Submitted to Biochimica et
Biophysica Acta

LBL-2645
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NICOTINIC CHOLINERGIC RECEPTOR IN BRAIN DETECTED BY BINDING OF [^{3}H]α-BUNGAROTOXIN

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Running Title: Cholinergic Receptor in Brain

SUMMARY

[^{3}H]α-Bungarotoxin ([^{3}H]α-Bgt) was prepared by catalytic reduction of iodinated α-Bgt with tritium gas. Crude mitochondrial fraction from rat cerebral cortex bound 40-60 · 10^{-15} moles of [^{3}H]α-Bgt per mg of protein. This binding was reduced by 50% in the presence of approx. 10^{-6} M d-tubocurarine or nicotine, 10^{-5} M acetylcholine, 10^{-4} M carbamylcholine or decamethonium or 10^{-3} M atropine. Hexamethonium and eserine were the least effective of the drugs tested. Crude mitochondrial fraction was separated into myelin, nerve endings, and mitochondria. The highest binding of toxin per mg of protein was found in nerve endings, as well as the greatest inhibition of toxin binding by d-tubocurarine. Binding of [^{3}H]α-Bgt to membranes obtained by osmotic shock of the crude mitochondrial fraction indicates that the receptor for the toxin is membrane bound. The capacity to bind was extracted by incubation with sodium deoxycholate. ^{125}I-labeled α-Bgt, prepared with Na^{125}I and chloramine T, was highly specific for the acetylcholine receptor in diaphragm; however, it was less specific and less reliable than [^{3}H]α-Bgt in brain. We conclude that a nicotinic cholinergic receptor exists in brain, and that [^{3}H]α-Bgt is a suitable probe for this receptor.

INTRODUCTION

After the pioneer work of De Robertis *et al.* on the characterization of synaptic receptors in rat brain[1] major progress in this area has been

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Abbreviations: AChR, acetylcholine receptor; ACh, acetylcholine; α-Bgt, α-bungarotoxin; [^{3}H]α-Bgt, tritiated α-bungarotoxin; fmoles, femtomoles = 10^{-15} moles.

achieved using peripheral systems. Cholinergic nicotinic receptors from electric organs and muscle have been studied and purified extensively. α-Type* neurotoxins from cobras or Bungarus multicinctus venoms have proved to be very specific reagents for the AChR in these systems[2]. However, information on a nicotinic AChR in brain remains scarce. Farrow and O'Brien[3] could not detect binding of nicotinic drugs to brain particles, but Moore and Loy[4] reported binding of an α-toxin to a sodium deoxycholate extract of brain particles. While the present work was in progress, a communication was published that describes the binding of ^{125}I-labeled α-Bgt to brain membranes as a function of age of the rat[5].

The experiments reported here describe the pharmacological characteristics of [^{3}H]α-Bgt binding to brain particles and the subcellular distribution of the toxin-binding component. ^{125}I-labeled binding was studied as well, and the striking difference in binding between the two labeled derivatives of the same toxin is discussed. A summary report of these results has been presented at the Third Annual Meeting of the Society for Neuroscience, San Diego, California (U.S.A.), November 1973[6].

MATERIALS AND METHODS

Crude venom from Bungarus multicinctus was obtained from Miami Serpentarium. CM-Sephadex C-50 was from Pharmacia, CM-Cellulose (CM-52) from Whatman. Nicotine hydrochloride, atropine and decamethonium iodide were obtained from K and K Laboratories, hexamethonium chloride dihydrate from Mann Research Laboratories, carbamylcholine chloride from Sigma Chemical

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*α-type toxins from snake venoms block impulse transmission by acting postsynaptically--that is, they bind to the receptors.

Co., and d-tubocurarine chloride from Calbiochem. Acetylcholine perchlorate was synthetized in our laboratory. BW-284C51 (1:5 bis(4-allyldimethylammoniumphenyl) pentan-3-one diiodide) was a gift from Burroughs Wellcome & Co., and promethazine a gift from Wyeth. Acetythiocholine was obtained from Calbiochem, and chloramine T from Matheson, Coleman and Bell. $Na^{125}I$ carrier free was purchased from New England Nuclear. Carrier free tritium was supplied by Lawrence Livermore Laboratory.

α-Bgt was isolated from crude venom of Bungarus multicinctus by ion exchange chromatography on CM-Sephadex and CM-Cellulose as described by Mebs et al.[7] It was distinguished from the other toxins of this venom by its amino acid composition, toxicity in mice and action on frog muscle, all of which compared favorably with the published values[7,8]. Upon electrophoresis in sodium dodecylsulfate-polyacrylamide gels, at least 90% of the material migrated in a single band, and the faint "contaminant" band was most likely a dimer of α-Bgt on the basis of its relative mobility.

$[^3H]$α-Bgt was prepared by catalytic reduction of iodinated α-Bgt with carrier free tritium gas (details to be published). Tritiated toxin was purified by gradient elution from a CM-Cellulose column. Its toxicity in mice after subcutaneous injection was similar to native toxin. The specific activity was 14.7 Ci/mmol, that is, 0.5 atoms of tritium per molecule of toxin. For storage purposes, 1 ml aliquots of $[^3H]$α-Bgt (30 μg/ml) in 0.2 M ammonium acetate, pH 5.8, were frozen in liquid nitrogen and kept at -10°C. After 6 months of storage, 6% of radioactivity was exchanged with the solvent, but the binding characteristics of the labeled toxin have not changed.

Subcellular fractionation of brain homogenates. Male Sprague-Dawley rats (from Simonson, Gilroy, California, U.S.A.) of 200-300 g were used. Subcellular fractionation was done following the conventional techniques of De Robertis[9] and Whittaker[10]. A 10% homogenate of cerebral cortex in 0.32 M sucrose, pH 7.0, was centrifuged at 1 000 x g for 10 min and the precipitate was discarded. The supernatant was then centrifuged at 17 300 x g for 20 min (Sorvall RC-2B). This precipitate, called crude mitochondrial fraction, was fractionated into nerve endings, mitochondria and myelin by a discontinuous sucrose gradient[10]. The microsomal fraction was obtained by centrifuging at 100 000 x g (Spinco L) for 90 min the supernatant from the crude mitochondrial fraction. The total particulate fraction was the precipitate after centrifuging the whole homogenate at 100 000 x g for 90 min. In some experiments the crude mitochondrial fraction was submitted to osmotic shock with 10 ml of distilled water per g of original weight, and membranes pelleted at 17 000 x g after 30 min were used. Extracts of these membranes were obtained by incubation with sodium deoxycholate as described by Changeux et al.[11]

Acetylcholinesterase (EC 3.1.1.7) was assayed by the method of Ellman et al.[12] with $6 \cdot 10^{-4}$ M acetylthiocholine as substrate and $5 \cdot 10^{-7}$ M promethazine to inhibit cholinesterase (3.1.1.8).

Binding assay. To determine the binding of α-Bgt, the precipitated particles, usually the crude mitochondrial fraction, were resuspended in Ringer's solution (115 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1.3 mM $MgSO_4$, 4 mM KH_2PO_4, 33 mM Tris-HCl (pH 7.4), and 3.3 mM glucose). This suspension was incubated with [^{3}H]α-Bgt in Ringer's solution at room temperature, in a final volume of 1.5-2 ml. The particle fraction contained 3-4.5 mg

protein per ml and consisted of the material obtained from 100 mg of original wet weight. When the effect of cholinergic drugs on the binding of α-Bgt was studied, 1 ml of suspension was incubated with 0.5 ml of the drug in Ringer for 60 min. [^{3}H]α-Bgt (0.5 ml) was then added and the incubation was continued for 15 min. Unbound toxin was removed by centrifugation at 39 000 x g for 10 min. The precipitate was washed 3 times by resuspension in 10 ml Ringer's solution. For the drug competition experiments, the washing solution contained 10^{-3} M competitor. No radioactivity was detected in the supernatant after the third wash. The precipitate was oxidized to ^3H_2O and CO_2 in a Packard 305 Sample Oxidizer and the product was counted in a Packard Tri-Carb scintillation spectrometer with 30-34% efficiency. Protein content of each tube was determined by the method of Lowry et al.[13] in an aliquot taken from the suspension after the second wash.

