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RADIOLABELED α-BUNGAROTOXIN DERIVATIVES: KINETIC INTERACTION WITH NICOTINIC ACETYLCHOLINE RECEPTORS

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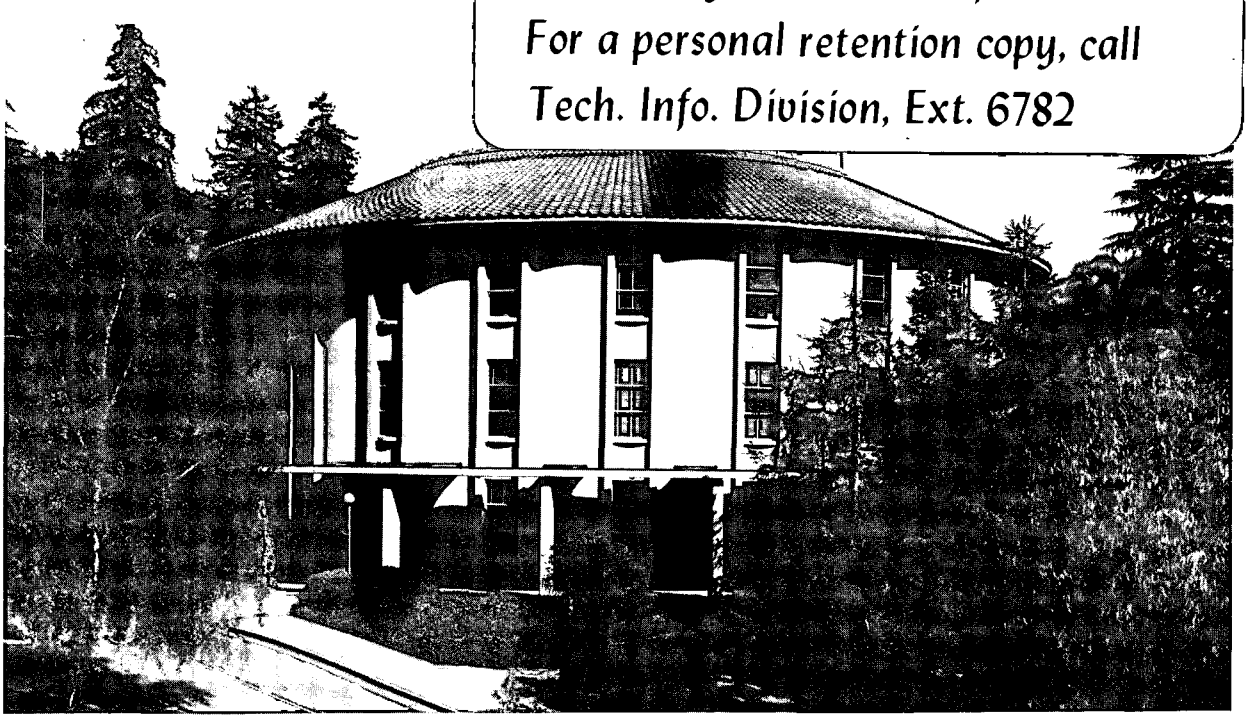

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Radiolabeled α-Bungarotoxin Derivatives: Kinetic Interaction with Nicotinic Acetylcholine Receptors

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A preliminary account of these results was presented at the Ninth Annual Meeting, Society for Neuroscience (Bennett *et al.*, 1979).

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1. Abbreviations used: α-Bgt, α-bungarotoxin; nAChR, nicotinic acetylcholine receptors; monoiodo α-Bgt, monoiodinated α-Bgt; diiodo α-Bgt, diiodinated α-Bgt; [^{3}H]α-Bgt, tritium-labeled α-Bgt; [^{125}I]-α-Bgt, ^{125}I-labeled monoiodinated α-Bgt; [$^{125}I_2$]α-Bgt, ^{125}I-labeled diiodinated α-Bgt.

ABSTRACT

The binding interactions of purified tritiated [^{3}H]α-Bgt, monoiodinated and diiodinated derivatives of α-bungarotoxin with membrane-bound nicotinic acetylcholine receptors (nAChR) from Torpedo californica electroplax and rat brain have been characterized by several kinetic and equilibrium techniques. By all criteria, [^{3}H]α-Bgt and ^{125}I-labeled monoiodinated α-Bgt ([^{125}I]α-Bgt) exhibited comparable specificities and affinities for nAChR. In contrast, affinity of nAChR for ^{125}I-labeled diiodinated α-Bgt ([$^{125}I_2$]α-Bgt) was reduced, and [$^{125}I_2$]α-Bgt-nAChR complexes showed anamolous biphasic dissociation kinetics. [^{125}I]α-Bgt and [$^{125}I_2$]α-Bgt binding was inhibited most potently by native α-Bgt as opposed to iodinated toxins. [^{3}H]α-Bgt was the radio-toxin most resistant to inhibitory influences. The use of well-characterized, chemically-modified α-Bgt derivatives may identify ligand binding microheterogenities and tissue-specific receptor sub-classes.

INTRODUCTION

Utilization of curarimimetic neurotoxins as specific probes for nAChR[1] has contributed to our knowledge of neurotransmitter receptor structure and function (Lee, 1971; Heidmann and Changeux, 1978). Despite wide diversity of available toxins, and a significant literature on effects of chemical modification on their toxic activity (Tu, 1977), use of different naturally-occurring or chemically-modified toxins to probe receptor properties has been limited.

In an earlier communication (Lukas[iewicz] *et al.*, 1978), we reported that column-purified native α-Bgt and tritiated, monoiodinated and diiodinated α-Bgt derivatives exhibit characteristically different ultraviolet and circular dichroism spectra, suggesting that progressive iodination of an exposed tyrosine residue(s) leads to alterations in toxin secondary structure. Radiotoxin binding competition data further suggested that iodination also leads to some disruption of receptor recognition properties presumably as a consequence of structural alterations.

In order to quantitatively document any such alterations in receptor binding characteristics, kinetic properties of the reaction of [^{3}H]α-Bgt, [^{125}I]α-Bgt and [$^{125}I_2$]α-Bgt derivatives with both membrane-bound nAChR from *Torpedo californica* electric organ and rat brain membrane functions were examined.

EXPERIMENTAL PROCEDURE

Materials

Crude venom from *Bungarus multicinctus* (Miami Serpentarium,Miami FL), d-tubocurarine chloride (Calbiochem), and carbamylcholine chloride (Sigma) were stored at -20°C. Wag/Rig rats were from the Lawrence Berkeley Laboratory rat colony. Liquid nitrogen-frozen chunks of *Torpedo californica* electric organ were obtained from Pacific Biomarine, Venice CA and stored at -80°C.

Preparation of α-Bgt followed procedures of Eterović *et al*. (1975b), as modified by Lukas(iewicz) *et al*. (1978). Iodination of α-Bgt and resolution of monolabeled, dilabeled and unlabeled toxins via ion-exchange column chromatography at pH 6.5 were carried out as described previously (Lukas[iewicz] *et al*., 1978). Tritiated α-Bgt was prepared according to Eterović *et al*. (1975a) using purified monoiodo α-Bgt, and resolved from residual iodinated species by ion-exchange chromatography at pH 6.5 (Lukas[iewicz] *et al*. 1978). Nonradiolabeled α-Bgt (25 μM) fractions were stored in 1 ml aliquots at -20°C until use, and kept at 0°C thereafter. Radiolabeled α-Bgt derivatives (10-25 μM) were stored at -20°C in the presence of 1 mg/ml bovine serum albumin.

