes a time dependent transformation of nAChR to a high-affinity state. It is desirable to find conditions that would enable one to "freeze" the receptor in high- or low-affinity forms, and thus more readily enable comparison of biochemical and physiological states of the receptor.

Toward elucidation of the molecular mechanisms underlying receptor function, chemical modification procedures and their consequences on the physiological properties of nAChR have been instructive. Karlin and Bartels (1966) demonstrated that reduction of Electrophorus electricus electroplax with DTT inactivated the physiological response to ACh. Application of NEM to DTT-reduced electroplax rendered this inactivation irreversible, but full response to ACh was restored on treatment with DTNB. Thus, the involvement of receptor thio-groups[2] in the biological activity of peripheral nervous system nAChR was established.

Another fundamental property of receptor function is the selectivity displayed toward ions to be passed through the membrane in the post-synaptic current. It is important to determine whether these ions also play roles in regulating receptor function. They might act directly, by binding to specific sites on the receptor, or indirectly, e.g. by catalyzing chemical modification of receptor. There is evidence that Ca^{++} may participate in the natural deactivation of receptor, denensitization (Nastuk & Parsons, 1970), and that Ca^{++} interacts with nAChR (see Chang & Bock, 1977 and references cited below).

On the foundation established by the elucidation of agonist-specific affinity alterations of the CNS nAChR (Lukasiewicz & Bennett, 1978a, 1978b), this report describes the effects of thio-group modification on the toxin-binding competition effectiveness of cholinergic drugs toward membrane-bound nAChR derived from rat brain subcellular fractions. In addition, studies are described on the effects of ionic composition and cation specificity of affinity state transitions of the CNS nAChR.

EXPERIMENTAL PROCEDURE

Methods for purification of α-Bgt from crude lyophilized venom of Bungarus multicinctus (Miami Serpenterium; Eterović et al., 1975a; Hanley et al., 1977) and for preparation of [^{3}H]α-Bgt (specific activity of 25 dpm/fmole, 95% bound by excess nAChR from Torpedo Californica electroplax) by catalytic reduction of iodinated α-Bgt under tritium gas (Eterović et al., 1975b; Lukasiewicz et al ., 1978) are as previously described.

Membrane fractions

Crude mitochondrial fraction membranes are prepared fresh daily from brain (cerebellum is discarded) of Wag/Rig rats (Lawrence Berkeley Laboratory rat colony). Unless otherwise noted, all manipulations are at 0-4°C. A 10% v/v homogenate is prepared in 0.32 M sucrose, 0.5 mM NaH_2PO_4, pH 7.5, 10 μM

phenylmethylsulfonyl fluoride with 10 strokes of a teflon pestle rotating at 1000 rpm (Sunbeam) within a pyrex homogenizer. Crude nuclear fractions and cellular debris are removed by centrifugation at 2000g for 15 min. Supernatants are collected, taking care to reject the white layer above the pellet. The pellet is resuspended in sucrose and centrifuged again. Supernatants are then pooled and subjected to centrifugation at 17,800g for 15 min (Sorvall RC-2B, SM-24 rotor). The pellet is resuspended in binding Ringers medium (115 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1.3 mM $MgSO_4$, 33 mM Tris, pH 7.4 with HCl) or, where indicated, in a Ca^{++}-free Ringers medium (1.8 mM $MgCl_2$ and 1-4 mM EGTA is substituted for $CaCl_2$), sedimented at 24,000g for 15 min and resuspended in Ca^{++}-containing or Ca^{++}-free Ringers medium again. Samples are then either divided into aliquots for binding assays, or are subjected to treatment with thio-group reagents prior to binding assay as described below or in figure and table legends. These preparations contain 15-25 fmole toxin sites per mg membrane protein. No further purification steps were followed as the yields of material are low and specific toxin binding activities are increased less than three-fold on sucrose density gradients (Hanley et al., 1978) or by other selective sedimentation procedures (Morimoto, Lukasiewicz and Bennett, unpublished).

Thio-group modification

In general, membrane preparations at ~20 mg mg/ml are treated with 0.3 mM DTT for 20 min. Some DTT-treated membranes are further reacted with 0.3 mM DTNB for 20 min, or with 0.3 mM NEM for 5 min followed by DTNB reaction. In all cases, thio-group reagent-treated membranes are diluted in the appropriate Ringers medium and subjected to centrifugation at 36,000g for 5-10 min to remove excess reagent prior to subsequent chemical treatment or preparation of samples for binding assays. Modifications of this general procedure, and details for experiments using other reagents are described in figure

or table legends. Unless specifically noted, all manipulations are performed in Ca^{++}-containing medium, and at 0-4°C.

Binding assays

The extent of specific [^{3}H]α-Bgt binding to membranes is determined as the difference in radioactivity bound to samples chased with 4 μM native toxin for 25 min following treatment with [^{3}H]α-Bgt, and blank samples treated with 4 μM native toxin for 30 min prior to the addition of [^{3}H]α-Bgt. The native toxin chase period serves to terminate [^{3}H]α-Bgt specific binding as well as to permit non-specific and short-lived pseudo-specific binding of [^{3}H]α-Bgt to equilibrate to values for native toxin-pretreated blank samples. Unless otherwise noted, the duration of exposure to [^{3}H]α-Bgt at 10 nM is 30 min. This compromise experimental paradigm is designed to increase specific/nonspecific binding sensitivity and to approach conditions of an initial toxin-binding rate assay ($\tau_{1/2} \simeq 11$ min for toxin-receptor association; Lukasiewicz & Bennett, 1978a). Following the native toxin chase period, membranes are subjected to two cycles of suspension in 3 ml of Ringer medium and centrifugation for 15 min at 36,000g. Supernatants are removed, pellets are drained to dryness, and then resuspended and quantitatively transferred to vials containing Aquasol-2 (New England Nuclear) for ^{3}H determination by liquid scintillation counting (Packard TriCarb 3375 or Beckman LS 9000; 30-40% efficiency). Typically, 500 counts per minute are specifically bound in the absence of cholinergic ligand out of 800-900 total cpm. Affinities of cholinergic ligands for membrane-bound nAChR are inferred from their ability to block toxin binding. Drug competition studies are carried out by one of two protocols. For preincubation experiments, cholinergic drug is added to membranes 30 min prior to the addition of [^{3}H] α-Bgt. For coincubation experiments, effector and [^{3}H]α-Bgt are simultaneously added to membrane samples. For experiments using cholinergic ligands that are

substrates for acetylcholinesterase, preincubation of membranes with 10 μM neostigmine or 100 μM eserine is sufficient to block enzymatic activity over the time course of the binding assay without affecting [^{3}H]α-Bgt binding.

Membrane proteins are determined by the method of Lowry et al. (1951).

Acetylcholine perchlorate was synthesized in our laboratory. Other chemicals and reagents are: Tris, phenylmethylsulfonyl fluoride, carbachol chloride, decamethonium bromide, trimethylphenylammonium iodide, lobeline HCl, β-mercaptoethanol, p-chloromercuribenzoate, NEM, EGTA (Sigma); d-tubocurarine chloride, eserine, S-acetylthiocholine, butyrylthiocholine iodide, glutathione, neostigmine, DTT (Calbiochem); tetraethylammonium chloride, nicotine HCl (K & K Laboratories); hexamethonium chloride dihydrate (Mann), gallamine triethiodide (ICN); DTNB (Aldrich); iodoacetamide (Nutritional) Biochemical); ascorbic acid (Baker).

RESULTS

Levels of [^{3}H]α-Bgt specifically bound to membrane-bound CNS nAChR decreases as the concentrations of competing cholinergic ligand increases. An apparent affinity of nAChR for ligand may be inferred from the concentration at which 50% of specific toxin binding is blocked (IC_{50}). The specific range of carbachol concentrations over which toxin binding is blocked varies depending on the duration of exposure of membranes to carbachol, on reaction of membranes with thio-group-directed reagents, and on the concentration of Ca^{++} in the assay medium (Figure 1). IC_{50} values for carbachol competition toxin binding are ∿20 μM when native membranes are preexposed to carbachol in the presence of Ca^{++}. IC_{50} values are also ∿20 μM for DTNB-treated membranes, where oxidation of sulfhydryl groups and promotion of disulfide bond formation is presumably favored. Thus, CNS nAChR is in a high-affinity state under these conditions. In contrast, IC_{50} values are increased when toxin and carbachol are simultaneously added to native membranes, and are ∿200 μM for

NEM-treated membranes, where sulfhydryl groups are irreversibly alkylated. Under these conditions, nAChR is in a low-affinity state toward carbachol. Ca^{++} causes increase in IC_{50} values for carbachol. Preincubation assay in Ca^{++}-free Ringers medium yields IC_{50} values of ~200 μM. These differences in nAChR state are observed only for cholinergic agonists. IC_{50} values are essentially the same (10 μM) for d-tubocurarine competition toward toxin binding to DTNB- or NEM-treated membranes, and for assays in Ca^{++}-free or Ca^{++}-containing Ringers medium (Figure 1). Thus, nAChR exists in one state with respect to antagonist competition.

DTT treatment, which cleaves disulfide bonds and maintains reduced sulfhydryls, fails to alter the IC_{50} (10 μM) for d-tubocurarine competition (Figure 2). However, for carbachol, DTT treatment of membranes gives rise to a marked reduction in competition effectiveness toward toxin binding on coincubation (Figure 2). IC_{50} values are about 1 mM. A similar large reduction in toxin binding competition potency ($IC_{50} \simeq 2$ mM) is observed for coincubation with ACh, indicating that DTT treatment also yields nAChR in a low-affinity state toward agonist. This effect is not a consequence of irreversible denaturation of nAChR. The affinity of DTT-treated membranes for agonists is sensitive to preincubation with agonist. IC_{50} values are 50 μM when DTT-treated membranes are preincubated with ACh prior to the addition of toxin (Figure 2). Furthermore, as indicated above, subsequent treatment with DTNB completely reverses the effects of DTT.