^{125}I-labeled α-Bgt was prepared from α-Bgt and Na^{125}I by oxidation with chloramine T as described by Berg et al.[14] The initial specific activities were in the range of 3-8 · 10^4 cpm/pmole. The binding assay was as described for [^{3}H]α-Bgt except that the washed pellet was resuspended in 1 ml Ringer solution and counted in a γ-ray NaI well counter from Nuclear Chicago with about 10% efficiency. ^{125}I-labeled α-Bgt standards were counted simultaneously to correct for decay of ^{125}I which has a half-life of 60 days. ^{125}I-labeled α-Bgt was always used within a month after iodination. Binding to diaphragm was assayed as described by Berg et al.[14], except that the tissue was counted without homogenization in the γ-ray counter.

Except for the studies using diaphragm, the reported values are means of duplicate determinations.

RESULTS

[^{3}H]α-Bgt binding to brain subcellular particles. High affinity binding of [^{3}H]α-Bgt to brain crude mitochondrial fraction was observed with 1-40 nanomolar toxin (Fig. 1). Maximum binding varied from 40 to 60 femtomoles per mg of protein for different preparations, depending probably on the purity of mitochondrial fractions. The equilibrium constant for this binding was $5.6 \cdot 10^{-9}$ molar. At higher toxin concentrations, a lower affinity binding was observed. In a similar assay, the total particulate fraction from Saccharomyces cerevicae bound only 8% of the value obtained for cortex.

The binding of [^{3}H]α-Bgt to brain particles was reduced by cholinergic drugs (Fig. 2). A nicotinic antagonist, d-tubocurarine, and an agonist, nicotine, protected 50% of toxin binding sites at concentrations near 10^{-6} M. Acetylcholine, carbamylcholine and decamethonium reached 50% protection between 10^{-5} and 10^{-4} M. A muscarinic antagonist, atropine, had little effect until 10^{-4} M, while concentrations of 10^{-9} M affect muscarinic receptors[15]. A ganglionic antagonist, hexamethonium, and an acetylcholinesterase inhibitor, eserine, had little effect even at high concentrations. Native α-Bgt at 10^{-7} M inhibited [^{3}H]α-Bgt binding by 80%. The remaining 20% was probably non-specific binding, since it was not affected by any of the drugs tested.

Unexpectedly, the microsomal fraction bound [^{3}H]α-Bgt as well (Table I). The bindings to mitochondrial and microsomal fractions were inhibited similarly by d-tubocurarine, suggesting that a similar kind of receptor is involved in both cases. Relative specific activity of acetylcholinesterase is also higher in microsomes (Table I, Ref. 9). Contamination of the microsomal fraction with cholinergic nerve endings is possible but

not necessary, as the presence of acetylcholinesterase in endoplasmic reticulum and cellular membrane has been demonstrated by histochemical methods[16]. Presence of AChR in axonal membrane of central neurons has been postulated[16] and an α-Bgt-binding molecule has been described in the axonal membrane of the lobster walking leg[17].

Upon fractionation of the crude mitochondrial fraction into myelin, nerve endings and mitochondria, the highest specific activity for $[^3H]\alpha$-Bgt binding was found in nerve endings (Fig. 3). Some of the binding to myelin and mitochondria is probably due to cross-contamination with nerve endings, since it is partially inhibited by d-tubocurarine.

Binding of $[^3H]\alpha$-Bgt to membranes obtained by osmotic shock of the crude mitochondrial fraction is illustrated in Fig. 4. The equilibrium constant for this binding, $3.2 \cdot 10^{-9}$ M, is very similar to the one found with crude mitochondria ($5.6 \cdot 10^{-9}$ M). This suggests that the same receptor is involved in both cases, and that it is a membrane-bound molecule. After incubation of the membranes with sodium deoxycholate the binding capacity was extracted, as revealed by incubation of the extract with $[^3H]\alpha$-Bgt. Excess toxin was removed in this case by filtration through Sephadex G-75; the bound toxin appeared with the high molecular weight material, slightly after the bulk of brain proteins (Fig. 5).

<u>^{125}I-labeled α-Bgt binding to brain and diaphragm</u>. Results obtained with two different preparations of ^{125}I-labeled α-Bgt are presented. One of these preparations (Prep. 3) showed saturable binding at 3 to 4 pmoles bound per g of original weight (approx. 70 fmoles/mg protein). This

amount is similar to the value obtained with [^{3}H]α-Bgt (40-60 fmoles/mg protein), but only 50% of this binding was inhibited by preincubation with 10^{-3} M d-tubocurarine (Fig. 6a). At saturating toxin concentrations maximum binding was reached in 10 min. The amount and the rate of ^{125}I-labeled α-Bgt binding showed little change with temperature between 0°C and 37°C (results not shown). When the same toxin was assayed for specific binding to rat diaphragm, more than 80% of bound radioactivity was found in the area containing end plates (Fig. 7a). These results confirm previous findings[14] that ^{125}I-labeled α-Bgt is highly specific for AChR in diaphragm. It is also shown that "old" ^{125}I-labeled α-Bgt differs from freshly iodinated toxin mainly by markedly decreased total binding due primarily to very large loss in the specific binding component. On the contrary, when "old" ^{125}I-labeled α-Bgt was incubated with brain particles non-specific binding was greatly increased.

A second preparation of ^{125}I-labeled α-Bgt (Prep. 4) showed only the characteristics of non-specific binding to brain even when freshly prepared (Fig. 6b). This binding was not saturable; it was not decreased in the presence of d-tubocurarine, or carbamylcholine, and the amount bound was increased about 5 times over previous results (Fig. 6b). However, the same toxin preparation showed highly specific binding to diaphragm. The saturation curve was very similar to the one shown on Fig. 7a. Preincubation with d-tubocurarine or carbamylcholine inhibited most of the binding to the area containing end plates. About half of the binding to the area without end plates was inhibited as well, suggesting that some AChR may exist in this area (Fig. 7b) (see also Ref. 14).

In conclusion, although ^{125}I-labeled α-Bgt seems to be specific for AChR from diaphragm, different toxin preparations gave inconsistent results with brain particles.

DISCUSSION

The binding of [^{3}H]α-Bgt to brain cortex as described here strongly suggests the presence of a nicotinic AChR in this tissue. The component(s) binding [^{3}H]α-Bgt is present in low concentration, in the order of pmoles/g. This result agrees with those reported in two previous communications (4,5); the value of 17.5 nmoles/g reported elsewhere[18] seems increasingly improbable.

Table II shows the striking similarities between pharmacological profiles of the AChR from ganglionic neurones in tissue culture[19] and the [^{3}H]α-Bgt binding to cerebral cortex reported here. Nicotinic receptors from muscle and electroplax were more sensitive to decamethonium, as was expected, but otherwise they were not grossly different from the neuronal receptors.

