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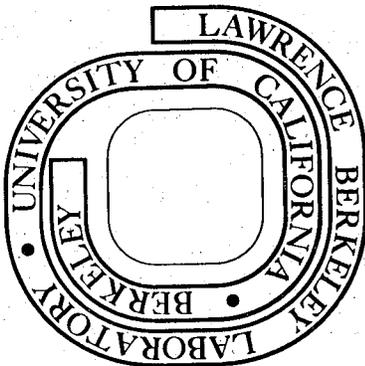
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AGONIST-INDUCED AFFINITY ALTERATIONS OF A CENTRAL NERVOUS SYSTEM
NICOTINIC ACETYLCHOLINE RECEPTOR*

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SUMMARY

Pretreatment of α -bungarotoxin (α -Bgt) binding sites from rat brain with cholinergic agonists causes transformation of sites to a high-affinity form toward agonist over a time course of minutes, consistent with identity of those sites as central nicotinic acetylcholine receptors (nAChR). This agonist-induced alteration in receptor state may be correlated with physiological desensitization. Agonist inhibition of toxin binding to the high-affinity state is non-competitive, suggesting the existence of discrete toxin-binding and agonist-binding sites on the central nAChR. These results thus offer a possible explanation of observed impotency of α -Bgt toward blocking in vivo cholinergic responses in the central nervous system.

INTRODUCTION

α -Bungarotoxin has been shown to interact with high affinity and specificity with non-uniformly distributed membrane-bound sites derived from rat brain. Toxin binding is uninhibited under physiological ionic strength conditions and is essentially 'irreversible' in nature (1). However, physiological experiments have demonstrated impotency of α -Bgt¹ toward blocking central cholinergic responses (2-4; however, see 5), and apparent differentiation between α -Bgt-binding sites and receptors mediating cholinergic responses has been demonstrated biochemically for cultured sympathetic neurons (6,7) and for ganglionic cells (8). Earlier results indicated that membrane-bound α -Bgt binding sites from rat brain exhibit unique sensitivity to cholinergic ligands (1), the nature of which is analogous to ligand-toxin interactions at peripheral nAChR sites (9-12). To provide evidence that α -Bgt-binding sites in brain are authentic cholinergic receptors rather than a

curious toxin-binding molecule, a detailed examination of the response of those entities to cholinergic ligands was undertaken.

MATERIALS AND METHODS

[¹²⁵I]-mono-iodinated α -Bgt and [³H] α -Bgt are prepared according to Lukasiewicz et al. (13). Preparation of 1000-17000g CMF from rat brain, methods used for specific α -Bgt binding assays, and sources for drugs used are as previously described (1) or are otherwise noted in legends and tables.

RESULTS

Ligand-putative CNS nAChR binding [$\tau_{\frac{1}{2}}^{\text{assoc}} \approx \text{msec}$; (14)] is assumed to equilibrate far faster than toxin-receptor binding [$\tau_{\frac{1}{2}}^{\text{assoc}} \approx 11 \text{ min}$, (1)]². Hence, any difference in ligand competition effectiveness toward toxin binding for the case where receptor is treated with ligand prior to exposure to toxin (preincubation) as opposed to the case where toxin and ligand are added simultaneously to receptor (coincubation), would reflect a ligand-induced alteration in receptor state. From the results shown in Figure 1, it is apparent that preincubation with ACh induces transformation of toxin receptors to a high-affinity form toward ACh. The apparent IC₅₀ on preincubation in this representative experiment is 40-fold lower than that for coincubation. The direction of the preconditioning effect precludes non-specific degradation of receptor or ACh during preincubation.