IC_{50} values for a variety of cholinergic ligands whose competition effectiveness toward toxin binding was ascertained under conditions as exemplified in Figures 1 and 2 are summarized in Table 1. The effect of the presence of Ca^{++} on agonist IC_{50} values is clearly shown in that the ratio of IC_{50} for assay in Ca^{++}-free Ringers medium to that in full Ringers medium is ~10 for ACh, S-acetylthiocholine, carbachol and nicotine. In

contrast, for antagonists, that ratio is ∿1 for d-tubocurarine and ∿3 for gallamine. The oxidation/alkylation state of membrane thio-groups also selectively effects agonist IC_{50} values. The ratio of the IC_{50} value for NEM-treated to that for DTNB-treated membranes is again ∿10 for ACh, S-acetylthiocholine, carbachol and nicotine, but is ∿1 for d-tubocurarine and ∿3 for gallamine. Further, the most marked decrease in ligand competition effectiveness toward toxin binding to DTT-treated membranes is observed for agonists. Ratios of IC_{50} for coincubation with DTT-treated membranes to IC_{50} for preincubation in Ringers medium are in excess of 100 for both ACh and carbachol, and about 10 for S-acetylthiocholine and nicotine. The ratio for d-tubocurarine is ∿2. Interestingly, the binding properties for some of the ligands tested do not fall into these empirical agonist or antagonist classifications, perhaps reflecting mixed agonist/antagonist potencies or local anesthetic properties.

As shown by data summarized in Table II, native and DTT-treated nAChR are responsive to pretreatment with ACh when assays are done in the presence of Ca^{++}. That is, IC_{50} values are lower for preincubation assay then for coincubation assay reflecting increased affinity for agonist on pretreatment with agonist. In contrast, DTT-treated membranes are not responsive to ACh pretreatment when assays are done in Ca^{++}-free medium, nor are DTNB- or NEM-treated membranes irrespective of Ca^{++} concentration. There is a small but significant change in ACh competition effectiveness on preincubation in Ca^{++}-free Ringers medium. Taken together, these results indicate that a high-affinity form (toward agonist) of nAChR is "frozen" by DTNB treatment. A low-affinity state is preserved by NEM treatment or by DTT treatment when assays are conducted in the absence of Ca^{++}. When assays are conducted in full Ringers medium, the low-affinity states found on coincubation of

native and DTT-treated membranes, respectively, are transient. The increase in agonist affinity for native and DTT-treated nAChR on preincubation is more marked in the presence of Ca^{++}.

The results of experiments where a variety of thio-group reactive compounds were tested for their effect on ACh inhibition of toxin binding are summarized in Table III. DTT is the most potent agent tested toward increasing IC_{50} values (decreasing affinity) for ACh. β-Mercaptoethanol, reduced glutathione and ascorbic acid, which have less disulfide-directed activity than DTT (Cleland, 1964; Gorin et al., 1968), are less effective than DTT. Treatment with p-chloromercuribenzoate alone gives rise to IC_{50} values seen for preincubation assays in full Ringers medium. In each case, prior treatment with DTT enhances the ability of these sulfhydryl group-directed reagents to decrease the competition potency of ACh. Alkylation of DTT-reduced membranes with iodoacetamide yields nAChR that display IC_{50} values similar to that for NEM-alkylated membranes. As for DTNB-treatment, exposure of DTT-reduced membranes to oxidizing agent potassium ferricyanide yields nAChR with minimum IC_{50} values for ACh competition. Thus, while sulfhydryl-directed reagents have limited ability to decrease ACh competition IC_{50} values, their potency is increased when preceded by treatment with DTT, indicating that formation and cleavage of disulfide bonds is important in manifestation of affinity state changes. Alkylating agents and mild reducing agents irreversibly leave DTT-reduced nAChR in a low-affinity state. Full diminution in ACh affinity is seen only for treatment with DTT or DTT and p-chloromercuribenzoate. Oxidizing agents reverse DTT effects. Also summarized in Table III are data illustrating the absence of any affinity alteration effects in K^{+}-, Na^{+}-, or Mg^{++}-free media, and specificity of Ca^{++} effects. More detailed data (not shown) indicate that 50% of toxin binding in the presence of 10^{-5} M ACh is blocked at 600 μM Ca^{++}. Toxin binding is

blocked to 75% of maximum at 2 mM Ca^{++}, and completely blocked at ~20 mM.

These agonist-specific receptor state transitions are primarily attributable to effects on the affinity for reversibly-associating ligands (Figure 3). The maximum level of toxin binding in the absence of cholinergic effector and K_D^{app} values are largely unaffected, regardless of thio-group treatment procedure and the presence or absence of Ca^{++} in the assay medium.

The differences in apparent affinity (IC_{50}) of ACh for thio-group modified nAChR and for assays conducted in Ca^{++}-containing and Ca^{++}-free media do not result in any striking alteration in the nature of ACh competition for [^{3}H] α-Bgt binding with respect to cooperative effects (Figure 4). Hill plots of ACh competition data yield Hill numbers of about 1.0 for preincubation assays in full Ringers medium and for assays using DTNB-treated membranes in Ca^{++}-free or Ca^{++}-containing buffer, indicating that there is no cooperativity in ACh blockage of α-Bgt binding to nAChR in a high-affinity state. While the Hill plot slope for preincubation assay in Ca^{++}-free Ringers medium is somewhat greater than 1.0, and $n_H \simeq 0.78$ for NEM-treated membranes, these values do not differ markedly from n_H for preincubation assays in full Ringers medium.

The nature of ACh inhibition of toxin binding is further elucidated by examination of its effects on toxin binding isotherms (Figure 5). Hofstee-Eadie plots of α-Bgt binding demonstrate that ACh inhibition of toxin binding to DTNB-treated membranes or to native membranes on preincubation assay in Ca^{++}-free Ringers medium is non-competitive. In these instances, as for preincubation assay with native membranes in full Ringer medium (Lukasiewicz & Bennett, 1978b), the maximum number of toxin sites is reduced, and K_D^{app} values are altered, reflecting the increased observed potency of ligand toward blocking toxin binding at low toxin concentrations, where the actual rate of toxin binding is slower. In contrast, ACh inhibits toxin binding to NEM-treated membranes and, on coincubation, to DTT-treated membranes

in a simple competitive fashion, as previously shown for coincubation assays with native nAChR in full Ringers medium (Lukasiewicz & Bennett, 1978b). That is, values of K_D^{app} are affected, but maximum binding levels are not altered in the presence of ACh. The advantage with NEM- and DTT-treated membranes is that the resultant low-affinity states are more stable relative to that for native membranes.

For the different receptor state manipulations used, the initial rate of toxin binding is also affected differently by ACh. In the absence of ACh, the linear dependence of toxin binding rate data, shown as a 'kinetic' plot in Figure 6, yields virtually identical slopes for assays in the absence of Ca^{++} and for DTT-, DTNB- and NEM-treated membranes. Thus, the rate constant for toxin binding is unaffected by Ca^{++} concentration and thio-group manipulation. However, when ACh is present, rates are decreased in every case, but with different ACh concentration dependencies as cited above, and in different manners. For DTNB-treated membranes and for preincubation assays in Ca^{++}-free or full Ringers medium (Lukasiewicz & Bennett, 1978b), the ordinate value is approached asymptotically in the displayed data, which is calculated assuming that the maximum number of toxin sites is unaltered in the presence of ACh. However, if the data are replotted using an experimentally-derived, reduced number of available toxin binding sites (maximum binding after 60 min incubation in the presence of ACh at concentration indicated), plots are linear (not shown), yielding an identical rate constant for toxin binding as control experiments. Thus, in these cases, the rate constant for toxin binding to available sites is unaltered, but the number of sites is depressed in the presence of ACh. As is the case for coincubation assays with native membranes, 'kinetic' plots plateau abruptly for coincubation assays with DTT-treated membranes, illustrating the transient nature of the low-affinity state detected under these conditions. There is a progressive increase in affinity for ACh as incubation times increase, so that while

the initial rate of toxin-binding is not markedly affected, at longer times, further toxin binding is prevented. In contrast to the above cases, for NEM-treated samples, the 'kinetic' plot is linear. Thus the number of available toxin binding sites is unaltered, but the rate constant for binding to those sites is diminished.

The results of toxin binding rate and saturation isotherm studies may be summarized as follows. For nAChR in high-affinity states, that is, for preincubation in Ca^{++}-free or Ca^{++}-containing Ringers medium or for DTNB-treated membranes, ACh has the predominant effect of non-competitively reducing the number of available toxin sites without having primary effects on K_D^{app} values or on the rate constant for toxin binding to available sites. The diminished affinity of ACh for native and DTT-treated membranes on coincubation assay is transient. However, the major effect of ACh in these cases is only to slow down the toxin binding rate and increase K_D^{app} values without altering the quantity of available toxin sites. These low-affinity states may be preserved by alkylation of reduced thio-groups with NEM.

An experiment conducted to estimate the time course of affinity state transition on addition or depletion of Ca^{++} is shown in Figure 7. On addition of Ca^{++}, the rate of transformation to a high-affinity state is on the order of minutes, similar to that seen for the rate of transition when the duration of preincubation with ACh in full Ringers medium is increased (Lukasiewicz & Bennett, 1978b). Reversal of the increase in ACh affinity, as determined from an experiment where depletion of Ca^{++} is effected by addition of EGTA, is much faster, as even on addition of EGTA simultaneous with initiation of the toxin binding assay, IC_{50} values are about those for ACh competition for toxin binding to membranes treated with EGTA throughout. Thus, the time course of manifestation of increase in nAChR affinity for ACh as mediated by Ca^{++} (or by preincubation with ACh) is slower than reversal of that process.

A variety of cholinergic ligands were used in attempts to prevent NEM-alkylation of DTT-reduced nAChR, i.e., permit the increase in affinity for ACh on subsequent treatment with DTNB. The results show no ability of either agonist or antagonist to protect from alkylation (Figure 8).

