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EVALUATION OF SEVERAL OXIMES AS REACTIVATORS OF UNAGED SOMAN-INHIBITED WHOLE BLOOD ACETYLCHOLINESTERASE IN RABBITS*

LARREL W. HARRIS,† DANA R. ANDERSON, WILLARD J. LENNOX, CLAUDE L. WOODARD, ANITA M. PASTELAK and BETH A. VANDERPOOL

Applied Pharmacology Branch, US Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD 21010-5425, U.S.A.

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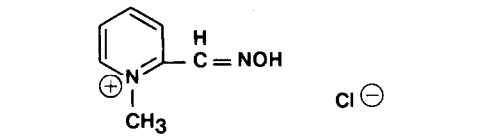
Abstract—The antidotal benefit of oximes against organophosphorus (OP) anticholinesterase intoxication is thought to be due to reactivation of the OP-inhibited acetylcholinesterase (AChE). This study was conducted to determine whether the antidotal efficacy against soman by the oximes 2-hydroxyiminomethyl-3-methyl-1-[2-(3-methyl-3-nitrobutyl oxymethyl)]-imidazolium Cl (ICD 467) and 1,1′-methylenebis[4-(hydroxyiminomethyl) pyridinium] di-Cl (MMB-4) resulted, in part, from reactivation of the inhibited AChE. These oximes were tested in parallel with pralidoxime Cl (2-PAM) and 1-(2-hydroxyiminomethyl-1-pyridinio-3-(4-carbamoyl-1-pyridinio)-2-oxapropane di-Cl (HI-6). Rabbits were atropinized (8 mg/kg, i.m.) and intoxicated with soman (13 μg/kg, i.v.; 1.2 × LD_{50}) 5 min later. Three minutes after soman, animals were treated with oxime (50, 100 or 150 μmol/kg, i.m.). Whole blood was collected from a catheter in the central artery of the ear just before soman, at 2 min after soman and at 2, 5, 10, 15, 30, and 60 min after oxime or vehicle for determination of AChE activity. Shortly thereafter, animals were anesthetized and exsanguinated with immediate flushing using heparinized saline. AChE activity was also determined on the cortex, medulla-pons and diaphragm to assess central and peripheral reactivation. Treatment with HI-6 or MMB-4 (50 μmol/kg, i.m.) resulted in significant ($P < 0.05$) reactivation of soman-inhibited whole blood AChE and diaphragm cholinesterase (ChE), but not brain AChE. In contrast, 2-PAM was completely ineffective in reactivating soman-inhibited AChE. HI-6 was significantly better than MMB-4 in reactivating blood AChE; they were essentially equal against soman-inhibited diaphragm ChE. Three animals exposed to soman and treated with ICD 467 died within 15 min. When animals not exposed to soman were treated with ICD 467 (25 μmol/kg, i.m.), whole blood AChE activity was depressed by 60% within 5–10 min after treatment. Furthermore, ICD 467 failed to reactivate significantly unaged soman-inhibited erythrocyte AChE, *in vitro*. These observations indicate that ICD 467 would be contraindicated as a therapy for anti-ChE intoxication and that the efficacy of HI-6 or MMB-4 can be explained, in part, by reactivation of soman-inhibited AChE.

Atropine sulfate and pralidoxime Cl (2-PAM) are available as antidotes for medical treatment of accidental organophosphorus (OP) insecticide exposure and are fielded as self-aid for the soldier who is exposed to nerve agents. Atropine, an antimuscarinic agent, when given before or after anticholinesterase intoxication, is effective by preventing excess acetylcholine (ACh) from producing its effects at all muscarinic cholinergic junctions. In contrast, oximes reactivate the inhibited cholinesterase. The effectiveness of atropine and oxime therapy depends on the anticholinesterase and the time lapse between poisoning and therapy [1]. 2-PAM is of little value in case of exposure to soman [2] because the phosphonylated acetylcholinesterase (AChE) rapidly becomes resistant to reactivation [3], a phenomenon called "aging". Even when 2-PAM is administered before aging is complete, no significant protection is afforded, probably because a high concentration of oxime is necessary to inhibit aging and reactivate the inhibited enzyme [4].

