TABLE 2

EFFECT OF PYRIDOSTIGMINE PRETREATMENT AND ATROPINE AND OXIME TREATMENT AGAINST SOMAN POISONING IN RABBITS

Pyr/Atr/2-PAM			Pyr/Atr/HI6		
Soman µg/kg	No. Responding	No. Sampled	Soman µg/kg	No. Responding	No. Sampled
21.0	0	1	61.9	0	1
28.3	0	2	82.5	0	2
29.7	2	2	110.0	0	2
40.0	1	2	127.4	1	2
41.9	0	2	135.0	0	1
56.5	3	3	143.9	1	2
59.2	0	2	146.7	1	2
79.8	1	1	160.4	1	2
80.0	2	2	180.0	4	4
83.6	1	1	240.0	2	2
LD50	42.0 µg/kg		LD50	144.5 µg/kg	
95% CI	0.0-102.1		95% CI	105.5 - 175.3	
Slope	3.8		Slope	15.0	
N	18		N	20	

TABLE 4

EFFECT OF ATROPINE AND OXIME TREATMENT AGAINST TABUN IN RABBITS

Atr/2-PAM			Atr/HI6		
Tabun µg/kg	No. Responding	No. Sampled	Tabun µg/kg	No. Responding	No. Sampled
294.7	0	2	166.4	0	2
416.3	0	2	235.0	0	2
552.7	0	2	331.9	0	2
588.0	0	2	369.7	0	2
674.1	0	2	468.9	0	2
736.7	1	2	477.3	1	2
801.2	1	2	492.9	2	2
830.6	0	2	567.2	1	2
952.3	2	2	657.0	2	2
982.0	1	2	662.3	1	2
1131.7	1	2	674.1	1	2
1173.2	2	2	801.2	1	2
1309.0	1	2	875.8	2	2
1345.1	1	2	952.3	2	2
1744.9	2	2	1167.4	2	2
LD50	986.5 µg/kg		LD50	558.6 µg/kg	
95% CI	770.6 - 1377.1		95% CI	416.1 - 707.4	
Slope	6.1		Slope	6.7	
N	30		N	30	

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TABLE 5

EFFECT OF PYRIDOSTIGMINE PRETREATMENT ON THE EFFICACY OF ATROPINE AND OXIME TREATMENT OF TABUN POISONING IN RABBITS

Pyr/Atr/2-PAM			Pyr/Atr/HI6		
Tabun µg/kg	No. Responding	No. Sampled	Tabun µg/kg	No. Responding	No. Sampled
375.2	0	2	294.7	0	2
530.0	1	2	416.3	0	2
798.6	0	2	588.0	0	2
952.3	0	4	601.8	0	2
1057.5	0	2	756.4	0	2
1131.7	0	2	802.2	1	2
1198.9	0	2	830.6	0	2
1345.1	0	2	952.3	0	2
1493.7	0	2	1069.3	1	2
1509.2	1	2	1173.2	0	2
1598.6	1	2	1198.8	1	2
1900.0	0	4	1425.3	1	2
			1509.2	0	2
			1900.0	3	4
LD50	> 2000 µg/kg		LD50	1576.2 µg/kg	
95% CI	-		95% CI	1171.3 - 8824.0	
Slope	-		Slope	4.0	
N	28		N	30	

TABLE 6

EFFECT OF PRETREATMENT AND TREATMENT THERAPY
AGAINST NERVE AGENT LETHALITY IN RABBITS

Pretreatment (mg/kg)	Treatment (mg/kg)	LD50 Soman (µg/kg)	LD50 Tabun (µg/kg)
-	Atr(13) + 2-PAM (25)	28.4 (24.0-37.9)	986.5 (770.6-1377.1)
-	Atr(13) + HI6 (37.8)	89.8[1] (64.6-354.8)	558.6 (416.1-707.4)
Pyrido (0.08)	Atr(13) + 2-PAM (25)	42.0 (0.0-102.1)	> 2000
Pyrido (0.08)	Atr(13) + HI6 (37.8)	144.5 (105.5-175.3)	1576.2 (1171.3-8824)
Pyrido (0.08)	Atr(13) + 2-PAM (25) + Diaz (1.0)	49.4 (39.2-66.6)	-
Pyrido (0.08)	Atr(13) + HI6 (37.8) + Diaz (1.0)	240.6[1] (191.7-361.9)	-

a. LD50 estimate and 95% CI based on modified data set. See Tables 1 and 3 for explanation.
LD50 GD = 15.2 (12.3-19.5). From —— Task 87-35
LD50 GA = 234.0 (172-318). From —— Task 87-35

TABLE 7

EFFECTIVENESS OF PYRIDOSTIGMINE, ATROPINE AND OXIMES ON 24 HOUR SURVIVAL FOLLOWING CHALLENGE WITH 10 X LD50 SARIN OR VX IN RABBITS

Pretreatment	Treatment (mg/kg)	No.dead/No.tested Sarin	VX
Saline	Atr(13) + 2-PAM(25.7)	4/8	0/6
Saline	Atr(13) + HI6(37.8)	0/6	1/6
Pyr(0.08)	Atr(13) + 2-PAM(25.7)	1/6	0/6
Pyr(0.08)	Atr(13) + HI6(37.8)	0/6	0/6

+ 81

D) MOUSE (3 studies)

Study D-1 Study of .

(Located in vol 1.19, p. 54+, of the NDA. This is the same study as rabbit study C-2 discussed above).

Dose of P = 0.13 mg/kg i.m. given 7.5 mn. before Soman (i.m.). (It was not stated if a vehicle control for P was used). Atropine (11.2 mg/kg) and 2-PAM (25.1 mg/kg) were given i.m. 10 seconds after Soman administration. Strain of mice = ICR-Swiss (males).

Results, expressed as PRs as defined earlier (based on 24 hour Soman LD 50), are shown in attached tables 4 (no P prophylaxis) and 7 (P prophylaxis). The PR (with 95% CL) for atropine/PAM alone was 1.1 (0.94, 1.3) and for P/atropine/PAM was 2.7 (1.4, 4.6). The report stated that P "significantly" enhanced the efficacy of the other treatments; however it is not clear that the effects of P were tested statistically. There was no overlap in the 95% CL for the 2 PR values given above. Survival broken down by Soman dose is shown in tables 2 (no P) and 5 (P prophylaxis); these limited data do suggest an effect of P.

Also note that a slight effect of atropine was seen when given alone but not when given with 2-PAM in non-P-pretreated mice (Table 4), and that P-pretreatment alone (i.e. no atropine/PAM given) did not appear to alter the LD 50 of Soman (Table 7). (However, note that the value for the LD 50 of Soman alone was apparently based on historical data, making the accuracy of these latter comparisons questionable).

It was stated that P reduces "whole blood cholinesterase" activity by approx. 30-70% at the dose of P used in this study; this was based on values cited in a literature report, i.e. enzyme activity was not measured in the present study.

STUDY D-1

Table 2. Data for Groups of 20* Mice Treated Intramuscularly 10 Seconds After Challenge with GD, Also Given Intramuscularly

Treatment	Challenge (mg/kg)	Median Time to Death in Minutes	Median Quality of Life of Survivors	No. of Survivors at 24 hours
GD Control**	0.0782	-	Not observed	10/10
	0.0984	6	" "	7/10
	0.124	11	" "	5/10
	0.156	6 1/4	" "	0/10
Atropine	0.0914	-	2	20
	0.145	11	3	13
	0.230	3 1/4	2-3	2
	0.364	3 1/2	-	0
PAM/Atropine	0.103	> 13	2	16
	0.162	7	3	3
	0.258	6	3	1
	0.409	2	-	0
TMB-4/Atropine	0.173	> 135	1	19
	0.230	> 360	2	14
	0.306	> 157	2	1
	0.409	4 3/4	-	0
Atropine/TMB-4/ Benactyzine	0.129	-	2	20
	0.205	163	3	15
	0.325	134	3	1
	0.515	4	-	0

* Except for GD controls.

** Data from previous experiment for comparative purposes; LD50 is 0.115 (0.103 - 0.129) mg/kg. On actual experimental days, agent LD50 varied from 0.0831 to 0.131 mg/kg, with a mean value of 0.102 mg/kg.

Table 4. Protective Ratios Found With Various Therapies. All Injections Were Intramuscular. Treatment Administered 10 Seconds After Challenge in Mice, At First Sign of Intoxication in Rabbits.

Treatment	Species	LD50 Multiples Treated with 95% Confidence Limits and Slope Value of Fitted Line*
GD Control	Mouse	1.0
Atropine · SO_4	Mouse	1.4 (1.3-1.6); Slope Value = -9.24
PAM · Cl Atropine · SO_4	Mouse	1.1 (0.94-1.3); Slope Value = -6.94
TMB-4 Atropine · SO_4	Mouse	2.1 (1.9-2.3); Slope Value = -13.7
TMB-4 Atropine · SO_4 Benactyzine · HCl	Mouse	2.0 (1.8-2.3); Slope Value = -11.8
GD Control	Rabbit	1.0
Atropine · SO_4	Rabbit	1.9 (1.6-2.3); Slope Value = -8.55
PAM · Cl Atropine · SO_4	Rabbit	2.2 (1.9-2.7); Slope Value = -9.04
TMB-4 Atropine · SO_4	Rabbit	1.6 (1.3-2.0); Slope Value = -6.90
TMB-4 Atropine · SO_4 Benactyzine · HCl	Rabbit	2.9 (2.4-3.5); Slope Value = -8.07

* An LD50 value of 0.115 mg/kg was used for mouse calculations, and a value of 0.0115 mg/kg for rabbit calculations. Agent potency was checked, using mice on each experimental day. The LD50 values varied from 0.0831 to 0.131 mg/kg, with a mean value of 0.102 mg/kg.

