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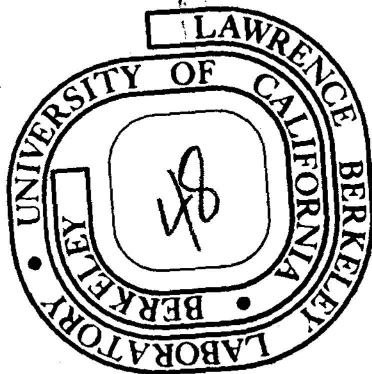

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Radioactive Labeling of α-Bungarotoxin with Tritium

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Running Title: Preparation of α-[^{3}H] Bungarotoxin

ABSTRACT

α-[^{3}H] Bgt was prepared by catalytic reduction of ^{125}I-labeled α-Bgt with tritium. Specific activities of 10-15 Ci/mmole were attained. The radioactive label as found in tyrosine. Tritiated α-Bgt appears to bind specifically to the cholinergic receptor of diaphragm, and a similar component of cerebral cortex. This specificity and the high specific radioactivity attained provide a useful tool for the study of ACh R in brain and other tissues with low receptor concentration.

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The widespread use of α-bungarotoxin (α-Bgt)[3] for the study of cholinergic receptors has prompted the preparation of several radiolabeled toxin analogs. Iodinated α-Bgt, labeled either with ^{125}I or ^{131}I has been prepared by the chloramine T method, by the lactoperoxidase method, with ^{125}ICl, and by Pressman and Eisen's method (1-5).

Iodinated analogs offer the attractive advantage of high specific activity, and relatively simple methods of preparation. They were found to inhibit the physiological response of muscle and nerve cells to acetylcholine, and to bind specifically to cholinergic receptors as judged from inhibition of binding by other cholinergic agents (see references given above). However, iodinated analogs present the disadvantage of short half-life of the isotopes used: 60 days for ^{125}I and 7 days for ^{131}I. Cumulative damage to the protein by the penetrating γ-rays from iodine was also observed. This necessitates frequent preparations and prompt use of the labeled toxin. ^{125}I-labeled α-Bgt[3] prepared by the chloramine T method was found unsuitable for studies of ACh R (acetylcholine receptor)[3] in brain (6).

[^{3}H] Acetyl α-Bgt with 0.4-0.8 acetyl groups per molecule was prepared by Barnard _et al._ (7). Chang _et al._ (8) have separated several acetylated derivatives, finding that N,O-di and N,N,O-tri [^{3}H] acetyl α-Bgts were specific reagents for the ACh R from diaphragm, while tri N, tetra and hexa acetylated products were increasingly less specific. The specific activities obtained were in the order of 1 Ci/mmole. This makes them unsuitable for biochemical work with tissues of low ACh R concentration.

This work describes the preparation and some properties of a high specific activity α-[^{3}H] Bgt, which seems to bind with high specificity to the ACh R from brain cortex and diapharagm.

MATERIALS AND METHODS

Crude venom from *Bungarus multicinctus* was purchased from Miami Serpentarium and stored at -20°C until used. CM-Cellulose (CM-52) was from Whatman, Viokase from Grand Island Biological Company, and ^{125}ICl (5 mCi/mmole) from New England Nuclear Corporation. Carrier-free tritium was supplied by Lawrence Livermore Laboratory. d-Tubocurarine chloride was from Calbiochem, carbamylcholine chloride from Sigma Chemical Co., and nicotine hydrochloride from K and K Laboratories. Palladium, 5% on powdered alumina, was from Matheson Coleman and Bell.

Preparation of ^{125}I-labeled α-Bgt

α-Bgt was prepared from crude *Bungarus multicinctus* venom as described previously (9,10). The purity of the α-Bgt preparations was estimated to be greater than 90% by disk electrophoresis in 10% polyacrylamide gels, following the procedure of Laemmli (11). The activities of acetylcholinesterase (EC 3.1.1.7), phospholipase A (EC 3.1.1.4), nucleotide pyrophosphatase (EC 3.6.1.9), and the phosphomonoesterase (EC 3.1.3.1) were measured by standard described methods (12-15).

←These enzymes are usually present as contaminants of α-Bgt after chromatography on CM-Sephadex, but their activities were found negligible in the final preparation. For desalting and concentrating of α-Bgt and its analogs, Amicon UM-2 membranes were used.

α-Bgt was iodinated with ^{125}ICl following the procedure of Menez *et*

al. (16). After reducing the excess ^{125}ICl with $Na_2S_2O_3$, the reaction mixture was promptly layered over a CM-Cellulose column (2.5 x 25 cm) equilibrated with 0.1 M ammonium acetate, pH 5.8. Washing with initial buffer eliminated unbound iodine and the remaining salts. Diiodo, mono-iodo and native α-Bgt (if any remained as such) were then separated by a linear gradient of 0.1 to 0.4 M ammonium acetate, pH 5.8 (4). Radio-activity of the effluent was measured in a liquid scintillation spectrometer. ^{125}ICl standards were counted simultaneously to correct for the radioactive decay of ^{125}I. For the determination of specific activities, protein concentration was estimated by multiplying the absorbance at 280 nm by a factor of 0.85 (9).

Tritiation of $^{125}I_2$-labeled α-Bgt[3]

The general procedure of Menez et al. (16) was followed. The tritium system shown in Fig. 1 is fabricated from stainless steel, glass and brass. The equipment is enclosed in a glove box, with the mechanical and diffusion pumps located on the outside. Both pumps are vented through a tritium oxidizer which, in turn, brings the exhaust back into the glove box. The main box exhaust is filtered through a number of silica gel filters (17). The exhaust line is also monitored before and after the filters. The two absolute pressure gauges used are both of the Wallace & Tiernan type, Model 62-075. To check the system for the absence of leaks, a Hastings DV 3M gauge was used. All the valves, except for the main pumpout valve and the one closest to the uranium tritide bed, are of the Nupro "H" series bellows valves, Model B4-H. The other two are of the Veeco forged brass angle valve, Model SL-38-S, and Hoke, Model 423506Y-316-55, respectively. The reaction flask had a small rotatory spoon to contain the catalyst during initial flushing with tritium (16); its total internal volume was 10 ml.