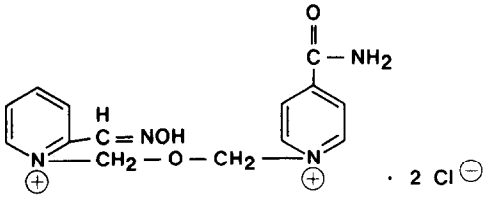
In an effort to improve therapy against soman, a batch of oxime, 1,1′-methylenebis[4-(hydroxyiminomethyl) pyridinium] di-Cl (MMB-4) [5], was synthesized in-house in the mid-seventies and later tested against soman; in the mouse screen, atropine and MMB-4 therapy provided significant protection against soman intoxication.‡ Also in the mid-seventies, a new class of oximes (H-oximes) was synthesized by Hagedorn [6]. Of these oximes, 1-(2-hydroxyiminomethyl-1-pyridinio -3- (3-carbamoyl-1-pyridinio)-2-oxapropane di-Cl (HS-6) and 1-(2-hydroxyiminomethyl-1-pyridinio -3- (4-carbamoyl-1-pyridinio)-2-oxapropane di-Cl (HI-6) have been shown to have therapeutic benefit against soman [7–9]. Since rather large doses of HI-6 have been used in previous efficacy studies and since HI-6 may not be sufficiently stable in aqueous solutions for field use, the search for better oximes has continued. In recent studies, the imidazolium oximes [e.g. (ICD 467)] have been shown to be more efficacious than 2-PAM against intoxication by soman [10, 11]. It is not known whether the antidotal benefit of ICD 467 or MMB-4 is due to reactivation of the soman-inhibited AChE, reversible inhibition of the AChE,

† Correspondence: Mr. Larrel W. Harris, Applied Pharmacology Branch, US Army MRICD, Aberdeen Proving Ground, MD 21010-5425.

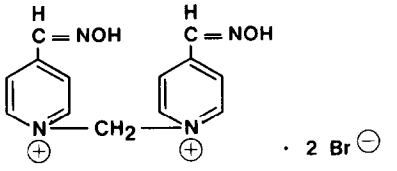
‡ Lennox WJ and Sultan WE, unpublished results.



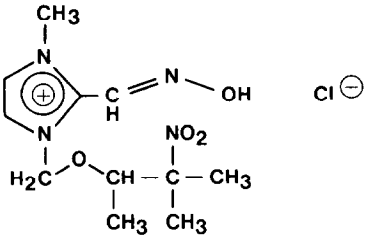
2-PAM



HI-6



MMB-4



ICD 467

Fig. 1. Structures of oximes tested.

or a combination of both. Thus, this report covers protection of the AChE and/or reactivation of the inhibited AChE by ICD 467, HI-6, MMB-4 and 2-PAM in rabbits intoxicated with soman. The structural formulas of the four oximes studied are given in Fig. 1.

MATERIALS AND METHODS

Materials. Male Hra:(NZW) SPF rabbits (2.8 to 3.2 kg) were used. The rabbits were housed singly in temperature–humidity-controlled quarters and were maintained on a 12-hr dark–light cycle with lights on at 6:00 a.m. Food and water were provided *ad lib*. Male mice [22–25 g; Crl: CD-1(ICR)BR] were used for potency checks on soman.

Therapeutic drugs were obtained from the repository at the Walter Reed Army Institute of Research; the original sources of the respective drugs were atropine sulfate, Sigma, St. Louis, MO; 2-PAM Cl, Ayerst Laboratory, St. Lauren, Quebec, Canada; HI-6, SRI International, Menlo Park, CA; MMB-4, Ash Stevens, Detroit, MI; and ICD 467, Starks Associates Inc., Buffalo, NY. The purities of the therapeutic drugs were all in excess of 99.5%. Soman was obtained from CRDEC, Aberdeen Proving Ground, MD, and its purity was >96%.

The drugs were prepared in twice distilled water just prior to use. Appropriate solutions of soman were prepared in normal saline; the solutions were stored on cracked ice during use.