Table 5. Data for Groups of 20 Mice* Administered Pyridostigmine Prophylaxis 7-1/2 Minutes Before Challenge, Challenged with GD, and Treated Intramuscularly 10 Seconds After Challenge.

Treatment	Challenge (mg/kg)	Median Time to Death in Minutes	Median Activity Level of Survivors	No. of Survivors at 24 hrs.
GD Control**	0.0782	-	Not observed	10/10
	0.0984	6	" "	7/10
	0.124	11	" "	5/10
	0.156	6 1/4	" "	0/10
None***	0.0727	6	1	18
	0.0914	9 1/2	1	18
	0.115	3 1/4	1	4
	0.145	2 1/4	-	0
	0.182	2	-	0
	0.230	1 1/2	-	0
Atropine	0.126	1 1/2	1	19
	0.189	6 1/2	2	13
	0.282	4	2	6
	0.423	3 1/2	-	0
PAM/Atropine	0.177	-	1	20
	0.266	3 1/2	2	9
	0.397	3 1/2	2	5
	0.594	2 1/2	1	3
TMB-4/Atropine	0.193	4 1/2	1	19
	0.289	4	1	18
	0.433	2 1/2	2	6
	0.647	2	2	1
Atropine/TMB-4/	0.199	23 1/2	2	19
Benactyzine	0.298	9	1	19
	0.446	4 1/2	2	11
	0.667	3 1/2	2	3

* Except for GD control

** Data from previous experiment for comparative purposes; LD50 is 0.115 (0.103-0.129) mg/kg. On actual experimental days, agent LD50 ranged from 0.0831 to 0.131 mg/kg, with a mean value of 0.102 mg/kg.

*** Distilled water injected in place of treatment mixture.

Table 7. Protective Ratios Found With Pyridostigmine Prophylaxis Alone or Combined With Various Therapies. All Injections Were Intramuscular, With Prophylaxis Administered 7.5 Minutes Before Challenge to Mice and 30 Minutes Before Challenge to Rabbits. Treatment Given 10 Seconds After Challenge to Mice, At First Sign of Intoxication to Rabbits.

Treatment	Species	LD50 Multiples Treated With 95% Confidence Limits and Slope Value of Fitted Line
GD Control	Mouse	1.0
Pyridostigmine · Br	Mouse	0.88 (0.59-1.3); Slope Value = -13.1
Atropine · SO_4	Mouse	1.9 (1.7-2.2); Slope Value = -6.87
PAM · Cl Atropine · SO_4	Mouse	2.7 (1.4-4.6); Slope Value = -5.0
TMB-4 Atropine · SO_4	Mouse	3.3 (2.9-3.8); Slope Value = -7.16
TMB-4 Atropine · SO_4 Benactyzine · HCl	Mouse	4.1 (3.5-4.8); Slope Value = -5.96
GD Control	Rabbit	1.0
Pyridostigmine · Br	Rabbit	<1.6
Atropine · SO_4	Rabbit	3.2 (1.9-7.3); Slope Value = -3.18
PAM · Cl Atropine · SO_4	Rabbit	3.1 (2.2-4.2); Slope Value = -3.16
TMB-4 Atropine · SO_4	Rabbit	3.4 (1.5-5.9); Slope Value = -2.03
TMB-4 Atropine · SO_4 Benactyzine · HCl	Rabbit	7.7 (5.3-11.0); Slope Value = -3.97

Study D-2 Study of Koplovitz, et. al.

(Located in volume 1.16, p. 226+, of the NDA. This is a published paper, the same as that described and included under the guinea pig studies [study B-5], above).

Dose of P = 0.2 or 0.82 mg/kg p.o. given 60 min. prior to Sarin, VX, or Tabun given i.m. Atropine (11.2 mg/kg) and 2-PAM (25 mg/kg) were given i.m. 10 seconds after nerve agent administration. Strain of mice = Swiss ICR (Charles River), males.

Results are shown in table 2. Results are expressed as PRs as defined above. (Based on 24 hr. LD 50s for nerve agents). Atropine/PAM alone gave PRs of 2.1, 7.8, and 1.3 for Sarin, VX, and Tabun, resp., all of which were said to be statistically different from untreated controls. For Sarin, P pre-treatment did not alter the effect of atropine/PAM. For VX, the low dose of P did not alter the effect of atropine/PAM, but the high dose decreased the PR from 7.8 to 3.9. (The latter was still statistically significantly greater than that in untreated controls). For Tabun, P produced a slight but statistically significant increase in the PR compared to atropine/PAM alone. (PR = 1.7 and 2.1 at the low and high P doses, resp., compared to 1.3 with atropine/PAM alone). Results presented in this paper were not broken down by dose of nerve agent.

AchE inhibition was not measured in this study; based on "unpublished data" it was estimated that the low and high doses of P would cause approx. 30 and 60% inhibition, resp. (Contradictory statements are made in the paper whether this refers to whole blood or RBC enzyme).

Study D-3 Study of Koplovitz and Romano (volume 1.16, p. 233+, of NDA) (Study in its entirety is attached).

Dose of P = 0.20 or 0.82 mg/kg p.o. given 30 or 60 minutes (see below) prior to administration of Soman given i.m. Atropine (11.2 mg/kg) and 2-PAM (25.0 mg/kg) were given i.m. 10 sec. after Soman. Strain of mice = ICR Swiss, gender not stated.

A group of mice was given a dose of 2 LD 50 Soman (the actual dose of Soman used was not given, nor was the method or results for determining the LD 50) followed by atropine + PAM. Another group was also pretreated with P (0.82 mg/kg p.o.) 30 min. prior to Soman. (A vehicle control for P was apparently not employed in this part of the study). Results shown in figure 2. P reduced Soman-induced lethality (at 24 hrs. post-Soman) by about half compared to atropine/PAM alone. Incapacitation (measured by horizontal screen test at 24 hrs. post-Soman) among survivors was also decreased by P (compared to atropine/PAM alone).

Figure 3 shows similar data using a variety of Soman LD 50 multiples (again, actual Soman dose and method for determining LD 50 not given); doses of P were either 0.20 or 0.82 mg p.o. given 60 min. prior to Soman. Atropine/PAM alone decreased Soman-induced lethality, but not incapacitation among survivors, at the lower Soman doses (compare to figure 1). At the lowest dose of Soman (1.08 LD 50) P did not add to the near-maximal protection against lethality caused by atropine/PAM but did reduce incapacitation among survivors compared to atropine/PAM alone. At Soman doses up to 2.16 LD 50, P caused both reduced lethality and reduced incapacitation among survivors compared to atropine/PAM alone. The 2 doses of P caused approximately the same magnitude of effect. Results at higher Soman doses are not clear since no results are shown for atropine + PAM alone. Note that the Ns in this study were not given.

AchE activity was not measured in this study.

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Protection Against Soman-Induced Lethality and Physical Incapacitation Following Pyridostigmine Pretreatment in Mice.

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Abstract

As part of an ongoing in vivo drug screening program, the incidence of physical incapacitation was investigated in mice 24 hours after soman poisoning using the horizontal screen test. The ability of pyridostigmine (PYR) pretreatment and/or atropine sulfate (ATR) + Pralidoxime (2-PAM) treatment to influence incapacitation was evaluated. Animals intoxicated with 0.9 LD50s of soman and higher displayed increasing mortality and a significant (> 50%) incidence of physical incapacitation in survivors at 24 hrs. Treatment with ATR and 2PAM significantly reduced the incidence of mortality from soman (1.08 LD50) but did not affect the incidence of incapacitation (49%). As the level of soman challenge increased, the efficacy of ATR and 2PAM against both lethality and incapacitation decreased rapidly. In contrast, animals orally pretreated with PYR (0.20 or 0.82 mg/kg) 30 min or 1 hour prior to soman (1.08 to 3.42 LD50) challenge and treatment with ATR and 2-PAM manifested significantly greater survivability ($p<0.01$) and significantly less incapacitation ($p<0.01$) than vehicle pretreated controls. Animals pretreated with either dose of PYR demonstrated significantly enhanced protection from lethality and incapacitation over a range of soman challenges from 1.3 to 2.1 LD50 compared to animals treated with ATR and 2-PAM. No difference in protection was observed between the two PYR dosages. The results of these studies indicate that, in the mouse, pretreatment with PYR affords significant advantages when added to conventional ATR and 2-PAM therapy.

Introduction. Soman is a highly lethal and debilitating organophosphorus (OP) CW agent. Acute, near lethal soman exposure results in significant CNS pathology (Petras, 1981; LeMercier et al., 1983) and persistent, severe alterations in the behavior (McDonough et al., 1986) of rats and non-human primates (Gause et al., 1986). The purpose of these studies was to assess the incidence of physical incapacitation in surviving mice 24 hrs after soman poisoning and to determine whether standard pretreatment therapy with PYR and/or treatment with ATR and 2-PAM influenced the incidence of debilitation.

