

FILE COPY

(2)

AD _____

AD-A223 952

MECHANISMS OF ACTION OF ANTICHOLINESTERASES
AND OXIMES ON ACETYLCHOLINE RECEPTORS

ANNUAL REPORT

MOHYEE E. ELDEFRAWI

JULY 23, 1988

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-85-C-5092

University of Maryland School of Medicine
Baltimore, Maryland 21201

Approved for public release; distribution unlimited

The findings in this report are not to be construed as an official
Department of the Army position unless so designated by other
authorized documents

DTIC
ELECTE
JUL 18 1990
S _C E D

90 07 17 009

MECHANISMS OF ACTION OF ANTICHOLINESTERASES
AND OXIMES ON ACETYLCHOLINE RECEPTORS

ANNUAL REPORT

MOHYEE E. ELDEFRAWI

JULY 23, 1988

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-85-C-5092

University of Maryland School of Medicine
Baltimore, Maryland 21201

Approved for public release; distribution unlimited

The findings in this report are not to be construed as an official
Department of the Army position unless so designated by other
authorized documents

REPORT DOCUMENTATION PAGE

Form Approved
OMB No 0704-0188
Exp Date Jun 30, 1986

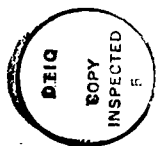
1a. REPORT SECURITY CLASSIFICATION Unclassified			1b. RESTRICTIVE MARKINGS	
2a. SECURITY CLASSIFICATION AUTHORITY			3. DISTRIBUTION/AVAILABILITY OF REPORT Unlimited	
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE				
4. PERFORMING ORGANIZATION REPORT NUMBER(S)			5. MONITORING ORGANIZATION REPORT NUMBER(S)	
6a. NAME OF PERFORMING ORGANIZATION University of Maryland School of Medicine		6b. OFFICE SYMBOL (if applicable)	7a. NAME OF MONITORING ORGANIZATION	
6c. ADDRESS (City, State, and ZIP Code) 660 West Redwood Street Baltimore, Maryland 21201			7b. ADDRESS (City, State, and ZIP Code)	
8a. NAME OF FUNDING/SPONSORING ORGANIZATION U.S. Army Medical Research & Development Command		8b. OFFICE SYMBOL (if applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER DAMD17-85-C-5092	
8c. ADDRESS (City, State, and ZIP Code) Fort Detrick, Frederick, Maryland 21701-5012			10. SOURCE OF FUNDING NUMBERS	
			PROGRAM ELEMENT NO. 62734A	PROJECT NO. 3M162. 734A875
			TASK NO. AI	WORK UNIT ACCESSION NO. 487
11. TITLE (Include Security Classification) (U) Mechanisms of Action of Anticholinesterases and Oximes on Acetylcholine Receptors				
12. PERSONAL AUTHOR(S) Mohyee E. Eldefrawi				
13a. TYPE OF REPORT Annual		13b. TIME COVERED FROM 4/15/85 TO 7/14/88	14. DATE OF REPORT (Year, Month, Day) 1988 July 23	15. PAGE COUNT
16. SUPPLEMENTARY NOTATION The view, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy, or decision, unless so designated by other documentation.				
17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)	
FIELD	GROUP	SUB-GROUP	Nicotinic and muscarinic acetylcholine receptors, oximes, binding of radioactive receptor ligands, <i>RA5</i>	
06	15			
06	01			
19. ABSTRACT (Continue on reverse if necessary and identify by block number) Oximes are strong nucleophiles, capable of activating phosphorylated acetylcholine (ACh)-esterase. In this report we provide evidence that, in addition to their action on the enzyme, oximes also react with nicotinic and muscarinic receptors. In our studies, we found 2-PAM, HI-6, and Toxogonin bound to both nicotinic and muscarinic ACh receptors. Modulation of binding of nicotinic receptor ligands in the Torpedo electric organ suggested that the oximes acted as nicotinic receptor antagonists. Inhibition of the receptor-regulated $^{22}\text{Na}^+$ transport supported this conclusion. These three oximes also bound to muscarinic ACh receptors of rat brain and displaced receptor agonists (e.g., [^3H]CD) and antagonists (e.g., [^3H]QNB and [^3H]PZ). The displacement kinetics suggested that the oximes bind to the same site that binds agonists and antagonists. However, their competitive inhibition of carbamylcholine-induced [^3H]c-GMP production in neuroblastoma cultures confirmed that oximes act as antagonists of muscarinic receptors.				
20. DISTRIBUTION/AVAILABILITY OF ABSTRACT ☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT. ☐ DTIC USERS			21. ABSTRACT SECURITY CLASSIFICATION Unclassified	
22a. NAME OF RESPONSIBLE INDIVIDUAL Mrs. Virginia Miller			22b. TELEPHONE (Include Area Code) 301-663-7325	22c. OFFICE SYMBOL SGRD-RMS

SUMMARY

During the third year of this contract, the interactions of three oximes with the nicotinic (n) acetylcholine (ACh) receptor of *Torpedo* electric organ and the muscarinic (m) ACh receptors of rat brain and neuroblastoma N1E-115 cultures were studied. The n-ACh receptors of *Torpedo* were labeled with [125 I] α -bungarotoxin (α -BGT), and [3 H]phencyclidine ([3 H]PCP), and the agonist regulated uptake of $^{22}\text{Na}^+$ into *Torpedo* microsacs was used as a functional assay for receptors. Muscarinic receptors of rat brain were labeled with [3 H]Quinuclidinyl benzilate ([3 H]QNB), [3 H]pirenzepine ([3 H]PZ), and [3 H]cis-methyl dioxolane ([3 H]CD). Carbamylcholine-stimulated [3 H]c-GMP synthesis in neuroblastoma N1E-115 cell cultures was used as a functional assay for m-receptors.

The three oximes 2-PAM, HI-6, and Toxogonin bound to n-ACh receptors of *Torpedo*, displaced the binding of [125 I] α -BGT, and inhibited the carbamylcholine-stimulated binding of [3 H]PCP. These data suggested to us that the oximes bound to the common recognition site which reacts with ACh, carbamylcholine, and α -BGT. The time dependent effect of these agents on the initial rate of binding of both [125 I] α -BGT and [3 H]PCP to resting receptors suggested that 2-PAM acts as a partial agonist, whereas HI-6 and Toxogonin act as competitive antagonists of the n-ACh receptor. The effect of the three oximes on receptor-regulated $^{22}\text{Na}^+$ transport supported the conclusion.

The oximes also bound to muscarinic receptors. Kinetic analysis of saturation binding of three m-ACh receptor ligands indicated that the oximes bound to the common recognition site that binds ACh and all the radiolabeled ligands studied. Interestingly, the order of affinity that m-ACh receptors of different types had for oximes differed. Inhibition of m-ACh-receptor-regulated synthesis of [3 H]c-GMP in neuroblastoma culture suggests that the oximes act as competitive antagonists of muscarinic receptor.



Accession For	
NTIS GRA&I	☒
DTIC TAB	☒
Unannounced	☐
Justification	
By _____	
Distribution/	
Availability Codes	
Dist	Avail and/or Special
A-1	

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985).

TABLE OF CONTENTS

SUMMARY.....	1
FOREWORD.....	3
1. STATEMENT OF PROBLEM.....	9
2. BACKGROUND AND LITERATURE REVIEW.....	10
3. RATIONALE USED IN CURRENT STUDY.....	13
4. EXPERIMENTAL METHODS.....	14
4.1 Nicotinic Receptor Binding.....	14
4.2 Nicotinic Receptor Function.....	14
4.3 Muscarinic Receptor Binding.....	15
4.4 Assay of Muscarinic Receptor Function.....	16
4.5 Data Analysis.....	18
5. RESULTS.....	19
5.1 Binding of Oximes to n-ACh Receptors.....	19
5.2 Effect of Oximes on n-ACh Receptor Function.....	20
5.3 Binding of Oximes to m-ACh Receptors.....	20
5.3-1 Effect of Oximes on [³ H]QNB Binding.....	20
5.3-2 Effect of Oximes on [³ H]PZ Binding.....	20
5.3-3 Effect of Oximes on [³ H]CD Binding.....	21
5.4 Effect of Oximes on m-ACh Receptor Function.....	21
6. DISCUSSIONS AND CONCLUSIONS.....	23
6.1 Effects on n-ACh Receptors.....	23
6.2 Effects on m-ACh Receptors.....	23
7. LITERATURE CITED.....	37
DISTRIBUTION LIST.....	41
ILLUSTRATIONS.....	25
Fig. 1.	Concentration-dependent inhibition of the initial rate of binding (40 sec) of [¹²⁵ I]α-BGT to <i>Torpedo</i> ACh receptors by three oximes. HI-6 (○), Toxogonin (Δ), and 2-PAM (□) were added simultaneously with the tissue and α-BGT and coincubated for 40 sec. HI-6 (●), Toxogonin (▲), and 2-PAM (■) were pre-incubated with the <i>Torpedo</i> membranes prior to measuring binding. Symbols are means of nine measurements and standard deviations were <10%.....
Fig. 2.	Concentration-dependent inhibition of the initial rate of binding of [³ H]PCP to activated <i>Torpedo</i> ACh receptors (i.e., binding in presence of 100 μM carbamylcholine) by oximes. HI-6 (○), Toxogonin (Δ),

	and 2-PAM (□) were coincubated for 30 sec. HI-6 (●), Toxogonin (▲), and 2-PAM (■) were preincubated with the membrane for 1 hr prior to binding assay. Symbols are means of nine measure- ments and standard deviations were <5%.....	26
Fig. 3.	Time-dependent binding of [³ H]PCP to resting <i>Torpedo</i> ACh receptors in absence (●) and presence of 10 ⁻⁴ M of HI-6 (○), Toxogonin (Δ), and 2-PAM (□). Symbols are means of six measurements and standard errors were <7%.....	27
Fig. 4.	Inhibition of n-ACh receptor-regulated ²² Na uptake into <i>Torpedo</i> microsacs induced by 100 μM carbamyl- choline in presence of HI-6 (○), Toxogonin (Δ), and 2-PAM (□). Symbols are means of six measure- ments and standard deviations were <12%.....	28
Fig. 5.	Concentration dependent inhibition of [³ H]QNB binding to rat brain muscarinic receptors by the oximes HI-6 (○), 2-PAM (□), and Toxogonin (Δ). Oximes and [³ H]QNB (0.2 nM) were coincubated with the tissue for 60 min in 1 ml of 50 mM phosphate buffer, pH 7.4. Symbols represent means of three experiments, having triplicate measurements. Standard deviations were <6%.....	29
Fig. 6.	Scatchard plots of the binding of [³ H]QNB to rat brain muscarinic receptors in absence of oximes (●) and in presence of 200 μM HI-6 (○), 60 μM 2-PAM (□), 20 μM Toxogonin (Δ).....	30
Fig. 7.	Concentration-dependent inhibition of [³ H]PZ bind- ing to rat brain muscarinic receptors by the oximes HI-6 (○), 2-PAM (□), and Toxogonin (Δ). Details are as described in legend to Fig. 5.....	31
Fig. 8.	Scatchard plots of the binding of [³ H]PZ to rat brain muscarinic receptors in the absence of oximes (●) and in the presence of 10 μM HI-6 (○), 15 μM 2-PAM (□), and 2.3 μM Toxogonin (Δ).....	32
Fig. 9.	Concentration-dependent inhibition of [³ H]CD bind- ing to rat brain muscarinic receptors by the oximes HI-6 (○), 2-PAM (□), and Toxogonin (Δ). Details are as described in legend to Fig. 5.....	33
Fig. 10.	Scatchard plots of the binding of [³ H]CD to rat brain muscarinic receptors in the absence of oximes (●) and in the presence of 100 μM HI-6 (○), 30 μM 2-PAM (□), and 10 μM Toxogonin (Δ).....	34
Fig. 11.	Concentration-dependent inhibition of carbamyl- choline-induced synthesis of [³ H]c-GMP in neuro- blastoma cells by Toxogonin (Δ) and HI-6 (○). Symbols are means of two experiments, each performed in triplicate (N = 6); bars represent ± SD.....	35
Fig. 12.	Concentration dependent synthesis of [³ H]c-GMP in neuroblastoma cells by carbamylcholine in absence (●) and presence (○) of 10 μM Toxogonin. The	

cells were preincubated with Toxogonin for 30 min
prior to addition of carbamylcholine. Symbols are
means of two experiments; bars represent $\pm$ SD..... 36

1. STATEMENT OF PROBLEM

The long-term objective of this research is to understand the effects of anticholinesterases and oximes and to improve therapy for poisoning by organophosphate (OP) nerve agents. Toxicity of OP and carbamate (CB) anticholinesterases is due mainly to their inhibition of acetylcholine (ACh) esterase, which leads to increased concentration of ACh in cholinergic synapses, which, in turn, causes excessive activation of ACh receptors and, consequently, their desensitization. Carbamate anticholinesterases may also protect ACh esterase from the irreversible inhibition caused by chemical warfare agents and by aging of ACh esterase, while the therapeutic action of oximes is due to their reactivation of the phosphorylated non-aged ACh esterase. However, several symptoms and effects that these agents produce are not solely explainable by their action on ACh esterase, which suggests that molecular targets in addition to ACh esterase must be involved. Our research and that of others has shown that some of these agents interact directly with, and activate or inhibit nicotinic (n) and muscarinic (m) ACh receptors. The objectives of this contract were to determine how OP, CB, and oxadiazolidinone anticholinesterases and oximes affect n-ACh and m-ACh receptor functions; to determine which mechanisms of interaction are involved; and to correlate these molecular events with the toxic and/or therapeutic effects of these agents. The information obtained will contribute to the understanding of the mechanisms involved in the toxicity of anticholinesterases and could aid development of an effective therapy against poisoning by OP nerve agents.

