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US Army Medical
Research and Development Command

Appendix 1

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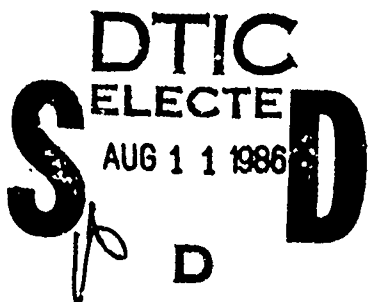

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29 - 31 May 1985

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FOREWORD

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US Army Medical
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Appendix 1

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Columbia, Maryland

29 - 31 May 1985

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The Fifth Annual Chemical Defense Bioscience Review was held at Johns Hopkins University Applied Physics Laboratory, Columbia, Maryland on 29-31 May 1985. The objective of this review was to present selected research findings in the area of medical defense against chemical warfare agents. This document constitutes the proceedings of that review. Contents of this volume include:

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1. Pyridostigmine

PYRIDOSTIGMINE PRETREATMENT OF SOMAN POISONING IN THE GUINEA PIG; CORRELATING EFFICACY WITH ACETYLCHOLINESTERASE INHIBITION (AChEI)

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ABSTRACT

The therapeutic efficacy of atropine sulfate/pralidoxime chloride (ATR/2-PAM) treatment (im) therapy and pyridostigmine bromide (PYR) pretreatmant (oral) therapy were evaluated in soman-challenged (SC) guinea pigs. ATR/2-PAM efficacy was assessed as protective ratio (PR = treated soman LD50/control soman LD50); PYR efficacy was assessed both as PR and by response surface modeling (RSM) techniques. The optimal ATR/2-PAM gives a PR of 3.78. PYR pretreatment (1 hr) produced a dose(log)-dependent (r = .96) inhibition of whole blood AChE and afforded significant ($p<0.05$) increase in PR (with doses greater than .12 mg/kg PYR) against soman when followed by 64 mg/kg ATR/100 mg/kg 2-PAM treatment. These PR's, however, were poorly correlated (r = .45) with the corresponding level of PYR-induced AChEI. In contrast, RSM analysis of efficacy indicated that the optimal ATR/2-PAM dose combination varied as a function of both the soman-challenge level and the PYR pretreatment dose. Efficacy was therefore evaluated for varying PYR pretreatment doses in combination with the appropriate optimal ATR/2-PAM treatment (as determined by RSM for each soman-challenge dose and PYR dose evaluated). When assessed in this manner, PYR efficacy (PYR) was found to be highly correlated (r = .97) with PYR-induced AChEI. Since percent AChEI was directly correlated with PYR dose (log), these results indicate that PYR pretreatment efficacy is highly correlated, dose-dependent phenomenon, providing ATR/2-PAM treatment is optimized.

MATERIALS and METHODS

1. Guinea Pigs: Mixed sex Duncan Hartley, 300 to 400 gms.; N=6 for each therapy/agent challenge combination.

2. Nerve Agent: Pinacolylmethylphosphonofluoridate (Soman; GD) diluted in saline and administered sc at a dose of 1.0 ml/kg.

3. Treatment: Atropine Sulfate (ATR) and Pralidoxime Chloride (2-PAM): Diluted in sterile water and administered im, 1.0 min. post-soman, at a dose volume of 1.0 ml/kg.

4. Pretreatment: Pyridostigmine Bromide (PYR): Diluted in sterile water and administered per os (oral gavage), 1.0 hour prior to soman, at a dose volume of 5.0 mg/kg.

5. Acetylcholine esterase inhibition (AChEI): Whole blood AChEI was assessed at 1.0 hour post-PYR, using the method of Siakotos, A. N., et al, (Biochem., 3:1, 1969).

6. Data Assessment: Lethality: Assessed at 24 hours post-soman; LD50's determined by probit analysis (Finney, D.J., 'Probit Analysis', 3rd Ed., Cambridge Univ. Press, 1971).
   Protective Ratios (PR): assessed as the ratio of the soman LD50 following therapy to the soman LD50 without therapy.
   Comparison of PR's: Statistical difference in PR's assessed by Newman-Keul's analysis of variance (Steel, R.G.D. and Torrie, J.H., 'Principles and Procedures of Statistics', Chap.7, MCGRAW-HILL, N.Y., 1960).

Correlation: Percent AChEI/PYR dose correlation was determined by PROPHET System non-linear regression analysis (NIH Pub. No., 80-2169, 1980); PR/PYR dose correlation and PR/% AChEI correlation were determined by linear regression analysis (Tallarida, R.J. and Jacob, L.S., 'the Dose-Response Relationship in Pharmacology', Springer-Verlag, N.Y., 1979).
Response Surface Modeling (RSM): Assessed for combination PYR pretreatment and ATR/2-PAM treatment of soman-induced lethality using the methods described by Carter, W.H. et al (Cancer Research, 39, 3446-3453, Sept. 1979). PYR pretreatment, ATR and 2-PAM treatment and soman challenge doses were evaluated as independent variables and probability of survival assessed as the dependant variable.

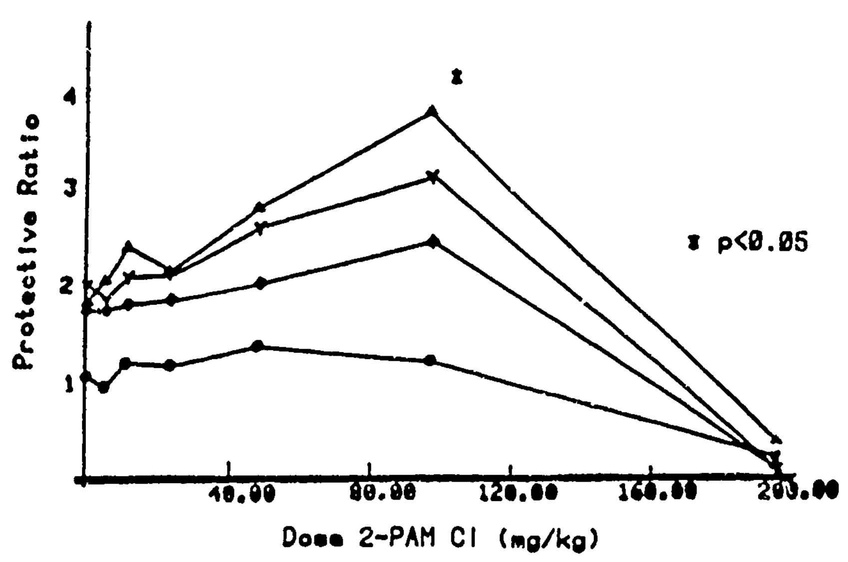

FIG 1. Optimal ATR/2-PAM dose combinations for treatment of Soman; ATR 64 mg/kg/2-PAM 100 mg/kg afforded significantly ($p<0.05$) greater protection (PR=3.78) than all other dose combinations evaluated.

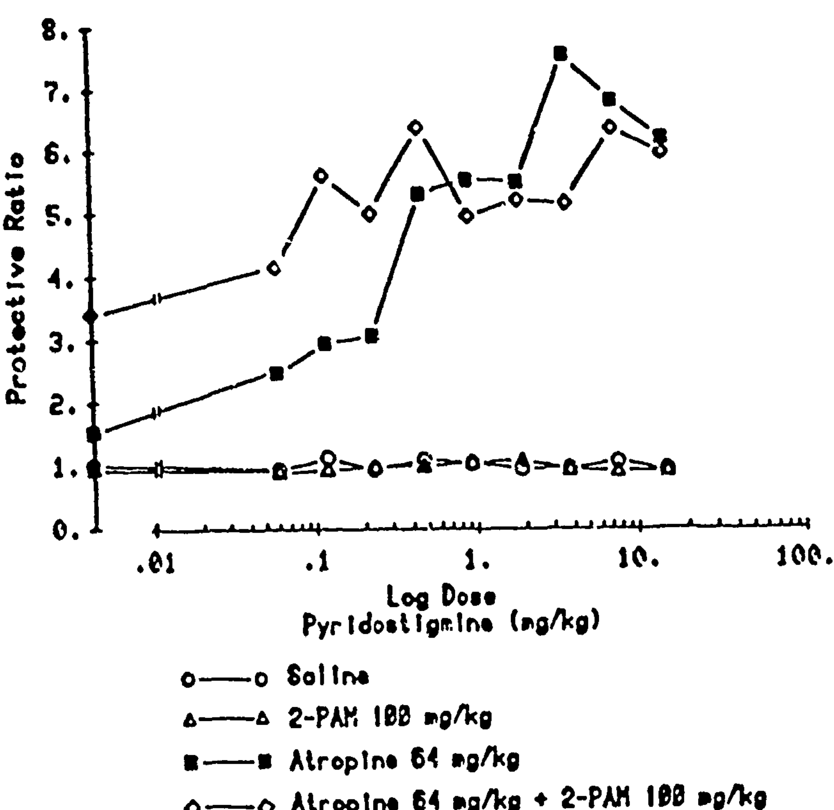

FIG 2. Pretreatment efficacy (PR) of PYR alone and in combination with 2-PAM, ATR, and ATR plus 2-PAM treatments. PYR alone and with 2-PAM had no effect on efficacy. In combination with ATR, PYR doses greater than 0.47 mg/kg afforded significantly ($p<0.05$) greater PR's than ATR without PYR. In combination with ATR plus 2-PAM, PYR doses greater than 0.12 mg/kg afforded significantly ($p<0.05$) greater PR's than ATR/2-PAM without PYR.

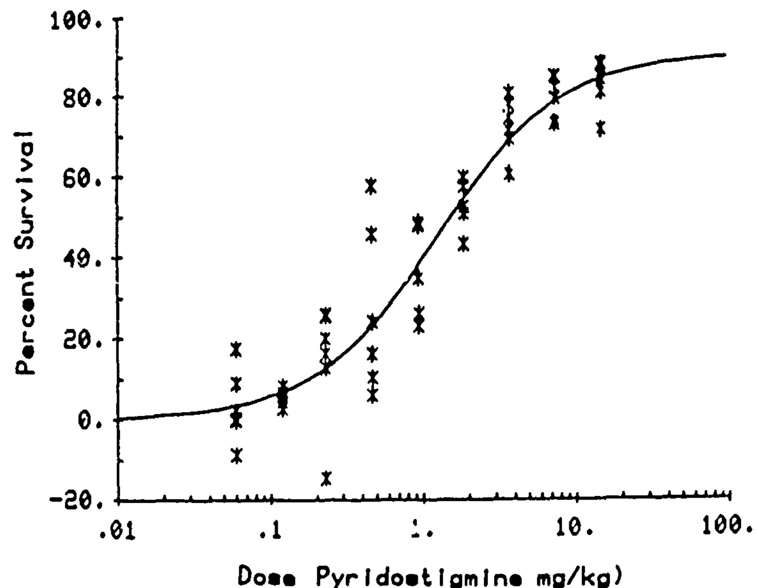

FIG 3. Percent AChEI as a function of PYR dose. Although PYR is a quaternary compound, oral administration induced a highly correlated dose dependent inhibition of whole blood AChE ($r=0.96$).

Dose Pyridostigmine (mg/kg)	% Inh AChE (Mean ± S.D)	Mean PR
0.06	3.11 ± 8.95	4.16
0.12	5.4 ± 2.1	5.62
0.23	16.4 ± 9.5	5.02
0.47	26.4 ± 20.6	6.38
0.94	34.0 ± 11.2	4.97
1.90	52.4 ± 6.5	5.21
3.75	71.9 ± 7.1	5.16
7.5	79.1 ± 5.2	6.33
15.0	82.2 ± 6.1	5.95

r = .96 (Dose vs. % Inh AChE); r = .48 (% Inh AChE vs. Mean PR); r = .45 (Dose vs. Mean PR)

FIG 4. Comparisons of % AChEI and efficacy (PR) afforded by PYR pretreatment: PR's were assessed following adjunct treatment with ATR 64 mg/kg plus 2-PAM 100 mg/kg. Although % AChEI was highly correlated with PYR dose (r=0.96), therapeutic efficacy (PR) was poorly correlated with both PYR dose (r=0.45) and % AChEI (r=0.48).

- A logistic model utilizing multifactorial quadratic regression analysis
- Provides means to assess multiple agent interactions
- Not constrained by the number of independent variables
- Permits identification of specific treatment variables
- Allows statistical assessment of each model parameter
- Allows statistical assessment of overall model fit

FIG 5. Characteristics of RSM analysis. As indicated, this analysis technique is ideally suited to evaluate combination therapy of nerve agent toxicity. Specifically, its utility is not limited by the number of different pretreatment/treatment/agent challenge modalities (independent variables).

INDEPENDENT VARIABLES	DEPENDENT VARIABLE
PYR (4) X ATR (4) X 2-PAM (4) X GD (5)	Probability of Survival

FIG 6. Multifactoral design for RSM analysis of combination PYR/ATR/2-PAM therapy of soman (GD). Four doses of PYR (0.12, 0.47, 1.9, 7.5 mg/kg) in combination with four doses of ATR (8, 16, 32, 64 mg/kg) in combination with four doses of 2-PAM (12.5, 25, 50, 100 mg/kg) were evaluated against five exposure levels of soman (at equal log increments) for each therapy combination.

$$p\,(\text{survival}) = \frac{1}{1 + e^{-X'B'}}$$

where

$$X'B' = B_0 + B_1X_1 + B_2X_2 + B_3X_3 + B_4X_4 + B_{11}X_1^2 + B_{22}X_2^2 + B_{33}X_3^2$$
$$+ B_{12}X_1X_2 + B_{13}X_1X_3 + B_{14}X_1X_4 + B_{23}X_2X_3 + B_{24}X_2X_4 + B_{34}X_3X_4$$
$$+ B_{123}X_1X_2X_3 + B_{124}X_1X_2X_4 + B_{134}X_1X_3X_4 + B_{234}X_2X_3X_4 + B_{1234}X_1X_2X_3X_4$$

and

X_1 = dose of 2-PAM (mg/kg)

X_2 = dose of ATR (mg/kg)

X_3 = dose of PYR (mg/kg)

X_4 = exposure level of GD (µg/kg)

FIG 7. Best-fit function for probability of survival by RSM analysis of combination PYR (X_3), ATR (X_2) and 2-PAM (X_1) therapy of soman (X_4) intoxification.

B_0 = an unknown parameter associated with the proportion of untreated animals surviving

B_1 = " " " " " " effect on survival of 2-PAM
B_2 = " " " " " " " " " " ATR
B_3 = " " " " " " " " " " PYR
B_4 = " " " " " " " " " " GD

B_{11} = " " " " " " toxicity of 2-PAM
B_{22} = " " " " " " " " ATR
B_{33} = " " " " " " " " PYR

B_{12} = " " " " " " interaction between 2-PAM & ATR
B_{13} = " " " " " " " " 2-PAM & PYR
B_{14} = " " " " " " " " 2-PAM & GD
B_{23} = " " " " " " " " ATR & PYR
B_{24} = " " " " " " " " ATR & GD
B_{34} = " " " " " " " " PYR & GD

B_{123} = " " " " " " " " 2-PAM, ATR, & PYR
B_{124} = " " " " " " " " 2-PAM, ATR, & GD
B_{134} = " " " " " " " " 2-PAM, PYR, & GD
B_{234} = " " " " " " " " ATR, PYR, & GD

B_{1234} = " " " " " " " " 2-PAM, ATR, PYR, & GD

FIG 8. β parameter estimates for each singular independent variable and for each possible independent variable interaction.

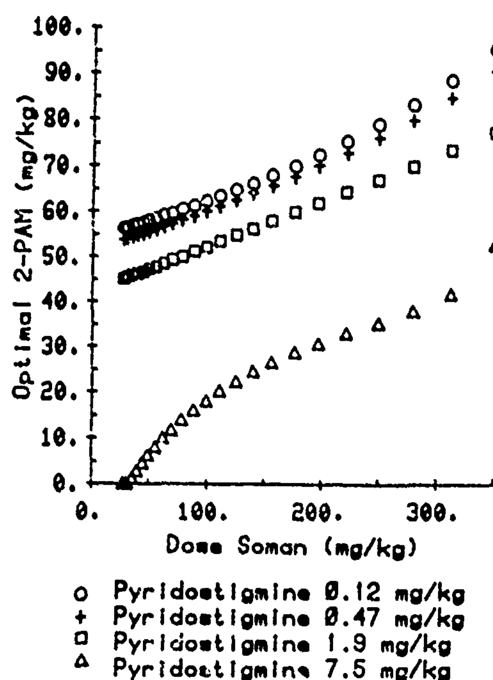

FIG 9. Optimal 2-PAM treatment as determined by RSM. Optimal 2-PAM therapy increased as a funtion of increasing soman exposure levels, but decreased as the PYR pretreatment therapy was increased.

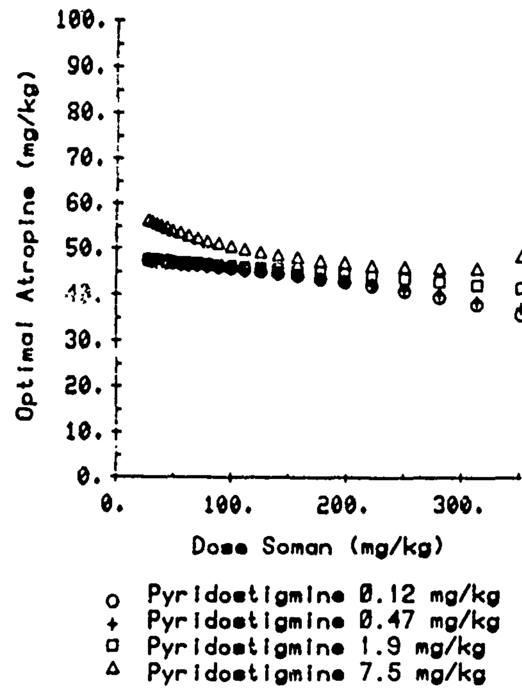

FIG 10. Optimal ATR treatment as determined by RSM. Unlike 2-PAM therapy (FIG 8 above), optimal ATR therapy remained relatively constant, independent of the PYR pretreatment dose and soman exposure level.

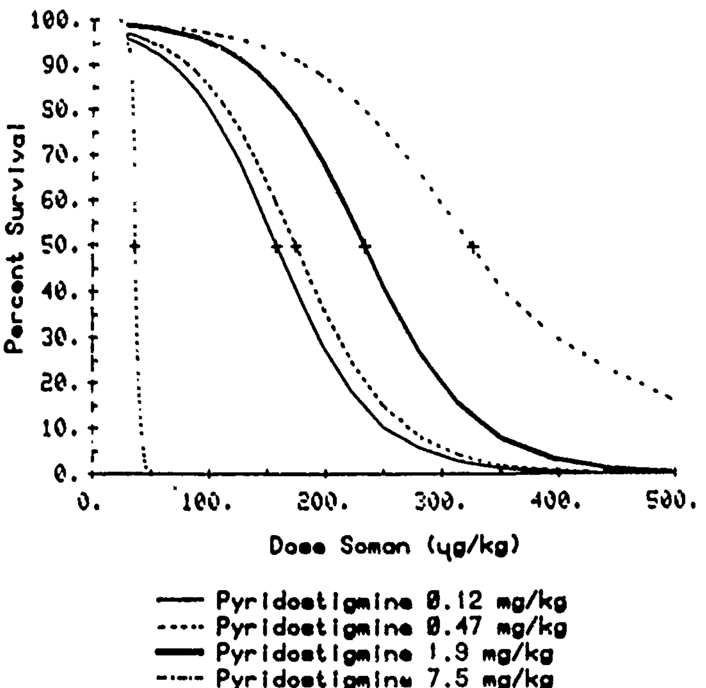

FIG 11. Percent survival with varying PYR pretreatment doses followed by optimal ATR and 2-PAM therapy. Percent survival decreased as the soman exposure level increased. Percent survival increased, however, (at all levels of soman exposure) as the PYR pretreatment dose was increased; (+) indicates the soman LD50 for each PYR pretreatment group.

Dose PYR (mg/kg)	% Inhib. AChE	PR
.12	5.4	5.6
.47	26.4	6.3
1.90	52.4	8.4
7.50	79.1	11.9

r = .96 — r = .97

r = .95

FIG 12. Comparison of PYR dose, PR, and % AChEI. PR's calculated using LD50 values for each PYR pretreatment in combination with optimal ATR/2-PAM treatment (as assessed by RSM; FIG 11). When PYR pretreatment was followed by optimal ATR/2-PAM therapy (for each agent exposure level and PYR pretreatment dose), therapeutic efficacy (PR) was highly correlated with both PYR dose (r=0.95) and with % AChEI (r=0.97).

SUMMARY

1. If ATR/2-PAM treatment is not optimized:
   - PYR pretreatment efficacy is not dose dependent (r=0.45).
   - PYR pretreatment efficacy is poorly correlated with % AChEI (r=0.48).

2. If ATR/2-PAM treatment is optimized:
   - Optimal ATR is independent of PYR and Soman.
   - Optimal 2-PAM is directly dependent on Soman and inversely dependent on PYR.
   - PYR efficacy is dose dependent (r=0.95).
   - PYR efficacy is highly correlated with % AChEI (r=0.97).

REVERSED PHASE ION-PAIR [illegible]UID CHROMATOGRAPHIC DETERMINATION OF PYRIDO[illegible]IGMINE IN PLASMA

E.T. Lin, O. Yturralde, W.L. Gee, L.Z. Benet, and L. Fleckenstein

INTRODUCTION

PYRIDOSTIGMINE IS AN ACETYLCHOLINESTERASE INHIBITOR AND IS USED TO REVERSE NON-DEPOLARIZING NEUROMUSCULAR BLOCKADE, TREATMENT OF MYASTHEMIA GRAVIS AND IS ALSO SUGGESTED AS A PROTECTIVE AGENT AGAINST ORGANOPHOSPHATE POISONING.

THE UNAVAILIBILITY OF RELIABLE AND SENSITIVE METHODS FOR THE ASSAY OF PYRIDOSTIGMINE HAS BEEN THE LIMITING FACTOR IN THE PHARMACOLOGIC AND PHARMACOKINETIC INVESTIGATION OF THE DRUG.

THIS REPORT WILL DESCRIBE A REVERSE-PHASE, ION-PAIR LIQUID CHROMATOGRAPHIC METHOD WITH UV ABSORPTION DETECTION AT 208 NM FOR THE ANALYSIS OF PYRIDOSTIGMINE BROMIDE IN PLASMA. THE CLEAN-UP PROCEDURE INVOLVES A PROTEIN PRECIPITATION STEP AND A COLUMN ELUTION STEP PRIOR TO SEPARATION BY ION-PAIR HPLC.

CH_3
CH_3 N C O
O
Br⁻
N^+-CH_3

Pyridostigmine Bromide

Cl⁻
CH_3
HO
$^+N-C_2H_5$
CH_3

Edrophonium Chloride
(Internal Standard)

FIG. 1

METHODOLOGY

INSTRUMENTS

- WATERS ASSOCIATES MODEL 6000A LIQUID CHROMATOGRAPH PUMP (WATERS ASSOCIATES, MILFORD, MA)
- KRATOS SPECTROFLOW 773 ABSORBANCE DETECTOR (KRATOS ANALYTICAL INSTRUMENTS, RAMSEY, NJ)
- WATERS INTELLIGENT SAMPLE PROCESSOR MODEL 710B (WATERS ASSOCIATES, MILFORD, MA)
- HEWLETT-PACKARD REPORTING INTEGRATOR #3392A (HEWLETT-PACKARD, SANTA CLARA, CA)

REAGENTS

- ALL SOLVENTS ARE HPLC GRADE.
- ALL CHEMICALS ARE REAGENT GRADE.
- ACETONITRILE - J.T. BAKER CHEMICALS (PHILLIPSBURG, NJ).
- SODIUM LAURYL SULFATE (SDS) - 99% PURITY - FLUKA CHEM. CO. (HAUPPAGUE, NY). RECRYSTALLIZED TWICE WITH MEOH.
- TETRAMETHYLAMMONIUM CHLORIDE (TMA+CL-) - FLUKA CHEM. CO. (HAUPPAGUE, NY).
- PYRIDOSTIGMINE BROMIDE - WALTER REED ARMY INSTITUTE OF RESEARCH (WAHSINGTON, DC).
- EDROPHONIUM CHLORIDE (INTERNAL STANDARD) - ROCHE LABORATORIES (NUTLEY, NJ).

SAMPLE PREPARATION

- PIPET 0.5 ML OF PLASMA SAMPLES INTO A CLEAN TEST TUBE.
- ADD 1.0 ML OF CH_3CN CONTAINING THE INTERNAL STANDARD EDROPHONIUM HCL (40 NG/ML) TO PRECIPITATE THE SAMPLES.
- VORTEX FOR 10 SECONDS AND THEN CENTRIFUGE AT 3000 X G FOR 10 MINUTES.
- POUR SUPERNATANT INTO A C-2 BOND ELUTE CARTRIDGE THEN WASH WITH 2 ML OF WATER AND 2 ML OF 100% ACETONITRILE TO REMOVE UNDESIRED ENDOGENOUS SUBSTANCES.
- WASH THE BOND-ELUT WITH 1 ML OF 95% CH_3CN CONTAINING 0.1% SDS AND 0.05% TMA^+CL^-.
- ELUTE WITH 3 ML OF 95% CH_3CN CONTAINING 0.1% SDS AND 0.05% TMA^+CL^-.
- EVAPORATE THE FRACTION COLLECTED UNDER N_2 TO DRYNESS AND THEN REDISSOLVE IN 30% CH_3CN (200 UL).
- TRANSFER TO WISP VIALS THEN INJECT ONTO COLUMN.

ASSAY CONDITIONS

- WAVELENGTH

  208 NM

- COLUMN

  ALTEX ULTRASPHERE - OCTYL COLUMN 4.6 MM X 25 CM

  C-8 5U BECKMAN INSTRUMENTS INC., BERKELEY, CA.

- SOLVENT SYSTEM

  ACETONITRILE: WATER (30:70) CONTAINING 0.1% SODIUM LAURYL SULFATE (SDS, WT/VOL) + 0.1% H_3PO_4 (VOL/VOL) + 0.0025 M TETRAMETHYLAMMONIUM CHLORIDE (TMA^+CL^-).

- FLOW RATE

  1.0 ML/MIN

- DETECTOR SETTING

  SENSITIVITY = 0.004

- STOCK SOLUTION

  A. PYRIDOSTIGMINE BROMIDE - 10 MG/100 ML IN H_2O

  B. EDROPHONIUM HCL - 10 MG/ML IN H_2O

RECOVERY

RECOVERY WAS DETERMINED BY COMPARING THE DIFFERENCE OF PYRIDOSTIGMINE SPIKED IN PLASMA AND IN WATER. EACH SAMPLE WAS PREPARED AS DESCRIBED EXCEPT THAT THE INTERNAL STANDARD WAS NOT ADDED UNTIL THE N_2 EVAPORATION STEP AND THE WATER DID NOT PASS THROUGH THE C-2 BOND ELUTE CARTRIDGE. RESULTS APPEAR IN TABLE 1.

$$\%RECOVERY = \frac{\text{PHR OF PYRIDOSTIGMINE BROMIDE IN PLASMA}}{\text{PHR OF PYRIDOSTIGMINE BROMIDE IN WATER}}$$

SAMPLE STORAGE

ALL SAMPLES WERE KEPT FROZEN AT -80°C BEFORE ANALYSIS AND THAWED AT ROOM TEMPERATURE.

- WORKING SOLUTION
  - A. PYRIDOSTIGMINE BROMIDE - 1 MCG/ML IN H_2O
  - B. EDROPHONIUM HCL - 1 MCH/ML IN H_2O

- RETENTION TIMES (SUBJECT TO CHANGE DEPENDING ON TEMPERATURE AND COLUMN PERFORMANCE)
  - A. PYRIDOSTIGMINE BROMIDE - 20.5 MIN.
  - B. EDROPHONIUM HCL - 30 MIN.

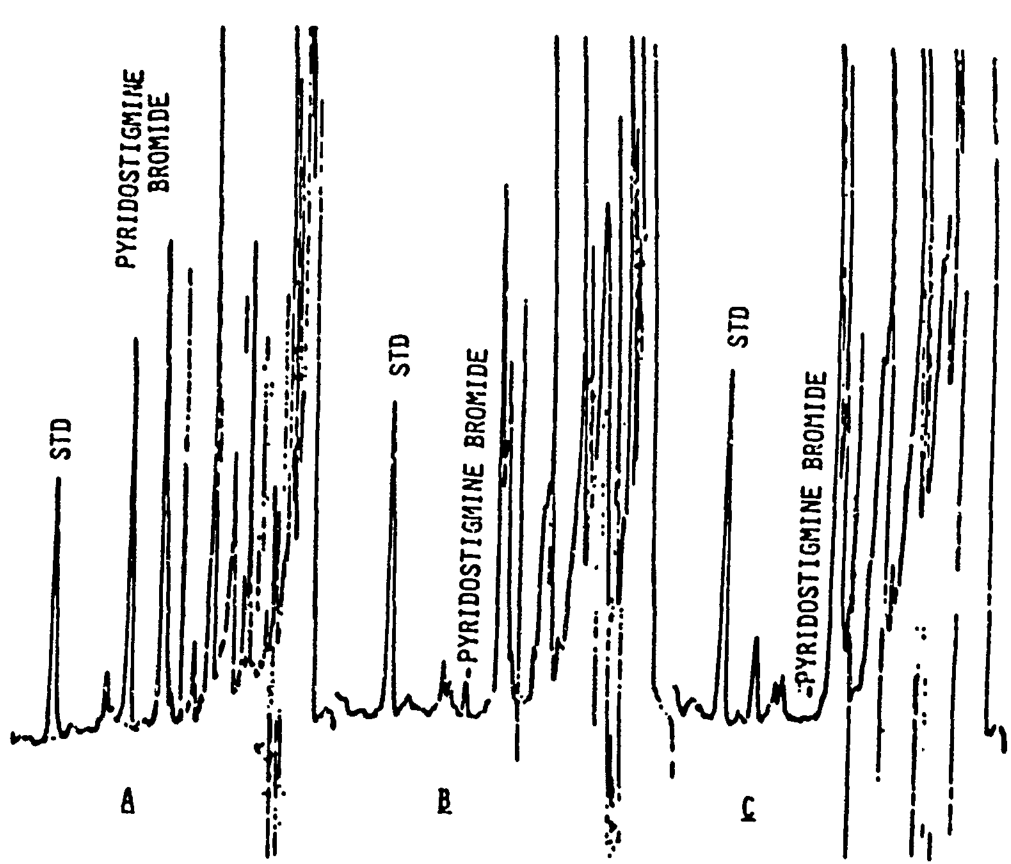

FIG. 2. CHROMATOGRAM OF PLASMA SPIKED WITH PYRIDOSTIGMINE BROMIDE AT CONCENTRATIONS

A = 70 NG/ML B = 5 NG/ML C = BLANK

CONDITIONS: DETECTOR SETTING: 208 NM, ABSORPTION RANGE = 0.004
RECORDER READING: 10 MV, 20 CM/MIN
SOLVENT SYSTEM: 30% CH_3CN, 0.1% SDS, 0.1% H_3PO_4, 0.0025 M TMA^+Cl^-

RESULTS

TABLE 1: RECOVERY OF PYRIDOSTIGMINE BROMIDE IN PLASMA

	PEAK HEIGHT RATIO	
	WATER SAMPLE	PLASMA SAMPLE
HIGH CONCENTRATION (70 NG/ML)		
SAMPLE 1	1.48	1.29
RECOVERY (1.29/1.48) = 87%		
MEDIUM CONCENTRATION (40 NG/ML)		
SAMPLE 1	0.86	0.68
RECOVERY (0.68/0.86) = 79%		
LOW CONCENTRATION (15 NG/ML)		
SAMPLE 1	0.34	0.25
RECOVERY (0.25/0.34) = 73%		
X-LOW CONCENTRATION (5 NG/ML)		
SAMPLE 1	0.10	0.08
RECOVERY (0.08/0.10) =80%		

TABLE 2: PLASMA PYRIDOSTIGMINE BROMIDE PEAK HEIGHT RATIO

CONCENTRATION	PEAK HEIGHT RATIO*
0	0
2	0.057
4	0.103
6	0.151
10	0.234
16	0.351
30	0.765
50	1.128
100	2.314

*REGRESSION EQUATION FOR PEAK HEIGHT RATIO:

$Y = 0.0230 X + 0.0087$ $R^2 = 0.9987$

TABLE 3: INTER-DAY PRECISION OF PYRIDOSTIGMINE BROMIDE PLASMA ASSAY

1	2	3	4	5	6	MEAN	SD	% CV
HIGH CONCENTRATION (SPIKED: 70.0 NG/ML)								
73.7	69.2	64.3	70.4	70.8	68.5	69.5	3.1	4.4
MEDIUM CONCENTRATION (SPIKED: 40.0 NG/ML)								
40.9	42.1	36.9	39.4	39.0	40.2	39.8	1.8	4.5
LOW CONCENTRATION (SPIKED: 15.0 NG/ML)								
13.7	14.1	15.7	13.9	14.3	14.9	14.4	0.7	4.9
X-LOW CONCENTRATION (SPIKED: 5.00 NG/ML)								
5.60	5.20	6.0	5.40	4.20	6.40	5.40	0.8	14.0

TABLE 4: INTRA-DAY PRECISION OF PYRIDOSTIGMINE BROMIDE IN PLASMA

1	2	3	4	5	6	MEAN	SD	% CV
HIGH CONCENTRATION (SPIKED: 70.0 NG/ML)								
70.4	65.5	63.1	65.9	73.0	68.4	67.7	3.6	5.3
MEDIUM CONCENTRATION (SPIKED: 40.0 NG/ML)								
39.4	39.0	39.7	42.2	44.0	38.6	40.5	2.1	5.2
LOW CONCENTRATION (SPIKED: 15.0 NG/ML)								
14.0	16.4	15.5	16.0	14.4	14.6	15.2	1.0	6.3
X-LOW CONCENTRATION (SPIKED: 5.00 NG/ML)								
5.00	4.70	4.60	4.70	4.80	4.90	4.80	0.2	3.1

PYRIDOSTIGMINE BROMIDE CONCENTRATION (NG/ML) AT -80° C

	5.00 NG	15.0 NG	40.0 NG	70.0 NG
DAY 0	6.20	16.2	38.8	63.4
1	4.60	15.2	37.6	67.1
2	5.20	15.1	35.5	67.9
3	5.40	14.9	38.8	69.7
6	4.10	14.0	39.2	72.3
13	5.50	14.4	35.7	65.5
20	5.10	15.1	36.1	64.6
29	4.60	14.6	40.9	70.6
57	5.95	18.3	43.4	71.8
99	3.70	12.6	38.2	63.9
135	6.14	15.7	42.7	66.1

TABLE 6: ACCURACY OF PYRIDOSTIGMINE BROMIDE ANALYTICAL METHOD

SAMPLE #	SPIKED LEVEL NG/ML	MEASURED LEVEL NG/ML	
19	0	0	
20		0	
25		0	
12	1.8*	2.7	
13		2.6	
15		2.7	MEAN = 2.52
18		2.3	± SD = 0.26
22		2.7	% CV = 10.17
23		2.1	BIAS = 40.0%
2	3.6	4.1	
3		4.1	
4		4.1	MEAN = 4.10
6		4.2	± SD = 0.06
8		4.0	% CV = 1.54
24		4.1	BIAS = 12.2%
1	17.6	15.4	
7		15.5	
9		15.8	MEAN = 15.62
11		15.3	± SD = 0.56
16		15.4	% CV = 3.71
21		15.3	BIAS = 11.25%
1	44.1	39.7	
10		41.9	
14		42.6	MEAN = 40.65
17		38.4	± SD = 1.50
26		40.5	% CV = 3.71
27		40.8	BIAS = 7.82%

* BELOW ASSAY SENSITIVITY

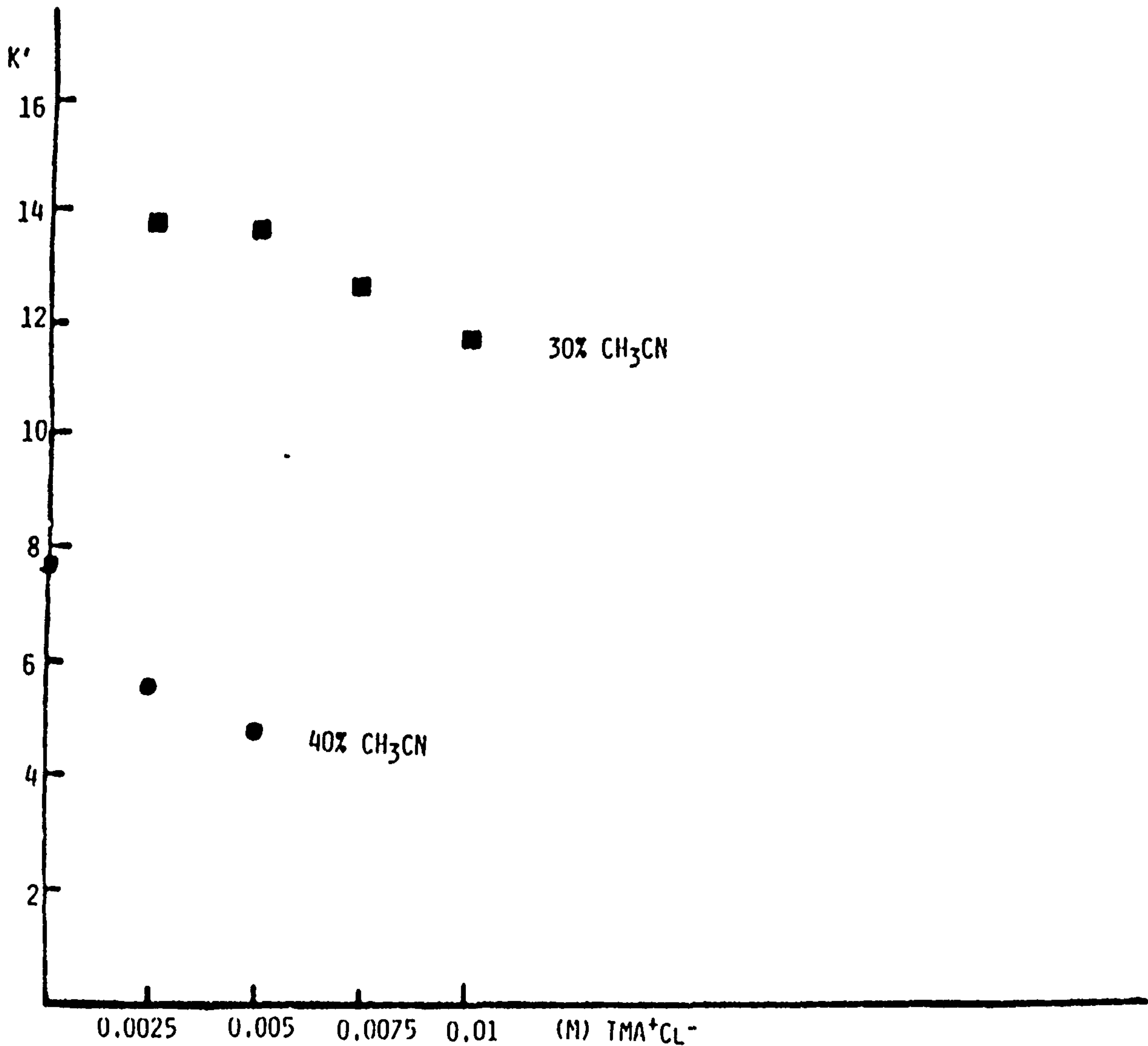

FIG. 3: RELATIONSHIP BETWEEN CAPACITY FACTORS(K') AND TETRAMETHYLAMMONIUM CHLORIDE ($TMA^{+}Cl^{-}$) CONCENTRATION SOLVENT SYSTEM: CH_3CN, 0.2% SDS, 0.1% H_3PO_4

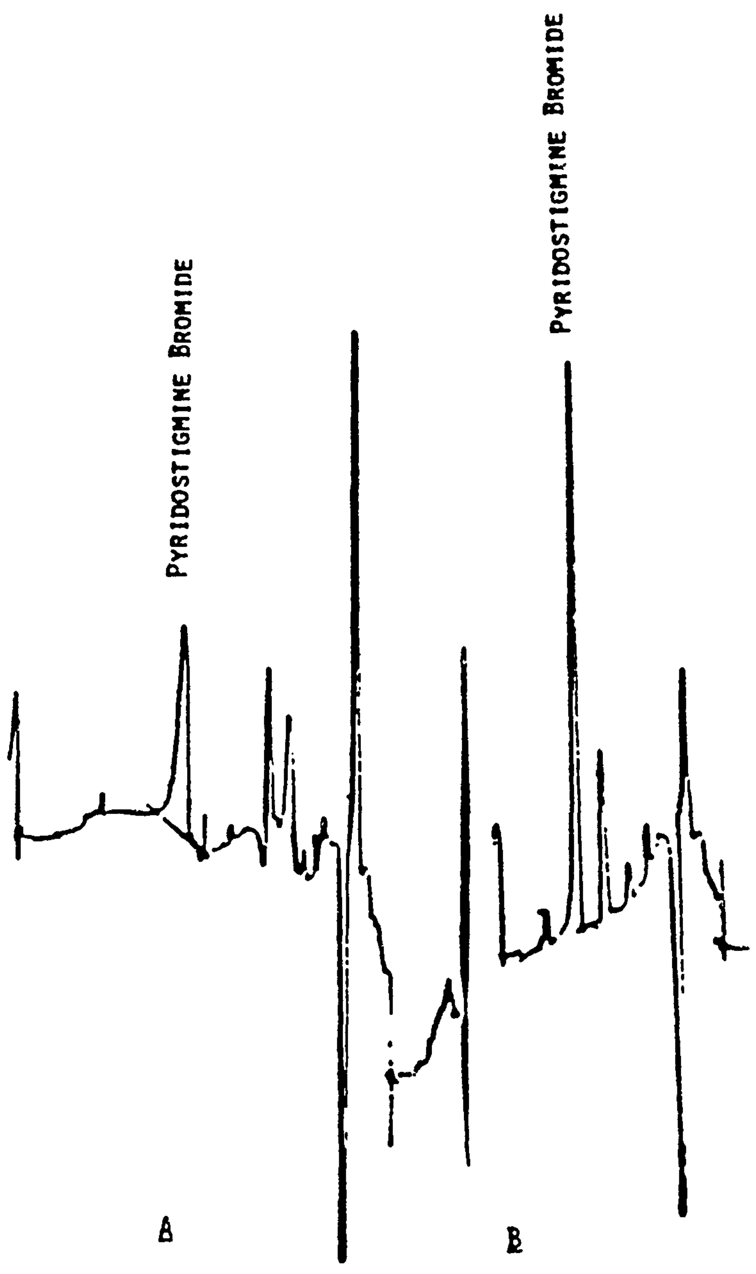

FIG. 4: COMPARISON OF PEAK SHAPE FROM A 10 NG PYRIDOSTIGMINE BROMIDE (A) WITHOUT $TMA^{+}CL^{-}$
B) 0.0025 M $TMA^{+}CL^{-}$ IN THE MOBILE PHASE (40% CH_3CN, 0.15% SDS, 0.1% H_3PO_4)

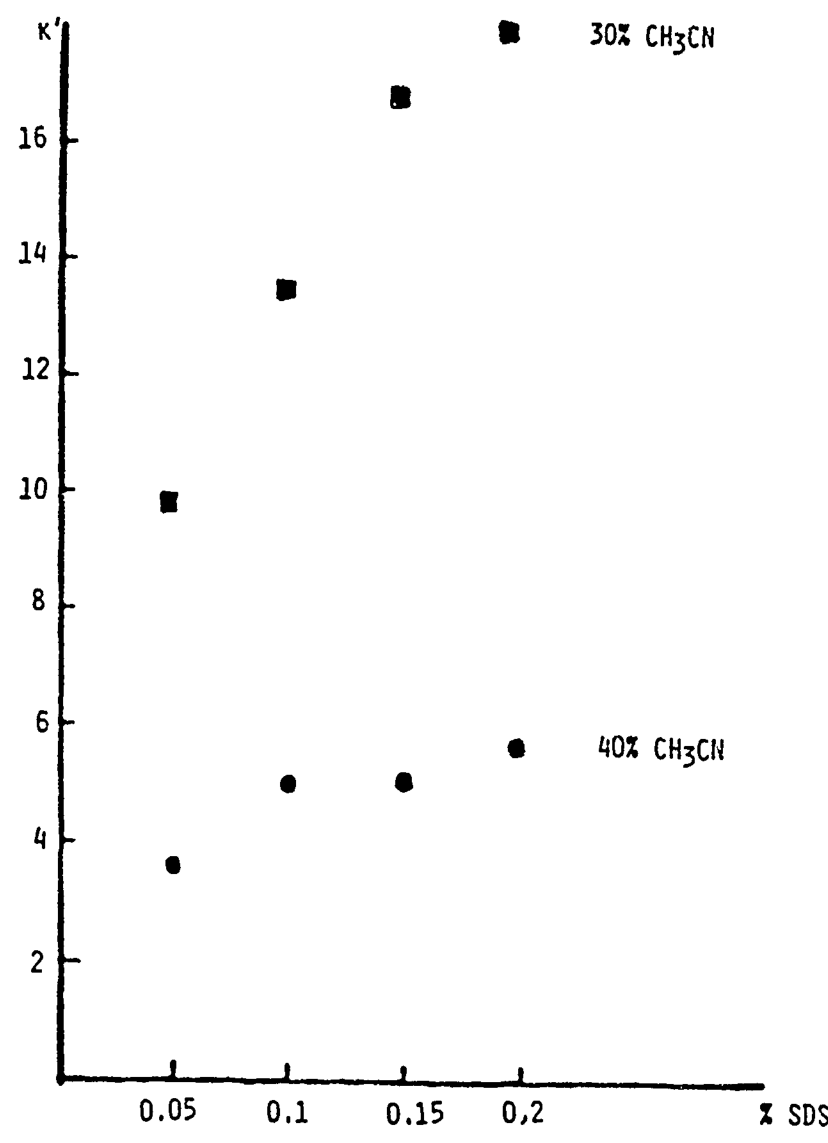

FIG. 5: RELATIONSHIP BETWEEN CAPACITY FACTORS (K') AND % SDS SOLVENT SYSTEM: CH_3CN, 0.1% H_3PO_4, 0.0025 TMA^+Cl^-

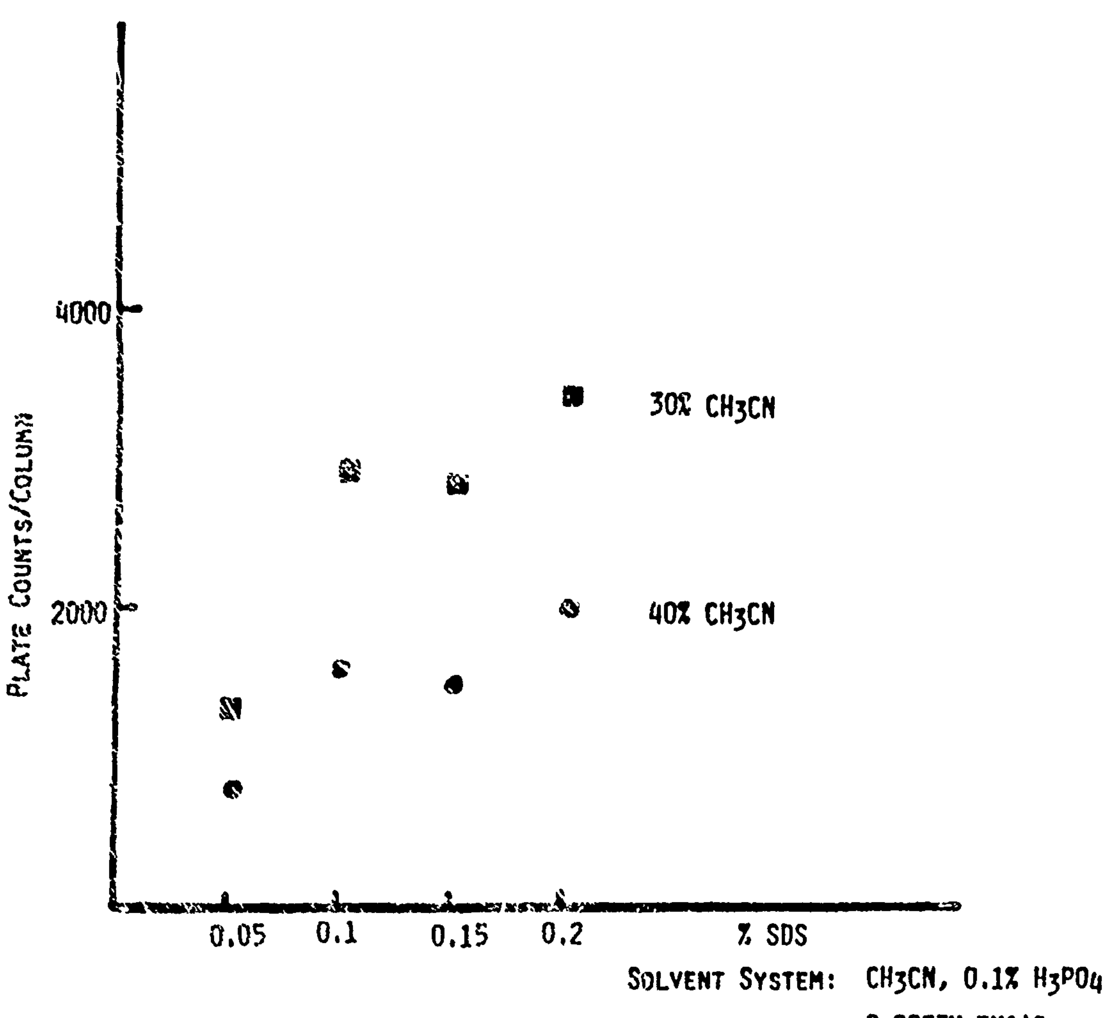

FIG. 6: Relationship between plate counts and %SDS

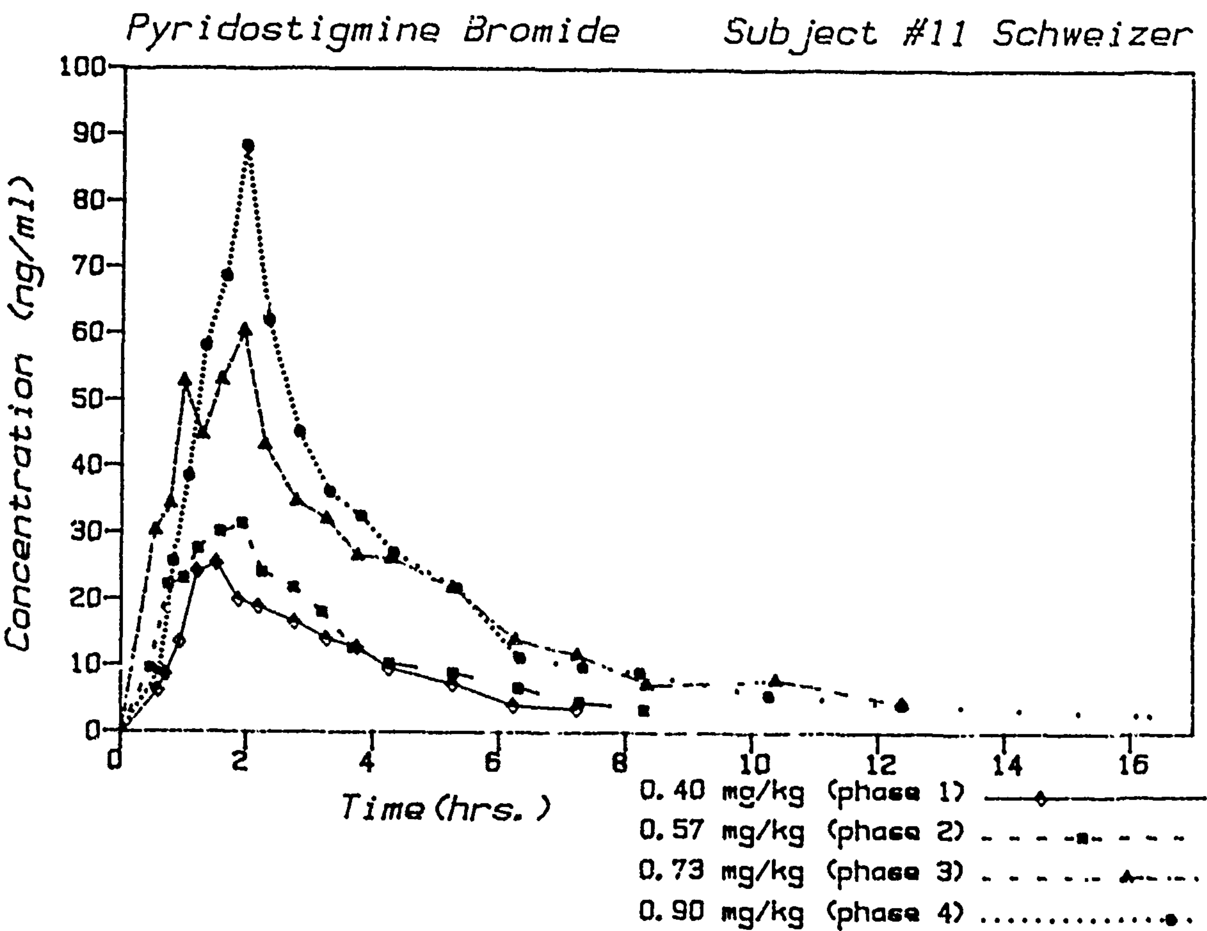

FIG. 8: TIME PROFILE OF ONE HUMAN VOLUNTEER GIVEN PYRIDOSTIGMINE BROMIDE IN SYRUP.

CONCLUSION

A HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF PYRIDOSTIGMINE HAS BEEN DEVELOPED FOR PLASMA. 0.5 ML SAMPLE IS NEEDED FOR ANALYSIS. SAMPLE PREPARATION INVOLVES PROTEIN PRECIPITATION, COLUMN PURIFICATION AND HPLC SEPARATION. THE ASSAY HAS A DETECTION LIMIT OF 2 NG/ML. ASSAY VARIABILITY IS BETWEEN 3% AND 14% AND RECOVERY IS APPROXIMATELY 80%. PYRIDOSTIGMINE SAMPLES ARE STABLE AT -80°C FOR UP TO FOUR MONTHS.

THE ASSAY HAS BEEN USED TO ANALYZE PLASMA SAMPLES FOLLOWING ORAL DOSAGE OF PYRIDOSTIGMINE BROMIDE.

THE EFFECT OF PYRIDOSTIGMINE INJECTIONS ON VITAL FUNCTIONS OF DOGS AT REST AND DURING EXERCISE

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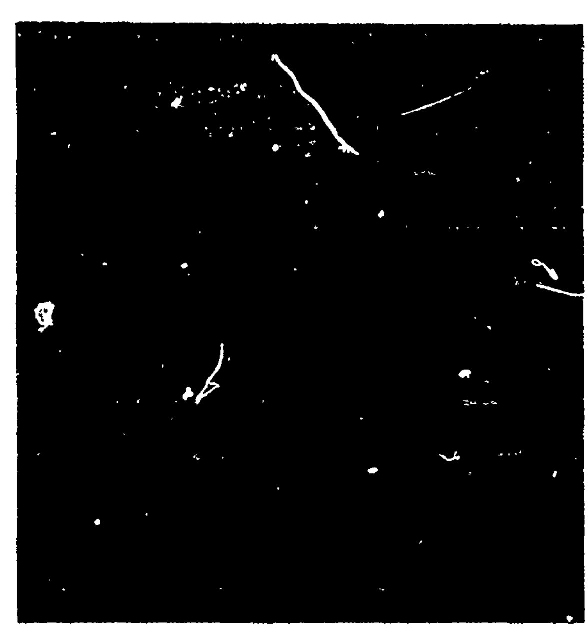
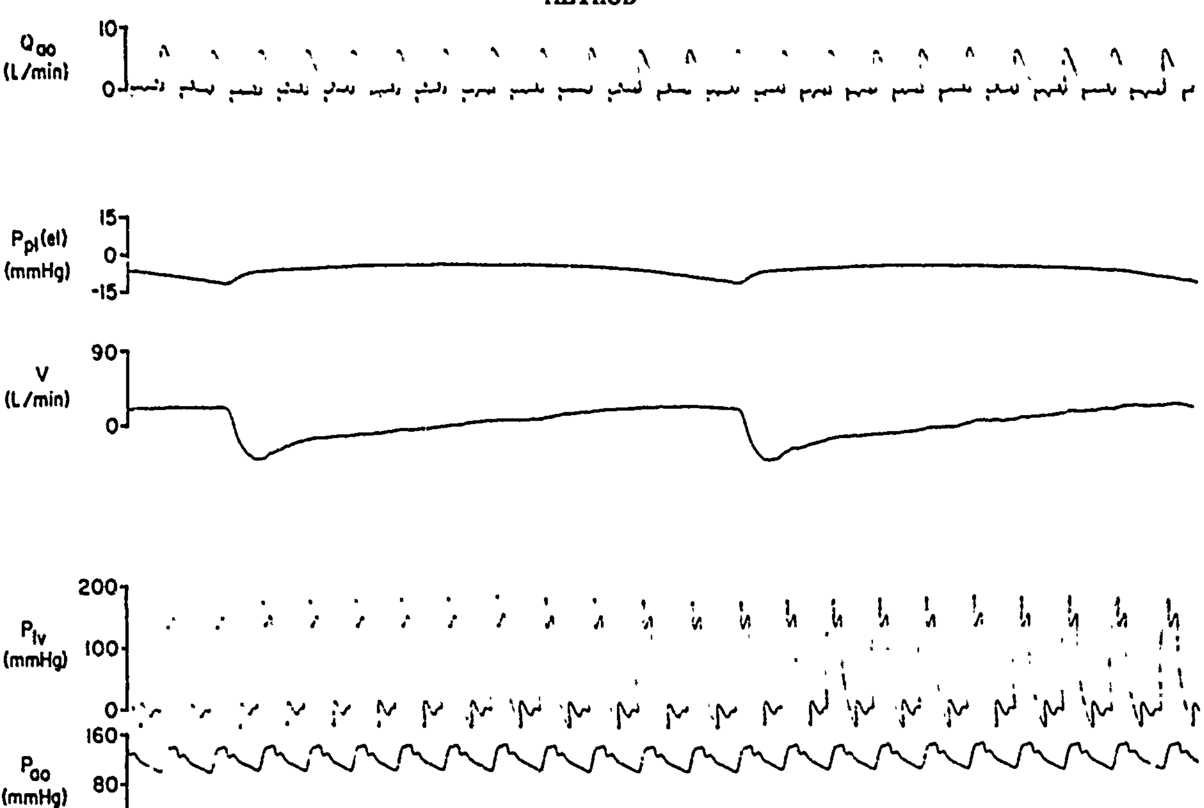
GENERAL CONCLUSION : LOW DOSE PYRIDO. DOES NOT COMPROMISE RESPIRATION AND CIRCULATION DURING EXERCISE MORE THAN IT COMPROMISES THESE FUNCTICTIONS IN THE RESTING STATE. DURING EXERCISE THE VITAL FUNCTIONS ARE RATHER LESS COMPROMISED.

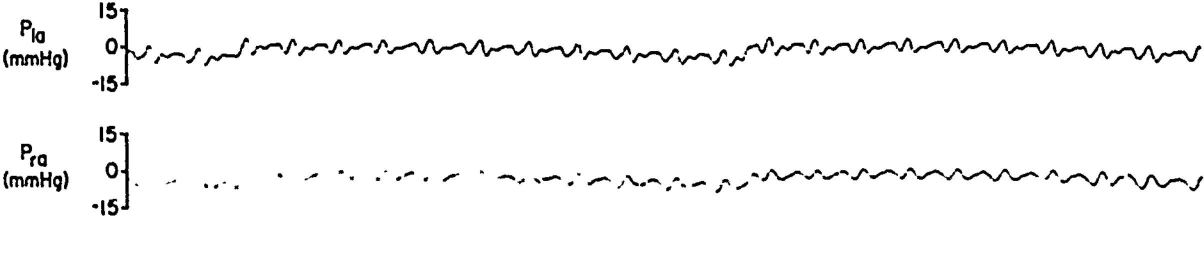
INTRODUCTION

THE EFFECT OF PYRIDOSTIGMINE ON CARDIOVASCULAR AND RESPIRATORY FUNCTIONS, ON BLOOD GASES, AND ON LACTATE AND PYRUVATE OF VENOUS BLOOD WAS STUDIED IN 43 EXPERIMENTS IN A TOTAL OF 10 TRAINED AND INSTRUMENTED DOGS AT REST AND DURING EXERCISE. FIFTEEN MINUTES AFTER I.M. INJECTION OF 1 MG · KG^{-1} OF PYRIDOSTIGMINE A STEADY STATE PLASMA CHOLINESTERASE LEVEL OF 40% OF CONTROL WAS ESTABLISHED.



IHE EFFECT OF A LOWER DOSE OF PYRIDOSTIGMINE ON CARDIOVASCULAR AND RESPIRATORY FUNCTIONS AND ON LACTATE AND PYRUVATE LEVELS OF VENOUS BLOOD WAS STUDIED IN 20 EXPERIMENTS IN A TOTAL OF 6 DOGS AT REST AND DURING EXERCISE. FIFTEEN MINUTES AFTER I.M. INJECTION OF 0.6 MG/KG OF PYRIDOSTIGMINE A STEADY STATE PLASMA CHOLINESTERASE LEVEL OF 55% OF CONTROL WAS ESTABLISHED.





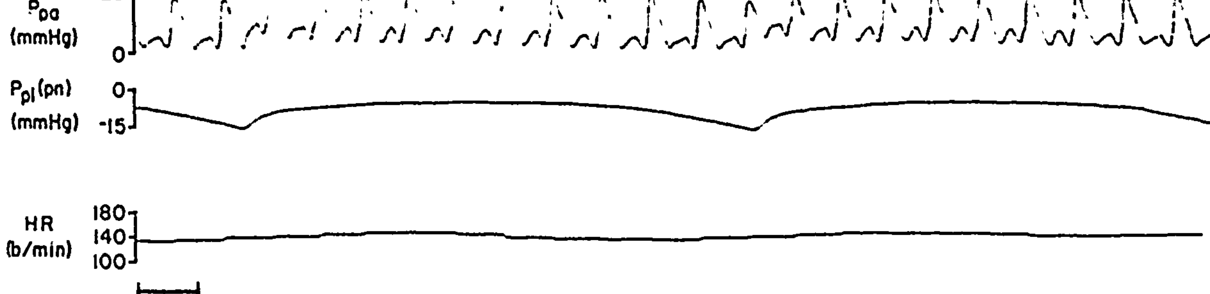


FIGURE 1: RECORDING OF CIRCULATORY AND OTHER FUNCTIONS AT REST.

QAO = AORTIC FLOW, PP(EL) = PLEURAL PRESSURE MONITORED BY PRESSURE TRANSDUCER IN SHELTERED SPACE WITHIN THE CHEST. $\dot{V}$ = RESPIRATORY AIR FLOW, PLV = LEFT VENTRICULAR PRESSURE. PAO = AORTIC PRESSURE, PLA = LEFT ATRIAL PRESSURE, PRA = RIGHT ATRIAL PRESSURE, PPA = PULMONARY ARTERIAL PRESSURE, PP(PN) = PLEURAL PRESSURE MONITORED IN SHELTERED SPACE WITHIN THORAX THROUGH CATHETER CONNECTED TO PRESSURE TRANSDUCER ATTACHED OUTSIDE THE CHEST, HR = HEART RATE.

DERIVED FUNCTIONS

CIRCULATION

(1) LEFT VENTRICULAR TENSION TIME INDEX

$$TTI = \int_{\substack{\text{SYSTOLES} \\ \text{ONE MINUTE}}} P_{LV} \cdot dT$$

(2) CARDIAC EFFECTIVENESS INDEX

$CEI = Q_{AO}/\bar{P}_{LV}$ AND Q_{AO}/TTI

(3) PRESSURE WORK INDEX

$$PW\ INDEX = K_1(P_{AO}SYS \cdot HR) + K_2(0.8 \times P_{AO}SYS + 0.2 \times P_{AO}DIA) \times HR \times SV/BW + 1.43$$

WHERE

$K_1 = 4.08 \times 10^{-4}$
$K_2 = 3.25 \times 10^{-4}$
BW = BODY WEIGHT

RESPIRATION

(4) DYNAMIC COMPLIANCE

$C_{DYN} = V_T/(P_{PL}$ END INSP FROM FRC)

(5) INSPIRATORY PULMONARY RESISTANCE

$R = (P_{PL}$ PK $\dot{V}$ FROM FRC $- 0.5 \times P_{PL}$ END INSP FROM FRC)/(INSP PK $\dot{V}$)

(6) PLEURAL PRESSURE TENSION TIME INDEX

$$PPTI = \int_{\substack{\text{INSPIRATIONS} \\ \text{ONE MINUTE}}} (P_{PL} - P_{PL} \text{ AT FRC}) \cdot dT$$

(7) BREATHING EFFECTIVENESS INDEX

$BEI = V_{MIN}/PPTI$

BLOOD CHEMISTRY

(8) OXYGEN CONSUMPTION

$VO_2 = O_2CT_{(A-V)} \times Q_{AO}$

RESULTS

CIRCULATION - REST

FUNCTION	UNITS	N	CONTROL	PYRIDO	ADJ DIFF	SIG	P
HR	BEATS MIN^{-1}	39	104.05 ± 4.05	97.05 ± 3.51	- 7.00 ± 4.09		0.0913
Q_{AO}	ML MIN^{-1}	38	2401.45 ± 99.16	2320.61 ± 92.59	-80.84 ± 86.46		0.3531
SV	ML	38	23.67 ± 0.68	24.43 ± 0.71	0.77 ± 0.57		0.1847
$P_{AO}SYS$	MMHG	39	139.83 ± 2.71	139.44 ± 3.07	- 0.39 ± 2.98		0.8957
$P_{AO}DIA$	MMHG	39	85.26 ± 2.71	82.65 ± 2.68	- 2.61 ± 2.84		0.3610
$\bar{P}_{AO}$	MMHG	39	106.88 ± 2.74	105.82 ± 2.83	- 1.06 ± 2.88		0.7130
$\bar{P}_{LA}$	MMHG	30	0.63 ± 0.78	2.13 ± 0.58	1.50 ± 0.65		0.0242
$\bar{P}_{RA}$	MMHG	31	-1.95 ± 0.61	-1.22 ± 0.54	0.73 ± 0.47		0.1256
$\bar{P}_{PA}$	MMHG	34	8.77 ± 0.85	11.05 ± 0.72	2.28 ± 0.59	*	0.0003
$\bar{P}_{LV}$	MMHG	35	39.58 ± 1.83	40.85 ± 1.99	1.27 ± 2.02		0.5316
$TM\bar{P}_{AO}$	MMHG	39	115.61 ± 2.57	115.21 ± 2.75	-0.40 ± 2.89		0.8905
$TM\bar{P}_{LA}$	MMHG	30	9.33 ± 0.61	11.40 ± 0.66	2.07 ± 0.75	*	0.0081
$TM\bar{P}_{RA}$	MMHG	31	6.26 ± 0.48	8.05 ± 0.52	1.79 ± 0.58	*	0.0034
$TM\bar{P}_{PA}$	MMHG	34	18.34 ± 0.71	21.14 ± 0.72	2.81 ± 0.60	*	0.0001
$TM\bar{P}_{LV}$	MMHG	35	49.25 ± 1.72	50.46 ± 1.84	1.21 ± 2.04		0.5564
TTI	MMHG	11	36.09 ± 1.75	36.90 ± 2.23	0.81 ± 2.85		0.7797
$Q_{AO}/\bar{P}_{LV}$	ML MIN^{-1} $MMHG^{-1}$	30	56.40 ± 2.05	57.17 ± 2.38	0.77 ± 2.09		0.7151
Q_{AO}/TTI	ML MIN^{-1} $MMHG^{-1}$	11	69.72 ± 5.12	67.96 ± 5.06	-1.76 ± 5.91		0.7692
PW INDEX	ML O_2 MIN^{-1}/100G	38	11.77 ± 0.51	11.14 ± 0.43	-0.64 ± 0.49		0.1985

CIRCULATION - EXERCISE

FUNCTION	UNITS	N	CONTROL	PYRIDO	ADJ DIFF	SIG	P
HR	BEATS MIN^{-1}	39	139.49 ± 4.58	122.26 ± 3.86	-17.23 ± 3.43	*	0.0001
Q_{AO}	ML MIN^{-1}	38	3323.05 ±116.50	3193.40 ±114.05	-129.66 ± 95.92		0.1810
SV	ML	38	24.20 ± 0.62	26.50 ± 0.68	2.30 ± 0.52	*	0.0001
$P_{AO}SYS$	MMHG	35	151.52 ± 2.96	151.56 ± 3.25	0.04 ± 3.58		0.9915
$P_{AO}DIA$	MMHG	39	92.57 ± 2.49	85.34 ± 3.01	-7.23 ± 3.04		0.0202
$\bar{P}_{AO}$	MMHG	39	117.02 ± 2.77	112.33 ± 2.95	-4.69 ± 3.21		0.1491
$\bar{P}_{LA}$	MMHG	32	0.19 ± 0.44	0.88 ± 0.54	0.69 ± 0.59		0.2448
$\bar{P}_{RA}$	MMHG	29	-2.52 ± 0.60	-1.84 ± 0.74	0.69 ± 0.60		0.2569
$\bar{P}_{PA}$	MMHG	34	12.04 ± 1.03	13.62 ± 1.04	1.58 ± 0.50	*	0.0026
$\bar{P}_{LV}$	MMHG	35	55.46 ± 2.36	50.35 ± 1.82	-5.11 ± 1.90	*	0.0093
$TM\bar{P}_{AO}$	MMHG	39	127.18 ± 2.74	123.37 ± 2.95	-3.81 ± 3.19		0.2375
$TM\bar{P}_{LA}$	MMHG	32	10.60 ± 0.54	12.12 ± 0.72	1.52 ± 0.80		0.0615
$TM\bar{P}_{RA}$	MMHG	29	7.73 ± 0.66	9.41 ± 0.76	1.69 ± 0.79		0.0389
$TM\bar{P}_{PA}$	MMHG	34	22.69 ± 1.10	25.34 ± 1.18	2.65 ± 0.72	*	0.0005
$TM\bar{P}_{LV}$	MMHG	35	66.19 ± 2.40	62.08 ± 1.93	-4.11 ± 2.07		0.0[illegible]4
TTI	MMHG	11	50.90 ± 2.47	48.19 ± 2.03	-2.09 ± 2.47		0.[illegible]
$Q_{AO}/\bar{P}_{LV}$	ML MIN^{-1} $MMHG^{-1}$	30	58.94 ± 2.25	62.08 ± 2.18	3.14 ± 2.15		0.14[illegible]
Q_{AO}/TTI	ML MIN^{-1} $MMHG^{-1}$	11	63.35 ± 7.74	71.38 ± 3.95	8.03 ± 7.44		0.2940
PW INDEX	ML O_2 MIN^{-1}/100G	38	16.73 ± 0.63	15.34 ± 0.62	-1.39 ± 0.64		0.0328

HR - HEART RATE, Q_{AO} = CARDIAC OUTPUT, SV STROKE VOLUME, $P_{AO}SYS$, DIA SYSTOLIC,DIASTOLIC BLOOD PRESSURE, $\bar{P}_{AO}$ - MEAN AORTIC PRESSURE, $\bar{P}_{LA}$ = MEAN LEFT ATRIAL PRESSURE, P_{RA} - MEAN RIGHT ATRIAL PRESSURE, $\bar{P}_{PA}$ MEAN PULMONARY ARTERY PRESSURE, $\bar{P}_{LV}$ = MEAN LEFT VENTRICULAR PRESSURE, TM... = TRANSMURAL ...., TTI - LEFT VENTRICULAR TENSION TIME INDEX, $Q_{AO}/\bar{P}_{LV}$ AND Q_{AO}/TTI = INDEXES OF EFFECTIVENESS OF LEFT VENTRICULAR WORK, PW INDEX = PRESSURE WORK INDEX, N = NUMBER OF COMPARISONS MADE, PYRIDO - AFTER PYRIDOSTIGMINE INJECTION, ADJ DIFF - ADJUSTED MEAN DIFFERENCE BETWEEN PYRIDOSTIGMINE VALUES AND CONTROL VALUES, SIG = SIGNIFICANCE, * = $P<0.02$

CIRCULATION

REST

Function	Units	n	Control	Pyrido	Adj Diff	sig	P
HR	beats min⁻¹	20	109.6 ± 5.4	105.4 ± 6.4	-4.2 ± 6.2		0.5007
Q_{ao}	ml min⁻¹	20	2306. ± 91.	2501. ± 216.	195. ± 220.		0.3741
SV	ml	20	22.4 ± 1.7	25.8 ± 3.1	3.4 ± 3.2		0.3004
P_{ao} sys	mm Hg	20	136.4 ± 3.9	137.4 ± 5.3	1.0 ± 4.0		0.8061
P_{ao} dia	mm Hg	20	85.1 ± 4.5	80.8 ± 4.5	-4.3 ± 5.4		0.4293
$\bar{P}_{ao}$	mm Hg	20	105.7 ± 4.1	104.2 ± 4.8	-1.5 ± 5.0		0.7717
$\bar{P}_{la}$	mm Hg	16	-0.72 ± 0.67	1.38 ± 0.51	2.10 ± 0.91	*	0.0454
$\bar{P}_{ra}$	mm Hg	15	-3.44 ± 0.34	-2.07 ± 0.38	1.37 ± 0.46	*	0.0012
$\bar{P}_{pa}$	mm Hg	20	9.77 ± 0.57	11.49 ± 0.75	1.72 ± 0.63	*	0.0093
$\bar{P}_{lv}$	mm Hg	20	43.4 ± 2.8	42.4 ± 3.1	-1.0 ± 2.9		0.7323
TM$\bar{P}_{ao}$	mm Hg	16	119.0 ± 4.4	117.3 ± 5.7	-1.7 ± 5.8		0.7670
TM$\bar{P}_{la}$	mm Hg	12	8.43 ± 0.84	11.05 ± 0.95	2.62 ± 1.06		0.0223
TM$\bar{P}_{ra}$	mm Hg	13	5.13 ± 0.85	7.79 ± 0.85	2.66 ± 0.93	*	0.0091
TM$\bar{P}_{pa}$	mm Hg	16	18.84 ± 0.68	21.4 ± 1.0	2.56 ± 1.11	*	0.0182
TM$\bar{P}_{lv}$	mm Hg	16	56.3 ± 3.2	54.7 ± 3.8	-1.6 ± 3.8		0.6789
TTI	mm Hg	20	37.5 ± 2.2	36.8 ± 2.5	-0.7 ± 2.2		0.7309
$Q_{ao}/\bar{P}_{lv}$	ml min⁻¹ mmHg⁻¹	20	56.9 ± 4.7	64.8 ± 6.5	7.9 ± 6.9		0.2612
Q_{ao}/TTI	ml min⁻¹ mmHg⁻¹	20	66.6 ± 5.5	72.4 ± 7.3	5.8 ± 7.5		0.4375
PW Index	ml s, mm⁻² /1000	20	11.82 ± 0.60	12.19 ± 0.90	0.37 ± 0.58		0.5288

EXERCISE

Function	Units	n	Control	Pyrido	Adj Diff	sig	P
HR	beats min⁻¹	20	147.4 ± 5.9	139.1 ± 4.6	-8.3 ± 5.4		0.1335
Q_{ao}	ml min⁻¹	20	3182. ± 114	3476. ± 202	294. ± 181		0.1144
SV	ml	20	22.5 ± 1.4	25.6 ± 1.7	3.1 ± 1.1	*	0.0085
P_{ao} sys	mm Hg	20	149.1 ± 5.2	147.4 ± 5.2	-1.7 ± 3.6		0.6374
P_{ao} dia	mm Hg	20	93.2 ± 4.7	83.8 ± 3.9	-9.4 ± 4.3		0.0358
$\bar{P}_{ao}$	mm Hg	20	117.0 ± 4.8	111.5 ± 4.4	-5.5 ± 3.9		0.1658
$\bar{P}_{la}$	mm Hg	16	0.48 ± 0.79	2.13 ± 0.80	1.65 ± 0.78		0.0487
$\bar{P}_{ra}$	mm Hg	15	-3.29 ± 0.57	-2.08 ± 0.67	1.21 ± 0.85		0.1666
$\bar{P}_{pa}$	mm Hg	20	13.68 ± 0.94	15.22 ± 0.95	1.54 ± 1.09		0.1662
$\bar{P}_{lv}$	mm Hg	20	58.0 ± 3.0	55.8 ± 2.5	-2.2 ± 2.7		0.4296
TM$\bar{P}_{ao}$	mm Hg	16	131.5 ± 5.5	124.3 ± 5.3	-7.2 ± 4.3		0.1052
TM$\bar{P}_{la}$	mm Hg	12	11.5 ± 1.0	13.1 ± 1.0	1.6 ± 0.9		0.0907
TM$\bar{P}_{ra}$	mm Hg	13	7.5 ± 1.2	8.42 ± 0.84	0.92 ± 1.45		0.5401
TM$\bar{P}_{pa}$	mm Hg	16	24.7 ± 1.3	25.18 ± 0.93	0.48 ± 1.58		0.7611
TM$\bar{P}_{lv}$	mm Hg	16	72.5 ± 3.0	67.6 ± 3.1	-4.9 ± 2.7		0.0840
TTI	mm Hg	20	50.3 ± 2.6	48.7 ± 2.3	-1.6 ± 2.4		0.5066
$Q_{ao}/\bar{P}_{lv}$	ml min⁻¹ mmHg⁻¹	20	59.2 ± 4.5	66.7 ± 4.3	7.5 ± 3.3		0.0311
Q_{ao}/TTI	ml min⁻¹ mmHg⁻¹	20	67.4 ± 5.0	73.5 ± 4.8	6.1 ± 3.3		0.0770
PW Index	ml s, mm⁻² /1000	20	16.82 ± 0.93	16.67 ± 0.97	-0.15 ± 0.84		0.8595

RESPIRATION - REST

FUNCTION	UNITS	N	CONTROL	PYRIDO	ADJ DIFF	SIG	P
$\bar{P}_{PL}$	MMHG	43	- 8.78 ± 0.48	- 9.51 ± 0.49	- 0.73 ± 0.50		0.1498
P_{PL} AT FRC	MMHG	43	- 8.57 ± 0.43	- 9.00 ± 0.51	- 0.43 ± 0.51		0.4052
P_{PL} PK EXP	MMHG	42	3.36 ± 0.61	4.88 ± 0.65	1.47 ± 0.55	*	0.0095
P_{PL} PK $\dot{V}$	MMHG	43	- 4.22 ± 0.29	- 5.07 ± 0.63	- 0.85 ± 0.63		0.1841
P_{PL} LOWEST	MMHG	43	- 6.49 ± 0.44	- 8.02 ± 0.48	- 1.53 ± 0.42	*	0.0004
P_{PL} END INSP	MMHG	43	- 4.35 ± 0.38	- 4.09 ± 0.66	0.27 ± 0.70		0.7035
PPTI	MMHG	40	1.27 ± 0.09	1.79 ± 0.11	0.52 ± 0.10	*	0.0001
V_{MIN}	$L \cdot MIN^{-1}$	40	14.88 ± 0.89	18.33 ± 1.29	3.45 ± 1.32	*	0.0113
F	MIN^{-1}	43	38.2 ± 3.5	60.5 ± 7.2	22.4 ± 5.1	*	0.0001
V_T	ML	40	467. ± 29.	437. ± 32.	-31. ± 23.		0.1886
INSP PK $\dot{V}$	$L \cdot MIN^{-1}$	42	52.2 ± 2.6	58.7 ± 3.2	6.5 ± 3.6		0.0754
TI/TT	—	41	0.42 ± 0.01	0.45 ± 0.011	0.032 ± 0.012	*	0.0104
VMIN/PPTI	$L \cdot MIN^{-1}$/MMHG	38	13.6 ± 1.0	11.9 ± 1.0	- 1.62 ± 0.95		0.0921
C_{DYN}	ML/MMHG	40	143. ± 20.	114. ± 12.	-29. ± 20.		0.1576
R	MMHG/$L \cdot MIN^{-1}$	37	0.0482 ± 0.0051	0.0665 ± 0.0054	0.0177 ± 0.0061	*	0.0047

RESPIRATION - EXERCISE

FUNCTION	UNITS	N	CONTROL	PYRIDO	ADJ DIFF	SIG	P
$\bar{P}_{PL}$	MMHG	43	- 10.59 ± 0.38	- 11.59 ± 0.48	- 1.00 ± 0.48		0.0415
P_{PL} AT FRC	MMHG	43	- 9.08 ± 0.44	- 9.66 ± 0.48	- 0.58 ± 0.47		0.2210
P_{PL} PK EXP	MMHG	43	6.54 ± 0.57	7.39 ± 0.55	0.85 ± 0.44		0.0564
P_{PL} PK $\dot{V}$	MMHG	43	- 5.34 ± 0.36	- 6.72 ± 0.43	- 1.38 ± 0.50	*	0.0070
P_{PL} LOWEST	MMHG	43	- 9.96 ± 0.62	- 12.55 ± 0.76	- 2.59 ± 0.68	*	0.0003
P_{PL} END INSP	MMHG	43	- 4.29 ± 0.39	- 5.64 ± 0.52	1.35 ± 0.48	*	0.0065
PPTI	MMHG	41	2.48 ± 0.17	3.01 ± 0.21	0.53 ± 0.21	*	0.0149
V_{MIN}	$L \cdot MIN^{-1}$	40	28.84 ± 1.85	28.92 ± 2.07	0.07 ± 1.65		0.9651
F	MIN^{-1}	43	83.49 ± 7.82	92.88 ± 9.50	9.40 ± 3.63	*	0.0116
V_T	ML	42	425. ± 28.	405. ± 28.	-20. ± 22.		0.3534
INSP PK $\dot{V}$	$L \cdot MIN^{-1}$	42	82.1 ± 4.2	86.3 ± 4.5	4.1 ± 4.3		0.3372
TI/TT	—	43	0.5206 ± 0.0096	0.511 ± 0.010	- 0.0097 ± 0.0093		0.2986
VMIN/PPTI	$L \cdot MIN^{-1}$/MMHG	41	13.9 ± 1.3	12.0 ± 1.4	- 1.9 ± 1.5		0.2079
C_{DYN}	ML/MMHG	42	155. ± 24.	125. ± 27.	-30. ± 30.		0.3160
R	MMHG/$L \cdot MIN^{-1}$	41	0.0431 ± 0.0036	0.0527 ± 0.0047	0.0096 ± 0.0058		0.1002

$\bar{P}_{PL}$ = MEAN PLEURAL PRESSURE, P_{PL} AT FRC = PLEURAL PRESSURE AT FRC, P_{PL} PK EXP** = PEAK EXPIRATORY PLEURAL PRESSURE, P_{PL} PK V** = PLEURAL PRESSURE AT PEAK INSPIRATORY FLOW, P_{PL} LOWEST ** = LOWEST PLEURAL PRESSURE, P_P END INSP ** = PLEURAL PRESSURE AT END INSPIRATION, PPTI = PLEURAL PRESSURE TENSION TIME INDEX, VMIN = MINUTE VOLUME, F = RESPIRATORY FREQUENCY, V_T = TIDAL VOLUME, INSP PK V = INSPIRATORY PEAK FLOW, T_I/T_T = INSPIRATORY TIME ÷ TOTAL RESPIRATORY CYCLE TIME, V_{MIN}/ PPTI = INDEX OF EFFECTIVENESS OF INSPIRATORY MUSCLE WORK, C_{DYN} = DYNAMIC COMPLIANCE, R = INSPIRATORY PULMONARY AIRWAYS RESISTANCE, N = NUMBER OF COMPARISONS MADE, PYRIDO = AFTER PYRIDOSTIGMINE INJECTIONS, ADJ DIFF = ADJUSTED MEAN DIFFERENCE BETWEEN PYRIDOSTIGMINE VALUES AND CONTROL VALUES, SIG = SIGNIFICANCE, * = $P<0.02$. ** - THESE VALUES ARE ALL TAKEN FROM FRC

RESPIRATION

REST

Function	Units	n	Control	Pyrido	Adj Diff	sig	P
$\bar{P}_{pl}$	mmHg	16	-8.62 ± 0.56	-9.49 ± 0.68	-0.87 ± 0.63		0.1911
P_{pl} at FRC	mmHg	16	-7.97 ± 0.60	-8.55 ± 0.70	-0.58 ± 0.58		0.3318
P_{pl} [illegible]	mmHg	16	1.47 ± 0.23	3.03 ± 0.62	1.56 ± 0.61	*	0.0161
P_{pl} [illegible] V	mmHg	16	-4.96 ± 0.46	-6.27 ± 0.98	-1.31 ± 0.71		0.0770
P_{pl} [illegible]	mmHg	16	-6.33 ± 0.82	-7.8 ± 1.3	-1.47 ± 0.85		0.1039
P_{pl} [illegible]	mmHg	16	-3.62 ± 0.79	-3.75 ± 0.95	-0.13 ± 0.57		0.8266
PPTI	mmHg	16	1.18 ± 0.16	1.59 ± 0.33	0.41 ± 0.16	*	0.0147
V_{min}	$L \cdot min^{-1}$	20	12.20 ± 0.86	14.8 ± 1.1	2.60 ± 1.09		0.0216
f	min^{-1}	20	38.1 ± 4.6	54.7 ± 6.6	16.6 ± 4.1		0.0003
V_T	ml	20	366. ± 26.	294. ± 19	-72. ± 23.		0.0044
[illegible] V	$L \cdot min^{-1}$	20	44.0 ± 2.7	50.3 ± 3.3	6.3 ± 3.4		0.0707
Ti/Tt		20	0.407 ± 0.012	0.433 ± 0.014	0.024 ± 0.016		0.1469
V_{min}/PPTI	$L \cdot min^{-1}$/mmHg	16	12.6 ± 1.3	13.3 ± 2.1	0.7 ± 1.7		0.6745
C_{dyn}	ml/mmHg	16	169. ± 34.	173. ± 35.	4. ± 35.		0.9121
R	mmHg/$L \cdot min^{-1}$	16	0.0720 ± 0.0070	0.096 ± 0.017	0.0240 ± 0.0110		0.1128

EXERCISE

Function	n	Control	Pyrido	Adj Diff	sig	P
$\bar{P}_{pl}$	16	-10.59 ± 0.60	-10.34 ± 0.75	0.25 ± 0.35		0.7405
P_{pl} at FRC	16	-9.43 ± 0.74	-9.32 ± 0.75	0.11 ± 0.94		0.8932
P_{pl} [illegible]	16	3.51 ± 0.41	4.54 ± 0.50	1.03 ± 0.46		0.0342
P_{pl} [illegible] V	16	-5.12 ± 0.54	-5.78 ± 0.60	-0.66 ± 0.60		0.2824
P_{pl} [illegible]	16	-7.64 ± 0.93	-8.07 ± 0.98	-0.43 ± 0.83		0.6056
P_{pl} [illegible]	16	-3.77 ± 0.67	-3.75 ± 0.67	0.02 ± 0.19		0.9700
PPTI	16	2.15 ± 0.33	2.16 ± 0.33	0.01 ± 0.27		0.9538
V_{min}	18	27.4 ± 2.8	27.9 ± 2.3	0.5 ± 2.2		0.8129
f	18	90 ± 12.	105. ± 11.	15. ± 9.		0.1135
V_T	18	342 ± 25.	298. ± 25	-44. ± 18.	*	0.0277
[illegible] V	18	88.7 ± 8.4	88.8 ± 6.5	0.1 ± 7.2		0.9797
Ti/Tt	20	0.535 ± 0.019	0.546 ± 0.019	0.011 ± 0.020		0.6056
V_{min}/PPTI	14	18.6 ± 2.8	17.9 ± 2.9	-0.7 ± 2.1		0.7304
C_{dyn}	14	139. ± 21.	141 ± 27	2. ± 30		0.9283
R	14	0.0364 ± 0.0051	0.057 ± 0.016	0.0206 ± 0.0117		0.1679

BLOOD CHEMISTRY - REST

Function	Units	N	Control	Pyrido	Adj Diff	Sig	P
pH_{AO}		40	7.389 ± 0.005	7.398 ± 0.005	0.009 ± 0.005		0.1056
$pH_{\bar{V}}$		40	7.361 ± 0.004	7.363 ± 0.005	0.002 ± 0.005		0.6716
$PO_{2\,AO}$	mmHg	40	85.81 ± 1.31	82.56 ± 1.24	- 3.24 ± 1.61		0.0481
$PO_{2\,\bar{V}}$	mmHg	40	41.92 ± 1.30	38.70 ± 0.80	- 3.22 ± 1.42		0.0266
$PCO_{2\,AO}$	mmHg	40	33.27 ± 0.47	31.28 ± 0.41	- 1.99 ± 0.54	*	0.0005
$PCO_{2\,\bar{V}}$	mmHg	40	38.30 ± 0.45	37.75 ± 0.55	- 0.55 ± 0.62		0.3790
O_2CT_{AO}	ml O_2/100ml of blood	40	15.24 ± 0.31	15.20 ± 0.32	- 0.05 ± 0.28		0.8663
$O_2CT_{\bar{V}}$	ml O_2/100ml of blood	40	9.60 ± 0.27	8.99 ± 0.29	- 0.61 ± 0.27		0.0254
$O_2CT_{(A-V)}$	ml O_2/100ml of blood	40	5.64 ± 0.20	6.21 ± 0.19	0.56 ± 0.20	*	0.0054
$\dot{V}O_2$	ml O_2 min^{-1}	35	135.9 ± 7.3	146.1 ± 7.4	10.2 ± 7.5		0.1780
LACT $\bar{V}$	mM	21	0.445 ± 0.046	0.609 ± 0.052	0.164 ± 0.046	*	0.0011
PYR $\bar{V}$	µM	11	89. ± 11.	97.9 ± 9.1	9. ± 14.		0.5542
LACT/PYR		11	5.89 ± 0.56	7.14 ± 0.73	1.25 ± 0.65		0.0703

BLOOD CHEMISTRY - EXERCISE

Function	Units	N	Control	Pyrido	Adj Diff	Sig	P
pH_{AO}		42	7.403 ± 0.006	7.411 ± 0.006	0.008 ± 0.007		0.2850
$pH_{\bar{V}}$		41	7.361 ± 0.006	7.362 ± 0.006	0.002 ± 0.006		0.7731
$PO_{2\,AO}$	mmHg	42	84.25 ± 1.03	83.65 ± 1.11	- 0.60 ± 1.32		0.6475
$PO_{2\,\bar{V}}$	mmHg	41	34.74 ± 0.69	34.68 ± 0.74	- 0.06 ± 0.89		0.9497
$PCO_{2\,AO}$	mmHg	42	31.73 ± 0.50	30.23 ± 0.53	- 1.51 ± 0.56	*	0.0083
$PCO_{2\,\bar{V}}$	mmHg	41	39.18 ± 0.62	38.83 ± 0.66	- 0.34 ± 0.69		0.6197
O_2CT_{AO}	ml O_2/100ml of blood	42	15.37 ± 0.31	15.23 ± 0.32	- 0.14 ± 0.28		0.6192
$O_2CT_{\bar{V}}$	ml O_2/100ml of blood	41	8.06 ± 0.33	7.64 ± 0.34	- 0.42 ± 0.26		0.1122
$O_2CT_{(A-V)}$	ml O_2/100ml of blood	41	7.27 ± 0.21	7.55 ± 0.18	0.28 ± 0.19		0.1434
$\dot{V}O_2$	ml O_2 min^{-1}	36	243. ± 12.	244. ± 11.	1. ± 12.		0.9243
LACT $\bar{V}$	mM	21	0.427 ± 0.048	0.557 ± 0.046	0.130 ± 0.044	*	0.0055
PYR $\bar{V}$	µM	11	72.0 ± 8.1	82.6 ± 6.2	11. ± 10.		0.3161
LACT/PYR		11	6.83 ± 0.54	7.79 ± 0.96	0.95 ± 0.78		0.2350

AO = ARTERIAL VALUES, $\bar{V}$ = MIXED VENOUS VALUES, O_2CT - OXYGEN CONTENT, (A-V) = A-V DIFFERENCE, $\dot{V}O_2$ = OXYGEN CONSUMPTION, LACT $\bar{V}$ = VENOUS LACTATE CONCENTRATION, PYR $\bar{V}$ = VENOUS PYRUVATE CONCENTRATE, N = NUMBER OF COMPARISONS MADE, PYRIDO = AFTER PYRIDOSTIGMINE INJECTIONS, ADJ DIFF = ADJUSTED MEAN DIFFERENCE BETWEEN PYRIDOSTIGMINE VALUES AND CONTROL VALUES, SIG = SIGNIFICANCE, * = P<0.02.

BLOOD CHEMISTRY

Function	Units	REST n	Control	Pyrido	Adj Diff	sig	P	EXERCISE n	Control	Pyrido	Adj Diff	sig	P
Lact	mM	11	0.640±[illegible]	0.695±0.038	0.055±[illegible]		0.2135	13	0.590±0.055	0.708±0.084	0.118±0.019		0.1144
PYR	uM	11	59.4±3.3	56.1±5.5	-3.3±6.1		0.6037	11	52.4±4.5	47.6±5.6	-4.8±6.9		0.4996
Lact/PYR		11	1.071±0.079	1.286±0.057	0.214±0.100		0.0462	11	1.036±0.055	2.6±1.4	1.564±1.331		0.1430

CONCLUSIONS

THE ADMINISTRATION OF 1 MG/KG PYRIDOSTIGMINE I.M. LOWERED CHOLINESTERASE IN PLASMA TO 40% OF CONTROL VALUES. IT CAUSED SALIVATION, MUCUS FORMATION, DEFECATION AND URINATION.

CIRCULATION:

AT REST AFTER PYRIDOSTIGMINE ADMINISTRATION, CARDIAC OUTPUT, LEFT VENTRICULAR WORK AND EFFECTIVENESS OF LEFT VENTRICULAR WORK REMAINED UNCHANGED. THE REASONS FOR THIS WERE PROBABLY THAT (A) THE EQUILIBRIUM OF AUTONOMIC ACTIVITY ON HEART WAS NOT GREATLY CHANGED (JUDGING BY THE FACT THAT HEART RATE DECREASE DID NOT REACH SIGNIFICANCE) AND (B) LEFT AND RIGHT ATRIAL PRESSURES WERE ELEVATED (FRANK-STARLINGS MECHANISM). AORTIC PRESSURE REMAINED UNCHANGED BUT PULMONARY ARTERY PRESSURE WAS ELEVATED.

DURING EXERCISE CARDIAC OUTPUT, LEFT VENTRICULAR WORK AND EFFECTIVENESS OF LEFT VENTRICULAR WORK REMAINED UNCHANGED EVEN THOUGH THERE WAS ENOUGH PREVALENCE OF THE PARASYMPATHETIC EFFECT ON THE HEART TO DIMINISH HEART RATE AND LEFT VENTRICULAR PRESSURE AGAINST THE VALUES AT CONTROL EXERCISE. DURING EXERCISE PYRIDOSTIGMINE DID NOT ELEVATE LEFT AND RIGHT ATRIAL PRESSURES, BUT IT ELEVATED PULMONARY ARTERY PRESSURE.

THE EFFECT OF THE EMPLOYED DOSAGE OF PYRIDOSTIGMINE ON CIRCULATION IS COMPATIBLE WITH THE EXPLANATION THAT PYRIDOSTIGMINE ENHANCES NOT ONLY MUSCARINIC ACTIVITY ON THE NERVE ENDINGS TO THE HEART BUT IT ENHANCES ALSO THE TRANSMISSION IN BOTH, THE PARASYMPATHETIC AND THE SYMPATHETIC GANGLIA. OUR FINDINGS SEEMS TO CONFIRM THAT PARASYMPATHETIC NERVOUS FUNCTIONS HAVE A CONSTRICTIVE EFFECT ON THE PULMONARY ARTERY.

RESPIRATION:

AT REST THE EFFECT OF PYRIDOSTIGMINE CONSISTS OF (A) AN INCREASE IN CENTRAL RESPIRATORY DRIVE, CHARACTERIZED BY AN INCREASE IN RESPIRATORY FREQUENCY AND IN MINUTE VOLUME AND (B) AN INCREASE IN RESPIRATORY RESISTANCE, JUDGED BY THE INCREASE IN DERIVED INSPIRATORY RESISTANCE. THE ELEVATION OF EXPIRATORY PLEURAL PRESSURE, THE LOWERING OF INSPIRATORY PLEURAL PRESSURE AND THE INCREASE IN THE RATIO OF INSPIRATORY TIME TO RESPIRATORY CYCLE TIME AND THE INCREASE IN THE PLEURAL PRESSURE TENSION TIME INDEX COULD BE CAUSED BY BOTH (A) AND (B).

DURING EXERCISE RESPIRATORY FREQUENCY IS INCREASED, BUT MINUTE VOLUME IS NOT CHANGED FROM CONTROL EXERCISE. THE INSPIRATORY PLEURAL PRESSURES ARE FURTHER FROM PLEURAL PRESSURE AT FRC THAN IN CONTROL EXERCISE. BUT EXPIRATORY PLEURAL PRESSURE AND DERIVED INSPIRATORY RESISTANCE ARE UNCHANGED FROM CONTROL EXERCISE.

THE INCREASE IN CENTRAL RESPIRATORY DRIVE COULD BE CAUSED REFLEXLY THROUGH AN EFFECT ON CHEMORECEPTORS IN THE ARTERIAL BED. THE INCREASE IN RESPIRATORY RESISTANCE COULD BE CAUSED PARTLY BY THE PARASYMPATHETIC EFFECT ON THE BRONCHIAL TREE, PARTLY BY ENHANCED MUCUS FORMATION. IT SEEMS THAT THE BRONCHIAL DILATION CONNECTED WITH EXERCISE MITIGATED THE ENHANCEMENT OF RESPIRATORY RESISTANCE CAUSED BY PYRIDOSTIGMINE.

BLOOD CHEMISTRY

DUE TO THE ENHANCED RESPIRATION AFTER PYRIDOSTIGMINE ADMINISTRATION THE ARTERIAL PCO_2 WAS LOWER THAN DURING CONTROL RESTING STATE AND EXERCISE. VENOUS BLOOD LACTATE WAS SIGNIFICANTLY ELEVATED. THE PYRUVATE TO LACTATE RATIO REMAINED UNALTERED. THIS INDICATES EITHER AN INCREASED GLYCOLYTIC ENERGY METABOLISM OR A DECREASED REUTILIZATION OF LACTATE AND PYRUVATE, BUT WITHOUT ADVERSE EFFECT ON MITOCHONDRIAL OXIDATION.

THE ADMINISTRATION OF 0.6 MG/KG PYRIDOSTIGMINE I.M. LOWERED CHOLINESTERASE IN PLASMA TO 55% OF CONTROL VALUES. THIS CAUSED LESS MUCOUS FORMATION AND SALIVATION AND LESS FREQUENT URINATION AND DEFECATION.

IN THE RESTING DOG, THE CIRCULATORY AND RESPIRATORY CHANGES OBSERVED WITH 0.6 MG/KG DOSE WERE IDENTICAL WITH THOSE OBTAINED WITH 1.0 MG/KG DOSE. WITH THE LOWER DOSE, THE INCREASE IN THE PULMONARY RESISTANCE AND THE DECREASE IN THE LOWEST PLEURAL PRESSURE DID NOT REACH A SIGNIFICANCE LEVEL. IN THE EXERCISING DOG, 0.6 MG/KG DOSE CAUSED NO SUBSTANTIAL CIRCULATORY AND RESPIRATORY CHANGES. THE CHANGES IN THE LACTATE AND PYRUVATE LEVELS IN VENOUS BLOOD WITH BOTH DOSES WERE SIMILAR BUT THE INCREASE IN THE LACTATE LEVELS WITH THE LOW DOSE DID NOT REACH A SIGNIFICANCE LEVEL BOTH AT REST AND EXERCISE.

WE CONCLUDE THAT THE INCREASE IN SYMPATHETIC NERVOUS ACTIVITY DURING EXERCISE OVERCOMES THE EFFECT OF PLASMA CHOLINESTERASE INHIBITION TO 55% OF CONTROL.

DECREASED TWITCH FORCE IN RAT SKELETAL MUSCLE FOLLOWING CONSTANT, SUBLIMINAL INFUSION OF PYRIDOSTIGMINE

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ABSTRACT

Recent work suggests that pyridostigmine is a more effective prophylactic treatment for organophosphorus intoxication than other carbamates. Its protective index is greater than that of physostigmine and, unlike physostigmine, its distribution is limited to non-CNS tissues. However, since pyridostigmine is an inhibitor of cholinesterases, it produces a variety of cholinergic signs including tremors and changes in neuromuscular function which lead eventually to myopathy. The purpose of this study was to determine if the physiologic responsiveness of skeletal muscle is also changed during pyridostigmine exposures which produce minimal cholinergic signs in the animal and no observable muscle wasting.

Two schedules of pyridostigmine dosing were used: single daily ip injections of 2 mg/kg and constant sc infusion via implanted Alzet pumps at an average daily dose of 5 mg/kg. Male Sprague Dawley rats were treated with each dose schedule, with groups of 5 animals withdrawn from the study after 1, 4, 10 and 20 days of treatment. Two saline control groups for each schedule were followed for 4 and 20 days. Each rat was anesthetized with urethane (1.5 g/kg, ip) and the hindlimb dissected to expose the sciatic nerve and triceps surae muscles. Bipolar electrodes were used to stimulate the sciatic nerve, resulting in a maximal twitch contraction which was recorded using a force displacement transducer. Ten second duration trains of 20, 50 and 100 Hz stimuli were also used to assess contracture and fatigue of the muscle.

Alzet pump infusion of pyridostigmine did not produce any overt signs of cholinergic intoxication and no change in muscle mass. However, pyridostigmine infusion produced significant reductions in supramaximal twitch tension, as well as in the total and peak tension generated by tetanic stimulation at 50 and 100 Hz. Muscle force remained at about 80% of control throughout the experimental period. IP administration of pyridostigmine, on the other hand, produced no significant changes in single twitch tension, tetanic contraction nor muscle mass. Immediately after each dose ip the animals exhibited some cholinergic signs (tremor, labored breathing). These attenuated in several hours and there were no overt signs 24 hours after the last dose of drug, the time at which muscle measurements were made. Neither dosing schedule significantly depressed plasma cholinesterase nor erythrocyte acetylcholinesterase activity at the time of the experiment. These results show that constant, low level exposure to pyridostigmine attenuates the ability of skeletal muscle to generate tension. This effect appears not to be related to inhibition of cholinesterases and is not a reflection of muscle atrophy. The data suggest that pyridostigmine pretreatment schedules must be carefully designed to avoid not only cholinergic signs but also detrimental effects on skeletal muscle contractility.

This work supported in part by the U.S Army Medical Research and Development Command under Contract DAMD-17-83-C-3187

PURPOSE

Pyridostigmine has been proposed as a prophylactic treatment for the acute cholinergic intoxication produced by irreversible cholinesterase inhibitors such as nerve agents. However, prolonged exposure to cholinesterase inhibitors produce changes in neuromuscular function and can lead to skeletal muscle myopathy. The purpose of this study was to determine whether the physiologic responsiveness of skeletal muscle was changed during exposure to pyridostigmine at doses which are below that necessary for development of cholinergic signs and muscle wasting.

METHODS

1. Male Sprague-Dawley rats were dosed with pyridostigmine in groups of 5 according to one of two schedules:
   (a) Single daily ip injection of 2 mg/kg; or
   (b) Alzet pump sc infusion at an average daily dose of 5 mg/kg.

2. Rats were withdrawn after 1, 4, 10 or 20 days of exposure. Saline control groups were compared to the 4 and 20 day groups.

3. Rats were anesthetized with urethane (1.5 g/kg) and the hindlimb dissected for recording muscle twitch tension from the triceps surae (see Figure 1).

4. Supramaximal pulses (0.05 msec duration) were applied to the sciatic nerve at a rate of 0.2 Hz. At intervals of 20 minutes the stimulus was increased to either 20, 50 or 100 Hz for a 10 second train.

5. Computer assisted analysis was used to calculate the twitch tension at onset and offset of the tetanic train, as well as the average tension during the train (integrated area/time).

6. Twitch tensions were corrected for skeletal muscle mass and are expressed as a percent change from the saline control.

7. At the end of the experiment the triceps muscle mass was determined, and the activity of plasma cholinesterase and erythrocyte acetylcholinesterase were determined using a modified Ellman assay

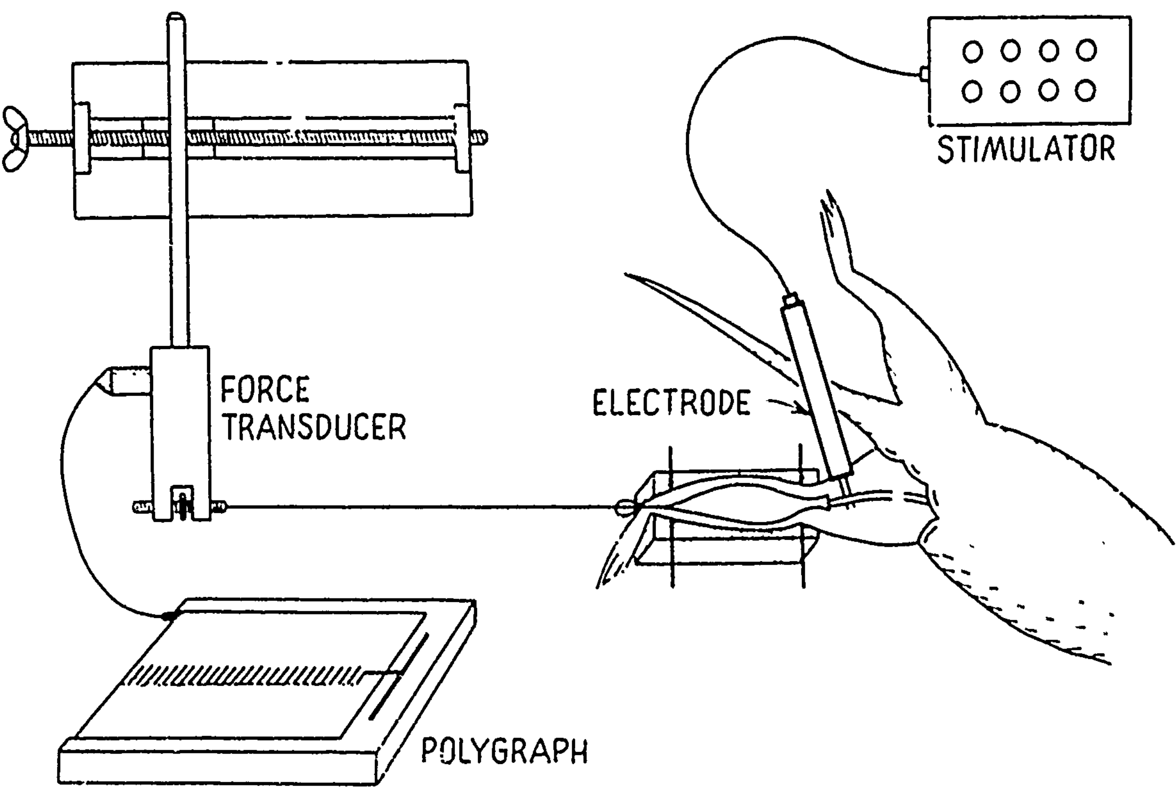
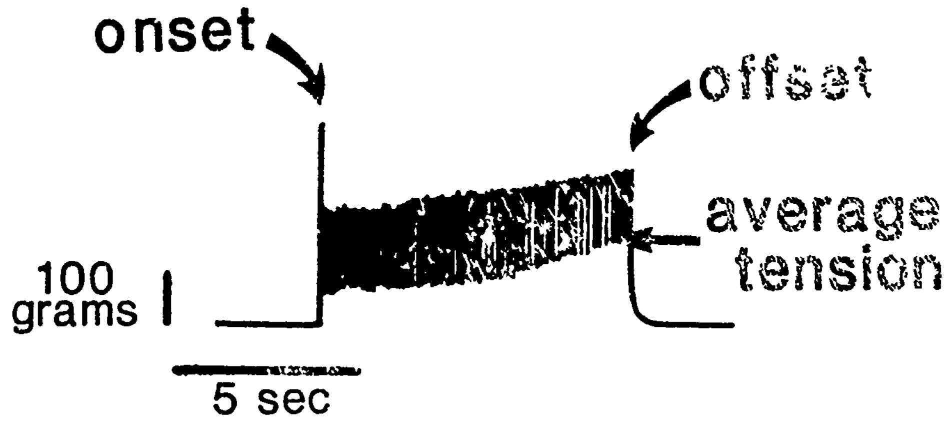
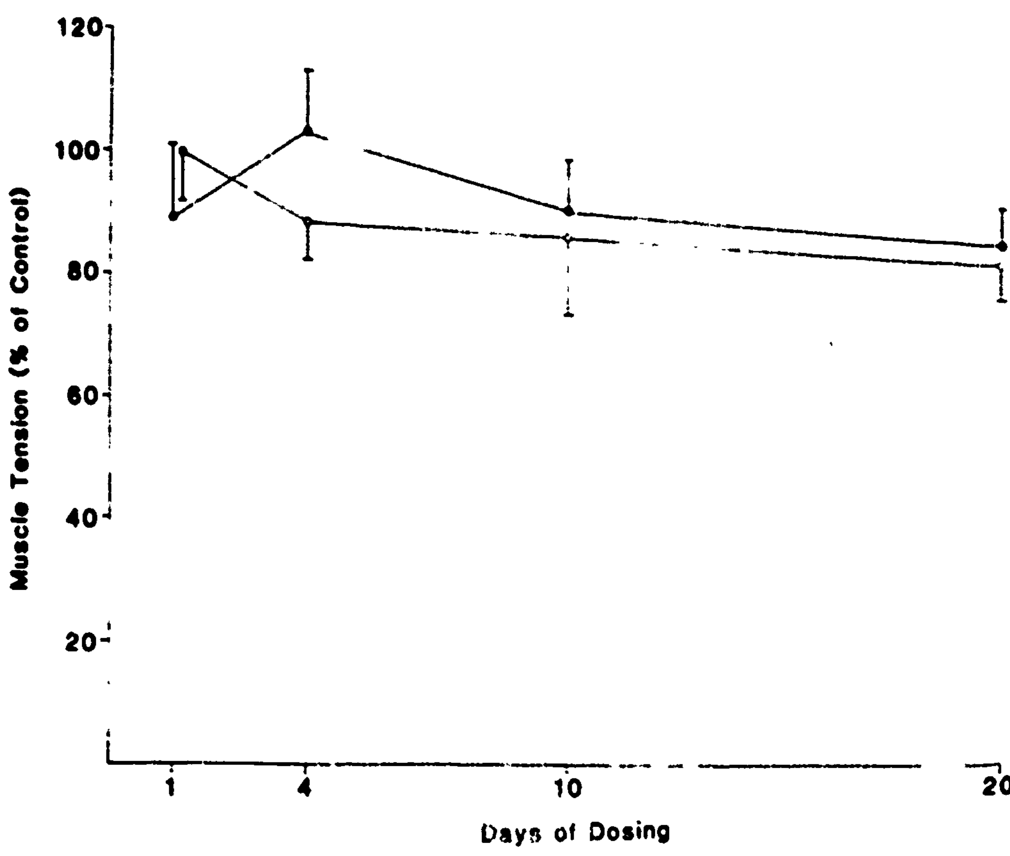


Figure 1. Schematic of the device for measuring muscle twitch tension from the triceps surae of the rat. Supramaximal stimuli were applied to the cut distal end of the sciatic nerve.





20 Hz-ONSET TENSION
Muscle Tension (% of Control)
120
100
80
60
40
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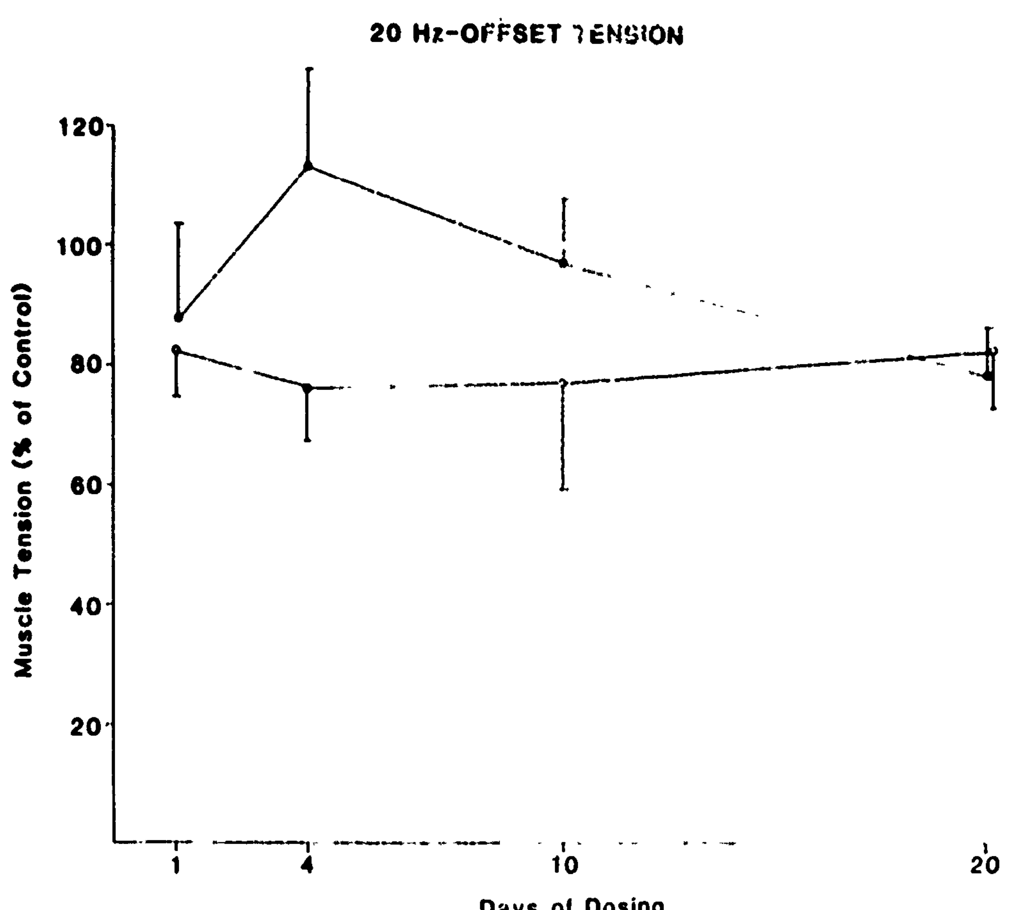
1
4
10
20
Days of Dosing



20 Hz-OFFSET TENSION
Muscle Tension (% of Control)
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1
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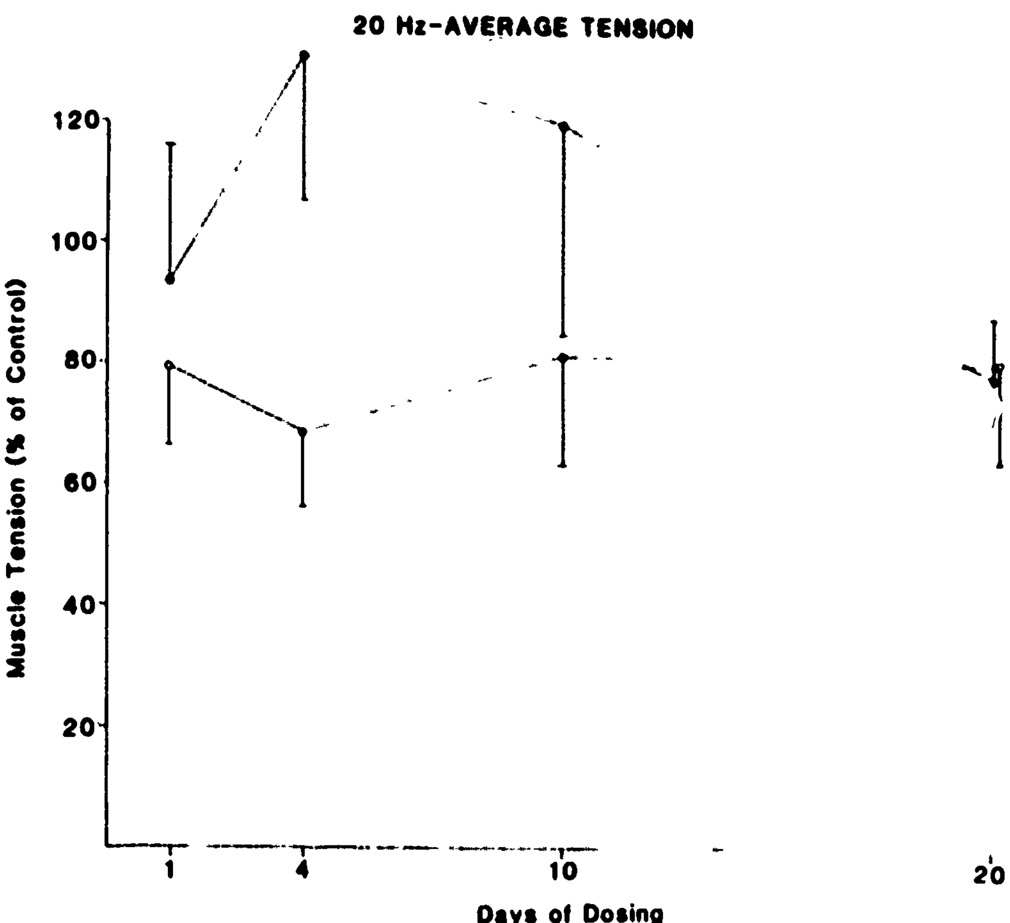
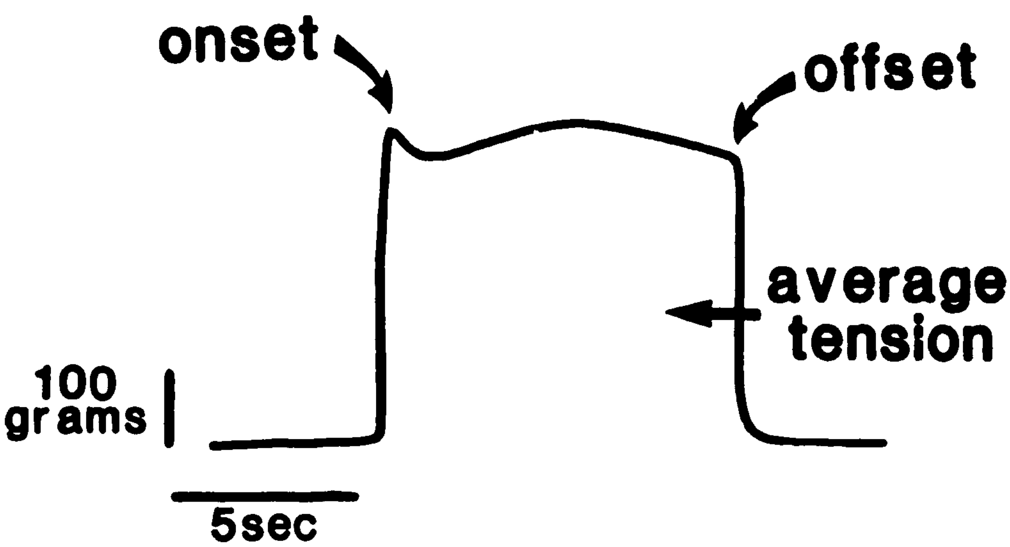
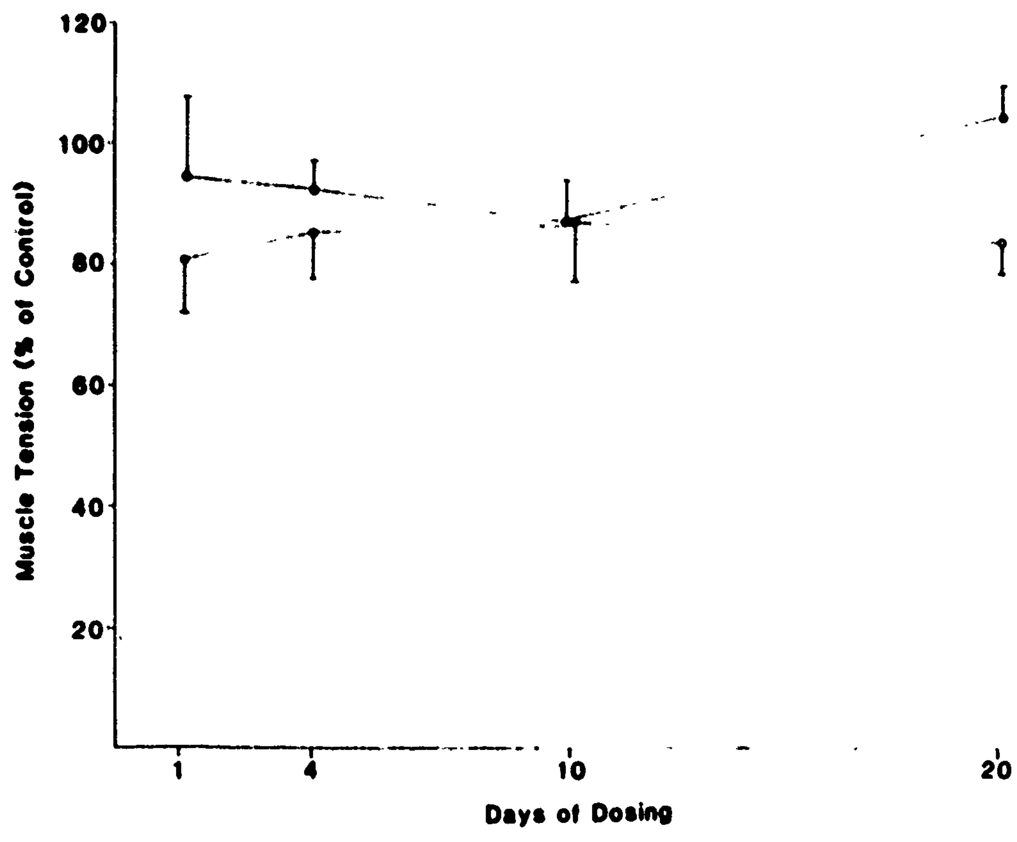
20
Days of Dosing



50 HZ




50 Hz-ONSET TENSION
Muscle Tension (% of Control)
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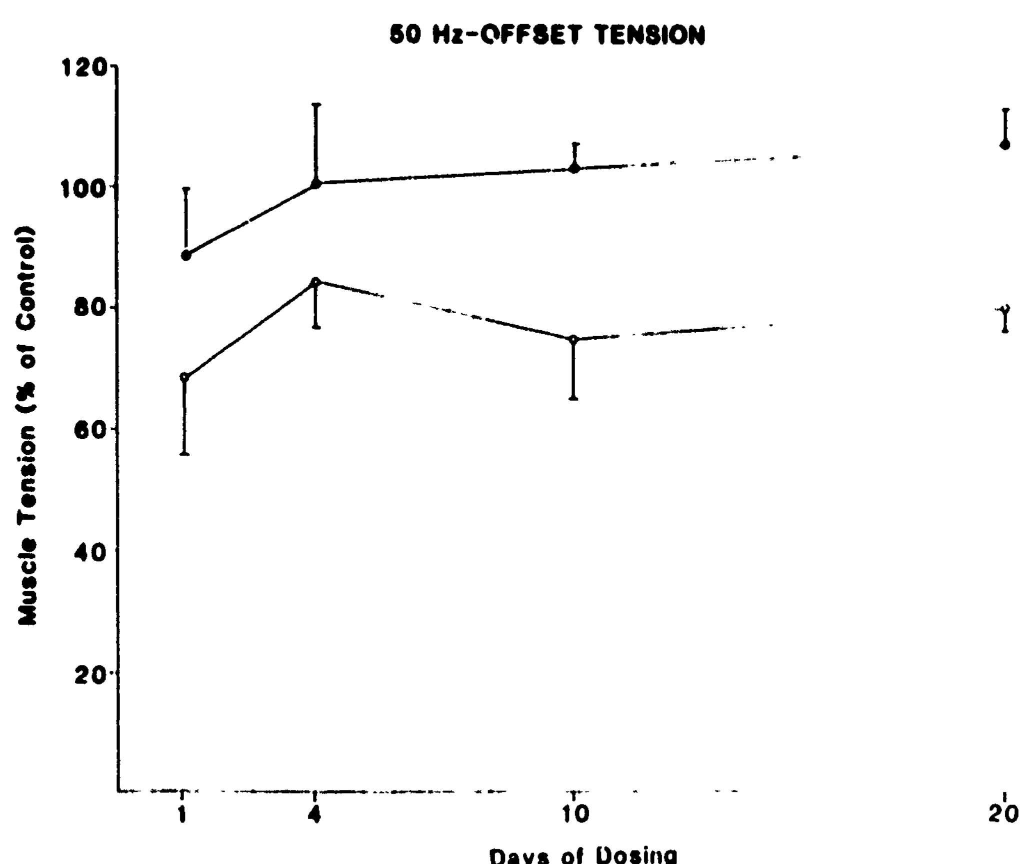
50 Hz-OFFSET TENSION
Muscle Tension (% of Control)
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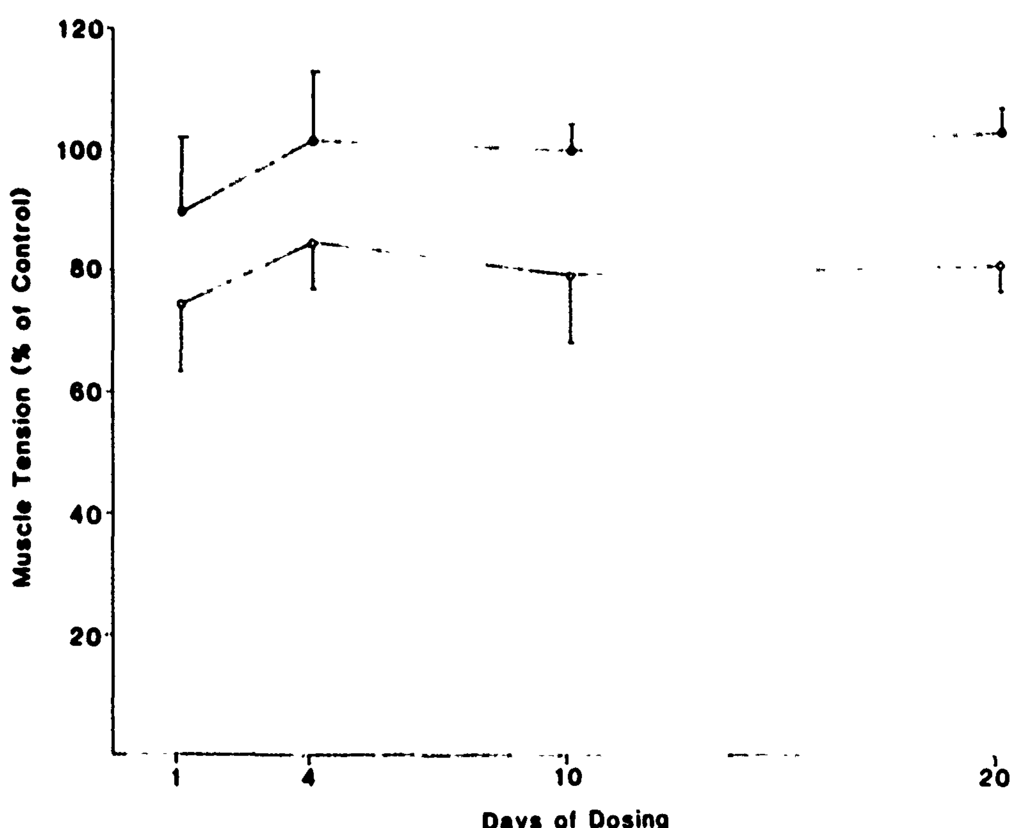
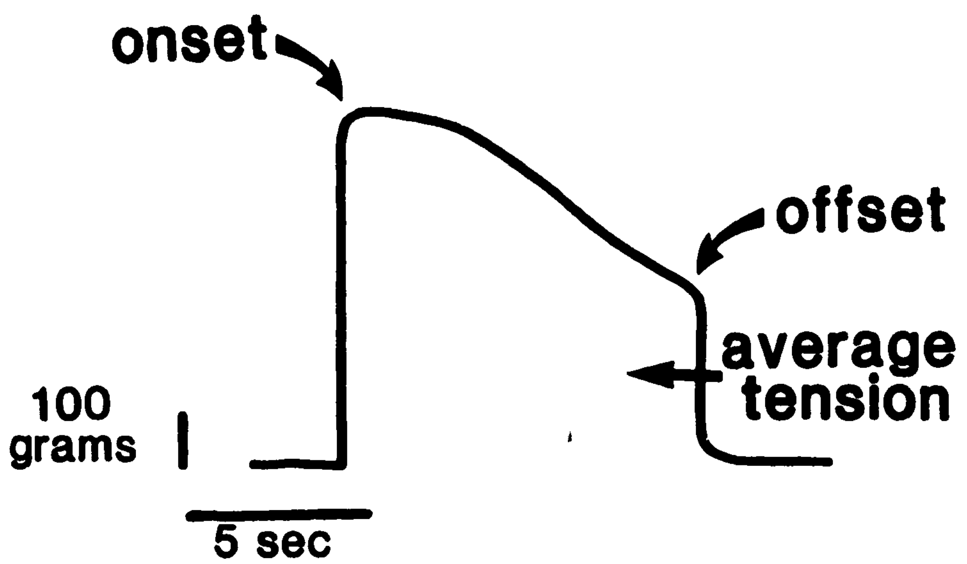
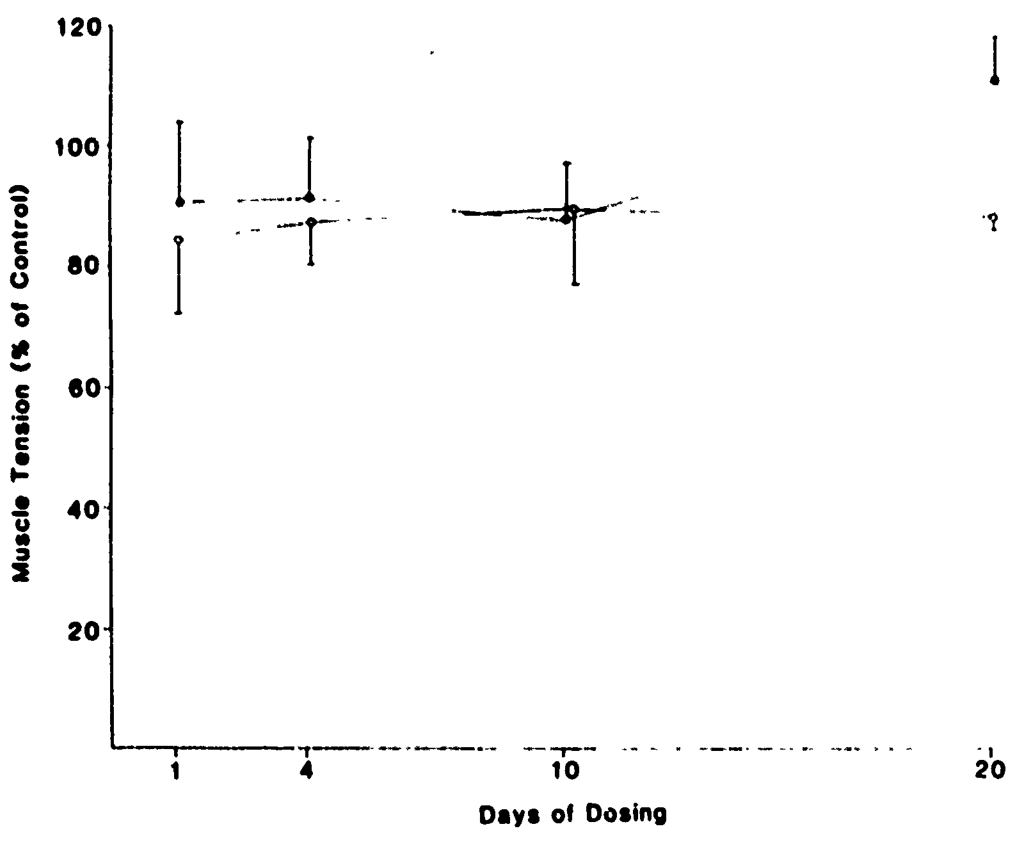
Days of Dosing



100 HZ




100 Hz-ONSET TENSION
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Muscle Tension (% of Control)
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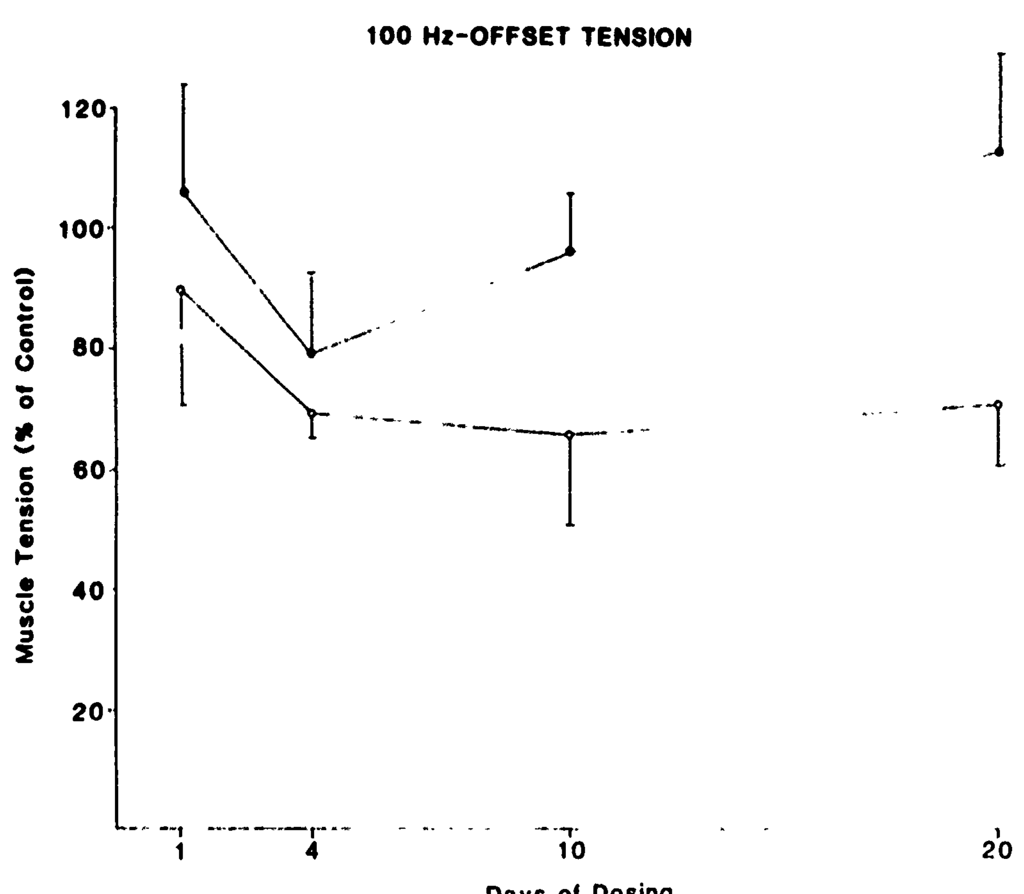
Days of Dosing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Muscle Tension (% of Control)
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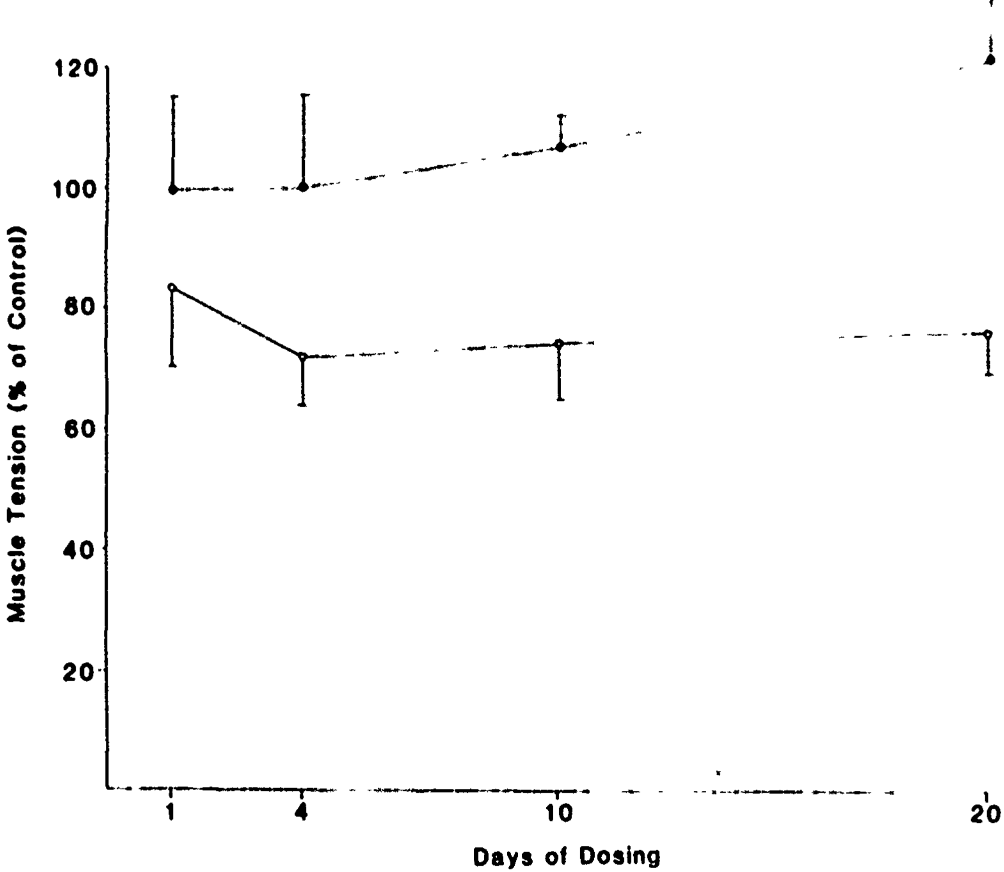

Figure 2. Effect of pyridostigmine on tetanic contraction of rat triceps surae muscle. The left panels show examples of individual experiments showing the tension generated during stimulation at 20, 50 and 100 Hz for 10 seconds. The points taken for measurement of twitch onset and offset are indicated. Average tension was measured as the integral of tension over the 10 second contracture. The remaining graphs are plotted as the mean ± SEM for the 5 rats per group and calculated as a percent of the vehicle control response. Solid circles indicate pyridostigmine given in daily ip doses (2 mg/kg/day) and open circles indicate Alzet pump infusion (5 mg/kg/day).

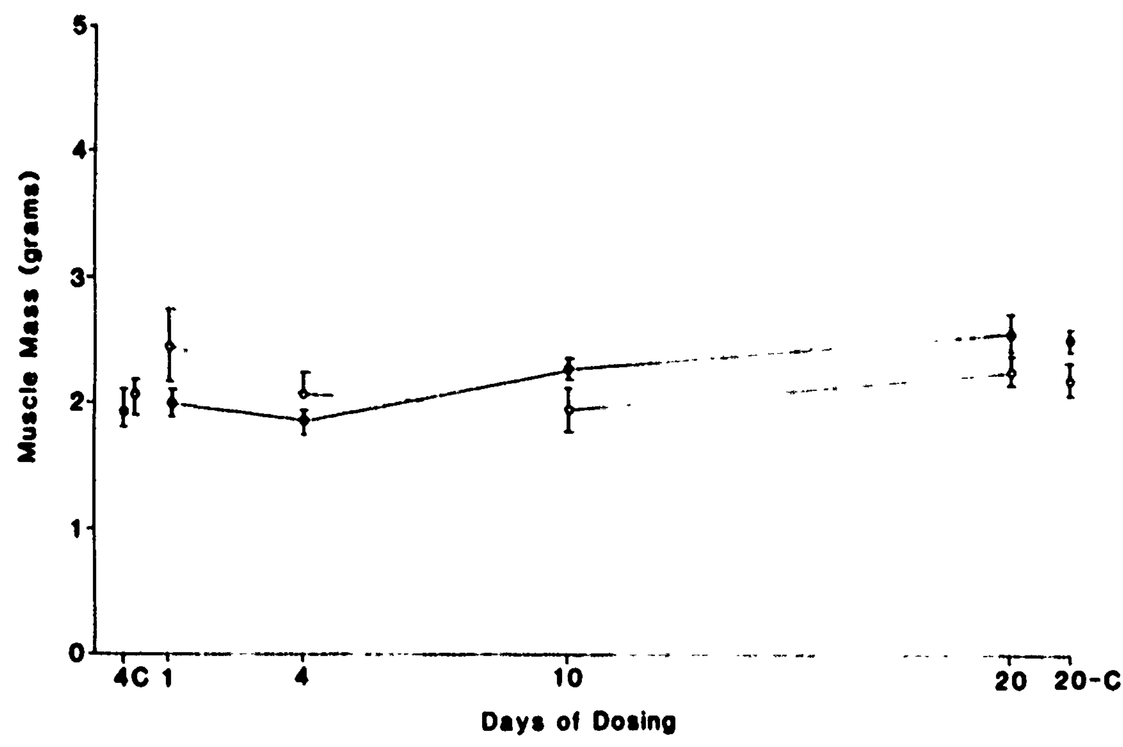

Figure 3. Effects of pyridostigmine on muscle mass. The muscle mass of each group of treated rats is plotted as the mean ± SEM (solid circles = ip pyridostigmine; open circles = Alzet pump infusion of pyridostigmine). The 4 day saline control values are plotted to the left (4–C) and the 20 day saline controls are plotted to the right (20–C). There were no significant effects of pyridostigmine on muscle mass.

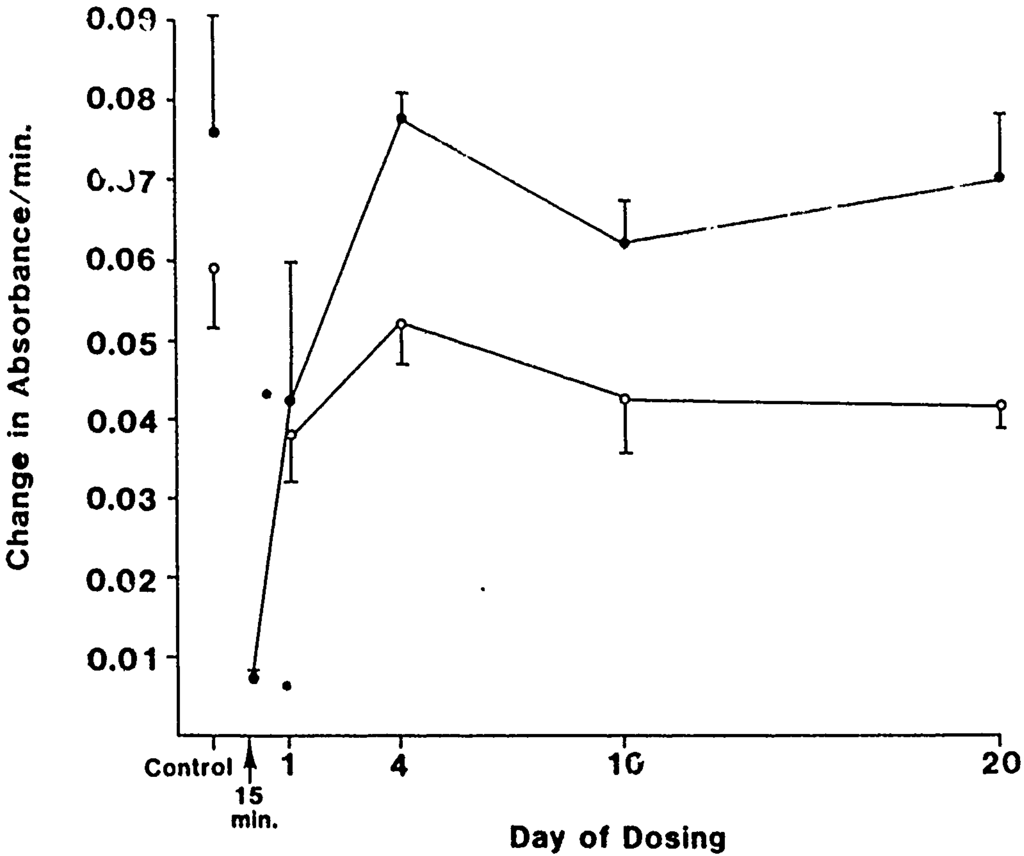

Figure 4. Effect of pyridostigmine on plasma cholinesterase. The activity of plasma cholinesterase is plotted for each treatment group (solid circles = ip pyridostigmine; open circles = Alzet pump administration). The 15 minute time point for the ip data was taken only for comparison. Muscle data were only taken from animals 24 hours after 1, 4, 10 or 20 ip doses Alzet pump infusion was continued to the end of the muscle recording session. Asterisks indicate statistical significance ($p<0.05$).

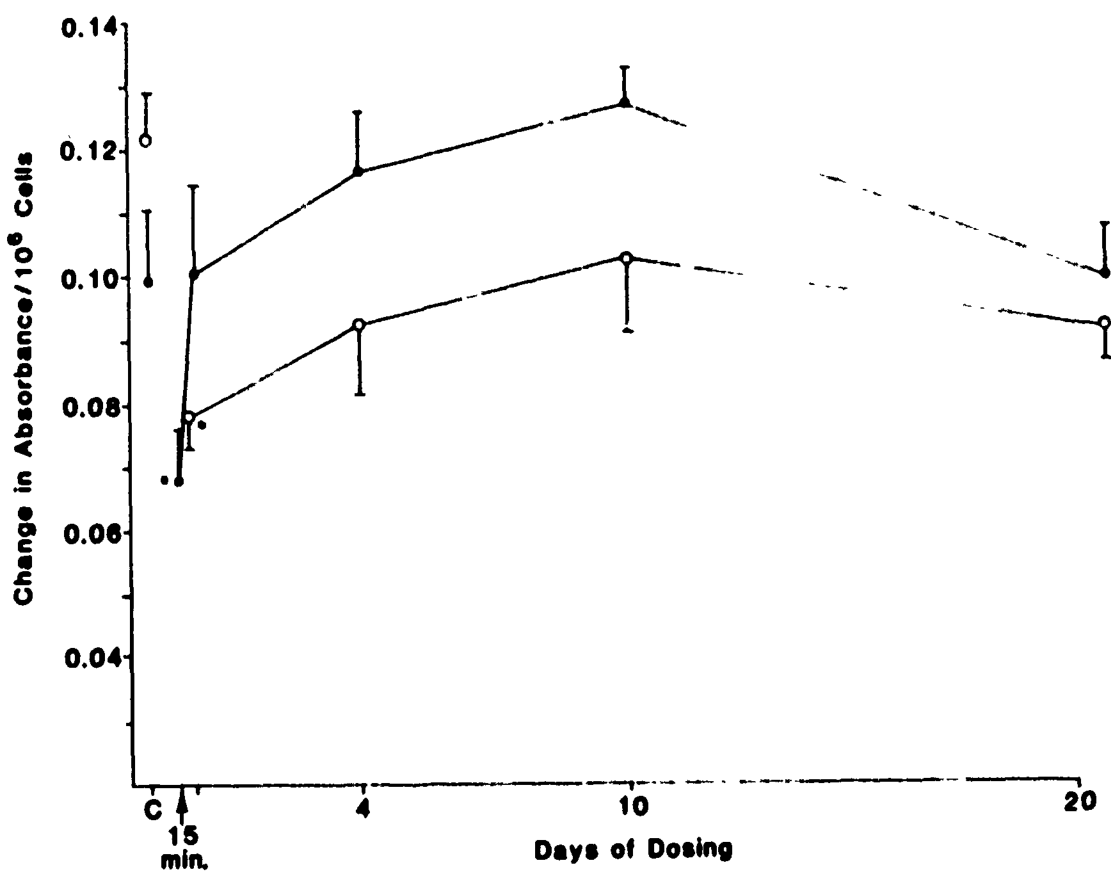

Figure 5. Effect of pyridostigmine on erythrocyte acetylcholinesterase. The enzyme activity is plotted for each treatment group (solid circles = ip pyridostigmine; open circles = Alzet pump administration). The 15 minute time point for the ip data was taken only for comparison. The measurements in the ip groups were made 24 hours after the last dose. Alzet pump infusion was continued until measurements were made. Asterisks indicate statistical significance ($p<0.05$).

