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13. ABSTRACT *(Maximum 200 Words)*

This research is focused on the use of various liposome-like drug carrier systems containing recombinant organophosphorus (OP) hydrolyzing enzymes (OPH = Organophosphorus Acid Hydrolase; OPAA = Organophosphorus Acid Anhydrolase) to prevent organophosphorus poisoning. The objective is to provide long term protection against OP intoxication by using OP-hydrolyzing enzymes with various liposome-based enzyme carrier systems such as sterically stabilized liposomes (SL) and modified liposome-like carriers (NT). Present research is focused on: studying and optimizing of the *in vitro* efficacy of the OP-comlex- hydrolyzing enzymes; optimizing the carrier systems; studying the *in vivo* efficacy of the encapsulated enzymes; studying the blood cholinesterase level in the presence of OPs, 2-PAM, and the OP-hydrolyzing enzymes and to monitor and attempt to predict the OP toxicity and antagonism. OPH enzyme has highly efficient substrate specificity to paraoxon but in general it is less efficient to diisopropylfluorophosphate (DFP), soman and sarin. However, OPAA can hydrolyze DFP, soman and sarin with a relatively high efficiency. DFP and paraoxon were used as model substrates to study the *in vitro* OP- hydrolyzing efficiency and the *in vivo* antidotal efficiency of the encapsulated OPAA and OPH. Hydrolysis of DFP was followed by measuring the amount of the fluoride ions formed by using a fluoride ion selective electrode. Hydrolysis of paraoxon was determined spectrophotometrically by measuring the p-nitrophenol formation. The paraoxon hydrolysis displayed saturation kinetics with increasing substrate concentration both with free OPH and encapsulated OPH. The kinetic parameters suggest that paraoxon can freely enter and exit the enzyme carrier systems. Preliminary toxicology studies indicate that the encapsulated OPH and OPAA, (NT-OPH and NT-OPAA), may strikingly enhance the antidotal effects of the clinically proven OP antidotes, 2-PAM and atropine. *In vitro* and *in vivo* measurements of plasma acetylcholinesterase (AChE) show correlation with the antidotal efficacy of the AChE reactivator, 2-PAM, and the OP hydrolyzing enzymes, OPAA and OPH.

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INTRODUCTION

This investigation effort was focused on antagonizing organophosphorus intoxication by employing recombinant enzymes encapsulated within biodegradable enzyme carrier systems. One of the mechanisms for detoxification of organophosphorus compounds should involve their degradation by OP-hydrolyzing enzymes. Once the OP compounds are hydrolyzed, they are inactive. The use of exogenous enzymes in OP antagonism was reported by Cohen and Warringa (1957) and Ashani *et. al.*, (1991). However, the injection of purified free enzyme preparations directly into the blood stream has serious limitations due to the possible immunologic reactions and various adverse physiological disposition factors (absorption, distribution, metabolism, excretion). Biodegradable liposome carriers, which are permeable to the toxic molecules, can provide sufficiently large amounts of the highly purified metabolizing enzymes to remain stabilized and protected in the circulation for a long period of time. Carrier erythrocytes encapsulating the rhodanese enzyme, were similarly successfully employed in cyanide antagonism (Cannon *et al.*, 1992, Leung *et al.*, 1986, 1991; Petrikovics *et al.*, 1994, 1995, Way *et al.*, 1985) and in OP antagonism (Pei *et al.*, 1994, 1995). McGuinn *et al.*, (1993) reported the encapsulation of squid- type diisopropylfluorophosphate-hydrolyzing enzyme (DFP-ase) into carrier erythrocytes. Sterically stabilized liposomes (SL) have been widely used as targeted drug delivery system in clinical therapy (Lasic and Papahadjopoulos, 1998). In the sterically stabilized liposome system, a drug is encapsulated into biocompatible carrier vesicles to circumvent the body's immune defences, thereby circumventing rapid uptake by the macrophage cells of the reticuloendothelial system (Allen, 1994, Papahadjopoulos *et al.*, 1991, Szoka and Papahadjopoulos, 1980, Woodle and Lassic, 1992).

Two other OP antagonists, pralidoxime (2-PAM) and atropine, exert their pharmacological protective effects by different mechanisms: 2-PAM reactivates the OP-inhibited AChE (Wilson *et al.*, 1955), and atropine competes with acetylcholine at the muscarinic receptor site. The combination of 2-PAM and atropine with an OP-hydrolyzing enzyme, in an appropriate protective carrier system, should provide for a more effective antidotal protection against the lethal effects of OP agents, as neither 2-PAM nor atropine can degrade the OP agent, but the OP-hydrolyzing enzyme does. These studies represent a new conceptional approach to protect against and also to treat chemical poisoning.

BODY

Previous studies reported the *in vitro* and *in vivo* protective effects of recombinant OPH enzyme encapsulated within carrier erythrocytes (Pei *et al.*, 1994, 1995) and sterically stabilized liposomes (SL) (Petrikovics *et al.*, 1999), and OPAA enzyme encapsulated within SL (Petrikovics *et al.*, 2000a and 2000b) to antagonize the lethal effects of paraoxon and DFP respectively. The prophylactic and therapeutic effects of (SL-OPH) carrier system have also been previously reported.

Present studies are to study the optimization of the *in vitro* efficacy of the OP hydrolyzing enzymes (Enzyme I: OPH, Enzyme II: OPAA) encapsulated in various liposomes and liposome-like carrier systems (Carrier I: SL; Carrier II: NT); to study the *in vivo* efficacy of the encapsulated enzymes; and to study the blood cholinesterase level in the presence of the OP agents, 2-PAM, and the OP-hydrolyzing enzymes to monitor and to attempt to predict the OP toxicity and the effects of these agents on OP antagonism.

Animals. Male Balb/C mice were purchased from Charles River Breeding Laboratories, Inc., (Wilmington, MA). All animal procedures were conducted in accordance with the guidelines in The Guide for the Care and Use of Laboratory Animals (National Academic Press, 1996), credited by AAALAC (American Association for the Assessment and Accreditation of Laboratory Animal Care, International).

Enzyme Sources, Enzyme Preparations.

Enzyme I. A recombinant OPH (EC 3.1.8) was purified from an *Escherichia coli* clone containing the plasmid expression vector pjK33 which was isolated from *Flavobacterium* sp (Serdar *et al.*, 1985, McDaniel *et al.*, 1988). This enzyme was obtained in preparative amounts and purified

over 1600 fold by the method of Omburo *et al.,* (1992) with minor modifications (Pei *et al.,* 1994, 1995).

Enzyme II. Purification of OPAA (EC 3.1.8.2) from *Alteromonas* strain JD6 has been previously reported by DeFrank, *et. al.* (1991) and Cheng, *et. al.* (1993, 1997).

Enzyme Activity Determination (with free enzymes and encapsulated enzymes).

Enzyme I. OPH activity was measured at room temperature by determining the increase in p-nitrophenol concentration in the presence of excess paraoxon. (Pei *et al.*, 1993,1994, 1995). Protein assays were done by the Bradford method (Bradford, 1976) using the Bio-Rad protein assay reagent (Bio-Rad, Richmond, CA). One unit of OPH is defined as that amount of enzyme which hydrolyzed 1 μmole of paraoxon to p-nitrophenol per minute.

Enzyme II. OPAA activity was measured by monitoring the production of fluoride ions from DFP with a fluoride ion selective electrode (Orion Research Inc., Boston, MA) (Hoskin and Roush, 1982). Protein assays were done by the Bradford method (Bradford, 1976) using BioRad protein assay reagents (BioRad, Richmond, CA).One unit of OPAA is defined as that amount of enzyme which hydrolyzed 1 μmole of DFP to fluoride ion and isopropyl phosphate per minute.

Encapsulation Process.

Sterically stabilized liposomes and other modified liposome-like enzyme carriers were prepared as previously described (Petrikovics *et al.*, 1999), using the method of Allen (1994) with some minor modifications. Enzyme activity within the carriers was determined as described above with some modifications, such as disrupting the carriers by sonication or by applying 1% (v/v) Triton-X detergent(Cohen *et al.*, 1991*)*. The encapsulation efficiency was calculated from the amount of encapsulated enzyme divided by the amount added times 100.

Prophylactic in vivo experiments.

Paraoxon Antagonism Studies. Male mice received OPH (free enzyme or encapsulated) one hour prior to receiving paraoxon (in 6% cyclodextrin and propyleneglycol solvent system) subcutaneously. Animals exposed to paraoxon with antagonists (atropine and/or 2-PAM and/or OPH) were determined by 24 hour mortality. Atropine and 2-PAM were administered intraperitoneally to mice prior to receiving paraoxon. The LD_{50} values were determined by the up-and-down method (simulated up-and-down study, Dixon, 1965), and the estimated 95% confidence interval was determined by the method of Bruce, (1985).

DFP Antagonism Studies. Male mice received OPAA (free enzyme or encapsulated) in maximum volume of 200 μl intravenously (dorsal tail vein injection) prior to receiving DFP subcutaneously. The LD_{50} value was obtained from five or more graded doses of DFP administered to five or more groups of six to eight mice, based on 24 hour mortality. Efficacies of the antagonists are expressed as the potency ratios. The LD_{50} values determined were based on a 24-hor mortality. Statistical calculations were conducted by the method of Litchfield and Wilcoxon (1949).

In vivo plasma cholinesterase studies.

I. With Paraoxon-OPH System. Male mice received OPH and 2-PAM in maximum volume of 200 μl intravenously (dorsal tail vein injection). Sublethal dose of paraoxon (0.7 mg/kg) was injected subcutaneously. Blood was taken two minutes after paraoxon injection, and it was centrifuged to obtain plasma. The AChE Activity was determined by the method of Ellman (1961) with minor modifications.

II. With DFP-OPAA System. Male mice received 100 units of OPAA and 2-PAM in maximum volume of 200 ul intravenously (dorsal tail vein injection). Sub-lethal dose of DFP was

injected subcutaneously. Blood was taken after DFP injection, and it was centrifuged to get the plasma. The AChE Activity was determined by the method of Ellman (1961) with minor modifications.

In vitro cholinesterase studies.

