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INHIBITION OF XENOBIOTIC-DEGRADING HYDROLASES BY ORGANOPHOSPHINATES

ANNUAL REPORT

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Pretreatment of mice by intramuscular injection of 4-nitrophenyl diphenylphosphinate inhibited 89% of liver carboxylesterase activity in 2 h. Hydrolysis of aspirin was not reduced in pretreated mice, although a transient increase in the salicylic acid hydrolysis product was observed. Pretreatment with 4-nitrophenyl methyl(phenyl)phosphinate had no significant effect on total liver carboxylesterase activity or on the metabolism of aspirin.

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Rabbit serum arylester hydrolase, which hydrolyzed 10 or 13 organophosphinates tested as substrates, was partially inhibited by 4-nitrophenyl isoprophy(phenyl)phosphinate and 4-nitrophenyl diphenylphosphinate, which were not substrates. Bovine pancreatic trypsin and α-chymotrypsin were stereoselective in reactions with 4-nitrophenyl isopropyl(phenyl)phosphinate, with more rapid loss of the longer retained enantiomer in high performance liquid chromatography employing the chiral phase D-3, 5-dinitrobenzoylphenylglycine. Trypsin, but not α-chymotrypsin, exhibited a similar stereoselective reaction with 4-nitrophenyl ethyl(phenyl)phosphinate; hydrolysis of this phosphinate was catalyzed with the same stereoselectivity by arylester hydrolase.

Summary

Pretreatment of mice by intramuscular injection of 4-nitrophenyl diphenylphosphinate inhibited 89% of liver carboxylesterase activity in 2 h. Hydrolysis of aspirin was not reduced in pretreated mice, although a transient increase in the salicylic acid hydrolysis product was observed. Pretreatment with 4-nitrophenyl methyl(phenyl)phosphinate had no significant effect on total liver carboxylesterase activity or on the metabolism of aspirin.

Rabbit serum arylester hydrolase, which hydrolyzed 10 of 13 organophosphinates tested as substrates, was partially inhibited by 4-nitrophenyl isopropyl(phenyl)phosphinate and 4-nitrophenyl diphenylphosphinate, which were not substrates. Bovine pancreatic trypsin and α-chymotrypsin were stereoselective in reactions with 4-nitrophenyl isopropyl(phenyl)phosphinate, with more rapid loss of the longer retained enantiomer in high performance liquid chromatography employing the chiral phase D-3, 5-dinitrobenzoylphenylglycine. Trypsin, but not α-chymotrypsin, exhibited a similar stereoselective reaction with 4-nitrophenyl ethyl(phenyl)phosphinate; hydrolysis of this phosphinate was catalyzed with the same stereoselectivity by arylester hydrolase.

Foreword

This report contains results of research conducted during the third year of a project originally scheduled for three years. A continuation of this project for one additional year with two additional objectives was approved.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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1. Introduction

Organophosphinates are candidate pretreatment agents in a continuing search for more effective protection from and therapy for poisoning by organophosphorus chemical agents such as soman (1). Both organophosphinates and carbamates such as pyridostigmine are reversible inhibitors of acetylcholinesterase: they act by sparing acetylcholinesterase from irreversible inhibitors such as soman.

These studies were undertaken to examine possible interactions of organophosphinates with certain hydrolases other than cholinesterases. It is important to investigate these related enzymes, since there are many hydrolases involved in the biotransformation of drugs and xenobiotics, particularly in mammalian liver, kidney and serum; therefore, there is potential for interaction of a pretreatment agent with subsequent drug therapy. In addition, xenobiotic-degrading hydrolases are involved in the pharmacokinetics of atropine and the toxicokinetics of soman and other chemical agents.

The pharmacokinetics of the organophosphinates themselves is likely to be influenced by hydrolases; in fact, we have found certain organophosphinates to be excellent substrates for rabbit serum arylester hydrolase (2). The choice of organophosphinate candidates for pretreatment agents should be made in consideration of these factors as well as efficacy.

Recent investigations have indicated that 15 organophosphinates, including some of those examined in this study, were not as effective as four carbamates and one phosphorinane when administered orally prior to intramuscular challenge by soman (3). While these results did not indicate the efficacy of the organophosphinates, it is possible that these compounds would be efficacious when administered by a different route. Oral administration exposes these compounds to the intestine prior to absorption and transport to sites of action. The intestine contains the highest levels of carboxylester hydrolase (carboxylesterase) activity among rat tissues (4,5), and we have found that organophosphinates react very rapidly with carboxylesterase (6). Since 4-nitrophenyl methyl(phenyl)phosphinate is five-fold more toxic when administered intramuscularly than orally in both mice and rats (vide 2, p. 11), perhaps it would be more efficacious against soman poisoning following intramuscular administration or some other route which avoids the gastrointestinal tract. Pyridostigmine and other carbamates are less likely to be metabolized hydrolytically (7).

In the first year of this contract, we described the inhibition and spontaneous reactivation of carboxylesterase by organophosphinates (8). Inhibition of porcine liver oligomeric carboxylesterase was extremely rapid, so that a 10-fold excess of most organophosphinates over the concentration of enzyme resulted in nearly complete inhibition in less than one min. Median inhibitory concentrations for a two min exposure were determined and these data indicated the potency of these compounds; e.g., the value for 4-nitrophenyl di-2-thienylphosphinate was 7.4×10^{-9} M (6). The aryl- and heteroaryl-containing phosphinates were more potent carboxylesterase inhibitors than seven 4-nitrophenyl dialkylphosphinates previously studied for inhibition of against horse liver carboxylesterase (9).

Phosphinylated rabbit liver monomeric carboxylesterase, upon separation from excess phosphinate inhibitor, was observed to reactivate spontaneously at a rate dependent on the chemistry of the attached phosphinyl group (2,6). In each of four series of analogous inhibitors, phosphinylated carboxylesterases recovered faster following inhibition by smaller compounds in the series. This carboxylesterase recovered from inhibition by most organophosphinates, although it did not recover from inhibition by paraoxon; this may have been due to dealkylation (aging) of phosphorylated monomeric carboxylesterase, which probably would not occur with phosphinylated enzyme (although we did not investigate this phenomenon).

Reactivation of ethyl(phenyl)phosphinylated rabbit liver carboxylesterase induced upon exposure to 1,1'-trimethylene-bis-(4-formylpyridinium bromide) dioxime (TMB-4) occurred at twice the rate of spontaneous reactivation discovered in the second year of this project (2,6). It is clear that carboxylesterase reacts rapidly with organophosphinates and therefore, interactions with xenobiotic biotransformation are possible upon organophosphinate administration. Spontaneous and oxime-induced reactivation of phosphinylated carboxylesterase indicates that the beneficial effect of protecting xenobiotic-degrading enzymes against chemical agents such as soman is another possibility associated with organophosphinate pretreatment.

In this report we discuss the comparative inhibition in vivo of murine liver carboxylesterases by two organophosphinates. Also reported herein are the results of tests for interactions of phosphinate pretreatment with subsequent metabolism of [carboxyl-^{14}C]-and [acetyl-1-^{14}C]-aspirin, a common drug containing a carboxylester which is normally hydrolyzed very rapidly.

In the second year of this contract, we reported that certain organophosphinates were excellent substrates for rabbit serum arylester hydrolase (2). In fact, 10 of 13 organophosphinates tested exhibited lower Michaelis constants for hydrolysis than paraoxon, which is commonly used to assess the activity of this enzyme in tissues. Specific activities of one arylester hydrolase preparation were up to 50-fold greater against certain organophosphinates than against paraoxon. It appears that enzymatic hydrolysis by this type of enzyme could be a major factor in the pharmacokinetics of organophosphinate pretreatment agents.

This report also includes results obtained with two organophosphinates whose hydrolysis by arylester hydrolase was negligible and which were tested subsequently for inhibition of arylester hydrolase activity. Further improvements in purification of this enzyme are also described.

Stereoselectivity of hydrolysis of 4-nitrophenyl ethyl(phenyl)phosphinate by arylester hydrolase was reported in the second year of the contract (2). Herein we report results of chiral selectivity in reactions of two organophosphinates with trypsin and α-chymotrypsin.

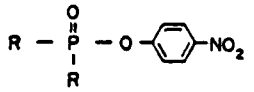
Three of four objectives of the original contract have been met, those being to determine the potentials for (i) inhibition of carboxylesterase, (ii) inhibition of arylester hydrolase (which led to the discovery of rapid enzymatic hydrolysis of organophosphinates), and (iii) stereochemical interactions of chiral organophosphinates with hydrolases. The fourth objective was to describe the interaction of organophosphinates with a mammalian fluorohydro-

lase. This has not been accomplished, although some preliminary progress has been made in purification of porcine kidney fluorohydrolase.

Future work will be performed to determine whether or not the high stereoselectivity observed in reactions with three hydrolases is a phenomenon common to hydrolases in general and to develop a method of resolving additional chiral organophosphinates by direct chromatographic techniques.

The organophosphinates used in these experiments were considered as four series of analogous compounds (Table 1). The chemical structure of every organophosphinate includes two carbon-phosphorus bonds. They were synthesized by Ash-Stevens, Incorporated, Detroit, MI, and provided to us by C. N. Lieske of the U.S. Army Medical Institute of Chemical Defense, Aberdeen Proving Ground, MD.

Table 1. Four series of organophosphinates used in studies of inhibition of xenobiotic-degrading hydrolases.

Compound		Number	Molecular Weight
Phenyl series:			
4-nitrophenyl methyl(phenyl)phosphinate		I	277
4-nitrophenyl ethyl(phenyl)phosphinate		II	291
4-nitrophenyl isopropyl(phenyl)phosphinate		III	305
4-nitrophenyl diphenylphosphinate		IV	339
Methyl series:			
4-nitrophenyl dimethylphosphinate		V	215
4-nitrophenyl methyl(2-furyl)phosphinate		VI	267
4-nitrophenyl methyl(2-thienyl)phosphinate		VII	283
4-nitrophenyl methyl(phenyl)phosphinate		(I)	277
4-nitrophenyl methyl(2-naphthyl)phosphinate		VIII	327
Heterocycle series:			
4-nitrophenyl methyl(2-furyl)phosphinate		(VI)	267
4-nitrophenyl di-2-furylphosphinate		IX	335
4-nitrophenyl methyl(2-thienyl)phosphinate		(VII)	283
4-nitrophenyl di-2-thienylphosphinate		X	351
Halogen series:			
4-nitrophenyl bis-chloromethylphosphinate		XI	284
4-nitrophenyl monochloromethyl(phenyl)phosphinate		XII	312
4-nitrophenyl dichloromethyl(phenyl)phosphinate		XIII	347
4-nitrophenyl trichloromethyl(phenyl)phosphinate		XIV	382
4-nitrophenyl methyl(trifluoromethylphenyl)phosphinate		XV	345

2. Inhibition of Mouse Liver Carboxylester Hydrolase

2a. Methods:

Organophosphinates I and IV from the phenyl series were compared for inhibition *in vivo* and *in vitro* of carboxylesterases of mouse liver. Male CF-1 mice (Charles River Breeding Laboratories) were injected intramuscularly with an LD_{01} dose of organophosphinate in polyethylene glycol 200 containing 1% glacial acetic acid (2). Mice were sacrificed 2 h later and livers were excised, minced and homogenized in 0.1 M sodium phosphate buffer, pH 7.5, in a glass-Teflon Potter-Elvehjem homogenizer mounted with an electric drill. The homogenate was centrifuged at 20,000 x g for 10 min and supernatant was collected as enzyme source. The activity remaining was determined 1 h after homogenization with 0.2 mM 1-naphthyl butyrate as substrate (8). Four replicates were performed, two on each of 2 days.

Further studies were initiated to determine the sensitivity to inhibition of various isozymes of carboxylesterase resolved by polyacrylamide gel electrophoresis. Mice were treated, sacrificed 2 h later and dissected, and liver homogenates were prepared as described above. The supernatant from centrifugation at 18,100 x g for 15 min was diluted 1:50 and 10 µl was placed in each well of 5% polyacrylamide slab gels for continuous electrophoresis. Gels were stained with 0.025% 1-naphthylacetate, 0.025% 2-naphthylacetate and 0.05% Fast Blue BB dye in buffer. One mouse was used for each of three treatments.

