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ENHANCEMENT OF HEPATIC MICROSOMAL ESTERASE ACTIVITY FOLLOWING SOMAN PRETREATMENT IN GUINEA PIGS*

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Abstract—Soman (pinacolyl methylphosphonofluoridate), a highly toxic organophosphate compound, has been found to be a strong inhibitor of hepatic microsomal carboxylesterase *in vitro*, but an enhancer of carboxylesterase when administered *in vivo*. In response to this paradoxical observation, the objective of this study was to determine if soman could cause true enhancement of the metabolism of drugs in the guinea pig and, if so, to characterize the enhanced enzyme activity. Following the pretreatment of guinea pigs with 90% LD_{50} soman, enhancement of microsomal esterase activity was noted 12 and 24 hr after pretreatment. Using Michaelis–Menten enzyme kinetic studies, enhancement was found to occur with liver carboxylesterase and procaine esterase, but not with aniline hydroxylase. Since the soman–enzyme complex was known to undergo aging with the release of pinacolyl alcohol and the subsequent formation of pinacolone, the effects of these metabolites on the activity of liver microsomal enzymes *in vitro* were explored. Pinacolone and pinacolyl alcohol produced enzyme enhancement *in vitro* in a manner similar to that produced by soman pretreatment. These effects were compared with those made by acetone in the same incubations, since the enhancing influence of acetone has already been well documented. Similarity was found between the *in vitro* effects of acetone and the effects of pinacolone and pinacolyl alcohol. Lastly, the *in vivo* effects of pinacolone on the activities of the same liver microsomal enzymes were studied following pretreatment of the guinea pigs with 90% LD_{Lo} (lowest published lethal dose) pinacolone. Pretreating guinea pigs with pinacolone prior to killing them enhanced liver microsomal carboxylesterase and procaine esterase activities, but had no effect on microsomal aniline hydroxylase activity. This pattern of enzyme enhancement was similar to that observed after soman pretreatment. Therefore, soman was found to enhance hepatic microsomal esterase activity in the guinea pig in a manner similar to that seen with its metabolites, as well as acetone. This information may give insight into how the efficacy and toxicity of therapeutic drugs, other xenobiotics, and endogenous materials may be altered in individuals who survive an exposure to soman.

Key words: enzyme enhancement, soman, pinacolone, pinacolyl alcohol, acetone, carboxylesterases, hydroxylases, esterases

Soman (pinacolyl methylphosphonofluoridate), a chemical warfare agent, has been found to inhibit microsomal carboxylesterase *in vitro*, but not after *in vivo* pretreatment. Preliminary studies indicated an increase in esterase activity in isolated liver microsomes from guinea pigs pretreated with soman prior to being killed [1]. Enzyme enhancement was not observed following pretreatment with paraoxon, another organophosphate compound [2].

Just as soman can react with hydrolytic enzymes in the blood, acetylcholinesterase at central or peripheral nerve synaptic sites, and a variety of esterases in tissues, soman can also bind to enzymes that metabolize drug substrates. As illustrated in Fig. 1, upon subcutaneous administration and after reaching the blood, soman can be defluorinated by hydrolytic enzyme reactions with plasma esterases and phosphatases to form an inactive metabolite, free pinacolyl methylphosphonic acid (PMPA)§ [3]. If soman reaches acetylcholinesterase (AChE) at central or peripheral nerve synaptic sites, it inhibits this enzyme by forming AChE-bound PMPA [4]. In a matter of minutes this complex can undergo aging by reaction with *O*-dealkylase to form the irreversibly AChE-bound methylphosphoric acid (MPA) [5]. If soman reaches esterases, such as carboxylesterases in organ tissues like the liver, protein-bound PMPA can be formed [6]. This complex can also undergo aging by reaction with *O*-dealkylase to form the irreversibly protein-bound MPA [5]. Soman is also known to bind to enzymes that metabolize drug substrates, specifically carboxylesterases that

* The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Navy, the Army, the Department of Defense, nor the U.S. Government. In conducting the research described in this report, the investigators adhered to the *Public Health Service Policy on Humane Care and Use of Laboratory Animals* (1986), as prepared by the National Institutes of Health.

† Portions of this work have been taken from a dissertation submitted to the faculties of Eastern Virginia Medical School and Old Dominion University, Norfolk, VA, in partial fulfilment of the requirements for the Degree of Doctor of Philosophy.

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§ Abbreviations: PMPA, pinacolyl methylphosphonic acid; AChE, acetylcholinesterase; MPA, methylphosphoric acid; LD_{LO}, lowest published lethal dose; FID, flame ionization detector; PABA, *p*-aminobenzoic acid; and PNPA, *p*-nitrophenyl acetate.

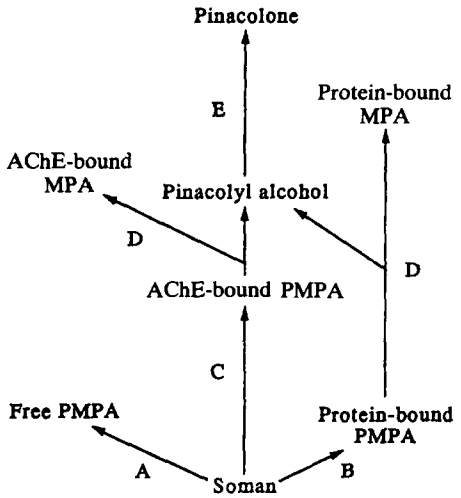

Fig. 1. Proposed pathway of soman metabolism. Key: soman (pinacolyl methylphosphonofluoridate) (four stereoisomers); PMPA (pinacolyl methylphosphonic acid) (inactive metabolite readily excreted into urine); MPA (methylphosphoric acid) AChE-bound MPA and protein-bound MPA are aged enzymes and irreversible complexes); A (hydrolytic enzymes primarily in plasma) (esterases and phosphatases); B (esterases primarily in organ tissues like liver); C (acetylcholinesterase at central and peripheral nerve synaptic sites) (AChE); D (*O*-dealkylase, an oxidase, splits bound PMPA); E (another oxidase).

hydrolyze aliphatic and aromatic esters of carboxylic acids, such as chloramphenicol succinate, prednisolone succinate, procaine and meperidine [1, 7]. During these dealkylation reactions, a leaving group, pinacolyl alcohol, is formed [8]. Since pinacolyl alcohol is a secondary alcohol, it may be oxidized by an oxidase to form a ketone, pinacolone.