Results of multiple experiments testing competition effectiveness of cholinergic ligands (quantitated as IC₅₀ values) on coincubation and preincubation, and a measure of their ability to induce a high-affinity form of receptor (ratio of coincubation IC₅₀ to preincubation IC₅₀) are shown in Table I. The data illustrate the agonist specific nature of the phenomena; IC₅₀ ratios are 1.0 for the antagonists lobeline, gallamine and d-tubocurarine and larger than 1.0 for cholinergic agonists. There is also an apparent rank order of affinity alteration effectiveness among the cholinergic agonists, with acetylcholine being the most effective agent tested.

The rate of transformation (Fig. 2) of receptor to the high-affinity form, determined from experiments where membranes are pretreated with 10^{-5} M ACh for various periods before initiation of toxin-binding reaction, has a half-time of about 2 minutes. From experiments where membrane preparations pretreated with ACh are diluted prior to initiation of toxin-binding reaction, reversal of that transformation is found to occur within the 30-minute toxin-binding assay period.

The rate of toxin binding is also affected differently by cholinergic agonist for preincubation and coincubation (Fig. 3). On preincubation, there is a monophasic approach to equilibrium binding and the maximum number of available sites decreases as ACh concentration increases. At a sufficiently high ACh concentration (10^{-5} M), no toxin binding is detected, even over a period of hours. For the coincubation condition, while the initial rate of toxin binding is largely unaffected by ACh at concentrations as high as 10^{-3} M, there is an ACh concentration-dependent phase of reversal of toxin binding as the duration of exposure to toxin and ligand is increased (Fig. 3). At long times, the extent of toxin binding at high ACh concentration approaches that for the preincubation condition. Since all toxin binding assays are terminated by the addition of an excess of native α -Bgt for a duration of 30 minutes prior to the centrifugation procedure used to separate free from bound radiolabeled toxin, these results indicate that prolonged exposure of toxin-membrane complexes to ACh leads to an accelerated decay of complexes, faster than that seen for the purely competitive action of native α -Bgt.

In order to examine the nature of agonist competition for toxin binding, the effect of 30 minute preincubation with carbachol on saturation of sites with α -Bgt was ascertained and found to deviate from simple mass action effects (Fig. 4). At low concentrations of carbachol (10^{-5} M), only apparent K_D is affected. As carbachol concentrations are increased, both K_D^{app} and apparent maximum binding are altered. The highest concentration of carbachol

blocks α -Bgt binding completely. These profiles are closely approximated by models assuming irreversible attachment of ligand during preincubation or transformation of sites to the exclusion of toxin binding. In contrast, the effect of coincubation with carbamylcholine on saturation of toxin sites with α -Bgt follows simple mass action models for a reversible competitive inhibitor of toxin binding, affecting only K_D^{app} values.

The different nature of agonist competition for α -Bgt binding sites on preincubation as opposed to coincubation is further demonstrated by double-reciprocal plots for acetylcholine competition (Fig. 5). On coincubation, apparent K_D values rise with increasing concentration of ACh, but the common ordinate intercept indicates that the number of maximum available sites is unaltered, hence the inhibition of toxin binding is competitive. In contrast, double reciprocal plots for preincubation with acetylcholine show upward curvature, and yield different values of K_D^{app} and maximum binding. These data fit models for non-competitive inhibition by a reversible ligand.

DISCUSSION

The α -Bgt binding entity derived from rat brain responds in a unique manner to cholinergic ligands. Pretreatment of sites with agonist, but not antagonist, transforms sites to a high-affinity form toward agonist. The agonist-specific affinity alteration is in agreement with other studies on the effect of cholinergic ligands on membrane-bound nAChR from rat skeletal muscle (9) or Torpedo electroplax (11,12), but differs from another report (10) where both agonists and antagonists were found to cause induction of the high-affinity state. From this result, it may be argued that α -Bgt binds to an authentic cholinergic receptor in the CNS with demonstrated nicotinic pharmacology (1).

Furthermore, the rank order of potency exhibited by cholinergic agonists on eliciting this change in receptor state, and the time course of its onset

suggest that these biochemical studies characterize CNS nAChR state transitions analogous to those expected for physiological desensitization (15).