In order to test the possibility that nAChR thio-group modification and Ca^{++} play sequential roles in receptor activation and desensitization, respectively, experiments were conducted testing temporal effects of thio-group modification and exposure to or removal of Ca^{++}. It is evident (Table IV) that the absence or presence of Ca^{++} during thio-group modification has no effect on those manipulations as DTNB- and NEM-treated membranes exhibit similar IC_{50} values when assays are conducted in a given Ringers medium. All solvent effects are therefore attributable to the absence or presence of Ca^{++} in the assay medium. For assays conducted in Ca^{++}-free medium, DTNB treatment yields membranes that display IC_{50} values for ACh competition toward α-Bgt binding that are about 10-fold higher than similarly treated samples assayed in the presence of Ca^{++}. That is, IC_{50} values are close to those seen for the low-affinity state, detected in Ca^{++}-containing Ringers medium, "frozen" by NEM-alkylation of DTT-reduced nAChR. However, the affinity of ACh for NEM-treated nAChR in Ca^{++}-free medium is also reduced ∿20-fold to IC_{50} values typical of coincubation assays with DTT-treated membranes. This result indicates that neither sulfhydryl modification nor Ca^{++} alone exclusively controls the affinity states of nAChR. Rather, the effects of Ca^{++} and thio-group manipulation appear to be additive.

DISCUSSION

From the results of toxin-binding inhibition studies, it has been demonstrated that the state of α-Bgt binding entities (which increasingly exhibit the properties of and may be identified as CNS nAChR) with respect to affinity for cholinergic agonists, is sensitive to manipulation of

(presumably receptor) thio-groups and to the presence of Ca^{++}. nAChR in a high-affinity form can be produced by preincubation with ACh, or by oxidation of DTT-reduced nAChR by DTNB. A permanent low-affinity state of nAChR results from treatment of DTT-reduced membranes with NEM, and transient low affinity states may be detected in coincubation experiments using DTT-reduced and native membranes. Affinities for agonist in each case are further diminished in the absence of Ca^{++}; Ca^{++} and thio-group effects appear to be additive. [^{3}H]α-Bgt binding to high affinity state(s) is inhibited non-competitively by ACh, but inhibition of toxin binding to low-affinity state(s) is competitive. Neither agonists nor antagonists protect nAChR from alkylation by NEM. None of these treatments alter toxin binding properties to membranes in the absence of ACh, nor are there marked alterations in cooperativity of agonist inhibition of toxin binding.

Despite the fact that this study deals with CNS nAChR and utilizes a toxin binding competition experimental design, parallels may be drawn between this work and results of other groups using different techniques to determine agonist affinities for peripheral nervous systems nAChR. Since the original description by Karlin & Bartels (1966) of alteration in the physiological response of Electrophorous electricus electroplax nAChR to cholinergic agonist, there have appeared reports detailing similar thio-group reagent effects on agonist affinity using direct binding assays (Eldefrawi & Eldefrawi, 1972; Schiebler et al., 1977). Thio-group modification also perturbs in vivo (Mittag & Tormay, 1970; Rang & Ritter, 1971; del Castillo et al., 1972; Ben-Haim et al., 1973; Lindstrom et al., 1973) and in vitro (Kasi & Changeux, 1971; Schiebler, et al., 1977) responses of chemically excitable nAChR to cholinergic agonists, and affects the ability of agonist to block toxin binding to T. californica electroplax nAChR (Lukasiewicz & Bennett, unpublished).

It may be concluded that CNS nAChR contain thio-groups that may parti-

cipate in the specific responses of nAChR to agonist that lead to receptor activation and/or desensitization. However, the detailed involvement of CNS nAChR thio-groups in those responses apparently differ from that in peripheral nAChR. For example, DTT treatment potentiates the response of eel electroplax nAChR to hexamethonium and decamethonium, but NEM-alkylation abolishes the response (Karlin & Bartels, 1966; Karlin, 1969). Our results show that the affinities of hexamethonium and decamethonium for native, reduced and alkylated CNS nAChR are about the same, and that both ligands actually bind with higher affinity to alkylated nAChR than to DTNB-treated membranes. Most of the evidence in this report suggests that disulfide bonds maintain CNS nAChR in a high affinity-state. Sulfhydryl group reagents, particularly p-chloromercuribenzoate, reduce agonist-mediated responses of electroplax nAChR (Karlin & Bartels, 1966), but are considerably less potent toward altering the affinity state of CNS receptor. Further, despite evidence that agonist treatment causes diminution of sulfhydryl group reactivity in Torpedo californica electroplax nAChR (Suarez-Isla & Hucho, 1977), and that a thio-group is near the active site of peripheral receptors and serves as a site for affinity-labeling of peripheral receptors (Karlin & Winnik, 1968; Froehner et al., 1977), our attempts to block NEM-alkylation of CNS nAChR with cholinergic agonists and antagonists failed. It may be the case that the CNS nAChR may be further distinguished, as is frog neuromuscular junction nAChR (Lindstrom et al., 1973), from other peripheral nAChR by differences in reactivity toward a thio-group affinity reagent, such as 4-(N-maleimido)-benzyltrimethylammonium.

There has recently emerged evidence for disulfide bond-controlled dimerization of nAChR components (Chang & Bock, 1977; Hucho et al., 1978; Hamilton et al., 1977; Witzemann & Raftery, 1978). Evidence has not evolved demonstrating that changes in receptor affinity for agonist on response to

agonist is related to the extent of disulfide-mediated dimerization (Schiebler et al., 1977; Gibson et al., 1976; however, see Chang & Bock, 1977). Our results, indicating a difference in ligand affinity for nAChR treated with DTT and with DTT-NEM in the presence of Ca^{++}, may suggest a complex involvement of multiple disulfide and sulfhydryl groups at different sites on CNS nAChR.

Further parallels may be drawn between the results reported here and those of other investigators using other experimental systems and procedures regarding effects of Ca^{++} on receptor function. In the presence of Ca^{++} the affinity of cholinergic agonists for membrane-bound nAChR from T. marmorata electroplax is increased (Cohen et al., 1974). There is also an acceleration of the usual time course of decrease in amplitude of the efflux of Na^{+} ions from excitable vesicles seen on exposure to carbachol (Sugiyama et al., 1976), an in vitro model of desensitization. Nevertheless, there is other evidence that Ca^{++} and cholinergic agonists may displace one another from binding sites on electroplax nAChR (Chang & Newmann, 1976; Eldefrawi et al., 1975; Rubmassen et al., 1976; Mihovilovic et al., 1977). The Ca^{++} concentration where all of these effects and the effects described in this paper are manifest are similar ($\sim$1 mM). Evidence exists suggesting that Ca^{++} may accelerate, if not induce, nAChR desensitization (Manthey, 1966; Nastuk & Parsons, 1970; Magazanik & Vyskocil, 1970; Schnitzler et al., 1975; Devore & Nastuk, 1977). It has been suggested that accumulation of Ca^{++} intracellularly may cause nAChR desensitization (Nastuk & Parsons, 1970), although interaction of Ca^{++} with any hidden sites exposed concomitantly with receptor activation may yield the same effect. Our data support the contention that Ca^{++} alters the nAChR affinity for agonist, and does so in a manner consistent with that expected for binding to a densensitized state, i.e., a receptor state with high affinity for agonists. An intriguing possibility that might explain all the Ca^{++} effect observations is that Ca^{++}-mediated desensitization is

accompanied by a net release of ACh from other (low-affinity) binding sites.

One purpose of this investigation is to draw on the results in order to relate the observed affinity states of CNS nAChR to physiological states of the receptor. The non-competitive nature of agonist competition for α-Bgt binding to nAChR in high-affinity states has several implications. First, the results show that agonist binding to a site other than the α-Bgt binding site is more effective at non-competitively blocking toxin binding than is agonist competitive inhibition of toxin binding to nAChR in low affinity states. This supports our initial suggestion (Lukasiewicz & Bennett, 1978b) that there may be structural, and perhaps functional, heterogeneity in agonist binding sites on CNS nAChR, offering a possible explanation of the observed impotency of α-Bgt toward central cholinergic responses (Miledi & Szczepaniak, 1975; Duggan et al., 1976). The results also support our conclusion that the apparent heterogeneity in agonist binding sites is mirrored in differences in α-Bgt and Dendroaspis viridis toxin 4.7.3 recognition by CNS nAChR (Hanley et al., 1978). Another pertinent implication of the present results is that, since desensitized nAChR is thought to be refractory to α-Bgt binding (Lester, 1972; Miledi & Potter, 1975), the high-affinity state may correspond to the desensitized state of the CNS nAChR. From the relation $K_I^{app} = IC_{50}\ (1 + T/K_D^{app})^{-1}$, where T = concentration of toxin (≃ 10 nM), $K_D^{app} \simeq 2$ nM, and K_I^{app} is an apparent inhibition constant for cholinergic ligand and an approximation of the ligand-nAChR dissociation constant, one calculates K_I^{app} for ACh binding to the high-affinity state of ∿1 μM. The K_I^{app} for ACh binding to NEM-alkylated nAChR is ∿10 μM, and for binding to the transient low-affinity state found on coincubation assay with DTT-treated membranes is ∿300 μM. While direct correlation of these data with ACh concentrations necessary to activate nAChR in vivo is difficult, the K_I^{app} values fall within the range of effective

physiological ACh concentrations (Lester et al., 1975; Katz & Miledi, 1973, Dionne & Stevens, 1975; Hartzell et al., 1975; Fertuck & Salpeter, 1976).