Experimental procedures. To ensure that the soman challenge was consistently lethal, a potency check was conducted using 10 male mice (5 pairs) each day that nerve agent was used. Each pair received, at random, one of five doses of soman (150, 120, 95, 76 or 60 μg/kg, i.m.) and 0.5 hr mortality was noted. The methods of Thompson [12] and Weil [13] were used to calculate the LD_{50}. The mouse assay revealed that the lethal potency of soman remained stable throughout this study.

Acetylcholinesterase assay. For the studies described below, the radiometric assay of Siakotos *et al.* [14], which was modified recently by our laboratory, was used. The radiometric assay for AChE is based on the adsorption of unhydrolyzed [1-^{14}C]acetyl-β-methylcholine (MCh) or [1-^{14}C]acetylcholine (ACh) [for diaphragm cholinesterase (ChE) activity] on IRP-69 resin suspended in dioxane [14] or twice distilled water. The supernatant solution containing the labeled product of hydrolysis was counted in a liquid scintillation spectrometer.

Briefly, the modified radiometric procedure was applied as follows. Each 100 μL combined volume of tissue/twice distilled water (TDW) (whole blood, 35 μL + 65 μL TDW; erythrocytes, 20 μL + 80 μL TDW; diaphragm, 100 μL of 11.1% homogenate in saline; cortex or medulla, 75 μL of 10% homogenate in saline + 25 μL TDW) was put into a tube containing 100 μL of 0.1 M PO_4 buffer (pH 7.8, and 0.3 M in NaCl) and 100 μL of a solution of labeled substrate (3×10^{-3} M). After incubating with labeled substrate for exactly 5 min at 37°, the reaction was stopped by the addition of 100 μL of 0.4 N perchloric acid solution followed by 5 mL of a slurry of IRP-69 resin in water. The mixture was then brought to 10 mL with additional water and centrifuged; 2 mL of the supernatant was transferred into 10 mL of scintillation fluor (Ecolume) for counting.

Oxime-induced reactivation of soman-inhibited whole blood AChE by oximes in vivo. This phase of work was conducted similarly to that described by Harris *et al.* [15]. Each rabbit was placed in a stainless steel rabbit restrainer. After aseptic preparation, a 25-gauge insert needle fitted with a 22-gauge polypropylene intravenous catheter was introduced into the central artery of the ear. The catheter was capped with a latex diaphragm (Jelco-Intermittent injection cap) once blood flow was obtained. The capped catheter was then secured to the rabbit's ear with 0.5-inch adhesive tape. Initially, and after sample collection, the intravenous catheter was flushed with a normal saline solution of heparin (10 mg sodium heparin/L) under 300 mm (Hg) pressure.

Rabbits were then dosed with atropine (8 mg/kg, i.m.) 5 min before poisoning with soman (13 μg/kg, i.v.; 1.2 LD_{50}s). Pretreatment with atropine ensured survival in animals not given oxime and markedly reduced signs of intoxication by soman during the test period. At 3 min after soman or vehicle administration, oxime (25, 50, 100 or 150 μmol/kg, i.m.) was administered. ICD 467 was found to be a moderate inhibitor of whole blood AChE; thus, the doses of ICD-467 were lowered to 25, 50 and 100 μmol/kg, whereas those for the other oximes were 50, 100 and 150 μmol/kg.

Blood (0.5 mL) was collected from the catheter in the central artery of the ear. Whole blood AChE activity was determined as described above after atropine (control), at 2 min post-soman, and at 2, 5, 10, 15, 30 and 60 min after oxime or vehicle administration. After removing the last blood sample at 60 min, animals were given 0.5 mL of heparin solution, i.v. deeply anesthetized with sodium pentobarbital (45 mg/kg, i.v.) and exsanguinated with immediate flushing using heparinized saline. AChE activity was determined on the cortex, medulla-pons and a peripheral muscle, the diaphragm, to assess central and peripheral reactivation. Four oximes (2-PAM, HI-6, MMB-4 and ICD 467) were each tested at three doses.