I. With Paraoxon-OPH System. The cholinesterase activity was determined by the method of Ellman (1961) with minor modifications. Substrate: Acetylthiocholine iodide (0.75 M); Reagent: DTNB (0.01M, 39.6 mg DTNB in 10 ml Phosphate Buffer pH=7.0, and 15 mg $NaHCO_3$); Enzyme: Cholinesterase; Paraoxon: (0.8 µl/ml solution); OPH (20units/ml); 2-PAM (1.72 mg/ml). When AChE inhibition by paraoxon was studied, 2-10 µl of the paraoxon solution was given to the system. When 2-PAM reactivation was studied, the 2-PAM solution (1.72 mg/ml) in 100 -500 µl volume was given to the system above, 1 min after adding the AChE, the substrate (0.75 M acetylthiocholine iodide) and DTNB reagent. When the effects of OPH were studied, 100 µl OPH solution was given 1 min after the AChE, substrate and DTNB. Absorbance was read at 412 nm.

II. With DFP-OPAA System. The cholinesterase activity was determined by the method of Ellman (1961) with minor modifications. Substrate: Acetylthiocholine iodide (0.75 M); Reagent: DTNB (0.01M, 39.6 mg DTNB in 10 ml Phosphate Buffer pH=7.0, and 15 mg $NaHCO_3$); Enzyme: Cholinesterase; DFP (1 µl/ml solution). When AChE inhibition by DFP was studied, 2-10 µl of the DFP solution (1 µl/ml) was given to the system. When 2-PAM reactivation was studied, the 2-PAM solution in 100 -500 µl volume was given to the system above, one minute after adding the AChE, the substrate (0.75 M acetylthiocholine iodide) and DTNB reagent. When the effects of OPAA were studied, 100 µl OPAA solution was given one minute after the AChE, substrate and DTNB . Absorbance was read at 412 nm.

A. In vitro Efficacy Studies with Encapsulated Enzymes.

Hydrophilic proteins can be encapsulated into liposome-like drug carriers with almost 100% encapsulation efficiency. The hydrophobic surface of the carriers allow efficient penetration of apolar molecules into the carriers where the encapsulated enzyme can hydrolyze the substrate molecules with the same efficacy as the non-encapsulated enzymes (Figure 1). As it is shown in Table I, the OPH is a highly efficient hydrolyzing enzyme for paraoxon, but it is not suitable for soman or sarin. OPAA is considered as an effective hydrolyzing enzyme for soman and sarin and also for DFP. The two enzyme - substrate systems ([paraoxon-OPH], and [DFP-OPAA]) served as model systems to predict the antidotal effects of OP hydrolyzing enzymes on various OP warfare agents.

A/I. In vitro studies with Enzyme I (OPH, NT-OPH).

The encapsulated OPH (NT-OPH) is capable of hydrolyzing paraoxon. The liposome like carrier (NT) without OPH does not hydrolyze paraoxon. Enzymatic hydrolyzis of paraoxon with varying amounts of (NT-OPH) is shown in Figure 2. The reaction is linear at the time range and the concentration range in which these experiments were conducted. Increases in p-nitrophenol formation is directly proportional to the amount of (NT-OPH). There is a direct linear relationship between the amount of (NT-OPH) and the rate of p-nitrophenol formation in the enzymatic reaction. No increase in p-nitrophenol concentration was detected when the liposome-like carrier (NT) did not contain OPH. Under the conditions of these experiments, increases in amount of (NT-OPH) is equivalent to increases in amount of OPH in the reaction mixture. Hydrolysis of paraoxon is linear to the amount of enzyme added to the samples as determined by the p-nitrophenol formation. Velocity of p-nitrophenol formation varies as a function of paraoxon concentration in the reaction mixture. OPH was essentially saturated when paraoxon concentration reached 0.1 mM (Figure 3

and 4). A double reciprocal plot of paraoxon hydrolysis by the free OPH or the encapsulated OPH (NT-OPH) is shown in the Figure 5 and Figure 6, respectively. The K_M values between the free OPH and (NT-OPH) correlate very well: free OPH=0.01 mM, (NT-OPH)=0.05 mM.

A/II. In vitro studies with Enzyme II (OPAA, NT-OAA).

In vitro studies with free and encapsulated OPAA (SL-OPAA) were previously reported (Petrikovics *et al.*, 2000a, 2000b). Enzymatic hydrolysis of DFP by various amounts of (NT-OPAA) is shown in Figure 7. The reaction is linear at the concentrations employed in these studies. Formation of fluoride anions is directly proportional to the amount of (NT-OPAA). There is no OPAA activity noted with the liposome-like carrier (NT) containing no OPAA. Under these conditions, increases in the amount of (NT-OPAA) is equivalent to the increases in the amount of OPAA in the reaction.

B. In vivo Efficacy Studies with the Encapsulated Enzymes.

B/I. Paraoxon Antagonism Studies with Encapsulated OPH.

OPH can hydrolyze Paraoxon with an extremely high efficacy *in vitro* (Table I). Earlier studies reported the antidotal effects of OPH in comparisson with, and /or in combination with 2-PAM and /or atropine when OPH was encapsulated within carrier erythrocytes (CRBCs) (Pei *et al.*, 1994, 1995) or within sterically stabilized liposomes (Petrikovics *et al.*, 1999). Paralleling the *in vitro* efficacy studies on isolated enzymes, the OPH alone gave a better protection than the 2-PAM + atropine combination. When OPH was given with 2-PAM and/or atropine, the protection of 2-PAM and/or atropine was strikingly enhanced. The magnitude of protection was over $1000LD_{50}$ when OPH was employed with the combination of 2-PAM and Atropine and when the OPH was encapsulated within a carrier. In our experimental conditions, [2-PAM ,atropine, OPH

(free or encapsulated), injected 15 minutes (ip), 45 minutes (ip), and 30 minutes (iv), respectively, prior to the administration of paraoxon (sc)], there is no statistical difference between the *in vivo* effects of the various OPH carriers (Figure 8, Table II). The antidotal potency ratios, expressed as the potention of the antagonized paraoxon over unantagonized paraoxon, illustrate the dramatic enhanced effects of the encapsulated OPH in antagonizing paraoxon intoxication particularly in combination with 2-PAM and/or atropine. The LD_{50} value of paraoxon of the control animal was used as a potency ratio of 1.0. The (CRBC-OPH) or (SL-OPH) alone caused an increase in the potency ratio to 126 or 139, respectively. When 2-PAM (potency ratio=5) was used in a combination of (CRBC-OPH) or (SL-OPH) the potency ratios increased to 630 or 611, respectively. Atropine (potency ratio=2) used in a combination with (CRBC-OPH) or (SL-OPH), exhibited an increase in the potency ratio to 625 or 600, respectively. When both atropine and 2-PAM (potency ratio=61) were used in a combination with (CRBC-OPH) or (SL-OPH), the potency ratio was elevated to 1043 or 1022, respectively. Figure 9 shows the preliminary *in vivo* antidotal effects with the (NT-OPH) system in comparison with the other OP-antidotes 2-PAM, atropine. The (NT-OPH) strikingly enhanced the protection of 2-PAM and atropine. These are very preliminary *in vivo* data with the (NT-OPH) systems. Further characterization of the liposome-like carrier systems, and additional *in vivo* studies with the (NT-OPH) systems are currently in progress.

B/II. DFP Antagonism Studies with Encapsulated OPAA . DFP antidotal effects of (SL-OPAA) alone and in combination with 2-PAM and/or atropine have been previously reported

(Petrikovics *et al.*, 200a and 200b). Parallel with the *in vitro* kinetic data, the DFP-OPA system is less effective than the Paraoxon-OPH system. The un-antagonized LD_{50} DFP is 4.2 mg/kg (control). Atropine gave an increase in LD_{50} to 5.7 mg/kg, and 2-PAM alone increased the protection to 7.7 mg/kg. When 2-PAM and atropine were combined, the LD_{50} was increased to 29.3 mg/kg. When free OPAA without liposomes was administered in combination with 2-PAM + atropine, the protection of 2-PAM and atropine combination was only slightly enhanced to 33.2 mg/kg. When sterically stabilized liposomes encapsulating OPAA was used in combination with 2-PAM and atropine, a dramatic enhancement was detected. Figure 10 shows the antidotal protection with (NT-OPAA) when it was combined with 2-PAM + atropine. The (NT-OPAA) enhanced the protection of 2-PAM and atropine against DFP intoxication. It should be emphasized that these *in vivo* antidotal data are very preliminary, and further investigation is necessary concerning the optimization of the carrier systems and the *in vivo* efficacy.

C. Monitoring AChE Levels.

In vitro and *in vivo* cholinesterase inhibition studies with DFP and reactivation with 2-PAM in the presence of OPAA was reported earlier as an indicator of the OP toxicity and anatagonism. 2-PAM also reactivates the paraoxon-inhibited AChE both *in vitro* (Figure 11) and *in vivo* (Figure 12). When OPH is given to the system which contains paraoxon, the paraoxon concentration decreases. The rate of AChE inhibition depends on the amount of paraoxon in the system: With higher OPH concentration less paraoxon remains in the system, therefore the AChE level is higher. Figure 11 shows the *in vitro* experiments (AChE activity measurements) with paraoxon, 2-PAM and the [2-PAM - OPH] combination. With the applied paraoxon concentration, the given AChE was inhibited by about 95%. The AChE reactivator 2-PAM remarkably increased the AChE activity

(remarkably lessened the inhibition by paraoxon), and the increase of activity (lessening the inhibition) was more substantial when 2-PAM and OPH were applied together. With this applied 2-PAM and OPH concentration, the 95% AChE inhibition was "released" to about 50%. Figure 12 shows the *in vivo* plasma AChE level in mice, after administering sublethal doses of paraoxon alone, or with 2-PAM or with 2-PAM and OPH combination. Blood samples from mice were taken two minutes after paraoxon administration. The paraoxon- inhibited the AChE activity by about 90 %, which was significantly released when 2-PAM was employed with the combination of different concentration of OPH.

FUTURE DIRECTIONS.

