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INHIBITION OF XENOBIOTIC-DEGRADING HYDROLASES BY ORGANOPHOSPHINATES

ANNUAL/FINAL REPORT

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An abridged synthesis of 4-nitrophenyl diphenylphosphinate is described in which this compound was prepared in two steps. Chromatographic characteristics of four series of 4-nitrophenyl organophosphinates are described including results on normal-phase, reversed-phase and chiral-phase columns. Enantiomers of four organophosphinates were separated on a commercial (R)-N-(3, 5-dinitrobenzoyl)phenylglycine chiral-phase column. Analysis of 4-nitrophenyl diphenylphosphinate was performed after administration to mice and after incubation with rabbit serum.

Porcine liver carboxylester hydrolase (EC3.1.1.1; carboxylesterase) was rapidly inhibited by 4-nitrophenyl organophosphinates containing aryl or heteroaryl groups directly bound to phosphorus. The most potent inhibitor was

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19. Abstract (cont'd)

4-nitrophenyl di-2-thienylphosphinate for which the median inhibitory concentration was 7.4×10^{-9} M. Rabbit liver monomeric carboxylester hydrolase was inhibited, separated from excess inhibitor by gel permeation chromatography and observed for spontaneous or 1,1'-trimethylene-bis-(4-formylpyridinium bromide) dioxime (TMB-4)-induced reactivation. Recovery was most rapid (k = 4 to 7 x 10^{-4} min^{-1}) from phosphinyl groups containing one alkyl substituent smaller than isopropyl and one aryl or heteroaryl group smaller than naphthyl. The di-2-thienylphosphinylated enzyme was an exception since recovery from it was rapid, although it lacked an alkyl substituent. Oxime reactivation by TMB-4 doubled the rates of recovery.

Concentrations of [carboxyl-^{14}C]procaine in blood of mice were increased three-fold for 27 min by exposure to O-4-nitrophenyl diphenylphosphinate 2 h prior to [carboxyl-^{14}C]procaine injection ip, while there was no effect of O-4-nitrophenyl methyl(phenyl)phosphinate pretreatment. There was no effect of either organophosphinate on the primary hydrolysis of [acetyl-1-^{14}C]aspirin when assessed by the expiration of [^{14}C]carbon dioxide; however, O-4-nitrophenyl diphenylphosphinate pretreatment produced transient increases in blood concentrations of both [carboxyl-^{14}C]aspirin and [carboxyl-^{14}C]salicylic acid following administration of [carboxyl-^{14}C]aspirin. Liver carboxylesterase activity in O-4-nitrophenyl diphenylphosphinate pretreated mice was 11% of control activity. These results indicate the potential for drug interaction with O-4-nitrophenyl diphenylphosphinate but not with O-4-nitrophenyl methyl-(phenyl)phosphinate. It appears that liver carboxylesterase activity has a minor role in hydrolysis of aspirin *in vivo*, but may be more important in procaine metabolism.

The substrate specificity of arylester hydrolase partially purified from rabbit serum was studied. It was found that 10 of 13 4-nitrophenyl organophosphinate compounds tested were substrates for the enzyme. Michaelis constants were determined and ranged from 0.021 mM for 4-nitrophenyl methyl-(2-furyl)phosphinate to 0.49 mM for 4-nitrophenyl bis-chloromethylphosphinate as compared to 0.61 mM for ethyl paraoxon. Specific activities for a number of other substrates were also determined for this enzyme.

Acetylcholinesterases from electric eel and from bovine erythrocytes were inhibited stereoselectively by P(+) 4-nitrophenyl ethyl(phenyl)phosphinate (EPP) and P(+) 4-nitrophenyl isopropyl(phenyl)phosphinate (IPP). Bovine pancreatic α-chymotrypsin was inhibited stereoselectively also, but the P(-) enantiomers of both EPP and IPP were more active than P(+). Trypsin, protease and carboxylester hydrolase exhibited less stereoselectivity than chymotrypsin, toward the (-) enantiomer of EPP or IPP. Arylester hydrolase was 9-fold more active in the hydrolysis of P(-) EPP than P(+). Chiral-phase HPLC was employed to isolate the enantiomers from racemic mixtures for experiments and to analyze reaction mixtures initiated with racemic EPP or IPP. Stereoselectivity was greater toward more inhibitory compounds, EPP and 4-nitrophenyl dichloromethyl(phenyl)phosphinate (DCMPP), than their less inhibitory homologs, IPP and 4-nitrophenyl trichloromethyl(phenyl)phosphinate (TCMPP).

Phosphorylase kinase from rabbit muscle appeared to have catalytic activity toward EPP, although it was at least 30-fold less active than arylester hydrolase; two other rabbit muscle transferases did not catalyze EPP hydrolysis. Phosphorylase kinase was stereoselective for (-)EPP.