Methods. A simple horizontal screen test (Coughenour et al 1977, Balster, 1980) was adopted to investigate the incidence of physical incapacitation after soman poisoning. The method simply involved placing mice individually on top of a 13 cm^2 wire mesh screen which was horizontally mounted on a metal rod. The rod was rotated 180° so that the mice were then oriented upside down on the bottom of the screen. The animals were observed for their ability to climb to the top of the screen in one min. Animals were considered incapacitated if they either fell off the screen or failed to climb to the top.

Four series of mice (ICR Swiss 20-30 gms) were evaluated for survival and physical incapacitation 24 hrs after soman poisoning. The first series consisted of animals which had received only soman challenge (0.57 to 1.42 LD50). The second series consisted of animals challenged IM with 2xLD50 soman and treated IM 10 sec later with ATR (11.2 mg/kg) + 2PAM (25.0 mg/kg). The third series of animals, in addition to receiving the above challenge and treatment were pretreated orally with 0.82 mg/kg of PYR 30 minutes prior to soman challenge. The final series of mice were pretreated orally with PYR (0.20 or 0.82 mg/kg) or vehicle 60 minutes prior to challenge with a range (1.08-3.42 x LD50) of soman doses and then treated IM 10 sec later with ATR +2-PAM. The survival data and the horizontal screen test data for physical incapacitation were analyzed by using a chi-square 2x2 or 2x3 contingency test (Siegel, 1956).

Results: Fig 1 summarizes the data from the first series of animals. At doses of 0.90 LD50 and greater, at least 50% of the

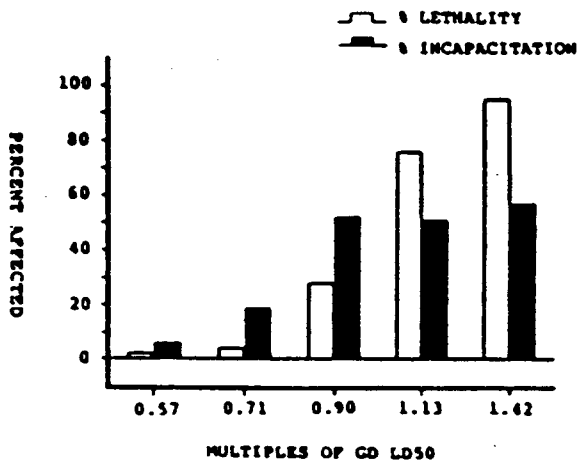

Figure 1. Effects of various dosages of soman on twenty-four hour lethality (⌐¬) and physical incapacitation (▬) in mice.

surviving animals remained physically incapacitated at 24 hrs. At 0.7, LD50s, 19% were incapacitated while 6% were incapacitated at 0.57 LD50s.

The results from the second and third series of mice are represented in Fig 2. The data shows that pretreatment with PYR (0.82 mg/kg) and treatment with ATR and 2-PAM provided significantly greater protection from soman-induced lethality (chi-square = 85.87, $p < 0.01$) and physical incapacitation (chi-square = 75.25, $p < 0.01$) than did the treatment regimen alone.

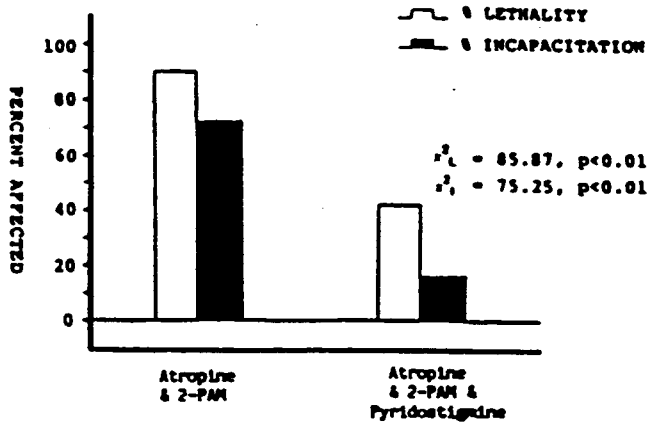

Figure 2. Effects of ATR + 2-PAM or PYR + ATR + 2-PAM on soman-induced lethality ( ) or incapacitation ( ). The results of chi-square analysis for lethality (χ^2_L) or incapacitation (χ^2_I) are shown above.

Fig 3 depicts the lethality and incapacitation data after multiple LD50s of soman in mice pretreated with PYR and/or treated with ATR and 2-PAM. Against 1.08 LD50 of soman, ATR and 2-PAM alone were effective

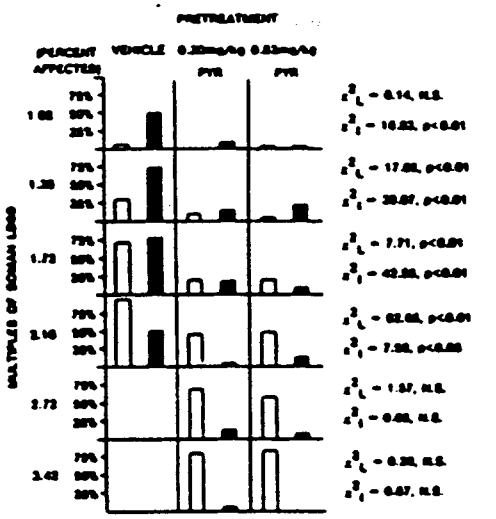

Figure 3. Effects of ATR + 2-PAM or two doses of PYR + ATR + 2-PAM against soman-induced lethality ( ) or incapacitation ( ). The results of chi-square analysis for lethality (χ^2_L) or incapacitation (χ^2_I) are shown in the figure.

in preventing lethality, but a high proportion (49%) of survivors remained incapacitated at 24 hrs. Pretreatment with PYR significantly reduced the amount of incapacitation (to 5-10%). As the challenge level of soman was increased, the efficacy of ATR and 2-PAM against both lethality and incapacitation decreased rapidly. In contrast, mice pretreated with PYR exhibited enhanced survival and continued resistance to the incapacitating effects of soman. In fact, even at soman challenge levels up to 3.42 LD50 we did not achieve incapacitation in more than 20% of surviving animals at 24 hrs. PYR (either 0.20 or 0.82 mg/kg) provided significantly greater protection from lethality and physical incapacitation over a regimen of ATR + 2-PAM administered without pretreatment. Furthermore, a comparison of 0.20 versus 0.82 mg/kg dose of PYR indicates that no further protection from either lethality or physical incapacitation is afforded by the higher dose of PYR.

Conclusions: The horizontal screen test is a useful means of rapidly assessing physical incapacitation in mice following soman poisoning. Soman produces substantial lethality and physical incapacitation in mice at 24 hrs with challenge levels of 0.90 LD50 or more. The incidence of lethality, but not of physical incapacitation, is affected by treatment with ATR and 2-PAM. On the other hand, PYR, when given as a pretreatment in conjunction with ATR + 2-PAM treatment, protects animals against both soman-induced lethality and physical incapacitation. This protection is significantly greater than that afforded by treatment with ATR and 2-PAM alone. Furthermore, no additional protection is afforded by use of dosages of PYR beyond that which results in 32% carbamylation of AChE (Koplovitz et al., 1985).

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E) RAT (2 Studies)

Study E-1 Study of Anderson, et. al.

(Located in vol. 1.4, p. 12+, of the NDA. This is a published paper, a copy of which is attached).

Dose of P = 131 ug/kg i.m. given 30 min. before Soman or VX given i.v. Atropine (16 mg/kg) and 2-PAM (100 u mol/kg) were given i.m. 30 sec. after nerve agent. Strain and gender of rats not clear, probably Sprague-Dawley.

Results were expressed as PRs (based on 24 hour LD 50s) as defined earlier. Results, shown in table 2, indicate that the PRs of atropine/PAM were not changed by pretreatment with P. (When the oxime HI-6 was used instead of 2-PAM, pretreatment with P did not change the PR of atropine/HI-6 against Soman; the PR against VX was slightly decreased [2.4 compared with 3.8 for atropine/HI-6 alone; this was said in the text to be statistically significant]). Note that results in this study were not broken down by dose of nerve agent.

P-induced AchE inhibition (whole blood, substrate = acetyl-β-methyl choline) averaged 67% at 28 min. post-dose (i.e. 2 min. before nerve agent challenge, although note that this was determined in separate groups of animals from those used in the "efficacy" study.).

The effects of P, 2-PAM, and HI-6 on AchE reactivation after dosing with Soman or VX were studied. (See p. 288 of the paper for design). It is interesting to note (Figure 1b) that significant reactivation of Soman-inhibited enzyme was seen when P and 2-PAM were given together, but no reactivation was seen when only 2-PAM was given. Since these 2 treatments had the same "efficacy" (i.e. little or none, see Table 2), it shows an apparent dissociation between efficacy and enzyme reactivation (the latter of which is claimed to be the mechanism of action for efficacy). (On the other hand, as seen in figure 2a, addition of P slightly reduced the reactivation of VX-inhibited enzyme caused by HI-6 [said to be statistically significant in the text], which corresponds to the slight decrease in efficacy caused by P [Table 2].).

Study E-2 Study of Lennox, et. al.

(Located in vol. 1.17, p. 39+, of the NDA. This is a published paper, the same as that described and included under the guinea pig studies [study B-2], above.