The liver of an untreated mouse was excised and homogenate prepared as above. Aliquots of this homogenate were incubated with several concentrations of I or IV, or with eserine sulfate to inhibit cholinesterases or paraoxon to inhibit both carboxylesterases and cholinesterases; the latter two inhibitors were used to characterize the isozymes since the subsequent staining procedure was not specific and revealed activity of several unrelated hydrolases. The inhibited homogenate was subjected to continuous electrophoresis on 5% polyacrylamide gels and enzyme activity was assessed by staining gels with a mixture of 1- and 2-naphthylacetate (1:1) and Fast Blue BB dye.

2b. Results

After 2 h exposure to injected organophosphinates *in vivo*, carboxylesterase activity of mouse liver was reduced 89% by IV (4-nitrophenyl diphenylphosphinate) and 20% by I (4-nitrophenyl methyl(phenyl)phosphinate); the latter result was not statistically significant (Table 2).

Electrophoresis of liver homogenates from mice treated as those above (*vide* Table 2) revealed in a control sample five bands of enzymatic activity toward 1- and 2-naphthylacetate mixture with mobilities relative to Bromphenol Blue tracking dye of 0.16, 0.22, 0.31, 0.46 and 0.58. Liver homogenates of one mouse treated *in vivo* with 100 mg/kg IV at 2 h prior to sacrifice produced only three bands of activity with relative mobilities of 0.15, 0.21 and 0.30, which were less intensely stained than those of the control; the two fastest running bands were completely lost. On the other hand, there was no visually discernible inhibition of

Table 2. Inhibition of carboxylester hydrolase activity in mouse liver 2 h following intramuscular injection of organophosphinates. The carboxylester hydrolase activity was assessed using 1-naphthyl butyrate as substrate.

	Treatment	Activity, μmoles 1-naphthol/min/mg protein ± s.e.
	Vehicle control	2.72 ± 0.36
I	4-nitrophenyl methyl(phenyl)phosphinate 1.11 mg/kg	2.17 ± 0.28
IV	4-nitrophenyl diphenylphosphinate 100 mg/kg	0.30 ± 0.16

carboxylesterase isozyme activity in the mouse treated with I: Its liver homogenate displayed bands with mobilities of 0.15, 0.22, 0.31, 0.46 and 0.60.

While the mice described above were exposed to equitoxic doses (the approximate LD_{01} of each), of I and IV, the actual dose of IV exceeded that of I by 90-fold on a weight basis and by 74-fold on a molar basis because I is much more toxic than IV. In order to compare the relative potencies of I and IV as inhibitors of mouse liver carboxylesterase more directly, a homogenate of the liver of an untreated mouse was prepared as above and aliquots were incubated with equimolar concentrations of I and IV prior to electrophoretic resolution of isozymes.

IV was at least 2-fold more potent than I as an inhibitor of mouse liver carboxylesterase isozymes when compared *in vitro* (Table 3). As observed also *in vivo*, the two fastest bands, with relative mobilities of 0.39 and 0.52, were most sensitive to inhibition. Resolution was improved in this gel so that a very slow band, with a mobility of 0.06, was also observed. This band was not resolved in the previous experiment.

Bands with mobilities of 0.14, 0.39 and 0.52 were identified as carboxylesterases because they were inhibited by 10^{-7} M paraoxon but not by 10^{-5} M eserine sulfate. Bands with mobilities of 0.06 and 0.19 were partially inhibited by eserine sulfate and completely inhibited by paraoxon; therefore, these bands probably contained cholinesterase activity, perhaps overlapping with carboxylester se activity. The band with 0.25 relative mobility was impervious both to paraoxon and to eserine sulfate, so that it contained either arylester hydrolase or acetylester hydrolase activity; these enzymes can be discriminated on the basis of the greater sensitivity of the former to heat or to 4-hydroxymercuribenzoate, but this was not pursued.

Carboxylesterase bands with mobilities of 0.14, 0.39 and 0.52 were all more sensitive to IV than to I, as observed when both organophosphinates were used at 5×10^{-8} M. These results were in agreement with the finding that porcine liver carboxylesterase was 2.4-fold more sensitive

Table 3. Inhibition of esterases from mouse liver following incubation with organophosphinates *in vitro*.

Band Number	1	2	3	4	5	6
R_f in Control	0.06	0.14	0.19	0.25	0.39	0.52
Inhibitor:		Relative intensity of activity stain:				
Control	++	+++	++	+++	++	++
eserine sulfate, 10^{-5}M	+	+++	+	+++	++	++
paraoxon, 10^{-7}M	-	-	-	+++	-	-
I[a], 2.5 x 10-8M	++	+++	++	+++	+	++
I, 5 x 10^{-8}	++	+++	++	+++	+	+
I, 1 x 10^{-7}M	+	+++	+	+++	-	-
IV[b], 2.5 x 10^{-8}M	++	+++	++	+++	-	-
IV, 5 x 10^{-8}M	+	++	+	+++	-	-
IV, 1 x 10^{-7}M	+	++	-	+++	-	-

[a] 4-nitrophenyl methyl(phenyl)phosphinate
[b] 4-nitrophenyl diphenylphosphinate

to IV than to I *in vitro* (6).

2c. Discussion

While I was 1000-fold more potent than IV as an inhibitor of acetylcholinesterase (1,10) and I was approximately 100-fold more toxic than IV (2), IV was more potent than I as an inhibitor of carboxylesterase. Furthermore, recovery of rabbit liver carboxylesterase from inhibition by IV was 6-fold slower than recovery from I (6). Because of its relatively low potency at inhibiting acetylcholinesterase, IV would have to be used at a much higher pretreatment dose than I in order to produce the desired protective effect; therefore, it should be considered that carboxylesterase inhibition would be a greater secondary effect of IV than of I.

Since 89% inhibition of mouse liver carboxylesterase activity was observed following pretreatment with IV (Table 2), it might be anticipated that enzymatic hydrolysis of a carboxylester-containing drug would be

reduced if it were administered following IV. An initial test of this hypothesis was performed with aspirin serving as a carboxylester-containing drug and the results are reported in the following section. Further tests employing procaine as a model carboxylester-containing drug are in progress.

It must be noted that phosphinate inhibition of enzymes other than acetylcholinesterase are secondary effects of pretreatment which could be either harmful or beneficial. Harm may be caused if deleterious drug interaction results; on the other hand, the reversible inhibition of xenobiotic-degrading hydrolases could serve to protect these important enzymes from complete and irreversible inhibition by soman or a similar agent of chemical warfare. We have demonstrated that phosphinylated carboxylesterases recover spontaneously and can be induced to more rapid reactivation by TMB-4 (2).

3. Aspirin Metabolism Following Organophosphinate Pretreatment in Mice

3a. Methods

Two experiments were performed to examine the effect of organophosphinate pretreatment upon the hydrolysis of aspirin in mice (Fig. 1); the experiments employed two different approaches to detecting the products of aspirin hydrolysis. In both experiments, male CF-1 mice weighing approximately 25 g were weighed and injected intramuscularly with an LD_{01} dose of an organophosphinate pretreatment agent 2 h prior to aspirin administration. Computed LD_{01} values and 95% confidence limits 1.11 (0.75-1.26) mg/kg for I and >100 mg/kg for IV; therefore, a dose of 100 mg/kg was used for IV. Control mice received vehicle only which was 0.076 ml polyethylene glycol-200 containing 1% glacial acetic acid and 10% methylene chloride.

In the first experiment, pretreated mice were injected intraperitoneally with 0.01 mCi (0.00138 mmole) [carboxyl-^{14}C]-aspirin in 0.076 ml 10 mM sodium bicarbonate. In this case, salicylic acid was the radioactive product.

Blood samples were drawn from the tail and sample volume determined at intervals commencing at 9 min; blood protein was immediately precipitated by addition of acetonitrile (containing nonradiolabeled aspirin and salicylic acid) and centrifugation. Supernatant was analyzed by high performance liquid chromatography (HPLC) (Fig. 2) in which the peaks corresponding to aspirin and salicylic acid were collected for liquid scintillation counting to measure radioactivity eluted.

This experiment was designed as a randomized complete block split plot in which the three main treatments (control and two organophosphinates) were repeated on four different days (blocks) so that 12 total mice were employed; subtreatments of the split plot were the time intervals at which repeated sampling of each mouse was performed. Total disintegrations per minute (dpm) of [^{14}C]-aspirin or [^{14}C]-salicylic acid per mouse were computed with the assumption that each mouse contained 0.0778 ml blood per g body weight (11). Data was analyzed by a general linear model computer routine (SAS Institute, Raleigh, NC).

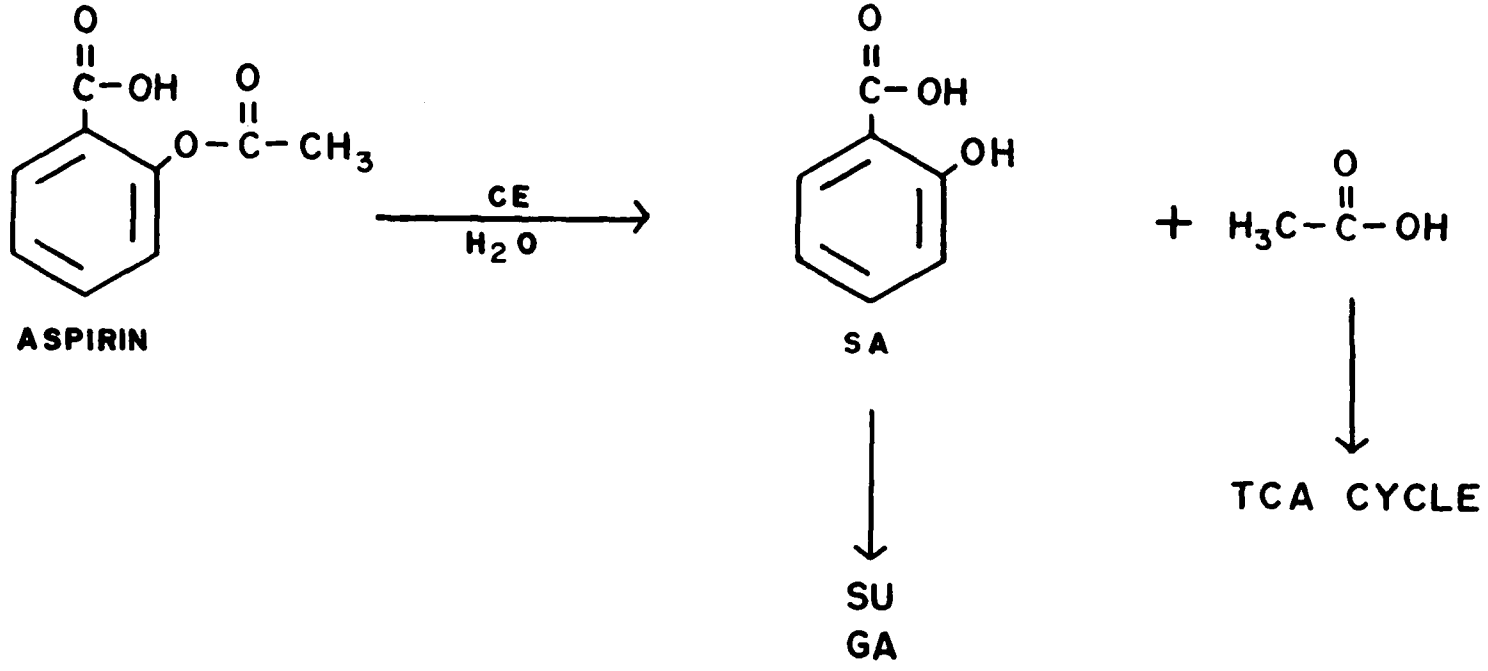


Figure 1. Pathways of aspirin metabolism in mammals: CE is carboxylester hydrolase, SA is salicylic acid, SU is salicyluric acid, GA is gentisic acid, and TCA is tricarboxylic acid. Two radiolabeled forms of aspirin were employed: (1) [carboxyl - ^{14}C]-aspirin, which yielded [carboxyl - ^{14}C]-SA, and (2) [acetyl-1-^{14}C]-aspirin, which yielded [1-^{14}C]-acetate, which gave [1-^{14}C]-carbon dioxide via the TCA cycle.