This report is concerned with a new conceptional approach of administering recombinant OP-hydrolyzing enzymes encapsulated within appropriate enzyme carrier systems to antagonize the toxic effects of organophosphorus compounds. It appears that this approach has reasonable potential to explore further scientific practicality. With the encapsulated OPH sytem, we achieved over 1000 LD_{50} protection when it was combined with the 2-PAM+ atropine combination. OPAA has moderate *in vitro* efficacy with various OP substrates (DFP, soman, sarin), but the encapsulated OPAA still showed remarkable protection with the combination of 2-PAM and atropine on the model compound, DFP. On the bases of the *in vitro* kinetic parameters, OPAA is expected to show even higher protections with the warfare agents, soman and sarin, *in vivo*. It should be noted, that more studies will need to be conducted employing this approach. The carrier systems that we employed in our experimental protocol must be optimized. More data regarding the carrier systems (penetrations though the carrier membranes, the stability of the carriers in the body, how long the carriers can keep the enzyme actively in the circulation) are necessary to characterize the mechanism of the systems. The enzyme amounts within the carriers also need to be optimized. Dose-response will be obtained to determined the optimal enzyme units for these activities. In the future, we plan to employ other routes than intravenous administration. Injecting the encapsulated enzymes intramuscularly would provide a more practical protective mechanism. For intramuscular injections, the carriers need to be smaller in size and they should have modified structural composition. The size of the liposomal vesicles formed, the life time, and the distribution profile of liposomal carriers in the body depends on their membrane structure (lipid-cholesterol ratio, chemical composition of the lipids, etc). Incorporating the lipid-cholesterol membrane with other molecules such as polyethylene glycol (PEG) can change the physiologic parameters of the carrier

systems. It is necessary to optimize the structure of the carriers to provide optimal administration route and lifetime for the metabolizing enzyme. We are exploring other routes of administration such as inhalation which includes many practical advantages. Cationic liposomes are expected to provide with modifications greater distribution to the lungs. The lifetime of 2-PAM in the body is also rather short, so we plan to attempt to extend the duration of time of 2-PAM by using an appropriate vesicle attached to the 2-PAM. Besides the prophylactic studies with these antidotal systems, we have initiated studies on a therapeutic basis. We also have other studies without the use of drug carries, which depends on the membrane properties and capsule structure and other release mechanisms. Preliminary data suggest that liposome-based carriers have great potential in military application as a delivery system to develop new therapeutic agents, as it may dramatically improve the efficacy of present drug therapy and drug antidotal therapy. It is apparent that OPAA with a turnover number of 2000 is more efficacious than butirylcholinestarase with a turnover number of one as a stoichiometric enzyme. With the present newer delivery systems and transport modifiers, a much more efficient, practical, efficacious antidotal system should be available to the combat soldiers in the near future.

KEY RESEARCH ACCOMPLISHMENTS

There is a paucity of information in evaluating the efficacy of antidotes on both prophylactic and therapeutic basis. These results represent an application of a new conceptional approach by using sterically stabilized liposome-based carriers (SL, NT) for OP-hydrolyzing enzymes (OPH, OPAA) to hydrolyze and antagonize the lethal effects of various organophosphorus compounds. The sterically stabilized long circulating liposomes are far superior to our earlier conventional liposomes. The sterically stabilized liposomes have much longer half-life than the conventional liposomes. These liposomes last for over 2-3 days in mice, whereas the duration of viability in conventional liposomes is less than a few hours. The encapsulation efficiency with sterically stabilized liposomes are almost quantitative when recombinant enzymes are encapsulated. The (NT-OPH) system, similarly to the (NT-OPAA) systems, was able to hydrolyze the OP molecules. The optimal enzyme-carrier system will probably be able to hydrolyze soman and sarin as well. We are aware of the toxic and nontoxic isomeric forms of these and how mutant enzymes of OPH and OPAA maybe employed in the future. Neither the SL or the modified NT carriers without enzymes can hydrolyze the OP compounds, and the rates of hydrolysis with the encapsulated enzymes were directly proportional to the amounts of enzyme loaded SL or NT, similarly to the experiments with free enzymes. The encapsulated enzymes behave the same as the free enzymes in the *in vitro* efficacy studies, which suggests that the substrate molecules can freely penetrate into the carriers and the hydrolysis products can freely diffuse out from the carriers. In the *in vivo* studies, the main difference between the free and the encapsulated enzymes was the free enzymes were not protected from the body's immune responses. Also, the duration and the degree of protection of the encapsulated enzymes depends on the structure and the stability of the carriers. Concerning OPAA, there is an increase in specific activity of the enzyme when encapsulated into certain carrier systems.

The mechanism of this enhancement both in biochemical and toxicological aspects are being explored. With the encapsulated OPH, the magnitude of the prophylactic protection was over 1000 LD_{50}, while based on the previous therapeutic experiments showed the therapeutic protection of 150 LD_{50}. This is an important evidence of the values of the prophylactic and therapeutic application of the bio-protected recombinant OP-hydrolyzing enzymes in OP antagonism, as they are far superior to the present combination of 2-PAM and atropine. These studies on the AChE level monitoring in the presence of the cholinesterase inhibitors, paraoxon or DFP, the OP-reactivator, 2-PAM, and the OP-hydrolyzing enzymes, OPH or OPAA, served as useful tools to monitor and possibly attempt to predict the biologic effects of these agents. This research is not just a theoretical scientific endeavor, as the PI feels that his program should lead to a practical superior prophylactic and therapeutic antidotal soldier's kit. Hopefully this antidotal effect should last for days or weeks without affecting the soldier's physical and mental performance. This demonstrates that the use of drug carriers, such as SL or NT, provide enhanced protections that are not-active or lesser activity with the free enzymes. Also, this new conceptual approach provides a remarkably greater protection against OP intoxication than any other antidotal combinations which presently exist. This development now permits the practical potential of developing prophylactic and therapeutic anti-OP agents.

REPORTABLE OUTCOMES

These studies serve as a new application of our previously successfully developed conceptual approach to antagonize the lethal effects of toxicants by employing encapsulated recombinant metabolizing enzymes. Earlier studies attempted to encapsulate the OP hydrolyzing enzymes within CRBCs and within conventional liposomes which are considered artificial red blood cells; however, the encapsulating efficiency was low and the half-life of these carriers was too short for our use in these studies. We have successfully modified the structures of the enzyme carriers and prepared sterically stabilized liposome carriers (SL) and modified liposome -like enzyme carriers (NT) which have better physiologic disposition factors than the conventional liposomes and CRBCs. We were also able to encapsulate OP-hydrolyzing enzymes successfully into these carriers. These enzyme-carriers maintain their properties to hydrolyze the OP compounds since the toxin molecules can penetrate through the membranes and the hydrolysis products can leave the carriers freely. The general importance of this concept is that there are less than half a dozen of specific antidotes which can antagonize over 5 LD_{50} doses. This approach now provides an OP antidote, which has a longer duration of action and it appears to be less toxic than the classic antidote of 2-PAM and atropine. Moreover, when the encapsulated enzymes were administered in combination with 2-PAM and atropine, there was a striking potentiating in the antidotal protection. Some of the *in vitro* properties of these (SL-OPAA) was reported in the peer reviewed society sponsored journal, *Drug Delivery,* (Petrikovics *et al.*, 2000b), and additional publication on the preliminary drug antagonism properties of (SL-OPAA) was published in the *Toxicological Sciences* (Petrikovics, *et al.*, 2000a). These are the top quality peer reviewed journals sponsored by our respective society.

The publication regarding the prophylactic and therapeutic efficacy of the 2-PAM and atropine and (SL-OPH) systems is in progress now. Publishing the results regarding the AChE level

monitoring in the presence of 2-PAM and/or OPH or OPAA with paraoxon or DFP, respectively as AChE inhibitors is also in progress. Data regarding the antidotal effects with the modified liposome-like enzyme carrier systems encapsulating recombinant OP-hydrolyzing enzymes (NT-OPH, NT-OPAA) are preliminary data. More experiments are necessary to complete these studies, but these very preliminary studies suggest that these new antidotal systems have considerable promise in the antidotal therapy.

CONCLUSIONS

These studies are based on our new conceptual approach to the antagonism of chemical toxicants. This approach employs fast recombinant enzymes which are known to degrade these toxicants after they are encapsulated within an appropriate enzyme carrier system. The rationale for this approach is that there are only a few antidotes which can antagonize over 5 LD_{50} doses of any chemical toxicants; therefore, the likelihood of developing a specific antidote is very optimistic, and there is a need for a more successful approach for treating chemical poisoning. Recent advancements in liposomal technology allow preparation of liposomal delivery system with minimal macrophage recognition, thereby extending the half-life of the conventional lipsomes from a few hours to a few days by using sterically stabilized, long circulating liposomes. This new technology, coupled with the rapid preparation of fast catalytic recombinant enzymes, permits the development of more effective prophylactic and therapeutic agents. These results represent an application of sterically stabilized liposomes and modified liposome-like enzyme carrier systems for the OP hydrolyzing enzymes to degrade OP agents and to antagonize the lethal effects of various OP agents. In these studies, recombinant OPH and OPAA enzymes were successfully encapsulated within the liposome-based enzyme carrier systems (SL, NT), and the *in vitro* and *in vivo* efficacy studies were conducted with these systems for the ability to hydrolyze/antagonize organophosphorus molecules. With these enzyme-carrier systems paraoxon and DFP were employed as model organophosphorus compounds. Earlier studies with OPH encapsulated within resealed carrier erythrocytes (CRBC) (Pei *et al.*, 1994, 1995) and within sterically stabilized liposomes (Petrikovics, *et al.*, 1999) were compared and indicated possible advantages of liposomal carriers over the CRBC systems. Liposomes are more stable and prior blood typing is not required for their use. With liposomes, the encapsulation efficiency is over 90% can be achieved; whereas with CRBCs the encapsulation efficiency was only

a maximum of 30%. When the antidotal protection with these two carrier systems (CRBC, SL) were compared, they had essentially the same protective effects (Table II, Figure 8). In paraoxon antagonism studies, (SL-OPH) alone was superior to the 2-PAM+atropine combination, and the (SL-OPH) with 2-PAM + atropine combination increased the protection by over 1000LD_{50} doses. The (SL-OPAA) combined with 2-PAM and atropine also enhanced the protection against DFP, but this enhancement was less dramatic than with the [(SL–OPH)-paraoxon] system. This observation is parallel with the *in vitro* kinetic data on isolated enzymes(Table I). The OPH is a more efficient hydrolyzing enzyme for paraoxon as a substrate than OPAA for DFP. The reason for choosing OPAA for these antidotal studies is that it can hydrolyze other OP agents, such as soman and sarin even more effectively. OPAA has a turnover number of 2,000, which indicates that 1 mole of enzyme will metabolize 2000 moles of sarin per second, or 120,000 moles/minute. This is in contrast to the stoichiometric enzymes which can sequester only a mole for mole basis on sarin. In a one minute interval, OPAA should be 120,000 times more active than the stoichiometric enzymes in removing sarin. This has both practical and theoretical advantages. Also, when red blood cells containing high concentrations of acetylcholinesterase were compared under our conditions, with liposomes with no cholinesterase, the protection of the liposomes and red blood cells showed no difference with the same content of OPAA. This suggests that, under the conditions of these experiments, the major advantage of OP antagonism is to use a hydrolytic rather than a sequestering mechanism. Based on the very preliminary studies with the (NT-OPH) and (NT-DFP) systems, they can enhance the protection of paraoxon or DFP intoxication , respectively(Figure 9 and 10). These new modified liposomal delivery systems would maintain even more favorable physiologic disposition and pharmacokinetics for the encapsulated enzymes in the circulation rather than the (SL-OPH) or (SL-OPAA) systems, which would allow other routes of introduction such as

intramuscular or inhalation. The *in vitro* studies show that the [OPH-paraoxon] and the [OPAA-DFP] enzyme reactions are liner within both enzyme carriers (SL and NT), and the product concentrations measured outside the carriers were the same whether the carrier membrane was intact or destroyed by sonication or 1% (v/v) Triton-X 100 detergents (Cohen *et al.*, 1991). This also means that the substrates paraoxon or DFP are freely available for the enzymes inside the carriers and the reaction products can penetrate through the carrier membranes. The reaction between OPH-paraoxon showed a saturation curve both with the free enzyme and with the NT-capsulated enzyme. The encapsulated OPH (NT-OPH) and OPAA (NT-OPAA) were capable of hydrolyzing paraoxon or DFP, respectively, the same way as the unencapsulated, free enzymes. The liposome- like carrier (NT) without enzymes will not hydrolyze paraoxon or DFP, respectively (Figure 2 and 7).