Toxin and Radio-Toxin Concentrations, Radio-Purity, and Specific Activities

Concentrations were determined from optical absorbance measurements (Cary 118 spectrophotometer) at 280 nm, corrected for scattering, using $\varepsilon_{280}^{0.1\%} = 1.32$ (Hanley *et al*., 1977), and confirmed with protein determinations according to Lowry *et al*. (1951). Radio-purity of labeled toxins was ascertained by titration of toxins (at concentrations in excess

of their apparent K_D) with increasing quantities of Torpedo nAChR. At large excess of receptor over toxin, the proportion of radioactivity sedimenting with receptor-containing membranes serves as a lower limit (due to nonquantitative precipitation of toxin-receptor complexes; see, also, Jones and Thompson, 1980) for the amount of radioisotope associated with toxin, and the biological activity of labeled species. Specific activity determinations based on direct counting of radiotoxin aliquots of known concentration were confirmed in toxin titration experiments, where membrane fragment-associated specific binding levels at saturation were indexed to the known concentration of toxin-binding sites. Over the course of the experiments described herein, the specific activity of the [^{3}H]α-Bgt preparation used was 12-14 dpm/fmol (5.4-6.4 Ci/mmol). The specific activity of the [^{125}I]α-Bgt preparations was about 15 dpm/fmole, and the specific activity of the [$^{125}I_2$]α-Bgt from the same preparation was twice that of the [^{125}I]α-Bgt at all times. Decay of the specific activity of the two radio-iodinated species exhibited the 60 day half-life of ^{125}I.

Radio-toxin Binding Assays

Rat brain crude mitochondrial fraction membranes were prepared, and toxin binding assays conducted fundamentally according to Lukas et al. (1979). Torpedo nAChR-rich membrane fragments were prepared essentially as described by Hazelbauer and Changeux (1974). Binding assays for Torpedo membrane fragments were done similarly to those for rat brain membranes, except that toxin-receptor complexes were sedimented at 100,000g for 30 min in a Beckman 40 rotor. In all data subsequently presented, binding levels are corrected for nonspecific binding (Lukas et

al. 1979). For nAChR titration experiments, radio-toxins at concentrations in excess of their apparent K_D were incubated with aliquots of Torpedo membranes containing increasing quantities of nAChR. For radio-toxin titration experiments, aliquots of membranes containing nAChR at concentrations less that the apparent K_D were incubated with aliquots of radio-toxin of increasing concentration for either 1 h, or overnight (20-24 h). Studies of dissociation of radio-toxin from toxin-receptor complexes followed two general paradigms. After overnight reaction with saturating levels of radio-toxin, and removal of unbound, free ligand, samples were either diluted in ten volumes of buffer (physical dilution), or supplemented with a large (100-fold) excess of unlabeled, native α-Bgt (chemical dilution). In some experiments, samples were supplemented with 1 mM d-tubocurarine or carbachol instead of native α-Bgt. Aliquots of each sample were then subjected to centrifugation at different times and assayed for the quantity of radio-toxin-receptor complex remaining.

For toxin association studies, reactions were carried out for specified periods of time until quenched with a large excess of native α-Bgt. Non-radiolabeled toxin competition assays were initiated by addition of an aliquot of membranes to solutions containing a fixed concentration of radio-toxin plus a variable concentration of competing ligand. The concentration of Torpedo nAChR, 4 nM, was approximately 8-10 times the concentration of the resumptive nAChR from rat brain. Non-specific binding contributions to total binding at 10 nM radiotoxin, after 1 h incubation are for [^{3}H]α-Bgt, [^{125}I]α-Bgt and [$^{125}I_2$]α-Bgt, respectively, 35%, 40%, and 42% for rat brain, and 9%, 5%, and 3% for Torpedo. Non-specific binding increased with time, especially for [$^{125}I_2$] α-Bgt incubated with rat brain membranes.

RESULTS

Results of nAChR titration experiments established that the radio-purity of [^{3}H]α-Bgt and [^{125}I]α-Bgt exceeds 80% (Fig. 1). The data also indicate that radio-purity of [$^{125}I_2$]α-Bgt approached that for the two other radio-toxins. An interesting feature of these results is the apparently higher avidity of [^{3}H]α-Bgt for nAChR relative to radio-iodinated toxins. Maximal binding of radio-toxin with receptor is achieved under the given conditions at the lowest nAChR concentration for [^{3}H]α-Bgt, and is not achieved for [$^{125}I_2$]α-Bgt even at the highest nAChR concentration tested.

Receptor saturation/toxin titration experiments were routinely carried out for 1 hr, or, in order to more closely approach equilibrium conditions, overnight. It should be pointed out that for both short and extended periods of reaction, apparent and true K_D values are valid measures of toxin-receptor affinities (Lukas _et al_. 1979). Results of a typical series of experiments (Fig. 2) illustrate that, in general, observed receptor affinities were highest for [^{3}H]α-Bgt, and lowest for [$^{125}I_2$]α-Bgt, and tended to increase with increased reaction time. On closer inspection of the data, as facilitated by Hofstee-Eadie-Scatchard analysis (Fig. 3), it is evident that apparent affinities are highest for receptor interaction with [^{3}H]α-Bgt and lowest for [$^{125}I_2$] α-Bgt. An exception was noted with overnight incubation with _Torpedo_ membranes, where affinities for [^{125}I]α-Bgt are about one-half of those for [^{3}H]α-Bgt. With the exception of radio-iodinated toxin binding to rat brain membranes, apparent K_D values are decreased on overnight incubation, as the reaction procedes to equilibrium.

Experiments were also performed to determine association rates of radio-toxins. Typically, membranes were incubated with radio-toxins at final concentrations of 2, 5, 10, 20, 40 and 80 nM, and the period of incubation varied from 10 sec to 24 h. Rates of toxin binding were determined from the slope of plots of ln[100 (% sites unoccupied)$^{-1}$ vs. time (Bylund, 1980). The values of these slopes, representing observed association rates, are plotted against radio-toxin concentration in Fig. 4. Over the range of radio-toxin concentrations 2 thru 40 nM, these plots are essentially linear, indicating that the apparent association rate constant is independent of toxin concentration over this range. Association rate constants are comparable for binding to rat brain membranes for all three species of radio-toxin. For interactions with Torpedo nAChR, rate constants for [^{3}H]α-Bgt and [^{125}I]α-Bgt are twice those observed for [$^{125}I_2$]α-Bgt. In addition, binding to brain of [^{3}H] α-Bgt and [^{125}I]α-Bgt is about six times more rapid than the binding to Torpedo membranes.

In order to further delineate the kinetic properties of radio-toxin-receptor interactions, dissociation rates of toxin from receptor sites were determined. For both rat brain membranes and Torpedo membrane bound nAChR, dissociation of receptor-toxin complexes on dilution is characterized by a single exponential process; monophasic decay curves fit the data for [^{3}H]α-Bgt and the [^{125}I]α-Bgt but a biphasic dissociation was found for [$^{125}I_2$]α-Bgt (Fig. 5). Dissociation of radio-toxin-receptor complexes is accelerated on exposure to a large excess of non-radiolabeled toxin. For dissociation of [^{3}H]α-Bgt from toxin binding sites, the process is again characterized by a single exponential decay. Experiments using [^{3}H]α-Bgt and rat brain membranes indicate that the dissociation

rates in the presence of 1 m*M* carbachol or d-tubocurarine are intermediate between those observed under chemical and physical dilution conditions. Another feature of the data is the observation that dissociation of radio-toxin upon chemical dilution follows a biphasic decay process for $[^{125}I]$α-Bgt, which is even more pronounced for $[^{125}I_2]$α-Bgt. The appearance of the biphasic decay process and relative quantities of quickly and slowly dissociating components was found not to be sensitive to the extent of receptor occupation. That is, decay profiles were strictly comparable whether receptor was incubated for 1 h with 4 n*M* or 40 n*M* toxin, or overnight with 4 n*M* or 6 n*M* toxin. Independent of the specific conditions used to measure radio-toxin dissociation, fastest rates are observed for dissociation from rat brain membranes with $[^{125}I_2]$ α-Bgt receptor complexes; $[^{3}H]$α-Bgt-receptor complexes are longest lived.