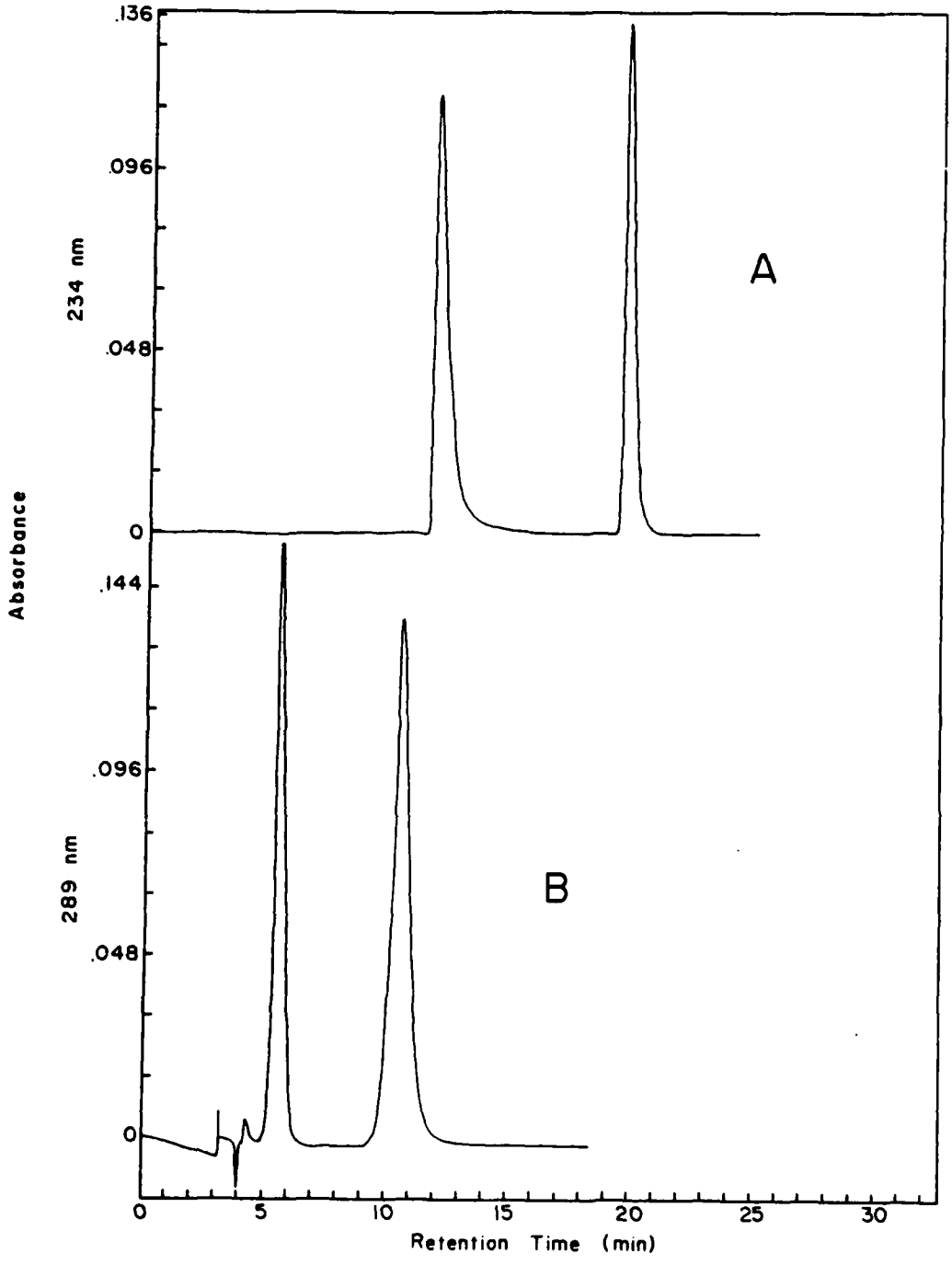


Figure 2. High performance liquid chromatography on octadecylsilyl reversed-phase column with mobile phases pumped at 1 ml/min; (A) Peak at 11.9 min was 0.0025 mg aspirin and peak at 20 min was 0.0025 mg salicylic acid; mobile phase was 0.2 M monopotassium phosphate, pH 2.5; water; and acetonitrile (40/35/25); (B) Peak at 6.0 min was 0.0025 mg 4-aminobenzoic acid and peak at 10.5 min was 0.0025 mg procaine HCl salt; mobile phase was 0.1 M sodium phosphate, pH 4.5, and acetonitrile (85/15) containing 0.005 M 1-pentane sulfonate.

In the second experiment, mice pretreated as described above were injected intraperitoneally with 0.001 mCi (0.0002 mmole) [acetyl-1-^{14}C]-aspirin. In this case, acetic acid was the radioactive product and it gave rise to radioactive carbon dioxide upon entering the tricarboxylic acid cycle.

Following injection, mice were held in a closed respiration apparatus in which air was passed through at 0.75 standard cu ft/h to exchange air in the residence chamber each 20 sec (Fig. 3). Expired air was passed through a gas-washing device containing 25% v/v Carbo-sorb® (United Technologies) in toluene/Triton X-100-based scintillation fluid (12) to collect [^{14}C]-carbon dioxide, which was measured by liquid scintillation counting. Each organophosphinate-pretreated mouse was compared to a control mouse in four replicates.

Since acetic acid, and not carbon dioxide, was the direct product of hydrolysis, an additional experiment was performed in duplicate, substituting [^{14}C]-acetic acid for [acetyl-1-^{14}C]-aspirin to assess the effect of pretreatment on the process of conversion of the primary hydrolysis product to carbon dioxide. There were two replicates in this experiment.

3b. Results

There was no reduction in the serum titre of the aspirin hydrolysis product [^{14}C]-salicylic acid when organophosphinate-pretreated mice were compared to control mice (Fig. 4). The only significant difference was an increase in [^{14}C]-salicylic acid levels at the 16 min sampling time in mice pretreated with IV, while the overall analysis indicated no significant effect due to treatment ($p > F = 0.228$). At the greatest concentrations reached in the serum, [^{14}C]-salicylic acid represented 38 to 49% of the total radioactivity administered as [carboxyl-^{14}C]-aspirin, indicating that very rapid aspirin hydrolysis had occurred in all treatment groups.

With IV pretreatment, there was an apparent transient increase in serum [carboxyl-^{14}C]-aspirin over control titre at 8 min but not at 16 min (Fig. 5); however, this does not indicate a major loss of aspirin hydrolysis in IV-pretreated mice, since the greatest serum concentration of [carboxyl-^{14}C]-aspirin represented only 2.9% of the applied dose and only 7.8% of the recovered radioactivity at 8 min. There was no significant effect due to treatment ($p > F = 0.118$). Had aspirin hydrolysis been prevented by IV pretreatment, [^{14}C]-salicylic acid serum titres would have been depressed, which was not observed (Fig. 4).

There was no effect of I on expiration of radiolabeled carbon dioxide following administration of [acetyl-1-^{14}C]-aspirin (Fig. 6) or [^{14}C]-acetic acid (Fig. 7), while approximately 70% of the applied dose of either compound was collected in 4 h. Similarly, there was no effect of IV on [acetyl-1-^{14}C]-aspirin metabolism (Fig. 8), while expiration of radiolabeled carbon dioxide from [^{14}C]-acetic acid was reduced significantly but only at 4 h (Fig. 9).

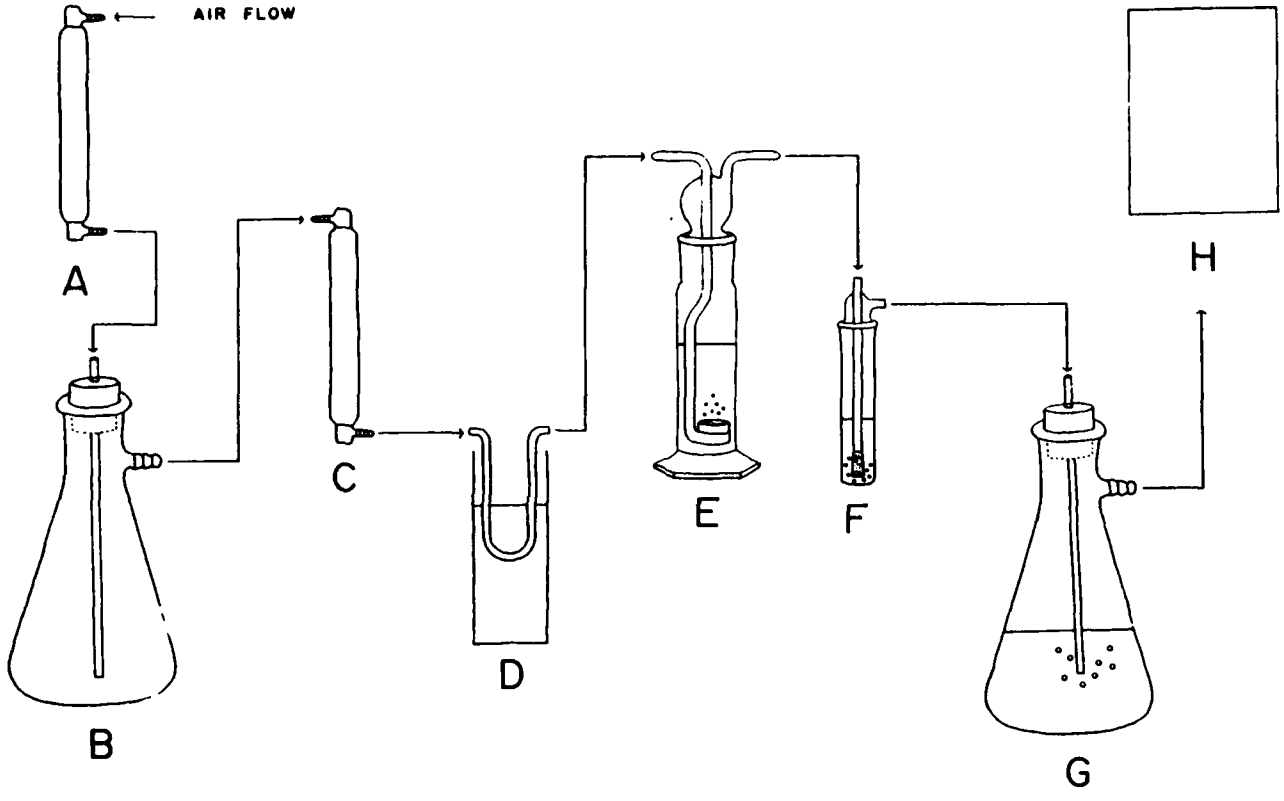


Figure 3. Apparatus for collection of [^{14}C]-carbon dioxide expired from mice: (A) Ascarite® column, (B) 250 ml flask for holding mouse, (C) Drierite® column, (D) dry ice and acetone bath for glass U-trap and expired organic compounds other than carbon dioxide, (E) primary trap for carbon dioxide containing 100 ml 25% (v/v) Carbo-sorb® in scintillation cocktail, (F) secondary trap for carbon dioxide containing 10 ml 25% (v/v) Carbo-sorb® in scintillation cocktail, (G) safety trap containing 5% aqueous potassium hydroxide, (H) diaphragm vacuum pump for pulling air through the system.