While cholinergic agonists are found to decrease the rate of toxin binding to peripheral nAChR, there is disagreement in the literature concerning the level of toxin binding to the high-affinity state at equilibrium (9,10,12). From the data presented herein, it is clear that both the initial rate of toxin binding and the equilibrium level of toxin-nAChR complexes are affected by preincubation with cholinergic agonist. It would appear that toxin binding and agonist binding to the high-affinity state of the CNS nAChR are mutually exclusive. Taken together with the demonstration of the relative impotency of cholinergic agonists toward displacement of toxin binding on coincubation and the non-competitive nature of agonist-mediated inhibition of toxin binding to the high-affinity form of nAChR, these results indicate a) that the high-affinity agonist binding site(s) and the α -Bgt binding site(s) on the CNS nAChR do not overlap, and b) that transformation to the high-affinity state is accompanied by allosteric changes in the CNS nAChR to the exclusion of toxin binding. If these interpretations be correct, the apparent impotency of α -Bgt toward blocking in vivo cholinergic responses in the CNS (2-4) may be explained by invoking the existence of toxin-insensitive, conductance-control, agonist-binding sites on the CNS nAChR.

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LEGENDS

- Figure 1. Extent of specific [^3H] α -Bgt binding (percent of control values) to rat brain CMF is plotted as a function of $-\log$ ACh concentration. CMF suspended in binding Ringers are treated with $100\ \mu\text{M}$ eserine and incubated in the presence of acetylcholine for 30 minutes prior to exposure to $10\ \text{nM}$ α -Bgt (preincubation, X), or ligand and toxin are added simultaneously to membranes (coincubation, O). The specific manner in which toxin, ligand and membranes were mixed and coincubation experiments started was found to have no quantitative effect on the data. The eserine concentration used was sufficient to block all cholinesterase activity over the complete time course of the experiment, yet did not alter α -toxin binding levels. Exposure to [^3H] α -Bgt is for 30 minutes before termination of reaction by addition of native toxin to $4\ \mu\text{M}$.
- Figure 2. Rate of transformation of receptor state in the presence of $10\ \mu\text{M}$ ACh is determined from plot of [^3H] α -Bgt specifically bound (percent of control) to CMF as a function of time preincubated (log scale) with ACh. Insert: recovery of specific binding activity on dilution of ACh to $1\ \mu\text{M}$. For both assays, duration of exposure to [^3H] α -Bgt ($10\ \text{nM}$) is 30 minutes.
- Figure 3. Extent of specific toxin binding to CMF as a function of duration of exposure to $5\ \text{nM}$ α -Bgt for 30 minute preincubation (left) and for coincubation (right) with ACh at concentrations indicated.
- Figure 4. Effect of carbachol on saturation of [^3H] α -Bgt binding sites for preincubation (left) and coincubation (right). Exposure to toxin is for 30 minutes, and carbachol concentrations are as indicated.
- Figure 5. Double reciprocal plot of the effect of acetylcholine on saturation of [^3H] α -Bgt sites for preincubation (left) and coincubation (right).