Summary

An abridged synthesis of 4-nitrophenyl diphenylphosphinate is described in which this compound was prepared in two steps. Chromatographic characteristics of four series of 4-nitrophenyl organophosphinates are described including results on normal-phase, reversed-phase and chiral-phase columns. Enantiomers of four organophosphinates were separated on a commercial (R)-N-(3, 5-dinitrobenzoyl)phenylglycine chiral-phase column. Analysis of 4-nitrophenyl diphenylphosphinate was performed after administration to mice and after incubation with rabbit serum.

Porcine liver carboxylester hydrolase (EC3.1.1.1; carboxylesterase) was rapidly inhibited by 4-nitrophenyl organophosphinates containing aryl or heteroaryl groups directly bound to phosphorus. The most potent inhibitor was 4-nitrophenyl di-2-thienylphosphinate for which the median inhibitory concentration was 7.4×10^{-9} M. Rabbit liver monomeric carboxylester hydrolase was inhibited, separated from excess inhibitor by gel permeation chromatography and observed for spontaneous or 1,1'-trimethylene-bis-(4-formylpyridinium bromide) dioxime (TMB-4)-induced reactivation. Recovery was most rapid (k = 4 to 7 $\times 10^{-4}$ min^{-1}) from phosphinyl groups containing one alkyl substituent smaller than isopropyl and one aryl or heteroaryl group smaller than naphthyl. The di-2-thienylphosphinylated enzyme was an exception since recovery from it was rapid, although it lacked an alkyl substituent. Oxime reactivation by TMB-4 doubled the rates of recovery.

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Arylester hydrolase has been partially purified from rabbit serum to a specific activity matching or exceeding all previous reports. Calcium ion is absolutely required for activity. The widely reported rapid loss of arylester hydrolase activity was overcome by combining this Ca^{2+} requirement with the presence of 0.02% sodium azide. A method has also been devised to store the enzyme for long periods of time (years). During size exclusion chromatography, the enzyme behaved as if it had a molecular weight of 180-200 kilodaltons. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis showed two bands of low molecular weight (40-45 kilodaltons and 47-54 kilodaltons) directly correlated with enzymatic activity, suggesting a possible $\alpha_2\beta_2$ structure for the native enzyme.

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Foreword

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

In conducting the research described in this report, the investigator(s) 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

1a. Background and Rationale

Organophosphinates are candidate pretreatment agents in a continuing search for more effective protection and therapy for poisoning by organophosphorus chemical warfare agents such as soman (1). Both organophosphinates and carbamates such as pyridostigmine are reversible inhibitors of acetylcholinesterase which can be spared 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 biotransformation of drugs and xenobiotics, particularly in mammalian liver, kidney and serum; therefore, there is potential for interaction of pretreatment agent with subsequent drug therapy in medical treatment of a soldier. In addition, xenobiotic-degrading hydrolases are involved in the pharmacokinetics of atropine and the toxicokinetics of soman and other chemical warfare 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). In addition, certain organophosphinates reacted rapidly with liver carboxylester hydrolases and pancreatic proteinases (3). The choice of organophosphinate candidates for pretreatment agents should be made with these factors included in consideration along with efficacy.

Recent investigations have indicated that 15 organophosphinates, including some of those in this study, were not as effective as 4 carbamates and 1 phosphorinane when administered orally prior to intramuscular challenge by soman (4). While these results did not indicate efficacy of organophosphinates, it is possible that these compounds would be efficacious when administered by a different means. 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 (5,6) and we have found that organophosphinates react very rapidly with carboxylesterase (7). Since 4-nitrophenyl methyl(phenyl)phosphinate (MPP) is five-fold more toxic intramuscularly than by oral administration in both mice and rats (vide Section 6b), perhaps it would be more efficacious against soman poisoning by intramuscular administration or some other means which avoids the gastrointestinal route. Pyridostigmine and other carbamates are less likely to be metabolized hydrolytically (8).

In the first year of this contract we described the inhibition and spontaneous reactivation of carboxylesterase by organophosphinates (9). 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 1 min. Median inhibitory concentrations for 2 min exposure were

determined and these data indicated the potency of these compounds; e.g., the value for 4-nitrophenyl di-2-thienylphosphinate (DTP) was 7.4×10^{-9} M (7). These aryl and heteroaryl containing phosphinates were more rapid carboxylesterase inhibitors than seven 4-nitrophenyl dialkylphosphinates previously studied against horse liver carboxylesterase (10).