There are only a few studies that report enzyme enhancement caused by soman. After soman poisoning in rats, the activity of ethylmorphine *N*-demethylase was enhanced [9]. Domschke *et al.* [10] noted that after rats were poisoned with soman (0.05 mg/kg) the original activity of plasma aliesterase was restored much more rapidly than after poisoning with paraoxon or diisopropylfluorophosphate. In the same study the activity of cholinesterase in the rat liver was initially depressed and then within a few hours it was increased to levels much greater than normal. The activities of liver threonine dehydrase and liver tryptophan pyrrolase were increased more than 2-fold within 2 hr after the administration of soman. The authors of this study concluded that soman may have had an inductive effect on all the enzymes occurring in the liver cell. In a recent study, 0.5 hr following administration of 50% LD_{50} soman in rats, liver microsomal carboxylesterase activity was not inhibited, but remained unaffected [11].

Based upon the current knowledge of the effects of soman, the phenomenon of enhancement following soman pretreatment has not been adequately explained. The soman-induced enhancement of enzymes noted in the above studies and in our preliminary *in vivo* studies has not been investigated *in vitro*. Likewise, the ability of soman to enhance other enzymes among the hepatic microsomal enzymes has not been studied. Also, the possible role of the ketone leaving group of soman in causing enhancement has not been explained.

In exploring this phenomenon, we first studied the effects of soman pretreatment on the activities of liver microsomal carboxylesterase, aniline hydroxylase, and procaine esterase. Next, the *in vitro* effects of pinacolone and pinacolyl alcohol, soman metabolites, were compared with those of acetone, a ketone like pinacolone, whose enhancing influence has already been reported in the literature [12]. Lastly, the effects of pinacolone pretreatment on the activity of liver microsomal enzymes were compared with those caused by soman.

MATERIALS AND METHODS

Care of animals. Experiments were conducted on adult male Hartley guinea pigs (300–500 g) from Charles River Laboratories (Wilmington, MA), because they provide sufficient liver tissue for multiple studies and are known to be sensitive to organophosphates. After entering the animal care facility, the animals received no drugs, and care was taken that they were never exposed to insecticides of any kind. They were housed two per cage in air-conditioned rooms in hanging cages without bedding and provided with food and water *ad lib*. The guinea pigs were killed by a blow to the head and then cervical dislocation at the time of use.

Handling of chemicals and treatment of animals. Dilute soman (2 mg/mL) was obtained from the U.S. Army Research Institute of Chemical Defense (Aberdeen Proving Ground, MD). It was a racemic mixture of all four soman stereoisomers. The 2 mg/mL dilution was divided into 200-μL aliquots, so that each contained 0.4 mg soman. The aliquots of soman were then stored at −80° for later use. Soman was prepared for injection by diluting the drug with Tris–KCl buffer. Then 90% LD_{50} soman [13] (22 μg/kg) was administered to two different groups of guinea pigs: in the first group 12 hr and in the second group 24 hr before the animals were killed. All drug injections were administered subcutaneously in the nape of the neck.

Pinacolone (3,3-dimethyl-2-butanone), pinacolyl alcohol (3,3-dimethyl-2-butanol), acetone, and all other chemicals in this study were obtained from the Sigma Chemical Co. (St. Louis, MO). For those studies using pinacolone, a 90% LD_{Lo} (lowest published lethal dose) of pinacolone [13] (630 mg/kg) was administered to the guinea pigs 12 hr prior to killing them.

Preparation of liver fractions. Using previously reported methods [7], liver microsomes, whole homogenate, 9,000 *g* supernatant, and the first 100,000 *g* supernatant were prepared from each guinea pig.

Protein determination. The spectrophotometric method of Lowry *et al.* [14] was used to determine the protein content of the liver fractions.

Determination of carboxylesterase activity. Overall

carboxylesterase activity was determined in liver fraction preparations spectrophotometrically by a modification of a well established method [15]. *p*-Nitrophenyl acetate (PNPA, 0.02 M) was used as the substrate, and carboxylesterase activity was calculated by measuring the absorbance change at 420 nm by following the release of *p*-nitrophenol at various time increments up to 10 min. Carboxylesterase activity was expressed as micromoles of *p*-nitrophenol per minute per milligram of protein.

Determination of aniline hydroxylase activity. The para-hydroxylation of aniline to *p*-aminophenol was used to assess the cytochrome P450-dependent oxidase system in liver fraction preparations spectrophotometrically by a modification of a well established method [16]. In this assay the *p*-aminophenol metabolite was chemically converted to a phenol–indophenol complex with an absorption maximum at 630 nm. The amount of *p*-aminophenol formed was expressed as nanomoles of *p*-aminophenol per minute per milligram of protein. Reduced nicotinamide adenine dinucleotide phosphate (NADPH) was a necessary cofactor for the para-hydroxylation of aniline.

Determination of procaine esterase activity. Procaine esterase activity was measured by determining the disappearance of procaine during the incubation of liver fraction preparations. The modifications of incubation conditions, sample preparation techniques, and HPLC procedures reported in an earlier study [2] were used. The activity of procaine esterase was expressed in terms of micromoles of procaine hydrolyzed per minute per milligram of protein.