During the first year of our investigation, the anticholinesterase properties of oxadiazolidinones were studied and the relative affinities of n-receptor and ACh esterase for six CB anticholinesterases were determined. In addition, the ability of the CBs to protect ACh esterase from irreversible inactivation by OP nerve agents and oxadiazolidinones was studied. During the second year, the study of the interaction of CB and OP anticholinesterases with the n-receptor of *Torpedo* electric organ was completed. VX was found to be a potent open-channel blocker and soman a weak partial agonist of n-receptors. The actions of lipophilic (apolar) CBs were compared to those of polar CBs. The data suggested that the apolar CBs methomyl, meobal and bendiocarb would be better therapeutic agents for OP poisoning (as ACh esterase protectants) than would neostigmine, pyridostigmine, or physostigmine. The study of the actions of OPs and CBs on m-receptors in rat brain and in neuroblastoma N1E-115 cells was also completed. A population of m-receptors with very high affinity for the agonist [³H]CD was particularly sensitive to the OP nerve agent VX and to the therapeutic OP echothiophate. Of the six CBs studied, the three polar CBs were the more effective inhibitors of m-receptor function.

During the third year, the effect of the three oximes 2-PAM, HI-6, and Toxogonin on the binding of radioactive ligands and on the functionality of n-receptors of *Torpedo* electric organs and m-receptors of rat brain and neuroblastoma cultures was studied. The results indicated that all oximes react with both n- and m- receptors.

2. BACKGROUND AND LITERATURE REVIEW

The toxicity of anticholinesterases is believed to be due mainly to inhibition of ACh esterase, the result of which is accumulation of ACh in cholinergic synapses, which causes repeated activation of m-receptors and activation, then desensitization, of n-receptors. The n-receptor is an allosteric protein that carries two sites which bind ACh and specific antagonists, as well as other sites that bind less specific antagonists-- most of which induce receptor desensitization. Early studies suggested that anticholinesterases have additional direct effects on n-ACh receptors, either activating or inhibiting them. Examples are tetraethylammonium (1) and m-hydroxyphenyltrimethylammonium (2), acting as agonists on denervated muscles; diisopropylfluorophosphate (DFP), paraoxon, and echothiophate at high concentrations inhibiting ACh receptor-induced depolarization in the electric eel (3), and DFP acting as a channel blocker (4).

The quaternary CB pyridostigmine (6) and the tertiary physostigmine (6, 7) have been shown to provide, via their anticholinesterase actions, some protection against soman poisoning in mammals. Binding of these CBs to n-receptors was demonstrated by their competitive inhibition of specific [^3H]ACh binding to these receptors in the electric organ of the electric ray, *Torpedo* sp., (8), and by the noncompetitive inhibition of the [^{125}I] α -BGT binding to the *Aplysia* ACh receptor (9). Detailed biochemical studies (10) revealed that in fact, pyridostigmine and neostigmine act as partial agonists of the n-receptor, while physostigmine acts as an inhibitor of the receptor in its open channel conformation. Organophosphates have also been found to interact with the n-receptor, as shown by electrophysiologic (11) and biochemical (12, 13) measurements. Albuquerque and coworkers investigated in detail the effect of carbamate and organophosphate anticholinesterases on n-ACh receptors of mammalian skeletal muscles. Their conclusions regarding the partial-agonist-like activity of carbamate (14, 15) and channel-blocking activity of some organophosphates (16) are in full agreement with our conclusions, which are based on biochemical rather than electrophysiological measurements.

Oximes are used therapeutically to treat organophosphate poisoning. These agents reactivate phosphorylated acetylcholinesterase. Successful use of these agents requires the injection of relatively high doses of oximes (5-10 mg/kg) (17). The question was, "What effects do oximes have on ACh receptors during enzyme reactivation?". During the last two years, Albuquerque and coworkers have published detailed studies of the effect of oximes on n-ACh receptors of mammalian skeletal muscles (18, 19). Their conclusion was that 2-PAM may both excite and inhibit the receptor. HI-6 was inhibitory only, whereas the non-oxime bispyridinium compound SAD-128 behaved more like a channel blocker. No investigation of their effects on muscarinic receptors was made.

Chronic administration of an OP anti-ChE has been shown to cause a decrease in n-receptor numbers in rat skeletal muscle (20) and a decrease in m-receptors in selected areas of rat brain, particularly the telencephalon (21). This receptor down-regulation is probably an action of the increased ACh in cholinergic synaptic gaps that results from ACh

esterase inhibition. However, down-regulation could also result from the direct action of OPs on m-receptors-- especially the sub-population that has high affinity for OPs (13). This effect is opposite of what occurs when the animal is exposed chronically to a muscarinic antagonist, such as scopolamine (22). At sublethal doses, OPs can also cause histological lesions and neuronal damage (23).

If the idea of using a CB to protect ACh esterase from inhibition by an OP (6) is to succeed, the CB should have little if any effect on ACh receptors and by itself produce no lesions; unless its direct action on these receptors opposes the effect of high ACh concentrations.

3. RATIONALE USED IN CURRENT STUDY

There are two major classes of ACh receptor: (1) n-ACh receptors, which regulate functions of skeletal muscle, autonomic ganglia, and certain nuclei in the brain, and (2) m-ACh receptors, which regulate the function of all parasympathetically-innervated organs (e.g., heart, smooth muscles of intestines, and blood vessels and glands) and most of the cholinergic function of the brain. These ACh receptors are exposed to oximes when the oximes are used to treat organophosphate poisoning. The current study was organized to find out if oximes present at therapeutic concentrations would react with n- and or m- ACh receptors and, if they did, to determine what effect such interactions are likely to produce.

Nicotinic and m-ACh receptors are detected in cellular and subcellular preparations by their binding of radiolabeled ligands and functional assays. Thus, an agent that bound to a receptor would displace binding of the radioactive ligand, demonstrating binding of the agent to the receptor. It is important to realize that multiple ligands that label different sites on the receptor or different receptor types ought to be used; otherwise, important interactions may be missed. The effect of the agent on receptor function (e.g., receptor-regulated ion transport or synthesis of a second messenger) indicates the effect that the agent is likely to produce after it binds to the receptor. Thus, it was reasoned that the combination of the two biochemical assays (i.e., binding and functional) could supply sufficient information to achieve the goals stated above.

4. EXPERIMENTAL METHODS

4.1. Nicotinic Receptor Binding:

Tissue preparations. Electric organs from *Torpedo nobiliana* (Biofish Associates, Boston, MA), stored at -90°C for less than 3 months, were homogenized in 50 mM Tris-HCl buffer, pH 7.4. The homogenates were centrifuged at $1000 \times g$ for 10 min and the supernatant fraction centrifuged at $30,000 \times g$ for 30 min. The pellets were reconstituted in the same buffer at a ratio of 1 g tissue/ml for [^3H]PCP assays; 10 g tissue/ml of 10 mM Tris-HCl buffer, pH 7.2, for $^{22}\text{Na}^+$ uptake assays; and 1:200 1 mM phosphate buffer, pH 7.4, for [^{125}I] α -BGT assays.

[^3H]PCP Binding. Binding of [^3H]PCP (Specific activity 48 Ci/mmol; New England Nuclear, Boston, MA) was determined as previously reported (24). The membrane preparation (100 μl) was incubated with 2 nM [^3H]PCP in a final volume of 1 ml of 50 mM Tris-HCl buffer, and after 30 sec (unless otherwise stated in this report) filtered over a Whatman GF/B glass-fiber filter (Maidstone, England) presoaked in 0.05% polyethylenimine (Sigma, St. Louis, MO) solution (to reduce nonspecific binding). The sample was washed on the filter with 10 ml of the ice-cold 50 mM Tris-HCl buffer, and the filter was placed in a mini-vial containing 4 ml of toluene-based scintillation fluid. The radioactivity was counted in a scintillation spectrometer after at least 8 hr. Nonspecific binding was determined as that occurring in presence of 5 mM amantadine.

[^{125}I] α -BGT Binding. The method of Kohanski et al. (25) was used. Disposable glass pasteur pipettes were filled with carboxymethyl cellulose (Whatman-52 Microgranular) preswollen and equilibrated in phosphate buffer (1 mM Na_2HPO_4 , 0.01% Triton X-100 (v/v), 0.03% NaN_3 (w/v)), pH 7.2. Membranes containing ≈ 0.5 pmol of receptor sites (previously treated or untreated with the tested compound) were incubated with 5 nM [^{125}I] α -BGT (> 100 Ci/mmol; New England Nuclear) for 40 sec at 23°C , then transferred to the minicolumn and washed through. The eluate containing [^{125}I] α -BGT bound to ACh receptors was collected and counted in an autogamma counter. Nonspecific binding was the amount of binding to membranes that had been exposed for 30 min to 10 μM *Naja* α -neurotoxin or added simultaneously to 1 mM *d*-tubocurarine.

4.2. Nicotinic Receptor Function:

$^{22}\text{Na}^+$ uptake measurement. The modification of the method of Epstein and Racker (26) was used to separate the sequestered from the free $^{22}\text{Na}^+$. Dowex 50 W-X8 resin, 100-200 mesh (BIO-RAD, Richmond, CA), was converted to the Tris form by equilibration in 10 mM Tris-HCl buffer, pH 7.2. One ml of the heavy slurry was packed into a pasteur pipette plugged with glass wool and kept under buffer at 4°C till use. The membrane suspension (≈ 1.5 mg protein in 100 μl) was mixed with 1 μCi $^{22}\text{Na}^+$ in 10 mM Tris-HCl buffer, pH 7.2, containing 10 mM NaCl, 2 mM KCl, and 0.2 mM CaCl_2 (in the absence or presence of 100 μM carbamylcholine) in a final volume of 400 μl . After 20 sec incubation, a 300 μl aliquot was filtered through the mini-column of Dowex resin and washed by 1 ml of 10 mM Tris HCl buffer. The eluate, containing the trapped $^{22}\text{Na}^+$ in the

microsacs, was counted in an autogamma counter. The n-receptor-stimulated influx was calculated as the difference in $^{22}\text{Na}^+$ content of Torpedo microsacs in the presence and absence of carbamylcholine.

4.3. Muscarinic Receptor Binding:

Preparation of rat brain membranes. Brains of adult Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) were rapidly removed after decapitation. Whole brains were homogenized at 4°C by Polytron (Brinkman, Rixdale, Ontario, Canada) in 50 mM Na/K phosphate buffer, pH 7.4, for the [^3H]QNB ([^3H]quinuclidinyl benzilate) experiments or in 5 mM HEPES buffer, pH 7.4, containing 1 mM NEM (N-ethylmaleimide, Sigma) for the [^3H]CD ([^3H]cis-methyldioxolane) experiments. The homogenates were centrifuged for 10 min at $1,000 \times g$, the supernatant centrifuged for 45 min at $40,000 \times g$, and the pellets were suspended in 50 mM Na/K phosphate buffer, pH 7.4 for the [^3H]QNB binding assay. However, for [^3H]CD binding, the pellets were suspended in 5 mM HEPES buffer, pH 7.4 containing 5 mM Ni^{2+} .