Results obtained with [^{3}H] α-Bgt and ^{125}I-labeled α-Bgt invite some comparisons. While [^{3}H]α-Bgt was a reliable reagent for brain AChR and its binding characteristics did not noticeably change after 6 months of storage, ^{125}I-labeled α-Bgt showed a marked tendency to progressively higher and higher non-specific binding in brain approximately a month after iodination. This behavior was clearly different from that exhibited with diaphragm where "old" toxin showed decreased binding. Moreover, some ^{125}I-labeled α-Bgt preparations did not show saturable binding to brain particles or competition with cholinergic drugs, even when the usual specific binding was seen

for diaphragm. Thus conditions for specific binding of α-Bgt to brain AChR seem to be more stringent than those required for diaphragm. Brain and muscle receptors are probably not identical as suggested by their sensitivies to drugs (Table II), but the difference in ^{125}I-labeled α-Bgt non-specific binding is more likely related to dissimilar biochemical composition of both organs. Salvaterra and Moore[5] reported specific binding of ^{125}I-labeled α-Bgt to brain particles. Their toxin was iodinated with ^{125}ICl instead of Na^{125}I and Chloramine T used by us. It is possible that failure of freshly prepared ^{125}I-labeled α-Bgt to bind specifically to brain AChR is due to some damage of the protein by the strong oxidant chloramine T.

The widespread existence of acetylcholine, cholineacetylase and acetylcholinesterase in central nervous system has prompted the search for central cholinergic pathways. Krnjević indicates that an ascending cholinergic system may mediate cortical arousal. However, the responses of most central neurones to iontophoretically applied acetylcholine were different from peripheral nicotinic synapses[22]. Alternative mechanism for acetylcholine central action have been proposed. According to Koelle's model, in some instances acetylcholine reacts with axonal AChR to facilitate the liberation of more acetylcholine or other neurotransmitters[16]. Since microsomal fraction contains mainly non-synaptic membrane[23], specific binding of [^{3}H]α-Bgt to this fraction could be an indication of the existence of extrasynaptic AChR.

ACKNOWLEDGMENTS

This work was supported, in part, by U. S. Atomic Energy Commission. One author (VAE) had a postdoctoral fellowship of Consejo Nacional de Investigaciones Cientificas y Tecnicas of Argentina. Thanks are given to Hiromi Morimoto for performing the assays for acetylcholinesterase and to Marie Hebert for assistance in the preparation of purified α-Bgt.

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TABLE I

[^{3}H]α-BUNGAROTOXIN BINDING TO PRIMARY FRACTIONS FROM RAT CORTEX

The preparation of the primary fractions, the binding assay and the enzyme assay were described in METHODS. Final particle concentrations in mg protein/ml were: total particulate 5.0, mitochondrial fraction 4.9, microsomal fraction 4.2. Final [^{3}H]α-Bgt concentration was $6.3 \cdot 10^{-9}$ M. Final d-tubocurarine concentration was 10^{-4} M. Relative specific activity for acetylcholinesterase is defined as specific activity in a given fraction divided by the specific activity of total particulate, which was 190 nmoles/min, mg protein.

	Total Particulate	Mitochondrial Fraction	Microsomal Fraction
	fmoles bound / mg protein		
Control	15	15	21
+ d-Tubocurarine	5	6	6
% Inhibition	67%	60%	71%
	Relative specific activity		
Acetylcholinesterase	1.00	0.81	2.00

TABLE II

DRUG AFFINITIES FOR ACETYLCHOLINE RECEPTORS FROM SEVERAL SOURCES

In the 4 initial columns protection against α-Bgt binding by the respective drug was measured. Figures indicate the concentration necessary to decrease toxin binding to 50%. Column 1 shows data from Fig. 2. Columns 2, 3 and 4 were obtained with ^{125}I-labeled α-Bgt (Refs. 19, 20 and 14 respectively). Data shown in column 5 were obtained by measuring changes in ionic fluxes of "microsacs" from electric organs upon the application of the drug; the figures are concentrations necessary for half of the maximal response. *: acetylcholine plus an inhibitor of acetylcholinesterase.

	Brain crude mitochondrial fraction	Chick sympathetic neurones	Chick muscle cells	Rat diaphragm	"Microsacs" from Electrophorus electricus
d-Tubocurarine	$7.4 \cdot 10^{-7}$	$2.5 \cdot 10^{-7}$	$7.4 \cdot 10^{-7}$	10^{-5}	$1.5 \cdot 10^{-7}$
Nicotine	$1.3 \cdot 10^{-6}$	$5 \cdot 10^{-7}$	$<10^{-5}$		
ACh + inhibitor*	$2.5 \cdot 10^{-5}$	$1.6 \cdot 10^{-5}$	$3.1 \cdot 10^{-7}$		$4 \cdot 10^{-6}$
Carbamylcholine	$1.4 \cdot 10^{-4}$		$\sim 10^{-5}$	10^{-4}	$3.3 \cdot 10^{-6}$
Decamethonium	$2.2 \cdot 10^{-4}$	$2 \cdot 10^{-4}$	$2.3 \cdot 10^{-7}$		$1.2 \cdot 10^{-6}$
Hexamethonium	$>10^{-3}$	$6 \cdot 10^{-4}$	$>>10^{-5}$		$6.2 \cdot 10^{-5}$
Atropine	$5.6 \cdot 10^{-4}$	$>10^{-3}$	10^{-4}	$>10^{-3}$	
Eserine	$>10^{-3}$		$3 \cdot 10^{-5}$		

FIGURE LEGENDS

Fig. 1. [^{3}H]α-Bungarotoxin binding to crude mitochondrial fraction. Details about preparation of the particles and the binding assay are given in METHODS. Final particle concentration was 100 mg original weight/ml, or 4.3 mg protein/ml. Incubation time was 1 h. The inset is the double reciprocal plot of original data, which gave a value for the equilibrium constant of $5.6 \cdot 10^{-9}$ M.

Fig. 2. Inhibition of [^{3}H]α-Bungarotoxin binding by cholinergic drugs. Crude mitochondrial fraction (3.0-4.5 mg protein/ml) was incubated with the competitor for 1 h. [^{3}H]α-Bgt was then added at a final concentration of $6.2 \cdot 10^{-9}$ M and the incubation was continued for 15 min. The samples were then processed as described in METHODS. The data relative to α-Bgt, d-tubocurarine and carbamylcholine were obtained with one mitochondrial preparation; those relative to nicotine, atropine and eserine with a second preparation, and those for decamethonium, hexamethonium, acetylcholine + BW-284c51, and BW-284c51 with a third. 100% binding was 18, 20 and 25 fmoles/mg protein respectively for the three preparations. +, α-Bgt; ▲, d-tubocurarine chloride; △, nicotine/hydrochloride; o, acetylcholine perchlorate + $1.8 \cdot 10^{-5}$ M BW-284c51; ●, carbamylcholine chloride; □, decamethonium iodide; ▽, atropine; ■, hexamethonium chloride; x, eserine salicylate; ⊙, BW-284c51.

Fig. 3. [^{3}H]α-Bungarotoxin binding to submitochondrial fractions. Submitochondrial fractions were prepared as described in METHODS. [^{3}H]α-Bgt final concentration was $6.2 \cdot 10^{-9}$ M. Protein concentrations in mg/ml were: myelin 2.3, nerve endings 3.2, mitochondria 4.0. ▒, Control binding; ▒, binding in the presence of 10^{-4} M d-tubocurarine; □, acetylcholinesterase relative specific activity.

FIGURE LEGENDS (Cont.)