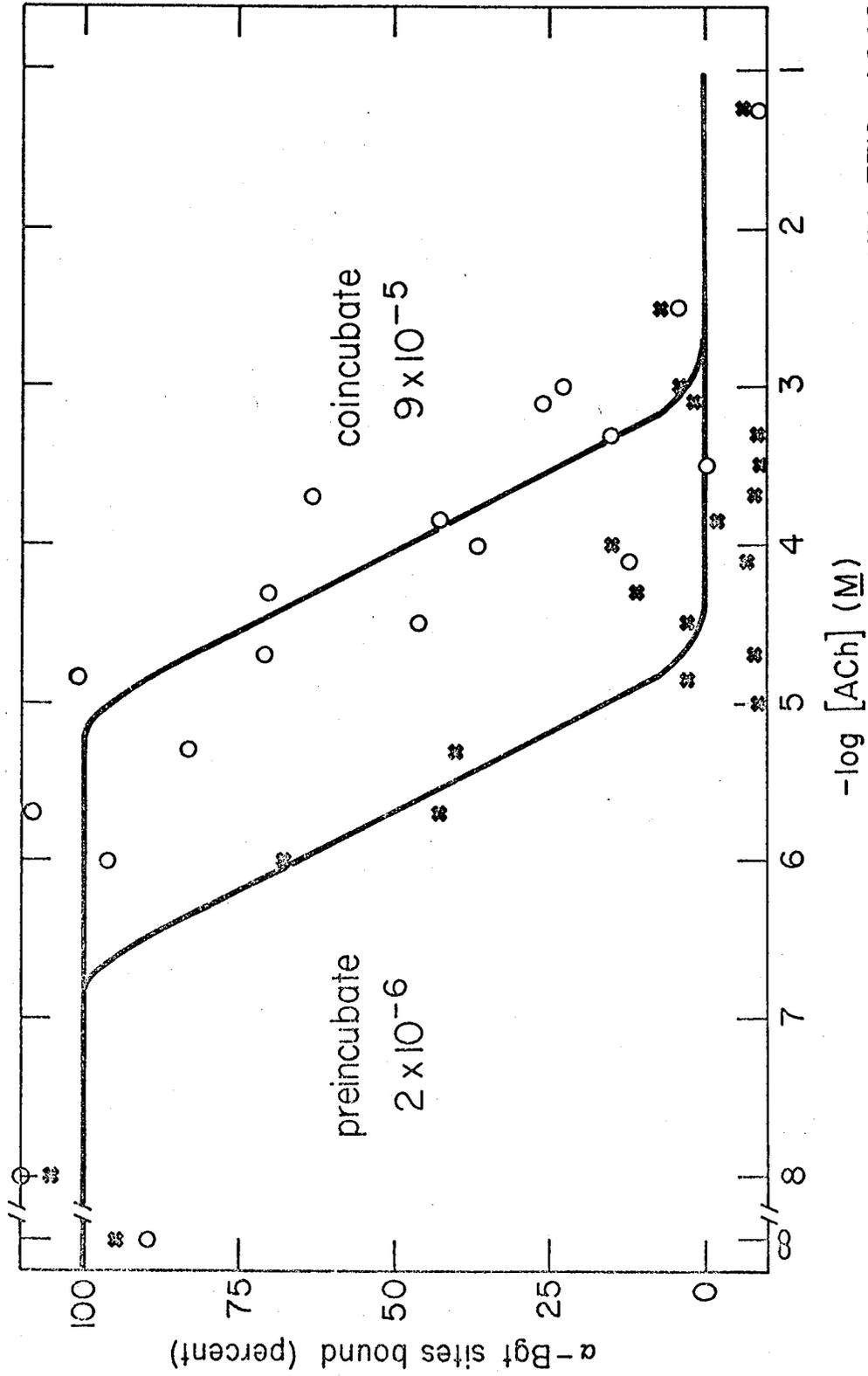
Table 1
SUMMARY OF LIGAND COMPETITION EXPERIMENTS

Drug	Coincubation IC ₅₀ (M)	Preincubation IC ₅₀ (M)	Ratio
acetylcholine	5x10 ⁻⁵	2x10 ⁻⁶	25
S-acetylthiocholine	2x10 ⁻⁴	1x10 ⁻⁵	20
butyrylthiocholine	5x10 ⁻⁴	5x10 ⁻⁵	10
decamethonium	5x10 ⁻⁴	6x10 ⁻⁵	8
carbachol	3x10 ⁻⁵	5x10 ⁻⁶	6
nicotine	5x10 ⁻⁶	1x10 ⁻⁶	5
trimethylphenylammonium	1x10 ⁻⁴	2x10 ⁻⁵	5
hexamethonium	1x10 ⁻²	5x10 ⁻³	2
gallamine	5x10 ⁻⁴	4x10 ⁻⁴	1.3
lobeline	3x10 ⁻⁵	3x10 ⁻⁵	1
d-tubocurarine	2x10 ⁻⁵	2x10 ⁻⁵	1