Phosphinylated rabbit liver monomeric carboxylesterase, upon separation from excess phosphinate inhibitor, was observed to reactivate spontaneously at a race dependent on the chemistry of the attached phosphinyl group (2,7). In each of four series of analogous inhibitors, phosphinylated carboxylesterases recovered faster following inhibition by smaller compounds in the series. This carboxylesterase recovered from most organophosphinates, although it did not recover from inhibition by paraoxon; this may have been due to dealkylation (aging) of phosphorylated monomeric carboxylesterase, which would not occur with phosphinylated enzyme, although we did not pursue this hypothesis. Herein we report the comparative reactivation of three different carboxylesterases.

Induced reactivation of ethyl(phenyl)phosphinylated carboxylesterase upon exposure to 1,1'-trimethylene-bis-(4-formylpyridinium bromide) dioxime (TMB-4) was twice the rate of spontaneous reactivation as discovered in the second year of this project (2,7). It is clear that carboxylesterase reacts rapidly with organophosphinates and therefore interactions with xenobiotic biotransformation are possible upon organophosphinate administration. Recovery and oxime-induced reactivation of phosphinylated carboxylesterase indicate that the beneficial effect of protecting xenobiotic-degrading enzymes is another possibility 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 interaction 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 lesser 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 organophosphinates pretreatment agents.

This report also includes results 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 described also.

Stereoselectivity of hydrolysis of 4-nitrophenyl ethyl(phenyl)-phosphinate (EPP) by arylester hydrolase was reported in the second year of the contract (2). Herein we report results of chiral selectivity in reactions of four organophosphinates with α -chymotrypsin, two organophosphinates with acetylcholinesterase and trypsin, and additional stereochemical studies with protease and carboxylester hydrolase. The inhibition of acetylcholinesterase was found to be a stereoselective reaction with opposite selectivity than the other reactions with enzymes and the catalyzed hydrolysis first reported.

1b. Objectives of this Contract

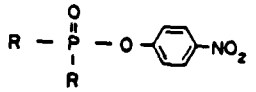
Three of four objectives of the original contract have been met; those being to determine the potentials for (i) inhibition of carboxylesterase, (ii) inhibition of arylester hydrolase (which led to discovery of rapid enzymatic hydrolysis of organophosphinates), and (iii) stereochemical interactions of chiral organophosphinates with hydrolases. The fourth objective was to describe the interaction of organophosphinates with a mammalian fluorohydrolase. This has not been accomplished due to difficulties encountered in purification of porcine kidney fluorohydrolase.

The first objective of the one-year continuation of contract was met when several additional enzymes, including acetylcholinesterase, were determined to be stereoselective toward certain organophosphinates. The other objective, to develop chiral-phase chromatographic methods for methyl series organophosphinates, was attempted without success; however, studies of stereospecificity were conducted with chloro-methyl organophosphinates for which chiral chromatography was developed. In addition, phosphorylase kinase was found to hydrolyze 4-nitrophenyl ethyl(phenyl)-phosphinate. This discovery has potential physiological significance and the stereoselectivity of hydrolysis was investigated.

1c. Names and Abbreviations of Organophosphinates Used in These Experiments

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.

Abbreviation	Compound		CAS[a] Number	Molecular Weight
	Phenyl series			
MPP	4-nitrophenyl methyl(phenyl)phosphinate		35691-25-9	277
EPP	4-nitrophenyl ethyl(phenyl)phosphinate		80751-40-2	291
IPP	4-nitrophenyl isopropyl(phenyl)phosphinate		80751-39-9	305
DPP	4-nitrophenyl diphenylphosphinate		10259-20-8	339
	Methyl series			
MFuP	4-nitrophenyl methyl(2-furyl)phosphinate		81425-59-4	267
MTP	4-nitrophenyl methyl(2-thienyl)phosphinate		91308-90-6	283
MPP	4-nitrophenyl methyl(phenyl)phosphinate		35691-25-9	277
MNP	4-nitrophenyl methyl(2-naphthyl)phosphinate		91308-91-7	327
	Heterocycle series			
MFuP	4-nitrophenyl methyl(2-furyl)phosphinate		81425-59-4	267
DFuP	4-nitrophenyl di-2-furylphosphinate		-	335
MTP	4-nitrophenyl methyl(2-thienyl)phosphinate		91308-90-6	283
DTP	4-nitrophenyl di-2-thienylphosphinate		81425-58-3	351
	Halogen series			
bCMP	4-nitrophenyl bis-chloromethylphosphinate		33714-89-5	284
CMPP	4-nitrophenyl monochloromethyl(phenyl)phosphinate		88144-99-4	312
DCMPP	4-nitrophenyl dichloromethyl(phenyl)phosphinate		81344-27-6	347
TCMPP	4-nitrophenyl trichloromethyl(phenyl)phosphinate		81344-26-5	382
TFPMP	4-nitrophenyl methyl(trifluoromethylphenyl)-phosphinate		81542-84-9	345

[a]Chemical Abstracts Service (vide Grothusen et al., 1986).