In vitro *studies with pinacolone, pinacolyl alcohol, and acetone.* During these studies, enzyme assays for carboxylesterase, aniline hydroxylase, and procaine esterase had pinacolone, pinacolyl alcohol, or acetone added to them. Concentrations of the three xenobiotics for *in vitro* use were first estimated from what might be expected to be the *in vivo* concentration after the 90% LD_{50} soman dose used in the soman pretreatment studies. Screening studies were performed with broad ranges of xenobiotic concentrations and different preincubation times, to identify concentrations and times that would cause enhancement of the enzyme *in vitro*.

Extraction procedures for these organic xenobiotics were developed and gas chromatographic techniques were used to quantify them. Typically, the contents of the incubation vessels were placed into closed midget impingers and then brought up to 5 mL with Tris–KCl buffer. Using tygon tubing, each impinger was connected to a 25-mL water trap, a drying tube containing sodium sulfate, and then a small charcoal tube (150 mg: a 100 mg forward section and a 50 mg backup section) (SKC, Inc., Eighty Four, PA). Using an oil-less high volume pump with a critical orifice for 14 L air/min, air was pulled through the impingers and the tubing system leading to the charcoal tubes for 5 min. Organics collected on each charcoal tube were desorbed using 1 mL carbon disulfide and then placed on a sample agitator for 30 min. Aliquots (1 μL) were injected into a gas chromatograph. The following gas chromatography parameters were used: a 20% SE-30 Packed Glass Column (8/100 Chromasorb® W, nonpolar, methyl silicone gum rubber, 6 ft length) for pinacolone and pinacolyl alcohol and a 10% SP-1000 Column (80/100 Supelcoport®, 20 ft in length, 1/8 in. in diameter) for acetone; a Hewlett Packard gas chromatograph (model 5890A), autoinjector (model 7673A), integrator (model 3396A), and controller (model 7673A); a GE hydrogen generator; and a flame ionization detector (FID).

Disappearance studies in open vessels were performed by using 2 mL total volume of xenobiotic and Tris–KCl buffer. One concentration of each organic xenobiotic was allowed to remain in the 37° shaking incubator for 0, 5, 10, 20, and 40 min. After the allotted time had passed, the contents of the open vessels were transferred into the closed midget impingers and 3 mL Tris–KCl buffer was added before extraction. In the case of disappearance studies in closed vessels, xenobiotic dilutions along with 3 mL Tris–KCl buffer were placed into the closed midget impingers at the beginning of the incubation period.

Data analysis. In studies where only one substrate concentration was used, the rate of enzyme reaction was calculated in terms of nanomoles product formed or substrate lost per minute per milligram of protein. In cases where three or more substrate concentrations were used, Michaelis–Menten constants, K_m and V_{max}, were calculated using Lineweaver–Burk plots. Since the liver fractions were not purified enzyme, the apparent K_m was calculated. In cases in which only two levels of the one experimental variable were assessed, the double-sided Student's *t*-distribution test was applied to determine if the means of the two groups were statistically different ($P < 0.05$). In cases where there were more than two levels of the one experimental variable, the one-way analysis of variance (ANOVA) test was used. Descriptive statistics, *t*-test, and ANOVA of the SPSSX (Statistical Package for the Social Sciences) Batch System in a DEC VAX-11/780 VMS V4.2 computer were used.

RESULTS

Precision, percent recovery and specificity studies. The average coefficient of variation (V) of within-day blank values of the enzyme assays was 5.8% and the average V of between-day control values was 8.4%. The percentage recovery of procaine in the procaine esterase assay was on average 93 ± 10% of the amount of procaine added at the beginning of the incubation period and an average of 3.6% spontaneous hydrolysis was detected. During the HPLC analysis, there were no interfering chromatographic peaks with the procaine and *p*-aminobenzoic acid (PABA) peaks. The average percentage recovery for pinacolone, pinacolyl alcohol and acetone during linearity studies was 91%. There were no interfering gas chromatographic peaks with pinacolone, pinacolyl alcohol and acetone peaks.

Linearity of reaction rates. The hydrolysis of PNPA during the carboxylesterase assay was a first-order reaction using 5–20 mM PNPA for up to 2 min. The para-hydroxylation of aniline was a first-order reaction between 2 and 10 mM aniline hydrochloride for up to 30 min. The hydrolysis of procaine was a

Table 1. Effect of soman pretreatment on guinea pig liver microsomal carboxylesterase activity*

Time of storage†	Apparent K_m (mM *p*-nitrophenyl acetate)	V_{max} (μmol *p*-nitrophenol/mg protein/min)	Apparent K_m/V_{max}‡
Control			
Day of killing	7.44 ± 0.53	8.14 ± 1.18	0.91 ± 0.12
One week	28.22 ± 5.24§	10.67 ± 1.40	2.60 ± 0.39§
Four weeks	27.03 ± 7.92§	10.67 ± 4.40	2.12 ± 0.35§
Eight weeks	19.40 ± 3.87§	9.48 ± 0.64	2.01 ± 0.32§
Twelve weeks	21.91 ± 5.27§	11.09 ± 1.86	1.91 ± 0.36§
12-hr Pretreatment‖			
Day of killing	21.85 ± 2.65¶	16.57 ± 2.51¶	1.87 ± 0.47¶
One week	20.21 ± 3.68	17.48 ± 5.05¶	1.69 ± 0.21¶
Four weeks	24.43 ± 3.04	18.55 ± 6.15¶	1.98 ± 0.35
Eight weeks	16.29 ± 2.99	11.03 ± 1.79§	1.46 ± 0.09¶
Twelve weeks	16.63 ± 2.69	10.96 ± 0.43§	1.50 ± 0.21
24-hr Pretreatment**			
One week	56.22 ± 17.43¶	15.40 ± 2.37¶	3.30 ± 0.61
Four weeks	67.96 ± 27.11¶	20.75 ± 10.69¶	3.48 ± 0.44¶
Eight weeks	71.61 ± 21.61¶	17.72 ± 4.82¶	3.95 ± 0.24¶