Experimentally-derived values for apparent K_D at 1 h and overnight incubation, and for dissociation and association rate constants and the resultant K_D's, are summarized in Table I.

In an earlier report, it was suggested that differences in radio-toxin receptor affinities were evident in binding competition experiments (Lukas *et al*. 1978). Data from similar experiments are shown in Fig. 6 in the form of modified Dixon plots (Dixon, 1953). In these transforms, a slope of one indicates that radio-labeled and non-radio-labeled toxins are equally effective as ligands interacting at receptor binding sites. Slopes greater than one suggest that receptor has higher affinity for the non-radiolabeled species, while slopes of less than one indicate that the receptor has higher affinity for the radiolabeled species. From the results using both brain and *Torpedo* membranes, it is evident that native α-Bgt competes most effectively for $[^{125}I_2]$α-Bgt

binding, and least effectively for [^{3}H]α-Bgt. Similar results are obtained when non-radiolabeled, monoiodo α-Bgt is used as competing ligand for sites on brain membranes. Dixon plot slopes are summarized in Table II. The results further illustrate that native α-Bgt is the most potent inhibitor of radio-iodinated toxin binding. Anomalous results were obtained with di-iodo α-Bgt vs. [^{3}H]α-Bgt and diiodo α-Bgt vs. [$^{125}I_2$]α-Bgt).

DISCUSSION

Monoiodinated, diiodinated and native (tritiated) α-Bgt derivatives, previously shown to be distinguishable on the basis of their ultraviolet and circular dichroism spectra (Lukas[iewicz] et al. 1978), exhibit characteristic kinetic properties on interaction with nicotinic-type acetylcholine receptors present in membrane preparations from rat brain and Torpedo electric organ. Detailed studies of receptor-radio-toxin binding properties yield quantitatively different values of preequilibrium apparent K_D and association and dissociation rate constants for different radio-labeled α-Bgt derivatives. The interaction of [^{3}H]α-Bgt with brain and Torpedo nAChR fits the simplest receptor binding mechanism, displaying monophasic association and dissociation profiles. [^{3}H]α-Bgt also binds to brain membrane sites with the highest affinity of tested α-Bgt derived radio-toxins. Affinity of [^{125}I]α-Bgt for receptor sites is only slightly less than that of [^{3}H]α-Bgt, except as binding to Torpedo nAChR approaches equilibrium. In addition, the dissociation rates for [^{125}I]α-Bgt-receptor complexes show a small degree of biphasic character. In contrast, affinity of receptor sites for [$^{125}I_2$]α-Bgt is lowest by all criteria, and

decay of $[^{125}I_2]$α-Bgt-nAChR complexes is clearly biphasic in the presence of excess native α-Bgt.

These results confirm and extend our previous findings (Lukas[iewicz) et al. 1978), and point to the kinetic bases of the observations, replicated herein, that native α-Bgt is the most effective inhibitor of radio-iodinated toxin binding, and that binding of $[^3H]$-α-Bgt is most resistant to inhibition. While differences between binding properties of $[^{125}I]$α-Bgt and $[^3H]$α-Bgt are small, our present evidence again suggests that major alterations in receptor binding activity shown by diiodo α-Bgt are probably a consequence of alterations in the conformation of the molecule induced by progressive iodination of an exposed tyrosine residue (Lukas[iewicz] et al. 1978). Provisional results of one experiment suggest that the susceptible residue is Tyr-54 (Hanley and Lukas, unpublished), in accordance with the interpretation of Blanchard et al. (1979) regarding iodination of α-Bgt via a nonenzymatic procedure similar to that described herein. In a recent report Wang and Schmidt (1980) have assigned the iodination to the corresponding tyrosine. It is evident, however, that while Tyr-54 is not one of the conserved residues thought to be fundamentally involved in receptor binding (Tsernoglou and Petsko, 1976) it may still influence receptor binding properties in a significant, if not predictable way (Hanley, 1978).

There are a number of reports in the literature documenting kinetic and equilibrium parameters for reaction of radiolabeled, curaremimetic neurotoxins with nAChR from Torpedo (Wang and Schmidt, 1980; James et al. 1980; Blanchard et al. 1979; Weiland, et al. 1976; Franklin and Potter, 1972), Electrophorous (Weber and Changeux, 1974; Mailicke et al. 1977; Fulpius et al. 1975; Bulger et al. 1977), cat muscle (Barnard et al. 1977)

rat muscle (Brockes and Mall, 1975; Kemp et al. 1980), chick muscle (Wang and Schmidt, 1980), and central (Wang and Schmidt, 1980; Morley and Kemp, 1981; Lowry et al. 1976; Moore and Brady, 1976; McQuarrie et al. 1976) neural tissues. Direct comparison of those data to the results described in this communication is difficult, however, due to important differences in incubation media, in the use of membrane-bound or solubilized receptor preparations, in the choice of the purified snake toxin used, and the nature of the radio-labeling modification reaction to non-overlapping ranges of toxin and receptor concentrations utilized, and insufficient documentation of the purity and characteristics of radio-toxin in some cases.

Four studies, in addition to ours, have dealt with differences in binding characteristics caused by labeling of α-Bgt with [^{125}I]. Vogel et al. (1972) first noted that [$^{125}I_1$]α-Bgt bound to cultured chick embryonic cells both more rapidly and with as much as three-fold lower K_D than did [$^{125}I_2$]α-Bgt. It appears from data given in their paper that the iodinated toxins contained little of the non-iodinated species. Recently, three other reports have appeared characterizing the binding of iodinated derivatives of α-Bgt (Blanchard et al. 1980; James et al, 1980; and Wang and Schmidt, 1980). Blanchard et al. used mixtures of [$^{125}I_1$]α-Bgt and native α-Bgt and concluded that the labeled toxin bound to membrane-bound receptor from Torpedo with the same rate constant as the unlabeled toxin. James et al compared the binding characteristics of non-labeled toxin, [$^{125}I_1$]α-Bgt labeled toxin and [$^{125}I_2$]α-Bgt. They also concluded that monoiodination of the toxin did not modify the binding properties to purified nAChR while [$^{125}I_2$]α-Bgt bound less rapidly to solubilized nAChR from Torpedo californica. Most recently Wang and Schmidt have compared

the binding properties of the diiodinated toxin to those of the monoiodinated toxin and the non-substituted toxin. They conclude that diiodination reduced the binding rate by a factor of 2 to 3, but did not change the dissociation rate ($t_{1/2}$ ~3 hrs) from the receptor obtained from chick optic lobes. No differences were reported between the association rates of native toxin and the mono-iodinatd toxin. (It should be pointed out, however, that the chromatographic step to purify labeled toxins was carried out at pH 7.4 and consequently a mixture of the mono-iodinated and non-iodinated toxin has been used in these three recent studies.) Thus, mono-iodination of α-Bgt tyr-54 is a relatively innocuous procedure. This can be contrasted with the labeling of α-Bgt with [^{3}H]-labeled pyridoximine phosphate which leads to a 9-fold decrease in receptor binding (James and Thompson, 1980). We would agree with the conclusion that [$^{125}I_2$]α-Bgt differs significantly from the native toxin and [$^{125}I_1$]α-Bgt, and that detailed kinetic studies of the binding of iodinated α-Bgt should not be made with mixtures of the two non-identical forms. It is also evident that contrary to some accounts in the literature (e.g., Blanchard et al. 1979) the dissociation of toxin-membrane nAchR complexes does have a measurable half-life, and toxin-receptor interactions consequently have measurable dissociation constants.