[^3H]QNB binding. Membranes ($\approx 100 \mu\text{g}$ of protein assay) were incubated with 0.2 nM [^3H]QNB (35.2 Ci/mmol; New England Nuclear), in a final volume of 1 ml of buffer, as previously described (27). In all assays, incubations were carried out for 90 min at room temperature, at which time equilibrium was attained. The binding reaction was terminated by filtration under vacuum through Whatman GF/B glass-fiber filters presoaked in 0.05% polyethylenimine, using a Cell Harvester (Brandel, Gaithersburg, MD). Filters were washed twice with 5 ml of ice-cold buffer, then each filter was placed into a minivial with 4 ml of toluene-based scintillation solution; then, the radioactivity was counted after at least 8 hr in a Beckman liquid scintillation counter. Nonspecific binding was that measured in the presence of 10 μM atropine.

[^3H]CD binding. Membranes ($\approx 500 \mu\text{g}$ protein per assay) were incubated with 5 nM [^3H]CD (55.5 Ci/mmol; New England Nuclear) in a final volume of 1 ml, using 5 mM HEPES buffer containing 5 mM Ni^{2+} , at $22^{\circ}\text{--}23^{\circ}\text{C}$ for 90 min to reach equilibrium. The binding reaction was terminated by filtration through Whatman GF/B glass-fiber filters presoaked in 0.05% ethylenimine solution, using the Brandel Cell Harvester. The filters were washed twice with 5 ml of buffer, then each was placed in a minivial with 4 ml of the scintillation solution and the radioactivity counted as detailed above. Nonspecific binding was measured in the presence of 10 μM atropine.

[^3H]PZ binding. Membranes from rat brain were prepared as described above for QNB binding. The specific binding of [^3H]PZ (80 Ci/mmol; New England Nuclear) was determined as described by Watson et al. (1983) (28). One hundred μl of brain membranes were incubated in a total volume of 1 ml of 10 mM phosphate buffer, pH 7.4, for 60 min at 23°C . Incubation was terminated by filtration over Whatman GF/B filters. Nonspecific binding was determined in the presence of 10 μM atropine.

4.4. Assay of Muscarinic Receptor Function:

Cell culture conditions:

Mouse neuroblastoma clone N1E-115 cells (passage 14-20) were grown as described by El-Fakahany and Richelson (29) in tissue culture flasks (75 cm²/250 ml; Falcon, Cockeysville) in 20 ml of Dulbecco's modified Eagle's medium (high glucose, no pyruvate) supplemented with 10% (v/v) fetal bovine serum (GIBCO Laboratories, New York). Cells were incubated at 37° in an atmosphere consisting of 10% CO₂ and 90% humidified air. The medium was changed on day 3 and day 5 and everyday thereafter by adding 10 ml of fresh medium and then removing 10 ml of the old. The medium was changed 24 hr before harvesting the cells. In these experiments, the cells were used 14 days after subculture for up to 20 days of age.

Receptor-mediated [³H]cyclic GMP assay. Cyclic GMP was assayed as previously described (29) using intact cells which were dissociated from the wall of the culture flask by incubating in Ca-free medium (138 mM NaCl, 5.4 mM KCl, 0.17 mM Na₂HPO₄, 5.5 mM glucose, and 48 mM sucrose; pH 7.35 and 335-340 mOsm) at 37°C for 10 min. The cells were collected by low-speed centrifugation (1000 rpm for 1 min), then suspended in working buffer made of HEPES, 20 mM; NaCl, 110 mM; KCl, 5.3 mM; CaCl₂, 1.78 mM; MgSO₄, 1 mM; glucose, 24 mM; sucrose, 50 mM-- pH 7.35 and 335-340 mOsm. This buffer (10 ml) was used at first to wash the cells; then, finally, the cells of one flask were suspended in 1 ml of this buffer and placed in a 25-ml Erlenmeyer flask. About 1 μM [³H]guanosine (≈10 μ Ci/ml; New England Nuclear) was added; then, the suspension was oscillated at 37°C for 50 min at 60 rpm using Orbit water bath shaker. The radioactive cell suspension was diluted to contain about 150,000-250,000 cells per assay. Equal aliquots were distributed into wells of a multi-well tray (Bacton Dickson, Oxnard, CA) and kept at 37°C at 80 oscillations/min for 10 min. To test the agonist activity of compounds, such as carbamylcholine, the compound was solubilized in the working buffer and added for only 30 sec; then 30 μl of 50% trichloroacetic acid solution was added, in order to terminate the reaction. Then, an aliquot of known activity of [¹⁴C]c-GMP was added as internal standard. When the effect of an inhibitor was tested, it was solubilized in the above buffer and incubated for 30 min at 37°C prior to the addition of carbamylcholine. The contents of each well were passed through a column (0.4 x 15 cm) of Dowex 50-H⁺ (AG 50 W-X2, 200-400 mesh, Bio-Rad Lab.) which had been washed with the following (in sequence): 1.25 N NaOH, water, 1.7 N HCl, water, and were, finally, equilibrated with 0.1 N HCl. Each well was washed with 0.5 ml of 5% (w/v) TCA; the wash was transferred to its column; and the column was then washed successively with 4.4 ml of 0.1 N HCl (eluate discarded), 1 ml of water (eluate discarded), and, finally, 1.4 ml of water-- which was collected in a microfuge tube. To this eluate, equal volumes (25 μl) of 3.2 M ZnSO₄ were added to 3.2 M Na₂CO₃ to precipitate any residual GDP or GTP. The tubes were then centrifuged in the microfuge and 0.5 ml of the supernatant was transferred to 5 ml of scintillation solution; the radioactivity was counted by an LKB (1218 RACKBETA; Gaithersburg, MD) liquid scintillation counter. All samples were corrected for the recovery of [¹⁴C]GMP, which was usually between 80 and 85%. The counting machine was programmed to

calculate the dpm for each sample for its [^3H] and [^{14}C] content.

4.5. Data Analysis:

Data were analyzed by iterative nonlinear regression analysis using the Equilibrium Binding Analysis (EBDA) computer program on an IBM PC (30). When more than one binding affinity was apparent, the best fits of the data to receptor models, consisting of one, two, or more binding sites, were compared, using the LIGAND program (31) adapted for use on the IBM PC.

5. RESULTS

Three oximes (2-PAM, HI-6, and Toxogonin) were studied for their ability to bind to and affect the function of nicotinic (n) and muscarinic (m) receptors. Evidence for binding to these cholinergic receptors was obtained from inhibition (and occasionally potentiation) of binding of radioactive ligands that bind to specific sites on the receptors. Analysis of the binding kinetics in the presence and absence of oximes is necessary, in some cases, to obtain information on the mechanism of interaction. The effects of oximes on the ability of n-ACh receptors to transport $^{22}\text{Na}^+$ and on the ability of m-ACh receptors to induce synthesis of c-GMP were studied to determine the results of oxime binding on receptor function.

5.1. Effects of Oximes on n-ACh Receptor Binding:

n-ACh receptor agonists and antagonists, which bind to the recognition site, reduce the initial rate of binding of [^{125}I] α -BGT to *Torpedo* receptors. Furthermore, receptor agonists, which induce receptor desensitization by prolonged exposure, stabilize the desensitized state of the receptor, which has an increased affinity for agonists. This is expressed in a leftward shift in the displacement curve of inhibition of [^{125}I] α -BGT binding by agonists following preincubation with the agonist.

HI-6 and Toxogonin inhibited the initial rate of binding of [^{125}I] α -BGT in a dose-dependent manner (Fig. 1). Preincubation of *Torpedo* membranes with these two oximes resulted in very little change compared to their simultaneous addition with [^{125}I] α -BGT. It suggested that HI-6 and Toxogonin were probably acting as competitive antagonists. On the other hand, 2-PAM, which was the least effective of the three oximes in inhibiting binding of [^{125}I] α -BGT, becomes a much more effective inhibitor if it is preincubated with the *Torpedo* membranes prior to the binding assay (Fig. 1). The significant increase in effectiveness of 2-PAM in displacing [^{125}I] α -BGT under preincubation conditions suggests that 2-PAM may act primarily as an agonist.

For further classification of the action of oximes on n-ACh receptors, their effects on the binding of the channel probe ([^3H]PCP) to activated and resting n-ACh receptor was studied. The binding of [^3H]PCP to activated n-ACh receptors is inhibited by competitive and noncompetitive receptor antagonists. Full agonists do not inhibit the binding, but partial agonists, at high concentration, do inhibit binding. Furthermore, preincubation of n-ACh receptor with agonists results in receptor desensitization, which reduces binding of [^3H]PCP. Receptor antagonists do not have such an effect.

HI-6 and Toxogonin inhibited [^3H]PCP binding to activated n-ACh receptor in a dose-dependent manner in the concentration range of 10 μM -1 mM (Fig. 2). Preincubation of the tissue with the oximes for 1 hr prior to binding measurement had no significant effect. However, 2-PAM, which produced a slight reduction at 1 mM, became quite effective in inhibiting [^3H]PCP binding after preincubation (Fig. 2). These results support the conclusion that all three oximes bind to the n-ACh

receptor, but, while 2-PAM may act as a partial agonist, HI-6 and Toxogonin appear to act mainly as competitive antagonists. The effect of the three oximes on binding of [3 H]PCP to resting n-ACh receptors was not conclusive (Fig. 3). Usually, agonists increase the initial rate of [3 H]PCP binding to the resting receptor by several orders of magnitude two to three while antagonists increase it only a little (< one order of magnitude). Thus, though 2-PAM enhanced binding more than the other two oximes did, it did not behave quite like a full agonist.

5.2. Effects of Oximes on n-ACh Receptor Function:

Receptor-regulated $^{22}\text{Na}^+$ uptake into *Torpedo* microsacs is an established functional assay used to distinguish between receptor agonists (which stimulate uptake) and antagonists (which do not stimulate uptake and inhibit agonist-induced uptake). HI-6, Toxogonin, and 2-PAM failed to stimulate $^{22}\text{Na}^+$ uptake into *Torpedo* microsacs when tested at concentrations of 10^{-6} to 10^{-3} M. On the other hand, all three oximes inhibited the $^{22}\text{Na}^+$ uptake induced by 100 μM carbamylcholine in a dose-dependent manner (Fig. 4). The concentrations that inhibited 50% of uptake were 3×10^{-4} M for HI-6 and 10^{-3} M for both Toxogonin and 2-PAM. Thus, 2-PAM, HI-6, and Toxogonin behaved as antagonists of the n-ACh receptor.

5.3. Binding of Oximes to m-ACh Receptors:

In human brain, muscarinic ACh receptors are classified into five subtypes (M_1 - M_5), depending on their selectivity to agonists and antagonists. All subtypes of m-ACh receptors bind [3 H]QNB with similar high affinity. M_1 binds [3 H]PZ with high affinity. The brain also has a small population of m-ACh receptors (probably M_2 type) which bind [3 H]CD with high affinity. The affinity of m-ACh receptors for the three oximes 2-PAM, Toxogonin, and HI-6 was determined from the effect of the oximes on the binding of radioactive ligands to rat brain m-ACh receptors.

5.3-1. Effects of oximes on [3 H]QNB binding. 2-PAM, HI-6, and Toxogonin displaced the binding of [3 H]QNB in a dose-dependent manner in the concentration range of 10^{-6} - 10^{-3} M (Fig. 5). The IC_{50} values for Toxogonin, 2-PAM, and HI-6 were 20, 60, and 200 μM , respectively. Scatchard plots of [3 H]QNB equilibrium binding in the absence and presence of the oximes showed that the oximes behaved competitively (Fig. 6). In other words, they bound to the same site as QNB. The calculated K_i values, obtained from the Scatchard plots, were 10 μM for Toxogonin, 69 μM for 2-PAM, and 96 μM for HI-6.