* The apparent K_m, V_{max}, and apparent K_m/V_{max} values are the means ± SEM of seven determinations with control guinea pigs and six determinations with each pretreated group. The enzyme reaction time was 1 min.
† Time of storage at −80° varied from 1 to 12 weeks following preparation of liver microsomes using Tris–KCl buffer.
‡ Apparent K_m/V_{max} represents the slope of the Lineweaver–Burk plot.
§ Statistically different ($P < 0.05$) from the day of killing value in the same treatment group.
‖ Six guinea pigs were pretreated with 90% LD_{50} soman 12 hr prior to being killed.
¶ Statistically different ($P < 0.05$) from the corresponding control value.
** Six guinea pigs were pretreated with 90% LD_{50} soman 24 hr prior to being killed.

first-order reaction using 0.1 to 1.6 mM procaine for up to 10 min. The determinations of pinacolone, pinacolyl alcohol, and acetone were linear at the following concentrations: pinacolone ($0.7–8 \times 10^{-4}$ M), pinacolyl alcohol ($0.7–8 \times 10^{-4}$ M), and acetone ($1.2–13.6 \times 10^{-4}$ M).

Effects of soman pretreatment on activity of liver microsomal enzymes. In vivo enhancement of carboxylesterase was demonstrated in guinea pigs pretreated with 90% LD_{50} soman 12 or 24 hr prior to being killed. As shown in Table 1, on the day of killing and 1, 4 and 8 weeks after freezing the microsomes at −80°, carboxylesterase activity was increased in both pretreated groups. However, at this same series of time points, aniline hydroxylase activity was not significantly different between control and pretreated groups (data not presented). At 1 and 4 weeks after freezing, procaine esterase activity was increased in the 12-hr pretreated group, but decreased in the 24-hr pretreated group (Table 2).

V_{max} values from control microsomes at 1, 4, 8 and 12 weeks were not significantly different from V_{max} values determined on the day microsomes were isolated for the three enzymes studied. However, corresponding apparent K_m values from control microsomes at 1, 4, 8 and 12 weeks were increased for carboxylesterase. Almost all apparent K_m and V_{max} values for pretreated microsomes at 1, 4, 8 and 12 weeks were not significantly different from apparent K_m and V_{max} values determined on the day of microsome isolation. Except for carboxylesterase apparent K_m values, pretreated and control liver microsomes were stored at −80° for up to 12 weeks prior to use, without significant changes in V_{max} and apparent K_m values.

There was no significant difference in the enzyme activities presented in Tables 1 and 2 when microsomes prepared with a single Tris–KCl buffer wash were compared with those prepared with multiple washes (data not presented).

In vitro *effects of pinacolyl alcohol, pinacolone, and acetone.* As recorded in Table 3, pinacolone *in vitro* at a concentration of 2.666×10^{-8} M caused an increase in apparent K_m and V_{max} over the control values in the microsomal carboxylesterase assay, with the enhanced V_{max} equal to 358% of the control V_{max}. However, with acetone there was no change in carboxylesterase activity. In the case of aniline hydroxylase activity, there was a striking difference in the activity of the enzyme following *in vitro* exposure to the xenobiotics, depending on whether the enzyme reaction had been initiated by the addition of essential cofactors prior to exposure to the xenobiotics. When the enzyme reaction was not initiated prior to the addition of pinacolone to the assay, there was little effect on enzyme activity (data not shown). However, as shown in Table 4, under conditions when the enzyme reaction was initiated, the activity of microsomal aniline hydroxylase from control animals was enhanced markedly. In Table 4 increased values of V for microsomal aniline hydroxylase activity are given after *in vitro* exposure to pinacolyl alcohol, pinacolone, or acetone. At the higher concentrations of pinacolone and acetone, V was over 300% of the control V. As reported in Table 5, acetone had an inhibiting effect upon procaine esterase. When microsomes from soman-

Table 2. Effect of soman pretreatment on guinea pig liver microsomal procaine esterase activity*

Time of storage†	Apparent K_m (mM procaine)	V_{max} (μmol procaine hydrolyzed/mg protein/min)	Apparent K_m/V_{max}‡
Control			
Day of killing	0.320 ± 0.063	0.043 ± 0.010	7.545 ± 0.438
One week	0.384 ± 0.080	0.046 ± 0.009	8.320 ± 0.187§
Four weeks	0.379 ± 0.094	0.047 ± 0.010	7.703 ± 0.441
Eight weeks	0.263 ± 0.035	0.033 ± 0.004	7.859 ± 0.568
Twelve weeks	0.262 ± 0.053	0.031 ± 0.006	8.241 ± 0.324
12-hr Pretreatment‖			
Day of killing	0.392 ± 0.133	0.052 ± 0.016	7.127 ± 0.644
One week	0.737 ± 0.214¶	0.078 ± 0.025¶	9.643 ± 1.016§
Four weeks	0.566 ± 0.167	0.090 ± 0.026¶	8.813 ± 1.198
Eight weeks	0.293 ± 0.146	0.044 ± 0.022	6.185 ± 0.773
Twelve weeks	0.509 ± 0.239	0.043 ± 0.009	8.367 ± 1.380
24-hr Pretreatment**			
One week	0.234 ± 0.053	0.026 ± 0.005¶	8.839 ± 0.490
Four weeks	0.230 ± 0.028	0.027 ± 0.003¶	8.474 ± 0.501
Eight weeks	0.203 ± 0.045	0.025 ± 0.004	7.771 ± 0.859