In vitro and *in vivo* measurement of plasma acetylcholinesterase (AChE) activity level shows a good correlation with the antidotal effects of different OP antidotes. These data suggest that measuring AChE activity may be a good monitor and possibly indicator to predict the *in vivo* antidotal protective effects of the AChE reactivator, 2-PAM, and the OP-hydrolyzing enzyme, OPH or OPAA. Reactivation of the OP- phosphorylated AChE was directly proportional to the 2-PAM concentration, and the AChE level was directly proportional to the concentration of cholinesterase inhibitor agents, paraoxon or DFP, respectively, and the OP- hydrolyzing enzymes, OPH and OPAA, respectively. Since the OP agents are AChE inhibitors, monitoring the AChE level in the biologic system can monitor and may be able to predict not only the toxic effects of the OP agents but the detoxification efficiency of the OP antidotes as well. Only the OP hydrolyzing enzymes can chemically destroy the OP agents. 2-PAM or other oximes with highly nucleophilic characteristics can reactivate the phosphorylated AChE, but when soman is used as a war gas, the soman will combine with AChE and an alkoxy reaction occurs, which is called "aging". Once aging occurs than

2-PAM can no longer reactivate the OP inhibited AChE. Therefore, a biodegradable capsule, encapsulating an OP-hydrolyzing enzyme would be attempted to maintain the duration of action of this antidotal system to terminate the pharmacological activity by chemically destroying the OP agents.

The use of external enzymes as drug antidotes is a new concept in the history of drug antidotal therapy, and the present and previously reported results suggest that this approach has a very bright future on a scientifically new conceptual basis. These studies indicate that improved delivery systems with longer half life and less macrophage recognition provide a basis to enhance the application of fast catalytic enzymes. These studies reaffirm that highly catalytic enzymes with appropriate new delivery systems may serve as new conceptual approach for protection and treatment against various chemical intoxications. These studies should lead to a superior OP antidotal kit which is much more effective, longer acting with less toxicity and side effects to the combat soldiers with minimal adaptation than presently is available. Much of this new science due to the recent rapidly emerging field of nano-technology and its related adaption to biomedical science.

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Figure 1.

OP Hydrolysis in enzyme carriers.

The OP-molecules can freely enter the carriers, and after they are hydrolyzed they can exit.

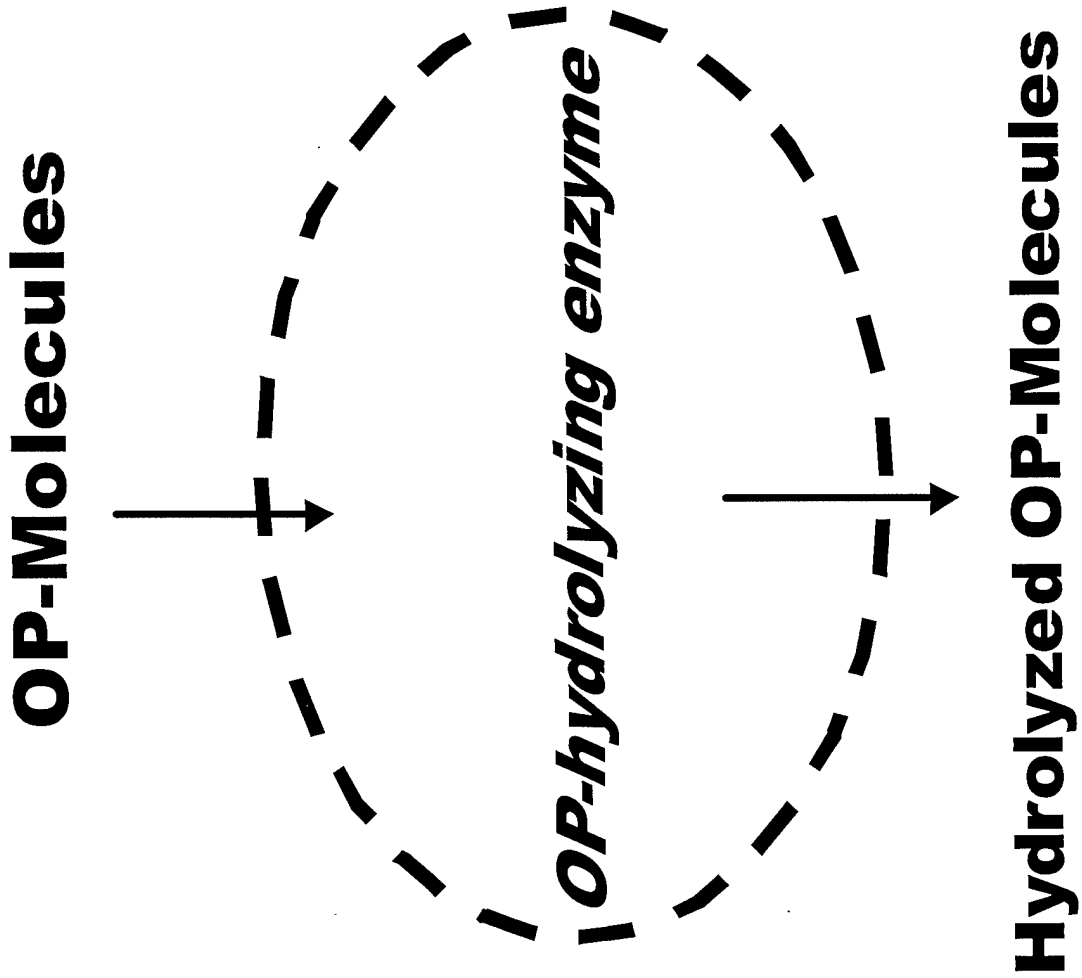


Figure 2.
Paraoxon Hydrolysis by (NT-OPH) as a function of volume of (NT-OPH).

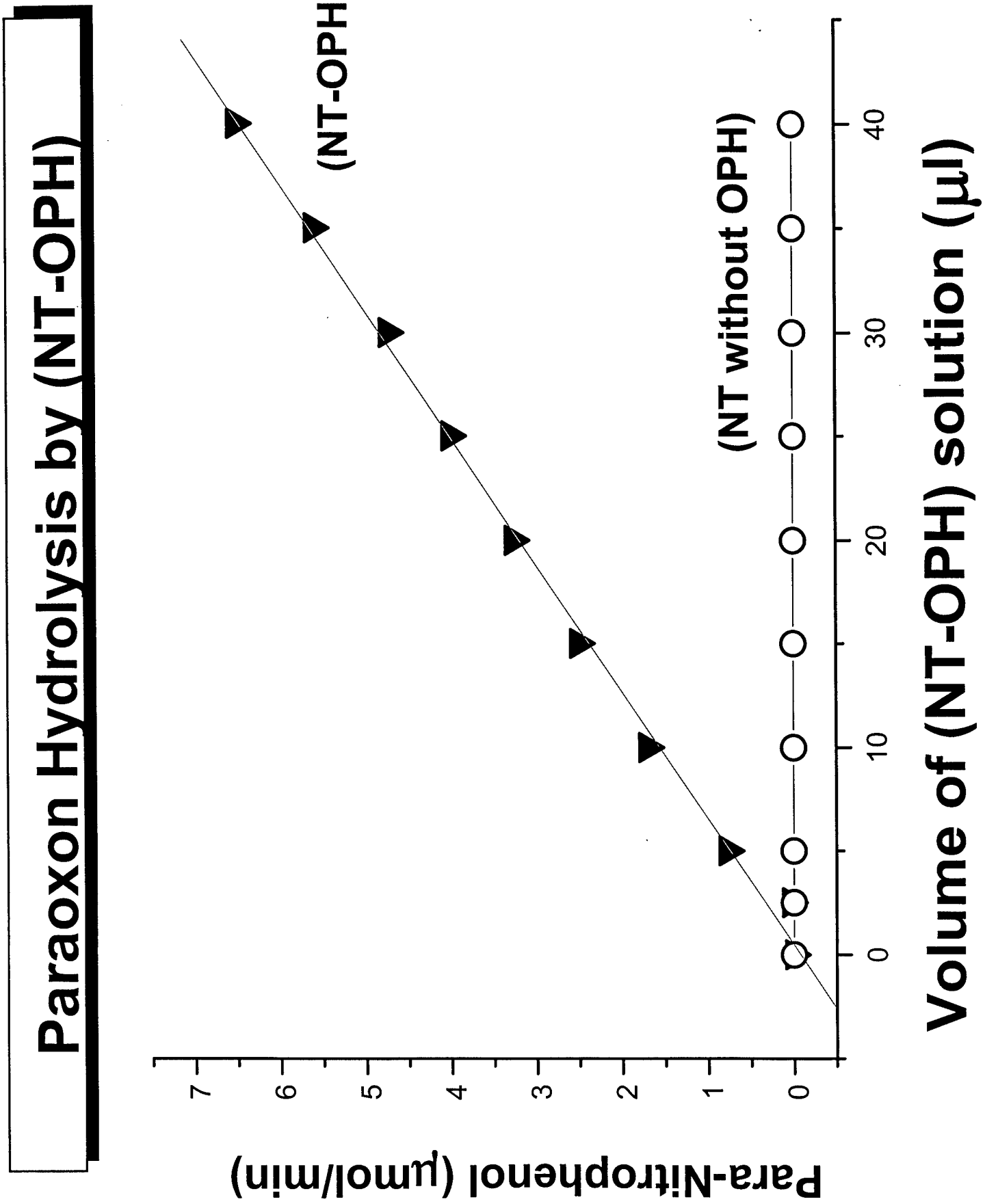


Figure 3.
Paraoxon Hydrolyzis by (NT-OPH) as a Function of Paraoxon Concentration. (Paraoxon saturation curve for encapsulated OPH).