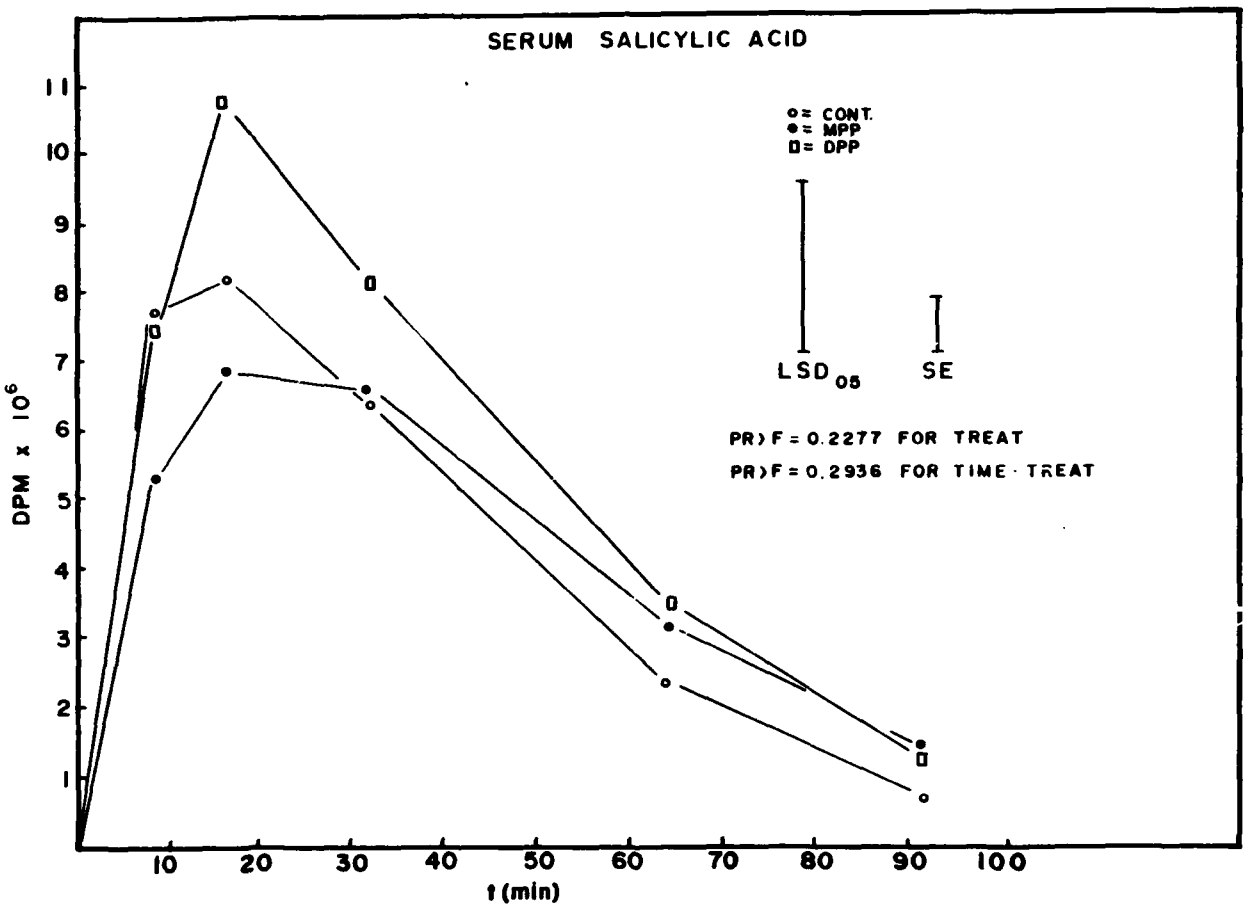


Figure 4. Radioactivity as salicylic acid in total body serum following intraperitoneal injection of 0.01 mCi [carboxyl-^{14}C]-aspirin into organophosphinate-pretreated mice. Mice were injected intramuscularly with LD_{01} organophosphinate 2 h prior to aspirin administration.

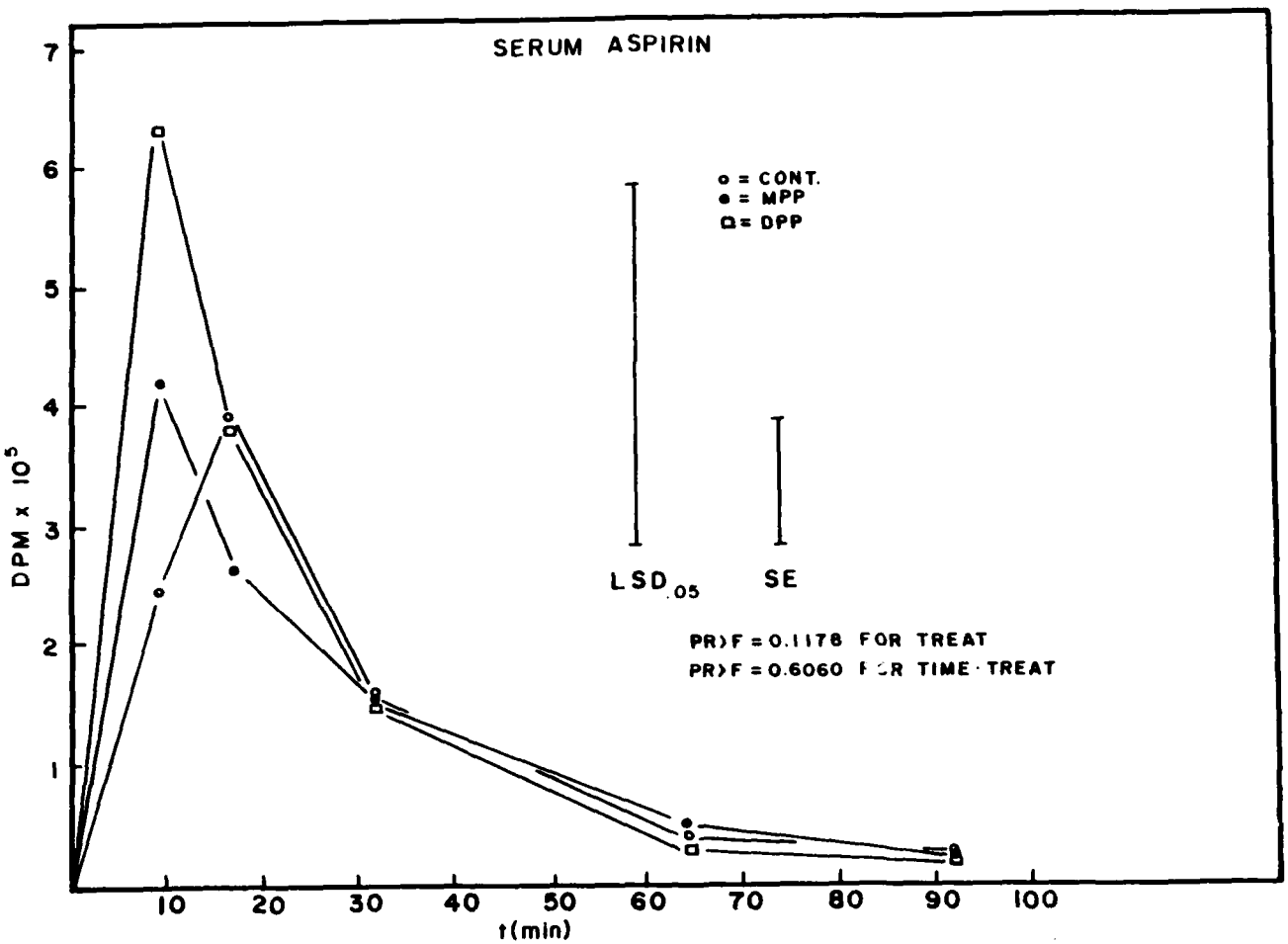


Figure 5. Radioactivity as aspirin in total body serum following intraperitoneal injection of 0.01 mCi [carboxyl-^{14}C]-aspirin into organophosphinate-pretreated mice. Mice were injected intramuscularly with LD_{01} organophosphinate 2 h prior to aspirin administration.

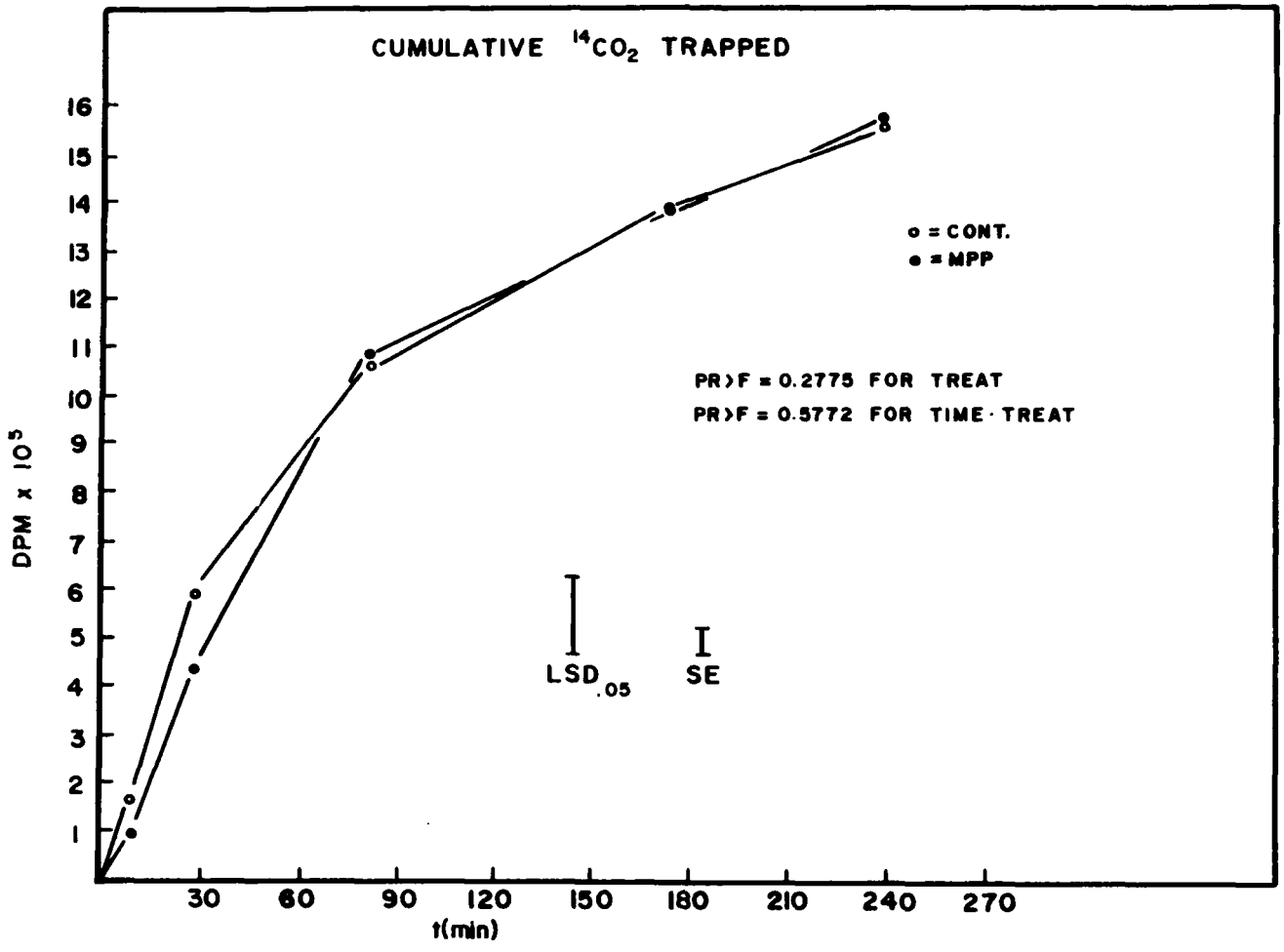


Figure 6. Radioactivity in carbon dioxide following 0.001 mCi [acetyl-1-^{14}C]-aspirin intraperitoneal injection of mice which had been injected intramuscularly 2 h earlier with 1.11 mg/kg 4-nitrophenyl methyl(phenyl)phosphinate.