* The apparent K_m, V_{max}, and apparent K_m/V_{max} values are the means ± SEM of seven determinations with control guinea pigs and six determinations with each pretreated group. The enzyme reaction time was 10 min.
† Time of storage at −80° varied from 1 to 12 weeks following preparation of liver microsomes using Tris–KCl buffer.
‡ Apparent K_m/V_{max} represents the slope of the Lineweaver–Burk plot.
§ Statistically different ($P < 0.05$) from the day of killing value in the same treatment group.
‖ Six guinea pigs were pretreated with 90% LD_{50} soman 12 hr prior to being killed.
¶ Statistically different ($P < 0.05$) from the corresponding control value.
** Six guinea pigs were pretreated with 90% LD_{50} soman 24 hr prior to being killed.

Table 3. *In vitro* effects of pinacolone or acetone on guinea pig liver microsomal carboxylesterase activity in closed vessels*

Enhancer concentration	Apparent K_m (mM *p*-nitrophenyl acetate)	V_{max} (μmol *p*-nitrophenol/mg protein/min)	Apparent K_m/V_{max}†	% Control V_{max}‡
Control	27.94 ± 4.31	12.81 ± 1.91	2.18	100.0
Pinacolone				
1.333×10^{-8} M	74.66 ± 37.67§	29.16 ± 11.38§	2.56	227.6
2.666×10^{-8} M	142.59 ± 47.17§	45.90 ± 10.20§	3.11	358.3
2.666×10^{-7} M	54.94 ± 12.37§	21.77 ± 5.15§	2.52	169.9
Acetone				
4.522×10^{-6} M	20.02 ± 5.36	10.69 ± 2.35	1.87	83.5
4.522×10^{-5} M	20.01 ± 3.61	11.41 ± 1.87	1.75	89.1
4.522×10^{-4} M	19.87 ± 2.82	10.85 ± 1.47	1.83	84.7

* The apparent K_m and V_{max} values are the means ± SEM of determinations with microsomes from four control guinea pigs. Microsomes were preincubated for 1 min with pinacolone or acetone. The enzyme reaction time was 1 min.
† Apparent K_m/V_{max} represents the average slope of four Lineweaver–Burk plots. It is apparent K_m divided by V_{max} from each line of data.
‡ V_{max} of pinacolone- or acetone-treated microsomes divided by V_{max} of control microsomes × 100%.
§ Statistically different ($P < 0.05$) from the corresponding control value.

pretreated guinea pigs were exposed to pinacolone *in vitro*, there was no further enhancement of procaine esterase activity (data not presented). In the case of microsomes from guinea pigs pretreated with 90% LD_{Lo} pinacolone, the three xenobiotics *in vitro* enhanced the activity of aniline hydroxylase, as shown in Table 4. In this case, the enzyme reaction was initiated prior to the addition of the xenobiotic. The three xenobiotics *in vitro*, however, inhibited the activity of procaine esterase (Table 5).

The disappearance studies of the xenobiotics in closed vessels determined the actual concentrations of the xenobiotics present during the *in vitro* studies. As shown in Table 6, with closed vessels the concentrations of the xenobiotics could be maintained over the period of the enzyme assays at a much higher and more stable level than if the vessels were open.

Neither pinacolyl alcohol nor pinacolone could be found in the liver fractions from soman-pretreated

Table 4. *In vitro* effects of pinacolyl alcohol, pinacolone, or acetone on guinea pig liver microsomal aniline hydroxylase activity in closed vessels*

Enhancer concentration	Velocity of reaction (V) (nmol p-aminophenol/ mg protein/min)	% Control V†
Control	0.11 ± 0.02	100.0
Pinacolyl alcohol		
2.650×10^{-4} M	0.15 ± 0.01‡	136.4
2.650×10^{-3} M	0.18 ± 0.01‡	163.6
2.650×10^{-2} M	0.19 ± 0.01‡	172.7
Pinacolone		
2.666×10^{-4} M	0.13 ± 0.01	118.2
2.666×10^{-3} M	0.13 ± 0.02	118.2
2.666×10^{-2} M	0.34 ± 0.02‡	309.1
Acetone		
4.613×10^{-3} M	0.13 ± 0.01	118.2
4.613×10^{-2} M	0.13 ± 0.03	118.2
4.613×10^{-1} M	0.34 ± 0.01‡	309.1
Pinacolone-pretreated	0.13 ± 0.02	118.2
Pinacolyl alcohol		
2.650×10^{-4} M	0.16 ± 0.01‡§	145.5 (123.1)‖
Pinacolone		
2.666×10^{-4} M	0.15 ± 0.01‡	136.4 (115.4)‖
Acetone		
4.613×10^{-3} M	0.17 ± 0.02‡§	154.5 (130.8)‖

* The velocity of reaction (V) values are the means ± SEM of determinations with microsomes from four control or four pinacolone-pretreated guinea pigs. The microsomes were not preincubated with the xenobiotic prior to addition of enzyme cofactors to begin the assay. Aniline HCl (6 mM) was used as substrate. The enzyme reaction time was 30 min.

† V of xenobiotic-treated microsomes divided by V of control microsomes × 100%.

‡ Statistically different ($P < 0.05$) from the control value.

§ Statistically different ($P < 0.05$) from the V of microsomes from pinacolone-pretreated guinea pigs.

‖ V of xenobiotic-treated microsomes divided by V of microsomes from pinacolone-pretreated guinea pigs × 100%.