Five other observations warrant specific comment. K_D^{app} values are typically 10 times as great as microscopic reversibility dissociation constants calculated from forward and dissociation rate constants. Consequently, in most cases, the receptor concentration used is about the same as true K_D, and the long half-times for dissociation of preformed toxin-receptor complexes confirms that true equilibrium has not been achieved. Thus, it is yet to be determined whether this discrepancy in apparent and microscopic K_D values are due to experimental conditions or

an inherent property of toxin-receptor interactions. Secondly, the results presented herein document decreased affinity of [$^{125}I_2$]α-Bgt for nAChR in both rat brain and Torpedo electroplax, in contrast to results of earlier experiments (Lukas[iewicz] et al. 1978) in which a decrease in affinity for Torpedo was not found. Thirdly, the acceleration of decay of radio-toxin-nAChR complexes on exposure to excess native α-Bgt may have a simple explanation, based on the assumption that the overall receptor-toxin interaction is comprised of numerous ionic, hydrophobic and Van der Waals contacts. In the absence of added non-radiolabeled ligand, the probability that enough receptor-toxin contacts will be simultaneously broken in the presence of solvent and solute molecules, with low affinity and specificity for those sites, will be small. Competing ligand, however, possesses sufficient affinity and specificity for those sites that the probability of simultaneous contact blockade is increased, particularly when the ligand shares specific contacts with radio-toxin. The intermediate ability of d-tubocurarine to accelerate dissociation of bound [^{3}H]α-Bgt from rat brain membranes suggests that it shares fewer contact points with [^{3}H]α-Bgt than does native α-Bgt. Of course, any such model must take into account steric hindrance limitations, and concede that the precise nature of toxin-receptor interactions at contact points may not be readily mimicked by solute or solvent molecules. The ability of carbachol to induce toxin dissociation may follow these considerations, but may also reflect contributions due to carbachol-induced alterations in receptor state. Fourthly, the explanation of toxin-binding inhibition results advanced in a previous communication (Lukas[iewicz] et al. 1978), which may also explain decreased affinity of nAChR for [$^{125}I_2$]α-Bgt as described herein, based on steric fit considerations and formation of

ternary toxin-receptor-toxin complexes is also consistent in the context of the present results. It is, however, curious, that some logical expectations of such a toxin binding model are not borne out by the present data--such as the absence of clear 1:1 stoichiometries for slowly and rapidly dissociating $[^{125}I_2]$α-Bgt-nAChR complexes under chase conditions. Lastly, the documented differences in receptor-toxin complex dissociation properties for $[^{3}H]$α-Bgt and $[^{125}I_2]$α-Bgt are subject to alternative explanations and prospecti. On the one hand, one might argue that evaluation of receptor-diiodo toxin interactions should be treated cautiously. In this regard, the apparent allosteric effects of diiodo-α-Bgt discussed by Bulger et al. (1977) may be attributed to the properties of the derivative itself. On the other hand, use of iodinated toxins might be useful in revealing microheterogeneities of receptor sites or toxin binding mechanisms.

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TABLE I

Kinetic Constants

		K_D^{app} (nM) 1 hr incub.	K_D^{app} (nM) overnight incub.	k_1 ($min^{-1}M^{-1}$)	k_{-1} (min^{-1}) dilution	k_{-1} (min^{-1}) chase	Calc. k_D (nM) dilution	Calc. k_D (nM) chase
Brain	[^{3}H]α-Bgt	1.6	0.9	$3.2 \cdot 10^6$	$2.4 \cdot 10^{-4}$	$4.6 \cdot 10^{-4}$	0.075	0.14
	[$^{125}I_1$]α-Bgt	2.1	2.6	$2.7 \cdot 10^6$	$2.7 \cdot 10^{-4}$	$3.9 \cdot 10^{-4}$(80) $1.2 \cdot 10^{-3}$(20)	0.10	0.14 0.44
	[$^{125}I_2$]α- Bgt	4.0	7.5	$2.7 \cdot 10^6$	$6.2 \cdot 10^{-4}$	$6.1 \cdot 10^{-4}$(45) $2.6 \cdot 10^{-3}$(55)	0.23	0.23 0.96
Torpedo	[^{3}H] α-Bgt	17	3.9	$5.6 \cdot 10^5$	$1.8 \cdot 10^{-4}$	$2.6 \cdot 10^{-4}$	0.32	0.46
	[$^{125}I_1$]α-Bgt	22	2.2	$5.0 \cdot 10^5$	$1.4 \cdot 10^{-4}$	$2.1 \cdot 10^{-4}$	0.28	0.42
	[$^{125}I_2$]α-Bgt	90	19	$2.5 \cdot 10^5$	$1.5 \cdot 10^{-4}$	$1.4 \cdot 10^{-4}$(80) $7.2 \cdot 10^{-4}$(20)	0.60	0.56 2.9

Empirically derived values of K_D^{app}, k_1 and k_{-1}, and calculated k_D values are from data shown in Figs. 3-5.

TABLE II

Dixon Plot Slopes

Non-radio-labeled toxin	Radio-toxin		
	[^{3}H]α-Bgt	[^{125}I$_1$]α-Bgt	[^{125}I$_2$]α-Bgt
Brain			
Native α-Bgt	1.02	1.72	2.26
Mono-iodo α-Bgt	0.49	0.98	1.52
Di-iodo α-Bgt	2.50	1.36	1.36
Torpedo			
Native α-Bgt	0.98	1.73	2.25
Di-iodo α-Bgt	-	-	0.56

FIGURE LEGENDS

Fig. 1. Receptor titration curves a - After a two hour incubation, quantity of radioactivity associated with the membrane pellet (percent of total added radioactivity) is plotted against concentration of Torpedo membrane-bound nAChR (nM) in order to assess radio-purity and biological activity of radio-labeled α-Bgt species. The concentration of each toxin was 140 nM in this experiment. b - Hofstee-Eadie-Scatchard transform of data in Fig. 1a. (○——○)-[^{3}H]α-Bgt; (▲- - -▲)-[^{125}I] α-Bgt; (□——□)-[$^{125}I_2$]α-Bgt. See methods for assay conditions and design.

Fig. 2. Receptor saturation curves. Quantity of radiolabeled toxin specifically bound (dpm x k) to membrane sites at fixed nAChR concentration is plotted against concentration of radiolabeled toxin (nM). (0,●)-[^{3}H]α-Bgt; (△,▲)-[^{125}I]α-Bgt; (□,■)-[$^{125}I_2$]α-Bgt. a - Rat brain membranes, 1 h incubation. b - Rat brain membranes, overnight incubation. c - Torpedo membranes, 1 h incubation. d - Torpedo membranes, 24 h incubation. k value for Figs. 2 and 3: for [^{3}]α-Bgt - a,b,c and d, k = 10^{-3}; for [^{125}I]α-Bgt - a and b, k = $6.25 \cdot 10^{-5}$; c and d, k = $1.33 \cdot 10^{-5}$; for [$^{125}I_2$]α-Bgt - a and b, k = $3.12 \cdot 10^{-5}$; c and d, k = $6.66 \cdot 10^{-6}$. See Methods for assay conditions and design.

Fig. 3. Hofstee-Eadie-Scatchard transforms of receptor saturation curves. Data in Fig. 2 replotted as radiolabeled α-Bgt bound (dpm x k) against [α-Bgt bound] [concentration of radiolabeled α-Bgt]$^{-1}$ (dpm x k ÷ n<u>M</u>). (o,●) - [^{3}H]α-Bgt; (△,▲) -[^{125}I$_1$]α-Bgt; (□,■) - [^{125}I$_2$]α-Bgt. a - Rat brain membranes, 1 h incubation. b - rat brain membanes, overnight incubation. c - <u>Torpedo</u> membranes, 1 h incubation. d - <u>Torpedo</u> membranes, overnight incubation. See legend to Fig. 2 for k values. Analysis of the data yields the following apparent K_D values for [^{3}H] α-Bgt, [^{125}I$_1$]α-Bgt and [^{125}I$_2$]α-Bgt binding respectively: a - 1.6, 2.1, 4.0 n<u>M</u>; b - 0.9, 2.6, 7.5 n<u>M</u>; c - 17, 22, 90n<u>M</u>, d - 3.9, 2.2, 19 n<u>M</u>.