It is attractive to speculate, based on the work of Karlin (1969) and Nastuk (1977) and their respective coworkers, that changes in receptor thio-group oxidation state accompany receptor activation, and that Ca^{++} mediates desensitization;

$$A + R \text{ (low affinity)} \xrightarrow{\text{SH modification}} AR^* \xrightarrow{Ca^{++}} AD \text{ (high-affinity)} \quad (1)$$

where A is cholinergic agonist, R is nAChR in the at-rest configuration, R* is activated nAChR and D is desensitized nAChR. One might predict that, in the absence of Ca^{++}, DDT-reduced, NEM-alkylated and DTNB-oxidized nAChR would have identical affinities for ACh as reduced and/or alkylated nAChR in the presence of Ca^{++}. Not all of these expectations are borne out, as Ca^{++} and thio-group effects appear to be additive for NEM- and DTNB-treated membranes and for preincubation assays with DTT-treated nAChR. However, the data are consistent with a simple model as shown in equation 1, if R* has an intermediate affinity for ACh, and if exposure to Ca^{++} potentiates ACh binding to all states of nAChR, including conversion of R* (oxidized nAChR) to D. Thus, a simple, but not unique, model might accommodate the present data and the implications of Karlin's and Nastuk's discoveries.

During preparation of this manuscript, a study appeared on the effects of sulfhydryl-group reagents on the affinity state of rat brain mAChR (Aronstam et al., 1978). It was demonstrated that treatment with NEM preserves mAChR in a high-affinity form. Pretreatment of mAChR with agonist promotes formation of mAChR in a low-affinity form toward agonist (Young, 1974). While there clearly are differences in the nature of mAChR and nAChR affinity changes on NEM treatment, it is remarkable that such treatment preserves both receptors in a non-desensitized form.

Regardless of the precise mechanism by which CNS nAChR are activated and desensitized, it is clear that thio-group modification and Ca^{++} effects

are involved in affinity state changes of nAChR. The exact relationship between the different agonist affinity states of CNS nAChR detected in these studies and the physiologically identifiable at-rest, activated and desensitized receptor remains unclear, but are subject to elucidation by carefully planned *in vivo* and *in vitro* studies of a homogeneous population of nAChR.

ACKNOWLEDGEMENTS

The authors express appreciation to M. R. Hanley for many helpful discussions and a critical reading of the manuscript, and to Beth Klingel and Gloria Goldberg for secretarial assistance.

TABLE I

IC_{50} Values (μM) for Competition vs. 10nM [^{3}H]α-Bgt

Drug	Rgr	Ca-free[a]	DTNB	NEM[b]	DTT[c]
acetylcholine	4	40 (10)	6	60 (10)	2000 (500)
S-acetylthiocholine	10	60 (6)	10	50 (5)	200 (20)
butyrylthiocholine	40	70 (2)	80	100 (1)	100 (3)
decamethonium	50	400 (8)	80	50 (0.5)	40 (1)
nicotine	1	10 (10)	3	30 (10)	10 (10)
carbachol	10	200 (20)	30	300 (10)	1000 (100)
trimethylphenylammonium	30	300 (10)	40	40 (1)	60 (2)
tetraethylammonium	80	800 (10)	80	800 (10)	5000 (60)
hexamethonium	1000	2000 (2)	1000	800 (0.8)	3000 (3)
gallamine	100	300 (3)	100	300 (3)	800 (8)
lobeline	20	200 (10)	30	60 (2)	60 (3)
d-tubocurarine	10	10 (1)	10	10 (1)	20 (2)

Characteristic IC_{50} values are determined from inspection of data plotted as in Figure 1. Preincubation assays are done for experiments done in full Ringers medium (Rgr), Ca^{++}-free medium (Ca-free) and for NEM-treated membranes (NEM). Coincubation assays are done for DTNB-treated membranes (DTNB) and for DTT-treated membranes (DTT). DTT, DTNB and NEM experiments are done in Ca^{++}-containing Ringers medium.. a-values in parenthesis denote ratios of IC_{50} value on preincubation in Ca^{++}-free medium to that on preincubation in Ca^{++}-containing Ringers medium. b-values in parenthesis denote ratios of IC_{50} value for inhibition of toxin binding to NEM-treated membranes to that for DTNB-treated membranes. c-values in parenthesis denote ratios of IC_{50} for DTT-treated membranes to that for preincubation assay in full Ringers medium. IC_{50} values for preincubation in full Ringers medium are 2000 μM for eseine and 400 μM for neostigmine.

TABLE II

Preincubation Responsiveness to ACh

Condition	IC_{50} pre (μM)	IC_{50} co (μM)
Ringers medium	4	50 (12)
DTT, Ringers medium	50	2000 (40)
DTNB, Ringers medium	5	5 (1)
NEM, Ringers medium	100	100 (1)
Ca^{++}-free medium	40	100 (2)
DTT, Ca^{++}-free	2000	2000 (1)

Comparison of IC_{50} values for preincubation and coincubation experiments for membranes treated as shown. a-values in parenthesis denote ratios of IC_{50} on coincubation to that on preincubation.

TABLE III

Summary of Thio-Group Directed Reagent and Ion Effects on ACh IC_{50} Values

Treatment	IC_{50} (μM)
0.3mM DTT	3000
1 mM β-mercaptoethanol	10
1 mM glutathione	5
1 mM ascorbate	4
1 mM p-chloromercuribenzoate	5
0.3 mM DTT- 1 mM glutathione	100
0.3 mM DTT-1 mM ascorbate	30
0.3 mM DTT-1 mM p-chloromercuribenzoate	2000
0.3 m_ DTT- 0.3 mM NEM	80
0.3 mM DTT-1mM iodoacetamide	60
0.3 mM DTT-0.3 mM DTNB	6
0.3 mM DTT-1 mM potassium ferricyanide	6
K^+-free	3
Na^+-free	2
Mg^{++}-free	2

Treatment is: DTT, β-mercaptoethanol, glutathione, ascorbate, p-chloromercuribenzoate, DTNB, potassium ferricyanide--20 min, coincubation assay; NEM, iodoacetamide--5 min, preincubation assay. For ion depletion experiments preincubation assays were used. All experiments performed in Ca^{++}-containing medium.

TABLE IV

Combined Solvent and Thio-Group Effects

Treatment Procedure			
Reaction Buffer	Thio Treatment	Assay Medium	IC_{50} (μM)
Ringer	NEM-DTNB	Ringer	100
	DTNB		4
	NEM-DTNB	Ca^{++}-free	3000
	DTNB		40
Ca^{++}-free	NEM-DTNB	Ringer	2000
	DTNB		5
	NEM-DTNB	Ca^{++}-free	2000
	DTNB		40

Crude mitochondrial fractions are suspended in Ca^{++}-containing Ringers medium (Ringer) or Ca^{++}-free medium (Ca^{++}-free) and subjected to 20 min exposure to 0.3 mM DTT followed by centrifugation and resuspension in appropriate reaction buffer. Treatment with DTNB or NEM , DTNB follows as described in METHODS, and final pellets, freed of excess thio-group reagent, are resuspended in indicated assay medium for coincubation assay (DTNB) or preincubation assay (NEM) under conditions as in Figure 1.

REFERENCES

Aronstam, R.S., Abood, L.G. & Hoss, W. (1978) Pharm. 14 575.

Barrantes, F.J. (1976) Biochem. Biophys. Res. Commun. 72, 479.

Ben-Haim, P., Landau, E.M. & Silman, I. (1973) J. Physiol. Lond. 234, 305.

Chang, H. W. & Bock, E. (1977) Biochemistry 16, 4513.

Chang, H.W. & Neumann, E. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 3364.

Cleland, W.W. (1964) Biochemistry 3, 480.

Cohen, J.B., Weber, M. & Changeux, J.-P. (1974) Molec. Pharm. 10, 94.

Colquhoun, D. & Rang. H.P. (1976) Molec. Pharm. 12, 419.

del Castillo, J., Bartels, E. & Sobrino, J.A. (1972) Proc. Natl. Acad. Sci. U.S.A. 69, 2081

Dionne, V.E. & Stevens, C.F. (1975) J. Physiol. Lond. 251, 245.

Duggan, A.W., Hall, J.G. & Lee, C.Y. (1976) Brain Res. 107, 166.

Devore, D. & Nastuk, W.L. (1977) Nature 270, 441.

Eldefrawi, M.E. & Eldefrawi, A.T. (1972) Proc. Natl. Acad. Sci. U.S.A. 69, 1776.

Eldefrawi, M.E., Eldefrawi, A.T., Penfield, L.A., O'Brein, R.D. & Van Campen, D. (1975) Life Sci. 16, 925.

Eterovic, V.A., Aune, R.G. & Bennett, E.L. (1975b) Anal. Biochem. 68, 394.

Eterovic, V.A., Hebert, M.S., Hanley, M.R. & Bennett, E.L. (1975a) Toxicon 13, 37.

Fertuk, H.C. & Salpeter, M.M. (1976) J. Cell Biol. 69, 144.

Froehner, S.C., Karlin, A. and Hall, Z.W. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 4685.

Gibson, R.E., O'Brien, R.D., Edelstein, S.J. and Thompson, W.R. (1976) Biochemistry 15, 2377.

Gorin, G., Fulford, R. and Deonier, R.C. (1968) Experientia 24, 24.

Hamilton, S.L., McLaughlin, M. & Karlin, A. (1977) Biochem. Biophys. Res. Commun. 79, 692.

Hanley, M.R., Eterovic, V.A., Hawkes, S.P., Hebert, A J. & Bennett, E.L. (1977) Biochemistry 16, 5840.

Hanley, M.R., Lukasiewicz, R.J. & Bennett, E.L. (1978) Soc. Neuroscience abstracts 4, in press.

Hartzell, H.C., Kuffler, S.W. & Yoshikami, D. (1975) J. Physiol. Lond. 251, 427.

Hucho, F., Bandini, G. & Suarez-Isla, B.A. (1978) Eur. J. Biochem. 83, 335.

Karlin, A. (1969) J. Gen. Physiol. 54, 2455.

Karlin, A. & Bartels, E. (1966) Biochim. Biophys. Acta 126, 525.

Karlin, A. & Winnik, M. (1968) Proc. Natl. Acad. Sci. U.S.A. 60, 668.

Kasai, M. & Changeux, J.-P. (1971) J. Memb. Bio. 6, 1.