Fig. 4. $[^3H]\alpha$-Bungarotoxin binding to brain membranes. Crude mitochondrial fraction was submitted to osmotic shock and the resulting membranes were incubated with $[^3H]\alpha$-Bgt for 2 h. Final membrane concentration was 4 mg protein/ml. The values for the equilibrium constant was $3.2 \cdot 10^{-9}$ M.

Fig. 5. $[^3H]\alpha$-Bungarotoxin binding to solubilized receptor. Membranes prepared by osmotic shock of crude mitochondrial fraction were incubated with 1% sodium deoxycholate for 90 min, following the procedure described by Changeux et al.[11]. Five ml of the extract (2.3 mg protein/ml) were incubated with $3.7 \cdot 10^{-8}$ M $[^3H]\alpha$-Bgt for 2 h; the incubate was then loaded on a Sephadex G-75 column (2.5 · 37 cm) and eluted with distilled water. Absorbance at 280 nm (x---x) and radioactivity (o——o) of the effluent are shown in the figure.

Fig. 6. ^{125}I-labeled α-Bungarotoxin binding to crude mitochondrial fraction. Particles at a final concentration of 100 mg original weight/ml were incubated with d-tubocurarine or Ringer's solution for 1 h. Upon addition of ^{125}I-labeled α-Bgt the incubation was continued for 10 min. Fig. 6a shows data obtained with ^{125}I-labeled α-Bgt from Preparation 3. Binding in the presence of $2.5 \cdot 10^{-4}$ M d-tubocurarine is also shown (-----). Fig. 6b represents a similar experiment performed with ^{125}I-labeled α-Bgt from Preparation 4. o, binding in the presence of $3.3 \cdot 10^{-4}$ M d-tubocurarine; •, binding in the presence of $3.3 \cdot 10^{-4}$ M carbamylcholine.

FIGURE LEGENDS (Cont.)

Fig. 7a. ^{125}I-labeled α-Bgt binding to rat diaphragm. Binding to diaphragm was assayed by the method of Berg et al.[14] ^{125}I-labeled α-Bgt from Preparation 3 was used. Incubation time was 105 min. The cpm bound upon incubation with "old" toxin were corrected for radioactive decay. ▲, binding of ^{125}I-labeled α-Bgt 1 day after iodination to the area containing end plates; △, binding of ^{125}I-labeled α-Bgt 1 day after iodination to the area without end plates; ●, binding of ^{125}I-labeled α-Bgt 80 days after iodination to the area containing end plates; o, binding of ^{125}I-labeled α-Bgt 80 days after iodination to the area without end plates.

Fig. 7b. Inhibition of the binding of ^{125}I-labeled α-Bgt to diaphragm by d-tubocurarine and carbamylcholine. Pieces of diaphragm were incubated with or without the competitors in Ringer's solution for 1 h. ^{125}I-labeled α-Bgt (Preparation 4) was then added at a final concentration of $8.75 \cdot 10^{-8}$ M and the incubation was continued for 105 min. Further procedure was as described by Berg et al.[14] ●, inhibition of binding to the area containing end plates by d-tubocurarine; o, inhibition of binding to the area without end plates by d-tubocurarine; ▲, inhibition of binding to the area containing end plates by carbamylcholine; △, inhibition of binding to the area without end plates by carbamylcholine.