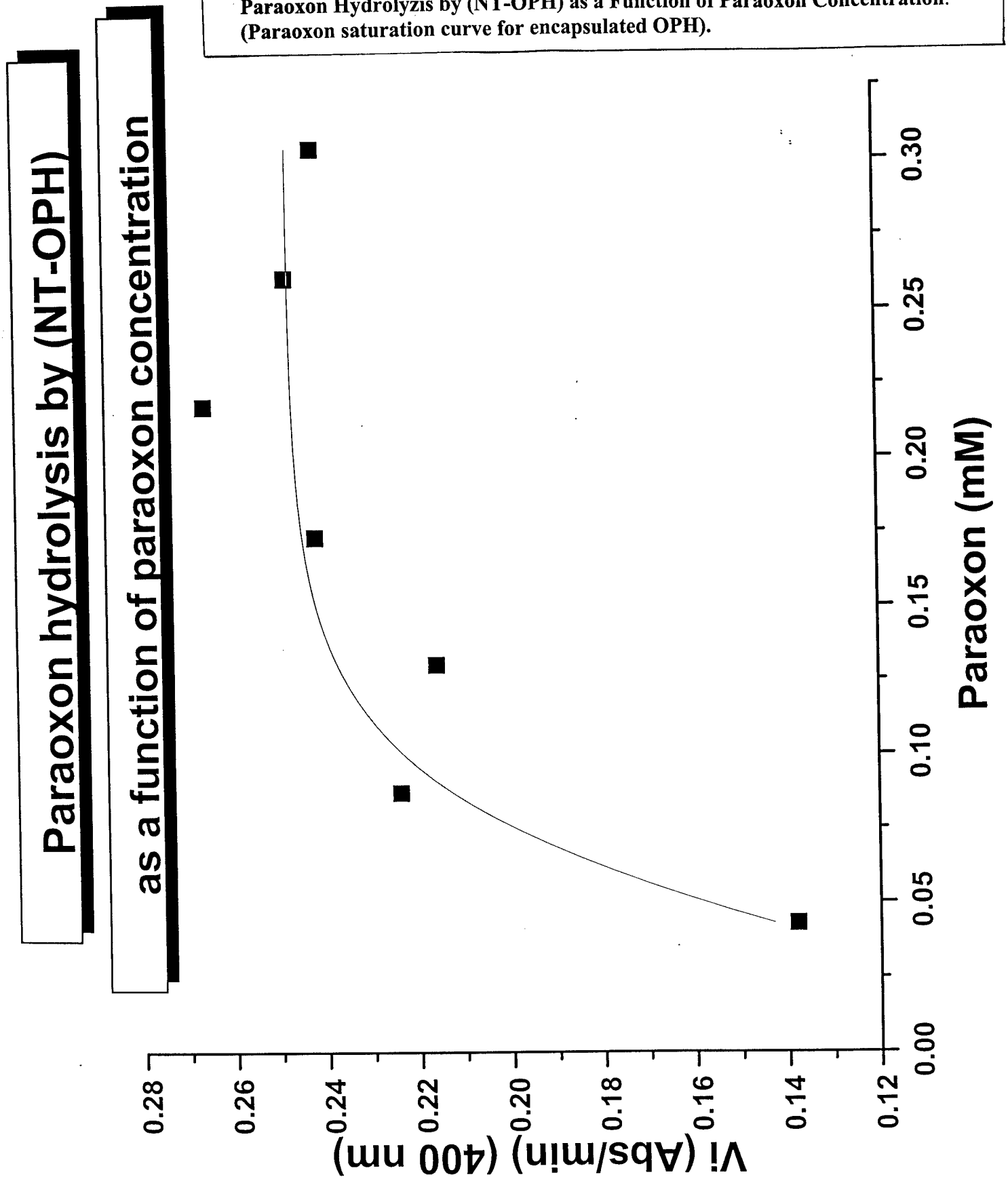


Figure 4.
Paraoxon Saturation Curve for Free OPH.

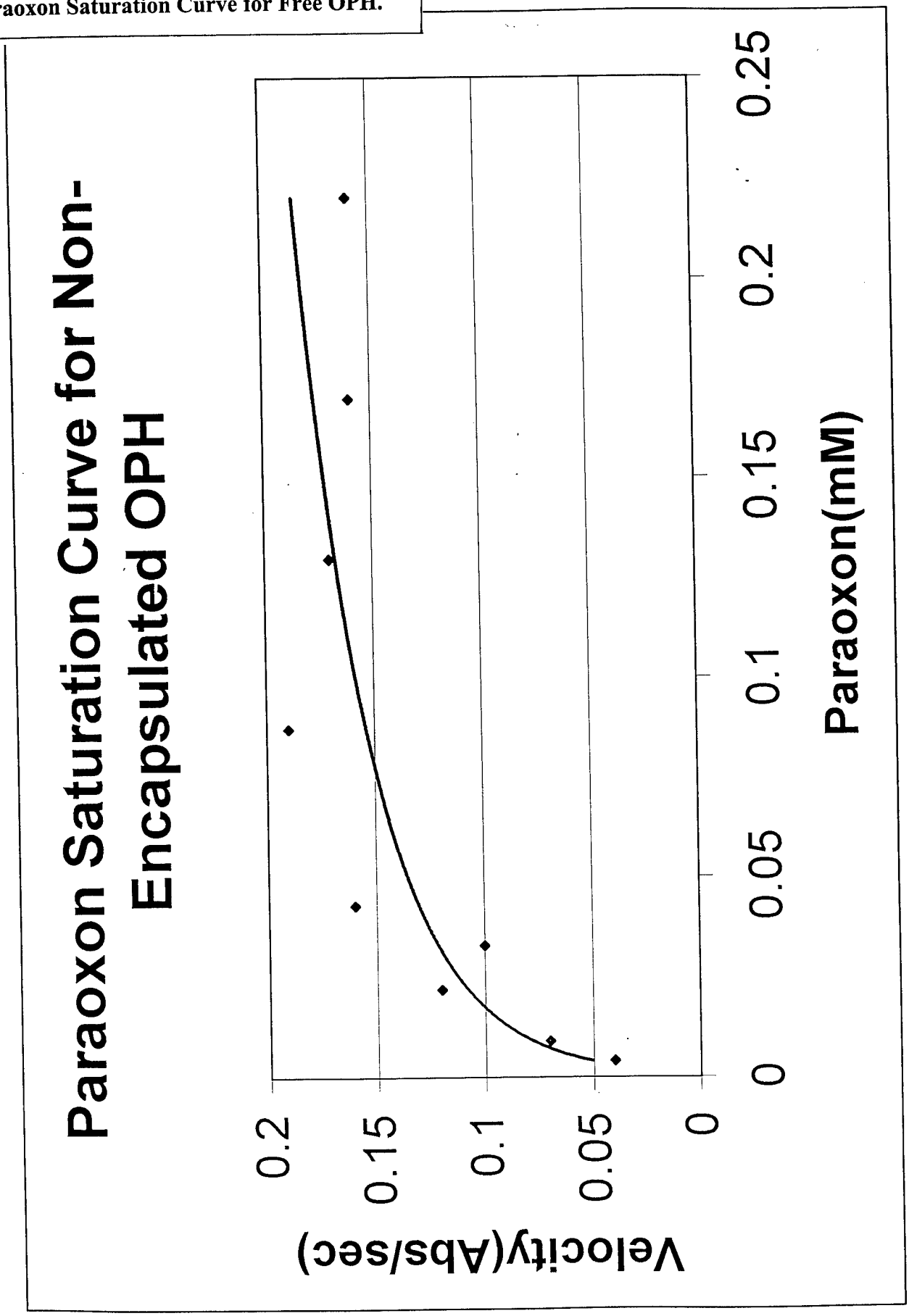


Figure 5.
Lineweaver - Burk Plot for (NT-OPH).