5.3-2. Effects of oximes on [3 H]PZ binding. The three oximes displaced the binding of [3 H]PZ in a dose-dependent manner, as expected (Fig. 7). Toxogonin was the most potent ($\text{IC}_{50} = 0.3 \mu\text{M}$), while HI-6 and 2-PAM were less potent (IC_{50} values were 10 and 15 μM , respectively). Scatchard plots of [3 H]PZ binding in the absence and presence of oximes indicated that displacement was competitive (Fig. 8). The calculated K_i values for Toxogonin, HI-6, and 2-PAM were 0.24 μM , 3.4 μM and 6.5 μM , respectively.

5.3-3. Effects of oximes on [³H]CD binding. The three oximes 2-PAM, Toxogonin, and HI-6 inhibited the binding of [³H]CD with IC₅₀ values of 30, 10, and 100 μM, respectively (Fig. 9). Scatchard plots of [³H]CD in the absence and presence of the oximes indicated that the oximes displaced [³H]CD binding noncompetitively (Fig. 10).

5.4. Effects of Oximes on m-ACh Receptor Function:

Initial screening indicated that at 1 mM, 2-PAM inhibited 40%, HI-6 inhibited 55%, and Toxogonin 93% of the carbamylcholine-stimulated c-GMP synthesis in neuroblastoma culture. Titration of the carbamylcholine-stimulated c-GMP synthesis by HI-6 and Toxogonin resulted in a concentration-dependent inhibition, with IC₅₀ values of 30 μM for Toxogonin and 500 μM for HI-6 (Fig. 11). The rightward shift of the carbamylcholine-stimulated c-GMP synthesis curve in the presence of 10 μM Toxogonin (Fig. 12) suggests that the oxime acts as a competitive antagonist of m-ACh receptors of the neuroblastoma cells.

6. DISCUSSIONS AND CONCLUSIONS

All three oximes bound to n- and m-ACh receptors, and affected their functions.

6.1. Effects on n-ACh Receptors:

The oximes bound to n-ACh receptors in the concentration range of 10 μM to 1 mM. They competed with and carbamylcholine for binding to the receptor's recognition site. The data suggest that both HI-6 and Toxogonin acted primarily as competitive antagonists of the n-ACh receptor. 2-PAM, on the other hand, appeared to act as a weak partial agonist.

These direct effects of oximes on n-ACh receptors ought to be beneficial from a therapeutic point of view. When given therapeutically, the oximes usually face a situation of excessive stimulation of n-ACh receptors as a result of irreversible inhibition of acetylcholinesterase by the organophosphate nerve agent. Therefore, if they are able to occupy the receptors and inhibit ACh action on those receptors until enough enzyme activity is recovered, their therapeutic action will be improved.

One wonders whether the effective concentrations observed in this study are physiologically relevant for the therapeutic actions of oximes. 2-PAM is administered at 10 $\mu\text{g/kg}$, which translates into a blood concentration of between 500 μM and 1 mM for a 70-kg man. Toxogonin, which is known to be a more potent enzyme activator than 2-PAM, is recommended at ≈ 5 mg/kg. In other words, Toxogonin will establish a peak blood concentration at somewhere between 200 and 500 μM . This means that, at peak concentration, the action of these two oximes on n-ACh receptors will contribute significantly to their therapeutic action.

6.2. Effects on m-ACh Receptors:

The three oximes bound to m-ACh receptors at concentrations well within the expected physiological concentrations needed to treat organophosphate poisoning. M_1 receptors, which are predominant in brain and autonomic ganglia, had the highest affinity for oximes. The M_1 and M_3 receptors of rat brain, which were labeled with [^3H]PZ, represent at least 50% of all m-ACh receptors of rat brain, which are labeled with [^3H]QNB. The affinity of M_1 receptors were highest for Toxogonin ($K_i = 0.24$ μM) and lowest for 2-PAM ($K_i = 6.5$ μM). These high affinities suggest that at physiological concentrations, the oximes are present at one to two orders of magnitude higher than the K_i values. In other words, occupancy of M_1 and M_3 subtypes of m-ACh receptors by oximes during oxime therapy is expected to be high. On the other hand, the M_2 subtype-- which is nearly the only m-ACh receptor in cardiac muscles, and is labeled specifically by [^3H]CD-- had much lower affinity for the oximes. Toxogonin was the most effective oxime in inhibiting [^3H]CD binding, (IC_{50} of 30 μM) compared to 2-PAM and HI-6 with IC_{50} values of 30 and 100 μM , respectively. These three oximes are charged and would not reach the brain.

These data suggest that 2-PAM may not have much of an effect on cardiac function, but will affect ganglionic function. Toxogonin, on the other hand, is expected to have a strong effect on cardiac and autonomic ganglia function at the physiological concentration established after a dose of 5 mg/kg.

ILLUSTRATIONS

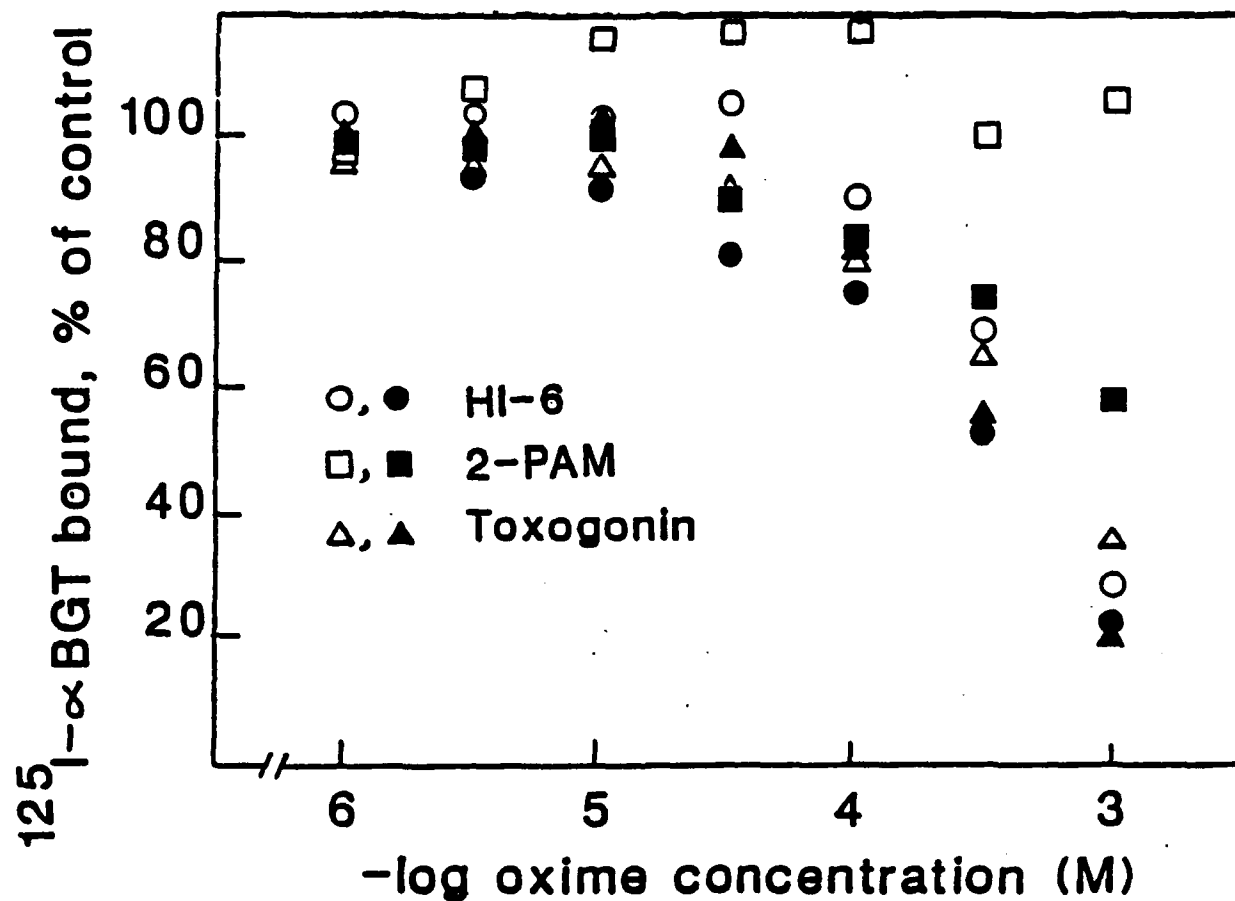


Fig. 1. Concentration-dependent inhibition of the initial rate of binding (40 sec) of [125 I] α -BGT to Torpedo ACh receptors by three oximes. HI-6 (O), Toxogonin (Δ) and 2-PAM ($\square$) were added simultaneously with the tissue and α -BGT and coincubated for 40 sec. HI-6 (●), Toxogonin ($\blacktriangle$), and 2-PAM ($\blacksquare$) were preincubated with the Torpedo membranes prior to measuring binding. Symbols are means of nine measurements and standard deviations were <10%.

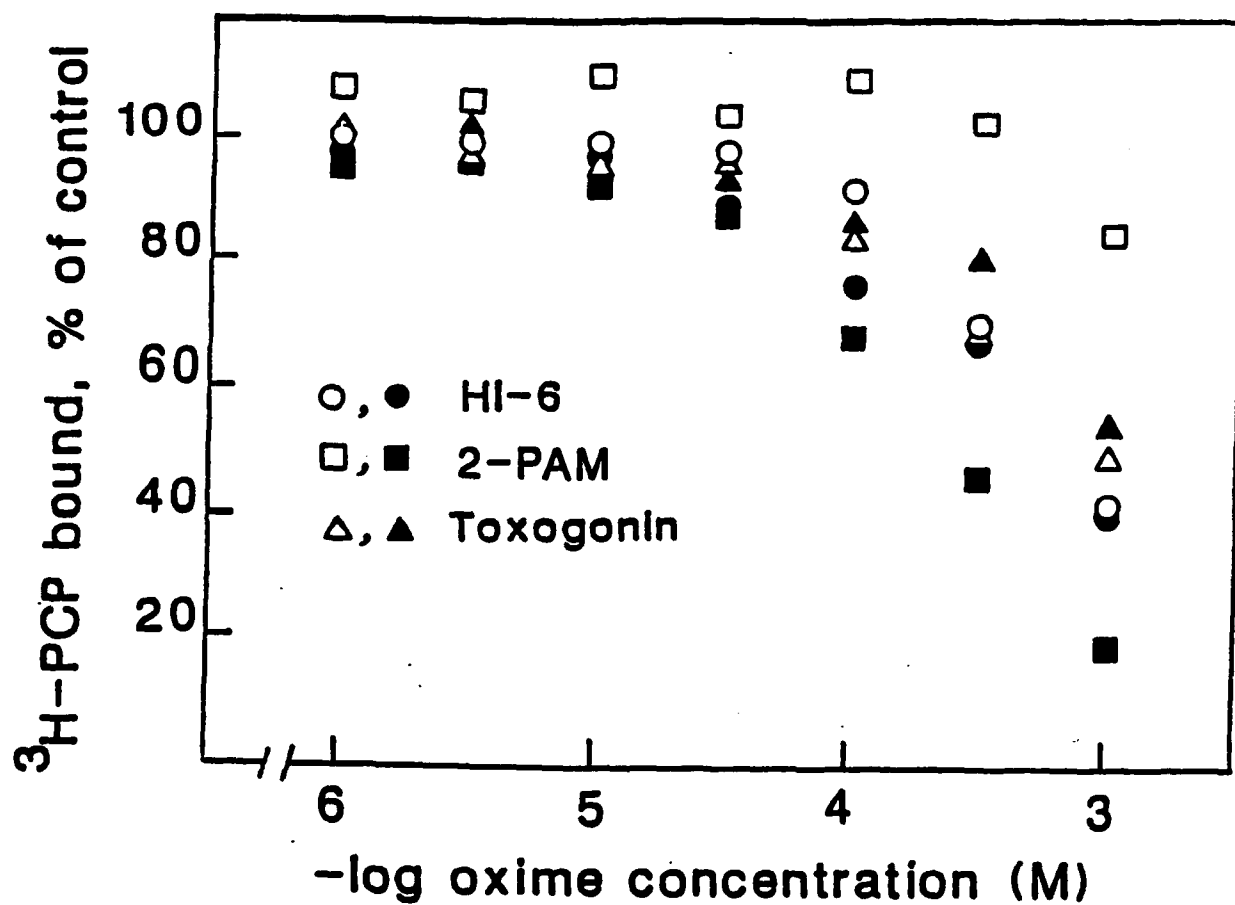


Fig. 2. Concentration-dependent inhibition of the initial rate of binding of [3 H]PCP to activated *Torpedo* ACh receptors (i.e., binding in presence of 100 μ M carbamylcholine) by oximes. HI-6 ($\circ$), Toxogonin ($\triangle$) and 2-PAM ($\square$) were coincubated for 30 sec. HI-6 ($\bullet$), Toxogonin ($\blacktriangle$) and 2-PAM ($\blacksquare$) were preincubated with the membrane for 1 hr prior to binding assay. Symbols are means of nine measurements and standard deviations were <5%.