Various doses of P were given i.m. 30 min. prior to Soman (given s.c.); actual doses of P were not stated but were based on a preliminary study done to determine doses causing 10, 20, 30, 45, and 70% inhibition of cholinesterase at 30 min. post-dose. (The dose range of P in the preliminary study was said to be 0.005 - 0.20 mg/kg). Atropine (16 mg/kg) and mecamylamine (0.8 mg/kg) were given i.m. at 1 min. post-Soman. (Note this study differed from all other "well-controlled" studies in this section in that mecamylamine was used, and PAM was not.) Strain of animals = [AMRI : (SDXWI)]BR stock (males).

Results are shown in table II of the paper. (Note that physostigmine was also studied). Results are expressed as PRs as defined earlier. (Based on 24 hr. LD 50s). Atropine + mecamylamine alone ("therapy control" in the table) did not have a significant effect. Only the group given the highest dose of P (i.e. that predicted to cause 70% AchE inhibition at the time of Soman administration) had a PR (2.4) which was statistically significantly different from that obtained with atropine and mecamylamine alone (1.3). (The next highest dose was predicted to cause a 45% inhibition of AchE.). Results as presented were not broken down by Soman dose.

As indicated in table II, there was no statistically significant correlation between "efficacy" and the degree of AchE inhibition. The relationship between these 2 variables is shown in figure 1 (open circles). (Note that the degrees of enzyme inhibition were not determined in the animals used in the efficacy study, but were predicted values based on a preliminary study. Also note that whole blood was used for the enzyme assay; substrate was acetyl-β-methylcholine.).

ANIMAL TOXICITY STUDIES

Dr. Sparenborg's review of these studies is attached.

Since Dr. Sparenborg's review, 3 animal studies have been performed to examine the potential for toxic interaction between pyridostigmine (P), permethrin (an insecticide), and DEET (N, N-dimethyl-m-toluamide, an insect repellant). These studies were performed in response to claims of illnesses or a "syndrome" among veterans of the Persian Gulf war, and that these illnesses were possibly caused by an interaction between the P and/or permethrin and/or DEET to which the soldiers were exposed. The 3 studies are as follows:

A) Acute oral lethality study in rats

This study was performed by the U.S. Army Center for Health Promotion and Preventive Medicine and was said to be conducted under GLP regulations. Male Sprague Dawley rats were used. Initially, 14 day LD 50 values were determined for each of the 3 compounds; results are shown in Tables 1 and 2, attached. Various combinations were then tested using the calculated LD_{16} values for 2 of the compounds in combination with varying doses (0, LD_{16}, LD_{30}, LD_{50}, LD_{70}, and LD_{84}) of the 3rd compound, and % mortality at 14 days was noted and compared to the expected mortality. This design is shown in Table 3, and results with the various combinations are shown in figures 1 (varying doses of P), 2 (varying doses of permethrin), and 3 (varying doses of DEET). It was concluded that a greater-than-additive effect was seen with P + DEET (first set of bars in figure 2) and with P + permethrin (first set of bars in figure 3); it was concluded that there was no interaction between DEET and permethrin (first set of bars in figure 1; the less than expected mortality was said to be not statistically significant). Combinations containing all 3 drugs also produced greater-than-additive effects (which did not appear to significantly greater from that seen with only P + DEET or P + permethrin although a ceiling effect was likely being approached). The mechanism for the observed interactions was not studied; the sponsor speculates (no evidence presented) on various PK interactions such as increased bioavailability of P (which is poorly absorbed by the gut) by DEET (which has been used as a transdermal carrier molecule), or inhibition of the degradation of permethrin (which is hydrolyzed by non-specific esterases) by P. The following points should be considered in interpreting these results:

1) The accuracy/reproducibility of the results may be questioned based on the relatively small Ns and variability of the data seen in the calculation of the various LD_x values. For example, note the actual data in Table 1 upon which the LD 50 values (and fractions thereof) used in the interaction study were based show that lethality was not always dose-proportional or even always dose-related. For example, the calculated LD_{30} for permethrin (511 mg/kg) actually caused 5/10 deaths. Some of

the apparent interactions seen with the various combinations may be at least in part due to such discrepancies.

2) Acute lethality is clearly not a model for a "Gulf War Syndrome" or the more chronic illnesses in the veterans. Also, it is not known if the interactions observed at lethal doses, if real, can be extrapolated to the lower exposures experienced in the Persian Gulf War. (Doses of P were 30 mg t.i.d. for short periods of time, a relatively low dose. There are apparently no hard data on exposure to DEET or permethrin, although the sponsor estimates this to be relatively low, particularly around the time of P administration. [Also note that permethrin and DEET were given p.o. in the rat study, and dermally in humans]).

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Table 1: Mortality associated with phase I. Dosage levels are spaced at 0.1 log intervals for PB and DEET and 0.2 log intervals for permethrin (Perm.).

Dosage (mg/kg)	Animals Treated	Pyidostigmine Mortality	Permethrin Mortality	DEET Mortality
PB 50 Perm. 316 DEET 2000	10	4	0	1
PB 63 Perm. 511 DEET 2510	10	3	5	3
PB 79 Perm. 794 DEET 3160	10	8	5	6
PB 100 Perm. 1260 DEET 3980	10	10	6	4
PB 126 Perm. 2000 DEET 5010	10	10	6	7

Table 2: Dosage levels used in phase II based on probit analysis of data from phase I.

Probit (% lethality)	PB mg/kg	Permethrin mg/kg	DEET mg/kg
4 (16%)	45.76	279	1946
4.5 (30%)	52.59	511	2628
5 (50%)	61.36	1000	3664
5.5 (70%)	71.59	1953	5109
6 (84%)	83.28	3576	6896

Table 3: Phase II study design: Expected mortality of dosage solutions when the vehicle contains the LD_{16} (additive LD_{32}) of two compounds.

Dose	First Cmpd. LD	Second Cmpd. LD	Third Cmpd. LD	Additive Cmpds. LD
1	16%	16%	0%	32%
2	16%	16%	16%	48%
3	16%	16%	30%	62%
4	16%	16%	50%	82%
5	16%	16%	70%	>100%
6	16%	16%	84%	>100%

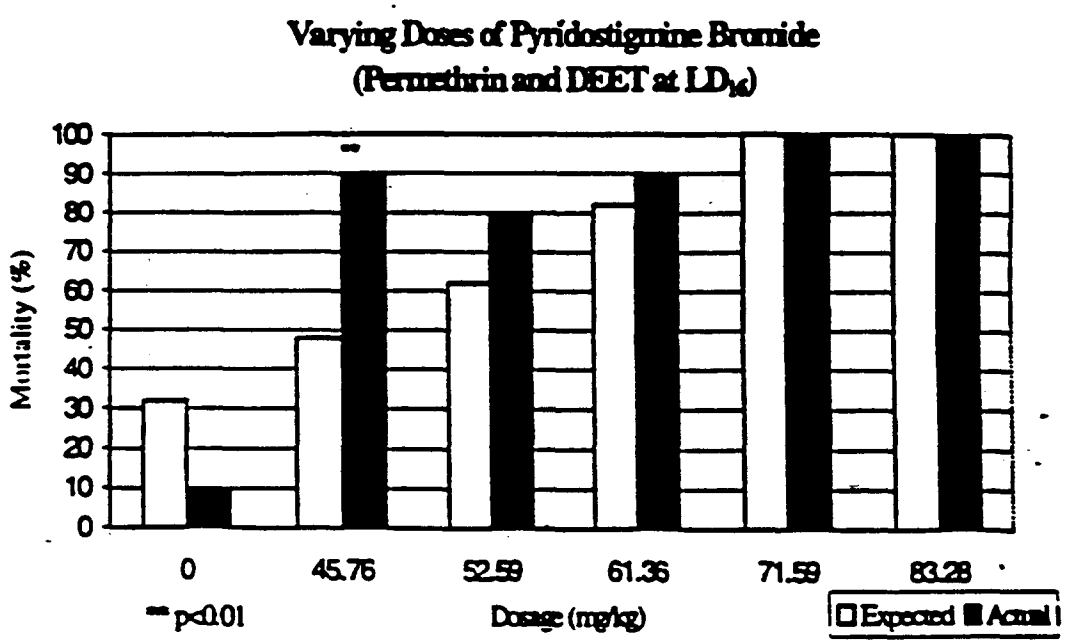

Figure 1: Response of rats given various doses of PB with LD_{16} of permethrin and DEET (additive LD_{32}) as compared to expected additive mortality. (n=10 per dose).