CONCLUSIONS

1. Alzet pump infusion of pyridostigmine produced a significant 20% reduction in the twitch tension generated during tetanic stimulation, despite no change in muscle mass and no cholinergic signs of intoxication in the animals at the time of the recording.

2. Daily ip administration of pyridostigmine produced no significant changes in tetanic twitch tension or muscle mass. Immediately after each dose the rats exhibited signs such as tremor and labored breathing. These attenuated in several hours and there were no overt signs at the time of muscle recording.

3. Although pyridostigmine decreased plasma cholinesterase and acetylcholinesterase activity immediately, this effect was attenuated during repeated administration. There were no significant changes in enzyme activity at the time of muscle recording.

4. Both treatment groups gained weight at the same rate as the control animals over the duration of the experiment.

5. These results show that constant infusion of pyridostigmine at doses which do not affect cholinesterase activity, muscle mass or change body weight gain nevertheless decrease the ability of the muscle to follow sustained, tetanic contraction. This effect may be a precursor to muscle atrophy which is known to occur during more ambitious dosing schedules with cholinesterase inhibitors.

ORAL PYRIDOSTIGMINE IN RATS: EFFECTS ON THERMOREGULATION, CLINICAL CHEMISTRY, AND PERFORMANCE IN THE HEAT

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Natick, Massachusetts 01760-5007

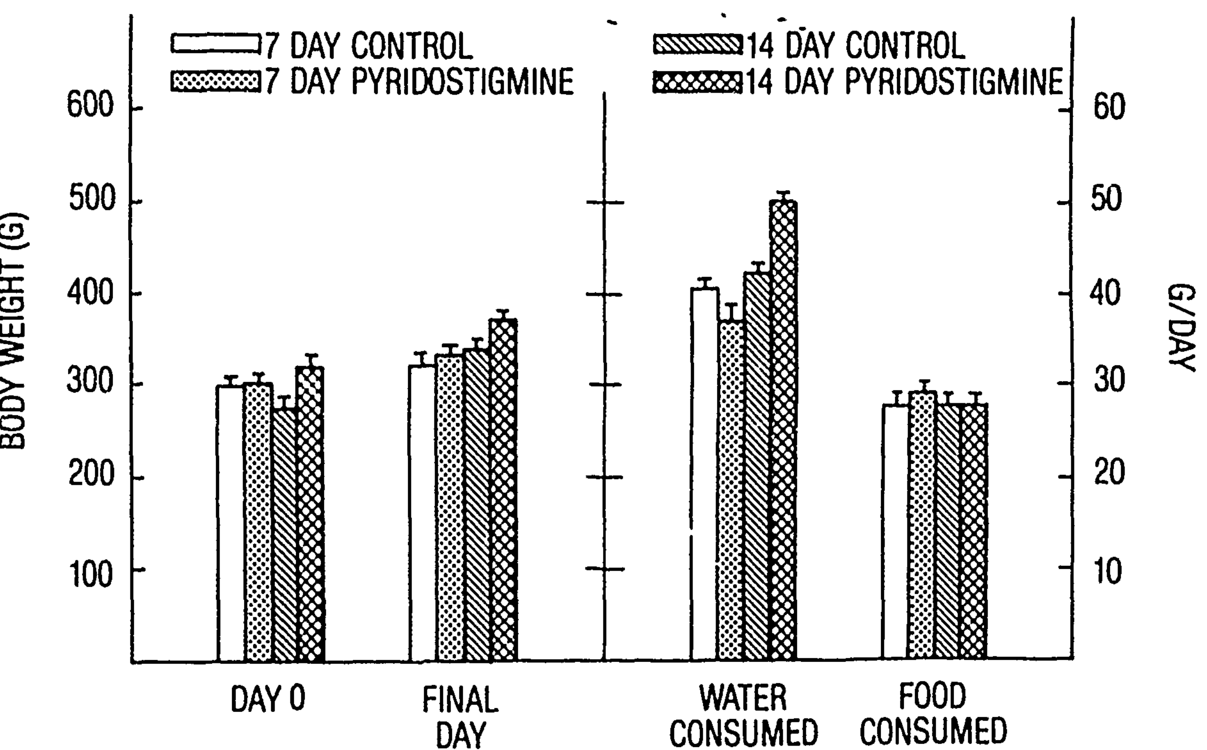
We have recently reported (J. Appl. Physiol 56, 891, 1984) that acute intraperitoneal administration of pyridostigmine bromide to rats resulted in significant decrements in physical performance in the heat, adverse thermoregulatory effects, and exacerbated elevation in several indices of heat/exercise injury, such as lactate and creatine phosphokinase (CPK). Circulating cholinesterase activity was inhibited by 64% in this earlier experimental protocol. To determine the effects of orally ingested and chronically administered pyridostigmine, Mestinon (pyridostigmine bromide, Roche Laboratories, Nutley, NJ) was dissolved (240 mg/930ml) in the drinking water of rats; experimental animals had free access to this solution as their sole source of drinking water for either 7 or 14 days prior to an exercise trial (9.14m/min, level treadmill) in the heat (35°C). In other groups of animals the clinical chemical effects of chronic pyridostigmine administration were determined without an exercise contingency. Water consumption, food consumption, body weights, and overt behavior were carefully monitored in all experimental and control animals.

The rats (n = 34) consuming pyridostigmine for 7 days (46.5 mg total consumption or 6.6mg/day) manifested a mean circulating cholinesterase activity of 21.8 units (μmoles acetic acid formed/h/ml plasma); control rats, treated identically but with tap water as the sole fluid source, demonstrated a mean circulating cholinesterase level of 28.3 units, p<.001. This treatment regimen had minor effects on food consumption (28.0 g/d control vs 28.9 g/d pyrido), water consumption (4[illegible]8 ml/d control vs 37.4 ml/d pyrido), and weight gain (3.4 g/d control vs 4.0 g/d pyrido) while circulating cholinesterase was inhibited by 23%. Pyridostigmine consumption for 7 days had no effects on a variety of clinical chemical indices of heat/exercise injury (CPK, sodium, potassium, lactate, urea nitrogen, creatinine, glucose, lactic dehydrogenase) prior or subsequent to exercise in the heat. Likewise, endurance was unaffected (28.5 min control vs 30.0 min pyrido) by the pyridostigmine consumption for 7 days.

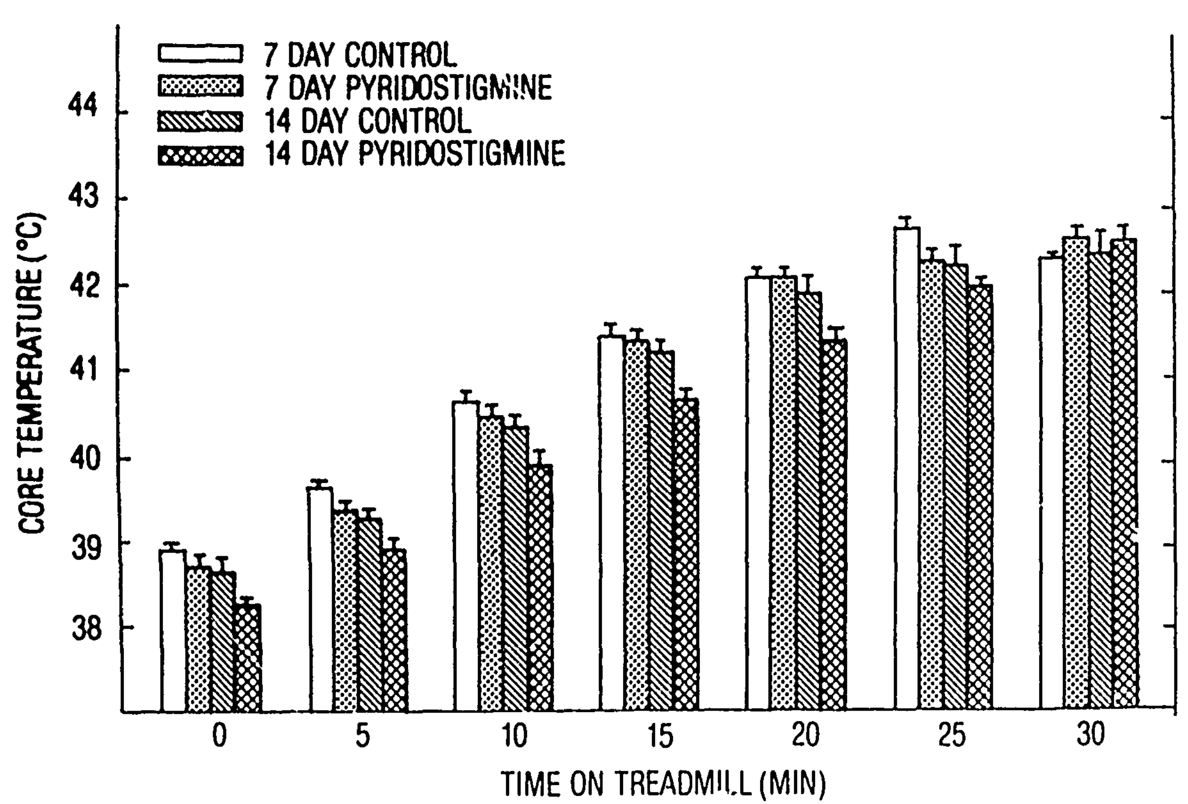
The experimental animals (n = 35) consuming pyridostigmine for 14 days had a mean cholinesterase activity of 15.3 units while their control counterparts (n = 33) had a mean level of 24.9 units, p<.001; thus, consuming pyridostigmine for the 14d interval elicited a 39% inhibition of circulating cholinesterase. During the 14 day period the experimental group consumed 124.3 mg pyridostigmine or 8.9 mg/day. Once again, consumption of the pyridostigmine had no significant effects on the clinical chemical indices of heat/exercise injury, nor were these indices exacerbated following the exercise regimen in the rats consuming the pyridostigmine. Rats consuming pyridostigmine manifested a mean endurance of 32.5 min while the 14d control group ran for 30.0 min; thus, no significant differences were observed in physical capacity as well as thermoregulatory variables. It is noteworthy that during exercise in the heat rats consuming pyridostigmine for 14d lost slightly more weight (water) than their control counterparts (15.6g vs 12.6g, p<.05); however, these rats were slightly, but not significantly, larger at the start of the run.

We have concluded from these studies that the oral administration of pyridostigmine in the drinking water of rats represents a quantitative and effective means to simulate human consumption of this proposed prophylaxis. Our results further indicate that due to the less intense cholinesterase inhibition or, more likely, due to the chronicity of the pyridostigmine inhibition, no undesirable thermoregulatory, metabolic, growth, or performance effects were observed.

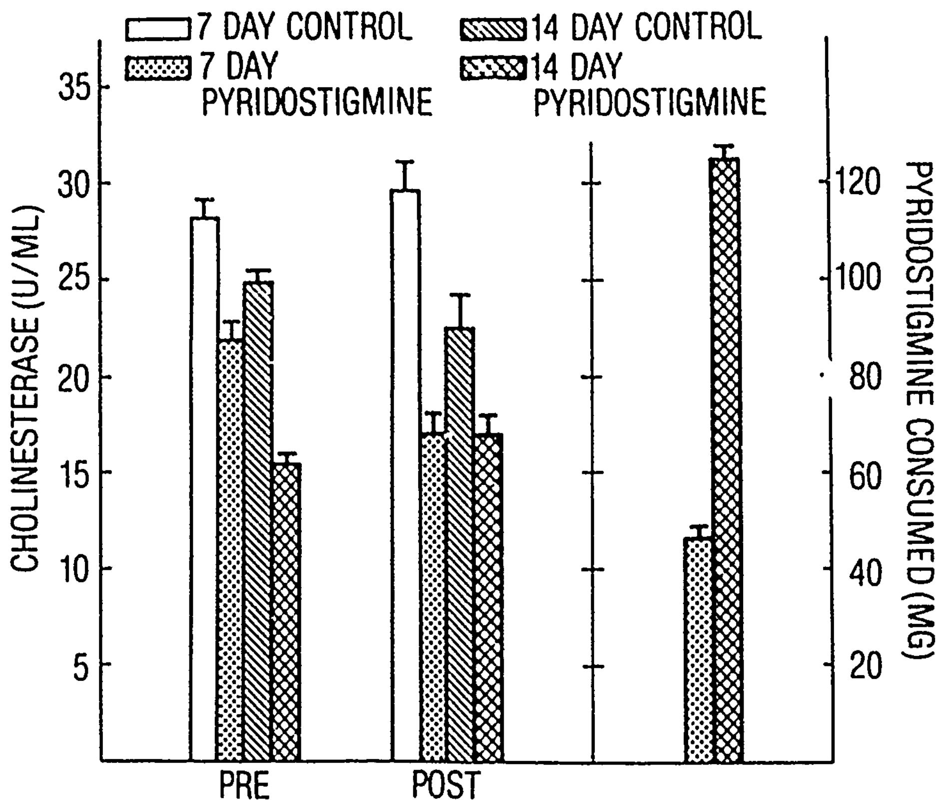
EFFECTS OF PYRIDOSTIGMINE CONSUMPTION ON WEIGHT GAIN AND WATER AND FOOD CONSUMPTION



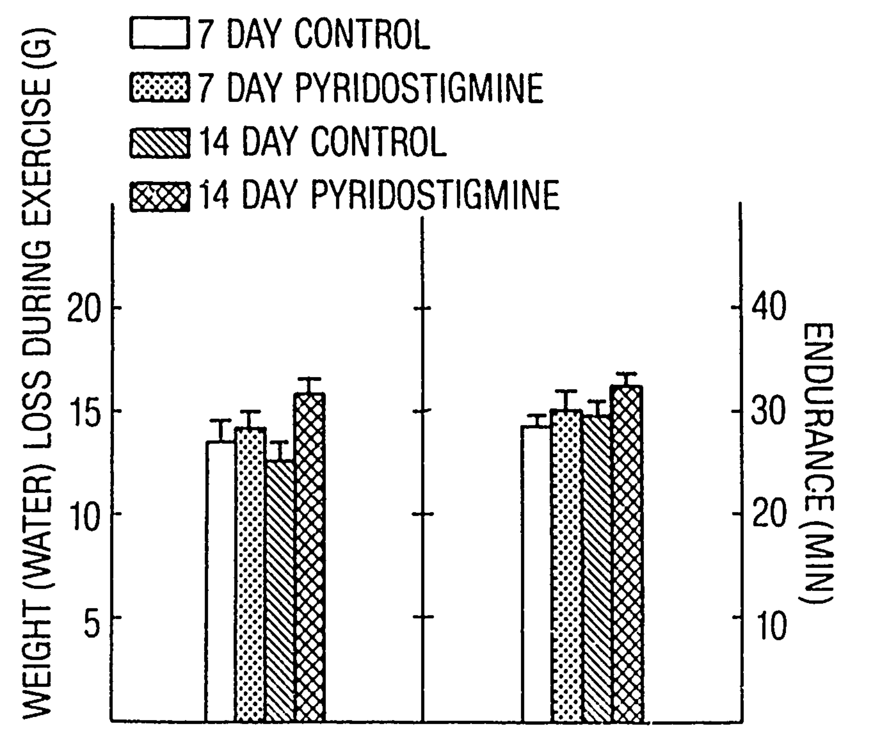
EFFECTS OF PYRIDOSTIGMINE CONSUMPTION ON THE CORE TEMPERATURE RESPONSE TO EXERCISE IN THE HEAT



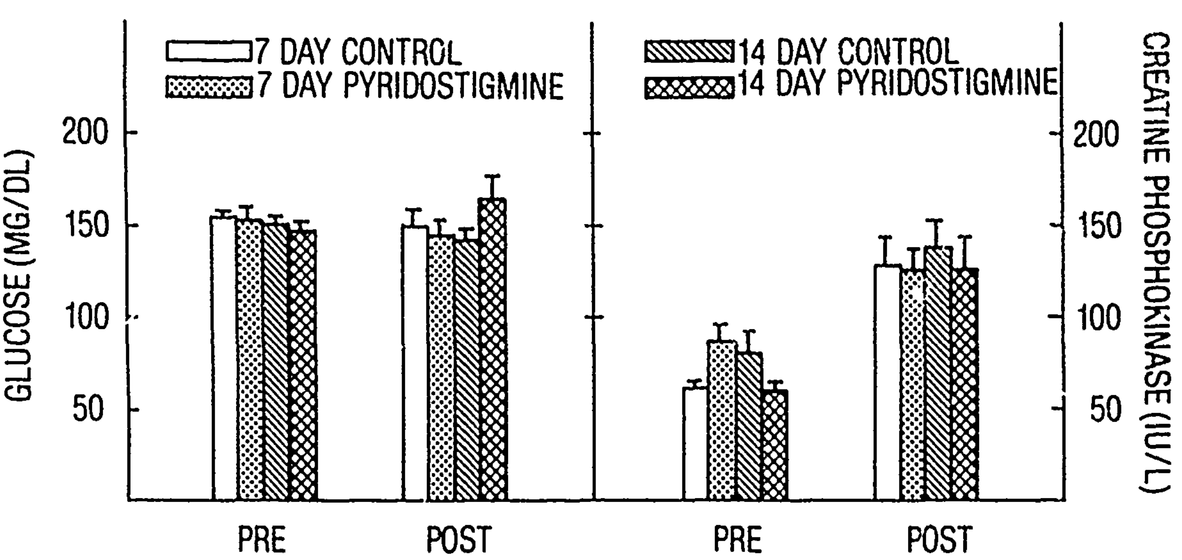
EFFECTS OF PYRIDOSTIGMINE CONSUMPTION ON CHOLINESTERASE ACTIVITY



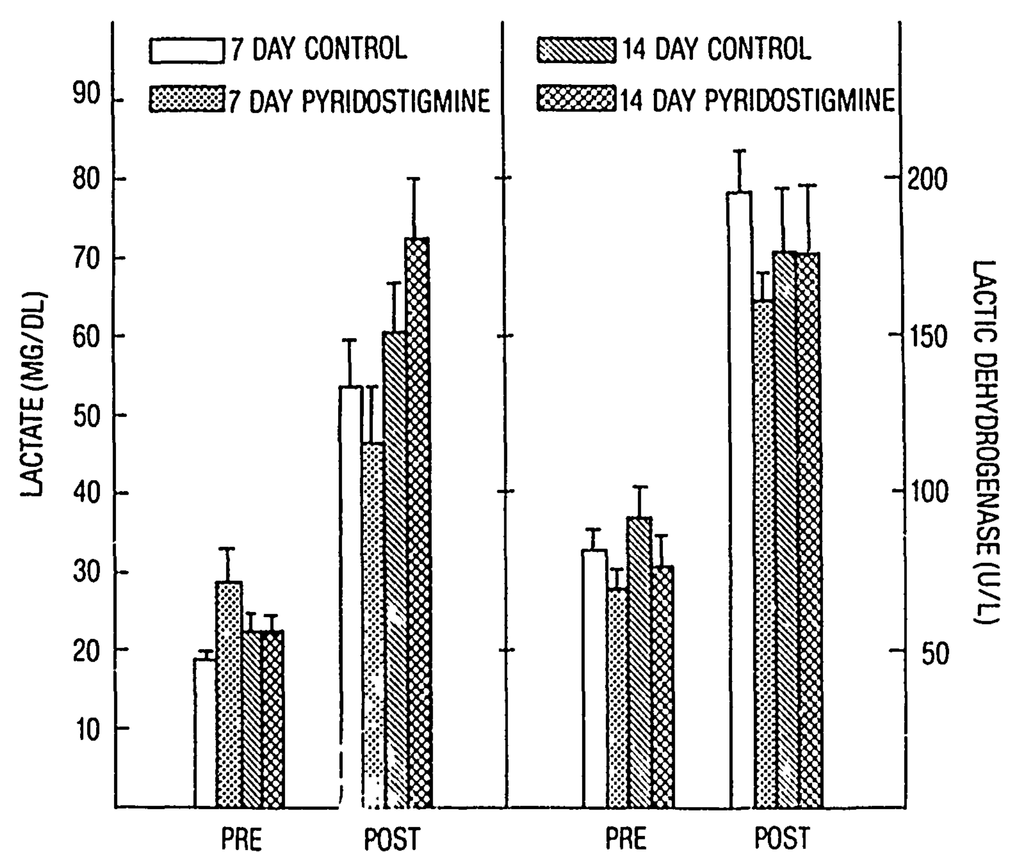
EFFECTS OF PYRIDOSTIGMINE ADMINISTRATION ON WEIGHT (WATER) LOSS DURING EXERCISE AND ENDURANCE



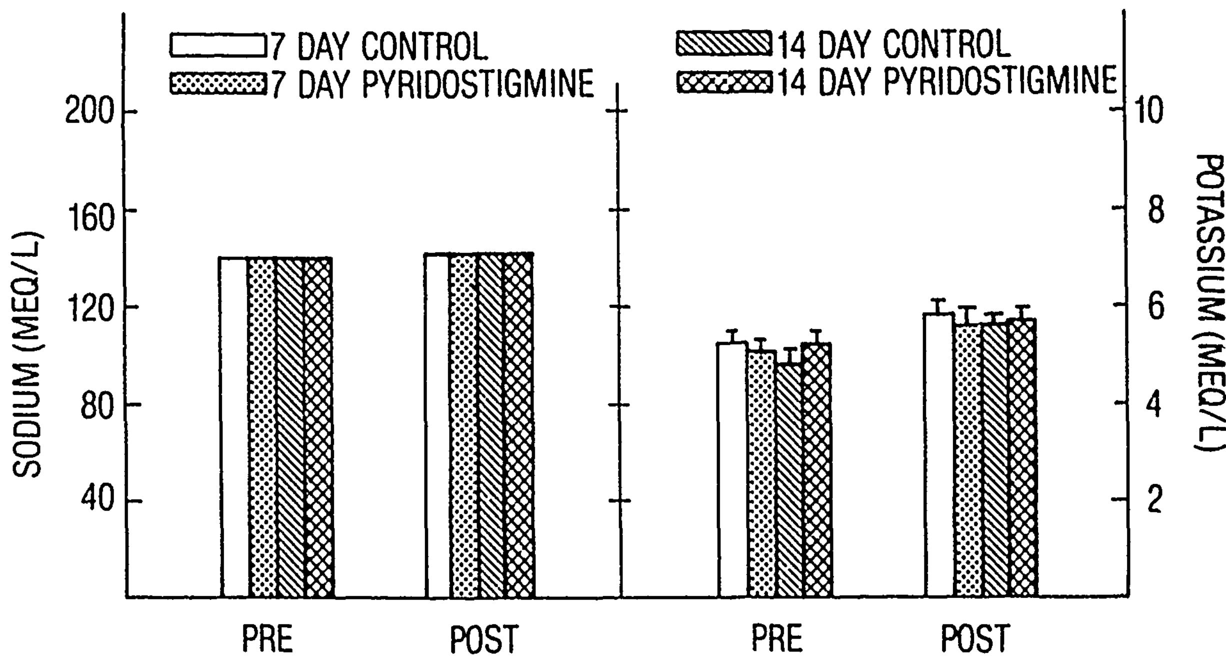
EFFECTS OF PYRIDOSTIGMINE CONSUMPTION ON PLASMA LEVELS OF GLUCOSE AND CREATINE PHOSPHOKINASE PRIOR AND SUBSEQUENT TO EXERCISE IN THE HEAT



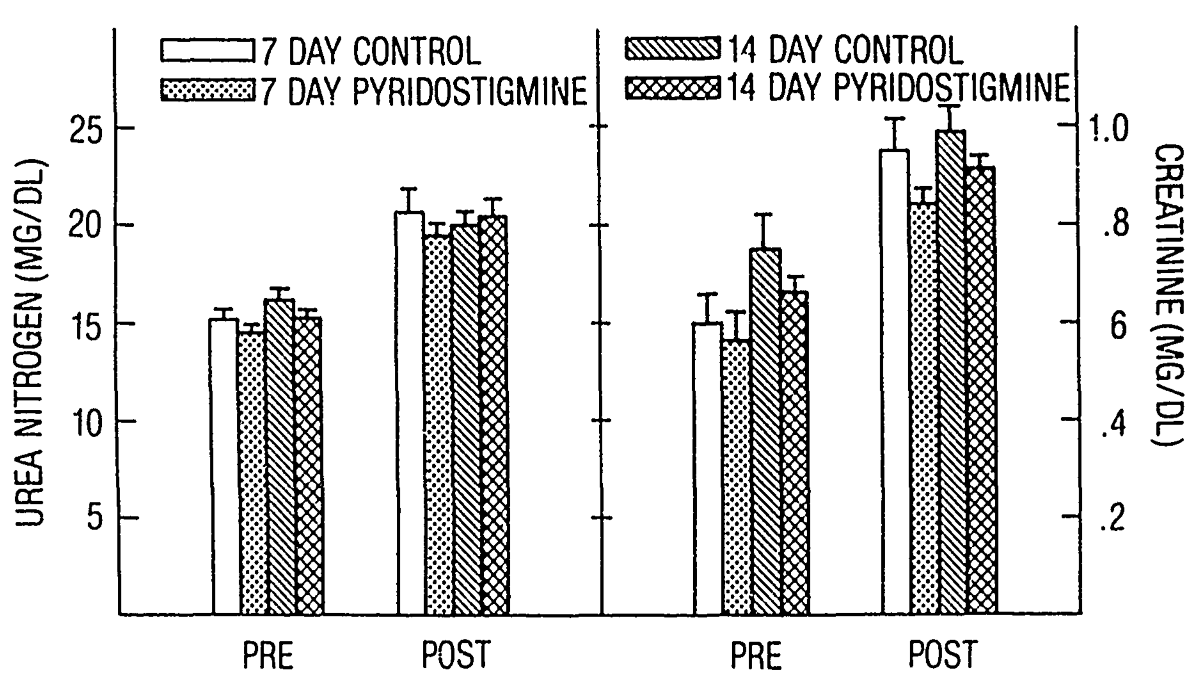
EFFECTS OF PYRIDOSTIGMINE CONSUMPTION ON PLASMA LEVELS OF LACTATE AND LACTIC ACID DEHYDROGENASE BEFORE AND AFTER EXERCISE IN THE HEAT



EFFECTS OF PYRIDOSTIGMINE CONSUMPTION ON PLASMA LEVELS OF SODIUM AND POTASSIUM BEFORE AND AFTER EXERCISE IN THE HEAT



EFFECTS OF PYRIDOSTIGMINE CONSUMPTION AND EXERCISE IN THE HEAT ON LEVELS OF CREATININE AND UREA NITROGEN



PROTECTION OF THE HOUSE FLY *(MUSCA DOMESTICA L.)* AGAINST DFP (DIISOPROPYL FLUOROPHOSPHATE) BY PRETREATMENT WITH PYRIDOSTIGMINE

Claudia F. Golenda, Robert A. Wirtz and Richard G. Andre
Department of Entomology, Walter Reed Army Institute of Research
Wasl . .

Introduction

Th, mode of action of carbamates, like organophosphates, is the inhibition of acetylcholinesterase (O'Brien 1967). The protective strategy of carbamates is related to their ability to temporarily carbamoylate acetylcholinesterase, preventing the enzyme from interacting with the organophosphate. Pyridostigmine has been shown to be one of the most effective carbamates in protecting mammals from the lethality of soman (Gordon et al. 1978). Pyridostigmine was evaluated as a protectant to diisopropyl fluorophosphate (DFP) using an insect model system (See Poster Stand # 40).

Methods

The toxicities of DFP and pyridostigmine to house flies were determined. A volume of 0.44 ul of DFP or pyridostigmine, diluted in buffered saline, was delivered by thoracic injection to individual flies. Control flies received only saline. Four concentrations of each material were tested against 30 flies (3 replicates, 10 flies per replicate) and tests were replicated 3 times. Mortality was recorded at 24 and 48 h. Mortality data (48-h) were pooled and subjected to probit analysis (Ray 1982) for estimation of the LD 50, 95, and slope values from probit regression lines for DFP and pyridostigmine.

Four doses of pyridostigmine (50, 125, 250, and 500 ug/g) were evaluated as protectants to DFP. Pyridostigmine was administered by thoracic injection 15 minutes prior to flies receiving a second injection containing a LD 95 dose of DFP. Each dose contained 3 replicates of 10 flies, and tests were replicated 4 times. Survival was recorded at 24 and 48 h. The 48-h survival data for each pretreatment dose (n=4) were pooled because a Chi Square analysis (P=0.05) indicated that replicated data were homogeneous. The mean proportion of survivors from each pretreatment dose were compared by a Chi Square difference and linearity test (Fleiss 1981). In other tests, 500 ug/g of pyridostigmine was given 15 minutes prior to flies receiving increasing doses of DFP (2.5, 5.0, 12.5, and 25 ug/g). Mortality was recorded at 24 and 48 h.

Results

The probit regression lines for DFP and pyridostigmine are presented in Figures 1 and 2. A Chi Square analysis indicated that the probit regression line for DFP (Prob>Chi Sq=0.7647), but not pyridostigmine (Prob>Chi Sq=0.001), fit the probit equation model, $\Phi^{-1}(y) + 5 = (A + Bx)$; however, the best fit line for pyridostigmine was used to estimate the LD 50 and 95 values. All pretreatment doses of pyridostigmine were sign-free.

While the lowest pretreatment dose (50 ug/g) did not afford protection to DFP (5 % survival), pretreatment doses of 125, 250, and 500 ug/g significantly increased the percentage of survivors to 28.3, 64.2, and 88.3 %, respectively. The relationship between survival and pretreatment dose of pyridostigmine was linear (Figure 3).

When flies were pretreated with 500 ug/g and challenged with increasing doses of DFP (2.5, 5.0, 12.5 and 25.0 ug/g), the mean percent mortality was 7.7, 28.9, 32.2, and 25.5 %, respectively (Figure 4), indicating that the relationship between DFP challenge dose and mortality in pyridostigmine pretreated flies did not fit a quantal dose-response line. Because of the ungraded nature of this response, a protective ratio at the LD 50 could not be determined.

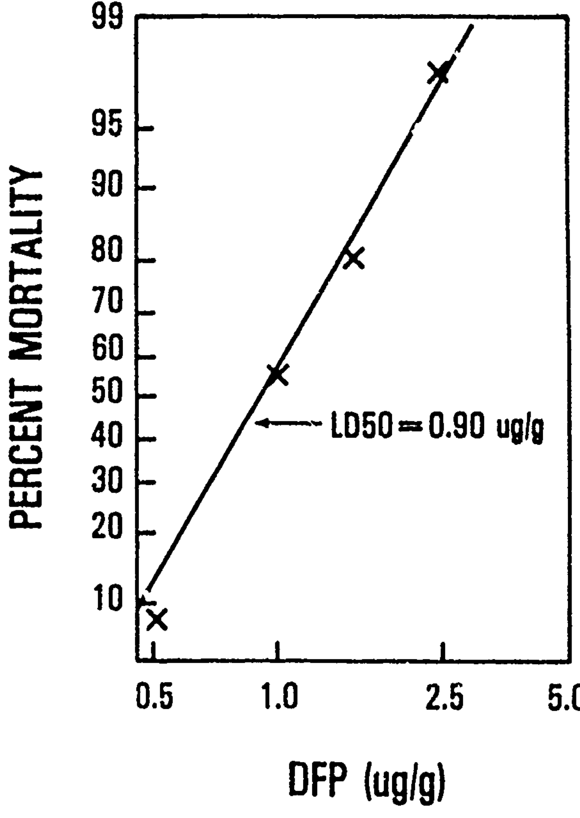


Figure 1. Probit regression line for diisopropyl fluorophosphate (DFP) in house flies.

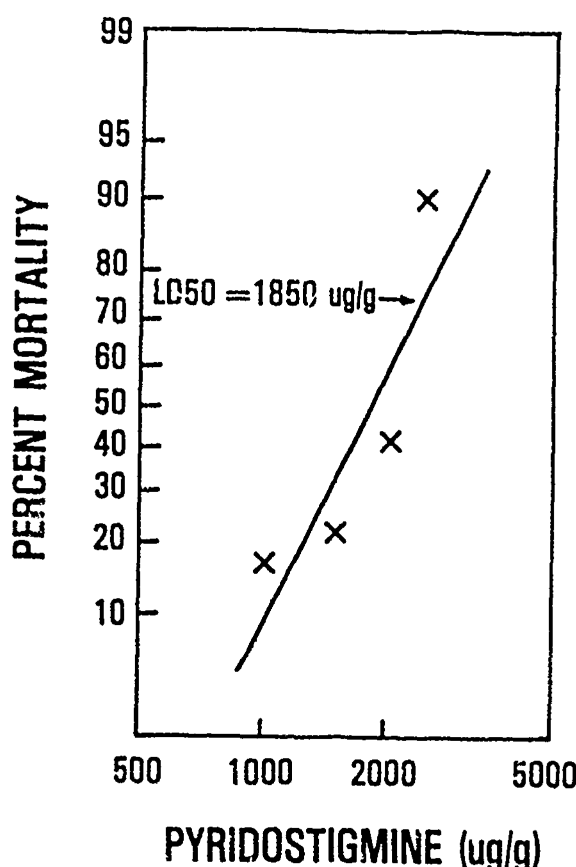


Figure 2. Probit regression line for pyridostigmine in house flies. Note: Relationship between mortality and dosage deviates from probit model.

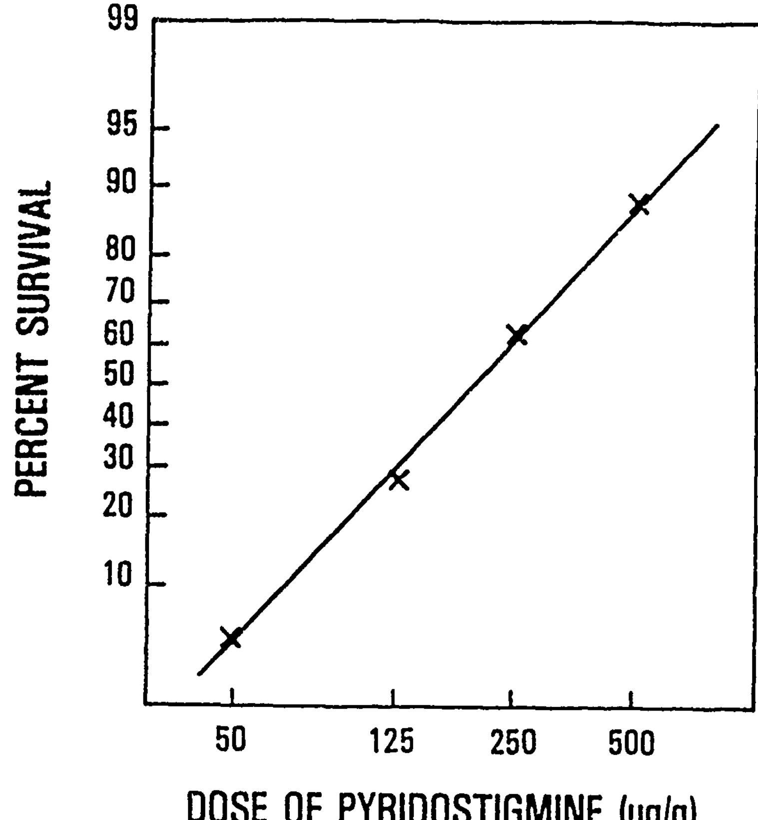


Figure 3. The relationship between pretreatment dose of pyridostigmine and survival in house flies challenged with a LD 95 dose of diisopropyl fluorophosphate (DFP).

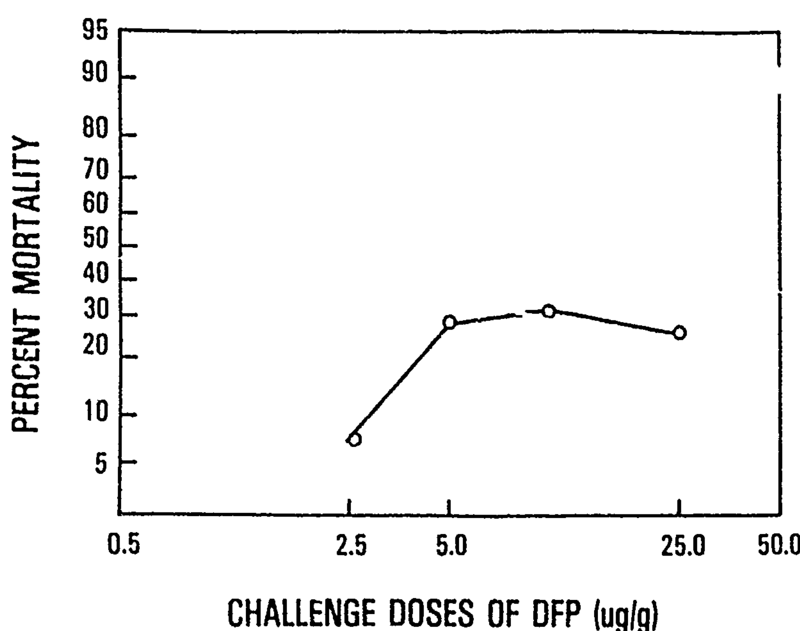


Figure 4. The effect of increasing challenge doses of diisopropyl fluorophosphate (DFP) on survival in house flies pretreated with 500 ug/g of pyridostigmine.

Discussion and Conclusions

Pyridostigmine protected house flies against the lethality of DFP. Similarly, pyridostigmine protected guinea pigs (Gordon et al. 1978) and monkeys (Dirnhuber et al. 1979) against soman. The experiments reported in this study support the hypothesis that pyridostigmine and DFP have a common site of action in house flies, and that this site can be protected by previous exposure to a carbamate. The ability to protect flies against DFP poisoning suggests that DFP produces a biochemical lesion in insects similar to that reported for mammals.

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THE EFFECTS OF PYRIDOSTIGMINE ON GUINEA PIG ILEUM MUSCLES

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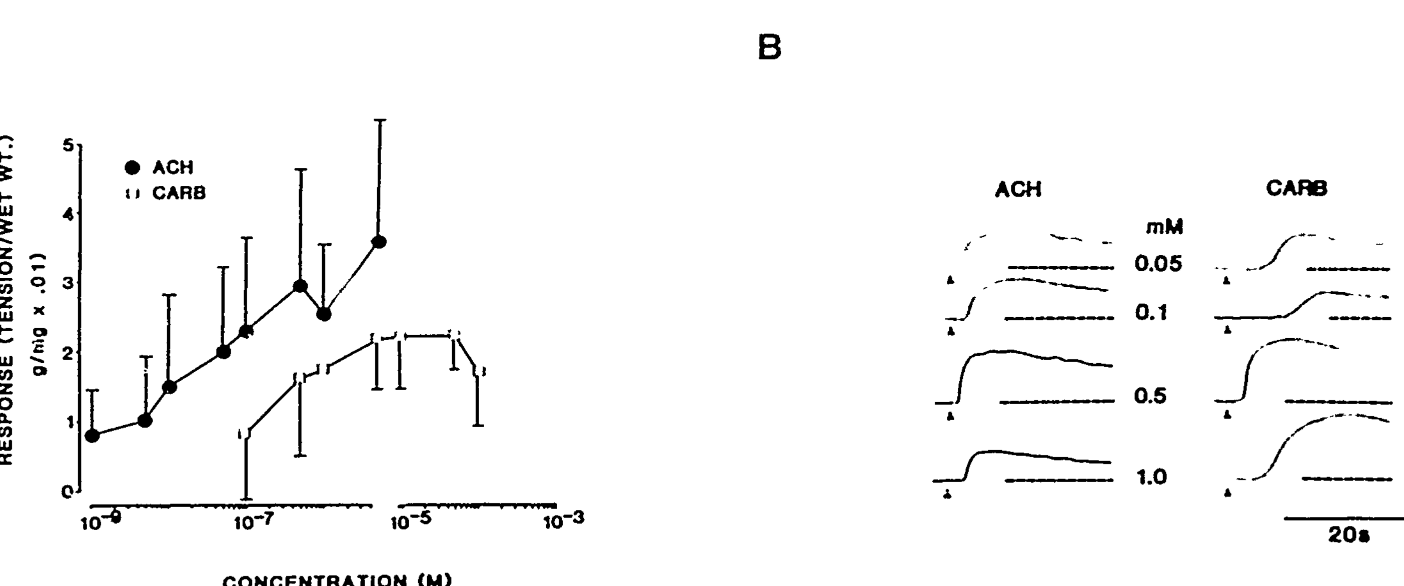
Aberdeen Proving Ground, MD

ABSTRACT

Pyridostigmine, a reversible inhibitor of acetylcholinesterase (AChE), has been used in the treatment of myasthenia gravis, and more recently, has been proposed for pretreatment protection of AChE against more potent and irreversible AChE inhibitors such as soman. It is known that pyridostigmine has a small agonist effect on nicotinic acetylcholine (ACh) receptors in skeletal muscle, but little is known of its effect on smooth muscle or the muscarinic ACh receptors (mAChR) that are more common on these tissues. The guinea pig ileum was selected to assay the effects of pyridostigmine on mAChR's and because this muscle has frequently been used to assay cholinergic drugs, including soman and sarin. Guinea pigs were either decapitated or etherized. The gut was exposed and a long piece of ileum removed, beginning approximately two inches from the cecum. The mesentery was cleaned from the ileum and the contents of the ileum, if any, were removed. The ileum was placed in Tyrodes solution that contained (mM): $CaCl_2$ 1.8, $MgCl_2$ 0.5, KCl 2.7, $NaHCO_3$ 12, NaCl 136, NaH_2PO_4 0.4, Dextrose 5.1, Hexamethonium Bromide 0.1, and Diphenhydramine 1μM (pH = 7.6). One inch pieces were cut and put into individual muscle baths where they were bathed with warm Tyrodes solution (34-37° C.). Dose-response curves obtained from acetylcholine (ACh) and carbachol (CARB) were similar to data that have been reported in the literature. ACh (1-5 nM) initiated a small contraction and a peak response at 0.5-1 μM. Concentrations of ACh greater than 1 μM caused a gradual decline in the contractile amplitude. In contrast, CARB initiated contractions at 5-10 nM, a peak contraction at 1-10 μM, and a decline in contractile amplitude at concentrations above that. Muscles exposed to pyridostigmine without any previous exposure to an agonist or antagonist, did not respond until the concentration was $>= 1$ mM. At 1-5 nm,pyridostigmine elicited a very dramatic relaxation in the muscle tone that persisted for the duration of the exposure. At these doses, the carbamate also abolished all spontaneous contractions in the muscle. When the muscle was washed with normal Ringer's solution, the muscle tone returned rapidly, and at a slower rate, the spontaneous contractions began again. When the muscle was first exposed to an agonist, used as a "primer," the responses were much more variable. After CARB, the muscle usually reacted much as it did to pyridostigmine without a primer. Its tone relaxed and the spontaneous contractions were always abolished at high concentrations of pyridostigmine. Occasionally, there was a slight increase in tone exhibited at concentrations of 1-2 μM. After ACh, pyridostigmine (1-100 μM) elicited a contraction in the muscle as well as causing the decrease in tone at high concentrations (1-5 mM). These data suggest that pyridostigmine may have some direct effects on the muscarinic ACh receptor of smooth muscle, but that its effects on the muscle are complex.

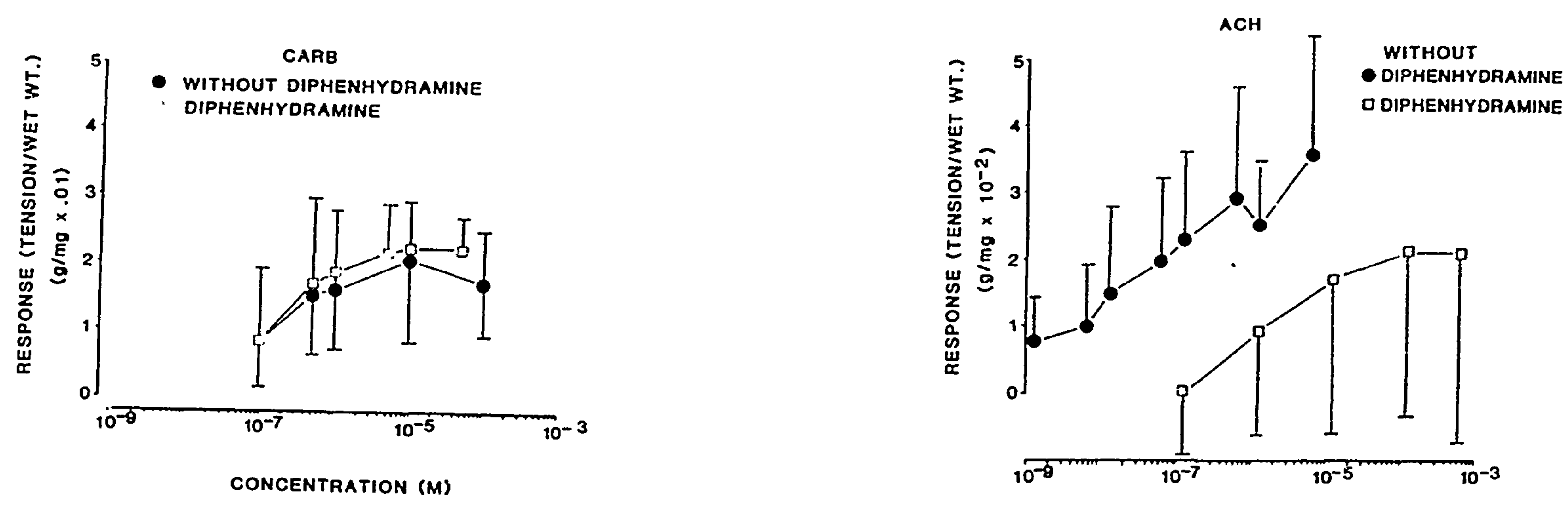
FIG. 1
AGONIST EFFECTS ON GUINEA PIG ILEUM



The contractions of guinea pig ileum tissue when exposed to two different agonists, ACh (left column) and CARB (right column), are shown in (A). Both ACh and CARB are illustrated at the same concentration in molar (M). The amplitudes are grams tension (force) divided by mg wet weight of the muscle. In this example, the muscle exposed to ACh had a stronger contraction because it was a smaller muscle.

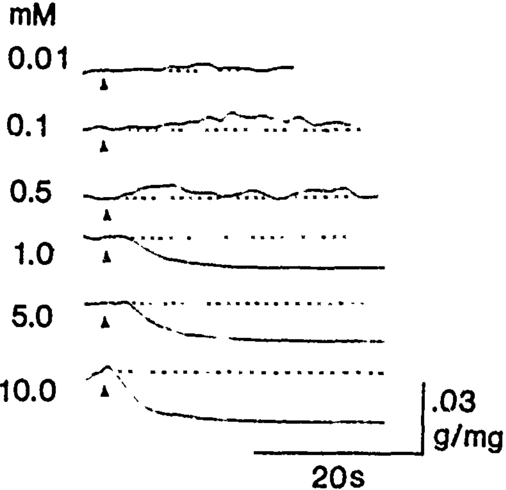
(B): In (B), the dose-response curves for ACh (closed circles) and CARB (open circles) are graphed. Each point is the mean ± the standard deviation. (For clarity, the SD was graphed in only one direction when it could overlap the other set of points graphed.) When there are no error bars, the SD was contained by the point graphed. For the ACh results, there are 10 muscle preparations from three animals; for the CARB results, there are 10 muscle preparations from six animals. The responses were measured as g-tension divided by mg wet weight of the tissue piece used. Concentrations are in molar (M).

FIG. 2
EFFECTS OF A HISTAMINE (H_1) RECEPTOR ANTAGONIST ON ACH AND CARB RESPONSES



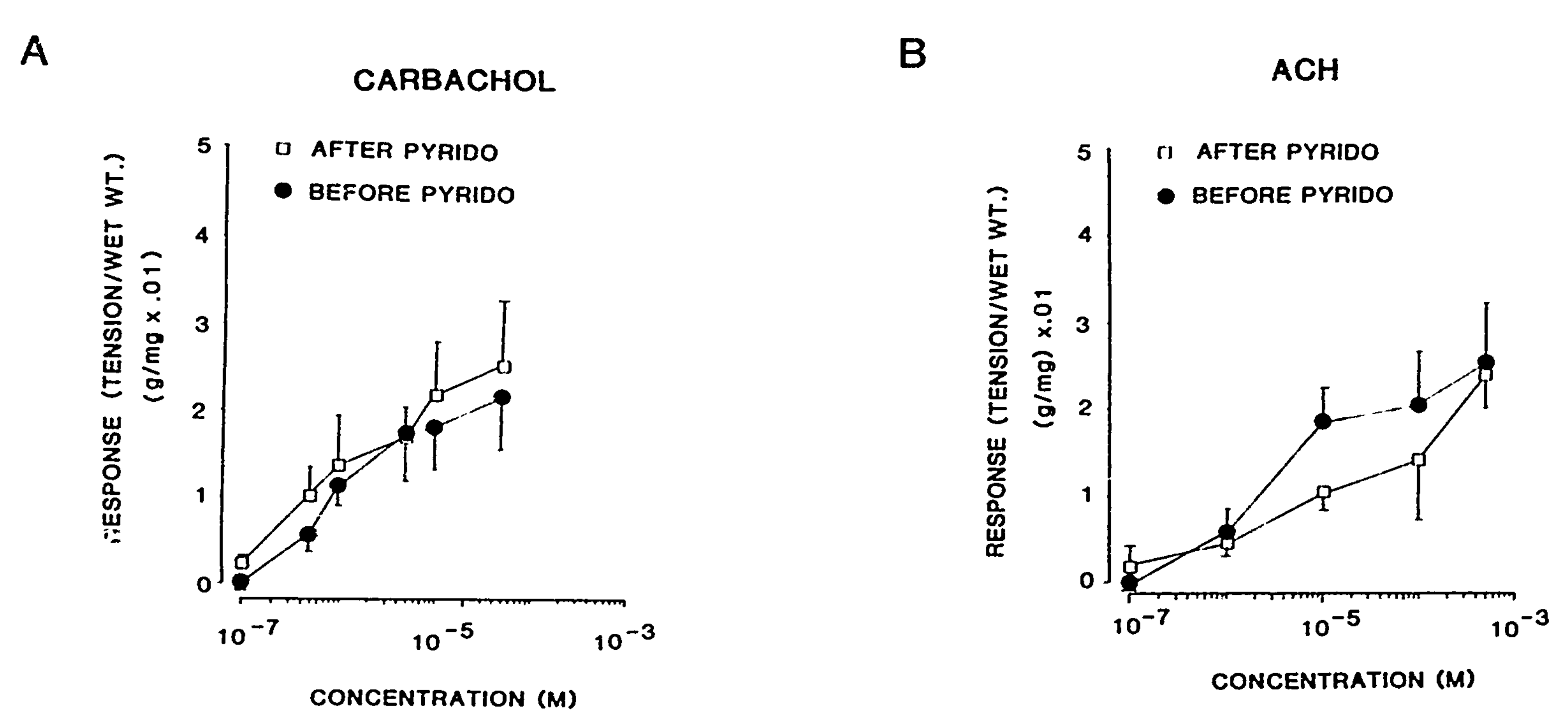
The response initiated by ACh and CARB on the guinea pig ileum is graphed to show how the presense of diphenhydramine in the Tyrodes affected these responses. Each point is the mean and the error bars are the SD. In A, the responses to CARB do not differ very much with or without the H_1 receptor antagonist. There were seven muscles tested from three animals (closed circles) without diphenhydramine and nine muscles tested from three animals (open squares) with diphenhydramine. In B, the responses to ACh demonstrate that diphenhydramine did affect the binding of this agonist. In diphenhydramine (open squares), there were 12 muscles tested from three animals and without diphenhydramine (closed circles) there were 10 muscles tested in three animals. The threshold to ACh was lower when diphenhydramine was not in the bath.

FIG. 3
PYRIDOSTIGMINE AFFECTS ILEUM MUSCLE TONE



The responses of one ileum muscle are illustrated at different concentrations of pyridostigmine presented with no "primer" drug. Vertical bars on the traces indicate where the drug was added to the bath. Upward deflections indicate an increase in muscle tone while downward deflections indicate a decrease in muscle tone. Most of the activity is spontaneous at the lower concentrations and unaffected by PYRIDO, but ≥ 1 mM there was a decrease in tone and an inhibition of spontaneous activity.

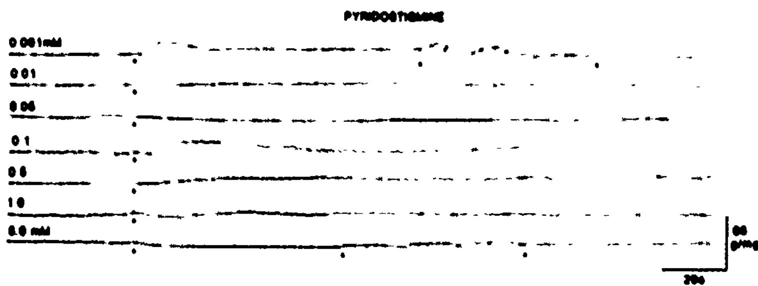
FIG. 4
AGONIST RESPONSES ARE UNAFFECTED BY PYRIDOSTIGMINE



Ilea were primed first with an agonist, CARB or ACh, then exposed to PYRIDO, then exposed to the agonist again. Neither CARB (A), nor ACh (B) responses were greatly affected by exposure to several concentrations of PYRIDO. In (A), the data points are the mean and SD of four muscles from one animal; in (B), the data points are the mean and SD of four muscles from one animal.

FIG. 5

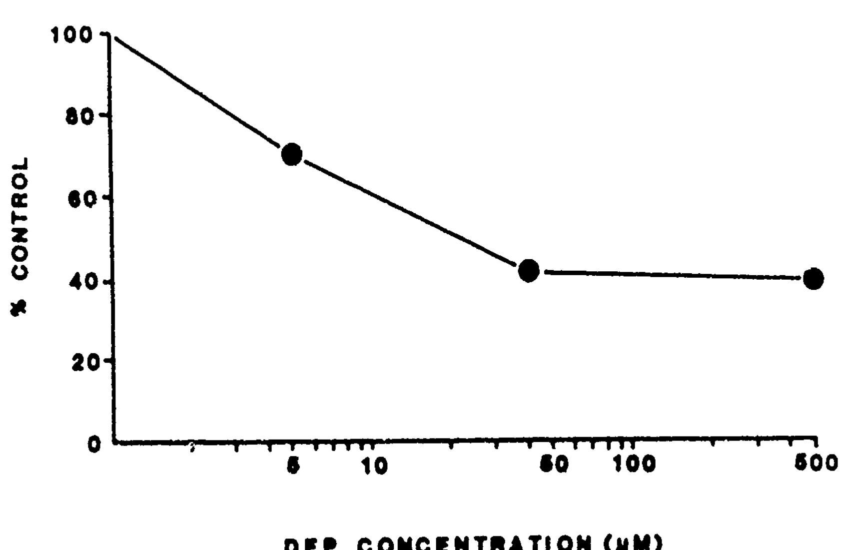
AFTER A "PRIMER", PYRIDOSTIGMINE CAUSED A VARIETY OF RESPONSES



Responses to PYRIDO at concentrations ranging from 1 µM to 5 mM illustrate that prior exposure to ACh reveals different responses that include a contraction at 1 µM and a small decrease in muscle tone at 1 mM. Arrows to the left identify when PYRIDO was added to the bath and the two sets of arrows to the right identify when the muscle was washed and returned to normal Tyrodes (top and bottom traces).

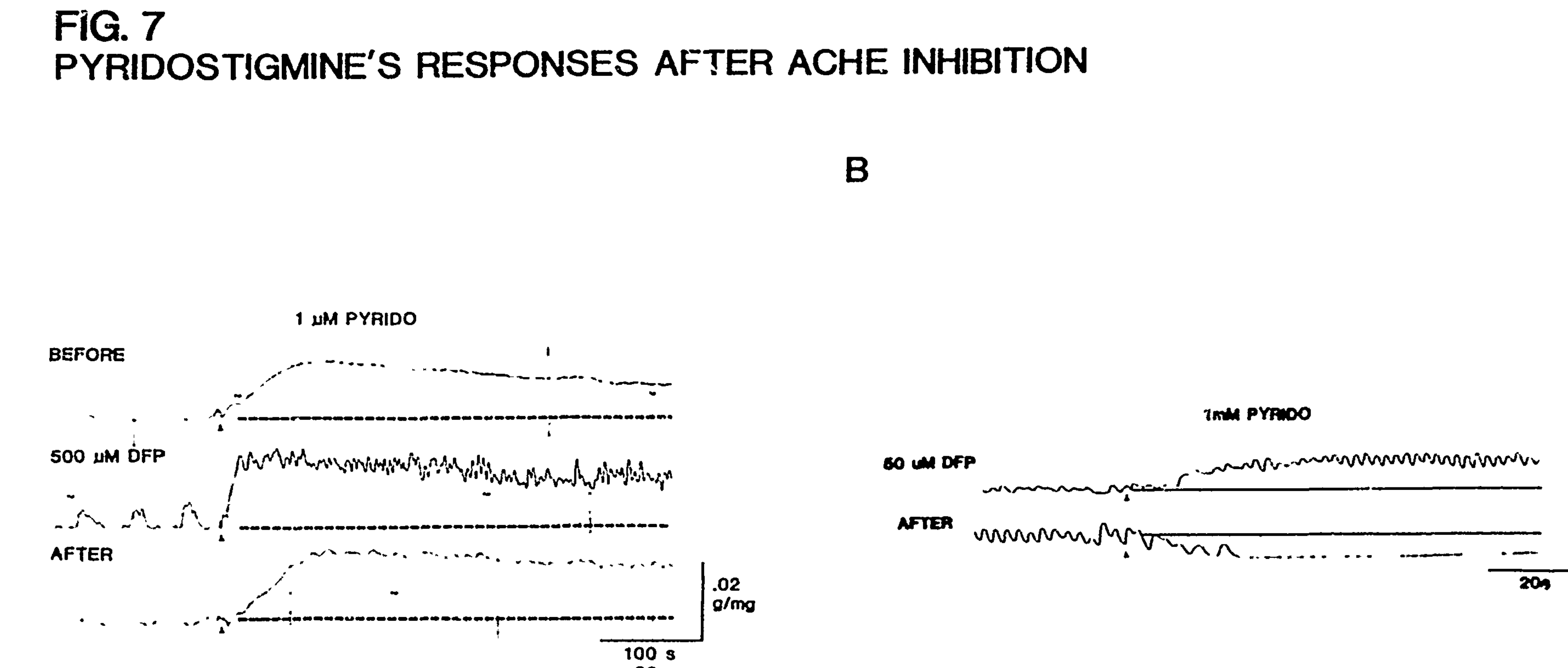
FIG. 6
INHIBITION OF ACHE BY DFP

AChE ACTIVITY



Ileum muscles exposd to diisopropylphosphorofluoridate (DFP) at different concentrations for 30 min. inhibited the Acetylcholinesterase (AChE) activity to a maximum of 39%. AChE activity was measured by the radiometric method of Siakotas, A.N., Filbert M.G., and Hester, R. (1969), BIOCHEM MED 3: 1-12.

FIG. 7
PYRIDOSTIGMINE'S RESPONSES AFTER ACHE INHIBITION



In (A), responses to 1 µM PYRIDO are illustrated before, during, and after exposure to 500 µM DFP. Arrows indicate where the PYRIDO was added to the baths. Note that the time base for the response with DFP in the bath is 100 s while the traces above and below are 20 s. DFP often initiated rhythmic contractions of the ileum muscle that were disrupted by PYRIDO. When DFP was not present, PYRIDO neither enhanced nor, in this case, abolished the spontaneous changes in tone. DFP was in the bath about 20 min when PYRIDO was added .

In (B), responses to 1 mM PYRIDO are illustrated during and after exposure to 50 uM DFP. Arrows indicate where PYRIDO was added to the bath. DFP had been in the bath for about 10 min (top trace) when PYRIDO was added. After washing, PYRIDO blocked all spontaneous activity and decreased the tone of the muscle, both responses are normal for this concentration.

CONCLUSIONS

1. Pyridostigmine affects the tone of ileum muscle tissues, causes contractions similar to traditional agonists (carbachol and acetylcholine), or relaxes the muscle tissue. This carbamate often inhibits spontaneous activity, which returns after washing with normal Tyrodes solution.

2. Pyridostigmine at low concentrations either has direct effects on muscarinic acetylcholine receptors or it stimulates release of acetylcholine from nerve terminals.

3. Pyridostigmine at high concentrations usually relaxed the tissue in a dose-dependent fashion, which suggests that it affects other types of receptors in smooth muscle membranes.

RELATIONSHIP BETWEEN PYRIDOSTIGMINE ORAL DOSES WITH PLASMA CONCENTRATION AND ERYTHROCYTE ACETYLCHOLINESTERASE INHIBITION IN THE MALE GUINEA PIG

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ABSTRACT

Potential relationships between the dose of pyridostigmine, erythrocyte acetylcholinesterase (AChE) inhibition and plasma concentration levels were investigated. Sixty-six fasting guinea pigs were divided into five groups of twelve and another group of six. Five groups received separate pyridostigmine doses: 0.12, 0.47, 0.94 (N=6), 1.90 and 7.50 mg/kg. The sixth group served as control. At 60 min post dose, the animals were sacrificed and blood collected with EDTA. A portion of the blood was centrifuged to obtain a packed erythrocyte sample for AChE activity determination. The remaining blood was then centrifuged, and the plasma removed for pyridostigmine assay. Erythrocyte AChE activity was determined by a colorimetric continuous flow method using acetythiocholine as substrate. Pyridostigmine concentration was measured by high performance liquid chromatography. There was a linear relationship between the reciprocal of the erythrocyte enzyme inhibition and the reciprocal of the dose that was administered ($r = 0.999$). A similar relationship occurred when plotting the reciprocal of the enzyme inhibition with the reciprocal of the concentration of pyridostigmine in plasma ($r = .995$). The relationship between drug levels in plasma and oral dose was linear ($r = 0.999$). The time relationships between plasma pyridostigmine levels and erythrocyte cholinesterase inhibition were also studied. At oral doses of 0.29 and 0.58 mg/kg the time of maximum inhibition of erythrocyte cholinesterase corresponded to the time of maximum concentration of pyridostigmine in plasma.

METHOD

1. Separate oral pyridostigmine doses were administered to five groups of male guinea pigs weighing 422.6 g (SD = 31.4g)

2. Sixty minutes after dosing, time of maximum erythrocyte cholinesterase (AChE) inhibition, blood was collected from decapitated animals using EDTA (Na_2) as anticoagulant

3. Blood was centrifuged to obtain plasma for pyrodistigmine determinations and erythrocytes for AChE activity

4. Plasma samples were frozen (-70°C) prior to determination of pyridostigmine concentrations by high performance liquid chromatography

5. Erythrocyte AChE activities were determined within five minutes of sample collection by a colorimetric continuous flow method using acetylthiocholine as substrate

6. In the in vitro experiment pyridostigmine was added to blood at 37°C to obtain wanted concentration in plasma volume

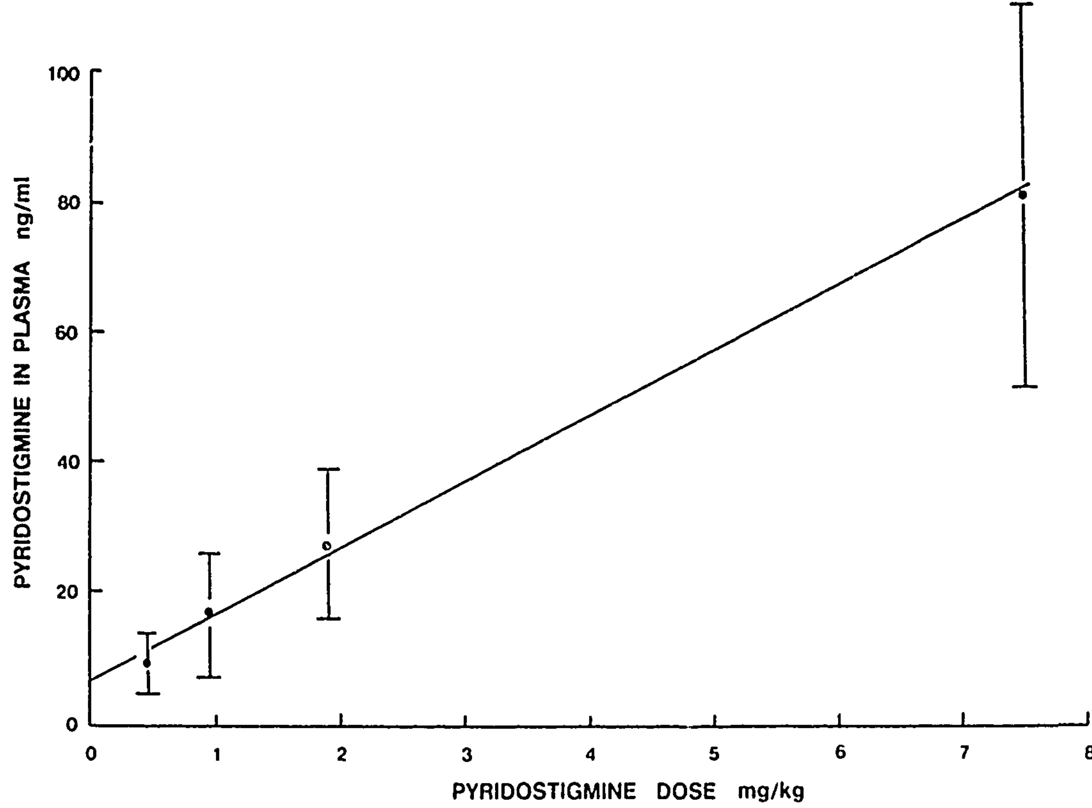
PURPOSE

To establish what relationships exist between oral doses of pyridostigmine, erythrocyte acetylcholinesterase inhibition and plasma concentration levels in the guinea pig

PYRIDOSTIGMINE PLASMA CONCENTRATION VS DOSE 60 MINUTES AFTER ORAL ADMINISTRATION

N	DOSE mg/kg	PLASMA CONC. ng/ml	(S D)
12	0.12	0	0
12	0.47	8.9	4.5
6	0.94	16.5	9.4
12	1.90	27.3	11.3
12	7.50	80.0	29.3

COEFF.OF CORRELATION = 0.999
INTERCEPT = 6.41
SLOPE = 9.98



CONCLUSIONS

1. There is a direct and linear relationship between pyridostigmine oral dose and measured pyridostigmine concentration in plasma

2. The reciprocal of ertyhrocyte AChE % inhibition relates to the reciprocal of dose administered

3. The reciprocal of erythrocyte AChE % inhibition relates to the reciprocal of pyridostigmine recovered in plasma

4. Pyridostigmine concentrations found in _in vivo_ studies at steady state and similar concentrations added to blood in _in vitro_ studies produced similar inhibitions of erythrocyte AChE

PERCENT ERYTHROCYTE AChE INHIBITION
VS PYRIDOSTIOGMINE DOSE 60 MINUTES
AFTER ORAL ADMINISTRATION

DOUBLE RECIPROCAL PLOT

NO. OF ANIMALS	DOSE mg/kg	1/DOSE x 5	AChE INHIB. (I) %	1/%I x 100
12	0.12	41.67	10.0	10.00
12	0.47	10.64	32.9	3.04
6	0.94	5.32	48.8	2.05
12	1.90	2.63	68.1	1.47
12	7.50	0.67	83.2	1.20

COEFF. OF CORRELATION 0.999
INTERCEPT 0.905
SLOPE 0.217

PERCENT ERYTHROCYTE AChE INHIBITION VS PYRIDOSTIGMINE PLASMA CONCENTRATION 60 MINUTES AFTER ORAL ADMINISTRATION

DOUBLE RECIPROCAL PLOT

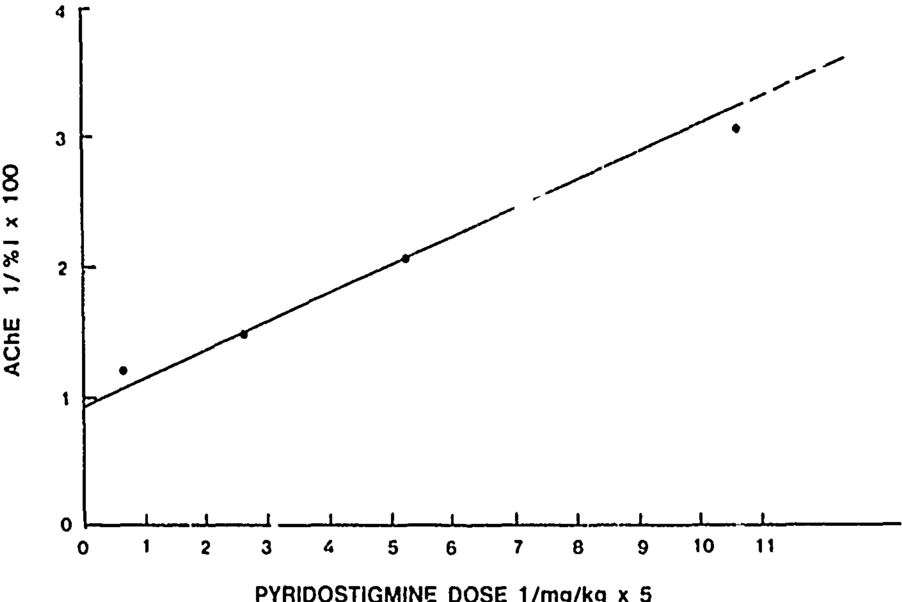
NO. OF ANIMALS	PLASMA PYRIDO.		1/ng/ml x 100	AChE INHIBITION		1/%I x 100
	ng/ml	(SD)		%	(SD)	
12	0			10.0		
12	8.9	4.5	11.26	32.9	13.3	3.04
6	16.5	9.4	606	48.8	14.2	2.05
12	27.3	11.3	3.66	68.1	9.0	1.47
12	80.8	29.3	1.24	83.2	4.6	1.20

COEFF. OF CORRELATION = 0.995
INTERCEPT = 0.887
SLOPE = 0.190

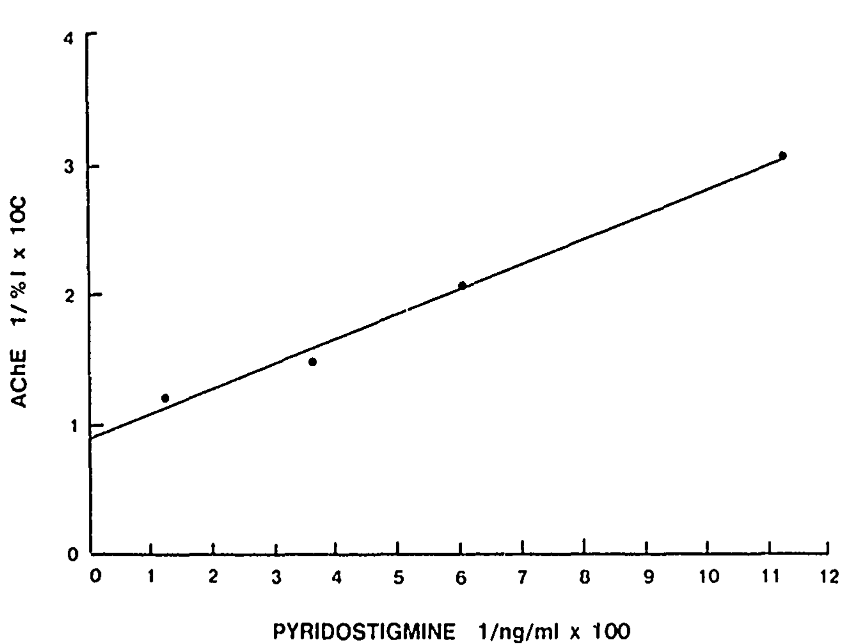
COMPARISON BETWEEN ERYTHROCYTE AChE ACTIVITIES AFTER ADDITION OR ORALLY ADMINISTERED PYRIDOSTIGMINE

DATA OBTAINED AT TIME OF MAXIMUM AChE INHIBITION

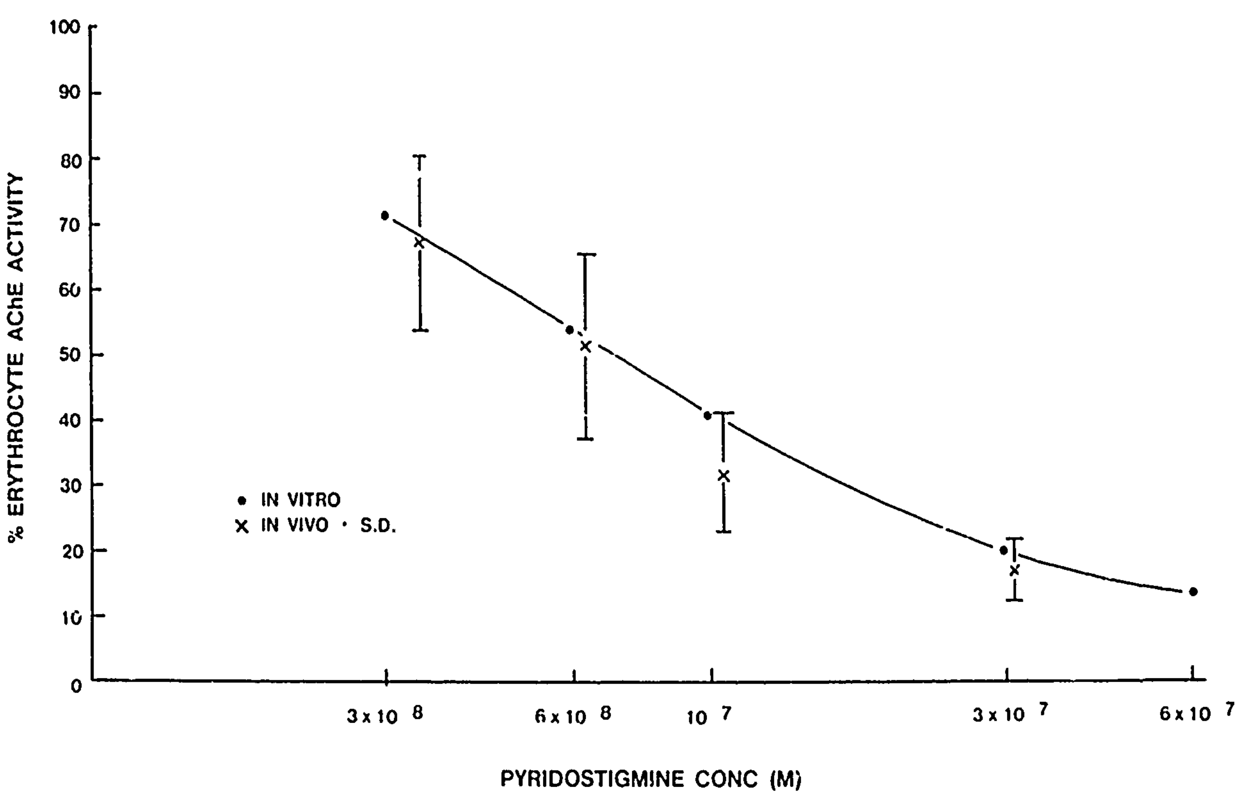
IN VITRO		IN VIVO		
PYRIDO. IN PLASMA (M)	AChE ACTIV. %	PYRIDO. IN PLASMA		AChE ACTIV. %
		ng/ml	M	
3×10^{-8}	71.4	8.9	3.4×10^{-8}	67.1
6×10^{-8}	53.9	16.5	6.3×10^{-8}	51.3
10^{-7}	40.5	27.3	1.04×10^{-7}	31.9
3×10^{-7}	19.7	80.8	3.1×10^{-7}	16.8



AChE 1/%I x 100
PYRIDOSTIGMINE DOSE 1/mg/kg x 5



AChE 1/%I x 10C
PYRIDOSTIGMINE 1/ng/ml x 100



100
90
80
70
60
50
40
30
20
10
0
% ERYTHROCYTE AChE ACTIVITY
• IN VITRO
X IN VIVO · S.D.
3 x 10 8
6 x 10 8
10 7
3 x 10 7
6 x 10 7
PYRIDOSTIGMINE CONC (M)

OXIME-INDUCED DECARBAMYLATION OF PYRIDOSTIGMINE-INHIBITED ACETYLCHOLINESTERASE

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ABSTRACT

Pretreatment with pyridostigmine (Py) followed by therapy with atropine and an oxime provides significant protection against poisoning by potent nerve agents (e.g soman). The rationale for this regimen is based on the regeneration of active enzyme from a pool of carbamylated enzyme. However, this rational therapy is clouded by the controversy concerning the use of oximes in the treatment of poisoning due to inhibition of acetylcholinesterase (AChE) by carbamate insecticides. Since Py and an oxime may be used in rapid sequence in the treatment of nerve agent poisoning, it is important to elucidate the combined effects of Py and oximes on the activity of the AChE enzyme. This study describes the effects of 2-PAM and HI-6 on carbamylation and decarbamylation of Py-inhibited AChE using whole blood (WB) and erythrocyte (RBC) AChE from guinea pigs and humans. Inhibited WB was incubated with vehicle, 2 x 10^{-4}M 2-PAM or HI-6; AChE activity was measured before and 60 min after the addition of oxime. 2-PAM and HI-6 increased WB-AChE activity from 45.9 (man) to 56.0 and 77.5% of control activity ($p < 0.05$), respectively. Similar data were obtained with guinea pig WB. To explore the possibility that oximes accelerate decarbamylation, control and Py-inhibited RBCs were washed free from excess Py and incubated with one of the following: phosphate buffer preparation (pH 7.3), 2-PAM (2×10^{-4}M) or HI-6 (2×10^{-4}M) at 37°C. Aliquots were removed from each for the measurement of AChE activity at 0, 15, 30, 45 and 60 min. First order rate constants were observed and compared to determine whether oximes accelerate the decarbamylation. The data suggest that decarbamylation is accelerated strongly ($p < 0.05$) by HI-6, and weakly by 2-PAM. In assessing the effects of 2-PAM on carbamylation of RBC AChE, washed RBCs were incubated for 10 min with buffer, alone or with concentrations of oxime ranging from 2×10^{-5}M to 5×10^{-3}M; Py was then added and the mixtures were incubated for 30 min, followed by centrifugation and determination of AChE activity. The data revealed that 2-PAM is capable of interfering with carbamylation in a dose-related fashion.

In summary, quaternary oximes are capable of increasing AChE activity in Py-inhibited blood by accelerating decarbamylation and/or interfering with recarbamylation of decarbamylated AChE. As a result, 2-PAM or HI-6 and Py should be compatible when given to animals at close intervals. Also, 2-PAM or HI-6 in combination with atropine therapy should provide antidotal value against Py intoxication.

INTRODUCTION

It is generally believed that both carbamate and organophosphorus (OP) insecticides are toxic by virtue of their ability to inhibit acetylcholinesterase (AChE) enzyme.[1] Pyridine-2-aldoxime (2-PAM) is used in conjunction with atropine for treatment of poisoning by OP insecticides.[2] The efficacy of 2-PAM and other oxime derivatives against OP intoxication is due to their ability to reactivate the OP-inhibited AChE.[3] However, with soman, the inhibited AChE rapidly becomes resistant to oxime reactivation due to a phenomenon called aging. Thus, pretreatment by carbamates (e.g., pyridostigmine) followed by therapy with atropine and oxime is required for significant protection against the class of rapidly aging OPs represented by soman, and the rationale for this regimen is based on the spontaneous regeneration of active AChE from a pool of carbamylated enzyme.[4,5] This novel pretreatment/therapy regimen is clouded by a controversy concerning the use of oximes in the treatment of carbamate intoxication.[6] Since pyridostigmine and an oxime may be used in rapid sequence in a poisonous chemical environment, it is important to elucidate the potential effects of oximes on the activity of carbamylated AChE in combination with known inhibitors. In this study 2-PAM was compared to the bisquaternary mono-oxime (HI-6) on carbamylation and decarbamylation of pyridostigmine-treated blood AChE.

1. O'Brien, R.D.: In: Insecticides: Action and Metabolism, Academic Press, N.Y., pp 88 (1967).
2. Fleisher et al: Toxicol. Appl. Pharmacol. 16:40 (1970).
3. Harris et al: Eur. J. Pharmacol. 14:38 (1971).
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5. Lennox et al: Fed. Proc. 43:564 (1984).
6. Reese: AM. Family Physic. 29:45 (1984).

METHODS

Samples of human (EDTA-K3 treated) and guinea pig whole blood (heparinized) were each incubated at 37° C, and pH 7.35 in 30 mM phosphate buffer containing pyridostigmine at 5×10^{-7} M and 1×10^{-7} M, respectively. The preparation with human blood also contained 2 μg phenylmethylsulfonylfluoride (PMSF). AChE activity, using acetyl-B-methylcholine as substrate, was determined shortly before and 60 min after the addition of carbamate and again 60 min after the addition of 2×10^{-4} M oxime. In determining the effects of the oxime on [illegible] erythrocyte AChE, blood was treated as described above before incubating with 10^{-6} M pyridostigmine or vehicle for 30 min. After centrifugation and discarding the plasma, erythrocytes (RBCs) were washed 4x with cold, heparinized saline to remove excess pyridostigmine. The RBCs (0.5 ml aliquots) were mixed with 1.0 ml phosphate buffer preparation (constituent concentrations: PMSF, 10 μg/ml; $CaCl_2$, 0.2 mM; $MgCl_2$, 0.1 mM; NaCl, 100 mM; phosphate buffer, 50 mM and pH 7.5) alone or with 2×10^{-4} M oxime at 37° C. Aliquots from the incubation mixture were assayed for AChE activity at 0, 15, 30, 45 and 60 min to permit calculation of % inhibition. After a logarithmic transformation of each value of percentage inhibition, a linear least squares regression analysis was applied to the data from each condition. control, 2-PAM and HI-6. Half times for decarbamylation were computed using the regression equations. The Wilcoxon signed ranks test was then applied to assess the changes in slope value between the conditions.

In assessing the effects of 2-PAM on carbamylation of AChE, 0.3 ml aliquots of washed RBCs were incubated for 10 min at 37° C with 1.3 ml of vehicle (phosphate buffer preparation) alone or with concentrations of 2-PAM ranging from 2×10^{-5} to 5×10^{-3} M; pyridostigmine (1×10^{-6} M) was then added to each preparation followed by an additional 30 min of incubation at 37°C. Each sample was then centrifuged, the supernatant was removed and 5 μl of RBCs were assayed for AChE activity. The factor of concentration of 2-PAM was tested for significance by doing an analysis of variance. The Newman-Keuls test was then applied to identify the lowest concentration of 2-PAM providing significant protection from carbamylation.

RESULTS

- The data in Table 1 show that oximes are capable of increasing AChE activity of pyridostigmine-inhibited whole blood.
- Fig. 1 illustrates the effects of 2-PAM and HI-6 on decarbamylation of pyridostigmine-inhibited erythrocyte AChE. Six additional replicates were generated. The median half-times (observed extremes) for decarbamylation in the presence of buffer, 2-PAM or HI-6 were 34.4 (27.6;46.4), 26.2 (13.6;48.4) and 12.5 (9.18;29.4) min, respectively. The signed ranks test applied to the sets of slope values revealed that an accelerated decarbamylation accurred in the presence of 2-PAM ($p=0.047$) and HI-6 ($p=0.01$). As illustrated in Fig. 1, the acceleration by HI-6 was dramatic. On average, the slope value in the HI-6 group was 182% larger than that in the control group, while the slope value in the 2-PAM group was only 47% larger.
- Fig. 2 depicts the effects of 2-PAM on carbamylation of human RBC AChE by pyridostigmine. The data show clearly that 2-PAM is capable of interfering with carbamylation in a dose related fashion.

TABLE 1

INCREASED ACETYLCHOLINESTERASE ACTIVITY OF PYRIDOSTIGMINE-INHIBITED WHOLE BLOOD AFTER OXIME TREATMENT

TREATMENT	INHIBITION TIME (MIN)	WHOLE BLOOD AChE ACTIVITY (% CONTROL ± S.D.)
	HUMAN BLOOD	
BUFFER + PYRIDOSTIGMINE + SALINE	60	45.4 (31.5 – 59.3)[1*]
BUFFER + PYRIDOSTIGMINE + SALINE	120	45.9 (36.2 – 55.6)[2*]
BUFFER + PYRIDOSTIGMINE + 2-PAM	120	56.0 (39.1 – 72.9)[3*]
BUFFER + PYRIDOSTIGMINE + HI-6	120	77.5 (63.9 – 91.9)[4*]
	GUINEA PIG BLOOD	
BUFFER + PYRIDOSTIGMINE + SALINE	60	47.9 (36.5 – 59.3)[1]
BUFFER + PYRIDOSTIGMINE + SALINE	120	48.3 (36.9 – 59.7)[2]
BUFFER + PYRIDOSTIGMINE + 2-PAM	120	67.4 (56.0 – 78.8)[3]
BUFFER + PYRIDOSTIGMINE +HI-6	120	84.9 (73.5 – 96.3)[4]

$P > 0.05$ FOR 1* VS 2* AND 1 VS 2
$P < 0.05$ FOR 2* VS 3* AND 2 VS 3
$P < 0.05$ FOR 3* VS 4* AND 3 VS 4

FIGURE 1

OXIME-INDUCED DECARBAMYLATION OF PYRIDOSTIGMINE-INHIBITED HUMAN ERYTHROCYTE AChE *IN VITRO*

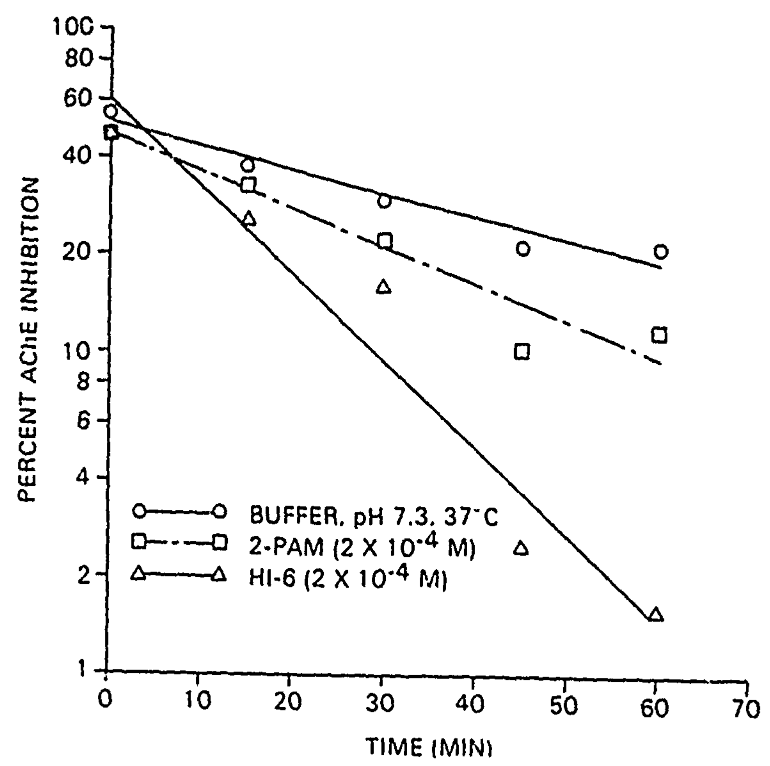
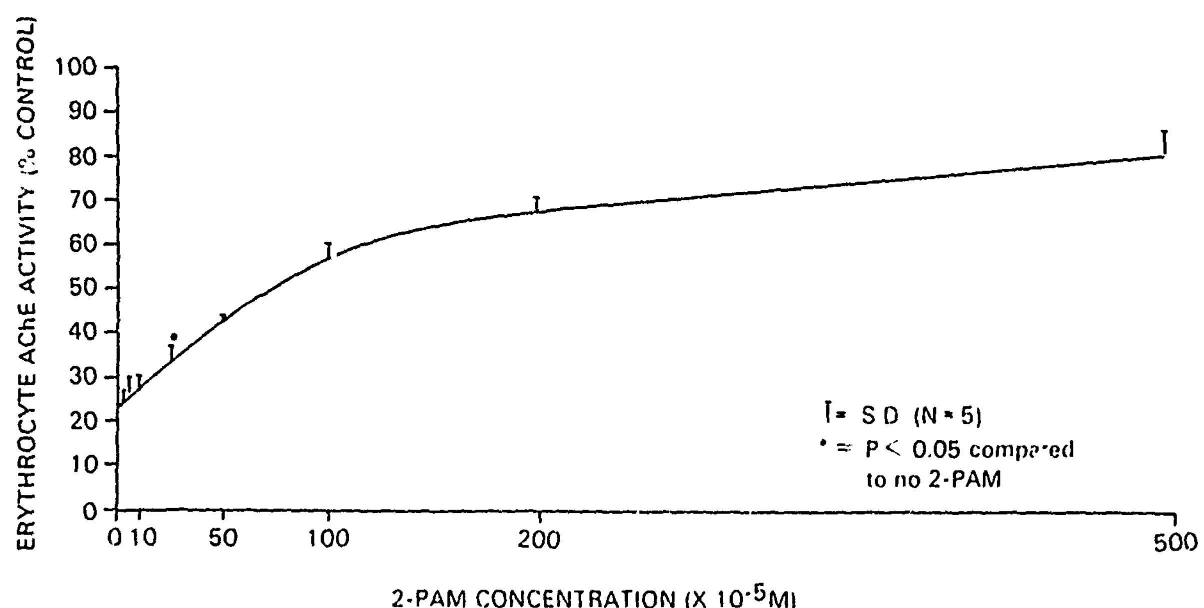


FIGURE 2

EFFECTS OF PRETREATMENT WITH 2-PAM ON INHIBITION OF HUMAN ERYTHROCYTE AChE BY PYRIDOSTIGMINE *IN VITRO*



SUMMARY

- Oximes can interrupt steady state conditions, i.e. interfere with recarbamylation of the decarbamylated acetycholinesterase.
- The data suggest that 2-PAM is weakly effective in accelerating decarbamylation.
- The data strongly suggest, for the first time, that carbamylated erythrocyte acetylcholinesterase can be reactivated by HI-6.
- The data for 2-PAM provide no evidence that this oxime would potentiate the toxicity of pyridostigmine. In fact, the present findings suggest that 2-PAM or HI-6 in combination with atropine would have antidotal value against pyridostigmine intoxication.

EFFECTS OF TIME, DILUTION AND PRESENCE OF SUBSTRATE ON DECARBAMYLATION OF PYRIDOSTIGMINE-INHIBITED ACETYLCHOLINESTERASE

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J.F. Glenn, W.G. Van Meter and H.H. Newball
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ABSTRACT

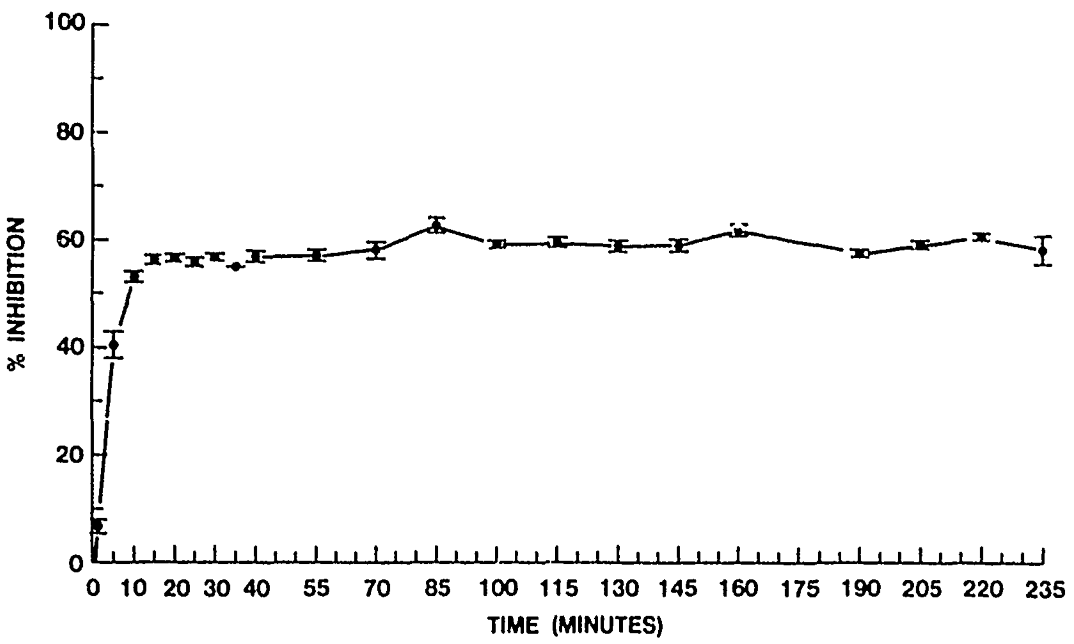
The influence of the variables, dilution, time, and presence of substrate on decarbamylation of pyridostigmine-inhibited acetylcholinesterase (AChE) was determined by two radioenzymatic assays (Newball, et al., The Pharmacologist 25:221, 1983; Siakotis, et al., Biochemical Medicine 3:1, 1969) by measurement of the hydrolysis of ^{3}H-MeCh (acetyl-β-methylcholine) and ^{14}C-ACh (acetylcholine). AChE from Electrophorus electricus was incubated in the presence of pyridostigmine (2×10^{-6}M, 0.1M phosphate buffer, pH 8, 37°C) and the kinetics of carbamylation determined by the measurement of the hydrolysis of ^{3}H-MeCh or ^{14}C-ACh (10^{-3}M). Steady state inhibition by pyridostigmine was achieved within 15 minutes and maintained for at least 4 hours, with an IC_{50} of 10^{-6}M.

To determine the effects of the variables, dilution and time, on decarbamylation, AChE was inhibited with 2×10^{-5}M pyridostigmine by incubation for 30 minutes at 37°C. At the end of the inhibition period, aliquots were diluted (range: 1:2 - 1:200) in buffer and incubated for periods up to 120 minutes. After dilution, a new steady state was established and maintained for several hours. Decarbamylation of inhibited enzyme was prevented by the presence of excess molar concentration of pyridostigmine as compared to that of enzyme. However, in the presence of substrate (10^{-3}M ACh or MeCh), the time of incubation of carbamylated enzyme became an important variable. This is probably due to a 3 - 4 log greater concentration of substrate compared to pyridostigmine. The observation that the half-time for decarbamylation at either 1:20 or 1:200 dilution was the same ($t_{1/2} = 35$ minutes) indicates that the rate of decarbamylation was independent of dilution.

When the incubation time in the presence of substrate is 5 minutes or less, both radiometric assays give an accurate in vitro measure of carbamylation of AChE by pyridostigmine. However, if the time of incubation in the presence of substrate becomes a significant fraction of the half-time for spontaneous decarbamylation, then the percent inhibition obtained must be corrected for the amount of decarbamylation of enzyme that takes place during substrate hydrolysis.

FIGURE 1.

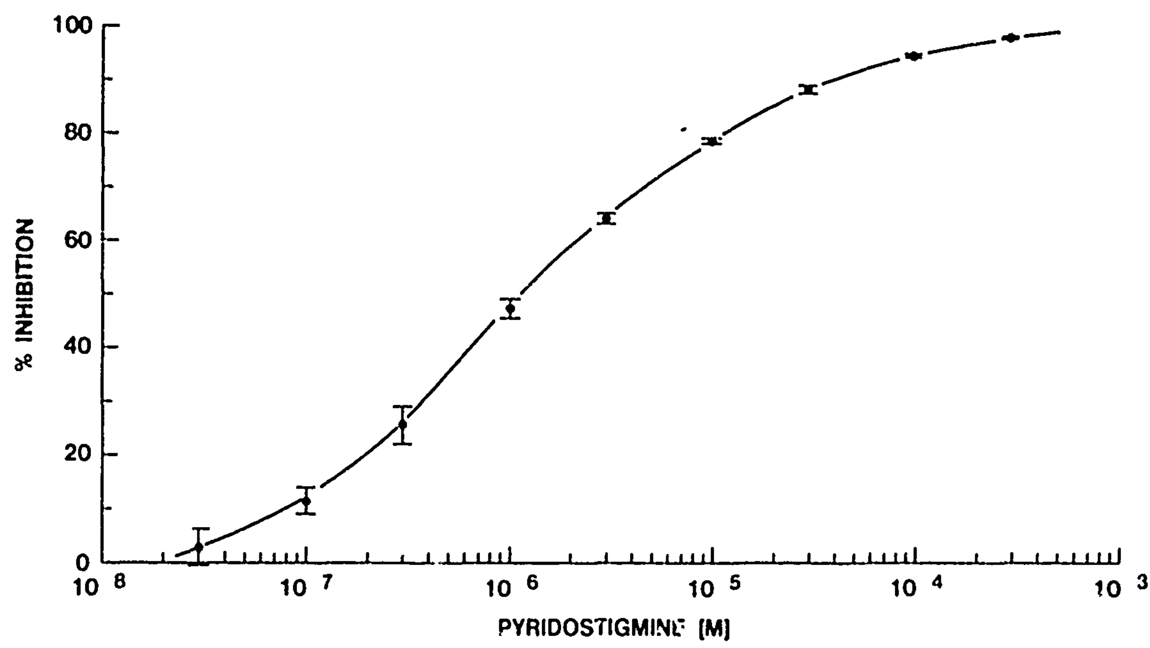
TIME COURSE OF CARBAMYLATION OF AChE BY PYRIDOSTIGMINE



The rate of inhibition of the hydrolysis of 10 ^{3}M ^{3}H-ACh or ^{3}H-MeCh by pyridostigmine was determined by incubation of eel AChE with 10 ^{6}M pyridostigmine at pH 8 and 37°C. Aliquots were assayed at the times indicated.

FIGURE 2.

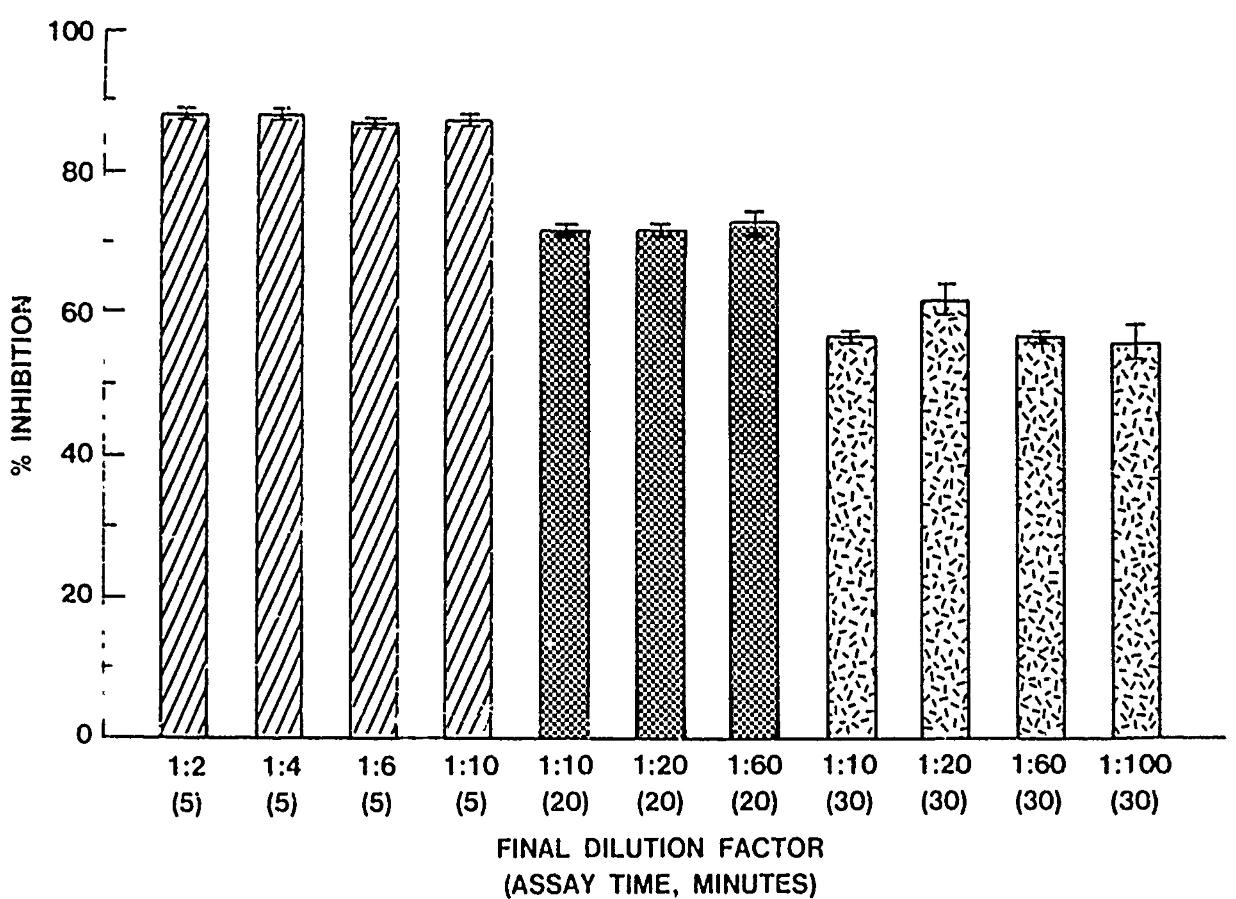
INHIBITION OF AChE AS A FUNCTION OF PYRIDOSTIGMINE CONCENTRATION



Eel AChE was incubated with various concentrations of pyridostigmine for 40 minutes at 37 C and pH 8. Enzyme activity was assayed by measurement of the hydrolysis of 10 ^{3}M ^{3}H-ACh. The IC_{50} for pyridostigmine under these conditions is 10 ^{6}M.

FIGURE 3.

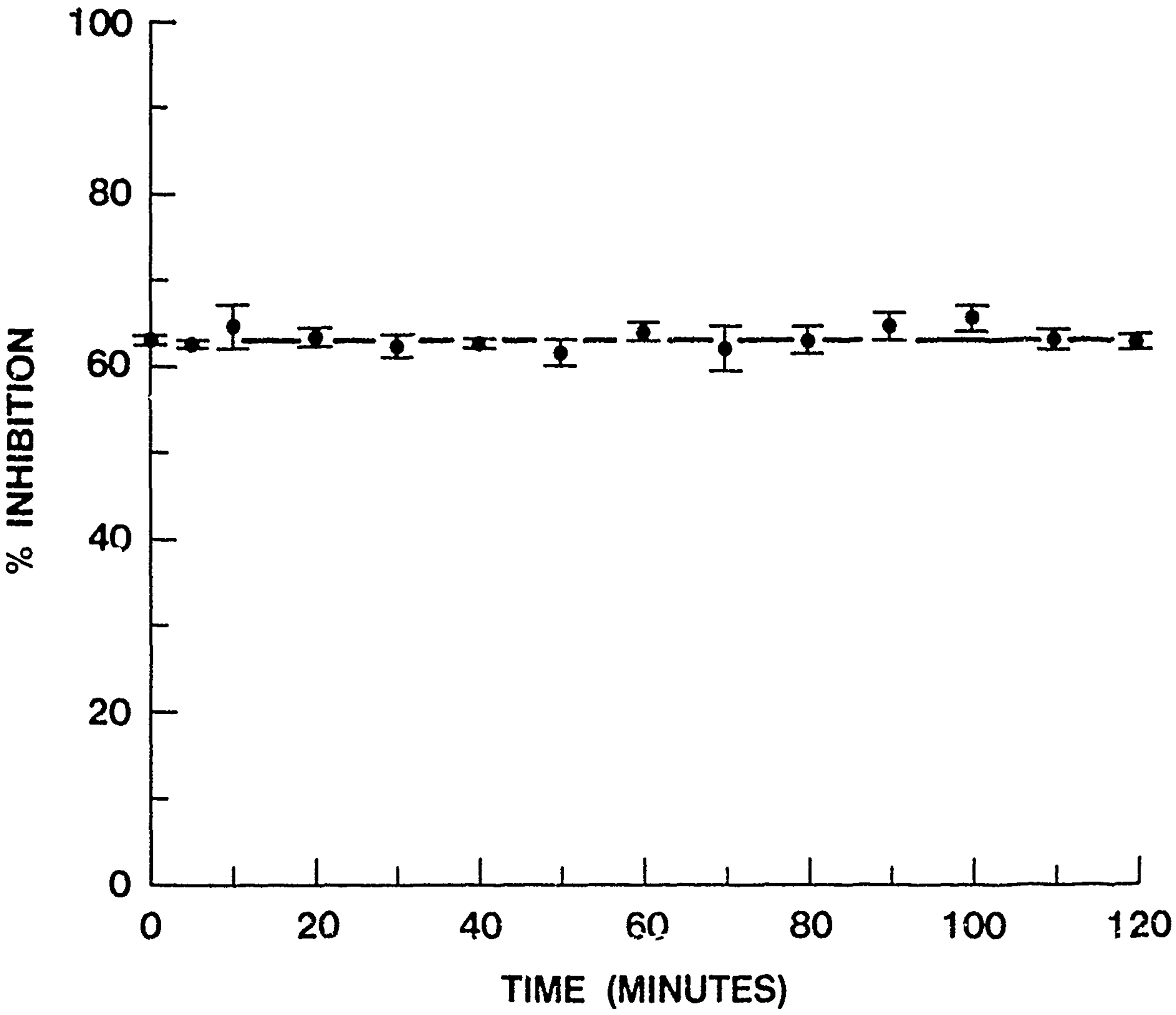
EFFECT OF DILUTION OF INHIBITED ENZYME PREPARATION ON THE STABILITY OF THE CARBAMYLATED AChE



Eel AChE was inhibited with 10^{-5}M pyridostigmine for 30 minutes at pH 8 and 37 C. Aliquots were diluted before assay and incubated with ^{3}H-MeCh for the periods indicated.

FIGURE 4.

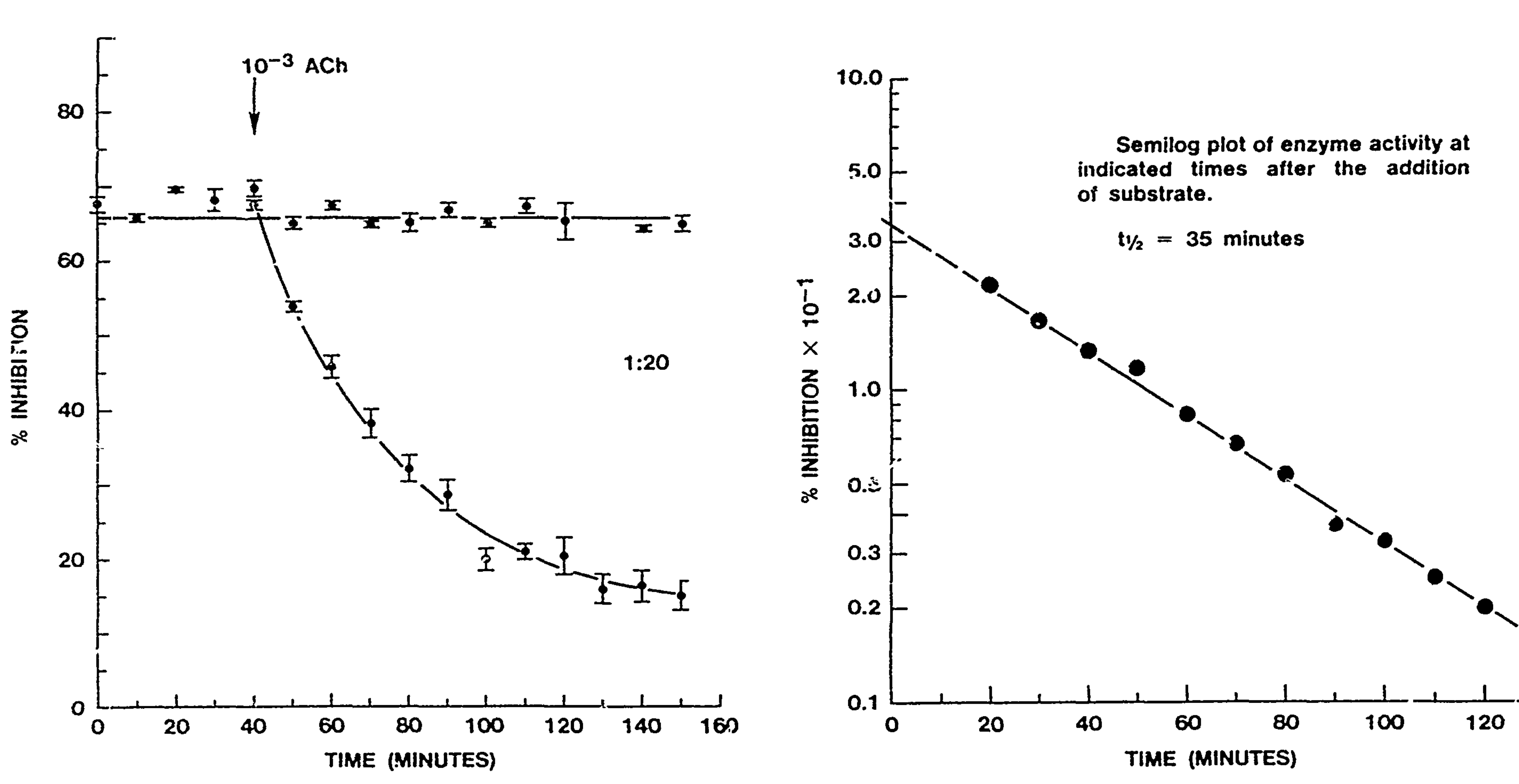
EFFECT OF TIME AFTER DILUTION ON THE STABILITY OF CARBAMYLATED ENZYME



Eel AChE was inhibited by incubation with 10^{-5}M pyridostigmine for 30 minutes at pH 8 and 37°C. Aliquots were [illegible]uted 1:20 - 1:200 and incubated at 37°C before the addition of substrate.

FIGURE 5-A.

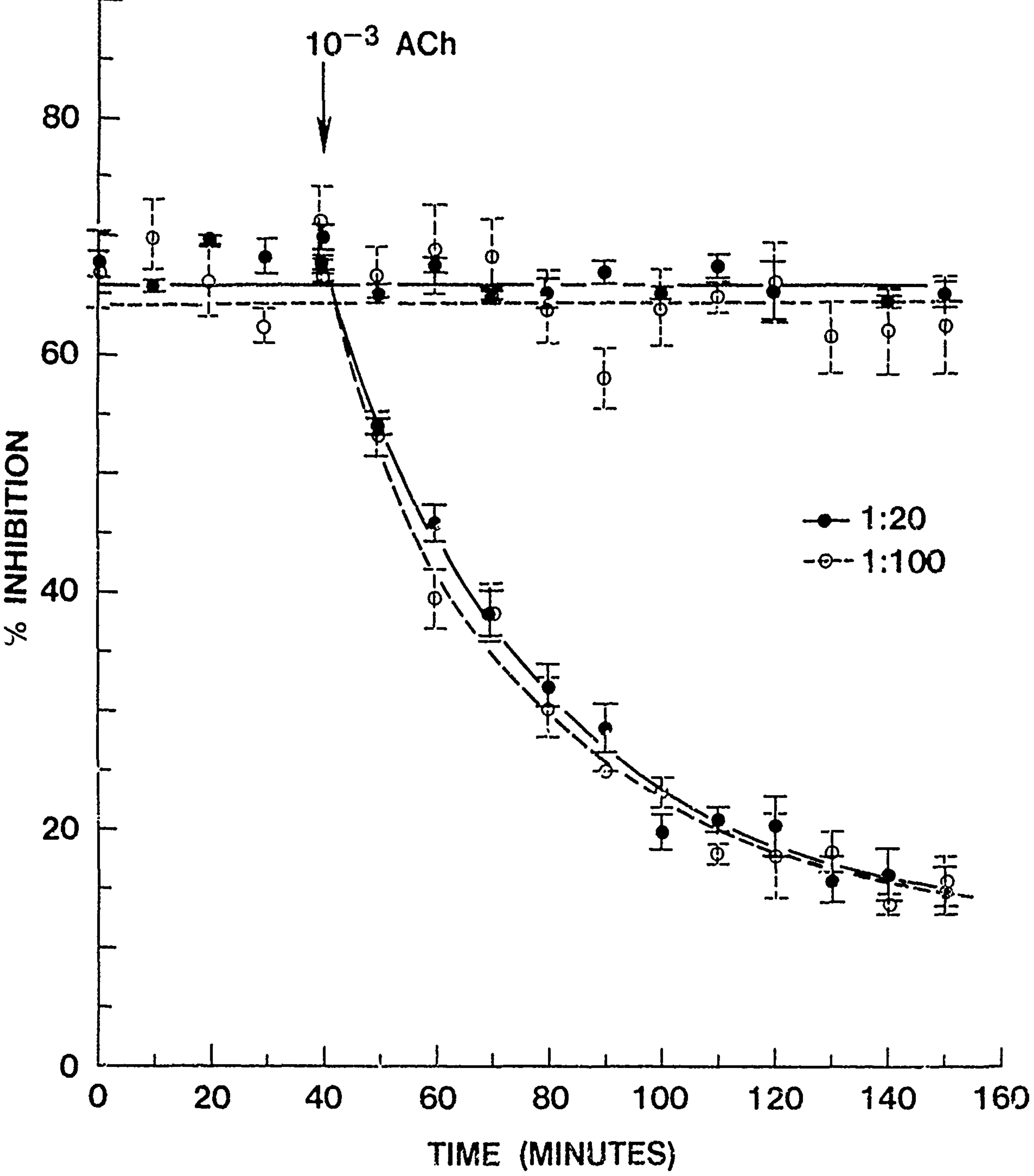
EFFECT OF THE PRESENCE OF SUBSTRATE ON THE STABILITY OF CARBAMYLATED AChE



Eel AChE was inhibited with 10^{-5}M pyridostigmine at 37°C and pH 8 for 30 minutes, then diluted 1:20 - 1:200. Substrate was added at the arrow. Aliquots were assayed for hydrolysis of ^{3}H-ACh at the times indicated.

FIGURE 5-B.

EFFECT OF THE PRESENCE OF SUBSTRATE ON THE STABILITY OF CARBAMYLATED AChE



The amount of dilution has no effect on the stability of carbamylated enzyme. Conditions identical to 5-A. Compare enzyme activity after dilution of 1:20 - 1:100.

SUMMARY

1. When incubation time in the presence of substrate is short (5 minutes or less) the radiometric assay procedures give an accurate *in vitro* measure of AChE after exposure to pyridostigmine.

2. When time of incubation in the presence of substrate is prolonged, a correction must be applied to account for spontaneous decarbamylation.

BLOOD AND SERUM CHOLINESTERASE INHIBITION BY PHYSOSTIGMINE AND PYRIDOSTIGMINE IN M. MULATTA AND M. FASCICULARIS

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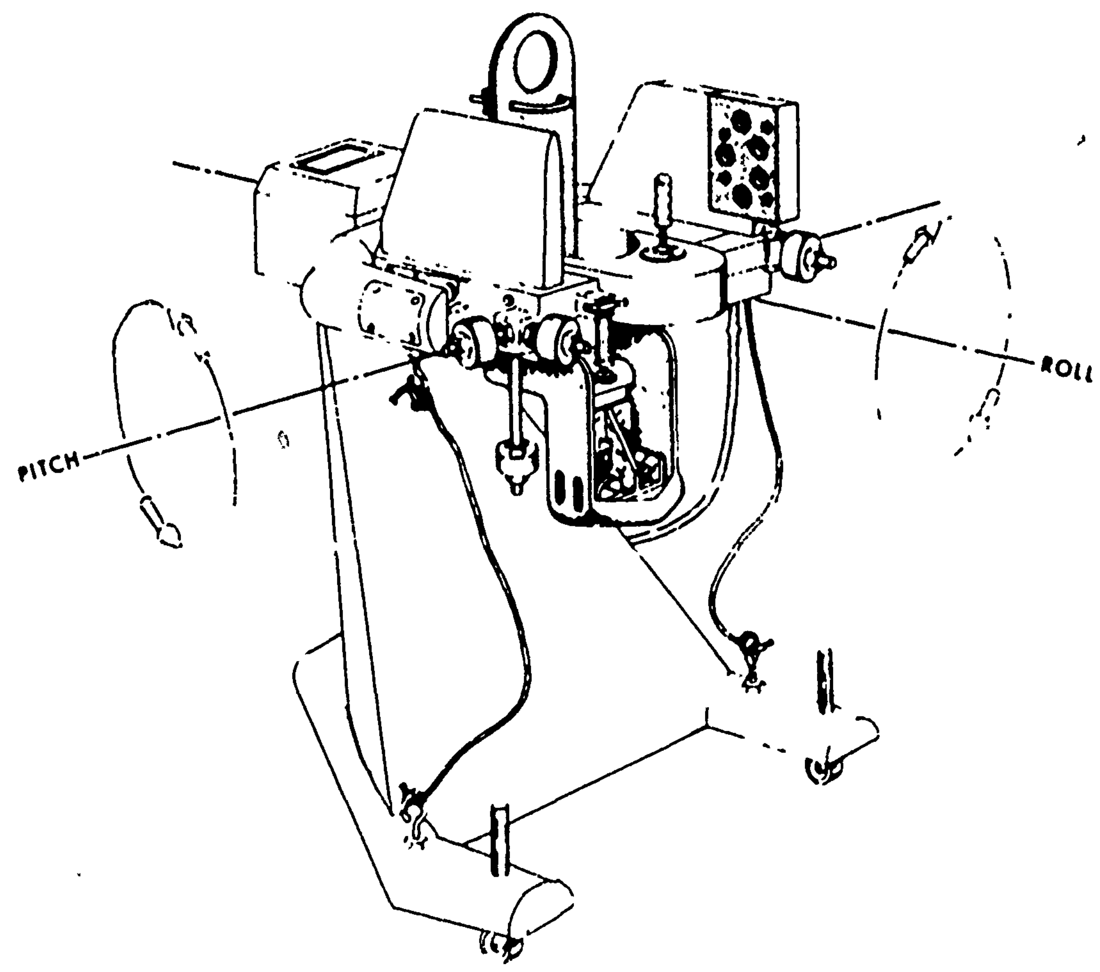
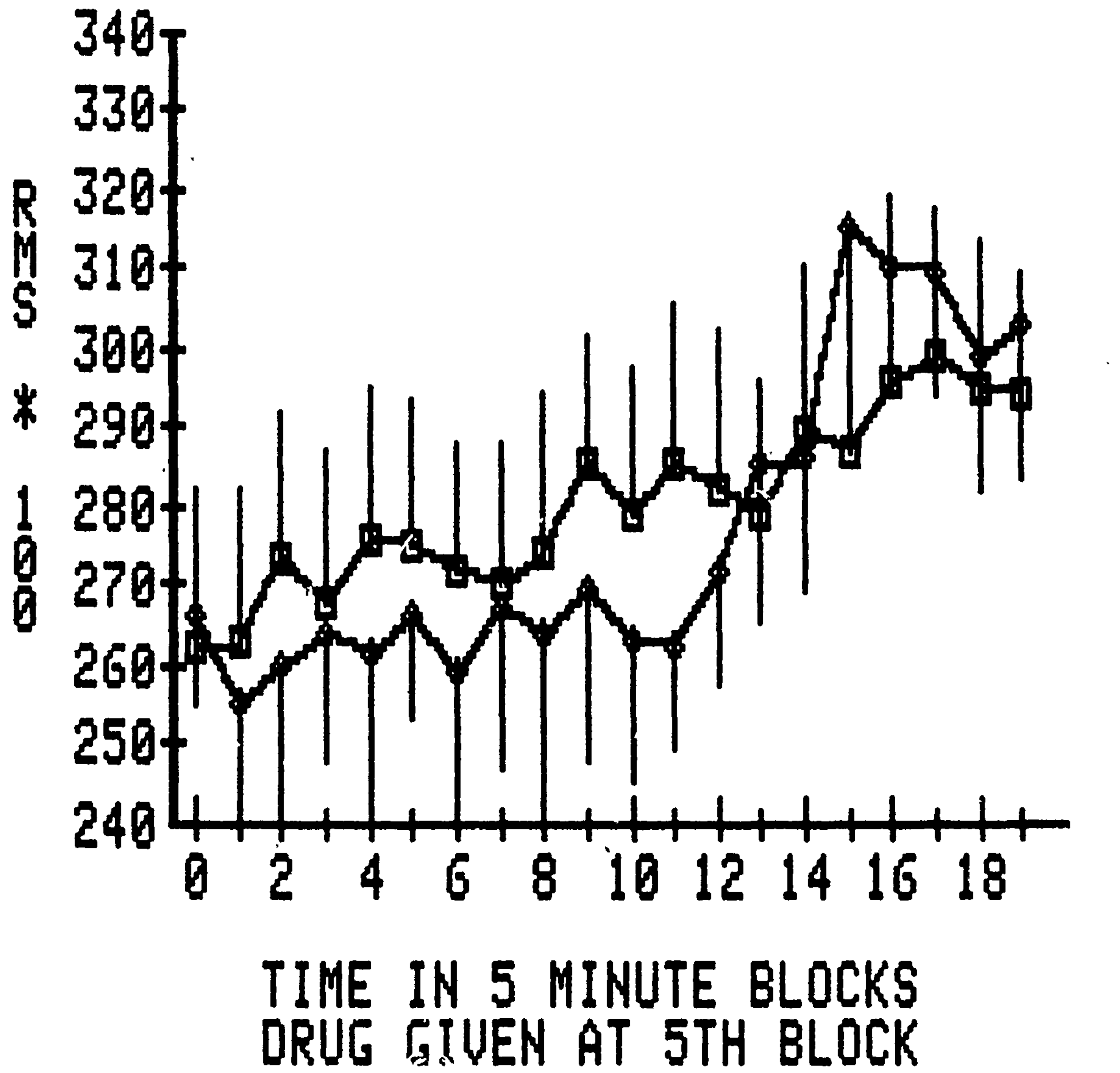
*US Army CDEC, Fort Ord, CA

INTRODUCTION

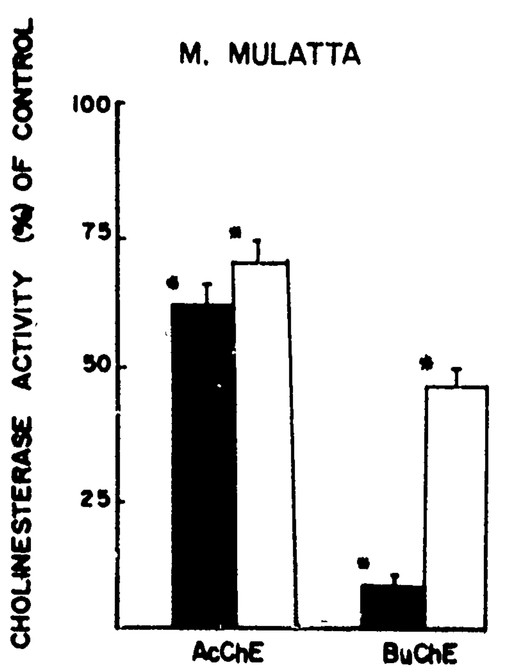
TWO SPECIES OF PRIMATES (N=4 ANIMALS EACH) WERE ADMINISTERED THE REVERSIBLE CARBAMATES, PHYSOSTIGMINE (0.13 MG/KG IM) OR PYRIDOSTIGMINE (0.15 MG/KG IM). BLOOD SAMPLES, TAKEN 30 MINS. POST-DRUG, WERE ASSAYED FOR WHOLE BLOOD AND SERUM CHOLINESTERASE LEVELS. SIMULTANEOUS BEHAVIORAL MEASUREMENTS ON THE PRIMATE EQUILIBRIUM PLATFORM (PEP) DID NOT SHOW PERFORMANCE DECREMENTS OVER 180-MINS PEP SESSIONS.

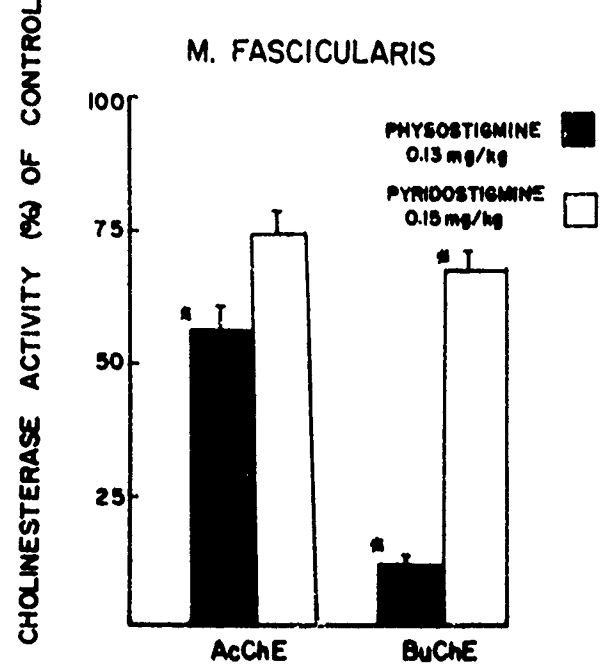


A VARIANT OF THE PRIMATE EQUILIBRIUM PLATFORM (PEP). FOR THIS STUDY, BOTH THE ROLL MODE AND THE RESPONSE TIME LIGHT PARADIGM WERE OMITTED.



REPRESENTATIVE DATA FOR PEP PERFORMANCE AFTER PYRIDOSTIGMINE (0.12 MG/KG) GIVEN AT BLOCK 5. THIS RECENT WORK WAS DONE ON A DIFFERENT SET OF PRIMATES THAN REPORTED IN THIS SESSION.





DATA SHOWING % INHIBITION OF WHOLE BLOOD AND SERUM CHOLINESTERASES IN THE TWO PRIMATE SPECIES POST-PHYSOSTIGMINE OR -PYRIDOSTIGMINE CHALLENGE. ASTERISKS DENOTE SIGNIFICANCE ($p<0.05$) VS. RESPECTIVE CONTROLS. IN ADDITION, BOTH SPECIES EXHIBITED SIGNIFICANT DIFFERENCES ($p<0.05$) BETWEEN SERUM CHOLINESTERASE INHIBITION DUE TO THE CARBAMATES.

CONCLUSIONS

PHYSOSTIGMINE AFFECTED WHOLE BLOOD AND SERUM CHOLINESTERASE COMPARABLY IN BOTH SPECIES, WITH THE LATTER ENZYME MORE HIGHLY INHIBITED. WHOLE BLOOD CHOLINESTERASE WAS 25-30% INHIBITED AT THE DOSE OF PYRIDOSTIGMINE GIVEN TO BOTH SPECIES. NEITHER CARBAMATE CAUSED SIGNIFICANT PERFORMANCE DECREMENTS ON THE PEP, AT THE DOSE LEVELS EMPLOYED.

PHYSIOLOGICAL AND PERFORMANCE EFFECTS OF PYRIDOSTIGMINE BROMIDE IN RATS AND MONKEYS

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Brooks AFB, Texas 78235-5000

Pyridostigmine bromide is included as a pretreatment drug in the chemical defense (CD) arsenal because it protects against OP agent lethality; its side effects are, at most, relatively innocuous at protective doses.

Our laboratory is conducting a series of behavioral experiments in rats and macaques to determine whether pyridostigmine bromide also protects against performance decrements induced by sublethal exposures to soman. Preliminary to efficacy studies in which both pyridostigmine and soman are given, we are investigating physiological and performance effects of pyridostigmine alone. Studies completed or in progress include:

Rats -- 1) LD_{50} determination, and 2) Dose-response functions for serum cholinesterase (ChE) inhibition, rectal temperature, tail-flick sensitivity, forelimb grip strength, shuttle-avoidance, and rotorod performance.

Rhesus Macaques; Dose-Response Functions -- 1) Effects of oral doses on serum ChE, 2) Effects of injected doses (IM) on primate equilibrium performance (PEP), and 3) Effects of IM doses on multiple avoidance performance (MAP).

Results:

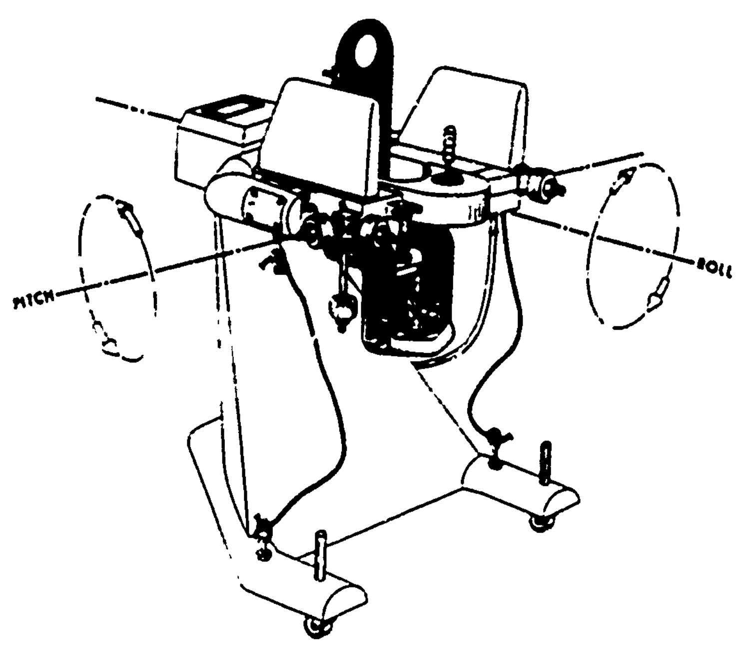
Rats -- 1) LD_{50} = 2.73 mg/kg IM injection (1 h and 24 h after injection). Most deaths occur in the first 30 min after injection. The most obvious and reliable signs of toxicity are fasciculations and red lacrimation. 2) Brain ChE activity is unaffected; serum ChE activity changes in a dose-related fashion, with 40% inhibition occurring at about 150 µg/kg; rectal temperature decreases significantly at the highest dose (1700 µg/kg); and tail-flick sensitivity, forelimb grip strength, shuttle-avoidance, and rotorod performance decline.

Rhesus Macaques -- 1) With oral doses, serum ChE inhibition peaks at about 2 h. ED_{50} dose for 30% peak inhibition was 1.5 mg/kg, but dose-related salivation during ingestion may have contaminated this result. 2) PEP is not reliably affected until high dose levels (5-10 times those required to produce a 40% inhibition of serum cholinesterase).

*(Contractor personnel supported by USAF Contract F33615-83-C-0606 to Systems Research Laboratories, Incorporated, P.O. Box 35313, Brooks AFB TX 78235.)

PRIMATE EQUILIBRIUM PLATFORM (PEP) PERFORMANCE

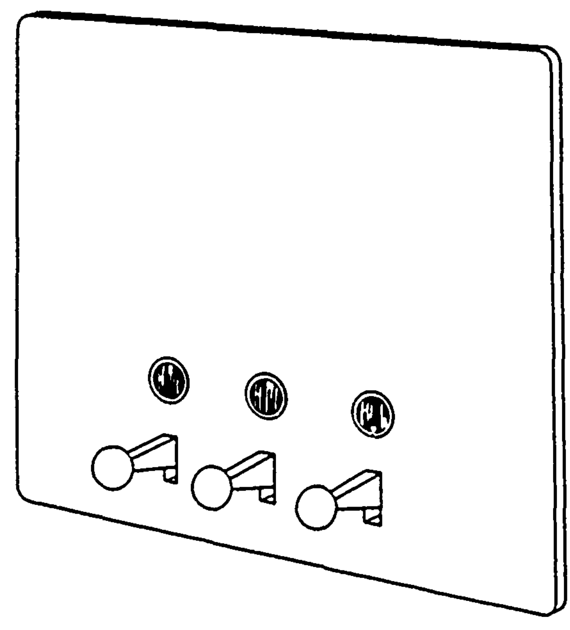
THE PEP IS A CONTINUOUS COMPENSATORY TRACKING TASK. THE EXPERIMENTAL SUBJECT IS SEATED IN A CHAIR THAT ROTATES ABOUT THE SUBJECT'S CENTER OF MASS. ROTATION ABOUT THE PITCH AXIS IS DRIVEN EXTERNALLY BY A RANDOM NOISE SIGNAL. THE SUBJECT'S TASK IS TO MANIPULATE A JOYSTICK CONTROL SO AS TO COMPENSATE FOR THESE UNPREDICTABLE PERTURBATIONS IN PITCH. PERFORMANCE IS MOTIVATED BY ELECTRIC SHOCK THAT IS DELIVERED TO THE SUBJECT'S TAIL EACH TIME THE POSITION OF THE CHAIR PLATFORM DEVIATES FROM THE HORIZONTAL BY MORE THAN 15 DEGREES. THE QUALITY OF THE SUBJECT'S PERFORMANCE IS MEASURED BY THE TIME VARIATION IN PLATFORM POSITION: THE ROOT-MEAN-SQUARED (RMS) DEVIATION ABOUT TIME-AVERAGE POSITION. THE RANDOM EXTERNAL INPUT IS ADJUSTED TO PROVIDE RMS VARIATION OF 12-15 DEGREES IN THE ABSENCE OF JOYSTICK INPUT. A TYPICAL, WELL-TRAINED SUBJECT MANIPULATES THE JOYSTICK SO AS TO REDUCE THIS VARIATION TO ABOUT 2-3 DEGREES, THUS RECEIVING ALMOST NO TAIL SHOCKS. PLATFORM POSITION IF SAMPLED BY COMPUTER 10 TIMES PER SECOND; RMS VARIATION IS AUTOMATICALLY COMPUTED AND STORED.



PEP APPARATUS

MULTIPLE AVOIDANCE PARADIGM (MAP) PERFORMANCE

The MAP is a 3-light, 3-lever, self-paced, discrete-trial avoidance task, in which the animal is repeatedly required to press one of three levers after the light above it is lighted. The animal is allowed up to two seconds to make the required response after the light appears at a randomly selected position on each trial. If the animal fails to respond within 2 s, or presses an incorrect lever, a brief shock (300 mA maximum) is delivered to his feet, and the light is extinguished. A correct response also extinguishes the light, and avoids the shock. As soon as a trial is ended (by a correct or incorrect response or by the expiration of the 2 s time limit), one of the other two lights is lighted, beginning the next trial. Several performance measures are available: latency or speed of response, accuracy or error rate, and frequency of failures to respond. For subtle treatment effects, latency is the most sensitive measure. Errors and failures to respond are extremely rare unless the subject is severely affected by a treatment.



MAP STIMULUS-RESPONSE PANEL

PROCEDURE - PEP AND MAP

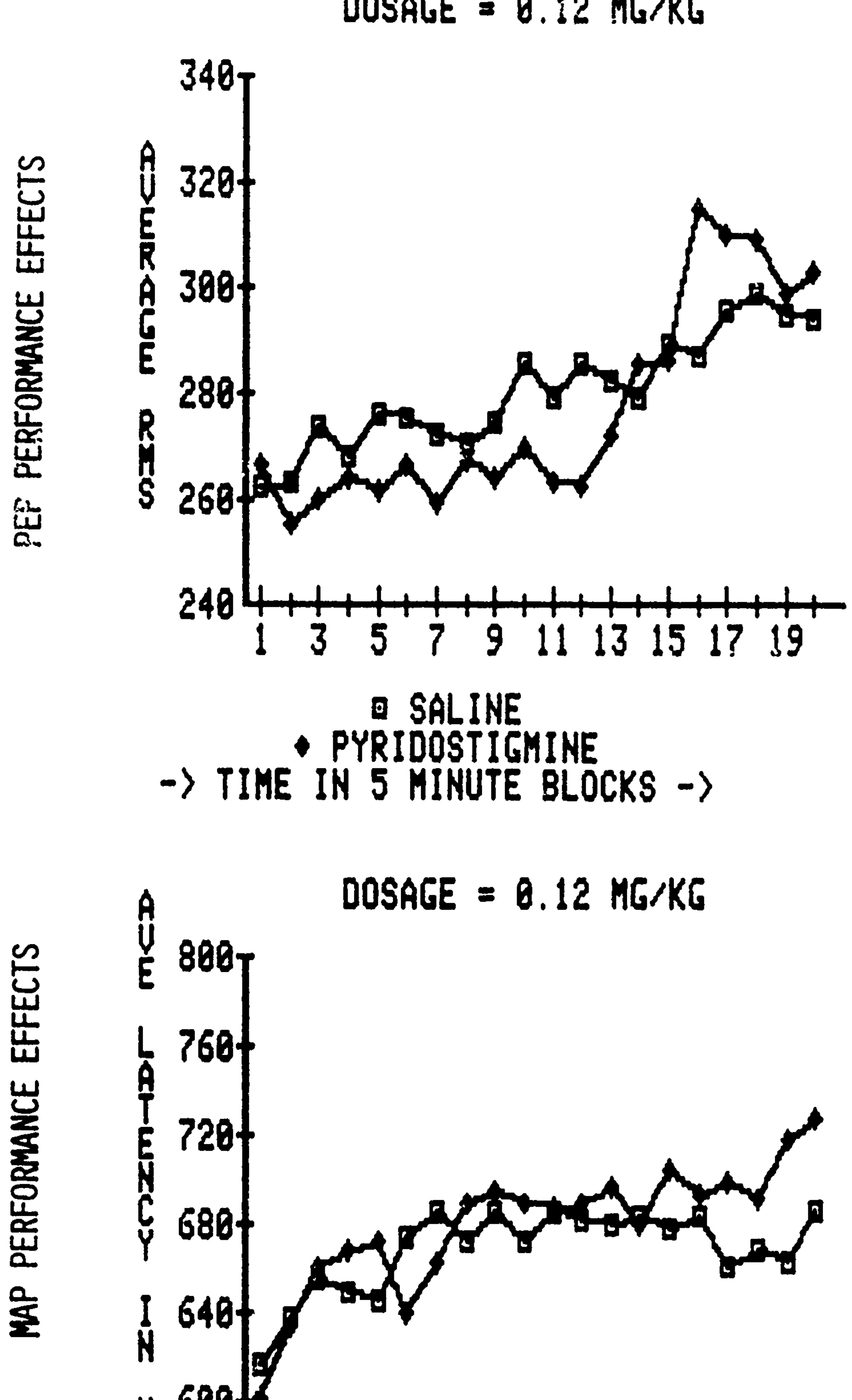
BASELINE AND DRUG TESTING SESSIONS WERE 2 H LONG. EACH SESSION WAS DIVIDED INTO 4 HALF-HOUR BLOCKS CONSISTING OF 25 MIN OF TESTING, FOLLOWED BY A 5 MIN REST PERIOD. INJECTIONS (I.M. SALINE OR PYRIDOSTIGMINE) WERE GIVEN DURING THE FIRST REST PERIOD. EACH OF 16 RHESUS MONKEYS (8 PEP & 8 MAP) RECEIVED 4 DOSES OF PYRIDOSTIGMINE BROMIDE: 0.12, 0.24, 0.48 & 0.96 MG/KG. DOSE ORDER FOR EACH MONKEY WAS DETERMINED BY A LATIN-SQUARE, REPLICATED TWICE FOR EACH TASK. THE MINIMUM INTERVAL BETWEEN DOSES WAS 7 DAYS.

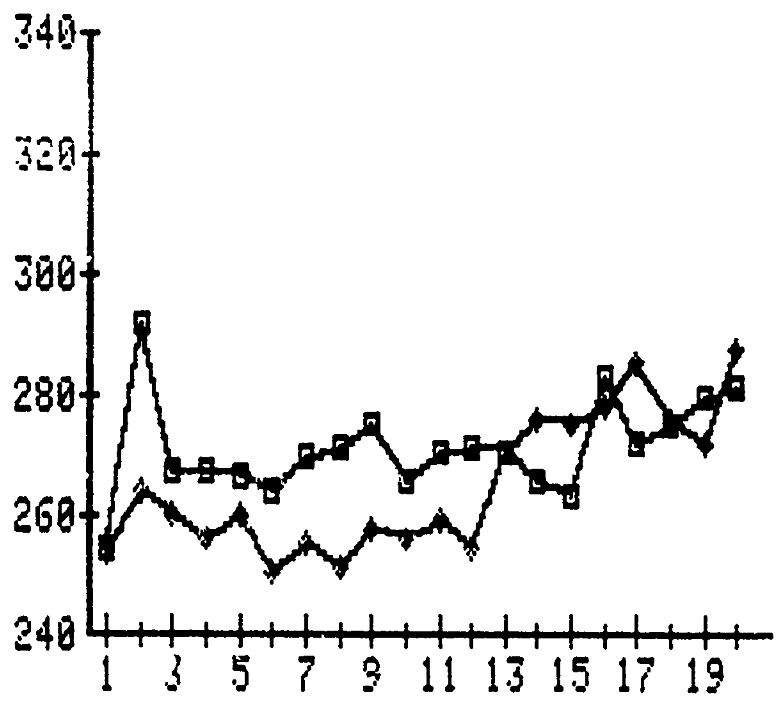
DATA ANALYSIS. MEAN RESPONSE LATENCY (MAP) AND RMS PLATFORM POSITION (PEP) FOR 20 5 MIN INTERVALS PER TEST SESSION WERE ANALYZED BY REPEATED MEASURES ANOVA, WITH DOSE AND TRIALS AS INDEPENDENT VARIABLES. DIFFERENCE SCORES (DRUG SESSION - BASELINE SESSION) AND RAW SCORES (DRUG SESSIONS ONLY) YIELDED SIMILAR RESULTS.

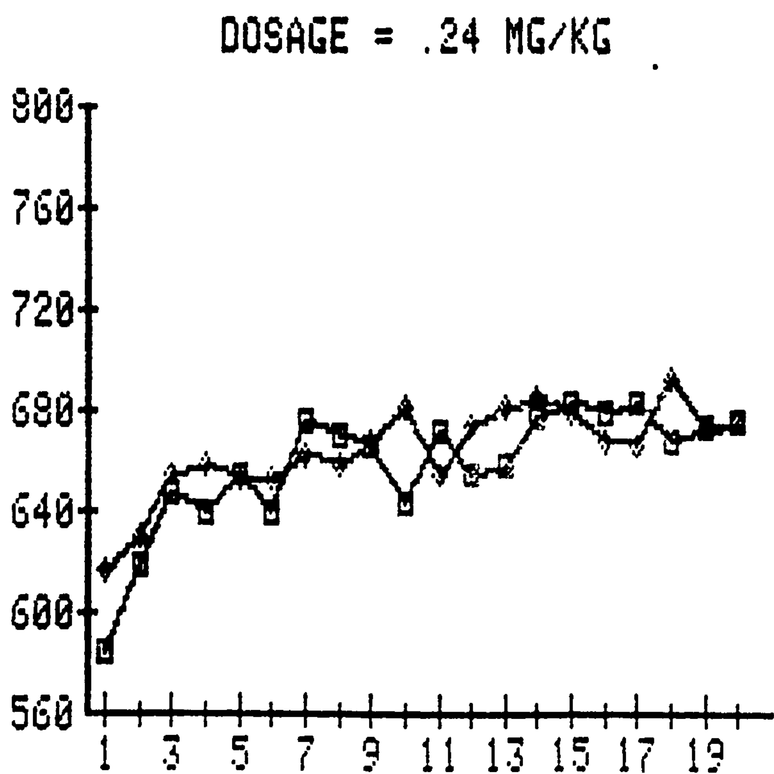
PEP - MAP RESULTS AND CONCLUSION

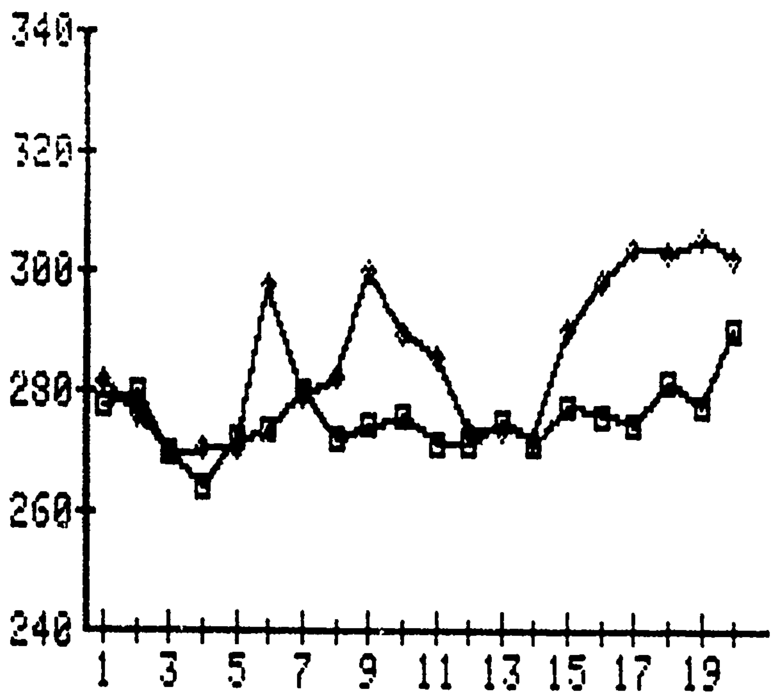
THE GRAPHS BELOW SHOW A SIMILAR PATTERN OF RESULTS FOR BOTH PEP AND MAP. NO PERFORMANCE DECREMENTS ARE OBSERVED AT THE 0.12 AND 0.24 MG/KG DOSES OF PYRIDOSTIGMINE. A SMALL DECREMENT OCCURS AT A DOSE OF 0.48 MG/KG; THE DECREMENT IS LARGER AT A DOSE OF 0.96 MG/KG. ANOVAS FOR BOTH TASKS SHOWED A HIGHLY SIGNIFICANT ($p < .001$) SUBJECT X DOSE INTERACTION, INDICATING SUBSTANTIAL INDIVIDUAL DIFFERENCES IN SENSITIVITY TO PYRIDOSTIGMINE. IN SPITE OF THIS VARIABILITY, THE DOSE EFFECT WAS SIGNIFICANT ($p < .05$) FOR PEP.

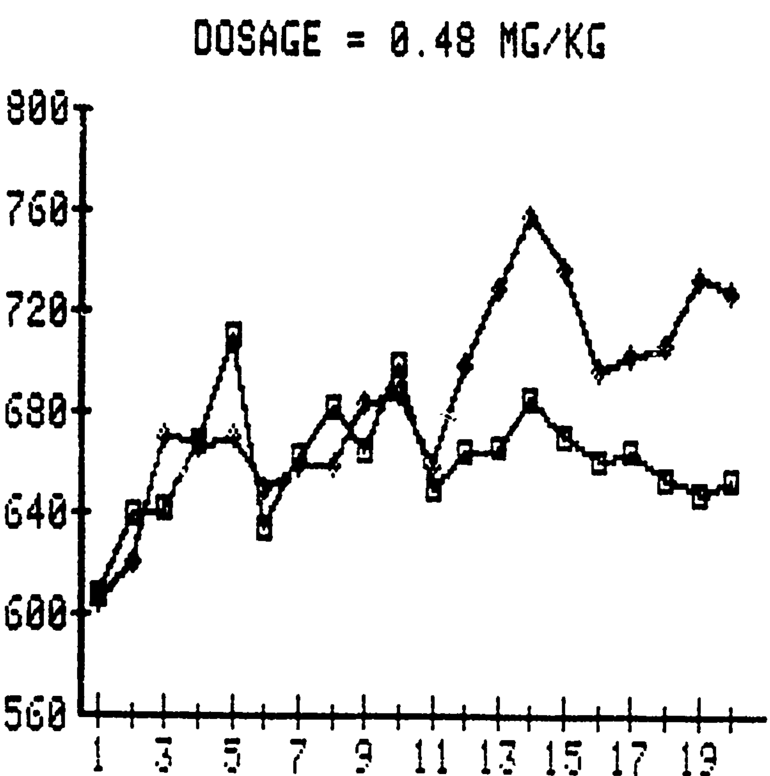
<u>CONCLUSION</u>. THE PERFORMANCE OF WELL-LEARNED TASKS BY LABORATORY PRIMATES IS UNAFFECTED BY DOSES OF PYRIDOSTIGMINE BROMIDE SUFFICIENT TO PROVIDE SUBSTANTIAL PROTECTION AGAINST THE LETHAL EFFECTS OF NEUROTOXIC ORGANOPHOSPHATE AGENTS. WHILE SUBSTANTIAL INDIVIDUAL DIFFERENCES IN SENSITIVITY TO PYRIDOSTIGMINE EXIST, DOSES AT LEAST 4 TIMES AS GREAT AS THOSE CONTEMPLATED FOR PROPHYLACTIC USE ARE SIGN-FREE IN TERMS OF OUR PERFORMANCE MEASURES, ON AVERAGE.