2. Synthesis and Analysis of Organophosphinates

2a. Abridged Synthesis of 4-Nitrophenyl Diphenylphosphinate

2a.i. Introduction

Organophosphinates are potential pretreatment agents for protection against organophosphate poisoning. This is due to the favorable properties of organophosphinates in phosphinylation of the active site of acetylcholinesterase; i.e., certain organophosphinates are very rapid inhibitors (11), certain phosphinylated acetylcholinesterases recover

rapidly either spontaneously or with oxime treatment (12), and phosphinylated acetylcholinesterase is not subject to O-dealkylation (aging) reactions known to hinder reactivation of phosphorylated acetylcholinesterase.

Isotopically labeled organophosphinates would be useful for investigations of pharmacokinetics in experimental animals and for studies of interactions of these compounds with acetylcholinesterase and other hydrolases, including stereochemical mechanisms. Organophosphinates have been synthesized previously (12,13,14). An alternative route of synthesis is available now for certain organophosphinates and it is described here as assembled and performed from known reactions.

2a.ii. Methods

Synthesis of 4-nitrophenyl diphenylphosphinate (DPP) was performed in two steps; first, diphenylphosphinyl chloride was prepared from diphenylphosphinous chloride (15). In a 100 ml three-neck flask, 53.9 mmol diphenylphosphinous chloride (Stauffer Chemical Co., Westport, CN) in 40 ml benzene was exposed to oxygen which was passed through at four bubbles/s for 6 h with condenser attached. Solvent was removed by distillation; then the product was distilled in a short path apparatus with vacuum at 63.2% yield. Product Rf was 0.34 by silica gel thin layer chromatography (TLC) in chloroform, methanol and water (80:30:0.5), which agreed with an authentic standard of diphenylphosphinyl chloride (Chemical Dynamics Corp., South Plainfield, NJ).

Synthesis of DPP from diphenylphosphinyl chloride and sodium 4-nitrophenoxide (Aldrich Chem. Co., Milwaukee, WI) was performed according to a previously described method (13) with modifications. In a 100 ml three-neck flask, 20 mmol sodium 4-nitrophenoxide was stirred with 20 mmol water and 20 ml acetone until completely yellow; then 18 mmol diphenylphosphinyl chloride in 12 ml acetone was added dropwise. The mixture was refluxed 3.5 h, stirred overnight, filtered and acetone removed *in vacuo*. The residue was dissolved in dichloromethane, washed with 0.5% sodium bicarbonate, then with water, and then dried through sodium sulfate. Dichloromethane was removed *in vacuo* and yield of oil was 94.1 ± 5.7%.

2a.iii. Results and Discussion

Oil was 63.1% title compound by octylsilyl reversed-phase high performance liquid chromatography (HPLC) with 50% acetonitrile in water (16). Product was crystallized from tetrahydrofuran and cyclohexane (1:1) and crystals melted at 127-140°F. Mass spectral analysis (Fig. 1) confirmed that the identity of the product was DPP.

The final yield of DPP by the synthesis described was 37% of theoretically possible yield. Yield could be improved by optimization of the first reaction to convert sufficient diphenyl phosphinous chloride to allow continuation with the second reaction in the same vessel [caution: unreacted diphenylphosphinous chloride might react violently with water or with sodium nitrophenoxide]; e.g., a fritted glass impinger will replace the pipette used to introduce oxygen into the reaction flask.