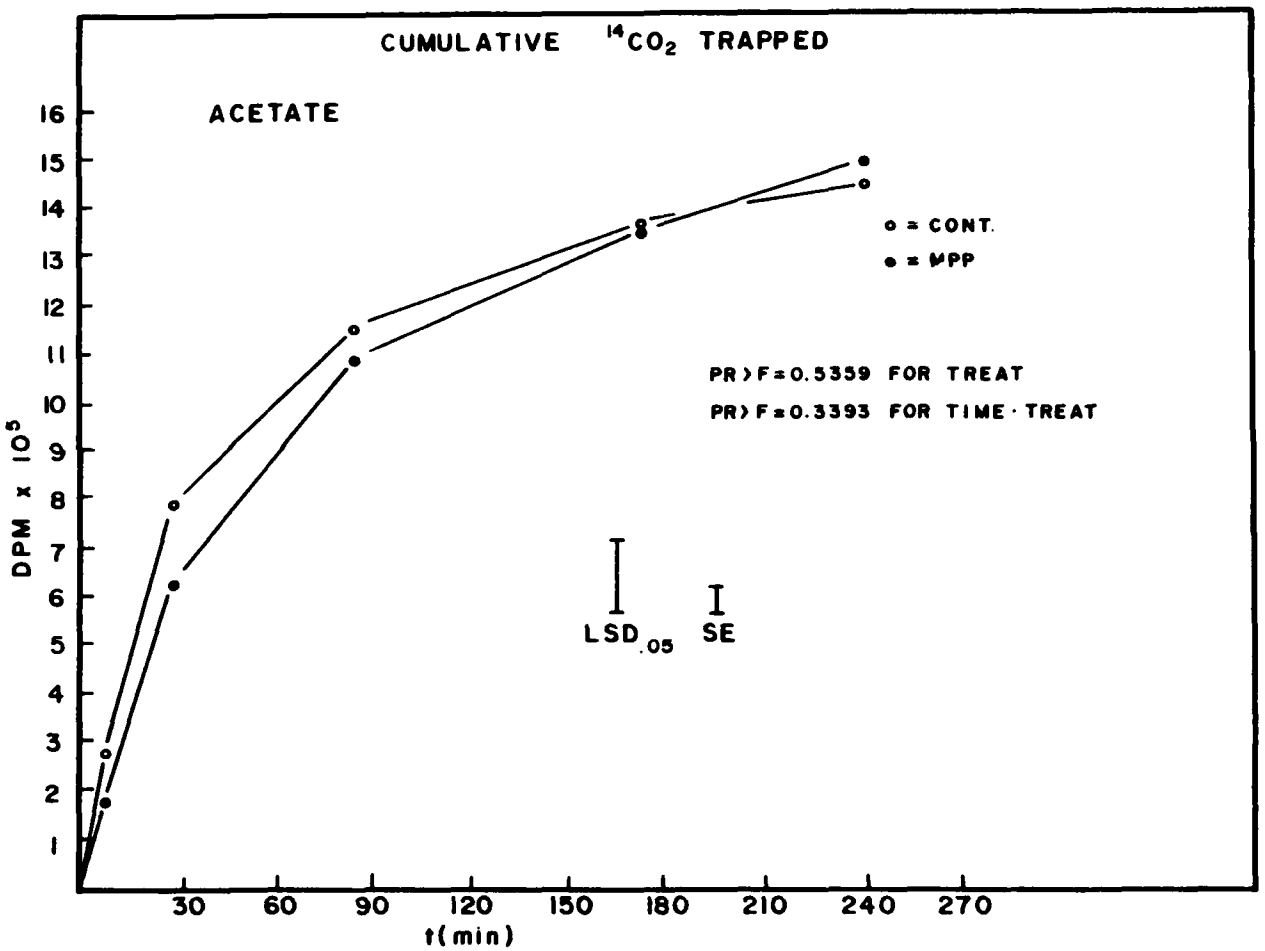


Figure 7. Radioactivity in carbon dioxide following 0.001 [1-^{14}C]-acetate intraperitoneal injection of mice which had been injected intramuscularly 2 h earlier with 1.11 mg/kg 4-nitrophenyl methyl(phenyl)phosphinate.

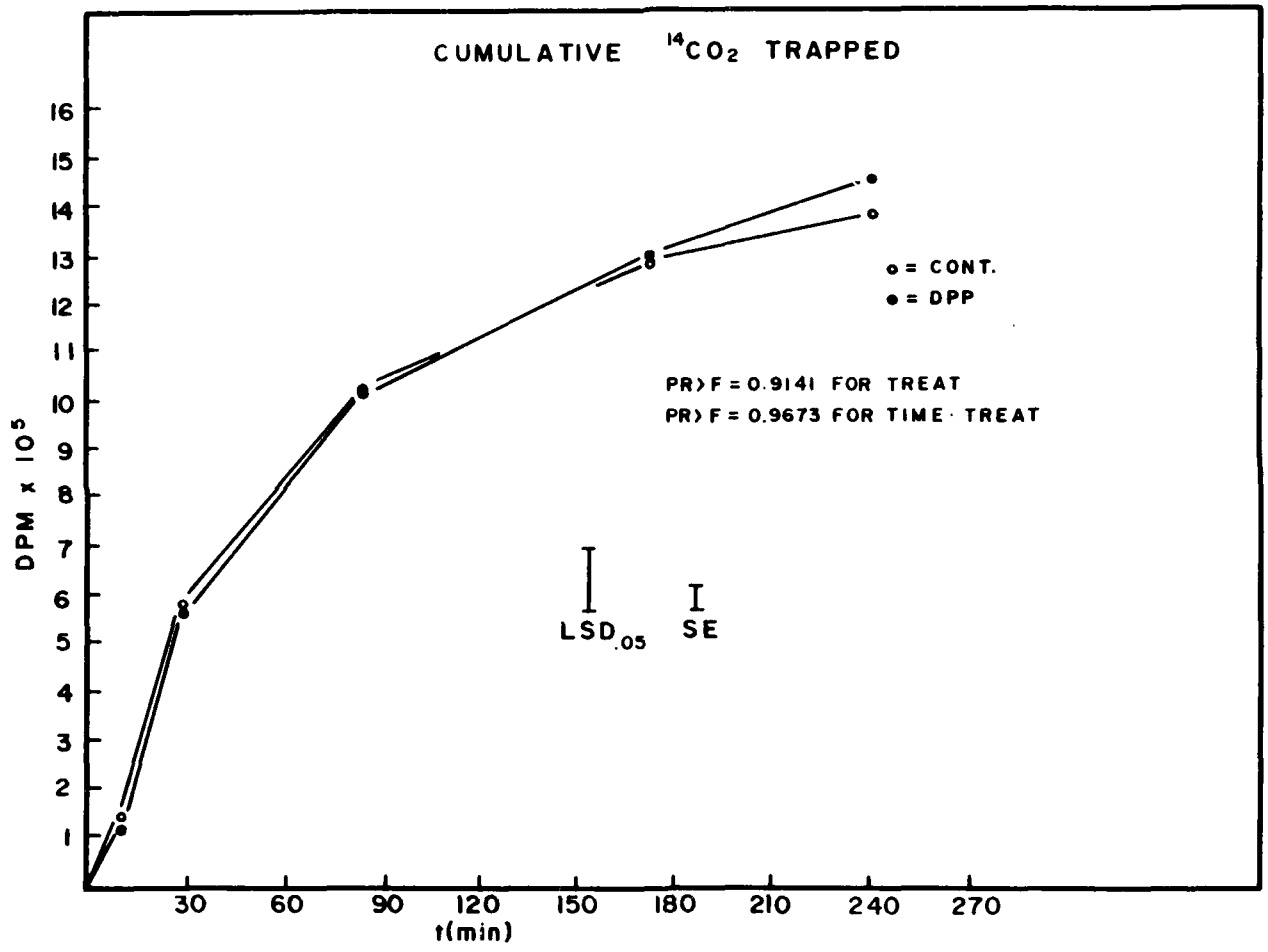


Figure 8. Radioactivity in carbon dioxide following 0.001 mCi [acetyl-1-^{14}C]-aspirin intraperitoneal injection of mice which had been injected intramuscularly 2 h earlier with 100 mg/kg 4-nitrophenyl diphenylphosphinate.

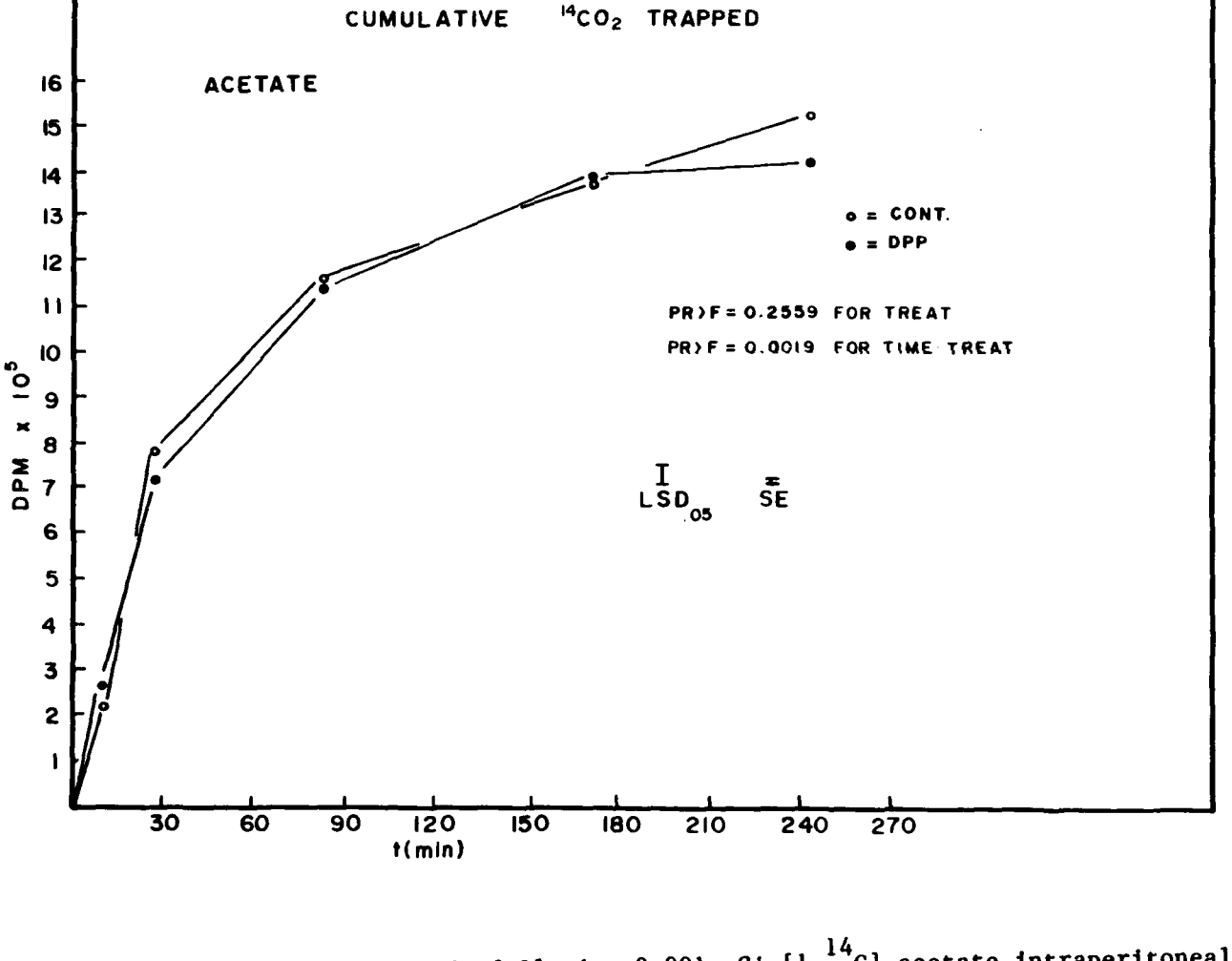


Figure 9. Radioactivity in carbon dioxide following 0.001 mCi [1-^{14}C]-acetate intraperitoneal injection of mice which had been injected intramuscularly 2 h earlier with 100 mg/kg 4-nitrophenyl diphenylphosphinate.

3c. Discussion

These results indicate that pretreatment agents I and IV did not prevent aspirin hydrolysis when administered at LD_{01} doses 2 h prior to aspirin administration. Since IV pretreatment was known to inhibit in vivo 89% of mouse liver carboxylesterase activity (vide Section 2b), these results suggest that aspirin metabolism via carboxylesterase may be a minor part of total aspirin hydrolysis in the mouse. Similar experiments are in progress with radiolabeled procaine as the model drug and high performance liquid chromatography (HPLC) analysis has been developed (Fig. 2).

There have been relatively few previous studies of aspirin metabolism despite its long history and continuing importance in medicine (13). It has been reported recently that four carboxylesterases purified from rat liver had differing hydrolysis profiles toward aspirin, procaine and other drugs (14).

4. Purification of Arylester Hydrolase from Rabbit Serum

4a. Methods

The purification procedure developed previously (8) was modified as follows: Blood was collected from rabbits and serum prepared as before, except that serum was adjusted to 2.5 mM Ca^{2+} and 0.02% sodium azide after filtration. Then 14-25% polyethylene glycol (PEG)-4000 fractionation was performed (replacing 12-18% PEG-4000 fractionation).

The PEG pellet was resuspended in buffer composed of 10 mM MOPS, pH 7.0, 2.5 mM Ca^{2+}, and 0.02% sodium azide (replacing sodium phosphate buffer), applied to a DEAE-Sepharose column, and eluted with a linear gradient to 400 mM NaCl in the MOPS buffer. Dialysis and a second DEAE-Sepharose column were added to the procedure. Protein of the active fractions was concentrated by 80% ammonium sulfate precipitation (replacing ammonium sulfate fractionation), resuspended in the MOPS buffer with sodium chloride added to 0.1 M, and applied to Ultrogel AcA34, from which one peak of arylesterase activity was eluted (the Matrex Red column formerly used as a final step was eliminated).

4b. Results

Improvements in procedures have increased the purification of rabbit serum arylesterase from 30-fold to 50-fold and have increased the yield of enzyme from 0.6% to 15.1% since the work described in the First Annual Report (8) (Table 4).

4c. Discussion

This is a relatively rapid procedure for preparation of arylesterase purified 50-fold. While a 385-fold purification from sheep serum was accomplished 25 years ago (15), the present procedure is easier to perform and more rapid.