DOSAGE = 0.12 MG/KG
PEF PERFORMANCE EFFECTS
AVERAGE RMS
340
320
300
280
260
240
1 3 5 7 9 11 13 15 17 19
SALINE
PYRIDOSTIGMINE
-> TIME IN 5 MINUTE BLOCKS ->
DOSAGE = 0.12 MG/KG
MAP PERFORMANCE EFFECTS
AVE LATENCY IN MSEC
800
760
720
680
640
600
560
1 3 5 7 9 11 13 15 17 19
SALINE
PYRIDOSTIGMINE
-> TIME IN 5 MINUTE BLOCKS ->

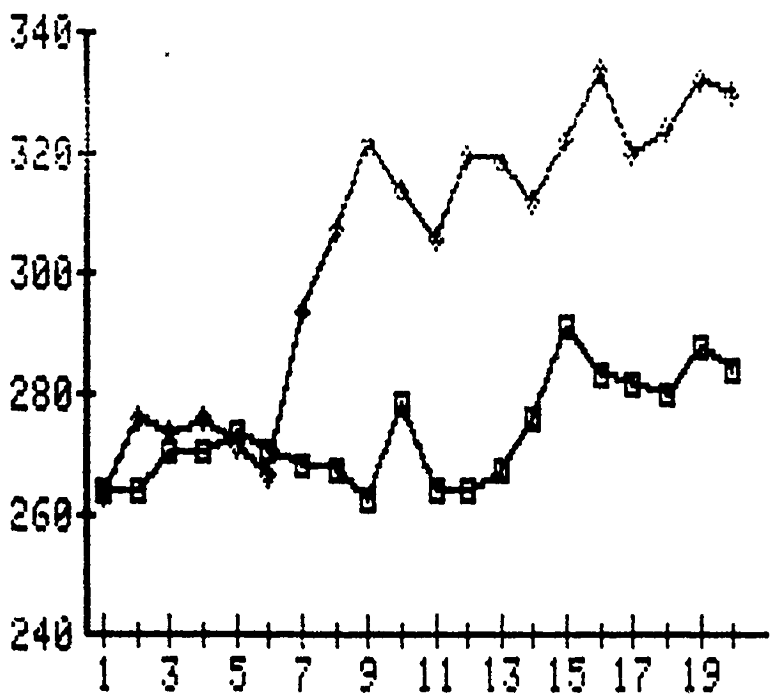

DOSAGE = 0.24 MG/KG
340
320
300
280
260
240
1
3
5
7
9
11
13
15
17
19

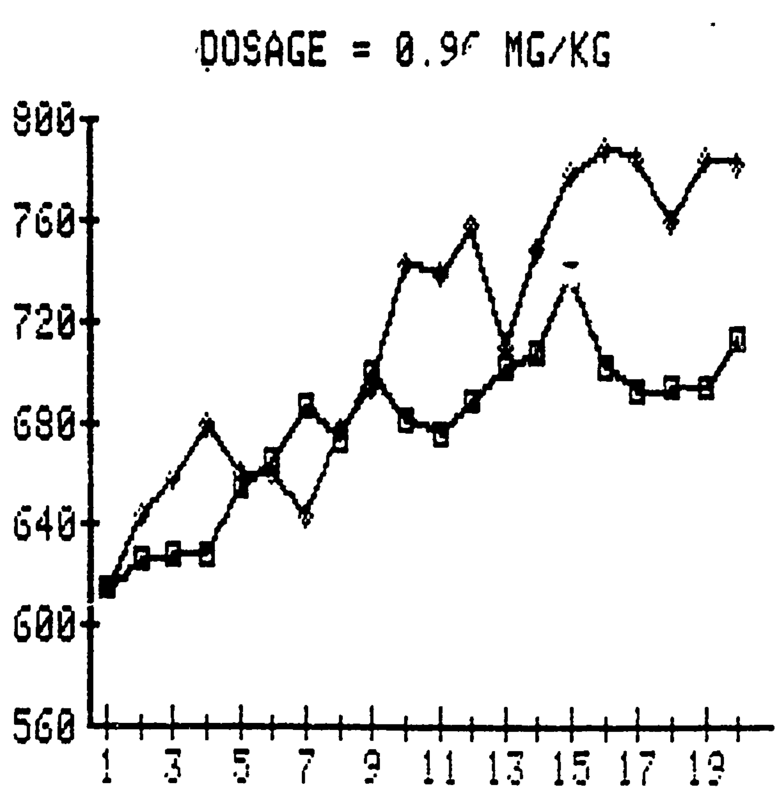

DOSAGE = .24 MG/KG
800
760
720
680
640
600
560
1
3
5
7
9
11
13
15
17
19

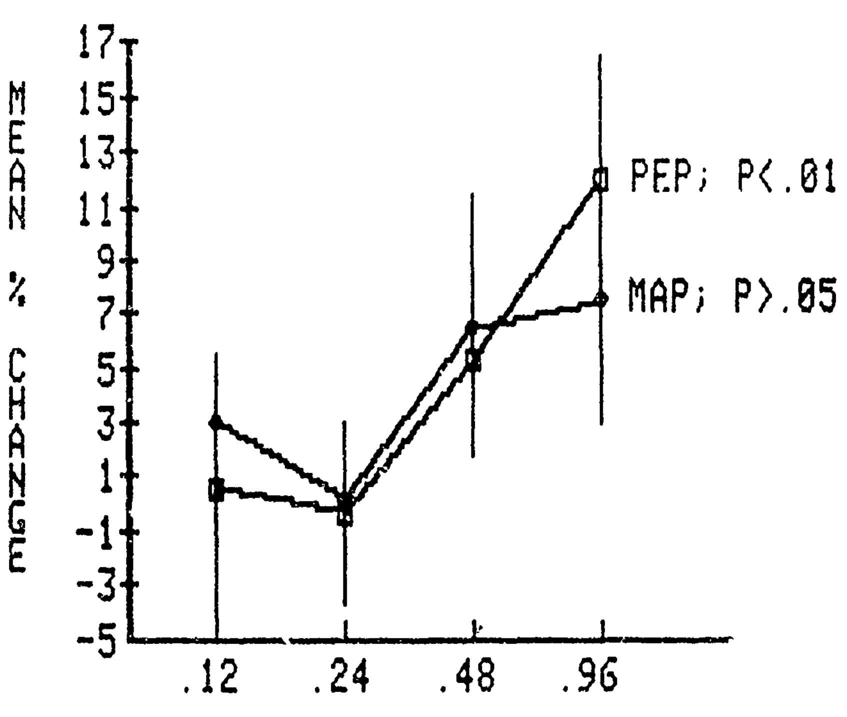

DOSAGE = 0.48MG/KG
340
320
300
280
260
240
1 3 5 7 9 11 13 15 17 19


DOSAGE = 0.48 MG/KG
800
760
720
680
640
600
560
1 3 5 7 9 11 13 15 17 19

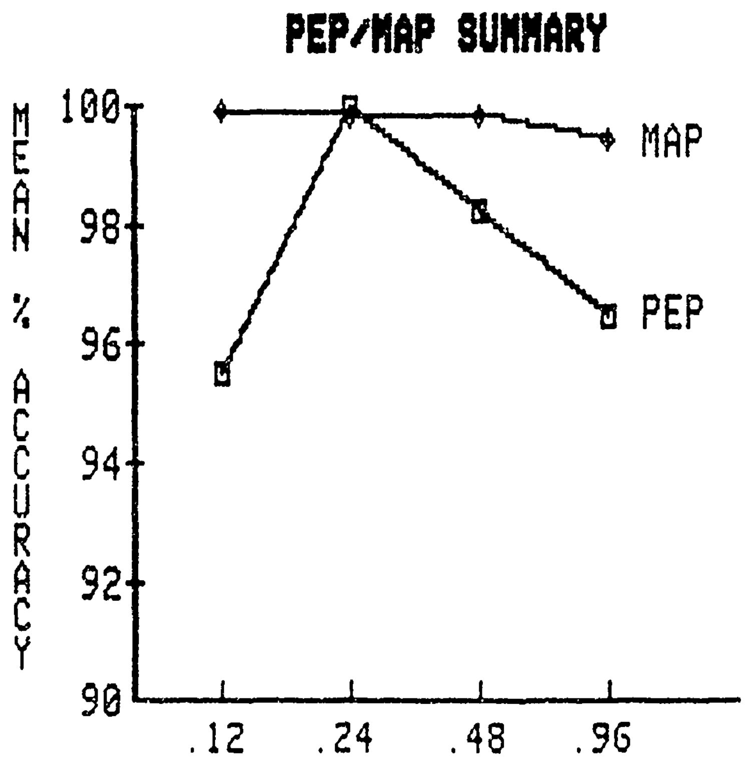
DOSAGE = 0.96 MG/KG







PYRIDOSTIGMINE (MG/KG)
PEP MEASURE = RMS OF POSITION
MAP MEASURE = RESPONSE LATENCY

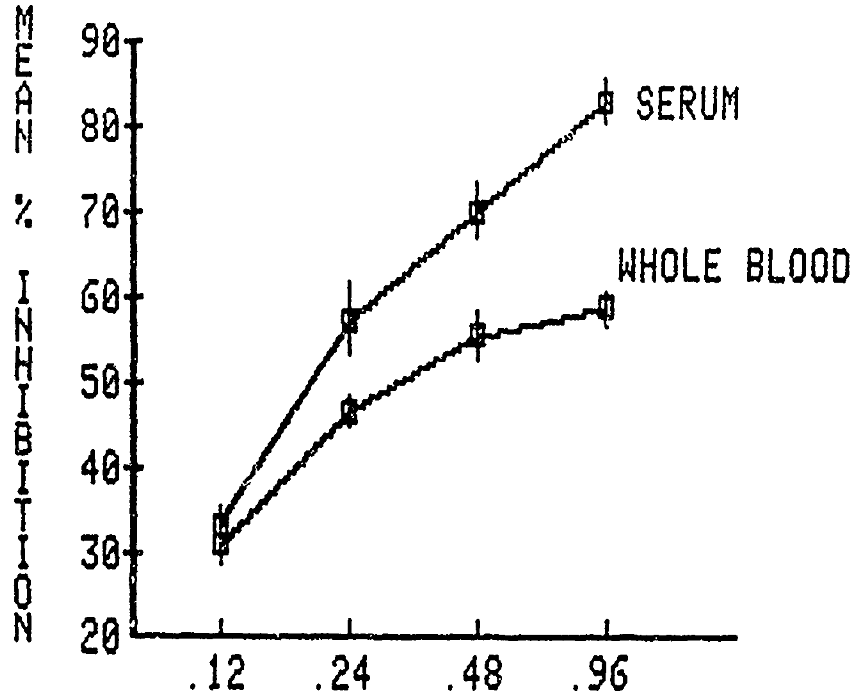


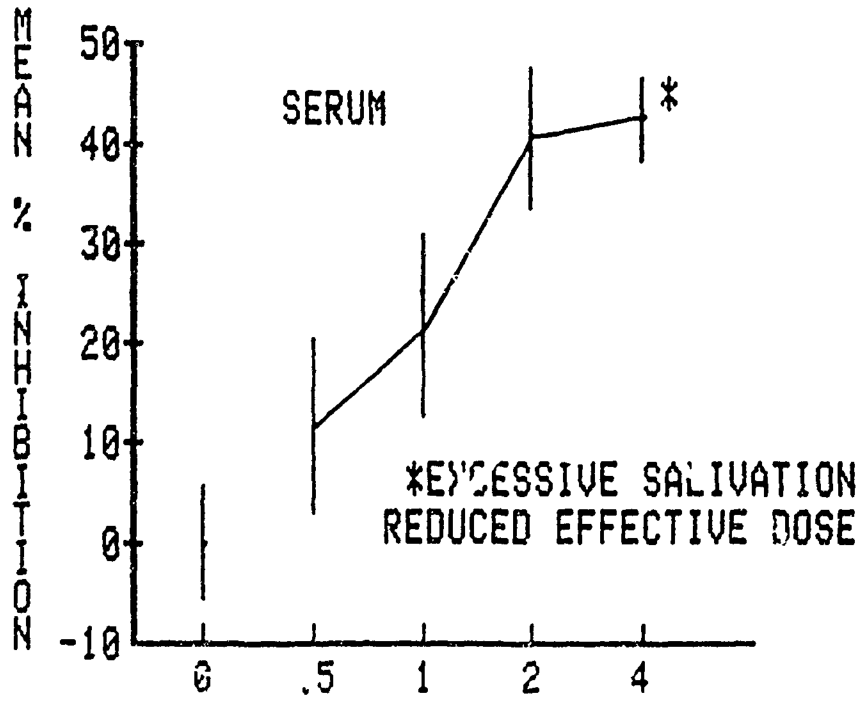
PYRIDOSTIGMINE (MG/KG)
PEP MEASURE = CRASH FREE PERIODS
MAP MEASURE = RESPONSE ACCURACY

CHOLINESTERASE ASSAYS

CHOLINESTERASE ACTIVITY IN BRAIN WAS MEASURED BY THE ENZYMATIC CONVERSION OF ACETYL-1-14C-CHOLINE IODIDE, AS DESCRIBED BY MICHALEK AND STAVINOKA (1978). RADIO-LABELED SUBSTRATE (0.10 μCI/RAT BRAIN) WAS ADDED TO A REACTION MIXTURE AND DILUTE BRAIN HOMOGENATE. THE REACTION WAS TERMINATED BY ADDING RESIN IN DIOXANE TO REMOVE UNHYDROLYZED SUBSTRATE. AFTER CENTRIFUGATION, AN ALIQUOT OF THE SUPERNATENT WAS ANALYZED BY LIQUID SCINTILLATION COUNTING.

SERUM AND ERYTHROCYTE CHOLINESTERASE ACTIVITIES WERE MEASURED USING A COMMERCIALLY AVAILABLE ASSAY KIT - BMC (BOEHRINGER CAT. NO. 124117). CHOLINESTERASES REACT WITH ACETYLTHEOCHOLINE IN A BUFFERED SUBSTRATE CONTAINING DITHIOBISNITROBENZOIC ACID TO FORM THIONITROBENZOIC ACID, WHICH WAS MEASURED PHOTOMETRICALLY BY ITS ABSORPTION IN THE REGION OF 410 NM. CHANGES IN ABSORBANCE PER UNIT TIME ARE DIRECTLY PROPORTIONAL TO CHOLINESTERASE ACTIVITY.


EFFECTS ON MONKEY CHOLINESTERASE
MEAN % INHIBITION
90
80
70
60
50
40
30
20
SERUM
WHOLE BLOOD
.12
.24
.48
.96
PYRIDOSTIGMINE (MG/KG)
INJECTION IN MUSCLE
BLOOD DRAWN 30 MIN POST INJ.


EFFECTS ON MONKEY CHOLINESTERASE
MEAN % INHIBITION
50
40
30
20
10
0
-10
SERUM
*
*EXCESSIVE SALIVATION
REDUCED EFFECTIVE DOSE
0
.5
1
2
4
PYRIDOSTIGMINE (MG/KG)
ORAL INGESTION
BLOOD DRAWN 2 HR POST INGESTION

RODENT TEST BATTERY

METHODS

ALL TESTS WERE STARTED OR ADMINISTERED 30 MIN AFTER I.M. INJECTION OF PYRIDOSTIGMINE BROMIDE OR SALINE CONTROL.

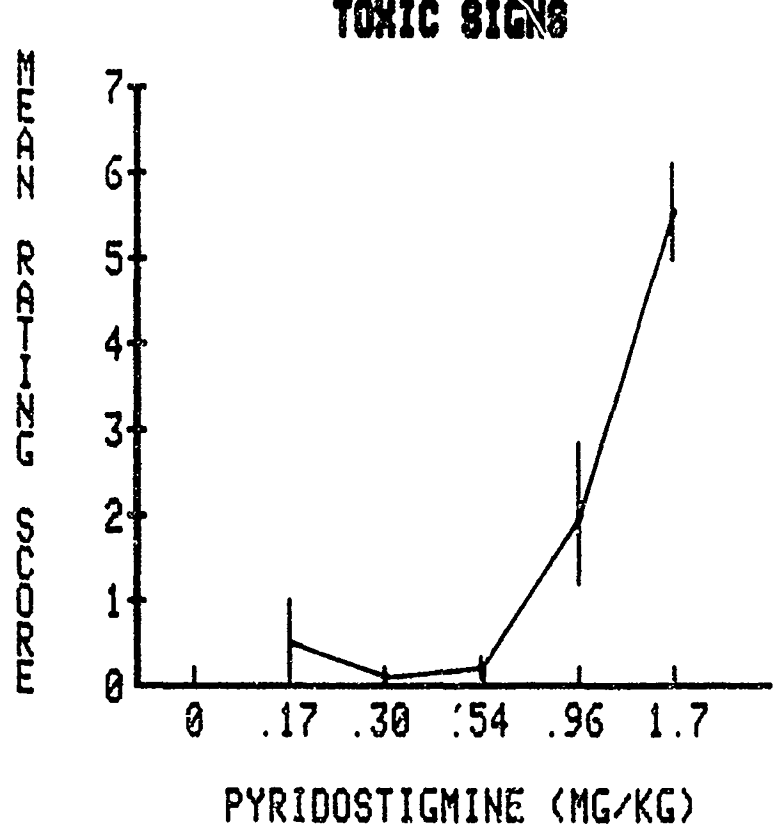
ED50 WAS CALCULATED USING THE METHOD OF PROBITS BASED ON THE CRITERION OF DIFFERING FROM THE CONTROL MEAN BY MORE THAN 1.96 X THE STANDARD DEVIATION OF THE CONTROL SCORES.

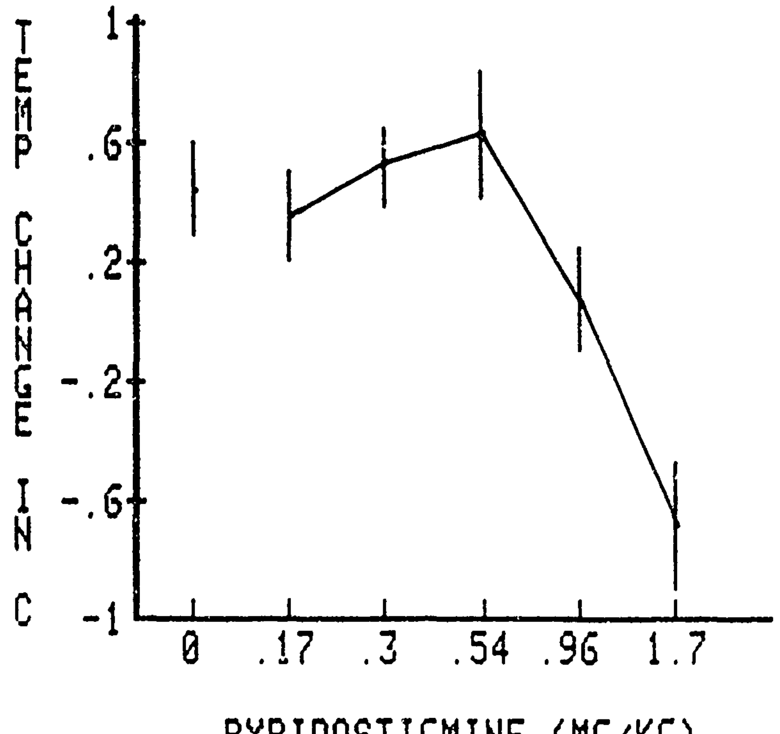
RESULTS AND CONCLUSIONS

THE MOST SENSITIVE BEHAVIORAL INDICATOR OF PYRIDOSTIGMINE TOXICITY WAS "GRIP STRENGTH", A MEASURE OF FORELIMB MUSCULAR STRENGTH, WITH AN ED50 AT 34% OF THE LD50. THIS TEST, ALONG WITH BLOOD CHOLINESTERASE LEVELS AND THE APPEARANCE OF TOXIC SIGNS, WERE THE ONLY THREE TESTS TO SHOW A DEFICIT AT THE 0.96 MG/KG LEVEL.

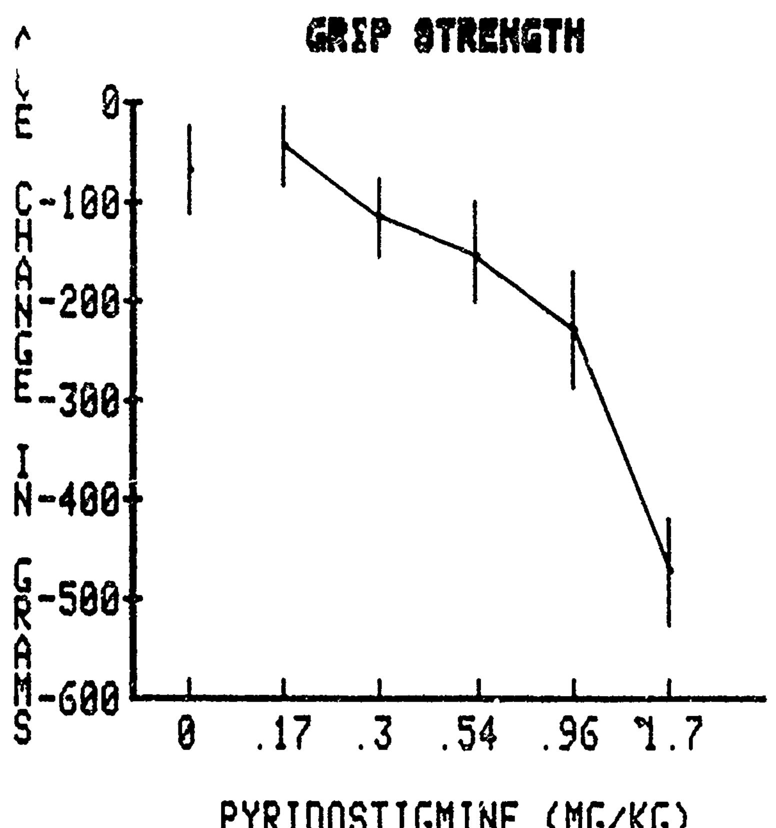
THE MORE "COGNITIVE", PRESUMABLY CENTRALLY MEDIATED, TASKS OF SHUTTLE AVOIDANCE AND PASSIVE AVOIDANCE WERE THE LEAST SENSITIVE TO PYRIDOSTIGMINE, CORRESPONDING TO THE DRUG'S LACK OF EFFECT ON BRAIN CHOLINESTERASE LEVELS.

RODENT TEST BATTERY	SUMMARY OF PYRIDOSTIGMINE EFFECTS			
			POST HOC DUNNETT'S TESTS	
PHYSIOLOGICAL	ED50	ANOVA p<	0 vs 1.7	0 vs 0.96
LETHALITY	2.73	--	--	--
TOXIC SIGNS	1.11	.001	.005	.01
RECTAL TEMPERATURE	1.74	.001	.005	NS
BIOCHEMICAL				
WHOLE BLOOD ChE	0.298	.001	p<.005 for all comparisons	
BRAIN ChE	INDETERM.	NS	NS	NS
STRENGTH AND SENSITIVITY				
GRIP STRENGTH	0.92	.001	.005	.025
THERMAL SENSITIVITY	2.21	.005	.05	NS
PERFORMANCE				
SHUTTLE ACQUISITION	INDETERM.	.01	.05	NS
SHUTTLE PERFORMANCE	1.79	.025	.025	NS
PASSIVE AVOIDANCE	INDETERM.	NS	NS	NS
ROTA-ROD	INDETERM.	.001	.005	NS

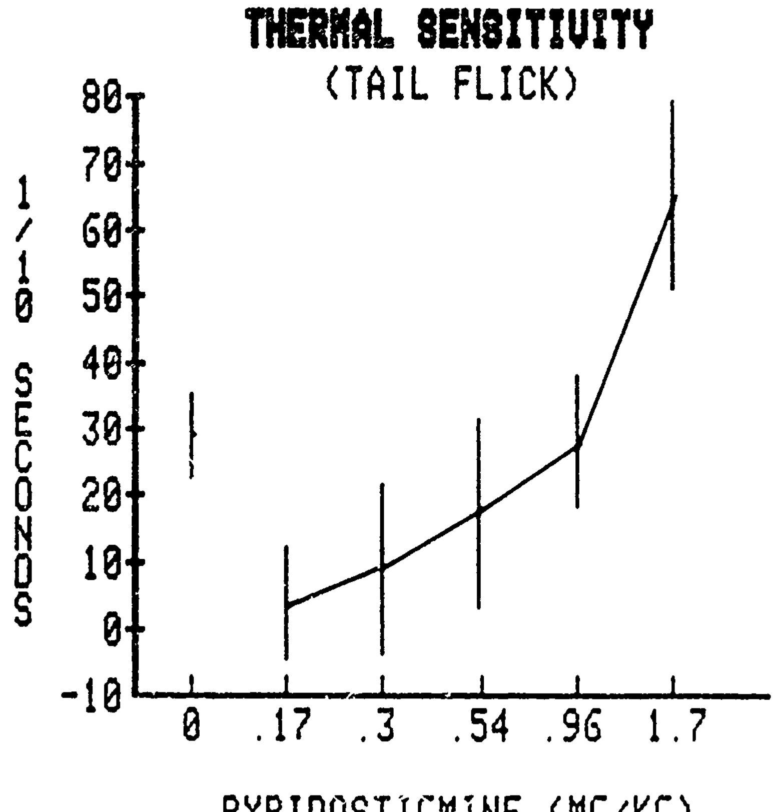




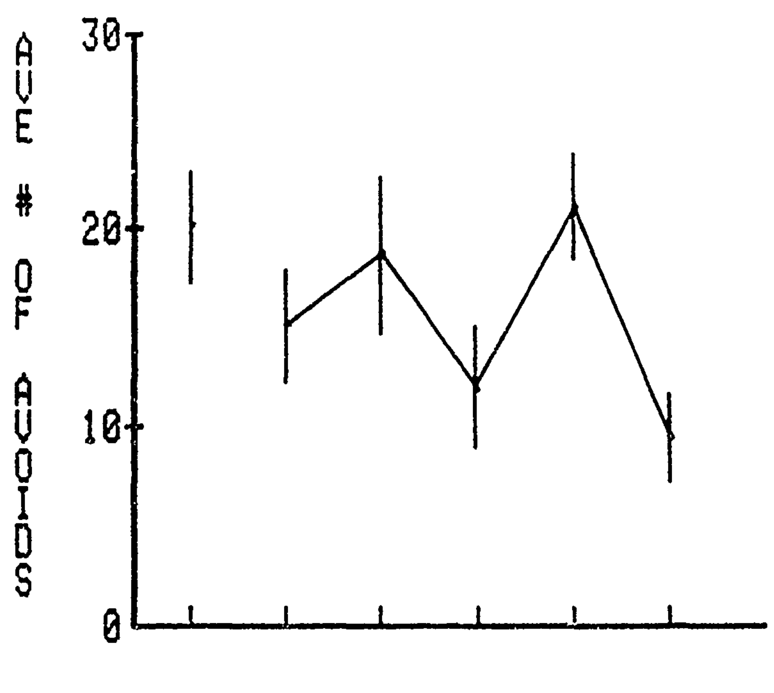
Y-AXIS = PRE/POST DIFFERENCE

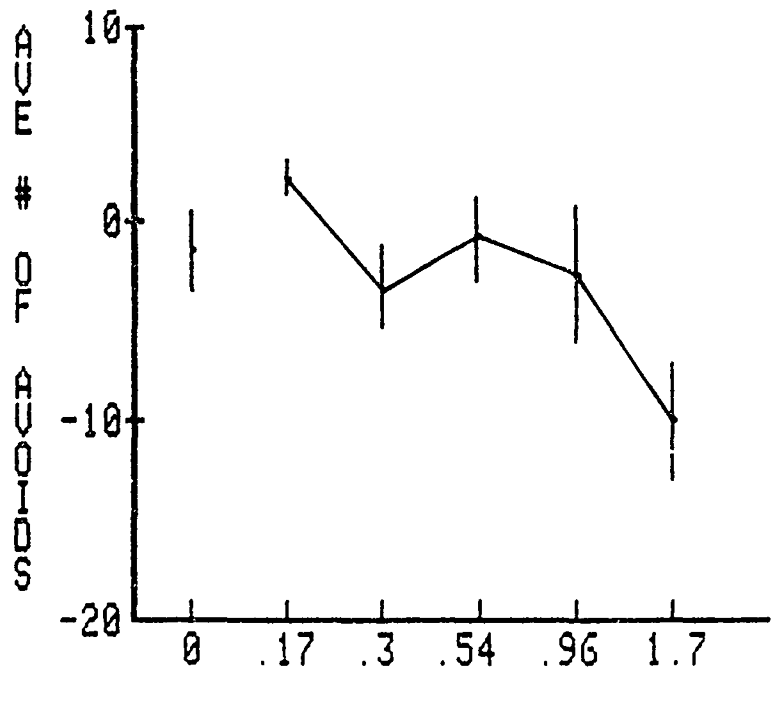


Y-AXIS = PRE/POST GRIP DIFFERENCE

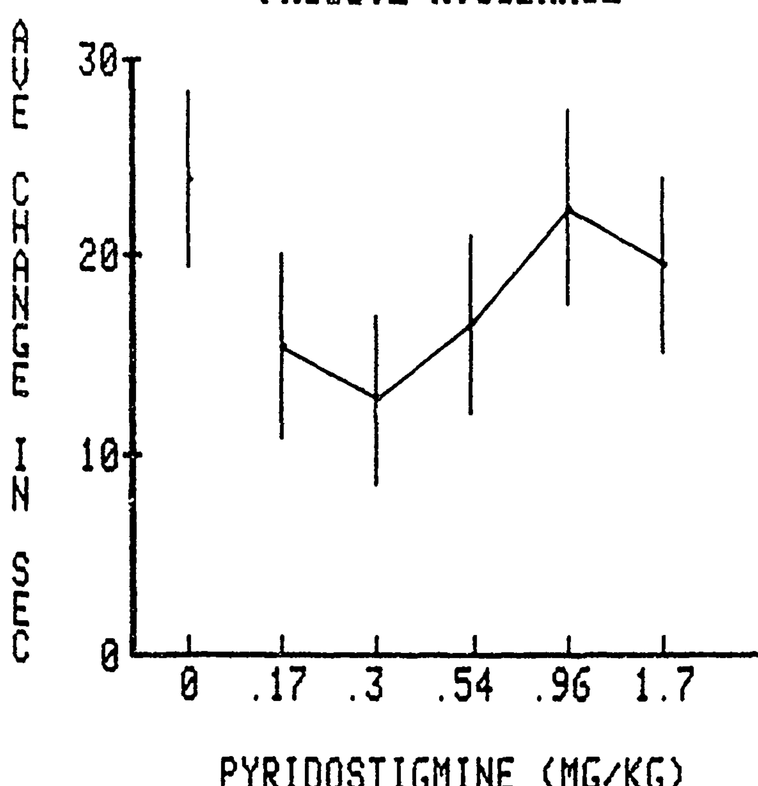


Y-AXIS = MEAN PRE/POST DIFFERENCE

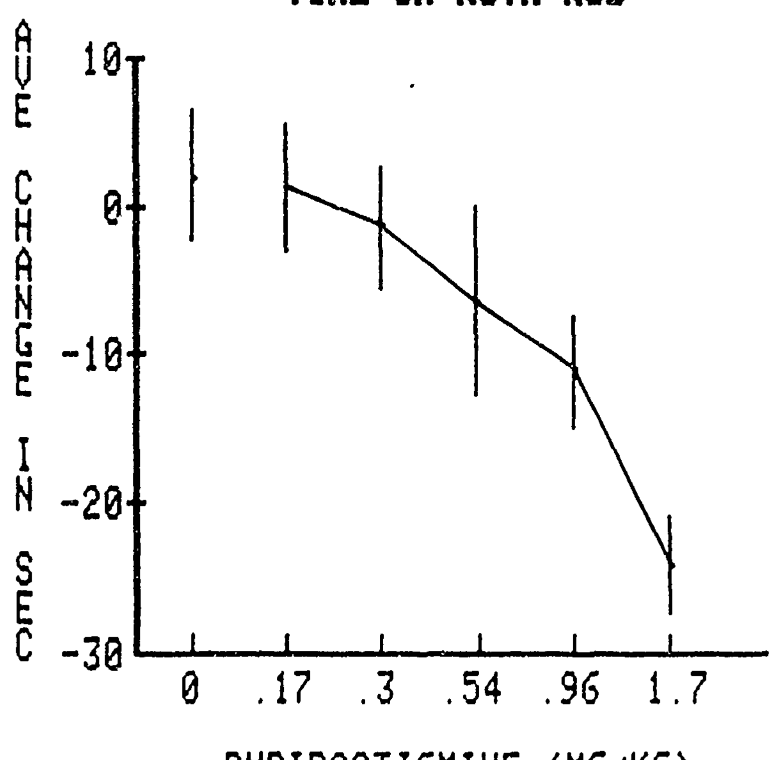




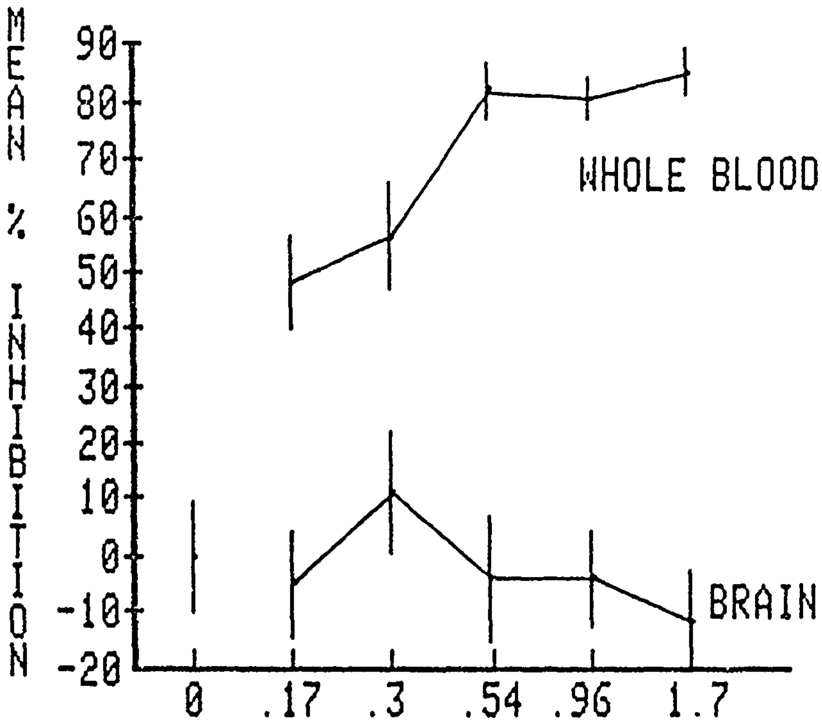
Y-AXIS IS CHANGE FROM PRETEST



Y-AXIS = TRAIN/TEST DIFFERENCE



Y-AXIS = PRE/POST DIFFERENCE


EFFECTS ON RAT CHOLINESTERASE
MEAN % INHIBITION
90
80
70
60
50
40
30
20
10
0
-10
-20
WHOLE BLOOD
BRAIN
0
.17
.3
.54
.96
1.7
PYRIDOSTIGMINE (MG/KG)
INJECTION IN MUSCLE
BLOOD DRAWN 30 MIN POST INJ.

TOXIC SIGNS OF PYRIDOSTIGMINE

	DOMINANT	LESS RELIABLE
RAT	FASCICULATION TREMOR OCULAR PORPHYRIN	DIARRHEA
MONKEY	FASCICULATION	SALIVATION DIARRHEA GAG/VOMIT

PRIMATE/RODENT COMPARISON

NOTE THE SIMILARITY BETWEEN RAT AND RHESUS MONKEY IN THE DOSE EFFECT OF PYRIDOSTIGMINE ON BOTH BLOOD CHOLINESTERASE AND BEHAVIORAL MEASURES.

THE PUTATIVE PROPHYLACTIC LEVEL OF 30 - 40% CHOLINESTERASE INHIBITION IS PRODUCED IN BOTH SPECIES AT APPROXIMATELY THE SAME DOSAGE OF PYRIDOSTIGMINE (ABOUT 0.15 MG/KG). MARGINAL BUT STATISTICALLY SIGNIFICANT BEHAVIORAL DEFICITS APPEAR IN BOTH SPECIES AT 0.96 MG/KG, ABOUT 6 TIMES THE PROTECTIVE DOSE.

RECOVERY OF RAT DIAPHRAGM, E.D.L. AND SOLEUS NEUROMUSCULAR JUNCTIONS FROM PYRIDOSTIGMINE-INDUCED ULTRASTRUCTURAL PATHOLOGY

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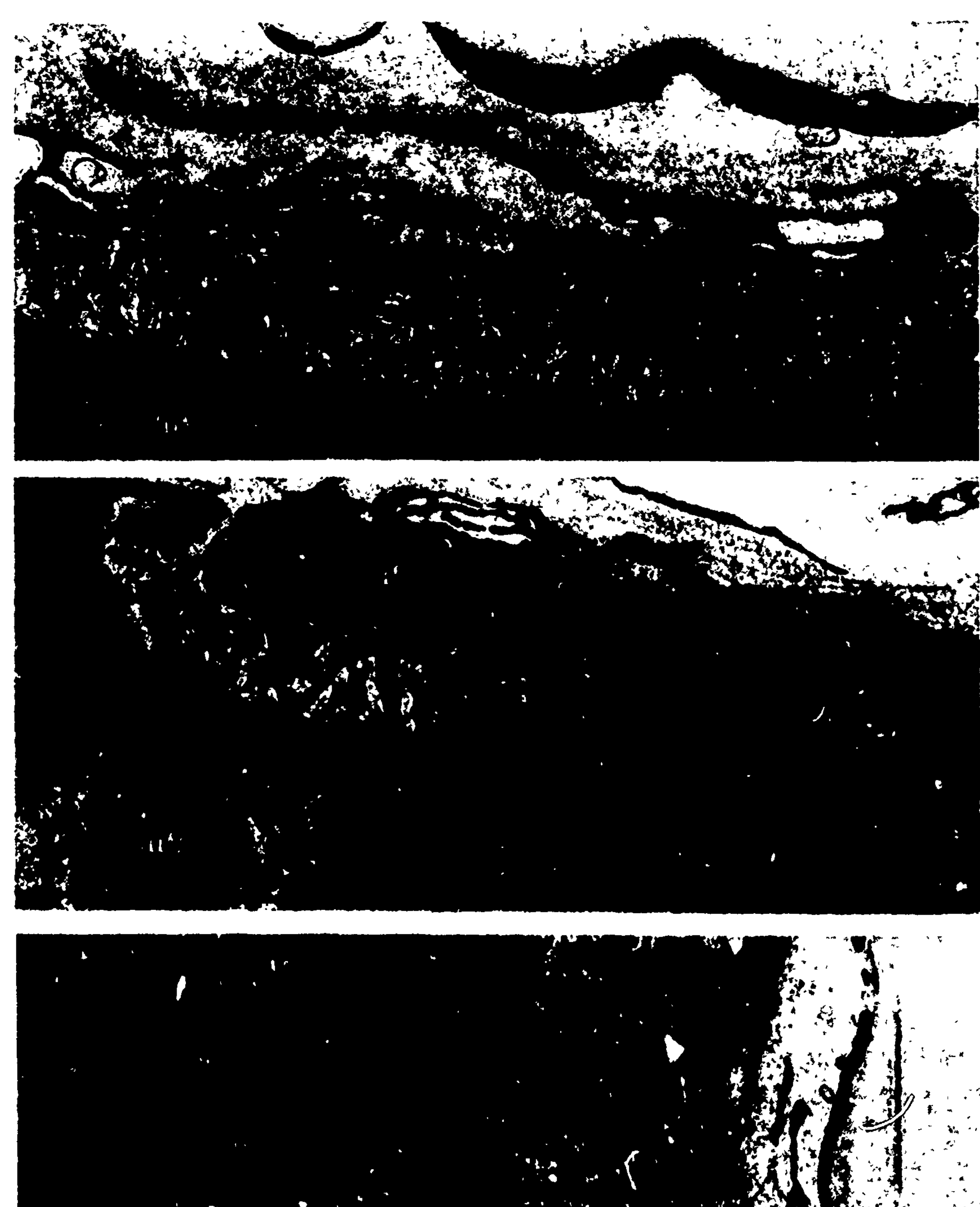
School of Medicine, University of Maryland, Baltimore, Maryland 21201

NEUROMUSCULAR JUNCTIONS IN DIAPHRAGM, EXTENSOR DIGITORUM LONGUS AND SOLEUS MUSCLES FROM MALE ALBINO RATS WERE ASSESSED FOR RECOVERY FROM MORPHOLOGICAL ALTERATIONS INDUCED BY EXPOSURE TO PYRIDOSTIGMINE BROMIDE IN MESTINON^R -EQUIVALENT BUFFER. ANIMALS RECEIVED ACUTE EXPOSURE (30 MIN) BY SINGLE SUBCUTANEOUS INJECTIONS OF DRUG (DOSES 0.0036 TO 3.6 MG/KG) OR SUBACUTE EXPOSURE (2-14 DAYS) BY SUBCUTANEOUSLY IMPLANTED 2 ML ALZET^R OSMOTIC MINIPUMPS THAT CONTAINED 1.5 OR 10 MG/ML OF PYRIDOSTIGMINE. RECOVERY WAS ANALYZED AT 7, 14, 21 AND 60 DAYS FOLLOWING WITHDRAWAL FROM DRUG. WHOLE BLOOD CHOLINESTERASE ACTIVITY LEVELS WERE MONITORED BY RADIOMETRIC ASSAY BEFORE AND DURING DRUG EXPOSURE FOR SOME ANIMALS. DRUG EXPOSURE CAUSED ULTRASTRUCTURAL CHANGES IN THE NEUROMUSCULAR JUNCTIONS OF ALL THREE MUSCLES. PRESYNAPTIC ALTERATIONS INCLUDED WITHDRAWAL OF THE NERVE TERMINAL FROM THE JUNCTIONAL FOLDS, INVASION OF THE SYNAPTIC CLEFT WITH SCHWANN CELL PROCESSES, AND ALTERATION OF AXON TERMINAL ORGANELLES. POSTSYNAPTIC ALTERATIONS OF THE MYOFIBRILLAR COMPONENTS AND THE MEMBRANE-BOUND ORGANELLES WERE ALSO PRESENT. THESE LESIONS WERE OBSERVED AS INDEPENDENT OCCURENCES IN SOME MUSCLE FIBERS BUT WERE PRESENT CONCURRENTLY IN OTHER FIBERS. THE EXTENT OF THE PATHOLOGY VARIED WITH FIBER TYPE, DOSE, AND ALSO WITH THE METHOD OF DRUG ADMINISTRATION. THE MOST SEVERE CHANGES IN ULTRASTRUCTURE WERE OBSERVED SUBSEQUENT TO HIGH ACUTE DOSES IN ALL THREE MUSCLES. THE MOST REMARKABLE ALTERATIONS, HOWEVER, OCCURRED IN SOLEUS FIBERS. HIGH SUBACUTE DOSES WHICH RESULTED IN SIMILAR DEPRESSIONS IN CHOLINESTERASE ACTIVITY PRODUCED MYOPATHIES QUALITATIVELY SIMILAR IN NATURE BUT GENERALLY LESS SEVERE IN EXTENT. THESE RESULTS SUGGEST THAT THE RATE OF CHOLINESTERASE CARBAMYLATION AFFECTS THE SEVERITY OF THE DAMAGE FOR A GIVEN TARGET LEVEL OF CHOLINESTERASE INHIBITION. RECOVERY FROM THE PYRIDOSTIGMINE-INDUCED PRE- AND POSTSYNAPTIC ALTERATIONS WAS BOTH TIME AND DOSE-DEPENDENT. IN ALL CASES EXAMINED, EVIDENCE OF RECOVERY WAS OBSERVED, BUT RECOVERY PROCESSES WERE NOT NECESSARILY COMPLETE AFTER 60 DAYS. MUSCLE ULTRASTRUCTURE FOLLOWING EXTENDED RECOVERY PERIODS, IS CURRENTLY BEING EXAMINED IN ALL THREE MUSCLES. THIS WORK SUPPORTED IN PART BY THE USAMRICD CONTRACT NO.DAMD 17-83-C-3126.

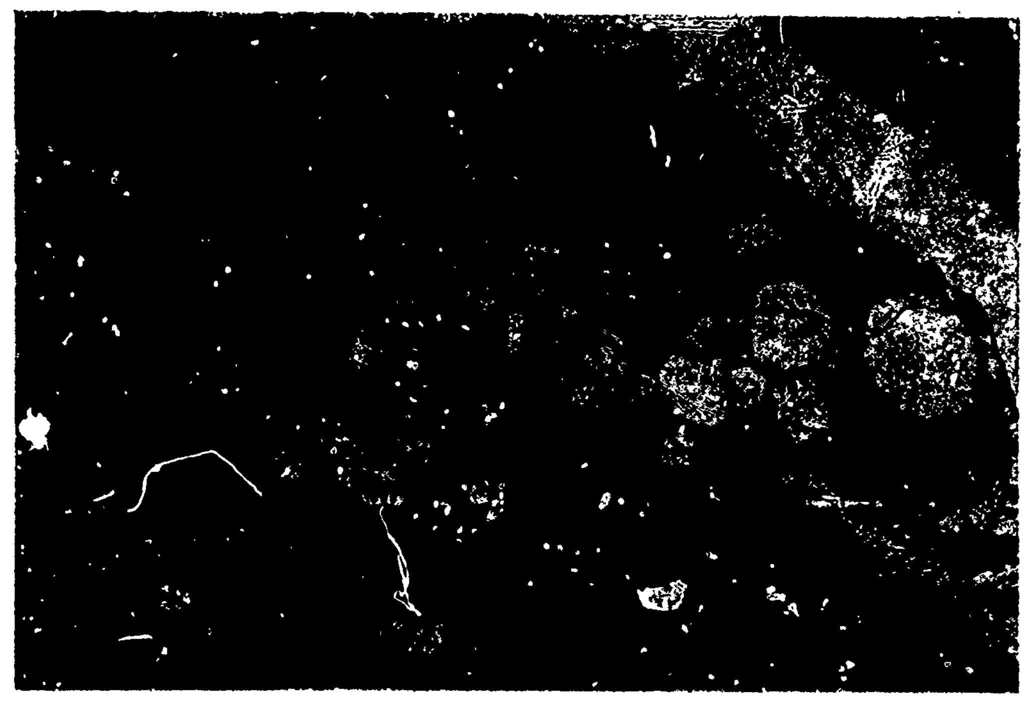
KEY

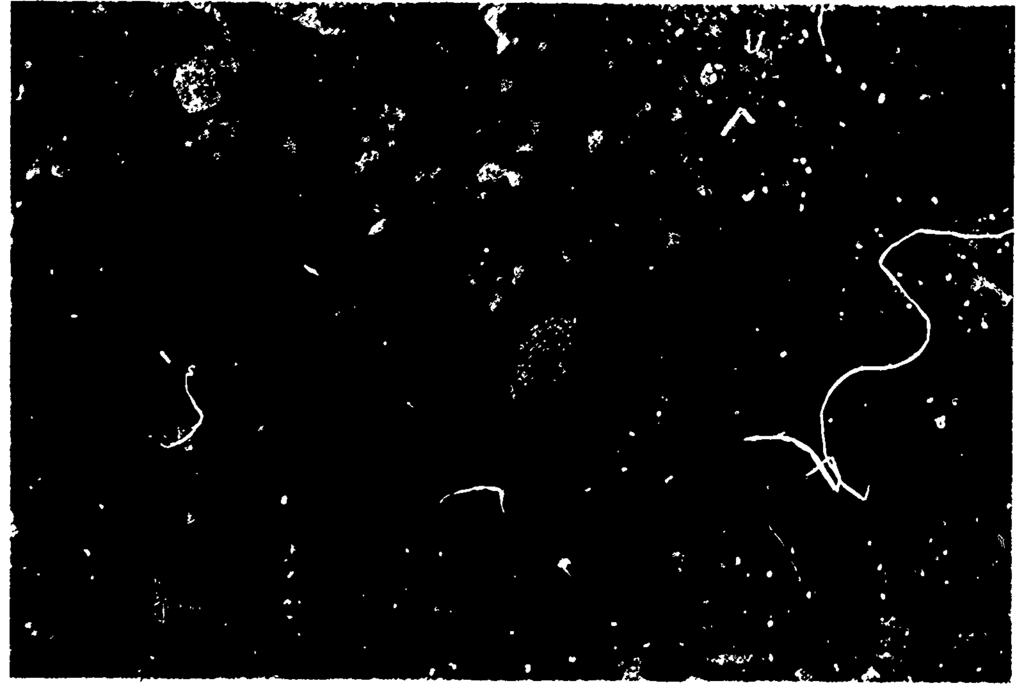
G = GOLGI	M = MITOCHONDRIAN	N = NUCLEUS
JF = JUNCTIONAL FOLD	MF = MYOFIBRILLAR APPARATUS	NT = NERVE TERMINAL

CONTROLS



FIGURES 1,2,3 NEUROMUSCULAR JUNCTIONS FROM CONTROL RATS FOLLOWING:
1) NO TREATMENT
2) 30 MINUTE EXPOSURE TO MESTINON-EQUIVALENT BUFFER BY SUBCUTANEOUS INJECTION.
3) 14 DAY EXPOSURE TO MESTINON-EQUIVALENT BUFFER VIA SUBCUTANEOUSLY IMPLANTED OSMOTIC MINIPUMP.

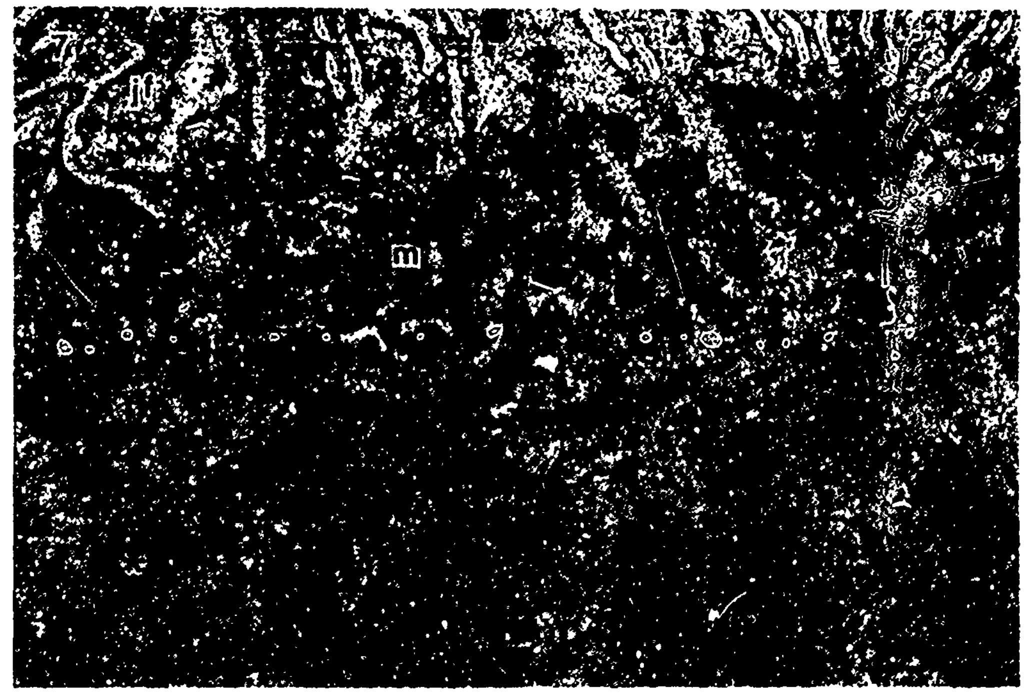




DIAPHRAGM

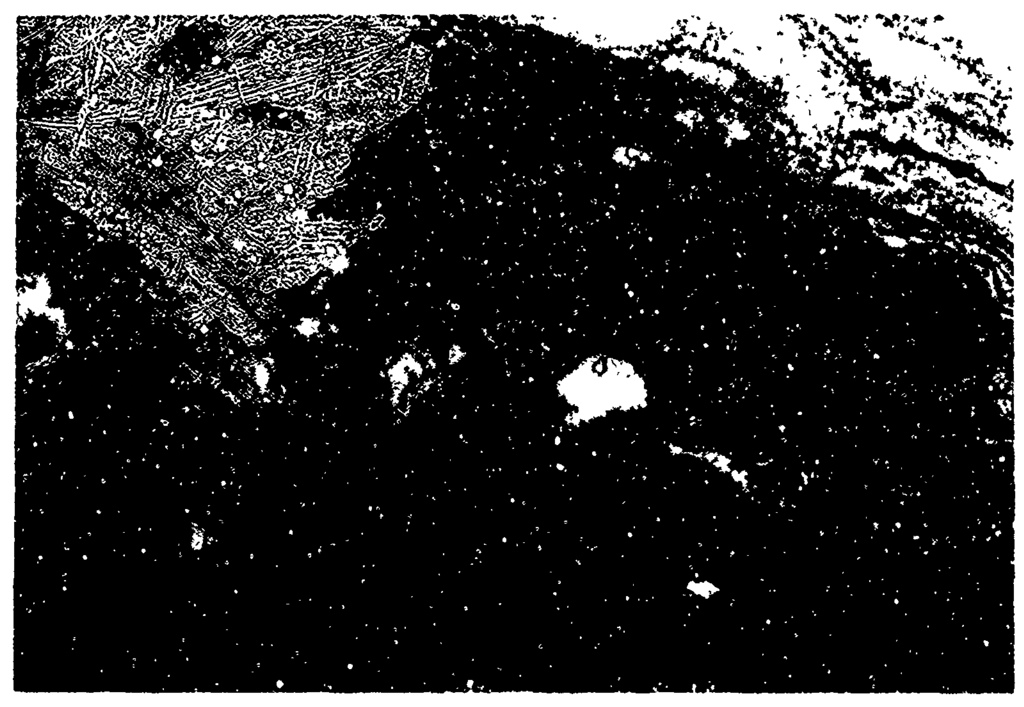
FIGURE 4 30 MINUTE EXPOSURE TO PYRIDOSTIGMINE (2.6 MG/KG) RESULTS IN SUPERCONTRACTION AND DISRUPTION OF SUBJUNCTIONAL SARCOMERES.

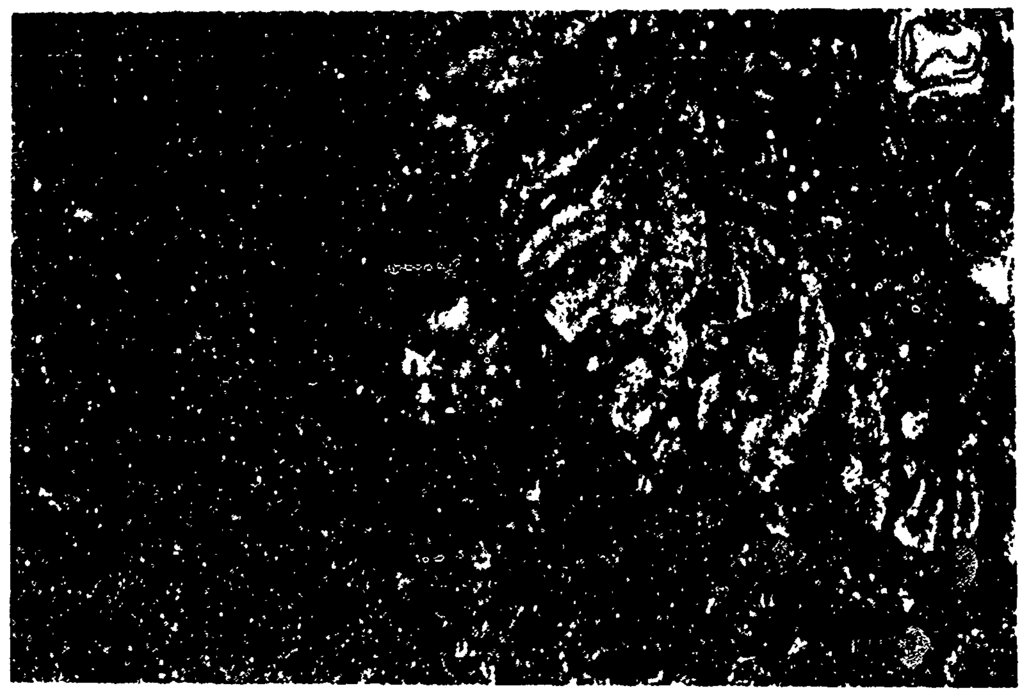
FIGURE 5 AT DOSES AS LOW AS 0.36 MG/KG SWELLING OF MITOCHONDRIA, ER AND THE NUCLEAR ENVELOPE IS PRESENT.



DIAPHRAGM

FIGURES 6, 7 FOLLOWING 60 DAYS OF RECOVERY FROM A SINGLE INJECTION OF 1 MG/KG PYRIDOSTIGMINE, THE NERVE TERMINAL HAS DENSELY PACKED SYNAPTIC VESICLES, AND OCCASIONAL LARGE VESICLES (ARROWS). SLENDER PROJECTIONS OF SCHWAAN CELL ARE PRESENT IN SOME AREAS OF THE PRIMARY CLEFT (ARROWHEAD). SOME MITOCHONDRIA POSSESS SWOLLEN OR LAMELLATED REGIONS. MYOFIBILLAR COMPONENTS ARE WELL RECOVERED. LYSOSOMAL-TYPE BODIES (ASTERISKS) ARE FREQUENTLY PRESENT SUBJUNCTIONALLY.

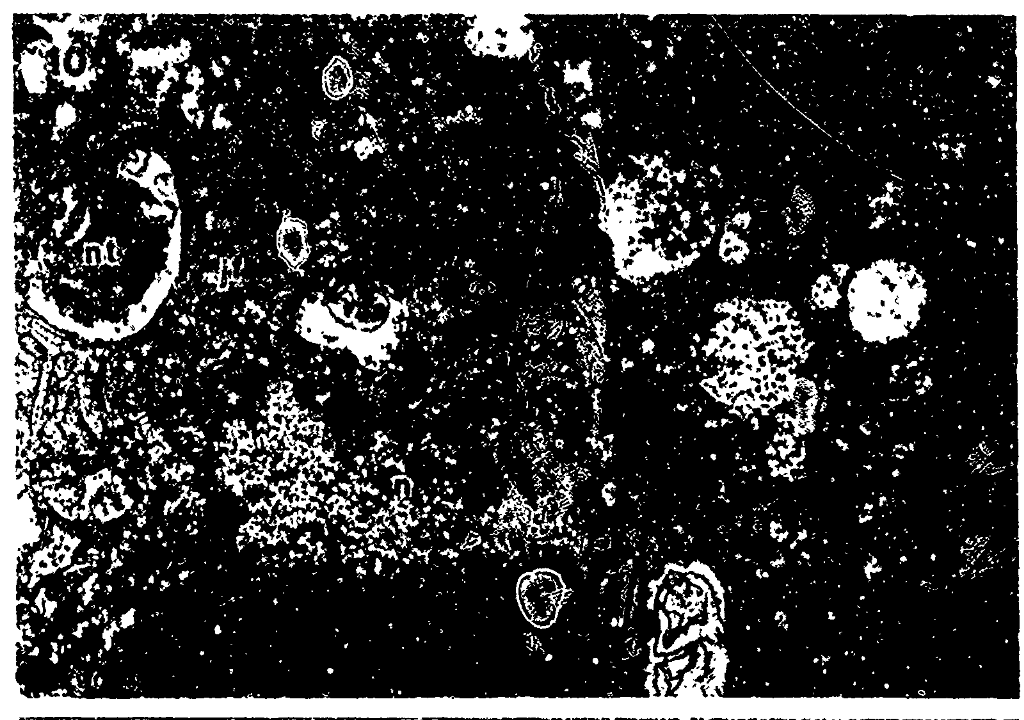


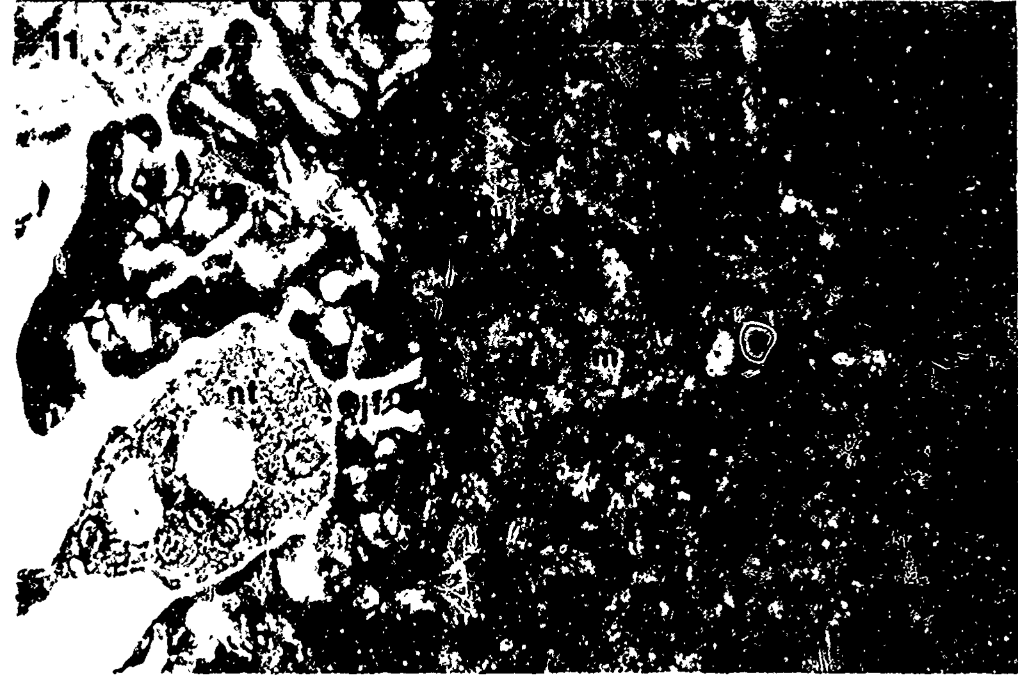


EDL

FIGURE 8 A 30 MINUTE EXPOSURE TO 3.2 MG/KG PYRIDOSTIGMINE ALSO AFFECTS THE NMJs OF EDL. POSTSYNAPTIC MITOCONDRIA, ARE OFTEN SWOLLEN. DISTENDED REGIONS OF NUCLEAR ENVELOPE (ARROW) AND DISRUPTED MYOFIBILLAR APPARATUS ARE ALSO PRESENT.

FIGURE 9 14 DAYS OF RECOVERY FROM A LOW DOSE (0.036 MG/KG) OF DRUG IS NOT SUFFICIENT TO COMPLETELY REVERSE DAMAGE. SOME PRESYNAPTIC MITOCHONDRIA REMAIN ALTERED. LYSOSOME-LIKE BODIES ARE PRESENT.

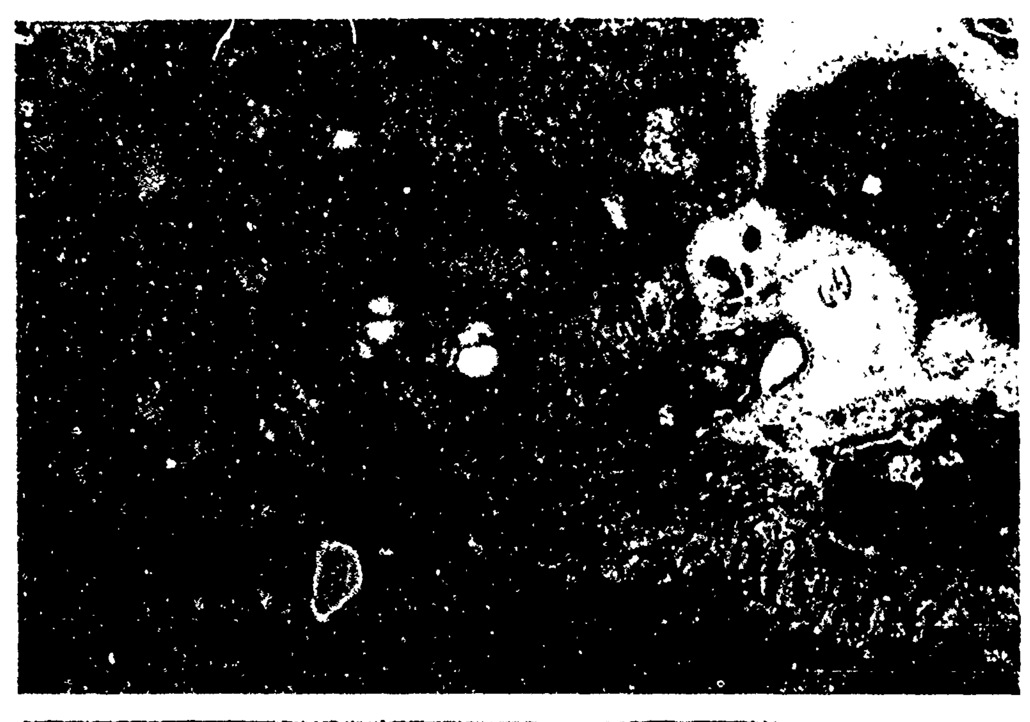


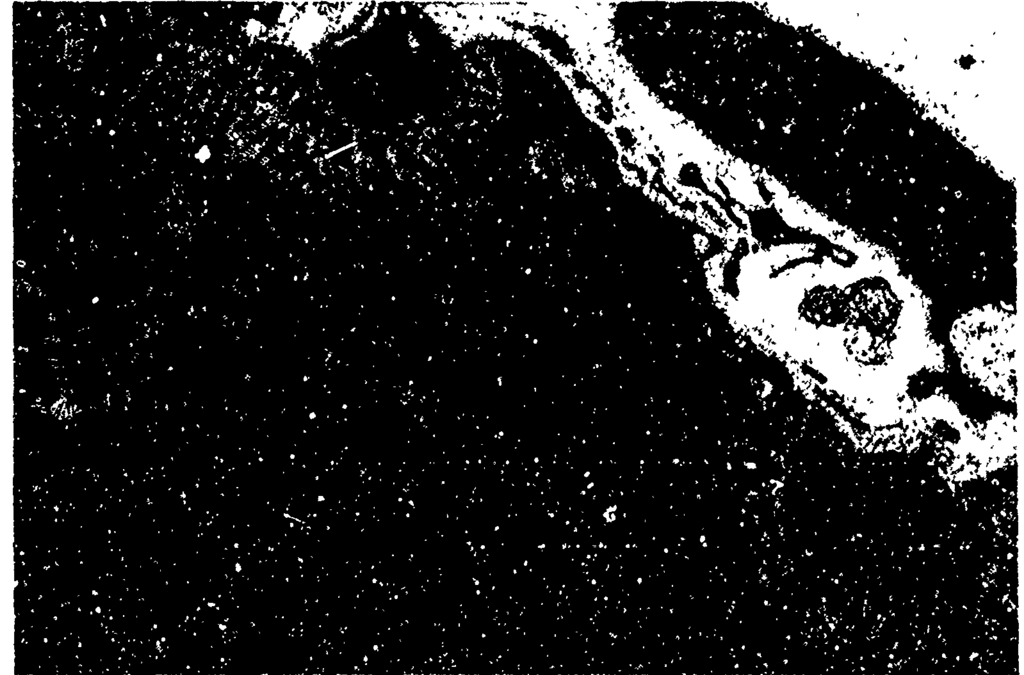


SOLEUS

FIGURE 10 SOLEUS NMJs ARE SIMILARLY AFFECTED BY 30 MINUTES EXPOSURE TO 3.2 MG/KG PYRIDOSTIGMINE. NOTE THE PORTION OF NERVE TERMINAL COMPLETELY ENVELOPED BY SCHWAAN PROCESSES (ARROWS).

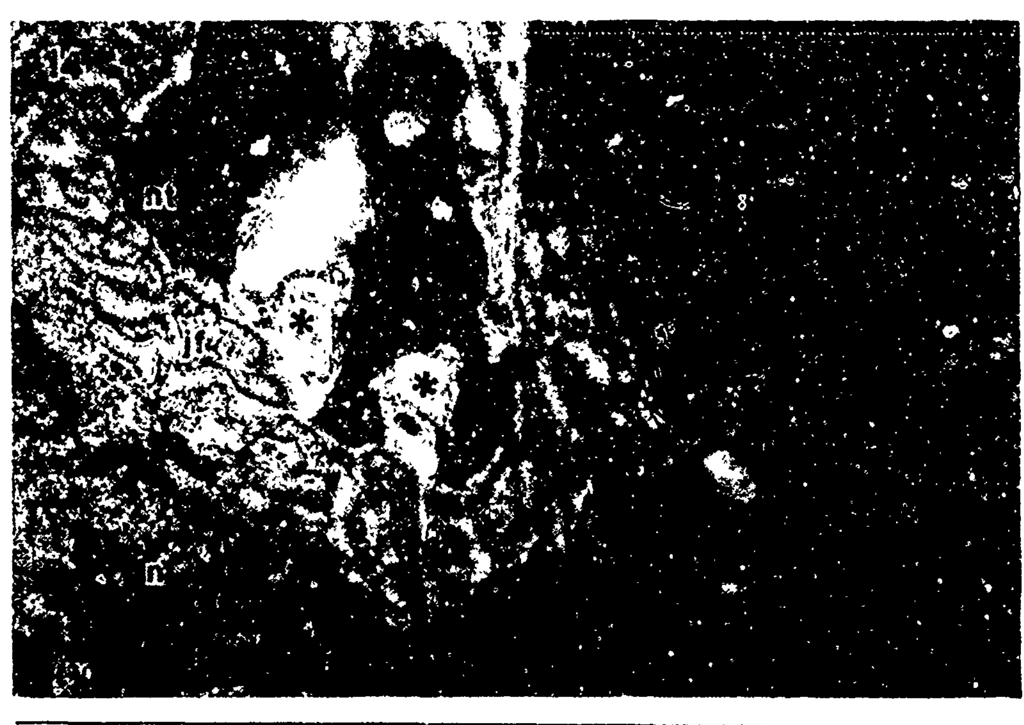
FIGURE 11 FOLLOWING 60 DAYS OF RECOVERY FROM A 1 MG/KG INJECTION, PRE- AND POST SYNAPTIC ALTERATIONS REMAIN (ARROWS) IN SOME SOLEUS FIBERS.

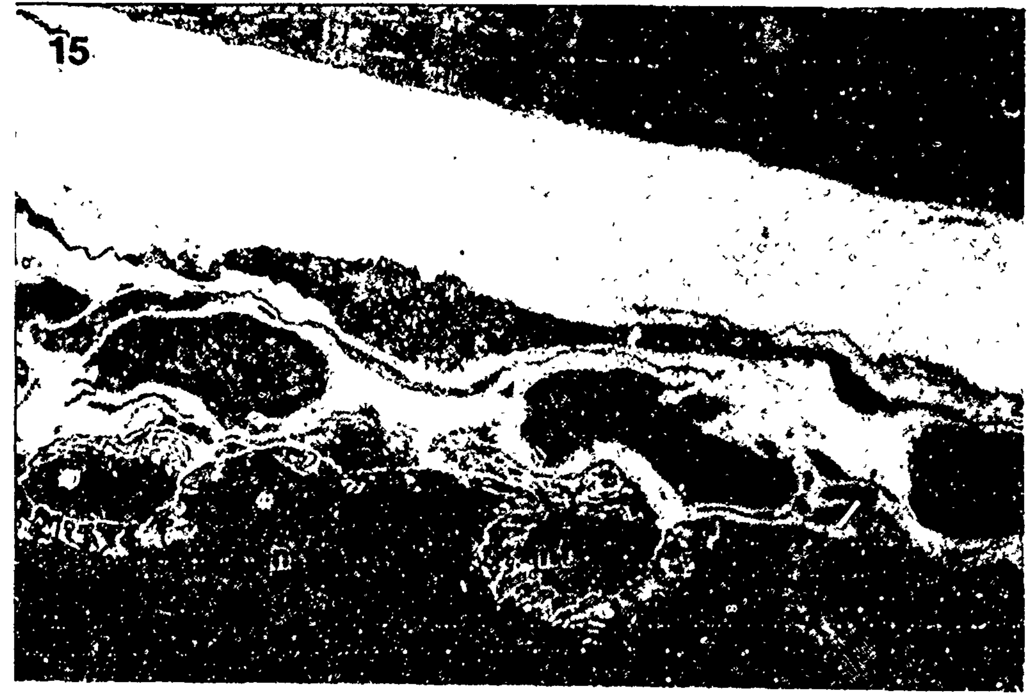




DIAPHRAGM

FIGURES 12, 13 2 DAY EXPOSURE TO PYRIDOSTIGMINE (10 MG/ML) VIA ALZET MINIPUMP MAY RESULT IN SOME SWOLLEN PRESYNAPTIC MITOCHONDRIA. POSTSYNAPTIC MYOFIBRILS MAY BE DISORGANIZED AND MITOCHONDRIA MAY BE SWOLLEN OR LAMELLATED (ARROWS). THE TWO LATTER CHANGES ARE NOT MUTUALLY DEPENDENT.





DIAPHRAGM

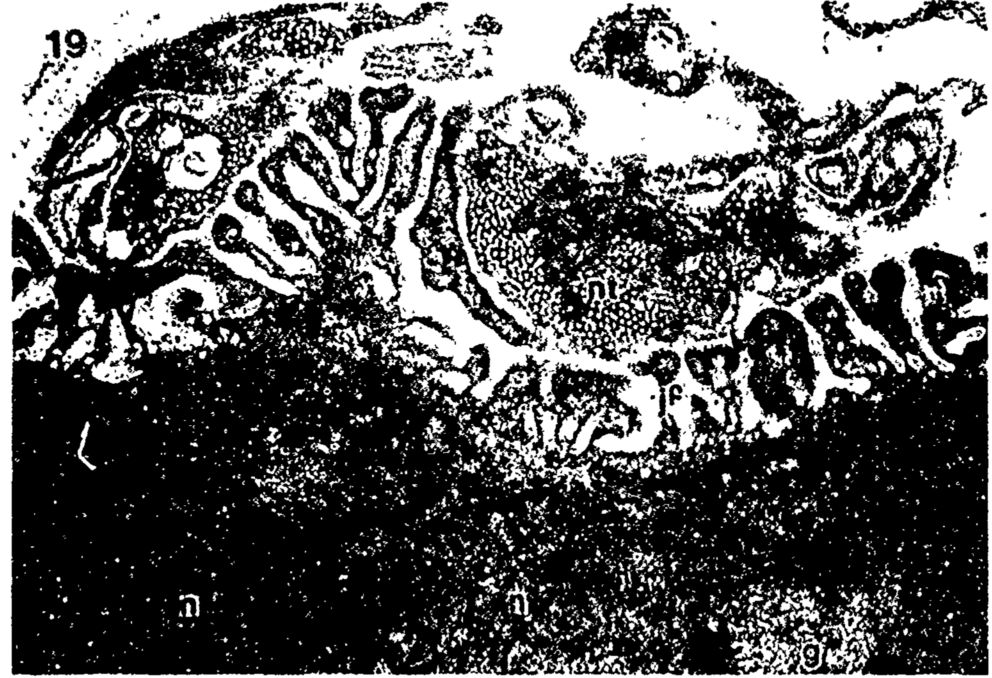
FIGURE 14 — 14 DAY EXPOSURE TO DRUG (10 MG/ML) VIA ALZET PUMP RESULTS IN PARTIAL WITHDRAWAL OF PORTIONS OF NERVE TERMINAL AND SUSTAINED SHORTENING OF SUBJUNCTIONAL SARCOMERES IN SOME FIBERS.

FIGURE 15 — THESE ALTERATIONS ARE SUBSTANTIALLY REVERSED FOLLOWING 60 DAYS RECOVERY FROM DRUG EXPOSURE.

EDL

FIGURE 16 14 DAY EXPOSURE TO PYRIDOSTIGMINE (10 MG/ML) VIA ALZET PUMP RESULTS IN PRE- AND POSTSYNAPTIC NMJ ALTERATIONS OF EDL AND SOLEUS (NOT SHOWN) MUSCLES.

FIGURE 17 60 DAYS OF RECOVERY FROM DRUG IS NOT SUFFICIENT TO COMPLETELY REVERSE THE DAMAGE. NOTE THE EXTENSIVELY INVAGINATED NUCLEUS WHICH IS ASSOCIATED WITH NUMEROUS GOLGI. SUCH NUCLEI ARE CHARACTERISTIC OF DRUG TREATED/RECOVERING CELLS.



SOLEUS

FIGURES 18, 19 FOLLOWING 60 DAYS OF RECOVERY FROM 14 DAYS OF SUBACUTE EXPOSURE TO DRUG (10 MG/ML), THE NERVE TERMINALS OF SOME SOLEUS MUSCLE FIBERS ARE SEPARATED FROM JUNCTIONAL FOLD CRESTS. NUCLEI ARE FREQUENTLY INVAGINATED AND ASSOCIATED WITH NUMBEROUS GOLGI.

CHEMICAL WARFARE PRETREATMENT DRUG INVESTIGATIONS UTILIZING PYRIDOSTIGMINE BROMIDE: LABORATORY METHODS FOR MONITORING PLASMA PYRIDOSTIGMINE AND ACETYLCHOLINESTERASE ACTIVITY LEVELS

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Rothe Development, Inc., San Antonio, Texas
School of Aerospace Medicine, Brooks AFB, Texas

SPECTROPHOTOMETRIC ASSAY OF HUMAN BLOOD ACETYLCHOLINEASTERASE ACTIVITY

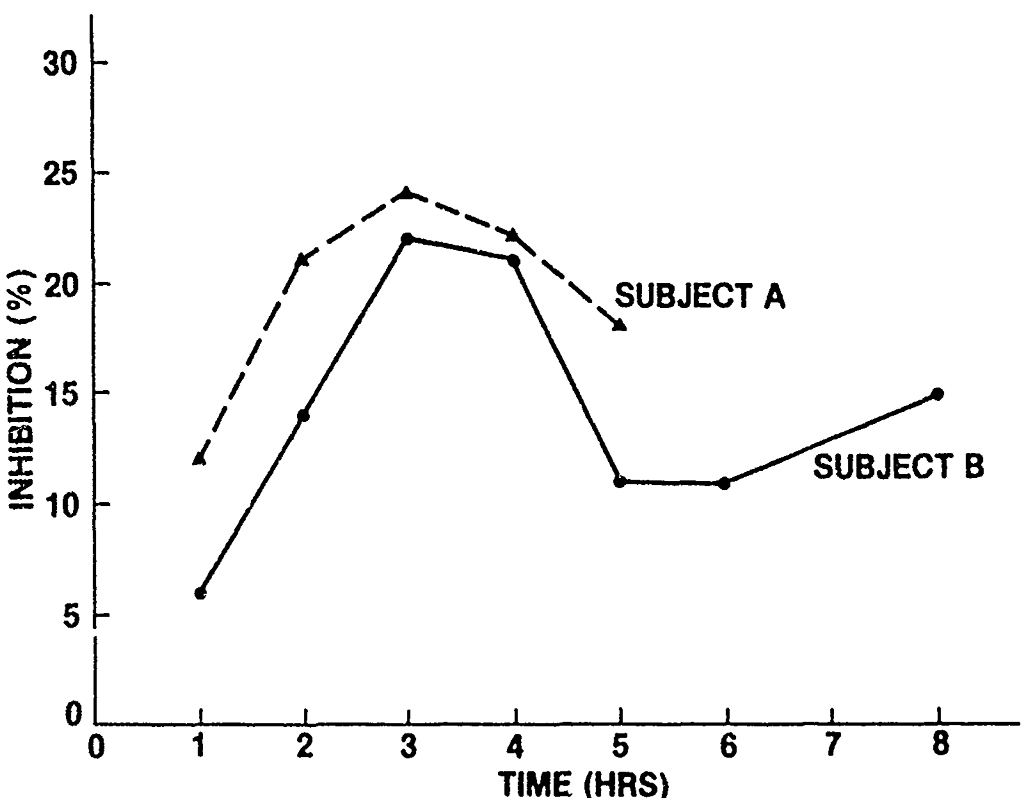


Figure 1. HOURLY PERCENT INHIBITION OF PLASMA CHOLINESTERASE ACTIVITY LEVELS FOLLOWING INGESTION OF 60 mg OF PYRIDOSTIGMINE BROMIDE.

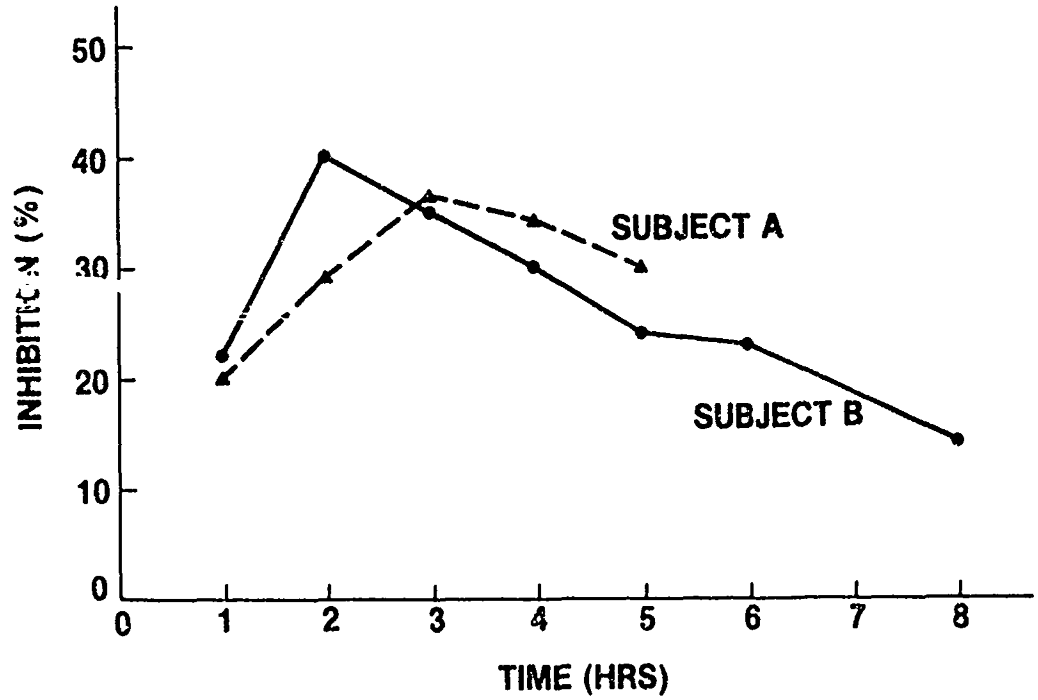


Figure 2. HOURLY PERCENT INHIBITION OF WHOLE BLOOD LYSAT CHOLINESTERASE ACTIVITY LEVELS FOLLOWING INGESTION OF 60 mg OF PYRIDOSTIGMINE BROMIDE.

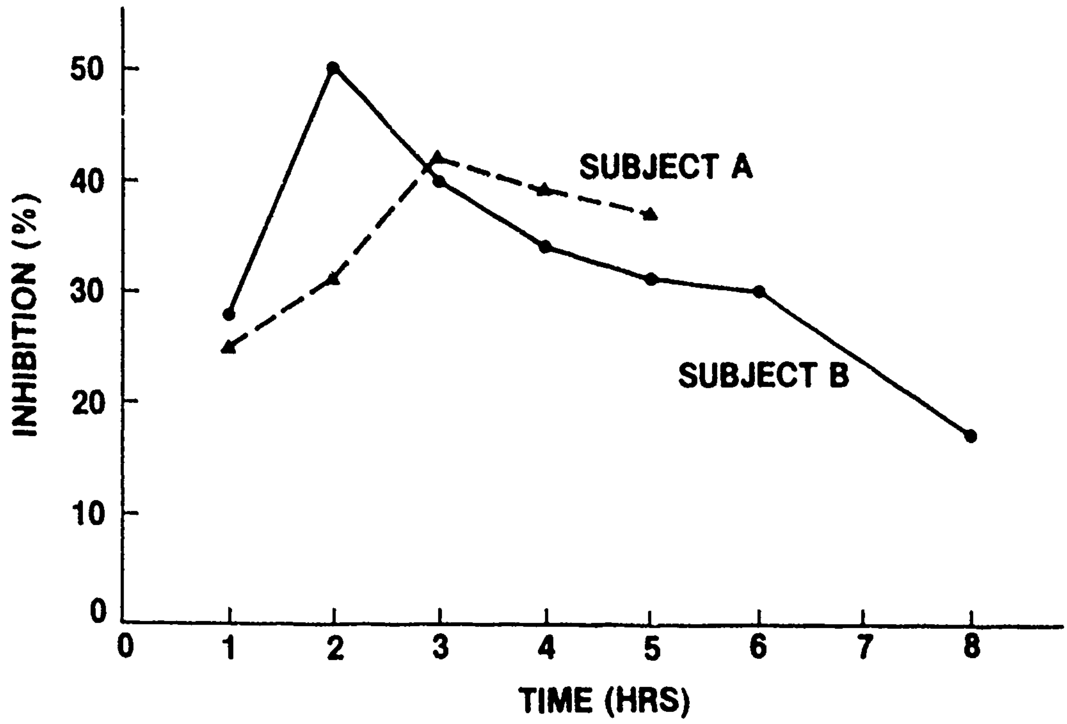


Figure 3. CALCULATED HOURLY PERCENT INHIBITION OF RED CELL ACETYLCHOLINESTERASE ACTIVITY LEVELS FOLLOWING INGESTION OF 60 mg OF PYRIDOSTIGMINE BROMIDE.

Table I. CHOLINESTERASE ACTIVITY LEVELS (EXPRESSED IN INTERNATIONAL ENZYME UNITS PER LITER, U/1) AND THE RESULTING PERCENT INHIBITION FOLLOWING INGESTION OF 60 mg OF PYRIDOSTIGMINE BROMIDE.

	SUBJECT A		SUBJECT B	
TIME (HRS)	ACTIVITY (U/1)	INHIBITION (%)	ACTIVITY (U/1)	INHIBITION (%)
		WHOLE BLOOD LYSAT		
* 0	6,435	--	6,277	--
1	5,207	20	4,899	22
2	4,623	29	3,804	40
3	4,150	36	4,097	35
4	4,308	34	4,435	30
5	4,513	30	4,793	24
6	--	--	4,849	23
7	--	--	--	--
8	--	--	5,418	14
		PLASMA		
0	3,577	--	2,269	--
1	3,173	12	2,141	06
2	2,845	21	1,971	14
3	2,729	24	1,783	22
4	2,798	22	1,812	21
5	2,954	18	2,034	11
6	--	--	2,022	11
7	--	--	--	--
8	--	--	1,950	17
		RED BLOOD CELL **		
0	10,072	--	11,275	--
1	7,643	25	8,135	28
2	7,030	31	5,750	50
3	5,852	42	6,829	40
4	6,193	39	7,514	34
5	6,382	37	7,842	31
6	--	--	7,974	30
7	--	--	--	--
8	--	--	9,408	17

* BASELINE, PRIOR TO INGESTION OF PYRIDOSTIGMINE BROMIDE.

** CALCULATED VALUE FROM:

$$\frac{\text{WHOLE BLOOD} - [\text{PLASMA} \times (1 - \text{HEMATOCRIT})]}{\text{HEMATOCRIT}}$$

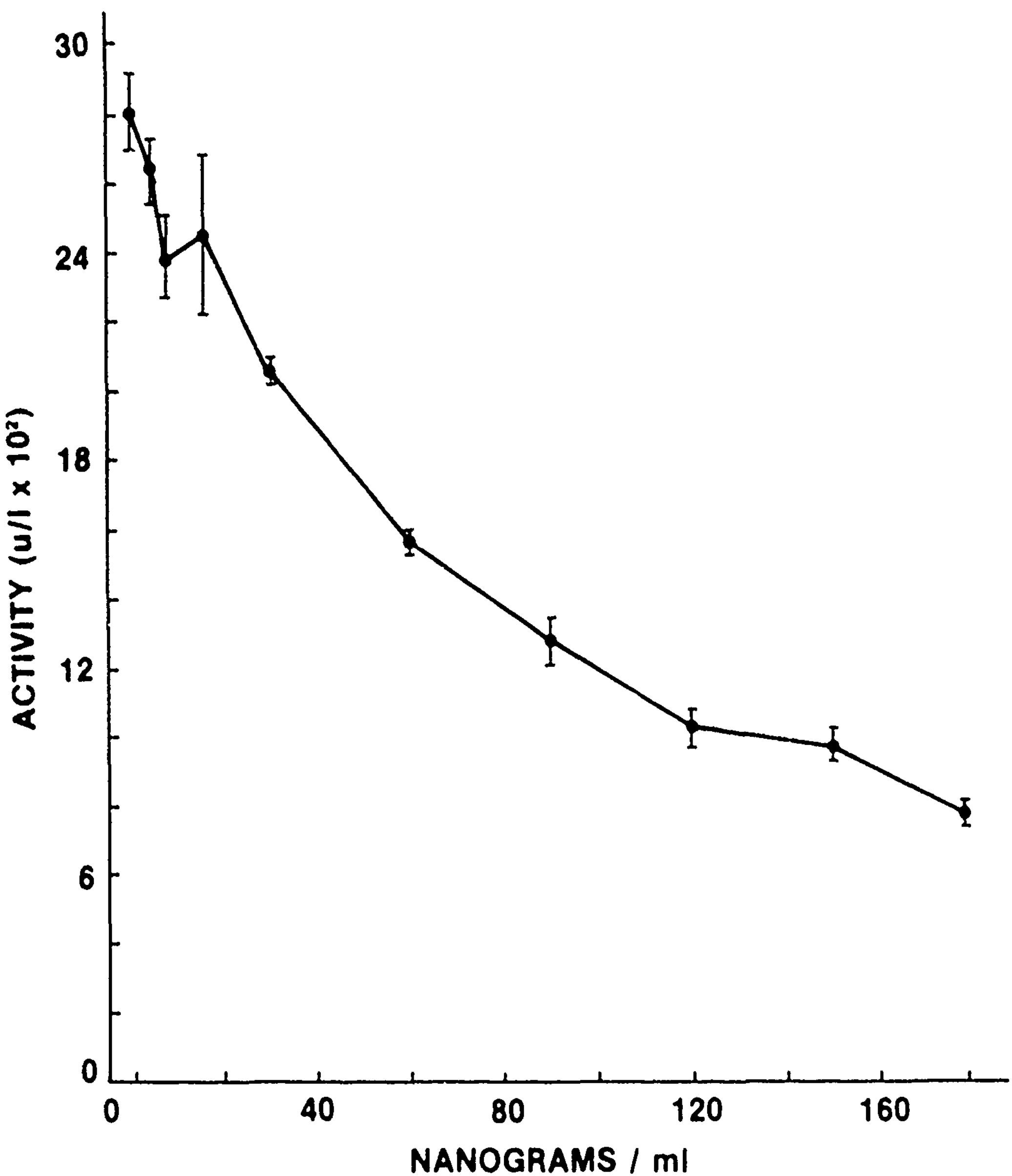


Figure 4. MEAN VALUE AND RANGE (N= 3) FOR POOLED PLASMA SPIKED WITH PYRIDOSTIGMINE BROMIDE. ACTIVITY DETERMINED AT 405 nm AFTER A TWO HOUR INCUBATION PERIOD.

EQUIPMENT

LKB ULTROSPECTROPHOTOMETER MODEL 4050
LKB AUTOFILL CONTROLLER WITH THERMOSTATED CUVETTE MODEL 4070
APPLE IIe COMPUTER
EPSON PRINTER
LKB ENZYME KINETICS SOFTWARE PACKAGE

ASSAY PROCEDURE

1. BLOOD IS COLLECTED IN EDTA TREATED VACUTAINER TUBE FROM THE CUBITAL VEIN OF HUMAN SUBJECTS AND IMMEDIATELY PLACED ON ICE.
2. HEMATOCRIT VALUE IS DETERMINED.
3. WHOLE BLOOD LYSAT PREPARATION: 1.8 ml WATER – .200 ml WHOLE BLOOD.
4. REMAINDER OF BLOOD SAMPLE IS CENTRIFUGED TO OBTAIN PLASMA.
5. TEST PROCEDURE - PIPETTE INTO TEST TUBE:
   a. 3.00 ml CHROMAGEN/BUFFER (50.00 mM PHOSPHATE BUFFER, pH 7.2) (0.25 mM DITHIOBISNITROBENZOIC ACID)
   b. 0 10 ml SUBSTRATE (156 mM ACETYLTHIOCHOLINE IODIDE)
   c. 0.02 ml WHOLE BLOOD LYSAT OR PLASMA
6. CHEMICAL REACTIONS

   $$\text{ACETYLTHIOCCHOLINE} \xrightarrow{\text{CHOLINESTERASES}} \text{THIOCHOLINE} + \text{ACETATE}$$

   $$\text{THIOCHOLINE} + \text{DITHIOBISNITROBENZOIC ACID} \longrightarrow \text{THIONITROBENZOIC ACID}$$

   ACETATE AND THIOCHOLINE REACT WITH DITHIOBISNITROBENZOIC ACID TO FORM THE YELLOW COLORED 2-NITRO-5-MERCAPTO BENZOATE THAT RESULTS IN AN INCREASING COLOR INTENSITY THAT IS DIRECTLY PROPORTIONAL TO CHOLINESTERASE ACTIVITY.
7. ALL TESTS CONDUCTED AT 25°C.
8. THE RATE OF CHANGE OF ABSORBANCE/MINUTE IS RECORDED AT 405 nm OVER A 3 MINUTE PERIOD.
9. RESULTS ARE GIVEN IN INTERNATIONAL ENZYME UNITS (U/1), WHICH IS DEFINED AS THE ACTIVITY OF ENZYME WHICH CONVERTS 1 UMOLE/1 OF SUBSTRATE IN 1 MINUTE AT STANDARD CONDITIONS.
10. ERYTHROCYTE ACETYLCHOLINESTERASE (RBC ACHE) ACTIVITY IS CALCULATED USING PLASMA AND WHOLE BLOOD LYSAT ACTIVITY AND SAMPLE HEMATOCRIT FROM THE FOLLOWING FORMULA:

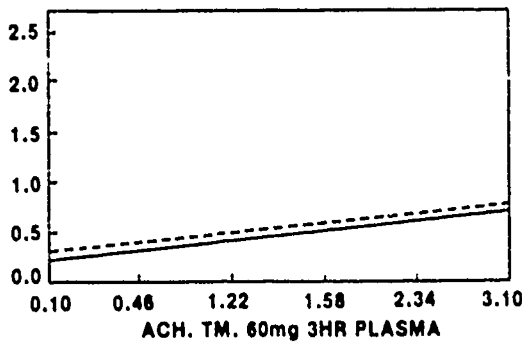
$$\text{RBC ACHE} = \frac{\text{WB} - [\text{PL} \times (1 - \text{HCT})]}{\text{HCT}}$$

WHERE: WB = CHOLINESTERASE ACTIVITY OF WHOLE BLOOD LYSAT
PL = CHOLINESTERASE ACTIVITY OF PLASMA
HCT = HEMATOCRIT EXPRESSED AS A DECIMAL EQUIVALENT

TYPICAL SPECTROPHOTOMETRIC PRINTOUT AND SAMPLE CALCULATION

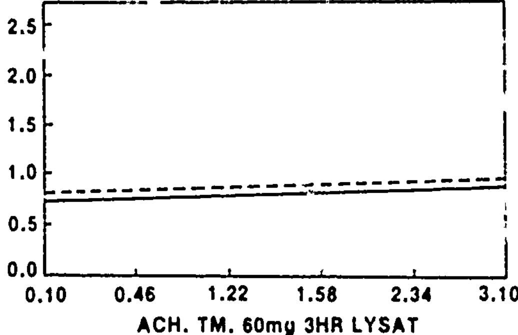
PLASMA

TITLE: ACH/TM/60mg/3HR/PLASMA
IDENTIFICATION: WHINNERY/1-10-85
LAG TIME: 0 MIN 10 SEC
DURATION: 3 MIN 0 SEC
MINIMUM ABS: 0
MAXIMUM ABS: 2.5
FACTOR: 11,700
WAVELENGTH: 405
SAMPLE NUMBER: 3
ABSORBANCE: INITIAL 0.215 FINAL 0.673 CHANGE 0.459
RATE OF CHANGE 0.152 A/MIN ACTIVITY 1773. IU STD. DEV. 0.002



WHOLE BLOOD LYSAT

TITLE: ACH/TM/60mg/3HR/LYSAT
IDENTIFICATION: WHINNERY/1-10-85
LAG TIME: 0 MIN 10 SEC
DURATION: 3 MIN 0 SEC
MINIMUM ABS: 0
MAXIMUM ABS: 2.5
FACTOR: 117.000
WAVELENGTH: 405
SAMPLE NUMBER: 3
ABSORBANCE: INITIAL 0.719 FINAL 0.824 CHANGE 0.104
RATE OF CHANGE: 0.035 A/MIN ACTIVITY 4092. IU STD DEV 0.002



$$\text{RBC ACHE} \quad \frac{\text{WB} - (\text{P} \times (1 - \text{HCT}))}{\text{HCT}}$$

WHERE: WB CHOLINESTERASE ACTIVITY OF WHOLE BLOOD LYSAT
PL CHOLINESTERASE ACTIVITY OF PLASMA
HCT HEMATOCRIT EXPRESSED AS A DECIMAL EQUIVALENT

$$\text{RBC ACHE} \quad \frac{4092 \text{ U/l} - (1773 \text{ U/l} \times (1 - .45))}{.45}$$

$$\text{RBC ACHE} \quad \frac{4092 \text{ U/l} - 975 \text{ U/l}}{.45}$$

RBC ACHE 6,926 U/l

ABSTRACT

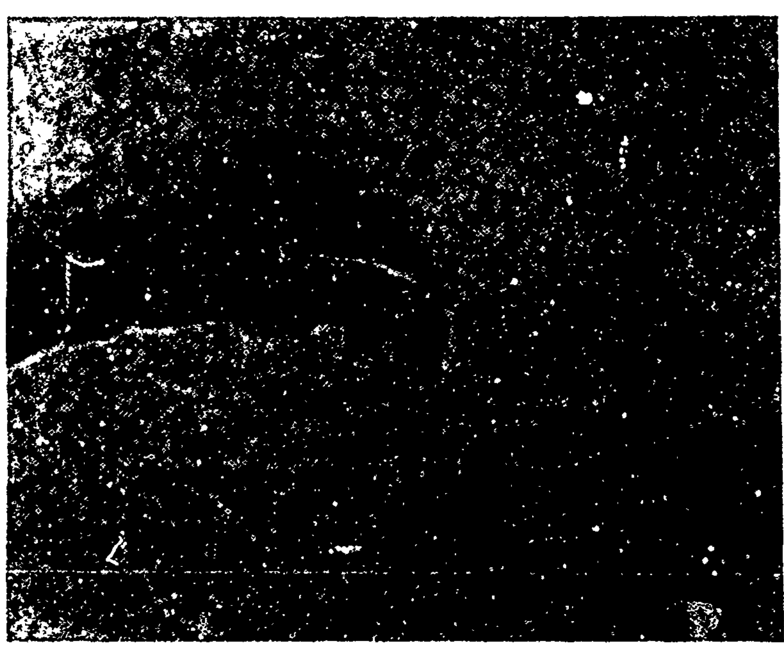
PYRIDOSTIGMINE BROMIDE (AN ACETYLCHOLINESTERASE INHIBITOR) IS A CANDIDATE PRE-EXPOSURE ANTIDOTE FOR PREVENTION OF UNDESIRABLE EFFECTS OF SPECIFIC CHEMICAL WARFARE AGENTS. OF PARAMOUNT CONCERN IN ATTEMPTS TO EVALUATE PSYCHOPHYSIOLOGICAL EFFECTS OF PYRIDOSTIGMINE ON HUMANS IN THE AEROSPACE ENVIRONMENT ARE RELIABLE, REPRODUCIBLE ASSAYS FOR MONITORING DRUG LEVELS IN PLASMA AND RESULTING ACETYLCHOLINESTERASE/ACTIVITY SUPPRESSION.

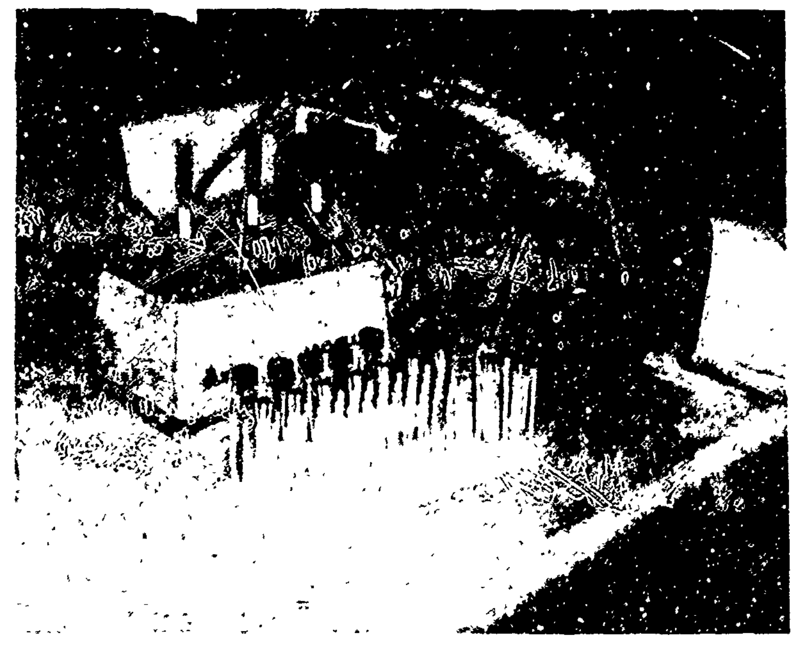
A REVERSE-PHASE, ION-PAIR LIQUID CHROMATOGRAPHIC METHOD WITH UV ABSORPTION DETECTION IS UTILIZED FOR THE ANALYSIS OF PYRIDOSTIGMINE BROMIDE IN PLASMA. THE TECHNIQUE INVOLVES THE USE OF AN INTERNAL STANDARD (NEOSTIGMINE BROMIDE), CLEAN-UP BY PROTEIN PRECIPITATION, AND CARTRIDGE ELUTION PRIOR TO SEPARATION ON A C-8 HPLC COLUMN.

ACETYLCHOLINESTERASE ACTIVITY IS DETERMINED BY A SPECTROPHOTOMETRICALLY COUPLED ASSAY. IN THE TWO STEP REACTION, ACETYLTHIOCHOLINE (AcSCH) IS THE SUBSTRATE AND DITHIONITROBENZENE, (DTNB) THE COLOR REAGENT; HYDROLYSIS OF AcSCH YIELDS THIOCHOLINE, WHICH REACTS WITH DTNB TO YIELD A YELLOW COLOR. THE REACTION IS MONITORED AT 405nm.

ACKNOWLEDGEMENT

THIS WORK SUPPORTED IN PART BY THE U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND





INSTRUMENTS

WATERS ASSOCIATES: MODEL 721 SYSTEM CONTROLLER
MODEL 730 DATE MODULE
MODEL 510 PUMP
MODEL 481 UV DETECTOR
MODEL TCM COLUMN HEATER
MODEL 710B SAMPLE PROCESSOR

ANALYTICHEM INTERNATIONAL: VAC ELUTE PROCESSING STATION
C-2 BOND ELUTE CARTRIDGE
C-8 SEPRALYTE COLUMN 5 um (4.6mm x 25cm)

HPLC PARAMETERS

FLOW RATE: 1 ml/MIN.
CHART SPEED: 0.5 cm/MIN.
DETECTOR SETTING: 208 nm
SENSITIVITY: .005
INJECTION VOLUME: 190 ul
RUNTIME: 35 MIN.
MOBILE PHASE: 30% CH_3CN, 70% H_2O CONTAINING .1% SDS, .1% H_3PO_4, .0025 M TMA-C1

COLUMN TEMPERATURE : 27°C

CHEMICALS

ACETONITRILE: FISHER HPLC GRADE
WATER: MILLIPORE MILLI-Q SYSTEM
SODIUM LAURYL SULFATE: FLUKA CHEMICAL 99% PURE
TETRAMETHYLAMMONIUM CHLORIDE: FLUKA CHEMICAL 98% PURE
PHOSPHORIC ACID: FISHER HPLC GRADE
PYRIDOSTIGMINE BROMIDE: LA ROCHE LABORATORIES
NEOSTIGMINE BROMIDE: SIGMA CHEMICAL

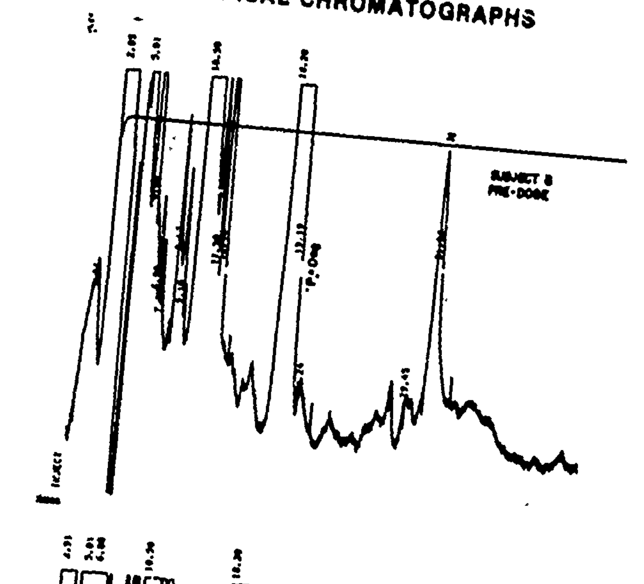
SAMPLE PREPARATION

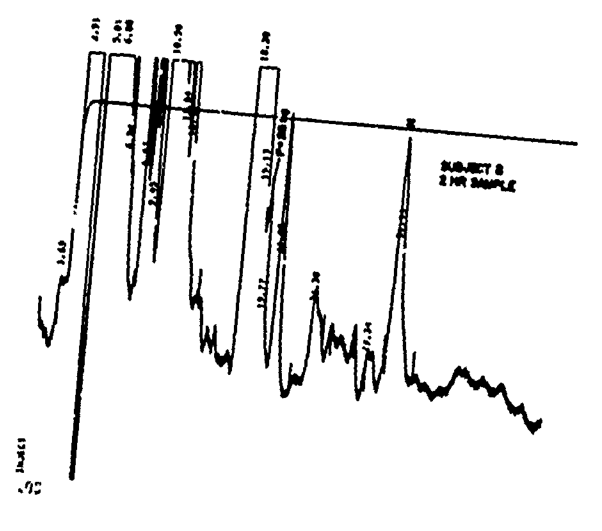
1. PIPET 0.5 ml OF PLASMA INTO A SCREW CAP CULTURE TUBE.
2. SPIKE PLASMA WITH 75 ng OF NEOSTIGMINE BROMIDE AND VORTEX FOR 10 SECONDS.
3. ADD 1.0 ml OF ACETONITRILE TO PRECIPITATE PLASMA PROTEINS.
4. VORTEX FOR 10 SECONDS; CENTRIFUGE AT 4000 x G FOR 10 MINUTES.
5. PIPET SUPERNATANT INTO PRE-CONDITIONED C-2 BOND ELUTE CARTRIDGE.
6. WASH WITH 2 ml OF WATER FOLLOWED BY 2 ml OF ACETONITRILE.
7. ELUTE WITH 1 ml OF 95% ACETONITRILE CONTAINING 0.1% SDS AND 0.05% TMA-C1.
8. REPEAT STEP Nr 7 WITH 2 ml AND RETAIN ELUTE IN SAME TUBE GIVING TOTAL VOLUME OF 3 ml.
9. EVAPORATE UNDER NITROGEN TO DRYNESS; REDISSOLVE IN 100 ul OF 30% ACETONITRILE.
10. TRANSFER TO LIMITED VOLUME INSERT AND INJECT 94 ul ONTO COLUMN.

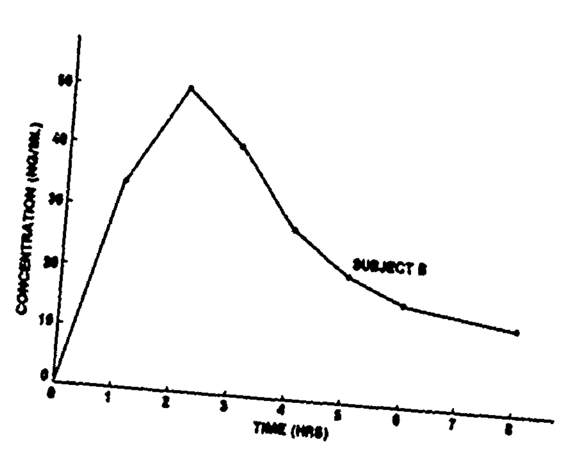
HPLC procedure adapted from an analysis developed by Dr. [illegible]

PLASMA PYRIDOSTIGMINE BROMIDE LEVELS FOR HUMAN SUBJECT INJESTING 60 mg TABLET

TYPICAL CHROMATOGRAPHS

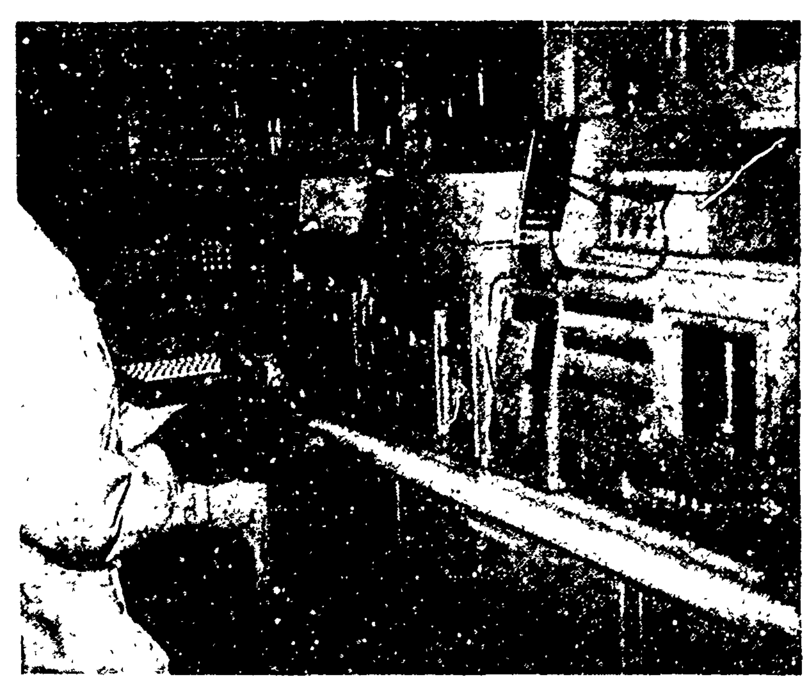




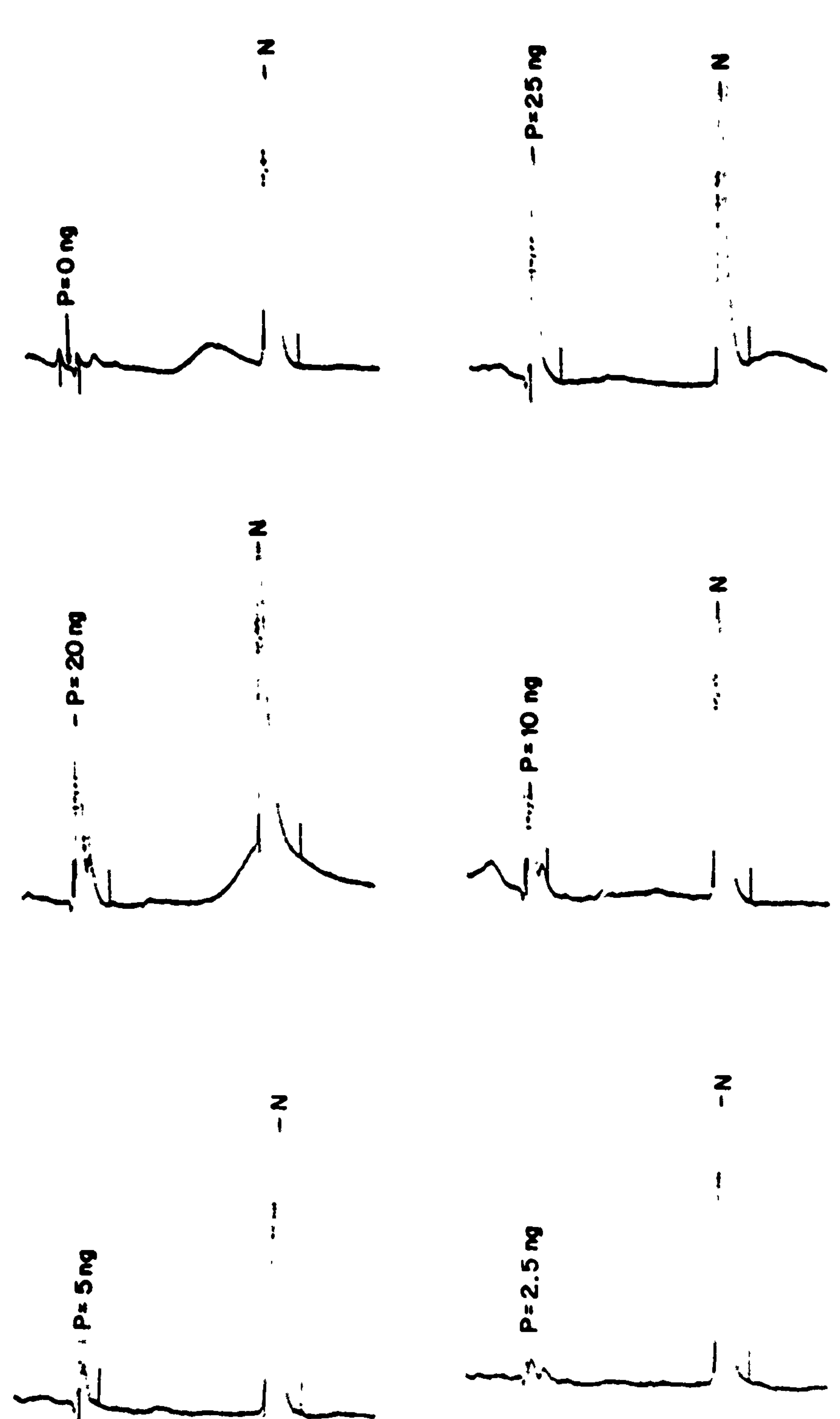


HPLC procedure adapted from an analysis developed by Dr. [redacted] E.T [illegible], et al

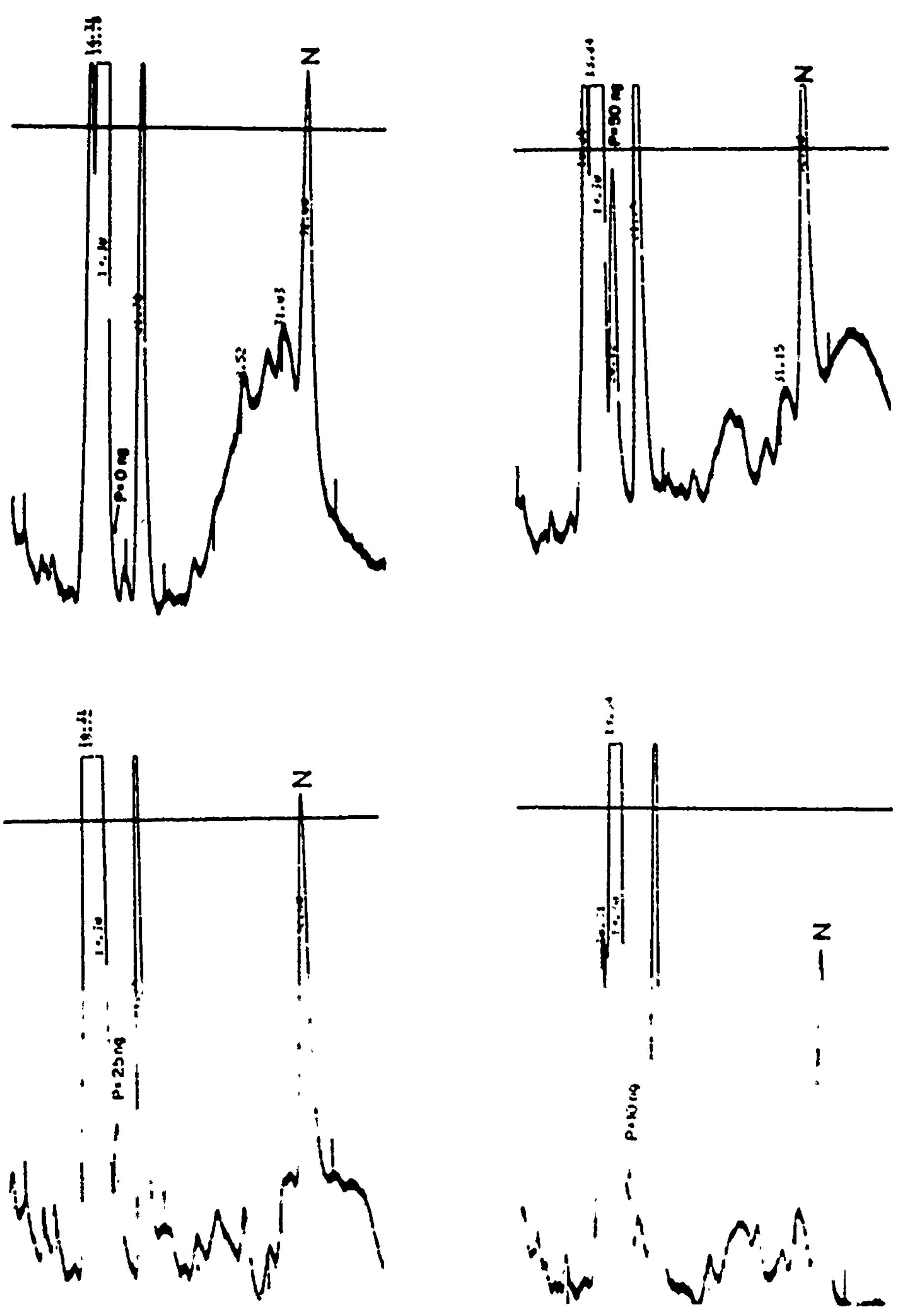
HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR QUANTITATIVE DETERMINATION OF PYRIDOSTIGMINE IN HUMAN PLASMA



TYPICAL CHROMATOGRAPHS OF AQUEOUS STANDARDS AT INDICATED PYRIDOSTIGMINE (P) CONCENTRATION. THE INTERNAL STANDARD NEOSTIGMINE (N) CONCENTRATION IS CONSTANT AT 50 ng.



TYPICAL CHROMATOGRAPH OF SPIKED PLASMA SAMPLES. PYRIDOSTIGMINE BROMIDE SPIKED AT INDICATED LEVELS AND NEOSTIGMINE BROMIDE AT 75 ng.



2. New Candidate Pretreatment/ Treatment Compounds

STUDIES ON THE MECHANISM OF ACTION OF THE ENHANCED PROTECTIVE EFFECT OF MULTIPLE VS. A SINGLE DOSE OF HI-6 AGAINST SOMAN POISONING

[illegible]
[illegible] Establishment [illegible], Canada

INTRODUCTION

Rats treated with atropine sulphate and a single dose of the oxime HI-6 can be protected initially from very high doses of soman. As time progresses the protective ratio falls rapidly and under conditions previously used in this laboratory stabilizes at about 3 x LD_{50} at 24 hours. Initially animals given high doses of soman with atropine and HI-6, convulse severely for a brief period, enter a period of relative sign free activity and then progressively deteriorate with returning signs of soman toxicity principally characterized by higher than normal frequency skeletal muscle activity, apparent deterioration of respiratory function and death.

We carried out experiments to determine whether these animals would survive if they were given additional doses of HI-6 at various time intervals following soman. The experiments were designed to determine whether any additional protection which might be observed was related to reactivation of (a) central or (b) AChE in the neuromuscular apparatus or in lieu of such activity whether other actions of HI-6 might be involved. In this way it was hoped to determine whether the return of toxic signs and death was related to AChE activity in one or both of the two compartments and whether multiple HI-6 injections might affect its distribuiton so that any additional beneficial effects might be due to factors not involved in its activity following one dose.

In addition, during the course of the investigation it became apparent that HI-6 had striking effects on skeletal muscle function in soman poisoned animals which might have been due either to central or peripheral actions of HI-6. AChE measurements were made in muscle and brain both during high frequency skeletal muscle activity and following its blockade by HI-6. It was hoped that we could determine whether this activity of HI-6 was due to

(a) reactivation of central AChE; (b) reactivation of skeletal muscle AChE; or (c) to direct effects on skeletal muscle function, all studies being related to the "in vivo" situation.

METHODS AND RESULTS

A. LD_{50} of soman in male rats was calculated by the proceedure of Finney.

All animals were given atropine SO_4 17.4 mg/kg and different groups were given various numbers of HI-6 injections (125 mg/kg, i.p.). One group was given HI-6 30-60 sec prior to soman. Mortality was measured for up to 14 days following [illegible] x LD_{50} soman.

A second group received additional doses of HI-6 following either 6 x LD_{50} or 9 x LD_{50} of soman. The additional doses were given at ½ hr, 1½ hr, 3 hrs and 6 hrs. A third group of animals was given 9 x LD_{50} soman and HI-6 at ½ hr, 1 hr, 2 hrs, 3 hrs, 4 hrs, 5 hrs and 6 hrs after soman. The mortality is recorded in Fig 1.

B. Rats were given HI-6 (125 mg/kg) and soman 6 x LD_{50}. One and one-half hrs later they were placed in a device to measure high frequency activity typical of tremors or fasciculations. A second group was given additional HI-6 at ½ hr and 1½ hrs following the soman dose. The results can be seen on Fig 2 (A.B.C.). Rats which received only one dose of HI-6 demonstrated much more high frequency activity then did those receiving 3 doses of HI-6.

In a second experiment, animals were given one dose of HI-6 and 6 x LD_{50} soman. After 1½ hrs of relatively symptom free activity there was a return of high frequency muscle activity. The reduction of this activity by a second dose of HI-6 is shown in Fig 2 (D.E.F.)

The lack of effect of diazepam (2.5 mg/kg) on this activity is seen in Fig 3 (A.B.C.).

Acetylcholinesterase from brain and peripheral muscle was measured by the radiometric assay of Siakotos 1967.

There was little indication that multiple doses of HI-6 reactivated AChE in the brains of rats treated with one or several doses of HI-6.

One dose of HI-6 reactivated or lead to AChE activity in the diaphragm that was higher than in control animals for about ½ hr but the activity returned to control values at subsequent time periods of measurement. Most animals survived much longer than would be predicted from diaphragm AChE activity which did not co-relate well with survival.

Similar effects were seen following multiple doses of HI-6. Diaphragm AChE activity was elevated above control values for a much longer period of time than was observed following one dose but eventually returned to control activity. Animals in this group all survived despite the low AChE activity.

DISCUSSION

In contrast to previously published data using the oximes (Rump and Faff, 1976; Jovanovic and Boskovic, 1983), it is evident that multiple HI-6 doses greatly increase the protective ratio against soman despite its well known property of rapidly aging AChE. Generally, more doses of HI-6 translated into better protection against soman in proportion to the amount of HI-6 given. Multiple injections of HI-6, following the regimen outlined in this presentation, were found to increase the protection ratio about 3 times above that expected from a single HI-6 injection (Lundy and Shih, 1983) previously described. It should be noted that no systematic examination was carried out to determine the optimal HI-6 regimen, so that the maximum protective ratio obtainable with HI-6 is as yet unknown.

About 1½ hrs after an initial insult with 6 x LD_{50} soman and 1 dose of

HI-6, animals began to suffer the return of symptoms typified by high frequency muscle activity, the origin of which was not initially clear. A second injection of HI-6 abolished the activity which was not accompanied by a rise in AChE activity in the muscles or the brain. Diazepam failed to affect this high frequency activity. The abolition of the high frequency muscle activity appeared to be therefore a direct effect of HI-6 and was not related to AChE reactivation in either compartment. This direct effect may not be similar to that seen on isolated skeletal muscle poisoned with soman "in vitro" since in those studies the return of neuromuscular function was related to increased AChE activity (French et al, 1983). It is suggested that the effect on the fasciculations may have been due to nicotinic blocking properties of HI-6 (Lundy and Tremblay, 1979; Clement, 1979).

There was no sustained or general increase in central AChE activity following a single or multiple HI-6 injections apparently ruling out the participation of this mechanism in the protective effects of the oximes. The reactivation of AChE in skeletal muscle was evident following one or several doses of HI-6. The survival of the animals however did not co-relate well with the maintenance of protection suggesting that enzyme reactivation was not the only factor involved in the protective effects of HI-6. It is higly unlikely that HI-6 could possibly reactivate enough enzyme, if in fact any unaged enzyme existed, 3-6 hrs after soman injection, to produce a beneficial effect.

It appears therefore that the protective effects of HI-6, as reflected by the multiple dose studies, were due in part to peripheral AChE reactivation, and partly to direct pharmacological activity although the proportion of either is unclear. These studies do not rule out the possibility that some of the protective effects of HI-6 may be due to inhibition by HI-6 of the egress of soman from its proposed storage site.

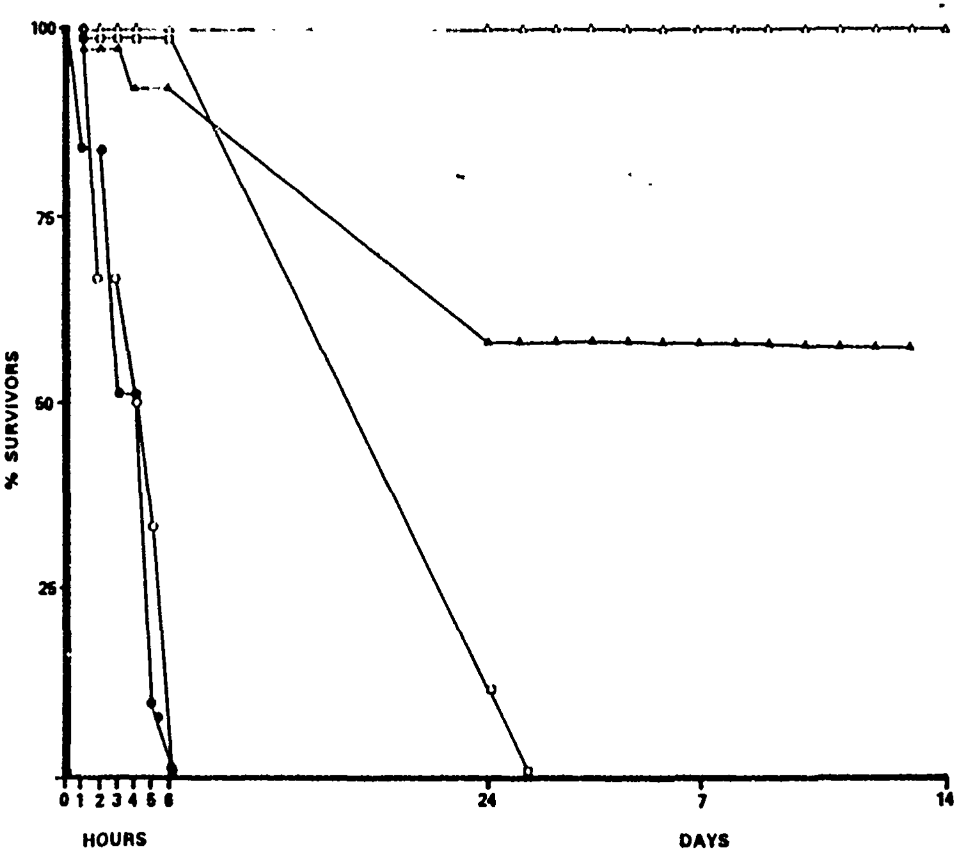


FIGURE 1. THE EFFECT OF VARIOUS DOSE SCHEDULES OF HI-6 AGAINST MULTIPLE LD50'S OF SOMAN IN THE RAT.

TABLE 1

Effect of Single vs. Multiple Doses of HI-6 on AChE Activity in Different Areas of Rat Brain

	Total HI-6 Doses	Time of HI-6 Administration	Time to Death/Sacrifice	AChE Activity[(a)] Brain Stem	Cortex	Hippocampus	Striatum
Control			-	8.87±0.13	5.18±0.17	8.78±0.43	34.78±1.75
Soman[(b)]			3.2 min.	1.07±0.09	1.23±0.05	0.91±0.045	1.06±0.08
Soman + Single Dose HI-6	1[(c)]	30-60S prior	½ hr	1.24±0.04	0.96±0.10	1.12±0.058	1.12±0.12
	1		1½ hr	1.33±0.06	1.30±0.14	1.28±0.07	1.29±0.10
	1		3 hr	1.24±0.09	1.20±0.11	1.42±0.016	1.45±0.08
	1		5 hr	0.61±0.06	0.79±0.10	0.67±0.06	1.00±0.03
Soman + Multiple Dose HI-6	1[(c)]	30-60S prior	½ hr	1.24±0.04	0.96±0.10	1.12±0.058	1.12±0.12
	2	+ ½ hr	1½ hr	1.58±0.15	1.63±0.15	1.59±0.13	1.53±0.10
	3	+ 1 hr	3 hr	1.57±0.16	1.38±0.15	1.76±0.14*	1.67±0.14
	4	+ 3 hr	5 hr	0.70±0.05	1.04±0.10	0.77±0.07	1.14±0.11

(a) AChE expressed as nmol ACh hydrolyzed/mg wet weight/min±SEM.

(b) Soman 650 μg/kg s.c. plus atropine SO_4 17.4 mg/kg i.p. 30 min prior to soman.

(c) HI-6 (125 mg/kg) 30-60S prior to soman.

AChE values of all soman groups irrespective of treatment were statistically different from controls.

* Denotes a value statistically different from soman without HI-6 ($p<.05$).

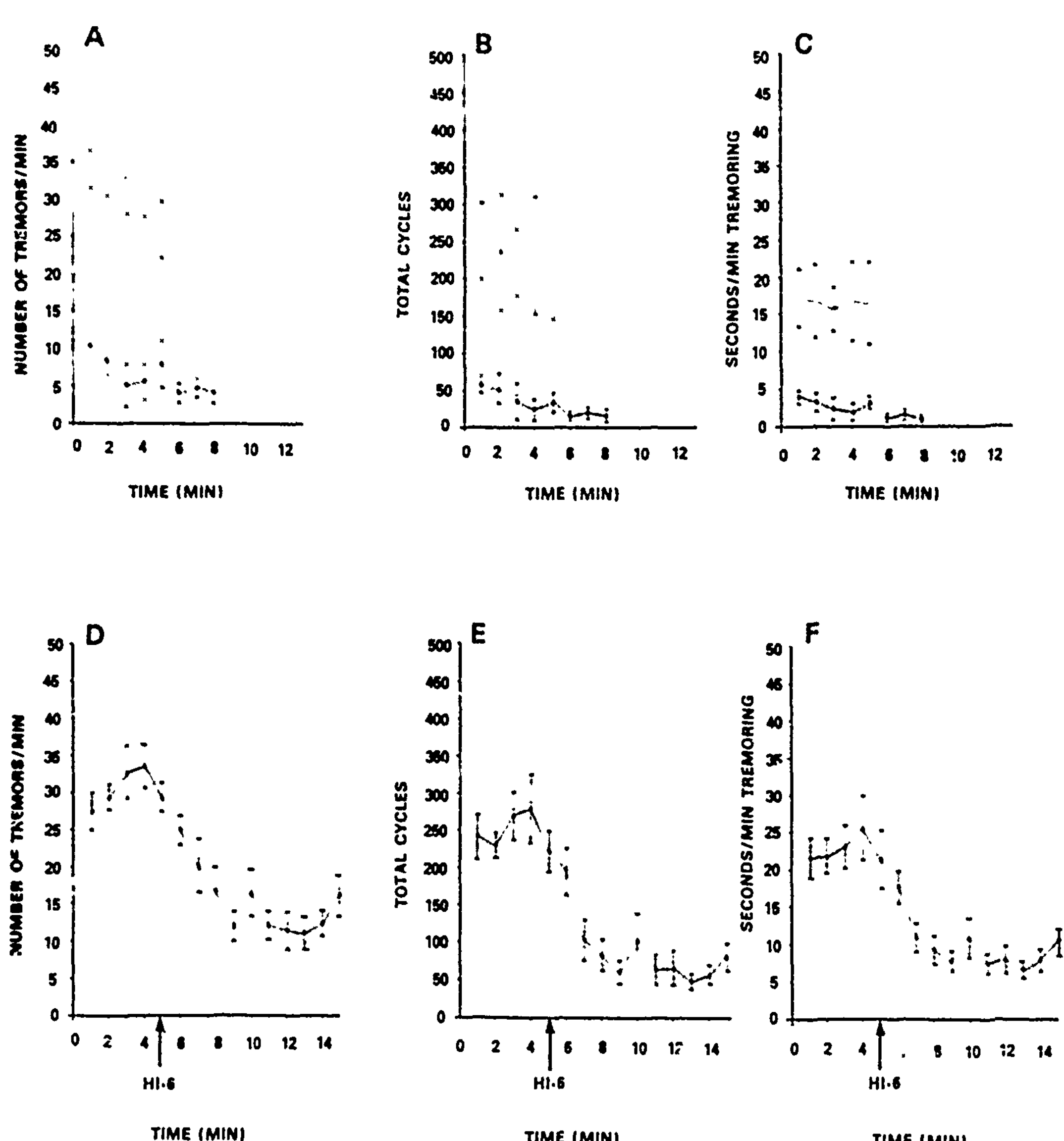


FIGURE 2. MEASUREMENT OF HIGH FREQUENCY MUSCLE ACTIVITY IN VIVO FROM RATS TREATED WITH DIFFERENT DOSE SCHEDULES OF HI-6 FOLLOWING SOMAN.

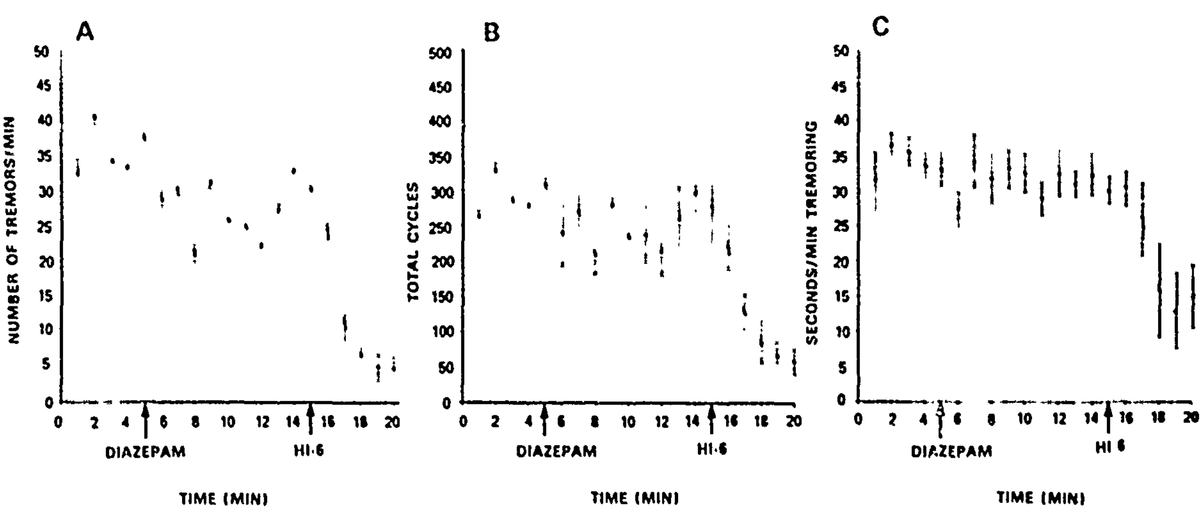


FIGURE 3. EFFECTS OF DIAZEPAM OR HI-6 ON HIGH FREQUENCY MUSCLE ACTIVITY IN VIVO FROM RATS TREATED WITH HI-6.

TABLE II

The Effect of Single vs. Multiple Doses of HI-6 on Rat Diaphragm Acetylcholinesterase

Treatment[a]	AChE Activity[b]	Time to Sacrifice Following Soman 650 μg/kg (hrs) ½	1½	3	5
Control	0.78±0.14(5)	-	-	-	-
Soman	0.006±.003(12)	no survivors	-	-	-
Soman + HI-6 (one dose)[c]		0.10±.022(12)*	0.036±.01(5)	0.016±.006(11)	0.015±0.011(6)
Soman + HI-6 (multiple doses)[d]		0.10±.022(12)*	0.15±.02(11)*	0.10±.019(11)*	0.031±0.026(6)

(a) All animals were pretreated with atropine sulphate 17.4 mg/kg i.p. ½ hour prior to soman 650 μg/kg s.c. (6 x LD_{50}).

(b) AChE activity expressed as nmol ACh hydrolyzed/mg wet weight/min + SEM (number of animals).

(c) 125 mg/kg i.p. HI-6 given 30-60S prior to soman.

(d) 125 mg/kg i.p. HI-6 given as in (c) plus at ½ hr, 1½ hr, and 3 hr.

* Values significantly different from control values. $p<0.05$.

TABLE III

AChE Activity in Fasciculating and Non Fasciculating

Muscle Groups and Brain From Soman Treated Rats Given HI-6

Tissue Type	T.F.L.	% Decrease	Rectus Abdominis	% Decrease	Brain	% Decrease
Control[a]	0.37±0.020	-	0.67±0.017	-	-	-
Fasciculating[b]	0.0021±0.0019	99.4	no activity	100	0.94±0.022	88.8
Non Fasciculating[c]	0.0059±0.0027	98.4	0.0086±0.004	98.7	1.08±0.085	87.1

(a) Animals given 17.4 mg/kg atropine SO_4 30 min. prior to soman 650 μg/kg.

(b) Treated as above with HI-6 30-60S prior to soman and sacrificed 2 hrs later.

(c) Treated as (b) at 2 hrs an additional dose of HI-6, 125 mg/kg i.p. was given and animals sacrificed 5 min later.

Number of tissues in all groups - 6.

Tensor fasciae latae (T.F.L.)

AChE, nmol ACh hydrolysed/my wet weight/min ± SEM

SUMMARY

1) MULTIPLE DOSES OF HI-6 GREATLY ENHANCE ITS PROTECTIVE EFFECT OVER ONE DOSE.

2) A SINGLE DOSE OF HI-6 INCREASES AChE ACTIVITY IN PERIPHERAL RESPIRATORY MUSCLE BUT NOT IN BRAIN FROM ANIMALS GIVEN 6 X LD_{50} SOMAN. PROTECTION IS INITIALLY GOOD BUT DECLINES RAPIDLY.

3) MULTIPLE DOSES OF HI-6 INCREASE AChE ACTIVITY IN PERIPHERAL RESPIRATORY MUSCLE BUT GENERALLY NOT IN BRAIN FROM ANIMALS GIVEN 6 X LD_{50} SOMAN.

4) INCREASE IN PERIPHERAL (DIAPHRAGM) AChE ACTIVITY DOES NOT CORELATE EXTREMELY WELL WITH THE PROTECTIVE EFFECT OF HI-6.

5) ANIMALS TREATED WITH A SINGLE DOSE OF HI-6 AND 6 X LD_{50} SOMAN CONVULSE FOR A SHORT PERIOD OF TIME AND REMAIN RELATIVELY INACTIVE FOR 1-1½ HOURS. THIS PERIOD IS FOLLOWED BY A RETURN OF ABNORMAL NEUROMUSCULAR ACTIVITY WHICH CAN BE ABOLISHED WITH A SECOND DOSE OF HI-6 BUT NOT DIAZEPAM. ANIMALS TREATED WITH MULTIPLE DOSES OF HI-6 FAIL TO DEVELOP THIS DELAYED NEUROMUSCULAR ACTIVITY. IT IS CONCLUDED THAT MOST OF THE ABNORMAL ACTIVITY IS DUE TO FASCICULATIONS.

6) AChE MEASUREMENTS INDICATE THAT BLOCKADE OF FASCICULATIONS BY HI-6 DOES NOT APPEAR TO BE THE RESULT OF REACTIVATION OF AChE ACTIVITY IN THE MUSCLES BUT IS DUE TO AN UNDEFINED DIRECT ACTIVITY WHICH MAY BE RELATED TO THE ANTICOTINIC EFFECTS OF HI-6.

7) IT IS CONCLUDED THAT THE BENEFICIAL EFFECTS OF MULTIPLE DOSES OF HI-6 IS NOT THE RESULT OF INCREASED LEVELS OF THE OXIME IN THE CNS. THE PERIPHERAL EFFECTS OF HI-6 MAY BE DUE TO SOME OR ALL OF THE FOLLOWING MECHANISMS: (A) REACTIVATION OF SOMAN INHIBITED ENZYME; (B) DIRECT EFFECTS ON FASCICULATING MUSCLES WHICH MAY OR MAY NOT BE ANALAGOUS TO DIRECT EFFECTS SEEN "IN VITRO"; AND/OR (C) INHIBITION OF THE EGRESS OF SOMAN BY HI-6 FROM A PROPOSED STORAGE SITE.

Clement, J.G. (1979). Pharmacological actions of HS-6, an oxime, in the neuromuscular junction. Eur. J. Pharmacol. 53, 135-141.

French, M.C., Wetherell, J.R. and White, P.D.T. (1983). The reversal by oximes and their oximinomethyl analogues of neuromuscular block produced by soman. Eur. J. Pharmacol. 91, 359-409.

Jovanović, D. and Bošković, B. (1983). Current problems in the experimental treatment of dimethoatc poisoning by cholinesterase reactivators. Acta Veterinaria 33, 21-28.

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SCREENING OF COMPOUNDS FOR ORAL PRETREATMENT EFFICACY AGAINST SOMAN POISONING IN MICE

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Aberdeen Proving Ground, MD 21010-5425
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ABSTRACT

Twenty-two compounds were evaluated for oral pretreatment efficacy against soman poisoning in mice. Included were 5 carbamates, 15 phosphinates, and 2 phosphorinanes. Compounds were administered by gavage at various time intervals (30-240 min) prior to soman challenge using doses based on either 1/4 fractions of the oral LD50 or solubility limits. At the appropriate time after pretreatment, mice were challenged intramuscularly (im) with 2xLD50 of soman. Supportive therapy with atropine (11.2 mg/kg) and 2-PAM (25 mg/kg) was administered im 10 seconds after soman challenge. Zero to 10 percent ($n=10$) of treated control mice survived 24 hours. Four carbamates and 1 phosphorinane significantly ($p<0.05$) increased survival (60-100%) at 24 hours. Although increased survival was observed following pretreatment with 3 of the phosphinates, protective levels were not significantly greater ($p>0.05$) than control (atropine/2-PAM). Effective compounds will be evaluated further as potential pretreatment drugs for nerve agent poisoning.

INTRODUCTION

Until a few years ago, the only effective pretreatment drugs for nerve agent poisoning were the classical carbamates. Recent evidence suggests that other classes of compounds may also be effective. Lieske (1) and Lawson (2) have described a series of organophosphinates exhibiting desirable inhibition kinetics and favorable rates of spontaneous and induced reactivation. Van Helden (3) has shown one of these phosphinates to be effective when administered intraperitoneally as a pretreatment for soman poisoning in rats. Ashani (4) recently reported the pretreatment efficacy of a novel cyclic organophosphate following subcutaneous administration to mice. Finally, Karlsson (5) has reported the pretreatment efficacy in mice of a novel ferrocene carbamate following subcutaneous administration.

All the above investigations were performed following parenteral drug administration. For field use it is neither feasible nor practical to administer a pretreatment by the parenteral route. Under these circumstances the oral route represents a logical alternative for pretreatment therapy.

The purpose of this work was to screen compounds belonging to the above named classes as oral pretreatments for soman poisoning in mice.

MATERIALS and METHODS

- Studies were conducted on male ICR Swiss mice (20-30 gms) quarantined in-house for at least 5 days prior to use. Subjects were fasted overnight with ad libitum access to water before experimentation.

- Experimental compounds were dissolved in the physiological vehicle in which they were most soluable and administered by gavage at 10 ml/kg. Soman challenge doses were prepared daily in sterile saline and injected intramuscularly (im) in a rear hind limb. Atropine and 2-PAM solutions were also prepared daily in deionized, distilled water, admixed and injected in the rear hind limb contralateral to the limb receiving soman. The dose volume for soman challenge and atropine/2-PAM treatments was 0.5 ml/kg. All drug dosages were administered using a randomized block design.

- Oral LD50's of test compounds were calculated by probit analysis (6) or the method of Thompson-Weil (7) based on 24 hour mortalities using 5 to 8 doses and 5-10 mice per dose.

- Initial pretreatment efficacy was determined by administering test drug dosages ranging from 1/4 to 1/256 of the oral LD50 or limits of solubility, if an oral LD50 could not be determined. Drugs were administered at intervals ranging from 30 to 240 minutes prior to soman challenge. Two groups of control animals were included. One group was pretreated with the vehicle and the other was pretreated 60 minutes prior to soman with 0.82 mg/kg pyridostigmine (opt. pretreatment dose) (8). At the appropriate time after pretreatment, mice were challenged with 2xLD50 of soman. Therapy with atropine (11.2 mg/kg) and 2-PAM (25.0 mg/kg) was administered 10 seconds post soman challenge. Survival was assessed at 24 hours.

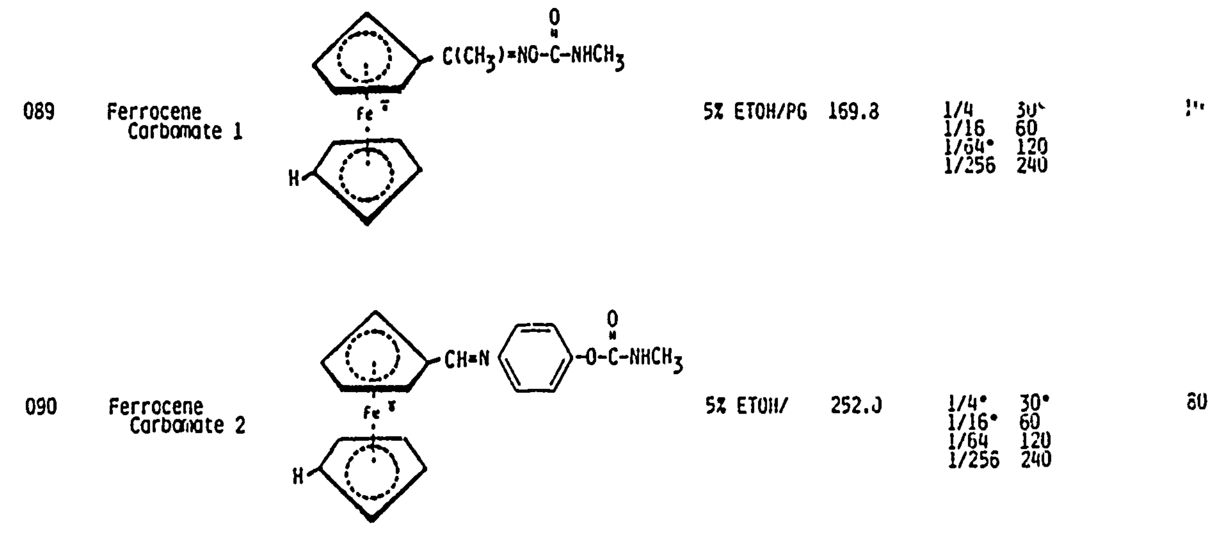
Phosphinates

ICD#	Designator	R	R_1	R_2	R_3	Vehicle	LD50 (mg/kg)	Dose[1]	PT[2] (min)	% Survivors[3] vs 2XLD50
088	Phosphinate 10	NO_2	O		CH_3	PG	51.07	1/4 1/16 1/64 1/256*	30* 60 120* 240	20
239	Phosphinate 11	NO_2	O		CH_2Cl	5% ETOH/ PEG-200	320.30	1/4 1/8 1/16*	30* 120*	10
240	Phosphinate 12	Cl	O	$N^+(CH_3)_3$	CH_3	5% ETOH/ PEG-200	2070	max 1/2 1/4	30 120	0
242	Phosphinate 14	NO_2	O	OCH_3	CH_2Cl	5% ETOH/ PEG-200	35.80	1/4 1/8 1/16	30 120	0
243	Phosphinate 15	NO_2	O	OCH_3	CH_3	5% ETOH/ PEG-200	77.10	1/4 1/8 1/16	30 120	0
064	Phosphinate 3	NO_2	O	CH_2Cl	CH_2Cl	5% ETOH/PG	857.90	1/4 1/16 1/64 1/256	30 60 120 240	0
062	Phosphinate 1	NO_2	O		CH_3	5% ETOH/ Peanut Oil	15.29	1/4 1/16 1/64 1/256	30 60 120 240	0
063	Phosphinate 2	NO_2	O		CH_2Cl	3% ETOH/ Peanut Oil	493.90	1/4 1/16 1/64 1/256	30 60 120 240	0
066	Phosphinate 5	F	O		CH_3	5% ETOH/ Peanut Oil	438.3	1/4 1/16 1/64 1/256*	30 60 120* 240	20
084	Phosphinate 6	NO_2	O		CH_2CH_3	0.8% ETOH/ Peanut Oil	5.90	1/4 1/16 1/64 1/256	30 60 120 240	0
241	Phosphinate 13	$N(CH_3)_3$	O		CH_2Cl	5% ETOH/ PEG-200	727.4	1/4 1/8 1/16*	30* 120*	10
086	Phosphinate 8	NO_2	O	Cl	CH_3	2% ETOH/ Peanut Oil	54.16	1/4 1/16 1/64 1/256	30 60 120 240	0
065	Phosphinate 4	NO_2	O			PEG-200	112.0	1/2max 1/4max	30 60 120 240	0
085	Phosphinate 7	NO_2	S	$CH_2(CH_2)_2CH_3$	$CH_2(CH_2)_2CH_3$	5% ETOH/ Peanut Oil	689.8	1/4 1/16 1/64 1/256	30 60 120 240	10
087	Phosphinate 9	NO_2	O	CH_3	CH_3	5% ETOH/PG	54.16	1/4 1/16 1/64 1/256*	30* 60 120 240	10

1. Dose expressed as either fraction of the LD50 or solubility limit.
2. Pretreatment time interval.
3. Difference between the maximum observed survival and the control survival.
* Pretreatment time interval and dose giving the highest survival.