Katz, B. & Miledi, R. (1973) J. Physiol. Lond. 231, 549.

Lee , T., Witzemann, V., Schimerlik, M. & Raftery, M.A. (1977) Arch. Biochem. Biophys. 183, 57.

Lester, H.A. (1972) Molec. Pharm. 8, 632.

Lester, H.A., Changeux, J.-P. & Sheridan, R.E. (1975) J. Gen. Physiol. 65, 797.

Lindstron, J.M., Singer, S.J. & Lennox, E.S. (1973) J. Memb. Biol. 11, 217.

Lowry, O.H., Rosenbrough, N.J., Farr, A.C. & Randall, R.J. (1951) J. Biol. Chem. 193, 265.

Lukasiewicz, R.J. & Bennett, E.L. (1978a) Biochim. Biophys. Acta. in press.

Lukasiewicz, R.J. & Bennett, E.L. (1978b) Biophys. J. 21, 52a.

Lukasiewicz, R.J., Hanley, M.R. & Bennett, E.L. (1978) Biochemistry 17, 2308.

Magazaniak, L.G. & Vyskocil, F. (1970) J. Physiol. Lond. 210, 507.

Manthey, A.A. (1966) J. Gen. Physiol. 49, 963.

Mihovilovic, M., Nowak, T., Raftery, M.A. & Martinez-Carrion, M. (1977) Biochem. Biophys. Res. Commun. 78, 525.

Miledi, R. & Potter, L.J. (1975) Nature 233, 599.

Miledi, R. & Szczepaniak, A.C. (1975) Proc. Roy. Soc. Lond. B. 190, 267.

Mittag, T.W. & Tormay, A. (1970) Fed. Proc. 29, 547a.

Nastuk, W.L. (1977) in Synapse, ed. Cottrell, Usherwood, Acad. Press, N.Y., p. 177-201.

Nastuk, W.P. & Parsons, R.L. (1970) J. Gen. Physiol. 56, 218.

Rang, H.P. & Ritter, J.M. (1971) Molec. Pharm. 7, 620.

Rubmassen, H., Hess, G.P., Eldefrawi, A.T. & Eldefrawi, M.E. (1976) Biochem. Biophys. Res. Commun. 68, 56.

Schiebler, W., Lauffer, L. & Hucho, F. (1977) FEBS Lett. 81, 39.

Schnitzler, R.M. DeBassid, W.A. & Parsons, R.L. (1975) Fed. Proc. 34, 407.

Suarez-Isla, B.A. & Hucho, F. (1977) FEBS Lett. 75, 65.

Sugiyama, H., Popot, J.-C. & Changeux, J.-P. (1976) J. Mol. Biol. 102. 485.

Weber, M., David-Pfeuty, T. & Changeux, J.-P. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 3443.

Weiland, G., Georgia, B., Wee, V.T., Chignell, C.F. & Taylor, P. (1976) Molec. Pharm. 12, 1091.

Weiland, G., Georgia, B., Lappi, S., Chignell, C.F. & Taylor, P. (1977) J. Biol. Chem. 252, 7648.

Witzmann, V. & Raftery, M.A. (1978) Biochem. Biophys. Res. Commun. 81, 1025.

Young, J.M. (1974) FEBS Lett. 46, 354.

FIGURE LEGENDS

Figure 1. Cholinergic ligand competition for [^{3}H] α-Bgt specific binding. Quantity of specific [^{3}H]α-Bgt binding sites occupied by toxin (percent of maximum) is plotted against the molar concentration (logarithmic scale) of carbachol (upper) and d-tubocurarine (lower). DTNB-treated membranes (●); preincubation assay in Ringers medium (▲); NEM-treated membranes (○); preincubation assay in Ca^{++}-free Ringers medium (△). Assays in 92mM NaCl, 4mM KCl, 1.4mM $CaCl_2$ (or 1.4 mM $MgCl_2$ and 4mM EGTA), 1.0mM $MgSO_4$, 26mM Tris, pH 7.4, 1% bovine serum albumin, 250 µl final volume, 21° with shaking. Samples typically contain 3-4 mg membrane protein. [^{3}H] α-Bgt concentration is 10nM and native toxin is at 4 µM at termination of assay.

Figure 2. Effect of DTT treatment on ligand competition effectiveness. Membranes are treated with DTT as described in METHODS, and washed free of DTT prior to initiation of binding experiment. Percent specific [^{3}H] α-Bgt sites occupied are plotted against the molar concentration of competing ligand on a logarithmic scale for DTT-treated membranes. Preincubation assay in the presence of ACh (●); coincubation assay in the presence of ACh (○); coincubation assay with d-tubocurarine (◇); coincubation assay with carbachol (□). Assay conditions as in Figure 1 in the presence of Ca^{++}.

Figure 3. [^{3}H] α-Bgt specific binding (fmole/mg membrane protein) is plotted against toxin concentration (nM) for NEM-treated (o) and DTNB-treated (o) membranes, and for assays conducted in Ca^{++}-free (Δ) and Ca^{++}-containing (Δ) Ringers media. Levels of specific toxin binding (fmole/mg membrane protein) to a different membrane preparation treated with DTT at concentrations indicated are 22.1, 0mM; 19.2, 0.1 mM; 18.4, 0.3mM; 19.6, 1.0mM. Assay conditions as in Figure 1.

Figure 4. Hill plots of apparent ACh binding as inferred from competition toward [^{3}H] α-Bgt binding. Assays conducted in Ca^{++}-free (Figure 4c, d) or Ca^{++}-containing (Figure 4a,b,e) Ringers medium. Hill numbers (± error slope determined from least squares linear regression analysis) are: a- DTNB-treated membranes, 1.01 (± 0.32); b- NEM-treated membranes, 0.78 (± 0.21); DTNB-treated membrnaes, 1.09 (± 0.24); d- preincubation assay in Ca^{++}-free Ringers medium, 1.18 (± 0.37); e- preincubation assay in full Ringers medium, 0.95 (± 0.32). Conditions as in Figure 1.

Figure 5. Mechanism of ACh inhibition of [^{3}H]α-Bgt binding. Hofstee-Eadie plots of specific toxin sites occupied vs. (sites occupied) (toxin concentration)$^{-1}$ yield values for v_{max} and slope, respectively (± standard error as determined from least squares linear regression analysis of the data), for: a- DTNB-treated membranes in the presence of 0 μM ACh, 85 ± 7, -4.2 ± 0.6 (Δ), 4 μM ACh, 69 ± 8, -4.7 ± 1.0 (o); b- NEM-treated membranes in the presence of 0 μM ACh, 70 ± 5, -4.5 ± 0.7 (o), 40 μM ACh, 70 ± 4, -8 ± 0.8 (Δ); c- preincubation assay in Ca^{++}-free medium in the presence of 0 μM ACh, 65 ± 3, -2.2 ± 0.2 (Δ), 40 μM Ach, 43 ± 4, -3.0 ± 0.5

(o); coincubation assay with DTT-treated membranes in the presence of 0 μM Ach, 113 ± 14, -15 ± 3 (o), 300 μM Ach, 118 ± 21, -19 ± 5 (Δ). Assay conditions as in Figure 1 or 2 for membrane protein concentrations of 14.4 mg/ml (a), 12.4 mg/ml (b), 11.8 mg/ml (c), 18.3 mg/ml (d). Duration of exposure to [^{3}H]α-Bgt is 60 min.

Figure 6. Effect of ACh on rate of toxin binding. The rate equation for interaction of toxin with receptor may be written $kt = (T_o-R_o)^{-1} \ln [R_o(T_o-RT)T_o^{-1} (Ro-RT)^{-1}]$ where R_o is the total receptor concentration (maximum number of toxin sites in the absence of cholinergic effector), T_o is the total toxin concentration, k is the binding rate constant, and RT is the quantity of toxin-receptor complexes at time of duration of exposure to [^{3}H]α-Bgt, t. For $T_o \gg R_o$, RT, the equation reduces to $kt = (T_o)^{-1} \ln [R_o-RT)^{-1}] = (T_o)^{-1} \ln (\%\ \text{toxin sites unoccupied})^{-1}$. Data are given for the rate of toxin binding in the presence of 10 nM [^{3}H]α-Bgt, yielding $k \simeq 8 \cdot 10^6$/min-mole for all preparations. a- DTNB-treated membranes in the presence of 0 μM ACh (o), 3 μM ACh (Δ); b- NEM-treated membranes in the presence of 0 μM ACh (o), 40 μM ACh, k 4·8-10^6 min-mole (Δ); c - preincubation assay in Ca^{++}-free medium with 0 μM ACh (o), 40 μM ACh (Δ); d- coincubation assay with DTT-treated membranes in the presence of 0 μM ACh (o), 2mM ACh (Δ). Assays as in Figure 1 or 2 except that duration of exposure is for time indicated on abscissa.

Figure 7. Rate of affinity state transformation as a function of duration of exposure to or removal of Ca^{++}. a- coincubation assay is initiated using membranes prepared in the presence of 1mM EGTA (△), or using EGTA-treated membranes after addition of Ca^{++} (4mM final total concentration) for 0 min (o), 1 min (□), 30 min (◈) prior to addition of ACh and toxin. b- preincubation assay is conducted using membranes prepared in Ca^{++}-containing Ringers medium (▲), or reaction is initiated by addition of toxin to membranes exposed to ACh for 30 min after exposure to EGTA for 0 min (o), 30 min (▣).