Fig. 4. Apparent association rate determination. Saturation curves from 2 to 80 nM in toxin concentration were determined as a function of time. Data from the first 10 min were transformed to plot ln [100 (percent sites unoccupied)$^{-1}$] against time. Apparent association rates for radio-toxin-receptor interactions (min^{-1}) are calculated and plotted against toxin concentration (n<u>M</u>). a - Rat brain membranes. b - <u>Torpedo</u> membranes. (o) - [^{3}H] α-Bgt, (△) [^{125}I$_1$]α-Bgt, (□) [^{125}I$_2$]α-Bgt. Values for k_1 (min^{-1} n<u>M</u>$^{-1}$) for [^{3}H]α-Bgt, [^{125}I$_1$]α-Bgt and [^{125}I$_2$]α-Bgt are, respectively, $3.2 \cdot 10^6$, $2.7 \cdot 10^6$ and $2.7 \cdot 10^6$ for rat brain, and $5.6 \cdot 10^5$, $5.0 \cdot 10^5$ and $2.5 \cdot 10^5$ for <u>Torpedo</u> membranes.

Fig. 5. Dissociation of radio-toxin-receptor complexes. Extent of receptor site occupation (percent maximal) is plotted against time (h): a - brain membranes. b - Torpedo membranes. Data shown are for [^{3}H]α-Bgt (upper panel), [$^{125}I_1$]α-Bgt (middle panel), [$^{125}I_2$]α-Bgt (lower panel). Receptor occupancy levels were determined following dilution in reaction buffer (●) or after addition of excess native α-Bgt (o, chase). Data are also shown for dissociation in the presence of 1 mM carbachol (◇) or d-tubocurarine (◆). Calculated values for the dissociation rate constant (min^{-1}) from brain membranes are: [^{3}H]α-Bgt, [^{125}I]α-Bgt, and [$^{125}I_2$]α-Bgt, respectively; dilution - $2.36 \cdot 10^{-4}$, $2.69 \cdot 10^{-4}$, $6.24 \cdot 10^{-4}$; chase - $4.62 \cdot 10^{-4}$, $3.86 \cdot 10^{-4}$ (80%) and $1.15 \cdot 10^{-3}$ (20%), $6.08 \cdot 10^{-4}$ (45%) and $2.56 \cdot 10^{-4}$ (55%. For [^{3}H]α-Bgt, $k_1 = 3.86 \cdot 10^{-4}$ (carbachol addition) and $2.75 \cdot 10^{-4}$ (d-tubocarine addition). Calculated values for the dissociation rate constant (min^{-1}) from Torpedo membranes are, for [^{3}H] α-Bgt, [$^{125}I_1$]α-Bgt, and [$^{125}I_2$]α-Bgt, respectively: dilution $1.75 \cdot 10^{-4}$, $1.38 \cdot 10^{-4}$, $1.48 \cdot 10^{-4}$; chase - $2.63 \cdot 10^{-4}$, $2.10 \cdot 10^{-4}$, $1.38 \cdot 10^{-4}$ (80%) and $7.22 \cdot 10^{-4}$ (20%).

Fig. 6. Radio-toxin binding competition. Data obtained on non-radiolabeled toxin inhibition potencies toward radio-toxin binding are plotted as modified Dixon transforms. a - Torpedo membranes: Native-Bgt vs [^{3}H]α-Bgt (●——●), vs [^{125}I]α-Bgt (△- - -△), vs [$^{125}I_2$]α-Bgt (■——■), diiodo α-Bgt vs [$^{125}I_2$]α-Bgt (△- - -△). Rat brain membranes using [^{3}H]α-Bgt (○——○; ●——●); [$^{125}I_1$]α-Bgt (◇- - -◇; ◆- - -◆) or [$^{125}I_2$]α-Bgt (□——□, ■——■; □——□. b - Competiton with non-radiolabeled diiodo α-Bgt. c - Competition with non-radiolabeled mono-iodo α-Bgt. d- Competition with non-radiolabeled diiodo α-Bgt.