Carbamates

ICD#	Designator	Vehicle	LD50 (mg/kg)	Dose[1]	PT[2] (min)	% Survivors[3] vs 2XLD50
023	Pyridostigmine	water	26.2	1/4 1/8 1/16 1/32 1/64 1/128 1/256 1/512	30 60 120 240 480	70
117	Neostigmine	water	16.4	1/4 1/8 1/16*	30 120*	60
118	Physostigmine	water	3.9	1/4* 1/16 1/64 1/256	30* 60 120 240	100



ICD#	Designator	Vehicle	LD50 (mg/kg)	Dose[1]	PT[2] (min)	% Survivors[3] vs 2XLD50
089	Ferrocene Carbamate 1	5% ETOH/PG	169.8	1/4 1/16 1/64* 1/256	30 60 120 240	[illegible]
090	Ferrocene Carbamate 2	5% ETOH/	252.0	1/4* 1/16* 1/64 1/256	30* 60 120 240	80

1. Dose expressed as either fraction of the LD50 or limit of solubility.
2. Pretreatment time interval.
3. Difference between the maximum observed survival and the control survival.
* Pretreatment time interval and dose giving the highest survival.

Phosphorinones

O O P R O

ICD#	Designator	R	X	Vehicle	LD50 (mg/kg)	Dose[1]	PI[2] (min)	% Survivors[3] vs 2XLD50
193	Phosphorinone 2	$-S(CH_2)_2NH(CH_2)_3NH_2$		Water	2132.00	1/8* 1/16	30 120*	20
034	Phosphorinone	O– (phenyl) –$^+N(CH_3)_3$	I^-	Water	527.00	max* 1/2* 1/4	30 120*	100

1. Dose expressed as either fraction of the LD50 or limit of solubility.
2. Pretreatment time interval.
3. Difference between the maximum observed survival and the control survival.
* Pretreatment time interval and dose giving the highest survival.

SUMMARY

1. The oral efficacy of several classes of pretreatment compounds was evaluated against soman poisoning in mice.

2. The results show that the classical carbamates, neostigmine, pyridostigmine, and physostigmine are all effective by the oral route.

3. Of the new classes of pretreatment compounds evaluated, both the ferrocene carbamates and cyclic organophosphates show promise as potentially useful therapeutic drugs.

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ORAL PRETREATMENT EFFICACY OF A NOVEL CARBAMATE AGAINST SOMAN POISONING IN MICE

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ABSTRACT

A novel ferrocene carbamate [[[4-[[(methylamino)-carbonyl]oxy]phenyl]imino]methyl]-ferrocene (FC) was evaluated orally in mice for acute toxicity, pretreatment efficacy against soman poisoning and inhibition of whole blood acetylcholinesterase (AChE). The 24-hour oral LD50 (95% CI) was 252 mg/kg (229-277). In initial efficacy studies, fractions ranging from 1/4 to 1/256 of the LD50 were administered by gavage at various time intervals (30-240 minutes) prior to intra-muscular (im) challenge with soman (2xLD50). Animals were treated im with atropine (11.2 mg/kg) + 2-PAM (25.0 mg/kg) 10 seconds after soman. Ten percent (1 of 10) of treated control mice (no pretreatment) survived 24 hours, while up to 90 percent survival was observed in mice pretreated with FC. Increased survival was observed at all pretreatment times. In studies to determine maximum protective efficacy, doses of FC ranging from 0.49 - 63 mg/kg increased the LD50 of soman 2.3 to 3.1 times, respectively, in mice treated with atropine and 2-PAM. In contrast, treatment with atropine and 2-PAM alone increased the LD50 of soman only 1.6 times. In addition, pretreatment with FC alone increased the LD50 of soman 1.4 times. Inhibition of whole blood AChE ranged from 1.0 % at 0.49 mg/kg to 71.4 % at 63 mg/kg and was correlated (r=0.98) with the increase in protective ratio (PR) observed over this dose range. These results indicate that compounds of this class are effective when given orally as a pretreatment against poisoning by organophosphorus nerve agents.

MATERIALS and METHODS

- Studies were conducted on male ICR Swiss mice (20-30 gms) quarantined for at least five days prior to use. Before testing subjects were deprived of food overnight with ad libitum access to water.

- The ferrocene carbamates were dissolved in PEG200 containing 5% ethanol and administered orally by gavage at 10 ml/kg. Soman challenge doses were prepared daily in sterile saline and injected intramuscularly (im) in a rear hind limb. Atropine and 2-PAM solutions were prepared daily in deionized, distilled water and injected im in the rear hindlimb contralateral to the limb receiving soman. The dose volume for soman challenges and atropine/2-PAM treatments was 0.5 ml/kg. All drug dosages were administered using a randomized block design.

- LD50's were calculated by probit analysis (6) based on 24 hour mortalities using 6-7 doses and 10 mice per dose.

- The initial pretreatment efficacy of each ferrocene carbamate was determined by administering doses (n=10/dose) from 1/4 to 1/256 of the oral LD50 at various time intervals (30-240min) prior to soman challenge. Two groups of control animals were included. One group was pretreated with the vehicle and the other was pretreated 60 minutes prior to soman with 0.82 mg/kg pyridostigmine (opt. pretreatment dose) (3). At the appropriate time after pretreatment, mice were challenged with 2xLD50 soman. Therapy with atropine (11.2 mg/kg) and 2-PAM (25.0 mg/kg) was administered 10 seconds post soman challenge. Survival was assessed at 24 hours.

- Optimal oral protection studies were performed using the pretreatment time interval from the initial efficacy study which gave the highest overall survival. Mice were pretreated with 1 of 8 doses of the ferrocene ranging from 1/512-1/4 LD50 or the vehicle and challenged im with one of 5 doses (n=10/dose) of soman for each pretreatment dose. Ten seconds after soman challenge, animals were treated with either saline, atropine (11.2 mg/kg), 2-PAM (25.0 mg/kg) or a mixture of atropine and 2-PAM. Mortality counts were taken at 24 hours, and soman LD50 for each pretreatment/treatment combination was calculated by probit analysis. A protective ratio for each combination was calculated by dividing the soman LD50 for untreated mice (vehicle pretreated-saline treated) into the soman LD50 for treated mice.

- In additional studies, activity of acetylcholinesterase in whole blood was measured 30 minutes after dosing (n=6/dose) using the method of Siakotos et al (7).

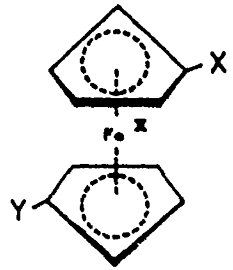
INTRODUCTION

Although carbamates such as pyridostigmine, neostigmine, and physostigmine have demonstrated significant efficacy as pretreatments for nerve agent poisoning when administered either parenterally (1,2) or orally (3), the search for new and better carbamates continues. Compounds are being sought which combine good oral availability without GI symptoms, ability to cross the blood brain barrier, and long duration of action with higher therapeutic index.

In 1980 Hetnarski et al (4) described a series of dicyclopentadienyliron or ferrocene carbamates exhibiting anticholinesterase activity with I50's in the range of 10^{-4}M to 10^{-6}M (panel 1). Hetnarski attributed the acetylcholinesterase inhibiting potency of these compounds on the ability of the ferrocene moiety to form charge transfer complexes with the enzyme.

Several years later Karlsson et al (5) reported the parenteral pretreatment efficacy of two of Hetnarski's compounds, #2 and #8, against soman poisoning in mice (panel 2). Compound #2 was ineffective. Compound #8 however, gave a protective ratio of 6 when administered ip at 5 mg/kg (1/30 LD50) 30 minutes prior to soman. Atropine (20 mg/kg) was also administered as a pretreatment 10 minutes prior to soman.

The purpose of this study was to determine the oral pretreatment efficacy of compound #2 (ICD# 089) and compound #8 (ICD# 090) against soman poisoning in mice.



Compound Number	X	Y	I_{50}* (M)
1	$C(H){=}NOC(O)NHCH_3$	H	1.0×10^{-4}
2	$C(CH_3){=}NOC(O)NHCH_3$	H	1.8×10^{-4}
3	$C(CH_3){=}NOC(O)NHCH_3$	$C(CH_3){=}NOC(O)NHCH_3$	1.0×10^{-5}
4	$C(C_2H_5){=}NOC(O)NHCH_3$	H	1.8×10^{-5}
5	$C((CH_2)_2CH_3){=}NOC(O)NHCH_3$	H	1.5×10^{-5}
6	$C(CH(CH_3)_2){=}NOC(O)NHCH_3$	H	2.0×10^{-6}
7	$C(C_6H_5){=}NOC(O)NHCH_3$	H	1.7×10^{-5}
8	$C(H){=}NC_6H_4OC(O)NHCH_3\text{-}p$	H	3.5×10^{-6}

Modified from Hetnarski, B. et al J. NeuroSci Res 5: 1-5 (1980)

* Bovine erythrocyte AChE

CH_3, C=N—O—C(=O)—N(H)—CH_3, Fe, H

ICD# 089

H, C=N, O—C(=O)—N(H)—CH_3, Fe, H

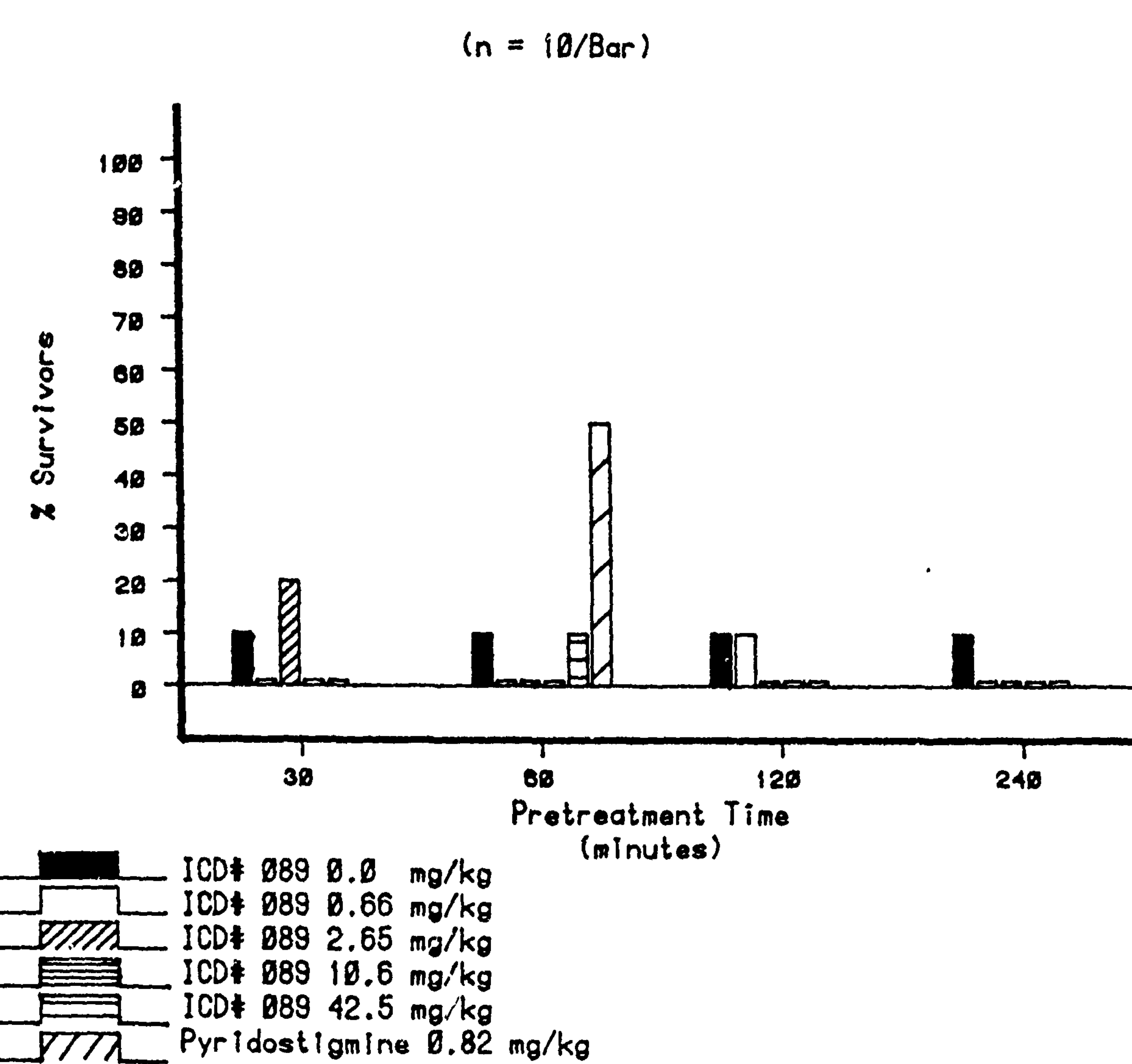
ICD# 090

ACUTE ORAL TOXICITY OF CARBAMATES IN MICE

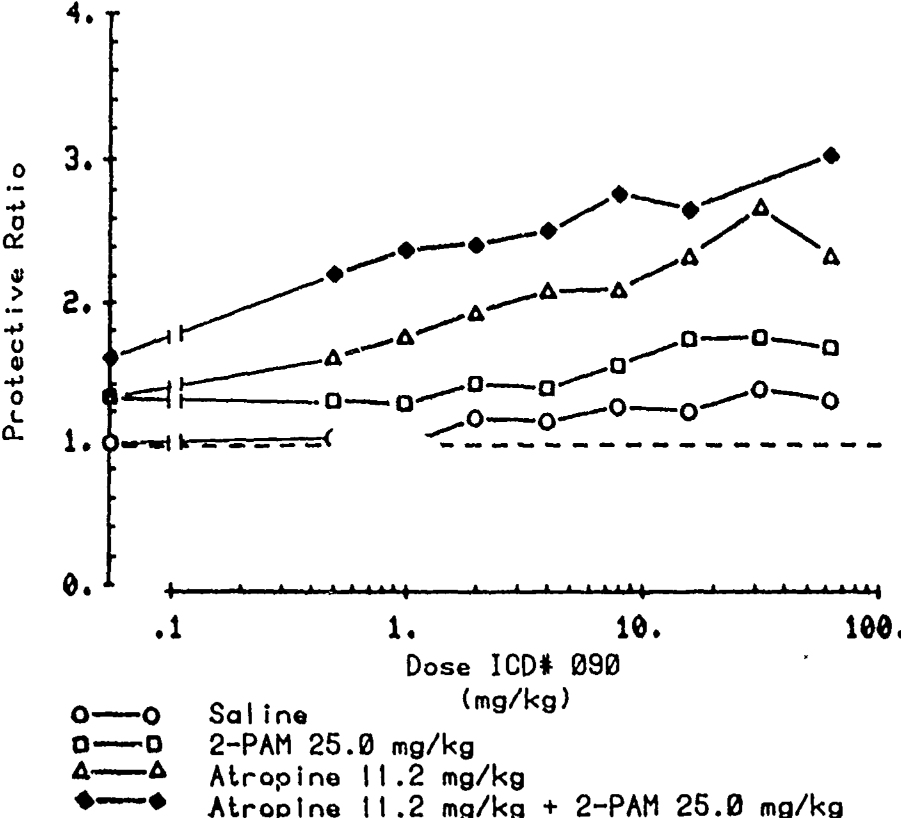
Compound	24 hr LD50	(95 % CI)
ICD# 090	251.9	(229.3 - 276.7)
ICD# 089	169.8	(142.9 - 201.7)
Pyridostigmine	26.2	(22.5 - 30.4)
Physostigmine	3.9	(3.5 - 4.3)

Initial Oral Pretreatment Efficacy ICD# 089 Against (2.0xLD50) GD in Mice Treated with Atropine/2-PAM

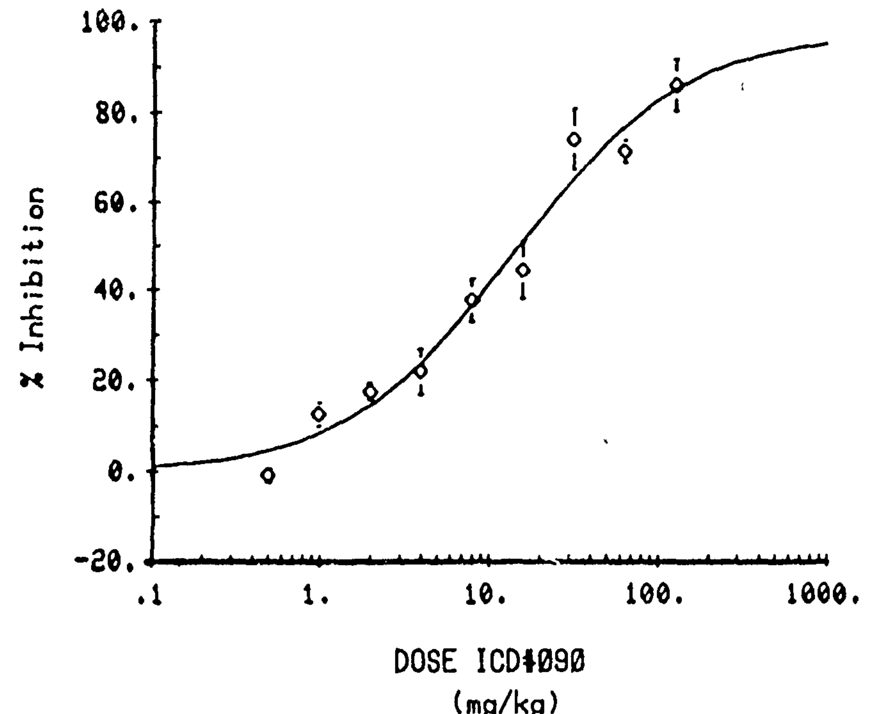
(n = 10/Bar)



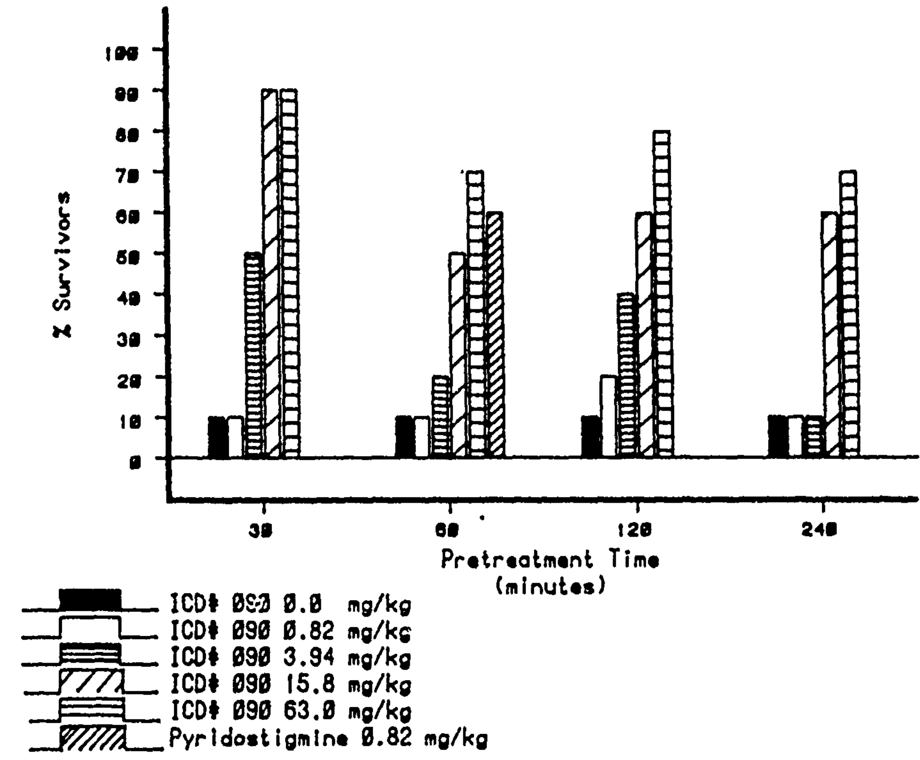
Protective Effect of ICD# 090 Oral Pretreatment
30 Minutes Prior to Soman in Mice



Inhibition of Whole Blood Acetylcholinesterase 30 Minutes
after Oral ICD#090 in the Mouse



Initial Oral Pretreatment Efficacy ICD# Ø9Ø Against (2.ØxLD5Ø) GD in Mice Treated with Atropine/2-PAM



SUMMARY

1. Oral toxicity and pretreatment efficacy of a new class of carbamates has been presented.

2. ICD# Ø9Ø was found to be a highly effective oral pretreatment in the mouse (PR = 3.1)

   - Exhibited significant ($p<0.05$) protection (PR = 1.4) alone.

   - Significantly ($p<0.05$) enhanced the efficacy of optimal atropine and 2-PAM therapy.

   - Significantly ($p<0.05$) better than pyridostigmine with atropine/2-PAM therapy.

3. Efficacy of ICD# Ø9Ø was directly correlated with acetylcholinesterase inhibition ($r = 0.98$).

4. Additional ferrocenes are being synthetized to more fully evaluate the therapeutic potential of this novel class of compounds.

Dose ICD# 090 (mg/kg)	% inh AChE (mean ± S.D.)	PR (95 % CI) (Atropine 11.2 + 2-PAM 25.0)
126.0	85.7 ± 3.5	– –
63.0	71.4 ± 6.3	3.07 (2.87 – 3.26)
31.5	73.9 ± 4.8	– –
15.8	44.5 ± 12.3	2.71 (2.56 – 2.87)
7.9	37.8 ± 11.8	2.81 (2.64 – 2.98)
3.94	21.9 ± 16.2	2.56 (2.43 – 2.70)
1.97	17.5 ± 15.3	2.47 (2.32 – 2.62)
0.98	12.6 ± 6.0	2.43 (2.29 – 2.57)
0.49	-0.8 ± 13.3	2.26 (2.14 – 2.38)
0.0	–	1.63 (1.53 – 1.72)

r = 0.98

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CENTRAL ANTIMUSCARINIC POTENCY OF CANDIDATE COMPOUNDS FOR ORGANOPHOSPHATE POISONING PRETREATMENT OR THERAPY

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INTRODUCTION

ATROPINE SULFATE IN COMBINATION WITH AN OXIME REACTIVATOR HAS LONG BEEN A STANDARD TREATMENT FOR POISONING BY ORGANOPHOSPHATE CHOLINESTERASE INHIBITORS (OP'S), BUT MANY INVESTIGATORS HAVE ATTEMPTED TO FIND A BETTER ANTIMUSCARINIC AGENT FOR THIS PURPOSE. APPARENTLY, THE RUSSIAN ARMY HAS SWITCHED TO APROPHEN (1) AS ITS PREFERRED ANTIDOTE. DURING THE 1960'S COLEMAN, ET AL., CARRIED OUT EXTENSIVE STUDIES COMPARING THE PROTECTION OF ATROPINE AND ABOUT 40 OTHER ANTIMUSCARINICS (ADMINISTERED WITH PRALIDOXIME METHOSULFATE) FOR MICE OR RATS GIVEN LETHAL DOSES OF SARIN AND OTHER OP'S [I. W. COLEMAN, P. E. LITTLE, AND R. A. B. BANNARD, CAN. J. PHYSIOL. PHARMACOL. 46, 109 (1968)]. ABOUT 1/4 OF THE COMPOUNDS GAVE SIGNIFICANT PROTECTION, AND SEVERAL WERE DISTINCTLY BETTER THAN ATROPINE. THE COMPOUND THEY CONSIDERED TO GIVE THE BEST PROTECTION IN A VARIETY OF ASSAYS WAS CARAMIPHEN (2).

ALTHOUGH A FALL IN BLOOD PRESSURE, EXCESSIVE SECRETIONS, BRONCHIAL CONSTRICTION, AND THORACIC MUSCLE WEAKNESS CONTRIBUTE TO RESPIRATORY DISTRESS IN OP POISONING, THE KEY TOXIC EFFECT FOR LETHALITY APPEARS TO BE THE ACTION OF ACETYLCHOLINE ON MUSCARINIC RECEPTORS IN THE CNS. IT HAS LONG BEEN RECOGNIZED THAT ATROPINE IS MUCH LESS EFFECTIVE AS AN ANTIMUSCARINIC AGENT CENTRALLY THAN PERIPHERALLY (WHEN ADMINISTERED PERIPHERALLY). THEREFORE, A POSSIBLE APPROACH TO A BETTER OP ANTIDOTE IS TO LOOK FOR AGENTS WITH A BETTER RATIO OF CENTRAL TO PERIPHERAL ANTAGONIST ABILITY. THIS IS SUPPORTED BY MADILL, ET AL., WHO FOUND THAT FOR A SET OF 9 ANTIMUSCARINIC AGENTS, PROTECTION OF GUINEA PIGS FROM SARIN POISONING PARALLELED THEIR CNS ANTI-

MUSCARINIC POTENCY, NOT THEIR PERIPHERAL POTENCY [M. D. MADILL, W. C. STEWART, AND M. L. SAVOIE, CAN. J. PHYSIOL. PHARMACOL. 46, 559 (1968)]. CARAMIPHEN (2) WAS FOUND TO BE THE MOST EFFECTIVE PROTECTING AGENT ALTHOUGH IT HAD ONLY 1/100 THE PERIPHERAL ANTI-MUSCARINIC ACTION OF ATROPINE.

BASED ON THE ABOVE CONSIDERATIONS, WE HAVE DECIDED TO EXAMINE APROPHEN, CARAMIPHEN AND A LARGE NUMBER OF OTHER ANTIMUSCARINIC AGENTS OF DIVERSE STRUCTURE FOR CNS BLOCKING ACTIVITY. WE HAVE CHOSEN THE ANTAGONISM OF TREMORS INDUCED BY THE MUSCARINIC AGONIST OXOTREMORINE AS A MEASURE OF THE CNS POTENCY OF THE COMPOUNDS. IN ADDITION TO A STRONG CNS ANTIMUSCARINIC POTENCY, A GOOD ANTIDOTE MUST POSSESS A SUITABLE ONSET AND/OR DURATION OF ACTION. IF A COMPOUND IS A POTENT AGENT WITH A SLOW ONSET OF ACTION, THEN THE OP MAY BE LETHAL BEFORE THE PROTECTIVE ACTION IS EXERTED. IF A COMPOUND IS TO BE USED AS A PRETREATMENT IN ADVANCE OF OP POISONING, THEN THE BLOCKING ACTION MUST PERSIST FOR A REASONABLE PERIOD. THEREFORE, WE HAVE EXAMINED CNS ANTI-MUSCARINIC ACTIVITY WHEN THE COMPOUNDS ARE GIVEN EITHER BEFORE OR AFTER THE OXOTREMORINE CHALLENGE.

COMPOUNDS

THIRTY-TWO COMPOUNDS HAVE BEEN COMPARED TO ATROPINE IN THE OXOTREMORINE TREMOR BLOCKING ASSAY. THE STRUCTURE OF THE COMPOUNDS (AS THE FREE BASE) IS SHOWN IN THE FIGURE TO THE RIGHT. THE COMPOUNDS WERE OBTAINED EITHER BY SYNTHESIS IN OUR LABORATORY BY STANDARD LITERATURE PROCEDURES OR AS A GIFT FROM PHARMACEUTICAL MANUFACTURERS THAT MARKET THE DRUGS. ALL OF THE COMPOUNDS WERE PREPARED AND TESTED AS WATER-SOLUBLE SALTS, GENERALLY THE HYDROCHLORIDES.

COMPOUNDS 15 - 25 ARE THE ONES SUPPLIED BY THEIR RESPECTIVE MANUFACTURERS AS FOLLOWS:

15 - TRIHEXYPHENIDYL HYDROCHLORIDE (ARTANE R)
LEDERLE LABORATORIES, WAYNE, NJ

16 - CYCLOPENTOLATE HYDROCHLORIDE (CYCLOGYL R)
ALCON LABORATORIES, FT. WORTH, TX

17 - OXYBUTYNIN HYDROCHLORIDE (DITROPAN R)
MARION LABORATORIES, KANSAS CITY, MO

18 - CHLORPHENOXAMINE HYDROCHLORIDE (SYSTRAL R)
ASTRA-WERKE AG, BIELEFELD, FEDERAL REPUBLIC OF GERMANY

19 - BENZTROPINE MESYLATE (COGENTIN R)
MERCK, SHARP & DOHME, WEST POINT, PA

20 - BIPERIDEN HYDROCHOLORIDE (AKINETON R)
KNOLL PHARMACEUTICAL CO., WHIPPANY, NJ

21 - TRIFLUPROMAZINE HYDROCHLORIDE (VESPRIN R)
E. R. SQUIBB & SONS, PRICETON, NJ

22 - METHIXENE HYDROCHLORIDE (TREST R)
DORSET LABORATORIES, LINCOLN, NE

23 - OXYPHENCYCLIMINE HYDROCHLORIDE (DARICON R)
BEECHAM LABORATORIES, BRISTOL, TN

24 - ETHOPROPAZINE HYDROCHLORIDE (PARSINDOL R)
WARREN LAMBERT CO. ANN ARBOR, MI

25 - ORPHENADRINE CITRATE (NORFLEX R)
RIKER LABORATORIES, NORTHRIDGE, CA

C_2H_5, C_2H_5 $N-CH_2-CH_2-O-\overset{O}{\overset{\|}{C}}-C(C_6H_5)(CH_3)-C_6H_5$

1

C_2H_5, C_2H_5 $N-CH_2-CH_2-O-\overset{O}{\overset{\|}{C}}-$ (cyclopentyl, C_6H_5)

2

CH_3-N (piperidyl) $-O-\overset{O}{\overset{\|}{C}}-$ (cyclopentyl, C_6H_5)

3

C_2H_5, C_2H_5 $N-CH_2-CH_2-O-\overset{O}{\overset{\|}{C}}-$ (xanthenyl, O)

4

C_2H_5, C_2H_5 $N-CH_2-CH_2-O-\overset{O}{\overset{\|}{C}}-C(OH)-$ (cyclohexyl, thienyl, S)

5

(piperidyl) $N-CH_2-CH_2-N(CH_3)-\overset{O}{\overset{\|}{C}}-C(C_6H_5)(OH)-C_6H_5$

6

CH_3-N (piperidyl) $-C(C_6H_5)(OH)-C_6H_5$

7

CH_3-N (piperazinyl) $N-CH_2-C(C_6H_5)(OH)-$ (cyclohexyl)

8

C_6H_5

$N-CH_2-CH_2-C-C(=O)-NH_2$

C_6H_5

9

C_6H_5

$C_6H_5-CH_2-N$ ... O, NH, O

10

C_2H_5, C_2H_5 $N-CH_2$ ... O, O, C_6H_5, C_6H_5

11

C_2H_5, C_2H_5 $N-CH_2$ ... O ... $C(C_6H_5)(OH)-C_6H_5$

12

$CH_2-O-C(=O)-CH(C_6H_5)-C_6H_5$

N, CH_3

13

CH_3-O ... $CH_2CH(CH_3)-N(C_2H_5)-CH_2-CH_2-CH_2-C(=O)$ ...

14

$N-CH_2CH_2-C(C_6H_5)(OH)$ ...

15

CH_3, CH_3 $N-CH_2-CH_2-O-C(=O)-CH(C_6H_5)$ ... OH

16

17

18

19

20

21

22

23

24

$(CH_3)_2N-CH_2CH_2-O-CH(C_6H_5)-C_6H_4(CH_3)$

25

26

27

28

$(C_2H_5)_2N-CH_2CH_2CH_2-O-C(=O)-C_5H_8(C_6H_5)$

29

$(C_2H_5)_2N-CH_2CH_2-O-C(=O)-C_6H_{10}(C_6H_5)$

30

31

32

METHODS

IN A PRELIMINARY EXPERIMENT, DIFFERENT DOSES OF OXOTREMORINE WERE ADMINISTERED S. C. TO GROUPS OF 10 MICE. THE PRESENCE OR ABSENCE OF TREMORS WAS THEN NOTED AT VARIOUS TIME INTERVALS. THE RESULTS ARE PRESENTED IN TABLE I. FROM THE DATA OF TABLE I, AN ED_{84} WAS CALCULATED FOR THE PRODUCTION OF TREMORS AT 10 MINUTES POST INJECTION. THIS DOSE OF OXOTREMORINE WAS SUBSEQUENTLY USED IN ALL EVALUATIONS OF ANTAGONISTS.

IN ONE SERIES OF EXPERIMENTS, EACH OF THE ANTAGONISTS WAS ADMINISTERED S. C. TO GROUPS OF 10 MICE 30 MINUTES PRIOR TO CHALLENGE WITH THE ED_{84} DOSE OF OXOTREMORINE. THE NUMBER OF MICE SHOWING TREMORS AT 10 AND 20 MINUTES AFTER THE OXOTREMORINE INJECTION WAS OBSERVED. THIS WAS REPEATED USING 3 TO 6 DIFFERENT DOSES OF THE ANTAGONIST UNTIL A GRADED ANTAGONISM WAS SEEN. FROM THIS DATA THE ED_{50} WAS CALCULATED AT EACH TIME POINT. THE ED_{50} VALUE OF EACH ANTAGONIST AND ITS POTENCY RELATIVE TO ATROPINE AT BOTH 10 AND 20 MINUTES ARE SHOWN IN TABLE II. IN A PARALLEL SERIES OF EXPERIMENTS, EACH ANTAGONIST WAS ADMINISTERED 1 MINUTE AFTER THE OXOTREMORINE INJECTION. AGAIN THE NUMBER OF MICE WITH TREMORS AT 10 AND 20 MINUTES WAS OBSERVED AND AN ED_{50} FOR ANTAGONISM OF THE TREMORS WAS CALCULATED. THE RESULTS ARE GIVEN IN TABLE III.

TABLE I -- THE PERCENTAGE OF MICE EXHIBITING TREMOR AT DIFFERENT TIMES AFTER SUBCUTANEOUS INJECTION OF THE INDICATED DOSE OF OXOTREMORINE.

	TIME (MIN)					
DOSE (MG/KG)*	5	10	20	30	60	90
0.1	10	10	10	0	0	0
0.2	10	40	50	10	0	0
0.4	40	80	100	80	10	0
0.6	50	90	100	80	10	0
0.8	70	100	100	100	60	0

*DOSE IS MG/KG OF OXOTREMORINE BASE. TEN MICE WERE USED AT EACH DOSE LEVEL.

TABLE II--THE ED_{50} VALUE AND RELATIVE POTENCY FOR ANTAGONISM OF TREMORS INDUCED IN MICE WHEN THE ANTAGONIST COMPOUND IS ADMINISTERED 30 MINUTES BEFORE OXOTREMORINE.

COMPOUND	ED_{50} (MG/KG)		POTENCY (ATROPINE = 100)	
	AT 10 MIN	AT 20 MIN	AT 10 MIN	AT 20 MIN
5	0.007	0.012	5400	2920
10	0.023	0.061	1640	574
27	0.065	0.25	582	138
19	0.15	0.27	255	132
32	0.22	0.62	173	56
16	0.25	0.67	149	52
3	0.32	0.50	119	70
ATROPINE	0.38	0.35	100	100
21	0.43	0.60	88	58
14	0.95	0.84	40	42
1	1.16	4.91	33	7
17	1.31	2.63	29	13
28	1.54	1.99	25	18
31	1.59	2.22	24	16
22	1.72	1.81	22	19

20	1.94	2.69	20	13
2	2.12	3.34	18	10
11	2.43	6.10	16	6
15	2.56	3.39	15	10
30	2.89	5.44	13	6
26	3.17	7.71	12	5
9	3.40	3.25	11	11
29	3.42	3.36	11	10
24	3.52	5.62	11	6
12	4.96	8.18	8	4
4	6.32	8.17	6	4
8	6.44	10.6	6	3
25	7.52	11.7	5	3
6	10.8	25.6	4	1
13	>20.0	>20.0	<2	<2
7	>20.0	>20.0	<2	<2
18	>20.0	>20.0	<2	<2
23	>20.0	>20.0	<2	<2

TABLE III--THE ED_{50} VALUE AND RELATIVE POTENCY FOR ANTAGONISM OF TREMORS INDUCED IN MICE WHEN THE ANTAGONIST COMPOUND IS ADMINISTERED 1 MINUTE AFTER OXOTREMORINE.

COMPOUND	ED_{50} (MG/KG)		POTENCY (ATROPINE = 100)	
	AT 10 MIN	AT 20 MIN	AT 10 MIN	AT 20 MIN
16	0,23	0,31	869	191
10	0,30	0,21	643	286
5	0,31	0,16	641	381
19	1,08	0,35	181	167
17	1,27	0,59	154	100
15	1,54	1,15	127	51
4	1,79	2,01	109	29
ATROPINE	1,96	0,59	100	100
20	2,16	1,36	91	43
14	2,31	1,30	85	46
1	2,49	1,87	78	32
22	2,93	1,33	67	44
2	3,29	1,87	59	32
29	3,81	2,90	51	20
11	3,83	3,17	51	19
31	4,50	4,26	43	14

30	4.86	5.32	40	11
6	5.10	3.39	38	17
21	6.29	2.14	31	28
24	7.89	6.22	25	10
26	8.26	5.39	24	11
12	8.70	6.33	22	9
13	8.88	11.3	22	5
25	15.2	12.2	13	5
18	>20.0	>20.0	<10	<3
23	>20.0	>20.0	<10	<3

RESULTS AND DISCUSSION

TABLE I SHOWS THE PERCENTAGE OF MICE WITH TREMORS AT VARIOUS TIMES AFTER S. C. ADMINISTRATION OF OXOTREMORINE IN DOSES RANGING FROM 0.1 - 0.8 MG/KG. THE PEAK EFFECT OF THE OXOTREMORINE APPEARS TO OCCUR BETWEEN 10 AND 20 MINUTES. EFFECTS AT ALL DOSES HAVE CEASED IN 90 MINUTES. AN ED_{84} WAS CALCULATED TO BE 0.48 MG/KG AT 10 MINUTES. THIS DOSE WAS CHOSEN FOR USE IN THE EVALUATION OF THE ANTAGONISTS.

TABLE II GIVES THE ED_{50} VALUE AND POTENCY RELATIVE TO ATROPINE FOR 32 COMPOUNDS AS ANTAGONISTS OF OXOTREMORINE-INDUCED TREMORS RANKED IN ORDER OF DECREASING EFFECTIVENESS AT A 10 MINUTE TIME POINT. SIX COMPOUNDS ARE DISTINCTLY MORE POTENT THAN ATROPINE. INSPECTION OF TABLE I SHOWS THAT AT 20 MINUTES (50 MINUTES AFTER GIVING THE ANTAGONIST), THE EFFECTIVENESS OF MOST OF THE ANTAGONISTS IS DECLINGING (ED_{50} RISES). ATROPINE, ON THE OTHER HAND, IS EQUALLY EFFECTIVE AT BOTH TIMES; THEREFORE, THE RELATIVE POTENCY OF MOST OF THE COMPOUNDS IS EVEN LESS FAVORABLE COMPARED TO ATROPINE AT 20 MINUTES THAN AT 10 MINUTES. ONLY 2 COMPOUNDS (5 AND 10) ARE STILL DECIDEDLY MORE ACTIVE ANTAGONISTS THAN ATROPINE AT 20 MINUTES. HOWEVER, EVEN COMPOUNDS WITH LESS CNS ANTAGONIST POTENCY THAN ATROPINE MIGHT BE USEFUL PRETREATMENT AGENTS IF THEY CAN BE GIVEN IN HIGHER DOSES WITHOUT TOXIC EFFECTS AND IF THE ACTION IS SUSTAINED FOR SOME TIME.

TABLE III PRESENTS THE ED_{50} VALUES AND RELATIVE POTENCIES FOR THE ANTAGONISTS WHEN THEY ARE ADMINISTERED 1 MINUTE AFTER THE OXOTREMORINE. THE ONSET OF CENTRAL ANTIMUSCARINIC ACTIVITY AS SEEN

IN THIS TEST IS MORE COMPARABLE TO THAT REQUIRED TO PROVIDE PROTECTION FROM THE CENTRAL EFFECTS OF OP POISONING. IT IS OBVIOUS THAT THE RANK ORDER OF THE COMPOUNDS IS DIFFERENT WHEN GIVEN AFTER THE OXOTREMORINE THAN WHEN GIVEN BEFORE. IT CAN BE SEEN THAT 5 OF THE COMPOUNDS (5, 10, 16, 17 AND 19) ARE MORE POTENT THAN ATROPINE FOR BLOCKING TREMORS IN THE 10 - 20 MINUTE TIME PERIOD. FOR MOST OF THE COMPOUNDS THE BLOCKING ACTION IS STILL INCREASING IN THAT TIME PERIOD (ED_{50} DECREASES). HOWEVER, ATROPINE SHOWS THE SHARPEST DECLINE IN ED_{50} VALUE. THEREFORE, EVERY ANTAGONIST SHOWED A DECREASE IN POTENCY RELATIVE TO ATROPINE AT THE 20 MINUTE TIME POINT COMPARED TO THE 10 MINUTE TIME POINT.

ACKNOWLEDGMENT

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PROTECTION AGAINST SOMAN (GD) TOXICITY BY CLONIDINE IN MICE

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Introduction

RECENT STUDIES IN OUR LABORATORY HAVE DEMONSTRATED THAT CLONIDINE, A CENTRALLY-ACTIVE $ALPHA_2$-ADRENERGIC AGONIST AFFORDS PROTECTION AGAINST THE TOXIC MANIFESTATIONS OF A REVERSIBLE CHOLINESTERASE INHIBITOR, PHYSOSTIGMINE. THIS PROTECTION IS MEDIATED THROUGH STIMULATION OF CENTRAL $ALPHA_2$-ADRENERGIC RECEPTORS WHICH DECREASES BRAIN ACETYLCHOLINE SYNTHESIS. THIS STUDY WAS DESIGNED TO DETERMINE WHETHER CLONIDINE PRETREATMENT AFFORDS PROTECTION AGAINST THE IRREVERSIBLE CHOLINESTERASE INHIBITOR, SOMAN (GD).

Results and Discussion

1. THE LD CURVE FOR GD OCCURRED OVER A VERY NARROW DOSE RANGE (0.1-0.2 MG/KG, TABLE 1 AND FIG. 1). THE EFFECTS OF GD ON OTT, LRR, ST AND SAL EXHIBITED A DOSE-RESPONSE RELATIONSHIP IN THIS RANGE (TABLE 1). SEVERAL DOSES OF CLONIDINE WERE ADMINISTERED 20 MIN PRIOR TO AN LD_{90} DOSE OF GD (TABLE 2). THE MICE APPEARED TO TOLERATE THE CLONIDINE WELL ALTHOUGH, ONE OBVIOUS EFFECT WAS PILOERECTION OF THE FUR. AS INDICATED IN TABLE 2, THE TOXIC MANIFESTATIONS OF GD WERE REDUCED IN ANIMALS PRETREATED WITH CLONIDINE FOLLOWING ALL DOSES (0.1-1 MG/KG). THE MAXIMAL PROTECTIVE ACTION WAS ELICITED BETWEEN 0.6 AND 1 MG/KG. WITH THE HIGHEST DOSE, LETHALITY WAS REDUCED 14%, OTT WAS INCREASED 5.5 FOLD, LRR WAS INCREASED AT LEAST 3 FOLD, THE OCCURRENCE OF ST WAS REDUCED TO 29% AND SAL WAS REDUCED TO 14%. ADMINISTRATION OF 0.3 MG/KG OF CLONIDINE AT DIFFERENT TIMES PRIOR TO GD INDICATED THAT THE CLOSER THE TWO INJECTIONS, THE MORE EFFECTIVE WAS THE PROTECTION AFFORDED (TABLE 3). THE MOST EFFECTIVE DOSE (1 MG/KG) AND PRE-TREATMENT TIME (5 MIN) FOR CLONIDINE WERE COMBINED, AND THE RESULTS INDICATED IN FIGURE 1. UNDER THESE CONDITIONS, CLONI-DINE SHIFTED THE LD CURVE FOR GD BY 0.51 MG/KG.

2. ATROPINE IS WELL KNOWN FOR ITS PROTECTIVE ACTIONS IN CHOLINESTERASE POISONING. UNDER THESE EXPERIMENTAL CONDITIONS, ATROPINE ALSO WAS EFFECTIVE IN REDUCING THE TOXIC MANIFESTATIONS OF GD (TABLE 4). PRETREATMENT WITH 25 MG/KG OF ATROPINE 5 MIN PRIOR TO GD PROVIDED THE MAXIMUM PROTECTION, REDUCING THE LETHALITY OF AN LD_{90} DOSE OF GD TO 38%.

Methods

OUTBRED, ICR MICE (25-34G) WERE EMPLOYED AND ALL INJECTIONS WERE MADE BY THE SUBCUTANEOUS ROUTE. DRUGS WERE DISSOLVED IN SALINE AND INJECTED IN A VOLUME OF 0.005ML/G BODY WEIGHT. LETHAL DOSE-RESPONSE (LD) CURVES WERE CONSTRUCTED USING 4-5 DOSES OF GD (8-12 ANIMALS/POINT). LD_{50} VALUES WERE OBTAINED BY LINEAR REGRESSION ANALYSIS OF LOG DOSE <u>VS</u>. PROBIT OF % LETHALITY PLOTS ($R>0.9$ IN ALL CASES). THE ONSET TIME TO APPEARANCE OF WHOLE BODY TREMORS (OTT), OCCURRENCE OF STRAUB TAIL (ST), OCCURRENCE OF SALIVATION (SAL) AND ONSET TIME TO LOSS OF RIGHTING REFLEX (LRR) WERE MEASURED. ONSET TIMES ARE GIVEN ONLY FOR LD_{90} DOSES OF GD.

ATROPINE INCREASED OT ONLY 2.4 FOLD AND WAS EFFECTIVE IN REDUCING THE OCCURRENCE OF ST AND SAL. HOWEVER, THE PREDOMINANT DIFFERENCE WITH REGARD TO CLONIDINE WAS THE PROLONGED LRR. THE LETHAL EFFECT OF GD IN MICE PRETREATED WITH ATROPINE USUALLY OCCURRED ON THE DAY AFTER THE EXPERIMENT (BUT LESS THAN 24 HR). IN CONTRAST, WHEN RATS WERE PRETREATED WITH CLONIDINE, IF THEY SURVIVED FOR AT LEAST 30 MIN FOLLOWING GD, NO FURTHER INCIDENCE OF LETHALITY WAS OBSERVED.

3. THE COMBINATION OF 1 MG/KG OF CLONIDINE AND 25 MG/KG OF ATROPINE IN A PRETREATMENT REGIMEN, RESULTED IN A GREATER THAN ADDITIVE PROTECTIVE RESPONSE (FIG. 1, TABLE 5). THE COMBINED TREATMENT RESULTED IN A SHIFT IN THE LD CURVE FOR GD WHICH WAS 15% GREATED THAN EXPECTED FROM THE SUM OF THE INDIVIDUAL SHIFTS (TABLE 5). ALSO, UNDER CIRCUMSTANCES WHERE THE DEGREE OF LETHALITY WAS SIMILAR BETWEEN GROUPS, (33-57%) THE COMBINATION OF CLONIDINE AND ATROPINE GREATLY PROLONGED LRR (TABLE 6). EVEN THOUGH MUCH HIGHER DOSES OF GD WERE EMPLOYED IN THE PROTECTED ANIMALS, OT AND LRR WERE SIGNIFICANTLY GREATER IN PROTECTED ANIMALS.

4. THESE RESULTS INDICATE THAT CLONIDINE EXERTS A MARKED PROTECTIVE ACTION AGAINST SEVERAL SIGNS OF GD TOXICITY, AND GREATLY POTENTIATES THE KNOWN PROTECTIVE ACTION OF ATROPINE. THE NATURE OF THIS POTENTIATION IS UNDER INVESTIGATION, HOWEVER, IT MAY BE RELATED TO THE PROPERTIES OF CLONIDINE AND ATROPINE AS, RESPECTIVELY, PRESYNAPTIC AND POSTSYNAPTIC INHIBITORS OF CHOLINERGIC ACTIVITY.

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Table 1

Soman Toxicity in the Mouse

DOSE (mg/kg)	n	% LETHALITY	OT (min)	LRR (min)	% ST	% SAL
0.100	10	0	5.8 ± 0.71	-	40	0
0.125	7	0	3.5 ± 0.31	-	71	29
0.150	10	40	3.7 ± 0.47	100.0 ± 20.0	100	80
0.175	12	83	3.5 ± 0.36	8.8 ± 0.87	92	83
0.200	12	100	3.4 ± 0.31	9.5 ± 1.02	100	100

LD_{50} = 0.156 mg/kg, OT = Onset to tremor, LRR = Time to loss of righting reflex, % ST = Occurence of straub tail, % SAL = Occurence of salivation.

Table 2

Effect of Clonidine Pretreatment on Soman[a] Toxicity

DOSE[a] CLONIDINE (mg/kg)	n	% LETHALITY	OT (min)	LRR (min)	% ST	%SAL
0	16	88	3.2 ± 0.27	8.0 ± 0.51	81	75
0.1	7	57	6.5 ± 1.08**	16.5 ± 1.35**	71	57
0.3	8	50	9.94 ± 2.42*	11.0 ± 7.45	50	50
0.4	7	43	10.9 ± 0.65**	23.9 ± 6.94*	57	43
0.6	7	14	13.3 ± 1.72**	N	57	14
1.0	7	14	17.7 ± 2.06**	N	29	14

a = Clonidine was administered 20 min prior to 0.175 mg/kg of Soman.

* = $p < 0.05$; ** = $p < 0.01$ compared to non-pretreatment, control mean.

N = Not sufficient for statistical analysis.

Table 3

Effect of Pretreatment Time on Clonidine-Induced Protection Against Soman[a] Toxicity

CLONIDINE PRETREATMENT TIME (min)[a]	n	% LETHALITY	OT (min)	LRR (min)	% ST	%SAL
5	7	14	12.8 ± 0.64	N	29	29
20	8	50	9.95 ± 2.42	22.0 ± 12.5	50	50
40	8	50	6.66 ± 0.43	15.0 ± 2.09	83	83
80	7	71	6.47 ± 0.68	14.9 ± 4.18	100	100

[a] = Clonidine was administered at specified times prior to 0.165 mg/kg of Soman.

N = Not sufficient for statistical analysis.

Table 4

Effect of Atropine on Soman[a] Toxicity

DOSE[a] ATROPINE (mg/kg)	n	% LETHALITY	OT (min)	LRR (min)	% ST	% SAL
0	9	89	2.5 ± 0.25	6.4 ± 0.72	100	89
1	9	78	4.0 ± 0.31*	> 6 h	67	11
5	9	78	4.8 ± 0.30*	> 6 h	100	0
25	8	38	6.0 ± 0.37*	> 6 h	13	0

a = Atropine was administered 5 min prior to 0.165 mg/kg of Soman.

* = $p < 0.01$ compared to non-pretreatment, control mean.

Table 5

Summary: The Protective Effects of Clonidine and Atropine on Soman-Induced Lethality in Mice

	(mg/kg)		
	LD_{50}	Change in LD_{50}	Protective Ratio
Soman	0.129		
Clonidine + Soman	0.180	0.51	1.40
Atropine + Soman	0.168	0.39	1.22
Clonidine+Atropine+Soman	0.264	0.135	2.04

Table 6

Comparison of the Protective Effects of Clonidine and Atropine Alone, or in Combination, Against Soman Toxicity

SOMAN	DOSE (mg/kg) CLONIDINE	ATROPINE	n	% LETHALITY	OT (min)	LRR (min)	%ST	%SAL
0.130	0	0	7	57	2.8 ± 0.25	14.3 ± 1.29	71	71
0.180	1[a]	0	14	50	11.3 ± 0.93*	21.8 ± 4.5	100	100
0.165	0	25[b]	8	38	6.0 ± 0.37*	> 6 h	11	0
0.250	1[c]	25[c]	9	33	7.2 ± 0.51*	> 6 h	100	0

a = Clonidine was administered 5 min prior to Soman.

b = Atropine was administered 5 min prior to Soman.

c = Clonidine was administered 5 min prior to atropine; and atropine; 5 min prior to Soman.

* = $p < 0.01$ compared to non-pretreatment, control mean.

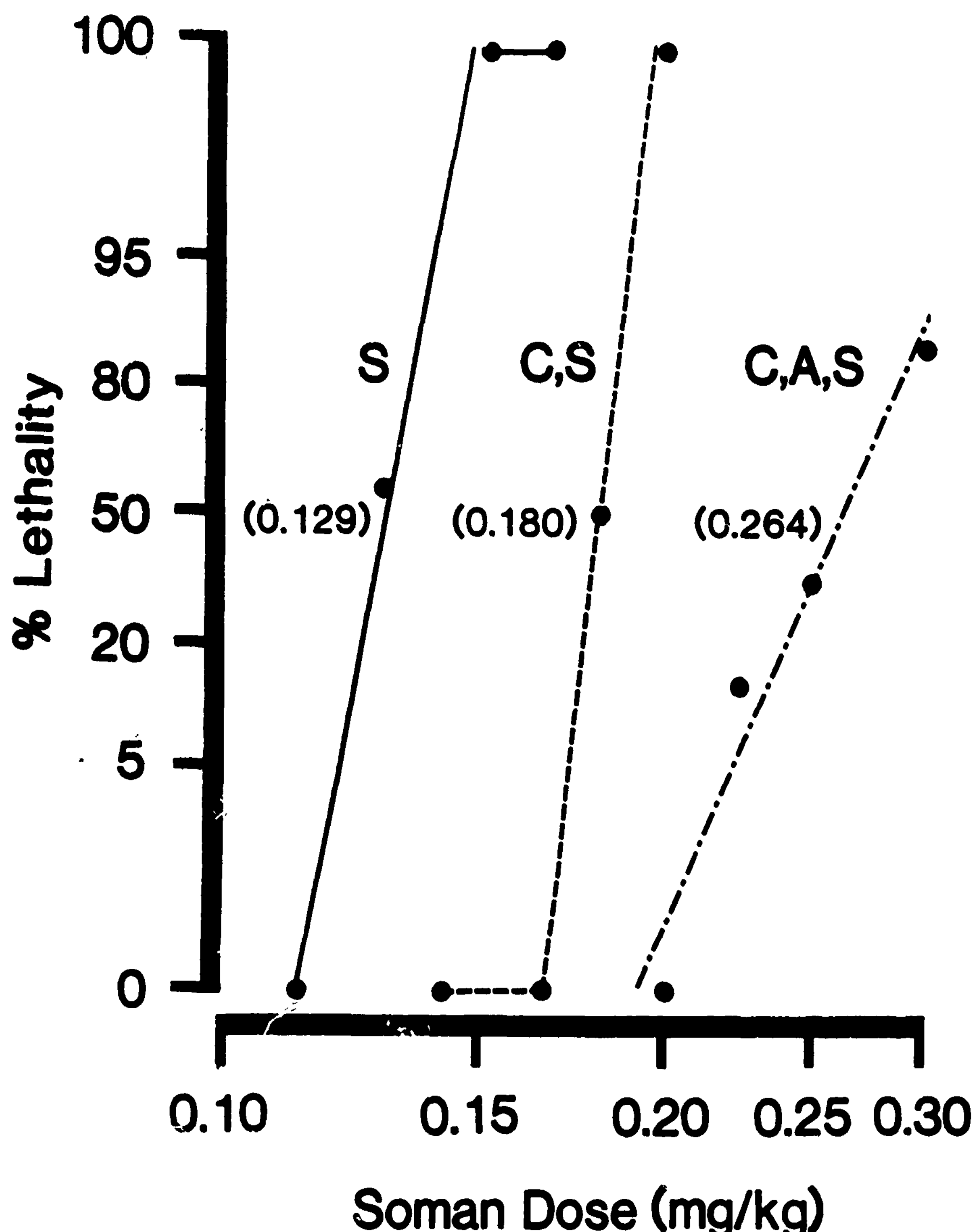

FIGURE 1. LOG-PROBIT PLOT OF SOMAN DOSE VS. % LETHALITY FOLLOWING S.C. INJECTION OF SOMAN. THE NUMBERS IN PARENTHESES ARE LD_{50} VALUES OBTAINED BY LINEAR REGRESSION ANALYSIS. S = SOMAN ALONE; C,S = CLONIDINE (1 MG/KG) FOLLOWED 5 MIN LATER BY SOMAN; C,A,S = CLONIDINE (1 MG/KG) FOLLOWED 5 MIN LATER BY ATROPINE (25 MG/KG) FOLLOWED 5 MIN LATER BY SOMAN.

CHEMOPROPHYLAXIS OF CYANIDE POISONING BY α-KETOGLUTARIC ACID

Arthur S. Hume, James C. Norris and Steven J. Moore

ABSTRACT

IN THIS STUDY α-KETOGLUTARIC ACID, AN α-KETOCARBOXYLIC ACID, WAS EVALUATED FOR ITS ABILITY TO COUNTERACT THE LETHAL EFFECTS OF CYANIDE. PRETREATMENT WITH α-KETOGLUTARIC ACID INCREASED THE LD50 VALUE OF CYANIDE (32 MG/KG) BY A FACTOR OF FIVE, A VALUE STATISTICALLY EQUIVALENT TO THAT ASCERTAINED IN MICE PRETREATED WITH SODIUM NITRITE AND SODIUM THIOSULFATE. THE COMBINATION OF α-KETOGLUTARIC ACID AND SODIUM THIOSULFATE INCREASED THE LD50 VALUE OF CYANIDE TO 105 MG/KG. ADDITION OF SODIUM NITRITE TO THE α-KETOGLUTARIC ACID/SODIUM THIOSULFATE REGIMEN INCREASED THE LD50 VALUE OF CYANIDE TO 122 MG/KG. NO INDUCTION OF METHEMOGLOBIN FORMATION WAS OBSERVED WITH α-KETOGLUTARIC ACID PRETREATMENT. IT APPEARS FROM THESE STUDIES THAT α-KETOGLUTARIC ACID IN CONJUNCTION WITH SODIUM THIOSULFATE PROVIDES A GREATER DEGREE OF PROTECTION THAN SODIUM NITRITE AND SODIUM THIOSULFATE WITHOUT THE DANGEROUS FORMATION OF METHEMOGLOBIN.

THE MECHANISM OF ACTION OF α-KETOGLUTARIC ACID COULD BE DUE TO A BINDING OF α-KETOGLUTARIC ACID WITH CYANIDE FOR IT HAS BEEN REPORTED THAT COMPOUNDS CONTAINING CARBONYL GROUPS CAN READILY REACT WITH CYANIDE. <u>IN VITRO</u> STUDIES WERE PERFORMED TO INVESTIGATE THIS POSSIBLE MECHANISM OF ACTION. BLOOD SAMPLES SPIKED WITH α-KETOGLUTARIC ACID AND/OR POTASSIUM CYANIDE WERE ANALYZED FOR THE RELEASE OF HYDROGEN CYANIDE FROM BLOOD. A GAS CHROMATOGRAPHIC TECHNIQUE WAS USED TO DETECT THE HYDROGEN CYANIDE IN THE HEADSPACE ABOVE THE SPIKED BLOOD SAMPLES. RESULTS FROM THIS STUDY SHOWED THAT A 0.5:1 MOLAR RATIO OF α-KETOGLUTARIC ACID:CYANIDE REDUCED HYDROGEN CYANIDE IN THE HEADSPACE BY 40% AS COMPARED TO

SAMPLES WITHOUT α-KETOGLUTARIC ACID. ALSO, THE ABILITY OF α-KETOGLUTARIC ACID TO PREVENT CYANIDE-INDUCED INHIBITION OF BRAIN CYTOCHROME OXIDASE ACTIVITY WAS INVESTIGATED. THESE DATA DEMONSTRATE THAT 0.04 M α-KETOGLUTARIC ACID COULD PREVENT THE COMPLETE INHIBITION OF CYTOCHROME OXIDASE ACTIVITY INDUCED BY 10^{-5} M POTASSIUM CYANIDE. THUS, THE PROTECTION OF α-KETOGLUTARIC ACID AFFORDED AGAINST CN-INDUCED LETHALITY COULD BE DUE TO BOTH THE RETAINING OF CYANIDE IN BLOOD AND THE PREVENTION OF CYANIDE-INDUCED INHIBITION OF BRAIN CYTOCHROME OXIDASE ACTIVITY.

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INTRODUCTION

CYANIDE IS A POTENT NUCLEOPHILE AND MAY REACT WITH CARBONYL GROUPS TO FORM CYANOHYDRINS (MORRISON AND BOYD, 1973). THUS, CYANIDE COULD REACT WITH THE CARBON IN THE KETONE GROUP OF α-KETOCARBOXYLIC ACID. GREEN AND WILLIAMSON (1937) ASCERTAINED THAT PYRUVIC ACID, AN α-KETOCARBOXYLIC ACID, REACTS WITH CYANIDE *IN VITRO* TO FORM CYANOHYDRIN. REDUCTION IN CYANIDE LETHALITY HAS BEEN OBSERVED AFTER THE ADMINISTRATION OF SODIUM PYRUVATE (CITTADINI *ET AL.*, 1972), WHICH SUGGESTS CYANOHYDRIN FORMATION CAN PROTECT AGAINST CYANIDE TOXICITY. FURTHERMORE, SCHWARTZ *ET AL.* (1979) HAVE REPORTED THAT SODIUM PYRUVATE ENHANCES THE EFFICACY OF SODIUM NITRITE AND/OR SODIUM THIOSULFATE IN ANTAGONIZING THE LETHAL EFFECTS OF CYANIDE. *IN VITRO* DATA INDICATE THAT α-KETOGLUTARIC ACID, ANOTHER α-KETOCARBOXYLIC ACID, BINDS CYANIDE (ALDOUS *ET AL.*, 1984). THUS IT WAS OF INTEREST TO DETERMINE IF α-KETOGLUTARIC ACID WOULD ALSO PROTECT AGAINST CYANIDE POISONING. SINCE CYANIDE TOXICITY OCCURS SO RAPIDLY, AND THE ABSORPTION AND DISTRIBUTION OF α-KETOGLUTARIC ACID IS NOT KNOWN, ALL ANTAGONISTS WERE ADMINISTERED PRIOR TO CYANIDE CHALLENGE.

Effects of Pretreatment Regimen on the LD_{50} Value and

Potency Ratio of Potassium Cyanide in Mice

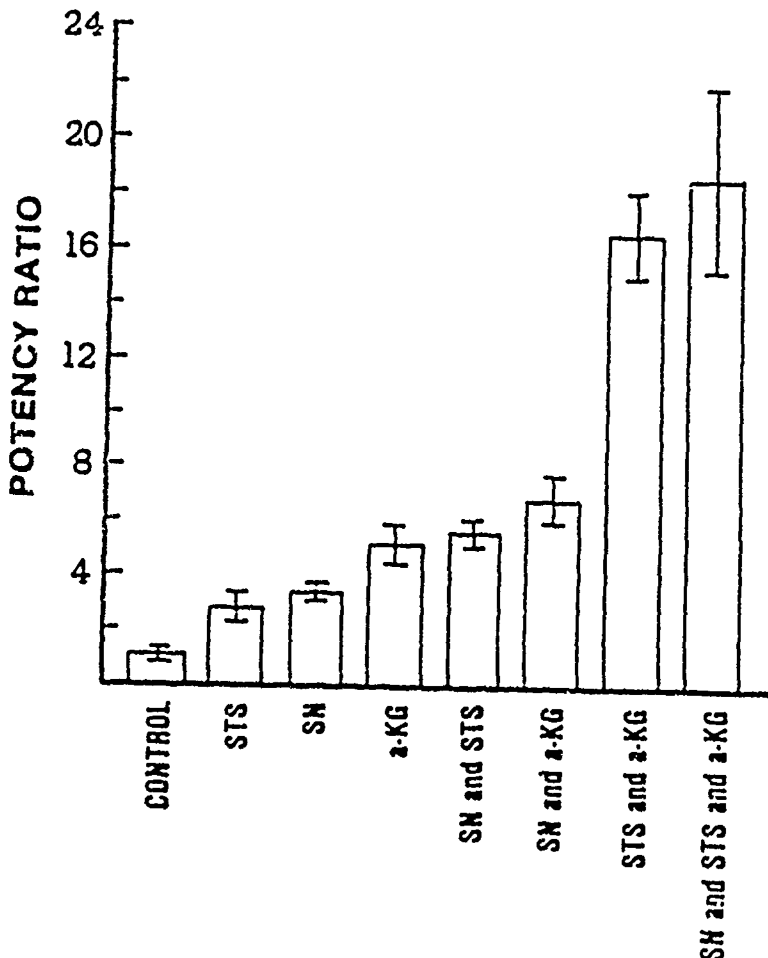
Pretreatment Regimen	LD_{50} Value[1] (mg/kg, i.p.) (95% Confidence Interval)	Potency Ratio[2] (95% Confidence Interval)
Controls	6.5 (6.3 - 6.6)	1.0 (0.8 - 1.25)
$Na_2S_2O_3$	17.0 (14.7 - 19.6)	2.64 (2.30 - 3.30)
$NaNO_2$	21.0 (20.2 - 21.8)	3.26 (2.93 - 3.62)
α-ketoglutaric acid	32.0 (28.1 - 36.5)	4.96 (4.31 - 5.70)
$NaNO_2$ + $Na_2S_2O_3$	34.7 (32.4 - 37.1)	5.38 (4.89 - 5.92)
$NaNO_2$ + α-ketoglutaric acid	42.8 (37.8 - 48.4)	6.64 (5.82 - 7.57)
$Na_2S_2O_3$ + α-ketoglutaric acid	105.0 (100.0 - 110.3)	16.28 (14.8 - 17.90)
$NaNO_2$ + $Na_2S_2O_3$ + α-ketoglutaric acid	122.0 (116.2 - 128.1)	18.97 (18.55 - 19.29)

[1] Lethality determined 12 hours after potassium cyanide administration

[2] Potency ratio = $\frac{LD_{50} \text{ Value of Potassium Cyanide with Antagonists}}{LD_{50} \text{ Value of Potassium Cyanide without Antagonists}}$

$P<0.05$ for confidence intervals which were exclusive

Protection against Lethal
Effects of Cyanide



Potency Ratio = $\frac{LD_{50} \text{ Value of KCN with Antagonists}}{LD_{50} \text{ Value of KCN without Antagonists}}$

STS = Sodium Thiosulfate
SN = Sodium Nitrite
a-KG = α-ketoglutaric acid

Comparison of the Effects of α-Ketoglutaric Acid

on Pretreatment Regimen

Group	Pretreatment Regimen	*Potency Ratio
A.	Controls	5.0 (4.5 - 5.5)
B.	α-ketoglutaric acid	
A.	$NaNO_2$	2.0 (1.9 - 2.2)
B.	$NaNO_2$ + α-ketoglutaric acid	
A.	$Na_2S_2O_3$	6.2 (5.4 - 7.1)
B.	$Na_2S_2O_3$ + α-ketoglutaric acid	
A.	$NaNO_2$ + $Na_2S_2O_3$	3.5 (3.2 - 3.9)
B.	$NaNO_2$ + $Na_2S_2O_3$ + α-ketoglutaric acid	

*Potency ratio = $\frac{LD_{50} \text{ Value of Potassium Cyanide in Group B}}{LD_{50} \text{ Value of Potassium Cyanide in Group A}}$

Effects of Pretreatment Regimen on the Production of Methemoglobin

Pretreatment Regimen	Grams % Methemoglobin
Controls	0.22 ± .16
α-ketoglutaric acid	0.28 ± .10[a]
$NaNO_2$	3.43 ± .32*
$NaNO_2$ + α-ketoglutaric acid	2.48 ± .22*[b]
$Na_2S_2O_3$	0.10 ± .10
$Na_2S_2O_3$ + α-ketoglutaric acid	0.19 ± .10[c]
$NaNO_2$ + $Na_2S_2O_3$	3.68 ± .40*
$NaNO_2$ + $Na_2S_2O_3$ + α-ketoglutaric acid	3.35 ± .69*[d]

*$P<0.05$ compared to values of control animals.

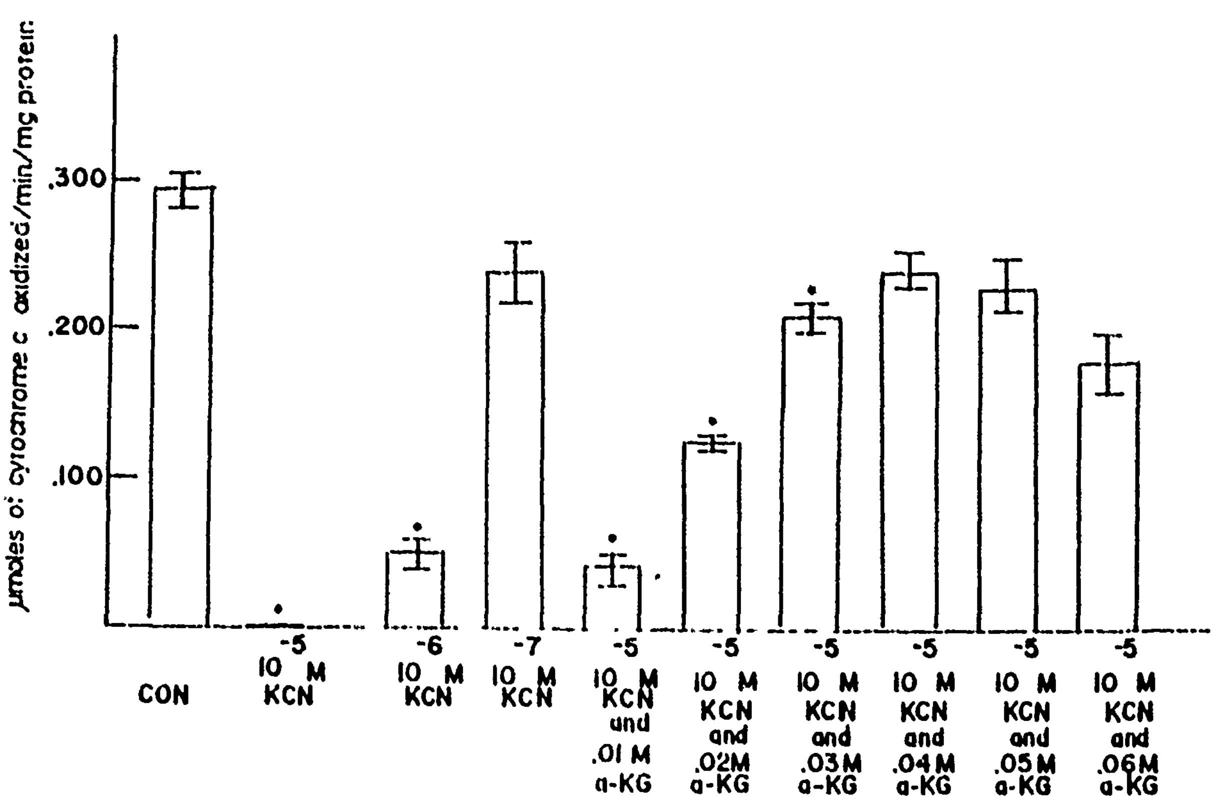
[a] $P>0.05$ compared to values of control animals.

[b] $P>0.05$ compared to values of $NaNO_2$ pretreated animals.

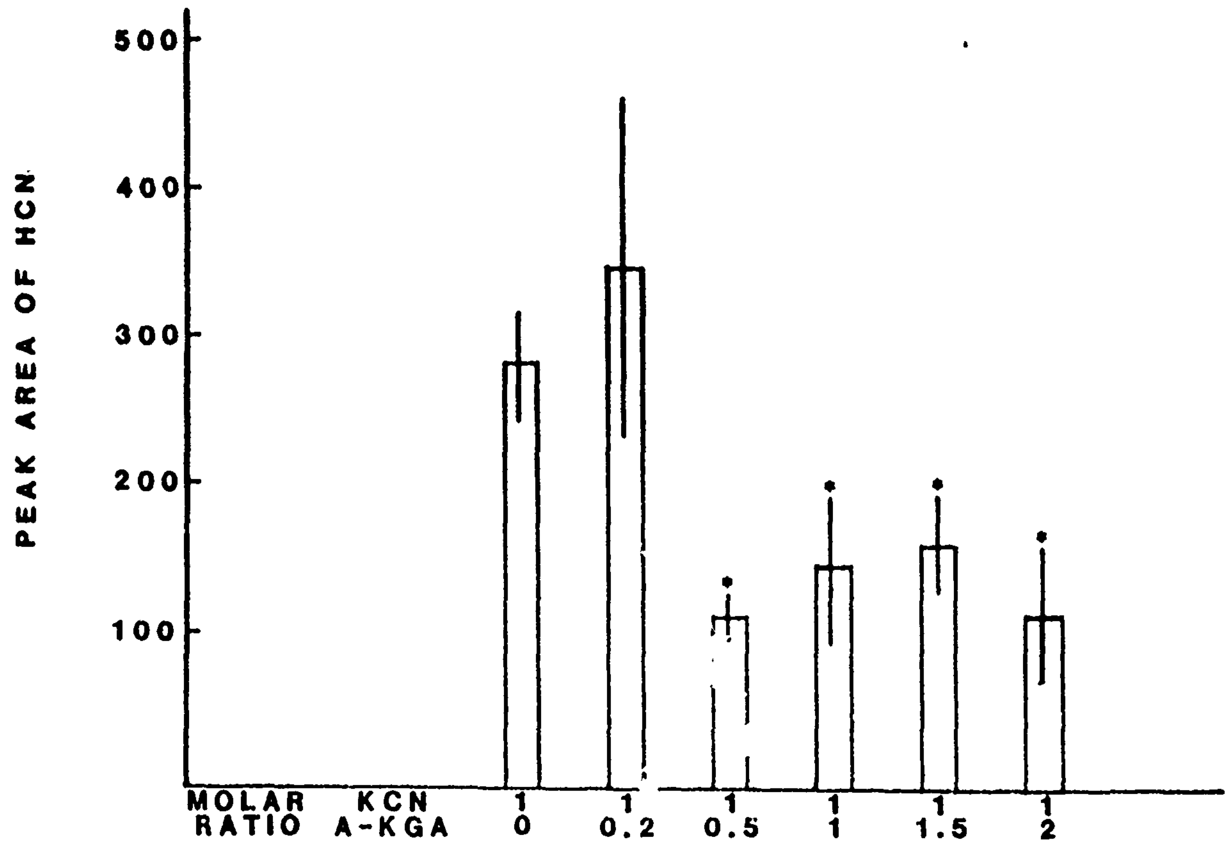
[c] $P>0.05$ compared to values of $Na_2S_2O_3$ pretreated animals.

[d] $P>0.05$ compared to values of $NaNO_2$ + $Na_2S_2O_3$ pretreated animals.

Inhibition of Cytochrome Oxidase by CN and
Protection by α-Ketoglutaric Acid



Retention of HCN in Blood by
α-Ketoglutaric Acid



CONCLUSIONS

1. ALPHA-KETOGLUTARIC ACID WAS AS EFFICACIOUS AS THE COMBINATION OF SODIUM NITRITE AND SODIUM THIOSULFATE IN PROTECTION AGAINST CYANIDE-INDUCED LETHALITY.

2. THE ADDITION OF SODIUM THIOSULFATE TO THE α-KETOGLUTARIC ACID PRETREATMENT REGIMEN RESULTED IN THE LARGEST INCREASE IN LD50 VALUE OF KCN AS COMPARED TO ANY OTHER SINGLE ADDITION.

3. THE _IN VITRO_ DATA SHOWED THAT α-KETOGLUTARIC ACID RETAINED CYANIDE IN BLOOD AND PROTECTED BRAIN CYTOCHROME OXIDASE ACTIVITY FROM CYANIDE-INDUCED INHIBITION.

4. THUS, IT CAN BE HYPOTHESIZED THAT α-KETOGLUTARIC ACID COULD PROTECT AGAINST CYANIDE-INDUCED LETHALITY BY PREVENTING CYANIDE FROM LEAVING THE VASCULAR SYSTEM INTO THE BRAIN AND/OR PROTECTING BRAIN CYTOCHROME OXIDASE ACTIVITY FROM CYANIDE-INDUCED INHIBITION.

ACETYLCHOLINE SYNTHESIS: A SEARCH FOR NEW INHIBITORS

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ABSTRACT

IN SITU ACH LEVELS ARE MAINTAINED WITHIN NARROW LIMITS. THE ENZYME CHOLINEACETYLTRANSFERASE (CHAT) DOES NOT APPEAR RATE-LIMITING AND SOME INVESTIGATORS (KUHAR AND MURRIN, 1978) HAVE SUGGESTED SODIUM-DEPENDENT HIGH AFFINITY CHOLINE UPTAKE FULFILLS THAT ROLE. TO ASSESS THE ROLE OF NA-DEPENDENT HIGH AFFINITY CHOLINE UPTAKE (HACHU), WE HAVE PREPARED ACETYLSECOHC-3 AND SEVERAL NOVEL QUINUCLIDINYL DERIVATIVES, MEASURING THEIR EFFECTS ON CHOLINE UPTAKE IN RAT BRAIN SYNAPTOSOMES. ACETYLSECOHC-3 AND SEVERAL QUINUCLIDINES REDUCED HACHU BY AS MUCH AS 80-90% AT LOW (10^{-7} TO 10^{-5}M) CONC. A SERIES OF 2-BENZYLIDENE-3-QUINUCLIDINONES WERE COMPARED WITH NVP AND BENZYLVINYLPYRIDINE AS INHIBITORS OF CHOLINEACETYLTRANSFERASE (CHAT). ALL OF THE QUINUCLIDINES DEMONSTRATED NONCOMPETITIVE INHIBITION VS CHOLINE AN A FIXED AC-COA CONC. THE MAJORITY OF K_I VALUES CALCULATED CAN BE CORRELATED WITH THE LIPOPHILLIC PARAMETER. STERIC FACTORS ALSO CONTRIBUTE ALTHOUGH THE REDUCTION OF THE KETO FUNCTION TO AN ALCOHOL MOST MARKEDLY (25 FOLD REDUCTION) AFFECTS INHIBITORY POTENCY, SUGGESTING A REQUIREMENT FOR A POLARIZED, REACTIVE OLEFIN AS SUGGESTED BY EARLIER INVESTIGATIONS OF CAVALLITO AND OF BAKER AND COLLABORATORS. THESE TERTIARY AMINES REPRESENT A STRUCTURAL DEPARTURE FROM PREVIOUSLY STUDIED CHAT INHIBITORS AND RESULTS IMPLY THAT ALTERNATIVE STRUCTURES POSSESSING REQUIRED ACTIVITY MAY PROVIDE NEW NOVEL CHAT INHIBITORS.

SOMAN POISONING IS BELIEVED TO RESULT IN THE INHIBITION OF ACETYLCHOLINESTERASE, AND THAT TOXICITY RESULTS FROM ACCUMULATION OF EXCESSIVE AMOUNTS, CENTRALLY AND PERIPHERALLY, OF ACETYLCHOLINE. IT WAS DESIRABLE TO DETERMINE WHETHER DRUGS WHICH INTERFERE WITH THE SYNTHESIS AND/OR RELEASE OF ACH WOULD IMPROVE SURVIVAL RATES FOLLOWING STANDARD TREATMENT WITH ATROPINE-2PAM. RESULTS WILL BE PRESENTED WHICH SUPPORT THIS CONCEPT AND SUGGEST A POSSIBLE MECHANISM BY WHICH THIS OCCURS.

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INTRODUCTION

THE CONCENTRATION OF ACETYLCHOLINE (ACH) IN BRAIN IS MAINTAINED WITHIN NARROW LIMITS EVEN THOUGH THE TURNOVER RATE IS SEVERAL FOLD THAT FOR OTHER NEUROTRANSMITTERS (CHENEY AND COSTA, 1978; JENDEN, 1979). THE PRECISE MECHANISMS REGULATING ACH METABOLISM ARE COMPLEX AND THERE IS CONSIDERABLE DISAGREEMENT AS TO WHICH FACTOR(S) IS RATE-LIMITING TO SYNTHESIS (SEE FISHER AND HANIN, 1980 FOR REVIEW). REGULATION OF ACH LEVELS CAN BE SEPARATED INTO SEVERAL CATEGORIES, NO ONE OF WHICH BY ITSELF IS CONTROLLING BUT COLLECTIVELY CAN REGULATE ACH SYNTHESIS: (1) CHOLINE ACETYLTRANSFERASE (CHAT) ACTIVITY; (2) AVAILABILITY OF ITS PRECURSORS, CHOLINE OR ACETYLCOA AND, (3) INDIRECTLY BY CHANGES IN ACH RELEASE (SEE FIG. 1: SCHEMATIC OF CHOLINERGIC TRANSMISSION).

THE ACUTE TOXICITY OF SOMAN, AN ORGANOPHOSPHOROUS CHOLINESTERASE INHIBITOR IS BELIEVED TO BE THE RESULT OF EXCESSIVE ACCUMULATION OF ACH AT CHOLINERGIC SYNAPSES, CENTRALLY AND PERIPHERALLY. IT WAS DESIRABLE TO DETERMINE WHETHER DRUGS WHICH INTERFERE WITH THE SYNTHESIS OF ACH WOULD IMPROVE SURVIVAL RATES FOLLOWING STANDARD TREATMENT WITH ATROPINE AND 2-PAM.

TO ASSESS THIS QUESTION, SPECIFIC INHIBITORS OF CHAT AND HIGH AFFINITY CHOLINE UPTAKE (HACHU) ARE NECESSARY. ACETYLSECO-HC AND SEVERAL NOVEL QUINUCLIDINYL DERIVATIVES WERE PREPARED AND EVALUATED FOR INHIBITION OF CHAT ACTIVITY, HACHU AND ACH SYNTHESIS, _IN VITRO_. THE MOST POTENT OF THESE COMPOUNDS WERE EVALUATED _IN VIVO_ FOR PROTECTION AGAINST SOMAN TOXICITY.

THE WORK WAS BASED UPON INITIAL REPORTS OF KUHAR AND MURRIN, (1978) AND DOWDALL, (1978).

THE CHEMISTRY PROGRAM HAS BEEN DIRECTED TOWARDS THE DESIGN AND SYNTHESIS OF COMPOUNDS AS INHIBITORS OF CHAT AND/OR HACHU, A PRIMARY GOAL BEING THE DEVELOPMENT OF COMPOUNDS CAPABLE OF ENTRY TO THE CNS AFTER SYSTEMIC ADMINISTRATION.

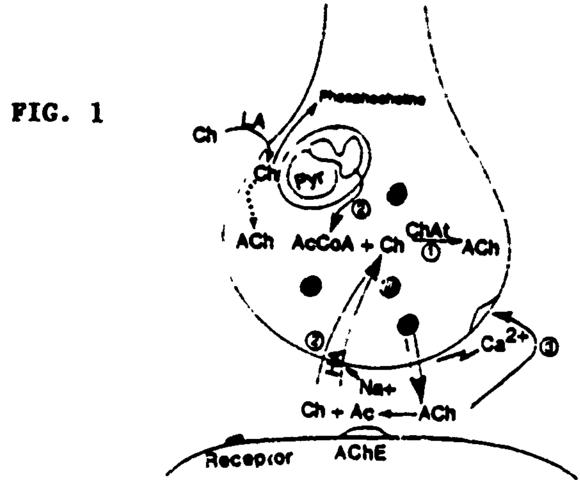
CAVALLITO AND CO-WORKERS (1970), BAKER AND GIBSON (1971, 1972), MALTHE-SORENSSEN ET AL (1974) AND ROWELL AND CHIOU (1976) HAVE ESTABLISHED PROTOTYPE STRUCTURES FOR CHAT INHIBITORS WITH HIGH _IN VITRO_ POTENCY. ALL OF THE COMPOUNDS SHARE A SIMILAR STRUCTURAL THEME, SPECIFICALLY, A POLARIZED DOUBLE BOND IN CONJUGATION WITH EITHER AN AROMATIC SYSTEM OR A CARBONYL; THE OLEFINIC SYSTEM IS SEPARATED FROM AN APPROPRIATELY SUBSTITUTED NITROGEN BY 3 OR 4 ATOMIC UNITS.

ALTHOUGH LIPOPHILIC AND STERIC CHARACTERISTICS PLAY A ROLE IN THE ASSOCIATION BETWEEN INHIBITOR AND ENZYME, THE REACTIVITY IMPLIED BY THE POLARIZATION OF THE DOUBLE BOND HAS BEEN SUGGESTED AS CONTRIBUTING TO THE INHIBITORY ACTIVITY (BAKER AND GIBSON, 1972). THIS HYPOTHESIS HAS BEEN RE-EXAMINED AND SUPPORTED WITH SOME MODIFICATION (CHWEH ET AL., 1984).

WE HAVE SYNTHESIZED A SERIES OF 2-BENZYLIDENE-3-QUINUCLIDINONES SHARING REACTIVITY CHARACTERISTICS SIMILAR TO THE AFOREMENTIONED INHIBITORS: A DOUBLE BOND IN CONJUGATION WITH AN AROMATIC RING, WHOSE POLARIZATION IS PROMOTED BY ITS POSITION RELATIVE TO THE CARBONYL (2A). THE QUINUCLIDINE NUCLEUS WAS CHOSEN BECAUSE IT IS A HIGHLY LIPOPHILIC TERTIARY AMINE ABLE TO PENETRATE THE BLOOD BRAIN BARRIER; IT IS PROTONATED AT PHYSIOLOGIC PH AND CAPABLE OF FORMING A CHARGED SPECIES SIMILAR TO THE REFERENCE QUATERNARY AMMONIUM COMPOUNDS; QUINUCLIDINYL COMPOUNDS ARE KNOWN TO HAVE EXCELLENT STRUCTURAL CHARACTERISTICS FOR INTERACTION WITH CHOLINERGIC ULTRASTRUCTURES (SCHULMAN ET AL., 1983); 3-QUINUCLIDINOL METHYL IODIDE, A RIGID ANALOG OF CHOLINE, HAS BEEN REPORTED TO BE AN INHIBITOR OF HACHU (DOWDALL, 1978). THIS LATTER POINT ALSO SERVES AS A BASIS FOR THE DEVELOPMENT OF INHIBITORS OF HACHU.

SYNTHETIC TRANSFORMATIONS INVOLVING THE 2-BENZYLIDENE-3-QUINUCLIDINONES ARE SHOWN IN 2B; ALTERATIONS IN THE STATE OF OXIDATION AND DEGREE OF UNSATURATION PERMIT ANALYSIS OF THE STRUCTURE-ACTIVITY REQUIREMENTS. IN ADDITION, A SERIES OF 2- AND 3-STYRYLQUINUCLIDINES HAS BEEN SYNTHESIZED (2C & 2D) IN ORDER TO DETERMINE THE CONTRIBUTIONS OF MOLECULAR ARCHITECTURE, AS OPPOSED TO REACTIVITY *PER SE*, ON THE INHIBITORY ACTIVITY OF THESE COMPOUNDS.

FIG. 1



METHODS

I. CHOLINE ACETYLTRANSFERASE ACTIVITY

McCaman and Hunt (1965), J. Neurochem. 12:253. Schrier and Shuster (1967), J. Neurochem. 14:977. White and Wu (1973), J. Neurochem 20:297.

REACTION:

$$\text{Choline} + {}^{14}\text{C-AcetylCoA} \xrightarrow{\text{ChAT}} {}^{14}\text{C-Acetylcholine}$$

ASSAY SYSTEM:

To determine A_{50}, Sodium phosphate buffer (pH 7.4), 50 mM; NaCl, 300 mM; EDTA, 0.1 mM; choline chloride, 1 mM; eserine sulfate 0.2 mM; BSA, 0.05% (w/v); $MgCl_2$, 20 mM; ^{14}AcetylCoA, 90 uM (S.A., 19 mCi/mmole), Triton X-100, 0.25%. To determine Ki, choline was varied from 0.25 - 2 mM.

SEPARATION:

Labeled ACh was isolated by anion exchange column chromatography (Bio Rad Ag 1 x 8) and counted by LSC.

ENZYME INHIBITION:

Enzyme activity (Km, Vmax) was determined in presence or absence of inhibitor by Eadie-Hofstee data analysis.

II. HIGH AFFINITY CHOLINE UPTAKE

A. PREPARATION OF SYNAPTOSOMES

Cotman (1974), In: Methods in Enzymology 31:445.

Synaptosomes from rat cerebral cortex prepared and purified by Ficoll gradient centrifugation (4%, 6% and 13%), 63,500 g, 60 min. Synaptosomal band (interface, 6% and 13%) pelleted at 50,000 g, 20 min.

B. CHOLINE UPTAKE

Simon and Kuhar (1975, Nature, Lond. 255:162. Simon et al (1976), J. Neurochem 26:909.

1. Synaptosomes suspended in Krebs-Ringer Bicarbonate Buffer (Total Uptake - 150 mM Na^+, Low Affinity - 30 mM Na^+)

2. Preincubated 5 min, 37°C; 2 uM ^{3}H-choline (1 uCi) added, samples incubated 4 min

3. Synaptosomes pelleted on Whatman glass-fiber filters and counted by LSC.

4. $$\frac{\text{pmol choline}}{\text{mg prot}} = \frac{(\text{DPM/mg prot})\ (\text{pmol choline added})}{\text{DPM choline added}}$$

HACHU = Total Uptake - LACHU (Low Na)

III. ACETYLCHOLINE SYNTHESIS IN VITRO

PROCEDURE: STERLING AND O'NEILL (1978), J. NEUROCHEM. 31:525.

ASSAY: FREEMAN ET AL (1975), J. NEUROCHEM. 24:729.

1. ACH SYNTHESIS WAS MEASURED IN RAT CEREBRAL CORTEX SLICES BY INCUBATION IN K-R BICARBONATE BUFFER CONTAINING D-GLUCOSE (10 MM), 5 UCI (U-^{14}C) GLUCOSE, CHOLINE CHLORIDE (0.3 UM), ESERINE SULFATE (0.4 MM), KCL (6 MM) W/INHIBITOR.

2. FOLLOWING AERATION, SAMPLES WERE INCUBATED 60 MIN, 37°C. INCUBATION WAS TERMINATED WITH ICE COLD PERCHLORIC ACID.

3. ACH WAS EXTRACTED BY ION-PAIR WITH DIPICRYLAMINE.

4. $$\text{NMOL ACH FROM } ^{14}\text{C-GLUCOSE} = \frac{(\text{DPM ACH})\ (\text{NMOL GLUCOSE})\ (3)}{(\text{DPM GLUCOSE})}$$

IV. PROTECTION STUDIES

TYPICAL PROTOCOL

GROUP I: SOMAN (125 UG/KG) AT TIME 0

GROUP II: SOMAN FOLLOWED BY ATROPINE SULFATE (16 MG/KG) AND 2-PAM (30 MG/KG) AT 1 MIN, I.M.

GROUPS III, ETC: SOMAN, A/P 1 MIN, INHIBITOR (I.M OR ICVT AT SPECIFIED TIME). IN MOST CASES, THE DOSE OF INHIBITOR CHOSEN WAS APPROXIMATELY THE KI VALUE FOR INHIBITION OF CHAT OR HACHU.

2 - SYNTHESIS

1a.

b.

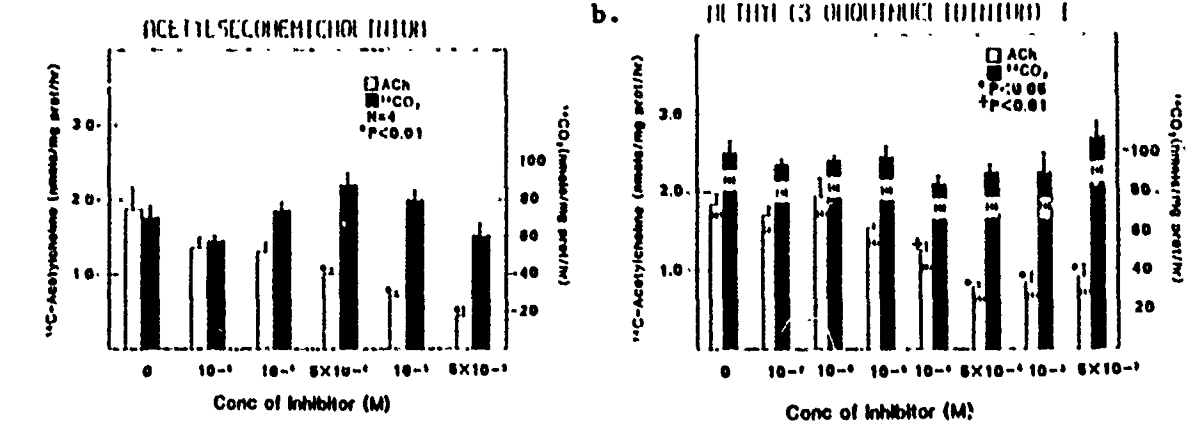
c.

Scheme A

d.

Scheme B

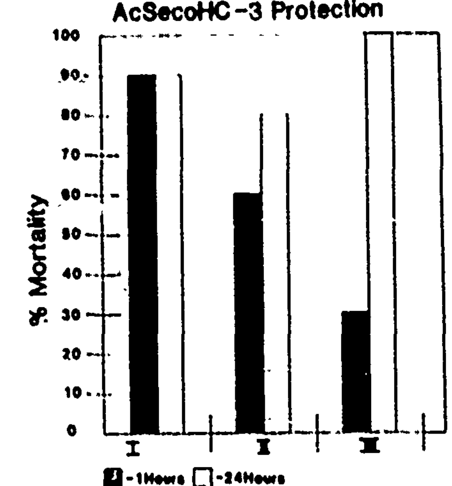
5 - ACh SYNTHESIS



To study the SAR of quinuclidinol derivatives directly on ACh synthesis, ACh and CO_2 production (as an estimate of respiration) from labeled glucose was measured. AcSecoHC-3, our reference compound produced a dose-related decline in ^{14}C-ACh synthesis to 23% of control at 5mM (Fig 5a). In contrast, there was no decrease in $^{14}CO_2$ production, N-methyl-3-quinuclidinol, our prototype quinuclidine derivative reduced ^{14}C-ACh production to a 40% of control at doses that did not reduce respiration. Our most potent quinuclidine derivative tested to date is N-methyl-3-quinuclidinone ($A_{50} = 2.4 \times 10^{-5}$M). To determine the necessity of the methyl functional group on the quinuclidinol ring, tissue was incubated with the tertiary amine, 3-quinuclidinol·HCl. This compound failed to reduce ^{14}C-ACh synthesis at concentrations up to 1 mM. With the exception of N-allyl-3-quinuclidinol which produced a modest decrease in ^{14}C-ACh to 80% of control at 0.1 mM other derivatives failed or minimally inhibited synthesis at concentrations up to 1 mM. It should be noted that several of these compounds inhibit HAChU and should not affect ACh synthesis from endogenous choline already present in the nerve terminal. Studies are currently being performed using ^{3}H-choline in addition to ^{14}C glucose to determine the relationship between inhibition of HAChU and inhibition of ACh synthesis.

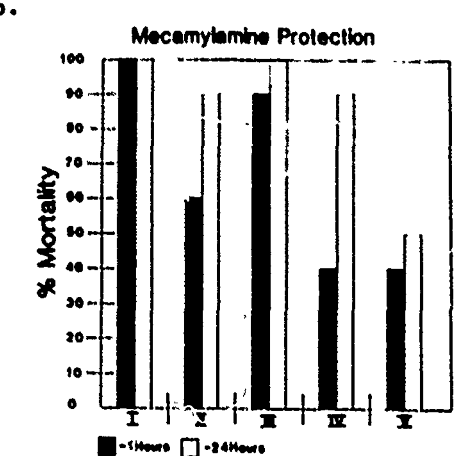
6 - PROTECTION VS SOMAN

a.



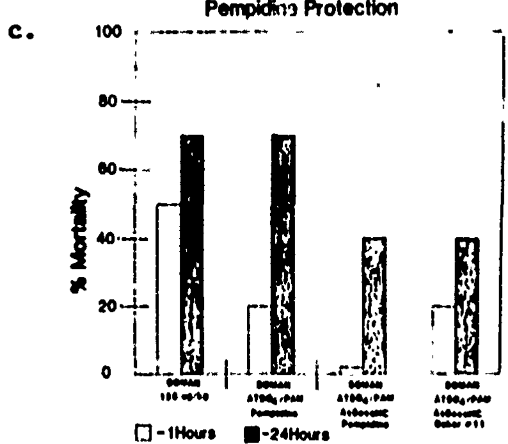
I SOMAN (128 UG/KG), 0 MIN
II SOMAN, ATROPINE (16 MG/KG)/PAM (30 MG/KG), + 1 MIN
III SOMAN, ATROPINE/PAM, ACETYLSECO-HC (ICVT), -30 MIN

b.



I SOMAN
II SOMAN, ATROPINE/PAM (-30 MIN)
II SOMAN, ATROPINE/PAM (+1 MIN)
IV SOMAN, ATROPINE/PAM (-30 MIN), MECAMYLAMINE (IM, -2 MIN)
V SOMAN, ATROPINE/PAM (+1 MIN), MECAMYLAMINE (IM, -2 MIN)

c.



SEVERAL OF OUR MORE POTENT CHAT- OR HACHU-INHIBITORS WERE EVALUATED IN VIVO FOR PROTECTIVE EFFECTS AGAINST SOMAN TOXICITY. THE DOSE OF INHIBITOR CHOSEN WAS APPROXIMATELY THE K_I VALUE FOR INHIBITION OF CHAT OR HACHU. ANIMALS WERE OBSERVED FOR UP TO 3 HRS FOLLOWING SOMAN ON THE DAY OF THE EXPERIMENT AND AT THE 24 HR MARK. THE LD VALUES AT 1 HR AND 24 HRS ARE PRESENTED.

ACETYLSECOHEMICHOLINIUM (100 UG/KG, -30 MIN ICVT), AN INHIBITOR OF HACHU AND CHAT, SIGNIFICANTLY REDUCED THE PERCENT MORTALITY WHEN GIVEN IN COMBINATION WITH A/P AT +1 MIN (FIG. 6A). THIS DATA VALIDATES PREVIOUS EXPERIMENTS IN OUR LAB SHOWING PROTECTION WITH ACETYLSECO-HC WHERE A/P WERE GIVEN AT -30 MIN. WHEN ATROPINE AND OXIME WERE COMPARED WHEN GIVEN 1 MIN AFTER OR 30 MIN PRIOR TO SOMAN (FIG. 6B), THE COMBINATION WAS MORE EFFECTIVE IN PROTECTING AGAINST SOMAN TOXICITY AT THE 1 HR TIMEPOINT WHEN GIVEN 30 MIN PRIOR TO SOMAN. NO SIGNIFICANT CHANGE WAS OBSERVED AT 24 HRS. THE MAIN PROTECTING AFFECT OF A/P IS THOUGHT TO BE BLOCKADE OF MUSCARINIC SITES. HEMICHOLINIUMS, IN ADDITION TO BLOCKING CHOLINE UPTAKE, HAVE GANGLIONIC BLOCKING ACTION, PRESUMABLY AT NICOTINIC RECEPTORS. TO SEE WHETHER PART OF THE CENTRAL ACTIONS OF ACSECOAC-3 MAY BE PARTIALLY THROUGH ANTAGONISM OF NICOTINIC RECEPTORS, A CENTRALLY ACTING GANGLIONIC BLOCKER, MECAMYLAMINE WAS TESTED. MECAMYLAMINE (IM, -2 MIN), REDUCED THE PERCENT MORTALITY TO 40% AT 1 HR REGARDLESS OF WHEN A/P WERE GIVEN. AT 24 HR, THE MECAMYLAMINE/A/P WAS MORE EFFECTIVE WHEN A/P WAS GIVEN AT 1 MIN POST SOMAN. STUDIES WITH ANOTHER NON QUATERNIZED NICOTINIC BLOCKER, PEMPIDINE DEMONSTRATED A SIMILAR PROTECTIVE EFFECT (6C).

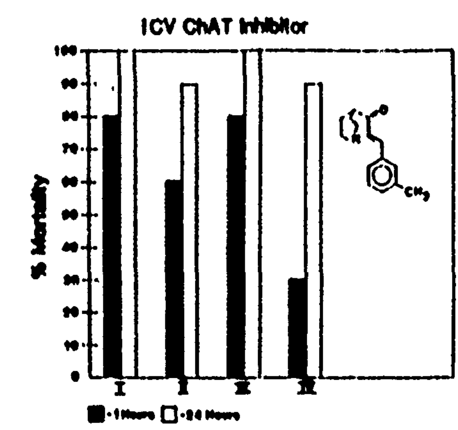
d.

ChAT-Inhibitor (ICV/IM)

% Mortality

■ -1Hours □ -24Hours

e.

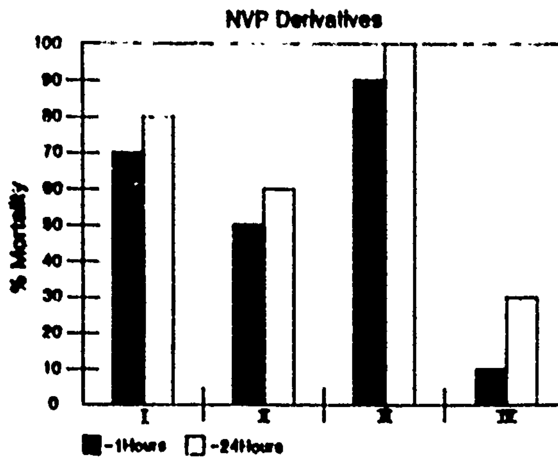


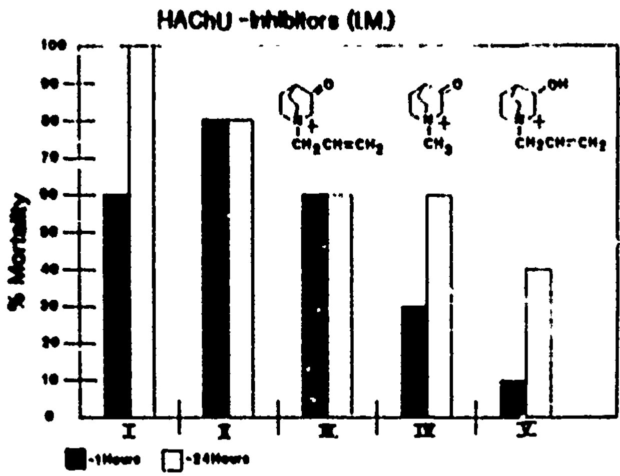
I SOMAN
II SOMAN, ATROPINE/PAM (+1 MIN)
III. SOMAN, ATROPINE/PAM, 2(M-METHYL)BENZYLIDENE-3-QUINUCLIDINONE (ICVT, -30 MIN, 1 UMOL/KG)
IV SOMAN, ATROPINE/PAM, 2(M-METHYL)BENZYLIDENE-3-QUINUCLIDINONE (IM,--30 MIN, 1 UMOL/KG)

I SOMAN
II SOMAN, ATROPINE/PAM (+1 MIN)
III SOMAN, ATROPINE/PAM, 2(M-METHYL)BENZYLIDENE-3-QUINUCLIDINONE (-30 MIN)
IV SOMAN, ATROPINE/PAM, 2(M-METHYL)BENZYLIDENE-3-QUINUCLIDINONE (-2 MIN)

We compared the effectiveness of 2-(m-methyl)benzylidene-3-quinuclidinone given ICVT vs I.M., and its effectiveness, ICVT given 30 min vs 2 min prior to Soman (Figs. 6d,e). When given at -30 min, the compound was more effective by the I.M. route of administration. However, the compound was effective ICVT when given at 2 min prior to Soman. Other benzylidene quinuclidinones were ineffective by the ICVT route. These studies may suggest an important peripheral vs central component to Soman toxicity, rapid brain metabolism of the compound or a redistribution phenomena.

Several benzylidene quinuclidinone derivatives, primarily inhibitors of ChAT activity, were compared for protective effects by the I.M. route. Only the 2-(m-methyl)benzylidene-3-quinuclidinone consistantly reduced the toxicity of Soman at both the 1 hr and 24 hr points. This represents an important difference in activity as a function of structure. It may be due to a number of factors including the appropriate concentration or perhaps the rate of metabolic transformation





I SOMAN (128 UG/KG), 0 MIN
II SOMAN, ATROPINE (16 MG/KG)/PAM (30 MG/KG) +1 MIN
III SOMAN, ATROPINE/PAM, NVP (1.7 UMOL/KG, I.M) -30 MIN
IV SOMAN, ATROPINE/PAM, MHENVP (1.7 UMOL/KG, I.M) -30 MIN

I SOMAN
II SOMAN, ATROPINE/PAM (+1 MIN)
III SOMAN, ATROPINE/PAM, N-ALLYL-3-QUINUCLIDINONE (IM, -30 MIN)
IV SOMAN, ATROPINE/PAM, N-METHYL-3-QUINUCLIDINONE(IM, -30 MIN)
V SOMAN, ATROPINE/PAM, N-ALLYL-3-QUINUCLIDINOL (IM, -30 MIN)

ANOTHER CHAT-INHIBITOR, N-HYDROXYETHYLNAPHTHYL-VINYL PYRIDINE (MHENVP, 1.7 UMOL/KG) SIGNIFICANTLY REDUCED THE TOXICITY OF SOMAN (FIG. 6F) WHEN GIVEN I.M. BUT HAD NO EFFECT BY ICVT ADMINISTRATION. THE NONQUATERNARY COMPOUND, NAPHTHYL-VINYL PYRIDINE FAILED TO REDUCE SOMAN TOXICITY WHEN GIVEN AT THE SAME DOSE.

FINALLY, WE EXAMINED SOME OF OUR MOST POTENT INHIBITORS OF HACHU (FIG. 6G). GIVEN I.M. AT 10^{-6} MOLES/KG, N-ALLYL-3-QUINUCLIDINOL DRAMATICALLY REDUCED SOMAN TOXICITY FROM LD_{60} TO AN LD_{10} AT 1 HR AND FROM LD_{100} TO LD_{40} AT 24 HRS POST SOMAN. THE N-ALLYL-3-QUINUCLIDINONE HAD NO EFFECT AT 1 HR WHEREAS THE N-METHYL-3-QUINUCLIDINONE REDUCED TOXICITY TO AN LD_{30} AT 1 HR. IT SHOULD BE NOTED THAT BEHAVIORALLY, THESE ANIMALS APPEARED NORMAL OR SOMEWHAT SEDATE; THEY DID NOT EXHIBIT THE TREMORS, PROFUSE SALIVATION AND GAGGING CHARACTERISTICALLY OBSERVED WITH THIS DOSE OF SOMAN. ALSO, THE COMPOUNDS HAVE QUATERNARY NITROGENS; THEREFORE, ONLY PERIPHERAL ACTIVITY IS EXPECTED WHEN GIVEN I.M. WHEN GIVEN ICVT, ONLY THE N-METHYL-3-QUINUCLIDINONE MODESTLY PROTECTED AGAINST SOMAN. THE TERTIARY AMINE, 3-QUINUCLIDINOL·HCl WHICH HAD NO EFFECT ON HACHU OR CHAT ACTIVITY ALSO FAILED TO PROTECT AGAINST SOMAN TOXICITY.

SUMMARY/CONCLUSIONS

A SYSTEMATIC APPROACH TO THE DESIGN, SYNTHESES AND EVALUATION OF COMPOUNDS WHICH INTERFERE WITH ACETYLCHOLINE SYNTHESIS HAS BEEN DESCRIBED. IT IS USUALLY ASSUMED THAT CHOLINEACETYLTRANSFERASE IS NOT AT THE RATELIMITING STEP. HOWEVER, BECAUSE, OF THE LIMITING AMOUNT OF BRAIN CHOLINE AVAILABLE, (TUCEK, J. NEUROCHEM. 1985) A POTENT INHIBITOR OF HIGH AFFINITY CHOLINE UPTAKE WOULD RESULT IN CHAT BECOMING THE RATE LIMITING STEP IN THE PROCESS.

IN THIS STUDY POTENT NEW INHIBITORS OF BOTH CHAT AND HACHU PROCESSES HAVE BEEN DESCRIBED. WHEN TESTED IN VIVO VERSUS LETHAL DOSES (LD_{50}+) OF SOMAN, THEY HAVE PROVIDED ADDED PROTECTION WHEN COMBINED WITH ATROPINE AND THE OXIME 2-PAM. THE NATURE OF THIS ADDED PROTECTION MAY BE OF CENTRAL OR PERIPHERAL ACTION. CERTAIN QUATERNARY COMPOUNDS GIVEN INTRAMUSCULARLY WERE MORE PROTECTIVE THAN WHEN GIVEN INTRACEREBROVENTRICULARLY WHEREAS OTHERS (ACETYL-SECO-HC3) WAS ACTIVE CENTRALLY SUGGESTING A POSSIBLE CENTRAL NICOTINIC INVOLVEMENT. TWO CENTRALLY ACTING NON-QUATERNARY GANGLIONIC BLOCKING DRUGS, MECAMYLAMINE AND PEMPIDINE, THOUGH ONLY VERY WEAKLY ANTI-NICOTINIC IN ACTION, PROVIDED A DEGREE OF ADDED PROTECTION WHEN GIVEN INTRAMUSCULARLY.

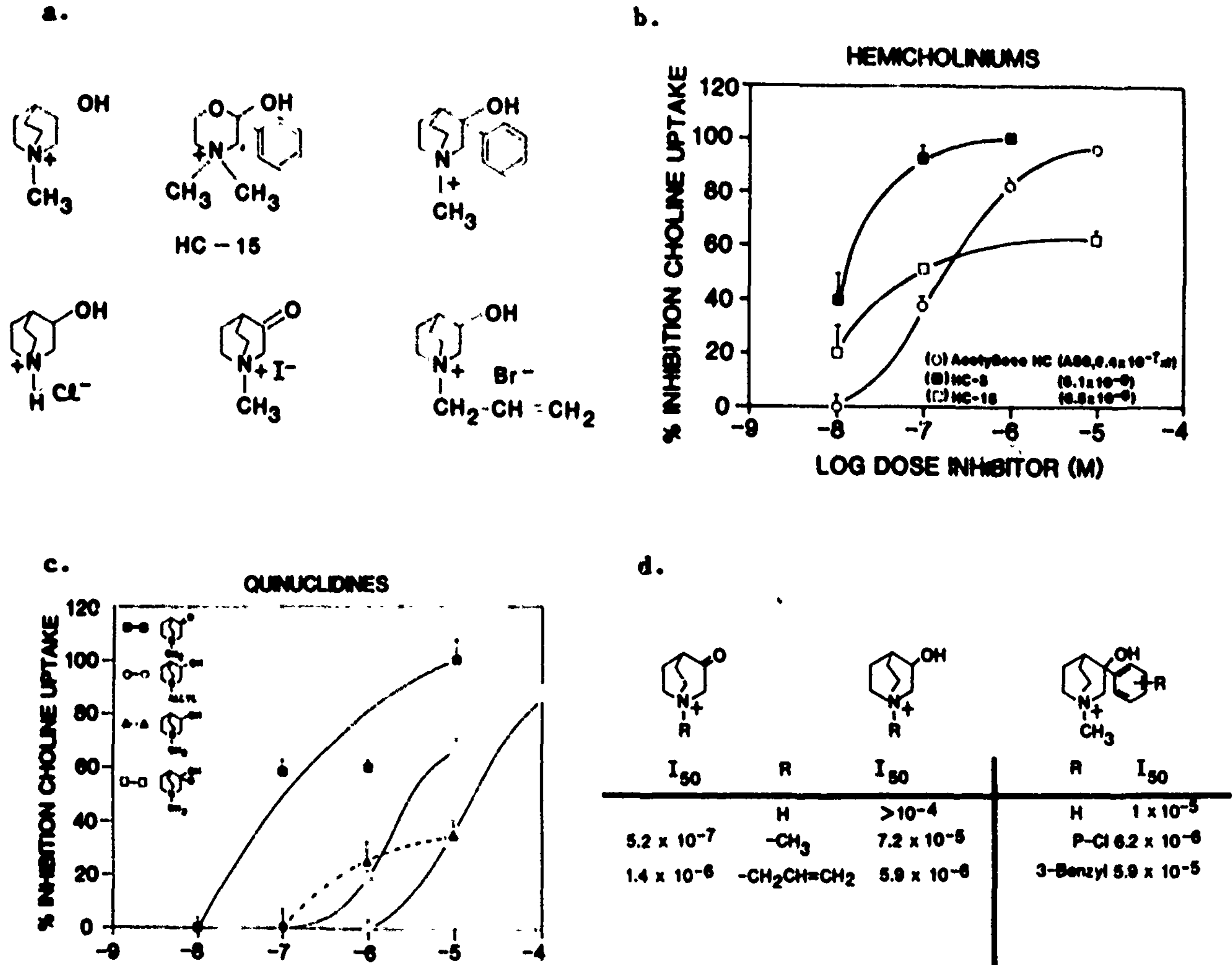
CLEARLY, COMPOUNDS SHOWING INTERFERENCE WITH ACETYLCHOLINE SYNTHESIS AND POSSIBLY WITH RELEASE REPRESENT A MOST PROMISING APPROACH IN DEVELOPING PROPHYLACTIC AND/OR THERAPEUTIC ADJUVANTS TO THE STANDARD ATROPINE-OXIME TREATMENT OF ORGANOPHOSPHONATE CASUALTIES. SUCH COMPOUNDS EVEN AFTER A SINGLE DOSE CAN PROVIDE THE ADDITIONAL TIME NEEDED FOR EVACUATION AND TREATMENT.

FURTHER WORK IS NEEDED TO:

A. ESTABLISH THE MECHANISM(S) OF THIS ADDED PROTECTION.

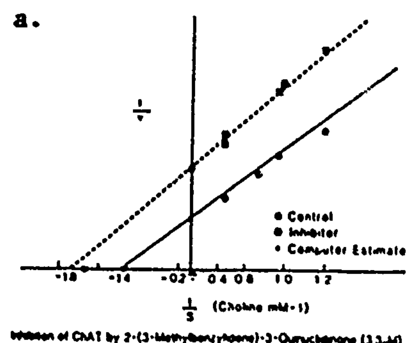
B. ESTABLISH THE PHARMACOKINETICS AND DOSAGE SCHEDULING.

C. TO DESIGN AND SYNTHESIZE ADDITIONAL COMPOUNDS WHICH CAN EFFECTIVELY EXPLOIT THE PROPERTIES INHERENT IN THE COMPOUNDS STUDIED THUS FAR AND PROVEN EFFECTIVE.



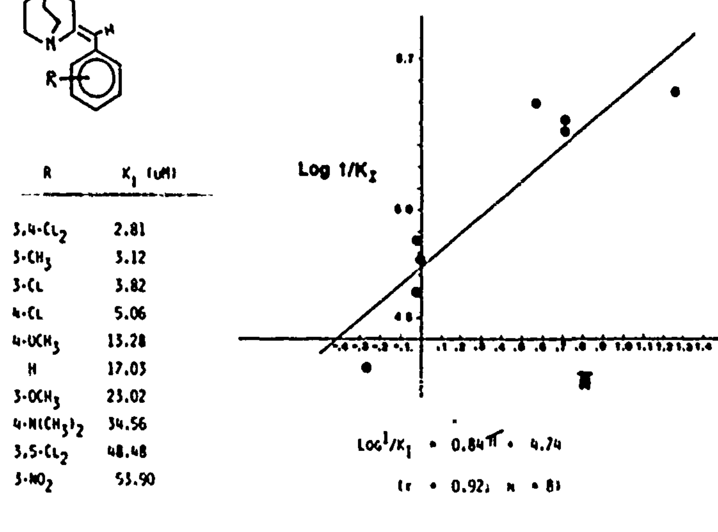
Choline uptake into cholinergic neurons for ACh synthesis is by a specific, saturable, sodium and temperature dependent transport mechanism (K_M = 2.1 uM; V_{MAX} = 47 pmol/4 min/mg protein). Several hemicholinium (HC) and quinuclidine derivatives were evaluated in a dose response manner. The 3-quinuclidinol methyliodide is known to inhibit HAChu (Dowdall, 1978; Kuhar and Murrin, 1978) and can be seen to share a structural similarity with HC-15, a potent inhibitor of HAChu (Fig 4a). To explore this similarity, the quinuclidinyl isostere of HC-15 was prepared. To explore differences in oxidation and N-substitution, keto and allyl derivatives were prepared. The A_{50} value represents that concentration of inhibitor which reduces HAChu by 50%. All HC compounds greatly reduced HAChu, HC-3 being the most potent (Fig 4b). N-methyl-3-quinuclidinone proved to be the most potent of the quinuclidine derivatives (Fig 4c,d). The A_{50} for this compound is a 100 fold greater than for the corresponding racemic alcohol. Increasing the size of the N-functional group from methyl to allyl did not significantly affect activity. However, removal of the quaternizing N-methyl group yielding the tertiary amine, greatly reduced its ability to inhibit HAChu. Of the 2-benzylidene-3-quinuclidinones, only the 3-chloro derivative significantly reduced HAChu. This serves as a lead in the development of tertiary-amine inhibitors.

a.



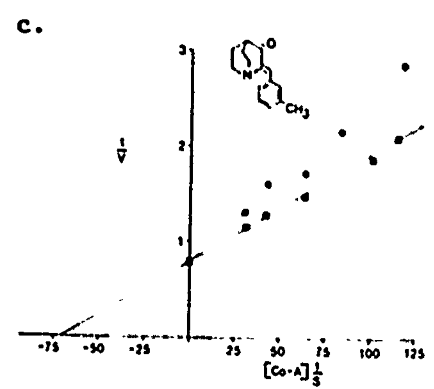
Inhibition of ChAT by 2-(3-Methylbenzylidene)-3-Quinuclidinone (3.3 μM)

b. INHIBITION OF CHOLINEACETYLTRANSFERASE BY 2-BENZYLIDENE-3-QUINUCLIDINONES



R	K_I (μM)
3,4-Cl_2	2.81
3-CH_3	3.12
3-Cl	3.82
4-Cl	5.06
4-OCH_3	13.28
H	17.03
3-OCH_3	23.02
4-$N(CH_3)_2$	34.56
3,5-Cl_2	48.48
3-NO_2	53.90

c.



ALL OF THE 2-BENZYLIDENE-3-QUINUCLIDINONES DEMONSTRATED NON-COMPETITIVE, OR MIXED, INHIBITION OF CHAT AGAINST VARIED CHOLINE AND FIXED ACETYL CO-A CONCENTRATIONS (SEE 3A). THESE COMPOUNDS ARE 10-100 TIMES MORE ACTIVE THAN THE ACRYLATE ESTERS, AND ARE WITHIN THE RANGE DEMONSTRATED BY THE STILBAZOLES. THE MAJORITY OF THE K_I VALUES CAN BE CORRELATED TO THE LIPOPHILIC PARAMETER π (3B). ALTHOUGH LIPOPHILICITY ACCOUNTS FOR A MAJOR PORTION OF THE ACTIVITY, STERIC FACTORS ALSO PLAY A ROLE AS EVIDENCED BY THE 17-FOLD LOSS IN ACTIVITY WHEN COMPARING THE 3,5-DICHLORO WITH THE 3,4-DICHLORO SUBSTITUTION. REDUCTION OF THE KETONE FUNCTION TO AN ALCOHOL YIELDS COMPOUNDS WITH A DIMINISHED POLARIZATION OF THE DOUBLE BOND AND LACKING THE "MICHAEL" REACTIVITY OF THE PARENT KETONES. PRELIMINARY EVALUATION OF THE ALCOHOLS CORRESPONDING TO THE UNSUBSTITUTED AND META-CHLORO KETONES DEMONSTRATED A 25 FOLD LOSS IN INHIBITORY ACTIVITY, SUPPORTING THE AFOREMENTIONED REQUIREMENT OF A POLARIZED OLEFIN.

IN ORDER TO BETTER ELUCIDATE THE MODE OF INTERACTION BETWEEN OUR INHIBITORS AND CHAT, WE HAVE ALSO BEGUN TO FOLLOW THE REVERSE REACTION CATALYZED BY CHAT USING THE SPECTROPHOTOMETRIC METHOD OF HERSH AND PEET ( ); THUS THE 2-BENZYLIDENE-3-QUINUCLIDINONE COMPETITIVELY INHIBITS UTILIZATION OF CA-A AT CONSTANT ACH CONCENTRATION (3C). ADDITIONAL EXPERIMENTS ARE PLANNED. THE 2 BENZYLIDENE 3-QUINUCLIDINONES REPRESENT A STRUCTURAL DEPARTURE FROM THE PROTOTYPE INHIBITORS AND SUGGEST THAT ALTERNATIVE STRUCTURES POSSESSING THE REQUIRED REACTIVITY CHARACTERISTICS MAY PROVIDE NEW AND NOVEL INHIBITORS OF CHAT.

STABILITY STUDIES OF HI-6 DICHLORIDE IN SIMPLE AND MIXED FORMULATIONS

Nesbitt Brown, R. Richard Gray, William Bone, Bhupendra Doctor and Ilse Hagedorn
Walter Reed Army Institute of Research and University of Freiburg

INTRODUCTION

COMPOUNDS SUCH AS N-METHYL PYRIDINIUM-2-ALDOXIME CHLORIDE (2-PAM • CL), OBIDOXIME CHLORIDE AND N, N'-TRIMETHYLEN-BIS(PYRIDINIUM-4-ALDOXIME) DIBROMIDE (TMB-4) HAVE BEEN FORMULATED INTO ANTIDOTAL PREPARATIONS FOR USE IN REACTIVATING INHIBITED ACETYLCHOLINESTERASE, POISONED BY ORGANOPHOSPHOUS NERVE AGENTS. HOWEVER, WHILE THESE COMPOUNDS ARE EFFECTIVE AGAINST ORGANOPHOSPHATES, SUCH AS SARIN (GB) AND TABUN (VX), THESE OXIMES HAVE BEEN PROVEN TO BE INEFFECTIVE AGAINST THE MORE POTENT AGENTS, SUCH AS SOMAN (GD).

HAGEDORN ET AL. SYNTHESIZED A SERIES OF BIS-PYRIDINIUM OXIMES WHICH ARE EXTREMELY EFFECTIVE IN ALLEVIATING SOME OF THE SHORTCOMINGS OF THE EARLIER OXIMES.

HI-6 DICHLORIDE [4-CARBAMOYL-2'-HYDROXYIMINOMETHYL-1,1'OXYDIMETHYLEN-DI(PYRIDINUM CHLORIDE)] IS ONE OF THE COMPOUNDS IN THIS CLASS OF REACTIVATORS, WHICH HAS BEEN USED SUCCESSFULLY IN REVERSING THE TOXIC EFFECTS OF GD POISONING IN EXPERIMENTAL ANIMALS.

AT THE SAME TIME, HI-6 HAS BEEN SHOWN TO UNDERGO CHEMICAL BREAK-DOWN DURING ITS EXPOSURE TO ELEVATED TEMPERATURES AND PH'S. IN THIS, STUDY, WE DEFINE THE CHEMICAL AND PHYSICAL PARAMETERS REQUIRED FOR MAINTAINING A STABLE FORMULATION WITH AN EXTENDED SHELF-LIFE.

OBJECTIVES

THE APPLICATION OF THE METHODOLOGIES EMPLOYED IN THIS STUDY, ALONG WITH THE DATA GATHERED ARE BEING UTILIZED IN OUR LABORATORY TO:

I. DETERMINE THE STABILITY AND SHELF-LIFE OF VARIOUS HI-6 FORMULATIONS DURING PROLONGED STORAGE.

II. DETERMINE THE DEGRADATION PRODUCTS FORMED IN THE FORMULATIONS AT ELEVATED TEMPERATURES.

III. DETERMINE THE REACTION KINETICS OF HI-6 AT VARIOUS TEMPERATURES AND CONCENTRATIONS.

IV. IDENTIFY BREAK-DOWN PRODUCTS OCCURRING DURING STORAGE FOR IND DATA REPORTS.

MATERIAL AND METHODS

I. WATERS' ALC/GPC-204 LC, INCLUDING;

- (A) 2-MODEL 6000 A PUMPS
- (B) MODEL 660 SOLVENT PROGRAMMER
- (C) U6K LOOP INJECTOR
- (D) 440 UV DETECTOR, SET AT 254nm

II. HOUSTON INSTRUMENT A 5000 RECORDER

III. COLUMBIA SCIENTIFIC - 3A INTEGRATOR

IV. KRATOS MS-50 MASS SPECTROMETER

PROCEDURE - (SEPARATION PARAMETERS)

I. PREPACKED MICRO - BONDAPAK C.18 COLUMN

II. MOBILE PHASE: (PIC B7) 0.01M 1-HEPTANE SULFONIC ACID/ACETONITRILE

III. FLOW RATE: 1.5 ML/MINUTE (ISOCRATIC MODE)

IV. AMBIENT TEMPERATURES

V. LOWER DETECTION LIMITS: 1ng (OXIMES)

VI. LINEAR RELATIONSHIP FOR ALL CONCENTRATIONS STUDIED

ACTIVE AND DEGRADATION COMPOUNDS OF HI-6 FORMULATIONS

I. ACTIVE COMPOUNDS

(a) HI-6 DICHLORIDE, [4-CARBAMOYL-2'-HYDROXYIMINOMETHYL-1,1' OXYDIMETHYLEN-DI(PYRIDINIUM CHLORIDE)]

(b) APROPHEN HYDROCHLORIDE

(c) ATROPINE SULFATE

II. PRESERVATIVES AND FUNGISTATIC AGENTS

(a) METHYL PARABEN

(b) PROPYL PARABEN

III. BY-PRODUCTS

(a) ISONICOTINIC ACID

(b) ISONICOTINAMIDE

(c) 4-CYANO PYRIDINE

(d) 4-PYRIDINE CARBOXALDEHYDE

(e) 4-HYDROXY-PYRIDINE

(F) 2-PYRIDINE ALDOXIME

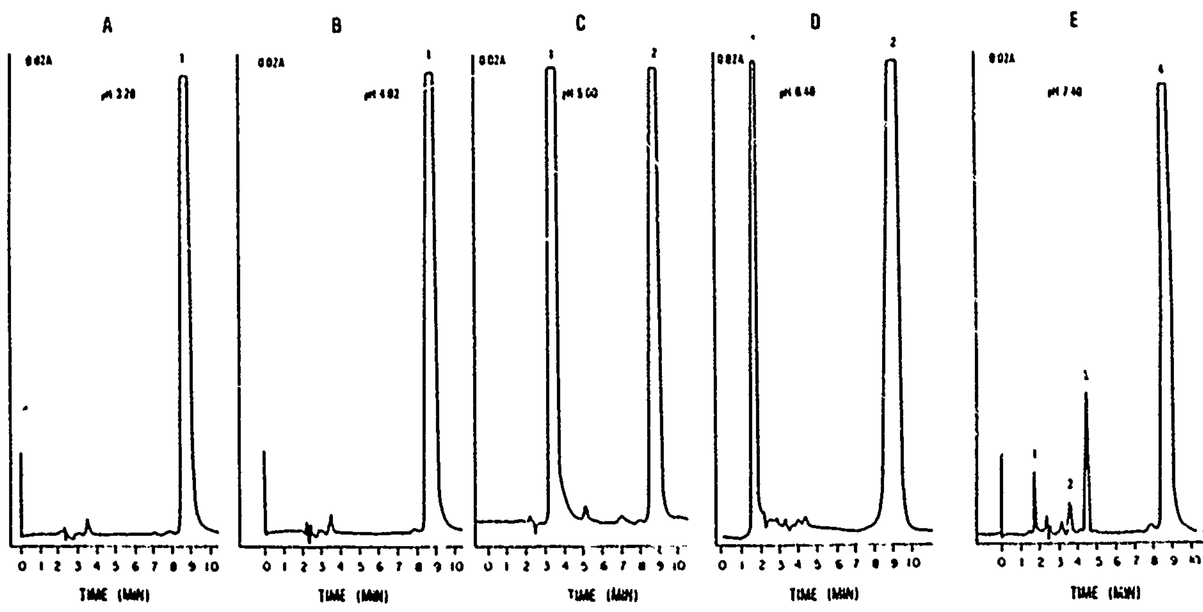


FIGURE 1. EFFECTS OF PH ON AN ACETATE BUFFERED SOLUTIONS OF HI-6. THE 12.5 mg/ml PREPARATIONS WERE INCUBATED AT 37°C FOR 60 MINUTES. PH 4.0 CONDITIONS PRODUCED THE LEAST AMOUNT OF HI-6 DEGRADATION WHEN EMPLOYED OVER EXTENDED PERIODS OF TIME. AMBIENT ROOM TEMPERATURES (18° - 20°C), WHEN USED AS AN OPTION TO REFRIGERATED TEMPERATURES (4°C) MAINTAINED STABILITY FOR MORE THAN 2 YEARS.

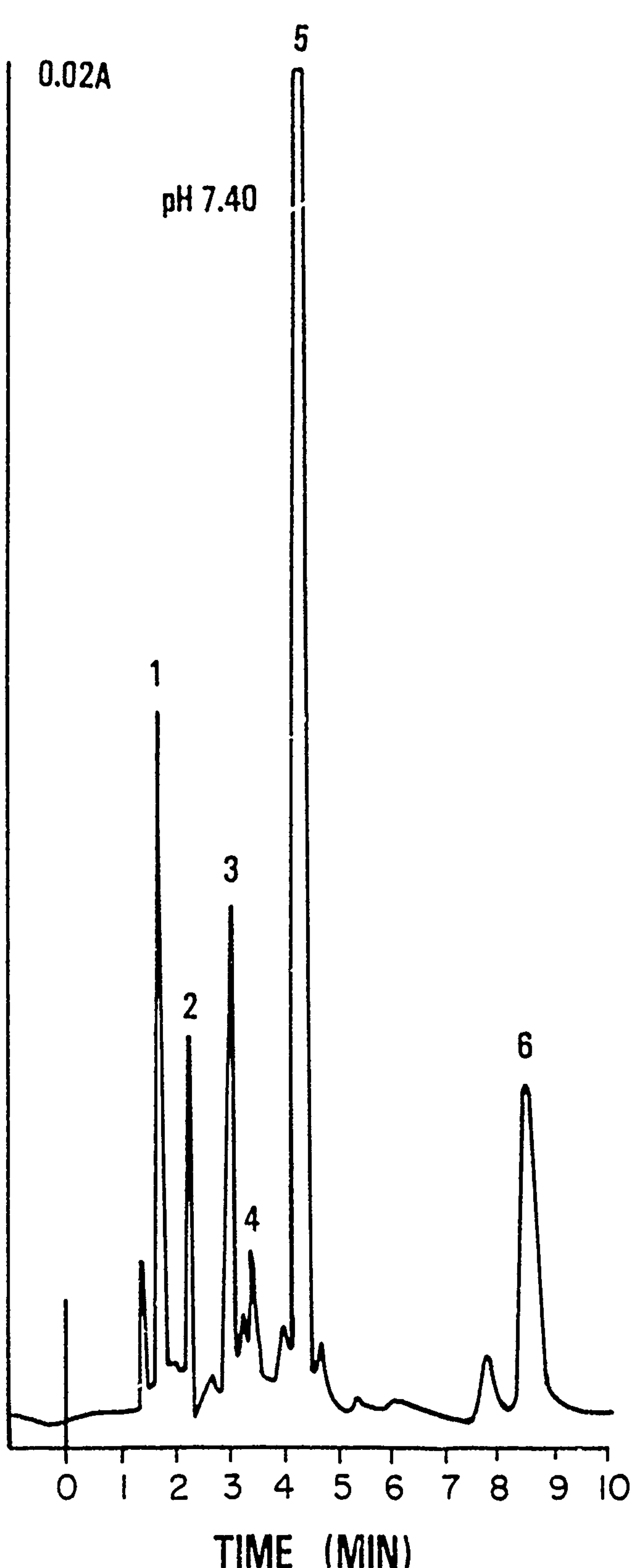


FIGURE 2. DEGRADATION OF HI-6 IN BUFFERED ACETATE SOLUTION AFTER 24 HOURS AT A TEMPERATURE OF 37°C. MORE THAN 13 MAJOR AND MINOR BY-PRODUCTS WERE OBSERVED IN THE HPLC CHROMATOGRAM. MASS SPECTROMETRY WAS USED TO IDENTIFY THE MAJOR SPECIES OF THIS DEGRADATION REACTION.

FIGURE 3 DEGRADATION SCHEME OF HI-6 AFTER 6 WEEKS OF STORAGE AT 37°C. IDENTIFICATION OF THE SUBSTITUTED PYRIDINE ANALOGS WERE DETERMINED BY PAIRED RETENTION TIMES OF HPLC AND THE MASS NUMBERS FROM MASS SPECTROMETRY.

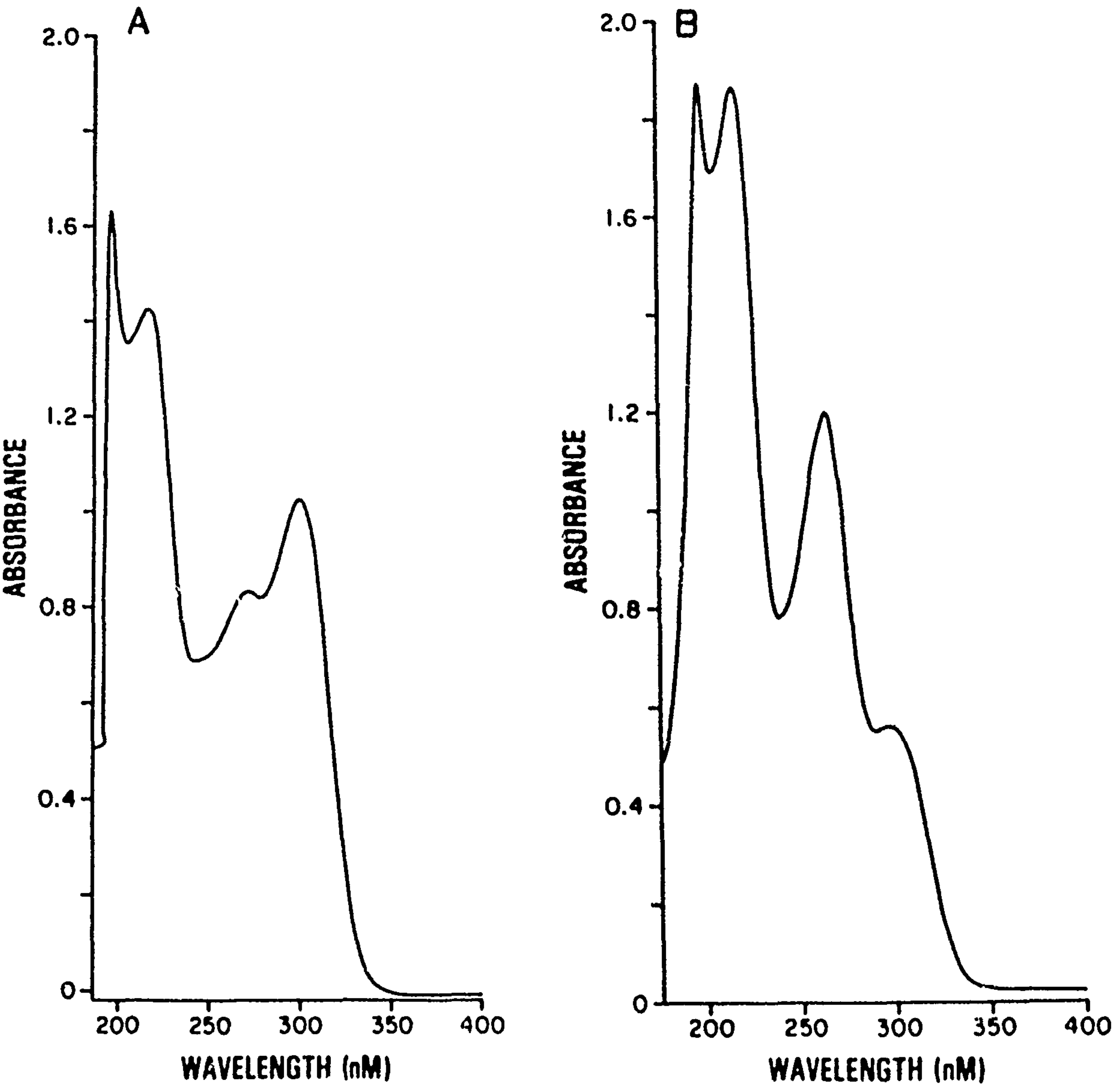


FIGURE 4. ABSORBANCE SPECTRA SHOWING; (A) AN HI-6 PREPARATION (ACETATE) AT DAY 1, (B) SIMILARLY PREPARED SAMPLE, STORED AT 40°C FOR SIX WEEK. THE CHANGE IN THE SPECTRA IS RESULT OF THE ACCUMULATION OF SUBSTITUTED PYRIDINE COMPOUNDS IN THE FORMULATION.

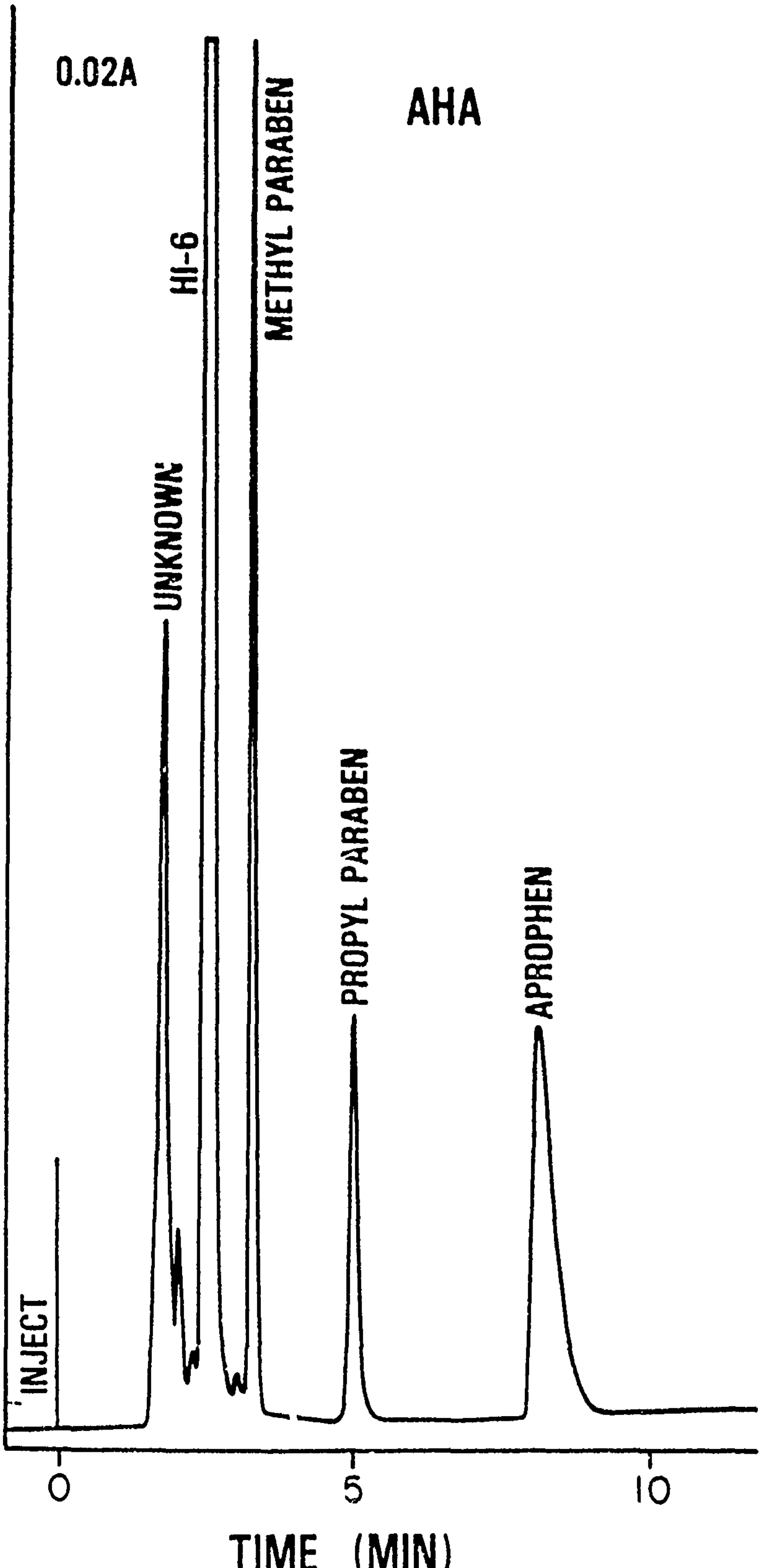
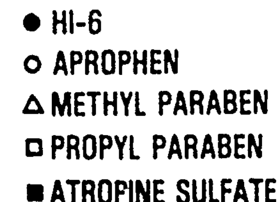


FIGURE 5. CHROMATOGRAM OF A NEW MULTICOMPONENT ANTICHOLINERGIC MIXTURE DEVELOPED IN OUR LABORATORY. HPLC METHOD WAS USED TO EVALUATE THE MIXTURE AT VARIOUS CONCENTRATIONS AT 4°C, 18°C AND 40°C. DATA SHOW THAT TEMPERATURES LESS THAN (18° - 20°C) MAINTAIN THE STABILITY OF THE MIXTURE, EXCEEDINGLY WELL.

STABILITY STUDY OF AHA IN CITRATE (AMBIENT TEMPERATURE)



PERCENT

0 10 20 30 40 50 60 70 80 90 100

0 4 8 12 16 20 24 28 32 36 40 44 48 52 56 60 64 68 72 78

TIME IN WEEKS

FIGURE 6 STABILITY CURVES OF HI-6 (•), APROPHEN (O), METHYL PARABEN (Δ), PROPYL PARABEN (□) AND ATROPINE (■), STORED FOR 78 WEEKS AT ROOM TEMPERATURE IN A PH 4.0 ACETATE BUFFER. ALL THREE ANTICHOLINERGIC COMPOUNDS MAINTAINED THEIR STABILITY (98%>) FOR MORE THAN 2 YEARS.

RESULTS AND DISCUSSION

HI-6 DICHLORIDE HAS BEEN STABILIZED IN SIMPLE AND MIXED FORMULATIONS AT VARIOUS TEMPERATURES AND CONCENTRATIONS. WHEN BUFFERED AQUEOUS SOLUTIONS OF HI-6 (12.5mg/ml) WERE STORED AT ROOM TEMPERATURES (18° - 20°C) FOR 24 MONTHS, 98% OF THE COMPOUND REMAINED UNCHANGED. SIMILAR RESULTS WERE OBSERVED FOR CONCENTRATIONS OF 250 mg/ml, STORED FOR 14 MONTHS, AT 4°C.

TEMPERATURES GREATER THAN 37°C PRODUCED DEGRADATION OF HI-6 TO SUBSTITUTED PYRIDINE ANALOGS. USING ION-PAIR REVERSE-PHASE HIGH PERFORMANCE LIQUID CHROMOTOGRAPHY AND FAST ATOMIC BOMBARDMENT MASS SPECTROMETRY, THE BY-PRODUCTS WERE SEPARATED AND IDENTIFIED.

THE CHROMATOGRAMS DISPLAYED SHOW THE RESULTS OF INCREASED TEMPERATURE AND PH ON THE ACTIVE THERAPEUTIC COMPOUNDS.

ANTICHOLINERGIC ACTIVITIES OF APROPHEN

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Aprophen, a potential antidote to organophosphate poisoning and an antispasmodic, was examined for its antimuscarinic activity [Biochem. Pharmacol. 32, 2979 (1983)] and its antinicotinic activity. When tested on guinea pig ileum, aprophen was about as potent as atropine ($K_B = 1 \times 10^{-8}$ M) in blocking the acetylcholine-induced contracton with a pA_2 of 8.5 ($K_B = 3 \times 10^{-9}$ M). As opposed to its antinicotinic effects on neural cell lines, the antimuscarinic activity of aprophen on the muscarinic receptors of N4TG1 neuroblastoma cells and of NG108-15 neuroblastoma x glioma hybrid cells was unaffected by the presence of anticholinesterase agents. The I_{50} values for aprophen were 5 and 1×10^{-6} M for the N4TG1 neuroblastoma cells and NG108-15 neuroblastoma cells, respectively.

In the absence of anticholinesterase agents, aprophen did not block the binding of [^{3}H]tubocurarine to the nicotinic receptors of NG108-15 neuroblastoma x glioma hybrid cells. However, in the presence of 10^{-6} M paraoxon, aprophen inhibited the binding of [^{3}H]tubocurarine to the nicotinic receptors of NG108-15 cells ($I_{50} = 2.4 \times 10^{-5}$ M).

Aprophen was found to be hydrolyzed by carboxyesterase and cholesterol esterase. It is thus speculated that the nicotinic receptors of NG108-15 neuroblastoma x glioma hybrid cells are well protected by active esterases, as opposed to the muscarinic receptors.

In addition, aprophen is highly cytostatic as well as cytotoxic to cells. The most sensitive cell types are those of neuronal or neuromuscular in origin, such as NG108-15 neuroblastoma x glioma cells, N4TG1 neuroblastoma cells, C6 glioma cells and H9c2 myoblasts. Aprophen exerts its cytotoxicity probably by its ability to compete for binding to the muscarinic and/or nicotinic sites, thus leading ultimately to cellular ionic imbalances.

INTRODUCTION

Aprophen (2-diethylaminoethyl 2,2-diphenylpropionate; Fig. 1), a cholinolytic and antispasmodic agent, which is administered prophylactically and therapeutically as an antidote to organophosphate poisoning. We previously demonstrated that aprophen is a muscarinic antagonist in N4TG1 neuroblastoma cells and NG108-15 neuroblastoma x glioma hybrid cells. The I_{50} values are approximately 1 and 5 µM respectively. In contrast, aprophen failed to affect the binding of [^{3}H]tubocurarine to the nicotinic receptors of the latter cells. We now present evidence that aprophen in the presence of an anticholinesterase is also an antinicotinic agent judging by its ability to inhibit the binding of [^{3}H]tubocurarine to NG108-15 neuroblastoma x glioma hybrid cells.

QNB

APROPHEN

FIGURE 4. CHEMICAL STRUCTURE OF QNB AND APROPHEN.

METABOLIC PATHWAY OF APROPHEN

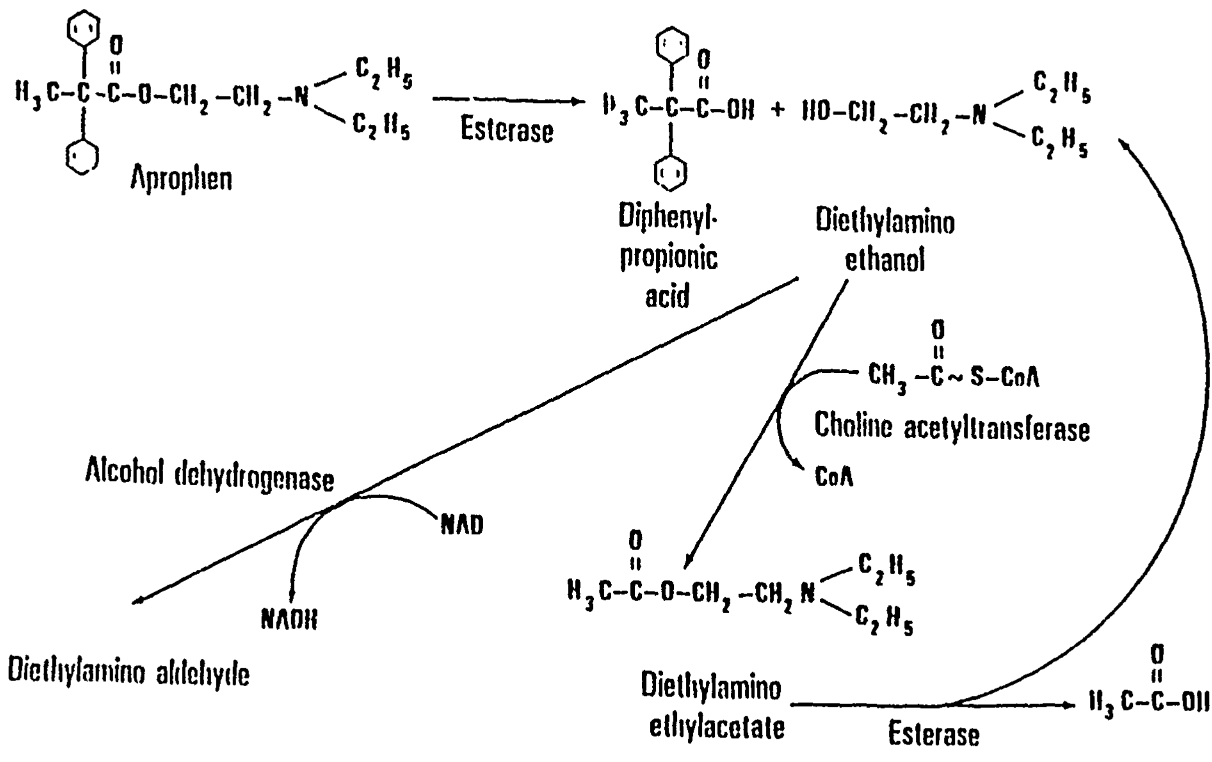


Fig. 1. Catabolism of aprophen by esterases to diphenylpropionic acid and diethylamino ethanol, and the further metabolism of diethylamino ethanol via alcohol dehydrogenase and choline acetyltransferase.