Potency of ICD 467 as an inhibitor of whole blood AChE activity in vivo. Rabbits were atropinized as described above and given 0.2 mL/kg of saline, i.v., instead of soman, followed by ICD 467 (25, 50 and 100 μmol/kg, i.m.) 3 min later. Whole blood AChE activity was monitored before and at 2, 5, 10, 15, 30, 45, 90 and 120 min after treatment with ICD 467.

Inhibition of whole blood AChE activity (IC_{50}) *by ICD 467* in vitro. The above potency studies revealed that ICD 467 was an inhibitor of rabbit blood AChE activity. To determine the IC_{50}, 200 μL of whole blood was incubated with 10 μL of ICD 467 solution (concentrations, when diluted with blood, ranged between 1.7 and 69×10^{-6} M) for 15 min at 37°, after which time 35 μL was removed for AChE assay as described above.

In vitro *reactivation of rabbit erythrocyte AChE inhibited by soman*. In the above *in vivo* experiments with soman, it was not possible to tell whether ICD 467 had reactivated soman-inhibited whole blood AChE. Since this is a very important issue, we studied the ability of 10^{-3} M ICD 467 or HI-6, *in vitro*, to reactivate soman-inhibited erythrocyte AChE. Briefly, three 0.5-mL aliquots of control rabbit whole blood were each mixed with 80 μL of 0.1 M phosphate buffer, pH 7.3, and 20 μL of 3×10^{-2} M oxime or saline; this was repeated on blood from the same rabbit at exactly 1 min following soman. The six blood samples were incubated for 30 min at 37°. After centrifuging and removing the plasma, the packed cells were washed twice with 12 mL of cold saline per wash; 20 μL of packed erythrocytes was assayed for AChE activity.

Oxime pretreatment as a means of protecting AChE from irreversible inhibition by soman. Rabbits were dosed with atropine (8 mg/kg, i.m.) in one thigh and oxime (MMB-4, ICD 467 or 2-PAM; 100 μmol/kg, i.m.) or vehicle in the other thigh; 5 min later, soman (13 μg/kg, i.v.) or vehicle was administered. Whole blood was removed for estimation of AChE activity before oxime, at 4 min post-oxime and at 2, 5, 10, 15, 30 and 60 min after soman or vehicle. At 60 min post-soman or vehicle, animals were anesthetized and exsanguinated as described above for determination of brain and diaphragm AChE activities.

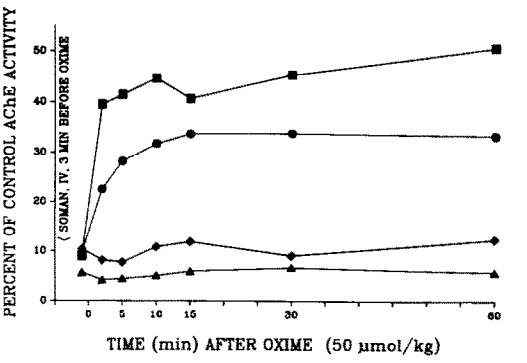


Fig. 2. Effects of oxime therapy on whole blood AChE activity in rabbits pretreated with atropine (8 mg/kg, i.m.) and intoxicated with soman. At 3 min after soman, i.v., the oximes [(■) HI-6, (●) MMB-4, (▲) 2-PAM] or (◆) H_2O were administered i.m. Whole blood was collected before and after soman and after oxime administration for AChE activity (N = 6 per study group). Control whole blood AChE activity was 17.2 ± 2.0 μmol MCh hydrolyzed/hr/mL (mean ± SD, N = 17).