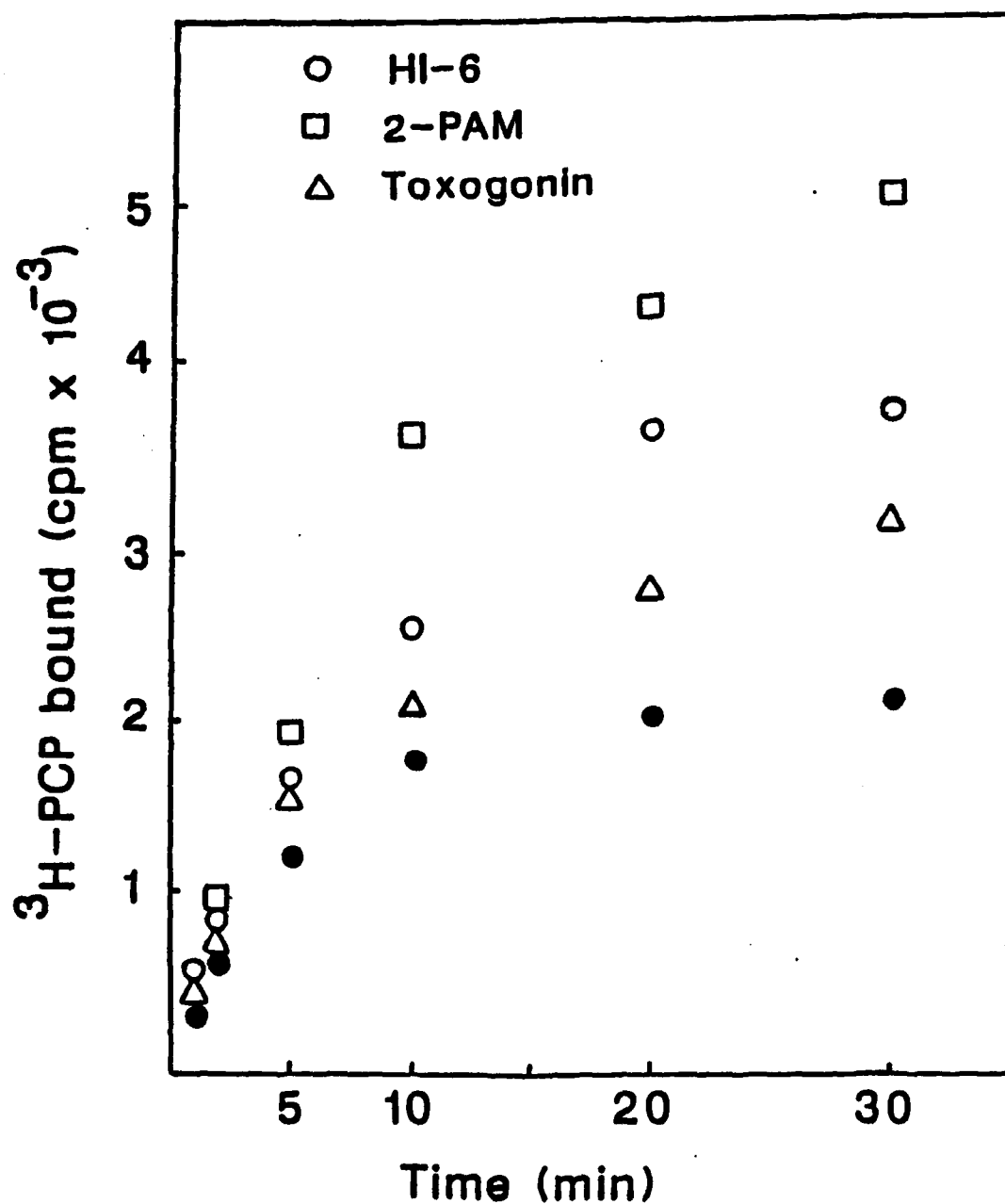


Fig. 3. Time-dependent binding of [^3H]PCP to resting *Torpedo* ACh receptors in absence (●) and presence of 10^{-4} M of HI-6 (○), Toxogonin (△), and 2-PAM (□). Symbols are means of six measurements and standard errors were <7%.

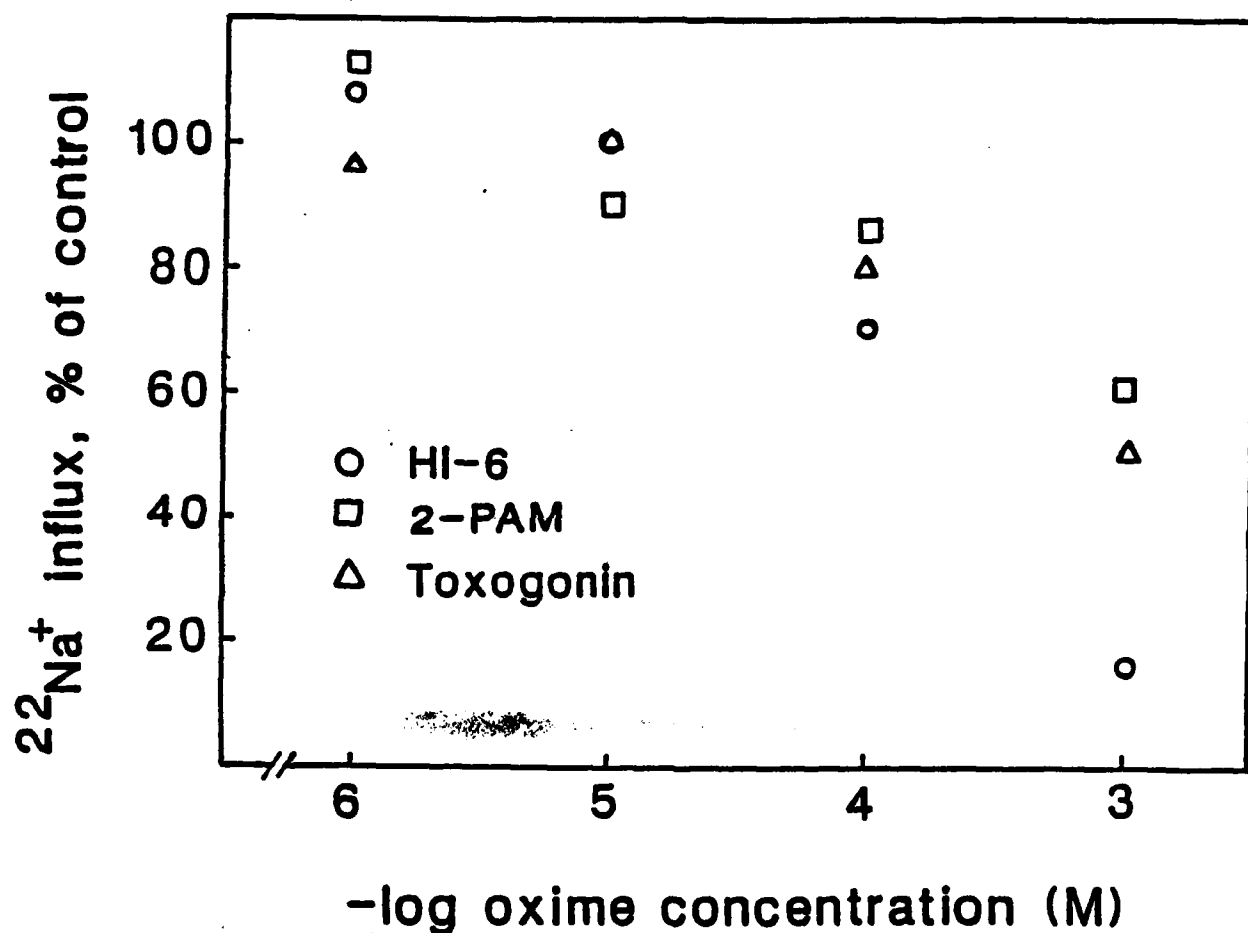


Fig. 4. Inhibition of n-ACh receptor-regulated ^{22}Na uptake into *Torpedo* microsacs induced by 100 μM carbamylcholine in presence of HI-6 (○), Toxogonin (△) and 2-PAM (□). Symbols are means of six measurements and standard deviations were <12%.

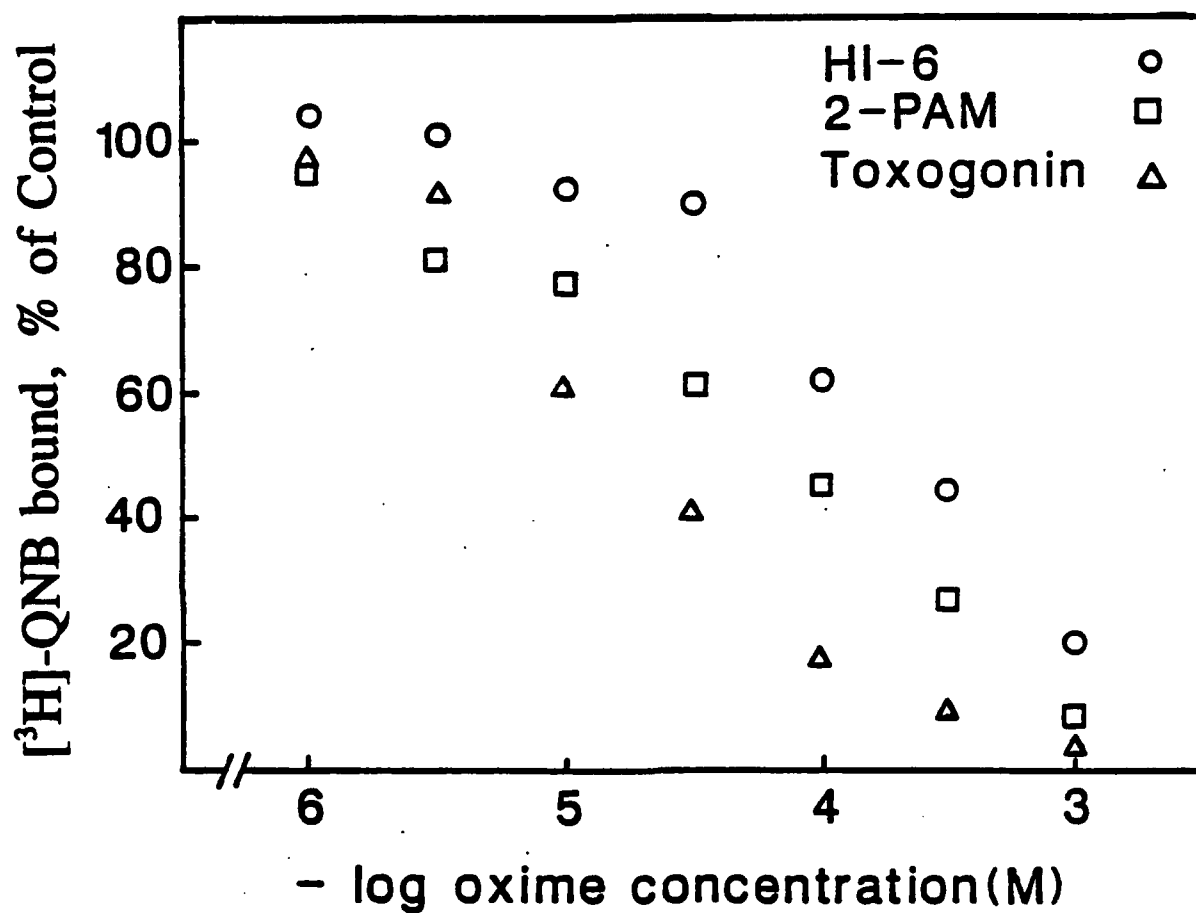


Fig. 5. Concentration-dependent inhibition of [3 H]QNB binding to rat brain muscarinic receptors by the oximes HI-6 (○), 2-PAM (□), and Toxogonin (△). Oximes and [3 H]QNB (0.2 nM) were coincubated with the tissue for 60 min in 1 ml of 50 mM phosphate buffer, pH 7.4. Symbols represent means of three experiments, each having triplicate measurements. Standard deviations were <6%.