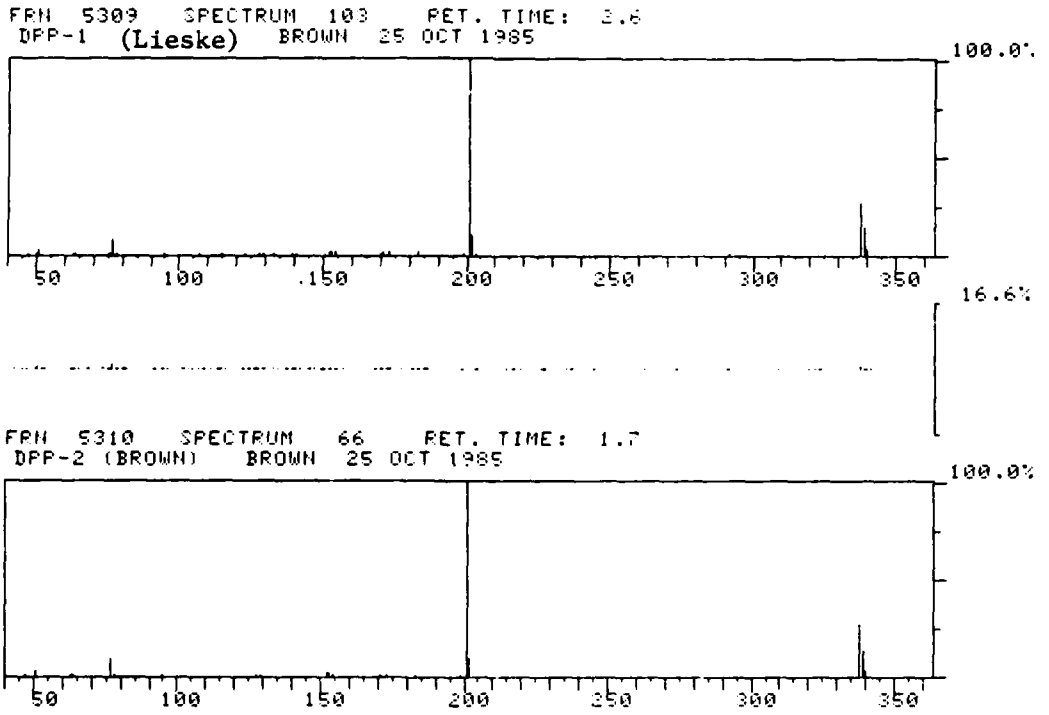


Figure 1. Mass spectra of authentic 4-nitrophenyl diphenylphosphinate (top panel) and the product of the abridged synthesis described in the text (bottom panel). Between the panels are bars representing the percent difference between authentic standard and synthesized product.

An advantage of this method is the potential for incorporating isotopes of oxygen into the molecule using the first reaction; isotopes of oxygen have been useful in stereoanalysis of phospho transfer enzymes (17). Methylphenylchlorophosphine is commercially available for the possible synthesis of MPP by this scheme.

2b. Normal-, Reversed-, and Chiral-phase High Performance Liquid Chromatography

2b.i. Introduction

Certain 4-nitrophenyl organophosphinates are rapid, transient inhibitors of acetylcholinesterase (18) and carboxylesterase (10,17). The stereospecificity of these reactions has not been investigated. While enantiomers of aryl hydroxy phosphonates have been separated directly by chiral-phase chromatography (19), organophosphinate enantiomers have not been resolved directly, but only after derivatization to diastereomers (20). We report the direct chromatographic separation of several 4-nitrophenyl organophosphinate enantiomers on a commercially available chiral phase column as well as the normal-phase and reversed-phase HPLC of four diverse series of these compounds. These techniques are useful for preparing small quantities of pure enantiomers and also for detecting organophosphinates in extracts of biological samples.

2b.ii. Materials and Methods

Organophosphinates were provided by the Institute of Chemical Defense of the U.S. Army Medical Research and Development Command as crystalline samples of 99% purity. Samples of 4-nitrophenol and diethyl-4-nitrophenylphosphate (paraoxon) were purchased from Sigma Chemical Company (St. Louis, MO). Unless specified differently, all chemicals were prepared as acetonitrile solutions. Acetonitrile, dichloromethane (Fisher Scientific Company, Pittsburgh, PA) water, 2-propanol and n-hexane (Burdick and Jackson Laboratories, Muskegon, MI) were HPLC grade.

The instrument consisted of a Tracor 980A solvent programmer (Tracor Insturments, Austin, TX), a Tracor 950 high-pressure pump, a Valco injector with 10 µl loop, a water jacket (Alltech Associates, Chicago, IL) with Neslab RTE-9 circulator bath (Neslab Instruments, Portsmouth, NH) for control of column temperature, a Tracor 970A ultraviolet detector, and a Fisher strip-chart recorder. Organophosphinates were detected by absorbance at 270 nm and retention times were measured from the time of injection. The limit of detection for most compounds was 20 ng; 2 µg of organophosphinate were injected.

Normal-phase HPLC was performed using a 10 µm Partisil 4.6 mm x 25 cm column held at 36°C, preceded by the use of a 30-38 µm Pellosil 4.6 mm x 6 cm guard column (Whatman Chemical Separation, Clifton, NJ). Solvents were degassed by sonication for 3 min, and then mixed by the programmer and pumped isocratically. Acetonitrile solutions of samples were diluted 1:19 (v/v) with 20% 2-propanol in hexane prior to injection.

Reversed-phase HPLC was performed on a 10 μm Lichrosorb RP-8 4 mm x 25 cm column at ambient temperature with a 30-40 μm Perisorb RP-8 4 mm x 2.5 cm guard column (E. Merck, Darmstadt, FRG). Solvents were mixed manually, degassed and pumped isocratically.