Desalted and lyophilized $^{125}I_2$-labeled α-Bgt in 0.5 ml of 4 mM sodium phosphate buffer, pH 7.4, was frozen in the reaction flask, and connected to the tritiation system. Ten mg of palladium 5% on alumina were placed into the upper compartment of the reaction flask, and flushed for 30 min with carrier-free $^3H_2$ from which any contaminating helium had been removed. The actual tritiation of the toxin took place at 400 mm of $^3H_2$, for 30 min. Tritium remaining in the system beyond the reaction flask was returned to the uranium trap. When the reaction was finished, excess $^3H_2$ was flushed with a N_2 stream and collected into evacuated cylinders. The catalyst was separated from the solution by centrifugation and washed once with 1 ml of distilled water. The combined supernatants were lyophilized twice. At this stage most adsorbed $^3H_2$ and ^3H_2O were eliminated and the solution was removed from the glove box. The reaction products were adsorbed on a CM-Cellulose column similar to the one used after the iodination step and washed with initial buffer until all ^3H_2O was eliminated. α-[^{3}H] Bgt was then separated from unreacted $^{125}I_2$-labeled α-Bgt and partially reacted $^{125}I_1$-labeled α-Bgt[3] by gradient elution. ^{125}I was determined in a γ-ray NaI well counter (Nuclear Chicago), to avoid interference from ^{3}H which had a specific activity several orders of magnitude higher. One ml aliquots of α-[^{3}H] Bgt (∿30 μg) in 0.2 M ammonium acetate, pH 5.8, were frozen in liquid nitrogen and stored at -20°C until used.

Characterization of α-[^{3}H] Bgt

α-[^{3}H] Bgt (0.7 μg) plus 500 μg of carrier α-Bgt were hydrolyzed with a mixture of pancreatic hydrolytic enzymes (Viokase) for 40 hr at 37°C (3), in sealed tubes. The hydrolysate was diluted to 3.5 ml and filtered

through a UM-2 membrane. The ultrafiltrate was lyophilized, and analyzed in a Beckman Automatic Amino Acid Analyzer, Model 120C, in which the effluent of ion exchange columns was diverted to a fraction collector to allow measurement of radioactivity in individual amino acids.

Lethalities to mice of native and labeled toxins were estimated by time to death after intraperitoneal injections (9). Twenty to thirty g male CD-1 Swiss mice were used.

For brain and diaphragm experiments, male Sprague Dawley rats weighing about 200 g were used. Binding to diaphragm was assayed following the technique of Berg *et al.* (1), except that the tissue was burned in a Packard Sample Oxidizer, Model 306, prior to scintillation counting of bound tritium. Binding to homogenates of cerebral cortex was measured as described previously (6), except for the fact that cortices were homogenized directly in Ringer solution instead of 0.32 M sucrose.

RESULTS AND DISCUSSION

Iodinated α-Bgt

Under the conditions described in METHODS, the CM-Cellulose column is able to separate native α-Bgt from monoiodo- and diiodotoxin (Fig. 2). However, with the proportions of ^{125}ICl to toxin used here (5:1 or 10:1) no unlabeled α-Bgt was detected. In several preparations, diiodotoxin was 80 to 100% of the recovered material, and the rest was monoiodotoxin. The recovery of protein was 75% and of radioactivity about 50%. The absorption spectrum of $^{125}I_2$-labeled α-Bgt was shifted slightly toward longer wavelengths, but otherwise was not significantly different from the spectrum of native α-Bgt (Fig. 3a). This red shift was observed previously with proteins iodinated on tyrosine and is expected since the absorption maximum

of tyrosine shifts from 275 nm to 287 nm upon diiodination (18). In addition, the pK_a of the phenolic hydroxyl is lowered to near neutral pH (18,19), and partial ionization could further increase the red shift (to 311 nm) as well as the extinction coefficient (6,250 $cm^{-1} \cdot M^{-1}$). Fluorescence of $^{125}I_2$-labeled α-Bgt was quenched to 20% of that of native toxin, and the emission maximum is shifted from 342 nm to 347 nm (Fig. 3b). The quenching of tryptophan fluorescence could arise from a direct interaction of the iodine atom with the indole side chain (20) or via resonance transfer of the excitation energy from tryptophan to an iodotyrosine residue with a lower fluorescence intensity.

Lethality to mice of $^{125}I_2$-labeled α-Bgt was similar to that of the native toxin (Fig. 4).

Tritiated α-Bgt

$^{125}I_2$-labeled α-Bgt was catalytically reduced with tritium, as described in METHODS. The reaction mixture was then separated into α-[^{3}H] Bgt and the remaining iodinated toxin analogs by a CM-Cellulose column. Under the conditions used, 50-70% of protein was recovered from the catalyst; 75% of this latter fraction was recovered from the column. Only 25% of the recovered material was α-[^{3}H] Bgt, which contained less than 0.2 ^{125}I atoms per molecule in the first preparation, and less than 0.4 ^{125}I atoms per molecule in the second. The rest was unreacted $^{125}I_2$-labeled α-Bgt or $^{125}I_1$-labeled α-Bgt. The specific activity attained was 0.36 to 0.49 tritium atoms per molecule--that is, 10.7 to 14.5 Ci/mmole. This specific activity is about 20% of the theoretical maximum of 2 atoms of tritium per molecule. Although dilution of tritium with hydrogen atoms from water has obviously occurred, the specific activity attained makes this derivative valuable for studies of AChR in very low concentrations.