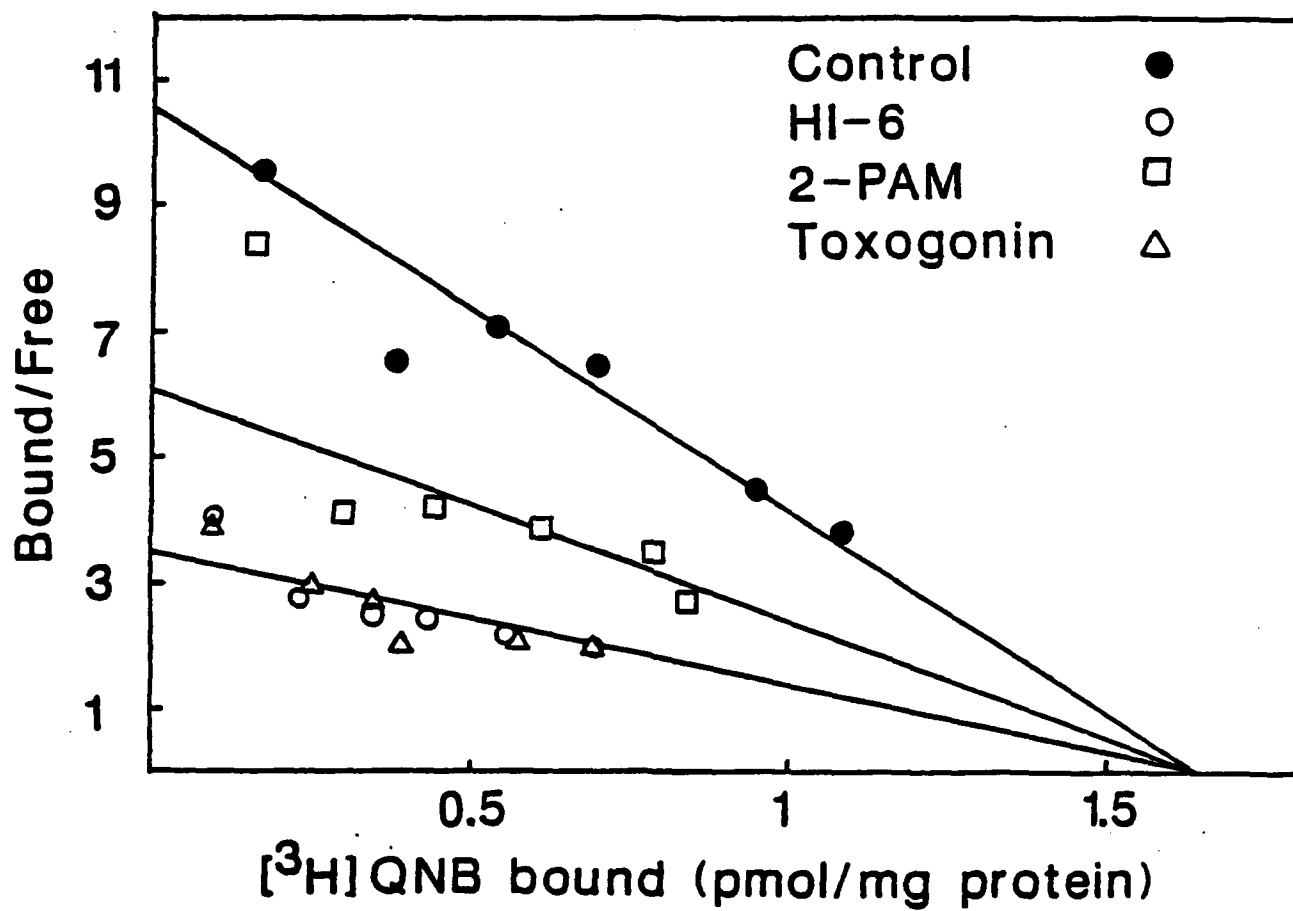


Fig. 6. Scatchard plots of the binding of $[^3\text{H}]\text{QNB}$ to rat brain muscarinic receptors in absence of oximes (●) and in presence of 200 μM HI-6 (○), 60 μM 2-PAM (□), 20 μM Toxogonin (△).

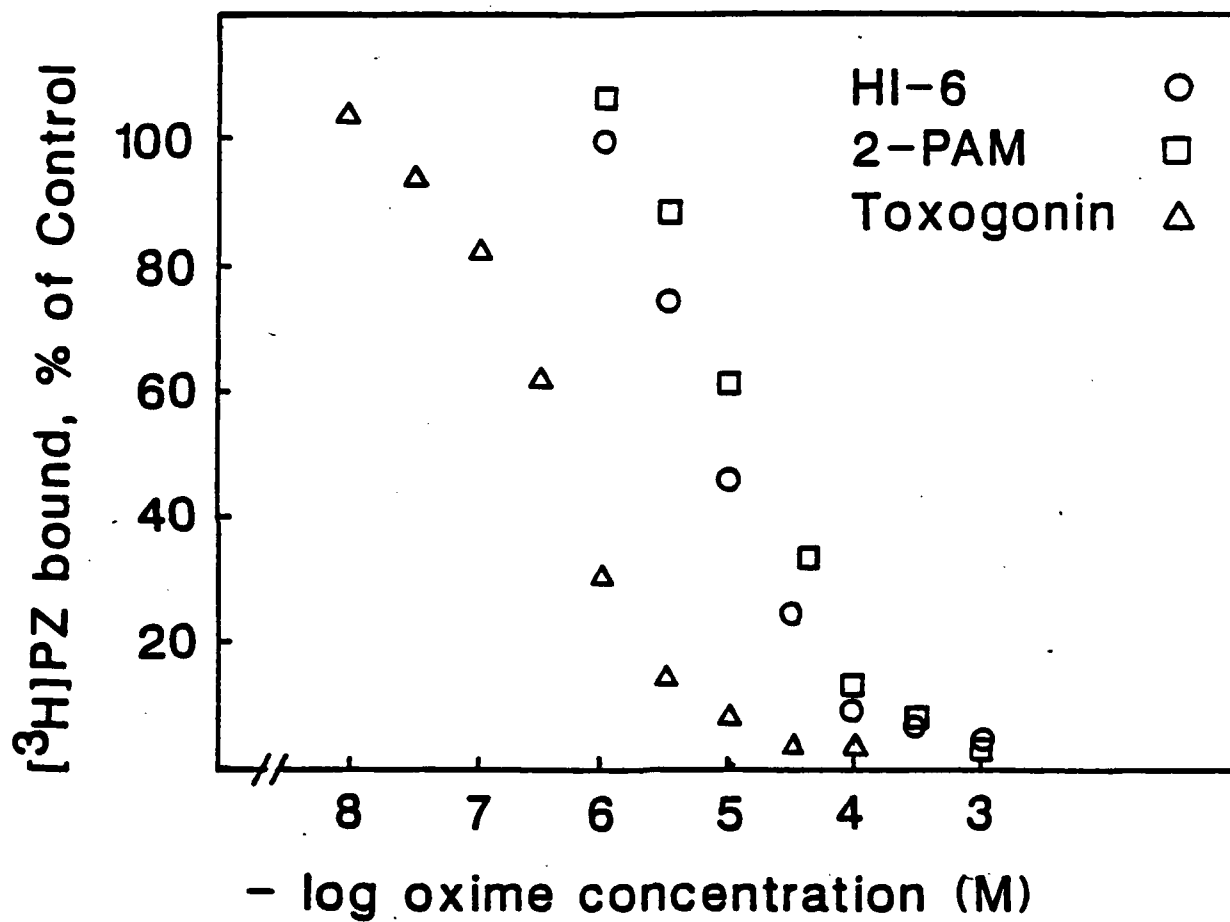


Fig. 7. Concentration-dependent inhibition of [3 H]PZ binding to rat brain muscarinic receptors by the oximes HI-6 (○), 2-PAM (□), and Toxogonin (△). Details are as described in legend to Fig. 5.

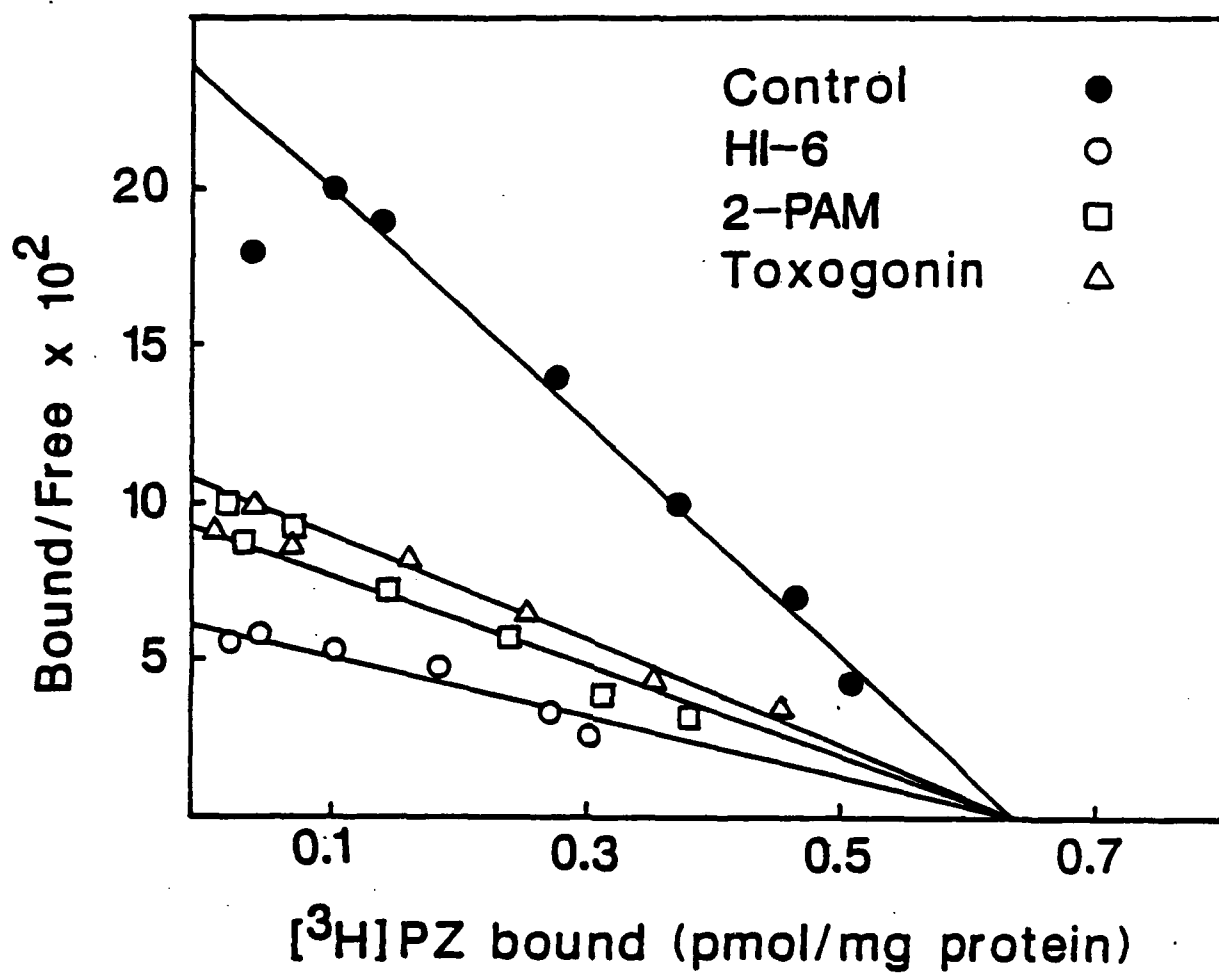


Fig. 8. Scatchard plots of the binding of [³H]PZ to rat brain muscarinic receptors in the absence of oximes (●) and in the presence of 10 μ M HI-6 (○), 15 μ M 2-PAM (□), and 2.3 μ M Toxogonin (△).