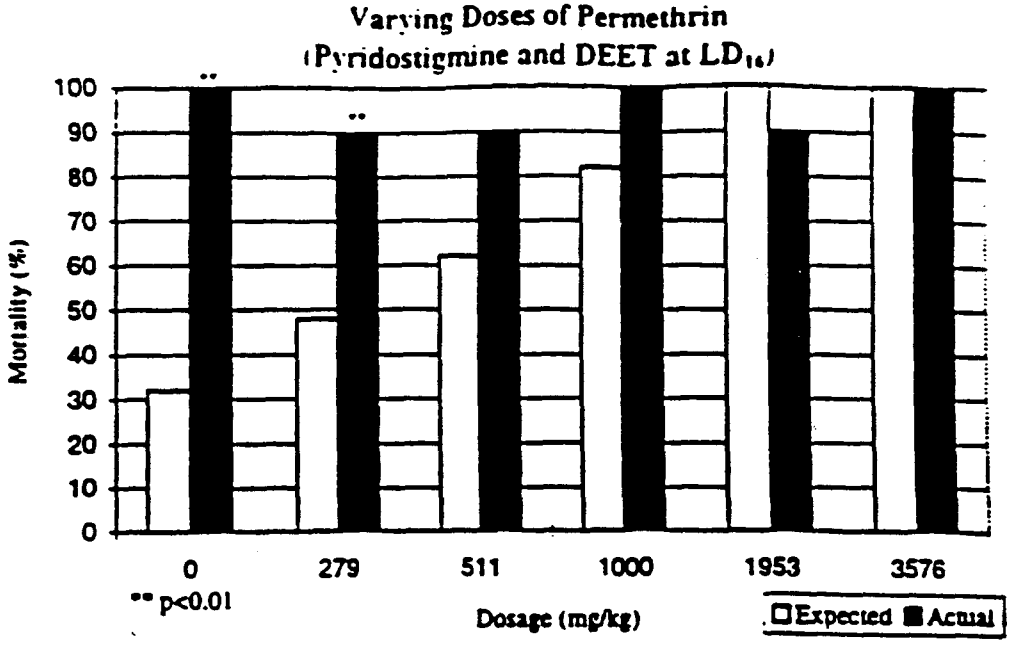

Figure 2: Response of rats given various doses of permethrin with LD_{16} of PB and DEET (additive LD_{32}) as compared to expected additive mortality. (n=10 per dose).

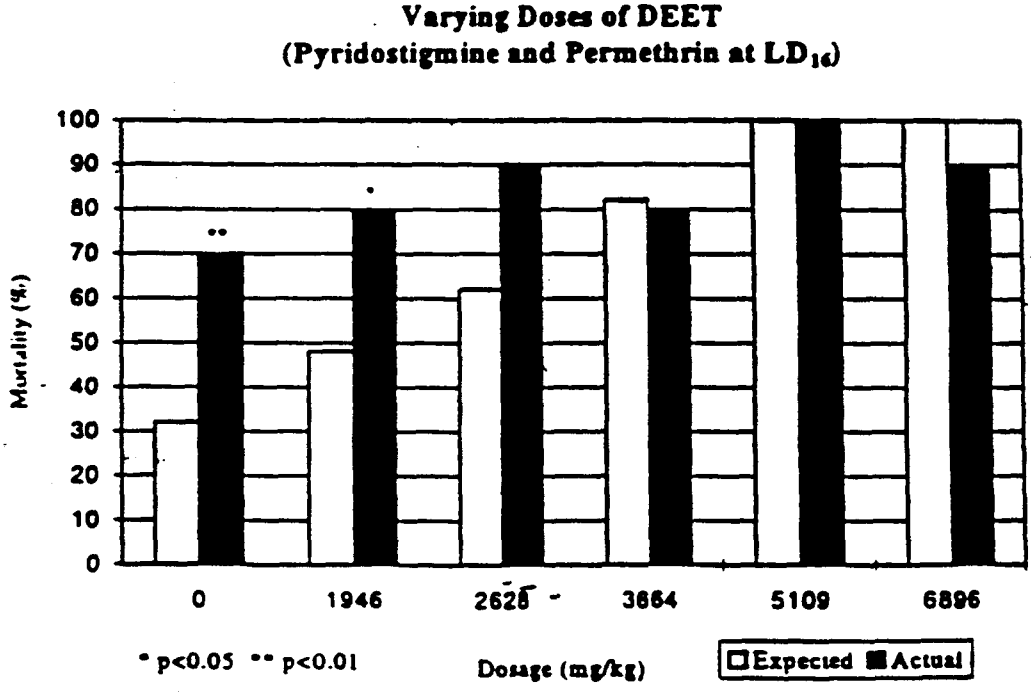

Figure 3: Response of rats given varying doses of DEET with LD_{16} of PB and permethrin (additive LD_{32}) as compared to expected mortality. (n=10 per dose).

B) Interaction study in hens

The study was performed by Abou-Donia, et.al., Duke University Medical Center; a copy of their paper is attached.

1. Three drugs, either singly or in combinations of 2 or 3, were given to hens each at a single dose level (P at 5 mg/kg p.o., DEET at 500 mg/kg s.c., and permethrin at 500 mg/kg s.c.) 5 days per week for 60 days. (These doses were based on a dose finding study in which they were said to cause "minimum changes in the clinical parameters".). Endpoints included clinical signs observed during treatment (particularly changes in motor function, e.g. motor activity, leg weakness, gait disturbances, postural changes, tremor, etc.) and histopathology of spinal cord and sciatic nerve after treatment. (Histological effects consisted of enlarged axons and, seen with the 3-drug combination only, axonal varicosities and prominent intraaxonal cytoskeletal aggregations said to be indicative of axonal damage.).

2. In general, for most of the drug combinations and most of the measured parameters, treatment with the 2-drug combos caused more adverse effect than treatment with individual drugs, and treatment with the 3-drug combo caused more adverse effect than treatment with the 2-drug combos. The authors imply that these are synergistic (as opposed to simply additive) effects, presumably because some of the effects seen with 3- and 2-drug combos were not seen with 2 drug combos and single drugs, respectively. The authors hypothesize that the mechanism for the observed interaction, at least that involving P, is a pharmacokinetic one, i.e. P inhibits the peripheral metabolism (by BuChE) of the other 2 compounds thus allowing more of them to enter the CNS and cause toxicity. No direct evidence is presented in support of this. The authors also consider pharmacodynamic hypotheses, i.e. the drugs might synergize since they have different (postulated) mechanisms of action, e.g. P by inhibiting AchE and permethrin by prolonging sodium channel opening (mechanism of DEET is apparently unknown; in this study it slightly inhibited plasma BuChE). (Incidentally, it is curious that the dose of P used in this study caused a huge inhibition of plasma BuChE [to 17% of control] yet had only minimal clinical effect; perhaps plasma BuChE is not a good indicator for nerve terminal AchE in this species).

3. The authors hypothesize that concomitant exposure to these 3 drugs (and presumably also to some of the 2-drug combinations) may be responsible for some of the symptoms seen in Gulf War veterans (particularly in those with genetically low BuChE and thus reduced insecticide-scavenging ability.). Reversibility of the effects seen in this study was not examined;

this would have been useful since the claimed neurological effects in the Gulf War vets have been lingering. (The histological effects seen in the hens might indicate a relatively long-lasting effect.).

4. As with the rat study, above, the relevance of these findings to the Gulf War veterans of course depends on their degree of exposure to these drugs.

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C) Interaction study in cockroaches

This study was performed by Dr. James I. Moss, a former research associate at the USDA in Gainesville, Florida. (It was not submitted by the sponsor; it was forwarded to us by OC 12/94). It was presented in extremely crude form and is difficult to evaluate independently. The study measured the lethality of P and DEET alone and in combination in German cockroaches. The drugs were applied topically in an acetone solution. Although not entirely clear, drug interaction was apparently evaluated by comparing the 48 hour LD_{50} and LD_{90} values of each compound given either (1) alone or (2) in combination with a non-lethal or low-lethal dose of the other compound. Results are shown in the attached table, which indicates "synergism ratios" (i.e., the number of times the LD_{50} or LD_{90} values were reduced when the drugs were given in combination) of about 2 to 6. (The "sum method" values appear to be more correct than the slightly higher "primary method" values since the latter apparently did not take into account the deaths produced by the synergist given alone).

This study is apparently non-GLP, and the question of the reproducibility of the results thus arises. It was described as "exploratory" by the investigator. It is also noted that only 48 hour lethality was evaluated because "these counts represented maximum effect of the chemicals"; one would have like to have an idea of the degree of mortality occurring beyond 48 hours and if it differed between groups; e.g., if mortality were more delayed after the drugs were given separately the degree of synergism would be overestimated.

Even when taking the conclusion of synergism at face values, it is difficult to extrapolate the result to humans at expected exposure levels. First, there is the usual problem of cross-species extrapolation, which is more acute in this case since the synergism was reported in insects. Secondly, it is not clear how interactions seen at high, lethal doses can be extrapolated to the lower exposures likely to have been encountered in humans.

It might be added that a supervisory review of this study stated that the synergism ratios between P and DEET were comparatively low, and concluded that "pyridostigmine was not a potent synergist for deet and that deet was similarly not a potent synergist for pyridostigmine". (It is also noted that a potential for interaction between P and permethrin was raised; the supervisory review stated that the cockroach data were insufficient on this point; at any rate any synergism observed would be subject to the same problems of interpretation as noted above. [It is noted that in the attached table, although there are no results for the P/permethrin combination, it can be seen that the additon of P plus permethrin to DEET resulted in synergism ratios which were equal to or lower than those obtained when only 2 of these drugs were combined]).

Table 1. The toxicity of permethrin, deet, and pyridostigmine bromide (PSB) to adult male German cockroaches when tested singly and combined.