Results of experiments as exemplified in Figure 1 designed to determine competition effectiveness of cholinergic drugs toward specific [³H]α-Bgt binding. Sources of drugs are S-acetylthiocholine iodide, butyrylthiocholine iodide (Calbiochem); decamethonium bromide, trimethylphenylammonium iodide, lobeline HCl (Sigma); gallamine triethiodide (ICN), and as cited in Lukasiewicz and Bennett (1).

¹Abbreviations used: α -Bgt, α -bungarotoxin; ACh, acetylcholine; nAChR, nicotinic acetylcholine receptor; [³H] α -Bgt, [³H]-labeled α -Bgt; CMF, rat brain crude mitochondrial fractions; IC₅₀, concentration of ligand at which 50% of specific toxin binding is inhibited; CNS, central nervous system.

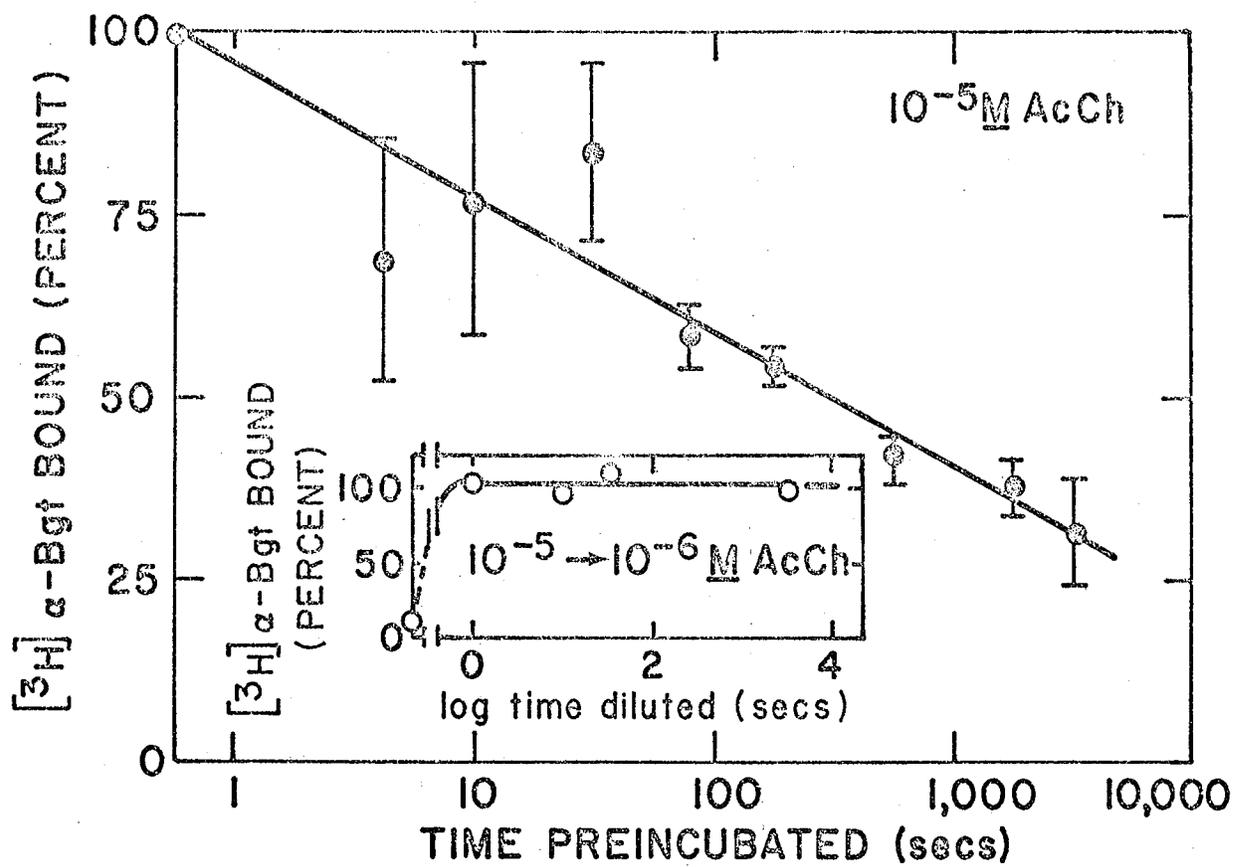
²Given the very slow rates of toxin-receptor association and dissociation, toxin binding in the experiments reported herein is never at true equilibrium. Hence, application of equilibrium kinetic analysis to the data is justified only as a first approximation. A more rigorous treatment of these phenomena would require analysis of initial rates of toxin binding. However, the low concentrations of receptor render such experiments difficult. Moreover, the results presented indicate that initial rates of toxin binding and the number of toxin sites occupied as equilibrium is approached are affected differently at a given agonist concentration for the coincubation condition, aside from the documented difference between preincubation and coincubation. Thus, the measured values of apparent ligand affinity for the receptor and the precise interpretation of the data is necessarily preliminary and qualitative. It is within this context that results are presented and the general interpretations of the data discussed.