Table 5. *In vitro* effects of pinacolyl alcohol, pinacolone, or acetone on guinea pig liver microsomal procaine esterase activity in closed vessels*

Xenobiotic concentration	Velocity of reaction (V) (μmol procaine hydrolyzed/mg protein/min)	% Control V†
Control	0.037 ± 0.002	100.0
Pinacolyl alcohol		
1.325×10^{-8} M	0.033 ± 0.002	89.2
2.650×10^{-8} M	0.038 ± 0.002	102.7
2.650×10^{-4} M	0.035 ± 0.003	94.6
Pinacolone		
1.333×10^{-8} M	0.037 ± 0.004	100.0
2.666×10^{-8} M	0.041 ± 0.002	110.8
2.666×10^{-4} M	0.038 ± 0.004	102.7
Acetone		
4.613×10^{-7} M	0.037 ± 0.004	100.0
4.613×10^{-3} M	0.036 ± 0.002	97.3
4.613×10^{-2} M	0.029 ± 0.002‡	78.4
Pinacolone-pretreated	0.033 ± 0.003	89.2
Pinacolyl alcohol		
1.325×10^{-8} M	0.030 ± 0.002‡	81.1 (90.9)§
Pinacolone		
1.333×10^{-8} M	0.031 ± 0.003‡	83.8 (93.9)§
Acetone		
4.613×10^{-7} M	0.032 ± 0.001‡	86.5 (97.0)§

* The velocity of reaction (V) values are the means ± SEM of determinations with microsomes from four control or four pinacolone-pretreated guinea pigs. The microsomes were preincubated with the xenobiotic for 16 min prior to the beginning of the assay. Procaine (0.8 mM) was used as substrate. The enzyme reaction time was 10 min.

† V of xenobiotic-treated microsomes divided by V of control microsomes × 100%.

‡ Statistically different ($P < 0.05$) from the control value.

§ V of xenobiotic-treated microsomes divided by V of microsomes from pinacolone-pretreated guinea pigs × 100%.

guinea pigs. In the case of pinacolone-pretreated guinea pigs, 1.65×10^{-5} M pinacolone was found in whole liver homogenate and 3.29×10^{-6} M pinacolone was found in the first 100,000 g supernatant.

Effects of pinacolone pretreatment on the activity of liver microsomal enzymes. In vivo enhancement of liver enzymes was demonstrated in guinea pigs pretreated with 90% LD_{Lo} pinacolone 12 hr prior to being killed (Table 7). Carboxylesterase activity was increased in the microsomal fraction, whole liver homogenate, and the 9,000 g supernatant. Aniline hydroxylase activity was not significantly different between control and pretreated groups. A significant increase in procaine esterase activity was found in the microsomal fraction.

DISCUSSION

The results show that pretreating guinea pigs with soman prior to killing them enhanced liver microsomal carboxylesterase and had mixed effects on procaine esterase activities. Microsomal aniline hydroxylase activity was not affected by pretreatment with soman. In the case of carboxylesterase activity, enhancement was found to be dependent upon the duration of time between administration of soman and sacrifice, since enhancement was greater in the 24-hr pretreatment group. However, since the enhancement of hepatic carboxylesterase activity was observed as early as 1 hr after pretreatment [1], the change occurs too quickly to be interpretable as an increased rate of enzyme synthesis.

The 12-week freezing studies demonstrated that

Table 6. Summary of xenobiotic disappearance studies

	[Xenobiotic] × 10^{-4}M*				
	Calculated Time 0	Measured Time 0	30 sec	10 min	30 min
Pinacolyl alcohol†					
Procaine esterase assay/Aniline hydroxylase assay (37°)					
Open vessels	2.662 (100%)	2.423 (91%)	2.686 (101%)	1.356 (51%)	0.102 (4%)
Closed vessels	2.662 (100%)	2.423 (91%)	1.966 (74%)	1.872 (70%)	2.050 (77%)
Pinacolone‡					
Carboxylesterase assay (25°)					
Closed vessels	2.666 (100%)	2.281 (86%)	1.903 (71%)	1.395§ (52%)	
Procaine esterase assay/Aniline hydroxylase assay (37°)					
Open vessels	2.666 (100%)	2.281 (86%)	1.108 (42%)	0.150 (6%)	0.030 (1%)
Open vessels with microsomes	2.666 (100%)	0.683 (26%)	0.585 (22%)	0.005 (0.2%)	0.000 (0%)
Closed vessels	2.666 (100%)	2.281 (86%)	1.266 (47%)	1.126 (42%)	1.120 (42%)
Closed vessels with microsomes	2.666 (100%)	2.281 (86%)	1.101 (41%)	0.945 (35%)	0.880 (33%)
Acetone					
Procaine esterase assay/Aniline hydroxylase assay (37°)					
Open vessels	4.545 (100%)	3.779 (83%)	2.679 (59%)	0.779 (17%)	0.300 (7%)
Closed vessels	4.545 (100%)	3.779 (83%)	2.879 (63%)	2.561 (56%)	2.680 (59%)

* Values in this table represent the molarity of the xenobiotic present in the incubation vessel followed by the percent of xenobiotic present compared with the calculated molarity present at time 0.

† 16 mm Hg vapor pressure.

‡ 13 mm Hg vapor pressure at 25°; 28 mm Hg at 37°.

§ Measured in an extraction impinger after time of incubation equals 4 min.

‖ 369 mm Hg vapor pressure at 37°.

no changes in enzyme activity occurred during long-term storage at −80°. Also, the studies of the effects of preparing microsomes with single or multiple washes demonstrated that the effects of soman pretreatment could not be removed. Therefore, this enzyme enhancement was found to be a true *in vivo* effect of soman that was not an artifact of storage or preparation of the liver microsomes.