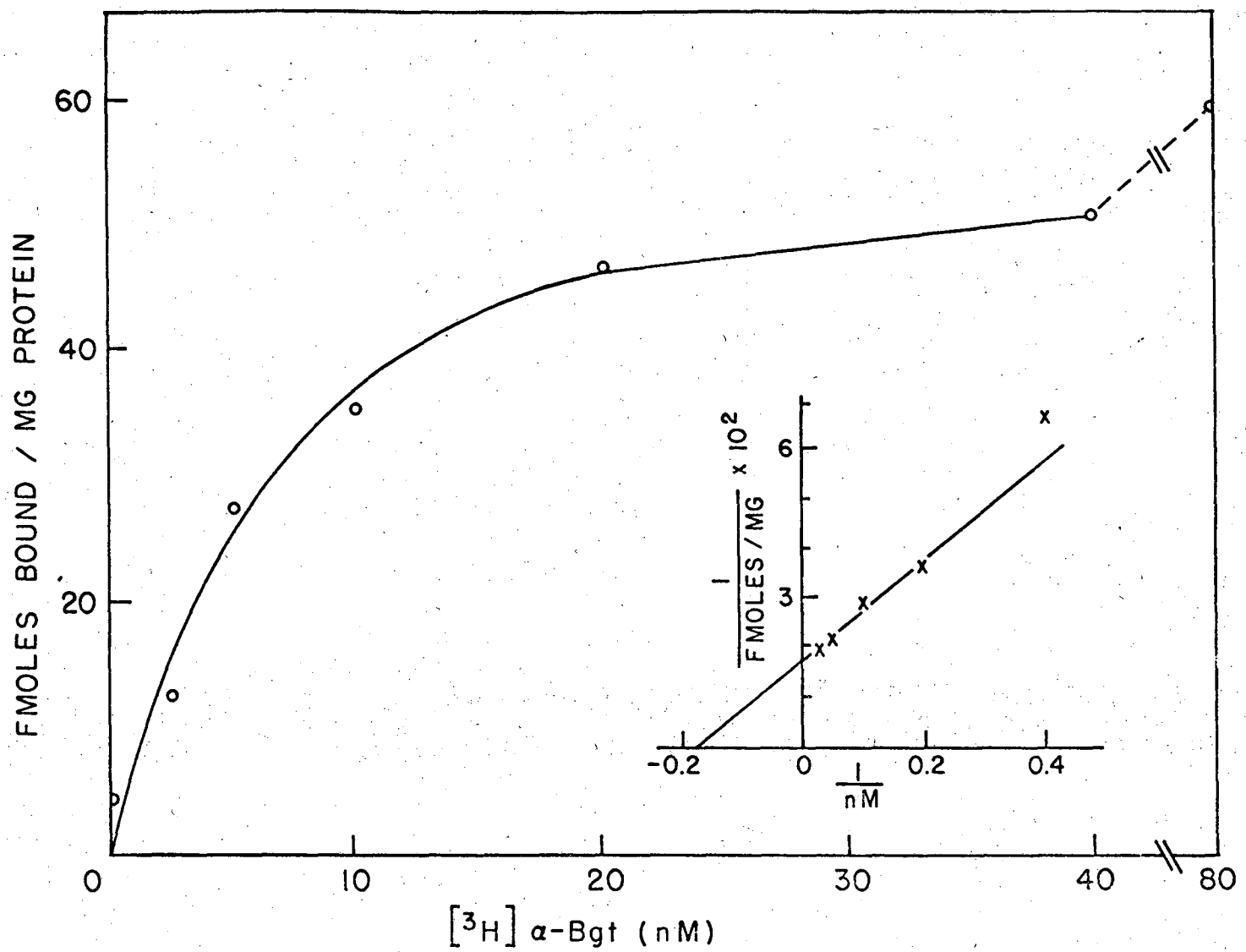

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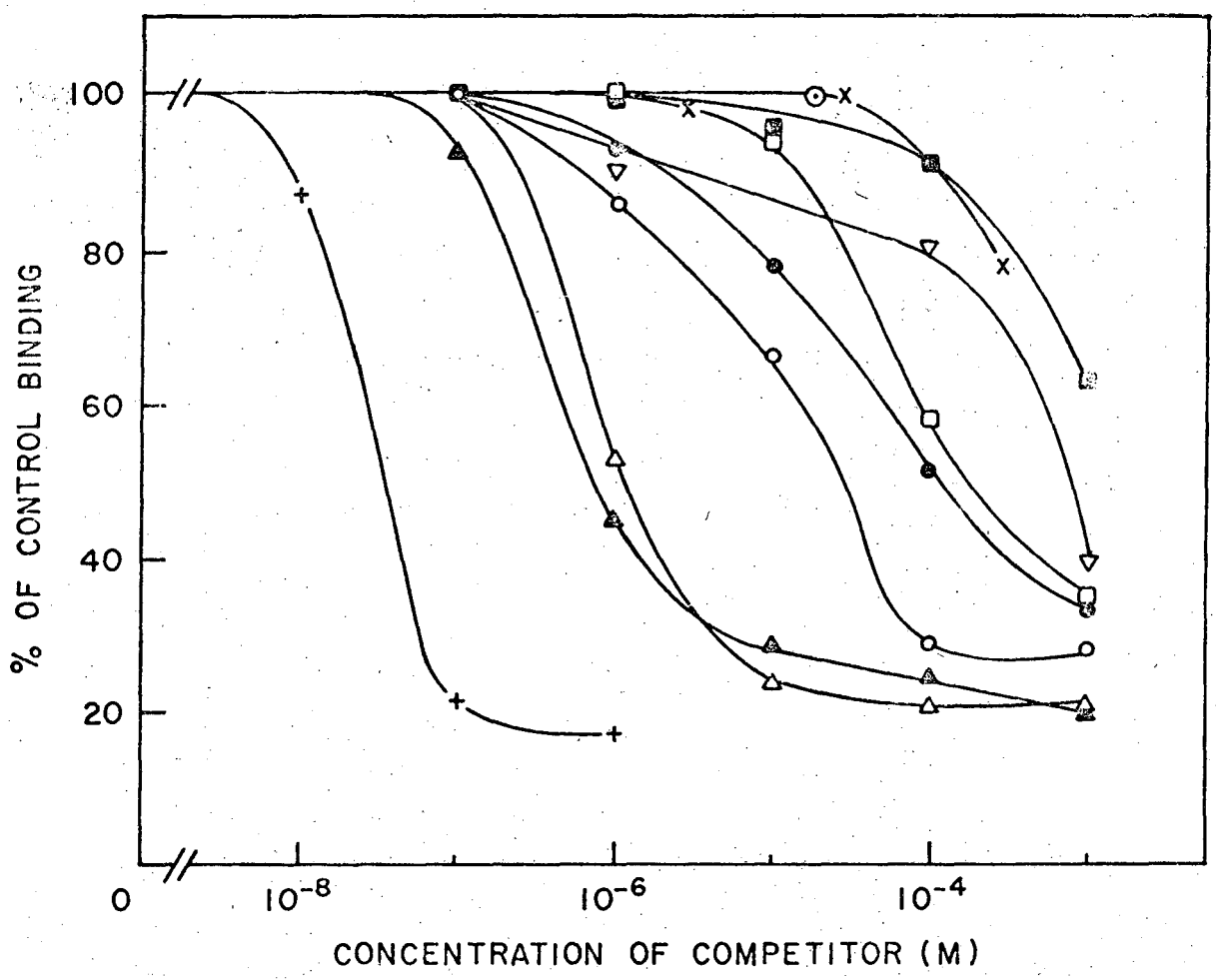
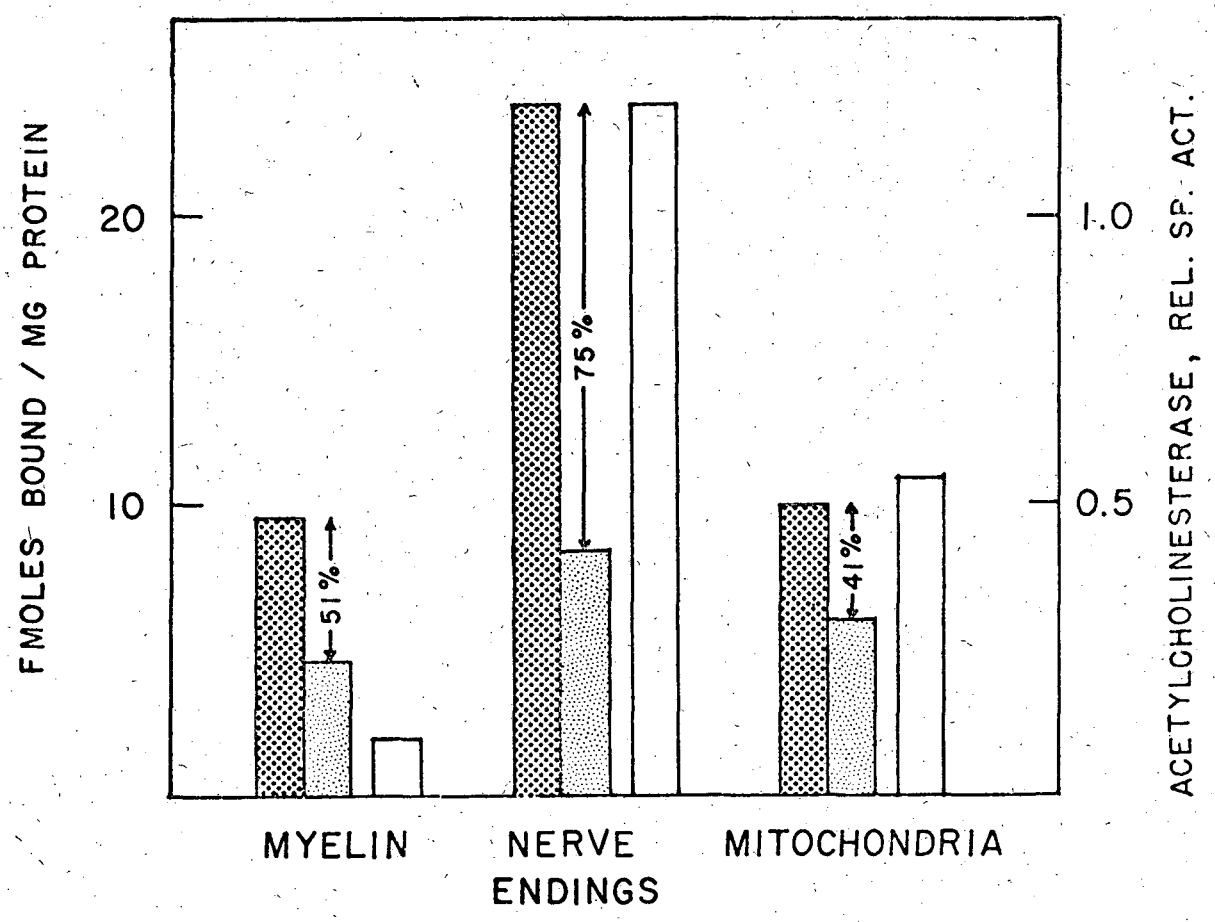
Fig. 1