RESULTS

The data in Fig. 2 illustrate the effectiveness of 50 μmol/kg, i.m., of each oxime for reactivation of soman-inhibited whole blood AChE activity. The percent control AChE activity is based on the activity of each animal prior to soman; thus, each animal served as its own control. The control whole blood AChE activity expressed as μmol MCh hydrolyzed/hr/mL = 17.2 ± 2.0 (mean ± SD; N = 17). Control blood from test animals in these experiments fell within ± 2 SDs. In animals not given soman and treated with oxime, only HI-6 caused significant inhibition (averaged approximately 20%) of blood AChE activity during the course of the study. Both HI-6 and MMB-4 produced marked reactivation ($P < 0.05$) of soman-inhibited whole blood AChE. An ANOVA revealed no significant differences in reactivation between dosages of 50 and 150 μmol/kg of oxime and that when each animal was used as its own control, HI-6 was found to be significantly ($P < 0.05$) better than MMB-4 in reactivating soman-inhibited whole blood AChE (Fig. 2). Also, the data revealed that inhibition of whole blood AChE was in excess of 80% at 2 min post-soman, or 1 min before oxime administration. Furthermore, the data clearly showed the inability of 2-PAM to reactivate unaged soman-inhibited AChE. Three animals intoxicated with soman and treated with ICD 467 died within 15 min after treatment; no noticeable reactivation (data not shown) was observed in the few samples of blood collected.

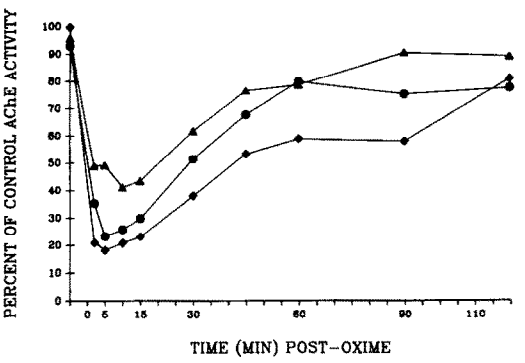


Fig. 3. Effects of administration of ICD 467 [(◆) 100 μmol/kg, N = 5; (●) 50 μmol/kg, N = 5; (▲) 25 μmol/kg, N = 4] on rabbit whole blood AChE activity. At 3 min after saline, i.v., ICD 467 was administered i.m. and whole blood AChE activity was monitored. See the legend of Fig. 2 for control value.

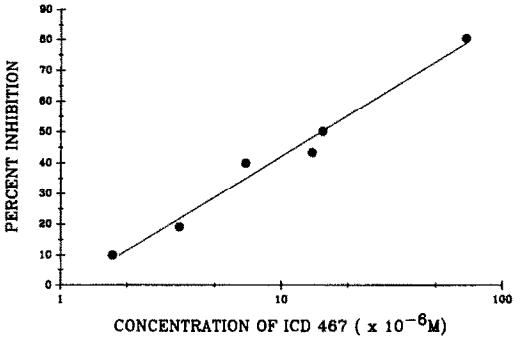


Fig. 4. Effects of ICD 467 on rabbit whole blood AChE activity *in vitro*. Whole blood was incubated with various concentrations of ICD 467 for 15 min at 37°, after which time AChE activity was determined. The percent relative inhibition was plotted as a function of concentration and the IC_{50} calculated [IC_{50} (95% confidence limits) = 15.4 (12.4–19.5) × 10^{-6} M].

The data in Fig. 3 depict the time variation of the inhibitory effects of ICD 467 on rabbit whole blood AChE activity. It can be seen that 25 μmol/kg, i.m., resulted in approximately 60% inhibition within 10 min after administration. Thereafter, inhibition decreased rapidly to approximately 20% by 60 min.

The *in vitro* concentration–AChE inhibition curve for ICD 467 is shown in Fig. 4. The calculated IC_{50} (95% confidence limits) was found to be 15.4 (12.4–19.5) × 10^{-6} M.

The effectiveness of the oximes in reactivating inhibited diaphragm cholinesterase (ChE) and brain AChE in rabbits intoxicated with soman is shown in Table 1. 2-PAM was ineffective in reactivating soman-inhibited diaphragm ChE; in contrast, MMB-4 and HI-6 produced significant reactivation. Also, none of the oximes tested significantly reactivated brain AChE.

The comparative effects of ICD 467 and HI-6, *in vitro*, in reactivation of soman-inhibited rabbit erythrocyte AChE are shown in Table 2. Even at high concentrations of ICD 467, no significant reactivation occurred. In contrast, HI-6 almost produced total reactivation of the inhibited erythrocyte AChE. The AChE activity of erythrocytes not exposed to soman (control cells) and processed in parallel with experimental cells and treated with ICD 467 (cells were washed to remove excess oxime) was similar to that of erythrocytes treated with buffer, indicating that the oxime had been effectively removed prior to determination of AChE activity.