Chemical(s)	LD-50 LD-90 Singly[a] (µg)	LD-50 LD-90 Combined[a] (µg)	Sum Method Synergism Ratio[b]	Primary Method Synergism Ratio[c]
Permethrin	0.14 (0.13-0.15) 0.20 (0.18-0.21)			
Deet	280 (240-340) 1,000 (720-1,650)			
PSB	338 (310-375) 791 (658-1,027)			
Deet + 100µg PSB	150 (120-190) 380 (270-720)	47 (33-56)[d] 92 (77-130)[d]	3.2 4.1	6.0 11.0
Deet + 200µg PSB	130 (Infinite) 1,240 (Infinite)	56 (5-82)[e] 220 (130->5,000)	2.3 5.6	5.0 4.5
PSB + 50µg Deet	325 (197-3,826) 1,064 (410->5,000)	97 (80-121)[d] 225 (162-539)[e]	3.4 4.7	3.5 3.5
Deet + 0.06µg Permethrin	180 (120-330) 800 (400->5,000)	61 (40-76)[d] 130 (100-200)[d]	3.0 6.2	4.6 7.7
Deet + 0.06µg Permethrin + 100µg PSB	190 (150-230) 850 (580-1,640)	51 (10-77)[d] 460 (220->5,000)	3.7 1.8	5.5 2.2

[a] Confidence limits at 95% level of probability in parentheses.
[b] Ratio is LD value of sum mortality of two or three chemicals tested singly divided by LD value of chemicals combined.
[c] Ratio is LD value of the primary chemical (underlined) divided by the LD value of the chemicals combined.
[d] Significantly different from the LD value for the primary chemical alone and the LD value for the primary (underlined) and secondary chemicals tested singly.
[e] Significantly different from the LD value for the primary chemical.

28a

SUMMARY

1) Results of "Efficacy" Studies

The sponsor has identified 15 animal studies considered to be "well-controlled" in support of the efficacy of pyridostigmine (P) as a prophylactic agent for the prevention of nerve agent-induced lethality. In general the design of these studies involves pre-treating the animals with P, followed by administration of nerve agent, followed by treatment with atropine + 2-PAM; survival is then measured at 24 or 48 hours post-dose. (Effect on survival usually expressed as a "protective ratio" ["PR"], i.e. the LD 50 of the nerve agent in the presence of the treatment regimen to that in its absence. In a few studies, only single, lethal doses of nerve agent were given.). The contribution of P to the above treatment regimen was evaluated by comparing the effect of the above regimen to that of atropine + PAM alone. (Atropine and PAM are already used in humans as a treatment for organophosphate cholinesterase inhibitor-induced poisoning: atropine to block the effect of excess acetylcholine at the cholinergic receptor, and PAM to reactivate the inhibited enzyme). (One rat study [#E-2], used the ganglionic blocking agent mecamylamine instead of PAM). (Note that in several of the "well-controlled" studies a vehicle control group for P was often not used. It is unlikely, however, that administration of vehicle would have a significant effect on the endpoint measured, i.e. lethality. [The sponsor's use of the term "well-controlled" refers to the use of an atropine/PAM only group to which to compare the effects of P + atropine/PAM , to distinguish these studies from those which only evaluated the effect of the entire regimen.]).

The "well-controlled" studies included 2 studies in rhesus monkeys, 5 in guinea pigs, 3 in rabbits, 3 in mice, and 2 in rats. In most of these studies the nerve agent used was Soman; this was the sponsor's primary interest since animal studies have shown this particular agent to be relatively resistant to atropine + PAM treatment. Other nerve agents (Sarin, VX, and Tabun) were used in some studies.

Results of these "well-controlled" studies are summarized in the attached table.

"WELL-CONTROLLED" STUDIES			
SOMAN	SARIN	VX	TABUN
+ (A-1)	0 (B-5)	0 (B-5)	+ (B-5)
+ (A-2)	+ ? (C-3)	0 ? (C-3)	0 (C-1)
+ (B-1)	0 (D-2)	- (D-2)	+ ? (C-3)
+ (B-2)		0 (E-1)	+ (D-2)
+ (B-3)			
+ (B-4)			
0 (C-1)			
+ ? (C-2)			
+ ? (C-3)			
+ (D-1)			
+ (D-3)			
0 (E-1)			
+ (E-2)*			

A = monkey
B = guinea pig
C = rabbit
D = mouse
E = rat

+ = protective effect of P shown
0 = no effect of P shown
– = P decreased protective effect of atropine + PAM
? = effect shown was equivocal

after letter refers to specific study discussed earlier in this review.
* mecamylamine used in place of PAM

P was clearly effective against Soman in monkey (2 of 2 studies) and guinea pig (4 of 4 studies). In monkey study A-1, P increased the PR of atropine/PAM alone from 1.64 to > 40. In monkey study A-2, P increased the PR of atropine/PAM alone from 1.4 to 28. Thus, adding P increased the PR of atropine/PAM 20 fold or more in these studies. The effect in guinea pigs was more modest: P increased the PRs of atropine/PAM alone approximately 2-4 fold (from ~ 1-3 to ~ 4-7). In contrast, effects in rabbits, mice, rats were inconsistent and/or small; where effects were seen the PR of atropine/PAM (or in the case of rat study E-2 of atropine/mecamylamine) was generally increased less than 2 fold (from ~ 1-2 to ~ 2-3). According to the sponsor, PRs in the range of 2-3 are an "unsatisfactory" level of effectiveness since they will "not provide protection against the up to 10 LD 50 experienced by the majority of troops under attack." (Volume 2.5 [Sponsor's volume number 2.47] p.47; based on sponsor's estimates of exposure to nerve agents).

In view of the fact that survival in the above-noted studies was only evaluated through 24-48 hours post-nerve agent administration, we requested (meeting of 4/6/95) that the sponsor submit any available data on longer-term outcome; such information was subsequently submitted for the monkey studies. For study A-1, the majority of monkeys alive at 48 hr. post-Soman were still alive at 1 week, either appearing normal or displaying various toxic signs. Fifteen P-treated monkeys were kept for further studies and have survived for at least several additional years generally in good health. In monkey study A-2, phases III and IV, where monkeys were given a single 5 LD 50 dose of Soman, animals were monitored through 10 days post-Soman; a majority of the animals alive at 48 hours were still alive at 10 days, either appearing normal or displaying various cholinergic signs. However, in phase V of this study (which used a PR paradigm) most of the P-treated monkeys which were alive at 48 hours had died by 10 days post-Soman.

The data on the other 3 nerve agents tested (Tabun, Sarin, and VX) were too minimal to make a firm conclusion regarding the effects of P. One paper (guinea pig study B-5/mouse study D-2) did show a small but statistically significant benefit of P against Tabun (atropine/PAM PR raised from 4.4 to 7.8-12.1 is guinea pigs and from 1.3 to 1.7-2.1 in mice); a study in rabbits also showed an equivocally positive effect. One study in mice (D-2) showed that P statistically significantly reduced the PR of atropine/PAM against VX from 7.8 to 3.9. (The PR of 3.9 was statistically significant different from untreated controls, i.e. there was still a net protection.). This was only seen at the higher dose of P used (estimated to cause 60% cholinesterase inhibition); it was not seen at a dose of P estimated to cause 30% cholinesterase inhibition.

The following points should be considered in the evaluation of the above-discussed "well-controlled" studies:

a) Aside from the monkey studies, the studies were non-GLP, primarily published studies, generally containing only summary results (e.g., only PRs given without showing lethality results broken down by nerve agent dose or individual animals), and sometimes lacking clear description of statistical methods or clear indication of which results were considered statistically significant.

b) The routes of nerve agent administration (various parenteral routes used) are not the same as anticipated in humans, which are presumably dermal and inhalation. The different tissue distribution and time course expected with different routes of administration would likely lead to a different pattern of toxic effects (e.g. see descriptions of sequence of events following vapor vs dermal exposure in volume 2.5 [sponsor's volume # 2.47], p. 35-36). These different patterns could be differentially susceptible to the effect of P. (Also note that the benefits of P were only shown with lethal doses of nerve agents. There are some animal data summarized in the NDA that suggest that P produces additive effects to sublethal doses of nerve agents.).

c) The route of P administration in several studies was i.m., which is not the route proposed for humans (p.o.).

d) No data were presented on the bioavailability of the high doses of nerve agents used. If bioavailability decreased with increasing doses of nerve agent, the increases in PRs due to P would be overestimated.

Several "supporting studies" were submitted (summarized in vol 1.3, p. 43+) which support the conclusion that P is effective as a prophylactic treatment for Soman-induced lethality in monkeys and guinea pigs. (In these supporting studies the effect of P was not separable from that of atropine + PAM; the conclusion of the efficacy of P rests on the historical lack of significant effect of atropine/PAM alone against Soman poisoning).

2) Species Differences In "Efficacy" Against Soman

As noted above, P was effective against Soman-induced lethality in rhesus monkeys, and to a lesser degree in guinea pigs, but had little or no effect in mice, rats, and rabbits. The sponsor has posited 2 hypotheses to explain these differences:

a) Differences in levels of carboxylesterase

The sponsor's argument (vol 1.3, p. 98-99) is that this enzyme detoxifies Soman and that species differences in the activity of this enzyme is the determinant of species differences of the ability of P to increase the PR of animals exposed to Soman and treated with atropine + PAM. It is stated that monkeys and humans have little or no carboxylesterase activity, and thus should respond similarly to the protective effects of P.