Table 4. Purification of arylester hydrolase from rabbit serum.

Fraction	Volume (ml)	Total Activity (μmoles/min)	Total Protein[a] (mg)	Specific Activity (μmoles/min/mg)	% Yield	Purification
Serum	200	182	14,300	0.0127	100	1
14-25% PEG	50	77.8	8,540	0.0091	42.7	0.72
First DEAE-Sepharose	85	119	5,050	0.0236	65.4	1.85
Second DEAE-Sepharose	98	106	335	0.317	58.4	24.9
80% Ammonium Sulfate	7.5	46	---	---	25.3	---
Ultrogel AcA34	1140[b]	27.5	42.8	0.642	15.1	50.5

[a]For all but the Ultrogel AcA34 effluent, the protein concentration was determined using a biuret assay. For the AcA34 effluent, a fluorescamine assay was required.

[b]Corrected for volume used. 0.5 ml of the ammonium sulfate resuspension was applied from a pool of 7.5 ml.

5. Inhibition of Arylester Hydrolase by Organophosphinates

5a. Methods

While 10 of 13 4-nitrophenyl organophosphinates tested were substrates of rabbit serum arylester hydrolase (2), III, IV and XIV were not hydrolyzed. Since the conventional substrate for arylester hydrolase is paraoxon, from which the chromophore 4-nitrophenol is a product of hydrolysis, organophosphinate substrates having the identical product have not been tested as inhibitors; however, III and IV could be tested as inhibitors in mixtures with paraoxon, with the assumption that the product could originate only from the substrate.

Arylester hydrolase was purified 25-fold from rabbit serum, following the procedure described in Section 4a through the second DEAE-Sepharose chromatography step. The assays were carried out in 2 ml of 0.1 M MOPS, pH 7.5, at 37°C. Substrates and inhibitors were each added in 10 μl of acetonitrile, and 10 μl of acetonitrile was added to the uninhibited controls. The enzyme solutions were added to the substrate and inhibitor mixture in a volume of 10 μl containing 0.342 mg protein. The increase

in 4-nitrophenol produced by substrate hydrolysis was followed at 405 nm. Spontaneous hydrolysis of inhibitors was negligible under these conditions. Experiments were performed in duplicate with paraoxon as substrate and again in duplicate with II as substrate. Substrate concentrations were near their Michaelis constants, 0.609 mM for paraoxon and 0.285 mM for II (2).

5b. Results

Arylester hydrolase was partially inhibited by III at 8.69×10^{-5} M and by IV at 4.35×10^{-5}M, respectively (Table 5). More concentrated solutions containing these inhibitors and substrate could not be made. The greatest inhibition of paraoxon hydrolysis observed was 42.5% when III was present at approximately one-fourth the concentration of paraoxon. The least inhibition observed was 6.7% when III was present at about one-tenth the concentration of paraoxon. Inhibition of arylester hydrolase by IV was observed when either paraoxon or II was the substrate (Table 5).

Table 5. Inhibition of rabbit serum arylester hydrolase by organophosphinates.

Inhibitor, mM	Substrate, mM	Activity, nmoles/min/mg ±s.e.		
		control	inhibited	% inhibition
III[a]	paraoxon			
0.0869	0.910	63.8± 3.6	59.5± 1.9	6.7
	0.607	46.7± 4.2	29.8± 2.9	36.2
	0.455	30.8± 3.9	25.0± 0.0	18.8
	0.304	20.0± 2.3	11.5± 3.8	42.5
IV[b]	paraoxon			
0.0435	0.910	63.8± 3.6	59.1±17.6	7.4
	0.607	46.7± 4.2	36.1± 3.4	22.7
	0.455	30.8± 3.9	22.6± 3.4	26.6
	0.304	20.0± 2.3	17.5± 2.1	12.5
IV[b]	II[c]			
0.0435	0.173	82.7±16.5	63.9±12.3	22.7
	0.115	49.2±16.6	34.4± 9.5	30.1
	0.087	25.9± 6.8	17.7± 4.2	31.7
	0.058	17.6± 8.7	11.0± 2.2	37.5

[a] 4-nitrophenyl isopropyl(phenyl)phosphinate
[b] 4-nitrophenyl diphenylphosphinate
[c] 4-nitrophenyl ethyl(phenyl)phosphinate

5c. Discussion

While arylester hydrolase was partially inhibited by III and IV at concentrations of 8.69×10^{-5}M and 4.35×10^{-5}M, respectively (Table 5),

this enzyme was much less sensitive to these compounds than carboxylesterase, for which 70% inhibition was observed upon 1 min exposure to IV at 4×10^{-8} (8). At the highest substrate concentrations tested, there was not more than 22.7% inhibition by either phosphinate. Data from experiments with III (Table 5) confirmed our previous report that certain organophosphinates were excellent substrates for arylester hydrolase (2).

The degree of inhibition was inversely related to the concentration of substrate present. This is expected in the case of a competitive mechanism of inhibition. It is possible that III and IV were competitive inhibitors acting by occupation of the active site of the enzyme; their structural similarity to II and to other organophosphinates which were substrates (2) could permit this hypothetical occupation of the active site.

There was no indication of progressive inhibition with time as would be expected if these compounds were irreversible inhibitors of this enzyme. In fact, there was no loss in the rate of enzymatic hydrolysis of either substrate over the usual 2-3 min recording period in the presence of either inhibitor. Experiments are in progress in which inhibitors are incubated with enzyme in the absence of substrate for various periods prior to measuring remaining activity; this will detect irreversible inhibition if it occurs.

6. Stereoselective Reaction of Organophosphinates with Proteinases

6a. Methods

Stereoselectivity of phosphinylation of proteolytic hydrolases was investigated by exposing enzyme to equimolar concentrations of organophosphinate followed by extraction and analysis of the remaining organophosphinate by chiral-phase HPLC. Bovine pancreatic trypsin (Type III-S, Sigma Chemical Company, St. Louis, MO) was obtained as the dialyzed and lyophilized commercial product. Bovine pancreatic α-chymotrypsin (Type II, Sigma Chemical Company) was obtained as the crystallized, dialyzed and lyophilized commercial product.

In 0.1 M MOPS buffer, pH 7.5, 0.34 mM phosphinate was incubated with 0.34 mM enzyme at 37°C. Aliquots were removed at intervals and two volumes acetonitrile/acetone (1:1) were added to precipitate the enzyme. The precipitate was removed by centrifugation at 9220 x g for 20 min. Solvents were removed from the supernatant by rotating evaporator and the remaining solution was extracted three times with dichloromethane. The extract was evaporated to dryness, redissolved with acetonitrile, and chromatographic analysis was performed from this solution.

Chiral-phase HPLC analysis of recovered phosphinate enantiomers was performed on both resolved D- and L-3,5-dinitrobenzoylphenylglycine (DBPG) covalently bound to an aminosilica column (Regis Chemical Company, Morton Grove, IL). Previously, we developed these separations on the analogous, ionically bonded D-DBPG column (2, 16). The present technique employed a 25 cm x 4.6 mm stainless steel column packed with 5 μm DBPG-aminosilica particles and protected by a preceding 6 cm x 4.6 mm i.d. guard column packed with 30-38 μm silica gel-bonded glass beads

(Pellosil, Whatman Inc., Clifton, NJ). The analytical column was contained within a water jacket for cooling to 18°C by water flowing from a circulating bath (model RTE-9, Neslab Instruments, Portsmouth, NH). Mobile phase was usually 7% 2-propanol in hexane degassed by sonication and was delivered at 1 ml/min from a Tracor 950 or 951 high-pressure pump (Tracor Instruments, Austin, TX) through a 2 μm line filter (Part A-315, Upchurch Scientific, Oak Harbor, WA) and an injector with a 10 μl loop (model C6U, Valco Instruments Company, Inc., Houston, TX) to the guard column and analytical column. Eluting enantiomers were detected by absorbance of ultraviolet light at 270 nm, using either a variable, single-wavelength detector (model 970, Tracor Instruments) or a variable, dual-wavelength detector (model 788, Micrometrics, Norcross, GA). Chromatograms were acquired from 1 volt detector output to a laboratory computer (model 9000, IBM Instruments, Danbury, CN) and stored after analog-to-digital conversion, using chromatography applications software (CAPS 2, IBM Instruments).

Experiments were performed in quadruplicate for analysis on the D-DBPG column and once for analysis on the L-DBPG column.

6b. Results

Stereoselectivity was observed in the reactions of III with trypsin (Fig. 10) and with α-chymotrypsin (Fig. 11). The greater-retained enantiomer of III was lost more rapidly than the lesser-retained enantiomer in reactions. The reaction was more selective and more rapid with α-chymotrypsin than with trypsin, so that the exposure to α-chymotrypsin for 40 min left only the lesser-retained enantiomer (Fig. 11). The chromatograms illustrated here were typical of results obtained from four replications.

Similar stereoselectivity was observed in the reaction of II with trypsin; however, there was no stereoselectivity in the loss of enantiomers of II after incubation with α-chymotrypsin (Fig. 12). When peak heights were measured for the enantiomers and the ratios calculated for the greater-retained peaks to the lesser-retained peaks after a 40 min exposure to proteinases, values for trypsin were 0.67 ± 0.10 for II and 0.65 ± 0.02 for III. However, for α-chymotrypsin, values were 0.96 ± 0.01 for II and 0.05 ± 0.05 for III, indicating the absence of stereoselectivity in the reaction of II with α-chymotrypsin.

A confirmatory experiment was conducted, employing a newly acquired HPLC column covalently bonded with the L-phenylglycine derivative, which was expected to reverse the order of elution of organophosphinate enantiomers. Disappearance of the lesser-retained enantiomer of III was observed upon chromatography of solutions incubated with trypsin or chymotrypsin (Fig. 13). These chromatograms from the L-DBPG column were reversed from those previously obtained on the D-DBPG column, as expected; therefore, this is additional confirmation of stereoselective hydrolysis of organophosphinates.

Additional evidence for the presence of enantiomers was found by comparative chromatography of a partially resolved sample of III on both D-type and L-type columns on the same day under identical conditions.

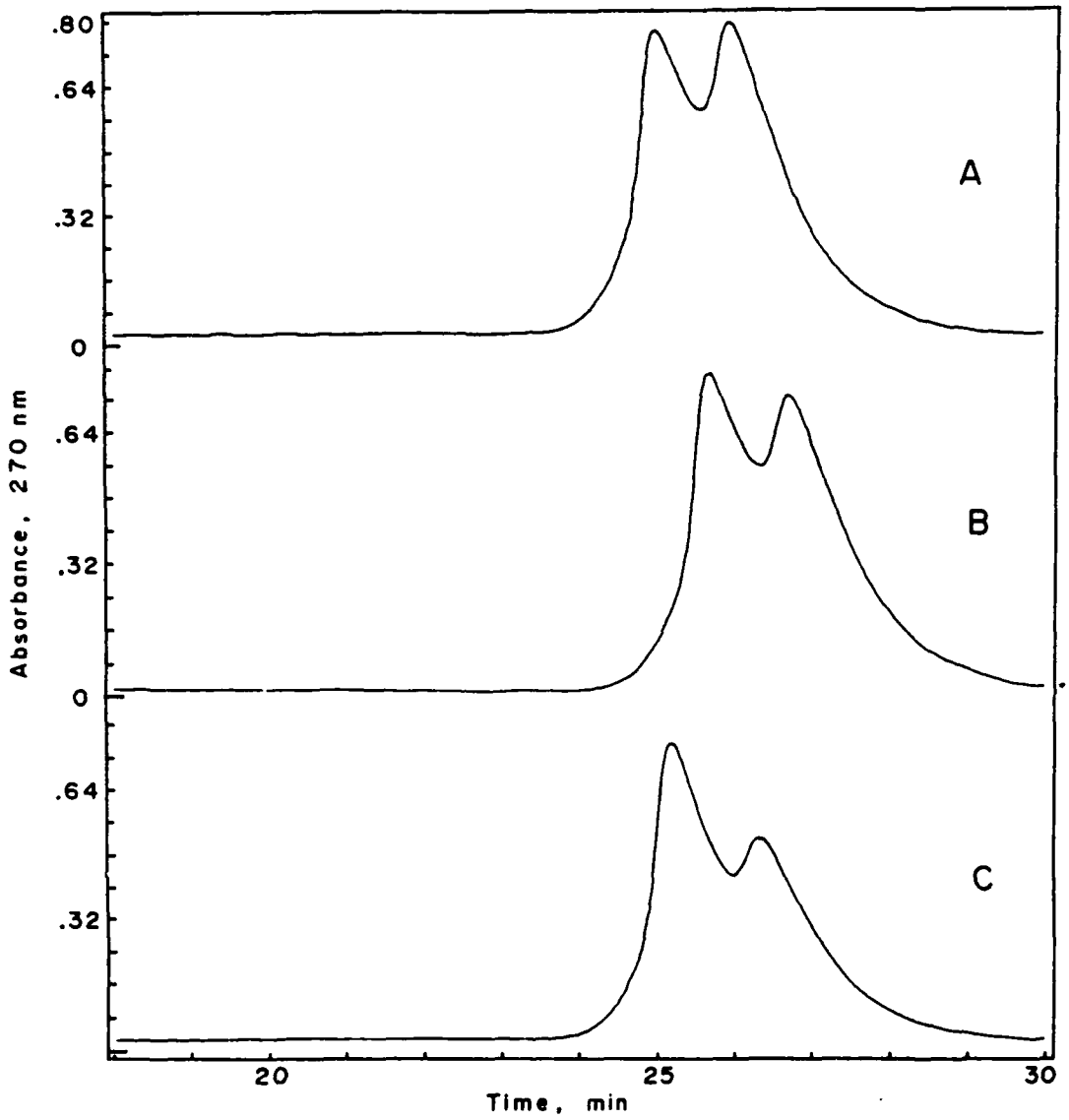


Figure 10. Stereoselective reaction of 0.34 mM racemic 4-nitrophenyl isopropyl (phenyl) phosphinate (III) with 0.34 mM trypsin in 0.1 M MOPS buffer, pH 7.5, at 37°C, as monitored by D-DBPG chiral-phase HPLC of extracts. (A) Control chromatogram of III in buffer without enzyme for 40 min. (B) Chromatogram of III following reaction with trypsin for 6 min. (C) Chromatogram of III following reaction with trypsin for 40 min.