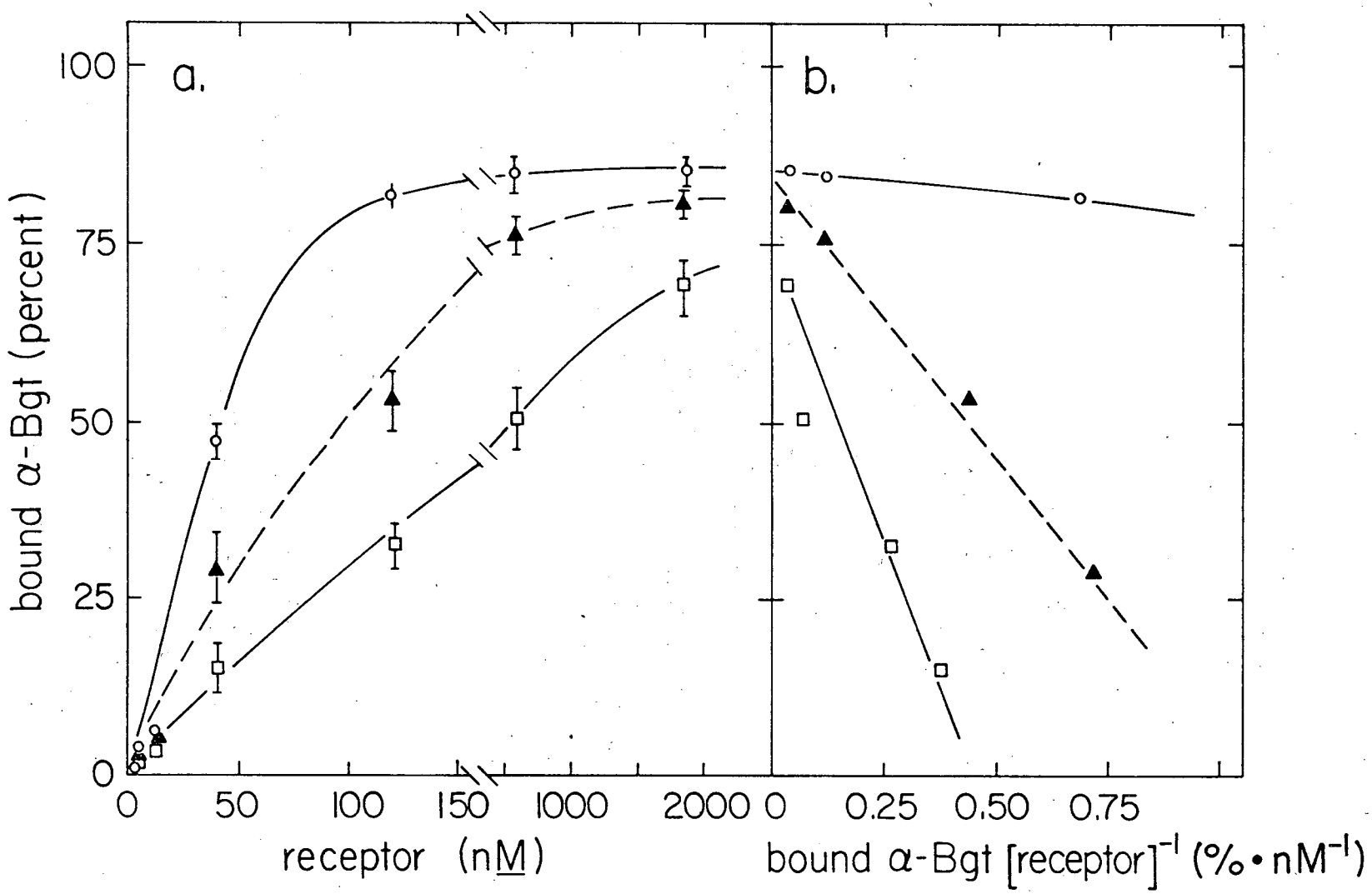

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Fig. 1

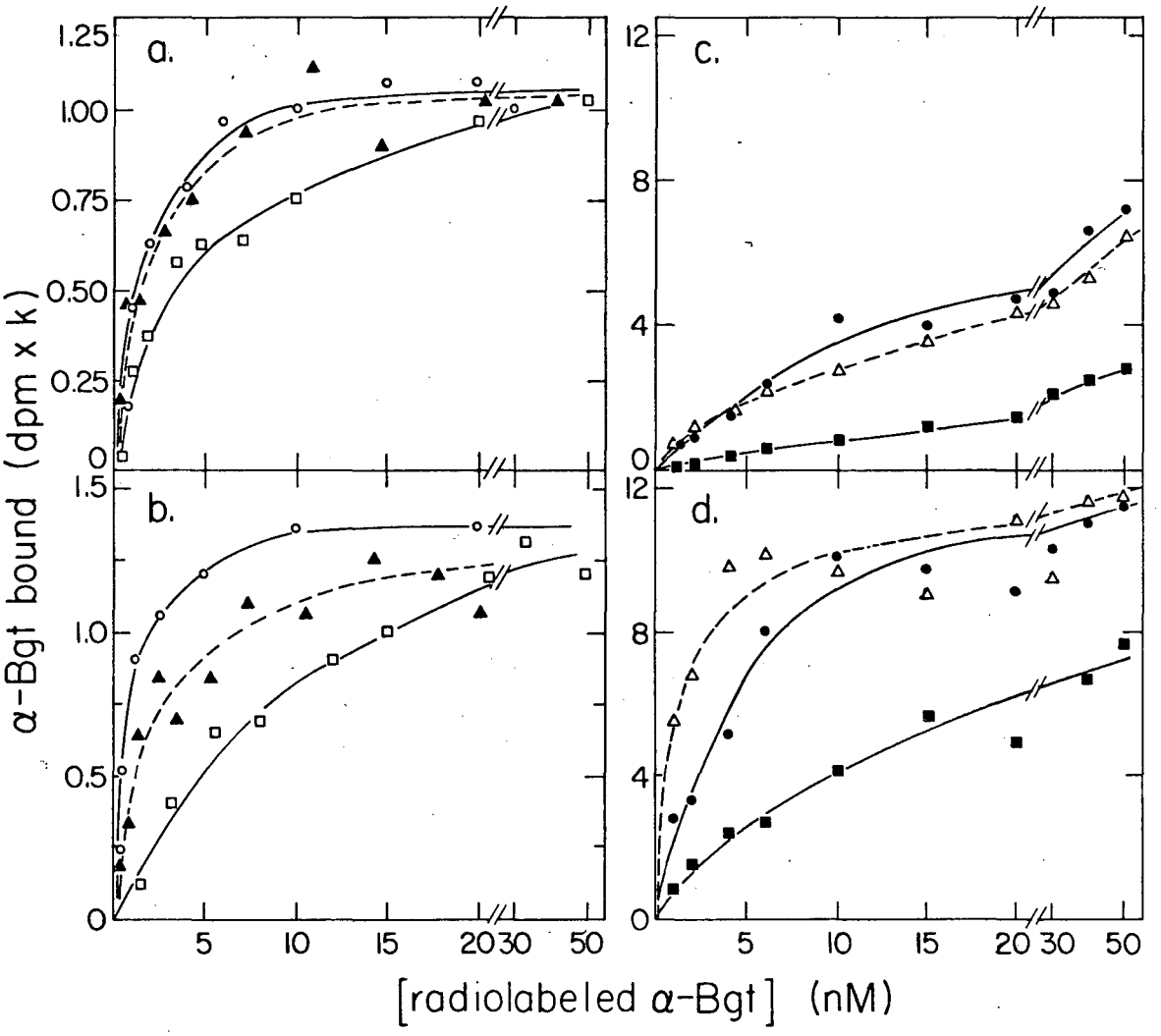

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Fig. 2

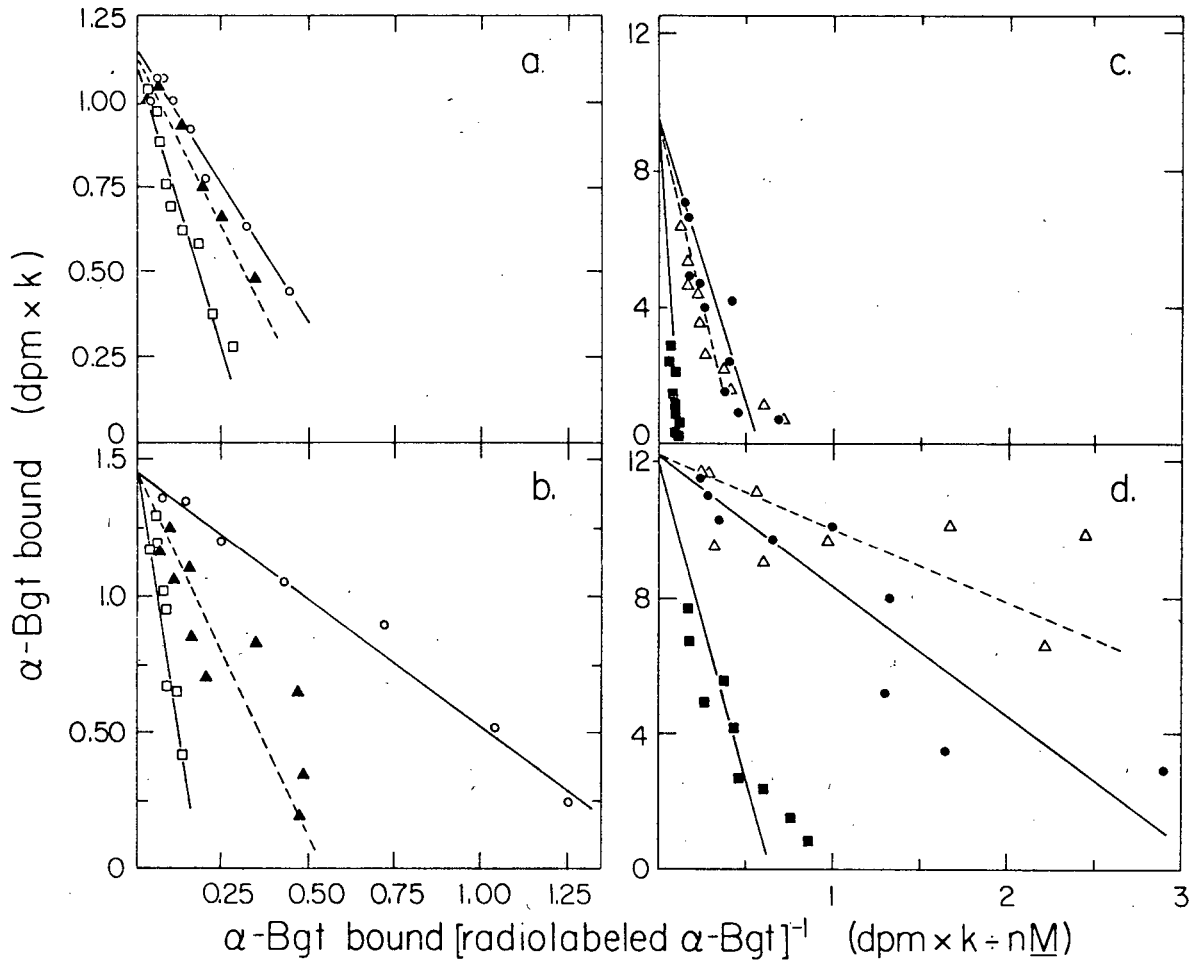