The data in Fig. 5 illustrate the effects of oxime pretreatment on whole blood AChE activity in rabbits intoxicated with soman. Even when given before soman intoxication, 2-PAM was completely ineffective in protecting the blood AChE. In contrast, MMB-4-treated rabbits maintained significant ($P < 0.05$) AChE activity following soman intoxication. In ICD 467-pretreated animals given soman, blood AChE activity showed partial recovery with time.

DISCUSSION

Soman is a highly toxic organophosphorus chemical warfare agent that induces an intoxication that is extremely difficult to treat for several reasons: (1) rapid aging of the inhibited AChE, (2) poor reactivation of the unaged AChE due to steric hindrance, (3) pronounced CNS effects, and (4) direct biochemical effects [3, 16–18]. Until the development of the H-series oximes by Hagedorn [6], no oxime had been reported to be effective in treating cases of soman intoxication in experimental animals. Certain H-oximes (e.g. HS-6 and HI-6) in conjunction with atropine are effective antidotes against soman intoxication [7–9, 15, 19].

Recently, ICD 467 [10] and MMB-4* have been shown to be effective against soman intoxication. However, there is still some question concerning the mode of protective action of these oximes, namely, whether *in vivo* they reactivate soman-inhibited AChE. The data presented in Fig. 2 and Table 1 indicate the comparative effectiveness of equimolar dosages of MMB-4, HI-6 and 2-PAM in reactivating soman-inhibited whole blood AChE; the blood AChE was inhibited approximately 90% and aged about 40% at the time of oxime administration (3 min after soman). The half-time for aging of soman-inhibited blood cholinesterase in rabbits is reported to be 7.6 min [15]. Thus, at 3 min after soman the inhibited AChE should largely be in the unaged form and should be receptive to effective oximes. Clearly, HI-6 was superior ($P < 0.05$) to the other oximes tested in reactivation of unaged soman-inhibited whole blood AChE. Also, MMB-4 administration effected significant reactivation of soman-inactivated AChE, but the extent of reactivation was certainly inferior ($P < 0.05$) to that of HI-6.

The effectiveness of MMB-4 against soman was somewhat surprising, since this oxime is similar to TMB-4 save for a 3 carbon bridge between the two pyridinium rings (TMB-4) versus a single carbon

* Lennox WJ and Sultan WE, unpublished results.

Table 1. Effects of oxime administration on tissue cholinesterase activity in rabbits intoxicated with soman*

Treatment	N	Tissue cholinesterase activity† (% control)		
		Diaphragm	Cortex	Medulla
Water	6	14.0 ± 9.0[a]‡	24.1 ± 7.2	18.8 ± 9.2
2-PAM	17	15.2 ± 9.8[b]	20.6 ± 12.0	23.0 ± 17.4
HI-6	18	39.7 ± 12.6[c]	22.4 ± 13.2	19.6 ± 11.1
MMB-4	18	34.2 ± 15.9[d]	22.2 ± 12.9	21.6 ± 14.9

* Rabbits were atropinized (8 mg/kg, i.m.) and then intoxicated with soman (13 μg/kg, i.v.; 1.2 LD_{50}s) 5 min later.

† The substrates used for ChE (diaphragm) and acetyl-ChE (cortex and medulla) determinations were acetylcholine (ACh) and acetyl-β-methylcholine (MCh), respectively; control AChE or ChE activities (μmol MCh or ACh hydrolyzed/hr/mg tissue (wet weight)] were: cerebral cortex, 105.5; medulla, 151.6; and diaphragm, 72.2. Values are means ± SD.

‡ a is similar to b ($P > 0.05$) and both of these differ significantly ($P < 0.05$) from c and d.

Table 2. Effects of oximes on soman-inhibited rabbit erythrocyte acetylcholinesterase activity*

Treatment†	N	Acetylcholinesterase activity‡ (% control)
Buffer	5	12.1 ± 10.1
ICD 467	5	16.5 ± 6.7
HI-6	5	98.9 ± 5.1

* Rabbits were atropinized (8 mg/kg, i.m.) and then intoxicated with soman (13 μg/kg, i.v.; 1.2 LD_{50}s) 5 min later.