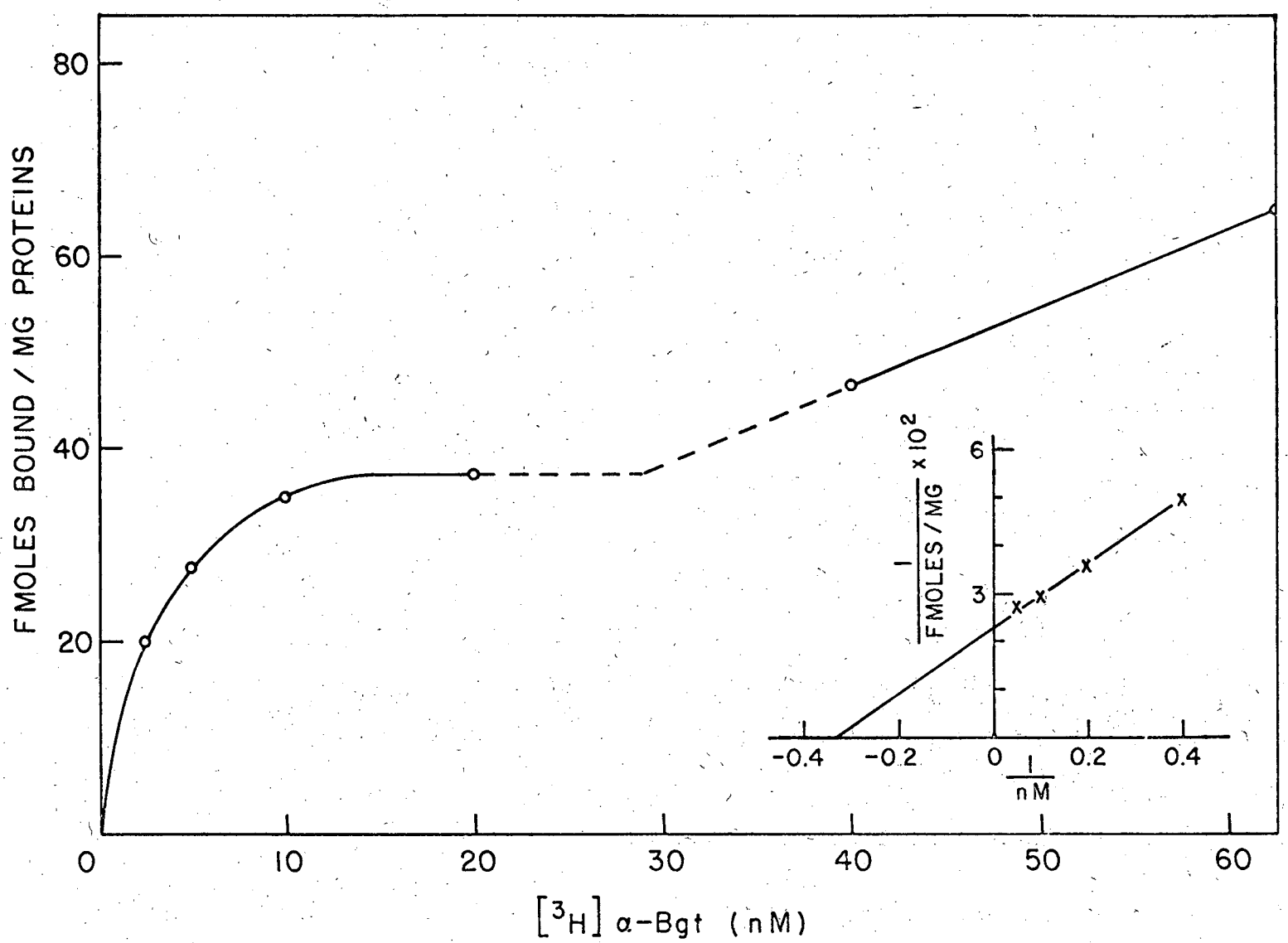
XBL742-5043

Fig. 2



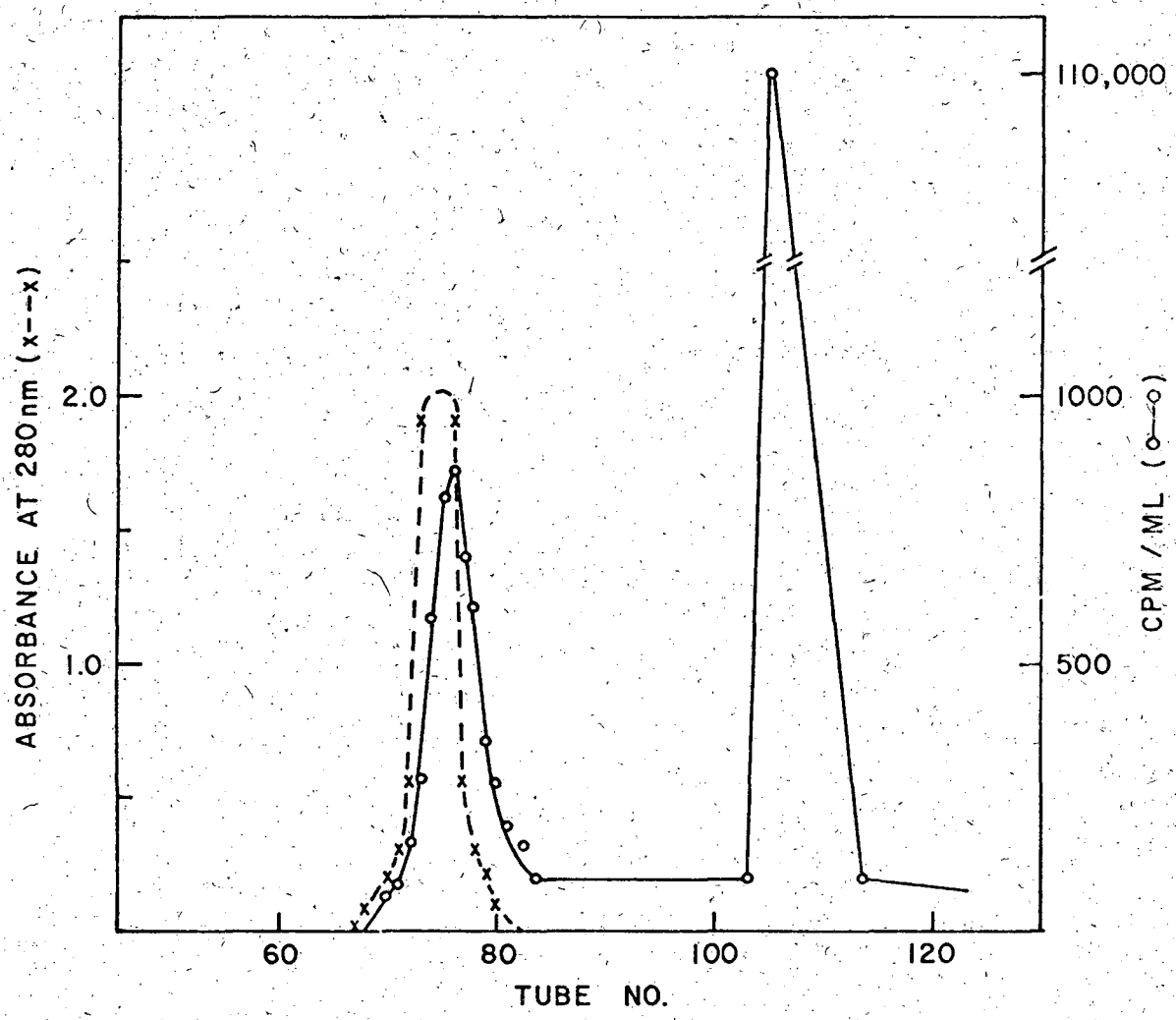
XBL742-5042

Fig. 3



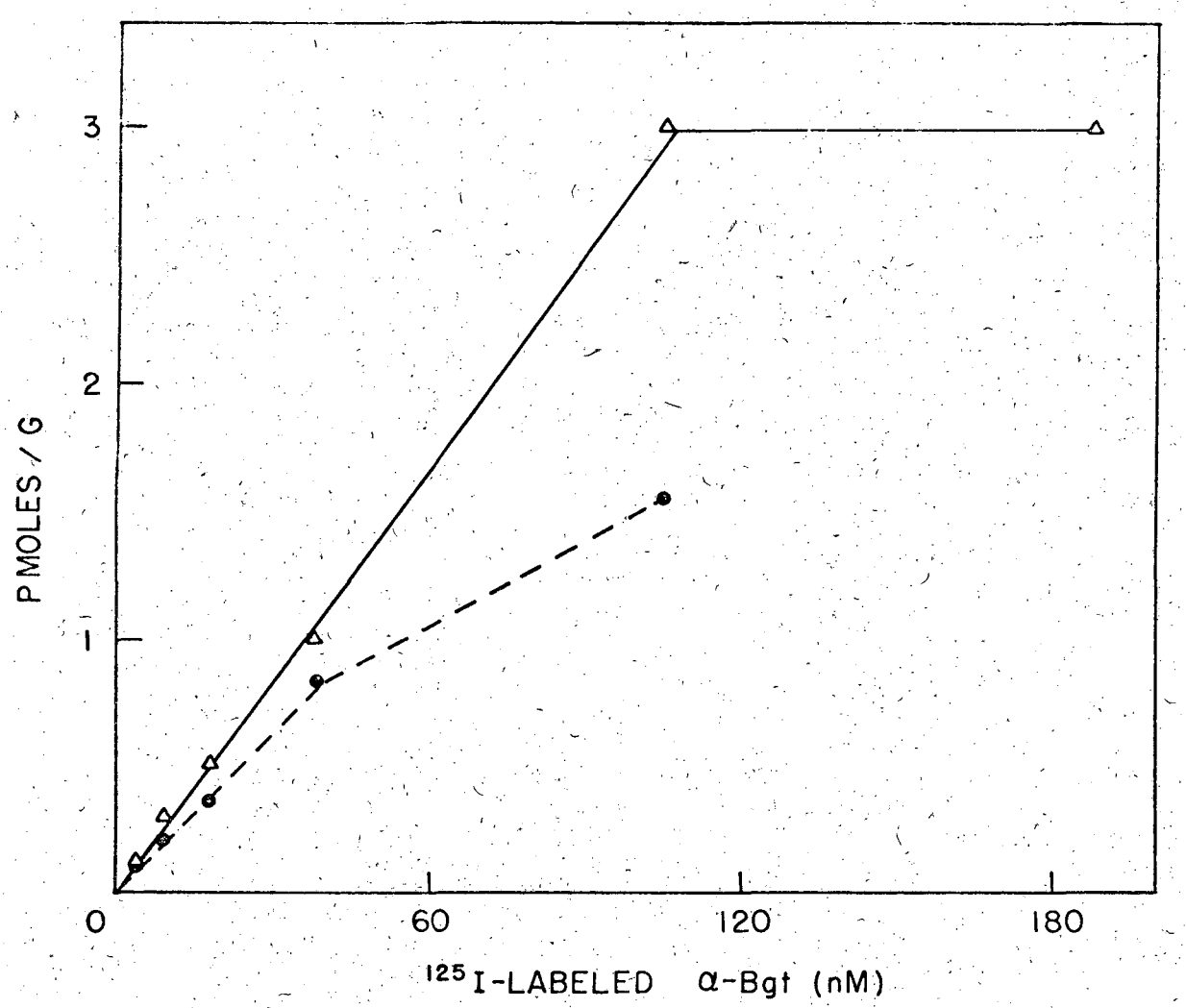