Figure 8. Effect of cholinergic ligand on alkylation of membrane-bound nAChR. Effectiveness of ACh toward blockage of α-Bgt binding for DTNB-treated (◇) and NEM-treated membranes (o), and for DTT-treated membranes exposed to ligand at listed concentrations, and for times indicated prior to addition of NEM. (△) - 3mM trimethylphenylammonium, 1-5 min; (□) - 1mM lobeline, 1 min; (▽) - 0.1-1mM d-tubocurarine, 1-5 min; (◈) - 1mM decamethonium, 1 min; (o) - 1mM carbachol, 1 min. Exposure to carbachol for longer times (30 min) prior to addition of NEM had no blocking effect. Assay conditions as in Figure 1. In all cases, ligand is not removed from membranes by dilution and centrifugation prior to addition of NEM. All NEM treatments are for 5 min and are followed by dilution and centrifugation sequence before addition of DTNB. Three cycles of dilution and centrifugation, with exposure to DTNB throughout, are used to reduce free ligand concentrations to levels that do not alter toxin binding before final resuspension in full Ringers medium for binding assay.

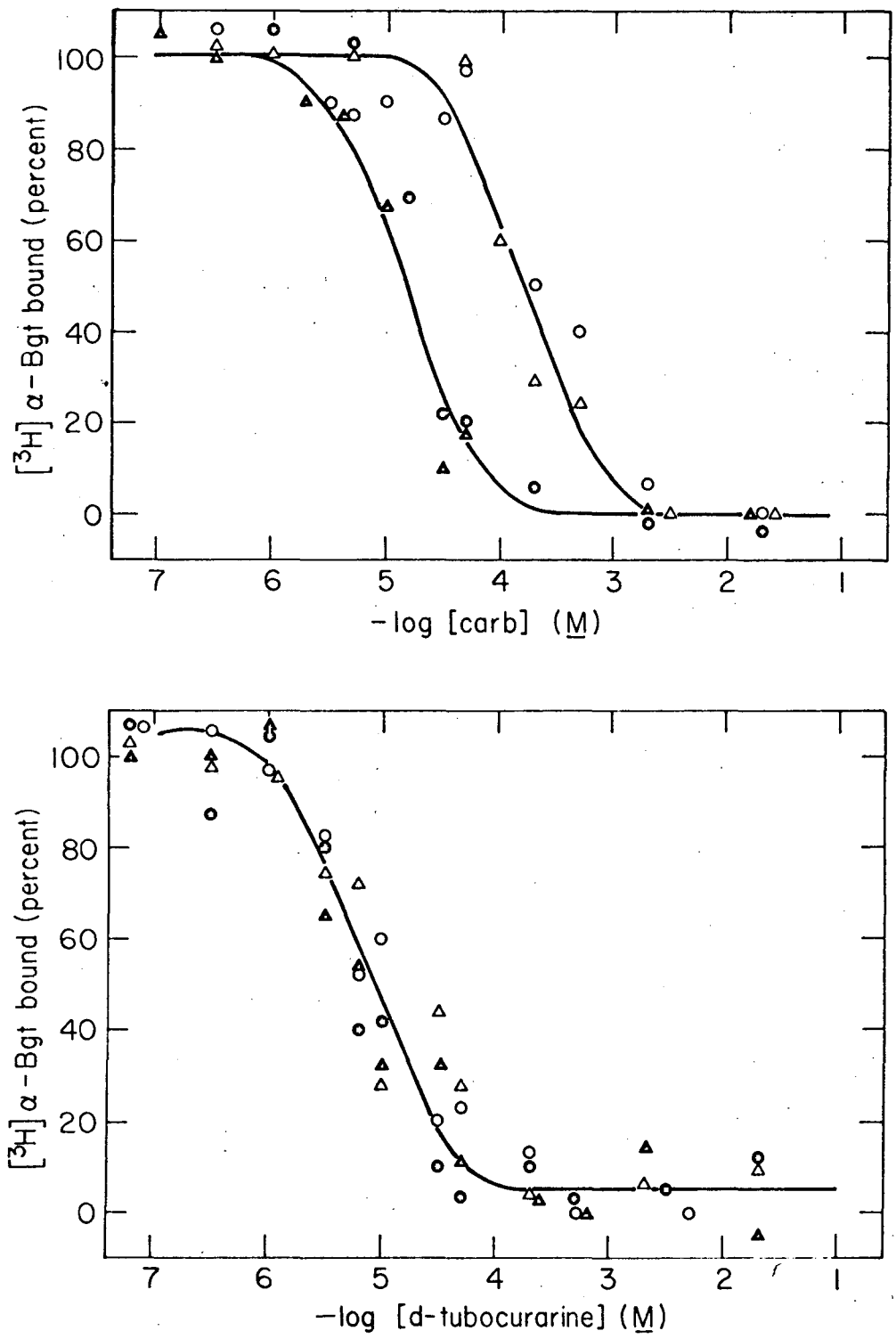

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Fig. 1

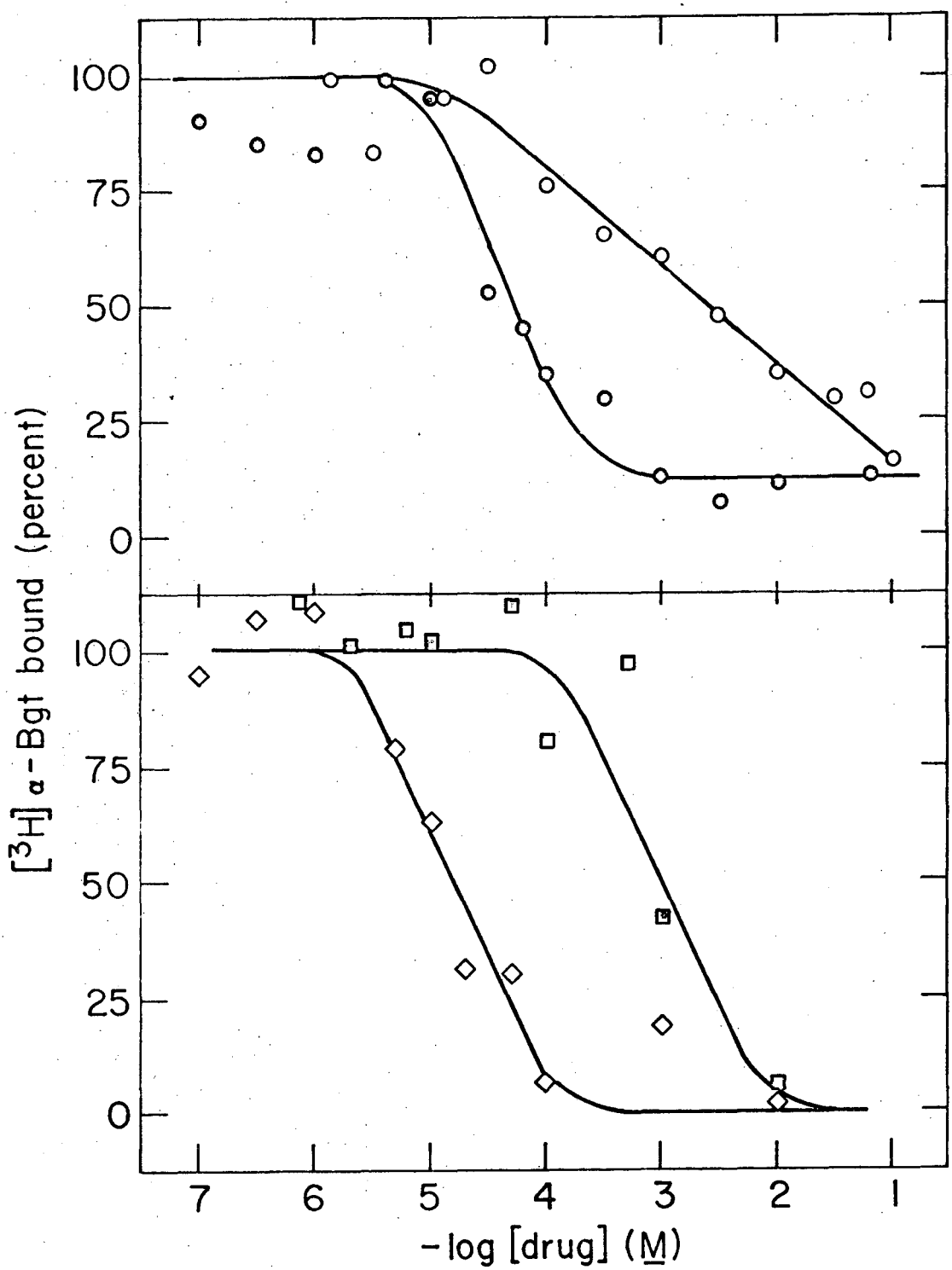

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Fig. 2

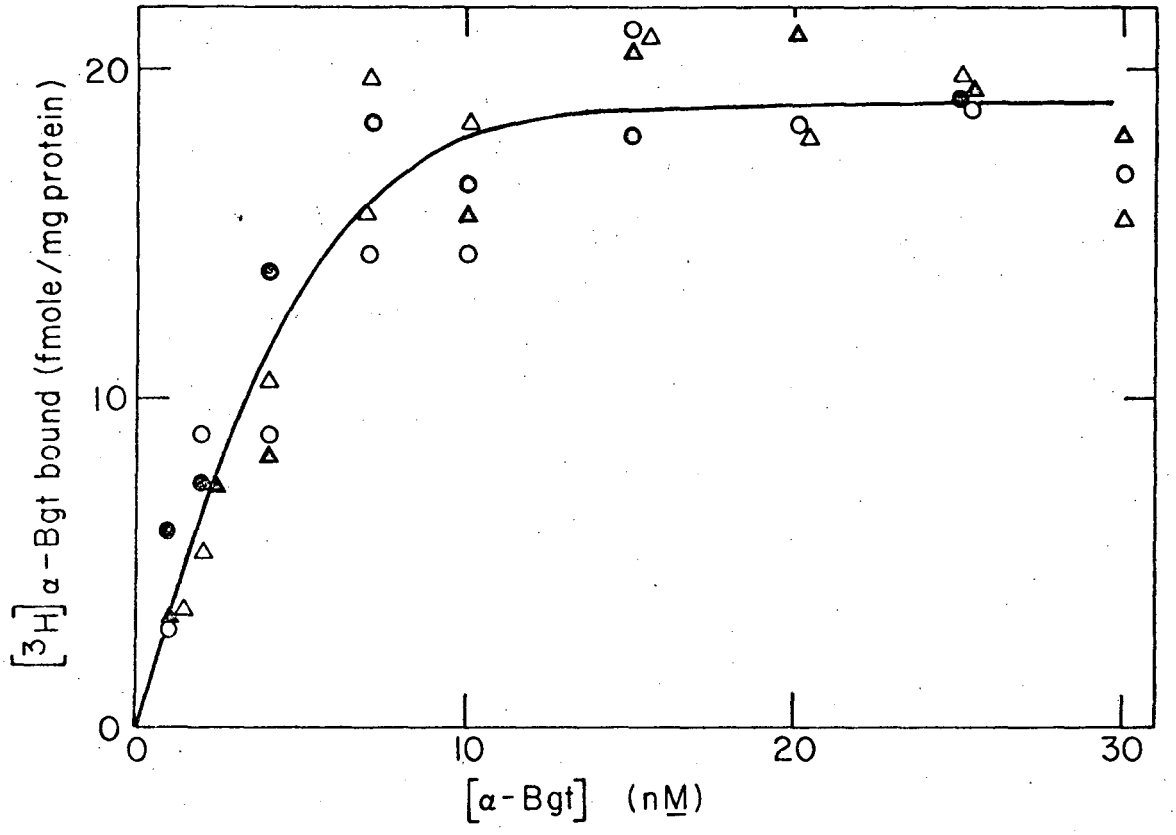