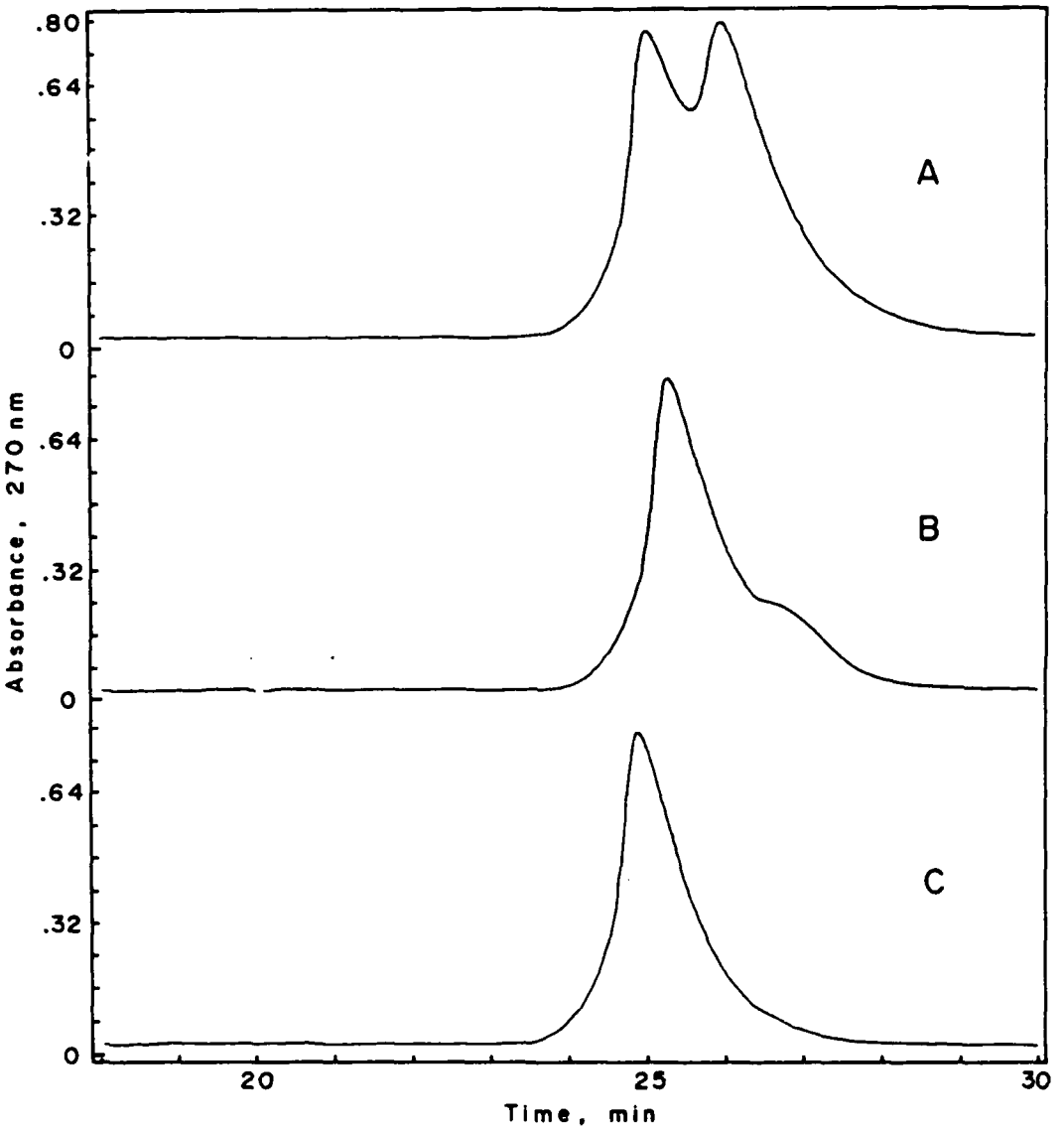


Figure 11. Stereoselective reaction of 0.34 mM racemic 4-nitrophenyl isopropyl (phenyl)phosphinate (III) with 0.34 mM α-chymotrypsin in 0.1 M MOPS buffer, pH 7.5, at 37°C as monitored by D-DBPG chiral-phase HPLC of extracts.
(A) Control chromatogram of III in buffer without enzyme for 40 min.
(B) Chromatogram of III following reaction with α-chymotrypsin for 6 min.
(C) Chromatogram of III following reaction with α-chymotrypsin for 40 min.

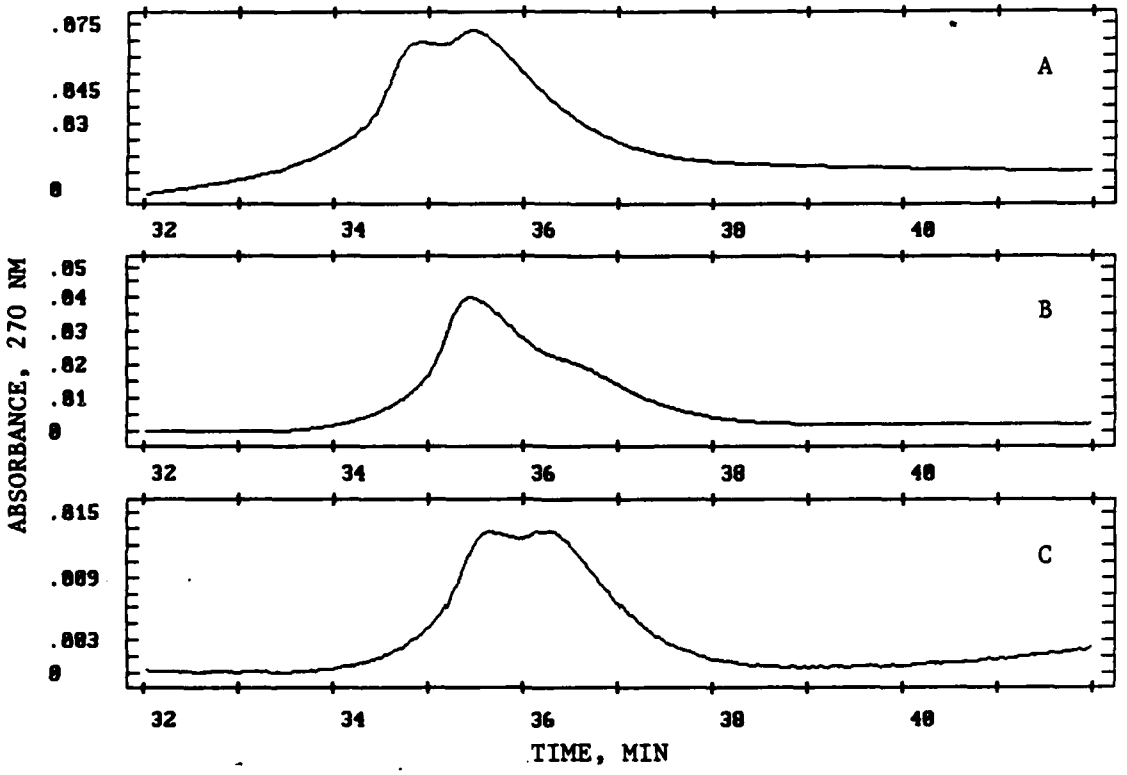


Figure 12. Reactions of 0.34 mM racemic 4-nitrophenyl ethyl(phenyl)phosphinate (II) with 0.34 mM proteinases in 0.1 M MOPS buffer, pH 7.5, at 37°C, as monitored by D-DBPG chiral-phase HPLC of extracts. (A) Control chromatogram of II in buffer without enzyme for 40 min. (B) Chromatogram of II following reaction with trypsin for 40 min. (C) Chromatogram of II following reaction with α-chymotrypsin for 40 min.

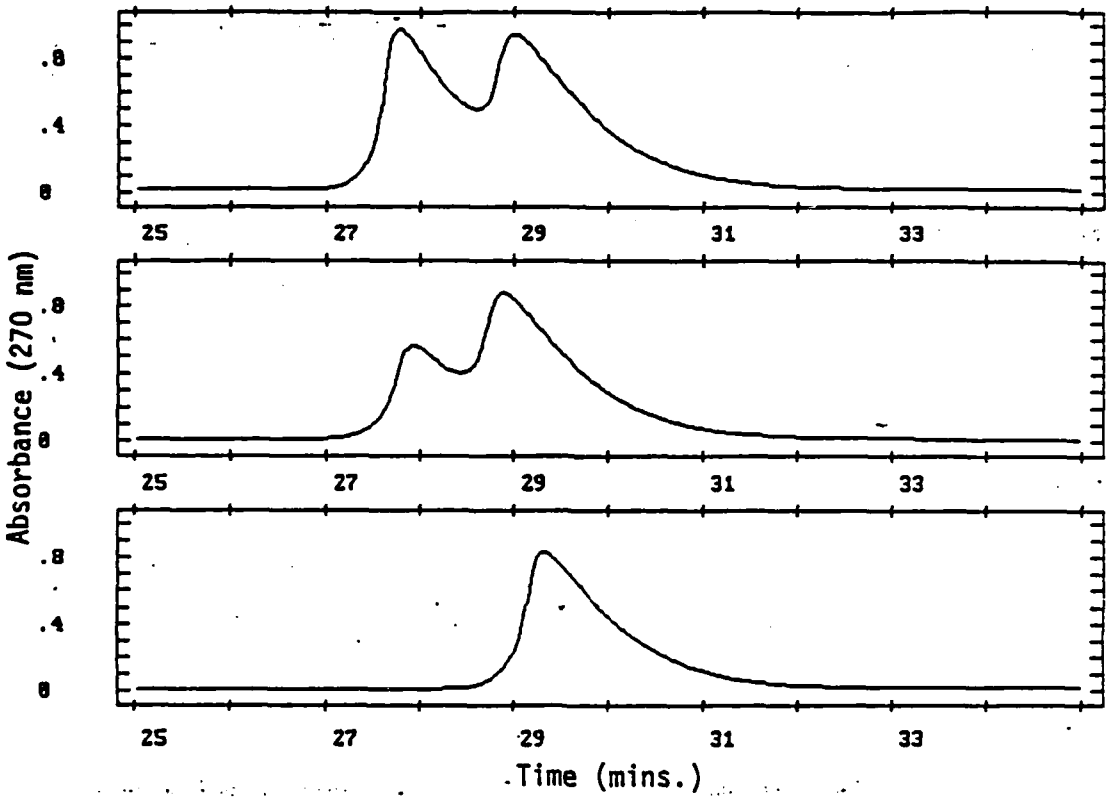


Figure 13. Stereoselective reactions of 0.34 mM racemic 4-nitrophenyl isopropyl (phenyl)phosphinate (III) with 0.34 mM proteinases in 0.1 M MOPS buffer, pH 7.5, at 37°C as monitored by L-DBPG chiral-phase HPLC of extracts. (A) Control chromatogram of III in buffer without enzyme for 40 min. (B) Chromatogram of III following reaction with trypsin for 40 min. (C) Chromatogram of III following reaction with α-chymotrypsin for 40 min.