In order to determine the position of tritium, α-Bgt was hydrolyzed enzymatically and the hydrolysate subjected to amino acid analysis. As shown by ultrafiltration through a UM-2 membrane, only 40% of the toxin was hydrolyzed to species of molecular weight less than 1000, after 40 hr of incubation. Eighty percent of the radioactivity in the ultrafiltrate

(To page 8)

was recovered in the effluent of the ion exchange columns: 4% in glycine and 76% in tyrosine. It was concluded that $[^3H]$ tyrosine is the principal labeled amino acid in α-$[^3H]$ Bgt, and that tritiation occurs by displacement of iodine from iodotyrosine as expected. No ^3H radioactivity was detected at the elution position of monoiodotyrosine.

Lethality of α-$[^3H]$ Bgt to mice was found to be about 50% that of native α-Bgt (Fig. 4). This lowered lethality may be due to partial reduction of disulfide bridges during the catalytic exchange. In vitro binding of tritiated toxin to rat diaphragm (Fig. 5a) was saturable and showed the characteristic preference for the end plate region (1,21,22). Inhibition of toxin binding by cholinergic agonists and antagonists in the diaphragm (Fig. 5b) also was as expected for α-Bgt (1). It should be pointed out that some loss of lethality with a concomitant retention of in vitro specificity for ACh R was observed previously for other toxins analogs (8,23).

Binding of α-$[^3H]$ Bgt to brain cortical homogenate was saturable at the expected low toxin concentrations, and strongly inhibited by the cholinergic agonist nicotine (Fig. 6). It was previously shown that α-$[^3H]$ Bgt binds to cortical crude mitochondrial fraction which contains the nerve endings, with all the characteristics of a nicotinic antagonist (6). Furthermore, the tritium label in α-$[^3H]$ Bgt was found to be very stable; only 6% of radioactivity was exchanged with the solvent in 6 months. Binding to brain and diaphragm of a α-$[^3H]$ Bgt preparation maintained frozen for one year was very similar to that observed with freshly tritiated α-Bgt. This behavior contrasts sharply with that observed for ^{125}I-labeled α-Bgt, for which specific binding to diaphragm was drastically

decreased, while the non-specific binding to brain was increased within a few months after iodination (6).

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FOOTNOTES

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[3]Abbreviations: α-Bgt, α-Bungarotoxin; ACh R, acetylcholine receptor.. ^{125}I-labeled α-Bgt, iodinated α-Bgt; $^{125}I_1$-labeled α-Bgt, monoiodinated α-Bgt; $^{125}I_2$-labeled α-Bgt, diiodinated α-Bgt.

FIGURE LEGENDS

Fig. 1. A schematic diagram of the equipment used for labeling α-bungarotoxin with carrier-free tritium. Abbreviations used: D.P., diffusion pump; G-1, 0 to 1 atmosphere absolute pressure gauge; G-2, 1 micron to 1 mm Hastings gauge; L.N., liquid nitrogen; M.P., mechanical pump; T.M., tritium monitor.

Fig. 2. Separation of α-Bgt from mono and diiodinated derivatives. 1.8 mg of α-Bgt plus 150 μg of a mixture of $^{125}I_2$-labeled α-Bgt and $^{125}I_1$-labeled α-Bgt (3.7×10^5 cpm), in 0.1 M ammonium acetate, pH 5.8, were separated by a column of CM-Cellulose (see METHODS). 2.4 ml fractions were collected. Peak I contained no radioactivity. Ninety percent of the radioactivity in peaks II and III was precipitable by 10% TCA. In other chromatographic separations with bigger samples, the specific activities of peaks II and III were determined, showing respectively one and two atoms of ^{125}I per molecule of toxin.

Fig. 3A. Absorption spectra of native and diiodinated toxin.

Fig. 3B. Fluorescence spectra of native and diiodinated toxin. Absorption spectra were obtained on a Cary 118C spectrophotometer, and have been normalized to the same optical densities. Fluorescence spectra were obtained with a Hitachi-Perkin Elmer MPF 2A recording fluorescence spectrometer with a Hamamatsu R 106 photomultiplier. The excitation band was at 280 nm; excitation and emission slitwidths were 4 nm. A_{280} of both solutions was 1.26.

Fig. 4. Lethality to mice of α-Bgt and its radioactive analogs. Three to five mice per group were used. Mortality was 100% in all cases. Potency is estimated from time to death (see METHODS). (o) native α-Bgt; (■) $^{125}I_2$-labeled α-Bgt; (▲) α-[^{3}H] Bgt.

Fig. 5. Binding of α-[^{3}H] Bgt to rat diaphragm.
a) α-[^{3}H] Bgt was incubated for 1 hr with radial sections of rat diaphragm, which were then washed, dissected and counted as described in METHODS. (■) binding to the area containing end plates; (□) binding to the area without end plates.
b) Inhibition of α-[^{3}H] Bgt binding by d-tubocurarine and carbamyl choline: Radial sections of diaphragm were incubated with the competitors for 1 hr. α-[^{3}H] Bgt was then added at a final concentration of 0.7 μg/ml, and the incubation was continued for 20 min. Further procedure was as described in METHODS. (■) α-[^{3}H] Bgt binding to the area containing end plates in the presence of carbamyl choline chloride; (o) binding to the area with end plates in the presence of d-tubocurarine.