The paper cited in support of the conclusion that species differences in carboxylesterase activity is a determinant of the efficacy of P is attached (Maxwell,

et.al.). Three species were used: guinea pig, rat, and rabbit. As noted, P was given to each species (at a dose calculated to cause 70% inhibition of "blood AchE") 25 min. before Soman; atropine was given 15 min. before Soman. (Note this is different from the protocols used in the "well-controlled" studies discussed earlier, where atropine [+PAM] was given after Soman). PRs found were 2.1, 5.2, and 6.1 in rat, guinea pig, and rabbit, resp. (Table 1). (Note that since there was apparently no atropine-alone group, the effect of P cannot be distinguished from that of atropine). (Also note the PR of 6.1 for rabbit is higher than those seen in the "well-controlled" rabbit studies discussed above). When animals were pretreated with CBDP, an inhibitor of carboxylesterase, the protective ratios were higher and more similar across species, i.e. 8.5, 10.0, and 11.4 in rat, guinea pig, and rabbit, resp. (Table 2.). (Note, table 2, that this was primarily due to a decrease [compared to non-CBDP-treated animals, Table 1] in the Soman LD 50 values in non-P/atropine pretreated animals). (In another paper by Maxwell [vol 1.17, p. 46+] a similar study was done in mice; the PR against Soman for P + atropine pretreatment in CBDP-treated mice was about 10; although a group of mice not given CBDP was not used for comparison, this PR value is much greater than that seen in other studies in mice).

There are several uncertainties in the above argument (i.e. concerning the role of species differences in carboxylesterase activity as an explanation for the species differences in sensitivity to the efficacy of P):

1) No data were presented to show that the stated species differences in carboxylesterase activity exist; the references cited did not contain this data.

2) Regarding the Maxwell et.al. study discussed above, in order to conclude that the observed species differences were due to differences in carboxylesterase activity, it would have to be known that the carboxylesterase inhibitor used did not have other actions which might have explained the results obtained. (It apparently did not inhibit AchE, as shown in the paper).

3) The Maxwell study did not use monkeys; this seems to be an important missing link in the argument regarding species differences. If monkeys are more susceptible than other species to the effects of P because they have low carboxylesterase levels, an inhibitor of carboxylesterase should have less of an effect in enhancing the efficacy of P in this species. (A lack of such an effect in monkeys would also support the conclusion that the P-enhancing effect of the carboxylesterase inhibitor seen in the other species was indeed due to such inhibition and not to some other action of the inhibitor.)

4) On theoretical grounds, it is not clear why the level of carboxylesterase activity would be a determinant of the efficacy of P. Carboxylesterase

presumably decreases effective plasma levels of Soman. Thus the effect of differing degrees of carboxylesterase activity is presumably a pharmacokinetic one with respect to Soman, i.e., it should result in differences in Soman plasma levels with a given dose, but not necessarily in differences in Soman plasma levels associated with the Soman LD 50. In other words, all else being equal, species with high carboxylesterase activity will need a greater dose of Soman to reach its LD 50 than species with low carboxylesterase (or in which carboxylesterase has been inhibited). Assuming plasma levels of Soman are related to its lethality, and P protects against this lethality by interfering with the effect of Soman on AchE, it is not clear why P would not give the same "protective ratio" in species with high carboxylesterase activity as in species with low activity (all else being equal); in the former case a proportionately higher dose of Soman would have to be given in the presence of P to achieve plasma levels sufficient to overcome the effect of P (i.e. the amount of Soman needed in plasma to overcome the effect of P should not depend on carboxylesterase activity). (As a simple illustration, assume species "A" has twice the carboxylesterase activity as species "B"; all other things being equal it is not clear why the LD 50 of Soman in species A would not be twice as great as that in species B in both non-P-treated and P-treated animals).

5) It is noted that in the Maxwell study, the effect of P was not separable from that of atropine. Thus, it cannot be ruled out that the efficacy-enhancing effects of the carboxylesterase inhibitor were due to enhancement of atropine rather than of P.

6) It is noted that several of the above points were mentioned to the sponsor at the meeting of 4/6/95, but were not addressed in the NDA.

b) Differences in decarbamylation rates

The sponsor's argument (vol. 1.3 p. 95-98) is that the rate of decarbamylation of P-inhibited AchE is a determinant of the efficacy of P. (The mechanism of action of P is hypothesized to be temporary carbamylation of the active site of AchE thus protecting it from more permanent phosphonylation by nerve agents; however, decarbamylation of the

enzyme must occur to provide enough active site for survival). It is claimed that the species order of decarbamylation rates parallels the species order of efficacy of P in protecting against Soman induced lethality. Monkeys and humans are said to have the fastest rates, and thus humans should respond to P as do monkeys. The data presented on decarbamylation rates are as follows:

1) Abstract of Harris, et.al. (vol 1.7, p. 148, attached)

As indicated, whole blood was incubated with P at a concentration of ~ 5 x 10^{-5} M, said to achieve ~70% AchE inhibition. RBCs were washed and assayed for AchE at various times. Half-times for "spontaneous decarbamylation" were approx. 39, 42, 48, and 63 min. for human, marmoset, guinea pig, and rat, resp. The abstract concludes that the half times for the non-human species "appear to be inversely related to the reported efficacy data." However, note the relatively large 95% CL; the only real difference in means appears to be between rat and the other species; also note that marmosets were not used in any of the "well-controlled" studies discussed earlier. Also note that the method used for AchE activity was not stated.

2) Paper of Ellin and Kaminskis (vol 1.6, p. 93+, attached)

In one experiment, whole blood was incubated with P (to give ~ 3-5 x 10^{-7} M in plasma fraction) after which RBC were washed and AchE activity determined at various times. Results (Table 3) show $T^{1/2}$ values which do not clearly correlate with the efficacy of P across species (31, 34, 46, 32, and 42 min. in human, monkey, guinea pig, rabbit, and rat, resp.).

In another experiment whole blood was incubated with P (to give 10^{-7} M in plasma fraction) but in contrast to the above, P was not washed out prior to assay of RBC for AchE. In this situation, P can re-carbamylate the enzyme after decarbamylation, so that the calculated rate of decarbamylation is only an "apparent" one. Results are shown in figure 1 and Tables 1 and 2. (The latter used 2 different methods of computation; results were similar). The calculated carbamylation rates do correlate with the efficacy of P across species, i.e. $T^{1/2}$ (Table 1) = 4, 5, 10, 14, and 31 hr. in human, monkey, guinea pig, rabbit, and rat, resp. (As noted in figure 1, the species with the fastest rates of enzyme recovery, human and monkey, also had the lowest degree of peak inhibition, raising the possibility that the faster rate of recovery was due more to a low rate of re-inhibition rather than to a faster rate of decarbamylation. This experiment would have been more useful if equal degrees of maximal inhibition were achieved across species).

3) Paper of Wetherell and French (vol 1.19 p. 166+, attached)

This paper studied decarbamylation rates of physostigmine-inhibited AchE in humans, monkeys, and guinea pigs. The approach was similar to that in the above paper; paradigms in which physostigmine either remained present in the incubation medium or was washed out/diluted were used. As shown, rates of enzyme recovery were fastest in humans followed by monkeys followed by guinea pigs. However, note that the relative efficacy of physostigmine in monkeys vs guinea pigs has apparently not been studied; thus it is not known if these rate differences correspond to differences in efficacy. Also, it would have been useful to study enzyme regeneration rates in rats, mice and rabbits which, if they are resistant to the efficacy of physostigmine as they are to that of P, would have provided stronger correlative data.

There are several uncertainties regarding the above argument (i.e. that species differences in the rate of decarbamylation of P-inhibited AchE account for species differences in the efficacy of P):

1) The data showing species differences in rates of enzyme regeneration after inhibition by P are rather limited, and not all of the studies showed such differences.

2) The studies presented examined enzyme activity in plasma and RBC; it is not known if the species differences seen are the same at nervous system sites critical for the efficacy of P.

3) The paradigms used in the above studies do not necessarily reflect enzyme regeneration rates in vivo. In one paradigm a relatively constant concentration of P was present in the medium, allowing for re-carbamylation of the enzyme. In another paradigm the P was washed out prior to measuring enzyme regeneration. The actual situation in vivo would be in between these two extremes, i.e. where the concentration of P is changing over time; this will differ between species depending on the PK profile of the drug. Also, in addition to species differences in intrinsic decarbamylation rate as a kinetic parameter, differences in carbamylation rate should also affect the time course of enzyme inhibition and regeneration observed in vivo.

4) Even if species differences in the rate of enzyme regeneration in the nervous system after administration of P occur, it still is not well-established if or how this plays a role in its efficacy. For example, one study (Harris et.al., vol 1.7, p. 65+) examined a

serious of homologs of physostigmine in guinea pigs; each compound was given at a dose sufficient to inhibit blood AchE by 70% at the time of Soman administration. The homologs were inferior to physostigmine in protecting against Soman-induced lethality. Since a slower rate of recovery of enzyme activity (in non Soman-treated animals) following the peak 70% inhibition had been shown to occur after administration of the homologs, the authors considered this to be evidence in support of the hypothesis that faster decarbamylation rates result in increased efficacy, and that "for successful efficacy the time frame for spontaneous decarbamylation probably lies between 0.5 and 2 hr." However in another study (Murphy et.al., vol 1.17, p. 67+), P was apparently effective ("apparently" because the effect of P was surmised from historical lack of effect of atropine + PAM with which it was given) in preventing Soman-induced lethality in rhesus monkeys when the P was given via implanted osmotic pumps designed to cause a constant amount of P-induced serum cholinesterase inhibition (~ 40%) from 4 days prior to Soman up to 2 hr. post-Soman exposure. It has also been hypothesized, from the results of studying the efficacy of a series of cholinesterase inhibitors against Soman in guinea pigs, that a decarbamylation rate of P-inhibited enzyme which is too fast will lead to loss of efficacy since it "might leave the enzyme exposed to Soman which had not yet been detoxified or excreted." (Berry and Davies, NDA vol 1.4, p. 39+).