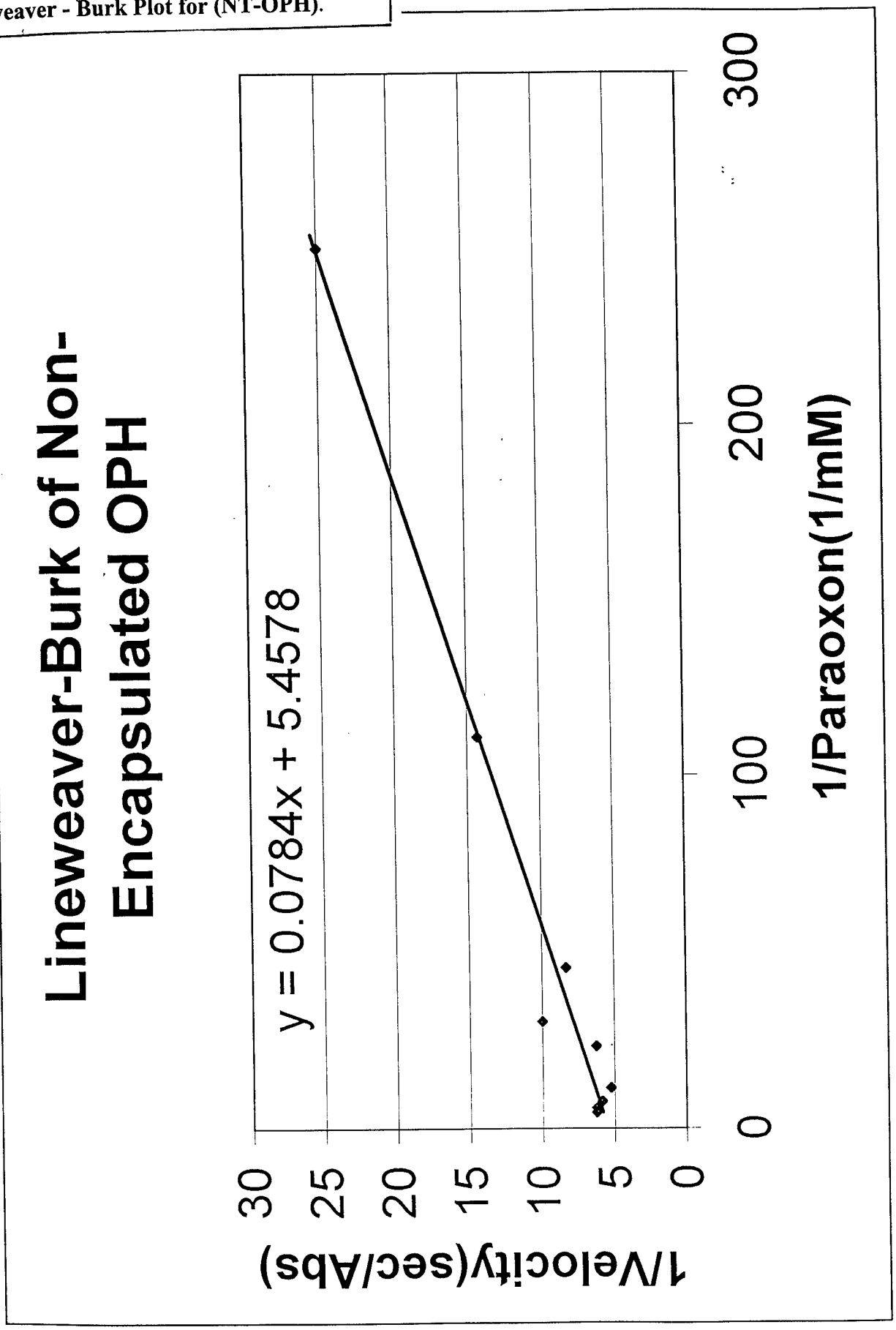


Figure 6.
Lineweaver - Burk Plot for Free OPH.

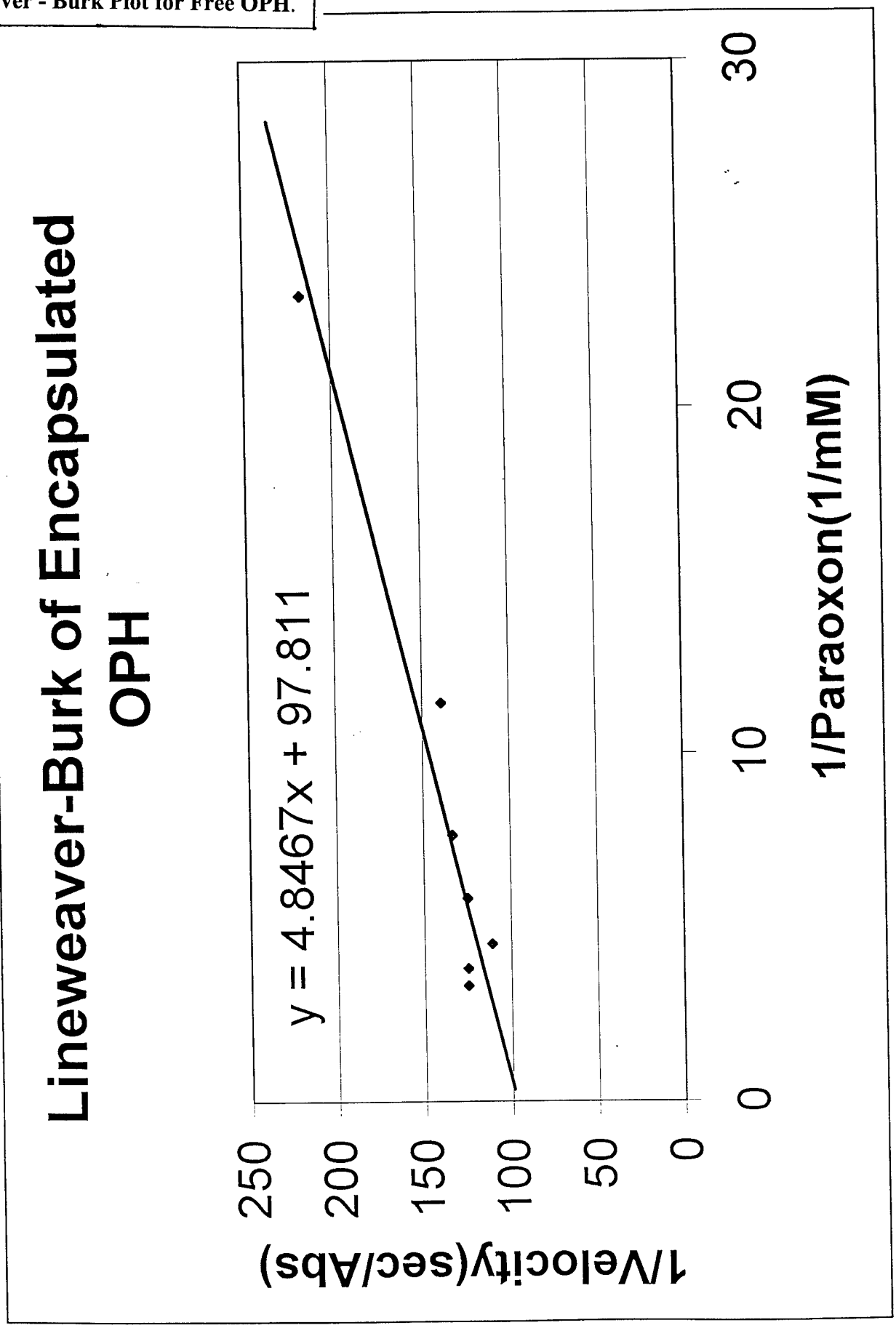
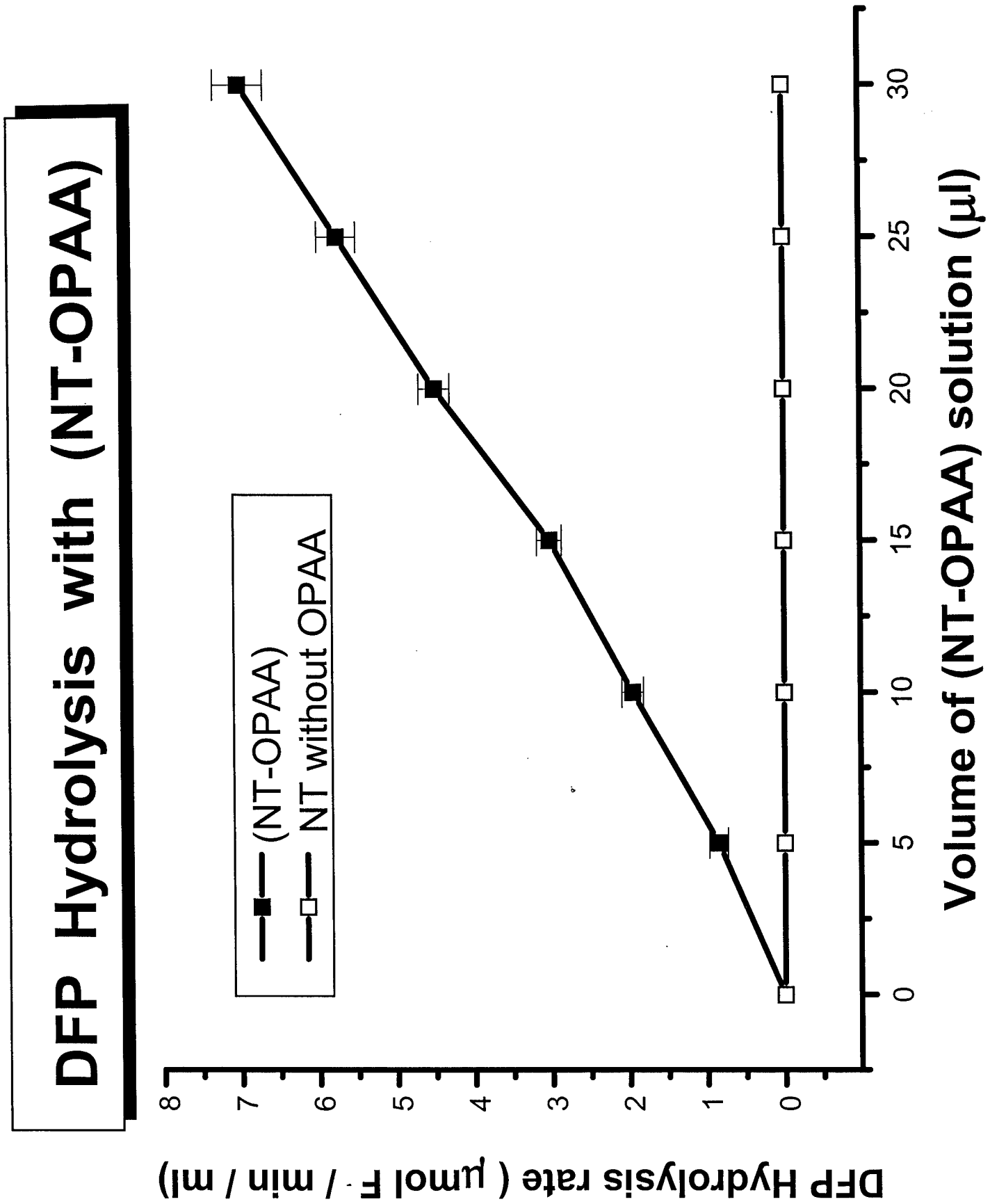


Figure 7.
DFP Hydrolyzis with (NT-OPAA) as a function of volume of (NT-OPAA).