Unfortunately, in our study higher doses of soman could not be used for pretreatment of the guinea pigs due to the high toxicity of soman. In future experiments it may be possible to dose guinea pigs with higher doses of the nontoxic $C(\pm)P(+)$ isomers of soman [17] to determine if enzyme enhancement increases. The toxic $C(\pm)P(-)$ isomers of soman [17] may cause toxicity without enzyme enhancement. Future experiments should attempt to determine if toxicity and enzyme enhancement caused by soman can be separated in this way. If enzyme enhancement can be traced to a specific isomer of soman, this information will provide insight into the relationship between dose and enhancement.

In future experiments, the levels of soman, pinacolyl alcohol and pinacolone in blood and liver fractions should be followed immediately after the administration of [^{3}H]soman to guinea pigs. Pinacolone and/or pinacolyl alcohol formation concurrent with enzyme enhancement would indicate sufficient release of xenobiotic from aged enzymes to produce soman-metabolite effects on the liver.

Studies have shown that pretreating guinea pigs with paraoxon, an organophosphate with no ketone leaving group, does not cause enzyme enhancement [2, 18]. Future experiments could involve pretreating guinea pigs with other organophosphates. Pretreating with another organophosphate that has a ketone

Table 7. Effect of pinacolone pretreatment on guinea pig liver enzyme activities*

Enzyme system and liver fraction	Apparent K_m†	V_{max}‡	Apparent K_m/V_{max}§	% Control V_{max}‖
Carboxylesterase activity				
Microsomal fraction				
Control	36.39 ± 9.80	16.26 ± 3.64	2.24 ± 0.55	
Pretreated	157.10 ± 67.59¶	56.46 ± 26.50¶	2.78 ± 1.25	347.1
Whole homogenate				
Control	33.14 ± 6.52	4.39 ± 0.46	7.55 ± 1.13	
Pretreated	77.34 ± 31.50¶	7.19 ± 2.32¶	10.76 ± 3.93	163.7
9,000 *g* Supernatant				
Control	82.46 ± 5.65	6.49 ± 0.18	12.70 ± 0.61	
Pretreated	162.14 ± 63.84¶	9.20 ± 3.75¶	17.62 ± 7.06	141.7
100,000 *g* Supernatant**	ND	ND	ND	
Aniline hydroxylase activity				
Microsomal fraction				
Control	2.58 ± 0.98	0.20 ± 0.01	13.11 ± 2.91	
Pretreated	1.61 ± 0.31	0.22 ± 0.02	7.42 ± 1.05¶	110.2
Procaine esterase activity				
Microsomal fraction				
Control	0.212 ± 0.017	0.035 ± 0.001	6.095 ± 0.349	
Pretreated	2.548 ± 0.609¶	0.082 ± 0.041¶	31.034 ± 11.477¶	235.9

* Values are means ± SEM of determinations with tissues from four control or four pinacolone-pretreated guinea pigs.

† Expressed in mM *p*-nitrophenyl acetate; mM aniline; or mM procaine.

‡ Expressed in μmol *p*-nitrophenol/mg protein/min; nmol *p*-aminophenol/mg protein/min; or μmol procaine/mg protein/min.

§ Apparent K_m/V_{max} represents the slope of the Lineweaver–Burk plot.

‖ V_{max} from pretreated divided by V_{max} from control animals × 100%.

¶ Statistically different ($P < 0.05$) from corresponding control value.

** Carboxylesterase activity was nondetectable (ND) in the 100,000 *g* supernatant from control or pretreated guinea pig liver.

leaving group smaller than the pinacolyl of soman may cause similar or different enzyme enhancement. Instead of large alkyl groups, such as pinacolyl, alkyl groups that are smaller in size could be selected. The size of the ketone leaving group may influence the degree of enzyme enhancement.

In vitro enhancement of enzymes by the addition of pinacolyl alcohol, pinacolone, or acetone to the enzyme assays was demonstrated in liver tissues from control guinea pigs, but not in liver tissues from soman-pretreated guinea pigs. The *in vitro* studies with acetone demonstrated enhancement of aniline hydroxylase. These findings duplicate reports of acetone in concentrations of 0.045 to 1.8 M enhancing aniline hydroxylation in rat, rabbit, mouse, and dog hepatic microsomes [19]. However, at concentrations of acetone as high as 0.46 M or higher, non-specific solvent effects may have contributed to the enhancement of aniline hydroxylation. Acetone, a xenobiotic that is known to cause enzyme enhancement *in vitro*, was used as a positive control in these studies to verify the presence of enzyme enhancement.

As expected, because of their high vapor pressures, the xenobiotics disappeared primarily by evaporation during the course of these enzyme assays. As recorded in Table 6, due to almost complete volatilization of the metabolites while using open vessels, closed vessels were selected for use. Also, the xenobiotics could not be found in liver fractions from soman-pretreated guinea pigs, because it is likely that during the use of the Polytron® homogenizer to prepare the liver fractions any pinacolone or pinacolyl alcohol present in the liver was vaporized quickly.

Since the microsomes from guinea pigs pretreated with soman 12 hr before they were killed already had procaine esterase enhancement, due to the release of pinacolone *in vitro* during the aging of soman-inhibited enzymes, further enhancement by pinacolone *in vitro* was not possible. Since there was no further increase in enhancement, all enhancement that was possible may have already occurred. In the case of microsomes from guinea pigs pretreated with 90% LD_{Lo} pinacolone, the three xenobiotics *in vitro* actually inhibited the activity of procaine esterase, but enhanced the activity of activated aniline hydroxylase. Since pinacolone-pretreated microsomes already had procaine esterase enhancement, further enhancement by pinacolone *in vitro* was not possible. However, with aniline hydroxylase, there was no enhancement following pinacolone pre-

treatment, so enhancement was possible after *in vitro* exposure.