Fig. 6. Binding of α-[^{3}H] Bgt to brain cortex homogenate.
a) Brain cortex homogenate (50 mg wet weight per ml) was incubated with α-[^{3}H] Bgt for 1 hr, in a total volume of 2 ml. Further processes were as described in METHODS.
b) 1 ml aliquots of homogenate (50 mg/ml) were incubated with 0.5 ml of nicotine for 1 hr; toxin was then added in 0.5 ml of Ringer at a final concentration of 6.25 x 10^{-9} M, incubated for 20 min, and further processed as in METHODS.

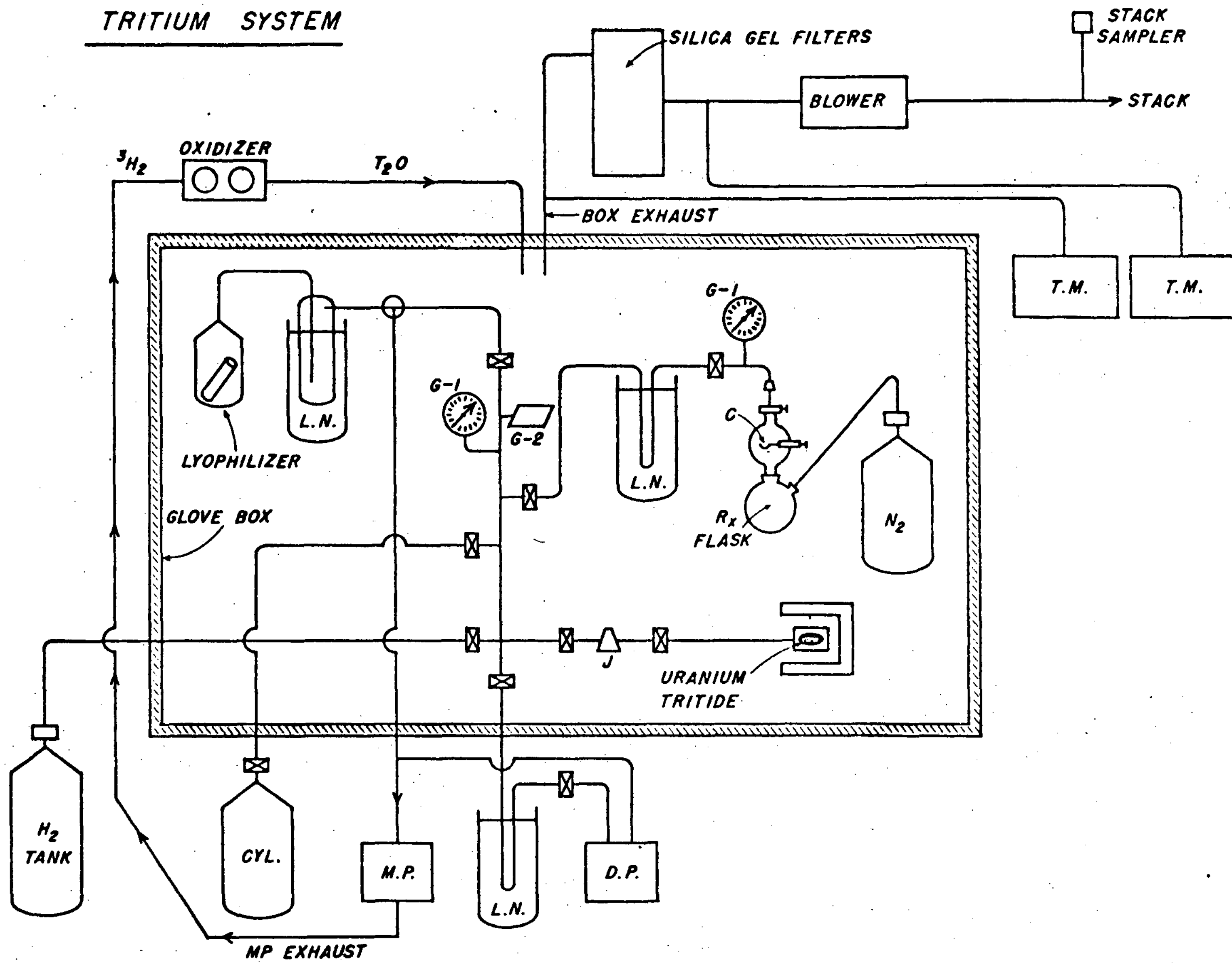

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Fig. 1.

