

RD-A148 222

REVERSAL OF ACETYLCHOLINESTERASE INHIBITOR TOXICITY IN
VIVO BY INHIBITORS. (U) SOUTH CAROLINA UNIV COLUMBIA
DEPT OF BASIC PHARMACEUTICAL SCI... J J FREEMAN ET AL.

1/1

UNCLASSIFIED

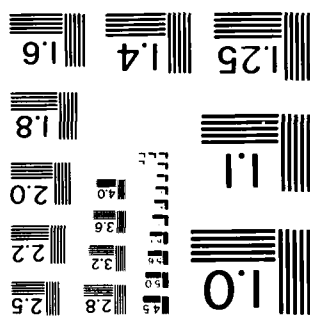
31 OCT 83 DAND17-82-C-2872

F/G 6/15

NL

							END						
							FILED						
							DEC						

MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS-1963-A



Report No.

AD _____

AD-A148 222

"Reversal of Acetylcholinesterase Inhibitor Toxicity
in vivo by Inhibitors of Choline Transport"

Final Report

John J. Freeman, Ph.D.
Joseph W. Kosh, Ph.D.

October 31, 1983

Supported by:

U.S. Army Medical Research
and Development Command

Fort Detrick, Frederick, MD 21701
Contract # DAMD 17-82-C-2072

College of Pharmacy, University of South Carolina
Columbia, SC 29208

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
S DEC 6 1984
A

DTIC FILE COPY

84 11 26 105

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER	2. GOVT ACCESSION NO. & RECIPIENT'S CATALOG NUMBER A148222	
4. TITLE (and Summary)		5. TYPE OF REPORT & PERIOD COVERED
Reversal of Acetylcholinesterase Inhibitor Toxicity <u>in vivo</u> By Inhibitors of Choline Transport		Final - 1 Mar. 1982 - 31 May, 1983
7. AUTHOR(s)		6. PERFORMING ORG. REPORT NUMBER
John J. Freeman Joseph W. Kosh		
9. PERFORMING ORGANIZATION NAME AND ADDRESS		8. CONTRACT OR GRANT NUMBER(s)
University of South Carolina College of Pharmacy, Dept. of Basic Pharmaceutical Sciences, Columbia, SC 29208		DAMD17-82-C-2072
11. CONTROLLING OFFICE NAME AND ADDRESS		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS
ATTN: SGRD-RMS U.S. Army Medical Research and Development Command Fort Detrick, Frederick, MD 21010		61102A.3M161102B510.EF.402
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)		12. REPORT DATE
U.S. Army Medical Research Inst. of Chemical Defense Attn: SGRDPLESS Aberdeen Proving Ground, MD 21010		October 31, 1983
		13. NUMBER OF PAGES
		15. SECURITY CLASS. (of this report)
		Unclassified
		16. DECLASSIFICATION/DOWNGRADING SCHEDULE
		NA
18. DISTRIBUTION STATEMENT (of this Report)		
Approved for public release distribution unlimited		
17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)		
19. SUPPLEMENTARY NOTES		
To be published in abstract form FASEB, St. Louis, MO. To be published in paper form.		
21. KEY WORDS (Continue on reverse side if necessary and identify by block number)		
Cholinesterase Inhibitor, diisopropylfluorophosphate Neostigmine, Physostigmine, Hemicholinium-3		
23. ABSTRACT (Continue on reverse side if necessary and identify by block number)		
The antagonism of acetylcholinesterase inhibitor toxicity was studied in mice. Hemicholinium-3 (HC-3) antagonized the toxicity of physostigmine and neostigmine by shifting the dose-response curve to the right, thus increasing the LD50. However, hemicholinium-3 failed to have a protective effect against diisopropylfluorophosphate (DFP), causing an enhancement of the toxicity. Hemicholinium-3 shifted the dose-response curve to the left of DFP's. Neostigmine, a quaternary nitrogen compound, increased the level of acetylcholine and choline in the brain. Since		

20. (Continued)

physostigmine also caused a similar response, the results indicated that neostigmine also crossed the blood-brain barrier. This was confirmed when neostigmine was shown to inhibit brain acetylcholinesterase following systemic administration.

Pretreatment, concurrent treatment and posttreatment with hemicholinium-3 (HC-3) did not consistently decrease the effects of physostigmine and neostigmine on acetylcholine levels in either the heart or brain. Thus, hemicholinium-3 may be exerting a different effect. The protecting effect of hemicholinium-3 on acetylcholinesterase inhibitor toxicity may be due to blockade of the post-synaptic receptor at the neuromuscular junction. The discrepancy between the effect of hemicholinium-3 on the reversible and irreversible inhibitors remains unexplained.

Report No.

AD _____

"Reversal of Acetylcholinesterase Inhibitor Toxicity
in vivo by Inhibitors of Choline Transport"

Final Report

John J. Freeman, Ph.D.
Joseph W. Kosh, Ph.D.

October 31, 1983

Supported by:

U.S. Army Medical Research
and Development Command

Fort Detrick, Frederick, MD 21701
Contract # DAMD 17-82-C-2072

College of Pharmacy, University of South Carolina
Columbia, SC 29208

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."

Forward

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council.

Table of Contents

	Page
Report Documentation Page - DD Form 1473	i
Title Page	ii
Foreward	iii
Table of Contents	iv
List of Figures	v
List of Tables	vi
Introduction	1
Methods	9
Results	13
Discussion	35
Bibliography	39
Distribution List	43

List of Figures

	Page
Figure 1. Plot of the effect of hemicholinium-3 on the lethal log dose-response of physostigmine	19
Figure 2 Probit Plot of the effect of hemicholinium-3 on the log dose-response curve of physostigmine.	20
Figure 3 Plot of the effect of hemicholinium-3 on the time to death of mice treated with increasing doses of physostigmine intraperitoneally.	21
Figure 4 Plot of the effect of hemicholinium-3 on the lethal log dose-response of neostigmine.	22
Figure 5 Probit plot of the effect of hemicholinium-3 on the log dose-response curve of neostigmine.	23
Figure 6 Plot of the effect of hemicholinium-3 on the time to death of mice treated with increasing doses of neostigmine intraperitoneally.	24
Figure 7 Plot of the effect of hemicholinium-3 on the lethal log dose-response curve of diisopropylfluorophosphate (DFP).	25
Figure 8 Probit plot of the effect of hemicholinium-3 on the log dose-response curve of diisopropylfluorophosphate (DFP).	26
Figure 9 Effect of hemicholinium-3 (HC-3) on the acetylcholine (ACh) concentration of mouse brain and heart.	27
Figure 10 Effect of hemicholinium-3 (HC-3) on the choline (Ch) concentration of mouse brain and heart.	28

List of Tables

Table 1.	Effect of hemicholinium-3 (HC-3) on the time to death of mice treated with intraperitoneal physostigmine	29
Table 2.	Effect of hemicholinium-3 (HC-3) on the time to death of mice treated with intraperitoneal neostigmine.	30
Table 3.	Effect of intraperitoneal hemicholinium-3 (HC-3) and physostigmine (LD50) on the acetylcholine (ACh) and choline (Ch) concentration of mouse brain.	31
Table 4.	Effect of intraperitoneal hemicholinium-3 (HC-3) and physostigmine (LD50) on the acetylcholine (ACh) and choline (Ch) concentration of mouse heart.	32
Table 5.	Effect of intraperitoneal hemicholinium-3 (HC-3) and neostigmine (LD50) on the acetylcholine (ACh) and choline (Ch) concentration of mouse brain.	33
Table 6.	Effect of intraperitoneal hemicholinium-3 (HC-3) and neostigmine (LD50) on the acetylcholine (ACh) and choline (Ch) concentration of mouse heart.	34

INTRODUCTION

Pharmacology of Acetylcholinesterase Inhibitors

The function of acetylcholinesterase (AChE) is to terminate the action of acetylcholine (ACh) at the cholinergic receptor. True acetylcholinesterase is located adjacent to the receptor while pseudocholinesterase is found in the blood. Stewart (1) and Douglas and Paton (2) suggested that the physiological effects of cholinesterase inhibitors are due to the increased interaction of acetylcholine with the receptor resulting from the inhibition of the enzyme acetylcholinesterase.

Acetylcholinesterase inhibitors can be divided into three categories depending on their duration of action and how they work. "True" cholinesterase inhibitors are competitive inhibitors of acetylcholine at the enzyme receptor. The second category, "reversible" cholinesterase inhibitors, form covalent bonds with the receptor in a reversible manner. The third category, "irreversible" cholinesterase inhibitors, also form covalent bonds with the receptor, but the bond that is formed is essentially irreversible.

The reversible cholinesterase inhibitors, physostigmine and neostigmine, have also been shown to inhibit the synthesis of ACh in guinea pig brain (3) in addition to their acetylcholinesterase inhibiting effect. Physostigmine but not neostigmine, has been shown to decrease the amount of ACh released from

the cholinergic nerve terminal following nerve stimulation (4). Neostigmine has also been shown to have a direct effect on the nicotinic receptor of mammalian neuromuscular junctions. Diisopropylfluorophosphate (DFP) is an example of an irreversible organophosphorous cholinesterase inhibitor. DFP has also been shown to produce an increase in the total acetylcholine content in the brain of the rat and rabbit.

The toxicity of cholinesterase inhibitors (whether reversible or irreversible) is due to excessive accumulation of acetylcholine which causes continuous stimulation of the post-synaptic receptor (5,6). Thus the receptor can be stimulated to the point where it can no longer respond resulting in paralysis. The cause of death is anoxia due to a combination of factors:

- 1) Central and peripheral respiratory paralysis, and
- 2) Severe bronchoconstriction complicated by excessive bronchial and salivary secretions.

The signs and symptoms of acute poisoning by anticholinesterase compounds have been reported to be the same whether the toxic substance is an irreversible or a reversible inhibitor of cholinesterase. The same symptomology has been reported for poisoning by physostigmine, neostigmine or irreversible compounds such as DFP. (7,8). The most important difference between reversible and irreversible cholinesterase inhibitors is that the equilibrium conditions are attained rapidly with the reversible inhibitors while inhibition produced by the irreversible inhibitor is slower to develop.

Cholinesterase inhibitors apparently have two mechanisms of action. At lower concentrations only inhibition of cholinesterase is seen, but at higher concentrations, a "curare-like" blockade is observed (9). Inhibition of acetylcholinesterase at myoneural junctions is more closely correlated with toxicity (10). Pharmacological observations have shown that the signs of poisoning produced by many inhibitors can be explained without postulating any direct interference with the central nervous system. Inhibition and recovery of brain acetylcholinesterase are not related to toxicity in any simple way.

General Pharmacology of Hemicholinium-3 (HC-3)

The hemicholiniums were first synthesized by Long and Schueler (11) in 1954. Chemically, the hemicholiniums are characterized by a choline moiety cyclized through hemiacetal formation. Pharmacologically, their most striking characteristic is their ability to cause respiratory failure (12). Schueler (13) first demonstrated that hemicholinium-3 (HC-3) produced a neuromuscular blockade in several skeletal muscle preparations.

The most likely mechanism of action of HC-3 includes a presynaptic as well as a postsynaptic action. A presynaptic action for HC-3 was first postulated by MacIntosh et al. (14) and later confirmed by Gardiner (15). Gardiner showed ACh synthesis was inhibited in intact but not in disrupted synaptosomes. Other

investigators have since confirmed that HC-3 blocks choline uptake into synaptosomes (16-18). Hemicholinium-3 has been shown to mainly inhibit the high affinity uptake system for choline in a competitive manner (19). The high affinity system is required for the synthesis of ACh (20-22).

Other evidence for a presynaptic effect of hemicholinium-3 has been provided by nerve stimulation studies and by choline uptake studies. In general, the higher the rate of nerve stimulation, the more effective hemicholinium-3 is in blocking cholinergic nerve transmission (23,12). The specific antagonism of HC-3 by choline suggested that HC-3 was interfering with choline transport into the nerve terminal (24). Furthermore, choline has been observed to antidote the toxic action of HC-3 at autonomic neuroeffector junctions, sympathetic ganglia, brain tissue and at neuromuscular junctions in anesthetized mice, rats, rabbits, cats, dogs and chickens (13, 23, 25).