† Blood was removed into oxime = 1×10^{-3} M, pH 7.3, before and exactly 1 min following soman administration; after incubating for 30 min at 30°, the cells were centrifuged and plasma was removed; the packed cells (0.2 mL) were washed two times with 12 mL of cold saline per wash followed by determination of erythrocyte AChE activity.

‡ Control blood received the same treatment as inhibited samples, but no soman; control erythrocyte AChE activity = 21.2 μmol MCh hydrolyzed/hr/mL. Values are means ± SD.

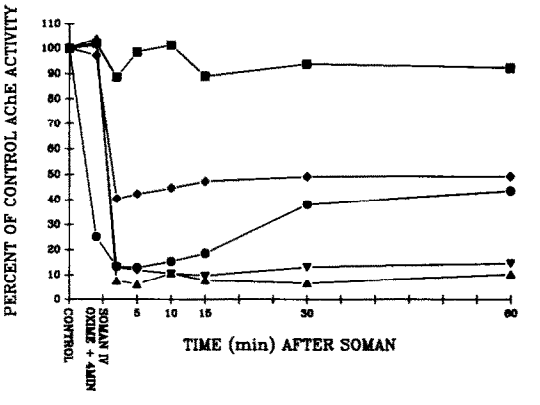


Fig. 5. Effects of oxime pretreatment on whole blood AChE activity in rabbits intoxicated with soman. Animals were dosed with atropine (8 mg/kg, i.m.) in one thigh and oxime (100 μmol/kg, i.m.) or vehicle in the other thigh 5 min before exposure to soman or vehicle, i.v. Key: (●) ICD 467, (▼) 2-PAM, (◆) MMB-4, (▲) H_2O, and (■) vehicle control. Whole blood AChE activity was monitored before and after oxime and after soman (N = 6 per study group). See the legend of Fig. 2 for control value.

bridge (MMB-4; see Fig. 1). TMB-4 is ineffective as an antidote against soman intoxication [2, 8]. As expected, our data confirmed studies where 2-PAM was shown to be ineffective against soman [2, 4]. The data on HI-6 also confirmed earlier reported findings *in vivo* and *in vitro*, namely, that this oxime reactivates unaged soman-inhibited AChE [19, 20].

The imidazolium oximes have been shown recently to be effective antidotes when given together with atropine against soman intoxication in mice [10, 11]. While the mode of protective action is not known, we postulated that it was due to reactivation of the inhibited AChE. However, when 25, 50 and 100 μmol/kg, i.m., of ICD 467 were administered to one rabbit each at 3 min after soman, each of the three animals died within 15 min after receiving the oxime. Further studies revealed (Figs. 3 and 4) that this imidazolium oxime was an inhibitor of AChE at the levels being used for reactivation. In fact, the anticholinesterase activity of ICD 467 appears to be several times more potent than that of TMB-4 or HI-6 [21]; this observation explained the difficulty in assessing induced reactivation in the presence of ICD 467. Efforts were then made to remove the oxime by washing the erythrocytes. Thus, whole blood was drawn before and at 1 min after soman, i.v., and put into oxime/buffer for reactivation of the inhibited erythrocyte AChE. After removing excess oxime by washing the erythrocytes and then assaying for erythrocyte AChE activity, it was found (Table 2) that ICD 467 (1×10^{-3} M) produced negligible reactivation of the soman-inhibited erythrocyte AChE when compared to HI-6 (1×10^{-3} M).

The data strongly suggest that the protection afforded by this imidazolium oxime is not related to reactivation of inhibited AChE. The protection may

be related to its reversible anticholinesterase potency. Clearly, the data in Fig. 5 support this supposition, because when ICD 467 was given before soman, the blood AChE was protected significantly from irreversible phosphonylation. Thus, it is possible that when this oxime was given i.m. to mice 10 sec following soman challenge [10], sufficient quantities of oxime reversibly inhibited the AChE before the soman irreversibly phosphonylated the enzyme.

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