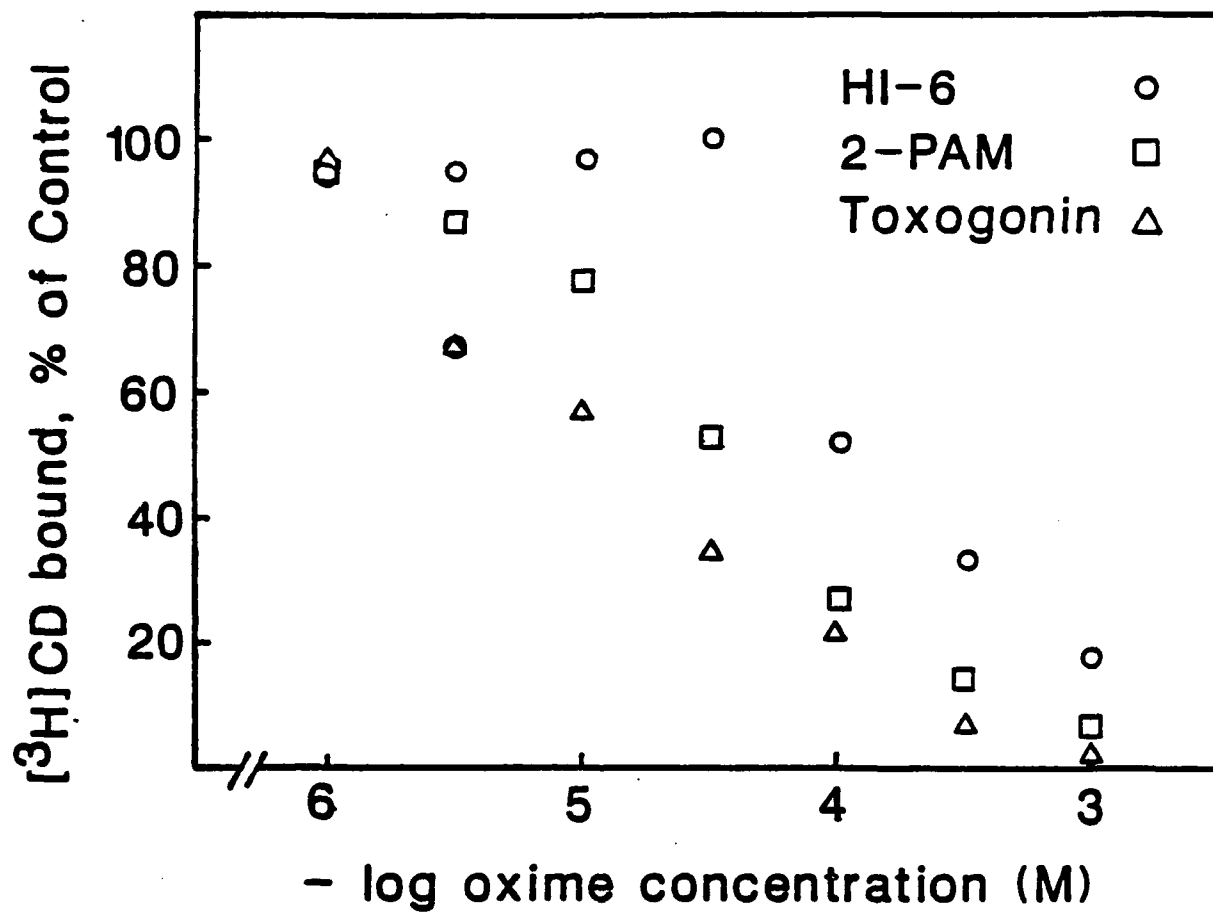


Fig. 9. Concentration-dependent inhibition of [3 H]CD binding to rat brain muscarinic receptors by the oximes HI-6 (○), 2-PAM (□), and Toxogonin (△). Details are as described in legend to Fig. 5.

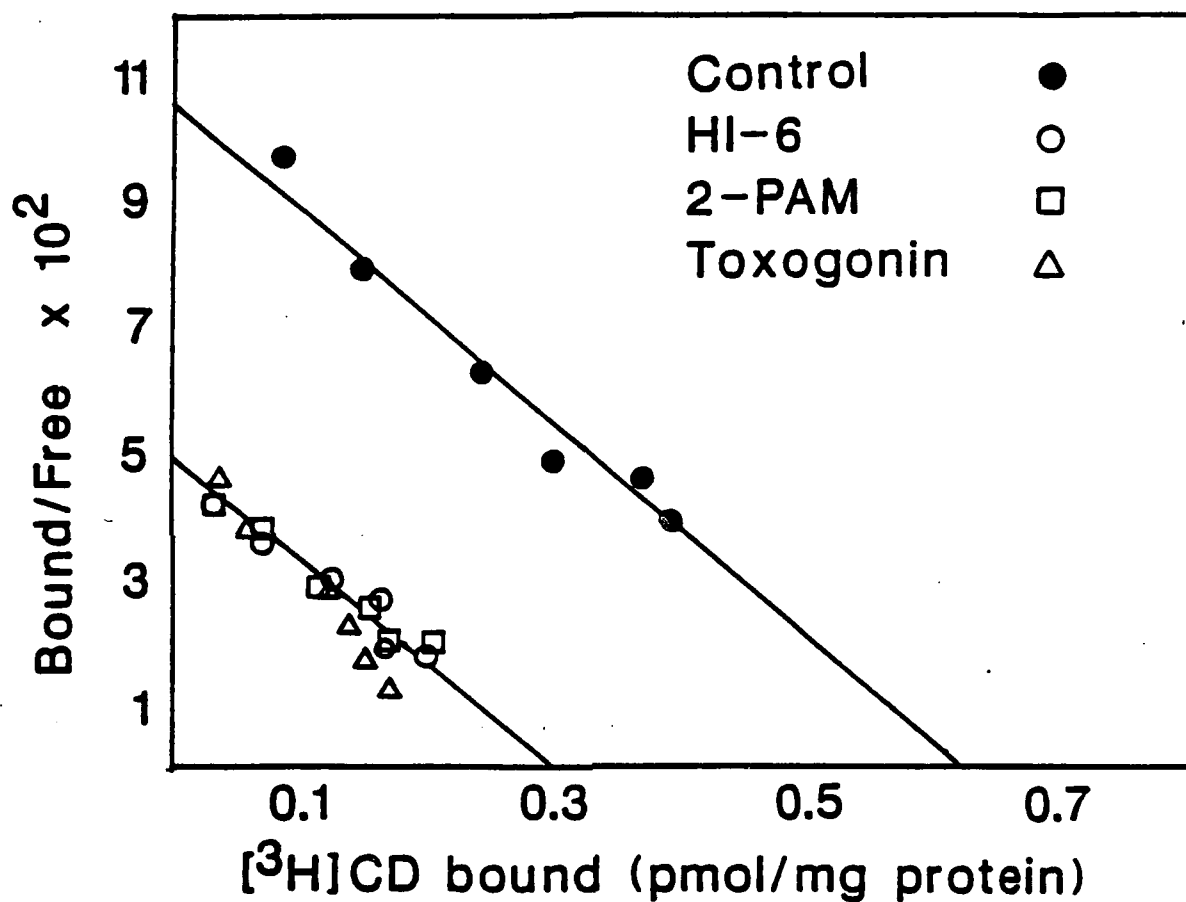


Fig. 10. Scatchard plots of the binding of $[^3\text{H}]\text{CD}$ to rat brain muscarinic receptors in the absence of oximes ($\bullet$) and in the presence of $100 \mu\text{M}$ HI-6 ($\circ$), $30 \mu\text{M}$ 2-PAM ($\square$), and $10 \mu\text{M}$ Toxogonin ($\triangle$).

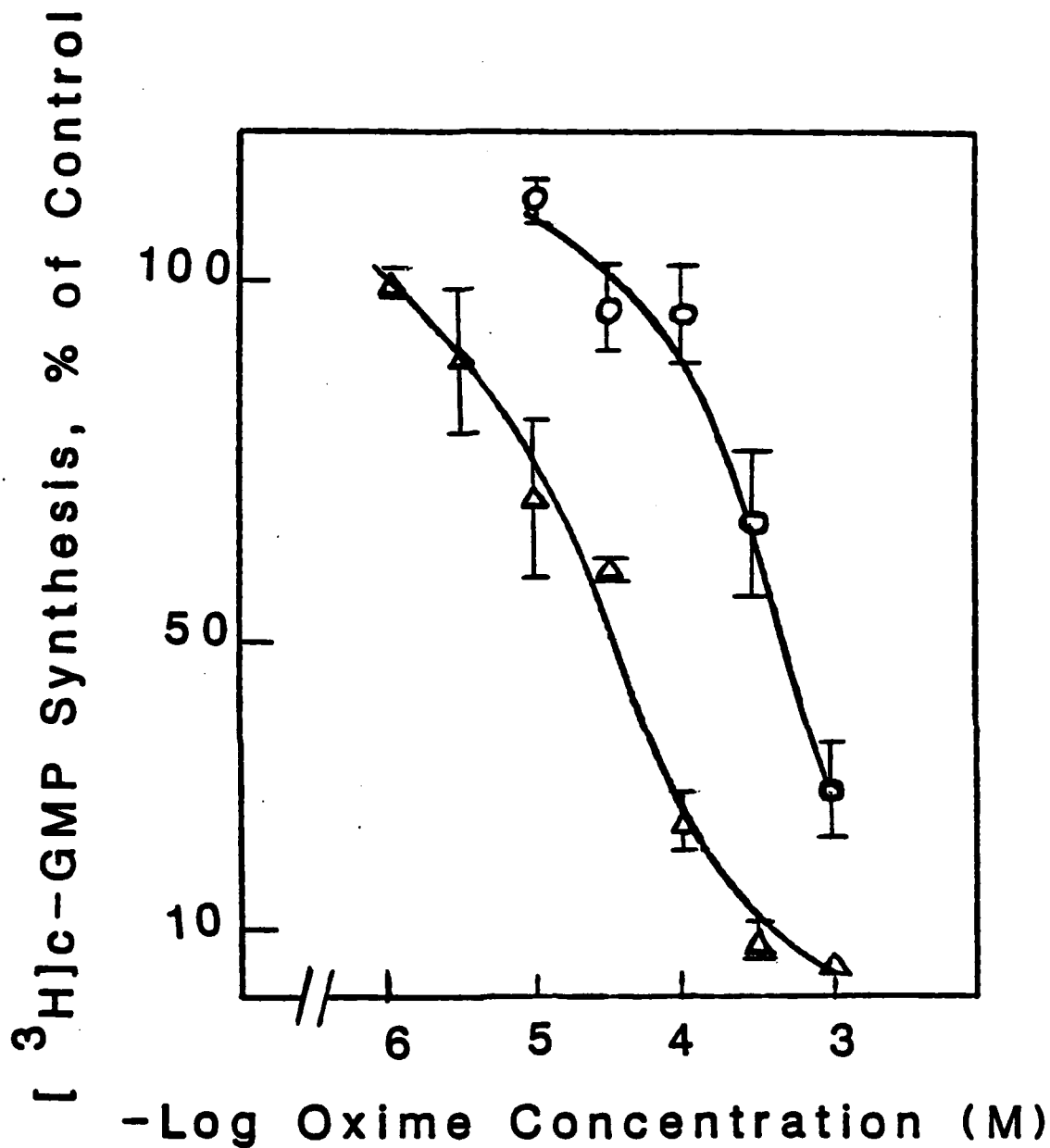


Fig. 11. Concentration-dependent inhibition of carbamylcholine-induced synthesis of $[^3\text{H}]c\text{-GMP}$ in neuroblastoma cells by Toxogonin (Δ) and HI-6 ($\circ$). Symbols are means of two experiments, each performed in triplicate ($N = 6$); bars represent $\pm$ SD.