Hemicholinium-3 has been shown to cause a decrease in the synthesis of acetylcholine by blocking the uptake of choline into the nerve terminal. This results in a decrease in the turnover of acetylcholine (26), as well as a decrease in the level of acetylcholine.

The site of HC-3 toxicity has always been controversial. Most investigators have suggested a peripheral site of action but several have concluded that the drug exerts its toxic action centrally. A peripheral site of toxicity for HC-3 was consistent with the well-known inability of quaternary ammonium compounds to pass through the blood-brain barrier.

A central site of action was first suggested by Schueler (13). Using a cross circulation experiment in anesthetized dogs, Schueler concluded that HC-3 was acting in the spinal cord to depress respiration. Kase and Borison (27) concluded that HC-3 depressed respiration centrally after stimulating the respiratory center in cats at the level of the brainstem. Using several centrally acting depressant drugs, Clement (28) concluded that HC-3 was also active centrally. Clement suggested that the anesthetic might be interfering with the central action of HC-3 thus protecting the animal against the central toxic effects of HC-3.

Studies which looked at the distribution of HC-3 after an i.p. dose have shown HC-3 does not readily penetrate the blood-brain barrier (29-31). Evidence by Schueler (13) substantiated the suggestion that HC-3 did not cross the blood-brain barrier into the brain since no alteration of the electroencephalogram was seen after an intraperitoneal injection. Longo (32) concluded that HC-3 toxicity was peripheral in nature since the effect of lethal doses of HC-3 on efferent phrenic nerve potentials were unaffected in spite of a failure of respiration. Holmes and Wilson (33) also concluded that HC-3 possessed a peripheral site of action. More recently, Freeman et al. (34) have demonstrated that hemicholinium-3 exerted its toxicity in the periphery following intraventricular administration.

Combination of Acetylcholinesterase Inhibitors and Hemicholinium-3

Atropine and oximes comprise the standard treatment for cholinesterase toxicity. However, treatment with atropine and an oxime is ineffective in animals poisoned by the organophosphorous cholinesterase inhibitor soman (35). This is due to the soman inhibited cholinesterase rapidly aging to a form which is resistant to oxime reactivation (36). Furthermore, atropine is ineffective in reversing the paralysis due to blockade of peripheral neuromuscular transmission which is the cause of the respiratory paralysis. For prophylaxis against aging, as well as treatment of toxicity, more fruitful approaches would be appropriate (37).

The most important cause of the acute toxicity of cholinesterase compounds is accumulation of acetylcholine subsequent to inhibition of cholinesterase. The acetylcholine which acts locally on various receptors originates largely by release from nerve endings (5, 6, 38). A reduction in the receptor interaction of acetylcholine should reduce the toxicity. A reduction could be accomplished by three mechanisms: -

- 1) Increase the activity of acetylcholinesterase
- 2) Block the receptor
- 3) Reduce the release of acetylcholine

Hemicholinium-3 is a well established inhibitor of choline transport in vitro. An inhibition of high affinity choline uptake at the cholinergic nerve terminal also appears to provide a satisfactory explanation of its effects in vivo (39). Therefore, in spite of the evidence for a postsynaptic action of HC-3, the predominant effect of HC-3 appears to be presynaptic.

Recent studies which have investigated the interaction between cholinesterase inhibitors and HC-3 have reversed the blockade of neuromuscular transmission produced by HC-3 with non-lethal doses of a cholinesterase inhibitor (40). Hemicholinium-3 was therefore used as a model for myasthenia gravis and cholinesterase inhibitors were used to reverse the muscle weakness.

The notion that inhibitors of choline uptake might be useful for treating cholinesterase toxicity is not new. Wills in 1970 (41) stated:

"Of the compounds that interfere with the production of acetylcholine within nerve cells, two that have been studied as possible adjuncts to atropine are the flavonoid morin and hemicholinium-3. Despite the fact that hemicholinium-3 has been found to lower concentrations of ACh in such tissues as auricle, intestine, iris and ciliary body, but not skeletal muscle, even those stimulated this compound has not been found to be effective therapeutically in cats poisoned with sarin (42)."

A search of the Wills article of 1963 (42) revealed that while in vivo neuromuscular studies were suggestive of a beneficial response, in vitro toxicity studies were not determined.

It is the purpose of this report to investigate mechanism number 3 above: the effect of reducing the release of acetylcholine on the toxicity of acetylcholinesterase inhibitors.

The hypothesis of the proposed study is that treatment of rats or mice with inhibitors of cholinergic transmission should decrease the toxicity of cholinesterase inhibitors. The rationale is that any compound which decreases the release of acetylcholine will decrease the interaction of acetylcholine with the receptor and decrease the toxicity of cholinesterase inhibitors.

A related hypothesis is that inhibitors of choline transport or inhibitors of cholinergic neurotransmission will retard the increase in acetylcholine levels caused by cholinesterase inhibitors. In some instances, the inhibitor of cholinergic neurotransmission should produce a reduction in the synthesis which should be detectable by a decrease in the level of acetylcholine. A reduction in the release of acetylcholine should correlate with a reduction in the toxicity of cholinesterase inhibitors.

METHODS

Animals

Male HA/ICR mice weighing between 25 and 35 grams were used in this study. Mice were supplied by Harlan Sprague Dawley Industries, Indianapolis, Indiana.

Toxicity Determination of Physostigmine and Neostigmine

Groups of ten mice were administered physostigmine intraperitoneally in eight doses ranging from 0.5 mg/kg to 1.2 mg/kg. Neostigmine was given in nine doses ranging from 0.3 to 1.1 mg/kg. Diisopropylfluorophosphate (DFP) was given in the dose range of 9.0 to 10.8 mg/kg. The number of mice killed in each group and time to death of each mouse were recorded.

Effect of Hemicholinium-3 (HC-3) on the Toxicity of Physostigmine, Neostigmine, and Diisopropylfluorophosphate

Nonlethal doses of hemicholinium-3 (HC-3) were administered to mice intraperitoneally in the schedule listed below:

- 1) HC-3 pretreatment + physostigmine (or neostigmine or DFP): HC-3 was given 30 minutes before physostigmine (or neostigmine or DFP).

- 2) HC-3 + physostigmine (or neostigmine or DFP) HC-3 was given simultaneously with physostigmine (or neostigmine).
- 3) Physostigmine (or Neostigmine or DFP + HC-3 posttreatment: HC-3 was given two minutes following physostigmine (or neostigmine).

In each treatment, percent of mice killed in each group and the time to death of each mouse were recorded. A "Least squares curve fit" program was used to draw the sigmoid curve of the log dose-response curves.

Probit Analysis

The percent of mice killed were transformed into a probit number according to the table of "Probit Transformation" found in the Science Tables (Documentia, Geigy) according to the method of Riggs (43). The log dose and probit number were also evaluated with the "Least squares curve fit" program to obtain a linear relationship.

Measurement of Acetylcholine (ACh) and Choline (Ch) in Mouse Brain and Heart

The gas chromatographic assay of Kosh et al. (44) was used to determine the level of ACh and Ch in brain and heart tissue. Mice were sacrificed with whole body microwave irradiation (2.0-2.4 seconds, full power). Hearts were washed with distilled water after removal from the body. Brains and hearts were then weighed and homogenized with a Polytron (Brinkman Instruments) in two ml of 15% 1N formic acid - 85% acetonitrile containing 5 nmoles butyrylcholine (BCh) as the internal standard. The sample was carried through the assay as previously described (44).

Two ul of the chloroform layer was injected into a Hewlet Packard 5880 gas chromatograph equipped with a nitrogen phosphorus detector (NPD). Area ratios (ACh/BCh and Ch/BCh) were obtained by dividing the ACh and Ch area by the BCh area.

Measurement of Acetylcholine (ACh) and Choline (Ch) Concentration in Mouse Brain and Heart Treated with Physostigmine (or Neostigmine or DFP) and Hemicholinium-3 (HC-3) Intraperitoneally

The measurement of ACh and Ch levels in mouse brain and heart were performed with physostigmine alone, HC-3 pretreatment + physostigmine, HC-3 + physostigmine concurrently or physostigmine + HC-3 posttreatment with the LD50 doses of physostigmine of 0.69, 0.72, 0.83 and 0.85 mg/kg respectively. (The LD50 doses of neostigmine when given alone, with HC-3 pretreatment, with HC-3 concurrent treatment and with HC-3 posttreatment were 0.55, 0.72, 0.70 and 0.80 mg/kg respectively). DFP was given in a dose range from 6 to 11.5 mg/kg. Mice were microwaved upon death or at the maximum time to death. Brain and heart were homogenized in two ml 15% 1N formic acid-85% acetonitrile and assayed according to the method described above.

Measurement of Acetylcholine (ACh) and Choline (Ch) Concentration in Mouse Brain and Heart Treated with Hemicholinium-3 (HC-3) Alone Intraperitoneally

The ACh and Ch levels in mouse brain and heart were measured in mice treated with 25 µg/kg HC-3 for 10, 20, 30 or 40 minutes. Mice were microwaved and brains and hearts were removed and assayed as above.

Protein Determination in Mouse Brain

The method of Bradford (45) was used to measure protein. Briefly, the assay involves the binding of Coomassie Brilliant Blue G-250 to protein which produces an absorbance at 595 nm.

Determination of Acetylcholinesterase Activity in Mouse Brain

The spectrophotometric method of Ellman et al. (46) was used to determine the acetylcholinesterase activity in mouse brain homogenates. Briefly, the enzyme activity was measured by following the increase in absorbance produced from thiocholine as it reacts with the dithiobisnitrobenzoate ion. The reaction rates were recorded with a Gilford spectrophotometer and recorder.

Determination of the Effect of Neostigmine (ip) on the Cholinesterase Activity in Mouse Brain

Mice were administered the LD50 dose (0.55-mg/kg) of neostigmine intraperitoneally. The brain was removed and homogenized in 0.25 ml phosphate buffer (pH 7.9) following their death. Surviving mice were sacrificed 780 seconds after the injection and the brain tissue then homogenized. The homogenate was centrifuged at 15,000 RPM for twenty minutes. Cholinesterase activity was determined according to the method described above. The amount of protein in the mouse brain supernatant was calculated according to the standard linear equation obtained from the assay of bovine serum albumin.

RESULTS

The Effect of Hemicholinium-3 (HC-3) on Physostigmine Toxicity

Figure 1 illustrates the log dose-response curve of physostigmine in mice and the effect of HC-3 on the curves. The curve shifted to the right and the LD50 of physostigmine was increased by administration of HC-3. The figure demonstrates that pretreatment, concurrent treatment and posttreatment with HC-3 antagonized the toxicity of physostigmine. Concurrent treatment of physostigmine and HC-3 had the most consistent effect of shifting the dose-response curve to the right. Two minutes posttreatment with HC-3 also had a pronounced effect on the dose-response curve by shifting it to the right.

Figure 2 is the probit analysis of the effect of HC-3 on physostigmine treatment in mice. By examining the probit value of 5, the LD50 of physostigmine with various treatments of HC-3 was obtained. The LD50 of physostigmine treatment alone was 0.69 mg/kg. The LD50 was increased to 0.72, 0.83 and 0.85 mg/kg m pretreatment, concurrent treatment and posttreatment respectively with HC-3.

Effect of HC-3 on the Time to Death of Mice Treated with Physostigmine

In addition to the lethal dose response (Fig. 1 and 2), the average time to death was also recorded (Table 5). Mice which were administered physostigmine usually died within 900 seconds. Behaviorally, death was preceded by gasping for approximately one minute followed by tonic-clonic convulsions. Mice which did not die within 1200 seconds survived without significant problems and did

not experience tonic-clonic convulsions. The number in parenthesis represents the number of deaths occurring in a group of 10 mice. There appeared to be a negative correlation between percent mortality and time to death. Figure 3 illustrates the linear relationship between the time to death plotted against the dose in mg/kg. Pretreatment and concurrent treatment tended to increase the time to death compared to physostigmine alone, especially at higher doses. The curve representing physostigmine plus HC-3 posttreatment is relatively short since the animals either survived or reached their LD100 in a very narrow range.