Chiral-phase chromatography was performed using a 5 μm Bakerbond™ 4.6 mm x 2.5 cm column (J. T. Baker Research Products, Phillipsburg, NJ) held at 18°C and preceded by the Whatman Pellosil guard column described previously. Solvents were mixed manually, degassed and pumped isocratically. For preparative HPLC, up to 250 μg of each chiral organophosphinate was injected repeatedly, the lesser retained enantiomer was collected to its peak, and the greater retained enantiomer was collected following its peak.

Mice (CF-1®, Charles River Breeding Laboratories, Wilmington, MA) of approximately 40 g each were injected in leg muscle with 4-nitrophenyl diphenylphosphinate in polyethyleneglycol (PEG) 200 (Sigma) with 10% dichloromethane and 0.1% glacial acetic acid (v/v) and euthanized with ethyl ether after 2 min. Legs were excised, skinned, covered with dichloromethane, frozen with liquid nitrogen and stored at -5°C. Thawed legs were homogenized in dichloromethane over 10 g sodium sulfate in a mortar and pestle and then extracted by Soxhlet apparatus (Fisher Scientific, Pittsburgh, PA) for 10 h. Extracts were concentrated to 0.5 ml *in vacuo* and transferred quantitatively to a 10 ml volume in 0.01% acetic acid in methanol (v/v). Samples were filtered through 0.45 μm nylon membrane (Rainin Instrument Company, Woburn, MA) and reversed-phase HPLC was performed as described previously. Data were acquired for quantitation on an IBM Instruments 9000 computer with chromatography application program CAPS2 (IBM Instruments, Danbury, CT). The limit of detection by HPLC was <0.12 μg and the detector response was linear to >1.0 μg.

Rabbit serum, to which 4-nitrophenyl diphenylphosphinate had been added at 0.1 mg/ml, was extracted by adding 0.1 ml to 2 ml ethyl acetate containing 0.2% (v/v) glacial acetic acid. This was mixed with 2 ml water and the ethyl acetate layer was collected, followed by two additional ethyl acetate extractions. Ethyl acetate was evaporated by nitrogen stream and the residue redissolved in 2 ml warm acetonitrile. Acetonitrile was partitioned against hexane three times, discarding the hexane layers. The acetonitrile was concentrated to 0.1 ml and a 10 μl sample was injected on the reversed-phase column.

2b.iii. Results and Discussion:

Retention times of organophosphinates on silica were directly related to polarity of the molecules, as observed when 10% 2-propanol in hexane was used to elute compounds of the phenyl series, halogen series and *bis*-substituted series (Table 2). The common decomposition product, 4-nitrophenol, and the organophosphate, paraoxon, were retained 9.8 and 12.8 min, respectively. Since compounds of the methyl series were absorbed strongly, it was necessary to increase the elutropic power of the mobile phase by doubling the 2-propanol content so that they were retained less than 20 min. Then 4-nitrophenol and paraoxon were eluted in 5.5 and 8.2 min, respectively.

Retention times of organophosphinates on the octylsilyl-bonded column were inversely related to polarity of the compounds and inversely correlated with retention times on silica (Tables 2 and 3). On the octylsilyl column, all organophosphinates were resolved from 4-nitrophenol, which was retained 4.2 min in 50% acetonitrile in water; paraoxon was eluted in 6.6 min. Resolution of methyl series compounds from each other was satisfactory by reversed-phase chromatography (Fig. 2), while these compounds were not resolved by normal-phase chromatography.

As indicated by HPLC, the most polar of these organophosphinates was the methyl(2-furyl) compound while the least polar was the trichloromethyl(phenyl) compound. In the halogen series, the addition of chlorine atoms dramatically influenced chromatographic behavior. There was a similar effect when the aliphatic substituent was enlarged in the phenyl series. Changes from aryl to heteroaryl substituents had less effect on the chromatographic characteristics of the organophosphinates.

Decomposition to a more polar product was observed when 4-nitrophenyl trichloromethyl(phenyl)phosphinate (TCMPP) was dissolved in methanol. TLC on silica gel PF-254 (E. Merck, Darmstadt, FRG) revealed a single spot at 0.4 Rf when TCMPP was dissolved in acetonitrile or in ethanol; however, spots at Rf 0.18 and 0.4 were observed upon dissolution in methanol. The second spot (Rf 0.18) was not 4-nitrophenol (Rf 0.30), 4-nitrophenyl dichloromethyl(phenyl)phosphinate (CMPP) (Rf 0.27) nor 4-nitrophenyl dichloromethyl(phenyl)phosphinate (DCMPP) (Rf 0.31). The decomposition product released 4-nitrophenol upon hydrolysis in potassium hydroxide.