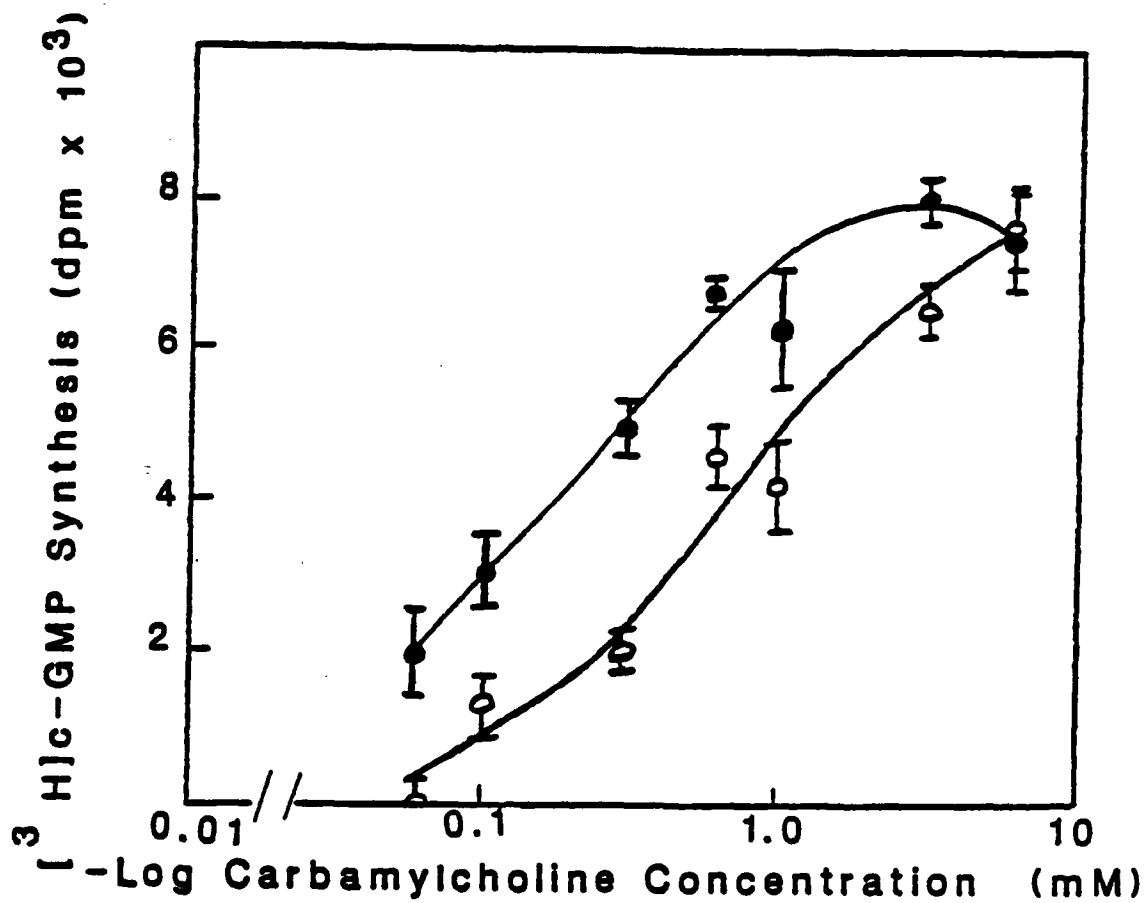


Fig. 12.

Concentration-dependent synthesis of [³H]c-GMP in neuroblastoma cells by carbamylcholine in absence (●) and presence (○) of 10 μM Toxogonin. The cells were preincubated with Toxogonin for 30 min prior to addition of carbamylcholine. Symbols are means of two experiments; bars represent ±SD.

7. LITERATURE CITED

1. Dale, H.H. and H.S. Gasser. The pharmacology of denervated mammalian muscle. I. The nature of the substances producing contracture. *J. Pharmacol. Exp. Ther.* 29:53-67 (1926).
2. Riker, W.F., Jr., and W.E. Wesco. Studies on the inter-relationship of certain cholinergic compounds. V. the significance of the actions of the 3-hydroxyphenyltrimethylammonium ion on neuromuscular junction. *J. Pharmacol. Exp. Ther.* 100:454-464 (1950).
3. Bartels, E. and D. Nachmansohn. Organophosphate inhibitors of acetylcholine-receptor and -esterase tested on the electroplax. *Arch. Biochem. Biophys.* 133:1-10 (1969).
4. Kuba, K., E.X. Albuquerque, J. Daly and E.A. Barnard. Study of the irreversible cholinesterase inhibitor, diisopropylfluorophosphate, on time course of end-plate currents in frog sartorius muscle. *J. Pharmacol. Exp. Ther.* 189:499-512 (1974).
5. Howard, J.F. and D.B. Sanders. The management of patients with myasthenia gravis, in *Myasthenia Gravis* (E.X. Albuquerque and A.T. Eldefrawi, eds.), Chapman and Hall, London, 457-489 (1983).
6. Koplovitz, I., D.E. Jones, D.E. Hilmas, L.W. Harris and C.J. Canfield. Efficacy of oral pyridostigmine against soman poisoning in mice. *Fed. Proc.* 43:565 (1984).
7. Deshpande, S.S., G.B. Viana, F.C. Kauffman, D.L. Rickett and E.X. Albuquerque. Effectiveness of physostigmine as a pretreatment drug for protection of rats from organophosphate poisoning. *Fund. Appl. Tox.* 6:566-77 (1986).
8. Seifert, S.A. and M.E. Eldefrawi. Affinity of myasthenia drugs to acetylcholinesterase and acetylcholine receptor. *Biochem. Med.* 10:258-265 (1974).
9. Carpenter, D.O., L.A. Greene, W. Shain and Z. Vogel. Effects of eserine and neostigmine on the interaction of α -bungarotoxin with *Aplysia* acetylcholine receptors. *Mol. Pharmacol.* 12:999-1006 (1976).
10. Sherby, S.M., A.T. Eldefrawi, E.X. Albuquerque and M.E. Eldefrawi. Comparison of the actions of carbamate anticholinesterases on the nicotinic acetylcholine receptor. *Mol. Pharmacol.* 27:343-348 (1985).
11. Albuquerque, E.X., S.S. Deshpande, M. Kawabuchi, Y. Aracava, M. Idriss, D.L. Rickett and A.F. Boyne. Multiple actions of anticholinesterase agents on chemosensitive synapses: Molecular basis for prophylaxis and treatment of organophosphate poisoning. *Fund. Appl. Toxicol.* 5:5182-5203 (1985).
12. Eldefrawi, M.E., J.J. Valdes and G. Schweizer. Interaction of organophosphate nerve agents with the nicotinic acetylcholine receptor. *Fifth Annu. Chem. Defense Biosc. Rev.* (1985).
13. Bakry, N.M.S., A.H. El-Rashidy, A.T. Eldefrawi, and M.E. Eldefrawi. Direct actions of organophosphate anticholinesterases on nicotinic and muscarinic acetylcholine receptors. *J. Biochem. Toxicol.* 3:235-259 (1988).
14. Pascuzzo, G.J., A. Akaike, M.A. Maleque, K.P. Shaw, R.S. Aronstam, D.L. Rickett, and E.X. Albuquerque. The nature of the interactions of pyridostigmine with the nicotinic acetylcholine receptor-ionic channel complex. *Mol. Pharmacol.* 25:92-101 (1984).
15. Shaw, K.P., Y. Arcava, A. Akaike, J.W. Daly, D.L. Rickett and E.X.

- Albuquerque. The reversible cholinesterase inhibitor physostigmine has channel-blocking and agonist effects on the acetylcholine receptor-ion channel complex. *Mol. Pharmacol.* 28:527-538 (1985).
16. Rao, K.S., Y. Arcava, D.L. Rickett and E.X. Albuquerque. Noncompetitive blockade of the nicotinic acetylcholine receptor-ion channel complex by an irreversible cholinesterase inhibitor. *J. Pharmacol. Exp. Ther.* 240:337-344 (1987).
 17. Gilman, A.G., L.S. Goodman, T.W. Rall and F. Murad. "The Pharmacological Basis of Therapeutics" 7th ed. MacMillan Publishing Co. N.Y. pp. 1839 (1985).
 18. Alkondon, M., K.S. Rao and E.X. Albuquerque. Acetylcholinesterases reactivators modify the functional properties of the nicotinic acetylcholine receptor ion channel. *J. Pharmacol. Exp. Ther.* 245:543-556 (1988).
 19. Alkondon, M. and E.X. Albuquerque. The nonoxime biophyrodinium compound 8AD-128 alters the kinetic properties of the nicotinic acetylcholine receptor ion channel: Possible mechanism for antidotal effects. *J. Pharmacol. Exp. Ther.* 250:842-852 (1989).
 20. Gupta, R.C., G.T. Patterson and W.-D. Dettbarn. Mechanisms of toxicity and tolerance to diisoprophylphosphorofluoridate at the neuromuscular junction of the rat. *Tox. Appl. Pharm.* 84:541-550 (1986).
 21. Chruchill, L., T.L. Pazdernik, J.L. Jackson, S.R. Nelson, F.E. Samson and J.H. McDonough, Jr. Topographical distribution of decrements and recovery in muscarinic receptors from rat brains repeatedly exposed to sublethal doses of soman. *Soc. Neurosci.* 4:2069-2079 (1984).
 22. Majocha, R. and R.J. Baldessarini. Tolerance to an anticholinergic agent is paralleled by increased binding to muscarinic receptors in rat brain and increased behavioral response to a centrally active cholinomimetic. *Life Sci.* 35:2247-2255 (1984).
 23. Churchill, L., T.L. Pazdernik, J.L. Jackson, S.R. Nelson, F.E. Samson, J.H. McDonough, Jr. and C.G. McLeod, Jr. Soman-induced brain lesions demonstrated by muscarinic receptor autoradiography. *Neurotoxicol.* 6:81-90 (1985).
 24. Eldefrawi, A.T., E.R. Miller, D.L. Murphy and M.E. Eldefrawi. [³H]Phencyclidine interactions with the nicotinic acetylcholine receptor channel and its inhibition by psychotropic, antipsychotic, opiate, antidepressant, antibiotic, antiviral, and antiarrhythmic drugs. *Molec. Pharmacol.* 22:72-81 (1982).
 25. Kohanski, R.A., J.P. Andrews, P. Wins, M.E. Eldefrawi and G.P. Hess. A simple quantitative assay of ¹²⁵I-labeled α -bungarotoxin binding to soluble and membrane-bound acetylcholine receptor protein. *Analyt. Biochem.* 80:531-539 (1977).
 26. Epstein, M. and E. Racker. Reconstitution of carbamylcholine-dependent sodium ion flux and desensitization of the acetylcholine receptor from *Torpedo californica*. *J. Biol. Chem.* 253:6660-6662 (1978).
 27. Ikeda, S.R., R.S. Aronstam and M.E. Eldefrawi. Nature of regional and chemically-induced differences in the binding properties of muscarinic acetylcholine receptors from rat brain. *Neuropharmacol.* 19:575-585 (1980).
 28. Watson, M., H.I. Yamamura and W.R. Roeske. A unique regulatory profile and regional distribution of [³H]pirenzepine binding in the

- rat provide evidence for distinct M_1 and M_2 muscarinic receptor subtypes. *Life Sciences* 32:3001-3011 (1983).
29. El-Fakahany, E. and E. Richelson. Temperature dependence of muscarinic acetylcholine receptor activation, desensitization, and resensitization. *J. Neurochem.* 34:1288-1295 (1980).
 30. McPherson, G.A. A practical computer-based approach to the analysis of radioligand binding experiments. *Computer Prog. Biomed.* 17:107-114 (1983).
 31. Munson, P.J. and D. Rodbard. LIGAND: A versatile computerized approach for characterization of ligand-binding systems. *Anal. Biochem.* 107:220-239 (1980).

DISTRIBUTION LIST

1 copy	Commander US Army Medical Research and Development Command ATTN: SGRD-RMI-S Fort Detrick, Frederick, Maryland 21701-5012
5 copies	Commander US Army Medical Research and Development Command ATTN: SGRD-PLF Fort Detrick, Frederick, Maryland 21701-5012
2 copies	Defense Technical Information Center (DTIC) ATTN: DTIC-DDAC Cameron Station Alexandria, VA 22304-6145
1 copy	Dean School of Medicine Uniformed Services University of the Health Sciences 4301 Jones Bridge Road Bethesda, MD 20814-4799
1 copy	Commandant Academy of Health Sciences, U.S. Army ATTN: AHS-CDM Fort Sam Houston, TX 78234-6100