Effect of Hemicholinium-3 (HC-3) on Neostigmine Toxicity

Figure 4 illustrates the lethal log dose-response curve of neostigmine in mice and the effect of pretreatment, concurrent and posttreatment of HC-3 on the curve. Regardless of the treatment schedule, HC-3 shifted the dose-response curve to the right enhancing the LD50. Posttreatment of HC-3 was the most effective treatment in shifting the curve to the right, since the LD50 was increased from 0.55 to 0.80 mg/kg.

Figure 5 represents the probit analysis of the effect of HC-3 on neostigmine toxicity in mice. The LD50 of neostigmine treatment alone was 0.55 mg/kg, and was increased to 0.70, 0.72 and 0.85 with concurrent treatment, pretreatment and posttreatment respectively.

Effect of Hemicholinium-3 (HC-3) on the Time To Death of Mice Treated with Neostigmine

The average time to death was also recorded for neostigmine when the toxicity curves were determined. Mice which were killed by neostigmine died within 1020

seconds of the injection (Table 6). Behaviorally, death was preceded by gasping for approximately one minute followed by tonic clonic convulsions. Animals which survived never exhibited tonic clonic convulsions. The number in parenthesis represents the number of deaths occurring in the group of 10 mice. There was a negative correlation between percent mortality and time to death (data not shown). Fig. 6 illustrates the linear relationship between the time to death and dose. The data illustrates that protection produced by HC-3 pretreatment, concurrent treatment, and posttreatment prolongs the time to death. Concurrent treatment with neostigmine and HC-3 prolonged the time to death to over 600 seconds.

The Effect of Hemicholinium-3 (HC-3) on the Lethal Log Dose-Response Curve of Diisopropylfluorophosphate (DFP)

Figure 7 illustrates the log dose response curve of diisopropylfluorophosphate (DFP). The curve was shifted to the left and the LD50 of diisopropylfluorophosphate decreased by pretreatment with HC-3. The figure demonstrates that pretreatment concurrent treatment and posttreatment of HC-3 potentiated the toxicity of hemicholinium-3. Posttreatment of DFP had the most dramatic effect of shifting the dose-response curve to the left.

Figure 8 is a probit plot of the effect of HC-3 on diisopropylfluorophosphate treatment in mice. The LD50 of diisopropylfluorophosphate (DFP) with various treatments of DFP can be obtained. The LD50 of DFP alone was 10 mg/kg and was decreased to 0.85 mg/kg by posttreatment.

The Effect of Hemicholinium-3 (HC-3) and Physostigmine on the Acetylcholine (ACh) and Choline (Ch) Concentration of Mouse Brain and Heart

The purpose of this experiment was to determine if the toxicity of physostigmine was related to the concentration of ACh and Ch in the heart and brain of mice (Table 1 and 2). The LD50 of physostigmine used for each treatment was taken from Fig. 2. Physostigmine alone caused a significant increase in the amount of ACh in the brain from 30.4 nmole/g to 43.1 nmole/g. HC-3 pretreatment, concurrent, and posttreatment did not decrease the elevation of ACh or Ch levels caused by physostigmine in the brain (Table 1).

Table 2 illustrates the effect of HC-3 and physostigmine on the ACh and Ch concentration of the mouse heart. The LD50 dose of physostigmine did not cause a significant change in the level of ACh in the heart but it did cause an increase in the Ch level of the heart. HC-3 pretreatment, posttreatment and concurrent treatment had similar significant effects on increasing Ch, but not ACh.

The Effect of Hemicholinium-3 and Neostigmine on the Acetylcholine (ACh) and Choline (Ch) Concentration of the Mouse Brain and Heart

The purpose of this experiment was to determine if the toxicity of neostigmine is related to the concentration of ACh and Ch in hearts and brains of mice. The LD50 for neostigmine used for each treatment was taken from Fig. 4.

Neostigmine significantly increased the amount of ACh and Ch in the brain to 41.5 nmole/g and 39.2 nmole/g respectively (Table 3). Pretreatment, concurrent

and posttreatment with HC-3 also caused increases in ACh and Ch. Treatment with HC-3 appeared to cause a more pronounced effect on choline than neostigmine alone, although all of the increases in choline were significant (Table 3). Neostigmine also caused a significant increase in the ACh level in the mouse heart (Table 4) but not choline. HC-3 pretreatment, concurrent and posttreatment caused variable effects on heart ACh, but caused consistent increases in heart choline. For instance, posttreatment with neostigmine increased choline in the heart to 58.5 nmole/g.

Effect of Neostigmine on the Cholinesterase Activity in Mouse Brain Following Systemic Administration

The average rate of cholinesterase activity in control mouse brain was 0.0921 ± 0.009 umole/min/mg. In mice treated with an LD50 of neostigmine (0.55 mg/kg, ip), the average rate of cholinesterase activity decreased significantly to 0.0632 ± 0.005 umole/min/mg. The inhibition of brain acetylcholinesterase activity in neostigmine treated mice showed that neostigmine had a significant effect in the brain following intraperitoneal injection.

Effect of Hemicholinium-3 (HC-3) on the Acetylcholine (ACh) and Choline (Ch) Concentration in Mouse Brain and Heart

The effect of HC-3 alone on the concentration of acetylcholine (ACh) and choline (Ch) in mouse brain and heart is shown in Fig. 9 and Fig. 10. The level of ACh and Ch is expressed as a percent of the control level. Neither the level of ACh nor the level of Ch changed significantly in either the brain or heart compared to control. However, the effect of HC-3 was more pronounced in the heart on both ACh and Ch even though it did not reach significance. No significant decrease

in Ach levels were observed in the brain and heart tissue following treatment with HC-3. But the ACh level in the heart tended to decrease. The Ch level in brain had no significant change either, while the Ch level in the heart increased but not significantly.

Fig. 1

Plot of the effect of hemicholinium-3 (HC-3) on the lethal log dose-response of physostigmine. Mice were administered non-lethal doses of HC-3 25 μ g/kg intraperitoneally (ip) 30 minutes before, concurrently, or two minutes after increasing doses of physostigmine ranging from 0.5 mg/kg to 1.2 mg/kg. The percent of mice killed (mortality) at each dose of physostigmine was plotted versus the log dose. A "Least squares curve fit" program was used to plot the sigmoid curves. (n = 10/dose)

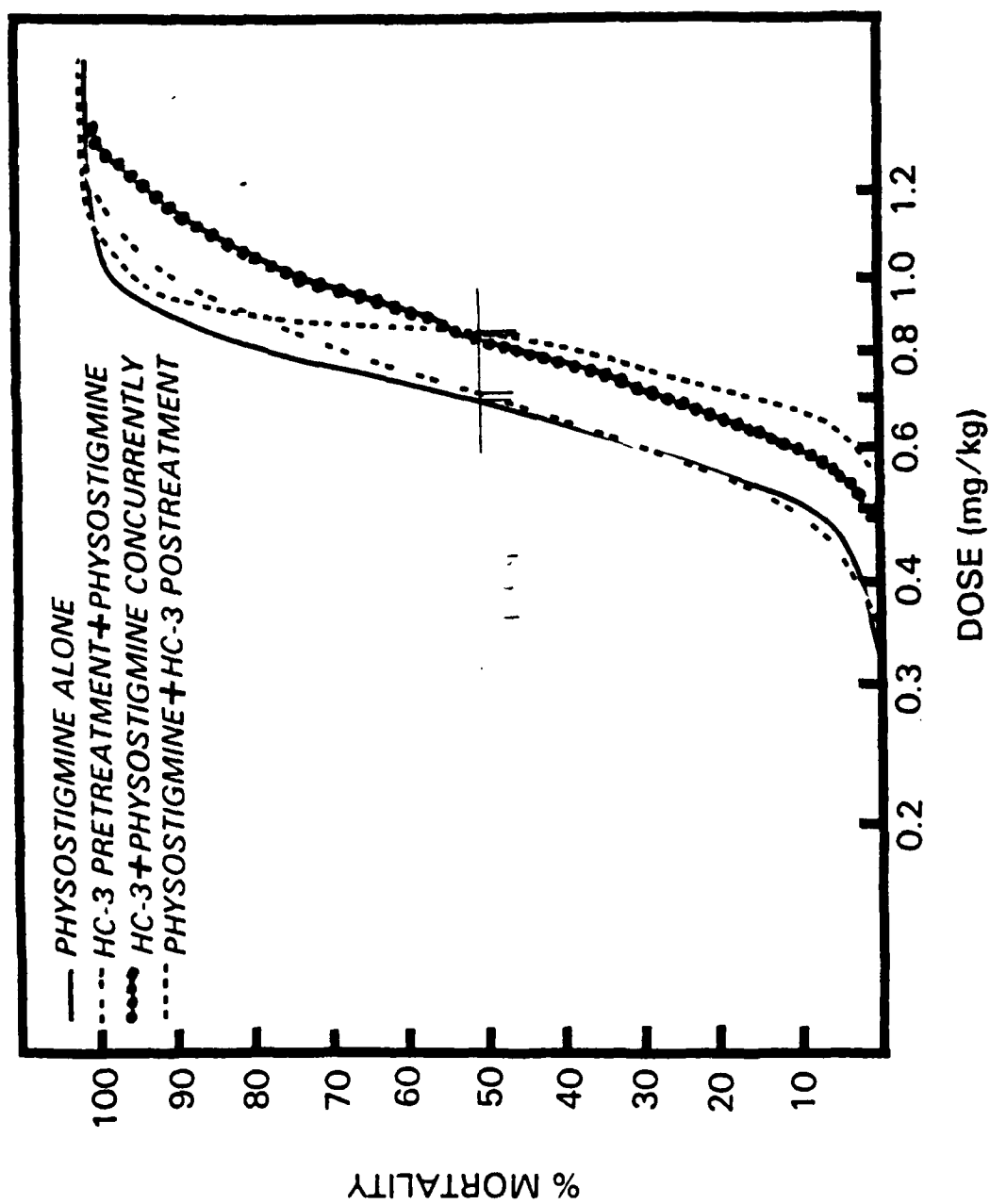


Fig. 2

Probit plot of the effect of hemicholinium-3 (HC-3) on the log dose response of physostigmine. The percent of mice killed were transformed to a probit number according to the "probit transformation table". A "Least squares curve fit" program was used to draw the straight lines. The drug dosage procedure was the same as in Fig. 1 ($n=10/\text{dose}$).