Decomposition product was isolated from TCMPP dissolved in methanol for several days, concentrated and passed through a Unisil silica chromatography column in hexane/ethyl acetate. Collected fractions containing product were identified by TLC, pooled, concentrated, and redissolved in acetonitrile. Hydrolysis of a measured aliquot in aqueous potassium hydroxide produced a strong yellow color indicating liberation of 4-nitrophenol which was measured spectrophotometrically and its concentration found from a standard curve of 4-nitrophenol concentration versus ultraviolet absorbance at 405 nm. Assuming complete conversion of product to 4-nitrophenol in the aliquot, the concentration of unknown product in a 5 ml solution in acetonitrile was calculated as 0.9 mM.

The decomposition product from TCMPP at 3.8×10^{-5} M inhibited 55% of rabbit liver monomeric carboxylester activity in 10 min at 37°C. The product was a moderate inhibitor but not as potent as eight organophosphinates tested against this enzyme (vide Table 6). A sample of this decomposition product was sent to the US Army Institute of Chemical Defense. Decomposition appeared to occur only in methanol, since the product was not seen from ethanol nor from acetonitrile solutions. There was no apparent decomposition on CMPP or DCMPP in methanol solutions. Methanol solutions were not used in any other portions of this contracted research; solutions were prepared in acetonitrile unless described otherwise.

Table 2. Normal-phase high performance liquid chromatography of 4-nitrophenyl organophosphinates on silica column in 2-propanol/hexane at a flow of 1 ml/min at 36°C.

Series	P-C Bonded substituents R_1	R_2	Retention time, min±s.e.	n	% 2-propanol in mobile phase
Methyl	CH_3	2-naphthyl-	12.80±1.05	4	20
	CH_3	2-thienyl-	18.45±1.20	4	20
	CH_3	phenyl-	19.30±0.941	6	20
Phenyl	$(CH_3)_2CH$	phenyl	9.26±0.265	7	10
	CH_3CH_2	phenyl	15.52±0.693	8	10
	CH_3	phenyl	30	2	10
Halogen	CCl_3	phenyl	5.71±0.125	7	10
	$CHCl_2$	phenyl	7.73±0.242	7	10
	CH_2Cl	phenyl	15.07±0.747	6	10
bis-substituted	phenyl	phenyl	5.6	1	20
			9.68±0.717	12	10
	2-thienyl	2-thienyl	12.42±0.802	9	10
	2-furyl	2-furyl	13.18±0.626	8	10

Decomposition of 1.45×10^{-3}M TCMPP in methanol was observed by HPLC on an octadecylsilyl column eluted at 1 ml/min 50% acetonitrile in water with detection at 210 nm (Fig. 3). The polar decomposition product increased with time and after 26 h there was no detectable parent TCMPP remaining while a control in acetonitrile produced no detectable decomposition product and less than 0.2% loss of parent in 26 h. The product was not 4-nitrophenol which was retained 4.4 min under these conditions.

Separation of enantiomers was observed for racemic mixtures of each of three chiral organophosphinates on the ionically bonded, chiral-phase column (Table 4). Elution on this column was in the same order as in normal-phase; i.e., the methyl series compounds were most highly retained. Since this column degenerates in polar mobile phase, enantiomers of the chiral, methyl series compounds could not be separated. Attempts to methyl series compounds on other separate commercially available chiral-phase columns were unsuccessful. An L-leucine column (Regis) and a urea derivative column (Supelco) were tested.

Table 3. Reversed-phase high performance liquid chromatography of 4-nitrophenyl organophosphinates on octylsilyl-bonded column in acetonitrile/water at a flow of 1 ml/min at 24°C.

Series	P-C Bonded substituents		Retention time, min±s.e.	n	% acetonitrile in mobile phase
	R_1	R_2			
Methyl	CH_3	2-naphthyl	8.81±0.071	4	50
	CH_3	phenyl	5.35±0.062	4	50
			8.34±0.075	2	40
	CH_3	2-thienyl	5.18±0.091	4	50
			7.54±0.040	2	40
	CH_3	2-furyl	4.62±0.024	4	50
			6.62±0.005	2	40
Phenyl	$(CH_3)_2CH$	phenyl	8.08±0.082	5	50
	CH_3CH_2	phenyl	6.55±0.093	5	50
	CH_3	phenyl	5.35±0.062	4	50
Halogen	CCl_3	phenyl	17.37±0.091	6	50
	$CHCl_2$	phenyl	10.39±0.056	7	50
	CH_2Cl	phenyl	7.24±0.038	7	50
	CH_3	4-CF_3 phenyl	9.13±0.051	5	50
	(second peak)		35.82±0.350		
bis-substituted	phenyl	phenyl	11.18±0.142	5	50
	2-thienyl	2-thienyl	9.17±0.111	5	50
	2-furyl	2-furyl	6.98±0.039	4	50
	CH_2Cl	CH_2Cl	5.33±0.058	4	50