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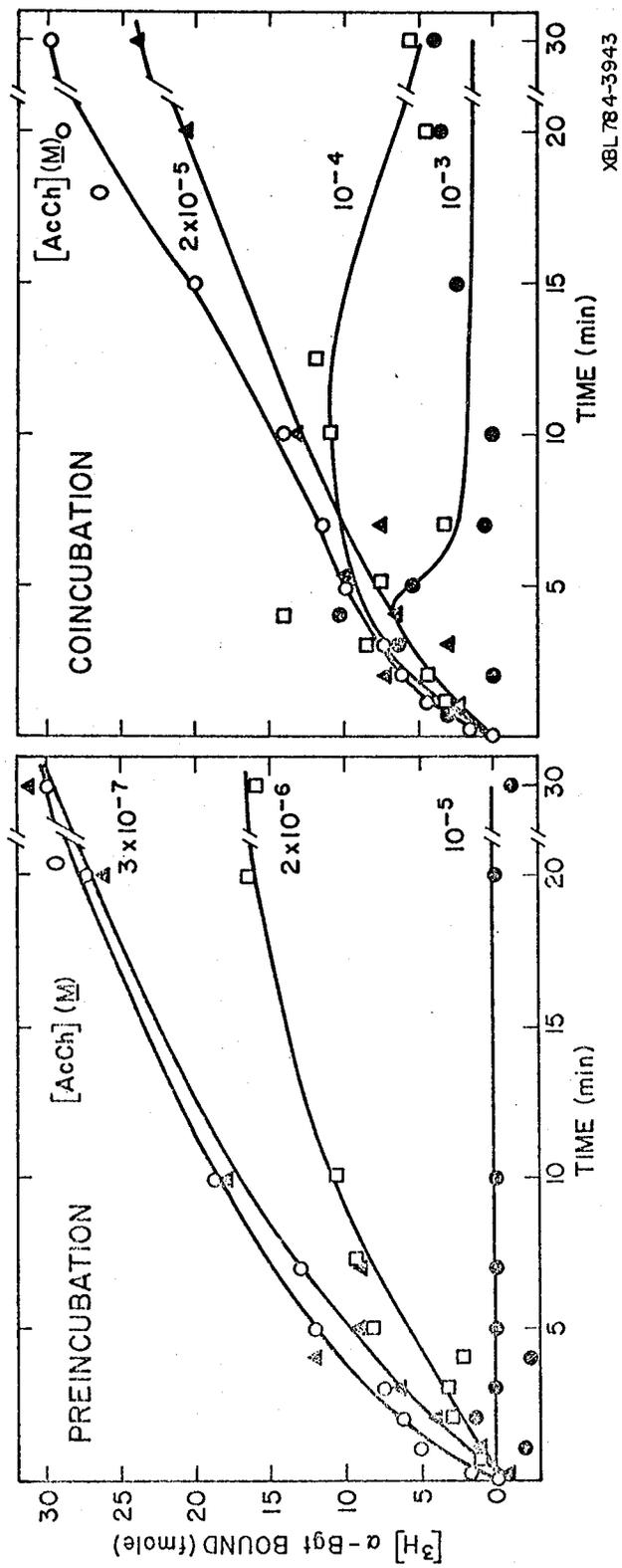
Figure 1