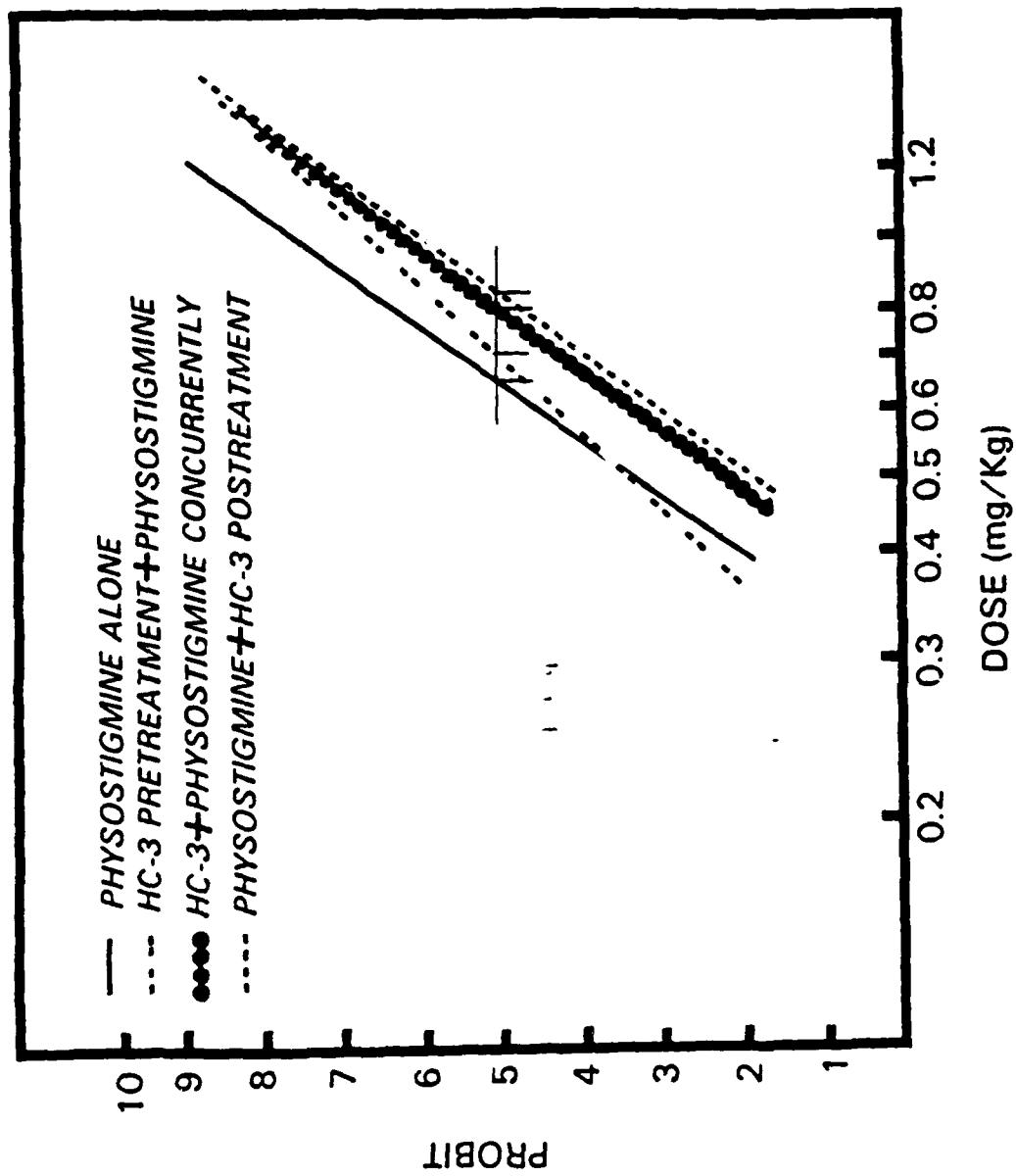


Fig. 3

Plot of the effect of hemicholinium-3 (HC-3) on the time to death of mice treated with increasing doses of physostigmine intraperitoneally (ip).

Male mice were administered nonlethal doses of 25 ug/kg HC-3 (ip) 30 minutes before, concurrently or two minutes after the dose of physostigmine (ip).

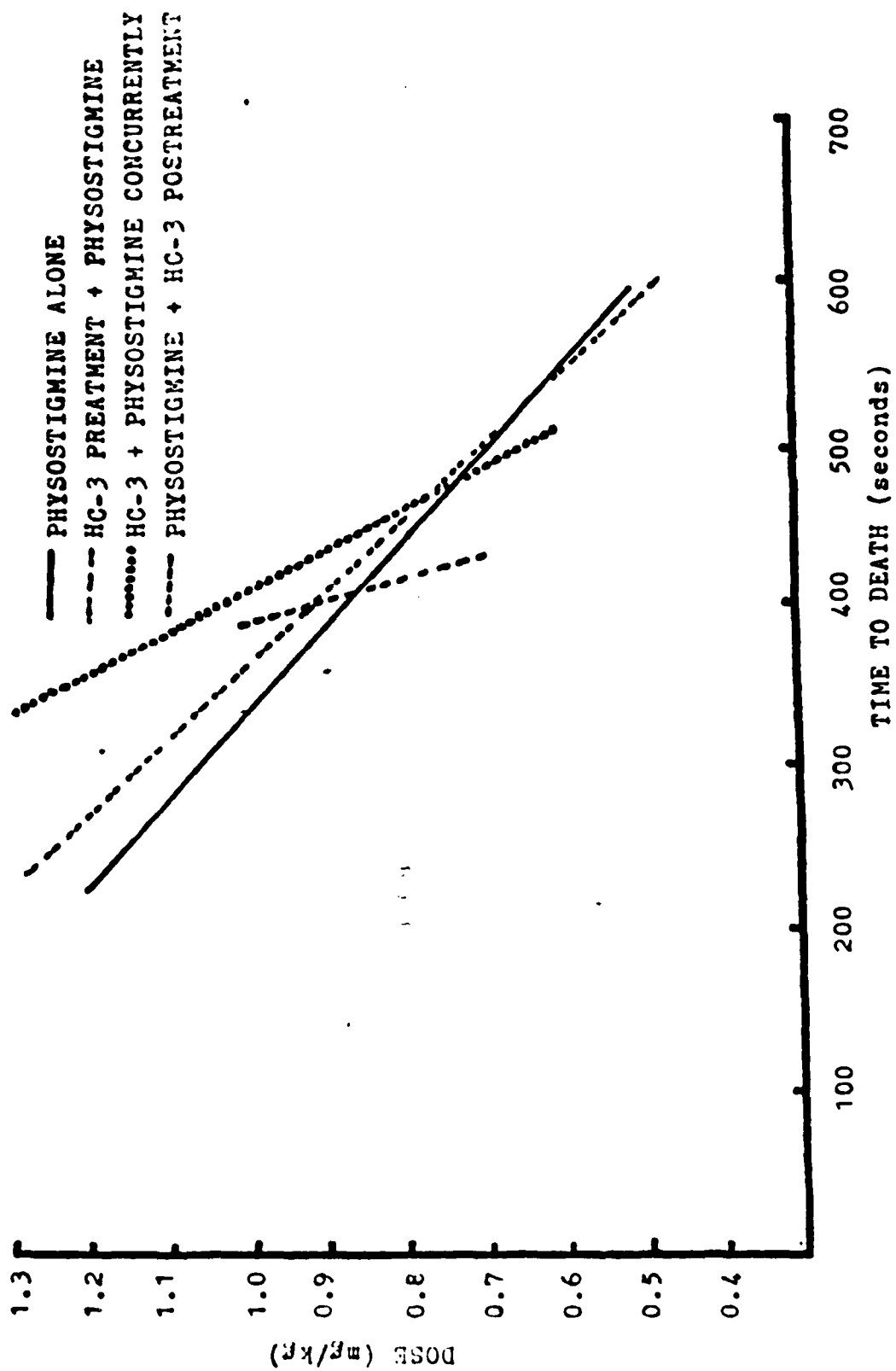


Fig. 4

Plot of the effect of hemicholinium-3 (HC-3) on the lethal log dose-response of neostigmine. Mice were administered non-lethal doses of HC-3 25 ug/kg intraperitoneally (ip) 30 minutes before, concurrently, or two minutes after increasing doses of neostigmine ranging from 0.3 to 1.1 mg/kg. The percent of mice killed (mortality) was plotted versus the log dose of neostigmine. A "Least squares curve fit" program was used to draw the sigmoid curves. (n=10/dose)

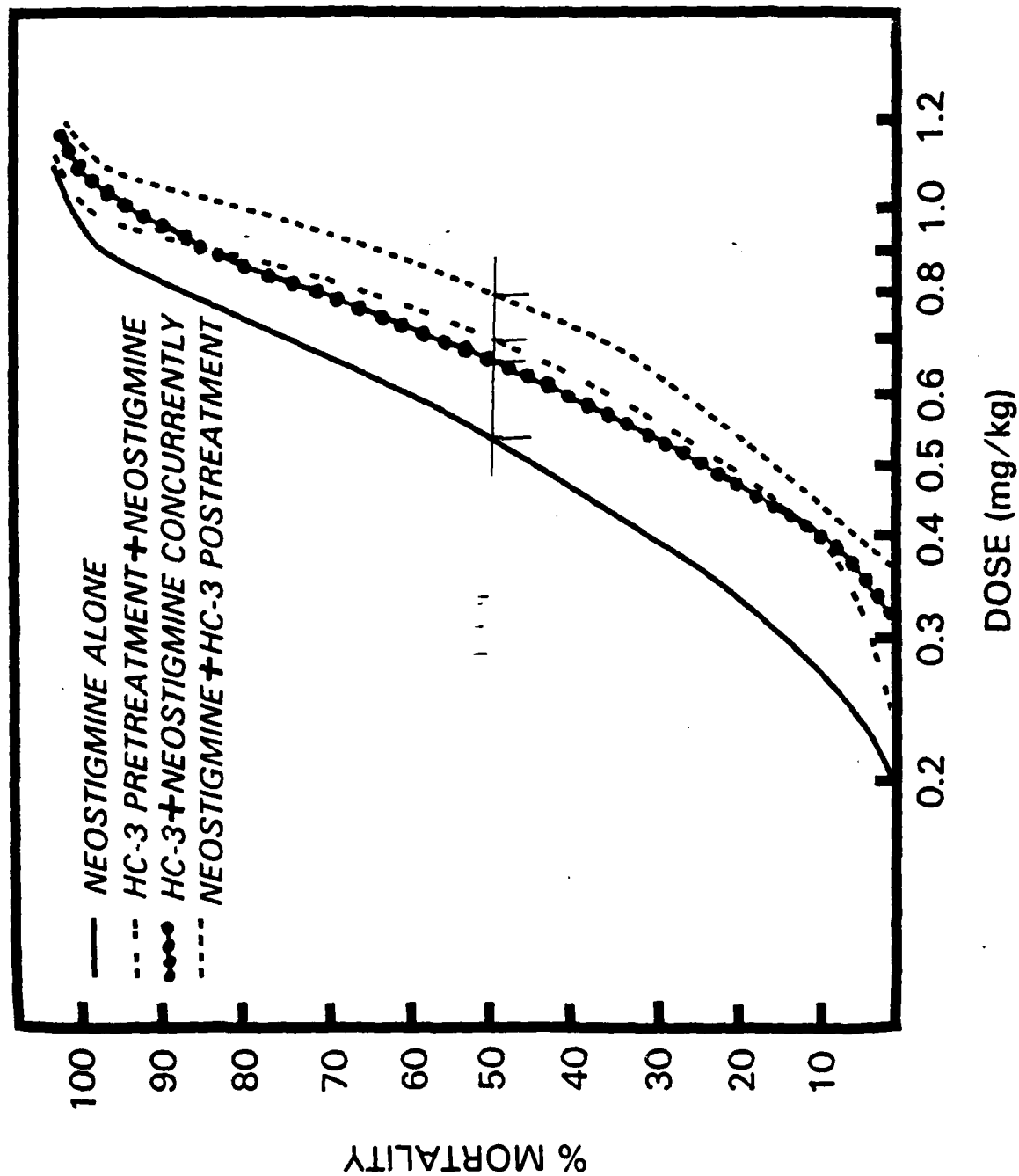


Fig. 5

Probit plot of the effect of hemicholinium-3 (HC-3) on the log dose response of neostigmine. The percent of mice killed was transformed into a probit number according to the "probit transformation table". A "Least squares curve fit" program was used to draw the straight lines. The drug dosage schedule was the same as Fig. 4 ($n=10/\text{dose}$).