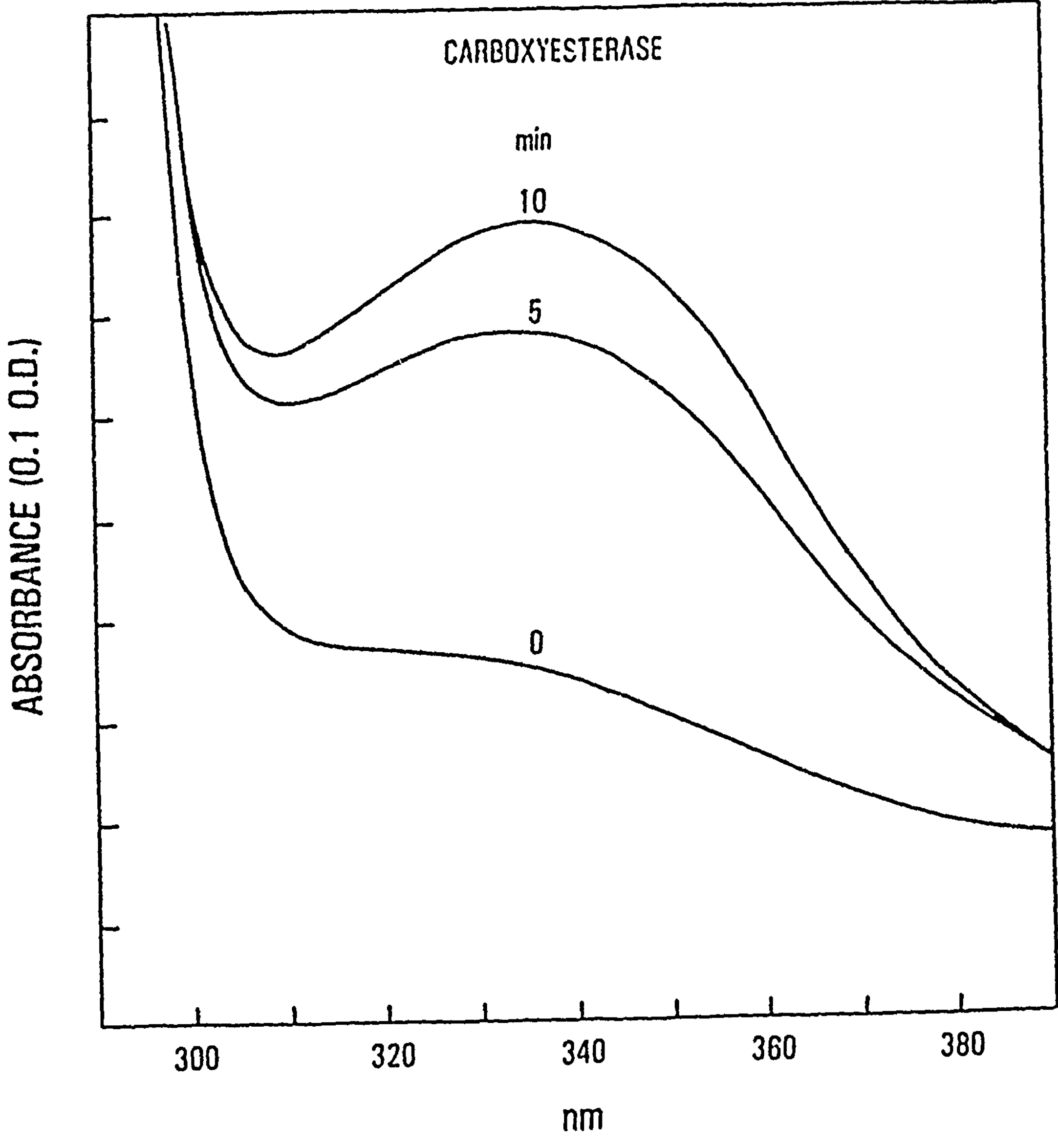


Fig. 3. Spectrophotometric method for assaying the hydrolysis of aprophen or compounds with aromatic ester bonds. The method is based on the hydrolysis of aprophen to diethylamino ethanol by a carboxyesterase and the subsequent oxidation of diethylamino ethanol using alcohol dehydrogenase in the presence of NAD. The reduction of NAD to NADH was monitored by spectral scans at the indicated times. The reaction mixtures contained in a final volume of 1 ml: 2mM aprophen, 2 mM NAD, alcohol dehydrogenase (40 U), 50 mM piperazine-N,N'-bis(ethanesulfonic acid) (pH 6.6), and carboxyesterase (35 U). The amount of aprophen hydrolyzed was 7.2 nmoles in 10 min.

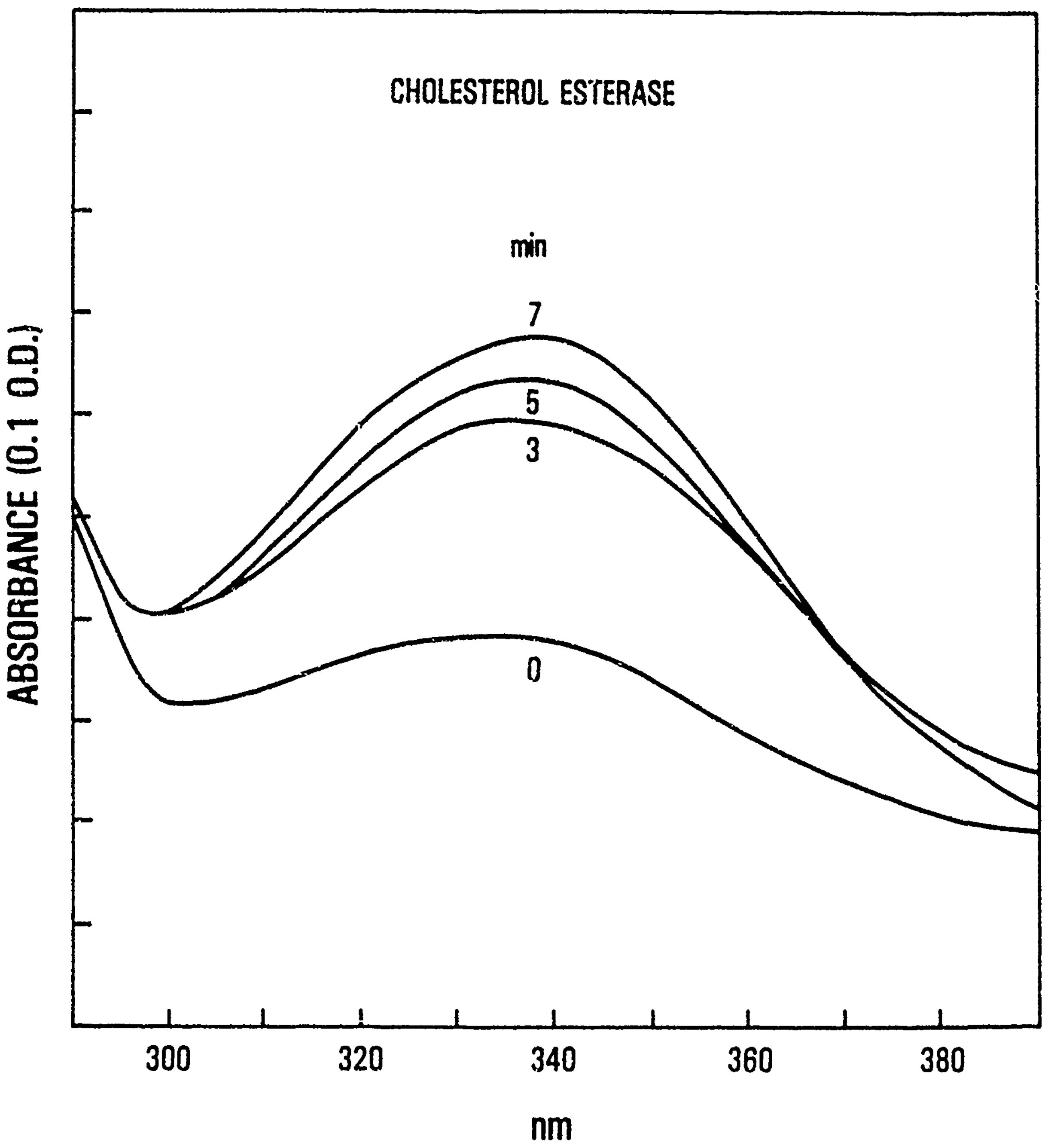


Fig. 4. Spectrophotometric method for the assay of aprophen. The assay components were described in Fig. 3, except that cholesterol esterase (3 U) was used in place of carboxyesterase. The amount of aprophen hydrolyzed was 4.8 nmol in 7 min.

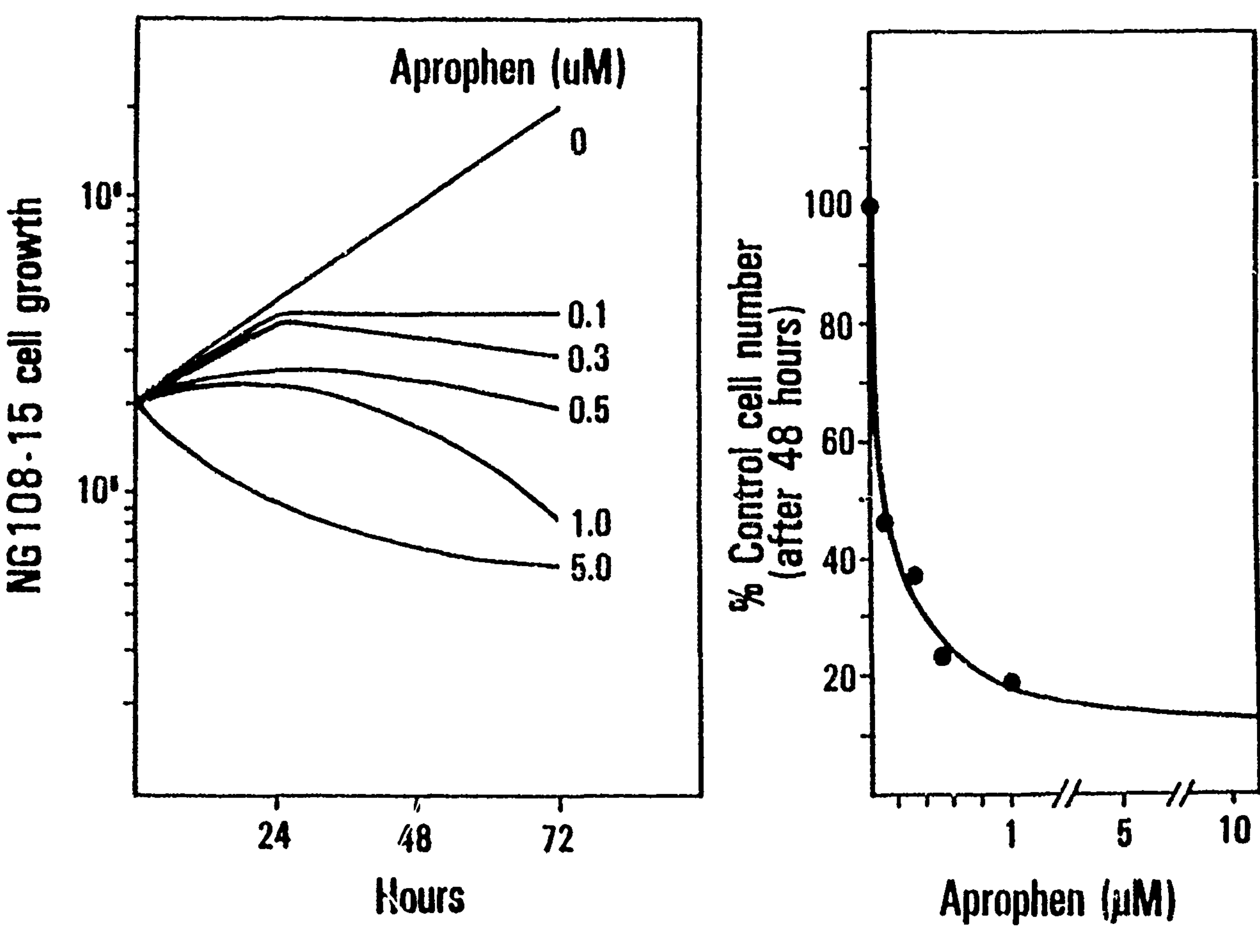


FIGURE 3. GROWTH OF NG108-15 NEUROBLASTOMA X GLIOMA HYBRID CELLS IN THE PRESENCE OF VARIOUS CONCENTRATIONS OF APROPHEN.

TABLE 1. CYTOTOXICITY OF APROPHEN

CELL TYPE	I_{50}*
	(µM)
NG108-15 NEUROBLASTOMA X GLIOMA	0.1
H9C2 MYOBLASTS	2.0
C_6 GLIOMA	2.0
N4TG1 NEUROBLASTOMA	3.0
3T3-C2 FIBROBLASTS	18.0
CLONE 9 LIVER CELLS	20.0

*I_{50} VALUES WERE DETERMINED AFTER 48 HRS OF INCUBATION WITH VARIOUS CONCENTRATIONS OF APROPHEN.

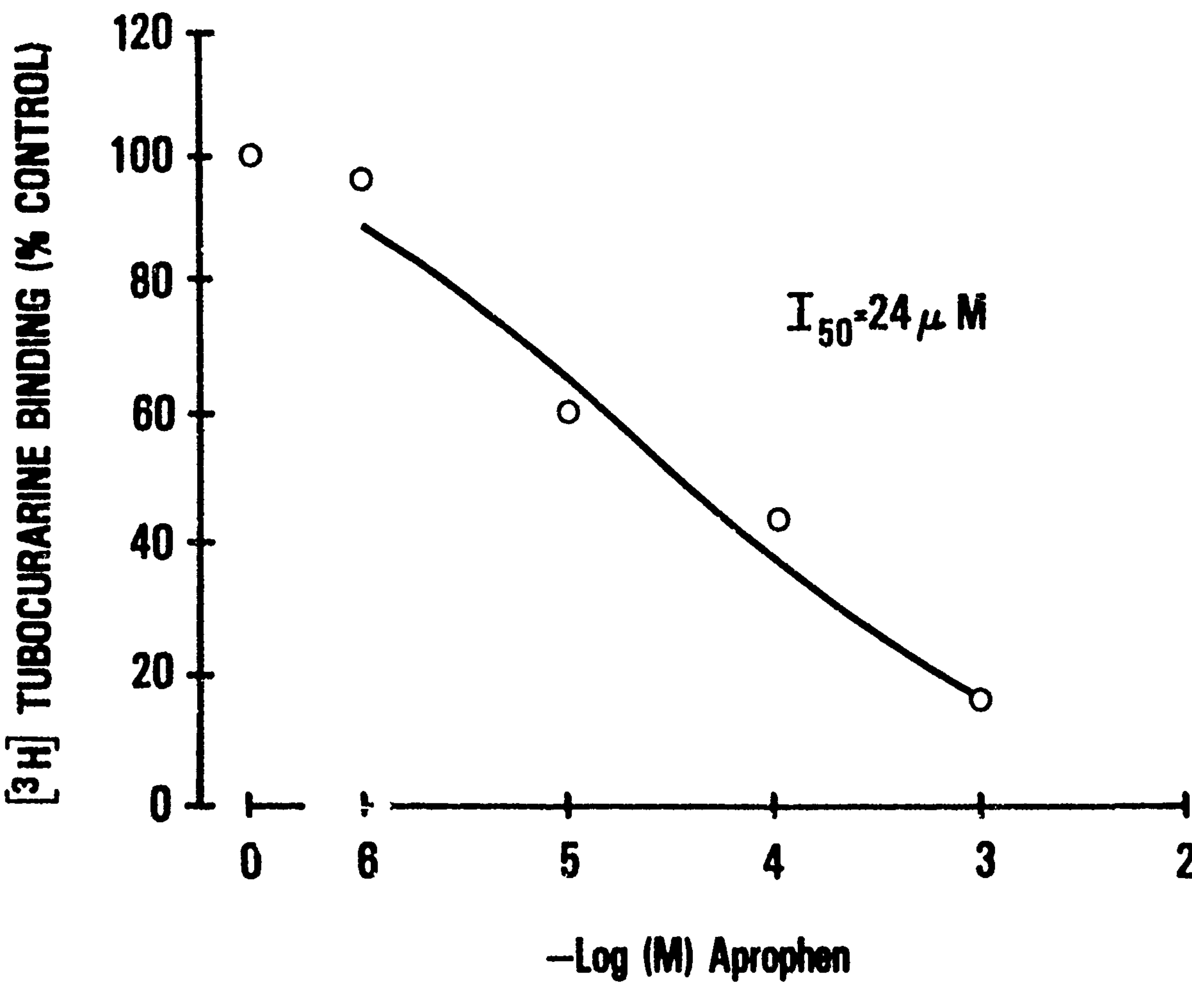
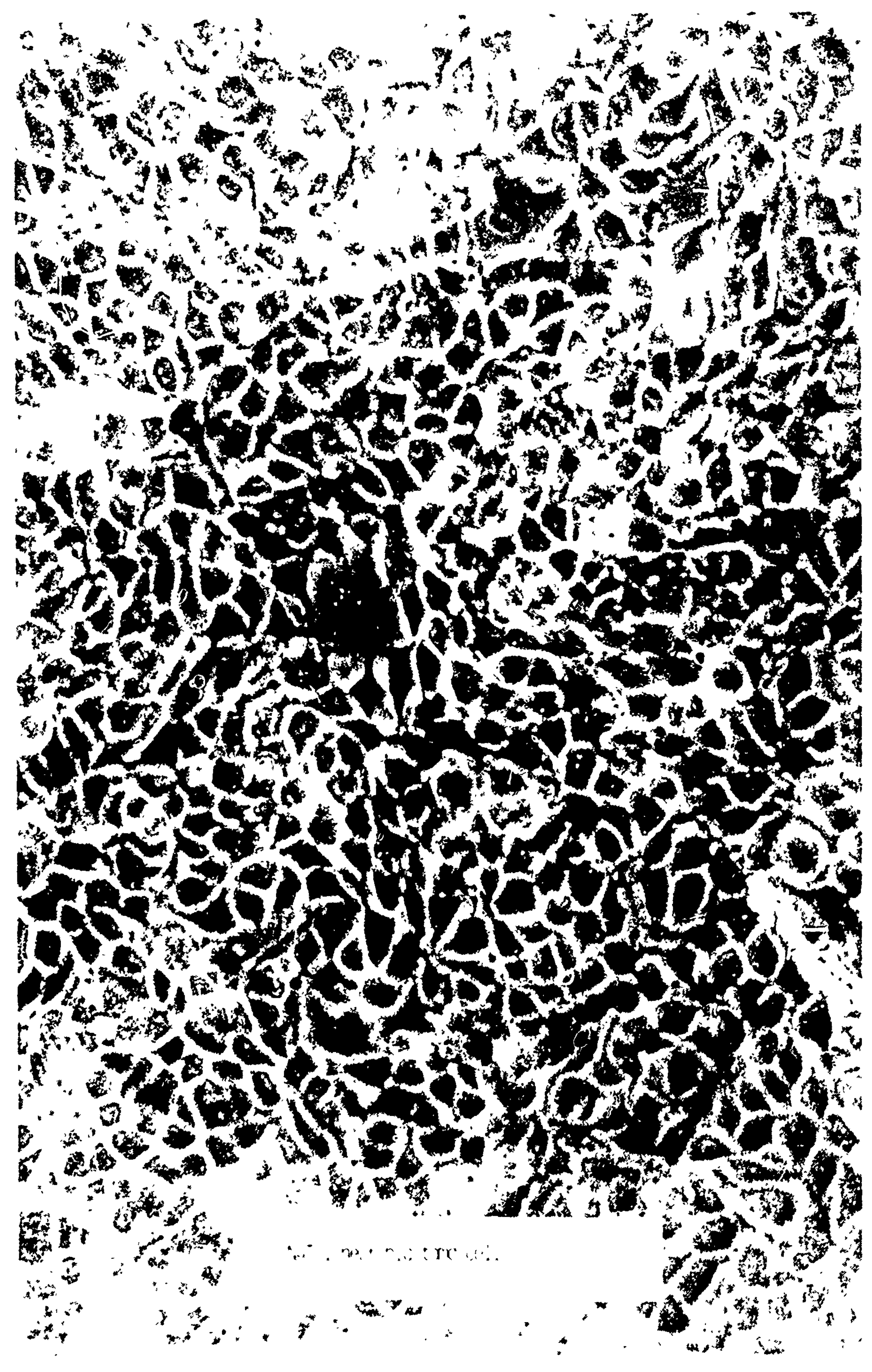
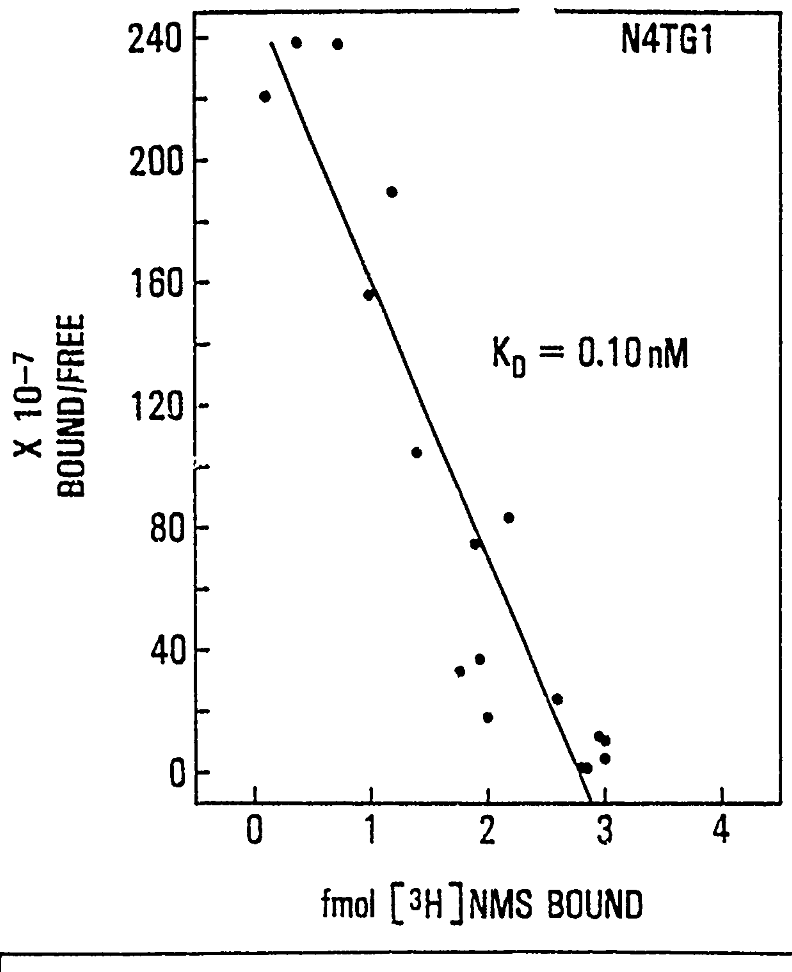
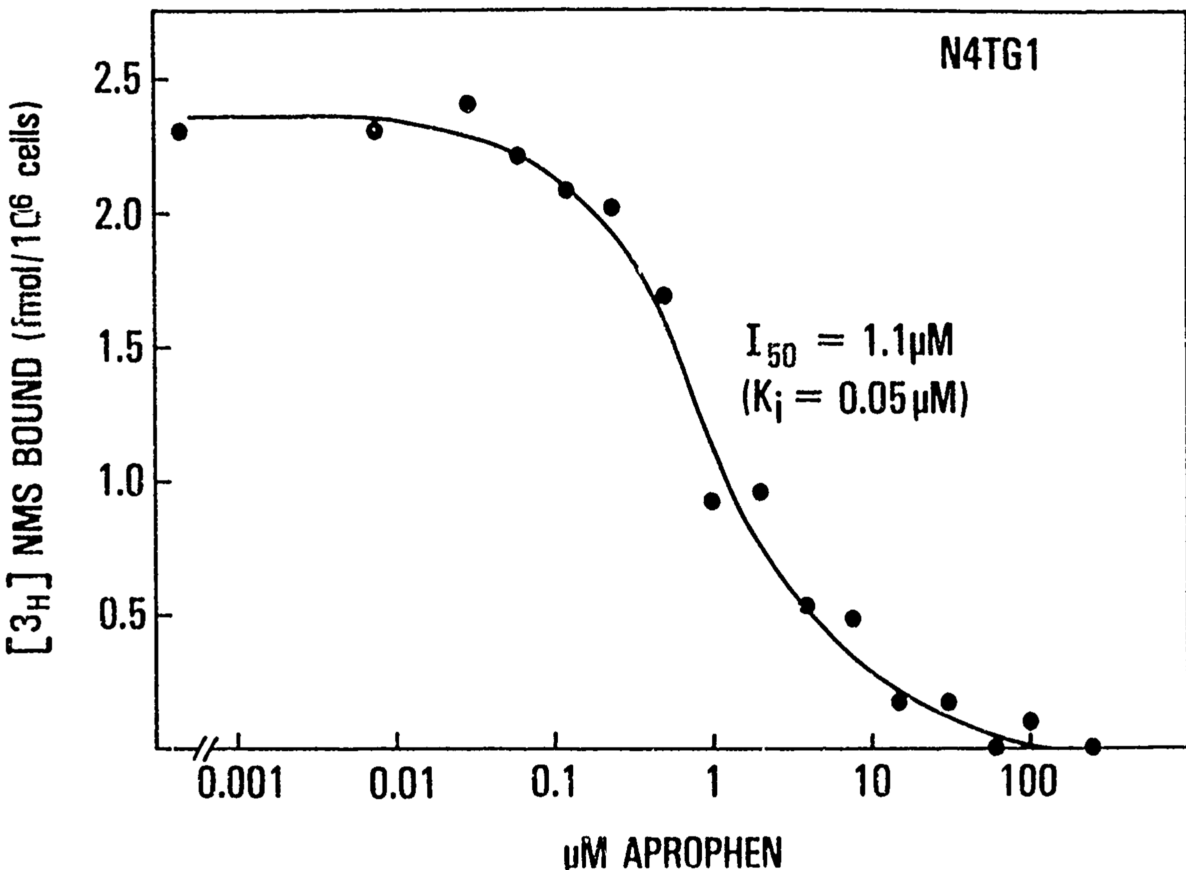


Fig. 2. Inhibition of [^{3}H]tubocurarine, a nicotinic antagonist, binding by aprophen to the nicotinic receptors of NG108-15 neuroblastoma x glioma hybrid cells. Cells (0.5 x 10^6) were incubated in Hank's medium with 2 nM [^{3}H]tubocurarine, 1 uM paraoxon, and varying doses of aprophen for 20 min at 22° C. The cells were pelleted through 500 ul silicone oil in an Eppendorf tube. The cell pellets were solubilized in 1.0 ml of Triton X-100. Radioactivity was determined by scintillation counting. An I_{50} of 24 uM for aprophen was determined using the GRAFIT computer program.

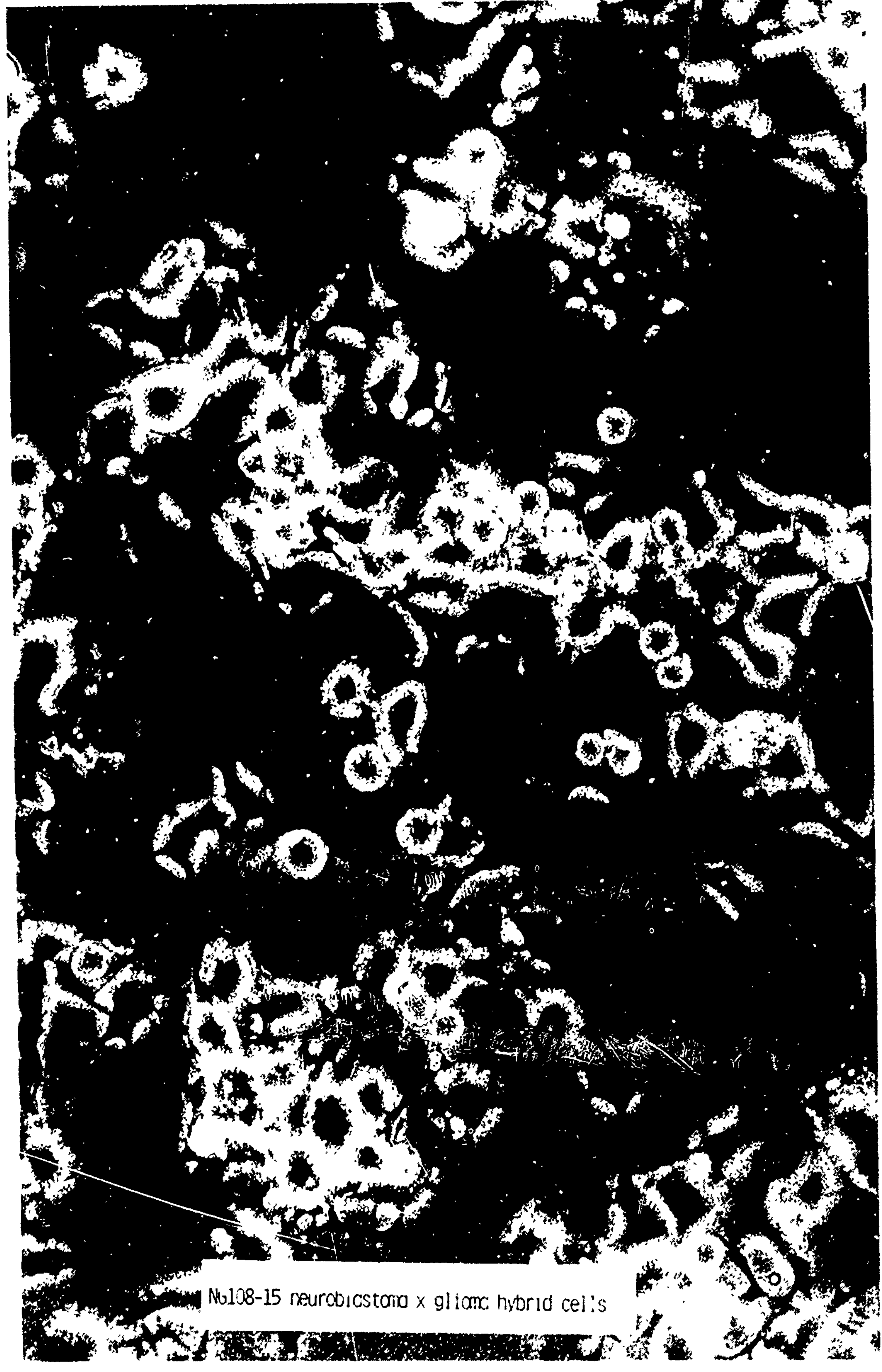
In contrast, aprophen at doses as high as 5 x 10^{-5} M was ineffective in blocking the contraction of frog rectus abdominis muscle, a pharmacological assay to measure antinicotinic potency, when induced by acetylcholine.







Scatchard plot of the binding of [N-methyl-^{3}H]scopolamine to the muscarinic receptors of N4TG1 neuroblastoma cells.



NG108-15 neuroblastoma x glioma hybrid cells

Table
Inhibition constants of muscarinic antagonists

Compounds	Ileum contraction		α-amylase release	NMS binding
	pA_2	$[K_B$ (M)]	I_{50} (M)	$K_{i(M)}$
Aprophen	8.5 ± 0.1	3.1×10^{-9}	1.1×10^{-8} ± 0.2	5.1×10^{-8} ± 1.1
Adiphenine	7.1 ± 0.3	9.2×10^{-8}	3.5×10^{-7} ± 0.3	4.4×10^{-8} ± 1.0
Benactyzine	8.2 ± 0.8	6.5×10^{-9}	3.0×10^{-8} ± 0.5	4.0×10^{-9} ± 1.1
Atropine	8.7 ± 0.1	2.0×10^{-9}	5.9×10^{-10} ± 5.7	8.9×10^{-10} ± 0.4
QNB	8.7 ± 0.9	2.0×10^{-9}	2.1×10^{-8} ± 0.6	5.3×10^{-10} ± 1.9
QNX	8.4 ± 0.5	4.0×10^{-9}	1.1×10^{-9} ± 1.5	6.4×10^{-10} ± 0.9
Pirenzepine	4.3 ± 0.4	5.0×10^{-5}	1.8×10^{-6} ± 1.2	1.7×10^{-7} ± 0.7

CONCLUSIONS

1. Antinicotinic activity of aprophen was demonstrated using $[^3H]$tubocurarine binding to nicotinic receptors of NG108-15 neuroblastoma x glioma hybrid cells in the presence of a cholinesterase inhibitor, paraoxon. An I_{50} of 24 uM for aprophen was determined. This is in contrast to previous work in our laboratory where aprophen in the absence of paraoxon was without a nicotinic effect.
2. In contrast, aprophen did not effect the acetylcholine-induced contraction of frog rectus abdominis muscle, even in the presence of an anticholinesterase.
3. Aprophen is a more potent antimuscarinic cholinergic (I_{50} about 5 uM) than antinicotinic cholinergic (I_{50} about 24 uM).
4. Because the antinicotinic effect of aprophen was detected only in the presence of an anticholinesterase, the nicotinic receptors in the NG108-15 neuroblastoma x glioma hybrid cells may be well protected by esterases. In contrast, muscarinic receptors can be readily assayed with whole cells in the absence of an anticholinesterase.
5. Aprophen was hydrolyzed by carboxyesterase and cholesterol esterase. Aprophen was not hydrolyzed by human plasma butyrylcholinesterase.
6. A spectrophotometric method for the assay of aprophen and other compounds with aromatic ester bonds was devised utilizing an esterase to cleave the ester bond to yield diethylamino ethanol which is then oxidized by alcohol dehydrogenase.

KINETIC INVESTIGATIONS INTO THE INTERACTIONS OF APROPHEN WITH RESPECT TO SERINE HYDROLASES

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INTRODUCTION:

We have studied the interactions between highly purified enzymes, including both cholinesterases (BuChE and AChE) and the carboxylesterases (CEs) with the benzilate aprophen. Aprophen is a potent reversible inhibitor of human serum BuChE. It is also hydrolyzed by BuChE as well as by rabbit liver CEs. AChE did not hydrolyze aprophen nor did this drug inhibit AChE activity.

Aprophen hydrolysis mediated by human serum BuChE does not appear to follow Michaelis-Menten kinetics. The substrate and inhibitor kinetics are complex and may involve allosteric and/or cooperative interactions.

Regeneration kinetics indicates that aprophen accelerates the regeneration of the DFP-BuChE complex and that aprophen protects human serum BuChE from inhibition by DFP. Neither the inhibition nor the regeneration kinetics of the fetal bovine serum AChE and the rabbit liver CE were altered.

Aprophen is a potent anticholinergic drug which interacts with specific serine hydrolases. The serine hydrolases kinetically form a homologous enzyme series; this series is composed of acetylcholinesterases (AChEs), butyrylcholinesterases (BuChEs) and carboxylesterases (CEs). These enzymes appear to follow identical reaction mechanisms with organophosphates and carbamate inhibitors. One focus of chemical defense research has been the protection of cholinesterases from organophosphate inhibition through use of slowly reversible carbamate inhibitors. The influence of aprophen on the interaction between serine hydrolases with organophosphates and carbamates is unknown. We have therefore initiated a systematic investigation designed to elucidate these potential interactions and to determine potential protection of serine hydrolases by aprophen.

Fetal bovine serum AChE is a typical mammalian G_4 enzyme. It is neither inhibited by nor does it hydrolyze aprophen. Diisopropylfluorophosphate (DFP) and eserine inhibition of this AChE is characterized by a bimolecular inhibition rate constant, k_i, of $7.7 \pm 1.3 \times 10^4$ M^{-1} min^{-1} and $2.9 \pm 0.5 \times 10^6$ M^{-1} min^{-1}, respectively. 2-PAM regeneration of the DFP-AChE complex exhibits a bimolecular regeneration constant, k_r, of 14.7 M^{-1} min^{-1}. Aprophen has essentially no effect on either the k_i or on the k_r.

Human serum BuChE is potently inhibited by the reversible inhibitor aprophen. The reaction is characterized by a competitive K_i of 3.7×10^{-7} M and further exhibits complex hyperbolic inhibition kinetics. Aprophen is also a substrate for BuChE and exhibits a very slow turnover number of 2.0×10^{-3} sec^{-1}. Human serum BuChE was partially protected from DFP inhibition by aprophen with no decernable change in the bimolecular inhibition rate constant, k_i, of $7.0 \pm 1.2 \times 10^5$ M^{-1} min^{-1}. 2-PAM regeneration of the DFP-BuChE complex was accelerated in the presence of aprophen by a factor of 2.7 over the 2-PAM control. The k_r was accelerated from 8.4 M^{-1} min^{-1} to 23.1 M^{-1} min^{-1} respectively.

The oligomeric CE from rabbit liver is a typical carboxylesterase. Little is known about aprophen inhibition of this enzyme although CEs hydrolyze aprophen. The turnover number is 1.4×10^{-3} sec^{-1}. The presence of aprophen did not alter either the inhibition (DFP) or the regeneration (2-PAM) kinetics observed with the CEs.

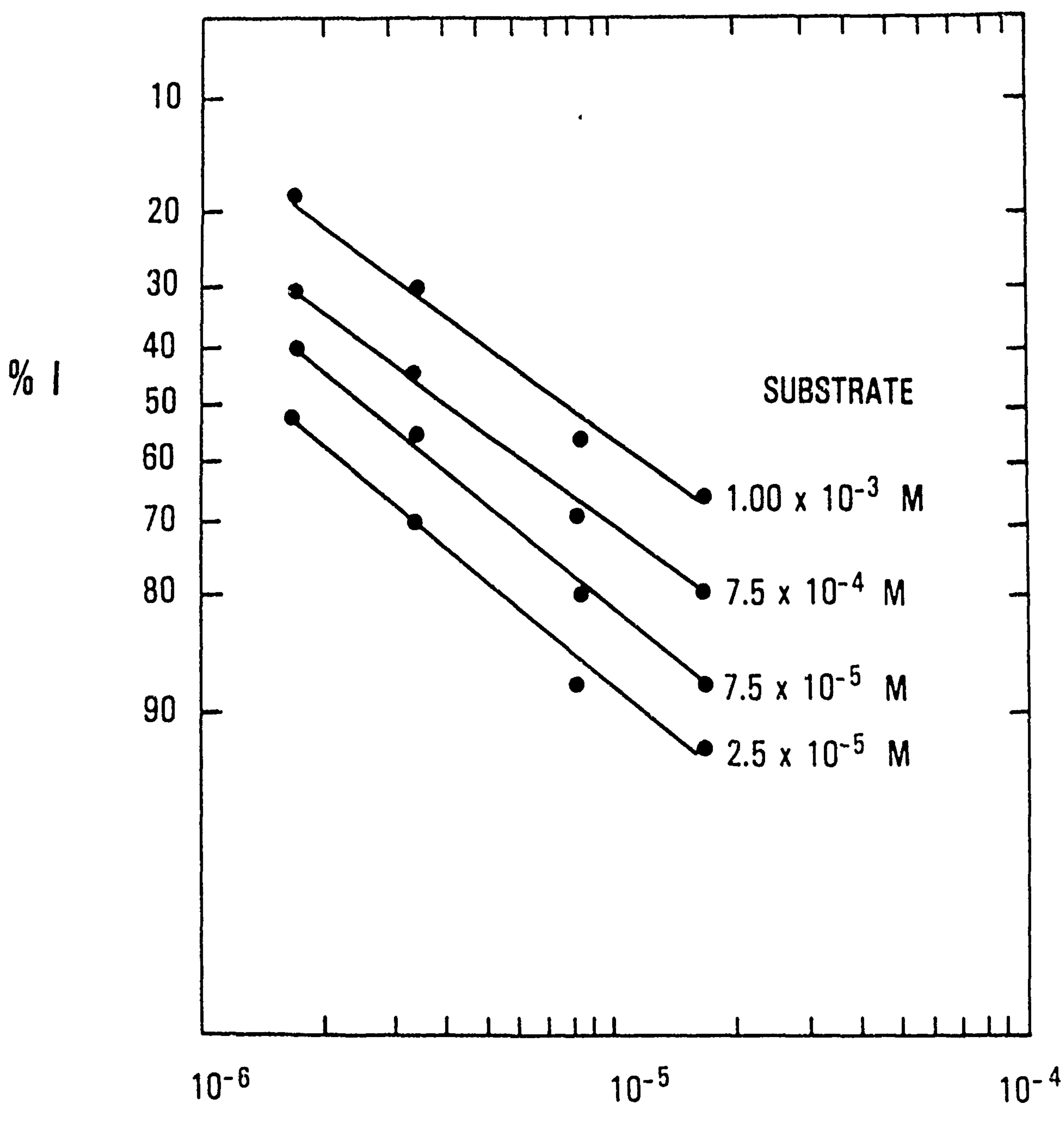


Fig 1. Probit Analysis of aprophen inhibition of human serum BuChE at fixed concentrations of butyrylthiocholine.

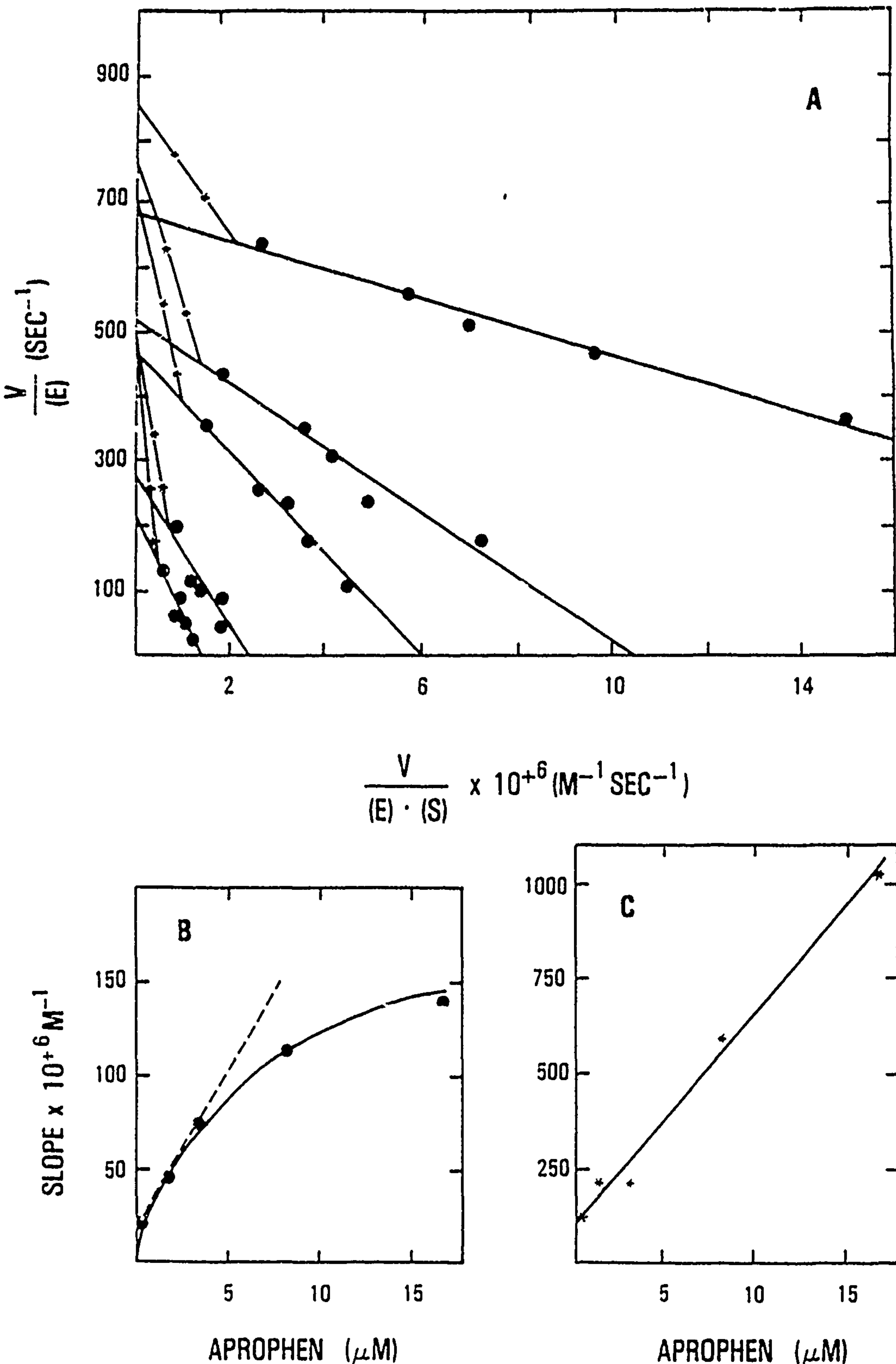
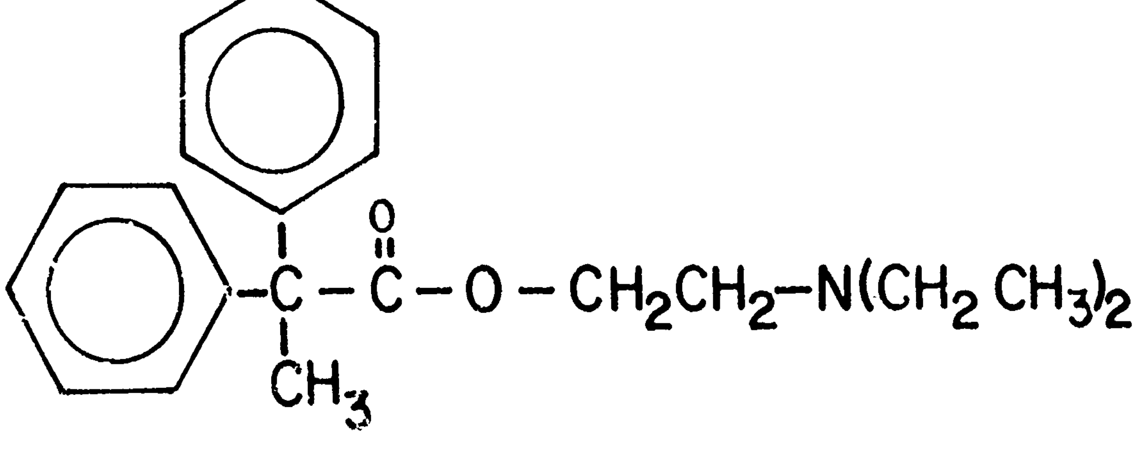


Fig 2. A: Hofstee plot depicting aprophen inhibition of pure human serum BuChE at fixed concentrations of aprophen.
B: Replot of the slope of the Hofstee plot in the low substrate region.
C: Replot of the slope of the Hofstee plot in the high substrate region.

Aprophen



|←— 9.12 Å —→|←— 4.47 Å —→|

Butyrylthiocholine

$CH_3CH_2CH_2-\overset{O}{\overset{\|}{C}}-S-CH_2CH_2-\overset{\oplus}{N}(CH_3)_3$

|←— 6.41 Å —→|←— 4.83 Å —→|

Fig 3. Structures and estimated bond distances of aprophen and butyrylthiocholine.

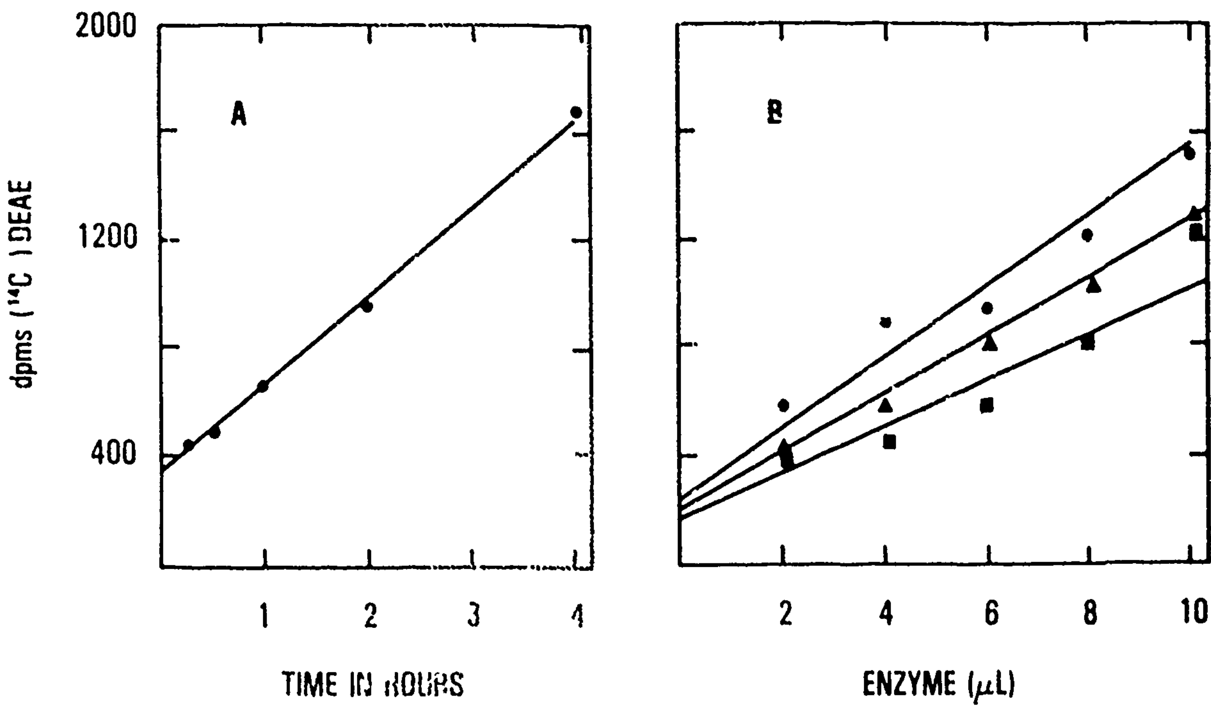


Fig 4. A: The enzyme concentration cure for the hydrolysis of pure human serum BuChE (●—●), horse serum (▲—▲), and rabbit liver homogenate (■—■).
B: Time course of aprophen hydrolysis by human serum BuChE.

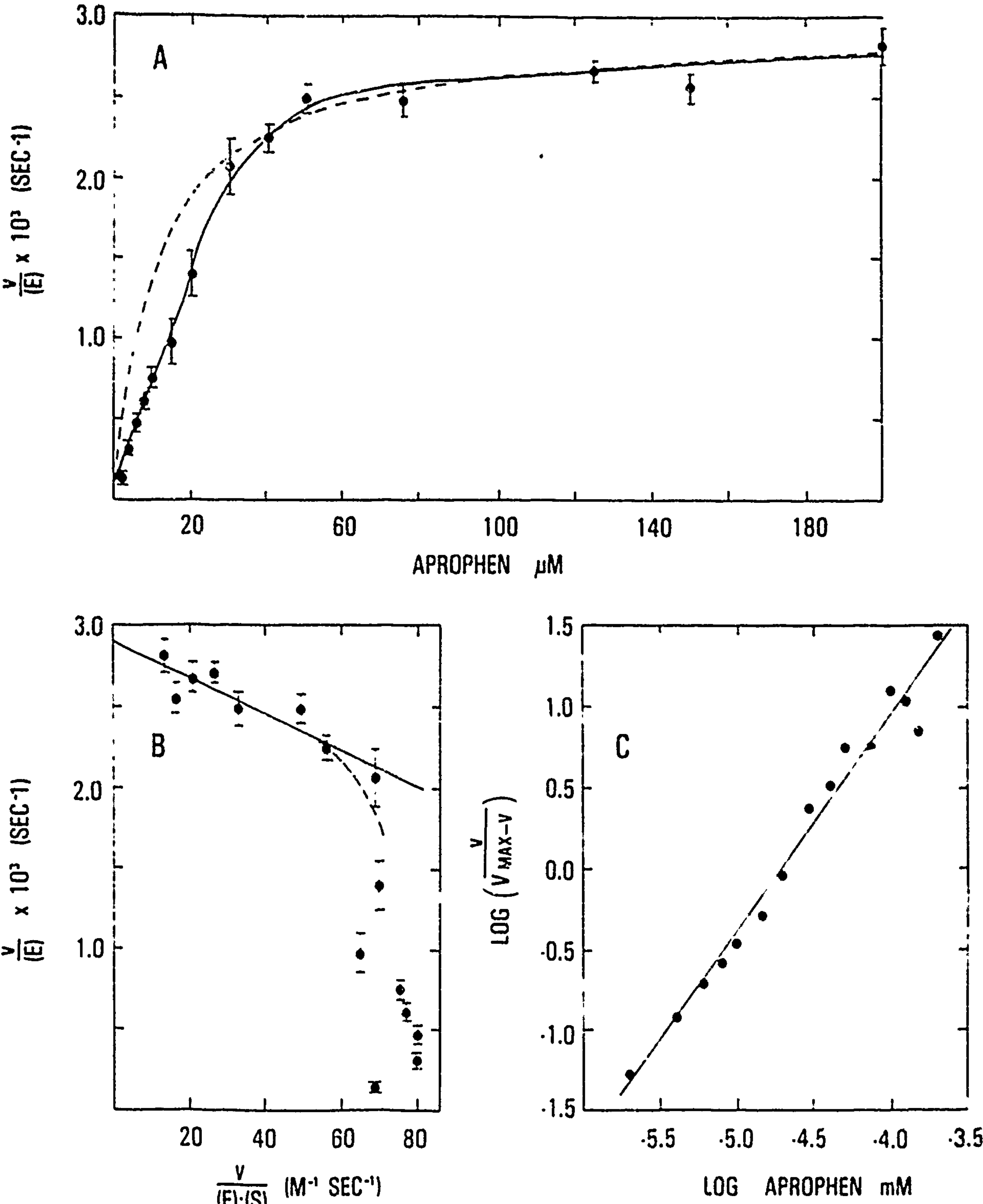


Fig 5. A: Saturation curve for the hydrolysis of aprophen by human serum BuChE.
B: Hofstee plot depicting nonlinearity in the low substrate region.
C: Hill plot depicting positive cooperativity of aprophen binding to BuChE.

Table 1

Turnover Constants and Substrate Ratios

	Substrate				
	APR k_{cat} (sec^{-1})	ΦTB k_{cat} (sec^{-1})	BTC k_{cat} (sec^{-1})	ΦTB/APR	BTC/APR
ENZYME					
HUMAN BuChE	$1.97\pm0.15 \times 10^{-3}$ (5)	600	632	3.05×10^{5}	3.21×10^{5}
Rabbit oCE	1.42×10^{-3} (2)	276	0.025	1.94×10^{5}	17.6
Rabbit mCE	$4.28 \pm 0.06 \times 10^{-4}$ (3)	195	1.13	4.56×10^{4}	254
Horse hsCE	$4.70 \pm 0.02 \times 10^{-4}$ (5)	4.25	---	9.05×10^{3}	---

APR, aprophen
ΦTB, phenylthiobutyrate
BTC, n-butyrylthiocholine

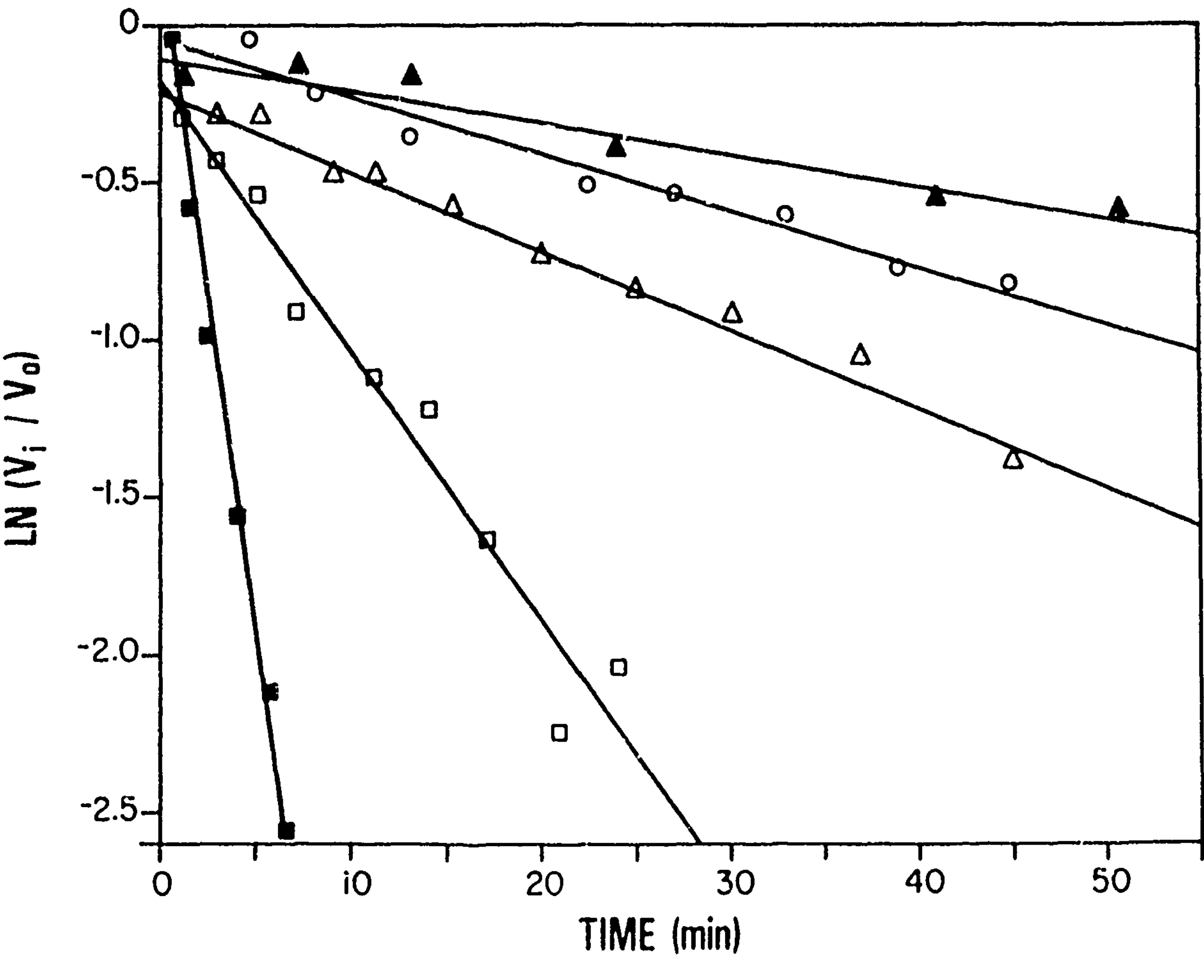


Fig 6. First order inhibition of fetal bovine serum AChE by DFP.

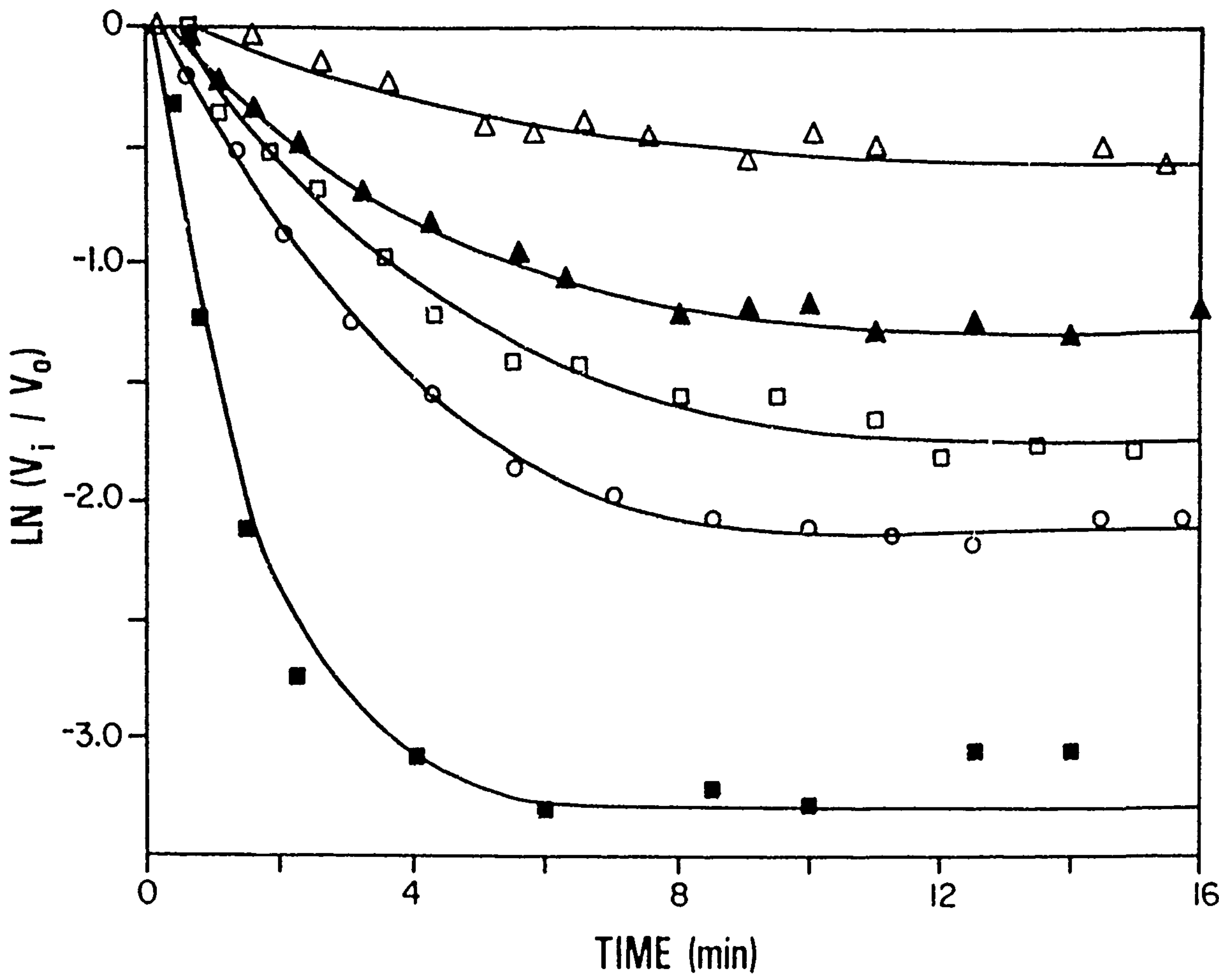


Fig 7. Second order inhibition of fetal bovine serum AChE by Eserine.

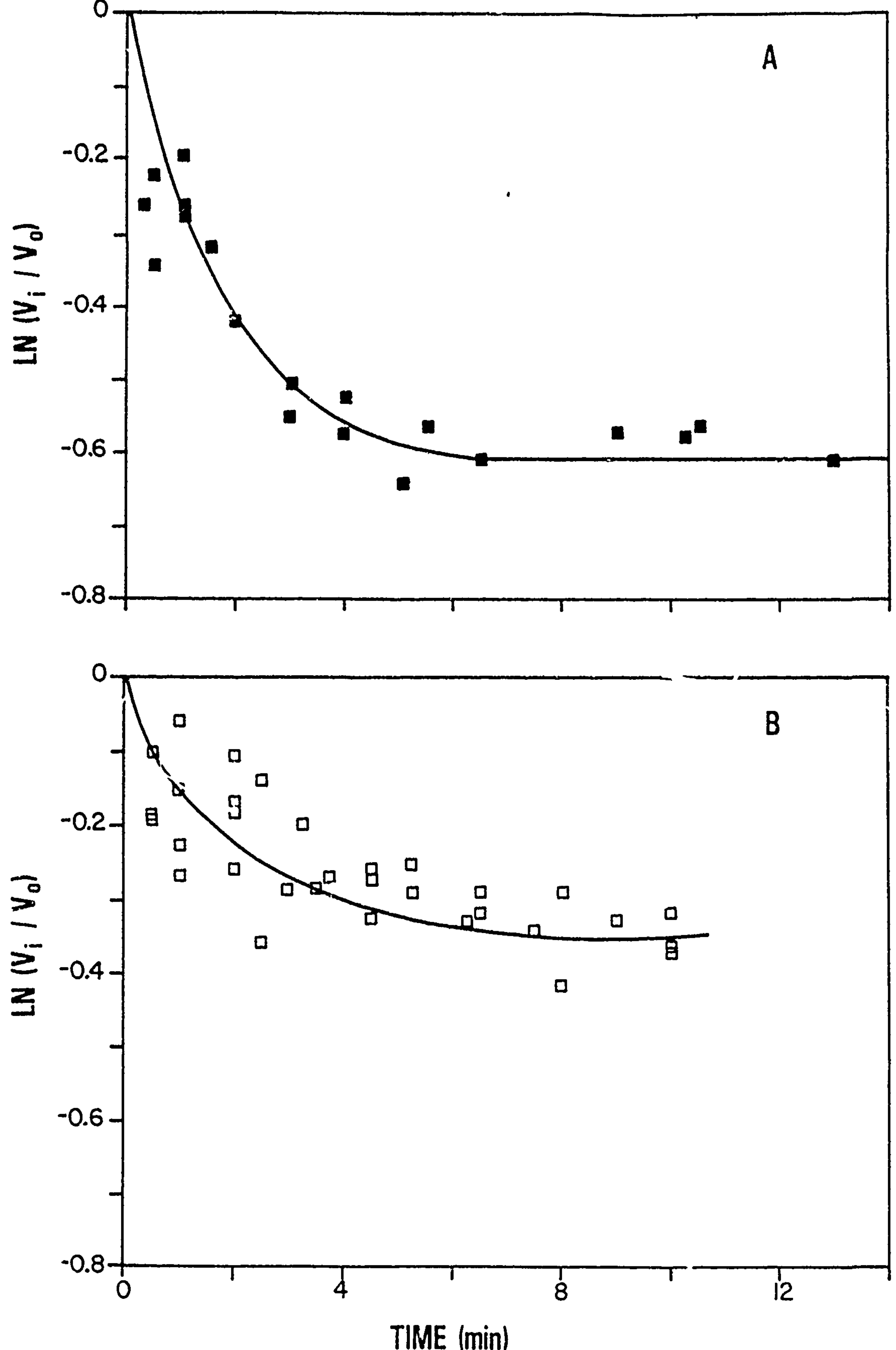


Fig 8. A: Inhibition of human serum BuChE by DFP.
B: Inhibition of human serum BuChE by DFP in the presence of aprophen.

TABLE 2

Bimolecular Inhibition Rate Constants in the Absence or Presence of Aprophen for Selected Serine Hydrolases.

Enzyme	Inhibitor	Aprophen	k_i, M^{-1} min^{-1}	T, °C	pH	Reference
AChE*	DFP	Absent	$7.70 \pm 1.33 \times 10^4$ (7)	37	8.0	
		Present	4.67×10^4	37	8.0	
	Eserine	Absent	$2.94 \pm 1.66 \times 10^6$ (4)	37	8.0	
		Present	3.20×10^6	37	8.0	
AChE**	DFP	Absent	$4.67 \pm 0.53 \times 10^4$ (4)	37	8.0	
	DFP	Absent	3.4×10^4	25	-	[15]
	Eserine	Absent	3.1×10^6		-	[15]
BuChE*†	DFP	Absent	$7.02 \pm 1.18 \times 10^5$ (6)	5	8.0	
		Present	$4.57 \pm 0.78 \times 10^5$ (4)	[illegible]	8.0	
BuChE†	DFP	Absent	1.10×10^6	5	-	[15]
CE ††	Eserine	Absent	5.3×10^6	37	7.4	[17]

* fetal bovine serum
** bovine erythrocyte
*† human serum
† horse serum
†† rat intestinal mucosa

TABLE 3

Bimolecular Regeneration Rate Constants in the Absence or Presence of Aprophen for Selected Diisopropylphospho-Serine Hydrolases.

Enzyme	Aprophen	k_r, M^{-1} min^{-1}	T,°C	pH	Reference
AChE*	Absent	14.72 ± 1.25(4)	37	8.0	
	Present	14.15 ± 1.92(4)	37	8.0	
AChE**	Absent	17	25	7.4	[15]
AChE†	Absent	140	25	7.4	[15]
BuChE††	Absent	8.36 ± 2.17(5)	37	8.0	
	Present	23.08 ± 2.55(5)	37	8.0	
CE*†	Absent	2.74 ± 0.54(3)	37	8.0	
	Present	3.62 ± 0.73(3)	37	8.0	

* fetal bovine serum
** human erythrocyte
† eel
†† human serum
*† rabbit liver

CONCLUSIONS:

1. BuChE is potently inhibited by aprophen; an apparent K_i of 3.7×10^{-7} M was determined.

2. BuChE hydrolyzes aprophen to diphenylpropionic acid and diethylaminoethanol.

3. CEs also hydrolyze aprophen.

4. The turnover rate for aprophen hydrolysis by the serine hydrolases is apparently 4×10^{-4} sec^{-1} per active site.

5. Aprophen hydrolysis mediated by BuChE is complex, probably involving both allosterism and cooperative binding of aprophen to the enzyme.

6. BuChE is apparently poartially protected from DFP inhibition by aprophen, although the inhibition kinetics was not altered; the k_i of $7.0 \pm 1.2 \times 10^5$ M^{-1} min^{-1} was determined.

7. Aprophen did not protect the fetal bovine serum AChE nor the rabbit liver CE from DFP inhibition; the k_i of $7.7 \pm 1.3 \times 10^4$ M^{-1} min^{-1} was determined for the AChE. The inhibition reaction rate could not be determined under our experimental conditions for the CE.

8. Aprophen accelerated the 2-PAM regeneration kinetics of the DFP-BuChE complex by a factor of 2.7 over the 2-PAM control; the k_r was 8.4 M^{-1} min^{-1} and 23.1 M^{-1} min^{-1} respectively.

9. Aprophen did not accelerate the 2-PAM regeneration kinetics of either the fetal bovine serum DFP-AChE or rabbit liver DFP-CE complex; the k_r was 14.7 M^{-1} min^{-1} and 2.7 M^{-1} min^{-1} respectively.

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THE PROPHYLACTIC EFFICACY OF VARIOUS SIMULATORS AGAINST SOMAN INTOXICATION: STRUCTURE-ACTIVITY STUDIES

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INTRODUCTION:

Earlier results indicated that in soman-intoxicated (6-8xLD_{50}) rats part of the AChE-inhibitor was stored in a depot. Gradual release of soman from the depot caused death in the 5-7 hr following an initially succesful oxime-therapy (Wolthuis et al., 1981a,b; Benschop et al., 1981).

This storage and the subsequent re-intoxication could partly be prevented by a prophylaxis with soman-simulators, i.e. compounds that resemble soman in chemical structure but that do not inhibit AChE.

It was found that the simulator obtained by replacing the fluoro atom of soman by a methyl group (simulator V) was most effective in preventing soman storage in muscle and death (Van Helden et al., 1983,1984).

Fig. 1

```
  C C   O              C C   O
  | |   ||             | |   ||
C-C-C-O-P-F          C-C-C-O-P-C
  |     |              |     |
  C     C              C     C

   soman              simulator V
```

QUESTION:

Would alterations in the pinacolyl moiety of simulator V further enhance its prophylactic efficacy? Additionally, three other structural variations (see Table) tested.

EXPERIMENT 1 (Fig. 1): NEUROMUSCULAR TRANSMISSION (NMT)

Hexobarbital-anaesthesized male Wistar rats (180-200g) were treated as shown at the top of figure 2.

Simulator: 36 µmol kg^{-1} i.v.; atropine: 50 mg kg^{-1} i.p.; soman: $8xLD_{50}$ (3.6 µmol kg^{-1} i.v.); HI-6: 150 µmol kg^{-1} i.v.

Dissected diaphragm strips were tested every 10 min in Krebs-Ringer buffer for their ability to sustain tetanic contractions upon indirect stimulation at four different frequencies (Wolthuis et al., 1981c). NMT was expressed as a percentage of NMT determined separately in untreated control preparations.

Changes in NMT during incubation were expressed as: $\%NMT_{t=0}-\%NMT_{t=50}$ (Fig. 3).

EXPERIMENT 2 (Table): SURVIVAL

Rats were treated as in exp. 1, except that $6xLD_{50}$ soman was given and the rats were kept intact. Survival times were measured.

RESULTS:

Figures 2 and 3 show that without simulator the oxime-induced recovery of NMT at t=0 was followed by a gradual failure of NMT in the subsequent 50 min (Control=C).

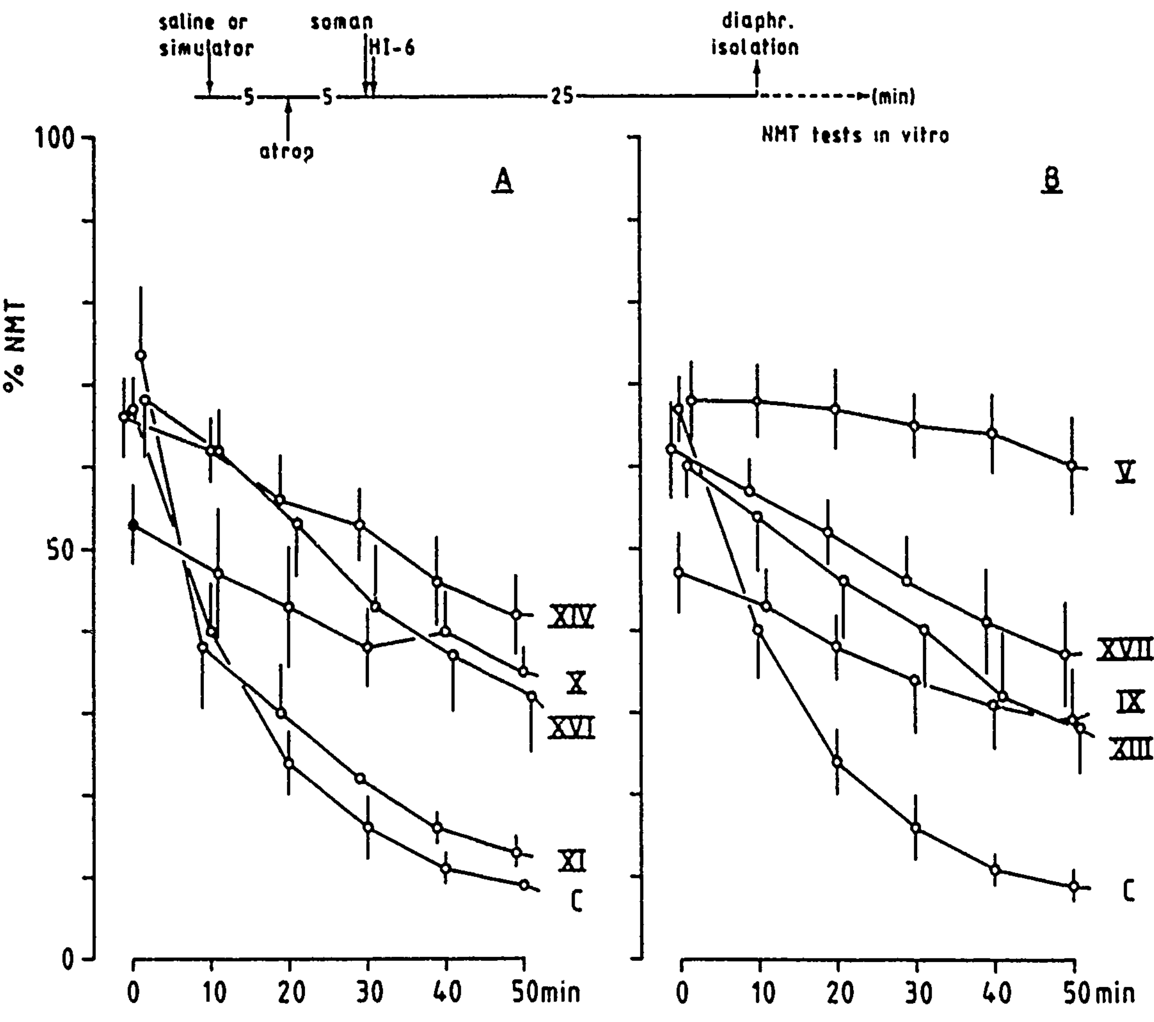
This failure of NMT could be prevented to varying degrees by simulator prophylaxis (except for compound XI). Again simulator V (Fig. 1B) was most effective. Alterations in its pinacolyl group decreased its efficacy (Fig. 1A: XIV,X,XVI and XI).

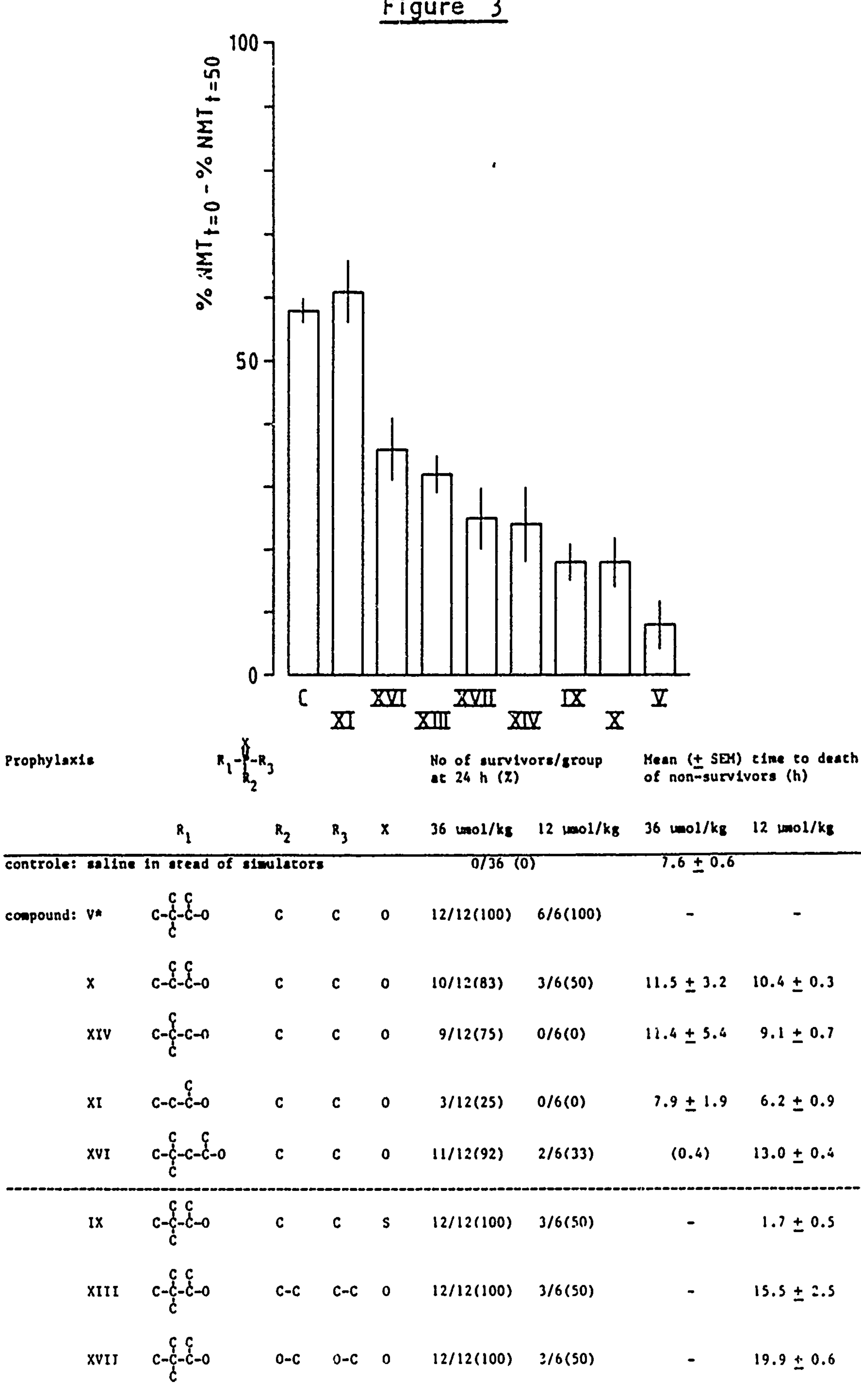
Compounds XVII,IX and XIII were also less effective than simulator V (Fig. 1B).

The same trends were found with regard to survival (Table). In all groups pretreated with 36 µmol kg^{-1} of the simulators, survival was significantly better than in control rats, except for XI. At 12 µmol kg^{-1} survival in all groups was better than the control group, except for XIV,XI or XVI. At 6 and 3 µmol kg^{-1} of compound V, the % survival was still 92% and 17% resp. (bottom of Table).

In an attempt to determine the i.v. LD_{50} of simulator V, it appeared that at a dose of 1.1 mmol kg^{-1} (200 mg kg^{-1}) none of the animals died.

Figure 2





Prophylaxis	$R_1-\overset{X}{\underset{R_2}{P}}-R_3$				No of survivors/group at 24 h (%)		Mean (± SEM) time to death of non-survivors (h)	
	R_1	R_2	R_3	X	36 umol/kg	12 umol/kg	36 umol/kg	12 umol/kg
controle: saline in stead of simulators					0/36 (0)		7.6 ± 0.6	
compound: V*	C C C-C-C-O C	C	C	O	12/12(100)	6/6(100)	-	-
X	C C C-C-C-O	C	C	O	10/12(83)	3/6(50)	11.5 ± 3.2	10.4 ± 0.3
XIV	C C-C-C-O C	C	C	O	9/12(75)	0/6(0)	11.4 ± 5.4	9.1 ± 0.7
XI	C C-C-C-O	C	C	O	3/12(25)	0/6(0)	7.9 ± 1.9	6.2 ± 0.9
XVI	C C C-C-C-C-O C	C	C	O	11/12(92)	2/6(33)	(0.4)	13.0 ± 0.4
IX	C C C-C-C-O C	C	C	S	12/12(100)	3/6(50)	-	1.7 ± 0.5
XIII	C C C-C-C-O C	C-C	C-C	O	12/12(100)	3/6(50)	-	15.5 ± 2.5
XVII	C C C-C-C-O C	O-C	O-C	O	12/12(100)	3/6(50)	-	19.9 ± 0.6

* at 6 umol/kg: no of survivors/group = 11/12(92%); mean time to death of non-survivors = 12 h
at 3 umol/kg: no of survivors/group = 1/6 (17%); mean time to death of non-survivors = 7.8 ± 2.9 h

CONCLUSION

All the synthesized and tested simulators appeared to be less active than compound V.

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SOLVOLYSIS OF NEUTRAL PHOSPHATE AND PHOSPHONATE ESTERS CATALYSED BY Co^{2+}-CHELATES OF TRIS-IMIDAZOLYL PHOSPHINES

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INTRODUCTION:

A LARGE NUMBER OF ENZYMES CONTAINING DIVALENT METAL IONS CATALYSE SUCH PROCESSES AS PHOSPHATE ESTER HYDROLYSIS OR PHOSPHORYL GROUP TRANSFER. AMONG THESE ARE THE Zn^{2+}-CONTAINING ALKALINE PHOSPHATASES (APASES) FROM MAMMALS OR BACTERIA AND PHOSPHO-DIESTERASES FROM SNAKE VENOM WHICH RESPECTIVELY CATALYSE THE HYDROLYSIS OF PHOSPHATE MONOESTERS OR DIESTERS AS IN EQ. 1.[1] IN ADDITION, CARBONIC

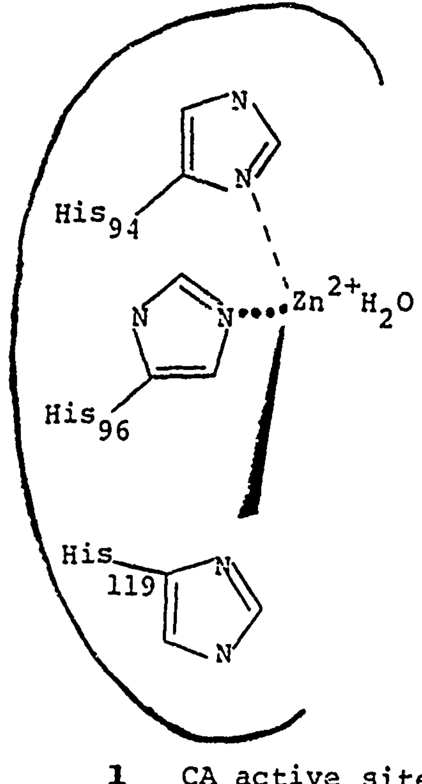
$$1. \quad \mathrm{RO\overset{\overset{O}{\|}}{\underset{\underset{O^-}{|}}{P}}\!-\!O(R')} + H_2O \underset{}{\overset{\text{ENZYME}}{\rightleftharpoons}} \mathrm{HO\overset{\overset{O}{\|}}{\underset{\underset{O^-}{|}}{P}}\!-\!O(R')} + HOR$$

R′ = (−) ALKALINE PHOSPHATASE

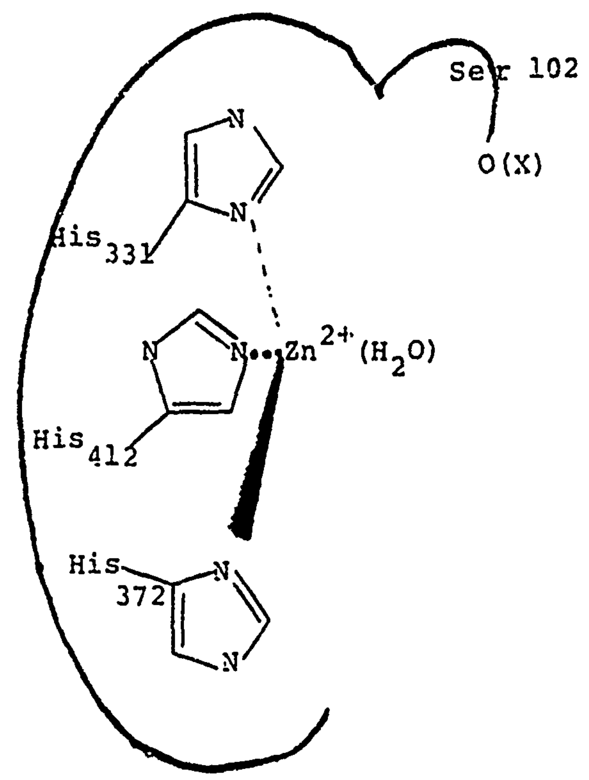
R′ = ALKYL PHOSPHODIESTERASE

ANHYDRASE, A Zn^{2+}-CONTAINING ENZYME FOUND IN ALL LIVING SYSTEMS, HAS BEEN SHOWN TO CATALYSE THE HYDROLYSIS OF A DIALKYL ARYL PHOSPHATE TRIESTER ANALOG OF PARAOXON[2], ALTHOUGH ITS PHYSIOLOGICAL FUNCTION APPARENTLY INVOLVES ONLY CATALYSING THE REVERSIBLE HYDRATION OF CO_2.

THE ACTIVE SITE OF CA CONSISTS OF A Zn^{2+}-ION BOUND TO THE ENZYME BY THREE HISTIDINE IMIDAZOLE UNITS IN A DISTORTED TETRAHEDRAL ENVIRONMENT AS IN 1.[3] ALTHOUGH INCOMPLETELY CHARACTERIZED AT PRESENT, THE ACTIVE SITE OF AP CONSISTS OF TWO Zn^{2+} IONS, ONE OF WHICH IS BOUND BY THREE IMIDAZOLE UNITS, AS WELL AS A SERINE OH UNIT, WHICH DURING THE COURSE OF CATALYSIS BECOMES PHOSPHORYLATED BY THE SUBSTRATE.[4]



1 CA active site



2 AP active site

X = H, resting state

X = $PO_3^{=}$, phosphorylated intermediate

QUESTION: CAN SMALL-MOLECULE-MIMICS OF THE ACTIVE SITES OF THESE TWO ENZYMES CATALYSE THE DECOMPOSITION OF A SELECTED NEUTRAL PHOSPHATE OR PHOSPHONATE SUBSTRATE?

TO TEST THIS WE PREPARED THE Co^{2+}-COMPLEXES OF TRIS-IMIDAZOLYLPHOSPHINES 3 AND 4[5] WHICH ARE SIMPLE, BUT REASONABLE APPROXIMATIONS FOR THE ACTIVE SITES OF CA AND APASE RESPECTIVELY. THE PHOSPHORUS SUBSTRATES

$3:Co^{2+}$ (X = imidazolyl)
(CA mimic)

$4:Co^{2+}$ (X = imidazolyl)
(APAse mimic)

WERE TRIS-2-PYRIDYLPHOSPHATE (5;TPP) AND ETHYL-p-NITROPHENYLMETHYLPHOSPHONATE (6;ENPMP).

5

6

METHOD:

We looked at the solvolysis of 5 and 6 catalysed by varying concentrations of Co^{2+} alone and equimolar Co^{2+} and ligands 3 or 4 in a medium of 80% ethanol-H_2O, this being required for solubility. Solutions were buffered (0.047M, μ= 0.047M $NaClO_4$) with CHES (pH 7.5-8.3) or MOPS (pH 5.4-7.2). "pH" values were measured with a Radiometer GK 2322C combination electrode immersed directly into the solutions before and after each reaction, and are not corrected for the organic solvent faction. (The corrections are small, amounting to a reductions from observed reading by ~ 0.2 units[6]). Reaction rates were monitored at 37.1°C by observing the rate of production of p-nitrophenylate at 400 nm for 6 and 2-pyridinol at 305 nm for 5 under pseudo-first-order conditions with catalyst in at least 10-fold excess. Reported values are averages of 2-3 determinations with a precision of ±5%. Product studies were conducted by comparing the UV-VIS spectra obtained after reaction with a mixture comprised of authentic catalytic components and anticipated hydrolytic products, (ethylmethylphosphonate + p-nitrophenol for 6 and bis-2-pyridylphosphate + 2-pyridinol for 5).

RESULTS:

TABLE 1. PSEUDO-FIRST-ORDER RATE CONSTANTS FOR THE SOLVOLYSIS OF 6 IN THE PRESENCE OF Co^{2+}:3 AND Co^{2+}:4 FOLLOWED AT 400 NM AT 37.1°C.[A,B]

PH	$k_{OBSD}(Co^{2+}:3) \times 10^5 s^{-1}$	$k_{OBSD}(Co^{2+}:4) \times 10^5 s^{-1}$
7.5	-	3.64
7.8	1.70	6.75
7.9	1.95	7.80
8.1	2.35	10.60
8.2	2.41	13.50
8.3	2.45	15.15

A. 80% ETHANOL-H_2O; 0.047 M CHES; μ = 0.047 $NaClO_4$.

B. 5×10^{-4}M Co^{2+}:3 OR Co^{2+}:4; 5×10^{-5}M 6.

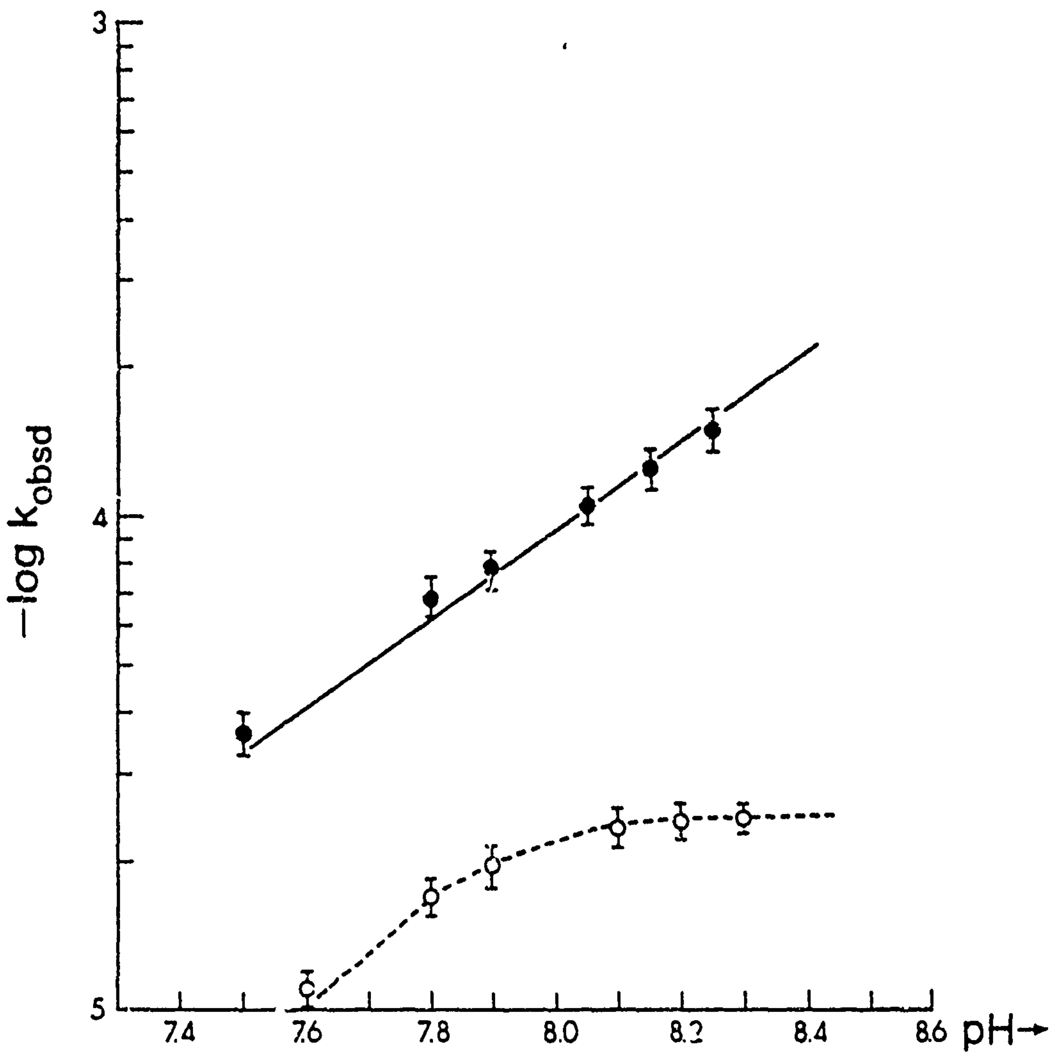


FIGURE 1. PLOT OF LOG K_{OBSD} VS PH FOR THE SOLVOLYSIS OF PHOSPHONATE 6 IN THE PRESENCE OF 5×10^{-4}M Co^{2+}:3 (○) OR Co^{2+}:4 (●). DATA OF TABLE 1.

TABLE 2. PSEUDO-FIRST-ORDER RATE CONSTANTS FOR THE HYDROLYSIS OF PHOSPHATE 5 CATALYSED BY Co^{2+}, Co^{2+}:3 AND Co^{2+}:4 FOLLOWED AT 305 NM AT 37.1°C.[A]

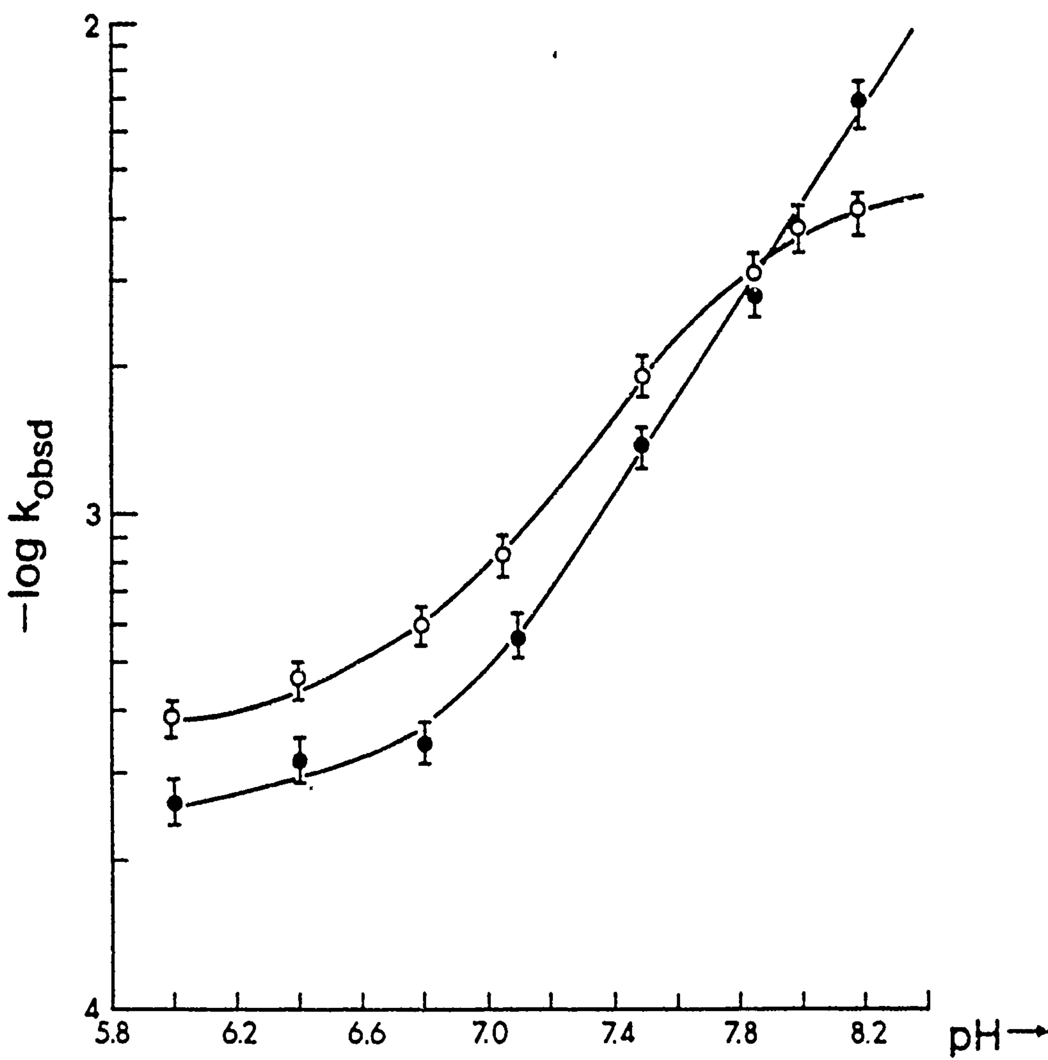
$k_{obsd} \times 10^4 s^{-1}$

pH	Co^{2+} [B]	Co^{2+}:3 [C]	Co^{2+}:4 [C]
5.4	4.52	-	-
5.8	4.19	-	-
6.0	4.55	3.82	2.66
6.4	4.37	4.55	3.17
6.8	5.02	5.93	3.44
7.1	6.31	8.28	5.61
7.45	13.1	19.0	13.7
7.85	20.0	30.4	27.0
8.1	-	38.4	-
8.15	-	41.1	68.4

A. 0.047 MOPS, pH 5.4-7.1; 0.047 CHES, pH > 7.1 80% ETHANOL H_2O

B. 4.93×10^{-4}M $CoCl_2$.

C. 4.84×10^{-4}M COMPLEX.



<u>Figure 2</u>. A plot of k_{OBSD} vs pH for the solvolysis of <u>tris</u>-2-pyridylphosphate (<u>5</u>) catalysed by Co^{2+}:<u>3</u> and Co^{2+}:<u>4</u>. Error bars represent deviations from the average of duplicate-triplicate determinations.

Discussion:

Uncatalysed, the hydrolysis of 6 is a very slow process, the second-order rate constant for OH^- attack being ~ $4 \times 10^{-2} M^{-1} s^{-1}$.[7] Hence at pH 7-8, the pseudo-first-order rate constant for spontaneous hydrolysis of 6 is ~ 4×10^{-9} - $4 \times 10^{-8} s^{-1}$. Both Co^{2+}:3 and Co^{2+}:4 facilitate the solvolysis significantly, and in a fashion which is dependent upon a basic form generated by deprotonation of the complex. However, from the shape of the pH vs rate constant profiles given [illegible]re 1, the mechanisms of catalysis are different. The same appears to be true for the solovolysis of tris-2-pyridylphosphate 5. Figure 2 shows that the process catalysed by Co^{2+}:3 is dependent upon ionization of a group associated with the complex having a pK_a ~ 8. However, catalysis afforded by Co^{2+}:4 increases linearly with pH up to at least pH 8.3, above which precipitation of the complex is observed.

We believe the data can best be accommodated by the processes shown in equations 2 and 3. Co^{2+}:3 probably reacts through the metal-bound OH^- as was found in a previous study.[5] Plots of k_{obsd} vs [Co^{2+}:3] or [Co^{2+}:4] for the solvolysis of either 5 or 6 at each pH value were stricly linear and showed no evidence for a pre-equilibrium complex. Quantitative monitoring of the solvolysis of 5 catalysed by Co^{2+}:3 or Co^{2+}:4 indicates only one molecule of 2-pyridinol is formed.

The kinetics of the solvolysis of 5 in the presence of equimolar Co^{2+}:4 adhere strictly to a pseudo-first-order rate law. This indicates that the [Co^{2+}:4] is invarient, and that Co^{2+}:4 is indeed a true catalyst. Most likely the mechanism of catalysis is general base, without the involvement of a long-lived covalent intermediate.

2.

Co^{2+}:3
X = 4,5-diisopropylimidazol-2-yl

$pK_a \sim 8$

5 → Co^{2+}:3 + (2-pyridyl-O)$_2$P(O)O$^-$ + 2-hydroxypyridine

6 → Co^{2+}:3 + EtO-P(O)(CH$_3$)-O$^-$ + $^-$O-C$_6$H$_4$-NO$_2$

3.

Co^{2+}:4
X = 4,5-diisopropylimidazol-2-yl

$pK_a > 8.3$

5 → Co^{2+}:4 + (2-pyridyl-O)$_2$P(O)O$^-$ + 2-hydroxypyridine

6 → Co^{2+}:4 + EtO-P(O)(CH$_3$)-O$^-$ + $^-$O-C$_6$H$_4$-NO$_2$

CONCLUSION:

Co^{2+}:4 IS ONE OF THE MORE EFFECTIVE REPORTED CATALYSTS REPORTED FOR THE BREAKDOWN OF PHOSPHONATE 6 UNDER HYDROLYTIC CONDITIONS. IT IS ROUGHLY AS EFFECTIVE AS 2-PAM. AT pH 8.3 80% ETHANOL-H_2O, T = 37°C THE SECOND ORDER RATE CONSTANT FOR CATALYTIC SOLVOLYSIS OF 6 BY Co^{2+}:4 IS $30.3 \times 10^{-2} M^{-1} s^{-1}$, EVEN THOUGH THE ACTIVE BASIC FORM IS NOT COMPLETELY GENERATED. BY WAY OF COMPARISON, REPORTED SECOND ORDER RATE CONSTANTS FOR OTHER SPECIES IN H_2O, T = 25°C ARE:

OH^- , $4 \times 10^{-2} M^{-1} s^{-1}$[7]

$(TME)_2Co^{3+}(OH^-)(H_2O)$, $5 \times 10^{-2} M^{-1} s^{-1}$[8]

$(TN)_2Co^{3+}(OH^-)(H_2O)$, $0.4 \times 10^{-2} M^{-1} s^{-1}$[8]

$(BPY)_2Co^{3+}(OH^-)_2$, $0.01\ M^{-1} s^{-1}$[8]

2-PAM , $23. \times 10^{-2} M^{-1} s^{-1}$[9]

ACKNOWLEDGEMENT: THE AUTHORS GRATEFULLY ACKNOWLEDGE THE FINANCIAL CONTRIBUTION OF THE UNITED STATES ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND (CONTRACT # DAMD-17-83-C-3091)

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CHARACTERISTICS OF HIGH AFFINITY CHOLINE UPTAKE INHIBITION BY A NEW HEMICHOLINIUM-3 ANALOG

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The characteristics of Na^+-dependent high affinity choline uptake inhibition in rat brain caudate preparations by hemicholinium-3 (HC-3) and its new analog, N-methyl 4-methyl piperidine derivative (A_5), were studied and compared. HC-3 is a potent inhibitor of choline uptake with a Ki value of 20 nM while the inhibitory potency of A_5 is ten times higher than HC-3. This inhibition of choline uptake by HC-3 and A_5 is reversible since washing of the drug treated caudate synaptosomal preparations with drug free buffer restores normal uptake of choline. However, the characteristics of the uptake inhibition by these two drugs are kinetically distinguishable. While HC-3 inhibits choline uptake competitively, A_5 inhibition is noncompetitive in nature. The apparent Km value of choline uptake increased from the control value of 1.4 ± 0.06 μM in the presence of 10nM HC-3, while 1 nM A_5 did not significantly affect the Km value (1.4 ± 0.09 μM). In contrast, the control V_{max} of 107 ± 12 pmol/4 min/mg protein changed to 87 ± 7 pmole/4 min/mg protein in the presence of 1 nM of A_5, while in the presence of 10 nM HC-3 the V_{max} (112 ± 8 pmol/4 min/mg protein) remained unaffected. To further characterize this difference in the mechanism of action of these two drugs, the caudate membrane preparations were labelled with [^{3}H]HC-3 (Sp. Activity 130-140 Ci/mmol) which is supposed to label the choline carrier sites in this membrane. [^{3}H]HC-3 binds with this membrane in the presence of NaCl in a saturable and reversible manner. Preliminary studies with saturation analysis indicate at least two binding sites for [^{3}H]HC-3. One is a high affinity low capacity binding site with K_d and B_{max} values of 3 nM and 80 fmol/mg protein respectively and other is a low affinity and high capacity binding site with K_d and B_{max} values of 25 nM and 400 fmol/mg protein respectively. The affinity of [^{3}H]HC-3 for the low affinity sites corresponds to its Ki for choline uptake inhibition.

In addition, displacement of [^{3}H]HC-3 from its binding sites by HC-3 and A_5 was studied. At 1 and 12 nM concentrations of [^{3}H]HC-3, the IC_{50} value for HC-3 was 8 nM and 30 nM respectively but the IC_{50} value for A_5 did not change markedly (2.5 nM to 3.5 nM) with the 12 fold change in [^{3}H]HC-3 concentrations.

These results suggest that (1) the low affinity [^{3}H]HC-3 binding sites may be choline uptake sites and (2) the mechanisms of choline uptake inhibition by HC-3 and A_5 are different. This work supported in part by the U.S. Army Medical Research and Development Command under Contract DAMD-17-83-C-3010.

A-5

METHODS

SODIUM DEPENDENT HIGH AFFINITY CHOLINE UPTAKE STUDY

CAUDATE TISSUE WAS HOMOGENIZED IN 0.32 M SUCROSE IN GLASS-TEFLON HOMOGENIZER AND THE HOMOGENATE WAS CENTRIFUGED AT 1000 x g FOR 10 MINUTES. THE RESULTING SUPERNATANT WAS RECENTRIFUGED AT 17,000 x g FOR 15 MINUTES AND THE PELLET WAS RESUSPENDED IN ORGINAL VOLUME OF 0.32 M SUCROSE AND USED AS A SOURCE OF SYNAPTOSOMES.

CHOLINE UPTAKE WAS STUDIED IN CAUDATE SYNAPTOSOMAL PREPARATIONS AT 37°C IN KREBS-RINGER PHOSPHATE MEDIA OF pH 7.4. FOR SODIUM INDEPENDENT UPTAKE, NA^{+} ION IN THE REACTION MEDIUM WAS REPLACED BY EQUIOSMOLAR AMOUNT OF SUCROSE. 100 uL OF SYNAPTOSOMAL SUSPENSION IN 890 uL OF APPROPRIATE MEDIUM WAS PREINCUBATED FOR 5 MINUTES. THE REACTION WAS INITIATED BY THE ADDITION OF 10 uL OF [^{14}C]CHOLINE (NEN, 50.5 CI/mMOL) AND TERMINATED 4 MINUTES LATER BY RAPID FILTRATION OVER MILLIPORE FILTER, FOLLOWED BY TWO 4 ml WASHES WITH COLD BUFFER.

SATURATION AND COMPETITIVE BINDING STUDY WITH [^{3}H]HC-3

CAUDATE TISSUE WAS HOMOGENIZED IN 10 mM NA^{+}/K^{+} PHOSPHATE BUFFER pH 7.4 (1:50 w/v) WITH POLYTRON FOR 20 SECONDS AT SETTING SEVEN. THE HOMOGENATE WAS CENTRIFUGED AT 50,000 x g FOR 10 MINUTES AND THE PELLET WAS WASHED TWO TIMES BY RESUSPENSION AND CENTRIFUGATION AT 50,000 x g BEFORE FINAL SUSPENSION IN 10 mM NA^{+}/K^{+} BUFFER pH 7.4 CONTAINING 150 mM NACL.

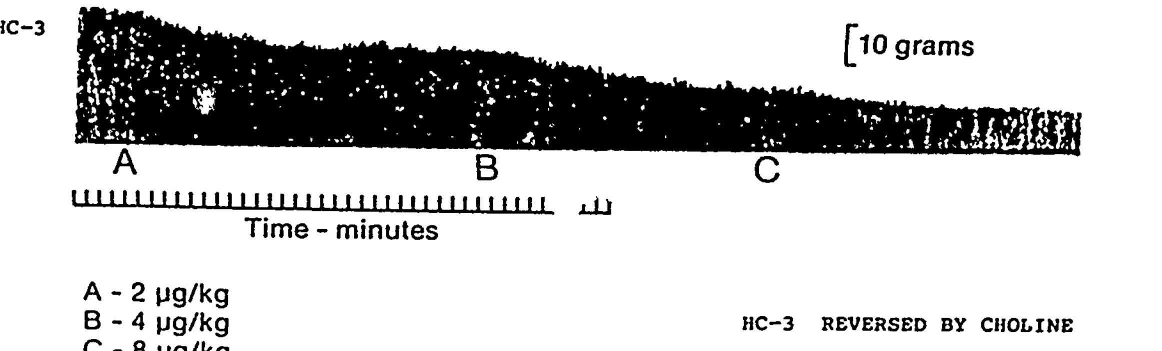
FOR [^{3}H]HC-3 (NEN, 134 CI/mMOL) BINDING 500 uL OF THE REACTION MIXTURE CONTAINING 0.5 mg OF TISSUE PROTEIN AND 20 uL [^{3}H]HC-3, WAS INCUBATED AT 25°C FOR 20 MINUTES AND RAPIDLY FILTERED THEREAFTER OVER GF/B FILTER FOLLOWED BY TWO 5 ml RINSES WITH COLD BUFFER. NONSPECIFIC BINDING WAS ESTIMATED IN PRESENCE OF 1 uM COLD HC-3.

INTRODUCTION

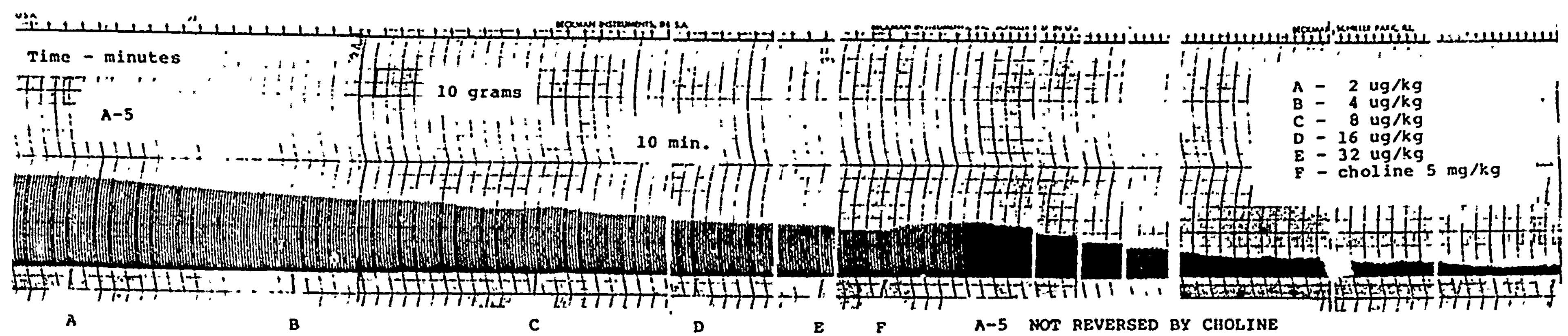
CHOLINERGIC NERVE TERMINALS HAVE A UNIQUE ABILITY TO ACCUMULATE CHOLINE INSIDE NERVE TERMINALS VIA A SODIUM DEPENDENT HIGH AFFINITY UPTAKE SYSTEM. HEMICOLINIUM-3 (HC-3), AN EFFECTIVE BLOCKER OF NEUROMUSCULAR TRANSMISSION, WAS SHOWN TO ACT VIA AN INHIBITION OF THIS UPTAKE SYSTEM. WE HAVE RECENTLY SYNTHESIZED AND EVALUATED A SERIES OF PIPERIDINO ANALOGS OF HC-3 AND FOUND ONE OF THESE ANALOGS 4,4'-BIS[1-HYDROXY-2(4-METHLY PIPERIDINO) ETHYL] BIPHENYL DIMETHOBROMIDE (A-5) TO BE EVEN MORE POTENT THAN HC-3 IN THE INHIBITION OF NEUROMUSCULAR TRANSMISSION AND UNLIKE HC-3, IT APPEARED TO BE POORLY REVERSED BY CHOLINE. WE, THEREFORE, EVALUATED THE POTENCIES AND KINETIC PROPERTIES OF A-5 AND HC-3 FOR THE INHIBITION OF SODIUM DEPENDENT HIGH AFFINITY CHOLINE UPTAKE IN RAT BRAIN CAUDATE SYNAPTOSOMAL PREPARATIONS. WE ALSO TESTED THE INTERACTIONS OF A-5 WITH SITES LABELLED WITH [^{3}H]HC-3 IN RAT BRAIN CAUDATE MEMBRANE PREPARATIONS SINCE HC-3 HAS BEEN POSTULATED TO BIND TO CHOLINE CARRIER SITES.

FIGURE 1

THE NEUROMUSCULAR BLOCKING ACTION OF HC-3 AND A-5 ON RABBIT SCIATIC - GASTROCNEMIUS PREPARATION



HC-3 REVERSED BY CHOLINE



A-5 NOT REVERSED BY CHOLINE

STIMULATION PARAMETERS: ONCE EVERY 10 SECONDS BY 200 Hz FOR 0.2 SEC., PULSE DURATION 0.2 ms., SUPRAMAXIMAL VOLTAGE

FIGURE 2

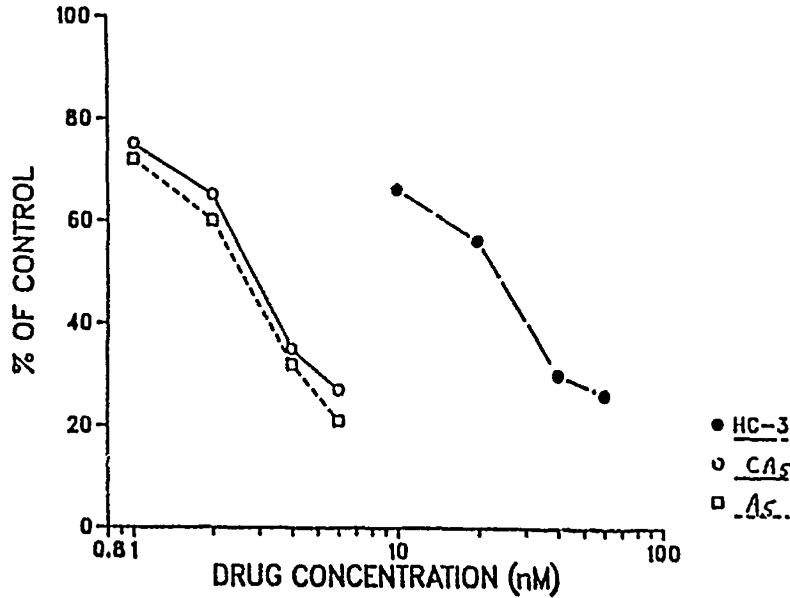


FIGURE 3

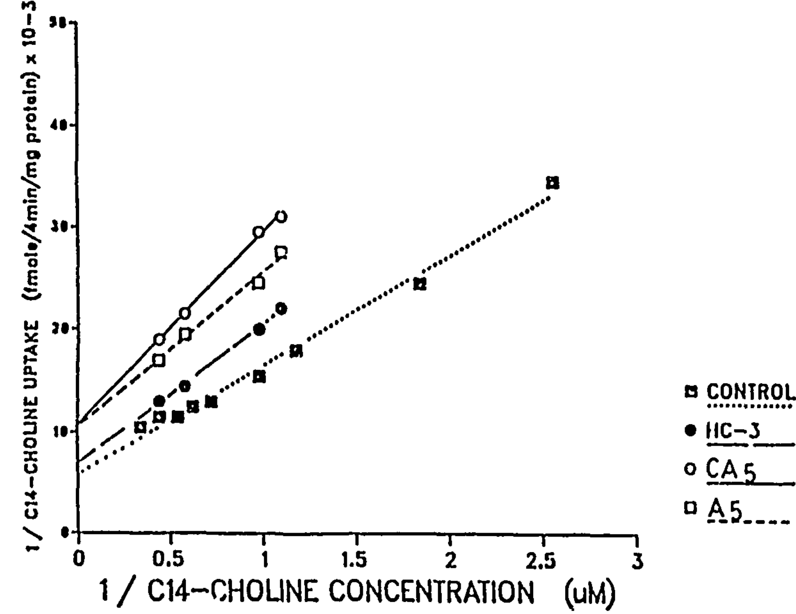


FIGURE 4

NaCl DEPENDENCE OF [^{3}H]HC-3 BINDING IN RAT BRAIN CAUDATE PREPARATIONS AT 25°C

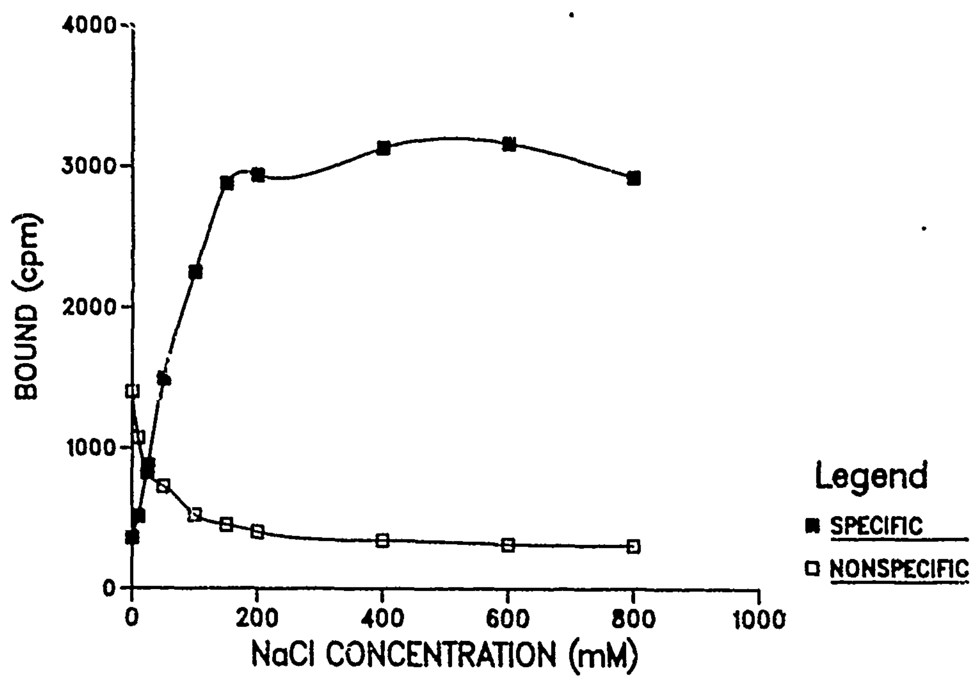


FIGURE 5

SCATCHARD PLOT OF [^{3}H]HC-3 BINDING IN RAT BRAIN CAUDATE PREPARATIONS AT 25°C

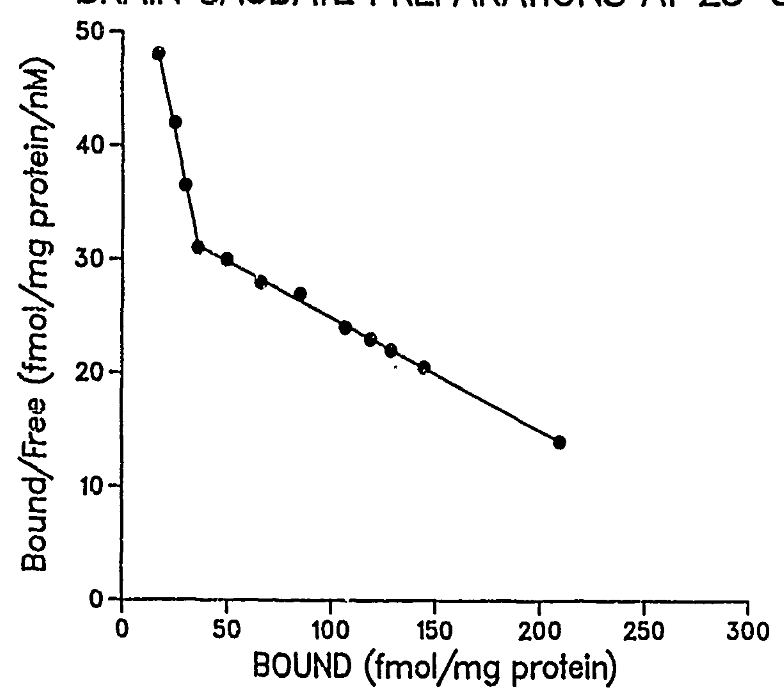


FIGURE 6

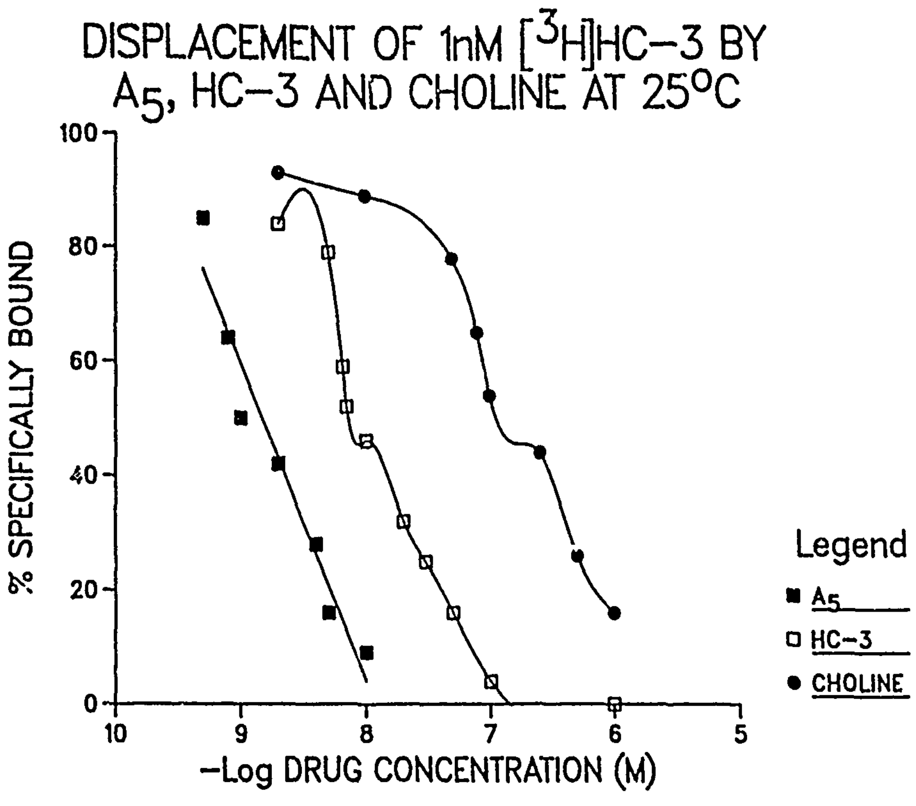


FIGURE 7

DISPLACEMENT OF 12nM [^{3}H]HC-3 BY A_5
HC-3 AND CHOLINE AT 25°C

% SPECIFICALLY BOUND

100
80
60
40
20
0

10
9
8
7
6
5

-Log DRUG CONCENTRATION (M)

Legend
A_5
HC-3
CHOLINE

TABLE 1

EFFECT OF HEMICHOLINIUM-3 AND A-5 ON Km AND Vmax VALUES FOR SODIUM DEPENDENT HIGH AFFINITY CHOLINE UPTAKE IN RAT CAUDATE SYNAPTOSOMAL PREPARATIONS

	Km (uM)	Vmax (pMOLE/4MIN/mg PROTEIN)
CONTROL (n = 3)	1.4 ± 0.06	107 ± 12
HEMICHOLINIUM-3 (n = 3)	2.5 ± 0.20*	112 ± 8
A-5 (n = 3)	1.4 ± 0.09	87 ± 7*

* SIGNIFICANTLY DIFFERENT FROM CONTROL, $p < 0.05$

TABLE 2

REVERSAL OF A-5 AND HC-3 INHIBITION OF SODIUM DEPENDENT HIGH AFFINITY CHOLINE UPTAKE BY [illegible]

ADDITION	Na+ DEPENDENT [C14]CHOLINE UPTAKE (pMOL/4min/mg PROTEIN)	
	BEFORE WASHING	AFTER WASHING
NONE	29.5	26.2
A-5	ND	27.8
HC-3	ND	27.3

SYNAPTOSOMAL SUSPENSIONS WERE PREINCUBATED WITHOUT OR WITH 0.1uM OF EITHER A-5 OR HC-3 AND AFTER 5 MINUTE OF PREINCUBATION [C14]CHOLINE UPTAKE WAS MEASURED BEFORE AND AFTER THREE WASHINGS WITH 0.32M SUCROSE (1:100 w/v)

TABLE 3

IC50 VALUES AND HILL-COFFICIENT (η H) OF [3H]HC-3 DISPLACEMENT BY A-5 AND COLD HC-3 AT 25°C

	1nM [H3] HC-3		12nM [H3] HC-3	
DRUG	IC50 (nM)	η H	IC50 (nM)	η H
A-5	2.5	1.2	3.5	1.2
HC-3	8.0	0.87	30.0	0.91

SUMMARY

1. BOTH A-5 AND HC-3 POTENTLY BLOCK CHOLINERGIC NEUROTRANSMISSION IN RABBIT NEUROMUSCULAR PREPARATIONS (FIGURE 1).

2. HC-3 BLOCK OF NEUROMUSCULAR TRANSMISSION IS READILY REVERSIBLE BY CHOLINE WHILE A-5 ACTION IS INSENSITIVE TO CHOLINE ADMINISTRATION (FIGURE 1).

3. BOTH A-5 AND HC-3 POTENTLY INHIBIT SODIUM DEPENDENT HIGH AFFINITY CHOLINE UPTAKE IN RAT BRAIN CAUDATE SYNAPTOSOMAL PREPARATIONS AND IN THIS SYSTEM A-5 APPEARS TEN TIMES MORE POTENT THAN HC-3 (FIGURE 2).

4. THE CHARACTERISTICS OF A-5 AND HC-3 INHIBITION OF SODIUM DEPENDENT HIGH AFFINITY CHOLINE UPTAKE ARE KINETICALLY DISTINGUISHABLE (FIGURE 3 AND TABLE 1).

5. A-5 AND HC-3 INHIBITION OF CHOLINE UPTAKE IS REVERSIBLE SINCE WASHING OF THE DRUG TREATED SYNAPTOSOMES WITH DRUG FREE BUFFER RESTORES NORMAL CHOLINE UPTAKE (TABLE 2).

6. BOTH [^{3}H]HC-3 BINDING WITH RAT CAUDATE MEMBRANE PREPARATION AND HIGH AFFINITY CHOLINE UPTAKE ARE SODIUM DEPENDENT (FIGURE 4).

7. [^{3}H]HC-3 LABELS AT LEAST TWO SITES IN THIS MEMBRANE PREPARATION; ONE WITH HIGH AFFINITY AND LOW CAPACITY AND OTHER WITH LOW AFFINITY AND HIGH CAPACITY (FIGURE 5). THE K_d FOR HC-3 BINDING AT LOW AFFINITY SITES CORRESPONDS TO ITS K_i FOR CHOLINE UPTAKE INHIBITION.

8. COMPETITIVE BINDING EXPERIMENTS WITH COLD HC-3/[^{3}H]HC-3 AND A-5/[^{3}H]HC-3 REVEAL THAT A-5 AND HC-3 INTERACTION WITH [^{3}H]HC-3 BINDING SITE IS COMPLEX, HAVING PSEUDO-HILL COEFFICIENTS FOR DISPLACEMENT OF 0.87 AND 1.2 FOR HC-3 AND A-5 RESPECTIVELY. AT 1nM [^{3}H]HC-3 CONCENTRATION, IC_{50} OF HC-3 AND A-5 WERE 8 AND 2.5nM RESPECTIVELY (FIGURE 6 AND TABLE 3) WHILE AT 12nM [^{3}H]HC-3 CONCENTRATION THESE VALUES WERE 30 AND 3.5nM RESPECTIVELY (FIGURE 7 AND TABLE 3).

CONCLUSIONS

1. A-5 IS MORE POTENT THAN HC-3 IN THE INHIBITION OF SODIUM-DEPENDENT HIGH AFFINITY CHOLINE UPTAKE.

2. A-5 INHIBITION OF CHOLINE UPTAKE IS NONCOMPETITIVE IN NATURE WHILE HC-3 INHIBITION IS COMPETITIVE.

3. PSEUDO-HILL COEFFICIENTS FOR A-5 AND HC-3 COMPETITION FOR [^{3}H] HC-3 BINDING SITES SUGGEST THAT WHILE HC-3 BINDING INDUCES HOMOTROPIC NEGATIVE COOPERATIVE SITE-SITE INTERACTION, A-5 MIGHT INDUCE HETEROTROPIC CO-OPERATIVE PHENOMENA.

4. LOW AFFINITY [^{3}H]HC-3 BINDING SITES MIGHT BE REPRESENTATIVE OF ACTIVE CHOLINE UPTAKE SITES.

NONQUATERNARY CHOLINESTERASE REACTIVATORS

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ABSTRACT

IN THE SEARCH FOR IMPROVED LIPOPHILIC CENTRALLY ACTIVE ACETYLCHLINESTERASE (AChE) ANTIDOTES, A SERIES OF NEW α-KETOTHIOHYDROXIMATES WAS PREPARED AND EVALUATED FOR THEIR ABILITY TO REACTIVATE AChEs INHIBITED BY ETHYL P-NITROPHENYL METHYLPHOSPHONATE (EPMP) AND SOMAN (GD). THE COMPOUNDS CONFORM TO THE GENERAL STRUCTURE 1 :

$$R-C_6H_4-C(O)C(NOH)S(CH_2)_N N^+R'_2 R'' \cdot X^-$$

WHERE R = H, CH_3, F, Br, Cl, OCH_3, CN; R' = CH_3, C_2H_5; R'' = H, CH_3; X = Cl, I; N = 2, 3. IN THIS SERIES, VARYING R SUBSTITUENTS ON THE ARYL RING PRODUCED COMPOUNDS WITH OXIME pK_a VALUES FROM 6.3 TO 8.0 OPTIMUM FOR AN AChE REACTIVATOR. INCREASING LIPOPHILICITY OF THE AMINE SEGMENT CORRELATED WITH REACTIVATOR POTENCY, AS DID ARYL SUBSTITUTED ELECTRON-WITHDRAWING GROUPS, PRESUMABLY DUE TO INCREASED BINDING TO HYDROPHOBIC SITES SURROUNDING THE AChE ACTIVE SITE. THE IN VITRO REACTIVATION POTENCY OF TYPE 1 COMPOUNDS APPROACHES AND EVEN SURPASSES THAT OF 2-PAM AND TOXOGONIN FOR GD-INHIBITED AChE. THESE INITIAL FINDINGS POINT TO ADDITIONAL STRUCTURE-ACTIVITY RELATIONSHIPS TO ASSIST US IN THE DESIGN OF IMPROVED ANTIDOTAL COMPOUNDS.

OBJECTIVES AND APPROACH

OBJECTIVES

- DEVELOPMENT OF NEW DRUGS AS ADJUNCTS TO OR REPLACEMENTS FOR CONVENTIONAL PYRIDINIUM OXIMES

GENERAL APPROACH

- SYNTHESIS AND IN VITRO EVALUATION OF NEW COMPOUNDS
- DETERMINATION OF STRUCTURE-ACTIVITY RELATIONSHIPS

SPECIFIC APPROACH

- NONQUATERNARY OXIMES AS AChE REACTIVATORS

BACKGROUND

- NONQUATERNARY REACTIVATORS SHOULD BE SUPERIOR TO PYRIDINIUM OXIMES AND WE WISHED TO DESIGN NOVEL COMPOUNDS BASED ON THIS PREMISE

- TO BE A POTENT REACTIVATOR, ANY COMPOUND MUST MEET THE FOLLOWING REQUIREMENTS:

1. NUCLEOPHILICITY

   a. $\text{RCHNOH} \underset{Ka}{\rightleftharpoons} \text{RCHNO}^- + \text{H}^+$

      OXIME → OXIMATE

   b. ONLY OXIMATE IS A GOOD NUCLEOPHILE

   c. EMPIRICALLY, WE KNOW THAT THERE IS AN OPTIMUM pK_a (= 8) FOR GOOD REACTIVATORS

2. AFFINITY FOR ChE ACTIVE SITE

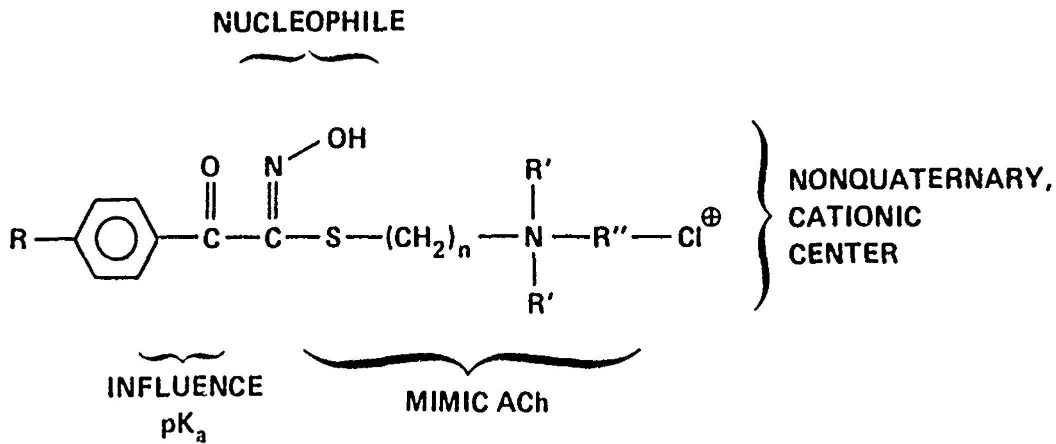
   a. MIMICK SUBSTRATE

   b. COULOMBIC EFFECTS (POSITIVELY CHARGED MOIETY)

   c. HYDROPHOBIC AND STERIC CONSIDERATIONS

CHEMISTRY

- WE COMBINED THE MOLECULAR REQUIREMENTS FOR NON-QUATERNARY REACTIVATORS INTO THE FOLLOWING GENERAL STRUCTURE:



WHERE $R = H, CH_3, F, Br, Cl, OCH_3$; $R' = CH_3, C_2H_5$; $R'' = H, CH_3$;
$n = 2\ 3$

SYNTHESIS OF COMPOUNDS

THE SYNTHESIS OF THESE α-KETOTHIOHYDROXIMATES WAS ACCOMPLISHED VIA TWO GENERAL ROUTES. FURTHER DETAILS ARE AVAILABLE UPON REQUEST.

NONQUATERNARY OXIMES-METHOD A:

R-C$_6$H$_4$-C(O)CH$_3$ $\xrightarrow[\text{HCl}]{\text{i-PrONO}}$ R-C$_6$H$_4$-C(O)C(NOH)Cl

+ HS(CH$_2$)$_n$NR'$_2$ ⟶ R-C$_6$H$_4$-C(O)C(NOH)S(CH$_2$)$_n$NR'$_2$

METHOD B:

R-C$_6$H$_4$-C(O)CH$_3$ $\xrightarrow{\text{Br}_2/\text{CCl}_4}$ R-C$_6$H$_4$-C(O)CH$_2$Br

+ HS(CH$_2$)$_n$NR'$_2$ ⟶ R-C$_6$H$_4$-C(O)CH$_2$S(CH$_2$)$_n$NR'$_2$

$\xrightarrow[\text{Base}]{\text{i-PrONO}}$ R-C$_6$H$_4$-C(O)C(NOH)S(CH$_2$)$_n$NR'$_2$

QUATERNIZATION:

R-C$_6$H$_4$-C(O)C(NOH)S(CH$_2$)$_n$NR'$_2$ $\xrightarrow{\text{R}'' \text{X}}$

R-C$_6$H$_4$-C(O)C(NOH)S(CH$_2$)$_n$N$^+$ R'$_2$ R'' · X$^-$

JA-4614-119

RELEVANT COMPOUNDS

ETHYL P-NITROPHENYL METHYLPHOSPHONATE (EPMP)

$$CH_3-P(=O)(OC_2H_5)-O-C_6H_4-NO_2$$

SOMAN (GD)

$$F-P(=O)(CH_3)-OCH(CH_3)C(CH_3)_3$$

2-PAM

$$\text{1-}CH_3\text{-pyridinium-2-}CHNOH \cdot Cl^-$$

HI-6

$$[C(O)NH_2\text{-4-pyridinium-1-}CH_2-O-CH_2\text{-1-pyridinium-2-}CHNOH] \cdot 2Cl^-$$

TOXOGONIN

$$[CHNOH\text{-4-pyridinium-1-}CH_2-O-CH_2\text{-1-pyridinium-4-}CHNOH] \cdot 2Cl^-$$

JA-4614-120

RESULTS

- ACIDITY:

  1. α-KETOTHIOHYDROXIMATE ACID-DISSOCIATION CONSTANTS (pK_a) CORRELATE WITH σ_p HAMMETT VALUES.

     WE FIND: $pK_a = 7.42 (\pm 0.04) - 1.22 (\pm 0.18) \sigma$

  2. ALL α-KETOTHIOHYDROXIMATES HAVE pK_a VALUES BETWEEN 6.8 AND 8.0.

- LIPOPHILICITY:

  1. THE OCTANOL-BUFFER PARTITION COEFFICIENTS (LOG P) FOR TYPE 1 COMPOUNDS RANGE OVER FIVE-ORDERS OF MAGNITUDE.

     WE FIND LOG Ps FROM -3.00 TO +2.00

  2. WITHIN EACH FAMILY OF DIALKYLAMINOALKYL OR THEIR QUATERNARY DERIVATIVES: INCREASING THE ELECTRON-WITHDRAWING CHARACTER OF THE BENZOYL SUBSTITUENT GENERALLY INCREASES LOG P VALUES. [IT IS OF INTEREST TO NOTE THAT THIS TREND IS ALSO OBSERVED WITH k_{HOX} VALUES AND % R_{MAX} VALUES DESCRIBED BELOW.]

RESULTS

- INHIBITION:

TO CORRECT FOR REVERSIBLE INHIBITION BY DRUG IN AChE ASSAY AND TO PROBE INTERACTIONS BETWEEN TEST COMPOUNDS AND ENZYME ACTIVE SITES WE DETERMINE REVERSIBLE AChE INHIBITION; REPORTED AS THE CONCENTRATION OF DRUG REQUIRED TO INHIBIT 50% OF THE ENZYME, IC_{50}

WE FIND THE α-KETOTHIOHYDROXIMATES ARE NOT VERY INHIBITORY: $IC_{50} > 1.0$ mM

- NUCLEOPHILICITY:

HYDROLYSIS OF PARA-NITROPHENYLACETATE AND AcSCh IS CATALYZED BY α-KETOTHIOHYDROXIMATES ACCORDING TO

$$\text{RATE} = [\text{OX}]\ [\text{SUBSTRATE}]\ k_n$$

k_n SHOULD CORRELATE WITH pK_a (BRONSTED RELATIONSHIP)

WE FIND: $\text{Log}(k_n) = -3.04\ (\pm 0.34) + 0.69\ (\pm 0.08)\ pK_a$ (p-Nitro)

: $\text{Log}(k_n) = -3.74\ (\pm 0.96) + 0.70\ (\pm 0.12)\ pK_a$ (AcSCh)

PROPERTIES OF α-KETOTHIOHYDROXIMATES

R —⟨phenyl⟩— $C(O)C(NOH)S(CH_2)_2N^+R'_2R'' \cdot X^-$

R	R'	R''	X	pK_a	LOG P[a]	IC_{50}, mM	EPMP[b]		GD
							k_{OX}	k_{HOX}	% R_{max}
H	C_2H_5	H	Cl	7.60	....	> 1.0	....	....	2.2
Br	CH_3	H	Cl	7.05	1.70	0.71	....	....	14.6
Br	C_2H_5	H	Cl	6.95	2.41	0.21	97.6	57.7	35.0
Br	CH_3	CH_3	I	7.18	-0.55	> 1.0	108	78.0	51.8
Br	CH_3	H	Cl	7.16	....	> 1.0	....	....	4.6
CH_3O	CH_3	H	Cl	7.88	1.07	> 1.0	35.7	11.9	0.0
CH_3O	C_2H_5	H	Cl	7.65	1.38	> 1.0	77.0	36.3	0.0
CH_3O	CH_3	CH_3	Cl	7.71	-1.96	> 1.0	53.7	23.4	5.6
CH_3O	C_2H_5	CH_3	Cl	7.82	-1.60	> 1.0	73.2	32.4	8.5
Cl	CH_3	H	Cl	7.00	1.74	> 1.0	48.5	38.8	8.0
Cl	C_2H_5	H	Cl	6.80	1.97	0.52	52.2	45.0	28.6
Cl	C_2H_5	CH_3	Cl	7.17	-0.42	> 1.0	203	148	59.4
F	CH_3	CH_3	Cl	7.73	-1.55	> 1.0	134	57.1	....
F	C_2H_5	CH_3	Cl	7.31	-1.17	0.72	251	166	....
CH_3	CH_3	H	Cl	7.76	1.52	> 1.0	93.7	38.6	....
CH_3	CH_3	CH_3	Cl	7.57	-1.44	> 1.0	79.3	41.0	....
	2-PAM			7.99	-3.24	> 0.5	2,470	717	28.2
	TOXOGONIN			7.54	>-3.0	> 0.5	2,600	1,400	48.6
	HI-6			7.27	>-3.0	> 0.5	7,200	4,900	79.6[c]

[a] Log P's Greater Than 3 Or -3 Are Not Accurately Determined By This Procedure.

[b] Reactivation of Human Erythrocyte RBC AChE In MOPS Buffer.

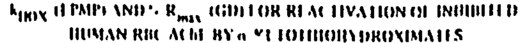
[c] GD Data Reported As Percent Maximum Reactivation At 1×10^{-3} M Except For HI-6 Which Was Determined At 1×10^{-4} M.

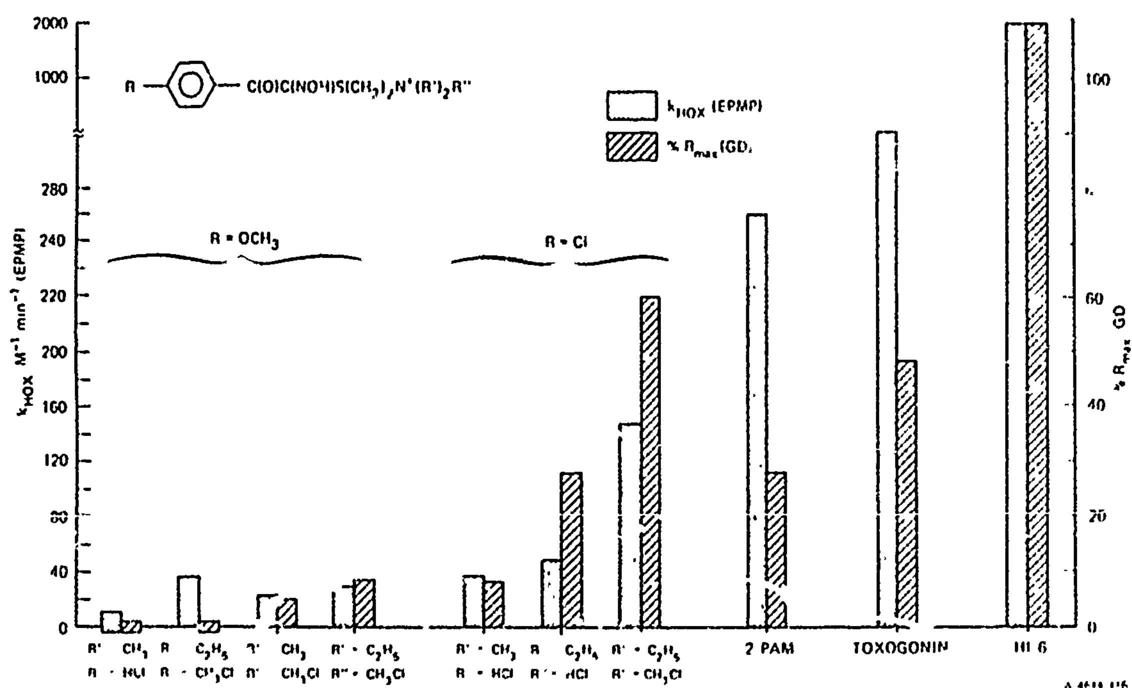
α-KETOTHIOHYDROXIMATE REACTIVATION OF ORGANOPHOSPHORUS INHIBITED EEL AND HUMAN AChE

OP COMPOUND/ENZYME	k_{OX} (RANGE)	k_{HOX} (RANGE)	2-PAM	TOXOGONIN	HI-6
NONQUATERNARY					
DFP/EEL	30-380	11-108	1,600		
EPMP/EEL	30-157	1.3-114	2,800	1,670	2,200
EPMP/HUMAN	35-90	10-60	2,470 717	2,600 1,400	7,200 4,900
[a]GD/HUMAN	0-35		28.2	48.6	79.6[b]
QUATERNARY					
EPMP/HUMAN	50-250	20-170	2,470 717	2,600 1,400	7,200 4,900
[a]GD/HUMAN	5-60		28.2	48.6	79.6[b]

[a]GD DATA REPORTED AS PERCENT MAXIMUM REACTIVATION AT 30 TO 60 MINUTES AND DRUG CONCENTRATION OF 1.0×10^{-3} M.

[b]MAXIMUM PERCENT REACTIVATION AT DRUG CONCENTRATION OF 1.0×10^{-4} M.





CONCLUSIONS

- PREPARATION OF α-KETOTHIOHYDROXIMATES IS SIMPLE AND ALLOWS FOR MANY STRUCTURAL VARIATIONS.

- pK_a AND NUCLEOPHILICITY OF α-KETOTHIOHYDROXIMATES CAN BE FINE-TUNED BY APPROPRIATE CHOICE OF SUBSTI-TUENTS.