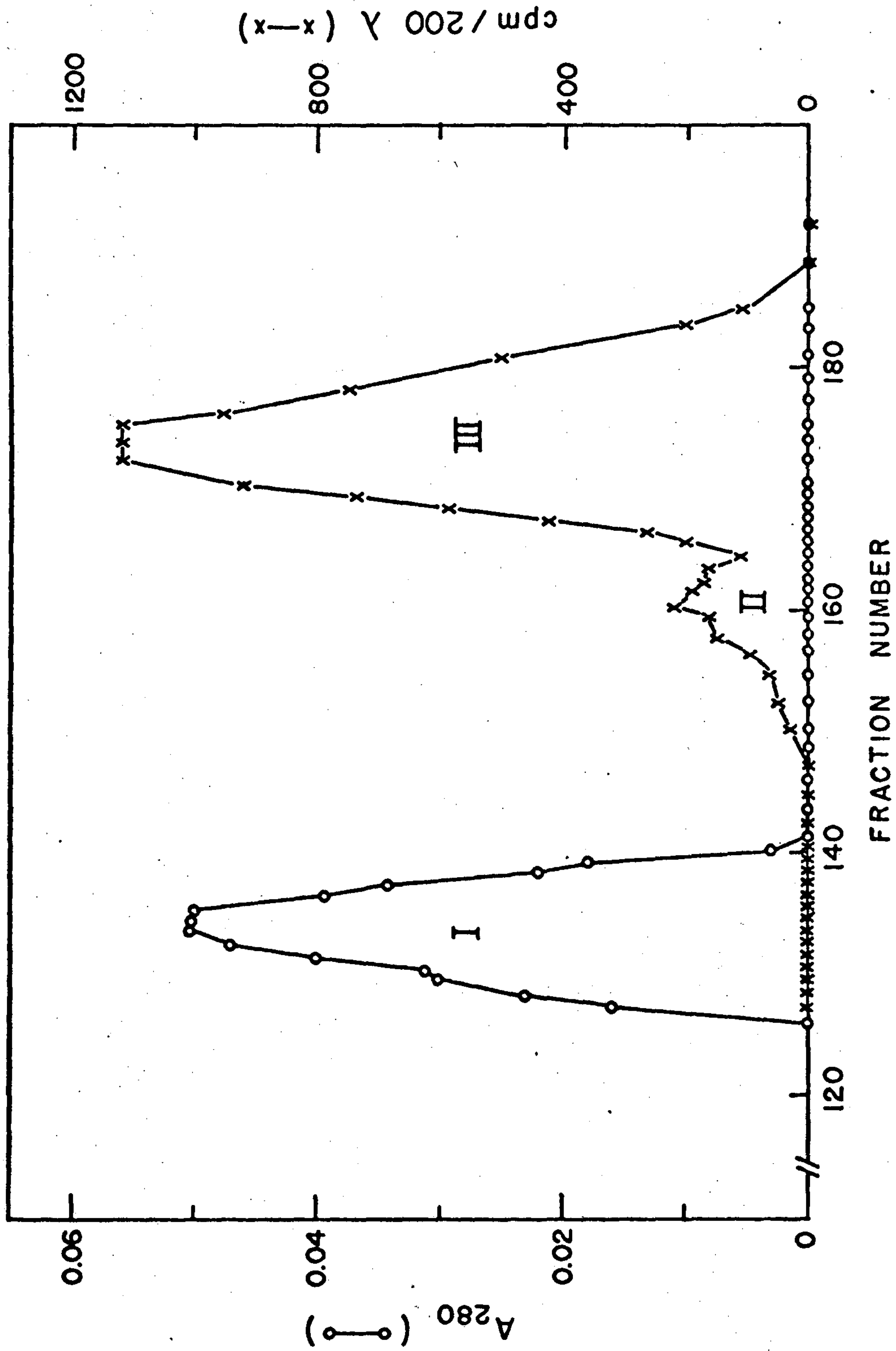

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Fig. 2.

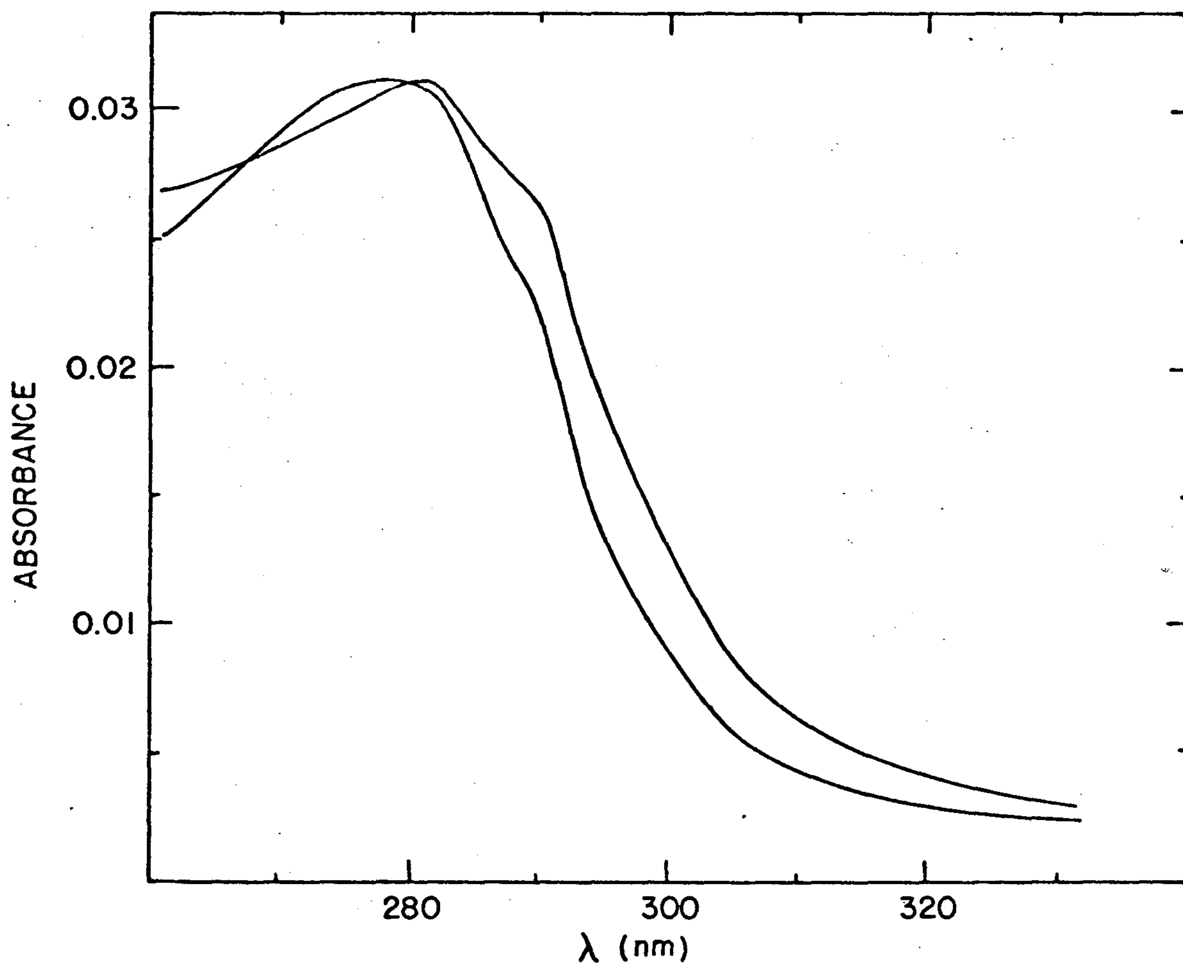


Fig. 3A.