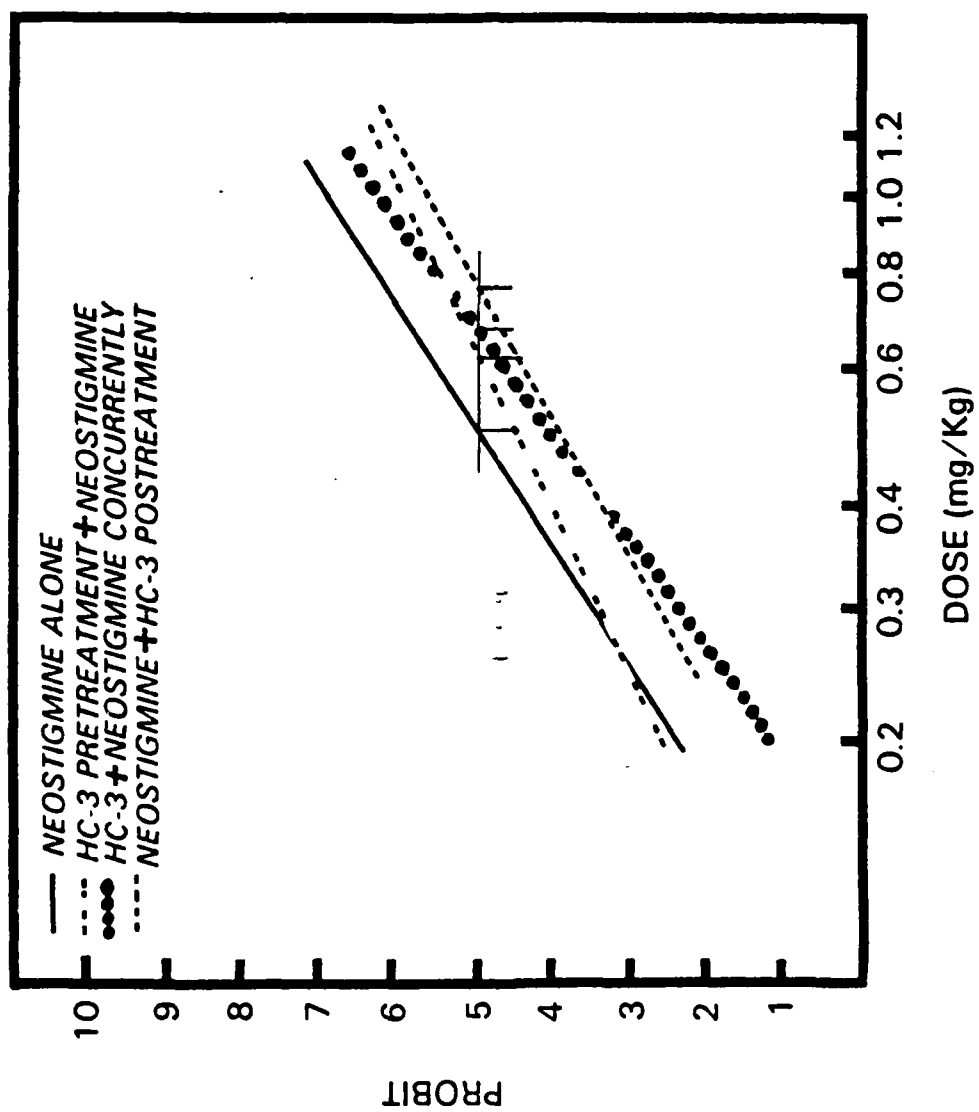


Fig. 6

Plot of the effect of hemicholinium-3 (HC-3) on the time to death of mice treated with increasing doses of neostigmine intraperitoneally (ip). Male mice were administered nonlethal doses of HC-3 25 ug/kg (ip) 30 minutes before, concurrently or two minutes after the ip treatment with neostigmine.

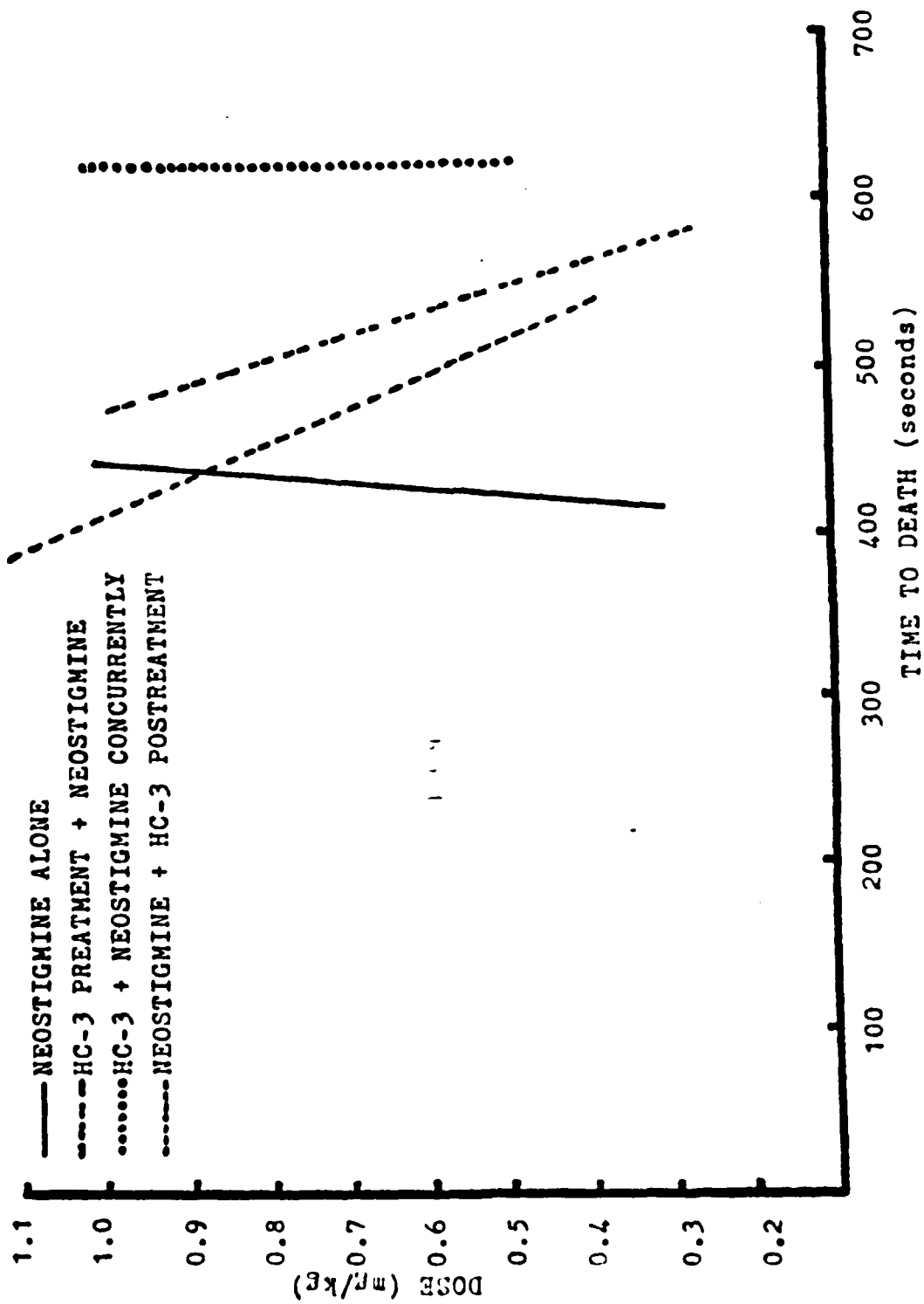


Fig. 7

The effect of hemicholinium-3 (HC-3) on the lethal log dose-response of diisopropylfluorophosphate (DFP). Mice were administered non-lethal doses of HC-3 25 μ g/kg intraperitoneally (ip) 30 minutes before, concurrently or two minutes after increasing doses of DFP ranging from 0.6 to 1.2 mg/kg. The percent mice killed (mortality) at each dose of DFP was plotted versus the log dose. A "Least squares curve fit" program was used to plot the sigmoid curves (n=10/dose).

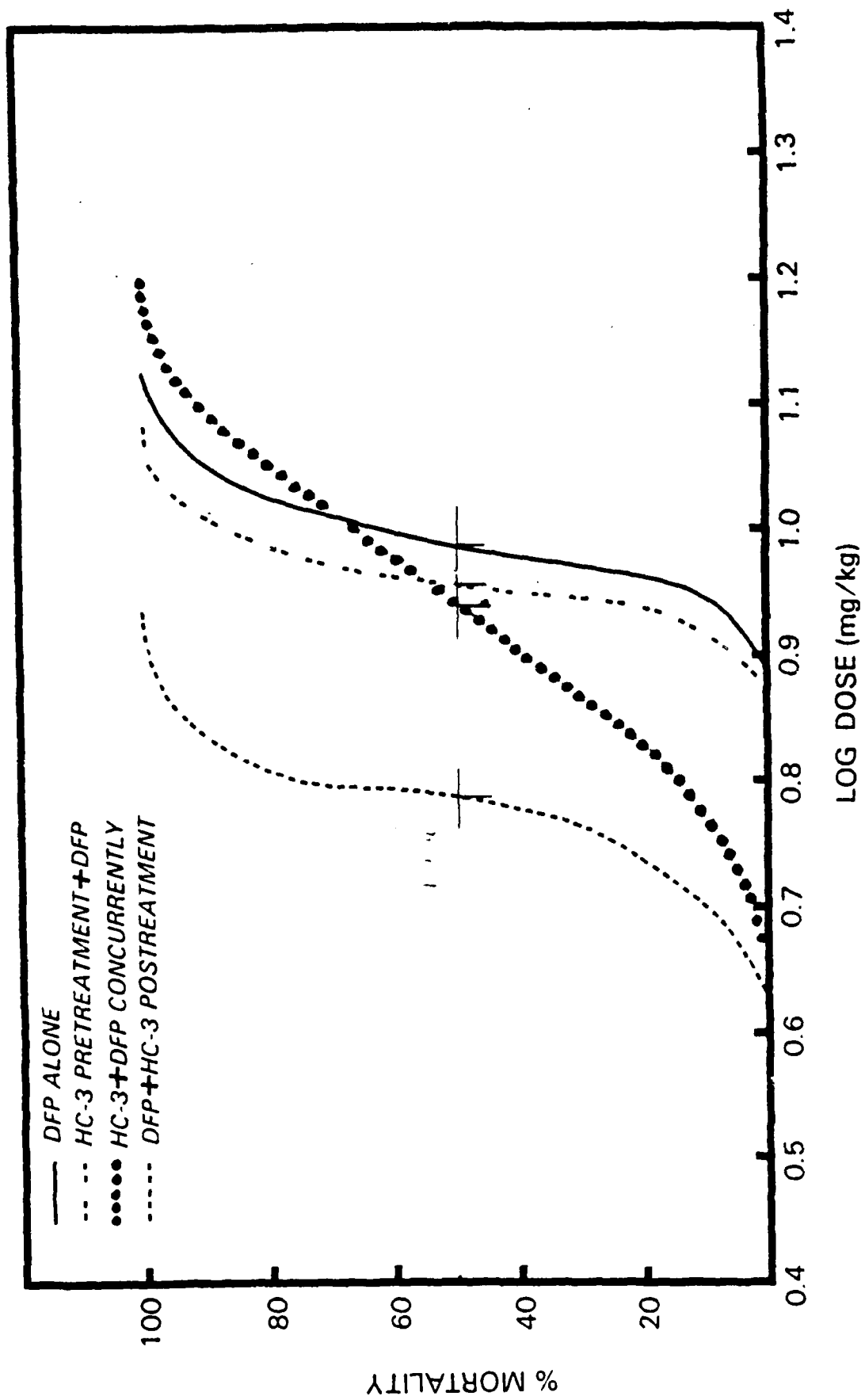


Fig. 8

Probit plot of the effect of hemicholinium-3 (HC-3) on the log dose response of diisopropylfluorophosphate. The percent of mice killed was transformed into a probit number according to the "probit transformation table". A "Least squares curve fit" program was used to draw the straight lines ($n=10/\text{dose}$).