Thus the protection of acetylcholinesterase from Soman by P is likely to be affected by several parameters which could differ across species, including intrinsic carbamylation and decarbamylation rates and the PK profile of P (especially in relation to the PK of the nerve agent). Also, both carbamylation and decarbamylation rates can be affected by PAM (Dawson, Neurochem. Int. 26(6): 643-54, 1995; cited in IND submission of 4/11/96); this effect might also vary across species. Even if species differences in all of these parameters (in the nervous system) were well-established, as noted above it has not been well worked out how or to what degree these parameters affect efficacy against nerve agent-induced lethality.

5) It is noted that several of the above points were mentioned to the sponsor at the meeting of 4/6/95, but were not addressed in the NDA.

3) Correlation of Efficacy of P against Soman-Induced Lethality with P-Induced AchE Inhibition

An evaluation of this correlation is important since P-induced RBC AchE inhibition is being proposed by the sponsor as a "surrogate marker" for efficacy. It is proposed that the inhibition of AchE in the peripheral nervous system by P (P is a quaternary N compound which presumably doesn't enter the CNS in significant amounts) is the first step in the mechanism of action by which it antagonizes Soman-induced lethality. It is hypothesized that such inhibition, which is reversible (involving carbamylation of the active site of the enzyme followed by spontaneous decarbamylation) protects a critical portion of the enzyme from the relatively permanent inhibition caused by Soman (which phosphonylates the active site) and that the amount of enzyme activity thus protected is adequate to allow for survival (provided atropine + 2-PAM are also given). (A diagram of the interactions of acetylcholine, neostigmine, and DFP with the active site of AchE is attached. [from Goodman + Gilman, 7th edition]. The mechanism of interaction of neostigmine and DFP with the active site is analogous to that for P and organophosphorus nerve agents, resp. The page following shows the chemical structures of the nerve agents studied, as well as those of atropine, 2-PAM, and P).

The attached table lists the "well-controlled" studies done with P. The study numbers correspond to those described earlier. For each study, the % AchE inhibition caused by P at the time of Soman administration is shown. If more than one dose of P was used, the % of AchE inhibition caused by each is shown. A * next to the % inhibition means that the inhibition was estimated from results in separate groups of animals in the study, i.e. animals different from those given Soman. A ** next to the % inhibition means that the inhibition was estimated from data from other studies. No asterisk means that the inhibition was measured in the same animals given Soman (i.e. the most reliable situation). Note that the method used to measure AchE varied from study to study, e.g. regarding substrate used, tissue sampled (e.g. RBC vs whole blood), etc., and in most cases the methods were not described in detail. It is not always clear if the assay was specific for AchE (e.g., vis a vis butyrylcholinesterase). This should be kept in mind when comparing % inhibition across studies. Also shown in the table are whether P was effective (+) or not effective (0); a ? means the result shown was equivocal. An indication of the magnitude of the efficacy seen is given as a protective ratio (PR) or survival fraction (SF) as defined in the legend to the table.

AchE Inhibition Due to Pyridostigmine in "Well-Controlled" Studies of the Efficacy of Pyridostigmine Against Soman					
SPECIES	STUDY#	% AchE INHIBITION	EFFICACY	PR	SF
Monkey	A-1	23	+	>25	
		37	+	>25	
Monkey	A-2	0.5‡	+		8/10
		4‡	+		8/10
		5-7‡‡	+	21	
		9‡	+		9/10
		26‡	+		7/10
Guinea Pig	B-1	40*	+	2.5	
Guinea Pig	B-2	10*	0		
		20*	0		
		30*	0		
		45*	0		
		70*	+	3.6	
Guinea Pig	B-3	3*	0		
		5*	0		
		16*	+	1.5	
		26*	+	1.9	
		34*	+	1.5	
		52*	+	1.5	
		72*	+	1.5	
		79*	+	1.9	
		82*	+	1.8	
Guinea Pig	B-4	not measured or estimated	+	4.0	

(Table continued on next page)

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AchE Inhibition Due to Pyridostigmine in "Well-Controlled" Studies of the Efficacy of Pyridostigmine Against Soman					
SPECIES	STUDY#	% AchE INHIBITION	EFFICACY	PR	SF
Rabbit	C-1	20-40*	0		
Rabbit	C-2	30-70**	+?	1.4	
Rabbit	C-3	20-40**	+?	1.5	
Mouse	D-1	30-70**	+	2.5	
Mouse	D-3	not measured or estimated	+	~2	
Rat	E-1	67*	0		
Rat	E-2	10*	0		
		20*	0		
		30*	0		
		45*	0		
		70*	+	1.8	

PR Protective Ratio (Soman LD 50 with pyridostigmine/atropine/PAM treatment ÷ Soman LD 50 with atropine/PAM treatment. [Study E-2 used mecamylamine instead of PAM].).

SF Survival fraction after a single lethal dose of Soman in groups given pyridostigmine/atropine/PAM. (Survival in groups given atropine/PAM alone was zero).

‡ value after subtraction of 3.0% inhibition seen in non-P treated controls.

‡‡ control value was 0%

*, ** see text for explanation of these and other symbols

Within the limitations of the data presented as noted above, the following can be seen in this table:

a) In monkeys there was no minimum level of AchE inhibition below which efficacy could not be demonstrated, i.e. efficacy was seen even in the absence of demonstrable enzyme inhibition.

b) In guinea pigs, results across studies were extremely variable, with the minimum level of AchE inhibition associated with efficacy ranging from 16 to 70%. (Although note that very slight, non-statistically significant effects on PR were seen at levels of inhibition below 70% in study B-2).

c) In rabbits, mice and rats it is difficult to make firm conclusions regarding levels of AchE inhibition associated with efficacy since efficacy data were limited and showed inconsistent results; also in several studies AchE inhibition was not measured but estimated from that seen in other studies. However, even in studies where efficacy was seen it was rather minimal in degree as discussed earlier, despite measured or estimated AchE inhibition of up to 70%.

Aside from the question of the minimum degree of AchE inhibition associated with efficacy, some of these "well-controlled" studies used more than one dose of P, and thus the relationship between the degree of P-induced enzyme inhibition at the time of Soman administration and degree of efficacy can be examined. The following are noted:

a) No relationship was seen in the monkey studies. In study A-1, doses causing either 23 or 37% mean enzyme inhibition caused identical efficacy. (Note that it is technically more correct to conclude that this study failed to provide evidence for a relationship between enzyme inhibition and efficacy than to conclude that evidence was provided for no relationship since higher doses of Soman, which could not be used due to practical limitations, could theoretically have distinguished between the efficacy of the two doses of P if such a difference existed). There was also no relationship in study A-2: doses causing mean AchE inhibition ranging from 0 to 26% caused identical efficacy.

Since, in the monkey studies, the degree of AchE inhibition was measured in the same monkeys given Soman in the efficacy studies, an examination of the correlation between enzyme inhibition and protection from lethality is possible in individual animals. In study A-1 such an examination is difficult because so few P-treated animals died. In this study, the 1 animal that died in the low-dose P group did have the lowest degree of AchE inhibition at the time of Soman exposure in this group; however the degree of AchE inhibition in the 3 animals which died in the high-dose P group was similar to that in animals which

survived. (data shown under description of study, earlier). In study A-2, examination of the individual data showed no clear correlation between P-induced AchE inhibition and survival, although in phase IV (intragastric administration of P) of that study, the 2 (of 10) monkeys which died by 48 hours had the highest pre-Soman enzyme inhibition. (Data shown under description of study, earlier).

b) In guinea pigs, dose response data were obtained in studies B-2 and B-3. In study B-2 the only statistically significant increase in PR due to P was at a P dose predicted to cause 70% enzyme inhibition. A "Spearman rank correlation procedure" showed a statistically significant association between predicted degree of enzyme inhibition and PR over the range of inhibition studied (10-70%) (See Table III from this study, attached; entire paper included earlier with description of study B-2).

In study B-3, the correlation between predicted enzyme inhibition (which ranged from 3-82%) and degree of efficacy was not statistically significant by linear regression analysis. (See Table I from this study, attached; entire paper included earlier with description of study B-3). (As discussed in the paper, modeling of the data by Response Surface Modeling, which uses predicted "optimal" doses for atropine and PAM, results in a correlation between P-induced AchE inhibition and efficacy).

In guinea pig study B-1 only one dose level of P was used. However, some information concerning the relationship between AchE inhibition can be obtained from the results with ondansetron, which was given at the same time as P in some groups. As described in more detail in the description of study B-1 earlier, ondansetron did not alter the efficacy of P despite that fact that AchE inhibition with ondansetron + P was greater than that with P alone. Although the difference was relatively small (50-55% vs 40% inhibition), it does not support an association between AchE inhibition and protection from Soman-induced lethality.

c) In mouse study D-3 two doses of P were used (HD = 4x LD); these caused equal efficacy (although AchE inhibition was not measured or estimated in this study).

d) In rat study E-2 doses of P were used which were predicted to cause a range of inhibition of AchE of 10-70% at the time of Soman administration. The dose of P causing 70% enzyme inhibition was the only one causing a statistically significant increase in PR; however, a "Spearman rank correlation procedure" failed to show a statistically significant correlation between predicted degree of enzyme inhibition and PR over the range of inhibition studied (10-70%). (See Table II from this study, attached; entire paper included earlier with description of Study E-2).