XBL742-5041

Fig. 4



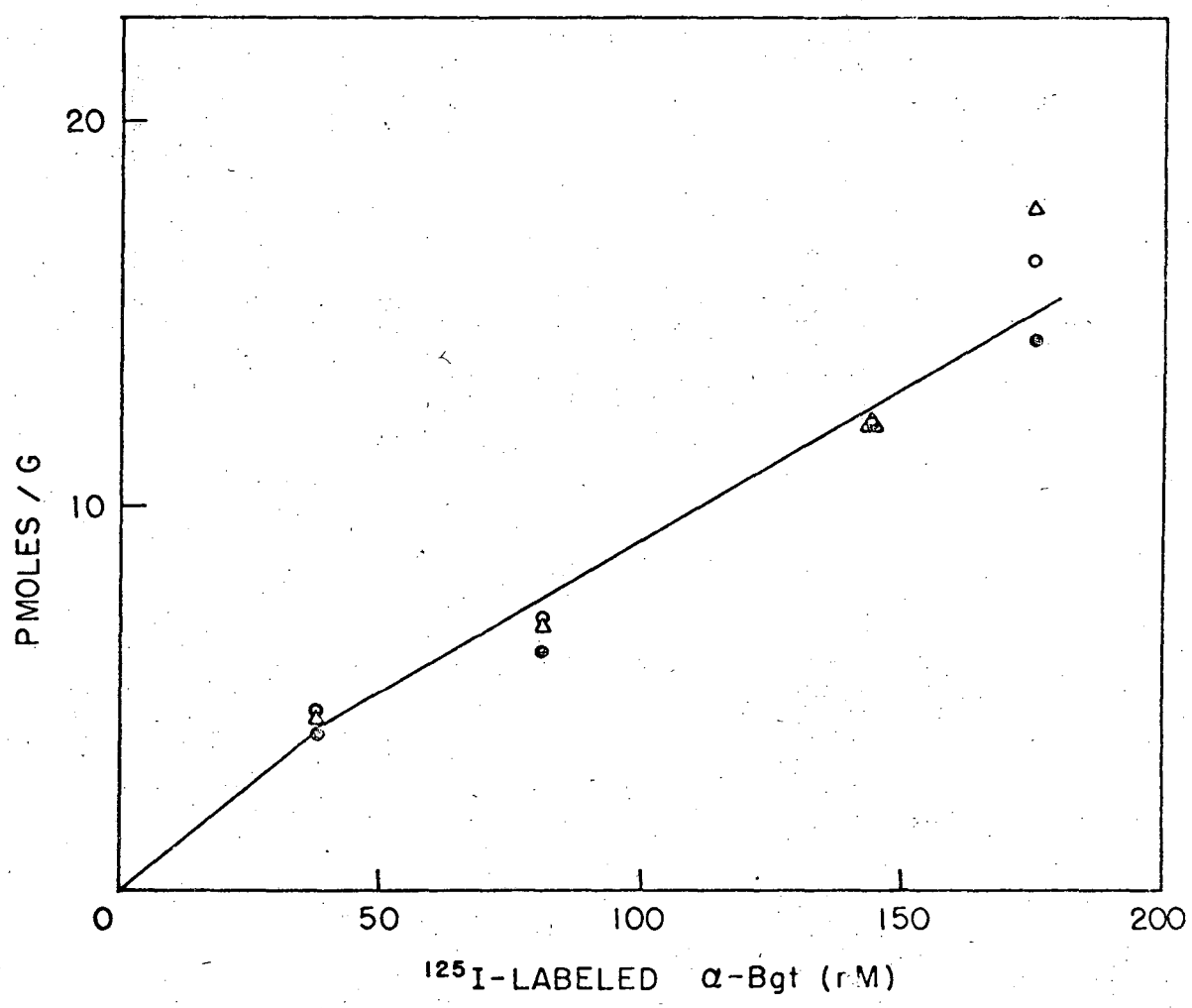
XBL742-5038

Fig. 5



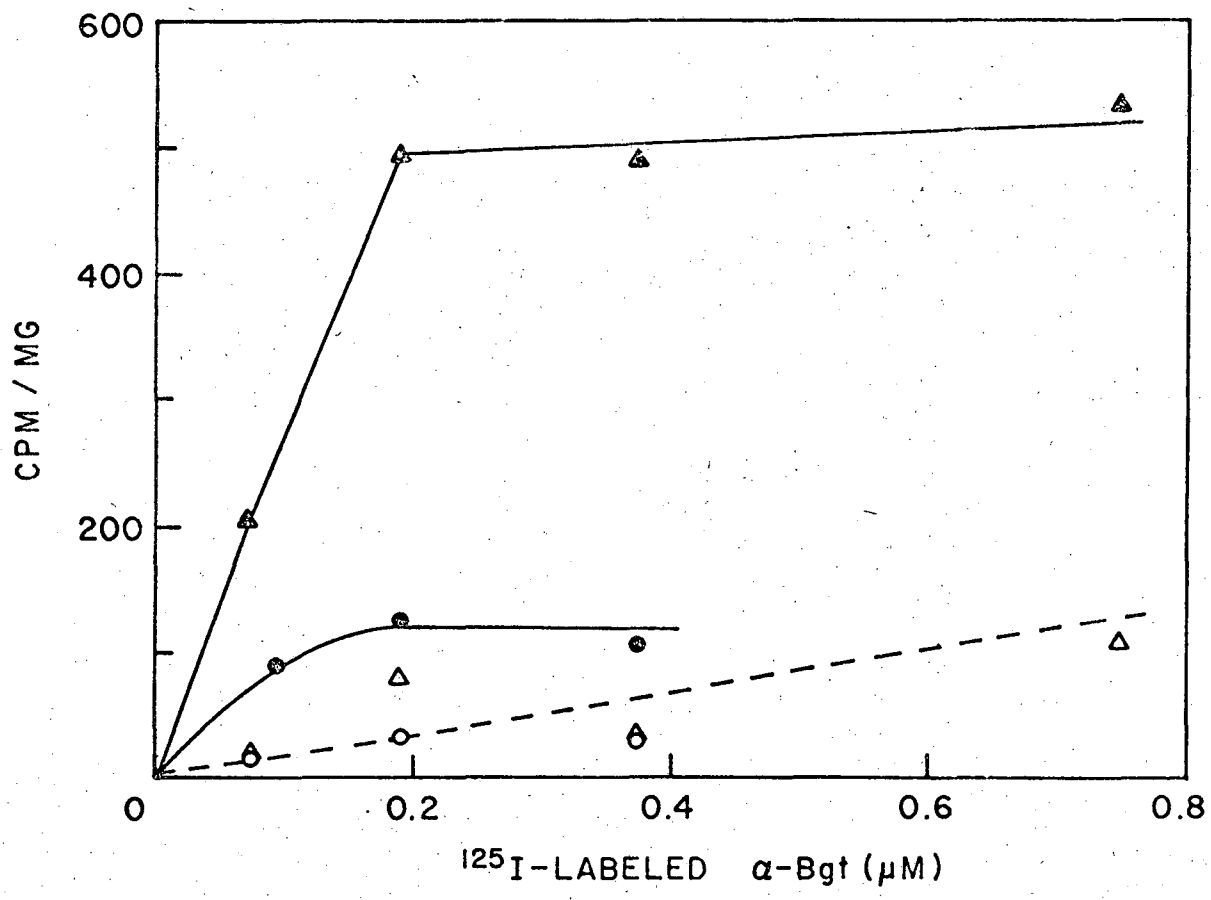
XBL742-5040

Fig. 6a