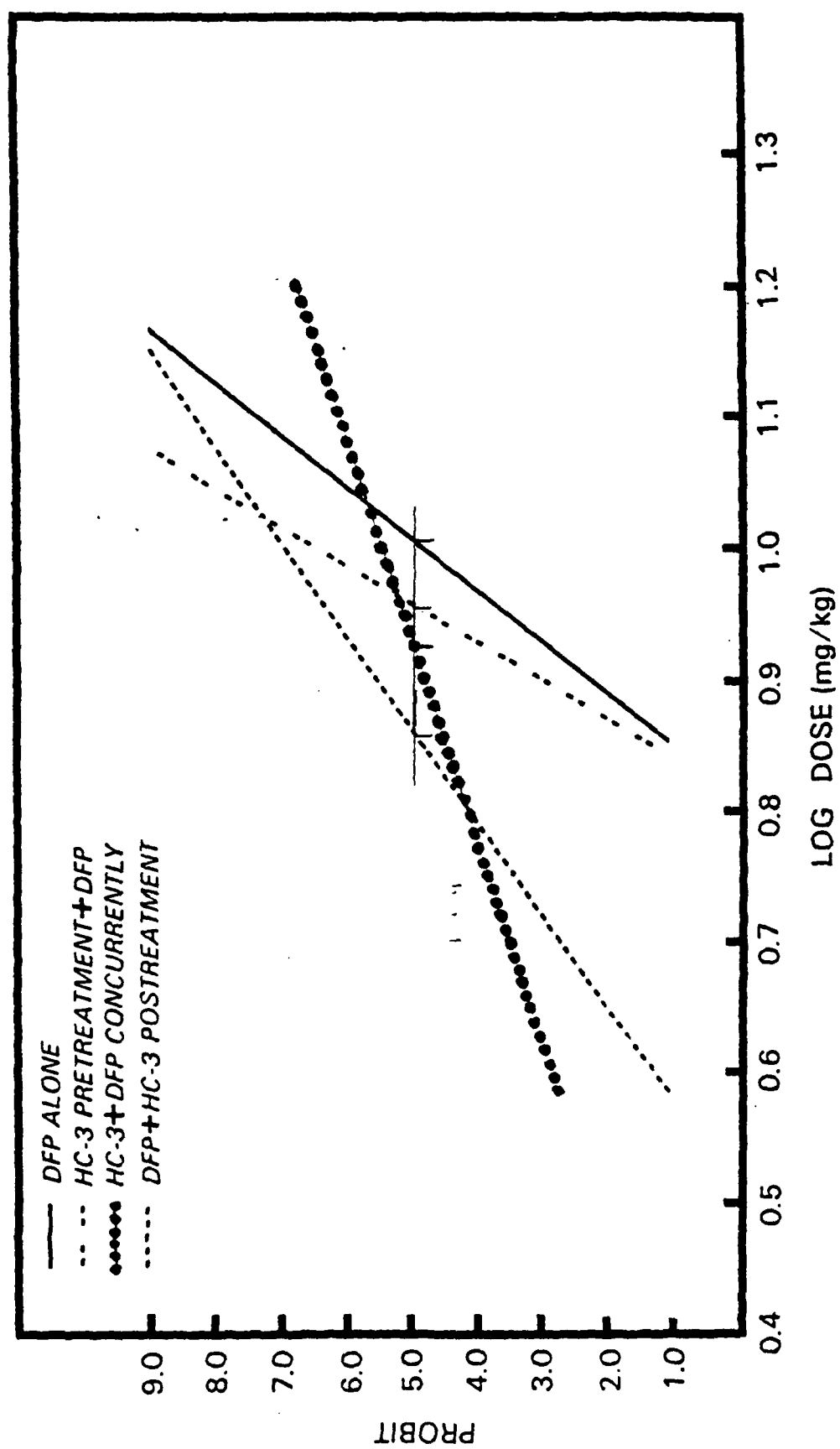


Fig. 9

Effect of hemicholinium-3 (HC-3) on the acetylcholine (ACh) concentration of mouse brain and heart. Animal whole bodies were microwaved 10, 20, 30 or 40 minutes after the intraperitoneal administration of hemicholinium-3 (HC-3) 25 µg/kg and the brains and hearts analyzed for ACh. Values are expressed as a percent of control level $\pm$ S.E.M. Control levels of ACh in brain and heart were 30.29 ± 2.40 nmole/g and 3.03 ± 0.45 nmole/g respectively. None of the values reached significance (n=6).

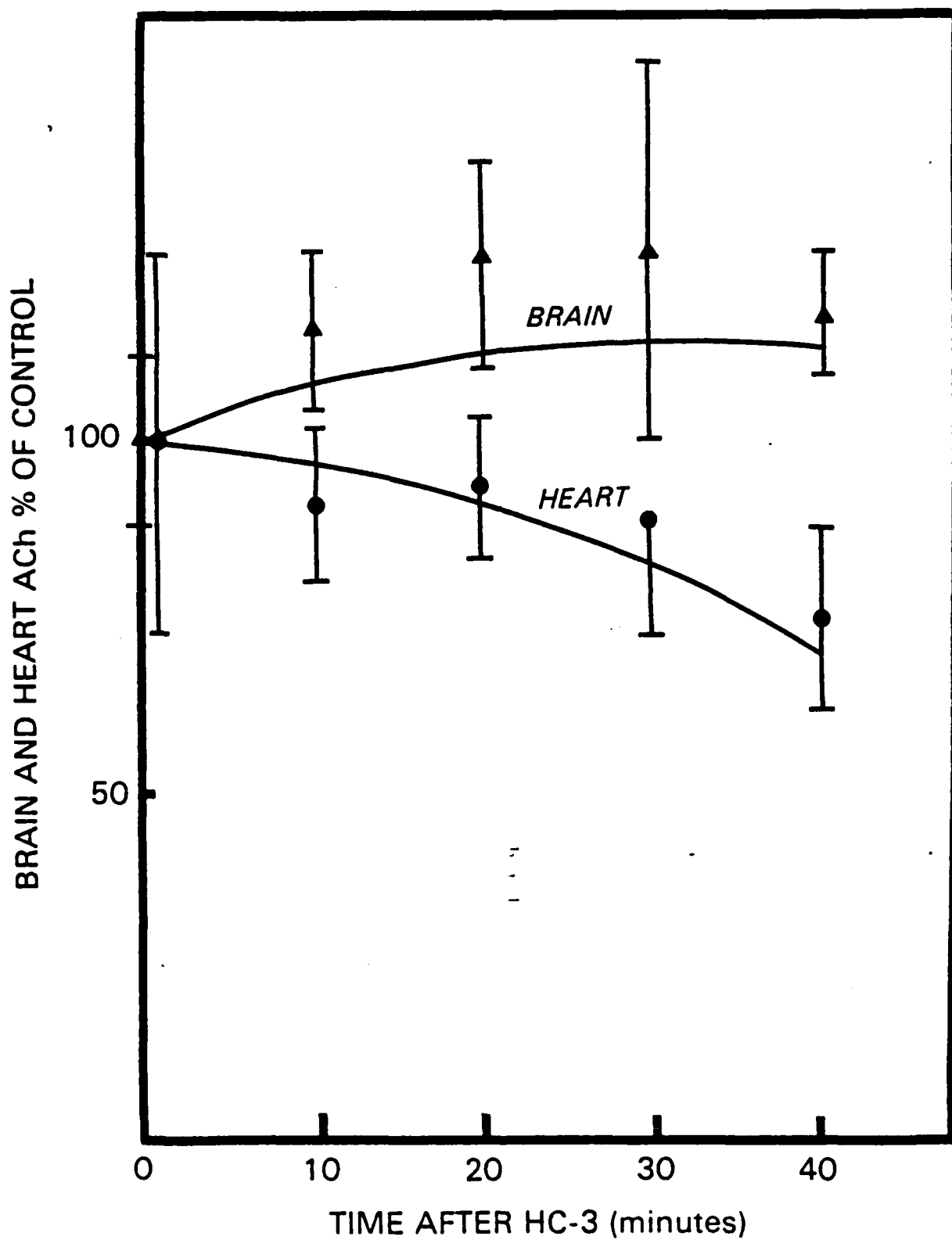


Fig. 10

Effect of hemicholinium-3 (HC-3) on the choline (Ch) concentration of mouse brain and heart. Animal whole bodies were microwaved at 10, 20, 30 or 40 minutes after the intraperitoneal administration of hemicholinium-3, 25 $\mu\text{g/kg}$ and the brains and hearts analyzed for Ch. Values are expressed as a percent of control level $\pm$ S.E.M. Control levels of Ch in brain and heart were 22.16 ± 2.45 nmole/g and 38.80 ± 3.53 nmole/g respectively. None of the values reached significance. (n=6).

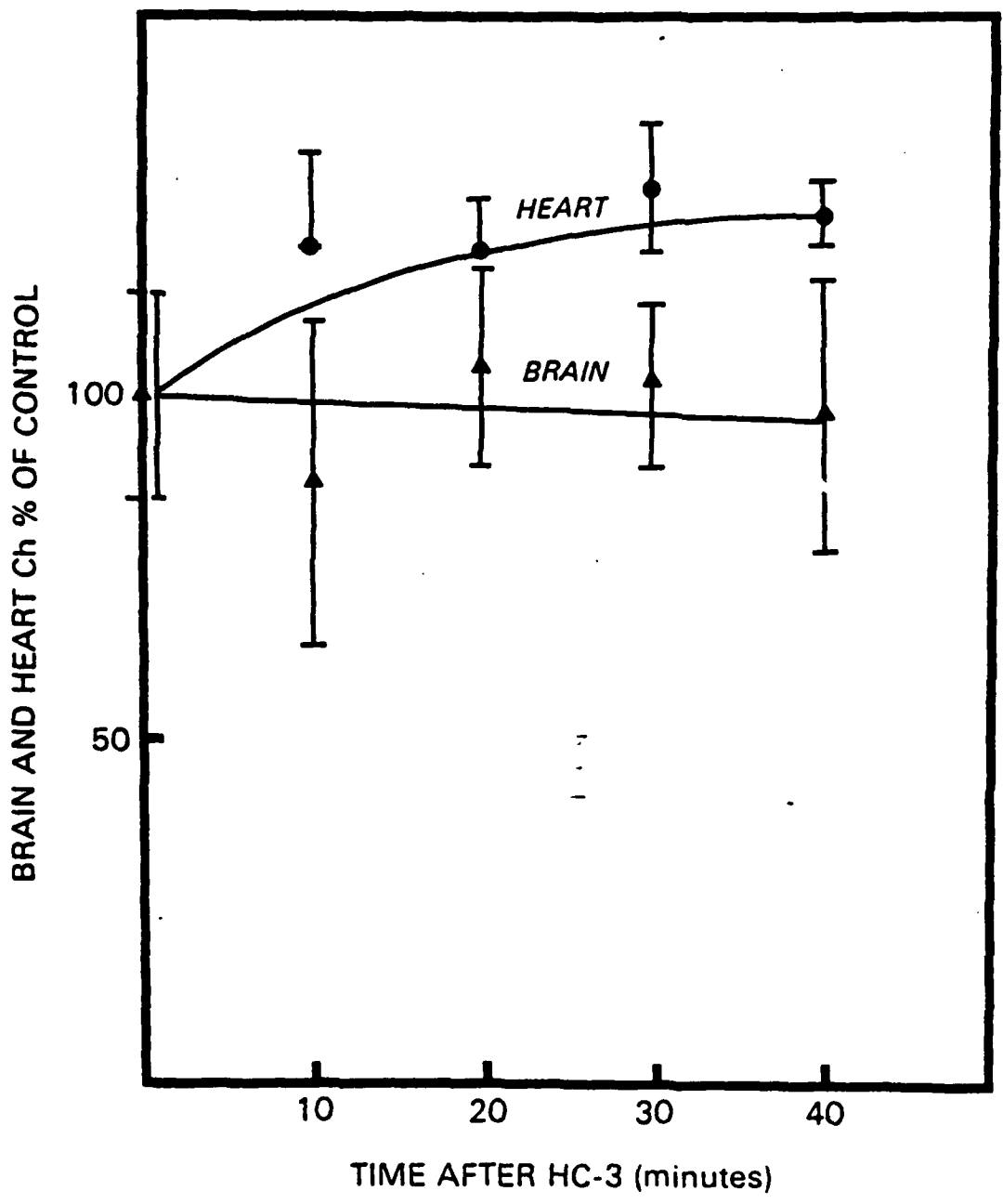


TABLE 1

Effect of Hemicholinium-3 (HC-3)^a on the Time to Death^b of Mice

Treated with Physostigmine

Dose Physostigmine mg/kg		HC-3 Pretreatment Physostigmine	HC-3 Physostigmine Concurrently	Physostigmine HC-3 Posttreatment
0.5	660(1) ^c	808(1)	α ^d	α
0.6	535(2)	584(1)	521(1)	α
0.7	693.00 $\pm$ 354.93(6)	211.50 $\pm$ 185.97(2)	465.00 $\pm$ 83.54(4)	380(1)
0.8	378.50 $\pm$ 22.49(7)	456.83 $\pm$ 129.72(6)	459.25 $\pm$ 41.55(4) ^f	495.00 $\pm$ 104.59(4)
0.9	348.33 $\pm$ 38.25(9)	421.44 $\pm$ 39.32(9)	417.60 $\pm$ 48.39(5)	379.83 $\pm$ 28.08(6)
1.0	306.70 $\pm$ 36.69(10)	363.67 $\pm$ 50.53(9)	451.89 $\pm$ 50.41(9) ^f	369.20 $\pm$ 25.67(10)
1.2	299.30 $\pm$ 16.93(10)	291.22 $\pm$ 15.87(9)	336.75 $\pm$ 31.71(8)	----- ^e
1.3	----- ^e	286.50 $\pm$ 24.37(10)	337.70 $\pm$ 43.57(10)	----- ^e

^aMale mice were administered 25 ug/kg HC-3 intraperitoneally 30 minutes before, concurrently or 2 minutes after increasing doses of physostigmine (ip).