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Fig. 3

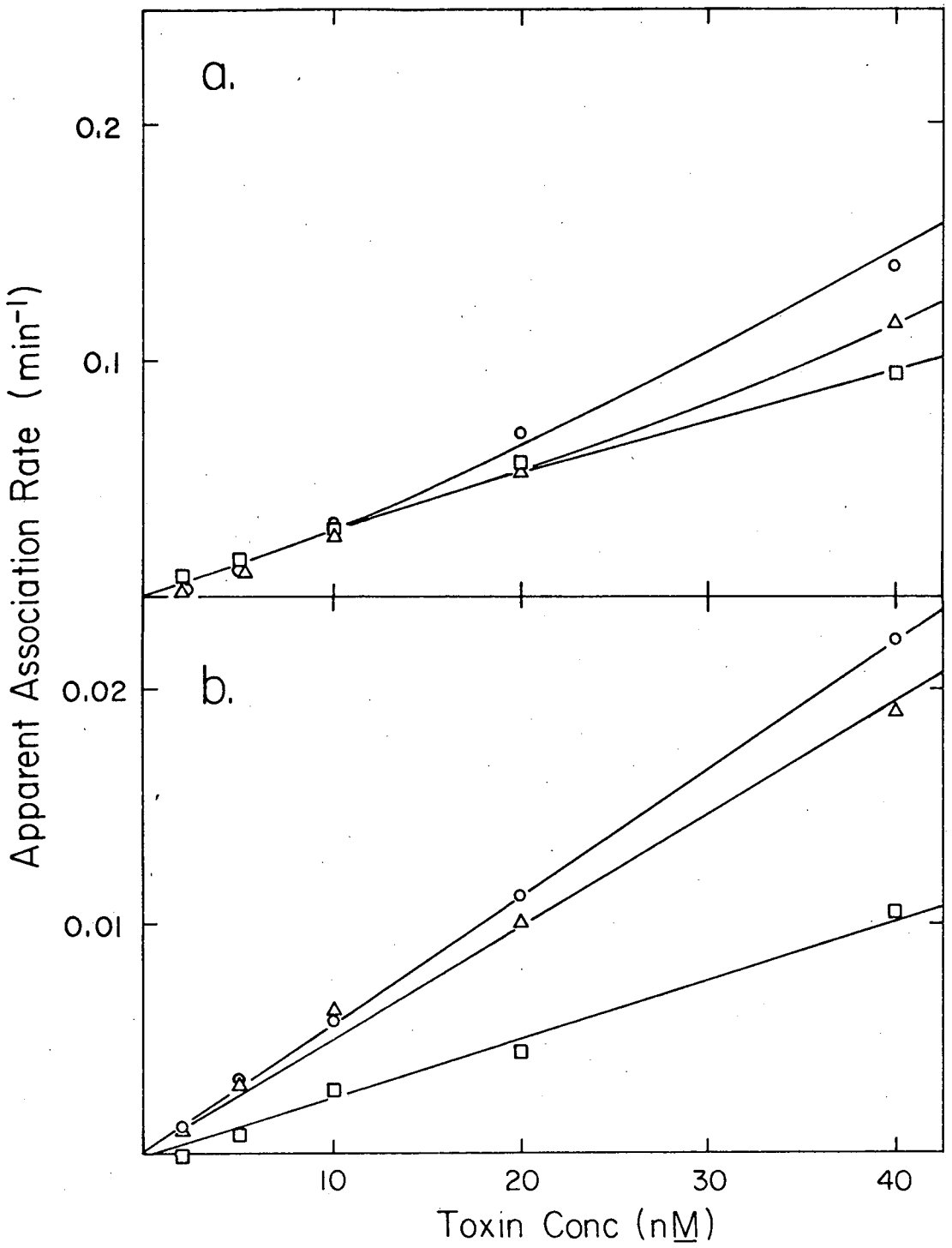

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Fig. 4
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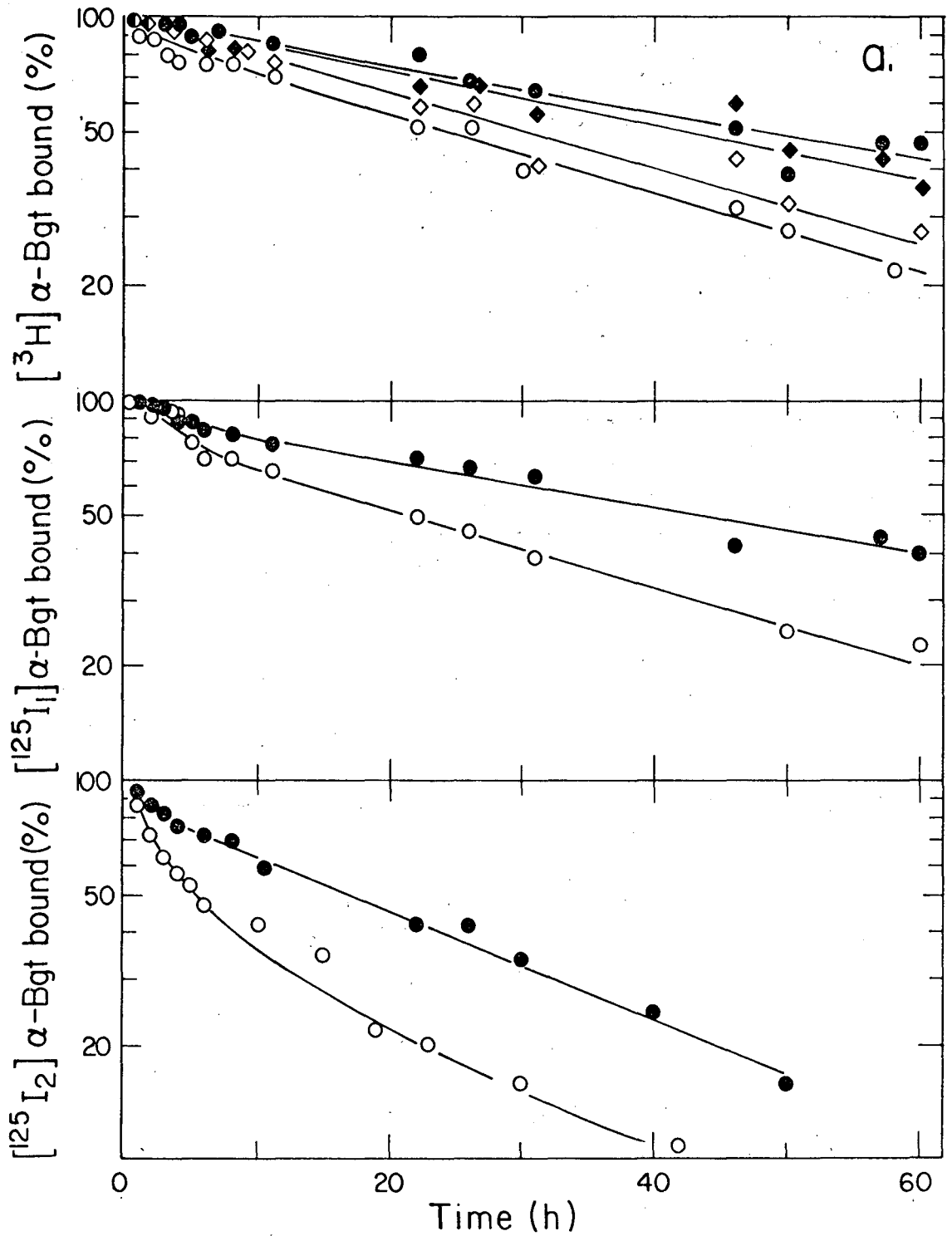