The chromatograms were complementary, as expected for the separation of enantiomers of one compound (Fig. 14). The L-DBPG column retained both enantiomers longer than the D-DBPG column and resolution was superior on the L-type column.

6c. Discussion

The stereoselectivity observed for reactions of organophosphinates with proteinases was qualitatively identical to that observed previously for the hydrolysis of II by arylester hydrolase (2); i.e., the reaction was more rapid with the greater-retained enantiomer on the D-DBPG column. Sutdies are in progress to examine this phenomenon in other hydrolases including acetylcholinesterase.

Phosphonylation of chymotrypsin with either C(+)- or C(-)-soman left only the P(+) isomer when analyzed by gas chiral liquid phase chromatography (17); therefore, the P(-) isomers reacted with chymotrypsin. The P(-) isomers also inhibit acetylcholinesterase more rapidly. In the case of soman, the P(-) isomers were not hydrolyzed by rabbit serum (presumably arylesterase) or by squid fluorohydrolase (18), while the P(+) isomers were. We observed that the same enantiomer of II reacted with both chymotrypsin (a B-esterase) and arylesterase (an A-esterase). Continuation of this approach may be useful in understanding the stereochemistry of the active sites of various hydrolases.

It was advantageous to use the more recently acquired L-type column in hydrolysis studies with the three enzymes we have studied because the enantiomer which was lost was observed more readily as the lesser-retained peak (Fig. 14). When it was the greater retained peak (on the D-type column), its loss was more difficult to observe due to skewed nature of the peaks which caused the tail of the lesser retained peak to interfere with the greater retained peak. Future investigations will employ the L-DBPG column for primary analysis and the D-DBPG column for confirmatory analysis only.

The reaction of organophosphinates with proteinases was probably a phosphinylation of the serine residue in the active site of the enzyme resulting in hydrolysis of the organophosphinate and inhibition of the enzyme. Investigations of organophosphinate inhibition of trypsin and α-chymotrypsin are in progress.

7. Conclusions

Organophosphinates clearly react with several hydrolases other than their intended target, acetylcholinesterase. Inhibition of liver carboxylesterases is very rapid, but it is transient with recovery rates dependent on the chemistry of the phosphinyl group bound to the enzyme. Pancreatic proteinases also react with organophosphinates and the reaction can be stereoselective. Certain organophosphinates are excellent substrates for serum arylester hydrolase.

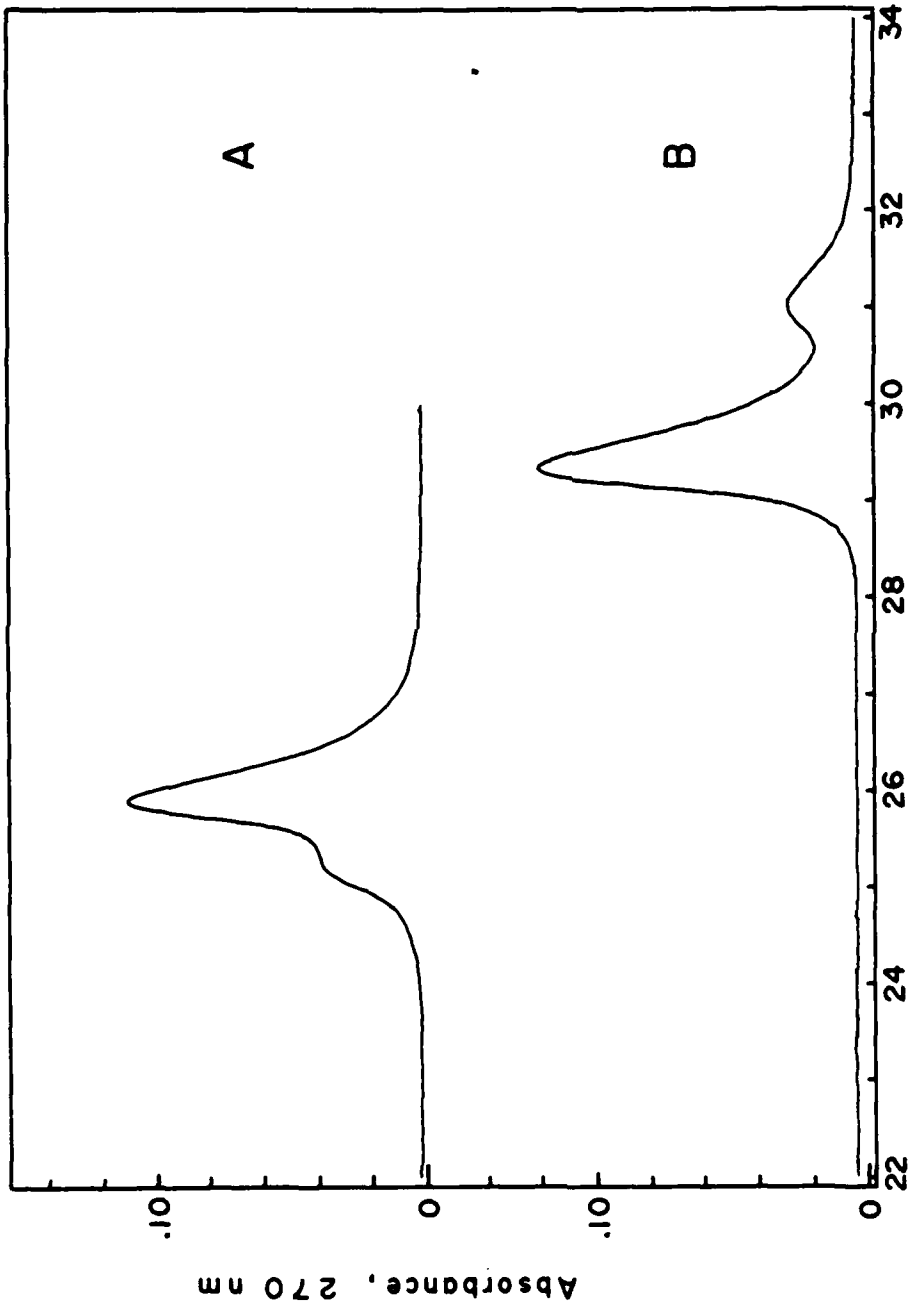


Figure 14. Comparison of D-DBPG and L-DBPG chiral-phase HPLC of identical samples of partially resolved 4-nitrophenyl isopropyl(phenyl)phosphinate (III). A sample of undetermined quantity was obtained by collection of the greater-retained enantiomer from repeated injections of racemic III on the D-DBPG column; in the process, a minor amount of the lesser-retained enantiomer was collected as a result of coelution. (A) Chromatogram of sample on D-DBPG column. (B) Chromatogram of sample on L-DBPG column.

While it is necessary for a pretreatment agent to inhibit acetylcholinesterase in a reversible manner in order to provide protection against chemical poisoning, knowledge of potential secondary reactions can be useful in understanding the total effect of the drug on the body. Interactions of the pretreatment agent, antidotes and other therapeutic drugs will be influenced by their reactions with xenobiotic-degrading hydrolases.

Direct experimentation in vivo with experimental animals is necessary to evaluate potential interactions of pretreatment agents with specific xenobiotics. While organophosphinate pretreatment significantly depressed liver carboxylesterase activity in mice, this did not reduce the primary hydrolysis of aspirin, as assessed by measuring aspirin and salicylic acid levels in serum and by measuring expired radiolabeled carbon dioxide originating from the acetyl moiety of aspirin or distribution of this hydrolysis product.

Arylester hydrolase catalyzed the hydrolysis of 10 of 13 organophosphinates tested, including several of the most rapid acetylcholinesterase inhibitors. Two organophosphinates which were not substrates appeared to be competitive inhibitors of this enzyme. Stereoselectivity of arylester hydrolase was discovered and the organophosphinate enantiomer which was the preferred substrate also reacted more rapidly with trypsin.

Design of an optimal organophosphinate pretreatment agent can be aided through the understanding of toxicokinetic mechanisms. Stereoselective reactions with B-esterases and arylester hydrolase are likely to influence the activity and selectivity of action of organophosphinate pretreatment agents.

8. Literature Cited

1. Lieske, C. N., J. H. Clark, H. G. Meyer, J. R. Lowe, M. A. Lawson, W. J. Lennox, W. E. Sultan, J. A. Kaminiskis, W. A. Groff, M. B. Shultz and A. Singer. 1981. The concept and chemistry of organophosphinates as prophylactic agents in organophosphorus intoxication. Amer. Chem. Soc. 181st Meeting. Atlanta, Georgia. Paper 71.

2. Brown, T. M., P. K. Bryson, J. R. Grothusen and J. K. Zimmerman. 1984. Inhibition of xenobiotic-degrading hydrolases by organophosphinates. Second Annual Progress Report Contract No. DAMD17-82-C-2193, U.S. Army Med. Res. Dev. Command. 25 pp.

3. Koplovitz, I., D. E. Jones, D. E. Hilmas, C. N. Lieske and C. J. Canfield. 1985. Screening of compounds for oral pretreatment efficacy against soman poisoning in mice. Abs. Fifth Ann. Chem. Def. Biosci. Rev. p. P19.

4. Maxwell, D. M., D. E. Lenze, W. A. Groff, A. Kaminskis and H. L. Froelich. 1985. A physiologically based toxicokinetic model of soman poisoning in rats. Abs. Fifth Ann. Chem. Def. Biosci. Rev. p. P42.

5. DeJong, B. J. P., C. Kalkman and W. C. Hulsmann. 1978. Partial purification and properties of monoacylglycerol lipase and two esterases from isolated rat small intestinal epithelial cells. Biochim. Biophys. Acta. 530:56-66.

6. Bryson, P. K. and T. M. Brown. 1985. Reactivation of carboxylester hydrolase following inhibition by 4-nitrophenyl organophosphinates. Biochem. Pharmacol. 34:1789-1794.

7. Matsumura, F. 1975. Toxicology of Insecticides. New York: Plenum Press. p. 228.

8. Brown, T. M., J. K. Zimmerman, P. K. Bryson and J. R. Grothusen. 1983. Inhibition of xenobiotic-degrading hydrolases by organophosphinates. First Annual Progress Report Contract No. DAMD17-82-C-2193, U.S. Army Med. Res. Dev. Command. 37 pp.

9. Ooms, A. J. J. and J. C. A. E. Breebaart-Hansen. 1965. The reaction of organophosphorus compounds with hydrolytic enzymes. The inhibition of horse liver aliesterase. Biochem. Pharmacol. 14:1727-1738.

10. Lieske, C. N. 1984. Personal communication.

11. Ahdaya, F. and F. Guthrie. 1982. Stomach absorption of intubated insecticides in fasted mice. Toxicology 22:311-317.

12. Brown, T. M. and A. W. A. Brown. 1980. Accumulation and distribution of methoprene in resistant Culex pipiens pipiens larvae. Ent. Exp. Appl. 27:11-22.

13. Junge, W. and K. Krisch. 1975. The carboxylesterases/amidases of mammalian liver and their possible significance. CRC Crit. Rev. Toxicol. 3:371-434.

14. Mentlein, R. and E. Heymann. 1984. Hydrolysis of ester- and amide-type drugs by the purified isozymes of nonspecific carboxylesterase from rat liver. Biochem. Pharmacol. 33:1243-1248.

15. Main, A. R. 1960. The purification of the enzyme hydrolyzing diethyl p-nitrophenyl phosphate (paraoxon) in sheep serum. Biochem. J. 74:10-20.

16. Brown, T. M. and J. R. Grothusen. 1984. High-performance liquid chromatography of 4-nitrophenyl organophosphinates and chiral-phase separation of enantiomers. J. Chromatogr. 294:390-396.

17. Benschop, H. P., C. A. G. Konings, J. Van Genderen and L. P. A. DeJong. 1984. Isolation, anticholinesterase properties, and acute toxicity in mice of the four stereoisomers of the nerve agent soman. Toxicol. Appl. Pharmacol. 72:61-74.

18. Hoskin, F. C. G. and A. H. Roush. 1982. Hydrolysis of nerve gas by squid-type diisopropyl phosphorofluoridate hydrolyzing enzyme on agarose resin. Science 215:1255-1257.

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