^bTime to death is expressed as mean $\pm$ S.E.M. in seconds.

^cNumber in parenthesis represents number of deaths per group (n=10).

^d α =all mice survived.

^e(-)=not measured, since LD₁₀₀ reached at a lower dose.

^f0.025, compared to physostigmine alone.

TABLE 2

Effect of Hemicholinium-3 (HC-3)^a on the Time to Death^b of Mice

Treated with Neostigmine

Dose Neostigmine mg/kg		HC-3 Pretreatment Neostigmine	HC-3 Neostigmine Concurrently	Neostigmine HC-3 Posttreatment
0.3	348(1) ^c	677(1)	α^d	α
0.4	282.60 $\pm$ 56.35(5)	616(1)	α	α
0.5	673.00 $\pm$ 453.47(3)	510.00 $\pm$ 265.87(2)	607.50 $\pm$ 203.54(4)	α
0.6	479.83 $\pm$ 73.56(6)	299.00 $\pm$ 24.04(2)	661(1)	519.33 $\pm$ 91.65(3)
0.7	442.00 $\pm$ 52.01(8)	570.00 $\pm$ 231.28(3)	576.50 $\pm$ 83.51(6)	429.17 $\pm$ 102.81(6)
0.8	410.44 $\pm$ 51.12(9)	601.13 $\pm$ 76.99(8)	667.11 $\pm$ 98.66(9)	651.33 $\pm$ 187.24(3)
0.9	479.80 $\pm$ 61.01(10)	389.25 $\pm$ 447.72(8)	786.80 $\pm$ 208.94(5)	415.25 $\pm$ 84.66(4)
1.0	----- ^e	583.10 $\pm$ 71.33(10)	623.10 $\pm$ 103.71(10)	418.43 $\pm$ 83.20(7)
1.1	-----	-----	-----	363.60 $\pm$ 63.24(10)

^aMale mice were administered 25 ug/kg HC-3 intraperitoneally (ip) 30 minutes before, concurrently or 2 minutes after increasing doses of neostigmine (ip).

^bTime to death is expressed as mean $\pm$ S.E.M. in seconds.

^cNumber in parenthesis represents number of deaths per group (n=10).

^d α =all mice survived.

^e(-)=not measured, since LD₁₀₀ reached at a lower dose.

TABLE 3

Effect of Intraperitoneal Hemicholinium-3 (HC-3)^a and Physostigmine (LD₅₀) on the Acetylation (ACh) and Choline (Ch) Concentration of Mouse Brain

	Control	Physostigmine (0.69 mg/kg) ⁱ	HC-3 Pretreatment + Physostigmine (0.72 mg/kg) ⁱ	HC-3 Physostigmine Concurrently (0.83 mg/kg) ⁱ	Physostigmine + HC-3 Post-treatment (0.85 mg/kg) ⁱ
ACh ^b nmole/g	30.29 ±2.40	43.14 ^c ±5.23	44.08 ^d ±3.12	58.66 ^{e,g} ±2.81	50.53 ^e ±2.90
Ch ^b nmole/g	22.16 ±2.45	32.33 ^c ±3.45	32.99 ^f ±1.87	47.91 ^{e,h} ±3.04	38.17 ^e ±2.70

^aMale mice were administered 25 ug/kg HC-3 intraperitoneally (ip) 30 minutes before, concurrently, or 2 minutes after LD₅₀ doses of physostigmine (ip).

^bValues are expressed as mean ± S.E.M. n=8-15.

^cp<0.05, ^dp<0.01, ^ep<0.001, ^fp<0.025, compared to control.

^gp<0.01, ^hp<0.001, compared to physostigmine alone.

ⁱThe LD₅₀ for physostigmine is shown in parenthesis as taken from fig. 2.

TABLE 4

Effect of Intraperitoneal Hemicholinium-3 (HC-3)^a and Physostigmine (LD₅₀)
on the Acetylation (ACh) and Choline (Ch) Concentration of Mouse Heart

	Control	Physostigmine (0.69 mg/kg) ^g	HC-3 Pretreatment + Physostigmine (0.72 mg/kg) ^g	HC-3 Physostigmine Concurrently (0.83 mg/kg) ^g	Physostigmine + HC-3 Post-treatment (0.85 mg/kg) ^g
ACh ^b nmole/g	3.03 ±0.45	3.35 ±0.60	3.10 ±0.37	4.34 ±0.75	4.22 ±0.45
Ch ^b nmole/g	38.80 ±3.53	53.40 ^c ±5.72	87.26 ^{d,e} ±4.78	73.75 ^{d,f} ±3.73	63.66 ^d ±4.55

^aMale mice were administered 25 ug/kg HC-3 intraperitoneally (ip) 30 minutes before, concurrently, or 2 minutes after LD₅₀ doses of physostigmine (ip).

^bValues are expressed as mean ± S.E.M. n=8-15.

^cp<0.05, ^dp<0.001, compared to control.

^ep<0.001, ^fp<0.005, compared to physostigmine alone.

^gThe LD₅₀ for physostigmine is shown in parenthesis as taken from fig. 2.

TABLE 5

Effect of Intraperitoneal Hemicholinium-3 (HC-3)^a and Neostigmine (LD₅₀)
on the Acetylation (ACh) and Choline (Ch) Concentration of Mouse Brain

	Control	Neostigmine (0.55 mg/kg) ⁱ	HC-3 Pretreatment + Neostigmine (0.72 mg/kg) ⁱ	HC-3 Neostigmine Concurrently (0.70 mg/kg) ⁱ	Neostigmine + HC-3 Post-treatment (0.80 mg/kg) ⁱ
ACh ^b nmole/g	30.29 ±2.40	41.48 ^c ±1.76	33.98 ^f ±2.26	43.75 ^e ±2.26	34.72 ^f ±2.05
Ch ^b nmole/g	22.16 ±2.45	39.17 ^d ±1.48	53.38 ^{d,g} ±2.55	48.86 ^{d,g} ±2.15	46.98 ⁿ ±1.44

^aMale mice were administered 25 ug/kg HC-3 intraperitoneally (ip) 30 minutes before, concurrently, or 2 minutes after LD₅₀ doses of neostigmine (ip).

^bValues are expressed as mean ± S.E.M. n=8-15.

^cp<0.01, ^dp<0.001, ^ep<0.005, compared to control.

^fp<0.025, ^gp<0.001, ^hp<0.01, compared to neostigmine alone.

ⁱThe LD₅₀ for neostigmine is shown in parenthesis as taken from fig. 5.

TABLE 6

Effect of Intraperitoneal Hemicholinium-3 (HC-3)^a and Neostigmine (LD₅₀)
on the Acetylation (ACh) and Choline (Ch) Concentration of Mouse Heart

	Control	Neostigmine (0.55 mg/kg) ⁱ	HC-3 Pretreatment + Neostigmine (0.72 mg/kg) ⁱ	HC-3 Neostigmine Concurrently (0.70 mg/kg) ⁱ	Neostigmine + HC-3 Post-treatment (0.80 mg/kg) ⁱ
ACh ^b nmole/g	3.03 ±0.45	5.64 ^c ±0.83	5.21 ^d ±0.57	3.02 ^h ±0.41	4.46 ±0.69
Ch ^b nmole/g	38.80 ±3.53	38.07 ±3.42	75.23 ^{e,g} ±4.51	55.79 ^{f,g} ±2.08	58.45 ^{f,g} ±4.21

^aMale mice were administered 25 ug/kg HC-3 intraperitoneally (ip) 30 minutes before, concurrently, or 2 minutes after LD₅₀ doses of neostigmine (ip).

^bValues are expressed as mean ± S.E.M. n=8-15.

^cp<0.05, ^dp<0.025, ^ep<0.001, ^fp<0.01, compared to control.

^gp<0.001, ^hp<0.05, compared to neostigmine alone.

ⁱThe LD₅₀ for neostigmine is shown in parenthesis as taken from fig. 5.

DISCUSSION

This study was based on the hypothesis that drugs which inhibit the uptake of choline will antagonize the toxicity of acetylcholinesterase inhibitors.

Hemicholinium-3 (HC-3) antagonized the toxic-lethality of physostigmine and neostigmine by shifting the lethal dose-response curve to the right, increasing the LD50 and increasing the time to death. The decrease in toxicity was not always associated with a decrease in acetylcholine (ACh) levels. Therefore, the hypothesis proved correct but the mechanism remains unclear.

Pretreatment (30 minutes) with HC-3 should be the most effective treatment schedule since HC-3 is a well known inhibitor of choline uptake into the nerve terminal (14, 15, 19) resulting in a long lasting depletion of acetylcholine. Therefore, concurrent treatment should be less effective and posttreatment (2 minutes) the least effective. However, the present study demonstrated that concurrent and posttreatment are more effective than pretreatment. The effectiveness of HC-3 in antagonizing the toxicity of physostigmine and neostigmine may not solely be due to interference with presynaptic choline uptake.

In addition to a presynaptic mechanism of action, HC-3 has also been shown to possess a postsynaptic mechanism by blocking the nicotinic receptor (46, 47). Further evidence for a different mechanism of action (other than an effect on uptake) was provided by the present combination studies involving acetylcholinesterase inhibitors and hemicholinium-3 (Tables 1-4). Since the dose of the cholinesterase inhibitor represented the LD50 dose taken from the dose-response curve, the

acetylcholine levels listed in table 3-6 may be related to the cause of death in mice. Hemicholinium-3 (HC-3) had variable effects on acetylcholine levels in brain and heart when combined with physostigmine and neostigmine. As expected, physostigmine alone caused an increase in acetylcholine (ACh) concentration in the brain and HC-3 did not reduce the increase in ACh concentration (Table 1). This would be anticipated since HC-3 has been shown not to cross the blood brain barrier (13, 32, 34). Physostigmine increased the level of acetylcholine proportional to the LD50.

Since cholinesterase inhibitors probably exert their toxicity by a combination of central and peripheral mechanisms, the effect of physostigmine and HC-3 was also studied in the heart. Physostigmine alone did not cause a significant increase in the concentration of heart acetylcholine, nor did HC-3 cause a reduction in the level of acetylcholine compared to control. Dieterich et al. (48), using an in vitro heart preparation demonstrated that a 10^{-4} M concentration of physostigmine had no effect on acetylcholine levels while a lesser 10^{-7} M concentration did cause an increase. These authors speculated that at the higher concentration (i.e. toxic doses in our experiment), physostigmine prevented the formation of "surplus" acetylcholine pools. Thus the data in table 2 may also be evidence for this paradoxical effect.

Unlike physostigmine, neostigmine alone did cause an increase in the level of acetylcholine in the heart. Concurrent HC-3 treatment and posttreatment did appear to reduce the increase in acetylcholine caused by neostigmine. However, this may be due more to the variability of the effect of neostigmine on the

heart than an effect of HC-3, since HC-3 alone had no effect on the heart (Fig. 7). Also, concurrent and posttreatment had a greater effect than pretreatment which would not be the expected result. It is also possible that the heart does not reflect the toxicity of cholinesterase inhibitors as well as diaphragm.

Neostigmine was selected as a peripheral control for physostigmine. Surprisingly, neostigmine alone caused an increase in acetylcholine levels in the brain following systemic administration. The consensus in the literature has shown that neostigmine does not get into the brain following systemic administration (49). There are, however, a few indications that neostigmine may cross the blood-brain barrier (50-52). The present study confirms and expands the previous studies and provides further evidence that neostigmine does cross the blood-brain barrier. In addition to demonstrating a significant effect on acetylcholine and choline in the brain (Table 3), neostigmine was also shown to have a significant effect on inhibiting acetylcholinesterase in the brain following systemic administration. Therefore, in high doses neostigmine does cross the blood-brain barrier.