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Fig. 3

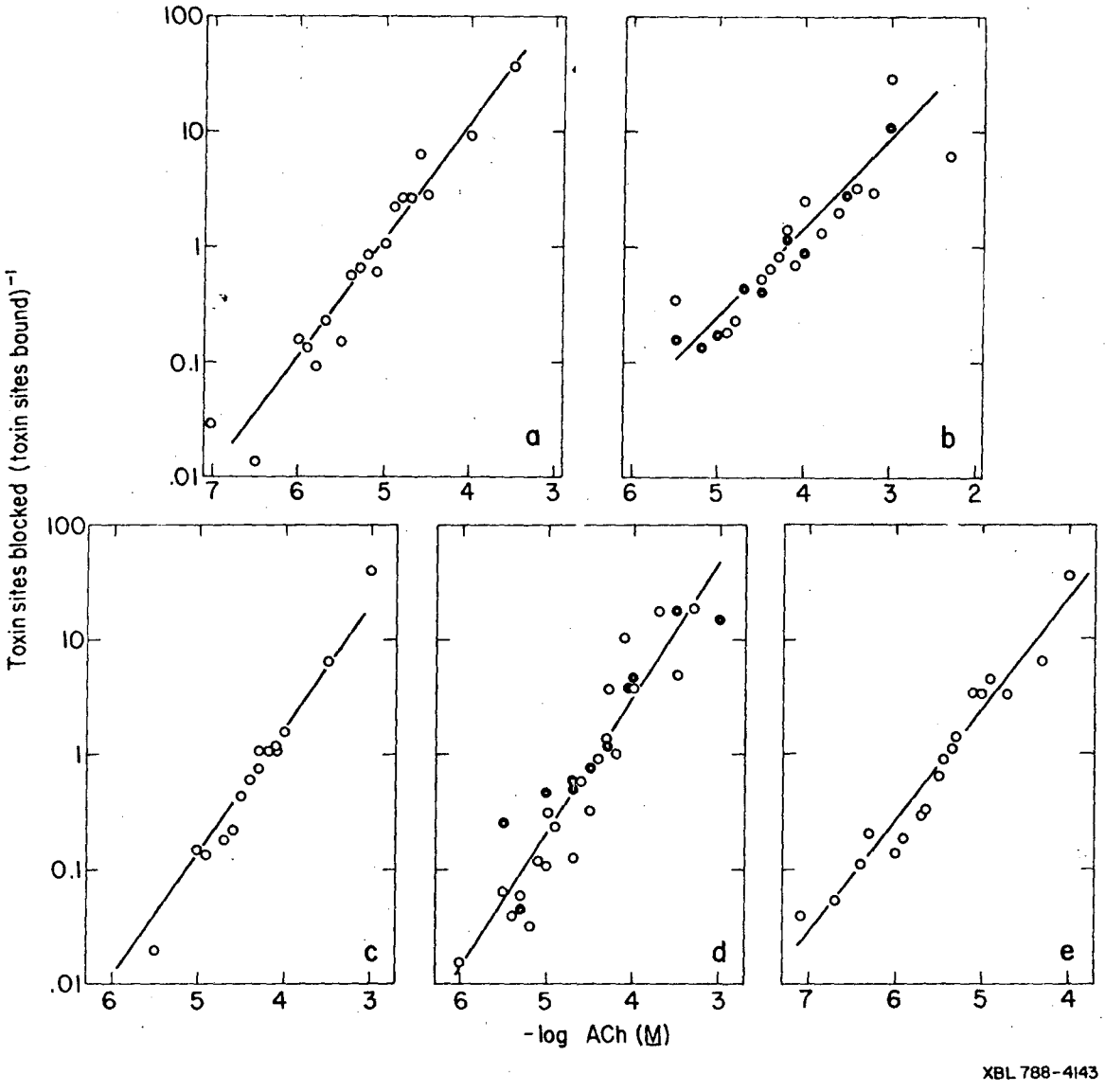

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Fig. 4

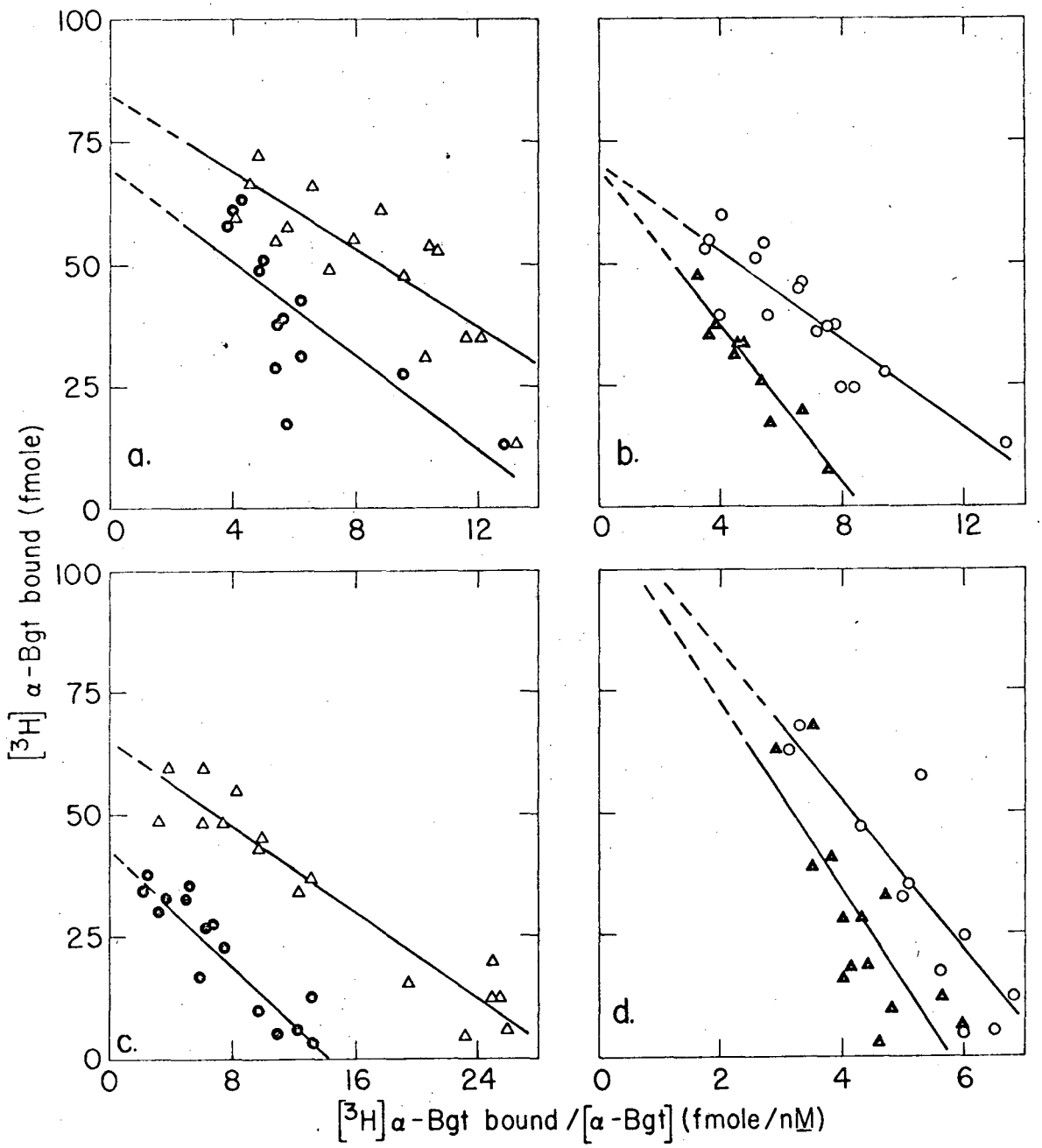

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Fig. 5

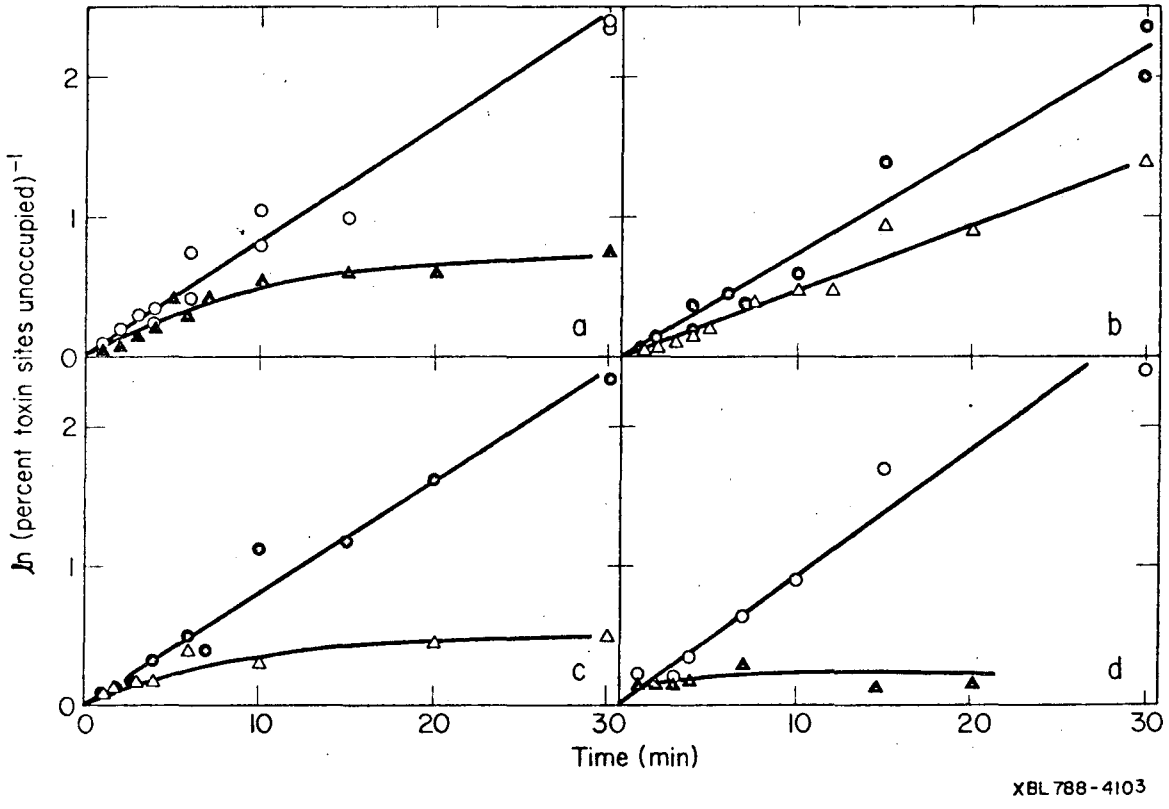

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Fig. 6

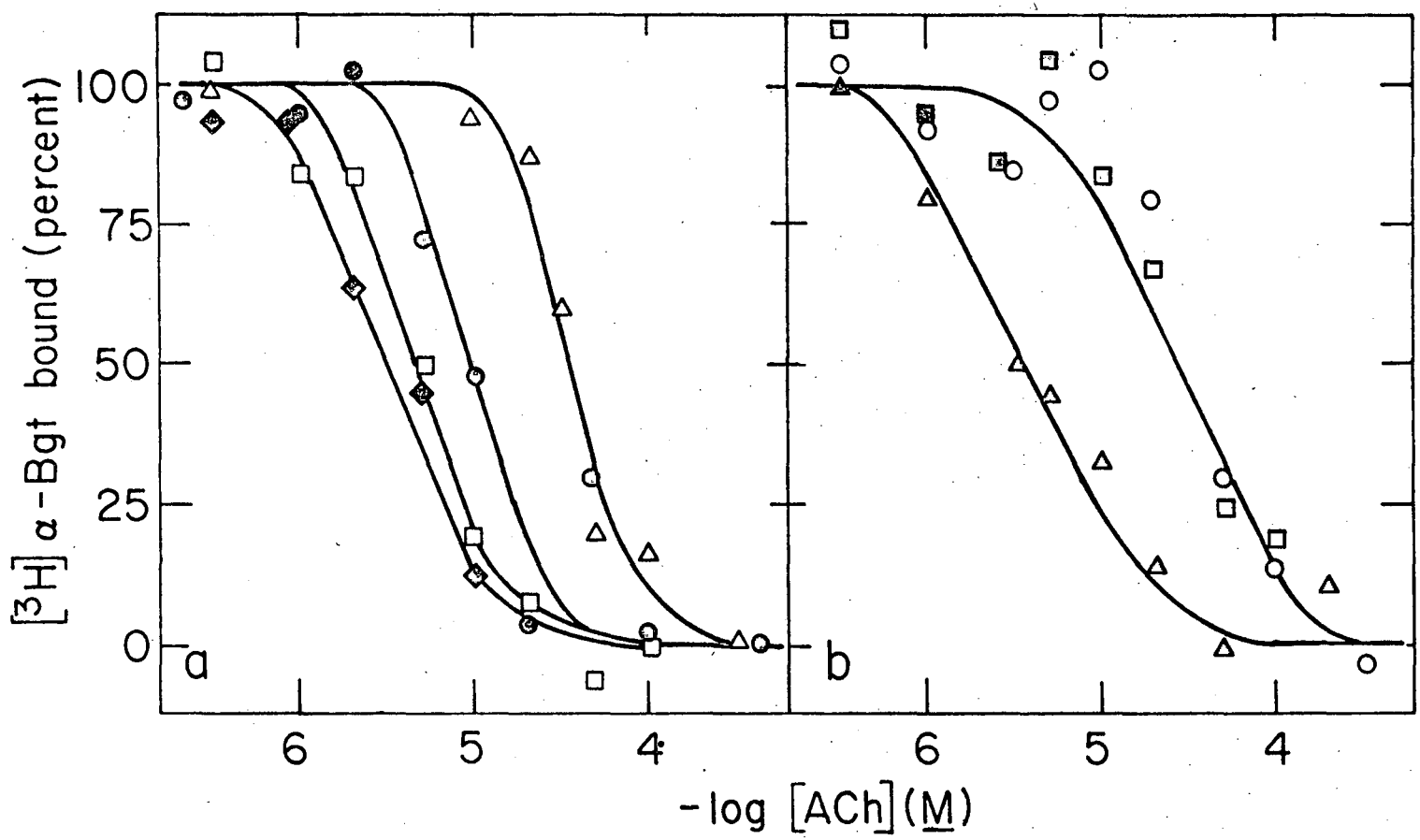

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Fig. 7

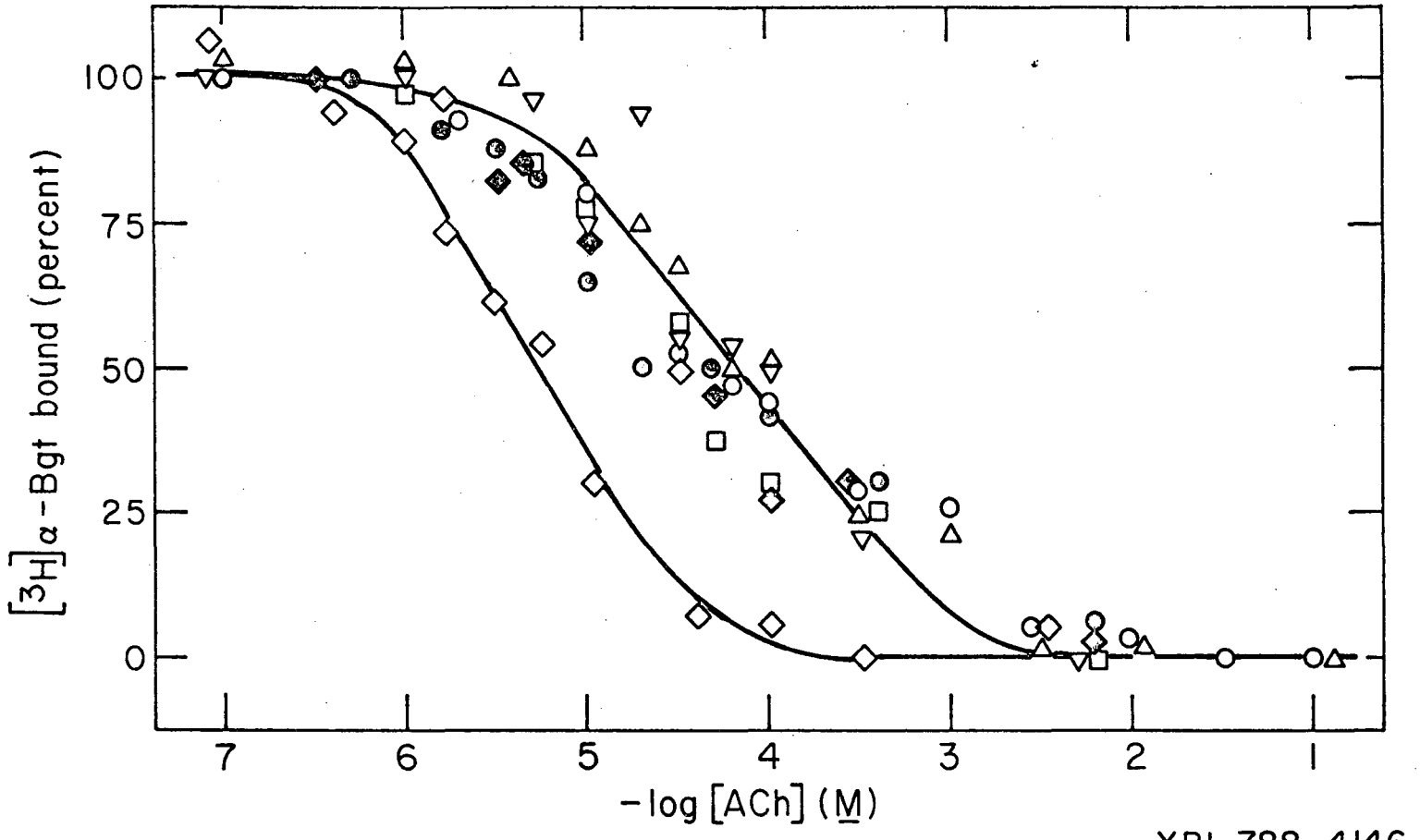

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Fig. 8

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