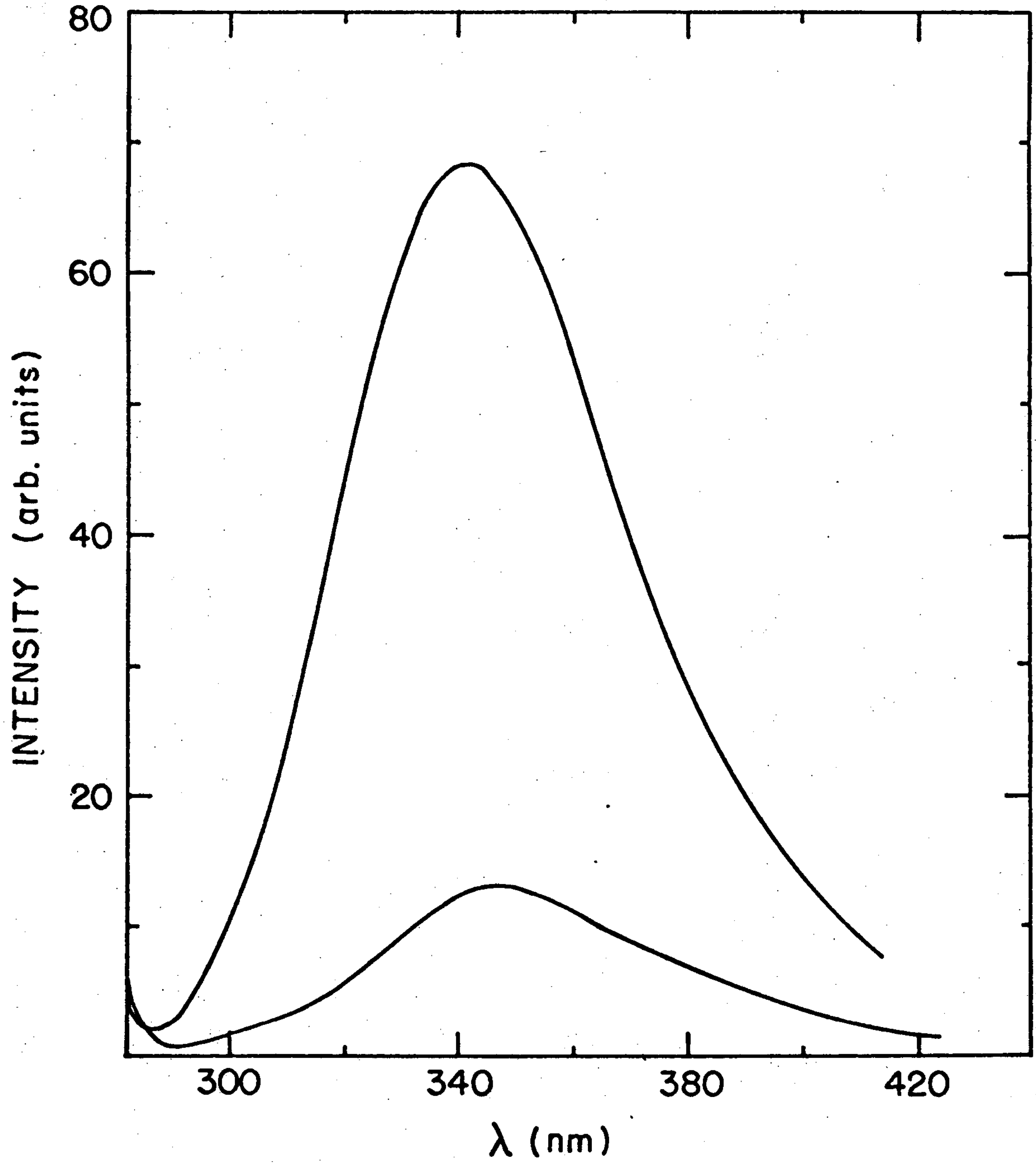


Fig. 3B.