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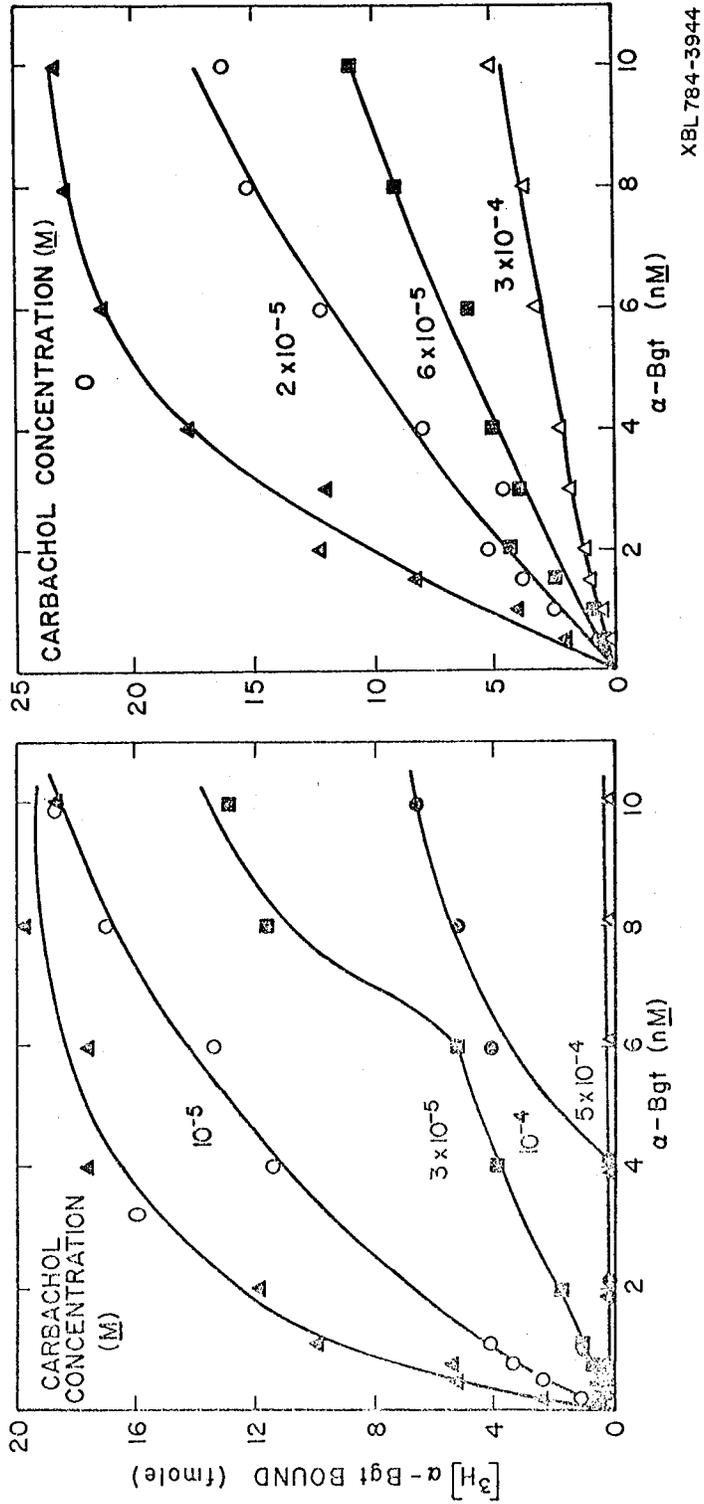
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Figure 2