- THE COMPOUNDS DESCRIBED HERE DO REACTIVATE A VARIETY OF INHIBITED AChE's, ALTHOUGH THEY HAVE ONLY A FRACTION OF THE IN VITRO POTENCY OF 2-PAM, TOXOGONIN AND HI-6

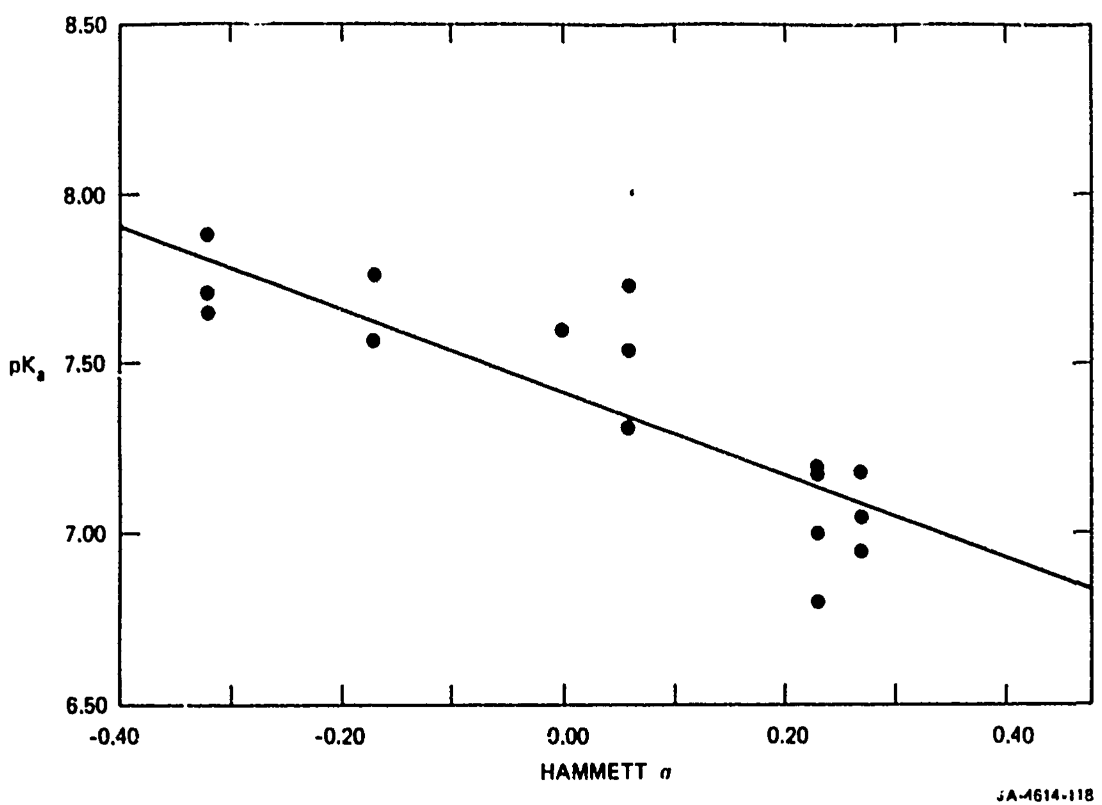
- BINDING AND ELECTRON-WITHDRAWING RING SUBSTI-TUENTS OF THESE COMPOUNDS TO LIPOPHILIC SITES ON AChE INFLUENCES RELATIVE EFFICACY AS REACTIVA-TORS.

- OUTSIDE OF pK_a ADJUSTMENT; THE DIALKYLAMINOALKYL THIOL DOES NOT ENHANCE AChE BINDING IN EITHER THE QUATERNARY OR NONQUATERNARY DRUGS STUDIED.

- ACTIVITY OF TYPE <u>1</u> COMPOUNDS AS REACTIVATORS OF GD-INHIBITED HUMAN RBC AChE IS HIGHLY DEPENDENT ON STRUCTURE

- SEVERAL OF THE TEST COMPOUNDS SURPASS THE IN VITRO POTENCY OF 2-PAM AND TOXOGONIN TOWARD GD-INHIBITED AChE.

- THIS SYSTEMATIC EVALUATION OF TYPE <u>1</u> COMPOUNDS HAS PROVIDED SIGNIFICANT INSIGHTS INTO THE MOLECULAR REQUIREMENTS FOR REACTIVATION OF OP-INHIBITED AChE, AND MAY RESULT IN USEFUL NEW DRUGS FOR THE TREATMENT OF ORGANOPHOSPHONATE POISONING.


8.50
8.00
pK$_a$ 7.50
7.00
6.50
-0.40
-0.20
0.00
0.20
0.40
HAMMETT σ
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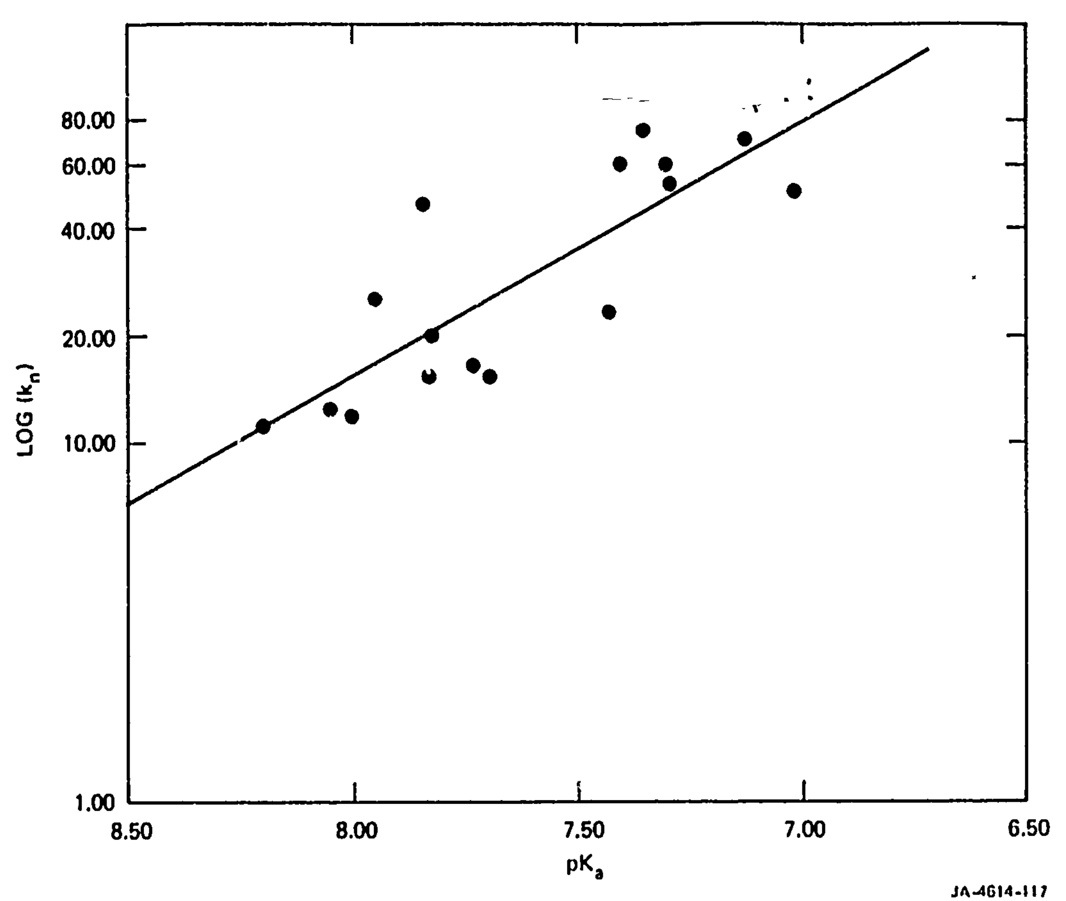
80.00
60.00
40.00
20.00
10.00
1.00
LOG (k$_n$)
8.50
8.00
7.50
7.00
6.50
pK$_a$
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REACTIVATION KINETICS

- BASED ON MUCH EVIDENCE FOR PYRIDINIUM OXIMES, WE CAN WRITE THE FOLLOWING MECHANISM FOR REACTIVATION

$$EOP + OX \underset{}{\overset{K_r}{\rightleftharpoons}} [EOP \cdot OX] \xrightarrow{k_r} EOH \quad (1)$$

WHERE

EOP = INHIBITED ChE

OX = OXIMATE FORM OF REACTIVATOR

[EOP · OX] = MICHAELIS COMPLEX BETWEEN REACTIVATOR AND ENZYME

EOH = ACTIVE ENZYME

K_r = AFFINITY CONSTANT = [EOP] [OX] / [EOP · OX]

k_r = DISPLACEMENT RATE CONSTANT

REACTIVATION KINETICS

- SINCE REACTIVATOR IS ALWAYS PRESENT IN LARGE EXCESS OVER INHIBITED ENZYME, [OX] DOES NOT CHANGE. UNDER THESE CONDITIONS THE KINETICS ARE GOVERNED BY:

$$\mathrm{LOG}\ (100\text{-}\%\ \mathrm{REACTIVATION}) = k_{OBS} \cdot t$$

- WHERE THE OBSERVED RATE CONSTANT (k_{OBS}) DEPENDS ON [OX] ACCORDING TO THE DOUBLE-RECIPROCAL RELATIONSHIP

$$(k_{OBS})^{-1} = K_r/k_r\ [OX]^{-1} + 1/k_r$$

- WE DEFINE TWO FURTHER INDICATORS OF REACTIVATOR ACTIVITY AS:

$k_{OX} = k_r/K_r$ = BIMOLECULAR RATE CONSTANT FOR REACTIVATION

$k_{HOX} = k_{OX}$ (FRACTION OF REACTIVATOR IN OXIMATE FORM)

$= k_{OX}\ [1 + \mathrm{ANTILOG}\ (pK_a - 7.6)]^{-1}$

= EFFECTIVE RATE CONSTANT FOR REACTIVATION

INTRODUCTION

ORGANOPHOSPHONATES IRREVERSIBLY INHIBIT ACETYLCHOLINESTERASE (AChE) BY PHOSPHONYLATING A SERINE HYDROXYL AT THE ENZYME ACTIVE SITE. APPROPRIATELY DESIGNED NUCLEOPHILIC COMPOUNDS CAN REVERSIBLY BIND TO THE PHOSPHONYLATED AChE AND DISPLACE THE PHOSPHORUS, THEREBY RESTORING ENZYME ACTIVITY. THE USE OF PYRIDINIUM ALDOXIMES SUCH AS 2-PAM FOR CHOLINESTERASE REACTIVATION IN THE TREATMENT OF ORGANOPHOSPHORUS ESTER POISONING IS WELL-KNOWN. HOWEVER, THESE HYDROPHILIC QUATERNARY PYRIDINIUM COMPOUNDS SUFFER SERIOUS LIMITATIONS DUE TO RESTRICTED ACCESS TO ACTIVE SITES IN VIVO CAUSED BY SLOW PENETRATION OF LIPOPHILIC BIOLOGICAL MEMBRANES. WE HAVE ATTEMPTED TO OVERCOME THIS LIMITATION BY DEVELOPING NONQUATERNARY LIPOPHILIC REACTIVATORS WITH APPROPRIATE NUCLEOPHILICITY (OXIME pK_a 6.8 - 8.0) AND HIGH SPECIFICY FOR THE AChE ACTIVE SITE(S).

A SECOND PROBLEM OF IMPORTANCE IS THE DIFFICULTY IN TREATING POISONING DUE TO SOMAN (GD, 3,3-DIMETHYL-2-BUTYL METHYLPHOSPHONOFLUORIDATE). 2-PAM IS ONLY MARGINALLY EFFECTIVE IN TREATING SOMAN POISONING. THE HAGEDORN OXIMES SUCH AS HI-6 DO HAVE REASONABLE EFFICACY, BUT ARE NOT VERY DESIRABLE AS DRUGS; THEY ARE DIFFICULT TO SYNTHESIZE, UNSTABLE IN SOLUTION, AND NOT EFFECTIVE AGAINST OTHER ORGANOPHOSPHONATES.

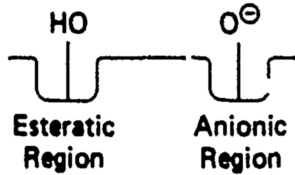
WE HAVE SYNTHESIZED A WIDE VARIETY OF NONQUATERNARY AND QUATERNARY α-KETOTHIOHYDROXIMATES, AND TESTED THEM AGAINST AChE INHIBITED BY ETHYL P-NITROPHENYL METHYLPHOSPHONATE (EPMP), A HIGHLY TOXIC ORGANOPHOSPHONATE (LD_{50} SC IN MICE 350 UG/KG).

WE HAVE ALSO MORE RECENTLY BEGUN TESTING THE α-KETOTHIOHYDROXIMATES FOR ABILITY TO REACTIVATE GD-INHIBITED AChE. SUCH EXPERIMENTS ARE COMPLICATED BY THE VERY RAPID DEALKYLATION ("AGING") OF ATHE INHIBITED ENZYME TO A NON-REACTIVATABLE FORM.

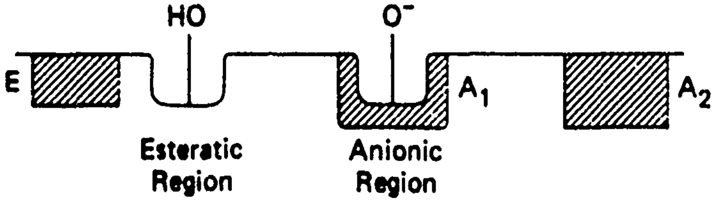
THE SYNTHETIC ROUTE TO THE α-KETOTHIOHYDROXIMATES AND THE RESULTS OF THE VARIOUS TESTS OF THEIR IN VITRO EFFECTS ARE REPORTED HERE.

INTRODUCTION

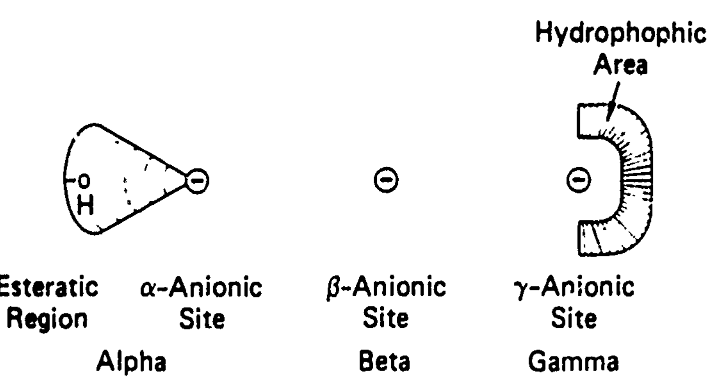
BELOW ARE SEVERAL MODELS OF THE ACHE SITE, DERIVED FROM STRUCTURE-ACTIVITY STUDIES OF SUBSTRATES, INHIBITORS, AND REACTIVATORS OF ACHE. THE RELATIVE EFFICACY OF OUR COMPOUNDS CAN BE EVALUATED IN THE CONTEXT OF THESE MODELS, BUT NONE OF THESE CONSIDER POSSIBLE 3-DIMENSIONAL ASPECTS OF THE ACTIVE-SITE REGION. OUR DATA APPEAR TO SUPPORT THE KABACHNIK MODEL WITH THE E LIPOPHILIC REGION AS A CLEFT, AND THE β-ANIONIC SITE OF THE ROUFOGALIS MODEL. OTHER INTERPRETATIONS ARE WELCOME.



SCHEMATIC REPRESENTATION OF WILSON MODEL OF THE AChE ACTIVE SURFACE



SCHEMATIC REPRESENTATION OF KABACHNIK MODEL FOR THE AChE ACTIVE SITE
Hydrophic Regions A_1, A_2 and E shown as shaded areas.



SCHEMATIC REPRESENTATION OF ROUFOGALIS MODEL FOR THE AChE ACTIVE SITE

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IN VITRO EVALUATION OF COMPOUNDS

- pK_a OF THE OXIME - DETERMINED SPECTROPHOTOMETRICALLY, FROM THE ABSORBANCE OF OXIME BASE AND ANION AT SEVERAL pH VALUES.

- LOG P - THE LOG OF THE PARTITION COEFFICIENT BETWEEN OCTANOL AND 0.1 M PHOSPHATE BUFFER, pH 7.4.

- AChE ASSAY - CARRIED OUT WITH HUMAN ERYTHROCYTE RBC AChE BY THE ELLMAN PROCEDURE (BIOCHEM. PHARMACOL. 7, 88, 1961), AT pH 7.6 IN A MORPHOLINOPROPANE SULFONIC ACID BUFFER (MOPS). IN THIS SPECTROPHOTOMETRIC ASSAY, THIOCHOLINE PRODUCED BY AChE FROM ACETYLTHIOCHOLINE REACTS WITH BIS-DITHIONITROBENZOIC ACID TO FORM A PRODUCT WITH MAXIMAL ABSORBANCE AT 412 nM. RATE OF INCREASE IN ABSORBANCE IS READ IN A GILSON-DU SPECTROPHOMETER WITH A 4-POSITION SAMPLE CHANGER, CONVERTED TO DIGITAL OUTPUT BY AN HP-MULTIMETER, AND RECORDED, ANALYZED AND PRINTED OUT BY AN HP-85 LABORATORY COMPUTER IN THE FORM SHOWN BELOW.

- NUCLEOPHILICITY TOWARD ACETYLTHIOCHOLINE (AcSCh) - EVALUATED AS THE RATE OF DRUG-INDUCED HYDROLYSIS OF AcSCh (MEASURED BY THE ELLMAN PROCEDURE), IN pH 7.6 MOPS BUFFER. FROM THE LINEAR REGRESSION LINE OF DRUG CONCENTRATION VS. THIOCHOLINE PRODUCTION RATE, THE THIOCHOLINE PRODUCTION PER MOLE DRUG PER MINUTE IS CALCULATED AS AN INDEX OF INHERENT NUCLEOPHILICITY OF THE DRUG, AND A CONTROL FOR SUBSTRATE HYDROLYSIS BY DRUG IN THE OTHER ASSAYS.

- AChE INHIBITION BY TEST COMPOUNDS - DETERMINED BY INCUBATING COMPOUNDS IN MOPS BUFFER WITH UNINHIBITED ENZYME, AND ASSAYING ALIQUOTS (BY THE ELLMAN PROCEDURE) AT LEAST 3 TIMES OVER 2 HOURS. INHIBITION DATA IS PRESENTED AS THE CONCENTRATION GIVING 50% INHIBITION OF AChE ACTIVITY (IC_{50}) IN pH 8.0 PHOSPHATE BUFFER, IN THE ELLMAN ASSAY, WHERE CONCENTRATION OF THE SUBSTRATE, AcSCh, IS .75 mM. IC_{50} IS DETERMINED FROM THE LINEAR REGRESSION LINE OF PERCENT OF CONTROL ENZYME ACTIVITY VS. THE LOG OF DRUG CONCENTRATION, AT ACTIVITY VALUES FROM 10 TO 90% OF CONTROL.

IN VITRO EVALUATION OF COMPOUNDS

- AChE REACTIVATION AFTER EPMP INHIBITION - AChE IS INHIBITED TO 10% OF CONTROL ACTIVITY WITH EPMP IN MOPS BUFFER, AND ALIQUOTS INCUBATED WITH THE TEST DRUG IN MOPS BUFFER AT 25°C. ALIQUOTS ARE REMOVED FOR AChE ASSAY AT 30 TO 240 MIN. REACTIVATION IS TIME- AND DOSE-DEPENDENT, UNCOMPLICATED BY SIDE REACTIONS SUCH AS DEALKYLATION (AGING) OF THE INHIBITED ENZYME AND REINHIBITION OF THE ENZYME BY PHOSPHONYLATED OXIME. THE KINETIC ANALYSIS INVOLVES CALCULATION OF THE EFFECTIVE RATE OF REACTIVATION FOR EACH DRUG CONCENTRATION, AND CORRECTION OF THE DATA FOR THE LOW RATE OF SPONTANEOUS REACTIVATION OF THE ENZYME, FOR THE HYDROLYSIS OF THE ASSAY SUBSTRATE BY THE TEST DRUG, AND FOR ANY INHIBITION OF THE ENZYME BY THE TEST DRUG. DRUG CONCENTRATION IS CORRECTED TO OXIMATE ANION (THE ACTIVE REACTIVATING MOIETY) CONCENTRATION, AND THE BIMOLECULAR REACTIVATION RATE CONSTANT (k_{OX}) CALCULATED, FOR A MEASURE OF THE INHERENT REACTIVITY OF THE OXIMATE ANION. RESULTS ARE ALSO EXPRESSED IN TERMS OF THE EFFECTIVE REACTIVATION POTENCY OF EACH DRUG (k_{HOX}), IRRESPECTIVE OF THE ACTUAL OXIMATE CONCENTRATION. A MORE COMPLETE DESCRIPTION OF THE CALCULATIONS AND THE COMPUTER-AUTOMATED KINETIC ANALYSIS IS AVAILABLE UPON REQUEST.

- AChE REACTIVATION AFTER INHIBITION BY GD - AChE IS TOTALLY INHIBITED BY GD, IN A 30-MIN INCUBATION IN pH 10.2, 0.04 M BARBITAL BUFFER. THE HIGH pH SLOWS "AGING" OF THE INHIBITED ENZYME AND DESTROYS EXCESS GD. ALIQUOTS ARE INCUBATED WITH DRUGS AT ONE FIXED CONCENTRATION IN pH 7.6 MOPS BUFFER AT 25°C, AND SAMPLED AT 5, 30, AND 60 MIN. REACTIVATION IS COMPLETE IN ABOUT 30 MIN BECAUSE THE AGING IS SO FAST AT pH 7.6 ($T^{1/2} < 10$ MIN), THAT ESSENTIALLY NO NONDEALKYLATED INHIBITED ENZYME REMAINS AFTER THIS TIME. THE SHORT TIME AVAILABLE FOR ENZYME ASSAY BEFORE THE REACTIVATION IS COMPLETE COMPLICATES EFFICIENT KINETIC STUDIES. WE CONSEQUENTLY REPORT HERE ONLY THE MAXIMAL PERCENT REACTIVATION ACHIEVED AT A 10^{-3} M CONCENTRATION OF EACH DRUG.

A NEW SYNTHESIS OF 1,4-DIARYLBUTANE-1,4-DIONES USING HYPERVALENT IODINE UNDER LEWIS ACID CONDITIONS. NOVEL ACETYLCHOLINESTERASE REACTIVATORS

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Summary

In the course of our work directed towards the synthesis of novel acetylcholinesterase reactivators we required a reliable synthesis of 1,4-diarylbutane 1,4-diones. This synthesis must allow extensive variation in the aryl portion. Ultimately the 1,4-dicarbonyl would be converted into the *bis*-oximino derivative. We have now discovered a new synthesis which involves the hypervalent iodine oxidation of silyl enol ethers using iodosobenzene, boron trifluoride etherate in dichloromethane at −40°C.

Synthesis of 1,4-Diarylbutane, 1,4-diones by Oxidation of Silyl Enol Ethers with Ph-I=O, $BF_3 \cdot Et_2O$.

$$2\ Ar-C(OSiMe_3)=CH_2 \xrightarrow{ii} Ar-C(=O)-CH_2-CH_2-C(=O)-Ar$$

ii = $BF_3 \cdot Et_2O$, PhIO

Ar = phenyl
= *p*-methoxyphenyl
= *p*-chlorophenyl
= *o*-hydroxyphenyl
= thienyl
= furyl
= benzofuryl

Mechanism

The mechanistic pathway by which this reaction takes place is proposed to be the following.

$$\underset{\underline{A}}{Ar-\overset{O}{\overset{\|}{C}}-CH_3} \xrightarrow{i} \underset{\underline{B}}{Ar-\underset{OSiMe_3}{\underset{|}{C}}=CH_2} = Ar-\overset{O}{\overset{\|}{C}}-CH_2^-$$

$$\underline{B} \xrightarrow{ii} \underline{C}: \left[Ar-\overset{O}{\overset{\|}{C}}-CH_2-\underset{C_6H_5}{\overset{OBF_2}{I}} \right] = Ar-\overset{O}{\overset{\|}{C}}-CH_2^+ \xrightarrow{ROH} \underline{D}: Ar-\overset{O}{\overset{\|}{C}}-CH_2-OR \quad (Ar{=}Ph)$$

$$Ar-\overset{O}{\overset{\|}{C}}-CH_2-CH_2-\overset{O}{\overset{\|}{C}}-Ar \xleftarrow{\underline{B}} \underline{C}$$

i = $ClSiMe_3$, Et_3N-DMF ·
ii = $BF_3 \cdot Et_2O$, PhIO ·

R = H, Me, Et

Silyl enol ether B is the synthetic equivalent of a carbanion. Reaction with the electrophile [PhI-OBF_2] generated from $BF_3 \cdot Et_20$, PhIO yields C. C is considered to be the synthetic equivalent of the α-ketocarbonium ion formed by ionization of the C-I bond. Coupling of the carbanionic and carbonium ion parts yields the 1,4-diketone. This reaction may be viewed as an umpolung of the enol system, and the validity of this description was indicated by addition of H_2O, MeOH, and EtOH to yield the α-substituted products (D).

Synthesis of Silyl Enol Ethers

$Ar-C(=O)-CH_3 \xrightarrow{i} Ar-C(OSiMe_3)=CH_2$

Important examples for the Reactivators:

2-acetylpyridine $\xrightarrow{i}$ 2-pyridyl-$C(OSiMe_3)=CH_2$

3-acetylpyridine $\xrightarrow{i}$ 3-pyridyl-$C(OSiMe_3)=CH_2$

4-pyridyl-$CH_3-C=O$ $\xrightarrow{i}$ 4-pyridyl-$CH_2=C-OSiMe_3$

$i = ClSiMe_3, Et_3N-DMF$

Synthesis of 1,4-Di(pyridinyl) butane 1,4-diones as precursors for Acetylcholinesterase Reactivators.

2-pyridyl-$C(OSiMe_3)=CH_2 \xrightarrow{ii}$ 2-pyridyl-$C(=O)-CH_2-CH_2-C(=O)$-2-pyridyl

3-pyridyl-$C(OSiMe_3)=CH_2 \xrightarrow{ii}$ 3-pyridyl-$C(=O)-CH_2-CH_2-C(=O)$-3-pyridyl

4-pyridyl-$CH_2=C-OSiMe_3 \xrightarrow{ii}$ 4-pyridyl-$C(=O)-CH_2-CH_2-C(=O)$-4-pyridyl

Oxidation of Silyl Enol Ethers with Ph-I=O, $BF_3 \cdot Et_2O$ and Nucleophiles, such as Water, Methanol and Ethanol. α-Functionalization of Ketones.

$Ar-C(=CH_2)-OSiMe_3$

ii · EtOH

ii H_2O

ii · MeOH

$Ar-C(=O)-CH_2-OEt$ $Ar-C(=O)-CH_2-OH$ $Ar-C(=O)-CH_2OMe$

Synthesis of α-Hydroxyketones as Intermediates for Potential Acetylcholinesterase Reactivators.

1. 2-pyridyl–C(OSiMe$_3$)=CH$_2$ —ii, ·HOH→ 2-pyridyl–C(=O)–CH$_2$OH —iii→

2-pyridyl–C(=NOH)–CH$_2$OH —iv→ N-methylpyridinium–C(=NOH)–CH$_2$OH $I^{\ominus}$

2. 3-pyridyl–C(OSiMe$_3$)=CH$_2$ —ii, ·HOH→ 3-pyridyl–C(=O)–CH$_2$OH —iii→

3-pyridyl–C(=NOH)–CH$_2$OH —iv→ N-methylpyridinium–C(=NOH)–CH$_2$OH $I^{\ominus}$

ii = $BF_3 \cdot Et_2O$, PhIO, CH_2Cl_2
iii = $NH_2OH \cdot HCl$, $NaHCO_3$
iv = MeI, THF

Reaction of Inhibited Acetylcholinesterase with <-Hydroxyoximes of Quaternary Pyridinium Compounds.

% REACTIVATION	100	58	85
K_I	0.07 ± 0.01MM	0.08 + 0.02MM	0.40 ± 0.02
PKA	7.5	8.7	8.4

% REACTIVATION	9.8	41
K_I	0.04 ± 0.01MM	0.15 ± 0.01MM
PKA	9.8	9.1

ANALYSIS OF ACETYLCHOLINESTERASE REACTIVATORS IN BIOLOGICAL TISSUES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A new and efficient high performance liquid chromatography (HPLC) assay has been developed for analysis of cholinesterase (AChE) reactivators in biological tissues. The AChE reactivator of specific interest in this study was N-methylpyridinium-2-carbaldoxime chloride (2PAM). Also of interest was N-methyl-1,6-dihydropyridine-2-carbaldoxime hydrochloride (Pro-2PAM) which has been used as a prodrug form of 2PAM (Bodor et al., Science, 190, 155, 1975). Pro-2PAM has been reported to be rapidly oxidized to 2PAM under neutral or alkaline conditions (Shek et al., J. Med. Chem., 19, 108, 1976). This chemical instability of Pro-2PAM has discouraged efforts to directly measure this prodrug in biological samples. However, it has been observed in our labortory that under acidic conditions the rate of conversion of Pro-2PAM to 2PAM is extremely slow. Taking advantage of the chemical stability of Pro-2PAM under acidic conditions, we were able to detect both 2PAM (retention time 4.6 min; flow rate 1 ml/min; detection wavelength, 312 nm; minimum detection on colum. 17 ng, 20 µl injection) and Pro-2PAM (retention time 6.1 min; flow rate 1 ml/min; detection wavelength, 251 nm) in physiologial or nonphysiological samples using a Brownlee reversed-phase PRP-1 column and elution with an acidic solvent system (0.1 M H_3PO_4, pH = 2.5). The results of these studies were quite surprising. For example, 15 min after administration of Pro-2PAM (50 mg/kg, IV) to mice, the brain level of Pro-2PAM (2.1% dose/gm tissue) was 3 times greater than the brain level of 2PAM (0.645% dose/gm tissue) which was generated in vivo by oxidation of the prodrug. In mice given an equivalent dose of 2PAM (50 mg/kg, IM) a substantially lower level (0.11% dose/mg tissue) of 2PAM was detected in brain. These results are qualitatively, but not quantitatively, consistent with those reported by Shek et al. (J. Med. Chem., 19, 113, 1976). Our data show a 5-fold increase in the actual brain levels of 2PAM from a dose of Pro-2PAM compared to the 13-fold increase reported by Shek et al. However, the surprising result was the observation that the major form of the reactivator in brain is Pro-2PAM rather than 2PAM. These results suggest that the conversion of the Pro-2PAM to 2PAM in biological samples is slower than originally reported. To confirm this hypothesis we have used our HPLC assay to study the conversion of Pro-2PAM to 2PAM in MOPS buffer (pH 7.4) and in buffered serum (pH 7.4). The results of these studies indicates that the rate and maximum percentage conversion of Pro-2PAM to 2PAM is not only dependent on pH, but also dependent on the initial concentration of the prodrug. These data show that Pro-2PAM does not oxidize to 2PAM as rapidly or completely as previously reported. These results help to explain our observation that the ED_{50} of the Pro-2PAM (20.3 mg/kg) is higher than that for 2PAM (7.9 mg/kg) in mice challenged with a dose (6.0 mg/kg, S.C.) of diisopropylfluorophosphate. This work supported in part by the U.S. Army Medical Research and Development Command under Contract DAMD-17-82-C-2078.

INTRODUCTION

The analysis of pyridinium oximes in biological tissues, particularly the blood and brain, is important because of the utility of these compounds as antidotes against organophosphate poisoning. Pyridinium oximes, such as N-methyl pyridinium 2-carbaldoxime chloride (2PAM), reactivate the enzyme acetylcholinesterase (EC 3.1.1.7) by displacing the organophosphoryl group from the active site of the enzyme (1). However, the therapeutic utility of 2PAM is limited because of the poor permeability of the drug through the blood brain barrier (BBB). To overcome this BBB permeability problem, Bodor *et al.* (2) and Shek *et al.* (3,4) synthesized a prodrug form of 2PAM by reduction of 2PAM to the dihydropyridine derivative (N-methyl-1,6-dihydropyridine-2-carbaldoxime hydrochloride, Pro-2PAM, Figure 1). Shek *et al.* (4) proposed that Pro-2PAM would penetrate the BBB better than 2PAM due to the increased lipophilicity of the prodrug and once in the brain Pro-2PAM would be oxidized to 2PAM, where the quaternary compound would be trapped. Evidence in support of this hypothesis includes the observation that administration of Pro-2PAM to mice results in a dramatic increase in the brain levels of 2PAM (4).

The increased levels of 2PAM (from Pro-2PAM) in the brain did not however result in an increase in the ability of the prodrug to protect the test animals against an organophosphate challenge (e.g., diisopropylfluorophosphate, DFP, 5,6). This discrepancy is difficult to explain. Pro-2PAM has been reported

TO BE OXIDIZED TO 2PAM RAPIDLY ($T_{1/2}$ = 1 MIN, 3). THEREFORE, THE PRODRUG SHOULD BE CONVERTED TO THE PARENT DRUG, 2PAM, SHORTLY AFTER BBB PENETRATION THUS PROVIDING A BETTER PROTECTIVE EFFECT TO BRAIN ACETYLCHOLINESTERASE. IN AN EFFORT TO RESOLVE THIS DISCREPANCY WE HAVE DEVELOPED HPLC SYSTEMS THAT ALLOW:

1) THE DETECTION OF 2PAM IN BIOLOGICAL TISSUES, INCLUDING THE BRAIN.
2) THE DETECTION OF PRO-2PAM, ITSELF, IN BIOLOGICAL TISSUES OR IN PHYSIOLOGICAL BUFFERS WITHOUT OXIDATION TO 2PAM DURING ANALYSIS.
3) THE DETERMINATION OF THE RATE AND EXTENT OF CONVERSION OF PRO-2PAM TO 2PAM *IN VITRO* AND *IN VIVO*.

NOH
N
CH_3
O_2
pH > 6
NOH
+N
CH_3
Pro-2PAM
1
2PAM
2

FIGURE 1. PRO-2PAM CONVERSION TO 2PAM IN PRESENCE OF OXYGEN.

EXPERIMENTAL

PREPARATION OF BIOLOGICAL SAMPLES

THE PARENT DRUG, 2PAM, WAS INJECTED I.M. (50 MG/KG IN WATER). (THERE WERE NO SIGNIFICANT DIFFERENCES IN THE BRAIN LEVELS OF THE DRUG AFTER I.M. OR I.V. ADMINISTRATION). THE PRO-2PAM (50 MG/KG IN 0.05 M CITRATE BUFFER, PH 3.0) WAS INJECTED I.V. IN THE LATERAL TAIL VEIN, UNLESS OTHERWISE NOTED.

STANDARD OUTBRED MALE MICE WERE DECAPITATED AT VARIOUS TIMES AFTER DRUG ADMINISTRATION. BLOOD WAS COLLECTED IN 0.15% EDTA TO PREVENT CLOTTING. THE SAMPLE WAS IMMEDIATELY CENTRIFUGED FOR 5 MINUTES AT 1000 X G. THE PLASMA WAS REMOVED AND PERCHLORIC ACID ADDED TO PRECIPITATE PROTEINS AND CENTRIFUGED. THE SUPERNATANT WAS FILTERED THROUGH A NITROCELLULOSE FILTER (0.45 μM), BASIFIED WITH NAOH AND STORED AT -20° C UNTIL DIRECT INJECTION ON THE HPLC. ALIQUOTS (100 μL) OF THE PACKED RED BLOOD CELLS (RBC) WERE DILUTED 5 FOLD WITH WATER AND FROZEN. PERCHLORIC ACID WAS ADDED TO THE THAWED SAMPLE TO PRECIPITATE PROTEINS AND THE SUPERNATANT WAS CENTRIFUGED, FILTERED AND BASIFIED AS ABOVE.

IN GENERAL BIOLOGICAL TISSUES WERE REMOVED SURGICALLY, WEIGHED AND HOMOGENIZED IN A ROTARY HOMOGENIZER FOR 30-45 SECONDS IN 1 ML OF WATER. IN THE ANALYSIS OF BRAIN SAMPLES FOR 2PAM FROM A DOSE OF 2PAM, CONCENTRATED PERCHLORIC ACID WAS ADDED AND THE SAMPLE CENTRIFUGED FOR 10-15 MINUTES (1000 X G). THE CLEAR SUPERNATANT WAS FILTERED (0.45 μM), BASIFIED AND STORED AS DESCRIBED ABOVE UNTIL THE SAMPLES WERE ANALYZED BY HPLC.

Since we noted that a strongly acidic media prevented the conversion of Pro-2PAM to 2PAM in the presence of oxygen or a strong oxidant (e.g., $AgNO_3$), it was then possible to analyze biological samples directly for both Pro-2PAM and 2PAM. To analyze brain samples for 2PAM and Pro-2PAM, the whole head was quickly immersed into a dry ice bath (methylene chloride, -70° C). The brain was then dissected out while submerged in a strongly acidic buffer (0.3 M phosphate buffer, pH = 1.5). The tissue was weighed in the acidic buffer and gently ground by hand with glass beads while in a sonication bath to avoid introducing excessive air. Perchloric acid was added and the sample was centrifuged and filtered as above. The samples were analyzed for 2PAM or Pro-2PAM by HPLC.

HPLC SYSTEM

COLUMN: PRP-1 reversed-phase - poly (styrene-divinylbenzene) by Hamilton Co. (4.6 mm X 20 cm). Guard column Brownlee cartridge system (4.6 mm X 3 cm). Usable pH range 1 to 13.

SOLVENTS: Acidic - 0.1 M H_3PO_4, pH = 2.5
Basic - 0.1 M Na_2CO_3, pH = 10.5

DETECTION: 2PAM; basic solvent - 334 nm
acidic solvent - 312 nm
Pro-2PAM; acidic solvent - 251 nm

RETENTION TIME: 2PAM - acidic and basic solvents - 4.6 minutes
Pro-2PAM - acidic solvent - 6.1 minutes
(Flow rate = 1.0 mL/min)

MINIMUM DETECTION: 2PAM - basic solvent - 10 ng on column
acidic solvent - 17 ng on column
(20 µL injection)

RESULTS

The HPLC systems developed in this study utilize a commercially available, polymer-backed reversed-phase column that allows th use of both strongly acidic or strongly basic buffers (pH range 1 to 13). Figures 2 and 3 show that these HPLC systems produce separations that allow for detection and quantification of 2PAM or Pro-2PAM in biological tissues.

These HPLC systems were used to determine the BBB permeability of Pro-2PAM and the extent of its conversion to 2PAM in the brain. Animals were given injections of either 2PAM (I.M., 50 mg/kg) or Pro-2PAM (I.V., 50 mg/kg) and then sacrificed at various times up to 15 minutes after injection and the brain levels of 2PAM or Pro-2PAM determined using the HPLC systems illustrated in Figures 2 and 3. Four important observations can be made about the data shown in Figure 4; (a) 2 minutes after administration of Pro-2PAM, the ratio of Pro-2PAM and 2PAM in the brain is approximately 2.7 (2.04 prodrug vs. 0.76 2PAM % dose/gm brain); (b) 15 minutes after administration of the prodrug there still exists significant levels of the Pro-2PAM (2.12% dose/gm brain); (c) administration of Pro-2PAM produces a higher brain level of 2PAM (5 times) than that achieved with a comparable dose of 2PAM, however, the magnitude of the increase is less than the 13-fold increase reported earlier by Shek et al. (4); and (d) the combined brain levels of Pro-2PAM and 2PAM from a dose of the prodrug is 20 times higher at 2 minutes after drug administration than the brain level of 2PAM from a dose of 2PAM (2.8 vs. 0.14 %, dose/gm brain).

Analysis of blood taken from the sacrificed animals showed that a dose of Pro-2PAM delivers 2PAM mainly to RBC's, whereas a dose of 2PAM is distributed mainly in the plasma (Figure 5). These results are consistent with those reported earlier by Shek *et al.* (4).

Analysis of the kidneys from sacrificed animals showed that at 2 minutes the kidney level of 2PAM from a dose of 2PAM was significantly higher than the level of 2PAM from a dose of Pro-2PAM. By 6 minutes the kidney levels of 2PAM from the two dosage forms were essentially equivalent.

In order to explain the apparent stability of Pro-2PAM in brain tissue (Figure 4), we studied the oxidation of Pro-2PAM to 2PAM in physiological buffers. As shown in Table 1 the rate of air oxidation of the prodrug to 2PAM is inversely proportional to the initial concentration of Pro-2PAM. As shown in Table 2 the maximum percent conversion of the prodrug to 2PAM in the presence of $AgNO_3$ is also inversely proportional to the initial concentration of Pro-2PAM.

Based on the data shown in Tables 1 and 2, we would predict that the percentage of dose per gram of brain tissue should be inversely proportional to the dose of Pro-2PAM administered. This hypothesis was confirmed by the data shown in Table 3. Although the brain levels of 2PAM increased with increasing doses of Pro-2PAM, the percentage of the dose in the brain is inversely proportional to the dose of the prodrug.

Our data, as well as literature data (Table 4), suggest that 2PAM is more effective than Pro-2PAM in protecting animals from an organophosphate challenge. These results are inconsistent with the observation that the prodrug produces a higher brain level of 2PAM. As shown in Figure 4 the prodrug also produces elevated brain levels of Pro-2PAM, but this dihydropyridine oxime probably does not contribute to the reactivation of brain acetylcholinesterase. It is noteworthy that Pro-2PAM given prophylactially 10 minutes prior to organophosphate exposure (Table 4), shows a better protective effect than 2PAM. These results might suggest that the T1/2 for conversion of the prodrug to 2PAM in biological tissues is longer than was previously anticipated, a result which would be consistent with the data shown in Table 1.

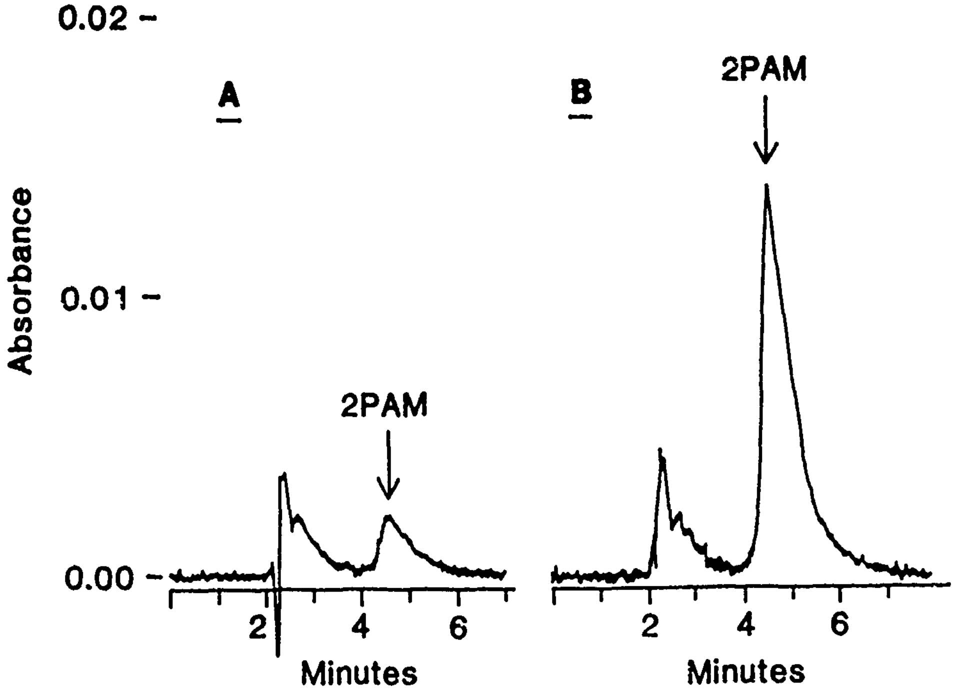

Figure 2. Typical elution profiles of 2PAM from a dose of 2PAM (A) (I.M., 50 mg/kg) and 2PAM from a dose of Pro-2PAM (B) (IV, 50 mg/kg) in brain tissue samples using a basic solvent system (100% Na_2CO_3 buffer, 0.1 M, pH = 10.5) and chromatography on a PRP-1 reversed-phase column. 20 µL injection; flow rate = 1.0 mL/min; detector wavelength = 334 nm.

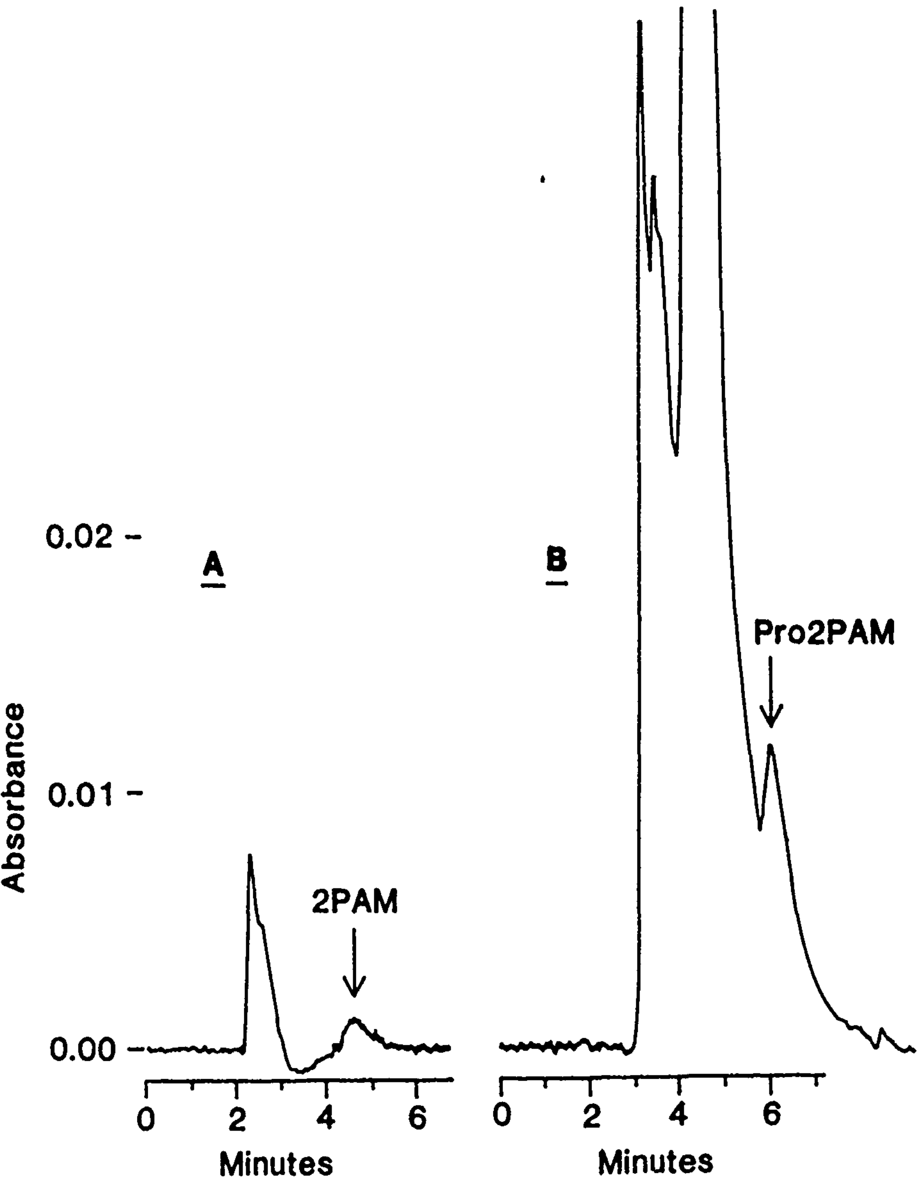

FIGURE 3. TYPICAL ELUTION PROFILES OF 2PAM (A) AND PRO-2PAM (B) FROM A DOSE OF PRO-2PAM (IV, 50 MG/KG) IN BRAIN TISSUE SAMPLES USING AN ACIDIC SOLVENT SYSTEM (100% H_3PO_4 BUFFER, 0.1 M, PH = 2.5) AND CHROMATOGRAPHY ON A PRP-1 REVERSED-PHASE COLUMN. 20 μL INJECTION; FLOW RATE 1.0 ML/MIN; DETECTOR WAVELENGTH: 312 NM (A), 251 NM (B).

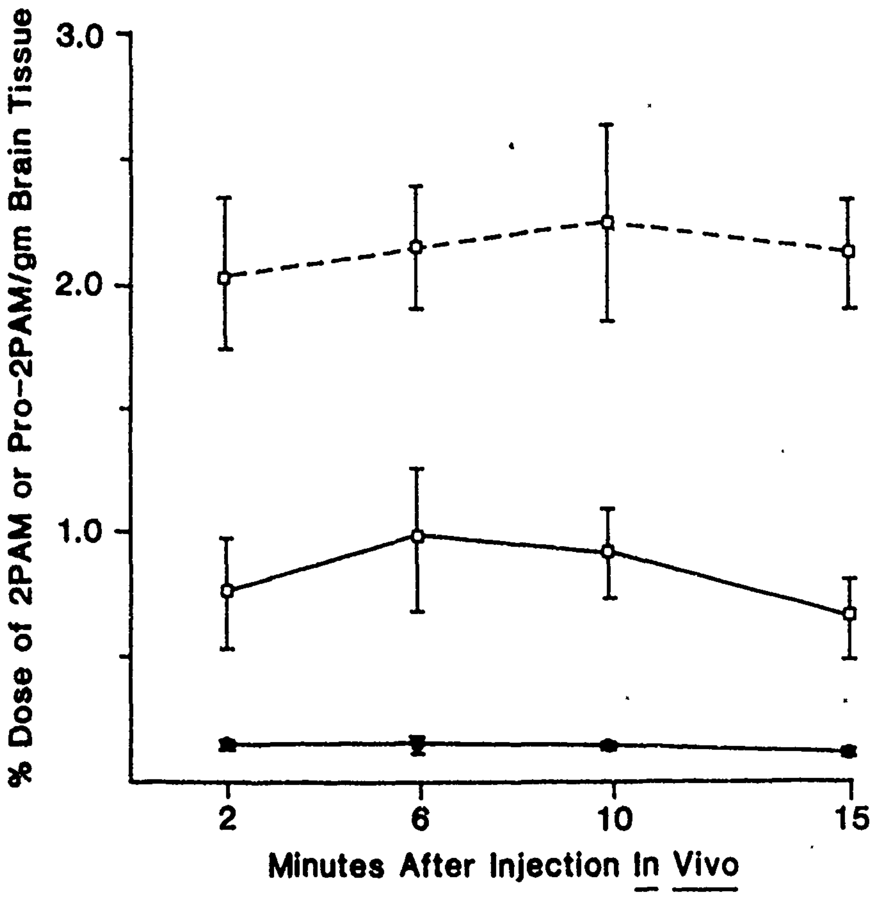

FIGURE 4. BRAIN TISSUE SAMPLES FROM ANIMALS GIVEN A 50 MG/KG DOSE OF 2PAM (IM) OR PRO-2PAM (IV). 2PAM INJECTION: 2PAM SAMPLES WERE ANALYZED IN THE BASIC SOLVENT SYSTEM (●—●, 0.1 M Na_2CO_3, pH = 10.5, 334 NM). PRO-2PAM INJECTION: BRAIN WAS QUICK FROZEN AND DISSECTED IN AN ACID BATH TO PREVENT CONVERSION OF PRO-2PAM TO 2PAM DURING ANALYSIS. SAMPLES WERE ANALYZED IN THE ACIDIC SOLVENT SYSTEM (0.1 M H_3PO_4, pH = 2.5) FOR PRO-2PAM REMAINING (□--□, 251 NM) AND 2PAM FORMED FROM PRO-2PAM (□—□, 312 NM).

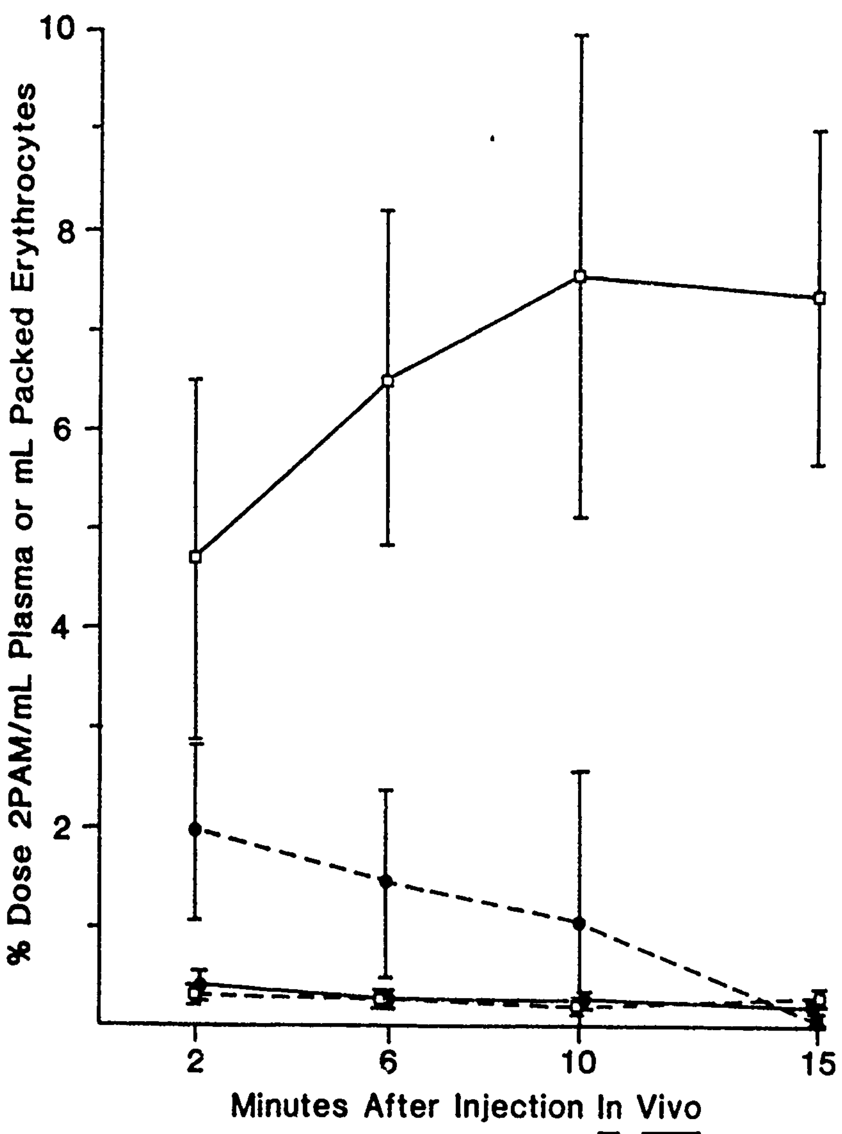

FIGURE 5. RBC AND PLASMA SAMPLES FROM ANIMALS GIVEN A 50 MG/KG DOSE OF 2PAM (IM) OR PRO-2PAM (IV). 2PAM INJECTION: RBC (●—●) AND PLASMA (●---●). PRO-2PAM INJECTION: RBC (□—□) AND PLASMA (□--□). ALL SAMPLES WERE ANALYZED IN BASIC SOLVENT SYSTEM (0.1 M Na_2CO_3, pH = 10.5, 334 NM).

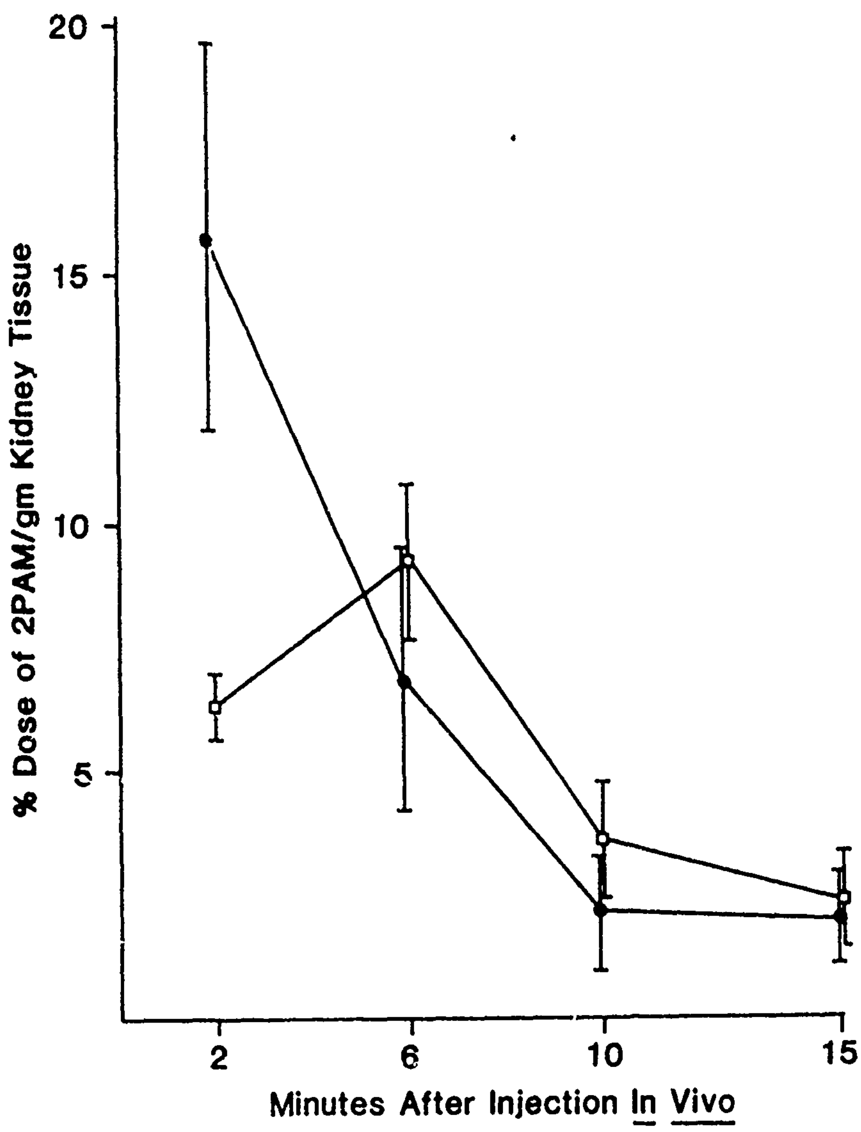

FIGURE 6. KIDNEY TISSUE SAMPLES FROM ANIMALS GIVEN A 50 MG/KG DOSE OF 2PAM (IM) AND PRO-2PAM (IV). 2PAM INJECTION (●—●) AND PRO-2PAM INJECTION (□—□) BOTH ANALYZED AS 2PAM IN BASIC SOLVENT SYSTEM (0.1 M Na_2CO_3, PH = 334 NM).

Table 1. Rate of Pro-2PAM Conversion to 2PAM in a Physiological Buffer.[1]

Pro-2PAM (M)	T1/2 (min)
10^{-5}	3.2
10^{-4}	10.0
10^{-3}	16.5
10^{-2}	31.3

[1]Experiments were carried out in MOPS buffer (0.1 M, pH = 7.4) containing bovine serum (1/1). At various times perchloric acid was added to samples. The samples were immediately filtered and analyzed by HPLC using the acidic buffer system (0.1 M H_3PO_4, pH = 2.5). 2PAM was detected at 312 nm.

Table 2. Extent of Pro-2PAM Conversion to 2PAM *In Vitro*[1].

Pro-2PAM (M)	Maximum Conversion (%)
10^{-5}	86
10^{-4}	70
10^{-3}	41
5×10^{-2}	31

[1]Pro-2PAM samples were oxidized with excess $AgNO_3$ at pH > 7. After 20 min the samples were filtered and analyzed by HPLC using the basic buffer system (0.1 M Na_2CO_3, pH 10.5). 2PAM was detected at 334 nm.

Table 3. Dependence of the 2PAM Brain Levels on the Dose of Pro-2PAM.

	2PAM present in brain[1]			
	2 Minutes		6 Minutes	
Dose mg/kg	µg/gm tissue	% dose/gm tissue	µg/gm tissue	% dose/gm tissue
100	32.6 (44.1-21.0)[2]	0.94 (1.33-0.55)	46.9 (56.1-37.6)	1.33 (1.55-1.10)
50	26.1 (36.1-16.2)	1.54 (2.03-1.04)	28.8 (34.1-23.6)	1.75 (2.09-1.41)
10	7.1 (8.4-5.8)	1.90 (2.22-1.59)	6.8 (7.7-5.9)	1.91 (2.09-1.73)

[1]Groups (n=3) of mice were given either 10, 50 or 100 mg/kg doses of Pro-2PAM (IV). The animals were sacrificed at 2 min or 6 mins after drug administration. The brains were quickly frozen in a -70° C dry ice bath and dissected under acidic conditions to prevent conversion of the prodrug to 2PAM. Samples were then analyzed by HPLC using the acidic solvent system (.1 M H_3PO_4, pH = 2.5). 2PAM was detected at 312 nm.

[2]95% confidence limits.

Table 4. Literature Reports of the Protective Ability of Pro-2PAM and 2PAM Against Organophosphate Challenge in Mice.

	2PAM	Pro-2PAM	Ratio	Ref
ED_{50} (mg/kg) 95% C.L.	7.9 (6.0-10.6)	20.3 (12.7-32.4)	2.5	-1
ED_{50} (mg/kg) S.E.	3.8 ±0.8	11.6 ±3.0	3.0	(6)[2]
#Died/#Tested	0/6	3/6	--	(5)[3]
Protective Ratio[4] (Oxime administered prophylactically)	1.41	2.20	0.64	(7)[5]

[1]Performed in this lab: 2PAM I.M., Pro-2PAM I.V.; vs. DFP (6.0 mg/kg S.C.).

[2]I.P. injection of oximes; vs. Paraoxon (0.9 mg/kg S.C.).

[3]34 mg/kg I.P. oxime dose; vs. Paraoxon (8 µmole S.C.).

[4]Protective Ratio: LD_{50} of prophylaxis and organophosphate/ LD_{50} of organophosphate.

[5]mg/kg I.M. oxime dose 10 min before DFP (S.C.) challenge.

CONCLUSIONS

1. 2PAM can be detected in the brain and in other tissues after administration of the parent drug (2PAM) or the prodrug (Pro-2PAM) using either a strongly acidic or strongly basic solvent system and chromatography on a PRP-1 reversed-phase HPLC column.

2. Pro-2PAM can be detected in the brain after administration of Pro-2PAM using the strongly acidic solvent system and chromatography on a PRP-1 reversed-phase HPLC system.

3. Pro-2PAM is present in the brain in significant quantities up to 15 minutes after injection of the prodrug.

4. Pro-2PAM delivers more 2PAM to the brain than the parent 2PAM, but less than previously reported. This smaller difference is apparently due to improved sample handling techniques. Our techniques prevent further oxidation of Pro-2PAM to 2PAM present in biological samples and thus our techniques give a more accurate estimate of the brain levels of 2PAM.

5. *In vitro* experiments demonstrated that in higher concentrations the Pro-2PAM oxidizes slower and affords lower yields of 2PAM than less concentrated solutions of the prodrug.

6. The brain level of 2PAM is proportional to the dose of Pro-2PAM but the higher the dose of the prodrug, the lower the percentage of dose that is delivered to the brain.

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3. Research Models and Methods

THE EFFECT OF AN ORGANOPHOSPHATE, A CARBAMATE AND A CHOLINOMIMETIC ON SALIVARY ENZYMES

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ABSTRACT

The objective of this study was to measure the effects of the organophosphate, diisopropylfluorophosphate (DFP), which is an irreversible cholinesterase inhibitor; the cholinomimetic, pilocarpine (PIL) and the carbamate, physostigmine (PHY), which is a reversible cholinesterase inhibitor, on salivary enzymes. Salivary cholinesterase, kallikrein, lysozyme and amylase levels were determined in eight rhesus monkeys treated with DFP, PIL and PHY. Parotid and extraparotid saliva were collected from the ketamine anesthetized animals for three successive 45 min. periods as follows: Period 1 - control; Period 2 - i.m. injection of either DFP (0.05 mg/kg), PIL (0.2 mg/kg) or PHY (0.3 mg/kg) at the begining of the period; Period 3 - repeated Period 2 dosage of the respective drug. Blood cholinesterase levels remained constant after exposure to PIL compared to the marked decrease observed after exposure to DFP (90% inhibited) and PHY (45% inhibited) in Period 3. Salivary cholinesterase levels also decreased after the second dose of DFP in both parotid and extraparotid saliva (72% and 85% inhibited respectively); however, extraparotid salivary cholinesterase levels increased after exposure to PIL from a mean control level of 3.02×10^{-3} units/ml to a mean level of 4.33×10^{-3} units/ml after the second dose ($p<0.025$). PHY also caused an increase in extraparotid salivary cholinesterase levels from a mean control level of 2.15×10^{-3} units/ml to 4.39×10^{-3} units/ml ($p<0.01$) after the second exposure. No significant changes were observed in parotid cholinesterase after exposure to either PIL or PHY. Exposure to PHY and PIL resulted in decreased salivary levels of the serine esterase, kallikrein, while exposure to DFP resulted in no significant change in kallikrein levels. Parotid salivary amylase levels were increased following exposure to PIL and PHY from mean control levels of 870×10^3 units/ml and 1600×10^3 units/ml to 2100×10^3 units/ml and 2500×10^3 units/ml, respectively, after the first dose. No significant changes in lysozyme levels were observed after exposure to the cholinergic drugs tested. Our results, to date, indicate the need to study the effects of specific chemical agents, such as sarin and soman, on salivary enzymes. Each of the three cholinergic compounds that we studied elicit a different salivary enzymatic response.

INTRODUCTION

The effects of cholinergic compounds on various salivary constituents have been described and reviewed in the literature. It has been established that there is a large normal variation in salivary compositon and that many components of saliva change when salivation is stimulated. While the actions of individual pharmacological compounds on salivary composition have been studied, very few direct comparisons between drugs have been documented. The object of this study was to compare the effects of three different cholinergic compounds on salivary enzymes. The compounds used in this study were: 1 - an organophosphate, diisopropylfluorophosphate which is an irreversible cholinesterase inhibitor; 2 - a carbamate, physostigmine which is a reversible cholinesterase inhibitor; and 3 - a cholinomimetic, pilocarpine which elicts a neuro-response similar to that of the anticholinesterase compounds without having the inhibitory effect on cholinesterase.

Of the enzymes examined, only cholinesterase and kallikrein have been shown to be directly inhibited by anticholinesterase compounds. Salivary cholinesterase has been shown to consist of both psuedo cholinesterase and acetyl cholinesterase, which has been reported to be derived in the glandular cells. Salivary kallikrein, a serine esterase has been demonstrated to be produced on the striated ductal cells.

Amylase was investigated because it has been described to amount to 50 per cent of the total salivary protein formed in the central acinar cells. The hydrolytic enzyme, lysozyme was studied because it was known to have a serine residue present at the active site of the enzyme.

The present study shows that the levels of salivary enzymes vary as the result of the cholinergic stimulant administered. This suggests the need to study the effects of specific chemical agents, such as sarin and soman, on salivary enzymes.

METHODS

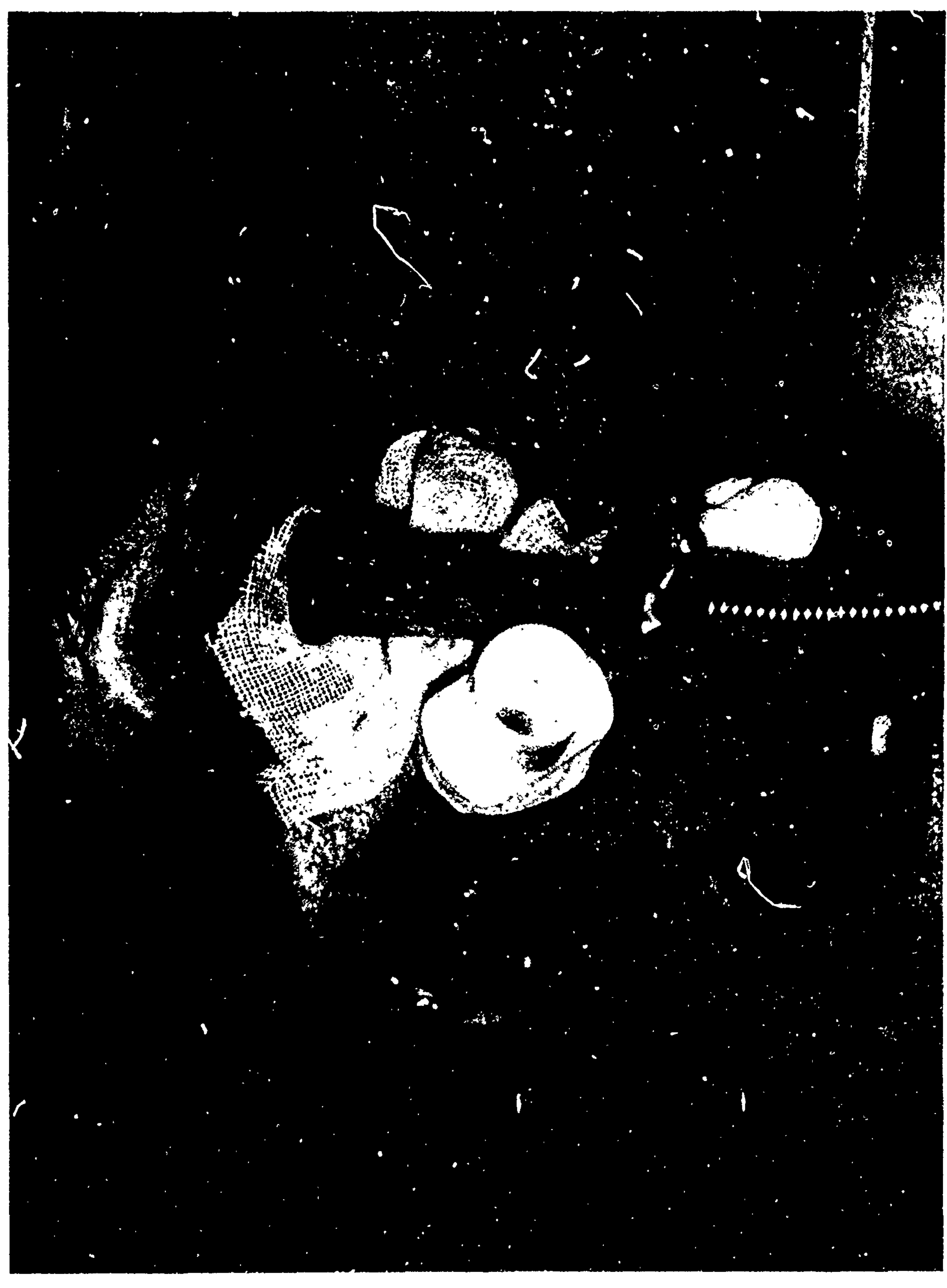
Eight adult male rhesus monkeys (Macaca mulatta) weighing between 6.5 kg and 13.0 kg were tested under the following regimen. Each monkey was fasted overnight and was sedated the following morning with ketamine (10mg/kg). Both parotid and extraparotid saliva were obtained during three successive 45-minute collection periods. Blood was drawn at the 30-minute mark for each period. Following a baseline collection period, the monkey was injected with either physostigmine (PHY) 0.3 mg/kg, pilocarpine (PIL) 0.2 mg/kg, or diisopropylfluorophosphate (DFP) 0.05 mg/kg. A second sampling period immediately followed. At this time, the initial dosage was repeated which was followed by a third collection period.

Parotid saliva was collected from both glands using Lashley cups which were modified to fit the rhesus monkey. Extraparotid saliva was obtained using a monojet syringe with a curved tip to collect the saliva from the oral cavity. The sample tubes were kept in crushed ice throughout the collection period, then stored at 20 C for later analysis.

The cholinesterase in blood was radiometrically assayed in three factions: whole blood, packed cells and plasma. Using the method of Johnson and Russell[1] the tritiated acetylcholine substrate was hydrolyzed by the enzyme and the labeled acetate group was extracted into scintillation cocktail [Liquifluor (50); isoamyl alcohol(300); toluene(700)] and counted. A modification of this procedure was used to measure cholinesterase in both parotid and extraparotid saliva, which not only has considerably less activity but very limited sample size.

The alpha amylase activity in saliva was determined by the method of Ceska et al[2]. In this method a starch is cross-linked to form an insoluble polymer substrate which is coupled to a blue dye. As a result of the action of amylase on the substrate, soluble fragments containing the blue dye are released which are measured on a spectrophotometer at 620 nm.

Salivary kallikrein levels were determined using the radiometric technique of Imarari et al[3]. This method used a tritiated N-alpha-tosyl-L-arginine methyl ester (^{3}H-TAME) as a substrate. The liberated methyl alcohol was extracted into the scintillation cocktail [liquifluor(50) and toluene(1000)] and activity was measured.



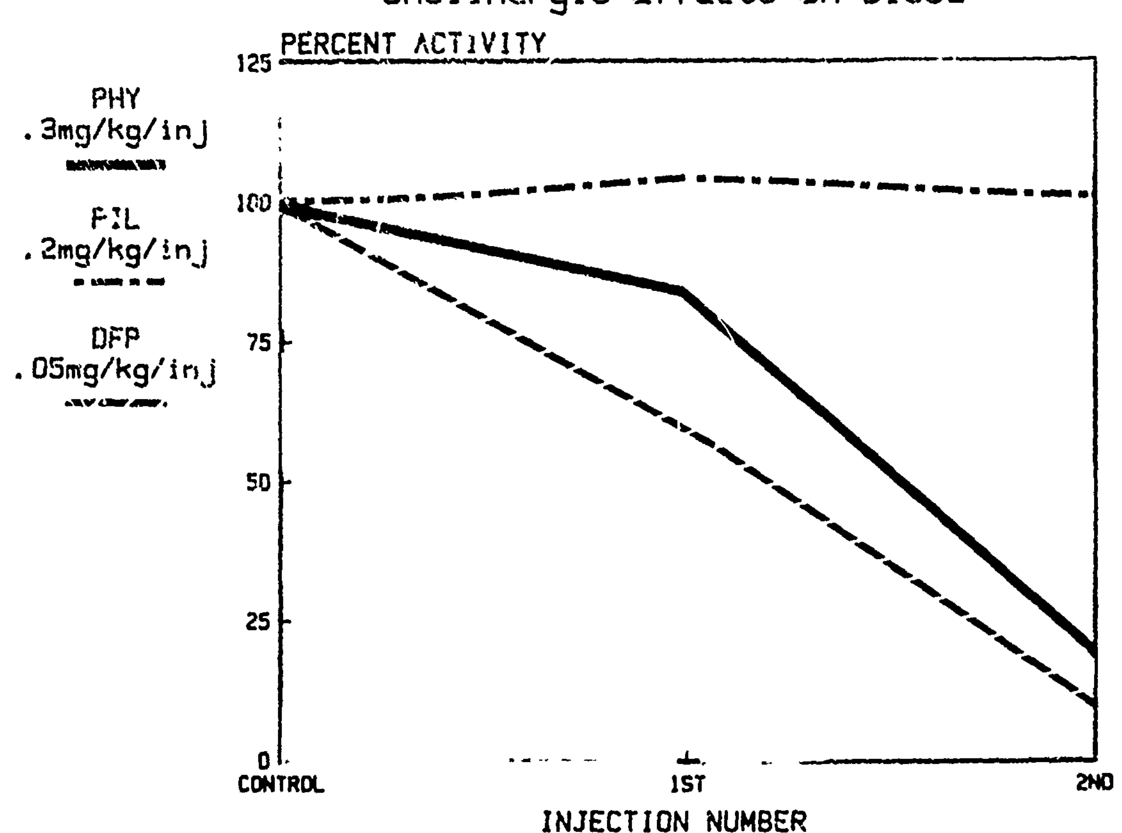
The lysozyme activity in saliva was assayed according to the modified method of Sugar[4]. The substrate used in this method was a suspension of <u>Micrococcus luteus</u>. Hydrolysis of the suspension was determined on a spectrophotometer at 450 nm.

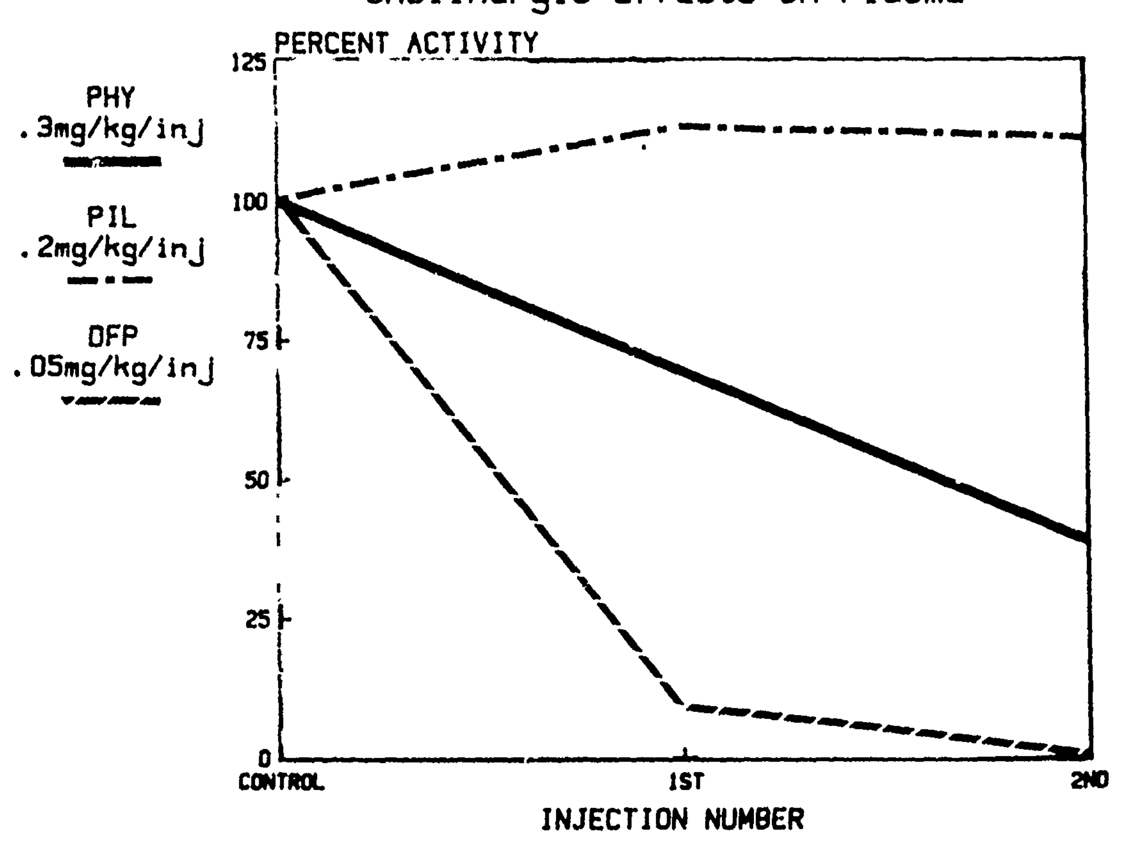
[1] Johnson, C. D. and Russell, R. L.: Anal. Biochem. 64, 229-238 (1975).

[2] Ceska, M., Birath, K. and Brown, B.: Clin. Chim. Acta. 26, 437 (1969).

[3] Imanari, T., Kaizu, T., Yoshida, H., Yates, K., Pierce, J. V. and Pisano, J. J. Symposium on Chemistry and Biology of Kallikrein-Kinin System in Health and Disease. J. J. Pisano, Ed. p205 (1978)

[4] Sugar, D.: Biochim. Biophysia. Acta, 3 303 (1952)




PERCENT CHOLINESTERASE ACTIVITY
Cholinergic Effects on Plasma
PERCENT ACTIVITY
125
100
75
50
25
0
PHY
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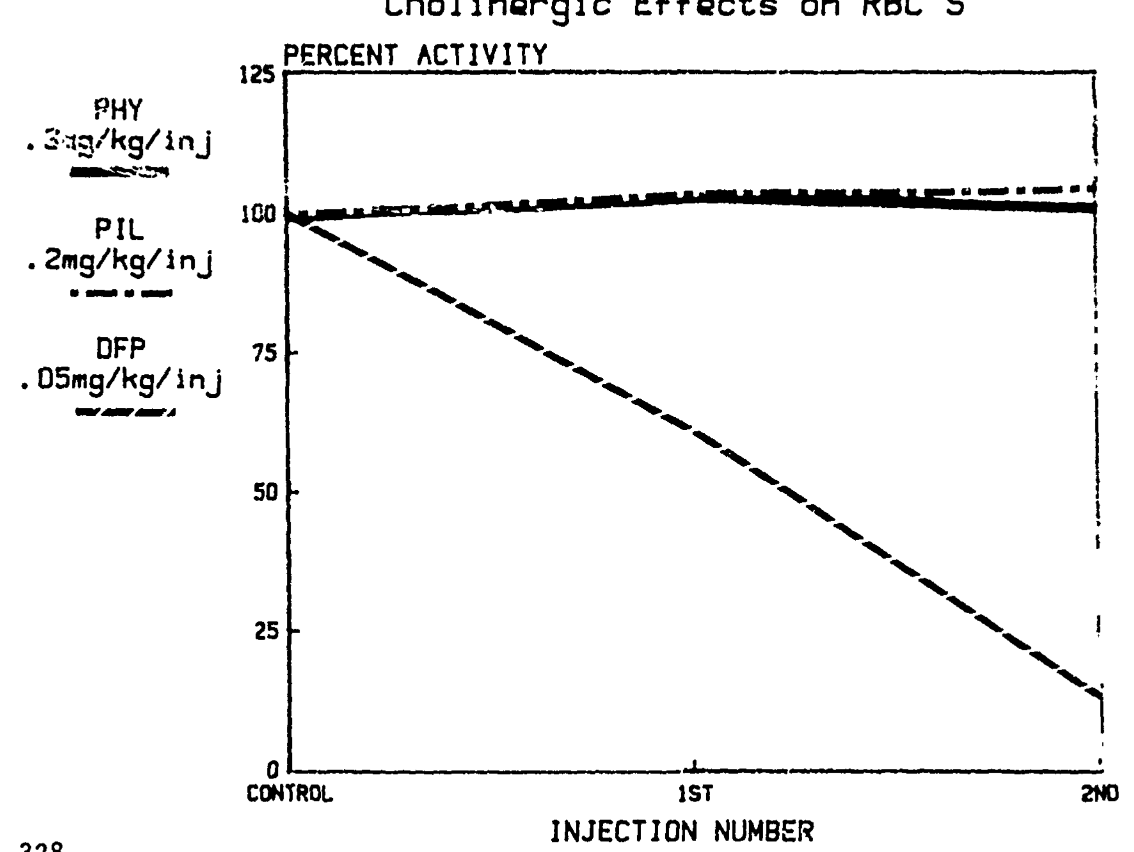
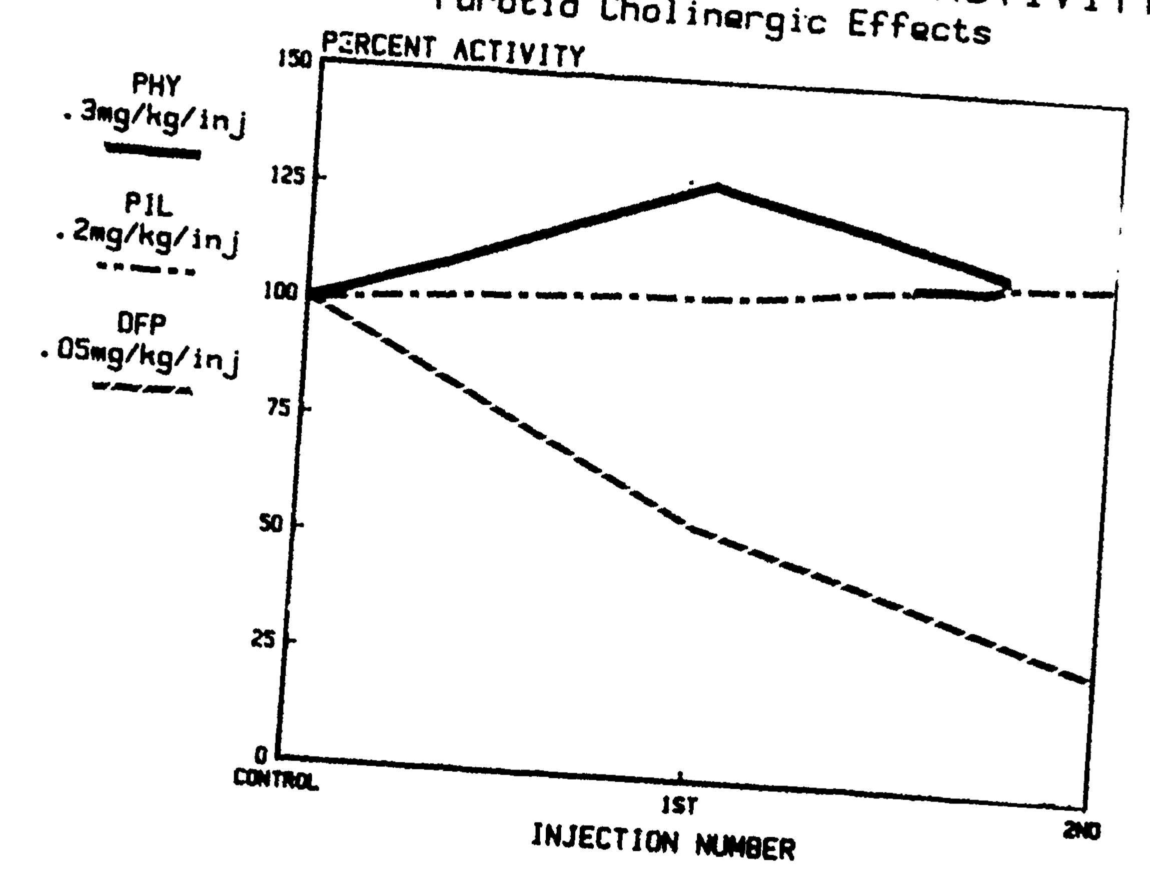
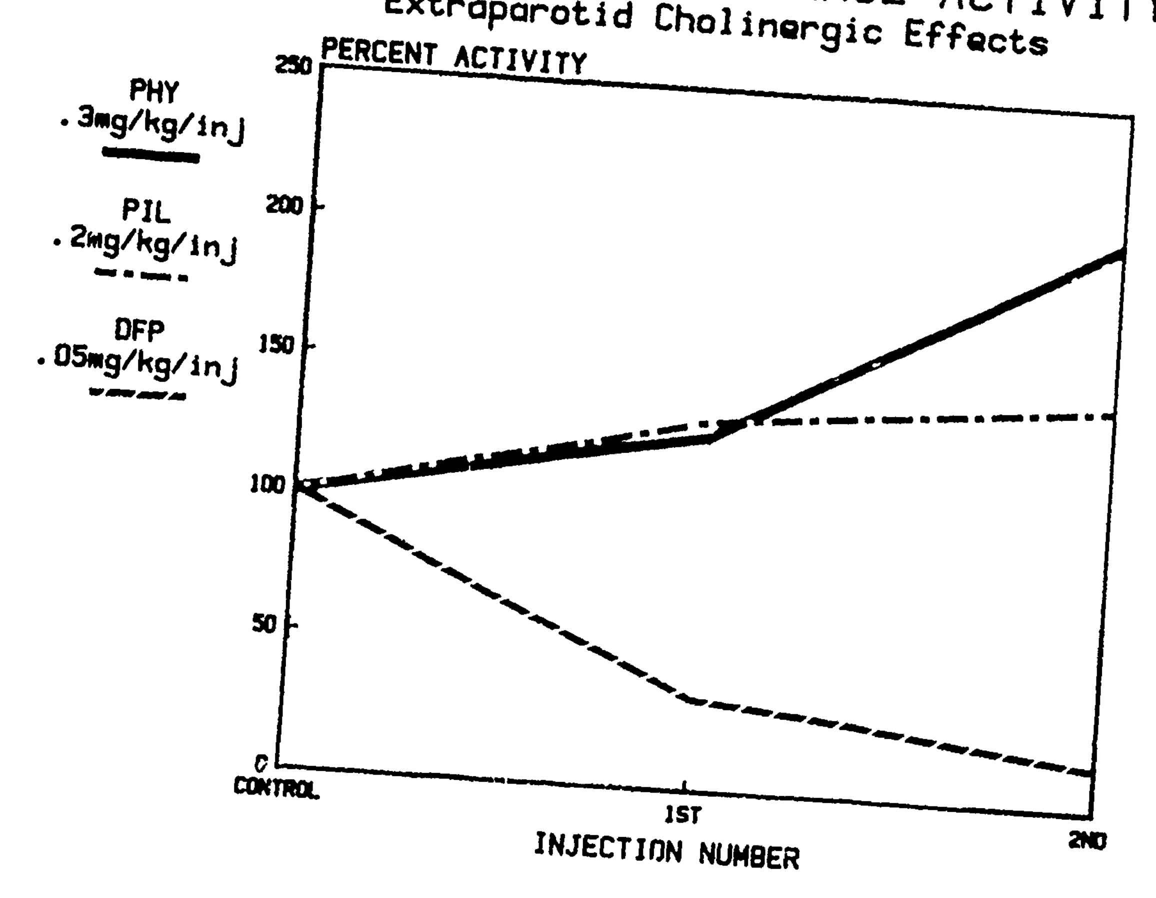
PIL
.2mg/kg/inj
DFP
.05mg/kg/inj
CONTROL
1ST
2ND
INJECTION NUMBER

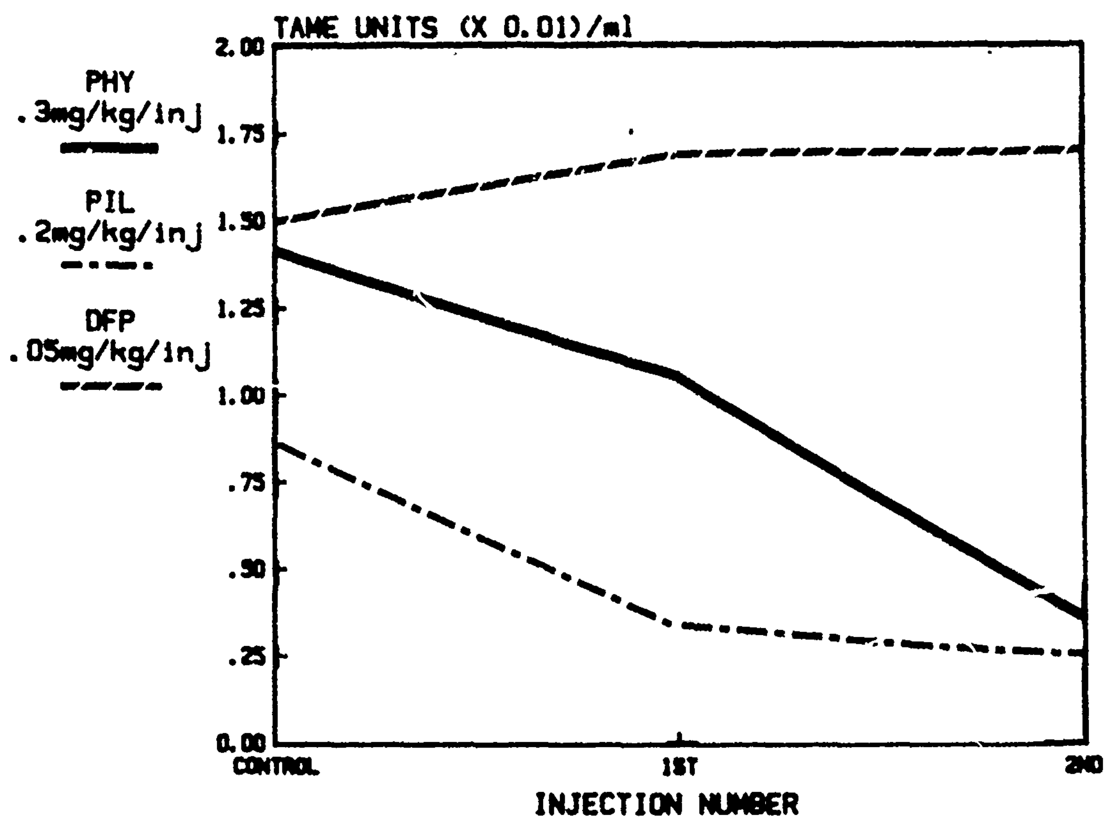

PERCENT CHOLINESTERASE ACTIVITY
Cholinergic Effects on RBC'S
PERCENT ACTIVITY
125
100
75
50
25
0
PHY
PIL
.2mg/kg/inj
DFP
.05mg/kg/inj
CONTROL
1ST
2ND
INJECTION NUMBER

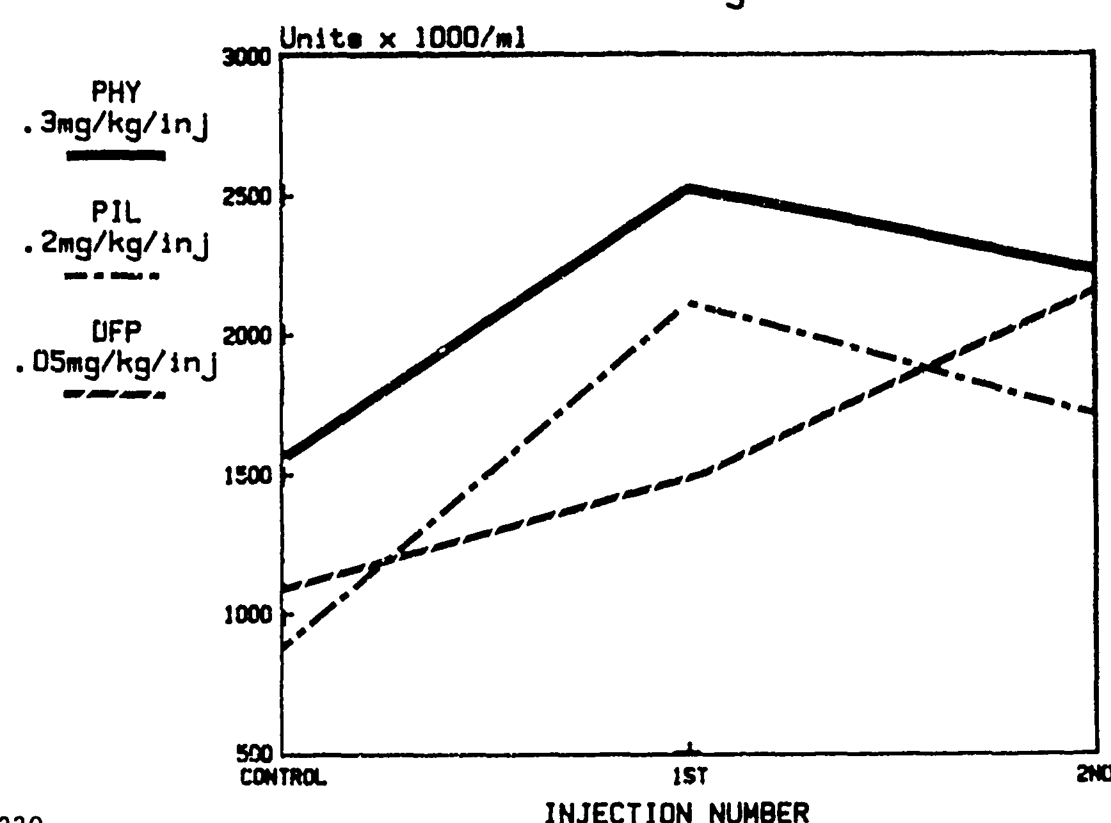
PERCENT CHOLINESTERASE ACTIVITY
Parotid Cholinergic Effects



PERCENT CHOLINESTERASE ACTIVITY
Extraparotid Cholinergic Effects




SALIVARY KALLIKREIN LEVELS
Parotid Cholinergic Effects
TAME UNITS (X 0.01)/ml
PHY
.3mg/kg/inj
PIL
.2mg/kg/inj
DFP
.05mg/kg/inj
2.00
1.75
1.50
1.25
1.00
.75
.50
.25
0.00
CONTROL
1ST
2ND
INJECTION NUMBER


SALIVARY AMYLASE LEVELS
Parotid Cholinergic Effects
Units x 1000/ml
PHY
.3mg/kg/inj
PIL
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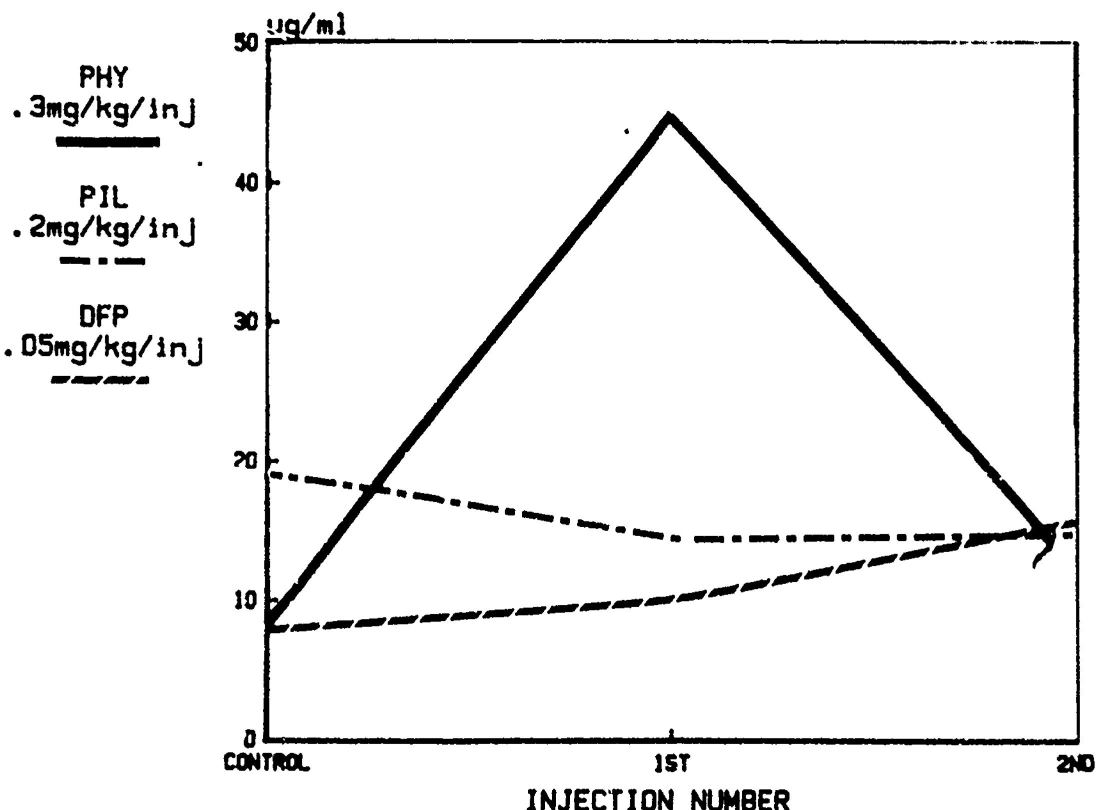
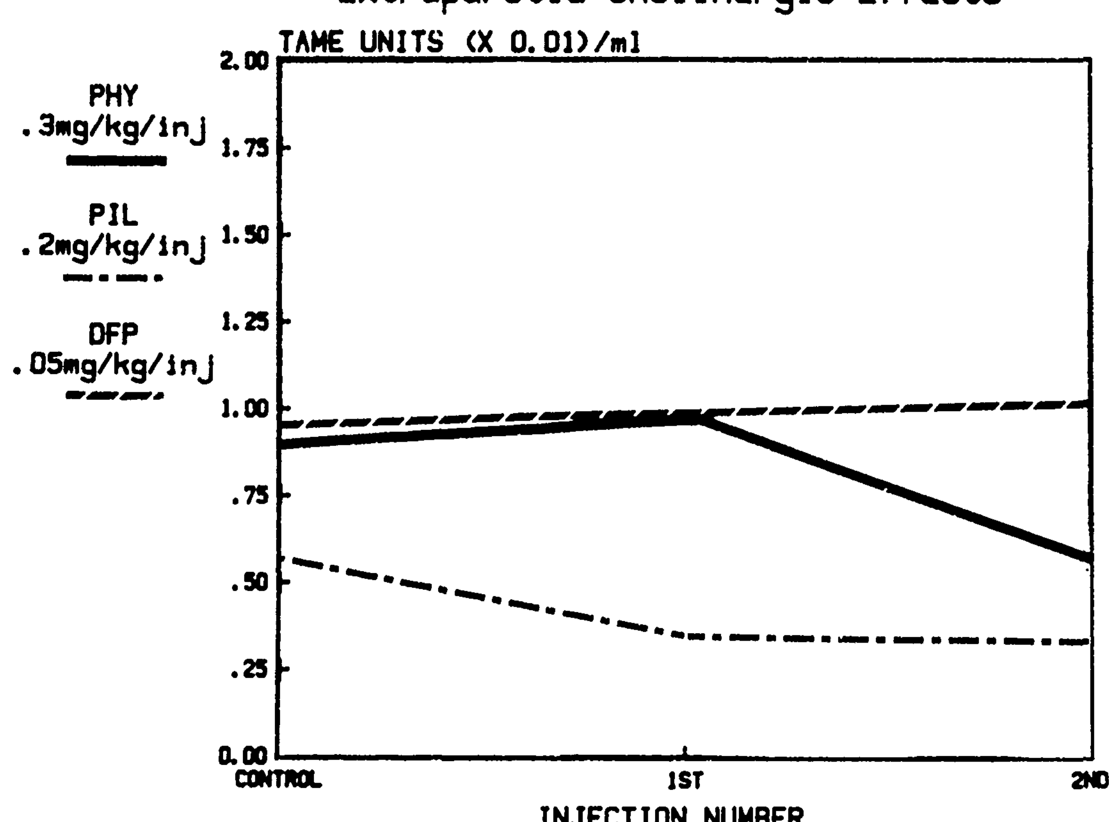
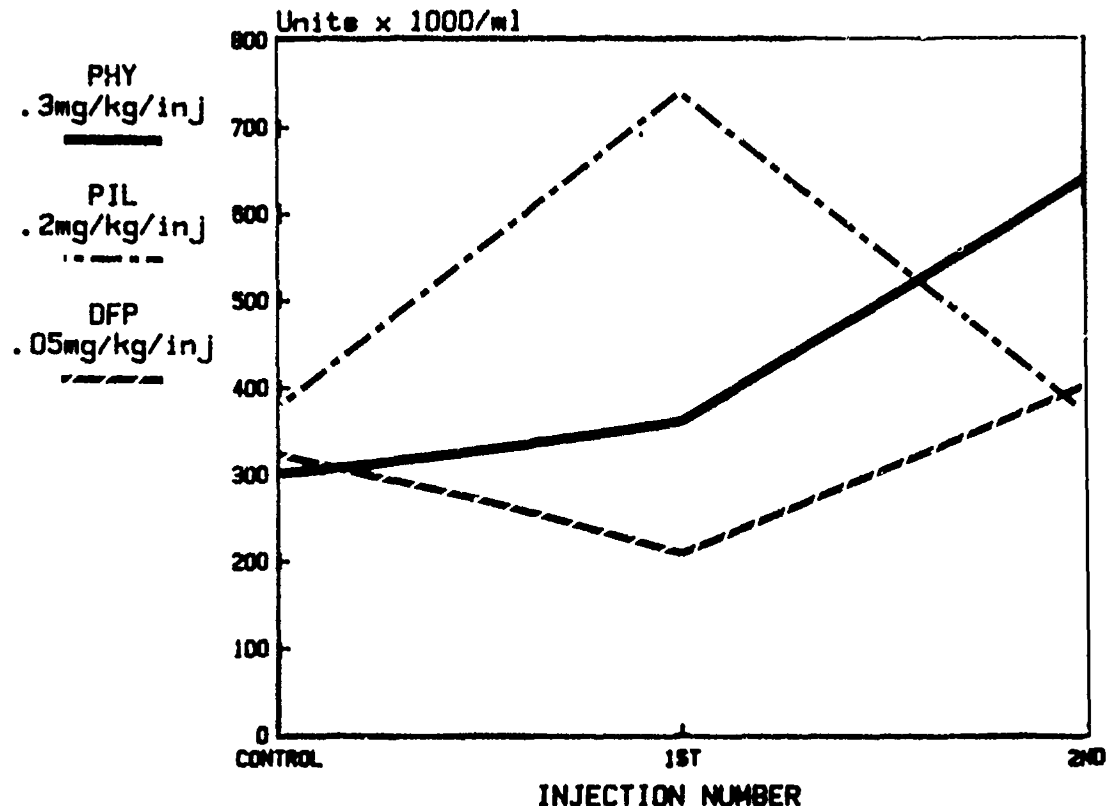
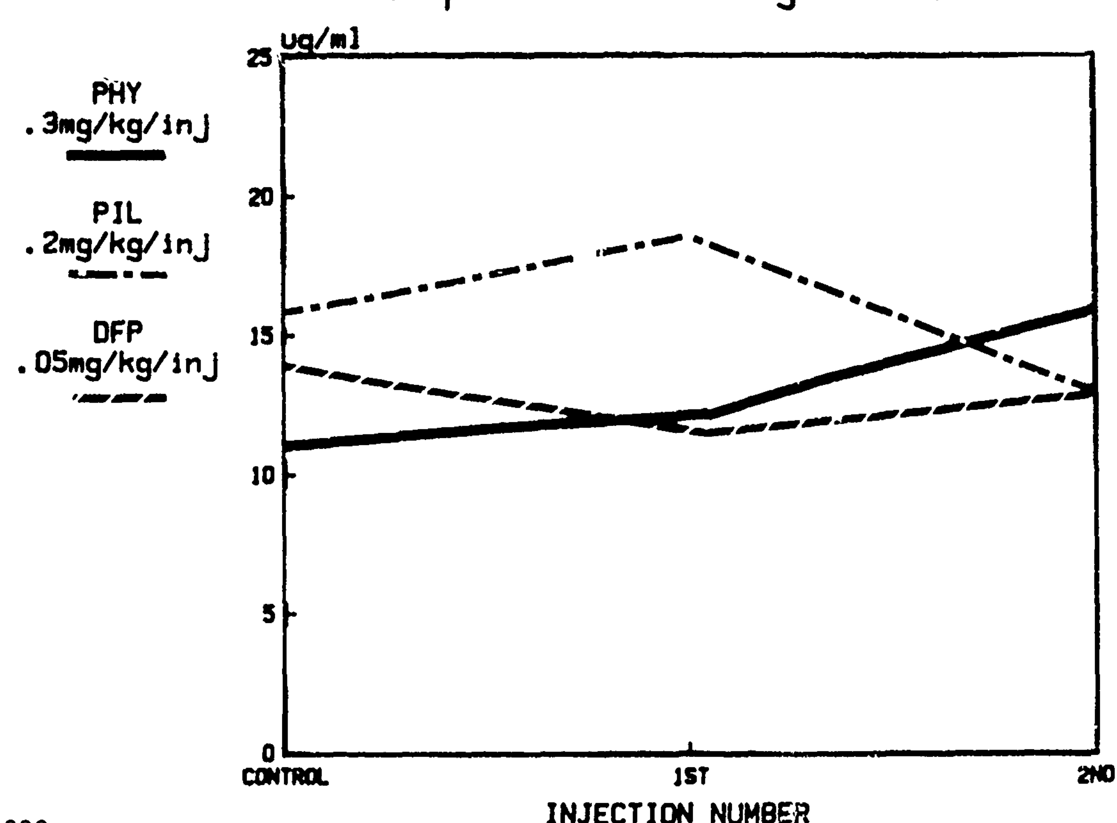
DFP
.05mg/kg/inj
3000
2500
2000
1500
1000
500
CONTROL
1ST
2ND
INJECTION NUMBER


SALIVARY LYSOZYME LEVELS
Parotid Cholinergic Effects
ug/ml
PHY
.3mg/kg/inj
PIL
.2mg/kg/inj
DFP
.05mg/kg/inj
50
40
30
20
10
0
CONTROL
1ST
2ND
INJECTION NUMBER


SALIVARY KALLIKREIN LEVELS
Extraparotid Cholinergic Effects
TAME UNITS (X 0.01)/ml
PHY
.3mg/kg/inj
PIL
.2mg/kg/inj
DFP
.05mg/kg/inj
2.00
1.75
1.50
1.25
1.00
.75
.50
.25
0.00
CONTROL
1ST
2ND
INJECTION NUMBER


SALIVARY AMYLASE LEVELS
Extraparotid Cholinergic Effects
Units x 1000/ml
PHY
.3mg/kg/inj
PIL
.2mg/kg/inj
DFP
.05mg/kg/inj
800
700
600
500
400
300
200
100
0
CONTROL
1ST
2ND
INJECTION NUMBER


SALIVARY LYSOZYME LEVELS
Extraparotid Cholinergic Effects
ug/ml
PHY
.3mg/kg/inj
PIL
.2mg/kg/inj
DFP
.05mg/kg/inj
25
20
15
10
5
0
CONTROL
1ST
2ND
INJECTION NUMBER

CONCLUSION

The results indicated that the anticholinesterase compounds inhibited blood cholinesterase levels, while the cholinomimetic had no significant effect on blood cholinesterase. The organophosphate also inhibited salivary cholinesterase, while both pilocarpine and physostigmine stimulated salivary cholinesterase secretion. All three compounds caused an elevation in the level of salivary amylase, while lysozyme levels remained relatively uneffected. Administration of both pilocarpine and physostigmine caused decreases in parotid kallikrien levels, while diisopropylfluorphosphate appeared to have no effect. The results from this study demonstrated the different effects on salivary enzyme levels following the administration of three different cholinergic compounds. It also indicated the need to study the effects of specific chemical agents on salivary enzymes.

EFFECT OF SARIN, SOMAN AND DFP ON PHOSPHOLIPID AND PROTEIN ASYMMETRY IN THE PLASMA MEMBRANE OF ELECTROPLAX FRO THE ELECTRIC EEL

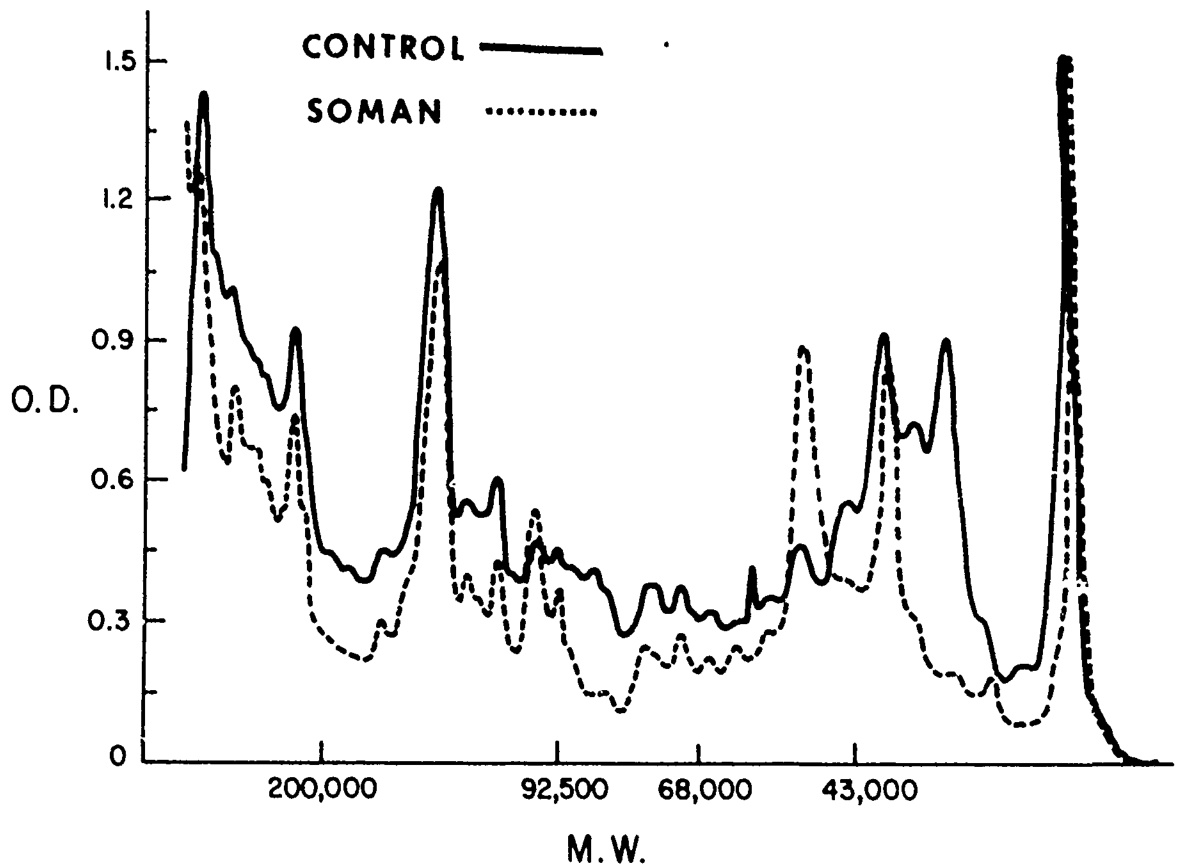
P. Rosenberg, K. Hubbard, H. Schupper, C. Supernovich and B.A. Weber
Section of Pharmacology and Toxicology, The University of Connecticut
School of Pharmacy, Storrs, CT 06268

SEVERAL LONG LASTING EFFECTS OF ANTICHOLINESTERASES (VISUAL, BEHAVIORAL, ELECTROENCEPHALOGRAPHIC) MAY NOT BE DUE TO CHOLINESTERASE INHIBITION, BUT COULD BE ASSOCIATED WITH CHANGES IN MEMBRANE COMPOSITION OR ORGANIZATION. THE ISOLATED SINGLE ELECTROPLAX FROM THE ELECTRIC EEL WAS USED IN THESE STUDIES BECAUSE IT IS AN EXCITABLE CELL WITH NICOTINIC JUNCTIONS AND IT IS POSSIBLE TO DISSECT SINGLE CELLS AND TO SPLIT THESE CELLS INTO INNERVATED, CONDUCTING, AND NON-INNERVATED, NON-CONDUCTING, SURFACES FROM WHICH PLASMA AND MITOCHONDRIAL ENRICHED MEMBRANE FRACTIONS CAN BE ISOLATED. MOST IMPORTANTLY, THIS PREPARATION ALLOWS VECTORIAL APPLICATION OF THE IMPENETRABLE MEMBRANE PROBES USEDIN THIS STUDY: LACTOPEROXIDASE(LPO) CATALYZED IODINATION, PHOSPHATIDYLCHOLINE (PC) EXCHANGE PROTEIN (PCEP) AND TRINITROBENZENE SULFONATE (TNBS).

INTACT CELLS WERE INCUBATED *IN VITRO* FOR 30 MINUTES WITH 10^{-3}M DFP, 10^{-4}M SARIN OR 10^{-4}M SOMAN WHILE LIVE EELS WERE EXPOSED *IN VIVO* FOR ONE WEEK TO 10^{-8}M ANTICHOLINESTERASES. INTACT OR SPLIT CELLS WERE THEN EXPOSED TO THE MEMBRANE PROBES, HOMOGENIZED AND FRACTIONS ISOLATED. THE INTACT ELECTROPLAX CELL WAS IMPERMEABLE TO RADIOACTIVE SUCROSE, TRIOLEIN, PC, TNBS AND SODIUM IODIDE AND SOMAN, SARIN AND DFP DID NOT INCREASE PERMEABILITY. FOLLOWING ORGANOPHOSPHATE EXPOSURE *IN VITRO*, CHOLINESTERASE IN ELECTRIC CELLS WAS INHIBITED 87 TO 100% WHILE FOLLOWING *IN VIVO* EXPOSURE INHIBITION IN VARIOUS TISSUES WAS ONLY 0 TO 30%. CONTENT OF PROTEIN, PHOSPHOLIPID AND CHOLESTEROL WAS NOT SIGNIFICANTLY AFFECTED BY THE ORGANOPHOSPHATES NOR WAS THE % DISTRIBUTION OF THE INDIVIDUAL PHOSPHOLIPIDS. *IN VITRO* EXPOSURE TO SOMAN AND TO A LESSER EXTENT, SARIN AND DFP,INCREASED THE CHOLESTEROL TO PHOSPHOLIPID RATIO IN THE INNERVATED PLASMA MEMBRANE FRACTION AND DECREASED IT IN THE NON-INNERVATED FRACTION WHILE FOLLOWING *IN VIVO* EXPOSURE TO SARIN A MARKED DECREASE IN CHOLESTEROL TO PHOSPHOLIPIDS WAS NOTED. THESE CHANGES WOULD BE EXPECTED TO ALTER MEMBRANE FLUIDITY. GEL SCANS OF MEMBRANE FRACTIONS (SDS-PAGE) SHOWED LITTLE ALTERATIONS, ALTHOUGH SOMAN CAUSED SOME CHANGES IN THE PROTEIN PATTERN OF THE NON-INNERVATED PLASMA MEMBRANE FRACTION. PCEP CATALYZES THE EXCHANGE OF ^{14}C-PC FROM SMALL UNILAMMELAR VESICLES TO EXPOSED PC IN THE ELECTROPLAX CELLS. LABELING IN HOMOGENIZED CELLS (90%) WAS GREATER THAN IN SEPARATED SURFACES (60%) OR INTACT CELLS (30%). PC LABELING WAS INCREASED IN THE INTACT CELLS EXPOSED *IN VITRO* TO SOMAN AND SARIN (FROM 30% TO 52% AND 60%, RESPECTIVELY). THERE WERE ALSO MARKED CHANGES IN PC LABELING FOLLOWING *IN VIVO* EXPOSURE OF THE EEL TO SOMAN OR SARIN AND DURING A SUBSEQUENT TWO WEEK RECOVERY PERIOD. TNBS COVALENTLY LABELS EXPOSED PHOSPHATIDYLETHANOLAMINE (PE) AND PHOSPHATIDYLSERINE (PS). LABELING OF PE(30%) WAS INCREASED (40%) IN THE INNERVATED PLASMA MEMBRANE FRACTION AFTER *IN VITRO* SOMAN EXPOSURE AND SLIGHTLY DECREASED FOLLOWING *IN VIVO* EXPOSURE. PS WAS NEITHER LABELED IN CONTROL NOR SOMAN TREATED CELLS. LPO CATALYZES IODINATION OF EXPOSED PROTEINS. IN THE INNERVATED AND NON-INNERVATED SURFACES BOTH SARIN AND DFP DECREASED LABELING OF HIGH AND INCREASED LABELING OF LOW MOLECULAR WEIGHT PROTEINS, WHILE IN THE SEPARATED PLASMA MEMBRANE FRACTION DFP MARKEDLY INCREASED LABELING.

THE RESULTS SUGGEST THAT CHOLINESTERASE INHIBITORS MAY CAUSE THE FOLLOWING MEMBRANAL CHANGES: ALTERED MEMBRANE FLUIDITY, ALTERED DISTRIBUTION OF PC AND PE IN THE OUTER AND INNER MONOLAYER OF THE PLASMA MEMBRANE AND A REORIENTATION OF PROTEINS. THESE CHANGES MAY NOT BE DUE TO CHOLINESTERASE INHIBITION BUT MAY BE ASSOCIATED WITH LONG LASTING EFFECTS OF ANTICHOLINESTERASES.

THIS WORK SUPPORTED IN PART BY THE U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND UNDER CONTRACT DAMD 17-82-C-2096.



Densitometric scan of the non-innervated plasma membrane fraction subjected to SDS-polyacrylamide gel electrophoresis.

EFFECT OF DFP, SOMAN AND SARIN ON CHOLINESTERASE ACTIVITY OF MEMBRANE FRACTIONS FROM THE INNERVATED AND NON-INNERVATED SURFACES OF ELECTROPLAX

FRACTION	CHOLINESTERASE ACTIVITY (μMOL/MIN/MG PROTEIN)			
	CONTROL	DFP	SOMAN	SARIN
CRUDE HOMOGENATE:				
INNERVATED	7.04±1.20	0.51±0.21	0.03±0.02	0.04±0.02
NON-INNERVATED	1.62±0.22	0.02±0.01	0.10±0.08	0.02±0.02
PLASMA MEMBRANE:				
INNERVATED	3.99±0.60	0.09±0.04	0.01±0.01	0.09±0.01
NON-INNERVATED	1.77±0.22	0.03±0.01	0.01±0.01	0.01±0.01

ELECTROPLAX WERE EXPOSED TO 1.0 mM DFP, 0.1 mM SOMAN OR 0.1 mM SARIN FOR 30 MIN AT ROOM TEMPERATURE. ALL RESULTS ARE PRESENTED AS MEANS ± S.E. (N=9 FOR CONTROL VALUES; N=3 FOR TREATED VALUES). ALL CONTROL VALUES ARE SIGNIFICANTLY DIFFERENT FROM THE CORRESPONDING TREATED VALUES ($p<0.001$).

EFFECT OF SOMAN AND SARIN ON THE CHOLESTEROL TO PHOSPHOLIPID MOLAR RATIO OF MEMBRANE FRACTIONS FROM THE INNERVATED AND NON-INNERVATED SURFACES OF ELECTROPLAX

FRACTION	CHOLESTEROL/PHOSPHOLIPID (MOLE/MOLE)		
	CONTROL	SOMAN	SARIN
PLASMA MEMBRANE:			
INNERVATED	0.78±0.09	1.14±0.25 *	0.85±0.12
NON-INNERVATED	0.58±0.06	0.47±0.12 *	0.54±0.05
MITOCHONDRIAL MEMBRANE:			
INNERVATED	0.94±0.12	0.96±0.24	0.90±0.13
NON-INNERVATED	0.65±0.11	0.56±0.10	0.51±0.20

ELECTROPLAX WERE EXPOSED TO 0.1 mM SOMAN OR SARIN FOR 30 MIN AT ROOM TEMPERATURE. ALL RESULTS ARE PRESENTED AS MEANS ± S.E. (N=8-11 FOR CONTROL VALUES; N=3-4 FOR TREATED VALUES). *SIGNIFICANTLY DIFFERENT FROM CORRESPONDING CONTROL VALUES (STUDENT'S PAIRED T-TEST; $p<0.05$).

Introduction

1. MEMBRANE EFFECTS OF ORGANOPHOSPHORUS COMPOUNDS: SEVERAL LONG-LASTING EFFECTS OF ANTICHE AGENTS (BEHAVIORAL, EEG, VISUAL) ARE INDEPENDENT OF CHE INHIBITION AND MAY BE ASSOCIATED WITH CHANGES IN COMPOSITION OR ORGANIZATION OF BIOELECTRICALLY EXCITABLE MEMBRANES.

2. ELECTROPLAQUE: THE ELECTROPLAQUE IS A SINGLE CELL DISSECTED FROM THE SACHS ORGAN OF THE ELECTRIC EEL, E. ELECTRICUS WHICH CONTAINS BOTH AN INNERVATED CONDUCTING AND NON-INNERVATED NON-CONDUCTING PLASMA MEMBRANE SURFACE. THIS IS A UNIQUELY SUITABLE MODEL IN WHICH TO STUDY THE EFFECTS OF ANTICHE AGENTS BECAUSE THESE CELLS IN ADDITION TO CONTAINING CHOLINERGIC JUNCTIONS CAN BE SPLIT IN HALF ALLOWING PROBES TO BE APPLIED SELECTIVELY FROM OUTSIDE OR INSIDE OF THE CELL. SPLITTING OF THE CELL AND DIFFERENTIAL CENTRIFUGATION ALLOWS US TO PREPARE PLASMA- AND MITOCHONDRIAL-ENRICHED MEMBRANE FRACTIONS FROM THE SURFACES AND THUS SELECTIVELY ANALYZE ANY ORGANOPHOSPHATE INDUCED CHANGE IN MEMBRANE ORGANIZATION.

3. PHOSPHATIDYLCHOLINE EXCHANGE PROTEIN (PCEP): PCEP IS A NONPERTURBING PROBE FOR ASSESSING PC COMPOSITION IN MEMBRANES. PCEP TRANSFERS A PC FROM THE OUTER MONOLAYER OF A DONOR MEMBRANE (SMALL UNILAMELLAR VESICLES, SUV, CONTAINING ^{14}C-PC) TO THE OUTER MONOLAYER OF AN ACCEPTOR MEMBRANE (ELECTROPLAX). THIS ALLOWS QUANTITATION OF THE PC LOCATED ON THE OUTER MONOLAYER OF THE PLASMA MEMBRANE OF ELECTOPLAX AND OF ANY EFFECT THAT ORGANOPHOSPHORUS COMPOUNDS MAY HAVE ON PC DISTRIBUTION.

4. <u>Trinitrobenzenesulphonic Acid (TNBS)</u>: TNBS labels the exposed primary amino groups of PE and PS to form trinitrophenyl-(TNP)-PE and TNP-PS. TNBS is a nonpenetrating probe under specified conditions in electroplax and thus can be used as a monitor for observing alterations in PE and PS distribution.

5. <u>Lactoperoxidase (LPO) Catalyzed Iodination</u>: LPO catalyzes the iodination of tyrosine and histidine groups in proteins. LPO is an impermeable probe in electroplax and thus can be used to study any effects that organophosphorus compounds may have on protein asymmetry in electroplax plasma membranes.

EFFECT OF SOMAN AND SARIN ON THE PHOSPHOLIPID DISTRIBUTION IN THE INNERVATED AND THE NON-INNERVATED SURFACES OF ELECTROPLAX

FRACTION	PHOSPHOLIPID (%)					
	SM	PC	PI	PS	PE	CL
CRUDE HOMOGENATE:						
INNERVATED						
CONTROL	7±1	48±1	5±1	9±1	28±1	3±1
SOMAN	7±2	49±2	4±1	10±1	26±1	4±1
SARIN	7±1	45±2	7±1	9±1	28±2	4±1
NON-INNERVATED						
CONTROL	5±1	54±2	6±1	9±1	23±1	3±1
SOMAN	3±1	59±2	7±1	6±1	23±2	3±1
SARIN	6±2	52±1	7±0.3	9±1	24±1	2±1
PLASMA MEMBRANE:						
INNERVATED						
CONTROL	4±1	50±2	6±1	9±1	29±1	2±1
SOMAN	4±0.4	48±1	6±1	10±1	30±2	4±1
SARIN	5±1	44±1	7±0.4	10±1	31±0.3	4±0.4
NON-INNERVATED						
CONTROL	2±1	50±2	8±1	9±1	26±2	2±1
SOMAN	1±1	50±1	7±1	8±1	30±0.4	4±1
SARIN	3±0.3	47±1	7±0.3	9±0.2	32±1	3±1

ALL RESULTS ARE SHOWN AS PERCENT OF TOTAL PHOSPHOLIPID AND ARE PRESENTED AS X ± S.E. (N=3 FOR TREATED VALUES. N=6 FOR CONTROL VALUES EXEPT FOR THE CONTROL NON-INNERVATED CRUDE HOMOGENATE WHERE N=5). NONE OF THE SOMAN OR SARIN VALUES ARE SIGNIFICANTLY DIFFERENT FROM THE CORRESPONDING CONTROL VALUES. ABBREVIATIONS: SM = SPHINGOMYELIN; PC = PHOSPHATIDYLCHOLINE; PI = PHOSPHATIDYLINOSITOL; PS = PHOSPHATIDYLSERINE; PE = PHOSPHATIDYLETHANOLAMINE; CL = CARDIOLIPIN.

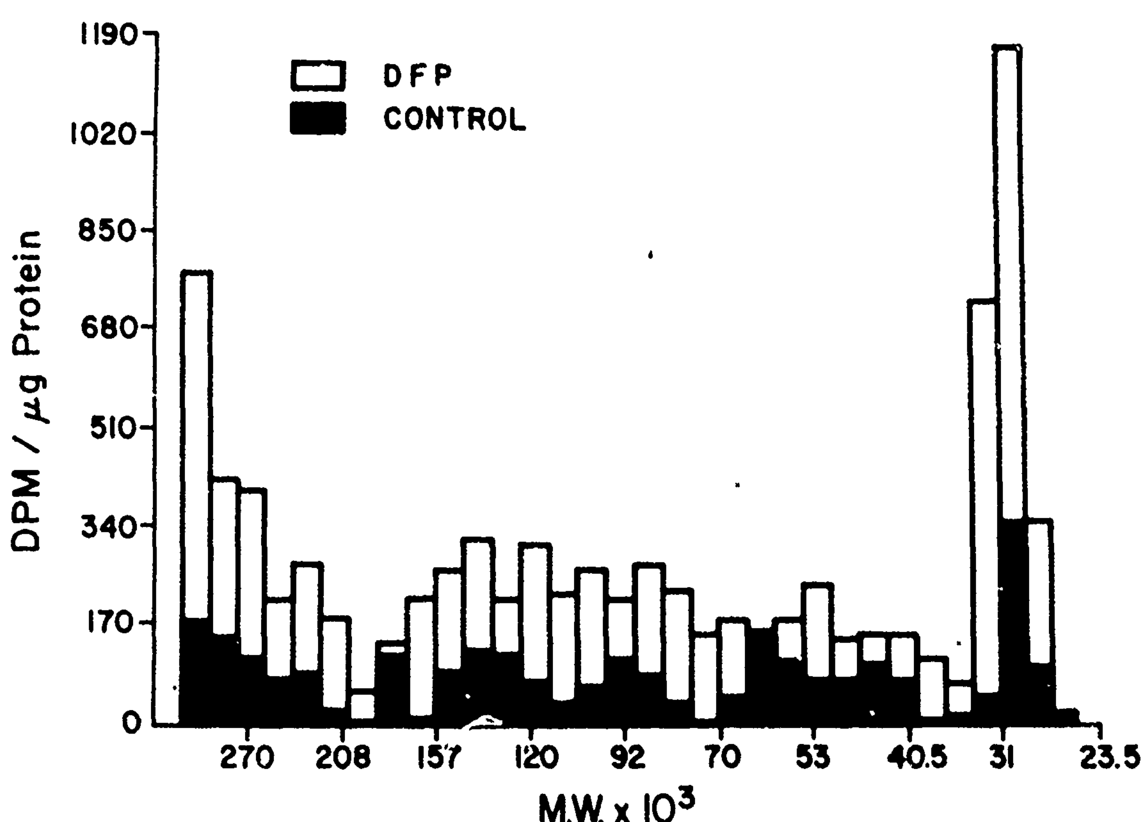
Objective

To determine if soman, sarin, or DFP alters the cholesterol to phospholipid ratio, pattern or accessibility of protein, or percentage of phosphatidylcholine (PC), phosphatidylethanolamine (PE) or phosphatidylserine (PS) on the outer monolayer of the plasma membrane. Such changes would suggest an effect on membrane organization that might explain certain long-lasting effects of anticholinesterases (i.e. behavioral, EEG, visual) which have not been correlated with cholinesterase (ChE) inhibition.

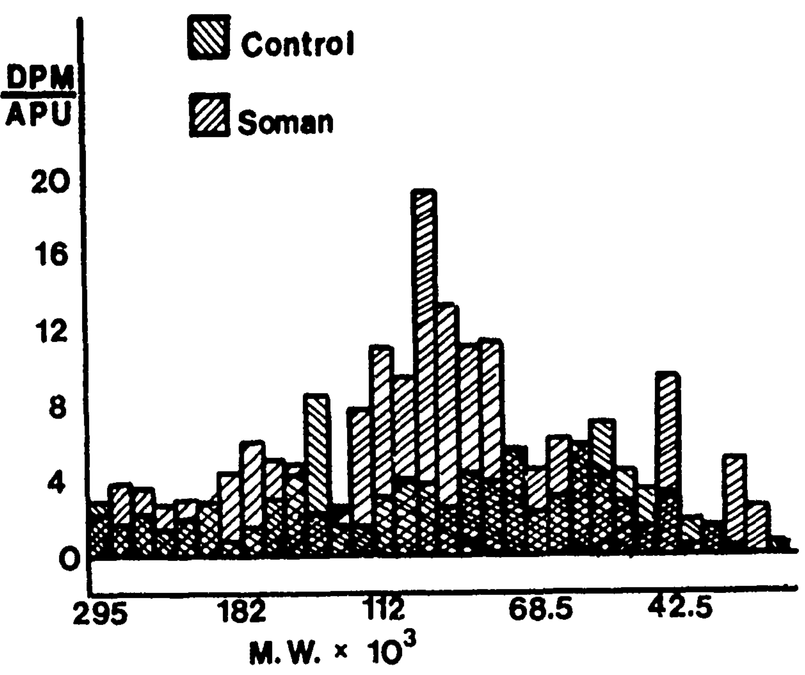
LACTOPEROXIDASE CATALYZED IODINATION

LACTOPEROXIDASE (LPO) CATALYZED IODINATION
OF PROTEIN IN ELECTROPLAX

CONDITION	LABELING	
	CPM/MG PROTEIN X 10^6	%
Homogenized Cells + LPO	5.1	100
Split Cells + LPO	2.6	51
Intact Cells + LPO	1.4	27
Intact Cells - LPO	0.07	1



Lactoperoxidase catalyzed iodination in the innervated plasma membrane fraction from the electroplax. DFP= 10^{-3}M, 30 minutes, in-vitro.



Lactoperoxidase catalyzed iodination of proteins in the innervated surface of the electroplax. Soman= 10^{-4}M, 30 minutes, in-vitro. APU= arbitrary protein unit.

Methods

1. <u>AntiChE Exposure-In Vitro</u>: Intact electroplax were incubated with control eel Ringer's solution or with antiChE agent (1mM DFP or 0.1mM soman or sarin) in eel Ringer's solution for 30 minutes at room temperature. <u>In vivo</u>: Eels were exposed for one week to 10^{-8} M soman or sarin in their aquarium water (pH 6.0). During this time the anticholinesterase potency of the water was checked by bioassay and additions of agent made as necessary.

2. <u>Membrane Isolation</u>: Plasma and mitochondrial membrane enriched fractions were isolated from the innervated and non-innervated surfaces of electroplax by differential centrifugation. Marker enzymes and electron microscopy assessed the enrichment of the fractions.

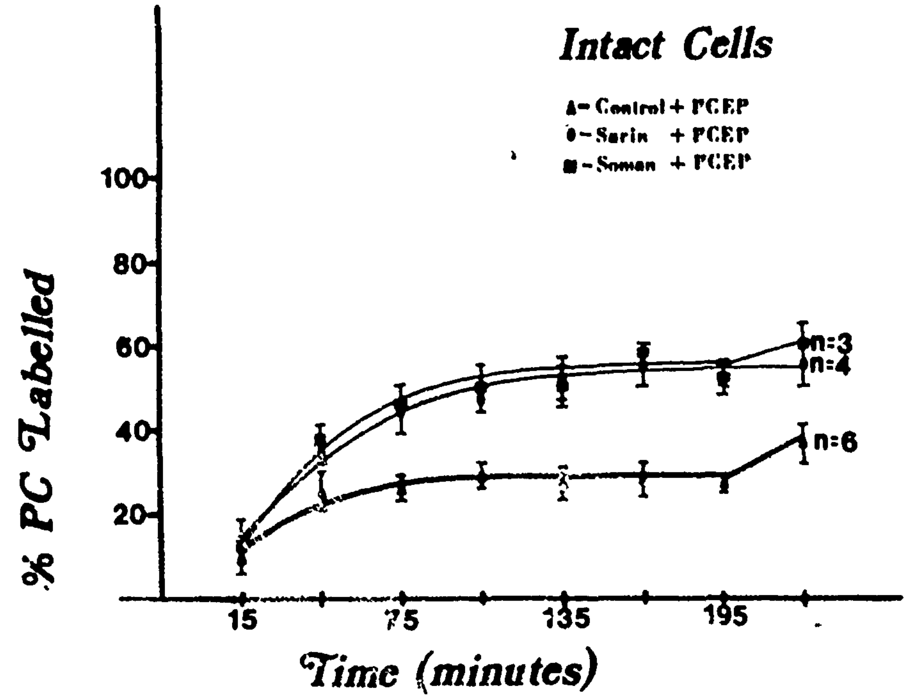
3. <u>Incubation Conditions</u>:

   A. <u>PCEP</u>:
   Tissues were incubated with PCEP and SUV in BSA-SET buffer (bovine serum albumin 5 mg/ml, sucrose 0.25 M, EDTA .001 M, Tris-HCl .05 M) at 37°C. PCEP was isolated from beef liver according to the method of Kamp *et al.* SUV(70 mole % phosphatidylcholine-includes ^{14}C-PC, 25 mole % PE, 5 mole % cardiolipin and .05 mole ^{3}H-Triolein-a non-exchangeable marker) were made by sonication according to the method of Bangham *et al.* The time course of labeling was determined by measuring the incorporation of radiolabeled PC in the cells at 30 minute time intervals. The final PC incorporation was corrected for the amount of ^{3}H-Triolein sticking.

   B. <u>TNBS</u>:
   Tissues were incubated with 5 ml of 0.5 mM TNBS for 20 minutes in 173 mM $NaHCO_3$ buffer containing 10 mM dextrose at pH 8.5 and at 4°C in the dark with shaking. Reaction was terminated by washing with pH 6.8 eel Ringer's solution.

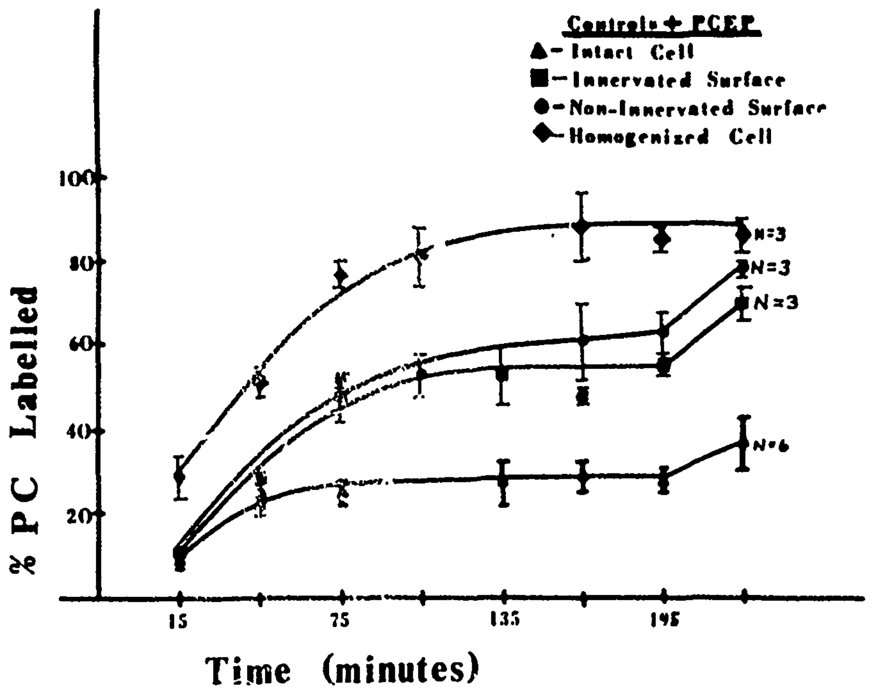
   C. <u>LPO</u>:
   Tissues were incubated in 5 ml eel Ringer's solution (pH 7.4) containing 55 µCi $Na^{125}I$, 5 mM glucose and 1 µM lactoperoxidase. Glucose oxidase (1 unit) is added to start the reaction and incubation is for 15 minutes at 25°C. The reaction is stopped by addition of termination solution (0.2 mM $Na_2S_2O_3$, 0.02 M NaI, pH 7.4) which contains protease inhibitors (5mM iodoacetamide, 5 mM Na azide, 5 mM EDTA, 0.5 mM PMSF).

PHOSPHATIDYLCHOLINE EXCHANGE PROTEIN



Effect of Soman and Sarin on PC Labelling

PC labelling in sarin and soman treated cells is significantly ($p < .05$) different from control cells.



PC Labelling in Electroplax

Homogenized cells are labelled to a greater extent than separated surfaces, which are in turn labelled to a greater extent than intact cells.

Conclusions

1. PCEP, TNBS AND LPO ARE USEFUL IMPENETRABLE MEMBRANE PROBES WHICH WERE USED TO STUDY ANTICHOLINESTERASE-INDUCED EFFECTS ON MEMBRANE ORGANIZATION AND ASYMMETRY IN A BIOLELECTRICALLY EXCITABLE TISSUE.

2. THE ANTICHOLINESTERASES:

   A. ALTER CHOLESTEROL TO PHOSPHOLIPID RATIO, SUGGESTING CHANGES IN MEMBRANE FLUIDITY.

   B. MODIFY EXPOSURE OF PROTEIN ON THE OUTER LEAFLET OF THE PLASMA MEMBRANE.

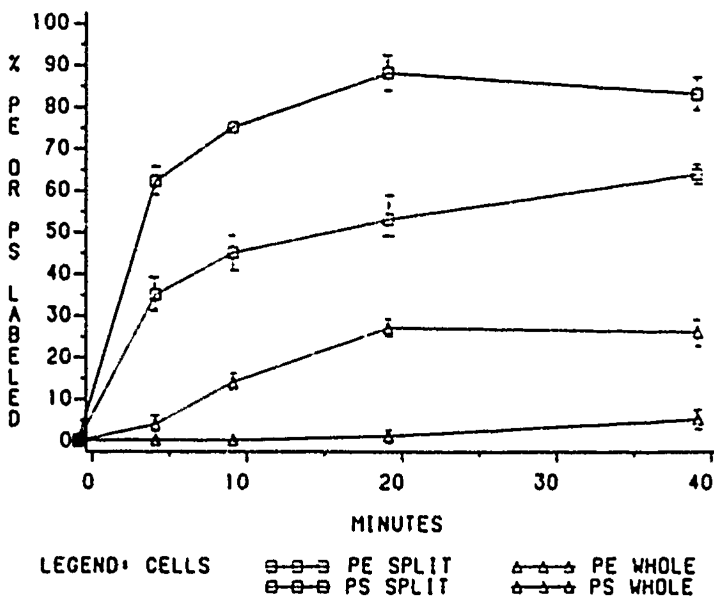
   C. CHANGE THE % OF PE AND PC WHICH ARE ON THE OUTER LEAFLET OF THE PLASMA MEMBRANE.

3. THE SPECIFIC CHANGES INDUCED BY SARIN AND SOMAN (10^{-4}M) *IN VITRO* ARE NOT IDENTICAL EVEN THOUGH THEY BOTH COMPLETELY INHIBITED CHOLINESTERASE WHILE *IN VIVO* (10^{-8}M) EFFECTS ARE OBSERVED EVEN WITH VERY LOW LEVELS OF CHOLINESTERASE INHIBITION.

4. THESE RESULTS SUGGEST THAT ANTICHOLINESTERASES MAY DRASTICALLY MODIFY MEMBRANE ORGANIZATION IN BIOELECTRICALLY EXCITABLE TISSUES BY ACTION(S) INDEPENDENT OF CHOLINESTERASE INHIBITION. SUCH EFFECTS COULD BE RESPONSIBLE FOR LONG-LASTING CLINICAL TOXICITIES OBSERVED WITH THESE AGENTS.

TRINITROPHENYLATION OF AMINOPHOSPHOLIPIDS

COMPARISON OF PE AND PS LABELING IN INTACT ELECTROPLAX TO THAT IN SPLIT ELECTROPLAX



Labeling with TNBS (0.5 mM) was carried out as described under methods section. $\bar{X} \pm$ S.E., N=3-6.

LABELING OF PE IN MEMBRANE FRACTIONS FROM INNERVATED AND NONINNERVATED SURFACES OF CONTROL, SOMAN AND SARIN TREATED ELECTROPLAX

FRACTION	TREATMENT	% PE LABELED	
		INNERVATED	NON-INNERVATED
CRUDE HOMOGENATE	CONTROL	22 ± 1	31 ± 1
	SOMAN	23 ± 4	30 ± 3
	SARIN	23 ± 3	34 ± 2
PLASMA MEMBRANE	CONTROL	25 ± 1	38 ± 2
	SOMAN	35 ± 1*	40 ± 5
	SARIN	27 ± 3	40 ± 2
MITOCHONDRIAL MEMBRANE	CONTROL	4 ± 2	4 ± 3
	SOMAN	8 ± 5	0
	SARIN	9 ± 5	0

CONTROL VALUES ARE BASED ON SIX DETERMINATIONS EXCEPT THE CONTROL NON-INNERVATED CRUDE HOMOGENATE VALUE WHICH IS BASED ON FIVE DETERMINATIONS. TREATED VALUES ARE BASED ON THREE DETERMINATIONS. $\bar{X} \pm$ SE. PS WAS NOT LABELED BY TNBS IN ANY OF THE FRACTIONS. *SIGNIFICANTLY ($P<.05$) DIFFERENT FROM CONTROL VALUE.

OTHER RESULTS

1. GENERAL:

   A. IN VITRO ORGANOPHOSPHATE TREATMENTS INHIBITED MORE THAN 90% OF ELECTROPAX ChE ACTIVITY. IN CONTRAST, IN VIVO TREATMENTS INHIBITED LESS THAN 50% OF ELECTROPLAX, PLASMA, AND LIVER ChE ACTIVITY WITHOUT INHIBITING RED BLOOD CELL, BRAIN OR HEART ChE ACTIVITY.

   B. THE PROBES USED IN THESE STUDIES WERE IMPERMEABLE IN ELECTROPLAX AS SHOWN BY:

      1. IMPERMEABILITY OF ELECTOPLAX TO ^{14}C-SUCROSE WAS NOT AFFECTED BY EXPOSURE TO THE ANTIChE AGENTS, PROBES OR INCUBATION MEDIA.
      2. ELECTROPLAX WERE IMPERMEABLE TO ^{125}NaI, ^{14}C-PC, ^{3}H-TRIOLEIN, ^{3}H-TNBS.
      3. LABELING OF PROTEIN, PC AND PE OR PS BY LPO, PCEP, AND TNBS, RESPECTIVELY, WAS GREATEST IN HOMOGENIZED, LESS IN SPLIT AND LEAST IN INTACT CELLS.

   C. THE ANTIChE AGENTS DID NOT MODIFY THE ELECTROPHORETIC PROTEIN PATTERN OF ELECTROPLAX FRACTIONS, EXCEPT FOR A SHIFT IN THE PATTERN OF THE NON-INNERVATED PLASMA MEMBRANE FRACTION AFTER SOMAN EXPOSURE.

   D. ORGANOPHOSPHATES DID NOT EFFECT PROTEIN CONTENT, TOTAL LIPID PHOSPHORUS, OR PHOSPHOLIPID DISTRIBUTION IN ELECTROPLAX FRACTIONS. HOWEVER, IN THE INNERVATED PLASMA MEMBRANE, SOMAN (10^{-4} M, IN VITRO) INCREASED CHOLESTEROL CONTENT AND CHOLESTEROL TO PHOSPHOLIPID MOLAR RATIO WHILE SARIN (10^{-8} M, IN VIVO) DECREASED THESE PARAMETERS.

2. PCEP:

A. PCEP did not alter cholesterol content, phospholipid content, or phospholipid distribution in electroplax.

B. Some labeling of PC, probably due to endogenous exchange proteins, occurred even in the absence of added PCEP.

C. In vivo exposure to soman (10^{-8} M) increased the percentage of PC in the outer leaflet of the plasma membrane, while sarin (10^{-8} M) decreased the percentage of PC. The effect of sarin, but not soman, reversed during the two week recovery period.

D. In vitro exposure to soman (10^{-4} M) increased the percentage of PC in the outer leaflet of the innervated plasma membrane and decreased the percentage of PC in the non-innervated plasma membrane.

3. TNBS:

A. TNBS was demonstrated to be impermeable in electroplax under specified conditions (0.5 mM TNBS at 4°C in the dark in a bicarbonate buffer at pH 8.5 for 20 min). These conditions resulted in labeling of only the PE on the outer leaflet of the plasma membrane in intact electroplax.

B. Mitochondrial aminophospholipids are not labeled by TNBS (0.5 mM) in intact cells. Apparent labeling is due to contaminating extracellular material.

C. Soman (10^{-8} M, in vivo) decreased labeling of PE in the innervated plasma membrane fraction.

4. LPO:

A. Sarin (10^{-4} M, in vitro) decreased accessibility of high molecular weight proteins and increased accessibility of low molecular weight proteins in the innervated and non-innervated surfaces.

B. Soman (10^{-4} M, in vitro) increased labeling of most of the proteins in the innervated and non-innervated surfaces.

C. DFP (10^{-3} M, in vitro) had effects, on the surfaces similar to those of sarin, while on the innervated and non-innervated plasma membrane fractions, it increased labeling of proteins.

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DO OXIMES HAVE EFFECTS OTHER THAN REACTIVATION OF ACETYLCHOLINESTERASE?