Paraoxon Antagonism with (CRBC-OPH) vs. (SL-OPH)

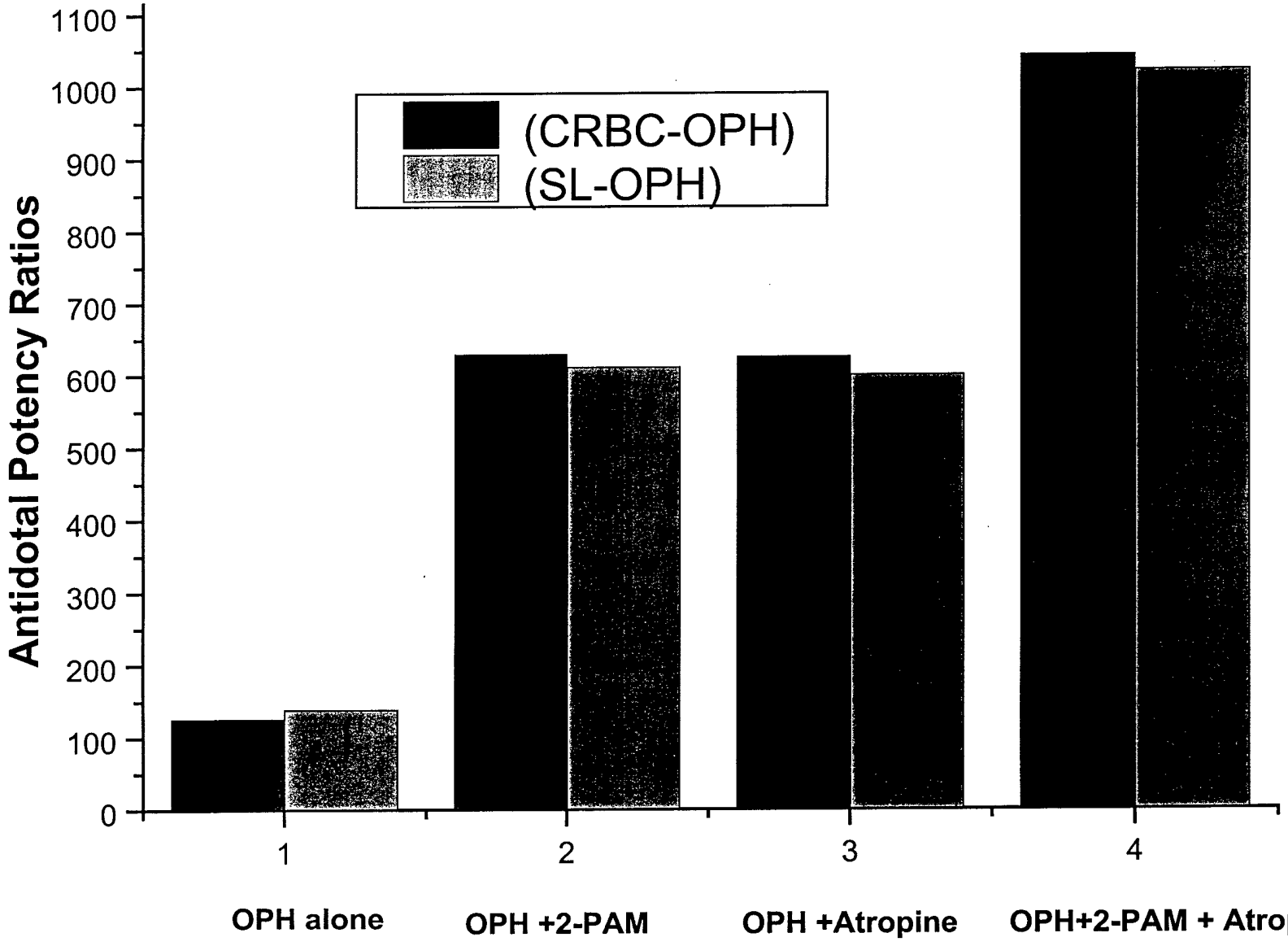


Figure 8. Paraoxon Antagonism with (CRBC-OPH) and (SL-OPH) when the two carriers have the same amounts of OPH.

Figure 9.
Paraoxon Antidotal Protection with (NT-OPH)

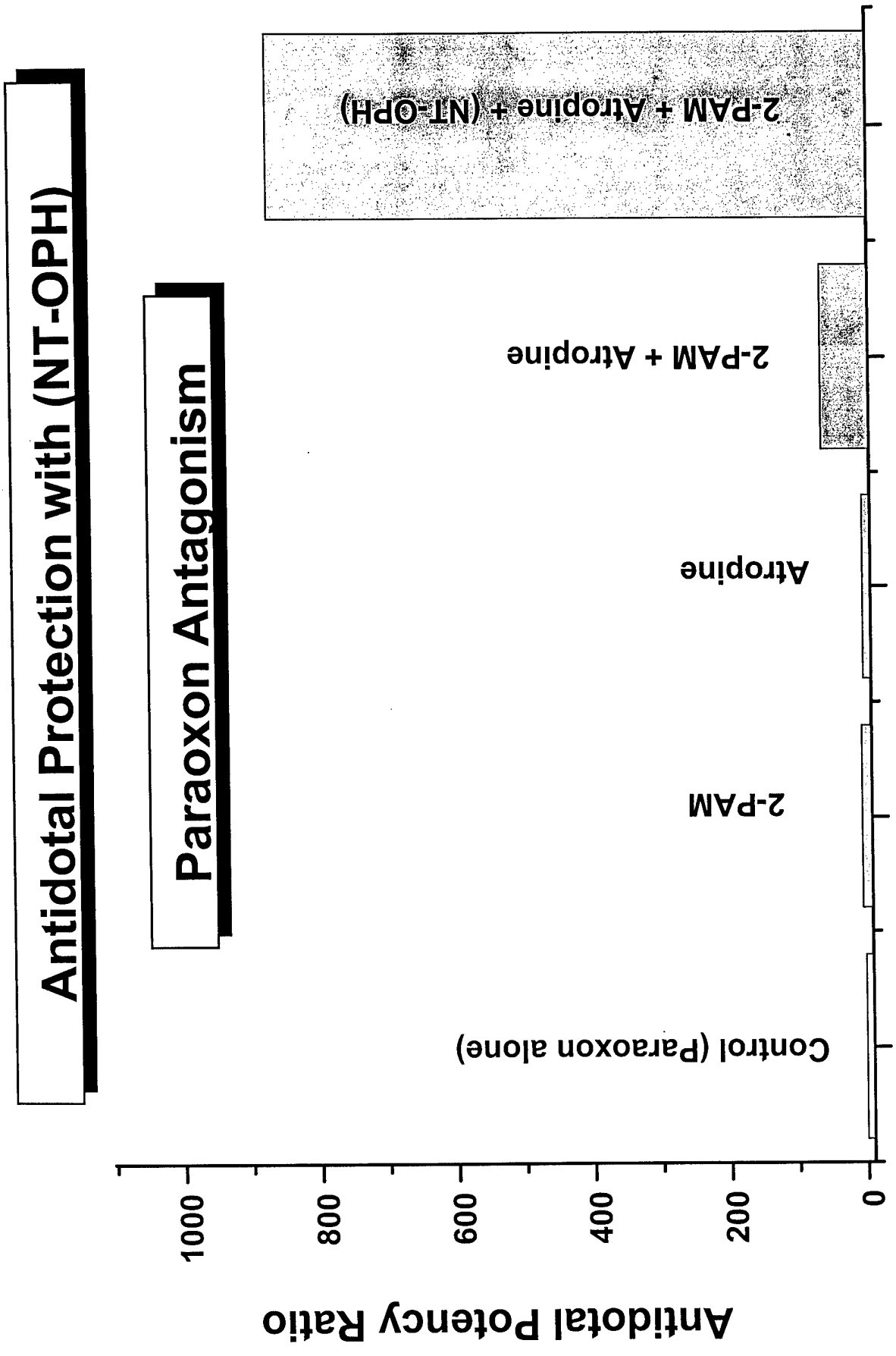


Figure 10.
DFP Antidotal Protection with (NT-OPAA).

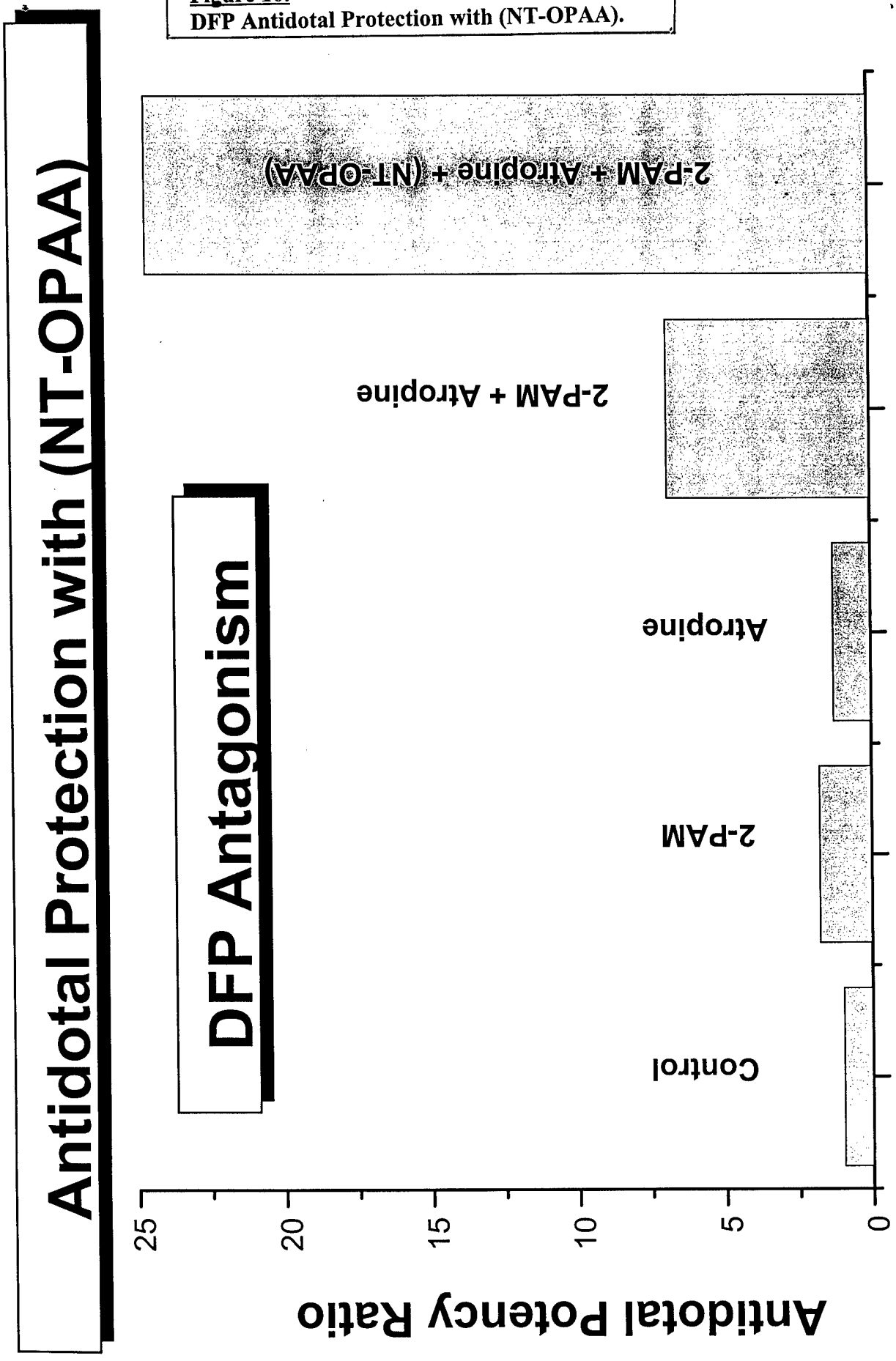


Figure 11.
In vitro **Cholinesterase Activity in the Presence of Paraoxon, 2-PAM and OPH.**