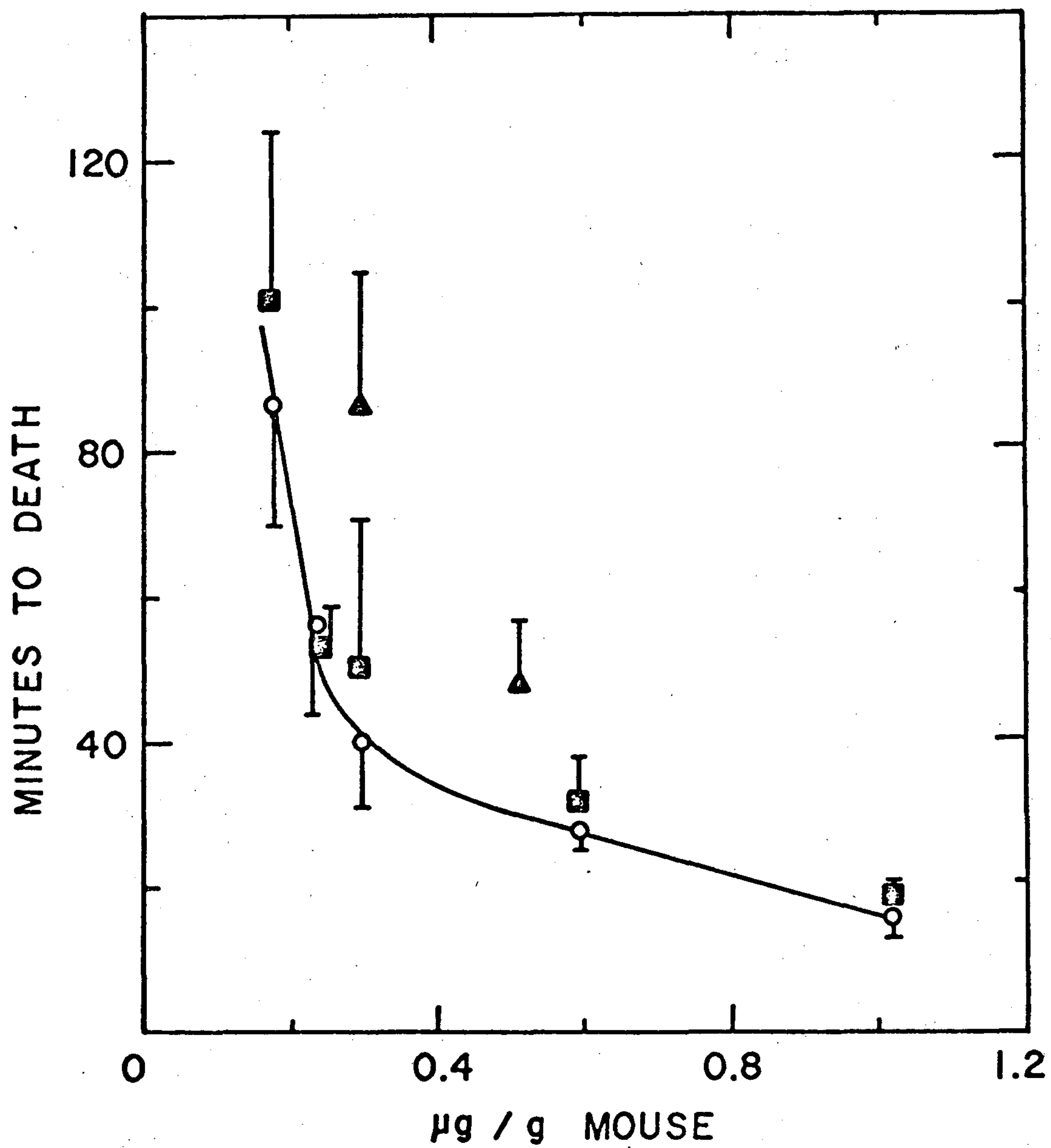


Fig. 4.