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INTRODUCTION

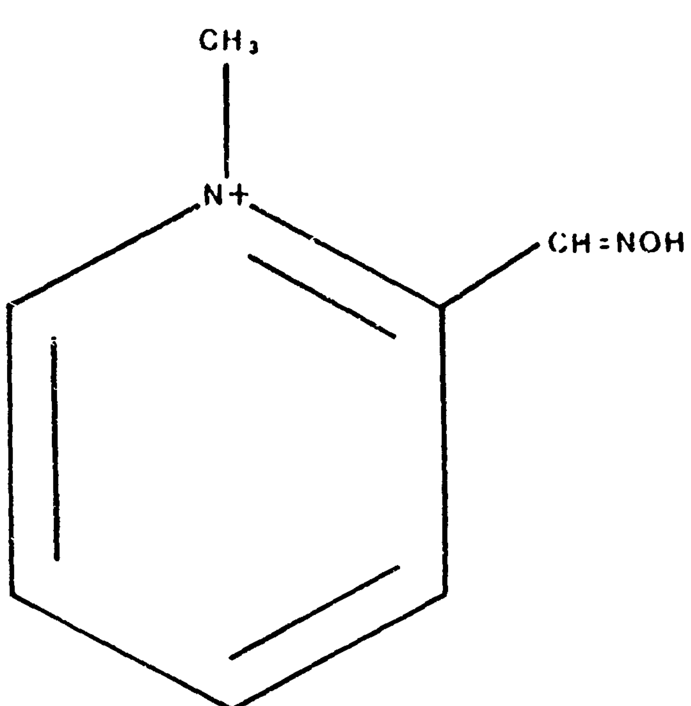
It is recognized, but not readily appreciated, that all cardiac glycosides do not exert their effect through the same molecular action (Bailey, et al., 1971). Qualitative as well as quantitative differences are observed among the glycosides not only with reference to their pharmacokinetics but also in relation to their distribution, toxicity, interaction with K^+ and Ca^{++}, and cardiac electrophysiology. A number of glycosides (i.e. ouabain, digoxin) appear to work through an interaction with Na^+ + K^+ ATPase. The cardiac glycoside binds to the phosphorylated enzyme form (E_2—P) of Na^+ + K^+ ATPase forming the glycoside phospho-enzyme complex (i.e. ouabain—E_2—P). The ouabain-enzyme complex is very stable and therefore, turns over (dephosphorylates) at a very slow rate. This reaction bears a mechanistic resemblance to the binding of anti-cholinesterase agents to acetylcholinesterase (AChE) and the subsequent inhibition of that enzyme.

Apparent similarities of effects of cardiac glycosides on the cholinergic nervous system (i.e. negative chronotropy, Godfraind, 1963) as well as a number of reports of an apparent interaction on cholinergic "receptors" or responses (Maheshwari, et al., 1971; Mozsik, 1969; Mozsik, et al., 1972) have also been described. From a physiological view, both of these enzymes, by independent mechanisms, regulate cellular K^+ concentrations and thus the excitability of cells. Chemicals that interact with these enzymes could influence either or both processes most likely in ways amenable to pharmacological intervention. It may be possible to develop second and third generation drugs directed to other phosphorylated enzymes. The clinical importance is evident. Na^+ + K^+ ATPase is thought to be the toxic receptor for digitalis, the sixth most widely used therapeutic drug in the United States. It is a drug used for certain heart arrhythmias and congestive heart failure. The drug has a very small therapeutic ratio and consequently, is quite toxic. It is ranked as the number one producer of iatrogenic disease in the hospital emergency room setting.

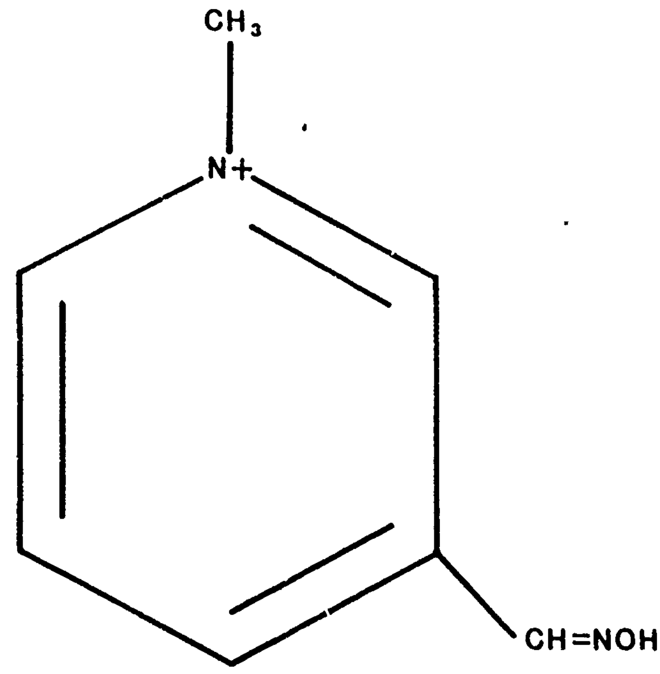
The oximes are a group of compounds a number of which are known to reactivate organophosphate-inhibited AChE by dephosphorylating the phosphorylated enzyme (Wilson and Froede, 1971). This dephosphorylation allows for release of the agent from the enzyme and reactivation. Although the oximes have the capacity to be specific for AChE a number of investigators have noted similarities between the enzymes Na^+ + K^+ ATPase and AChE (Akera and Brody, 1971). This interaction of oximes with other phosphorylated enzymes has not been well studied. It has been proposed that a reciprocal relationship exists between these two enzymes, however, documentation has been lacking. These data lead to a new understanding of how the electrogenic cell membrane Na^+ pump could be related to the sodium ion permeability depolarization produced by the interaction of acetylcholine at the cell membrane.

ABSTRACT

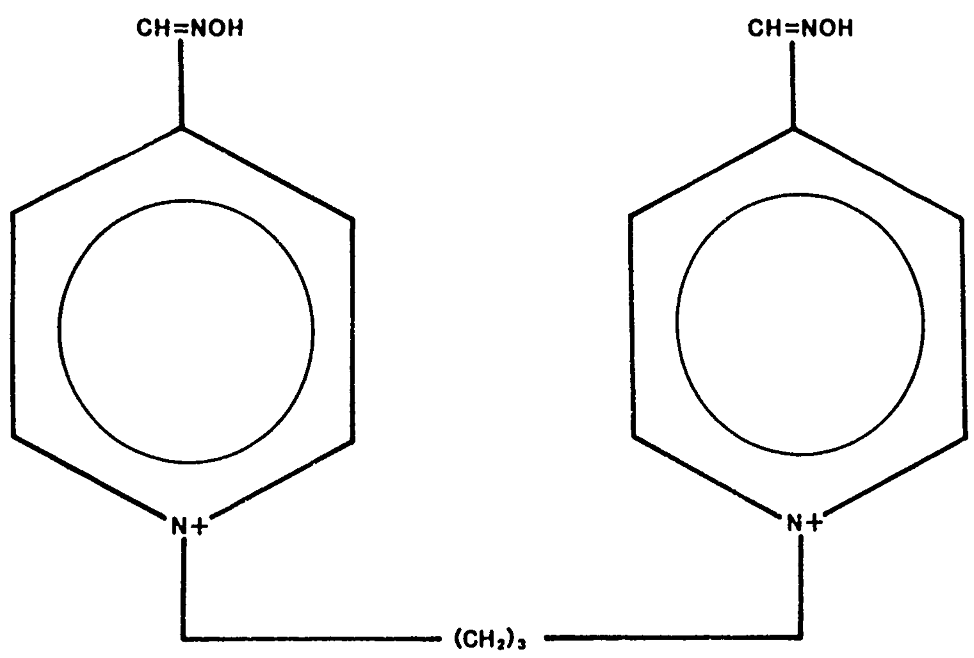
It is known that oximes (e.g. pralidoxime methiodide (2-PAM), 3-pyridine aldoxime methiodide (3-PAM), 1,1'-trimethylene bis (4-formyl-pyridinium bromide) dioxime (TMB-4), (1-[[[4-aminocarbonyl)-pyridino] methoxy] methyl]-2[hydroxyimino) methyl] pyridinium dichloride (HI-6)) reactivate organophosphate (OP)-inhibited acetylcholinesterase by dephosphorylating the OP-enzyme complex. Since other enzymes have similar serine phosphate bonds, it was proposed that additional chemical-enzyme complexes might be likewise affected. Binding of ouabain (OU) to the phosphorylated form of ATPase was selected as an appropriate test system. Guinea pig heart ATPase was prepared using methods of van Alstyne et al., 1980. In vitro ATP-dependent and independent OU binding (6×10^{-7} M) was performed using methods of Akera et al., 1973. In control studies, 94, 125, 157 and 176 nmol OU/mg protein were bound at 1, 3, 5, and 10 min, respectively. TMB-4 and 3-PAM (2×10^{-3} M) were found to decrease the binding level of OU to ATPase by 34.1 and 27.1%, respectively at 10 min. 2-PAM in the presence of ATP decreased the OU binding at 15.8% at 10 min. HI-6, likewise decreased ATP-dependent OU binding by 22.8% at 10 min. It is proposed that these oximes decrease OU binding by dephosphorylation of the OU-ATPase complex. These data suggest that this mechanism could be responsible for our earlier observation that oximes could reverse the arrhythmias caused by ouabain in the isolated heart (Baskin et al., 1984).



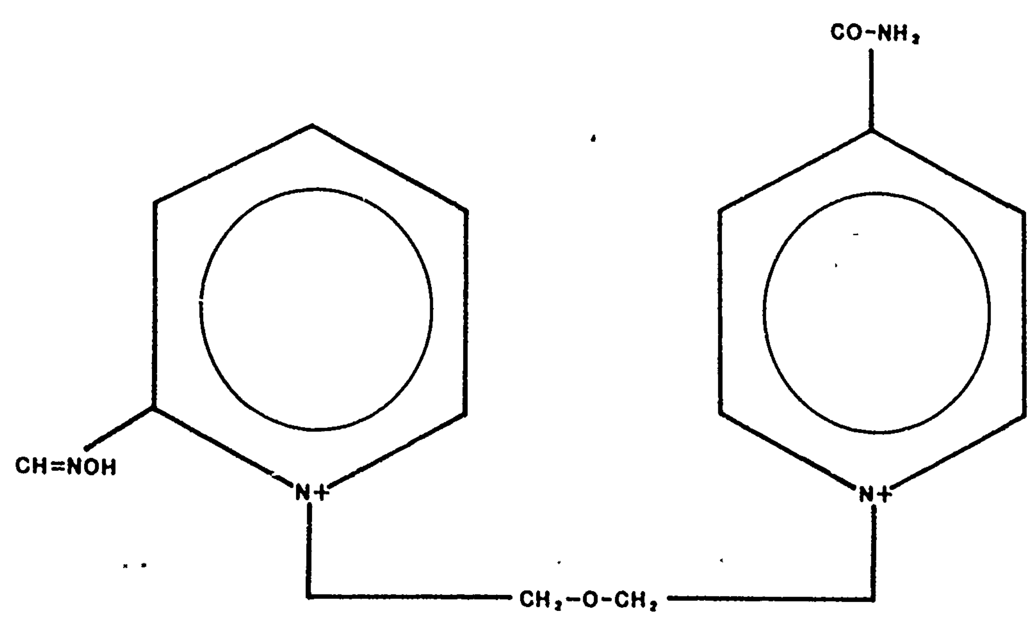
pralidoxime methiodide (2-PAM)



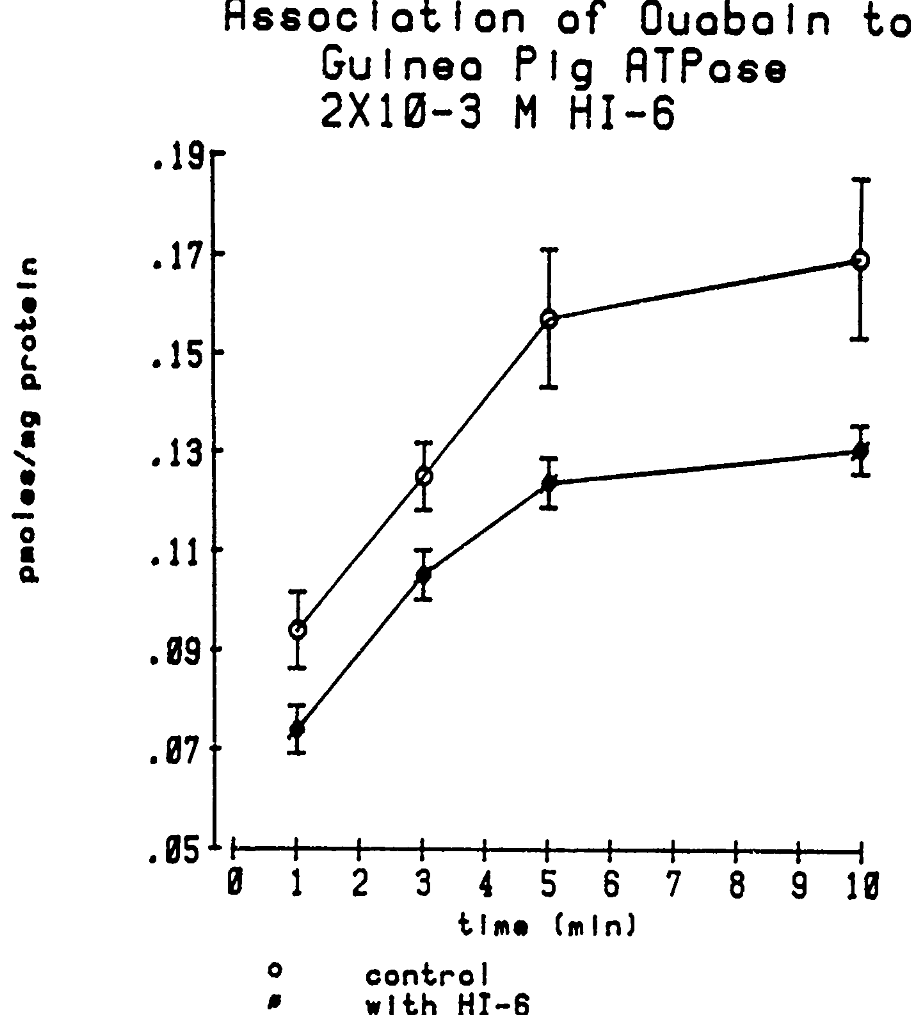
3-pyridine aldoxime methiodide (3-PAM)

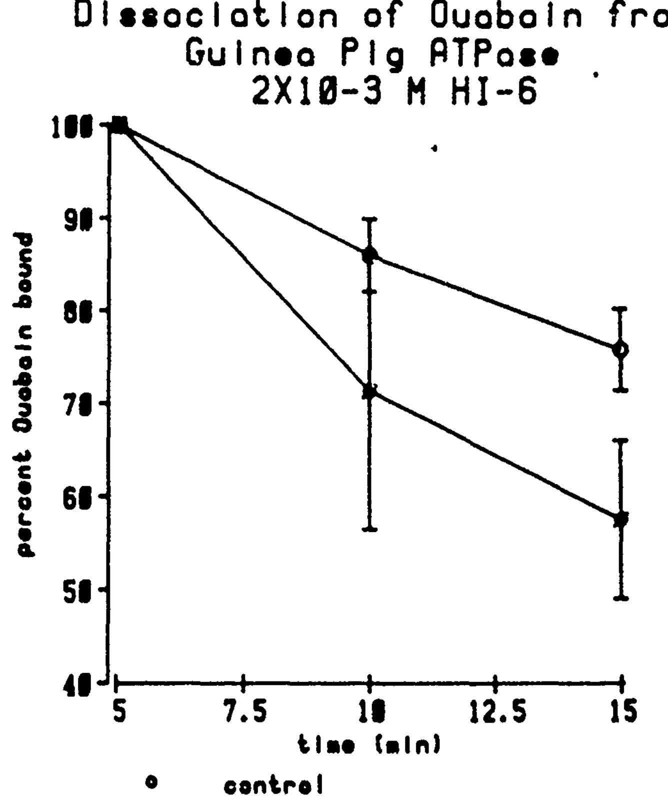


1,1'-trimethylene bis (4-formyl-pyridinium bromide) dioxime (TMB-4)

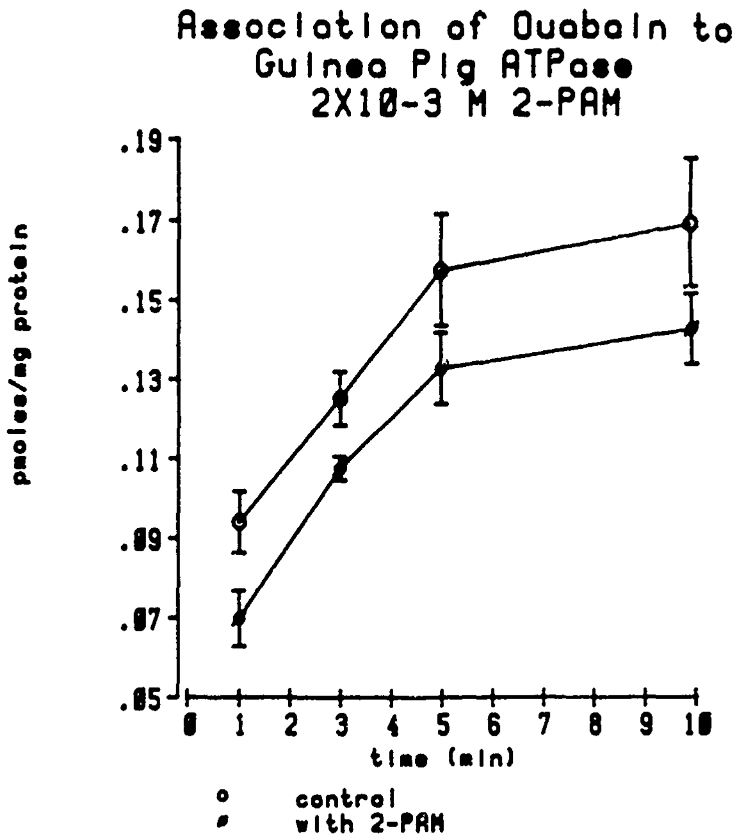


(1-[[[4-aminocarbonyl)-pyridino]methoxy]methyl]
-2[hydoxyimino methyl] pyridinium dichloride (HI-6)

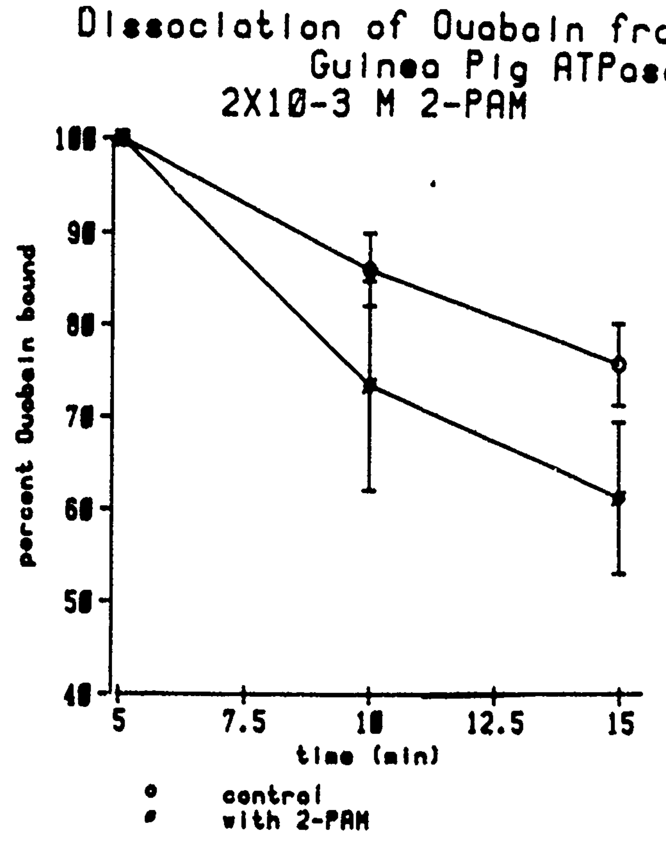


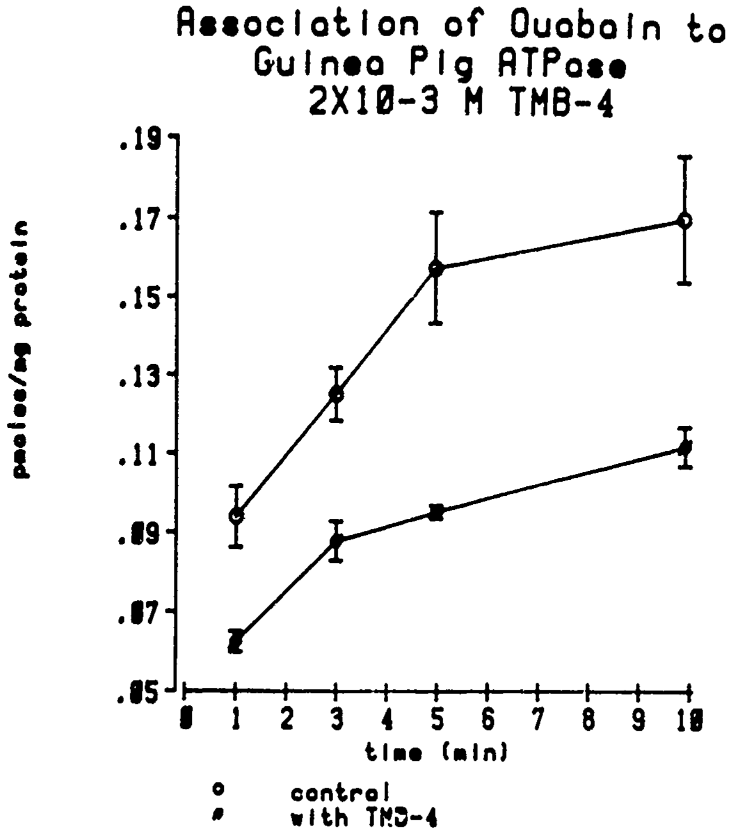


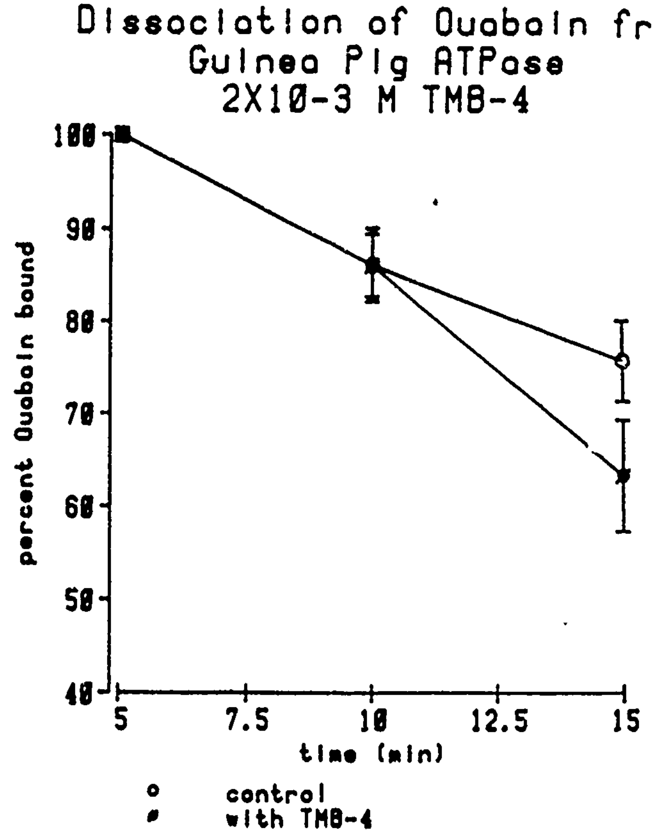
Dissociation of Ouabain from
Guinea Pig ATPase
2X10-3 M HI-6
percent Ouabain bound
100
90
80
70
60
50
40
5
7.5
10
12.5
15
time (min)
o control
* with HI-6



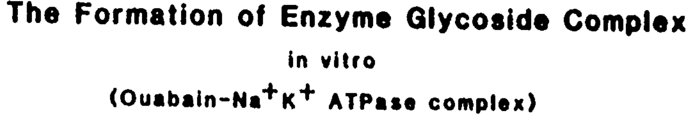
Association of Ouabain to
Guinea Pig ATPase
2X10-3 M 2-PAM
pmoles/mg protein
.19
.17
.15
.13
.11
.09
.07
.05
0
1
2
3
4
5
6
7
8
9
10
time (min)
o control
* with 2-PAM


Dissociation of Ouabain from
Guinea Pig ATPase
2X10-3 M 2-PAM
percent Ouabain bound
100
90
80
70
60
50
40
5
7.5
10
12.5
15
time (min)
control
with 2-PAM

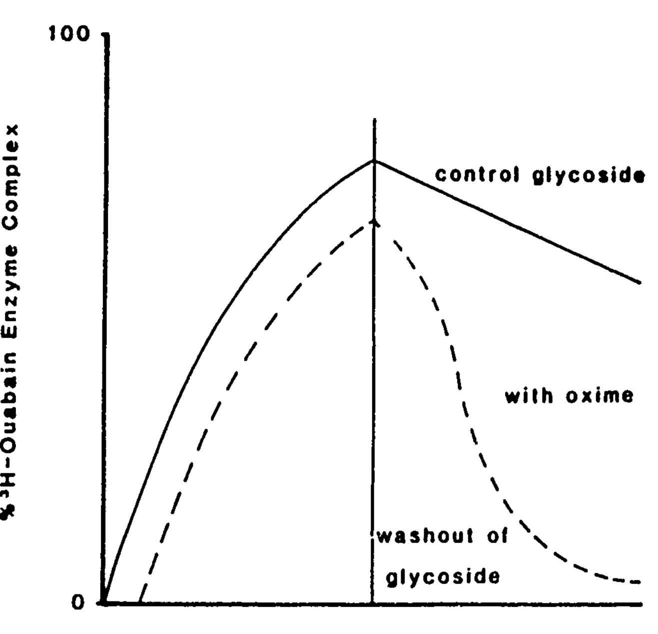

Association of Ouabain to
Guinea Pig ATPase
2X10-3 M TMB-4
pmoles/mg protein
.19
.17
.15
.13
.11
.09
.07
.05
0
1
2
3
4
5
6
7
8
9
10
time (min)
control
with TMB-4



Dissociation of Ouabain from
Guinea Pig ATPase
2X10-3 M TMB-4
percent Ouabain bound
100
90
80
70
60
50
40
5
7.5
10
12.5
15
time (min)
control
with TMB-4

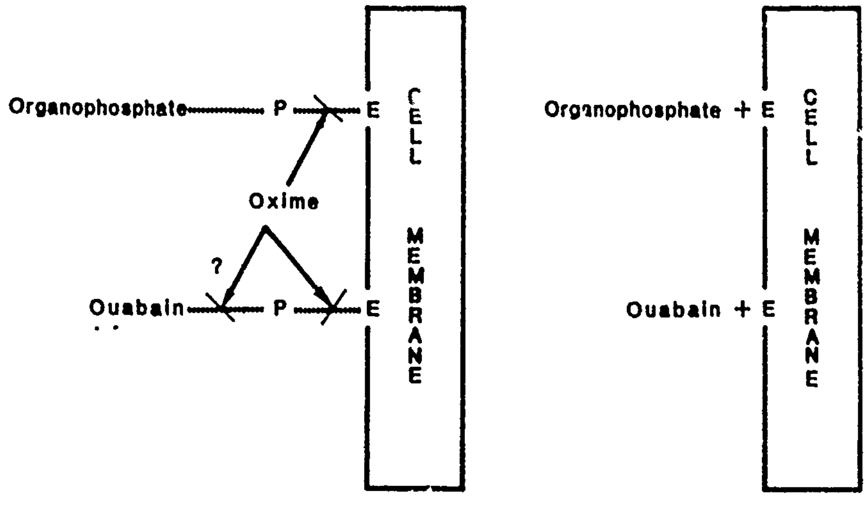


The Formation of Enzyme Glycoside Complex
in vitro
(Ouabain-Na+K+ ATPase complex)



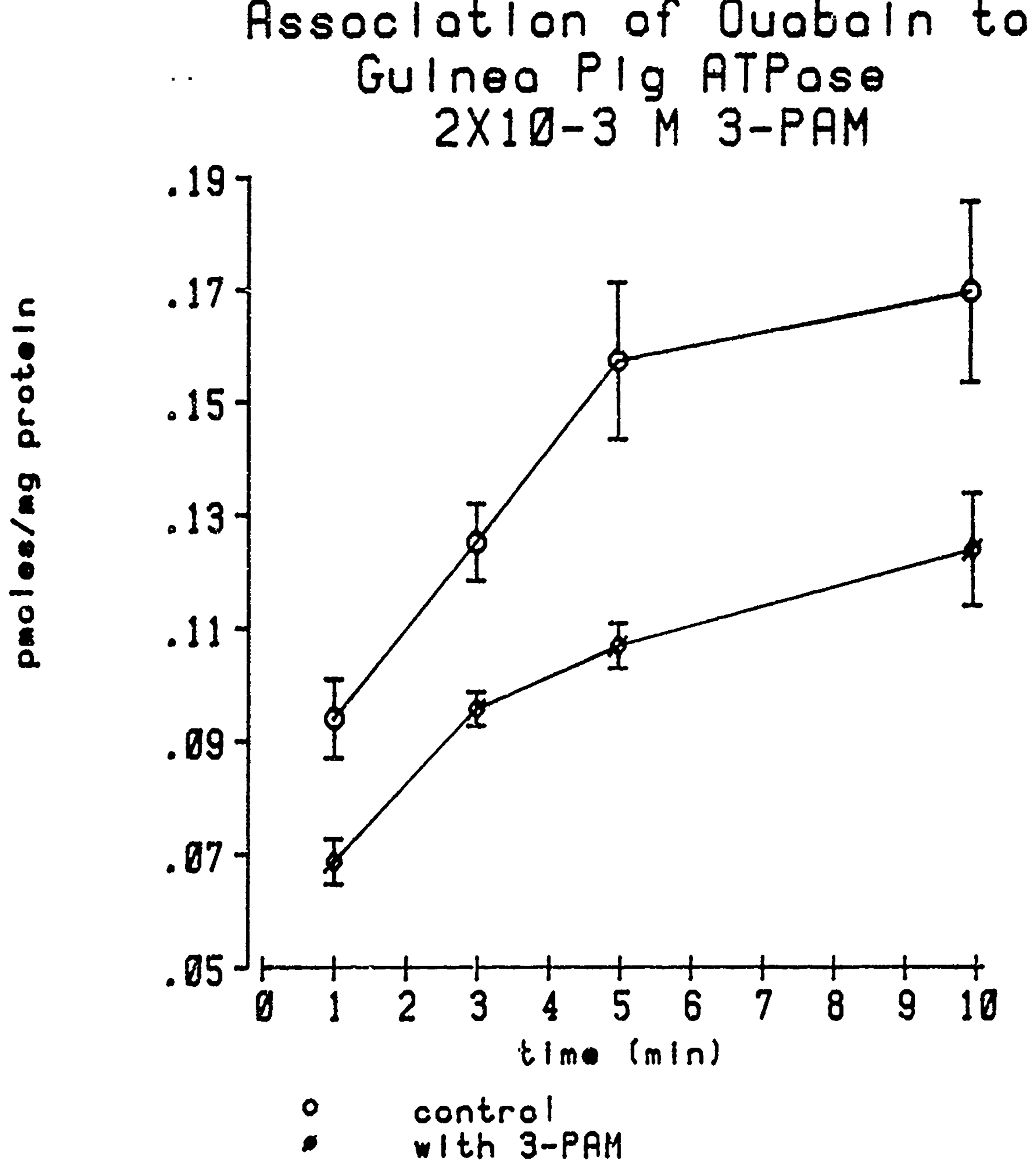
100
0
% 3H-Ouabain Enzyme Complex
control glycoside
with oxime
washout of
glycoside
Time (min)

Interaction Between Oximes and Drug Receptor Complexes



MATERIALS AND METHODS

$Na^+ + K^+$ ATPase was prepared from male guinea pig hearts (van Alstyne, et al., 1980) and incubated with ^{3}H-ouabain using standard conditions (Akera and Brody, 1971; Baskin, et al., 1973) until the ^{3}H-ouabain-enzyme was formed. The drug-enzyme complex was isolated and incubated under conditions at which dissociation occurs (Akera, et al., 1973). The rate of release of the glycoside from $Na^+ + K^+$ ATPase was measured in the presence or absence of oximes.



RESULTS AND DISCUSSION

The data indicate that the oximes [i.e. 2-PAM, 3-PAM, TMB-4 and HI-6 (see structures)], appeared to inhibit the binding of ^{3}H-ouabain to a Na^+, ATP-dependent binding site. This binding site is thought by most to be identical to Na^+ + K^+ ATPase. Since ouabain is associating to and dissociating from the ATP-dependent binding site during binding, the ^{3}H-ouabain — ATP-dependent binding site complex was prepared and the dissociation of ^{3}H-ouabain from the ^{3}H-ouabain — ATP-dependent binding site was measured. It was found that the oximes, agents known to dephosphorylate proteins, appear to increase the release of ^{3}H-ouabain from the ^{3}H-ouabain — ATP-dependent site. Any quantitative differences that exist among the oximes are unclear at this time. To rule out if ATP could act as a phosphate donor for oximes or that oximes could reduce the concentration of ATP, the effect of oximes on ATP concentration was measured. In data not shown, no significant effect of oximes was observed. The concentration of 2×10^{-3} M oxime was chosen as this is thought to be within the therapeutic range for this chemical (Green, unpublished).

The effect of the oximes on the binding of ouabain to Na^+ + K^+ ATPase is interpreted that the oximes not only dephosphorylate the organophosphate ligand to the acetylcholinesterase but may act to release other ligands to available phosphorylated enzyme sites. Thus, one can envisage that oximes may "specifically" dephosphorylate other phosphorylated enzymes that may bind organophosphates or other chemicals that bind to phosphorylated enzymes (see figure below). It is proposed that oximes that could increase the rate of release of cardiac glycosides under in vivo conditions could serve to act as antidotes for these potentially toxic groups of compounds (Wiggins, et al., 1980; Baskin, et al., 1984). Furthermore, it may be reasoned that drug interactions between oximes and cardiac glycosides would not be unexpected. The observation that oximes may regulate this type of drug receptor interaction may provide for further understanding of molecular rationale of drug receptor relationships.

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CONCLUSIONS

1. Oximes (i.e. 2-PAM, TMB-4, HI-6) appear to decrease the Na^{+}, ATP -dependent binding of ouabain to cardiac membranes.
2. Dissociation studies suggest that the effects of oximes are due to, in part, the dephosphorylation action of these compounds.
3. It is proposed that these mechanisms are responsible, in part, for the reversal of the toxic effects of ouabain by oximes (Baskin et al., 1984).
4. Oximes may prove to be useful tools for examining chemical ligand between drugs and enzyme as well as provide a possible antidote for digitalis toxicity.

EFFECTS OF ACETYLCHOLINESTERASE INHIBITORS ON HIGH AFFINITY SEROTONIN UPTAKE AND MUSCARINIC RECEPTOR MEDIATED EFFECTS OF ACETYLCHOLINE IN ASTROCYTE CULTURES

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PRIMARY ASTROCYTE CULTURES PREPARED FROM NEONATAL RAT BRAIN SHOW Na^+ DEPENDENT UPTAKE OF [^{3}H]LABELLED SEROTONIN ([^{3}H/5HT) WHICH IS INHIBITED BY SEVERAL CLINICALLY EFFECTIVE ANTIDEPRESSANTS WITH A RANK ORDER WHICH IS CONSISTENT WITH A HIGH AFFINITY (HA) UPTAKE PROCESS (H.K. KIMELBERG AND D. KATZ, SOC. NEUROSCI. ABST. 10:766, 1984). MAXIMAL HA UPTAKE OF [^{3}H/5HT REQUIRES INHIBITION OF CELLULAR MAO BY HIGH CONCENTRATIONS OF PARGYLINE OR LOW CONCENTRATIONS OF CLORGYLINE, CONSISTENT WITH INHIBITION OF MAO-A BEING THE MAJOR EFFECT. WE HAVE NOW CONFIRMED THIS UPTAKE TO ASTROCYTES BY SIMULTANEOUS AUTORADIOGRAPHY AND STAINING FOR THE ASTROCYTE-SPECIFIC MARKER, GLIAL FIBRILLARY ACIDIC PROTEIN (GFAP).

SOME OF THE CENTRAL NERVOUS SYSTEM (CNS) EFFECTS OF ACETYLCHOLINESTERASE (ACHE) INHIBITORS ARE THOUGHT TO BE UNRELATED TO THEIR EFFECTSON ACHE. SINCE THESE EFFECTS MAY, IN PART, BE DUE TO EFFECTS ON GLIA AND BECAUSE HA UPTAKE OF [^{3}H/5HT IS POTENTIALLY AN IMPORTANT PROPERTY OF THESE CELLS, WE DECIDED TO SEE IF ACHE INHIBITORS AFFECTED [^{3}H/5HT UPTAKE IN ASTROCYTE CULTURES. PHYSOSTIGMINE INHIBITED HA, Na^+ DEPENDENT UPTAKE OF 10^{-7}M [^{3}H/5HT WITH AN IC_{50} OF AROUND 50 μM. HOWEVER, PHYSOSTIGMINE HAD NO EFFECT ON Na^+ INDEPENDENT UPTAKE. PARATHION SHOWED 50% INHIBITION OF HA [^{3}H/5HT UPTAKE AT AROUND 10 μM, AND ALSO HAD NO EFFECT ON Na^+ INDEPENDENT UPTAKE. INHIBITION BY PARATHION WAS NOT REVERSED BY AT LEAST 60 MINUTES INCUBATION IN INHIBITOR-FREE MEDIUM FOLLOWING INCUBATION OF THE CELLS WITH PARATHION FOR 60 MINUTES. THE PARATHION METABOLITE, PARAOXON, WAS ALSO EFFECTIVE IN INHIBITING [^{3}H/5HT UPTAKE AND WAS ALSO NOT REVERSIBLE WITHIN 60 MINUTES. IN CONTRAST, THE POTENT ACHE INHIBITOR DIISOPROPYL FLUOROPHOSPHATE (DFP) HAD NO EFFECT ON [^{3}H/5HT UPTAKE, EVEN UP TO 1 MM CONCENTRATION AND AFTER 1 HR PREINCUBATION. THUS, THE EFFECTS OF PHYSOSTIGMINE AND PARATHION ON [^{3}H/5HT UPTAKE IN ASTROCYTES, AND PERHAPS INHIBITION OF SUCH UPTAKE IN NERVE TERMINALS, MAY EXPLAIN SOME OF THE CNS EFFECTS OF THESE COMPOUNDS WHICH ARE INDEPENDENT OF ACHE INHIBITION.

IN ADDITION, WE HAVE FOUND THAT PRIMARY ASTROCYTE CULTURES SHOW AN ACETYLCHOLINE (ACH) INDUCED PARTIAL INHIBITION OF A NOREPINEPHRINE-STIMULATED CAMP INCREASE, WHICH IS ITSELF DUE TO β-RECEPTOR STIMULATION. THIS ACH-INDUCED INHIBITION OF THE NE-STIMULATED CAMP RESPONSE IS SENSITIVE TO ATROPINE SO THAT IT APPEARS TO BE DUE TO ACTIVATION OF MUSCARINIC RECEPTORS. THIS EFFECT IS POTENTIATED BY 10^{-6}M PHYSOSTIGMINE. BASED ON THE WORK OF OTHERS, THE INHIBITION BY ACH MAY BE DUE TO STIMULATION OF PHOSPHOLIPASE C DUE TO ACTIVATION OF MUSCARINIC RECEPTORS, AND SUBSEQUENT HYDROLYSIS OF PHOSPHOINOSITIDE WITH STIMULATION OF PHOSPHOKINASES. THE SUBSTRATE FOR PHOSPHORYLATION DUE TO PHOSPHOKINASE STIMULATION APPEARS TO BE CYCLIC NUCLEOTIDE PHOSPHODIESTERASE, SINCE INHIBITION OF CAMP STIMULATION BY ACH IS NOT SEEN IN THE PRESENCE OF THE PHOSPHODIESTERASE INHIBITOR IBMX (ISOBUTYL-1-METHYLXANTHENE). WE HAVE ALSO OBSERVED ACH (10^{-5}M) INDUCED DEPOLARIZATION OF THE MEMBRANE POTENTIAL OF THESE CELLS. DEPOLARIZATION OF UP TO 10MV (RESTING MEMBRANE POTENTIALS = 72MV) WAS FOUND, WITH PARTIAL DESENSITIZATION UPON A SECOND EXPOSURE TO ACH. THIS SUGGESTS THAT ASTROCYTES IN MAMMALIAN CNS HAVE RECEPTORS FOR ACH AND SOME OF THE EFFECTS OF INCREASED LEVELS OF ACH DUE TO ACHE INHIBITION MAY BE ON GLIAL CELLS.

(SUPPORTED BY DOD CONTRACT DAMD 17-84-C4022).

A. PRIMARY ASTROCYTE CULTURES SHOW HIGH AFFINITY, Na^+ DEPENDENT UPTAKE OF [^{3}H]—SEROTONIN

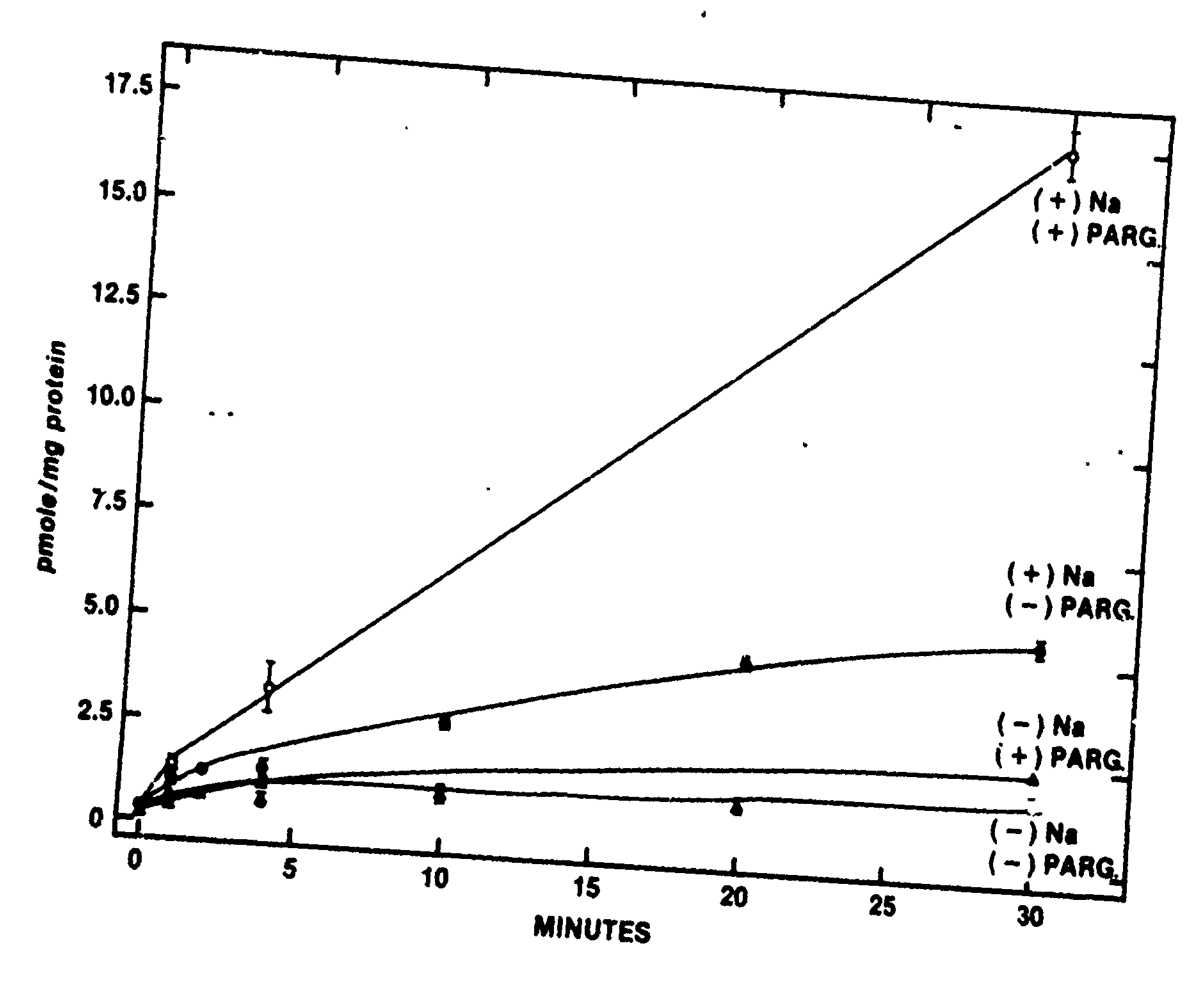


FIG. 1. EFFECT OF OMISSION OF MEDIUM NA^+ AND PARGYLINE ON [^{3}H] 5HT UPTAKE. [^{3}H] 5HT WAS PRESENT AT CONCENTRATION OF 10^{-7}M. WHEN NA^+ IN THE MEDIUM WAS OMITTED IT WAS REPLACED WITH CHOLINE. FINAL MEDIUM [K^+] WAS 4.5MM. CELLS WERE 28 DAYS OLD. SEE FIG. 4 FOR FURTHER EXPERIMENTAL DETAILS. VALUES ARE MEANS ± S.E.M., N = 3 WELLS.

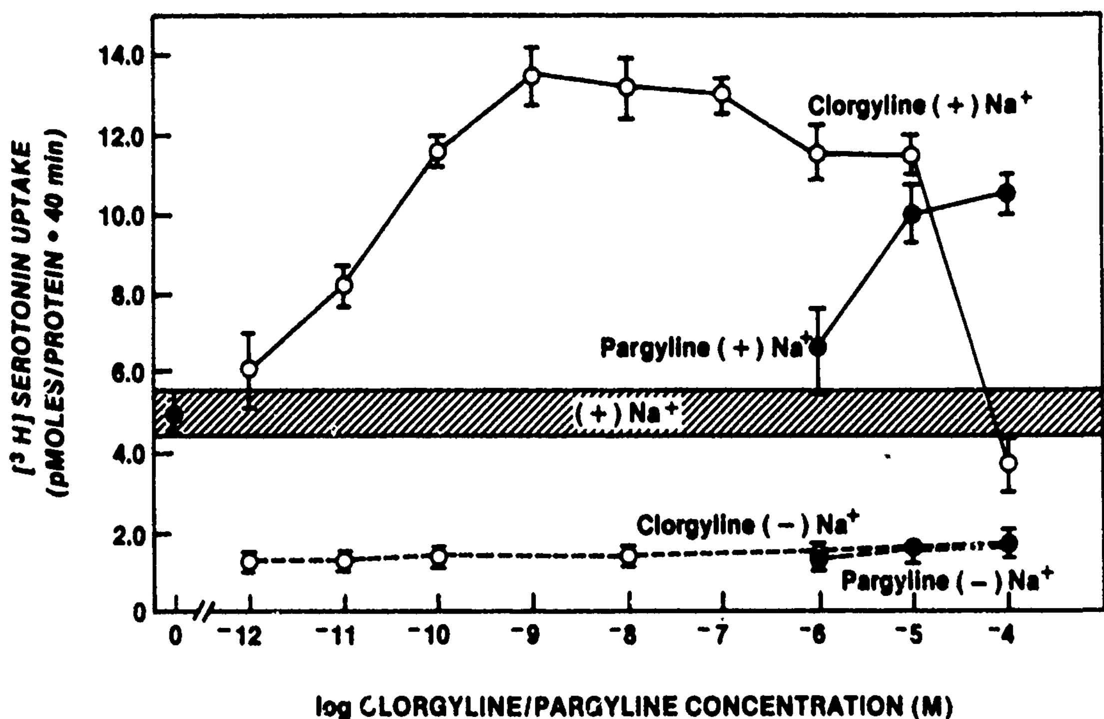


FIG. 2. EFFECT OF CLORGYLINE AND PARGYLINE ON [^{3}H] SEROTONIN UPTAKE

UPTAKE OF [^{3}H] SEROTONIN MEASURED FOR 40 MINUTES WITH VARYING AMOUNTS OF CLORGYLINE (PREFERENTIAL INHIBITOR OF MONOAMINE OXIDASE (MAO) ISOENZYME TYPE A) OR PARGYLINE (PREFERENTIAL INHIBITOR OF MAO-B) PRESENT. HATCHED LINE SHOWS UPTAKE (SEE FIG. 4) IN PRESENCE OF NA^+ ((+) NA^+) OR ABSENCE OF NA^+ ((-) NA^+) AS (○, ●). UPTAKE OF [^{3}H] SEROTONIN IS POTENTIATED BY LOW CONCENTRATIONS OF CLORGYLINE (10^{-8}M TO 10^{-7}M) CONSISTENT WITH MAXIMAL UPTAKE OF [^{3}H] SEROTONIN REQUIRING INHIBITION OF [^{3}H] SEROTONIN METABOLISM AND SUCH METABOLISM BEING MAINLY DUE TO THE MAO-A ISOENZYME. (N = 3 ± S.E.M.).

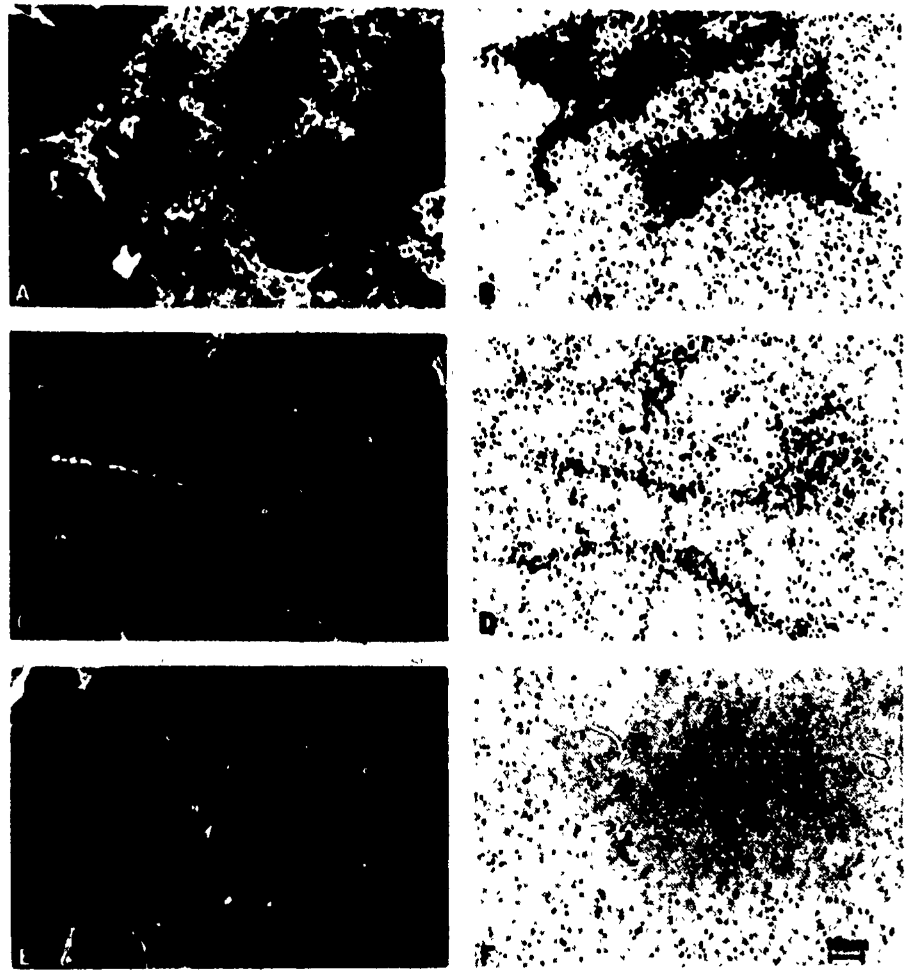


FIG. 3. GFAP IMMUNOCYTOCHEMISTRY AND AUTORADIOGRAPHY OF [^{3}H] 5HT UPTAKE UNDER CONTROL AND Na^+-FREE CONDITIONS. GFAP IMMUNOFLUORESCENCE (A,C,E) AND BRIGHTFIELD [^{3}H] 5HT AUTORADIOGRAPHY (B,D,F).

UPTAKE OF [^{3}H] 5HT UNDER CONTROL (+ Na^+) CONDITIONS; VIEW OF FLAT CELLS (A,B) AND PROCESS-BEARING CELLS (C,D). UPTAKE OF [^{3}H]5HT IN Na^+-FREE MEDIUM (Na^+ REPLACED WITH CHOLINE). E,F. PRIMARY ASTROCYTE CULTURES WERE PREPARED FROM THE CEREBRAL CORTICES OF NEWBORN RAT PUPS AFTER CAREFUL REMOVAL OF THE MENINGES, AND GROWN ON GLASS COVERSLIPS IN EAGLE'S BME + 10% FETAL CALF SERUM PLUS SUPPLEMENTAL VITAMINS AND AMINO ACIDS AND CONTAINING PENICILLIN AND STREPTOMYCIN AND USED WITHIN 4-6 WEEKS. FOR COMBINED AUTORADIOGRAPHY (ARG) AND IMMUNOCYTOCHEMISTRY, GROWTH MEDIUM WAS REMOVED. THE CELLS WERE WASHED AND UPTAKE OF 0.3µM [^{3}H]5HT (5 HYDROXY G-^{3}H TRYPTAMINE CREATININE SULFATE) WAS PERFORMED AS DESCRIBED IN FIG. 2. AFTER UPTAKE THE CELLS WERE RAPIDLY WASHED 7 TIMES WITH 2ML ICE-COLD PBS (240MM NACL, 3.5MM NaH_2PO_4, 12MM Na_2HPO_4, PH 7.25). THE CELLS WERE THEN FIXED IN 4% PARAFORMALDEHYDE + 0.25% GLUTARALDEHYDE, WASHED AND PERMEABILIZED IN -10°C ACETONE FOR 3 MINUTES AND THEN RINSED TWICE IN PBS. THEY WERE THEN STAINED WITH MONOCLONAL MOUSE ANTI-HUMAN GFAP IGG (AMERSHAM), AND RHODAMINE LABELLED RABBIT ANTI-MOUSE IGG. THE COVERSLIPS AFFIXED TO THE SLIDES WERE THEN DIPPED IN KODAK NTB2 AT 42°C AND LEFT AT 4°C FOR 11 DAYS. THEY WERE THEN DEVELOPED AND FIXED. THE CELLS WERE VIEWED WITH A NIKON LABOPHOT MICROSCOPE USING A 50X OIL IMMERSION LENS FOR BRIGHT-FIELD AUTORADIOGRAPHY AND A XENON 75W LIGHT SOURCE AND APPROPRIATE FILTERS FOR RHODAMINE EPIFLUORESCENCE. CONTROLS IN WHICH ANTI-GFAP ANTIBODY WAS ABSORBED WITH PURIFIED BOVINE GFAP SHOWED NO DETECTABLE FLUORESCENCE.

KINETIC CONSTANTS FOR [^{3}H] 5HT UPTAKE

Conditions	K_m (μM)	Vmax (pmoles/mg protein/4 min)
Na^+ sensitive	0.40 ± 0.11	6.42 ± 0.85
Na^+ insensitive	NON-SATURABLE	

TABLE 1.

INITIAL RATE OF UPTAKE OF / ^{3}H / 5HT MEASURED FOR 4 MINUTES. DATA WAS FITTED TO A COMPUTER PROGRAM AND VALUES SHOWN ARE MEAN (± SEM).

8. HIGH AFFINITY ^{3}H—SEROTONIN UPTAKE IS INHIBITED BY PHYSOSTIGMINE, PARATHION AND PARAOXON, BUT NOT BY DFP

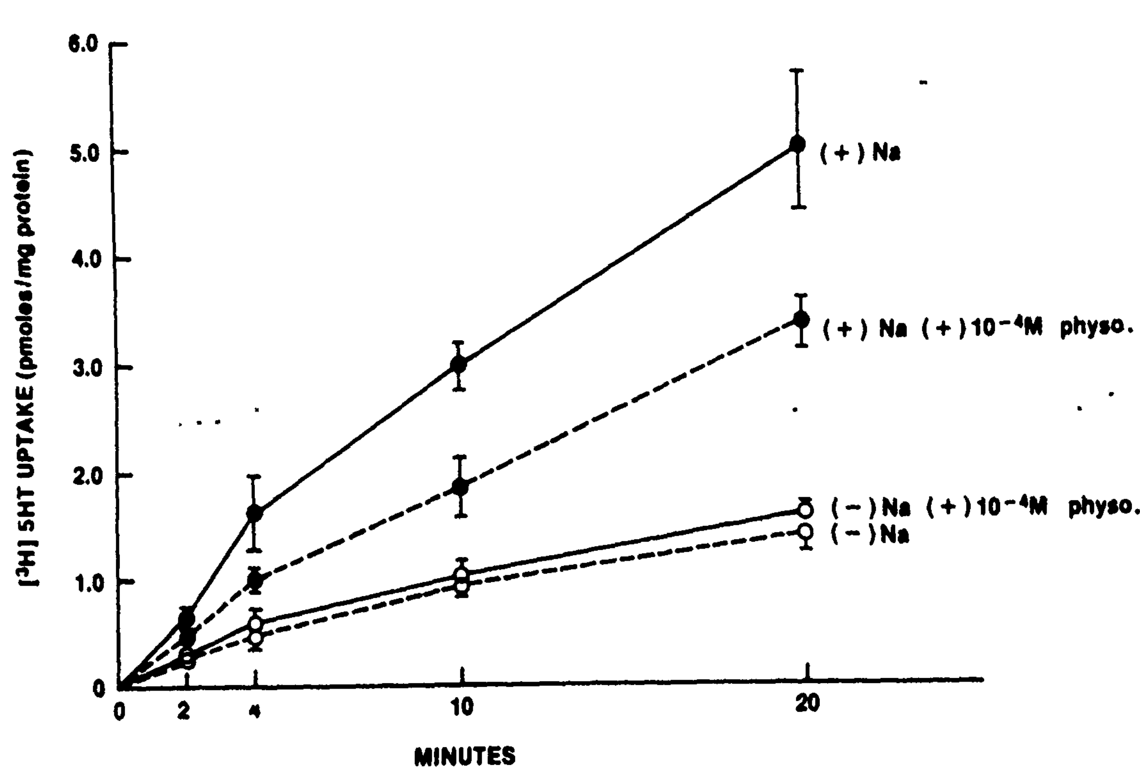


FIG. 4 TIME COURSE FOR UPTAKE OF [^{3}H] 5HT AND EFFECT OF 10^{-4}M PHYSOSTIGMINE

CELLS WERE GROWN IN 12 WELL, MULTIWELL TRAYS. GROWTH MEDIA WAS REMOVED AND THE CELLS WERE PRETREATED WITH 10^{-4}M PHYSOSTIGMINE FOR 20 MINUTES IN HCO_3^- BUFFERED MEDIUM (Na^+, 150; K^+, 4.5; Mg^{2+}, 0.4; Ca^{2+}, 1.3; Cl^-, 123; SO_4^{2-}, 1.2; HCO_3^-, 25 AND GLUCOSE 10 ALL IN MMOL/L) AT 37°C IN A 5% CO_2/95% AIR ATMOSPHERE. [^{3}H] 5HT WAS ADDED AT 0 TIME AND UPTAKE MEASURED FOR THE TIMES SHOWN IN THE PRESENCE (+ Na^+) AND ABSENCE (0 Na^+) OF Na^+ IN THE MEDIUM. FINAL CONCENTRATION OF [^{3}H] 5HT WAS 10^{-7}M (1µCi IN 1ML TOTAL REACTION VOLUME). THE REACTION MEDIUM ALSO CONTAINED 10^{-4}M PARGYLINE TO PREVENT OXIDATIVE DEAMINATION OF 5HT BY THE CELLS AND 10^{-5}M ASCORBATE. IN Na^+ FREE MEDIUM Na^+ WAS REPLACED BY CHOLINE AND $NaHCO_3$ BY TRIETHYLAMMONIUM HCO_3^-. AFTER UPTAKE THE MEDIUM WAS RAPIDLY ASPIRATED AND THE CELL MONOLAYER WASHED 7 TIMES (TOTAL WASHING TIME 10-15 SECS) WITH COLD 0.32M SUCROSE, 50MM TRIS HCL (PH 7.4) SOLUTION. THE CELLS WERE THEN SOLUBILIZED WITH 1N NAOH AND ALIQUOTS TAKEN FOR MEASURING PROTEIN AND ^{3}H. A ZERO TIME UPTAKE WHEN ^{3}H 5HT WAS ADDED AND IMMEDIATELY REMOVED WAS ALWAYS SUBTRACTED. N = 3 WELLS ± S.D.

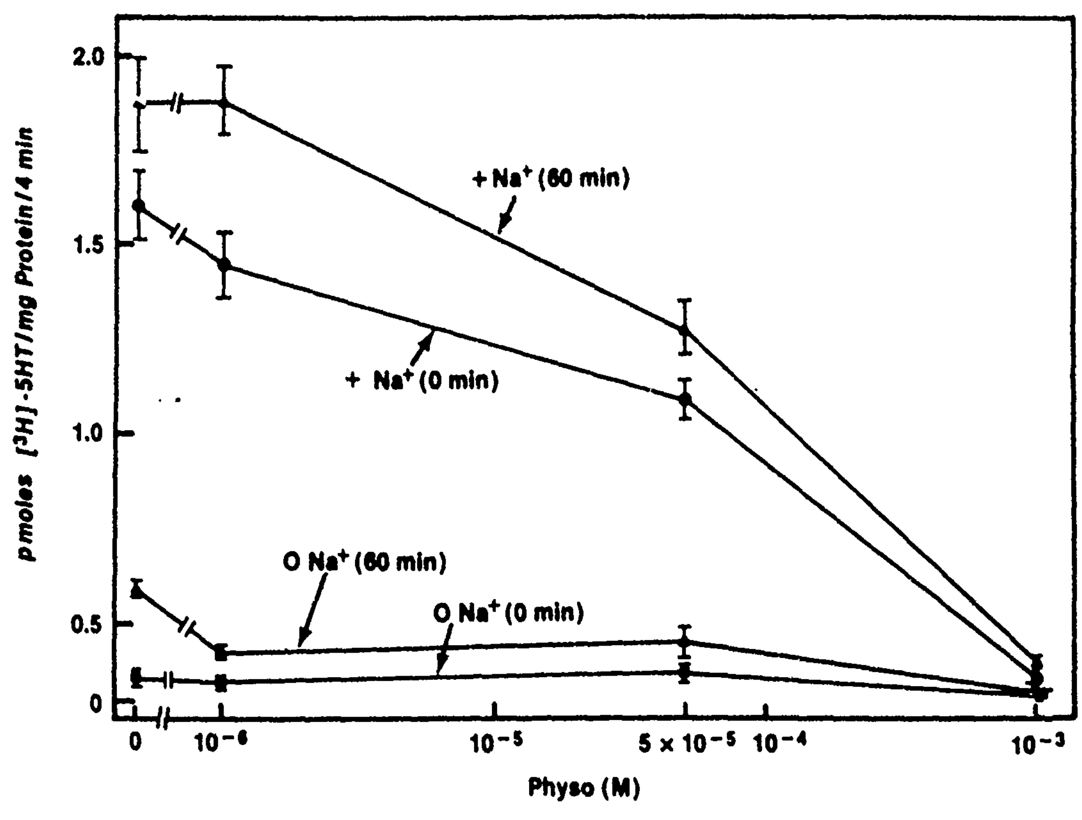


FIG. 5 DOSE RESPONSE CURVE FOR PHYSOSTIGMINE INHIBITION OF [^{3}H] 5HT UPTAKE

AFTER REMOVAL OF MEDIUM THE CELLS WERE PREINCUBATED FOR 60 MINUTES WITH THE DIFFERENT CONCENTRATIONS OF PHYSOSTIGMINE (60 MIN) OR PHYSOSTIGMINE WAS ADDED AT 0 TIME WITH 10^{-7}M [^{3}H] 5HT. UPTAKE WAS MEASURED FOR 4 MIN. OTHER CONDITIONS AS IN FIG. 4.

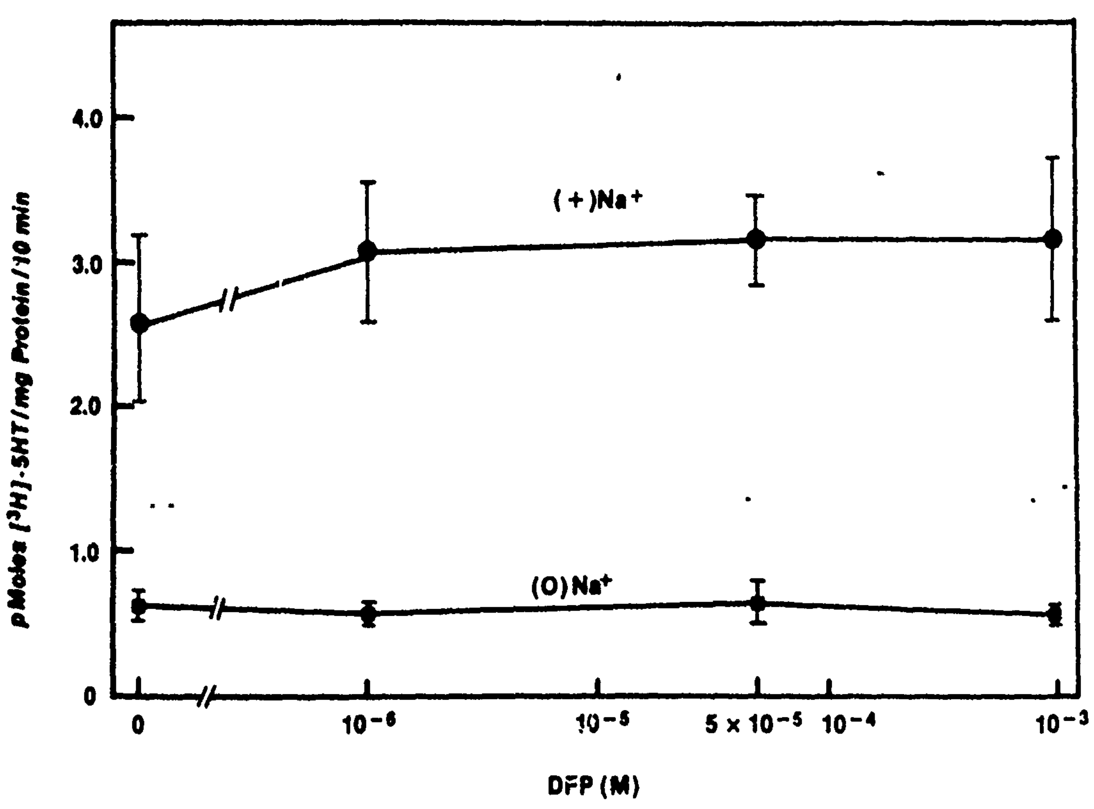


FIG. 6 DOSE RESPONSE CURVE FOR DFP AND [^{3}H] 5HT UPTAKE

10 MINUTE UPTAKE OF [^{3}H] 5HT IN THE PRESENCE AND ABSENCE OF VARYING CONCENTRATIONS OF DFP IN THE PRESENCE (+) AND ABSENCE (0) OF NA^+ IN THE MEDIUM AS DESCRIBED IN FIG. 4. [^{3}H]5HT AND DFP ADDED AT 0 TIME, CELLS (29 DAYS OLD) PREINCUBATED IN $\pm$ NA^+ CONTAINING HCO_3^- BUFFERED MEDIUM FOR 20 MINUTES PRIOR TO THIS.

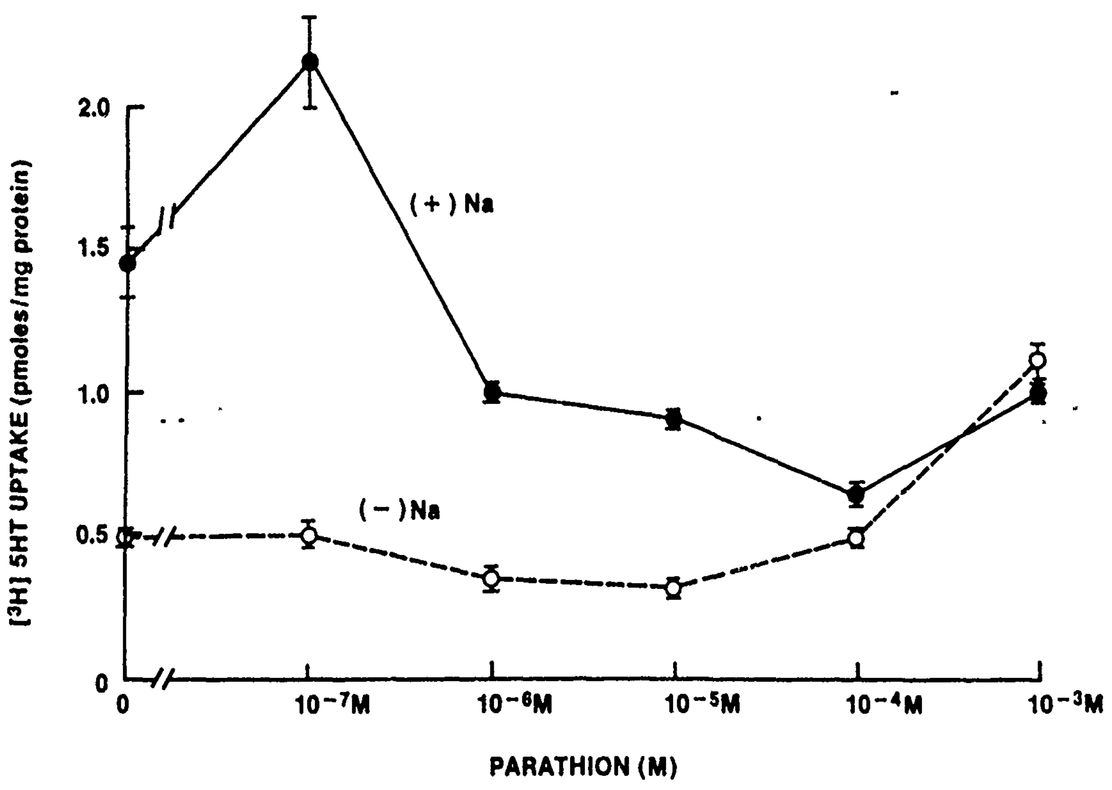


FIG. 7 Dose Response Curve for Inhibiton of $[^3H]$ 5HT by Parathion

Uptake of 10^{-7}M $[^3H]$ 5HT measured over 4 minutes in HCO_3^- buffered medium with or without Na^+ (see fig. 4). Concentrations of parathion shown present in 20 min preincubation period. Uptake measured as described in fig. 4.

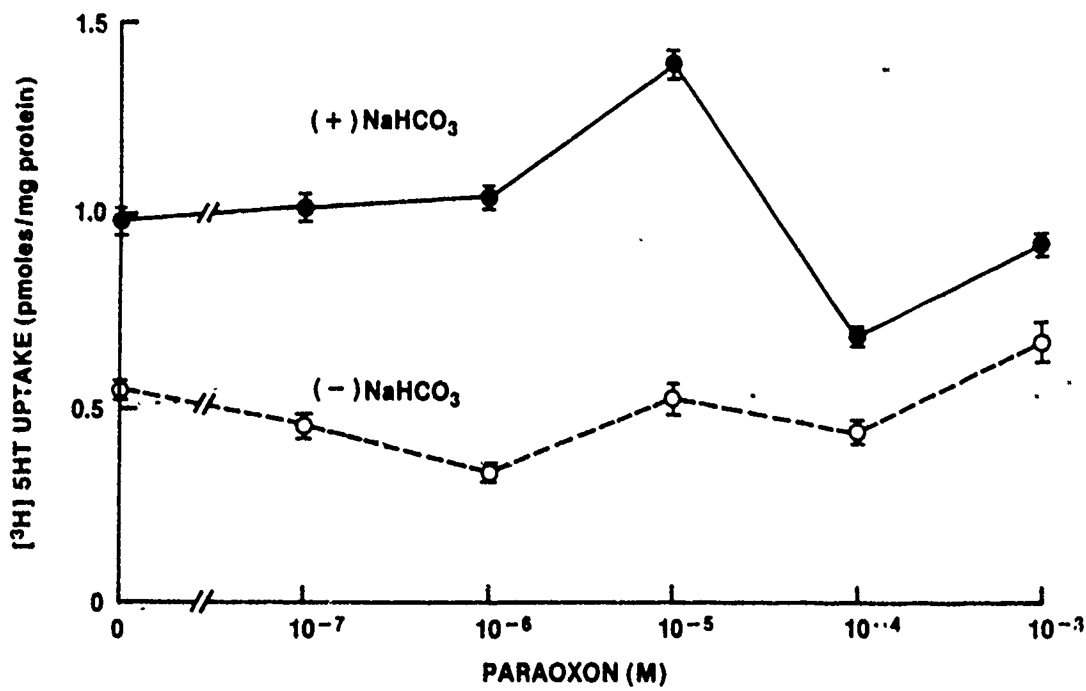


FIG. 8 DOSE RESPONSE CURVE FOR INHIBITION OF $[^3H]$ 5HT UPTAKE BY PARAOXON

SAME CONDITIONS AS IN FIG. 7.

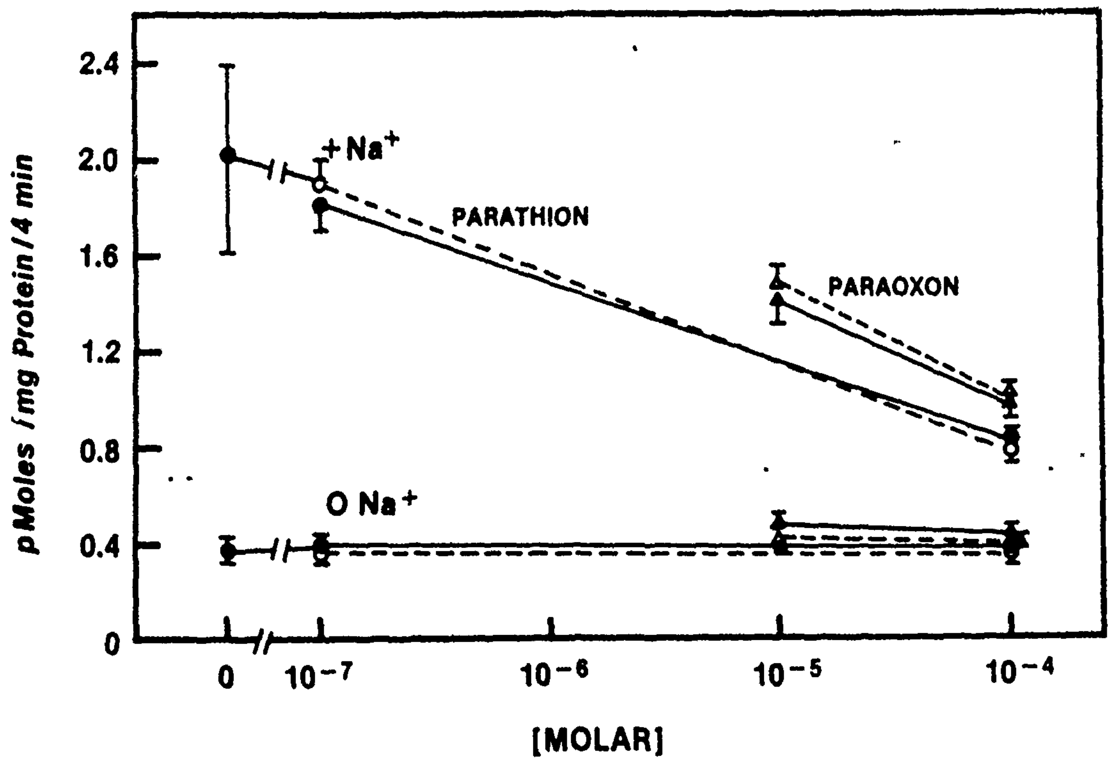


FIG. 9. DOSE RESPONSE CURVE FOR PARATHION AND PARAOXON INHIBITION OF [^{3}H] 5HT UPTAKE

EFFECT WHEN [^{3}H] 5HT WAS ADDED IMMEDIATELY AFTER 60 MIN PREINCUBATION WITH INHIBITORS (O——O, PARATHION, ▲——▲, PARAOXON) OR AFTER INCUBATING FOR A FURTHER 60 MINUTES IN INHIBITOR-FREE MEDIUM (O······O), PARATHION, △······△, PARAOXON). LOWER CURVES ARE FOR UPTAKE MEASURED IN Na^+ FREE MEDIUM. OTHER CONDITIONS AS IN FIG. 4. N = 6 ± S.D.

C. ACETYLCHOLINE INHIBITS β —RECEPTOR STIMULATED cAMP CONTENT AND DEPOLARIZES THE CELLS

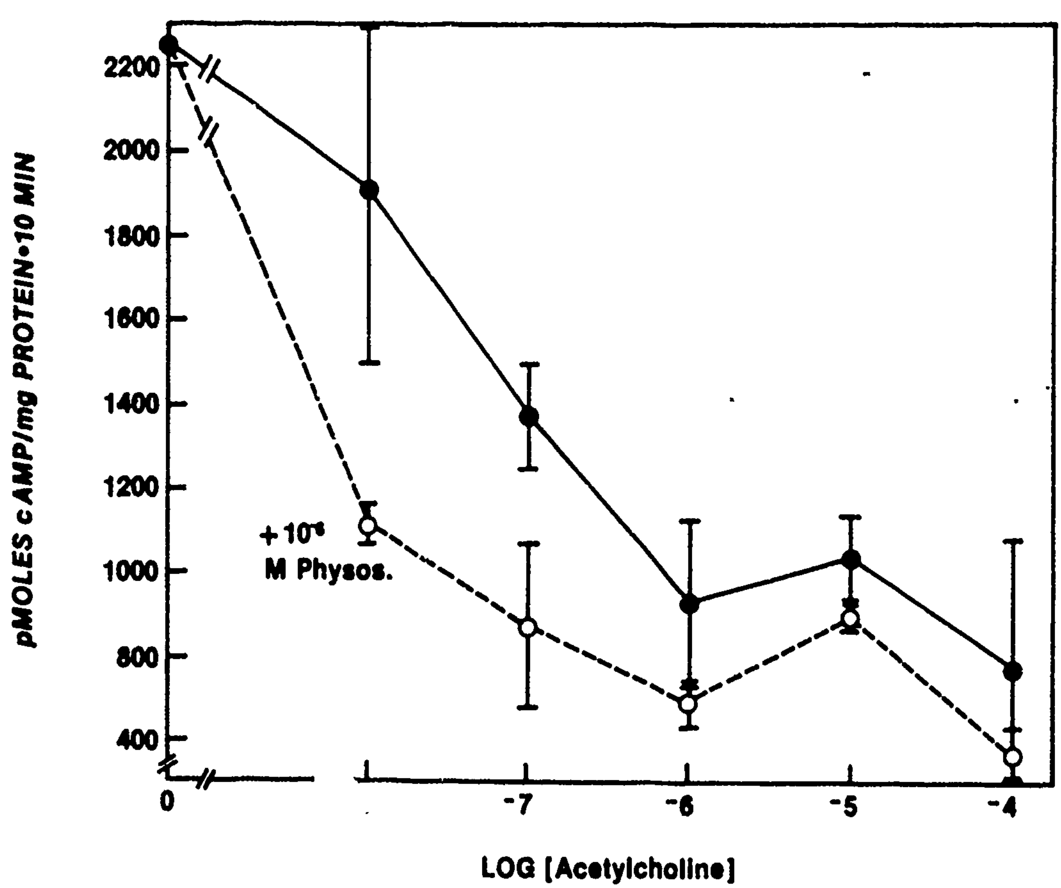
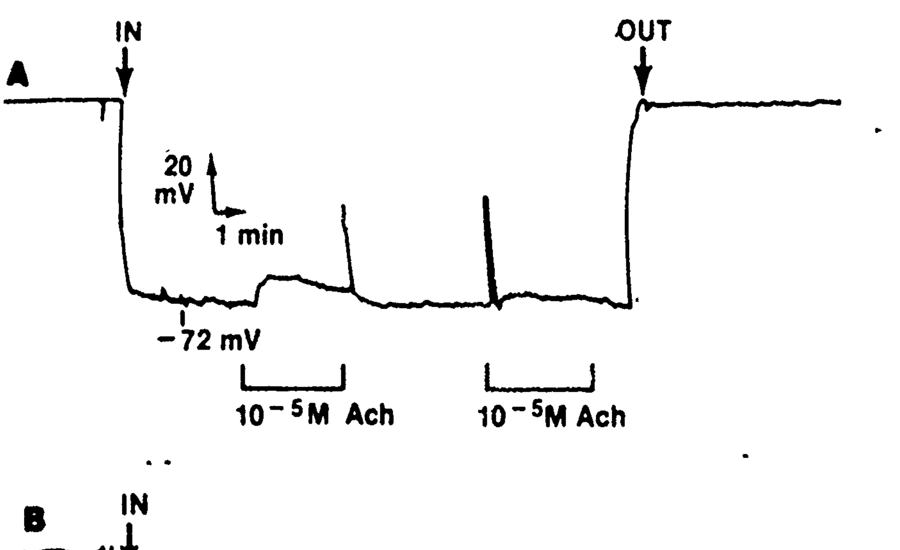


FIG. 10. STIMULATION OF cAMP CONTENT IN CELLS BY 10^{-5}M NE IS INHIBITED BY INCREASING CONCENTRATIONS OF ACETYLCHOLINE.

CELLS ARE INCUBATED WITH 10^{-5}M NOREPINEPHRINE FOR 10 MINUTES AND THE CYCLIC AMP CONTENT IS MEASURED USING PROTEIN BINDING ASSAY. THE CONCENTRATIONS OF ACETYLCHOLINE SHOWN ARE PRESENT SIMULTANEOUSLY WITH NOREPINEPHRINE WITH OR WITHOUT 10^{-6}M PHYSOSTIGMINE AS SHOWN. IT CAN BE SEEN THAT PHYSOSTIGMINE POTENTIATES THE INHIBITORY EFFECT OF ACETYLCHOLINE, ESPECIALLY AT LOW ACH CONCENTRATIONS, CONSISTENT WITH BREAKDOWN OF ACH BY ACHE BEING RATE-LIMITING. EACH POINT REPRESENTS MEAN ± S.D. (N = 3).



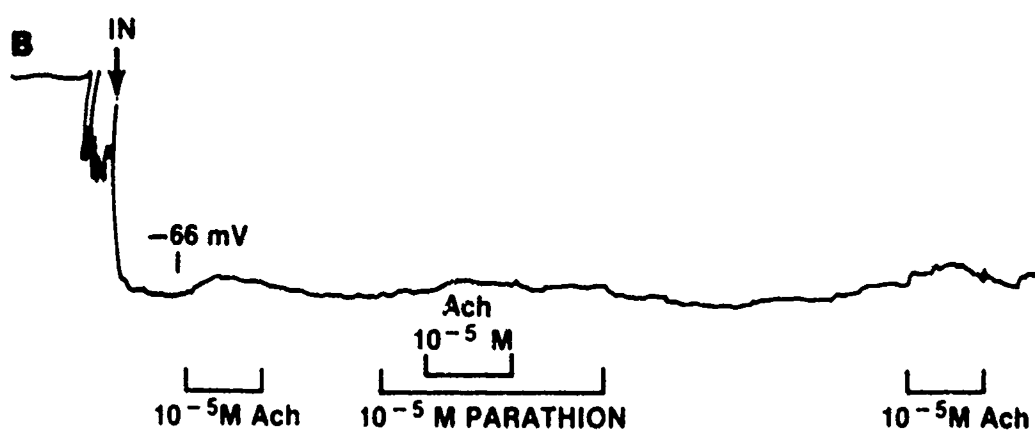


FIG. 11. ACH (10^{-5}M) DEPOLARIZES MEMBRANE POTENTIAL OF CULTURED ASTROCYTES BY UP TO 10MV

A. RESPONSE TO SECOND ADDITION OF ACH SHOWS LESS DEPOLARIZATION SUGGESTING DESENSITIZATION. MEMBRANE POTENTIAL OF CELLS RECORDED BY STANDARD ELECTROPHYSIOLOGICAL TECHNIQUES. IN REFERS TO IMPALEMENT OF CELL BY THE ELECTRODE WHICH MEASURES A SHARP CHANGE IN POTENTIAL FROM 0 TO -72MV. FIRST DEPOLARIZATION IS ABOUT 10MV, SECOND 4MV. OUT - ELECTRODE WITHDRAWN.

B. 10^{-5}M ACH AGAIN DEPOLARIZES IN SECOND CELL BY 7MV. IN THE PRESENCE OF PARATHION THE RESPONSE APPEARS TO BE ONLY 4MV WHILE A THIRD PERFUSION WITH 10^{-5}M ACH BY ITSELF GIVES AN APPARENT 7 TO 8MV DEPOLARIZATION AGAIN.

CONCLUSIONS

1. PRIMARY ASTROCYTE CULTURES SHOW HIGH AFFINITY (HA) SEROTONIN UPTAKE.

2. THIS UPTAKE CAN BE INHIBITED BY ACHE INHIBITORS SUCH AS THE TERTIARY AMINE CARBAMATE PHYSOSTIGMINE, AND THE NITROPHENYL PHOSPHOROTHIONATE PARATHION, WITH IC_{50} VALUES OF 50 AND 10μM RESPECTIVELY. THE PARATHION METABOLITE PARAOXON IS LESS EFFECTIVE THAN PARATHION. THE POTENT ORGANOPHOSPHATE ACHE INHIBITOR, DIISOPROPYL FLUOROPHOSPHATE (DFP), IS INEFFECTIVE UP TO 10^{-3}M.

3. ONLY THE MAJOR NA^{+} DEPENDENT COMPONENT IS AFFECTED.

4. THESE EFFECTS ON ASTROCYTIC HA SEROTONIN UPTAKE APPEAR UNRELATED TO ACHE INHIBITION BUT MAY REPRESENT OTHER SITES OF ACTION OF THESE COMPOUNDS IN THE MAMMALIAN CNS.

5. FUNCTIONAL EFFECTS SO FAR STUDIED OF ACETYLCHOLINE RECEPTORS (PROBABLY MUSCARINIC) PRESENT ON THESE CELLS ARE A) INHIBITION OF β RECEPTOR STIMULATED ADENYL CYCLASE AND B) DEPOLARIZATION OF THE RESTING MEMBRANE POTENTIAL.

AN *IN VITRO* ASSAY SYSTEM FOR ANTICHOLINESTERASES, REACTIVATORS AND CHOLINERGIC LIGANDS USING HOUSE FLY HOMOGENATE

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ABSTRACT

An *in vitro* assay system for anticholinesterases, reactivators and cholinergic ligands was developed using homogenate of adult house flies (*Musca domestica* L.). The model assay involved 8.43×10^{-7} M diisopropylfluorophosphate (DFP) and 1.55×10^{-4} M pralidoxime chloride (PAM) as the toxicant and reactivator, respectively, with the acetylcholinesterase (AChE) activity monitored spectrophotometrically. The net increases in the AChE activity by addition of PAM 3 min before or after DFP were 48.5% and 50.5%, respectively. Complementary *in vivo* studies involving PAM injection (31 μg/fly) 1 hr before or after that of DFP showed protective ratios of 50 and 162, respectively, calculated as the elevation factors of the baseline LD_{50} value (14 ng/fly).

The rapid and typical response to the reactivators (PAM, Toxogonin and HI-6) and certain ligands (atropine sulfate, acetylcholine chloride, edrophonium chloride, gallamine triethiodide and decamethonium bromide) suggests that the cholinergic system in the house fly is readily accessible and amenable to therapeutic tests and evaluation of candidate chemicals.

INTRODUCTION

Organophosphate poisons are believed to disrupt the nerve function primarily by inactivating an enzyme, acetycholinesterase (EC 3.1.1.7). The basic mechanism of cholinergic transmission among animal species is so much alike that the biochemical lesion due to nerve poisons underscores the morphological and taxonomic differences. It is not surprising, therefore, that some of the earliest insecticides found more use in chemical warfare than in agriculture or public health. As an analogy to this, it was proposed (Das,1984) that insects be used to test therapeutic compounds against anticholinesterases.

Insects, because of their dimunitive size, require extremely low amounts of expensive test compounds, can be treated in large numbers, and yield data of greater statistical confidence. We report here an *in vitro* assay system developed with the house fly homogenate. The *in vivo* assay system has been described elsewhere (Das et al.,1985), although some relevent data have been cited here.

MATERIALS AND METHODS

Test insect and chemicals

House flies (Ohio State Standard strain, OSS) were reared at 22-25°C and continuous fluorescent light conditions. Three- to five-day old female flies were used for the tests. All chemicals were purchased from Sigma Chem. Co., St. Louis, MO, except 2-pyridine aldoxime methochloride (pralidoxime chloride, PAM) which was purchased from Aldrich Chem. Co., Milwaukee, WI. Toxogonin, HI-6 and PAM-PAM were obtained from our Division of Experimental Therapeutics. A stock solution of DFP was made in anhydrous isopropanol (10 mg/ml) which was diluted with distilled water to desired concentration at the time of application. For dosages of 800 ng/fly and higher, DFP was directly dissolved in distilled water to eliminate isopropanol toxicity. All other chemicals were used as aqueous solutions.

In vitro procedures

Adult flies, fresh or frozen, were homogenized in a Polytron PT 10/35 homogenizer (Brinkmann Instruments Co., Westbury, NY) for 3 min in potassium phosphate buffer (pH 8.8, 0.1 M)(5 ml/g flies) and cleared through a 16-layer cheesecloth. The resulting crude extract possessed ca. 1 unit AChE activity per ml.

AChE activity was measured in a spectrophotomete·· using acetylthiocholine (ASCh) as the subs e (Ellman et al., 1961). A typical reaction consisteu of 3.0 ml buffer, 100 µl dithiobisnitrobenzoic acid (DTNB), 20 µl ASCh and 100 µl extract. Each reaction was repeated 5-6 times.

In vivo procedures

Thoracic injections (0.44 ul/fly) were made posterio-dorsally using a 10-µl syringe (701N) mounted on a dispenser (PB 600)(Hamilton Co., Reno, NV) after briefly anesthetizing with carbon dioxide gas. Each treatment was replicated at least 3 times, with 10 adult flies per replication. The mortality data were subjected to probit analysis (Finney,1964) and the response curves were drawn from simple linear regression.

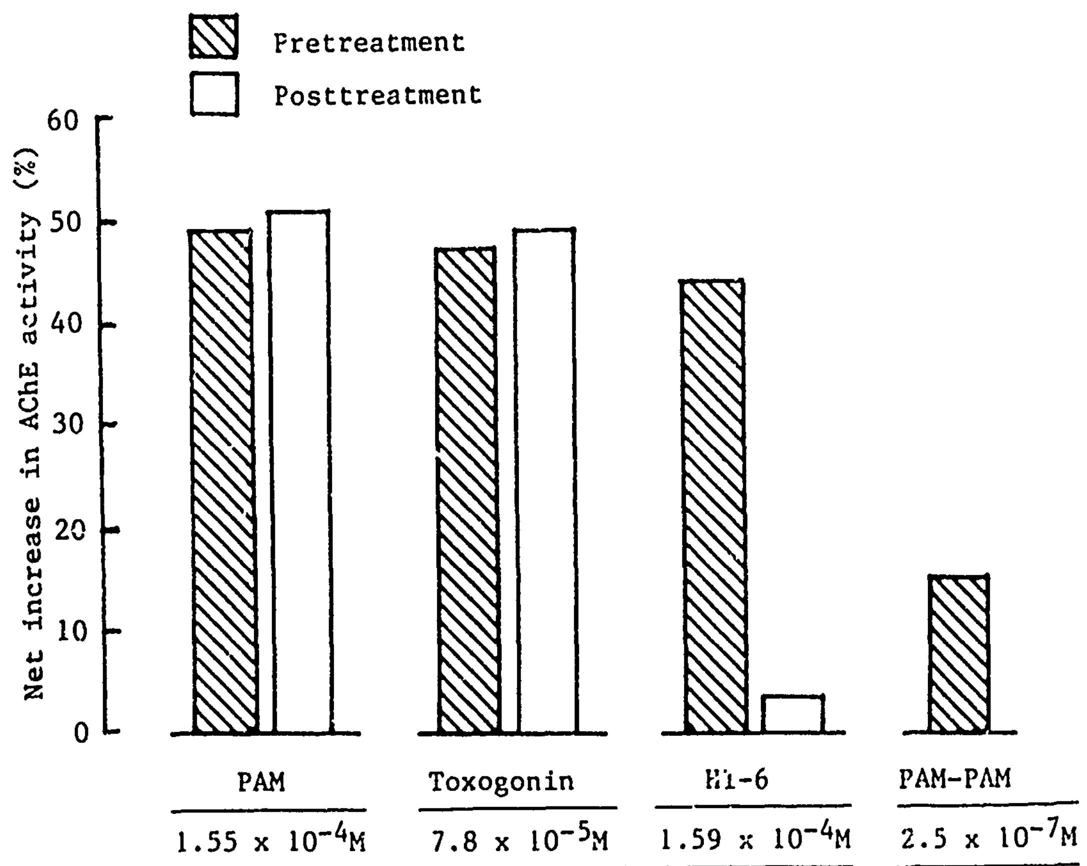

Figure 1. *In vitro* protection by oximes to the house fly AChE against the inactivation by DFP.

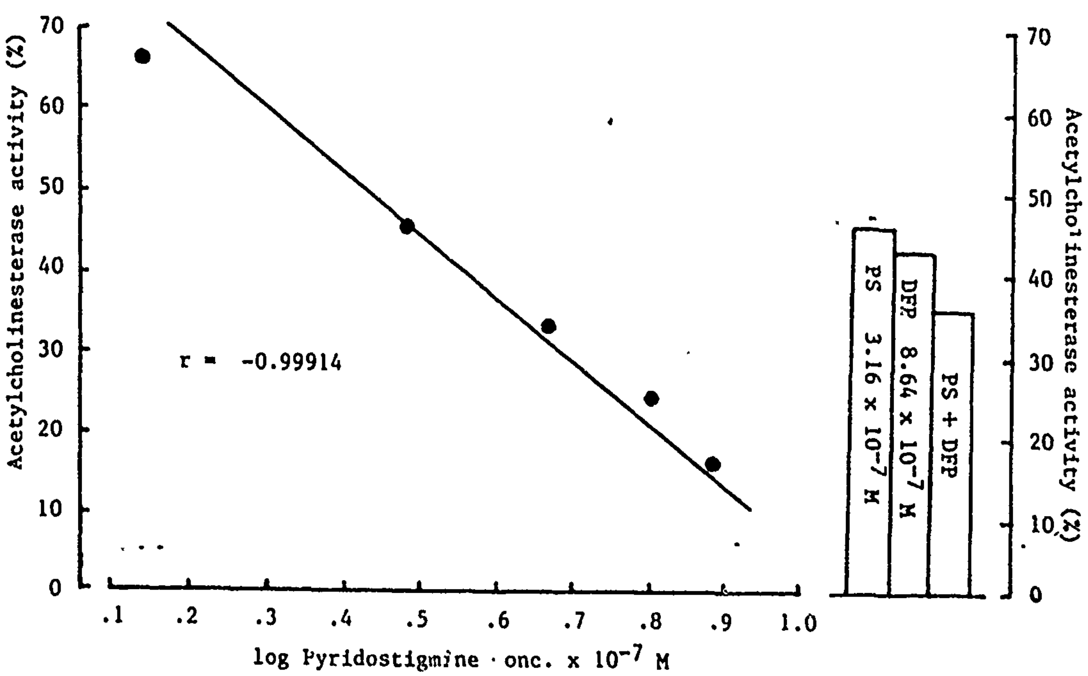

Figure 2. In vitro effects of pyridostigmine bromide and DFP on the house fly acetylcholinesterase activity.

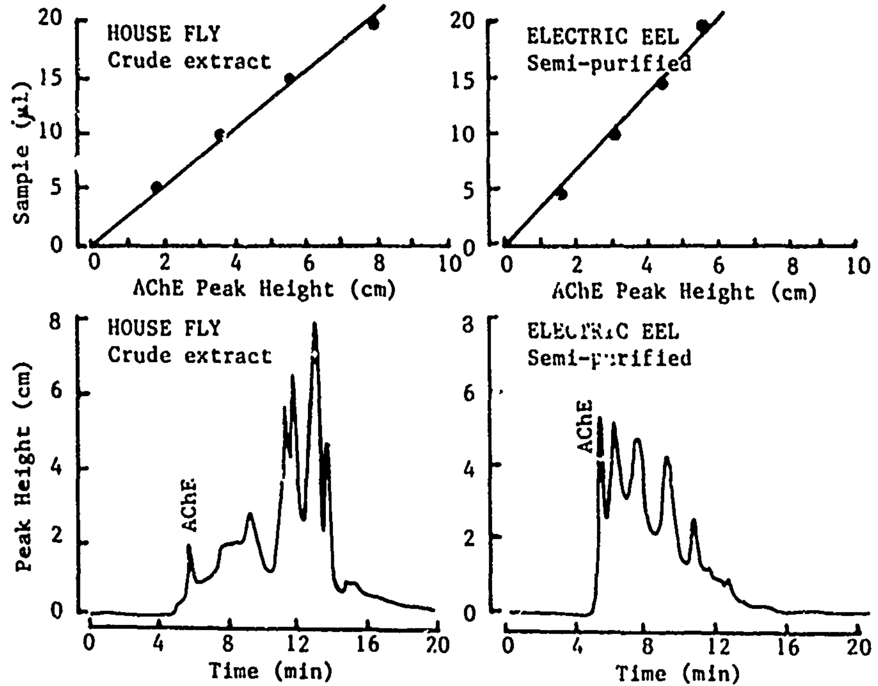

Figure 3. HPLC analyses of house fly and electric eel acetylcholinesterases. Protein-Pak 300 SW column was used with potassium phosphate buffer (pH 8.0, 0.05 M) as the mobile phase.

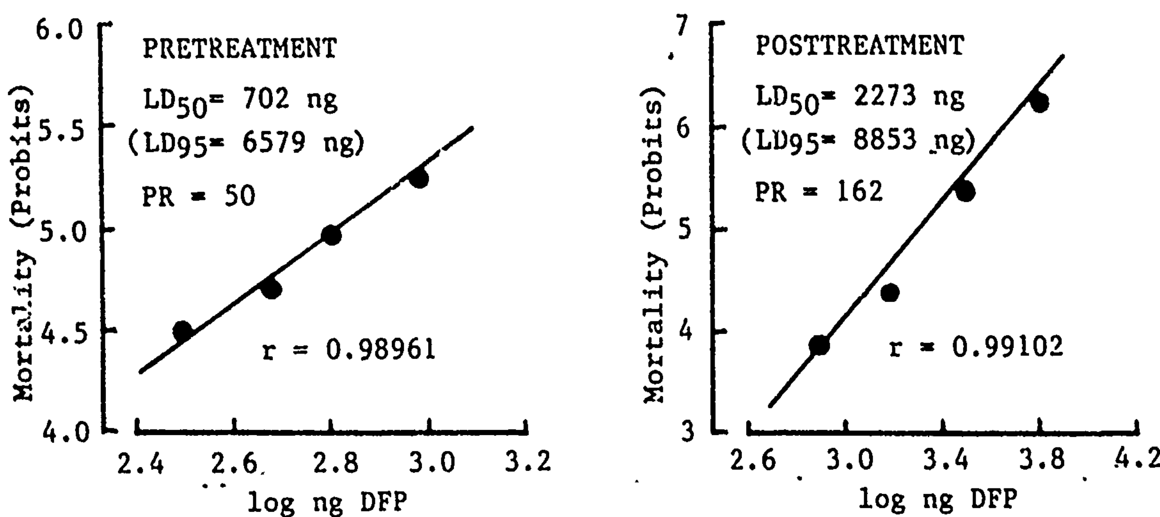

Figure 4. _In vivo_ protection by PAM to house flies against DFP. PR = Protective Ratio, calculated as the elevation factor of baseline LD_{50}.

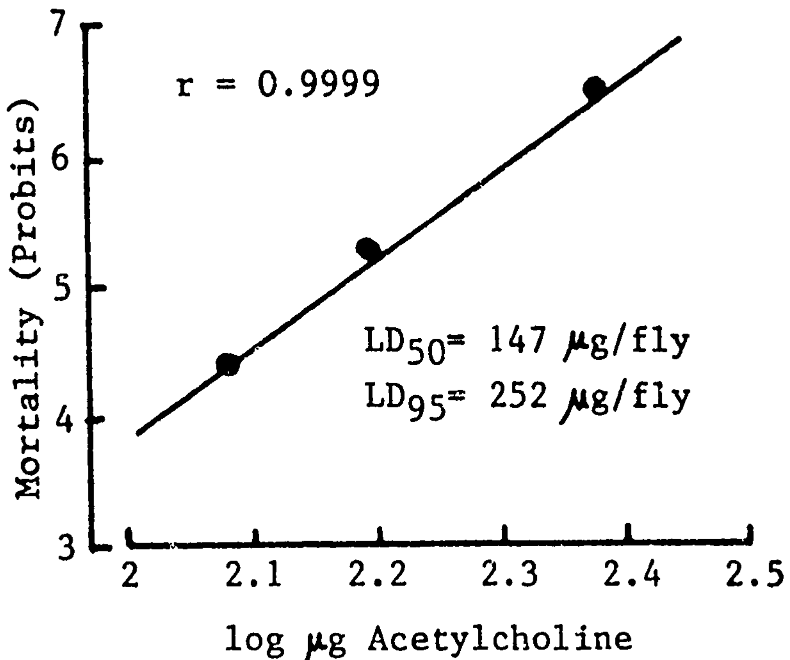

Fig.5. Dosage-mortality response of acetylcholine chloride.

RESULTS AND DISCUSSION

In vitro tests

A 3-min incubation of the crude extract with DFP (8.43×10^{-7} M) resulted in 41.5% of the initial activity. The addition of PAM (1.55×10^{-4} M) 3 min before or after DFP resulted in net increases in AChE activity of 48.5% and 50.5%, respectively (Fig.1). In similar tests, toxogonin (7.8×10^{-5} M) showed net activity increases of 47.1% and 49.1% in pretreatment and posttreatment, respectively, to DFP (6.7×10^{-7} M); HI-6 (1.6×10^{-4} M) showed 43.9% and 3.3% in pretreatment and posttreatment, respectively, to DFP (1.73×10^{-6} M); and PAM-PAM (2.5×10^{-7} M) showed 14.7% in pretreatment to DFP (1.1×10^{-6} M) (Fig.1).

The house fly homogenate was sensitive also to a medicinal carbamate, pyridostigmine bromide (Fig.2). This chemical, although a temporary (reversible) inhibitor of AChE, caused some additive effect to DFP when present together *in vitro*. Therefore, a different *in vitro* technique is being developed to evaluate such compounds.

HPLC analysis of centrifuged (100,000*g*, 1 hr) extract showed 8 different components as compared with 5 from the semi-purified electric eel AChE (Type V-S) (Fig.3). However, the AChE activity resided entirely in identical (first) peaks.

In vivo tests

In vivo studies were made by thoracic injection of selected compounds. Based on the 24-hr LD_{50} value of DFP (14 ng/fly), protective ratios of 50 and 162 were

obtained with PAM (31 µg/fly) when given 1 hr before or after DFP, respectively (Fig.4).

Edrophonium chloride injection (88 µg/fly) resulted in leg tremors, paralysis and death in ca. 12 hr. This syndrome was comparable to DFP-poisoning, except it was insensitive to PAM injection (30 µg/fly) either preceding or following edrophonium injection. Atropine sulfate (40 µg/fly) caused leg tremors and paralysis but no mortality. Gallamine triethiodide (53 µg/fly) produced signs similar to atropine but without leg tremors. Decamethonium bromide (25 µg/fly)-treated flies exhibited no signs of toxicity. The LD_{50} of acetylcholine chloride was 147 µg/fly (Fig.5) with toxic signs similar to those of DFP or edrophonium. It appears that the cholinergic system in the house fly is readily accessible to the chemicals tested here, providing an economical, convenient and comprehensive assay system for the screening of therapeutic agents.

The junctional transmission in insects being non-cholinergic, most of the toxicity is believed to be centrally mediated. This implies that the blood-brain barrier, which is known to exist in some insects for certain compounds (Eldefrawi et al., 1968), was ineffective in the house fly towards DFP and PAM. Nevertheless, some contribution by the junctional presynaptic acetylcholine receptors (Fulton and Usherwood, 19/7) and/or the glutamate receptors (Idriss and Albuquerque, 1985) cannot be disregarded.

...owledgement.-- The authors wish to thank Dr.Claudia F. Golenda of our Department for the supply of house flies.

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AN INSECT BIOASSAY FOR ASSESSING PROTECTIVE AND THERAPEUTIC AGENTS IN ORGANOPHOSPHATE POISONING

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Introduction

A bioassay system using insects was developed to provide a rapid, reliable, low cost screen for nerve agent antidotes. Initial studies were designed to compare the house fly model with test systems using rodents and nonhuman primates. Compounds shown to be protective and/or therapeutic in mammalian studies were used as a basis for comparison between the insect and mammalian models. These compounds included: 2-pyridinealdoxime methochloride (2-PAM), atropine, and physostigmine. The challenge organophosphate was diisopropyl fluorophosphate (DFP).

Methods

The toxicities of DFP, 2-PAM, atropine, and physostigmine were determined in house flies. Anesthetized flies were held by forceps on the stage of a dissecting microscope, and injected into the medial posterior of the thoracic dorsum using a 10-ul syringe (Figures 1 and 2). A volume of 0.44 ul containing the test materials or DFP, diluted in buffered saline, was dispensed into the body cavity of each insect. Control flies received only buffered saline. For each toxicity test, at least 3 doses were used, and there were 3 replicates of 10 flies per dose. Tests were replicated at least 3 times. Treated flies were housed in disposable petri dishes lined with filter paper (Figure 3), and kept in an incubator at 25°C. The 48-h mortality data were pooled from replicated tests and subjected to probit analysis (Ray 1982). The LD 50, 95, and slope values were estimated from the probit regression lines, and the LD 50 and 95 confidence limits calculated (P=0.05).

In protection and therapeutic studies using atropine (1000 ug/g), 2-PAM (1500 ug/g), atropine plus 2-PAM (1000 ug/g plus 1500 ug/g, repectively), and physostigmine (25 ug/g), the forementioned injection procedure was used; however, flies received two injections. In protection studies the first injection dispensed either atropine, PAM, PAM plus atropine, or physostigmine into the insect body cavity, while the second injection dispensed DFP; in therapeutic studies the order was reversed. Both injections were administered in the same puncture hole made in the insect cuticle. Atropine, 2-PAM, or a combination of atropine plus 2-PAM were administered 5 minutes prior to DFP, while physostigmine was administered 15 minutes prior to DFP. In therapeutic studies, at.opine plus 2-PAM was administered 1, 3, and 6 hours after flies were poisoned by an LD 95 dose of DFP (2.5 ug/g). Physostigmine was given 15 minutes after DFP poisoning. Control flies received saline pre- or post-treatments.

Probit regression lines for DFP were determined from the 48-h mortality data from flies receiving: 1) no antidotal pretreatment 2) a 2-PAM plus atropine pretreatment, and 3) a physostigmine pretreatment. The effect of administering antidotes after DFP poisoning was evaluated by a Chi Square analysis (Fleiss 1981) in which the proportion of 48-h survivors was compared to flies receiving no therapeutic injection.

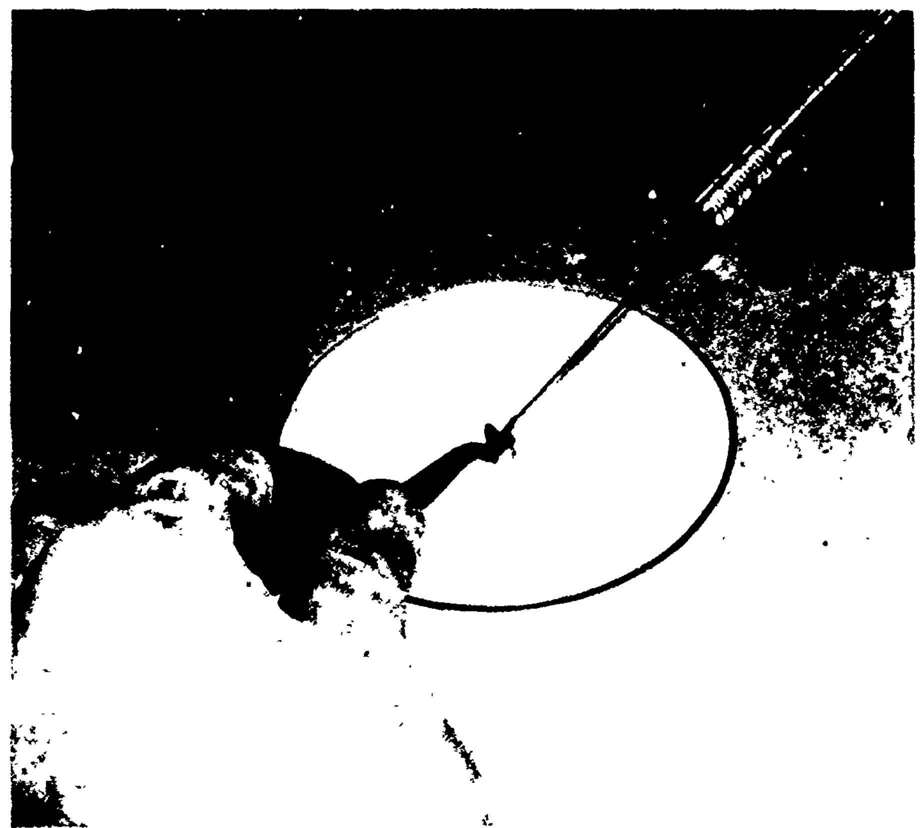

Figure 1: Injection procedure used to administer antidotes and organophosphate to house flies.

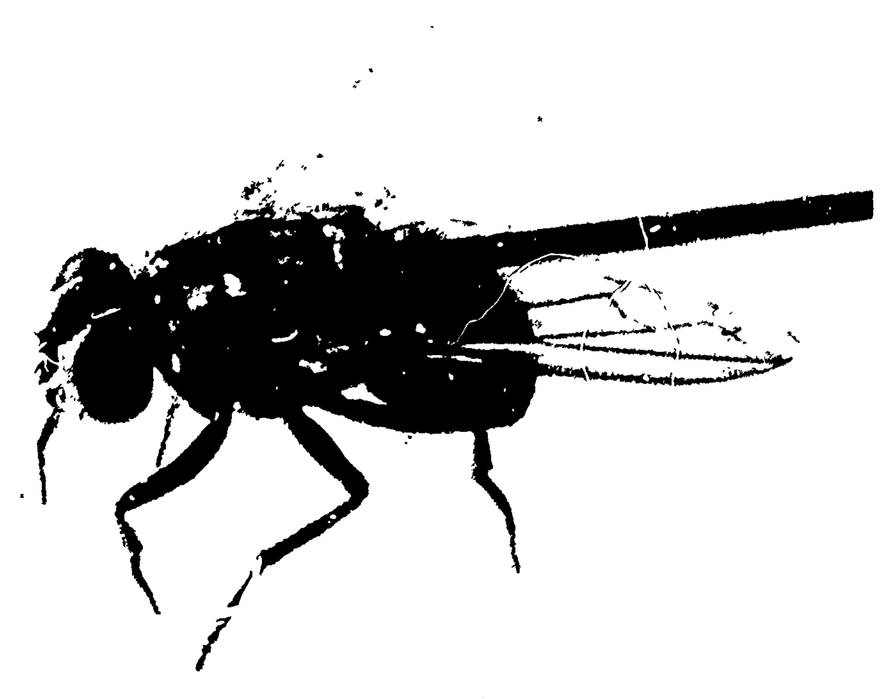

Figure 2: Injection, showing the position of 10-ul syringe in the medial posterior of the thoracic dorsum of the house fly.

Results

The relationship between the doses of atropine (1000 ug/g), PAM (1500 ug/g), and physostigmine (25 ug/g) used in protection and/or therapeutic studies and their toxicities to house flies is illustrated in Figure 4. Initial studies determined that both 2-PAM (77.8 % survival), and 2-PAM plus atropine (88.3 % survival) protected flies from DFP, but atropine (3.3 % survival) did not (Chi Square analysis, P=0.05). When 2-PAM plus atropine was administered 5 minutes prior to flies receiving increasing challenge doses of DFP, the LD 50 for DFP significantly increased. In flies receiving no pretreatment, the LD 50 for DFP was 0.85 ug/g while in flies pretreated with 2-PAM plus atropine the LD 50 for DFP was 13.5 ug/g (Figure 5); a protective ratio of approximately 16. Physostigmine also protected house flies against the lethality of DFP. The LD 50 for DFP in flies receiving no pretreatment was 0.95 ug/g, while in flies receiving physostigmine 15 minutes prior to DFP, the LD 50 increased to 3.9 ug/g (Figure 6); a protective ratio of 4.1.

Survival of house flies poisoned by a LD 95 dose of DFP was dependent on the time at which 2-PAM plus atropine was administered (Figure 7). Administering 2-PAM plus atropine 1, 3, or 6 hours after exposure to DFP resulted in survival rates of 86, 54, and 15 %, respectively. Physostigmine, when given after exposure to a LD 50 dose of DFP, did not significantly decrease mortality (Chi Square analysis, P=0.05). In comparing three post treatments of buffer, 12.5 ug/g, and 25 ug/g of physostigmine, the mean percent mortalities from three tests were 68, 46, and 58 %, respectively.

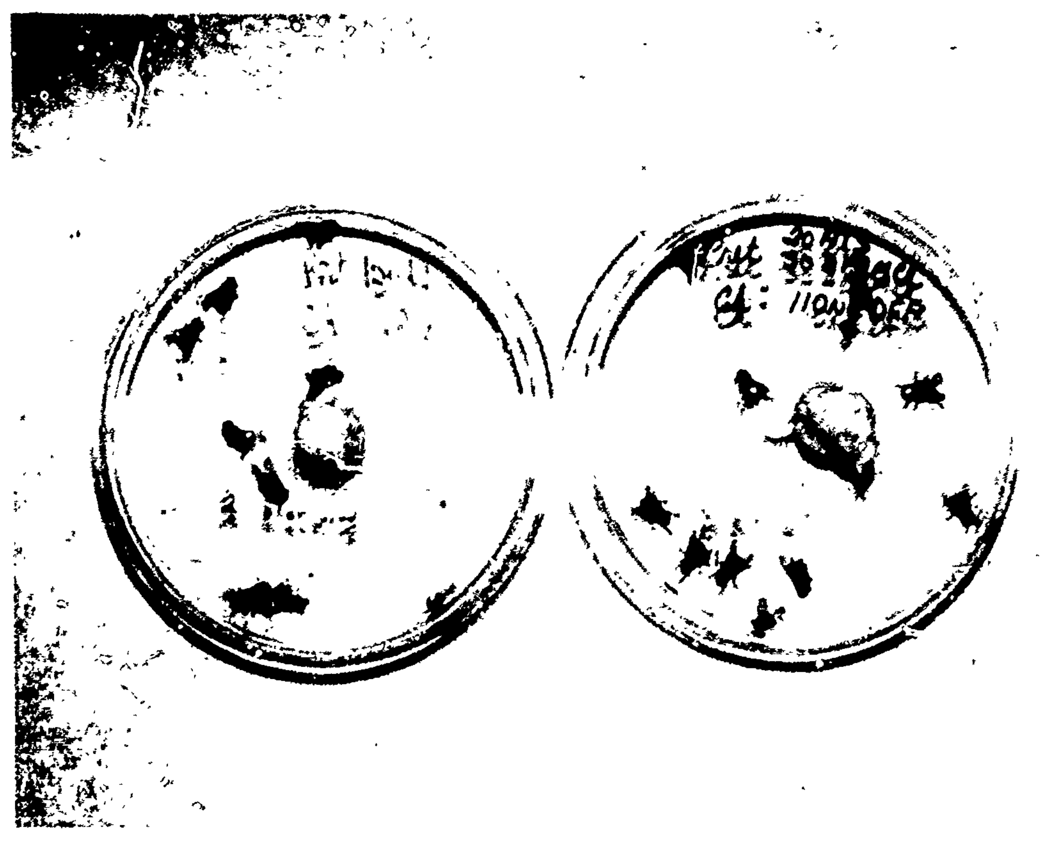

Figure 3: Petri dishes that housed treated flies during observation period after treatment.

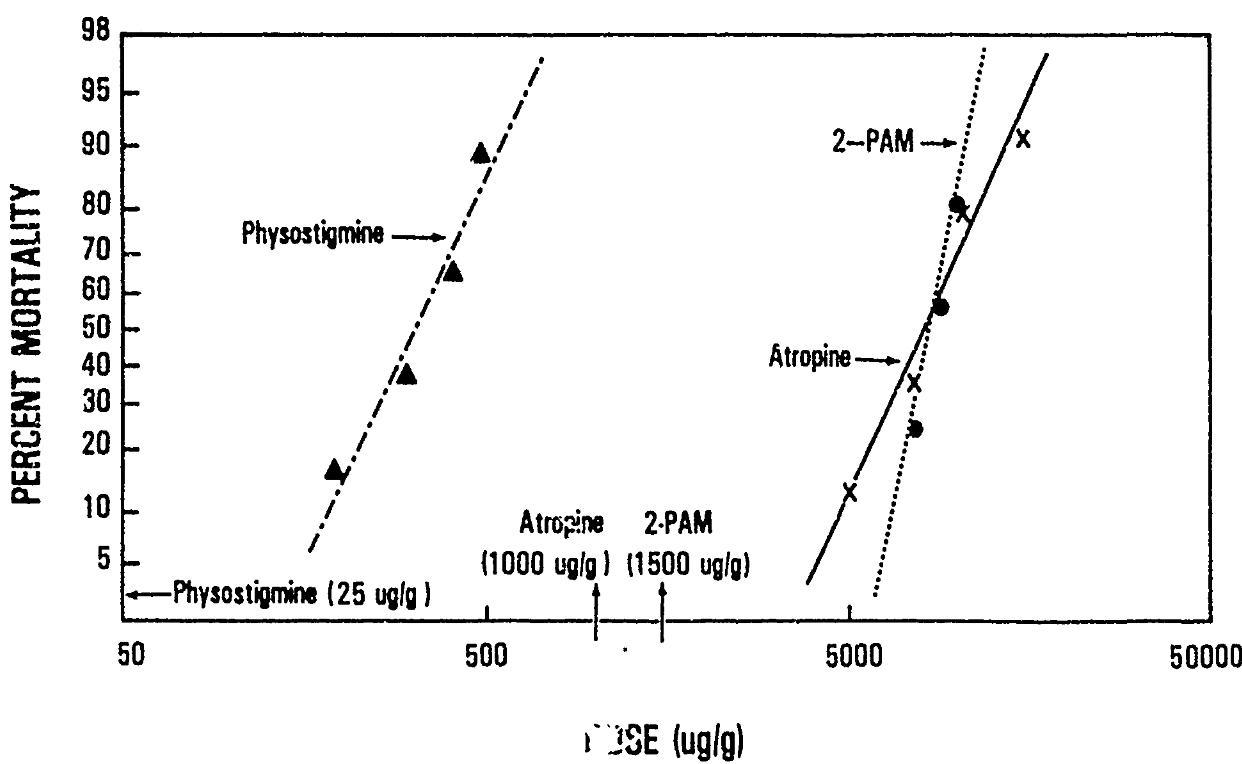

Figure 4: Probit regression lines for compounds used as protectants and/or therapeutics to DFP.

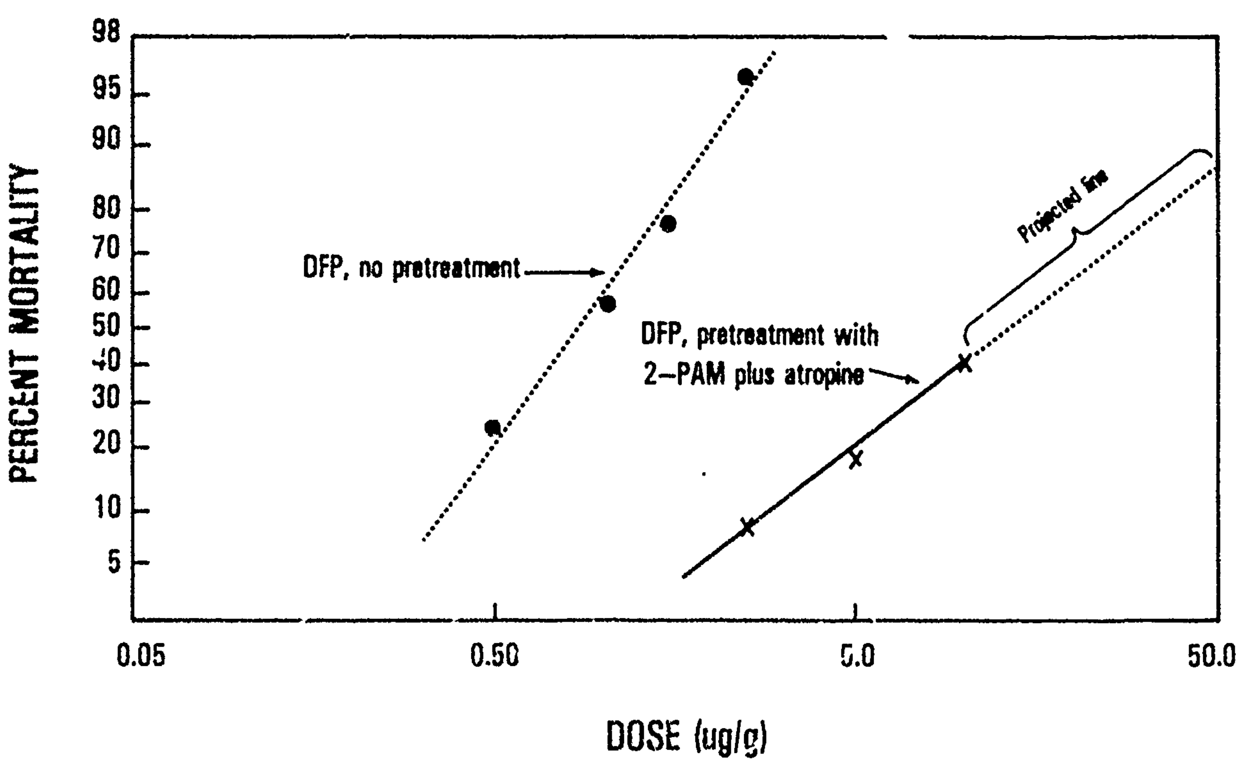

Figure 5: Probit regressions lines for DFP in flies receiving no pretreatment, and flies receiving a 2-PAM plus atropine pretreatment 5 minutes prior to DFP.

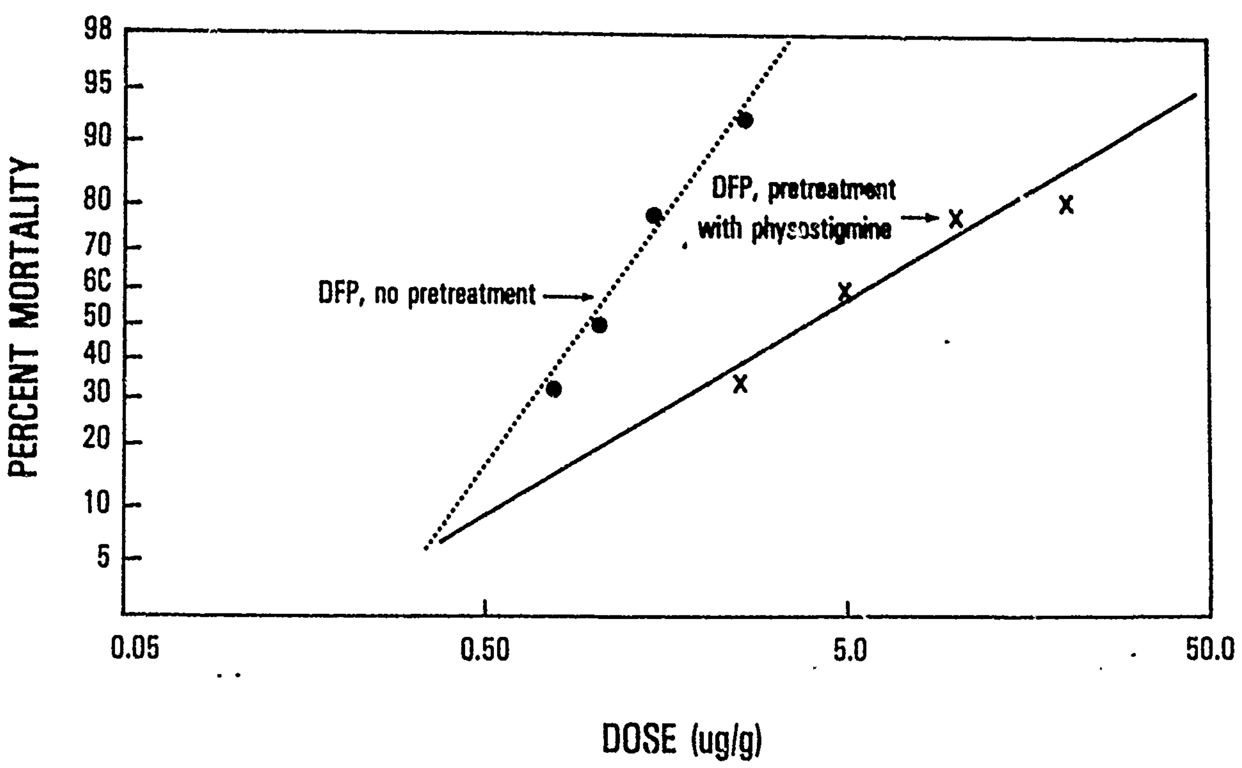

Figure 6: Probit regressions lines for DFP in flies receiving no pretreatment, and flies receiving a physostigmine pretreatment 15 minutes prior to DFP.

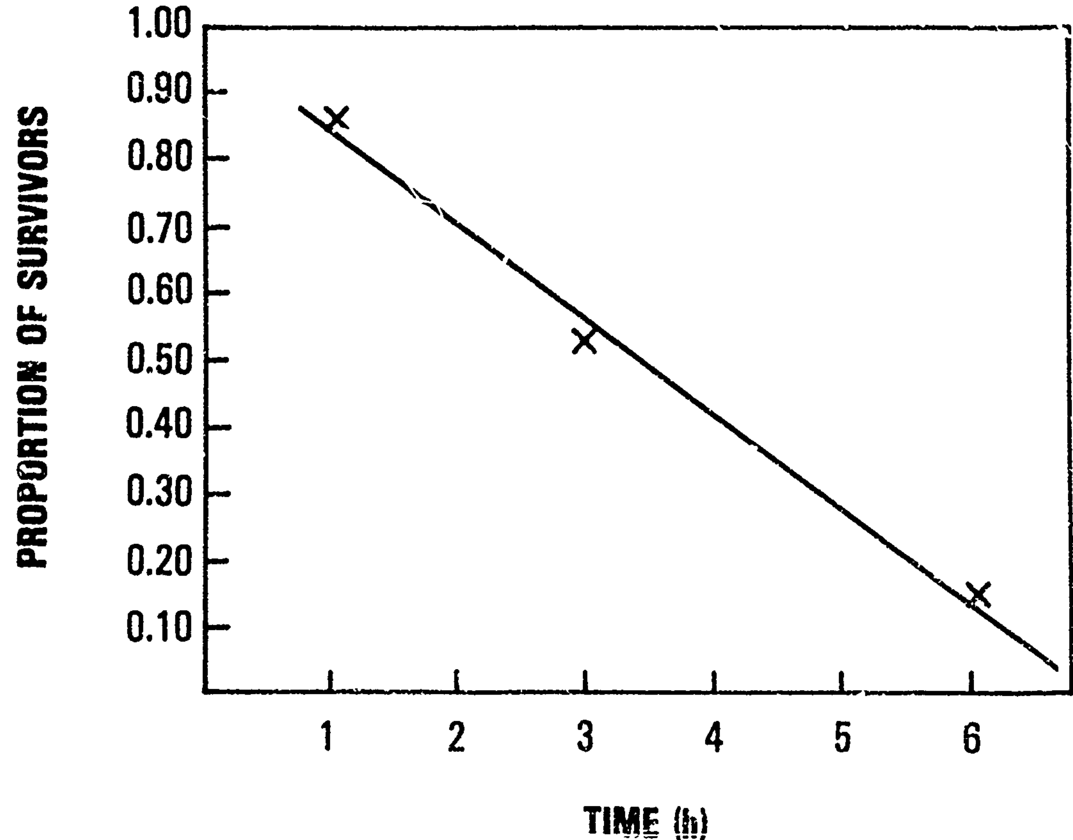

Figure 7: The effect of time on antidote administration when flies received atropine plus 2-PAM 1, 3, or 6 h after exposure to a LD 95 dose of DFP.

Discussion and Conclusions

PAM, atropine plus PAM, and physostigmine protected house flies against the lethality of DFP. Similar findings have been reported for PAM and atropine plus PAM in mice (Hobbiger 1957, Ramachandran 1966), and for physostigmine in cats (Koster 1946) and guinea pigs (Berry and Davies 1970).

The therapeutic effect of atropine plus PAM in house fly was time dependent with survival rate being greatest when therapy occurred soon after poisoning. The importance of immediate treatment to individuals poisoned by organophosphates has been described in mammals, including man (Hayes 1975). In cats (Koster 1946), physostigmine had no therapeutic effect when given after DFP poisoning had occurred. Similarly, in house fly physostigmine had no therapeutic effect when given 15 minutes after flies were poisoned by a LD 50 dose of DFP.

Insect based tests are rapid and relatively inexpensive since rearing and handling facilities and personnel can be greatly reduced. Furthermore, only small amounts of test materials are necessary, and this is particularly important in reducing the risk factor associated with working with nerve agents. The ability to use large numbers of insects makes these tests particularly applicable to statistical analysis. While more testing of the insect model is required, all indications are that the insect bioassay will provide a rapid, reliable, low cost primary screen for nerve agent antidotes, and will augment the mammalian screens now in use.

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MOLECULAR ORIGINS OF SELECTIVE TOXICITY: PROTONIC REORGANIZATION IN THE PHOSPHORYLATION OF SERINE HYDROLASES

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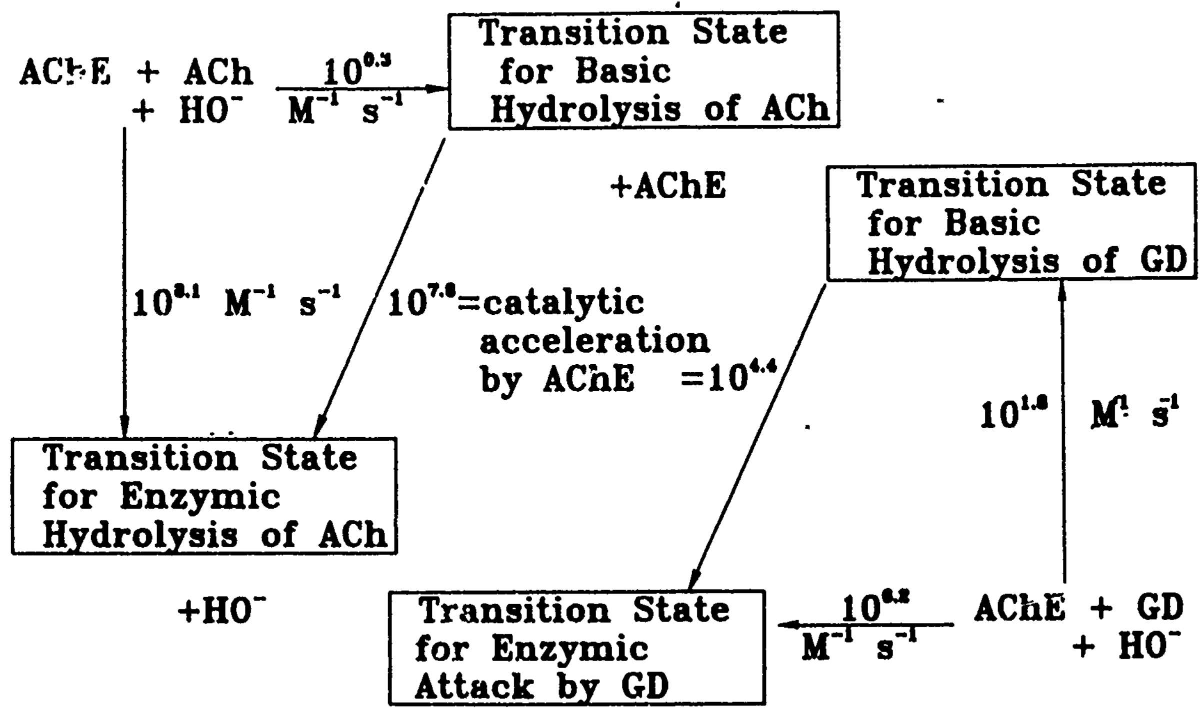


HOW DO ORGANOPHOSPHORUS TOXINS RECRUIT THE CATALYTIC POWER OF TARGET ENZYMES ?

DO INTERACTIONS BETWEEN TOXIN AND ACTIVE SITE ACTIVATE THE ACID–BASE CATALYTIC MACHINERY ?

IF SO – THIS SHOULD BE REFLECTED IN SOLVENT ISOTOPE EFFECTS (V_{HOH}/V_{DOD})

Kinetic Methods

1. Inhibitor/Substrate Competition

$$\mathrm{EI} \xrightarrow{k_{3I}} \mathrm{E^* + P_2}$$
$$k_{1I} \Uparrow\Downarrow k_{2I}$$
$$\mathrm{S+E+I}$$
$$k_{1S} \Uparrow\Downarrow k_{2S}$$
$$\left.\mathrm{ES} \xrightarrow{k_{3S}} \mathrm{E+P_1}\right\} \text{ monitored}$$

$$k_{obs} = \frac{k_{3I}[I_0]}{K_I + [I_0]} \cdot \frac{K_m}{K_m + [S]} \qquad K_I = \frac{k_{2I}+k_{3I}}{k_{1I}} \qquad K_m = \frac{k_{2S}+k_{3S}}{k_{1S}}$$

$$k_{obs}^{-1} = \frac{K_I + [I_0]}{k_{3I}[I_0]} \cdot \left[1 + \frac{[S]}{K_m}\right] \text{ if } [I_0] \gg K_I$$

k_{3I} obtained directly

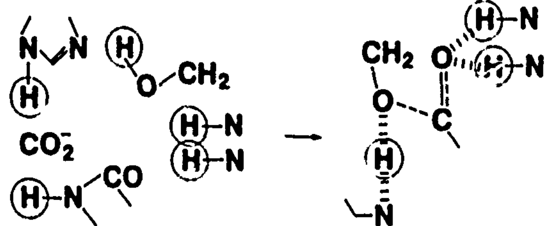
2. Direct Inhibition – P_2 monitored

a. $v_0 = k_{3I}[E_0][I_0]$ $\quad [E_0]$ and $[I_0] > 5\times10^{-5}$ M

b. $v_0 = k_{obs}[I]$; $\quad k_{obs} = k_{3I}[E_0]$

c. $v_0 = k_{obs}[E]$; $\quad k_{obs} = k_{3I}[I_0]$

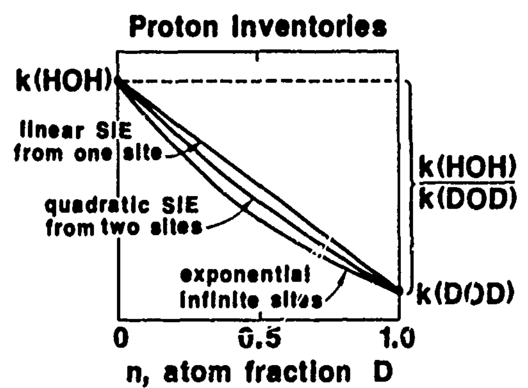
Protonic sites H→D in DOD

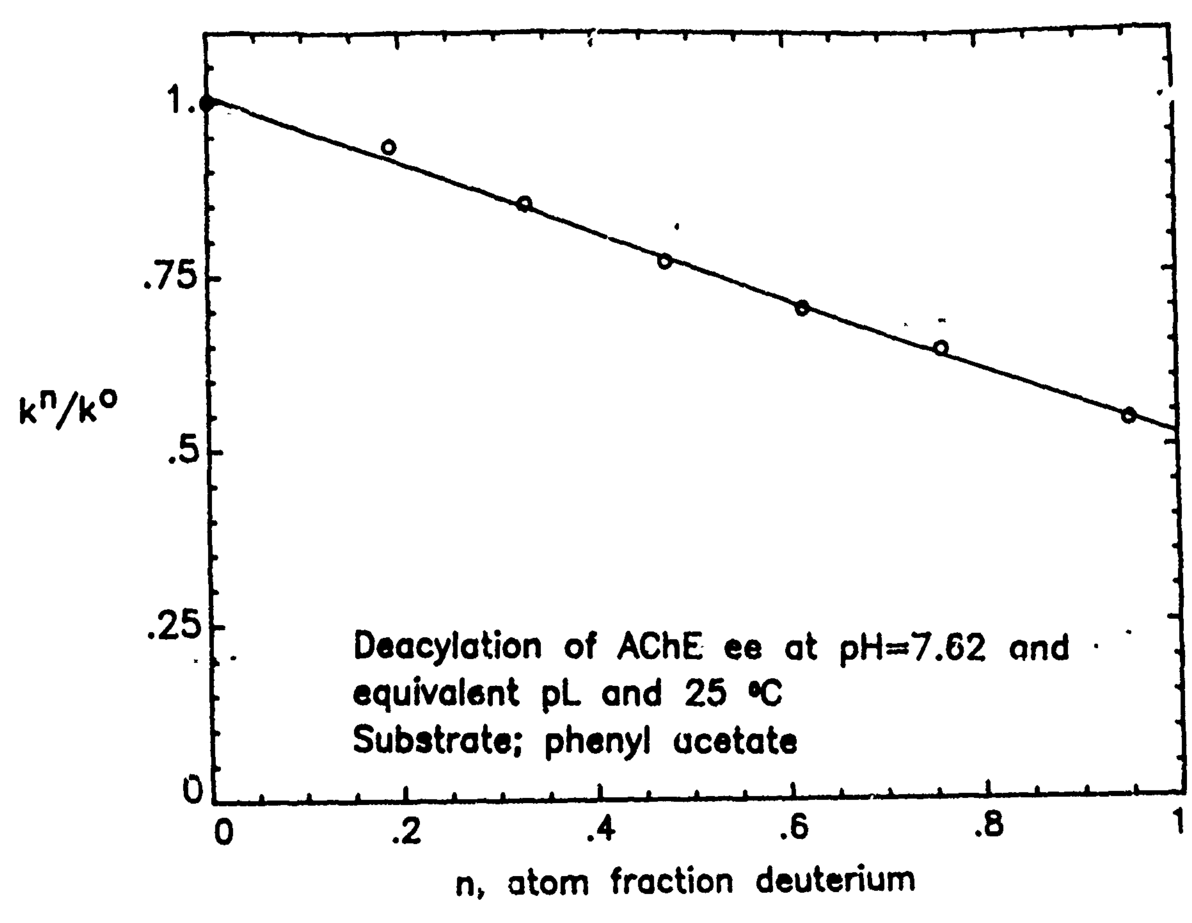


reactant state ⟶ transition state

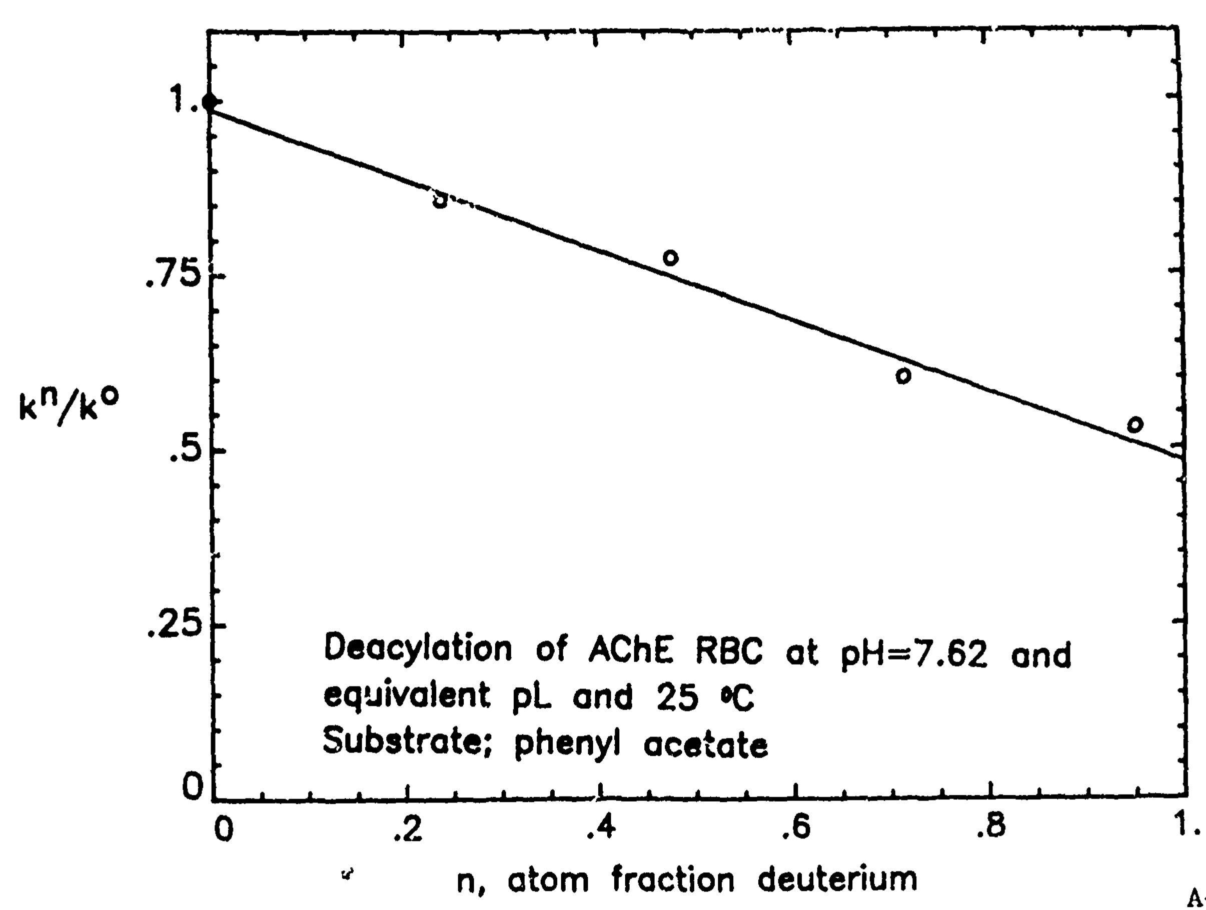
k(HOH)/k(DOD) (SIE) reflect bonding changes at exchangable sites

Proton Inventories





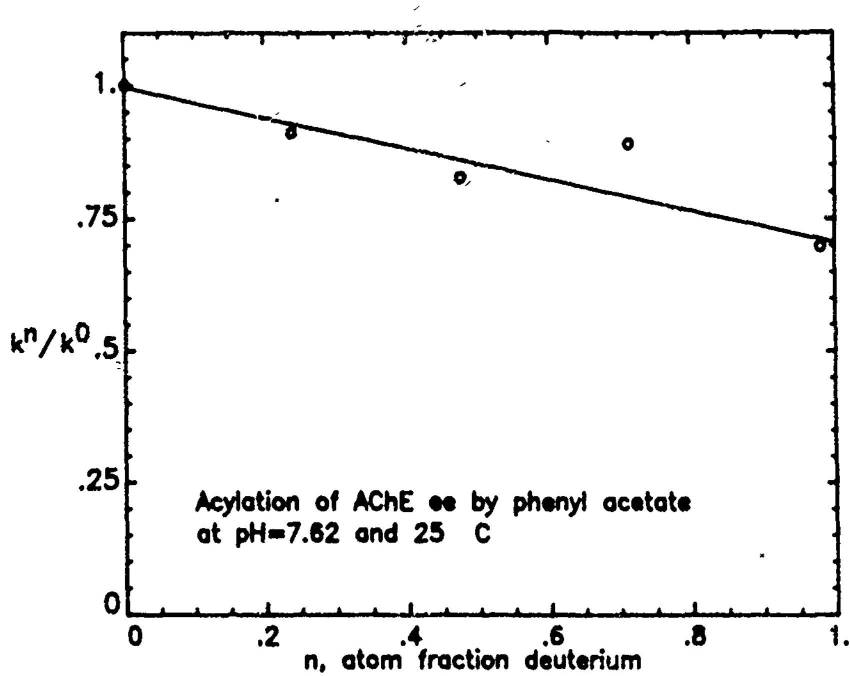
1.
.75
.5
.25
0
k^n/k^o
0
.2
.4
.6
.8
1.
n, atom fraction deuterium
Deacylation of AChE ee at pH=7.62 and
equivalent pL and 25 °C
Substrate; phenyl acetate



1.
.75
.5
.25
0
k^n/k^o
0
.2
.4
.6
.8
1.
n, atom fraction deuterium
Deacylation of AChE RBC at pH=7.62 and
equivalent pL and 25 °C
Substrate; phenyl acetate

SIE ~ 2

General base catalysis in the rate determining step.