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Fig. 5 a

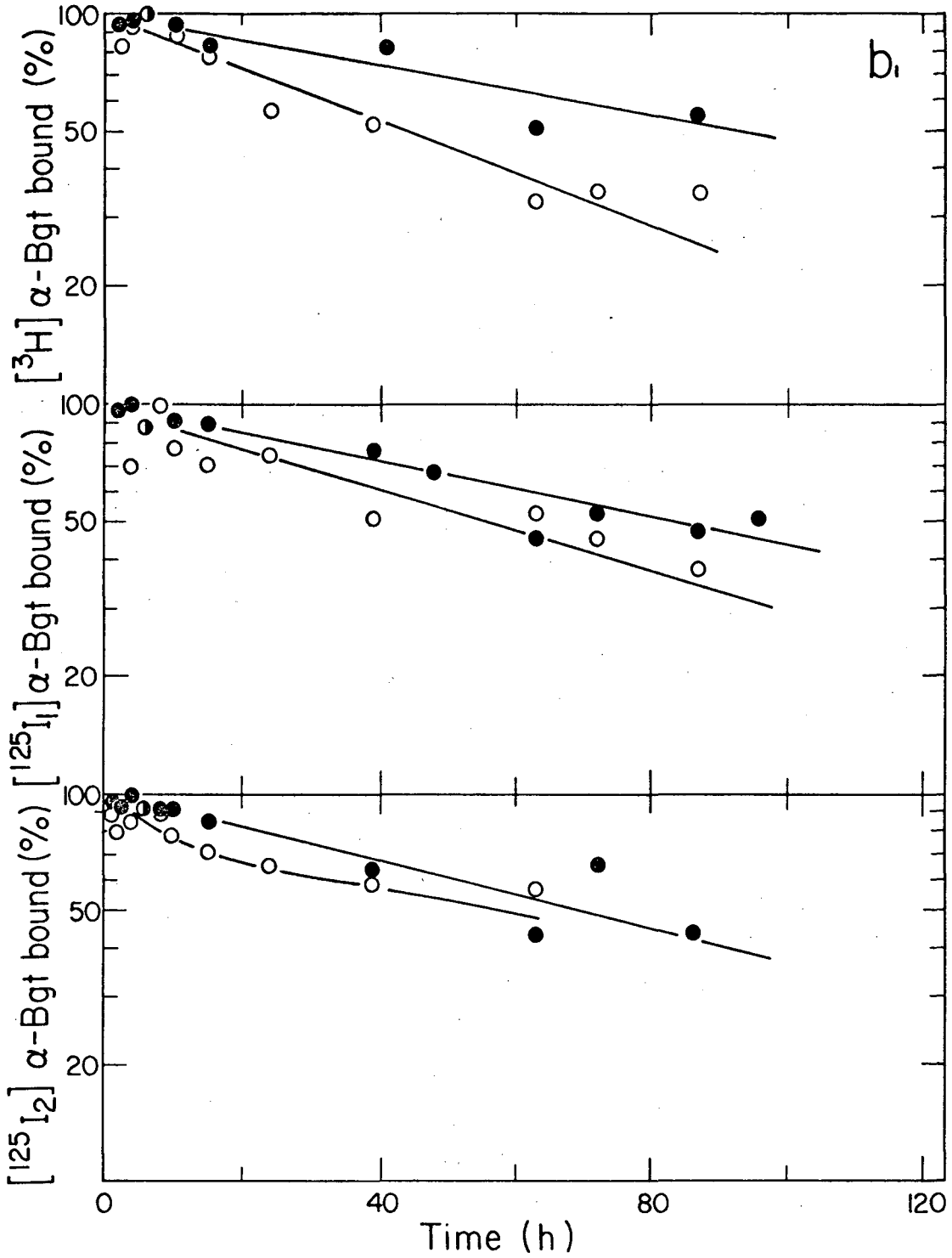

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Fig. 5 b

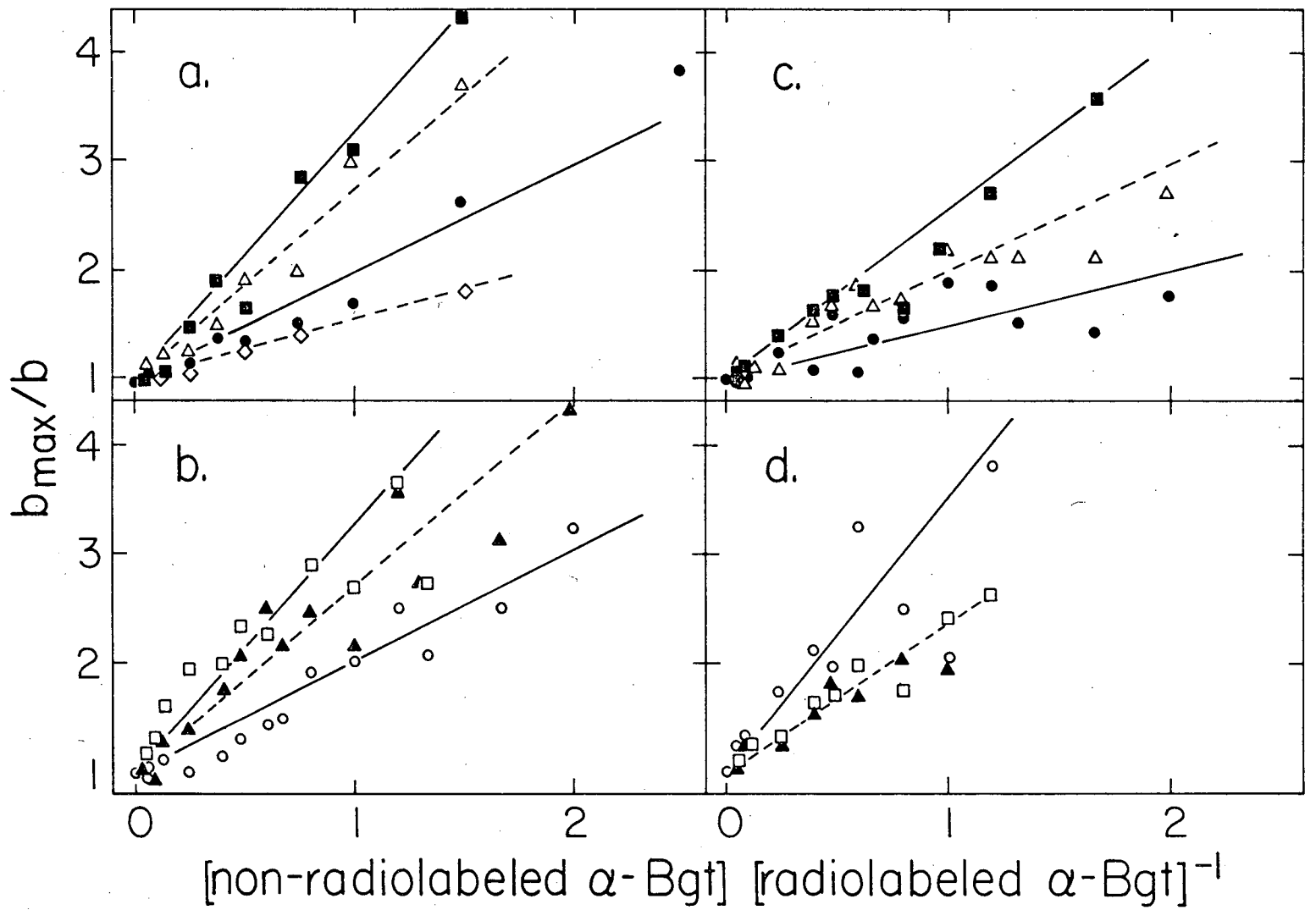

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Fig. 6

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