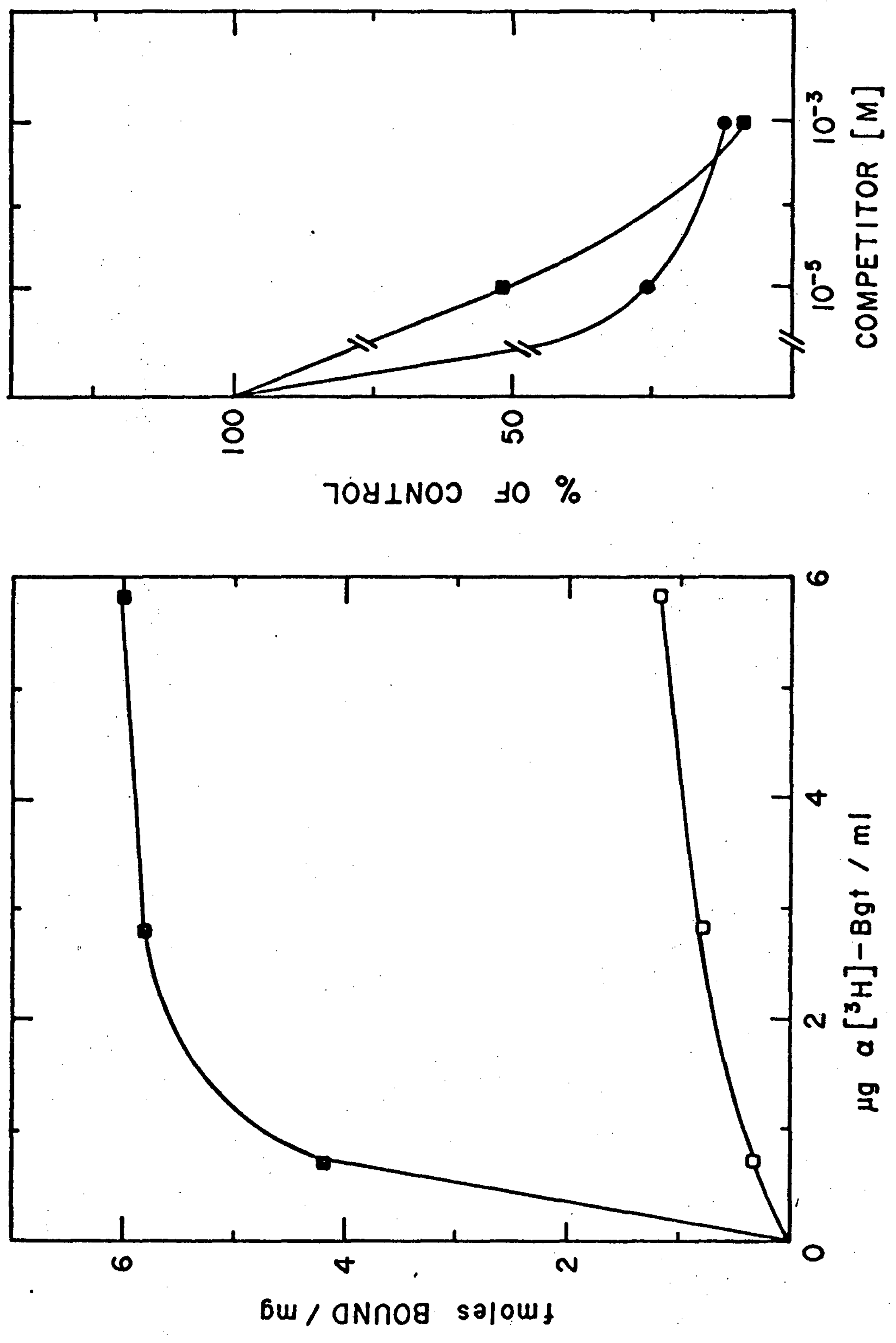
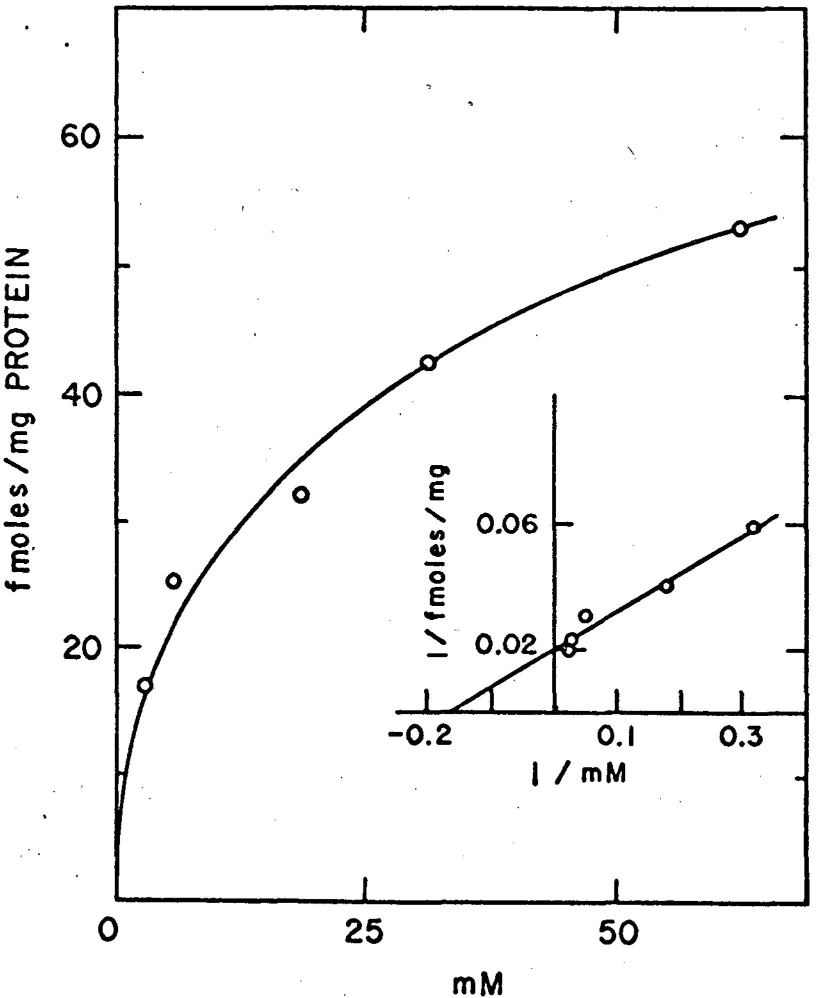


Fig. 5:



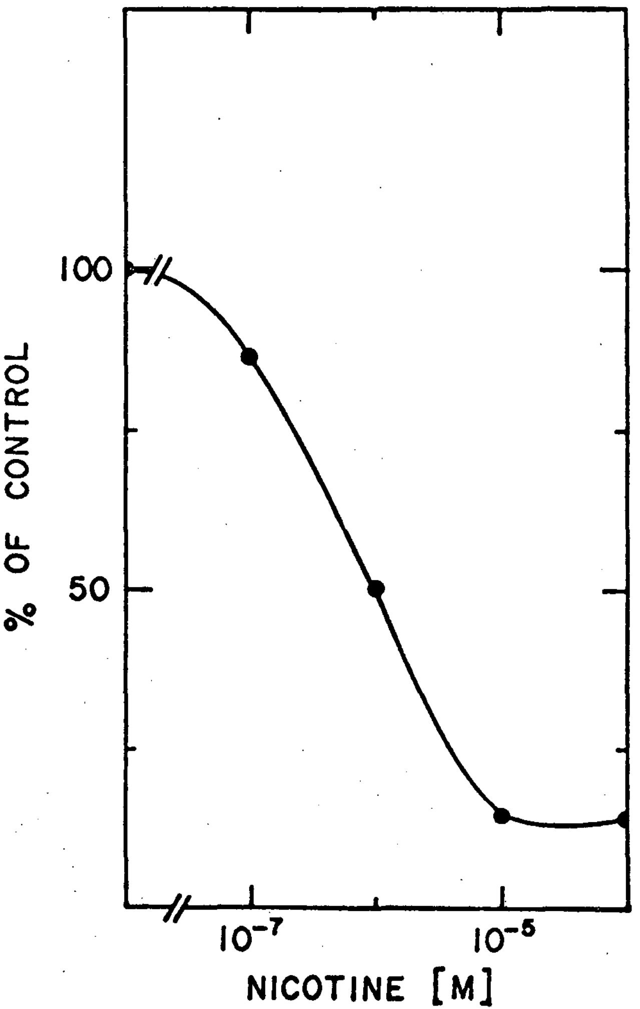


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Fig. 6.

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