SIE=1.46

in perfect agreement with Rosenberry, T. L. Croatia Chemica Acta 1975, 47, 235

Incursion of other steps then general base catalysis, such as conformational changes, in rate limiting process

GD

$CH_3-P(=O)(F)-O-CH(CH_3)-C(CH_3)_3$

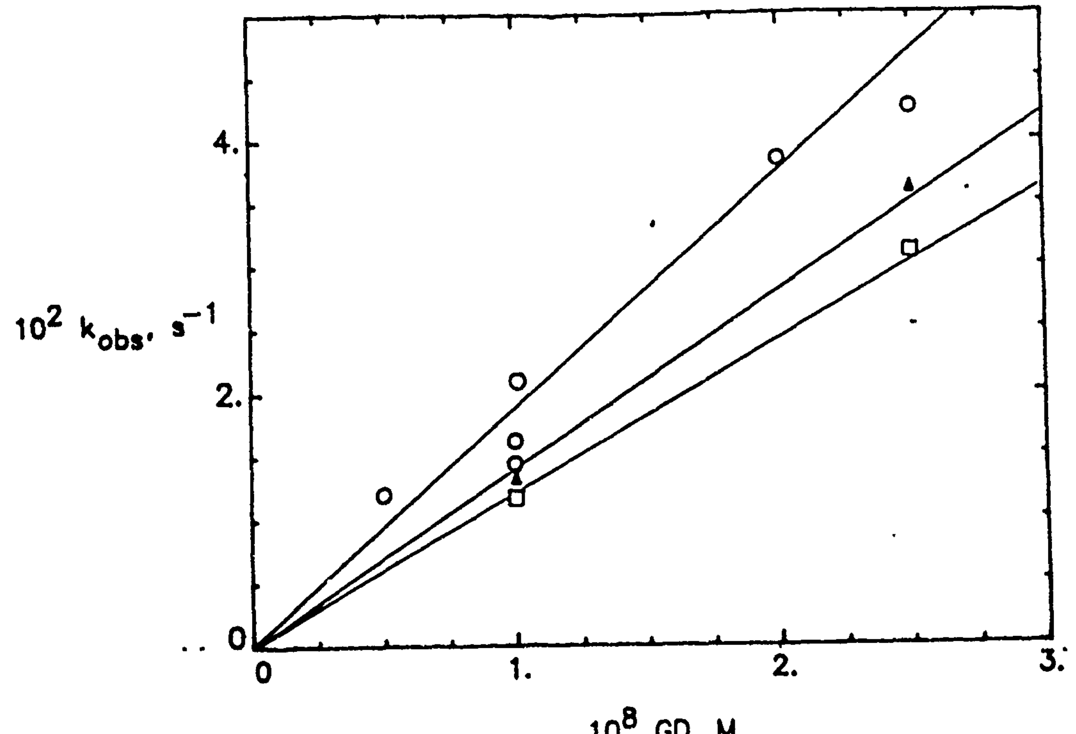
MPN

$CH_3CH_2CH_2-P(=O)(OCH_3)-O-C_6H_4-NO_2$

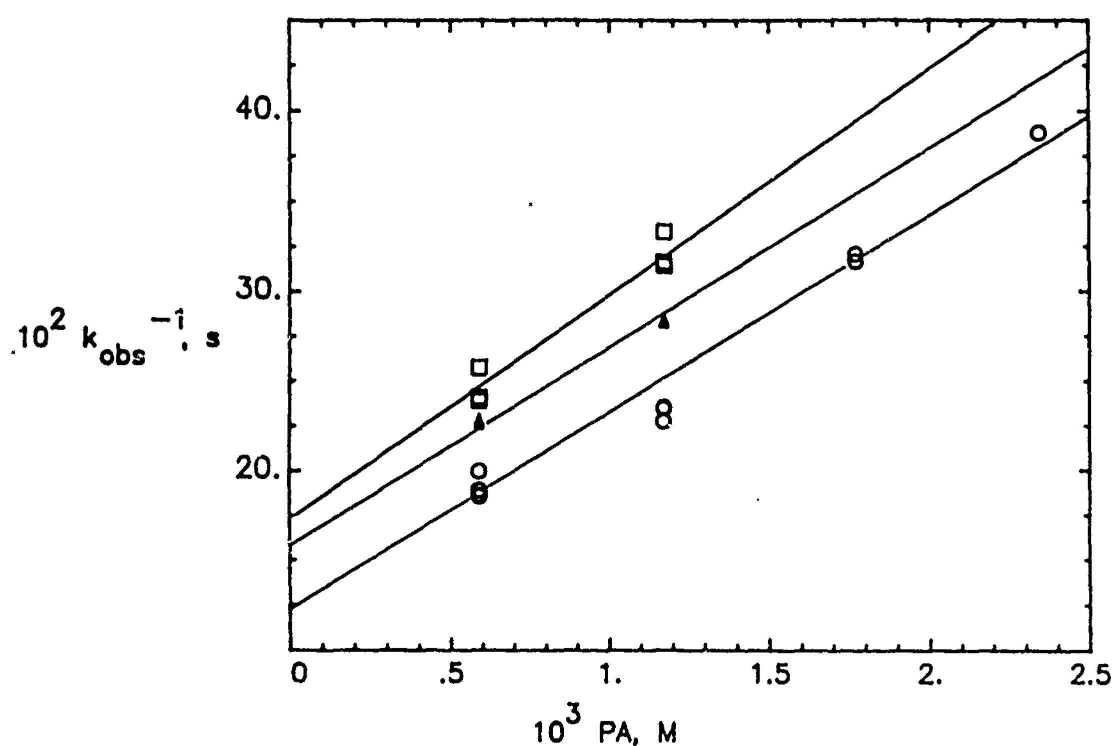
NPN

$CH_3CH_2CH_2-P(=O)(O-C_6H_4-NO_2)-O-C_6H_4-NO_2$

INHIBITORS

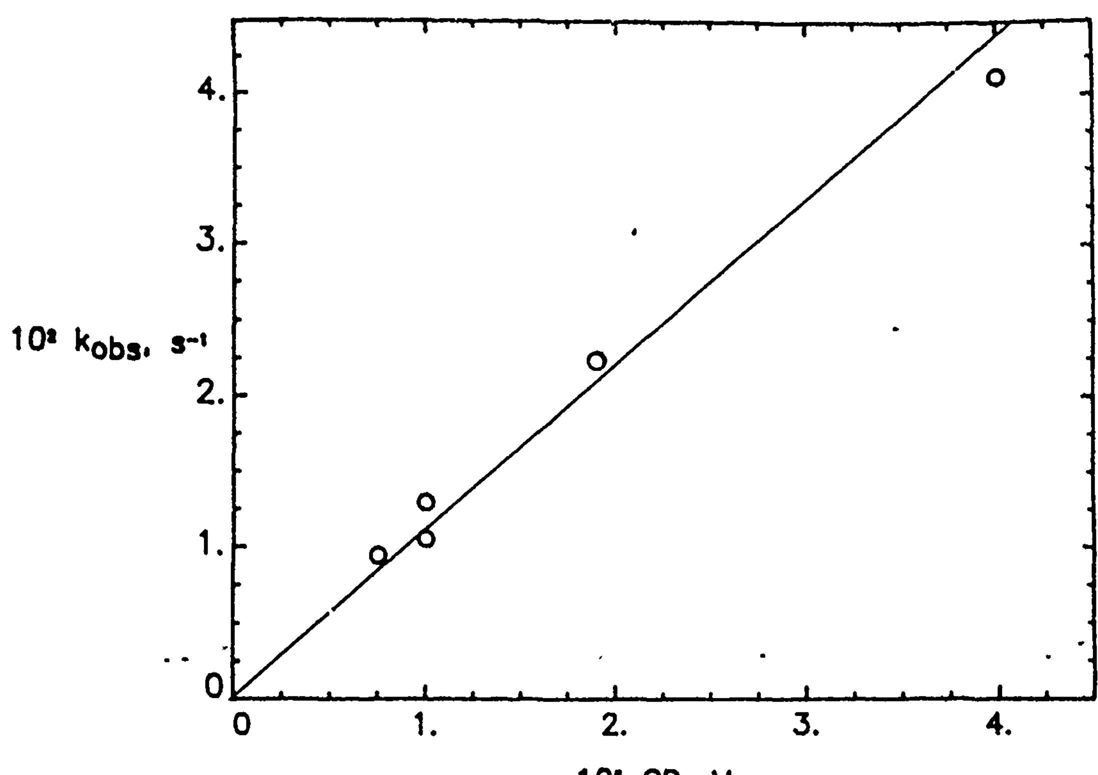


First order rate constants for the inhibition of AChE ee as a function of GD concentration at pH=7.62 and 25 °C, in the presence of 10^{-3} M phenyl acetate

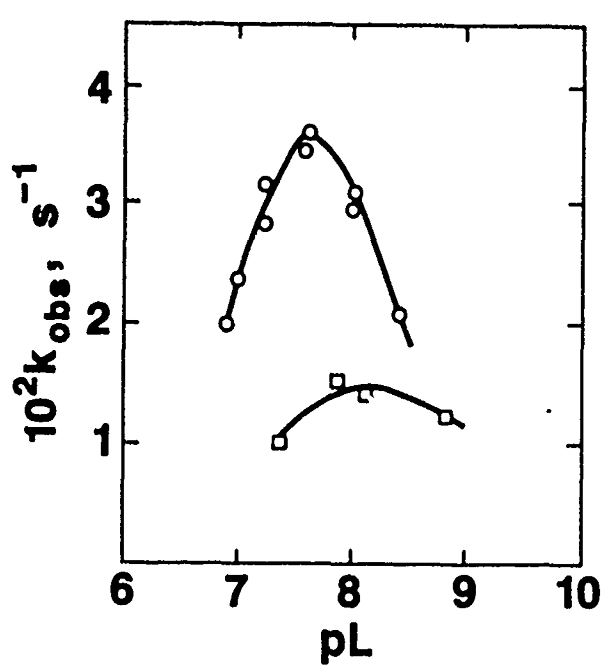


Dependence of the rate of inhibition of AChE ee by GD, on the concentration of phenyl acetate at pH=7.62 and 25 °C

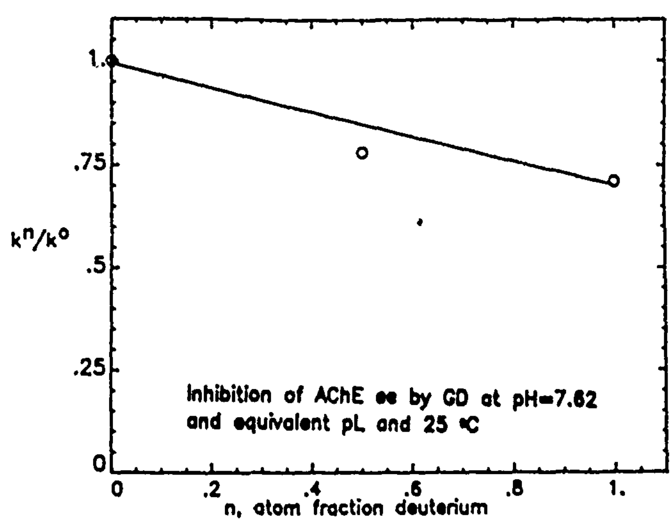
○= HOH, □= DOD, ▵= HOH : DOD = 1 : 1



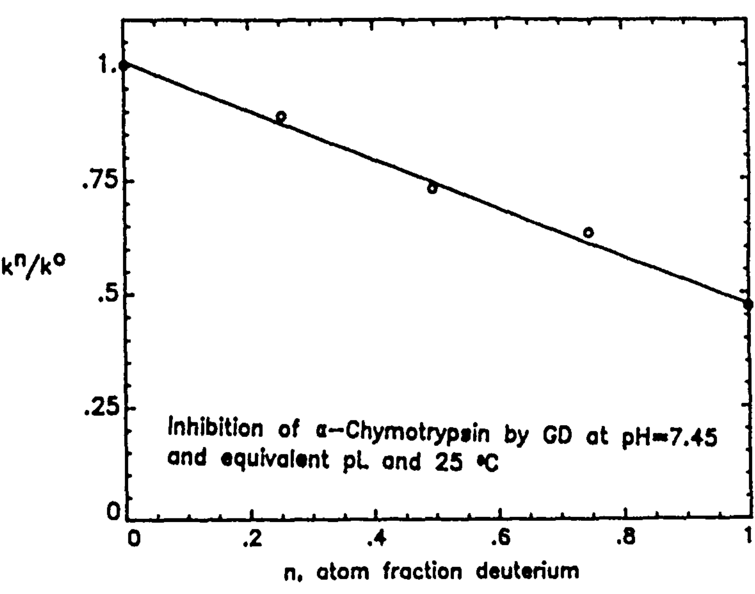
First order rate constants for the inhibition of α-Chymotrypsin as a function of GD concentration at pH=7.28 and 25 °C in the presence of 10^{-4} M substrate.

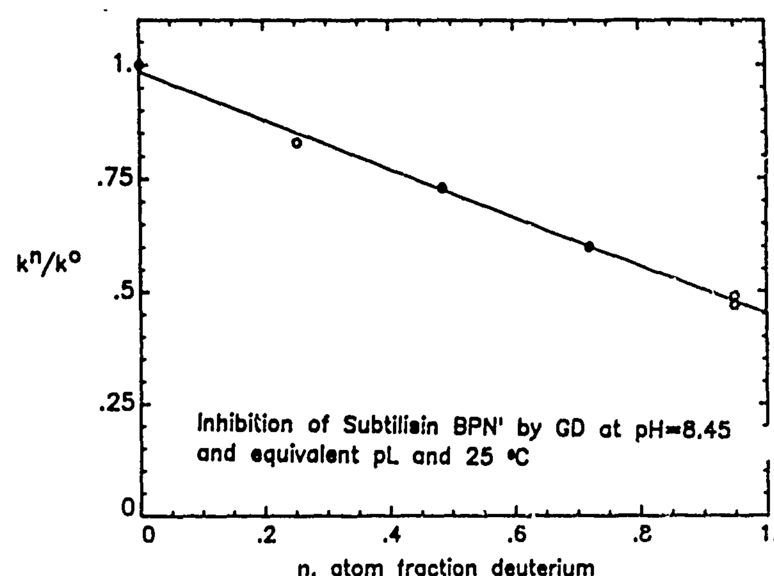


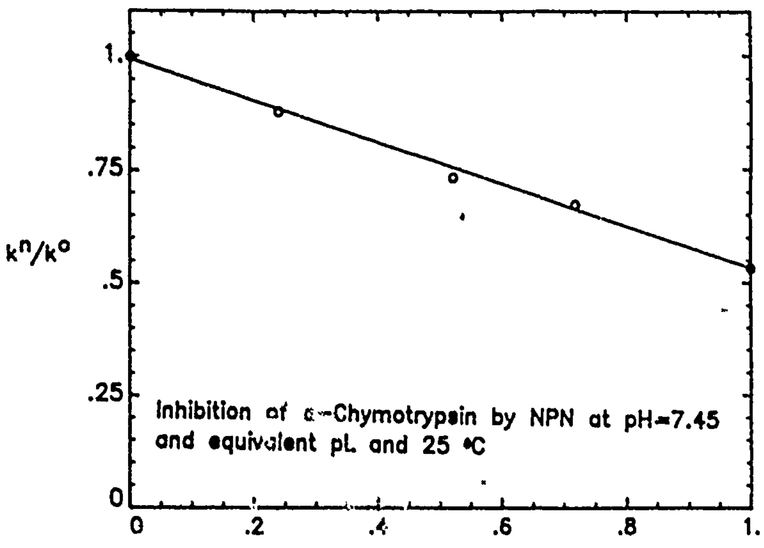
pL–rate profile for the inhibition of α–Chymothrypsin by NPN; ○HOH, □DOD

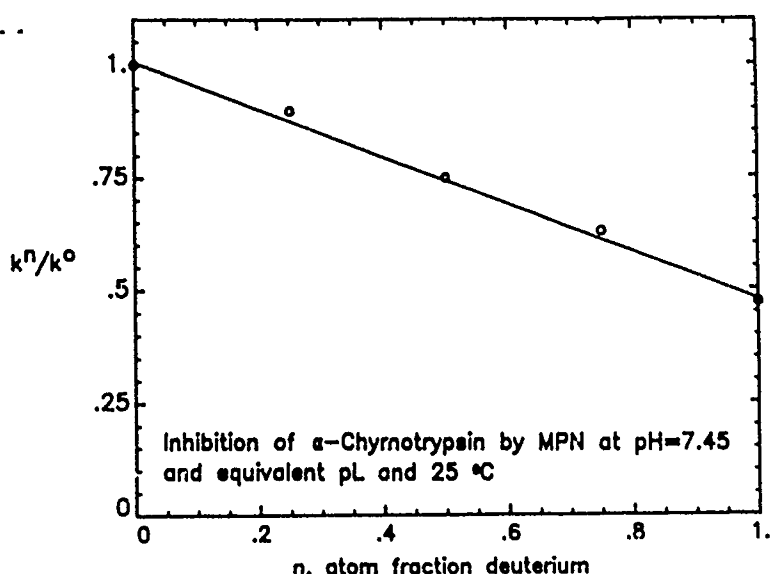


SIE = 1.41
Incursion of other steps (conformational change)
then generc: base catalysis in rate limiting process



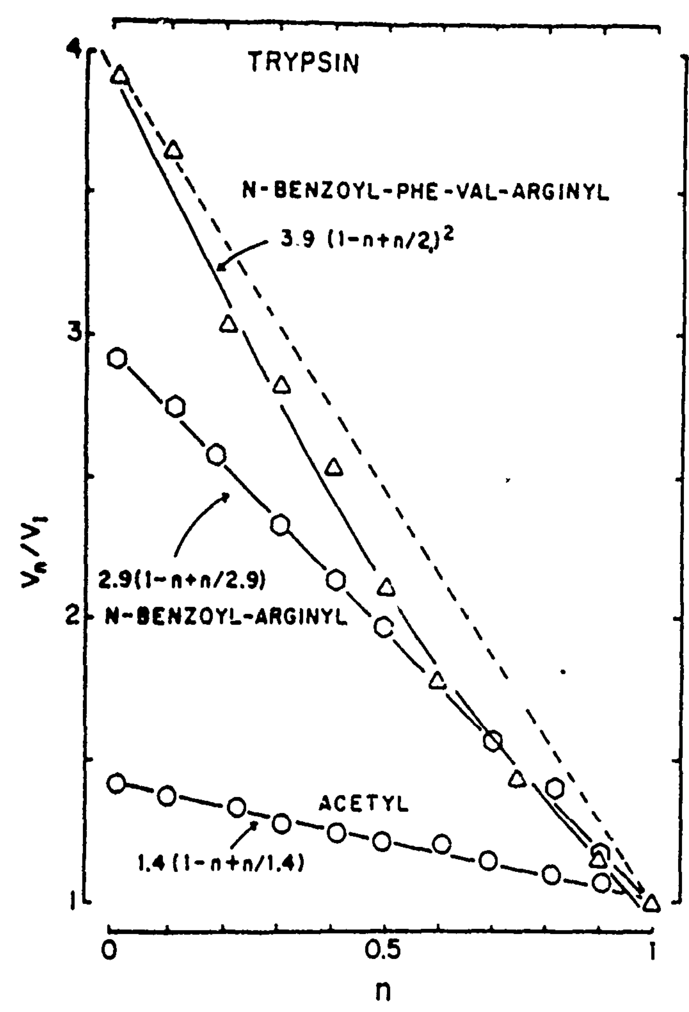




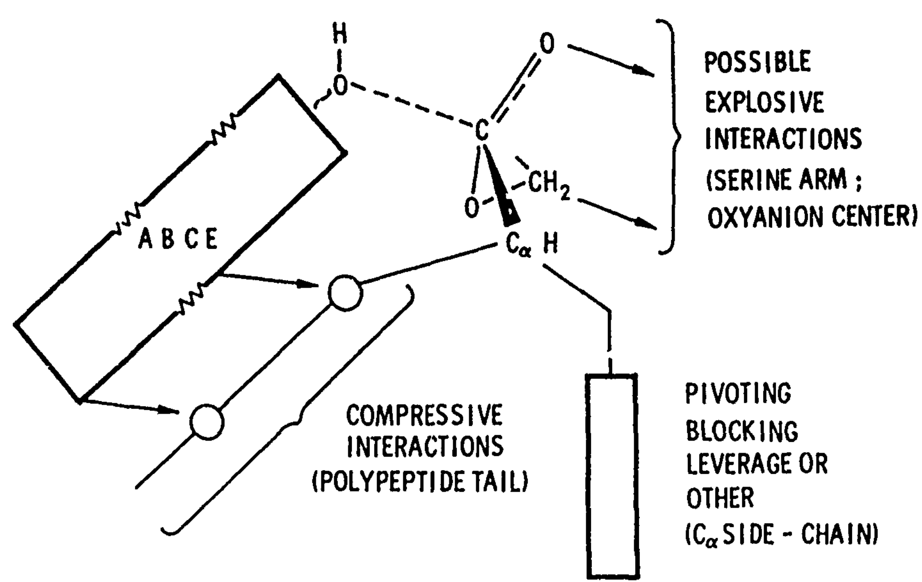


SIE ~ 1.7−2.1

General base catalysis in the rate determining step.



TRYPSIN
N-BENZOYL-PHE-VAL-ARGINYL
3.9 (1-n+n/2)²
2.9(1-n+n/2.9)
N-BENZOYL-ARGINYL
ACETYL
1.4 (1-n+n/1.4)
V_n/V_1
n



H
O
O
C
O
CH2
Cα H
A B C E
POSSIBLE
EXPLOSIVE
INTERACTIONS
(SERINE ARM ;
OXYANION CENTER)
COMPRESSIVE
INTERACTIONS
(POLYPEPTIDE TAIL)
PIVOTING
BLOCKING
LEVERAGE OR
OTHER
(Cα SIDE - CHAIN)

Bimolecular Rate Constants and SIEs for the Inhibition of Serine Hydrolases at 25 °C

Agent	k(HOH), M^{-1} s^{-1}	k(HOH)/k(DOD)
1. AChE ee; pH=7.70, 0.05 M Phos. B.		
GD	$1.67 \pm 0.20 \times 10^{6}$	1.41 ± 0.14
MPN	354 ± 26	1.43 ± 0.03
NPN	7.32 ± 0.01	1.3 ± 0.1
2. AChE RBC; pH=7.70, 0.05 M Phos. B.		
GD	$6.71 \pm 0.21 \times 10^{5}$	1.60 ± 0.17
MPN	922 ± 50	1.50 ± 0.10
3. α–Chymotrypsin; pH=7.45, 0.05 M Phos. B.		
GD	959 ± 60	1.94 ± 0.13
MPN	0.87 ± 0.02	2.12 ± 0.05
NPN	2260 ± 59	1.92 ± 0.06
4. Trypsin; pH=8.48, 0.05 M Tris B.		
MPN	n.r.	
NPN	263.7 ± 7.9	1.83 ± 0.06
5. Subtilisin BPN'; pH=8.45, 0.05 M Tris B.		
GD	20.5 ± 0.42	2.03 ± 0.03
MPN	2.57 ± 0.01	2.06 ± 0.05
NPN	1330 ± 49	1.85 ± 0.06
6. Porcine Elastase; pH=8.45, 0.05 M Tris B.		
MPN	3.19 ± 0.20	2.01 ± 0.15
NPN	1840 ± 50	1.65 ± 0.05

CHARACTERISTICS OF INACTIVATION

Serine proteases: *partial recruitment* of catalytic power of enzymes; major rate limiting event is serine attack assisted by protonic catalysis at a single site.

AChE-s: more complex mechanism; *partial recruitment* of catalytic assistance from a single site or possible multi proton participation accompanying a rate limiting conformational change.

Comparison with substrates:

1. Serine proteases; similarity to nonspecific amide substrates.
2. AChE-s; similarity to acylation by phenyl acetate in the magnitude of SIE and for GD, in the rate of reaction.

A PHYSIOLOGICALLY BASED TOXICOKINETIC MODEL OF SOMAN POISONING IN RATS

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ABSTRACT

The lack of a quantitative analytical procedure capable of measuring in vivo tissue levels of soman in animals receiving one LD50 has prevented the development of a comprehensive model of soman pharmacokinetics. However, a toxic event resulting from the in vivo presence of soman (i.e. inhibition of cholinesterase) is readily measured and therefore the toxicokinetics of soman can be described. A comprehensive toxicokinetic model of soman poisoning must incorporate the anatomical, physiological and biochemical parameters which influence soman toxicity. To develop this model organ volumes, blood flow rates and soman-detoxification enzymes were measured in brain, diaphragm, heart, lung, skeletal muscle, kidney, gut, liver, spleen and blood. The time-course of soman levels and cholinesterase inhibition in each tissue after administration of soman were then predicted by simultaneous solution of ten differential equations each representing the mass transport and detoxification of soman in a particular tissue. The toxicokinetic model was tested by comparing the predicted cholinesterase inhibition with the observed cholinesterase inhibition in rats receiving soman. This mathematical toxicokinetic model was capable of predicting the time-course of cholinesterase inhibition in all major tissues of the rat. Scale-up of this model for soman poisoning in other species including man appears possible if the necessary in vitro tissue measurements of detoxification enzymes and cholinesterase can be obtained.

PURPOSE

To develop a toxicokinetic model which can predict soman concentrations and cholinesterase inhibition in tissues of animals receiving 1 LD50.

METHODS

CHOLINESTERASE (ChE) was measured by a colorimetric procedure using acetylthiocholine as substrate. Tissues were homogenized in 1% Triton X-100 and ChE assays were performed on the resulting 10,000 xg supernatants. The relative amounts of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), which comprise the total ChE in each tissue, were estimated from the ChE activity in the presence and absence of 10^{-5}M iso-OMPA, a specific inhibitor of BChE.

CARBOXYESTERASE (CaE) was measured by a titrimetric procedure using tributyrin as substrate.

TOTAL TISSUE SITES for AChE, BChE and CaE were estimated by dividing total activity of each of these enzymes by their turnover rates of substrate per active site.

BLOOD FLOW was measured with radiolabelled microspheres.

BIMOLECULAR RATE CONSTANTS were determined in vitro for the reaction of soman with brain AChE, plasma BChE and plasma CaE.

SOLUTIONS OF DIFFERENTIAL EQUATIONS were obtained using DGEAR software (Hindmarsh, 1974) on a UNIVAC 1100. Predicted tissue concentrations of soman were calculated directly by DGEAR. Predicted ChE vs. time (t) were calculated from the recursive equation $E_t = (E_0 - \sum_{2}^{n} E_{n-1})\, C_{n-1} K \Delta t$ where E_t = ChE concentration at time t, Δt is the reaction time interval, $n = t/\Delta t$, K = bimolecular rate constant for reaction of soman with ChE, and C = soman concentration.

MODEL TESTING was accomplished by comparing tissue ChE levels predicted by the toxicokinetic model with ChE levels measured in 250 gm male rats receiving 100 μg/kg soman, im.

ASSUMPTIONS IN MODEL

1) Soman undergoes only 3 reactions – irreversible reaction with ChE, irreversible reaction with CaE and hydrolysis. The toxic P(–) isomers react only with ChE and CaE. The nontoxic P(+) isomers react only with hyrolytic enzymes.

2) Soman injected intramuscularly is completely absorbed by a first order process into the plasma.

3) Each tissue or organ is a well-mixed, homogeneous compartment.

4) Each compartment is interconnected only through the circulatory system.

5) Soman does not bind reversibly to tissues.

6) $$\begin{bmatrix}\text{Rate of change}\\ \text{of soman in}\\ \text{compartment}\end{bmatrix} = \begin{bmatrix}\text{Rate of}\\ \text{soman}\\ \text{in flow}\end{bmatrix} - \begin{bmatrix}\text{Rate of}\\ \text{soman}\\ \text{out flow}\end{bmatrix} - \begin{bmatrix}\text{Rate of}\\ \text{soman}\\ \text{metabolism}\end{bmatrix}$$

DIFFERENTIAL EQUATIONS USED IN MODEL

For each tissue (i) a differential equation of the following form was written:

$$V_i \frac{dC_i}{dt} = Q_i \left(C_p - \frac{C_i}{R_i}\right) - K_i V_i C_i$$

Where:

V_i = Volume of tissue (ml)

Q_i = Blood flow in tissue (ml/min)

R_i = Partition coefficient (tissue/plasma)

C_i = Soman concentration (M) in plasma (p) or tissue (i)

K_i = Rate constant (min^{-1}) for reaction with CaE

t = time

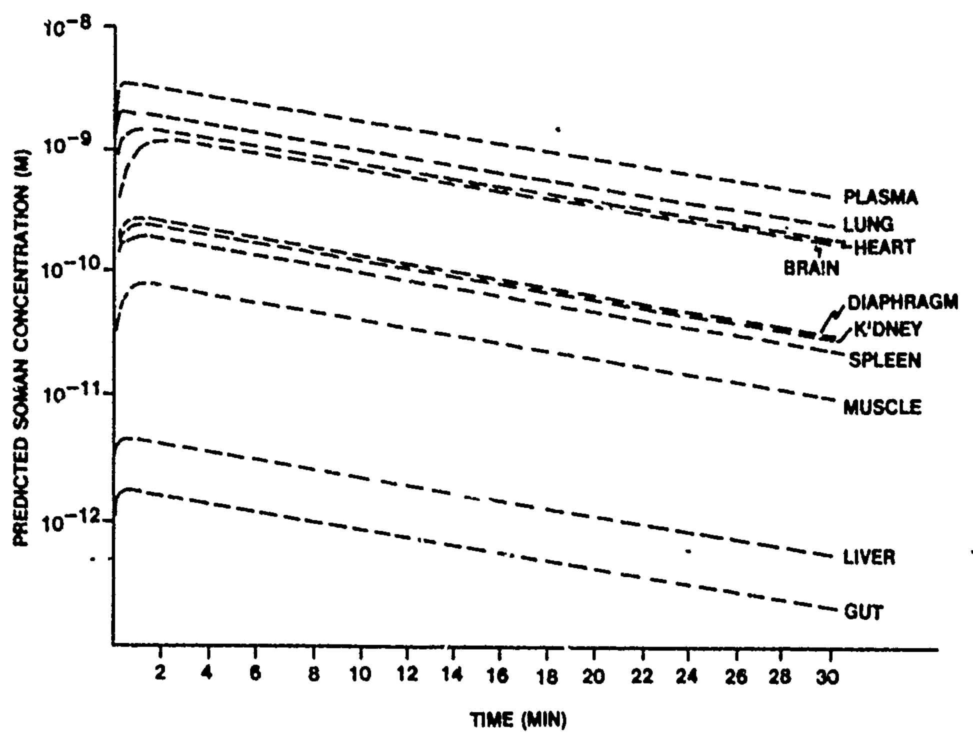
INPUT DATA FOR MODEL

	TISSUE* VOLUME (ml)	BLOOD FLOW (ml/min)	TOTAL AChE (nanomoles)	TOTAL BChE (nanomoles)	TOTAL CaE (nanomoles)
BRAIN	2.9	1.5	.112	.025	1.6
LUNG	1.5	45	.003	.024	19.5
SPLEEN	0.7	0.28	.004	.006	2.1
SKELETAL MUSCLE	127	11.3	.864	.521	285.8
DIAPHRAGM	0.7	0.25	.004	.005	1.9
INTESTINE	5.6	4.5	.021	.377	1200
KIDNEY	2.1	4.4	.001	.004	34.7
HEART	0.7	1.8	.016	.004	1.4
LIVER	10.0	5.8	.009	.055	480
PLASMA	9.8	45	.005	.026	41

*(Gerlowski et al, 1983)

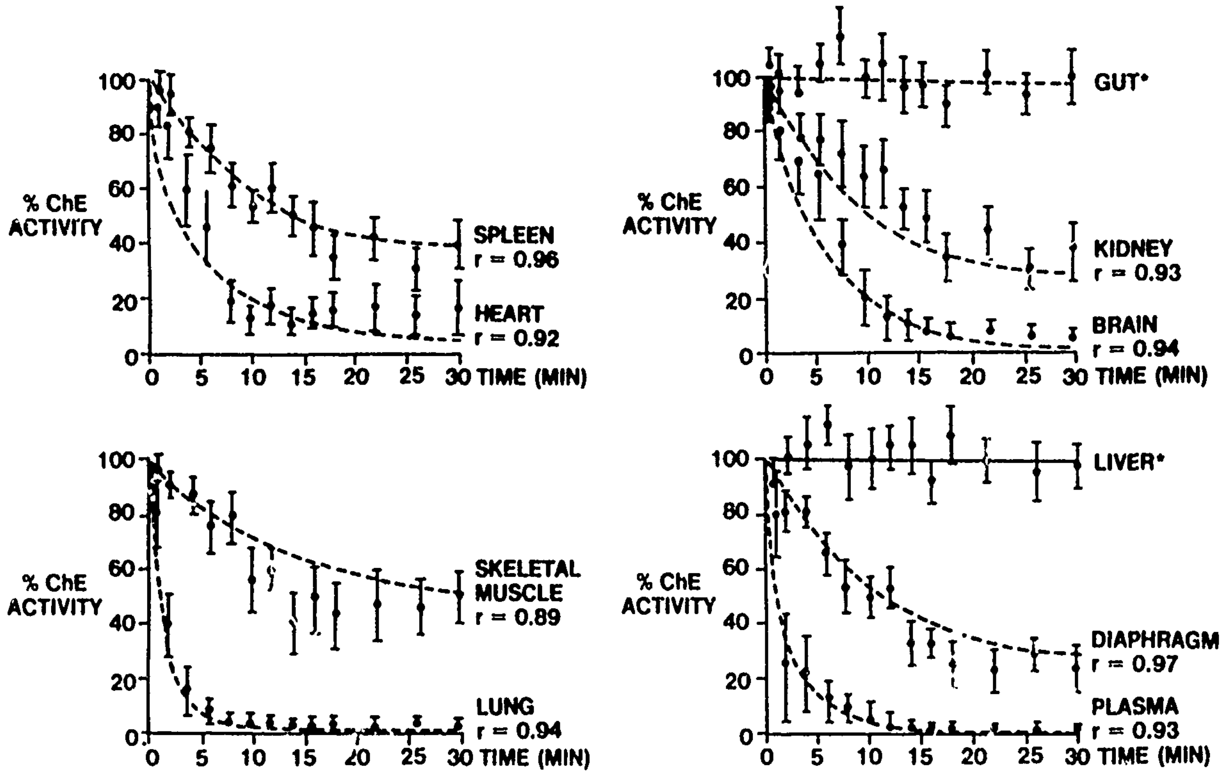
	BIMOLECULAR RATE CONSTANTS FOR REACTION WITH SOMAN (M^{-1} min^{-1})	ENZYME TURNOVER RATES (substrate/min/active site)
AChE	3.6×10^7	1.9×10^5 (WANG _et al_, 1982)
BChE	3.4×10^7	0.9×10^5 (LEE _et al_, 1971)
CaE	1.2×10^6	1.8×10^3 (IKEDA _et al_, 1977)

MODEL PREDICTIONS OF SOMAN CONCENTRATIONS IN RATS AFTER 100 µg/kg, DOSE IM.



MODEL TESTING

COMPARISON OF PREDICTED AND OBSERVED ChE



* r NOT CALCULATED SINCE SLOPE ≃ 0

--- ChE PREDICTED BY TOXICOKINETIC MODEL

Ⅰ Ⅰ ChE MEASURED IN RAT AFTER [illegible] µg/kg SOMAN IM (MEAN + S.D.)

CONCLUSIONS

1) Large intertissue differences exist in the time course of ChE inhibition of rats receiving soman. These intertissue differences are not the result of differences in the relative amounts of AChE and BChE in each tissue since bimolecular rate constants for reaction of soman with AChE and BChE are equivalent.

2) The time course of soman concentrations in tissues and the resulting ChE inhibition can be predicted by a conservative mathematical model composed of differential equations describing the mass transport (i.e. blood flow) and metabolism (i.e. reaction with CaE) of soman. The ChE values predicted by this mathematical model agree with experimentally determined ChE values with correlations from 0.89 to 0.94.

3) Scale-up of this model for soman toxicokinetics in other species is possible if the necessary blood flow and in vitro tissue measurements of detoxification enzymes and ChE can be obtained.

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INVESTIGATIONS OF CYANIDE ANTIDOTE ACTION AT THE CELLULAR LEVEL

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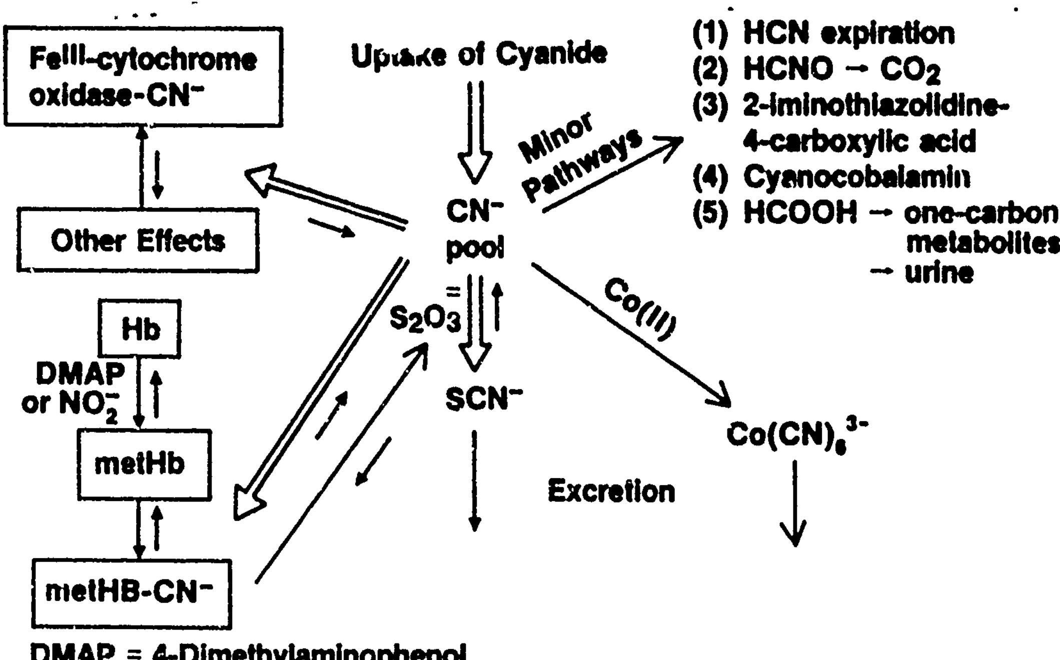
INTRODUCTION

In vitro approaches can be used to test mechanistic hypotheses for toxin and antidote action. In the case of cyanide toxicity, several detoxication pathways are available, depending on the antidote (1-5), as shown in Figure 1.

Figure . CYANIDE DETOXICATION PATHWAYS



DMAP = 4-Dimethylaminophenol

In vitro studies of antidote action generally have been limited to evaluating reversal of cytochrome oxidase inhibition and induction of methemoglobin by methemoglobin formers. Studies of antidote efficacy and mechanisms of antagonism to cyanide action at the cellular level do not appear to have been reported. Now that methods are available for the isolation and maintenance in culture of many cell types from various species, such studies are desirable. We report here on experience and findings with a novel coincubation system comprising hepatocytes, which possess detoxication enzymes for cyanide, and erythrocytes developed for these kinds of applications.

METHODS

REACTION FLASK

Air-tight reaction flask

Inlet stopper

Culture dish containing:

- **Hepatocyte monolayer (1.5×10^{6} cells/dish)**
- **Waymouth medium + albumin (0.2%) + rat RBCs (1.6 g Hb/dL)**

BASIC PROTOCOL

1. Attach rat hepatocytes in culture dishes (2-3 hr)
2. Form monolayer (20 hr)
3. Change to fresh medium + RBCs ±KCN (1.0 mM)
4. Transfer to flasks and gas with air: CO_2 (95:5)
5. Incubate for x min at 37° C with gentle shaking
6. Aspirate medium and fix hepatocyte ATP with Releasing Agent
7. Measure cyanide metabolites or complexes in the supernatant

ATP: luciferin-luciferase; results expressed as μM/culture

Urea synthesis: measure *in situ* with NH_4Cl (10 mM) and ornithine (10 mM)

Lactate dehydrogenase (LDH): autoanalyzer

Cyanide / Thiocyanate: batch Lewatit separation + modified Konig reaction

Cyanmethemoglobin: KCN + $K_3 Fe (CN)_6$

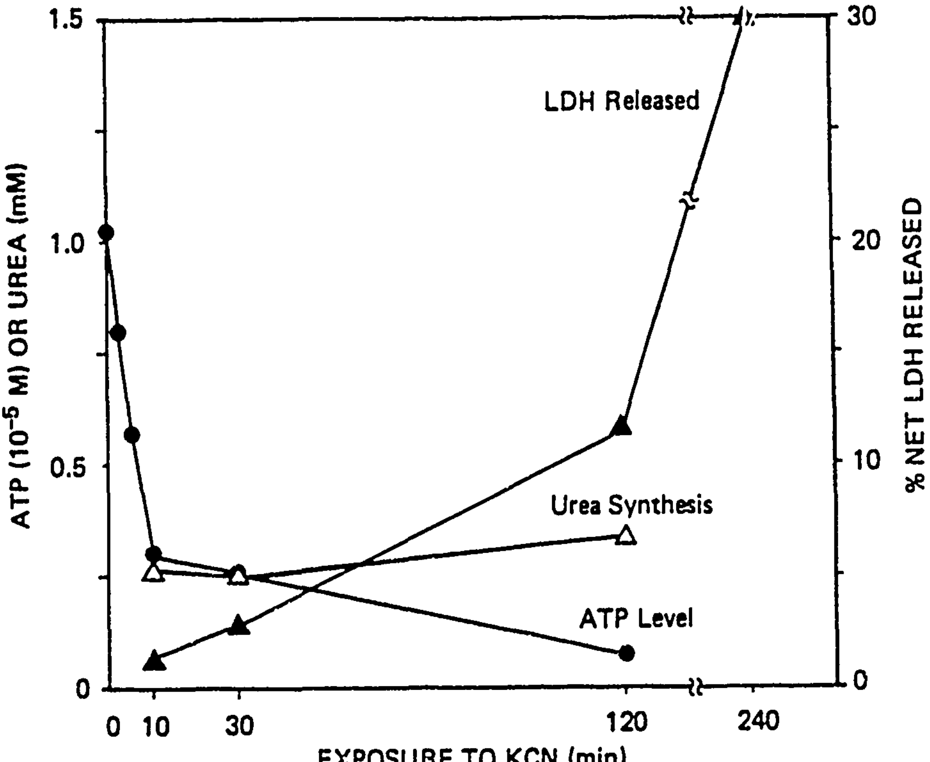


Figure 2 RESPONSE OF RAT HEPATOCYTES AFTER 24-HR CULTURE TO KCN (1.0 mM)

RESULTS

Figure 2 shows the effect of KCN on rat hepatocyte functional parameters and viability under the incubation conditions. ATP depression is an early, sensitive indicator of cyanide cytotoxicity. On removal of cyanide from the medium after short exposure (10 min), ATP recovers to control levels (data not shown); thus it is a suitable indicator for assessing antidote efficacy. (At 1.0 mM KCN, erythrocyte ATP, lactate/pyruvate, and LDH content were unaffected over a 120-min incubation period.)

Figure 3 presents data on the capability of classical antidotes to reverse cyanide-induced cytotoxicity. $NaNO_2$, $Na_2S_2O_3$, and their combination substantially reversed ATP depression at the indicated concentrations (but not 2-fold lower concentrations).

Table 1 summarizes results of an experiment to evaluate the need for erythrocytes for $NaNO_2$ efficacy. With hemoglobin at or greater than stoichiometric levels for complexation with cyanide in the flasks (1.6 g Hb/dL at 1.0 mM KCN; 0.4 g Hb/dL at 0.25 mM KCN), $NaNO_2$ substantially reversed hepatocyte ATP depression; below stoichiometric levels, $NaNO_2$ was ineffective.

Figure 4 shows the influence of erythrocyte and albumin content in the medium on ATP recovery with $Na_2S_2O_3$. Increased levels of each result in increased recovery rates.

Figure 5 shows that the increased recovery rates are accompanied by increased rates of $CN^- \rightarrow SCN^-$ conversion.

Table 2 summarizes data on the disposition of CN^- with various antidotes in the coincubation system.

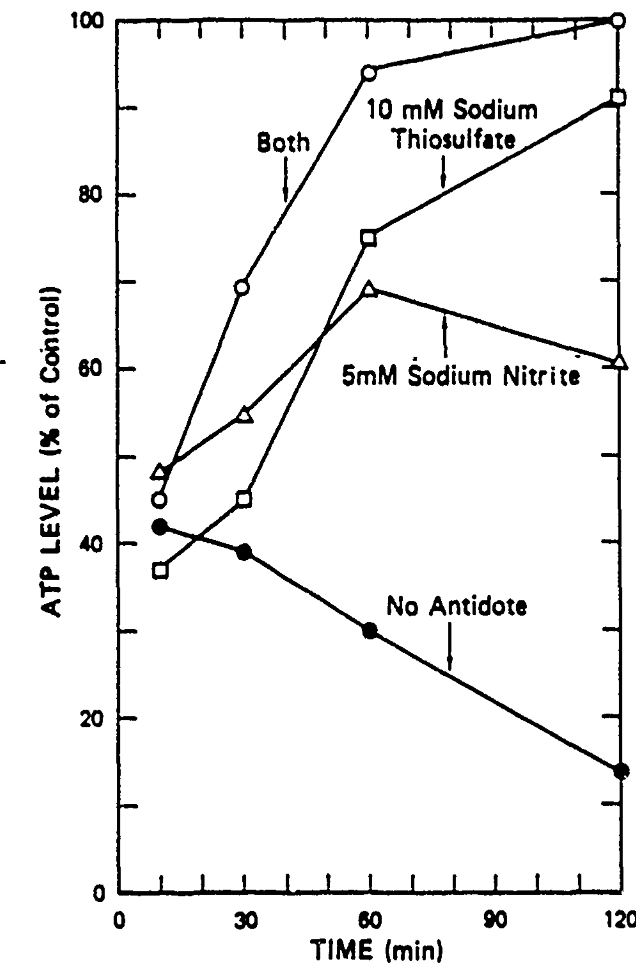


Figure 3 RESPONSE OF HEPATOCYTE ATP TO CYANIDE ANTIDOTES AFTER 10-MIN EXPOSURE TO KCN (1.0 mM) IN THE COINCUBATION SYSTEM

Table 1
DEPENDENCE OF EFFECTIVENESS OF $NaNO_2$ ON ERYTHROCYTE CONTENT IN THE CULTURE MEDIUM[a]

KCN (mM)	ATP Levels (μM) Erythrocyte content: 0	0.4[b]	0.8[b]	1.6[b]
0.0	6.1[c]	6.6	7.0	7.2
0.25	4.0	6.4	6.4	6.4
1.0	0.3	0.7	1.0	4.6

[a] $NaNO_2$ (5.0 mM) was added after a 10-min incubation with the indicated KCN concentration, and the reaction continued for 120 min more before hepatocyte ATP was assayed.

[b] Erythrocyte content corresponds to 0.4, 0.8, and 1.6 g Hb equivalents/dL.

[c] With no antidote, hepatocyte ATP levels were 5.7, 3.5, and 0.3 μM in flasks containing 0.0, 0.25, and 1.0 mM KCN, respectively.

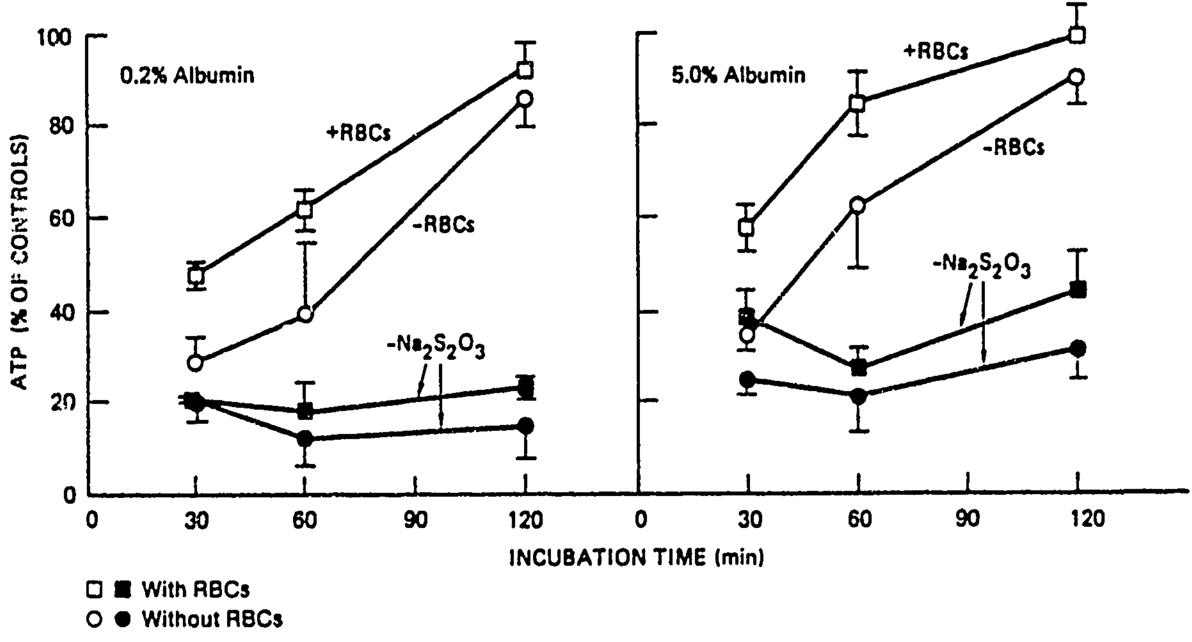


Figure 4 INFLUENCE OF ERYTHROCYTE AND ALBUMIN IN THE MEDIUM ON RATE OF REVERSAL OF CYANIDE-INDUCED HEPATOCYTE ATP DEPRESSION BY SODIUM THIOSULFATE (10 mM)

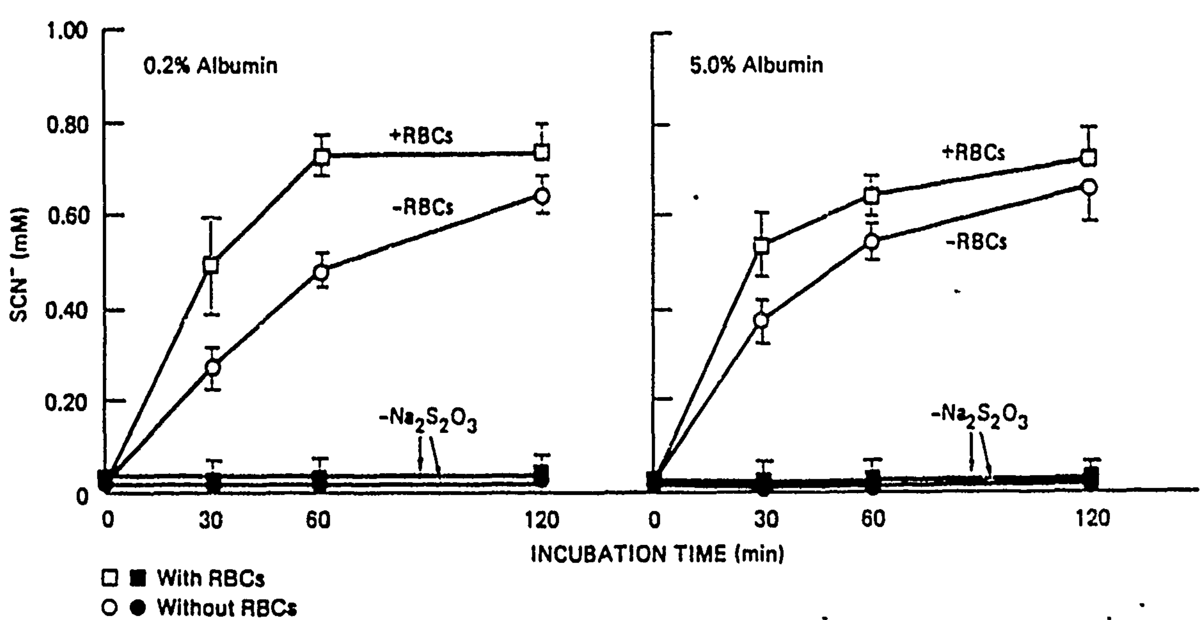


Figure 5 THIOCYANATE FORMATION FROM CYANIDE AND SODIUM THIOSULFATE (10 mM) IN RELATION TO ERYTHROCYTE AND ALBUMIN CONTENT IN THE MEDIUM

Table 2
CYANIDE METABOLITES FROM DIFFERENT ANTIDOTES IN THE COINCUBATION SYSTEM

Antidote (mM)	KCN[a] (mM)	ATP[b] (μM)	Cyanmet-hemoglobin[b] (%)	SCN^- (μM)
None	0	9.5 ± 3.4	0	--[c]
None	1.0	2.8 ± 1.5	0	5.8
$CoCl_2$ (0.25)	1.0	9.5 ± 2.5	4 ± 7	10.2
DMAP (0.25)	1.0	8.3 ± 2.0	83 ± 11	2.5
$NaNO_2$ (5.0) + $Na_2S_2O_3$ (10.0)	1.0	8.2 ± 2.5	84 ± 28	380
$Na_2S_2O_3$ (10.0)	1.0	6.4 ± 2.6	3 ± 5	--[c]
$NaNO_2$ (5.0)	1.0	5.2 ± 1.8	85 ± 7	7.6

[a]KCN added 10 min before antidote and incubation continued 60 min more.
[b]Mean ± SD for four experiments.
[c]Not determined.

DISCUSSION

Sodium nitrite and 4-dimethylaminophenol. These antidotes induced methemoglobin formation and, with cyanide present, cyanmethemoglobin formation with substantial recovery in hepatocyte ATP toward control cell levels after a 10-min incubation with KCN. Without erythrocyte hemoglobin, no recovery was observed. The classical methemoglobinemia hypothesis therefore can explain the action of these antidotes in the coincubation system in reversing cyanide-induced cytotoxicity in target cells. The relative potencies of the antidotes *in vitro* (Table 2) are similar to their relative capabilities for inducing methemoglobin and protecting against cyanide toxicity in various species *in vivo* (4, 6, 7).

Cobaltous chloride. This antidote was effective in the coincubation system without formation of significant methemoglobin or thiocyanate. It is inferred that its effectiveness depends on direct complexation with cyanide ion in the medium, as has been proposed (8).

Sodium thiosulfate. This antidote was effective in the coincubation system, but erythrocytes and albumin enhanced the recovery rate. Methemoglobin was not formed, but the rate of SCN^- formation was increased and this increase appears to underlie reversal of cyanide toxicity. The contribution to recovery occurred earlier with erythrocytes than with albumin, and it is proposed that sulfurtransferase activity in erythrocytes may also contribute to thiosulfate antidote action.

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ASSESSMENT OF SOMAN ANTIDOTAL EFFICACY OF ATR/2-PAM: THE USE OF RESPONSE SURFACE MODELLING (RSM)

R.A. Carchman, W.H. Carter, Jr., and D.E. Jones

ABSTRACT

The dose reponse surface associated with the treatment of SOMAN exposure with 2-PAM and ATR in combination was estimated using the logistic model for various levels (30 ug/kg, 42.4 ug/kg, 60.0 ug/kg, 84.6 ug/kg) of SOMAN. The method of maximum likelihood was used to estimate the model parameters. It was shown that the observed data and model predictions were in agreement. As a result, the estimated relationship was used to describe particular aspects of the dose response relationship such as regions in the treatment space associated with constant probability of survival, the relative importance of the treatment agents with changing levels of SOMAN, and the dosage level of each drug associated with maximum probability of survival for the varying levels of SOMAN exposure. Optimum treatment combinations of ATR and 2-PAM were determined with their 95% confidence regions as a function of the various SOMAN exposures. In addition, estimates of the optimum treatments within these regions were also calculated and compared with the different SOMAN exposures. RSM provides a powerful statistical modeling procedure which is not geometricly restricted by the number of test variables and, when used in conjunction with a variety of experimental designs (e.g., fractional factorial), can provide optimal therapeutic modalities subject to constraints (e.g., behavioral toxicity).

This work supported in part by the US Army Medical Research and Development Commnd under Contract DAAD05-84-M-M816.

METHODS

Animals: Mixed sex, 300-400 g Dimean Hartley albino guinea pigs from Charles River Breeding Laboratories were used in all experiments. Containment and test facilities were maintained at constant temperature (71 ± 3°C), humidity (55 ± 7%) and lighting (12 hour light/dark cycle).

Nerve Agent: Soman (GD; pinacolyl methylphosphorofluoridate) was prepared by the Chemical Research and Development Center, Aberdeen Proving Ground, MD. Purity was determined (by nuclear magnetic resonance) to exceed 95% for each Soman lot. Soman challenge doses were prepared in sterile isotonic saline and injected s.c. in the dorsal cervical area at a dose volume of 1.0 ml/kg.

Therapy Compounds: Atropine sulfate (ATR) was purchased from C.H. Boehringer and Sons, Ingelheim, West Germany (Lot No. 352). Pralidoxime chloride (2-PAM) was purchased from Ayerst Laboratories, New York, NY (Lot No. Z-799). ATR and 2-PAM concentrations (both single and combination therapies) were prepared in distilled, deionized water and injected i.m. (rear limb muscle mass) at a dose volume of 1.0 ml/kg, given 1.0 minutes post-Soman challenge. Combination therapy injections of 2-PAM and ATR were admixed and administered as a single therapy injection.

Preparation of Test Compounds: All test compound concentrations (both nerve agent and therapy) were prepared by dilution from a single master stock concentration of the appropriate compound. This procedure insured that all agent-challenge and therapy doses were accurate to the same level of significance.

Soman LD50 Determination: The Soman lethality responses for both untreated (control) and treated (2-PAM and/or ATR) guinea pigs were assessed from 24 hour mortality results, based on five Soman challenge doses administered at equally spaced logarithmic (log) intervals to six guinea pigs per challenge dose. Lethality data were assessed by probit analysis (1).

Optimal ATR/2-PAM Therapy Study: Soman/antidote efficacy was assessed for singular and combination therapy doses of ATR and/or 2-PAM. ATR doses ranging from 0.00 to 256 mg/kg were combined with 2-PAM doses ranging from 0.00 to 200 mg/kg and administered (as described above) to guinea pigs challenged with four different Soman challenge doses. This study was conducted in six equal segments, one segment per day, with each portion individually randomized with respect to administration of both Soman challenge and ATR/2-PAM therapy doses.

Statistical Analysis: As a result of the nature of the agents used, it would be expected that the proportion of animals surviving would increase to a point where treatment toxicity would exceed the therapeutic effect, and then decrease as treatment levels increased beyond that point. As levels of GD increased, it would be expected that this proportion would continue to decrease. The logistic model, quadratic in its argument, has been used successfully to describe such relationships (2). The quadratic term for GD exposure, X_3^2, was not included in the model because the effect of GD is such that the proportion surviving will continue to decrease with increasing levels of GD. Hence, the dose-response model becomes

$$p = [1 + \exp-(\beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_{11}X_1^2+\beta_{22}X_2^2+\beta_{12}X_1X_2+\beta_{13}X_1X_3+\beta_{23}X_2X_3+\beta_{123}X_1X_2X_3]^{-1}$$

where p is the proportion of surviving animals;
X_1 is the dose of 2-PAM in mg/kg;
X_2 is the dose of ATR in mg/kg;
X_3 is the dose of GD in μg/kg;
β_0 is an unknown parameter associated with the probability of untreated animals surviving;
β_1 is an unknown parameter associated with the therapeutic effect of 2-PAM;
β_2 the therapeutic effect of ATR;
β_3 the effect of GD;
β_{12} the interaction of 2-PAM and ATR;
β_{13} 2-PAM and GD;
β_{23} ATR and GD;
β_{123} 2-PAM, ATR, GD;
β_{11} and β_{22} are unknown parameters associated with the toxic effects of ; 2-PAM and ATR, respectively.

The model parameters were estimated from the experimental data by the method of maximum likelihood. The p value for the likelihood ratio test for the significance of the model is < 0.0001. The parameter estimates are given in Table 1. It should be noted that, with this model, positive coefficients are associated with agents or effects that tend to increase the probability of survival, while negative coefficients are associated with agents or effects that tend to decrease the probability of survival.

Estimated optimal treatment levels were obtained from the fitted dose-response model by using the direct optimization method of Nelder and Mead (3).

Table 1

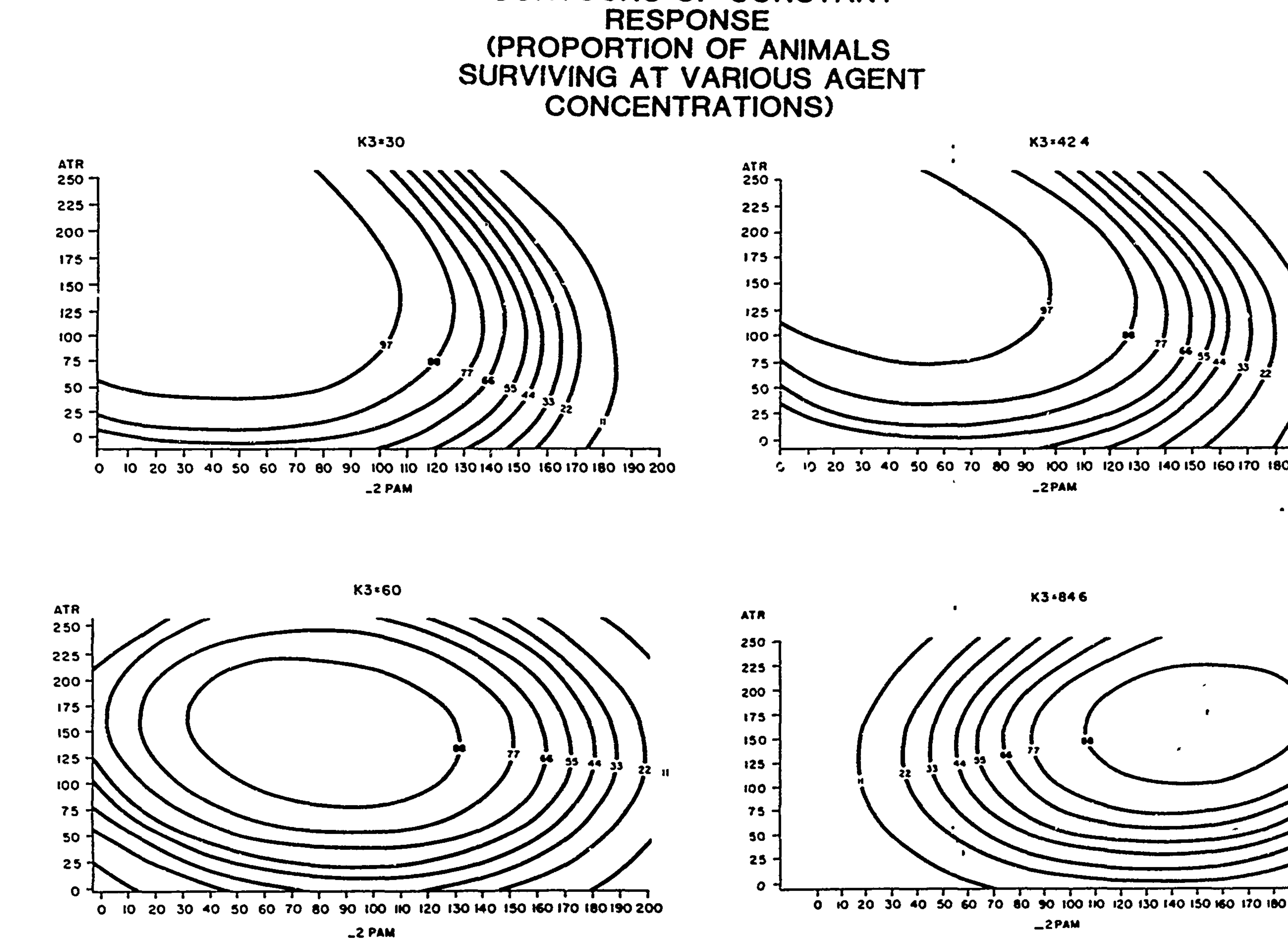
Estimation of Model Parameters

Parameter	Estimate	Standard Error of Estimate
β_0 (intercept)	3.0486	0.6084
β_1 (2-PAM)	0.0179	0.0097
β_2 (ATR)	0.0773	0.0114
β_3 (GD)	-0.1095	0.0137
β_{11}	-0.0003	0.000032
β_{22}	-0.0001	0.000020
β_{12}	-0.0004	0.000122
β_{13}	0.0006	0.000170
β_{23}	-0.0005	0.000180
β_{123}	0.000006	0.0000021

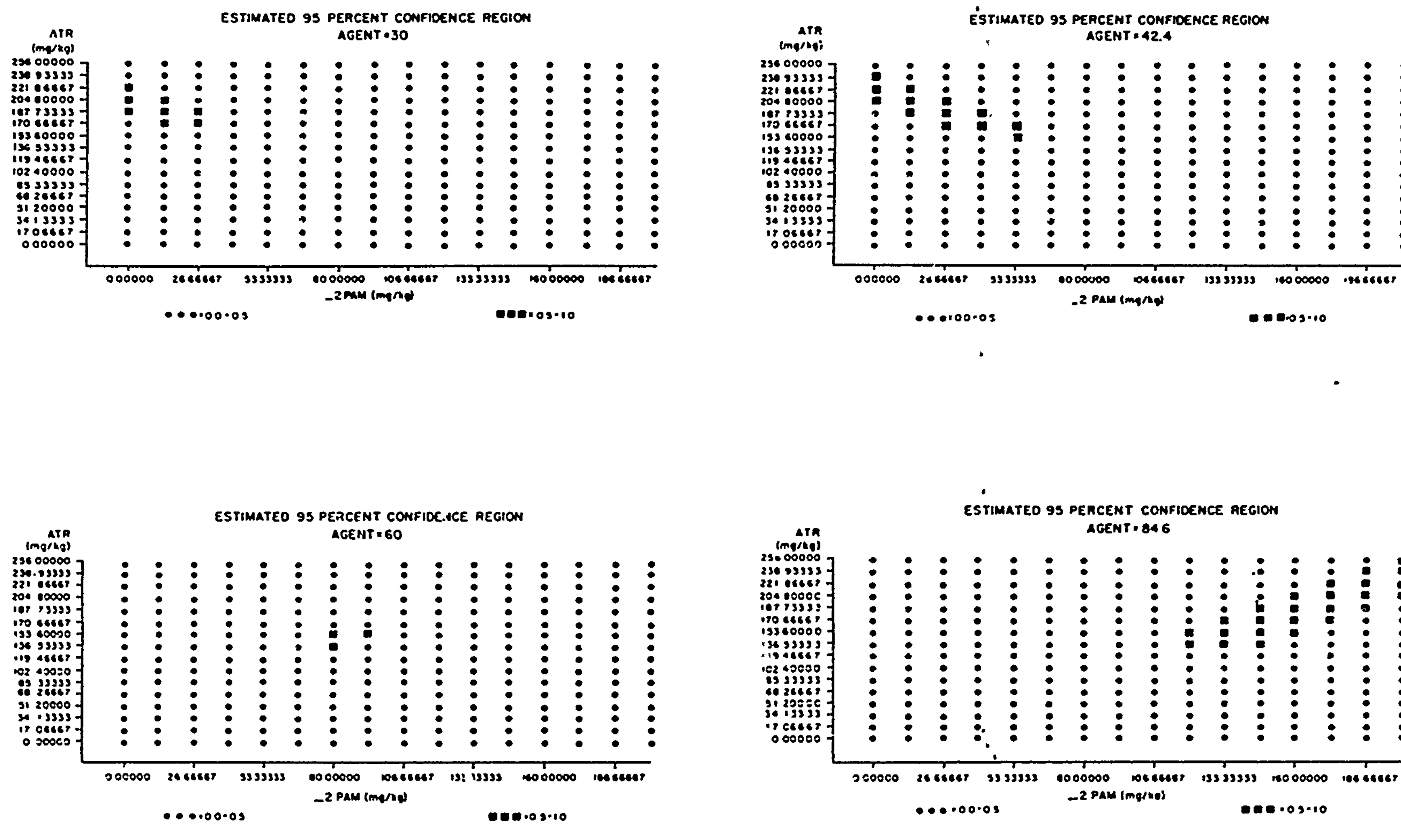
Table 2

Estimation of Optimal 2-PAM/ATR Combinations as a Function of GD Exposure

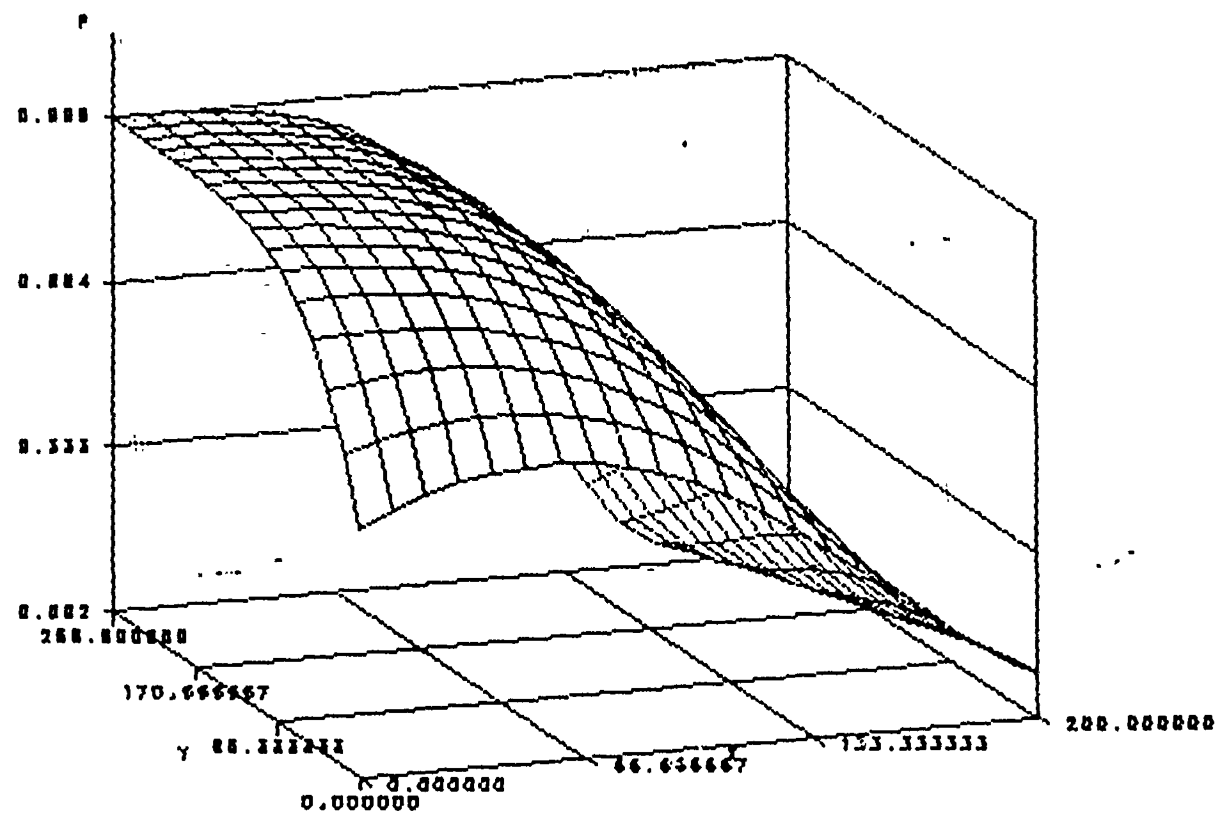
GD (μg/kg)	2-PAM (mg/kg)	ATR (mg/kg)
30	0.000006	217.4
42.4	29.4	179.2
60	83.1	148.5
84.6	150	168


CONTOURS OF CONSTANT
RESPONSE
(PROPORTION OF ANIMALS
SURVIVING AT VARIOUS AGENT
CONCENTRATIONS)
K3=30
K3=42.4
K3=60
K3=84.6
ATR
250
225
200
175
150
125
100
75
50
25
0
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200
-2 PAM
97
88
77
66
55
44
33
22
11

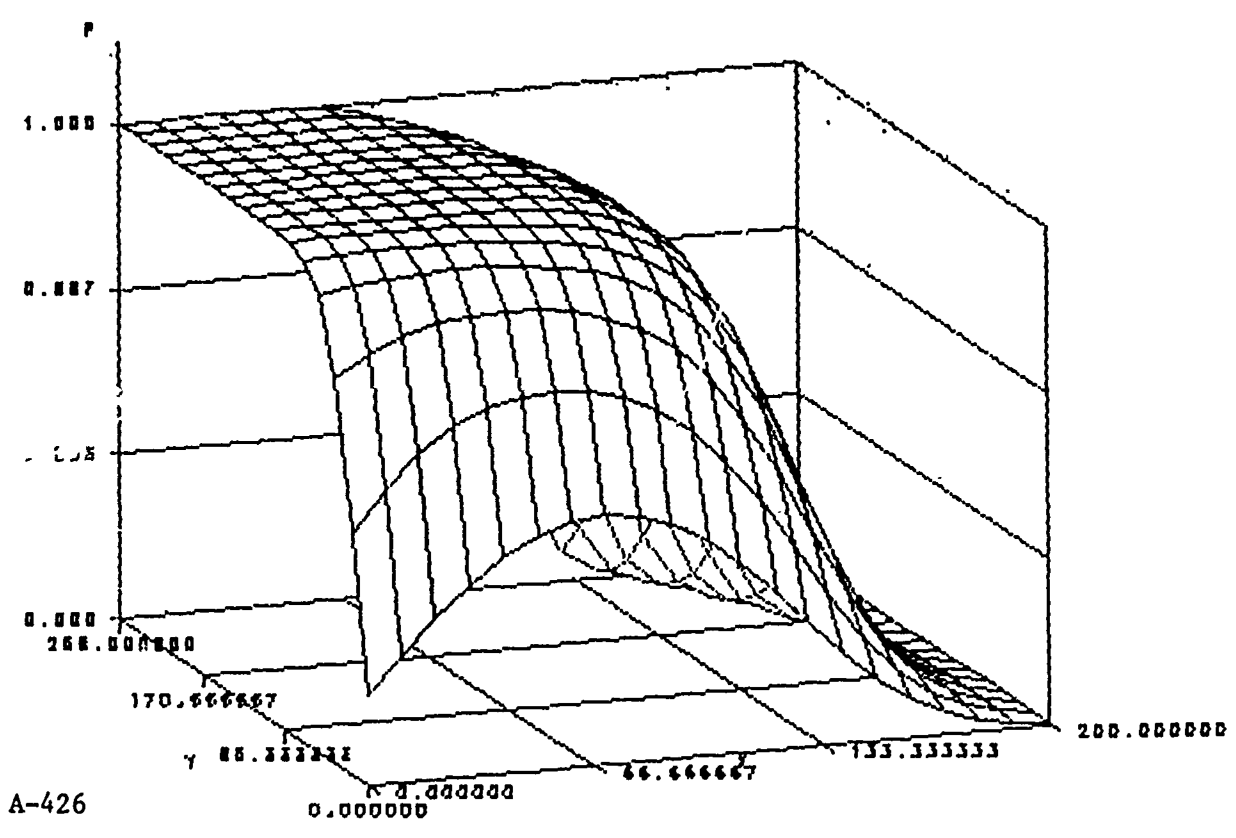
OPTIMUM TREATMENT REGION FOR −2PAM AND ATR AT VARIOUS AGENT CONCENTRATIONS



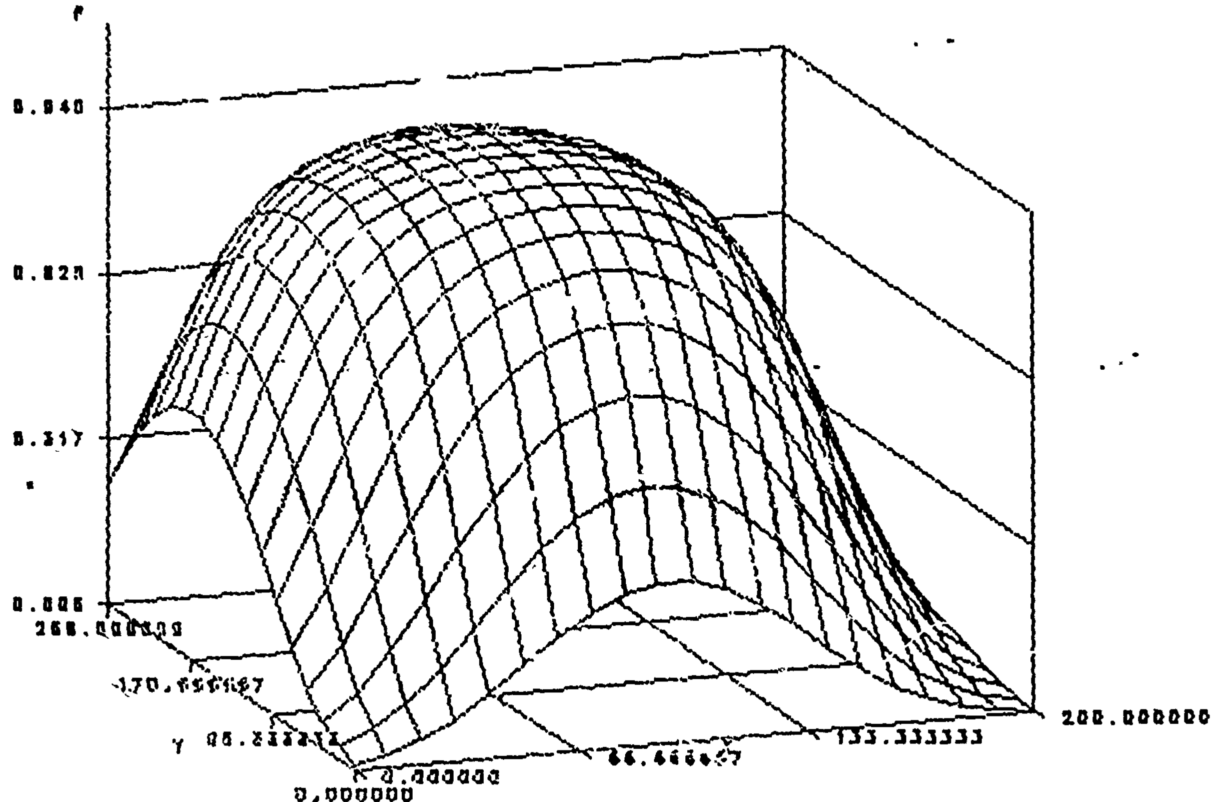
3D PLOT FOR 2PAM/ATR – GD LEVEL 30.0



3D PLOT FOR 2PAM/ATR – GD LEVEL 42.4



3D PLOT FOR 2PAM/ATR – GD LEVEL 60.0



CONCLUSION

The advantages associated with the use of RSM to model systems such as the one presented in this study include:

1) the ability to provide levels of statistical significance for the test parameters (e.g., incorporate the possible interactions between the treatment variables;

2) no theoretical limit to the number of agents in the combination being evaluated;

3) the use of experimental designs (e.g., central composite) to reduce the number of treatment combinations required for an experiment;

4) the ability to determine optimal levels of treatment for a given exposure or over an entire exposure range;

5) modeling any quantitative values (e.g., pretreatment or post-exposure intervals); and

6) generating hypotheses (e.g., as to the mechanism(s) which account for the behavior of 2-PAM and ATR over the range of GD exposure levels).

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USE OF A CELL CULTURE SYSTEM TO STUDY CHEMICAL TOXICITY

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ABSTRACT

Cells in culture offer a viable alternative to the use of animals as biological test systems. Cell culture systems also offer some additional advantages such as (a) defined and versatile experimental conditions, (b) homogeneous clonal cell population, (c) in vitro genetic, metabolic, morphologic, and functional manipulations etc. We are using the clonal neuroblastoma-glioma hybrid NG108-15 cells as a neuronal model to study the mechanisms of toxicity of organophosphorus compounds. The different neuronal properties of these cells such as differentiation, neurotransmitter metabolism, excitability, ion channel functions, membrane receptors, and synaptic interactions make this cell line an ideal experimental system to study mechanisms of chemical toxicity. Two separate aspects of our studies using this cell line are presented below.

1. Characterization of acetylcholinesterase (AChE) and its inhibition by organophosphorus compounds. Cholinesterase activity of NG108-15 cells is predominantly AChE. Specific AChE activity is increased several fold in NG108-15 cells differentiated by treatment with 1 mM dibutyryl cAMP compared to untreated control cells. Two molecular forms of AChE with sedimentation coefficients of approximately 5S and 10-11S, comparable to brain AChE, are present in NG108-15 cells. During development, the relative amount of the 10-11S AChE molecular form is increased compared to the 5S molecular form. The activity and molecular forms of AChE are regulated in NG108-15 cells, and these regulatory mechanisms may possibly be involved in neuronal development and differentiation. Based on similarities in types and properties of AChE in NG108-15 cells and brain, the NG108-15 cells may be used as a potential neuronal model system for studies on AChE.

We prepared separate pools of detergent solubilized 5S and 10-11S AChE molecular forms by sucrose density gradient fractionation, and studied their relative sensitivity toward inhibition by different organophosphorus compounds such as DFP, soman, sarin, and tabun. Soman, sarin, and tabun were much more potent inhibitors than DFP. However, no difference in the inhibitory potency of any of these compounds was seen between the solubilized 5S and 10-11S molecular forms.

2. Characterization of a specific soman hydrolyzing enzyme activity. NG108-15 cells, grown in culture, possess an enzymatic activity that hydrolyzes soman with an efficiency and specificity comparable to, or higher than, other presently known sources of this kind of enzyme. The relative rate of hydrolysis of sarin or tabun is only 10% that of soman, while DFP, paraoxon, and a phosphinate (p-nitrophenyl methyl(phenyl)phosphinate) are not hydrolyzed by this enzyme under the test conditions. Analysis of the kinetics of soman hydrolysis at substrate concentrations between 0.05 mM and 4 mM reveals two components of the enzyme activity. One component saturates below 1 mM soman with an apparent K_m and V_{max} of 0.2 mM and 13 nmoles/min/mg protein respectively. At 1 mM soman and above the rate of hydrolysis increases sharply with an apparent K_m and V_{max} of 5 mM and 200 nmoles/min/mg protein respectively. The enzyme activity is stable at low temperature, is localized almost exclusively in the soluble fraction of these cells, and is enhanced significantly by Mn^{++} ions and by chemical differentiation of these cells in culture. The NG108-15 cell line is thus a suitable system for further studies of this enzyme. The observed specificity of the enzyme for soman may be helpful in its purification as well as in the application of molecular biological techniques to produce large quantities of this enzyme, which may be useful for soman detection and/or detoxication.

OBJECTIVE

To test the clonal neuroblastoma-glioma hybrid NG108-15 cell line as a neuronal model for studying the mechanisms of toxicity of organophosphorus compounds we characterized two separate aspects of this cell line:

1. **Cholinesterase activity**
2. **Soman hydrolyzing enzyme activity**

METHODS

1. CELL CULTURE. NG108-15 CELLS WERE CULTURED IN A MEDIUM (pH 7.2 TO 7.4) OF 95% (V/V) DULBECCO'S MODIFIED EAGLE'S MINIMAL ESSENTIAL MEDIUM (DMEM) AND 5% (V/V) FETAL BOVINE SERUM. THE MEDIUM ALSO CONTAINED 100 μM HYPOXANTHINE, 1 μM AMINOPTERINE AND 16 μM THYMIDINE. THE AMBIENT CONDITIONS OF INCUBATION WERE A HUMIDIFIED ATMOSPHERE OF 10% CO_2-90% AIR, AND A TEMPERATURE OF 37°C. TO PROMOTE DIFFERENTIATION, CULTURES WERE FED WITH GROWTH MEDIUM SUPPLEMENTED WITH 1 mM Bt_2cAMP FOR DAYS INDICATED.

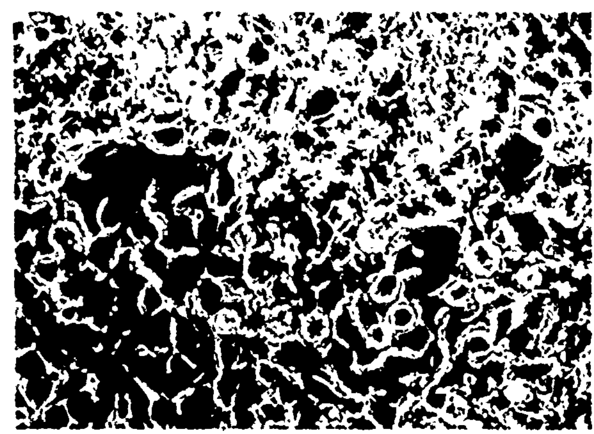
2. ASSAY AND CHARACTERIZATION OF AChE. AChE ACTIVITY WAS ASSAYED BY A RADIOISOTOPIC METHOD USING [^{14}C]-ACETYLCHOLINE IODIDE AS SUBSTRATE. THAT THE ENZYME ACTIVITY WAS TRUE ACETYLCHOLINESTERASE WAS DETERMINED BY USING THE SPECIFIC AChE INHIBITOR COMPOUND BW284C51 (SIAKOTOS, A.N., *et al.*, BIOCHEM, MED., 3: 1-12, 1969)

3. ANALYSIS OF AChE MOLECULAR FORMS. MOLECULAR FORMS OF AChE WERE CHARACTERIZED BY THEIR SEDIMENTATION COEFFICIENTS IN 5-20% SUCROSE DENSITY GRADIENTS USING *E. coli* ALKALINE PHOSPHATASE, p(6.1S); BEEF LIVER CATALASE, c(11.3S); AND *E. coli* β-GALACTOSIDASE, g(16S) AS INTERNAL MARKER ENZYMES.

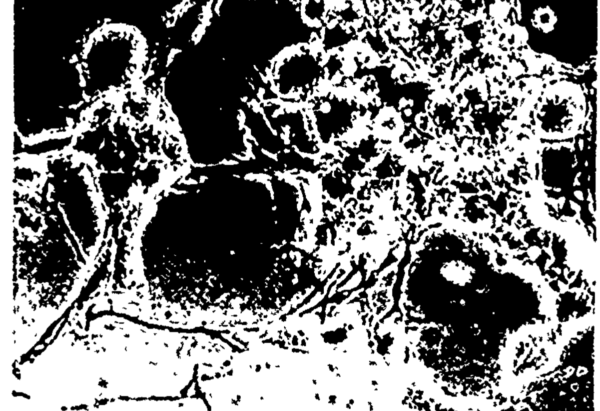
4. ASSAY OF PROTEIN. PROTEIN WAS DETERMINED BY A MODIFICATION OF THE METHOD OF LOWRY, *et al.* USING BOVINE SERUM ALBUMIN AS STANDARD.

PHOTOGRAPHS OF CELLS FROM NG108-15 AND PARENT LINES

NG108-15 (UNTREATED)

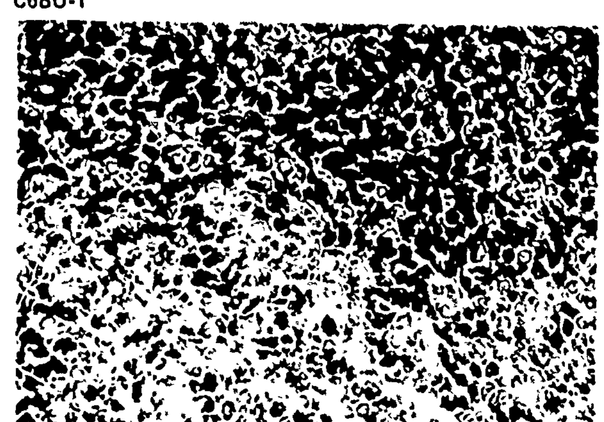


NG108-15 (Bt_2cAMP-TREATED)



N18TG-2

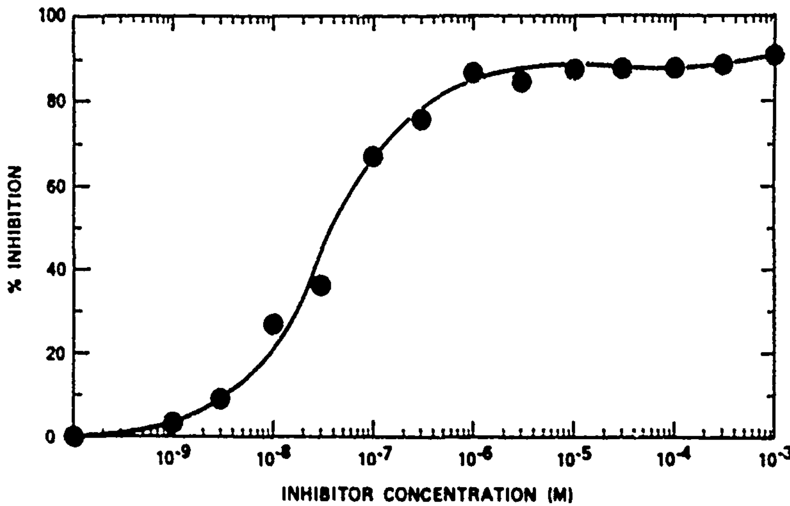
C6BU-1



PHASE CONTRAST PHOTOMICROGRAPHS (320×) OF CELLS FROM LINES INDICATED ARE SHOWN ABOVE. HYBRID CLONE NG108-15 (UPPER LEFT) WAS ORIGINALLY DERIVED BY HYBRIDIZING THE MOUSE C1300 NEUROBLASTOMA CLONE N18TG-2 (LOWER LEFT) WITH THE RAT GLIOMA CLONE C6BU-1 (LOWER RIGHT). ALL CULTURES WERE GROWN FOR 6 DAYS TO CONFLUENCY. SOME 50% CONFLUENT CULTURES OF NG108-15 CELLS WERE TREATED WITH 1 mM Bt_2cAMP FOR 3 DAYS FROM DAY 3 TO DAY 6 TO PROMOTE DIFFERENTIATION. Bt_2cAMP-TREATED NG108-15 CULTURES HAD ENLARGED CELL BODIES AND EXTENSIVE NEURITE FORMATION (UPPER RIGHT).

CHOLINESTERASE ACTIVITY OF NG108-15 CELLS IS PREDOMINANTLY AChE

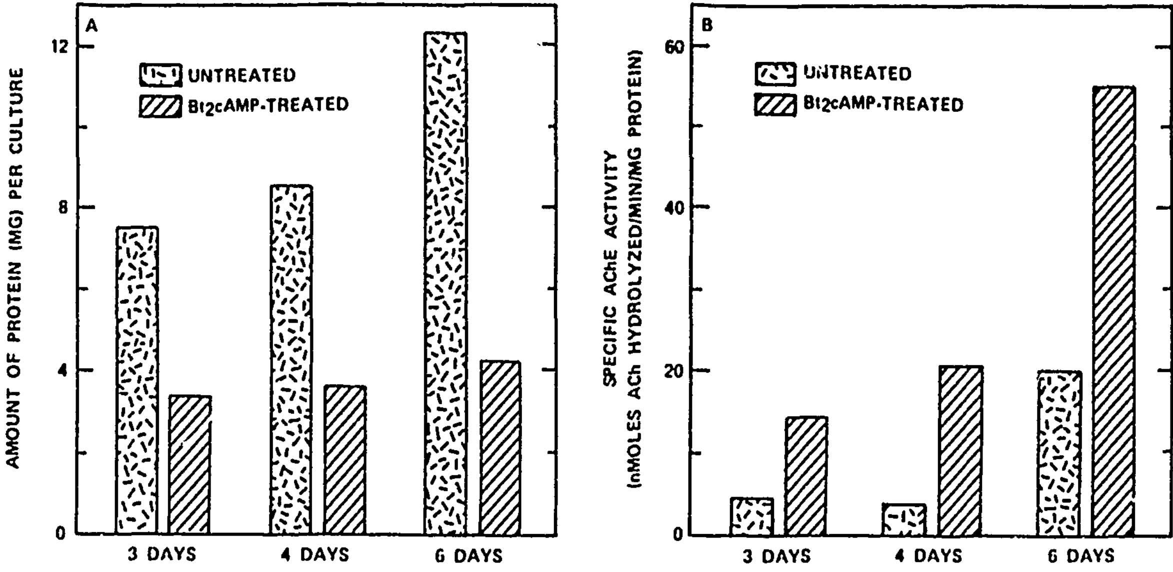
INHIBITION OF CHOLINESTERASE ACTIVITY OF NG108-15 CELLS BY THE SPECIFIC AChE INHIBITOR COMPOUND BW284C51



TOTAL CHOLINESTERASE ACTIVITY OF NG108-15 CELLS WAS INHIBITED 90% BY THE SPECIFIC AChE INHIBITOR COMPOUND BW284C51 (IC_{50} = 3-4 × 10^{-8}M) INDICATING THAT THE ENZYME ACTIVITY WAS PREDOMINANTLY AChE.

SPECIFIC AChE ACTIVITY INCREASES SEVERAL FOLD IN NG108-15 CELLS DURING DEVELOPMENT AND DIFFERENTIATION

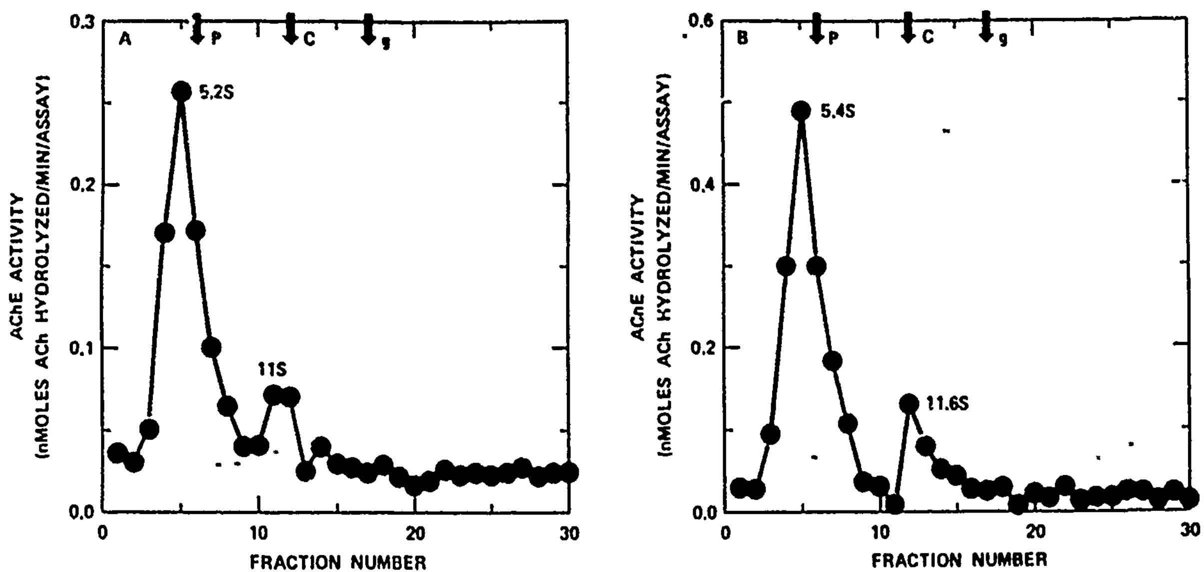
PROTEIN CONTENT (A) AND AChE ACTIVITY (B) OF UNTREATED AND Bt2cAMP-TREATED NG108-15 CELL CULTURES



SEPARATE CULTURES OF NG108-15 CELLS, GROWN IN THE ABSENCE OR PRESENSE OF 1mM Bt_2cAMP FOR DAYS INDICATED, WERE ANALYZED FOR TOTAL PROTEIN AND SPECIFIC AChE ACTIVITY. AFTER 6 DAYS, Bt_2cAMP-TREATED CULTURES CONTAINED 1/3 THE AMOUNT OF PROTEIN OF UNTREATED CULTURES (A) BUT THE SPECIFIC AChE ACTIVITY OF THE TREATED CULTURES WAS THREE TIMES THAT OF THE UNTREATED CULTURES (B).

NG108-15 CELLS CONTAIN TWO MOLECULAR SPECIES OF AChE - 5S AND 10-11S

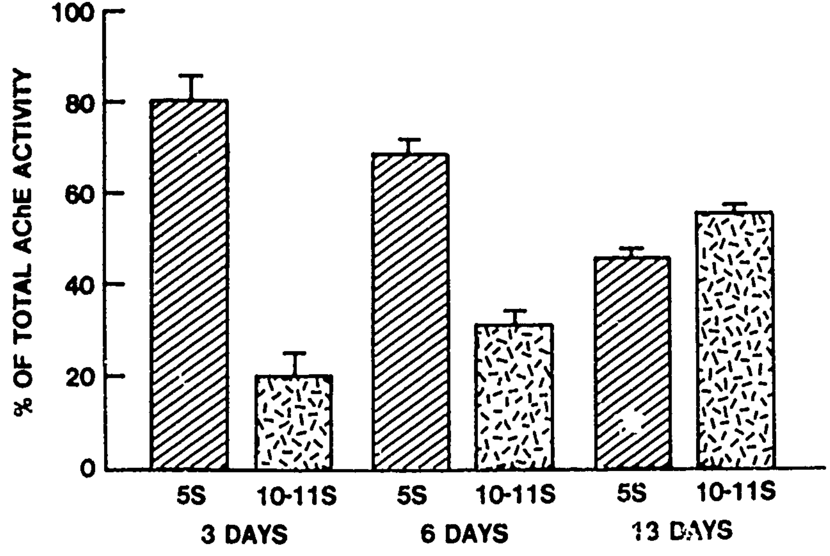
AChE MOLECULAR FORMS IN NG108-15 CELLS AFTER 3 DAYS OF CULTURE IN THE ABSENCE (A) OR PRESENCE (B) OF 1mM Bt2cAMP



THE MOLECULAR FORMS OF AChE WERE EXTRACTED SEPARATELY FROM RESPECTIVE CELL CULTURES BY SONICATION IN BUFFERED MEDIUM CONTAINING 2 mM EDTA, 1 M NaCL, 1% EACH OF TRITON X-100 AND SODIUM CHOLATE, AND WERE CHARACTERIZED BY THEIR SEDIMENTATION COEFFICIENTS IN 5-20% SUCROSE DENSITY GRADIENTS. TWO MOLECULAR SPECIES OF AChE WITH SEDIMENTATION COEFFICIENTS OF APPROXIMATELY 5S AND 11S WERE FOUND IN UNTREATED (A) AS WELL AS Bt_2cAMP-TREATED (B) CULTURES.

THE 10-11S AChE INCREASES COMPARED TO THE 5S AChE DURING DEVELOPMENT AND DIFFERENTIATION OF NG108-15 CELLS

CHANGES IN THE RELATIVE AMOUNTS OF AChE MOLECULAR FORMS DURING DIFFERENTIATION



The relative amounts of the 5S and 10-11S AChE molecular forms were determined in NG108-15 cell cultures grown in the presence of 1 mM Bt_2cAMP for 3, 6 and 13 days. The 5S form progressively decreased, while the 10S form proportionately increased during differentiation in culture.

SOLUBILIZED 5S AND 10-11S AChE ARE EQUALLY INHIBITED BY ANTICHOLINESTERASES

INHIBITION OF DETERGENT SOLUBILIZED AChE MOLECULAR FORMS OF NG108-15 CELLS BY ORGANOPHOSPHORUS ANTICHOLINESTERASES

ANTICHOLINESTERASE COMPOUND	IC_{50}(M) 5S AChE	IC_{50}(M) 10-11S AChE
DFP	2.5×10^{-6}	2.9×10^{-6}
SOMAN	4.6×10^{-10}	4.5×10^{-10}
SARIN	3.5×10^{-9}	4.0×10^{-9}
TABUN	3.0×10^{-8}	2.0×10^{-8}

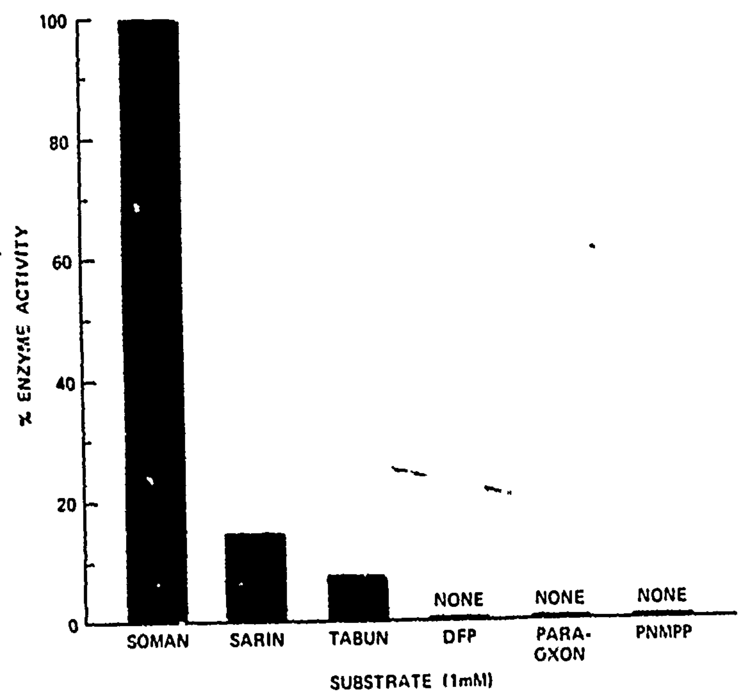
Concentrations of different anticholinesterase compounds required to inhibit 50 percent of total AChE activity in an assay (IC_{50}) were identical for either the 5S AChE or the 10-11S AChE indicating that the two molecular species are equally inhibited when solubilized.

ASSAY METHODS

1. THE RATE OF HYDROLYSIS OF SOMAN AND OTHER ORGANOPHOSPHORUS COMPOUNDS WAS MEASURED BY CONTINUOUSLY TITRATING THE AMOUNT OF ACID GENERATED DUE TO HYDROLYSIS WITH A PREVIOUSLY STANDARDIZED 10mM NaOH SOLUTION. TITRATIONS WERE CARRIED OUT IN UNBUFFERED 0.15M SALINE CONTAINING 0.1mM $MnCl_2$ INSIDE A WATER JACKETED (25°C) TITRATION CELL, PURGED WITH N_2 TO ELIMINATE CO_2, BY AN AUTOMATIC RADIOMETER pH METER-TITRATOR-AUTOBURETTE pH STAT SYSTEM AT A CONSTANTLY HELD pH 7.4.

2. AMOUNT OF PROTEIN WAS ESTIMATED BY A MODIFICATION OF THE METHOD OF LOWRY, et al. USING BOVINE SERUM ALBUMIN AS STANDARD.

NG108-15 CELLS CONTAIN A SOMAN HYDROLYZING ENZYME ACTIVITY



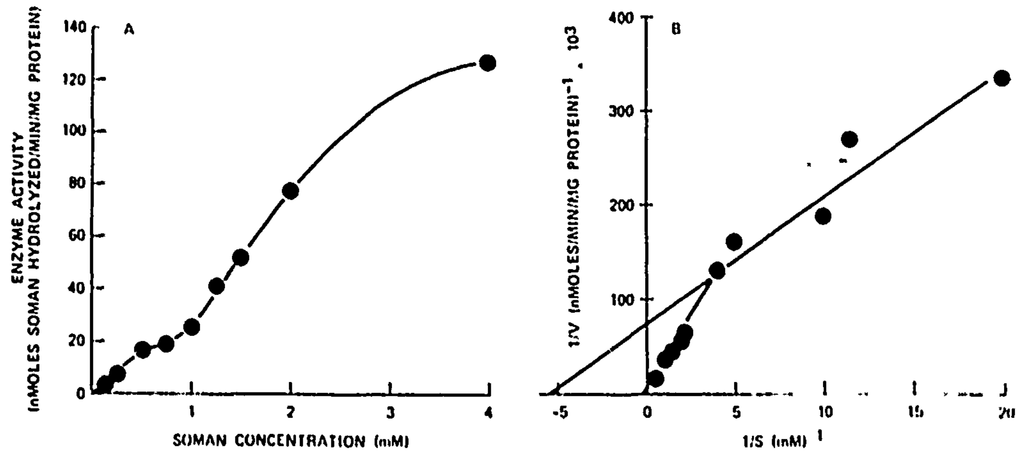
ENZYMATIC HYDROLYSIS OF ALL THE COMPOUNDS SHOWN BY NG108-15 CELLS-SONICATE WAS MEASURED USING 1mM SUBSTRATE CONCENTRATION. THE RELATIVE RATE OF HYDROLYSIS OF SARIN OR TABUN WAS APPROXIMATELY 10% OF SOMAN, WHILE DFP, PARAOXON, AND PNMPP WERE NOT HYDROLYZED, INDICATING THE SPECIFICITY OF THIS ENZYMATIC ACTIVITY FOR SOMAN

PROPERTIES OF SOMANASE ACTIVITY OF NG108-15 CELLS

1. NO LOSS OF ENZYME ACTIVITY AFTER KEEPING THE CELLS-SONICATE ON ICE OVERNIGHT.
2. HEATING THE CELLS-SONICATE IN A BOILING WATER BATH FOR 5 MINUTES REDUCES ENZYME ACTIVITY BY 90% OR MORE
3. THE ENZYME ACTIVITY IS STIMULATED BY Mn^{++} IONS.

SOMANASE ACTIVITY OF NG108-15 CELLS APPEARS TO HAVE TWO COMPONENTS

KINETICS OF HYDROLYSIS OF SOMAN BY NG108-15 CELLS



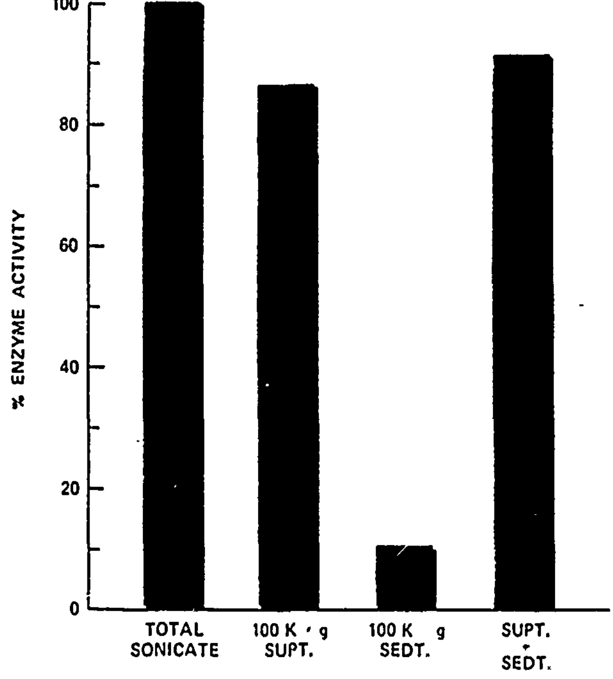
RATES OF HYDROLYSIS OF SOMAN BY NG108-15 CELLS-SONICATE AT INCREASING SUBSTRATE CONCENTRATION ARE SHOWN IN PANEL A, A PLOT OF 1/V vs 1/S IS SHOWN IN PANEL B. TWO COMPONENTS OF THE ENZYME ACTIVITY ARE SEEN

K_{m1} = 0.2mM, V_{max1} = 13 nMOLES/MIN/MG PROTEIN

AND K_{m2} = 5mM, V_{max2} = 200 nMOLES/MIN/MG PROTEIN

SOMANASE ACTIVITY IS ALMOST EXCLUSIVELY LOCALIZED IN THE SOLUBLE FRACTION OF NG108-15 CELLS

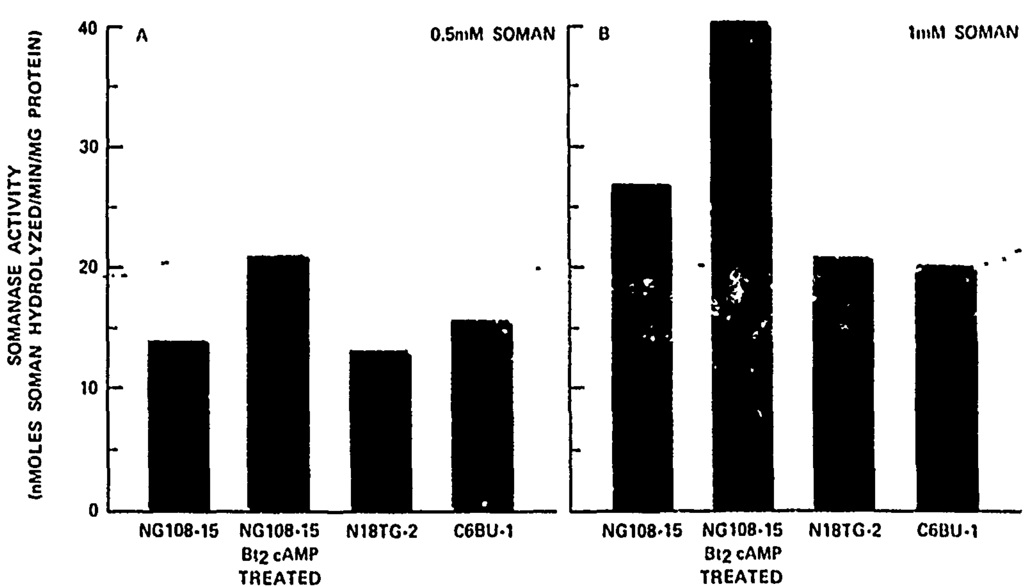
SUBCELLULAR LOCALIZATION OF SOMANASE ACTIVITY IN NG108-15 CELLS



EQUIVALENT AMOUNTS OF TOTAL SONICATE, 100,000 · g SUPERNATANT, 100,000 · g SEDIMENT, AND RECONSITITUTED SUPERNATANT PLUS SEDIMENT WERE ASSAYED FOR THEIR ACTIVITY TO HYDROLYZE 1mM SOMAN IN SEPARATE EXPERIMENTS. THE ENZYME ACTIVITY WAS FOUND TO BE ALMOST EXCLUSIVELY (90%) LOCALIZED IN THE SOLUBLE FRACTIONS OF THESE CELLS.

SOMANASE ACTIVITY OF NG108-15 CELLS IS DEVELOPMENTALLY REGULATED

SOMANASE ACTIVITY OF NG108-15 AND ITS PARENT CELL LINES



THE HYBRID CELL CLONE NG108-15 AS WELL AS ITS PARENTS, THE MOUSE C1300 NEUROBLASTOMA CLONE N18TG-2 AND THE RAT GLIOMA CLONE C6BU-1, WERE TESTED FOR THEIR SOMANASE ACTIVITY AT TWO DIFFERENT SUBSTRATE CONCENTRATIONS: 0.5mM AND 1mM SOMAN. ALL OF THESE CELL LINES WERE FOUND TO POSSESS EQUAL ACTIVITY OF THIS ENZYME. DIFFERENTIATED NG108-15 CELLS, TREATED WITH 1mM Bt2 cAMP FOR 3 DAYS, SHOWED A 50% INCREASE OF THIS ENZYME ACTIVITY.

CONCLUSIONS

1. AChE of NG108-15 cells is comparable to mammalian brain AChE with respect to molecular forms and developmental regulation (S C. Sung and B.A. Ruff, NEUROCHEM. RES., 8:303-311, 1983).
2. Some properties of NG108-15 cell somanase such as preference for soman and stimulation by Mn^{++} are comparable to mammalian tissue organophosphatase activity (F.C.G. Hoskin, M.A. Kirkish and K.E. Steinmann, FUNDUM. APPL. TOXICOL., 4:S165-S172, 1984).
3. These results suggest that the NG108-15 cell line may be a suitable model for studies on AChE and somanase.

ORGANOPHOSPHATE TOXICITY IN CULTURED CELLS

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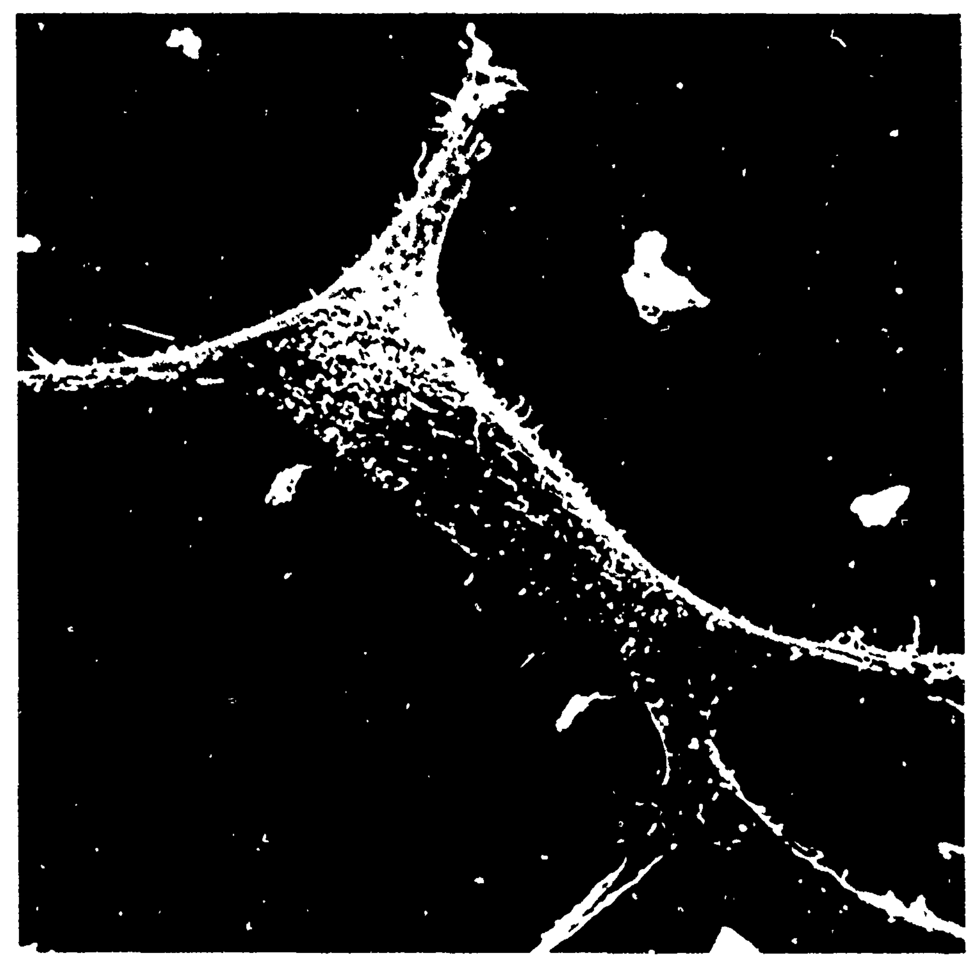
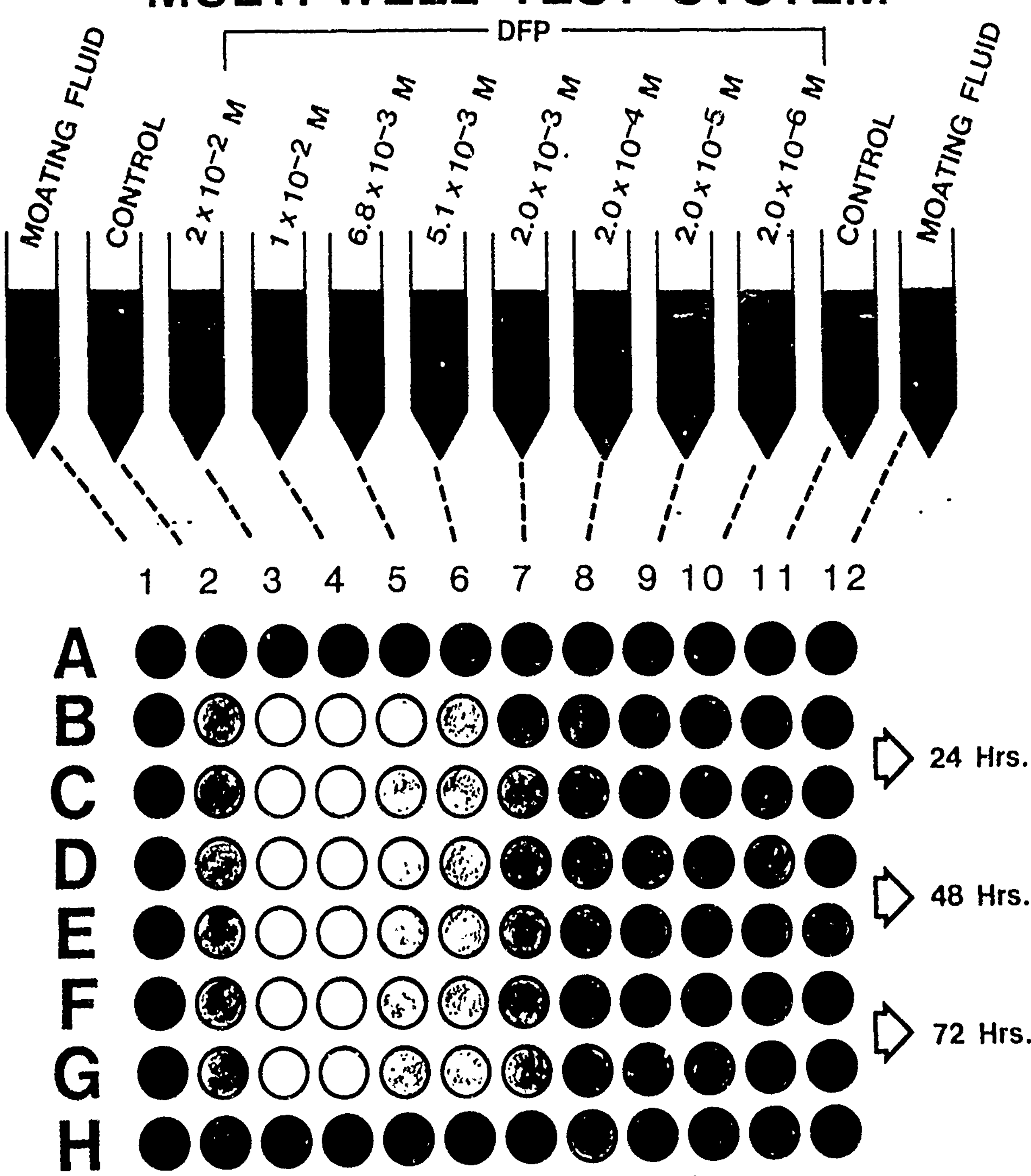
ABSTRACT

In vitro strategies for effective screening of OP-poisons and related drug treatments require development and standardization of reliable test systems. Toward that end, we have investigated xenobiotic induction of cytotoxicity using diisopropylfluorophosphonate (DFP) and mammalian cell cultures. Exponentially growing populations of established mouse fibroblasts were seeded into 96 well tissue culture plates at replicate densities of 4000 ± cells/well. Seventy two hours after attachment and growth in fresh medium, these acetylcholinesterase producing cells were exposed to media containing DFP at concentration ranging from 10^{-2} to 10^{-8} M. Dose response curves equating population growth kinetics (viability, mitotic activity, proliferation and population density) with corresponding DFP concentrations indicate that the ID_{50} producing 50% inhibition of growth is approximately 3×10^{-3} M DFP, and is comparable to the oral LD_{50} for mice. This encouraging correlation suggests that, our in vitro model shares some commonality with fundamental mechanisms affecting cytotoxicity in the mouse and perhaps in other living tissues. Subsequent attempts were made to establish criteria for determining in vitro and in vivo toxicity relationships. Photomicroscopy, electrophysiology, visual and biochemical studies revealed a matrix of morphological and pathophysiological changes which are quantifiable and amenable to computer analyses. Results clearly show that cytotoxicity and recovery are not strictly dose dependent. Morphology and growth kinetics following acute and chronic exposures to DFP, indicate that duration of exposure increases non-specific effects and complicates the potential for full physiological recovery. When electronically assessed for size according to dose, duration of exposure and recovery potential, corresponding alterations in cell volume appear to be long lived, lasting for at least 48 hours in fresh medium after removal from DFP. These changes persist despite the fact that growth potential is restored. It is reasonable to speculate that full recovery of in vivo systems could be similarly compromised. If in vitro and in vivo comparisons can be validated, then appropriate culture systems provide an obvious potential for identification of specific and non-specific lesions. Likewise, development of effective drug treatments might be screened and graded against a battery of the most sensitive test criteria.



ATTACHED WRL-10A CELLS: Populations of this cell line were derived from a clone of the L-929 mouse fibroblast. (Glinos, A.D. & Hargrove, D.D. (1965) Exp. Cell Res., 39, 249-258)

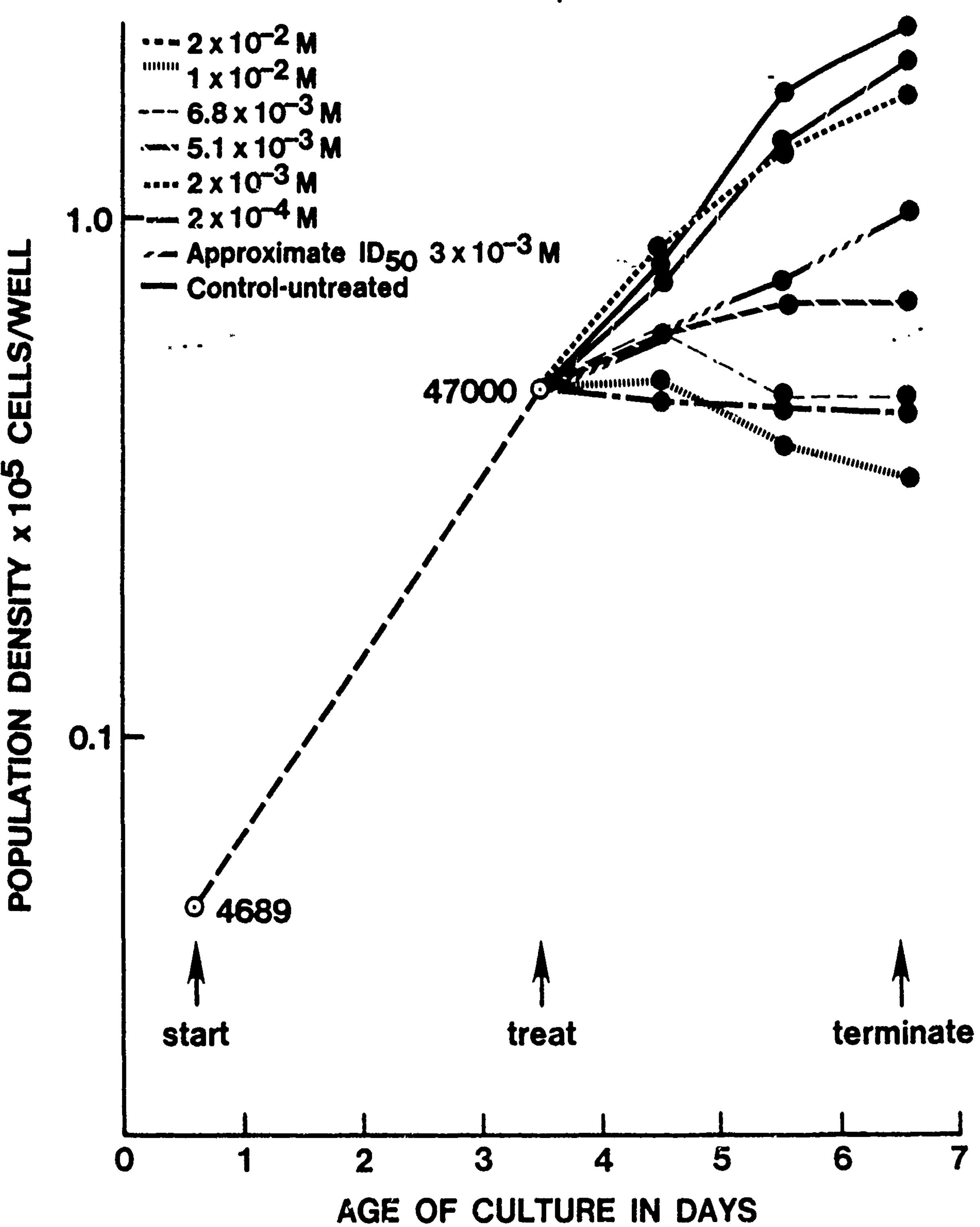
TOXICITY EXPERIMENTS were performed in a multi-well test system. Replicate populations of 4000 ± WRL-10A cells were seeded into each well of a 96-well culture plate. Cells were allowed to grow in freshly prepared media for 3 days before being challenged with DFP. Media consisted of EMEM (Eagles Minimum Essential Medium) plus 20% fetal calf serum, 1% L-glutamine and 100 μg/ml of gentamicin. DFP concentrations were graded according to intended dose and then distributed in verticle rows #3-10 to fit the design of each experiment. At the start of every experiment, each vertical row contained replicate wells with identical concentrations of cells, media and DFP. It was possible with this plate organization to harvest pairs of wells from each vertical row at 24, 48 and 72 hours following treatment and to determine effects of DFP on these cells as a function of dose and duration of exposure.



96 Well Tissue Culture Plate

MEDIA pH: In each experiment an acid shift in medium pH was observed after the wells had been treated with DFP and incubated in an atmosphere of 5% CO_2 and air. Color intensity in the shift from red to yellow was dose-related and due almost entirely to the combined presence of serum and CO_2. No acid shift was observed in the absence of 5% CO_2. Acidified media recovered very slowly toward a normal pH under room atmosphere conditions.

GROWTH RESPONSE TO CONTINUOUS DFP EXPOSURE



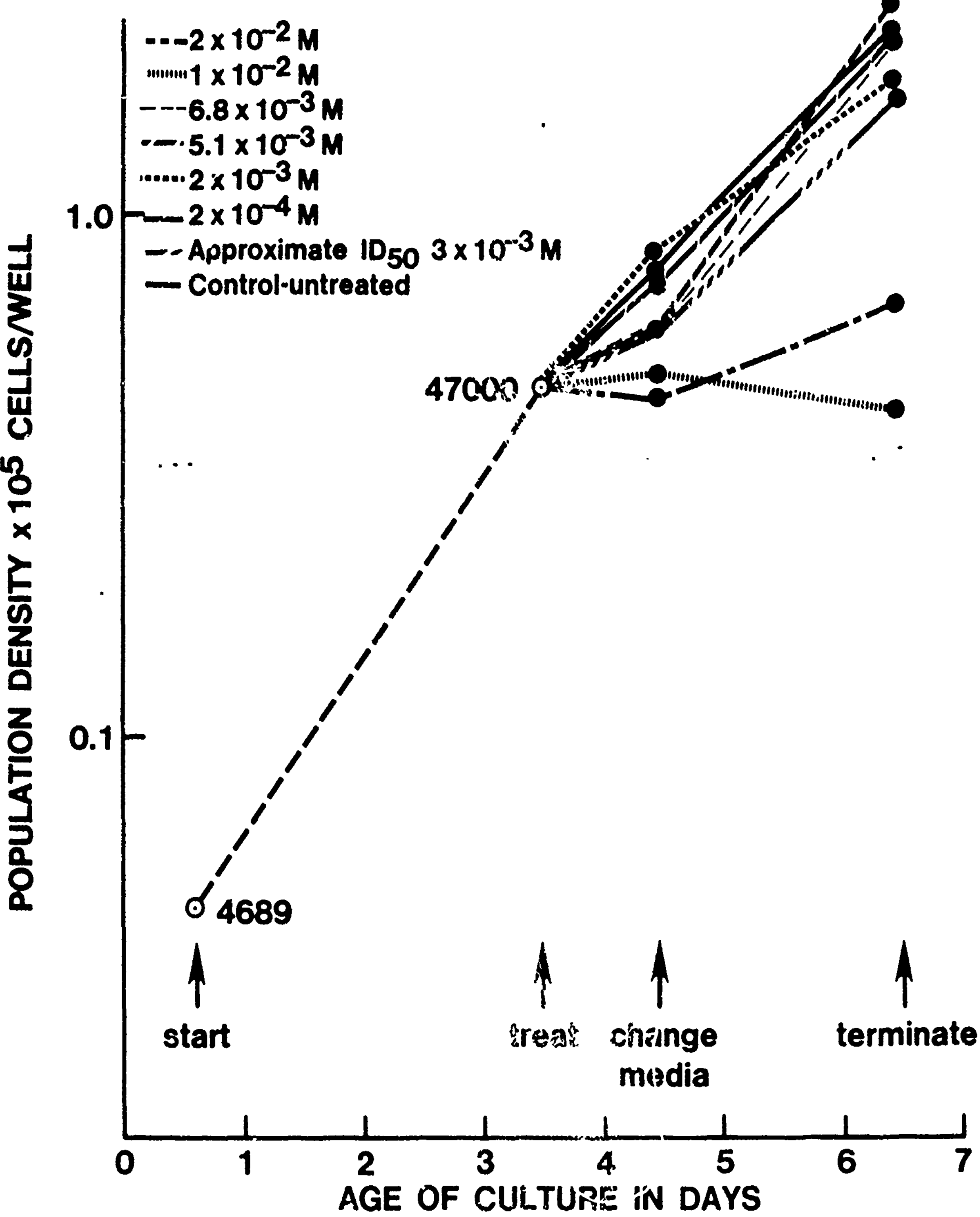
Growth Response to Continuous Exposure: Wells inoculated with 4,689 cells on Day 0, increased in density to an average of 47,000 cells/well before the start of DFP treatments on Day 3. From the point of treatment on Day 3 to the moment of termination on Day 6 , the pattern of growth inhibition was clear.

Populations exposed to 2×10^{-4} and 2×10^{-3} M DFP grew nearly as well as controls and increased to 190,000 and 170,000 cells/ well, respectively.

Populations exposed to 10^{-2} and 2×10^{-2} M DFP stopped growing immediately after treatment and then decreased in density.

Populations exposed to 6.8 and 5.1×10^{-3} M DFP showed a reduced ability to proliferate during their initial 24-hour post treatment period. Subsequently, populations treated with 5.1×10^{-3} M DFP leveled at 68,000 cells/well and populations treated with 6.8×10^{-3} M DFP decreased to 42,000 cells/well. The ID_{50} for these populations was determined to be approximately 3×10^{-3} M DFP.

RECOVERY POTENTIAL

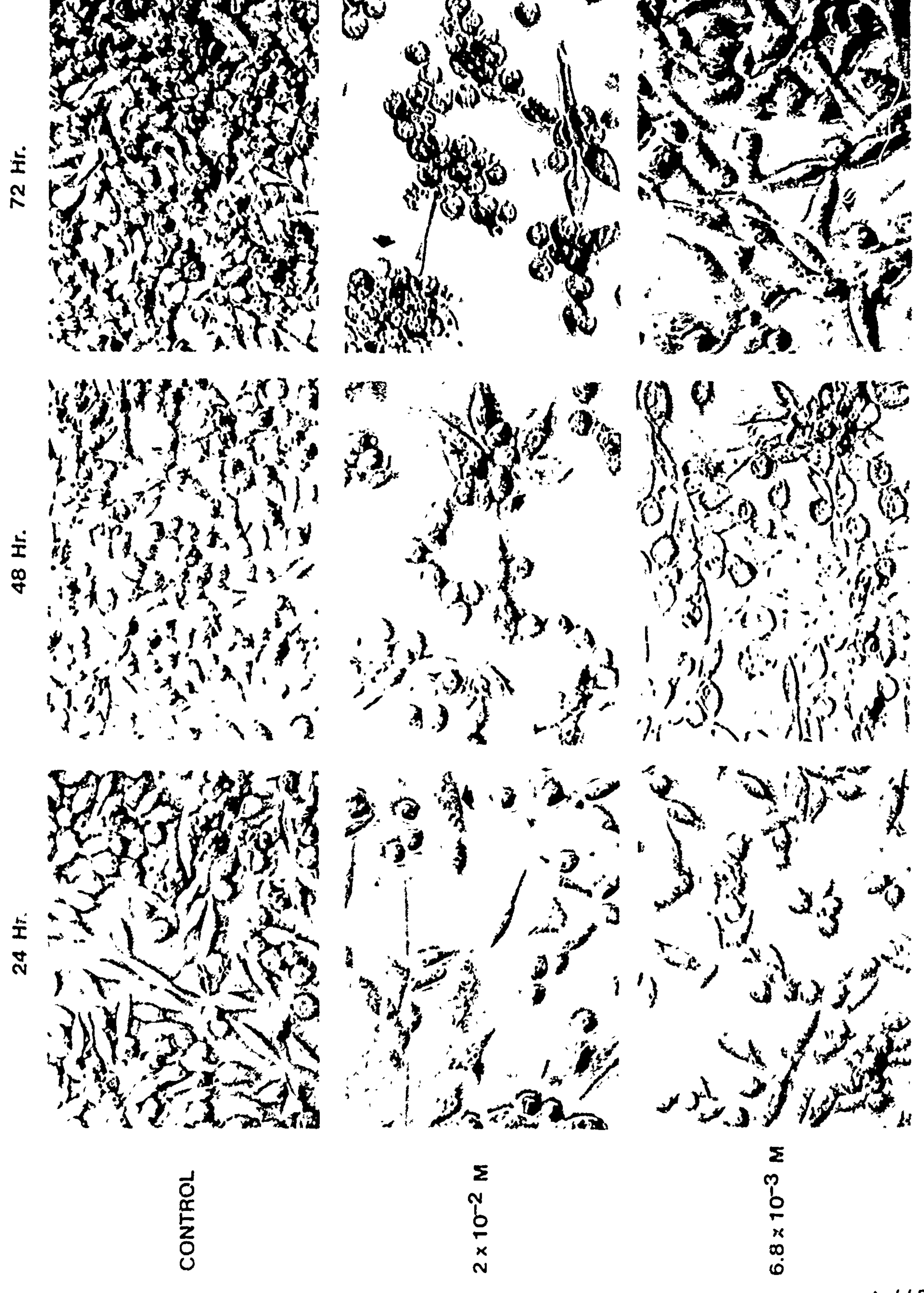


Recovery of Growth Potential: Twenty-four hours after exposure to DFP, cells were tested for recovery potential by replacing all well supernatants with fresh media. After 48 hours in fresh media,

Populations exposed to 10^{-2} and 2 x 10^{-2} M DFP showed little or no potential for recovery.

Populations exposed to 6.8 and 5.1 x 10^{-3} M DFP showed a remarkable potential for recovery and achieved densities that equalled or surpassed those of the controls.

CONTINUOUS EXPOSURE



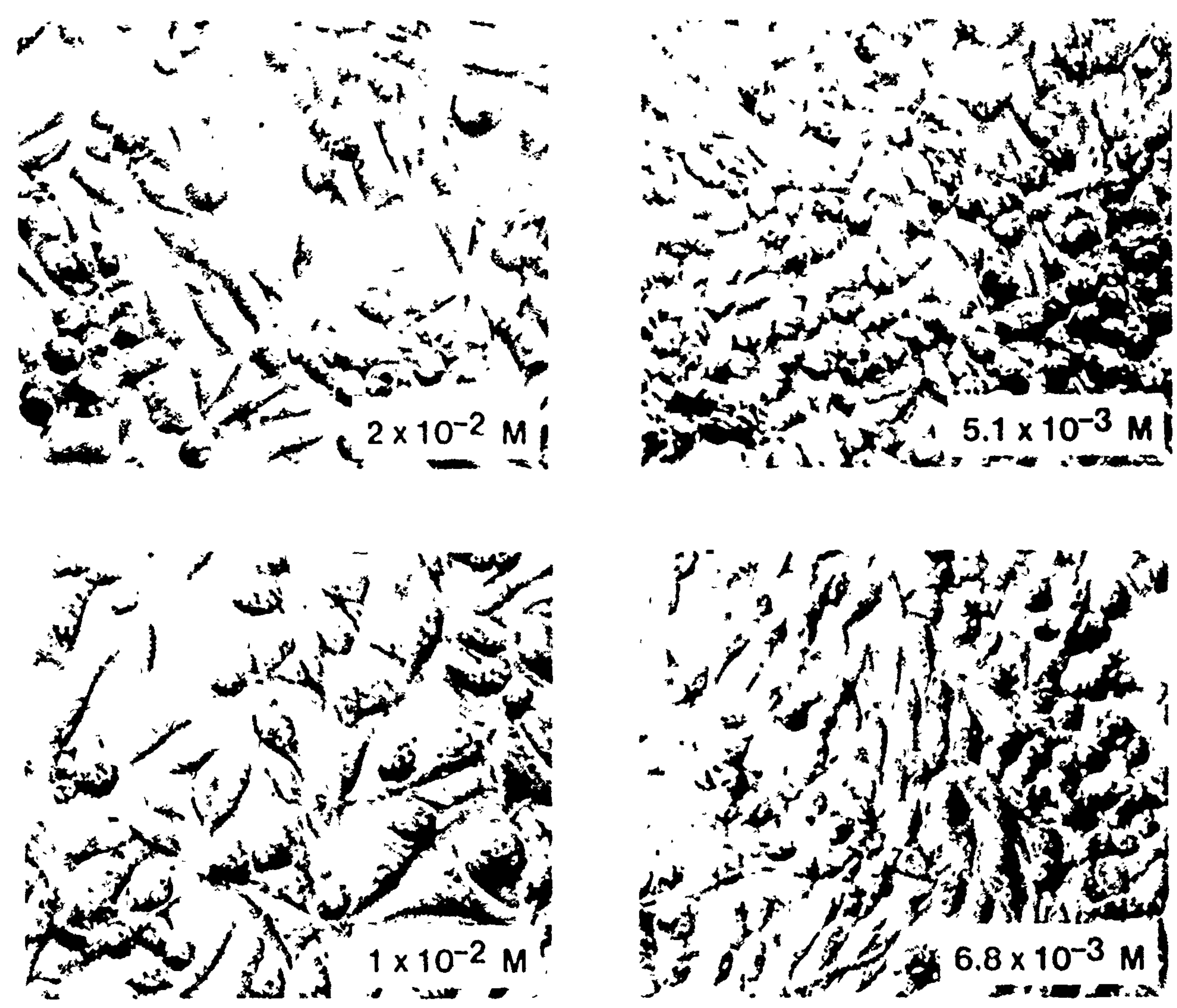
Photomicroscopy: twenty-four hours after initiation of continuous DFP treatment:

Untreated control populations were well attached, moderately dense, and contained a typical mixture of spindle, round and stellate shaped cells. At 48 hours, they were crowded and confluent and by 72 hours they were overgrown into multilayers.

Populations exposed to 10^{-2} and 2×10^{-2} M DFP developed gross cytopathology during the first 24 hours of exposure. These cells grew abnormally large, assumed an atypical morphology and developed roughened and knobbled surface membranes. At 48 hours, many of the cells had rounded and begun to stick together, forming cellular islands. At 72 hours these islands had developed into scattered grape-like clusters.

Populations exposed to 6.8 and 5.1×10^{-3} M DFP also developed gross cytopathology, but only after 48 hours of exposure. The salient feature is that structural changes first observed in these populations at 48 and 72 hours include changes that were observed at 24 hours with more toxic doses. Of particular note is the appearance of grossly enlarged and knobbled giant cells at 72 hours.

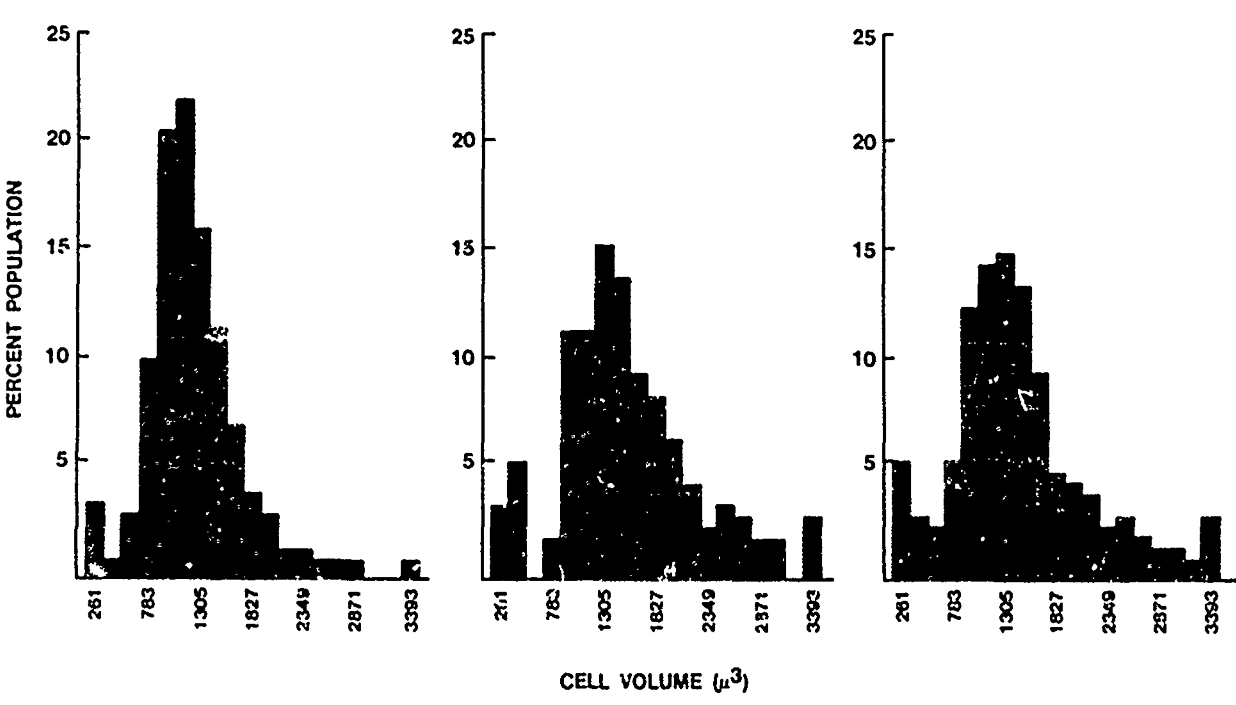
48 Hr. RECOVERY



Photomicroscopy (Recovery Studies): After 48 hours in fresh media, populations previously exposed to cytotoxic concentrations of DFP for 24 hours remain swollen and enlarged.

CELL VOLUME CHANGES

LOG CONTROL	72 HR. EXPOSURE	48 HR. RECOVERY
UNTREATED	5.1 x 10^{-3} M DFP	5.1 x 10^{-3} M DFP
MEAN POPULATION VOLUME	MEAN POPULATION VOLUME	MEAN POPULATION VOLUME
$1331.69 \pm 1.65\mu^3$ S.D.M.	$1678.79 \pm 1.93\mu^3$ S.D.M.	$1543.25 \pm 1.36\mu^3$ S.D.M

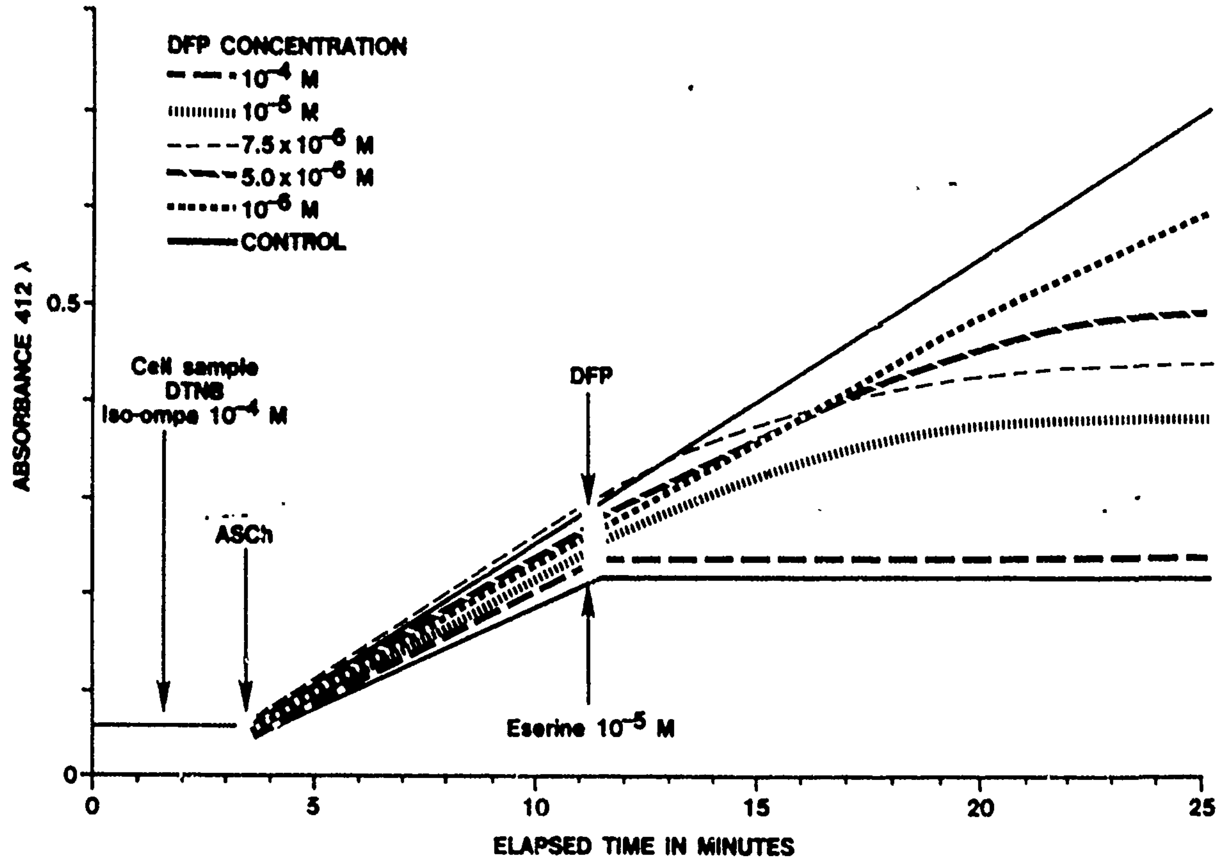


DFP-induced changes in cell volume have been assessed by Coulter count and computer analyses. Results clearly show that:

Cells from untreated control populations are tightly grouped in a narrow modal range with a mean population volume of $1331.69 \pm 1.65\,\mu^3$.

Populations continuously exposed to 5.1 x 10^{-3} M DFP for 72 hours are less tightly grouped, 25% larger, and have a mean cell volume of $1678.79 \pm 1.93\,\mu^3$.

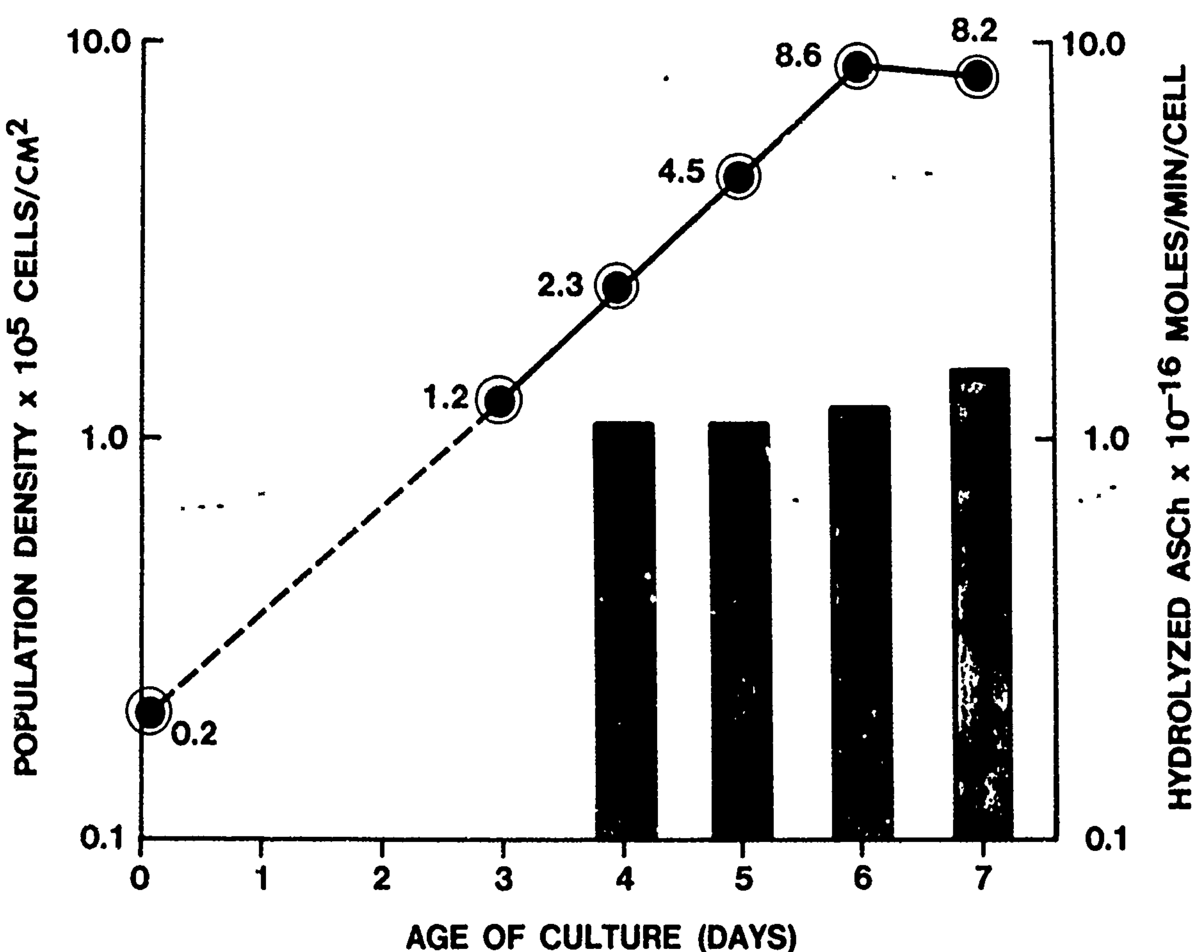
Recovery populations treated with identical concentrations of DFP for 24 hours remain larger than normal after 48 hours in fresh medium and register 16% larger than the controls with a mean cell volume of $1543.25 \pm 1.36\,\mu^3$. Microscopic and photographic evidence indicates that average volume changes are more dramatic and long lived at higher concentrations.



Acetylcholinesterase (AChE) activity of WRL-10A cells was determined by the colorimetric assay of Ellman et al. (Biochem. Pharmacol. 7:88-95, 1961). Acetylthiocholine (ASCh) hydrolysis and absorbancy changes at 412 λ proceeded in the continuous presence of 10^{-4} M iso-OMPA. Addition of 10^{-5} M eserine blocked hydrolysis completely, indicating true cholinesterase activity.

Despite the hysteresis, acetylcholinesterase activity was completely inhibited by DFP at doses down to 5×10^{-6} M. This latter value is in close agreement with the established sensitivity of murine AChE (Silver, A., The Biology of Cholinesterases, North-Holland Publishing Co., 1974).

AChE ACTIVITY OF ATTACHED CULTURES



Cells from attached cultures hydrolyzed approximately 10^{-16} M of ASCh min^{-1} $cell^{-1}$. Acetylcholinesterase activity showed little or no change with increasing density of these populations.

DISCUSSION AND CONCLUSIONS

1. DFP-induced cytopathology can be graded in cell culture according to dose and duration of exposure.

2. Doses approximating the ID50 produce the same structural lesions as higher doses, but require longer periods (48-72 hours) of intoxication.

3. Populations exposed to 5.1 and 6.8 x 10^{-3} M DFP for 24 hours recover their growth potential when placed in fresh medium for 48 hours, but do not recover structural integrity.

4. Photomicroscopic observations and computer analyses of cell volume changes suggest that similar progressions of reversible and irreversible changes may occur _in vivo_. Such long lived structural and functional changes _in vivo_ could disrupt normal metabolism and cell-to-cell communication, and could compromise recovery of the victim by leading to delayed neuropathies, myopathies, cardiac arrhythmias and ultimately to death.

5. Our results clearly indicate: (a) that a physiological range exist for structural, functional and biochemical sensitivities to DFP-induced lesions, (b) there is a reproducible progression toward irreversible tissue damage,and (c) it is possible to investigate these fundamental relationships by developing appropriate tissue culture models and strategies.