Neostigmine and physostigmine had significant effects of increasing brain and heart choline (Table 3-6). This effect is apparently not due to HC-3 since HC-3 alone did not affect brain or heart choline significantly (figure 8). Karlen et al. (53) also observed increased levels of choline in the mouse brain as a result of physostigmine. These authors speculated that physostigmine may impair the efflux rate of choline from the brain. However, the present study has demonstrated that acetylcholinesterase inhibitors also increase choline levels in the heart. The mechanism for this observed increase in

choline production is not known. Any alteration must be a result of a change in either the generation or efflux of choline. Since only physostigmine, not neostigmine, has been shown to inhibit the release of acetylcholine (4), it is unlikely that this is a satisfactory explanation for the observed actions.

The failure of hemicholinium-3 (HC-3) to have the desired effect on diisopropyl-fluorophosphate (DFP) toxicity is unexplained (Fig. 7 and 8). In fact, various treatments with hemicholinium-3 actually increased the toxicity. The purity of the DFP was questionable even though two suppliers and several batches were used. The LD50 (10 mg/kg) never did equal the LD50 reported in the literature, but this does not adequately explain the discrepancy in the data between the reversible inhibitors and irreversible inhibitors.

In conclusion, HC-3 has been shown to antagonize the toxicity of reversible, but not irreversible acetylcholinesterase inhibitors with variable effects on acetylcholine levels. Data suggests that the mechanism of action of hemicholinium-3 may be related more to a post-synaptic rather than to a pre-synaptic effect. The discrepancy between the effect of hemicholinium-3 on the reversible and irreversible inhibitors remains unexplained.

BIBLIOGRAPHY

1. Stewart, W.C. (1952). Accumulation of acetylcholine in discrete regions of the brain after dichlorvos treatment. *Journal of Neurochemistry* 27:1375-1378.
2. Douglas, W.W. and Paton, W.D.M. (1954). The mechanism motor end-plate depolarization due to a cholinesterase inhibiting drug. *Journal of Physiology* 124:325-344.
3. Shelley, H. (1956). The inhibition of acetylcholine synthesis in guinea-pig brain slices by eserine and neostigmine. *Journal of Physiology* 131:329-340.
4. Alderdice, M.T. (1979). Physostigmine, but not neostigmine, inhibits acetylcholine release. *Brain Research* 178:596-599.
5. Holmes, R. (1953). The physiological mechanism involved in poisoning with anticholinesterases. *Proc. Roy. Soc. Med.* 46:799-800.
6. Emmelin, N. and MacIntosh, F.C. (1956). The release of acetylcholine from perfused sympathetic ganglia and skeletal muscles. *Journal of Physiology* 131:477-496.
7. Hamblin, D.O. and Marchand, J.F. (1952). Phosphate ester poisoning, a new problem for the internist. *Ann. Int. Med.* 36:50-55.
8. Holmes, J.H. (1964). Organophosphorus insecticides in Colorado. *Arch. Environ. Health* 9:445-453.
9. Kordas' M., Brzin M. and Majcen Z. (1975). Comparison of the effect of cholinesterase inhibitors on end-plate current and on cholinesterase activity in frog muscle. *Neuropharmacology* 14:791-800.
10. Heath, D.F. (1961). Organophosphorus-poisons; anticholinesterase and related compounds. Oxford, New York, Pergamon Press.
11. Long, J.P. and Schueler, F.W. (1954). A new series of cholinesterase inhibitors. *Journal of American Pharmaceutical Association (Sci. ed.)* 43:79-86.
12. MacIntosh, F.C. (1961). Effect of HC-3 on acetylcholine turnover. *Federation Processings* 20:562-567.
13. Schueler, F.W. (1955). A new group of respiratory paralyzants. I. The 'hemicholiniums'. *Journal of Pharmacology and Experimental Therapeutics* 115:127-143.
14. MacIntosh, F.C., Birks, R.I. and Sastry, P.B. (1956). Pharmacological inhibition of acetylcholine synthesis. *Nature (London)* 178:1181-1187.
15. Gardiner, J.E. (1961). The inhibition of acetylcholine synthesis in brain by a hemicholinium. *Biochemistry* 81:297-303.

16. Marshbanks, R.M. (1968). The uptake of ¹⁴C-Choline into synaptosomes in vitro. *Biochemistry Journal* 110:533-541.
17. Potter, L.T. (1968). The uptake of choline by nerve endings isolated from the rat cerebral cortex. In "The interaction of drug and subcellular compounds of animal cells" p.293-304. Churchill, London.
18. Hemsworth, B.A. (1971). The effect of a hemicholinium analogue, HC-15, on neuromuscular transmission. *European Journal of Pharmacology* 15:91-100.
19. Guyenet, P., Lefresne, P., Rossier, J., Beaujouan, J. and Glowinski, J. (1973). Effect of sodium, hemicholinium-3 and antiparkinson drugs on ¹⁴C acetylcholine synthesis and 3H choline uptake in rat striatal synaptosomes. *Molecular Pharmacology* 9:630-639.
20. Haga, T. and Noda, H. (1973). Choline uptake systems of rat brain synaptosomes. *Biochimica et Biophysica Acta* 291:564-575.
21. Yamamura, H.I. and Snyder, S.R. (1973). High affinity transport of choline into synaptosomes of rat brain. *Journal of Neurochemistry* 21:1355-1374.
22. Suszkiw, J.B., Beach, R.L., Pilar, G.R. (1976). Choline uptake by cholinergic neuron cell somas. *Journal of Neurochemistry* 26:1123-1131.
23. Reitzel, N.L. and Long, J.P. (1959). The neuromuscular blocking properties of a,a,dimethylethanolamino 4,4,bisacetophenone (hemicholinium). *Archives of International Pharmacodynamics* 69:20-30.
24. Long, J.P. (1961). Hemicholiniums: structure activity relationships and actions on the peripheral nervous system. *Federation Proceedings* 20:583-586.
25. Reitzel, N.L. and Long, J.P. (1959). Hemicholinium antagonism by choline analogues. *Journal of Pharmacology and Experimental Therapeutics* 127:15-21.
26. Freeman, J.J., Choi, R.L. and Jenden, D.J. (1973). The effect of hemicholinium-3 on behavior and on brain acetylcholine in the rat. *Psychopharmacology Communication* 1:15-27.
27. Kase, Y. and Borison, H.L. (1958). Central respiratory depressant action of 'Hemicholinium' in the cat. *Journal of Pharmacology and Experimental Therapeutics* 88:58-66.
28. Clement, J.C. (1978). Effect of ethanol, pentobarbital, and morphine on the toxicity of hemicholinium-3 and d-tubocurarine in mice. *Archives of International Pharmacodynamics* 236:60-65.
29. Domer, F.R. and Schueler, F.W. (1960). Synthesis and metabolic studies C-14 labelled hemicholinium number three. *Journal of American Pharmacology Association (Sci. ed.)* 49:553-580.
30. Dren, A.T. and Domino, E.F. (1968). Effect of hemicholinium-3 (HC-3) on EEG activation and brain acetylcholine in dog. *Journal of Pharmacology and Experimental Therapeutics* 161:141-154.

31. Domino, E.F., Cassano, G.B., Placidi, G. (1974). Autoradiographic distribution of ¹⁴C-hemicholinium-3 in mouse whole body and dog brain. *Journal of Pharmacology and Experimental Therapeutics* 188 (1):77-85.
32. Longo, V.G. (1959). Actions of hemicholinium No. 3 on phrenic nerve action potentials. *Archives of International Pharmacodynamics* 119:1-9.
33. Holmes, R. and Wilson, H. (1960). The action of a 'hemicholinium' compound (HC-3) on the central inspiratory discharge and diaphragm activity in the cat. *Journal of Physiology* 153:62-63.
34. Freeman, J.J., Kosh, J.W. and Parrish, J.S. (1982). Peripheral toxicity of hemicholinium-3 in mice. *British Journal of Pharmacology* 77:239-244.
35. Heilbronn, E. and Tolagen, B. (1965). Toxogenin in sarin, soman and tabun poisoning. *Biochemical Pharmacology* 14:73-77.
36. Fleisher, J.H. and Harris, L.W. (1985). Dealkylation as a mechanism for aging of cholinesterase after poisoning with pinacolyl methylphosphonofluoridate. *Biochemical Pharmacology* 14:641-650.
37. Karczmar, A.G., Usdin, E. and Willis, J.H. (1970). Anticholinesterase Agents. In *International Encyclopedia of Pharmacology and Therapeutics*, ed. by G.B. Koelle, P. 363-469, Pergamon Press, New York.
38. Fedorchuk, I.G. (1959). Mode of action of organic phosphorus anticholinesterase compounds (Russian). *Fiziol. Zh. SSSR* 45:1004-1008.
39. Freeman, J.J., Macri, J.R., Choi, R.L. and Jenden, D.J. (1979). Studies on the behavioral and biochemical effects of hemicholinium in vivo. *The Journal of Pharmacology and Experimental Therapeutics* 210:91-97.
40. Leeuwijn, R.S., Veldsema-Curie, R.D. and Wolters, E. (1978). The effect of cholinesterase inhibitors and corticosteroids on rat nerve-muscle preparations treated with hemicholinium-3. *European Journal of Pharmacology* 50:393-401.
41. Wills, J.H. (1970). In *International Encyclopedia of Pharmacology and Therapeutics*. pp. 359-443.
42. Wills, J.H. (1963). Pharmacological antagonists of the anticholinesterase agents. In "Cholinesterase and Anticholinesterase Agents" Koelle, G.B. (Ed.) p. 883-920.
43. Riggs, D.S. (1970). In the mathematical approach to physiological problems. MIT Press, pp. 292-295.
44. Kosh, J.W., Smith, M.B., Sowell, J.W., and Freeman, J.J. (1979). Improvements on the gas chromatographic analysis of acetylcholine and choline. *Journal of Chromatography* 163:206-211.
45. Bradford, M.M. (1976). Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72:248-254.

46. Ellman, G.L., Courtney, K.D., Andres, V. Jr. and Featherstone, R.M. (1961). A new and rapid colorimetric determination of acetylcholinesterase toxicity. *Biochemical Pharmacology* 7:88-95.
47. Martin, A.R. and Orkand, R.K. (1961). Postsynaptic effects of HC-3 at the neuromuscular junction of the frog. *Canadian Journal of Biochemistry and Physiology* 39:345-349.
48. Thies, R.E. and Brooks, V.B. (1961). Postsynaptic neuromuscular block produced by hemicholinium no. 3. *Federation Proceedings* 20:569-578.
49. Dieterich, H.A., Kaffei, H., Kilbinger, H. and Loffelholz, K. (1976). The effect of physostigmine on cholinesterase activity, storage and release of acetylcholine in the isolated chicken heart. *Journal of Pharmacology and Experimental Therapeutics* 199:236-246.
50. Harris, L.W., Heyl, W.C., Stitcher, D.L. and Moore, R.D. (1978). Effect of atropine and/or physostigmine on cerebral acetylcholine in rats poisoned with soman. *Life Sciences* 22:907-910.
51. Brown, B.B., Taylor, E. and Warner, H.W. (1950). A comparative study of tetramethoquin, a new parasympathetic stimulant, neostigmine and physostigmine. *Archives Internationales de Pharmacodynamie* 81:276-289.
52. Paulet, G., Marsol, H. and Coq, H. (1957). Central effects of cholinesterase inhibition. *Journal of Physiology (Paris)* 49:342-347.
53. Irwin, R.L. and Hein, M.M. (1962). The inhibition of rat brain cholinesterase after administration of the dimethylcarbamates of deoxy-demethyl-lycoramine, neostigmine or physostigmine. *Journal of Pharmacology and Experimental Therapeutics*. 136:20-25.
54. Karlen, B., Lundgren, G., Lundin, J. and Holmstedt, B. (1979). Effect of physostigmine and atropine on acetylcholine turnover in mouse brain. *Archives of Pharmacology* 308:61-65.

DISTRIBUTION

4 copies: Commander

U.S. Army Medical Research
and Development Command

Attn: SGRD-RMS

Fort Detrick

Frederick, MD 21701

12 copies: Administrative

Defense Technical Information Ctr.

Attn: DTIC-DDA

Cameron Station

Alexandria, VA 22314

1 copy: Superintendent

Academy of Health Sciences, U.S. Army

Attn: AHS-COM

Fort Sam Houston, TX 78234

1 copy: Dean, School of Medicine

Uniformed Services University

of the Health Sciences

4301 Jones Bridge Rd.

Bethesda, MD 20014

END

FILMED

1-85

DTIC