As proposed for future soman pretreatment studies, the nontoxic $C(\pm)P(+)$ isomers and toxic $C(\pm)P(-)$ isomers should also be used *in vitro* with control microsomes. When enzyme assays were performed *in vitro* with a racemic mixture of soman stereoisomers present, enzyme activities were inhibited [1]. However, if an NADPH-generating system were present along with the soman, then oxidation of soman would be possible with the release of pinacolyl alcohol and pinacolone. With the release of these metabolites, subsequent enzyme enhancement may be measurable. Since we know the nontoxic isomers are quickly metabolized by the liver [17], higher concentrations of these two isomers should be used. These nontoxic isomers may cause enzyme enhancement without toxicity *in vitro*.

Although there have been several studies reporting the enhancement *in vivo* of aniline hydroxylase following administration of a single dose of acetone in rats [20] and in mice [21], there have been no studies reporting the enhancement of esterases following administration of acetone. Although there have been abundant studies documenting enzyme induction and enhancement following chronic exposures to organic solvents [22], this study is the first to report the *in vivo* effects of a single exposure to pinacolone on hepatic microsomal enzymes.

As reported in Table 7, pretreating the guinea pigs with pinacolone 12 hr prior to killing them caused enhancement of both carboxylesterase and procaine esterase in a manner similar to that seen following pretreatment with soman 12 hr prior to killing them. Neither pinacolone pretreatment nor soman pretreatment enhanced aniline hydroxylase activity.

As shown in Table 5, with microsomes from pinacolone-pretreated guinea pigs, further exposure to pinacolone *in vitro* did not cause additional enhancement of procaine esterase, but just the opposite, inhibition. The additional pinacolone added *in vitro* combined its effects with pinacolone from pretreatment, resulting in inhibition. As with most enhancers, once a specific concentration is exceeded, it no longer causes enhancement, but begins to cause inhibition. However, as shown in Table 4, with aniline hydroxylase, further exposure to pinacolone *in vitro* caused enhancement. Therefore, pinacolone, if added in the proper concentrations *in vitro*, can have an enhancing effect on microsomal enzymes from guinea pigs pretreated with pinacolone.

The enhancement of aniline hydroxylase *in vitro* by the addition of pinacolone, but not after *in vivo* pretreatment with soman or pinacolone, seems paradoxical. The estimated concentration of pinacolone present in the liver following pretreatment with 90% LD_{Lo} pinacolone (1.57×10^{-2} M) was close to the highest concentration of pinacolone used *in vitro* in a closed system (2.666×10^{-2} M). The estimated concentration of pinacolyl alcohol or pinacolone present in the liver following pretreatment with 90% LD_{50} soman (3×10^{-7} M) was slightly greater than the lowest concentration of pinacolone used *in vitro* in a closed system (1.333×10^{-8} M). Two possible reasons for the lack of enhancement seen after soman or pinacolone pretreatment may be (1) low concentrations of xenobiotic reaching the liver, and (2) preparation techniques for the liver tissue fractions that resulted in loss of xenobiotic. Another possibility is the lack of sufficient NADPH in the microsomal fractions from pretreated guinea pigs to support an enhanced enzyme reaction. In the case of our *in vitro* assays, an abundance of pinacolone and NADPH was provided in each assay.

The 90% LD_{50} for soman (22.5 μg/kg) is much smaller than the 90% LD_{Lo} for pinacolone (630 mg/kg) in the guinea pig. Soman is extremely toxic even in small doses, whereas pinacolone is toxic only in high doses. As specified above, the estimated molarity of pinacolyl alcohol or pinacolone in the liver is much greater following pinacolone pretreatment than following soman pretreatment. However, by means of the aging of soman-inhibited esterases in nervous tissues and other tissues throughout the body, sufficient pinacolone was released to exert an effect in the liver. As shown in Table 3, the concentration of pinacolone estimated to be present *in vivo*, 3×10^{-7} M, caused V_{max} to be 170% of the control value following *in vitro* exposure. Future studies with nontoxic isomers of soman may establish enzyme-enhancing doses of these isomers equivalent to the lowest enhancing dose of pinacolone in *in vivo* pretreatment.

Therefore, pretreating guinea pigs with pinacolone prior to killing them enhanced liver microsomal carboxylesterase and procaine esterase activities, but had no effect on microsomal aniline hydroxylase activity. Interestingly, this pattern of enzyme enhancement was similar to that following soman pretreatment in guinea pigs. In both cases, an increase in apparent K_m values was reported for carboxylesterase and procaine esterase, implying less enzyme affinity for substrate. This observation was unexpected and cannot be fully explained by these studies. The change in K_m, however, suggests that the enhancement cannot be explained by a simple increase in the amount of enzyme [12].

Without further study, the exact mechanism(s) of enzyme enhancement by pinacolyl alcohol and pinacolone cannot be known precisely. From the present findings, it is clear that soman pretreatment will cause enzyme enhancement *in vivo* and the metabolites of soman, known to be produced *in vivo*, will cause enhancement *in vitro*. It is also clear that the metabolites of aging, pinacolyl alcohol and pinacolone, act in a manner similar to that of acetone. Pinacolone, just as acetone, may have a primary effect upon the properties of enzymes *in situ*, by changing the spatial configuration of the microsomes or by interacting with specific functional groups of the enzymes [12]. Pinacolone may act by increasing the rate of breakdown of an enzyme–activator–substrate complex relative to the enzyme–substrate complex, as proposed for acetone [12, 19]. By a simple solvent effect, pinacolone may be unmasking additional active sites, so enhancement results [23]. It may be possible for pinacolone to cause the liberation of enzymes from the vesicular membrane by breaking the very weak linkage between the enzymes and the inner surface of the

vesicular membrane, as reported for acetone [24]. All of these possible mechanisms require further study before definitive conclusions about the mechanism(s) used by pinacolyl alcohol or pinacolone can be reached.

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