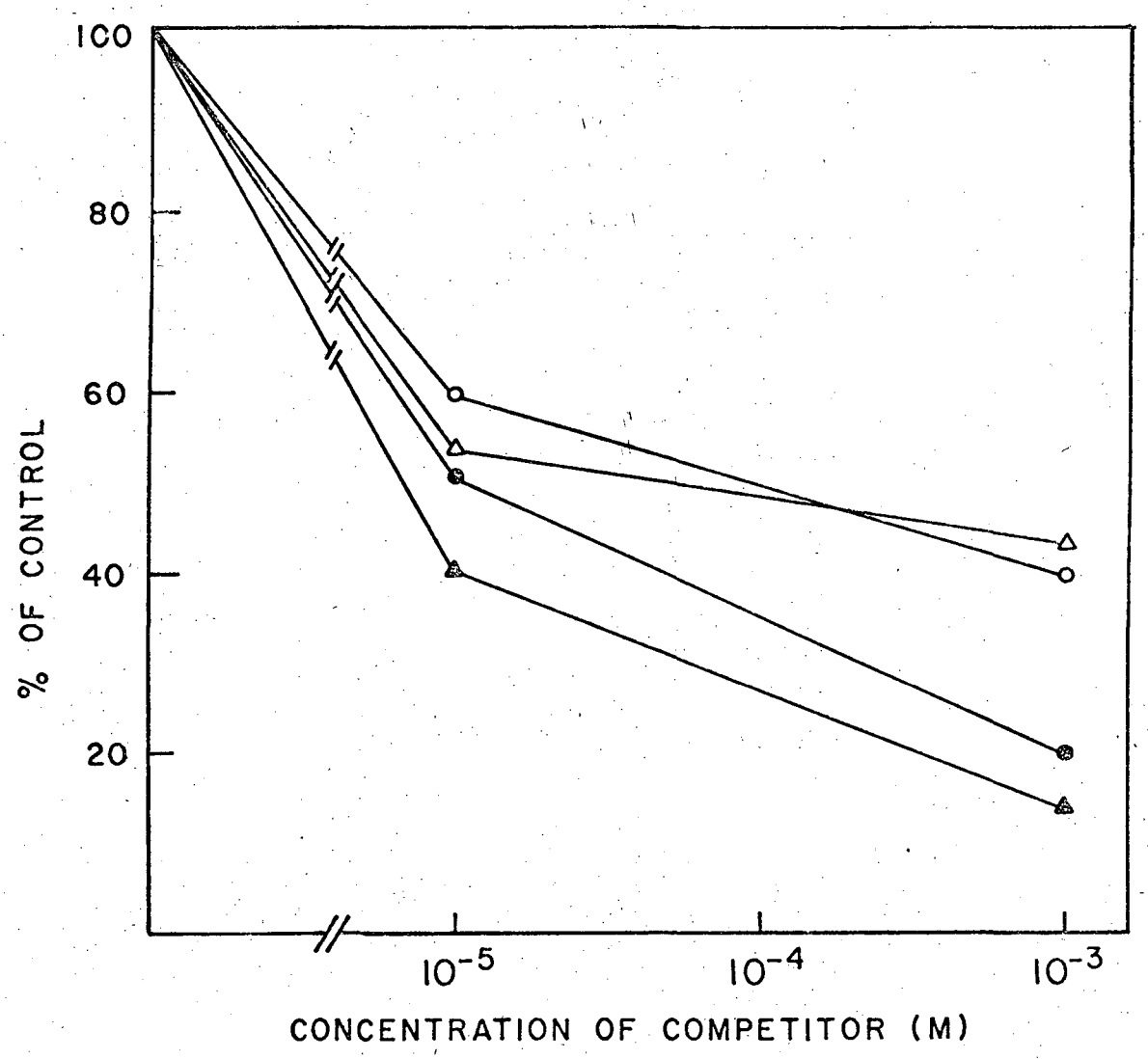
XBL742-5039

Fig. 6b



XBL742-5037

Fig. 7a



XBL742-5044

Fig. 7b

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