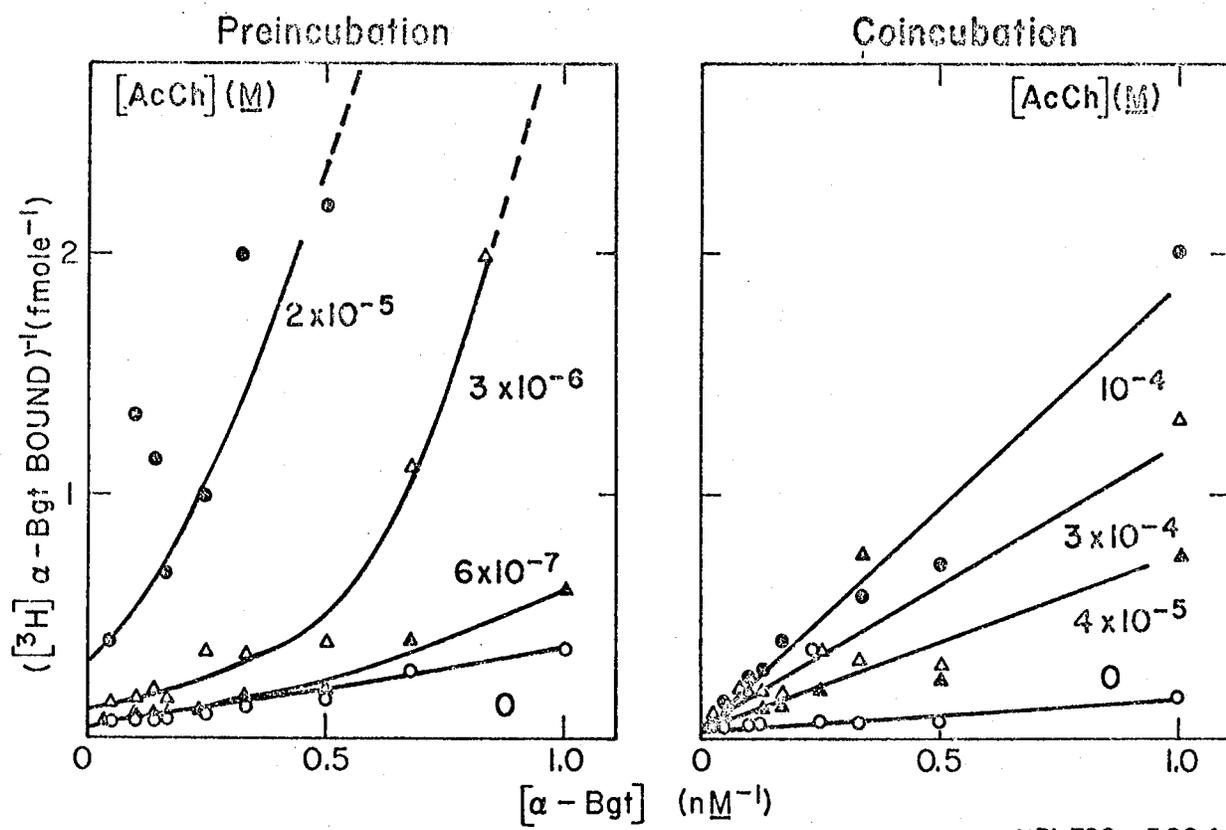
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Figure 3



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Figure 4



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Figure 5