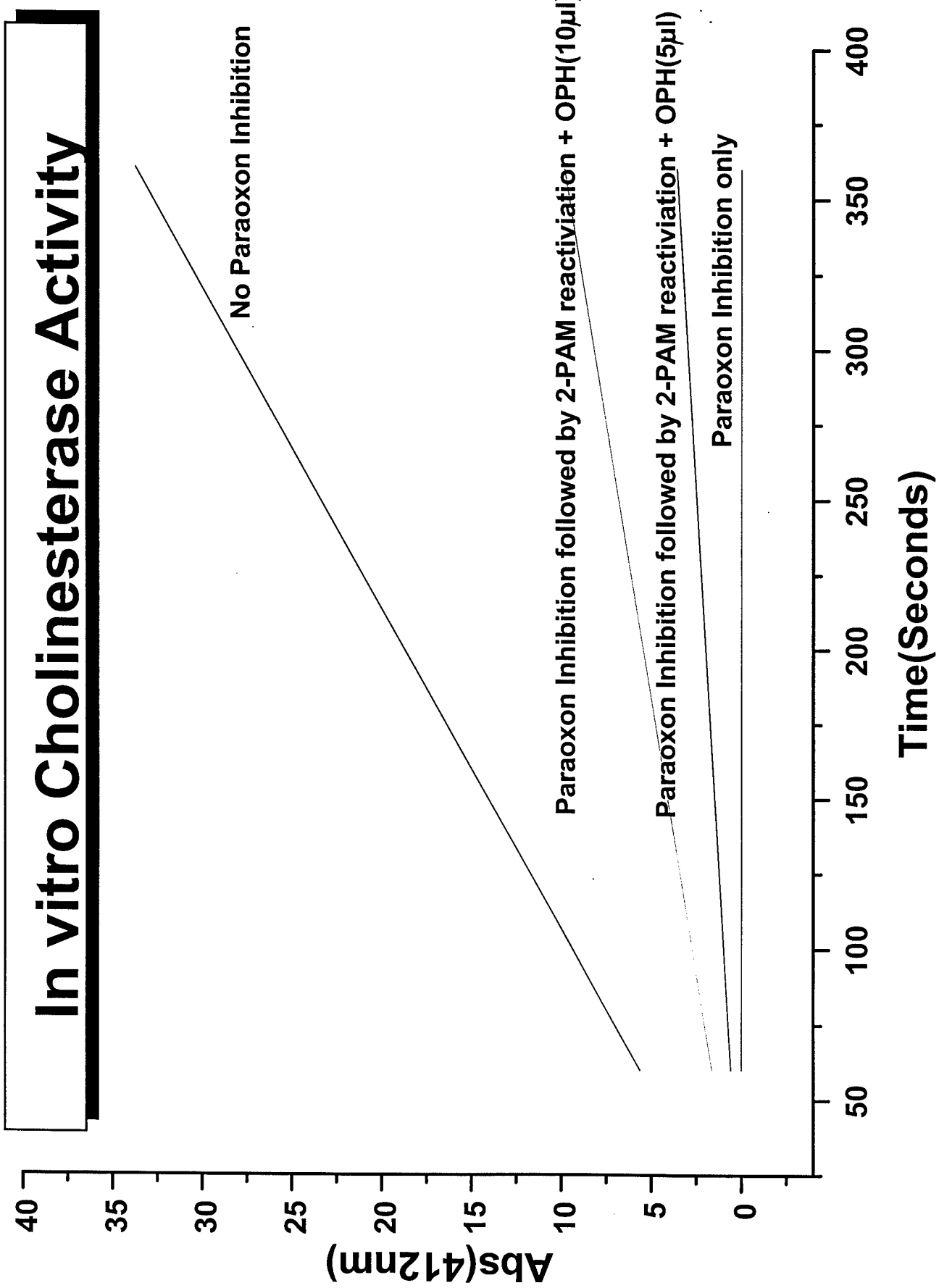


Figure 12.
Blood Cholinesterase Activity in mice when Paraoxon and/or 2-PAM and/or OPH were injected.

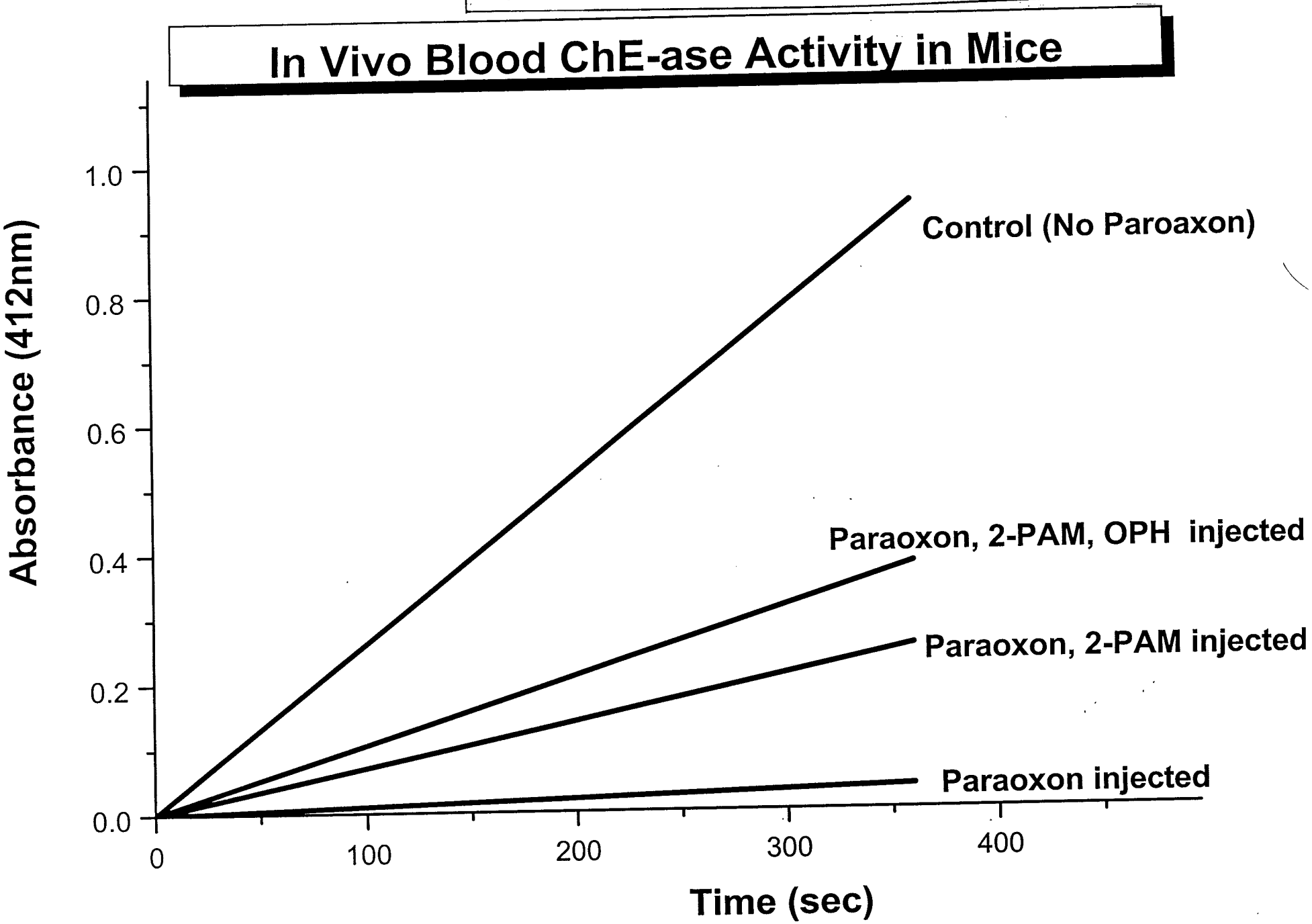


Table I
IN VITRO EFFICIENCY OF OPH AND OPAA ON SOME OP COMPOUNDS*

Substrate: / Enzyme	Paraoxon	DFP	Sarin	Soman
OPH (Enzyme I)	+++++	++	++	+
OPAA (Enzyme II)	+	+++	+++	+++

*Based on the following publications: Pei et al., (1995); Dumas (1990); Cheng et al., (1993)

Table II: PARAOXON ANTIDOTAL EFFECTS WITH OPH ENCAPSULATED IN CRBC AND SL***

	LD_{50} (mg/kg)	
Carriers	**CRBC***	**SL****
OPH alone	119.9 (91.22 - 157.58)	125.2 (91.4 - 170.5)
OPH & 2-PAM	597.7 (525.7 - 679.5)	550.2 (401.6 - 753.8)
OPH & Atropine	594.3 (548.6 - 644.6)	540.3 (394.5 - 740.2)
OPH & 2-PAM & Atropine	991.2 (891.4 - 1103.2)	920.0 (671.0 - 1260.4)

* LD50 values were obtained from five or more graded doses of Paraoxon administered to 5 or more groups of six or more mice/group. The LD50 values were determined by the method of Litchfield and Wilcoxon (1949), and determined by the program PHARM/PC Version 4.1 (Tallarida and Murray, 1987). All statistical procedures were performed at the 95% confidence level.

**LD50 values were determined by the up-and-down method (simulated up-and-down study, Dixon, 1965), and estimated 95% confidence interval was determined by the method of Bruce (1985). The LD50 values ere calculated from the equation: log(LD50) Log(Dosefinal) + k log(d), where does is the final dose administered, k is the tabular value from table (Dixon, 1965), and d is the dose interval between doses.

***OPH, Atropine, and 2-PAM were administered (60 min) (iv) 45 min. (ip), 15 min. (ip), prior to the administration of paraoxon (sc). Paraoxon (0.6-1200 mg/kg) dissolved in 6% cyclodextrin and/or propylene glycol solvent solution (40% propylene glycol, 10% ethanol, 50% water, v/v/v).