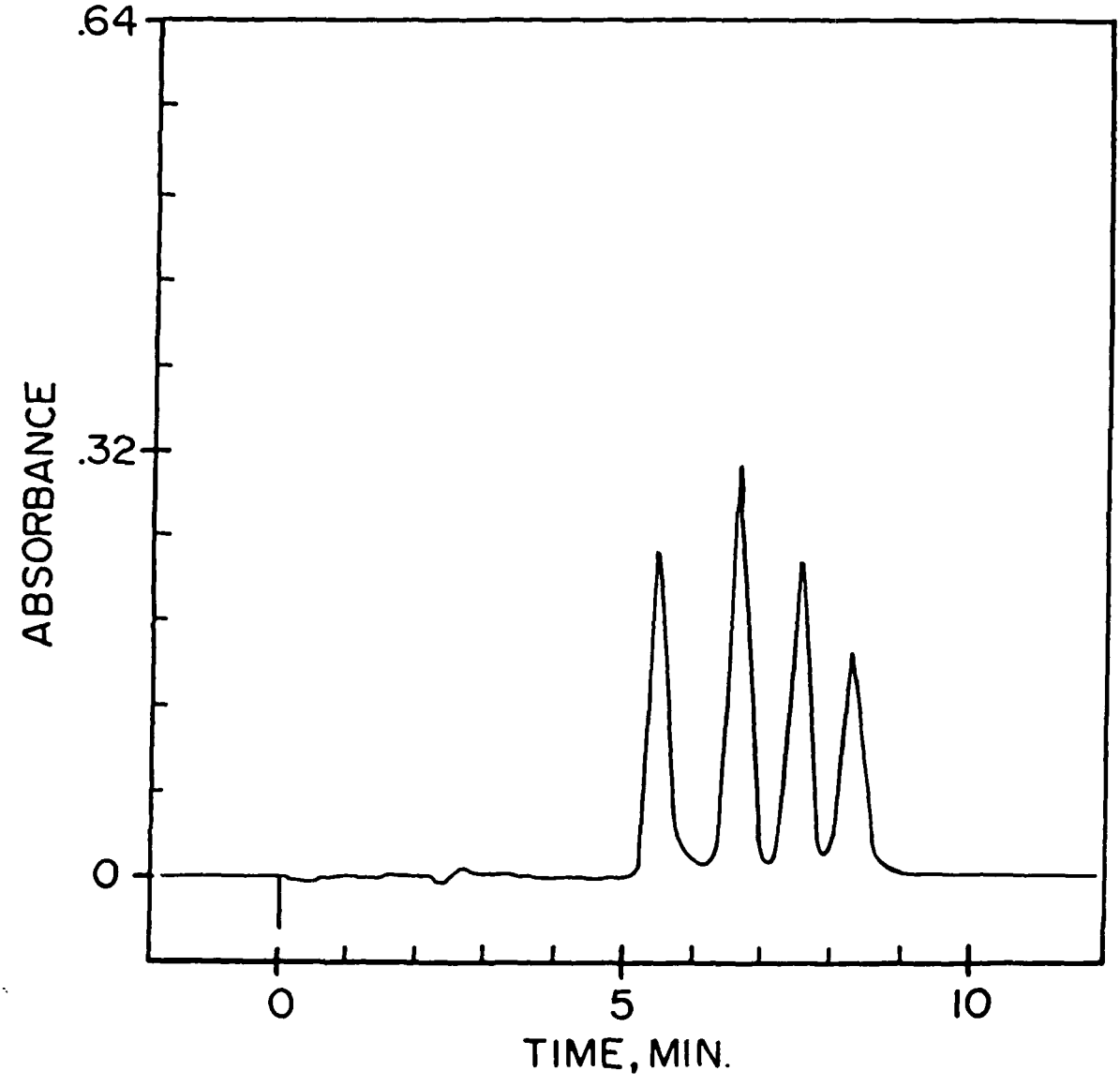


Figure 2. Reversed phase chromatography of 4-nitrophenyl organophosphinates on an octylsilyl column with acetonitrile-water (40:60) as the mobile phase; detection at 270 nm. Peaks in order of elution are 4-nitrophenol, 4-nitrophenyl methyl(2-furyl)phosphinate, 4-nitrophenyl methyl(2-thienyl)phosphinate and 4-nitrophenyl methyl(phenyl)phosphinate.

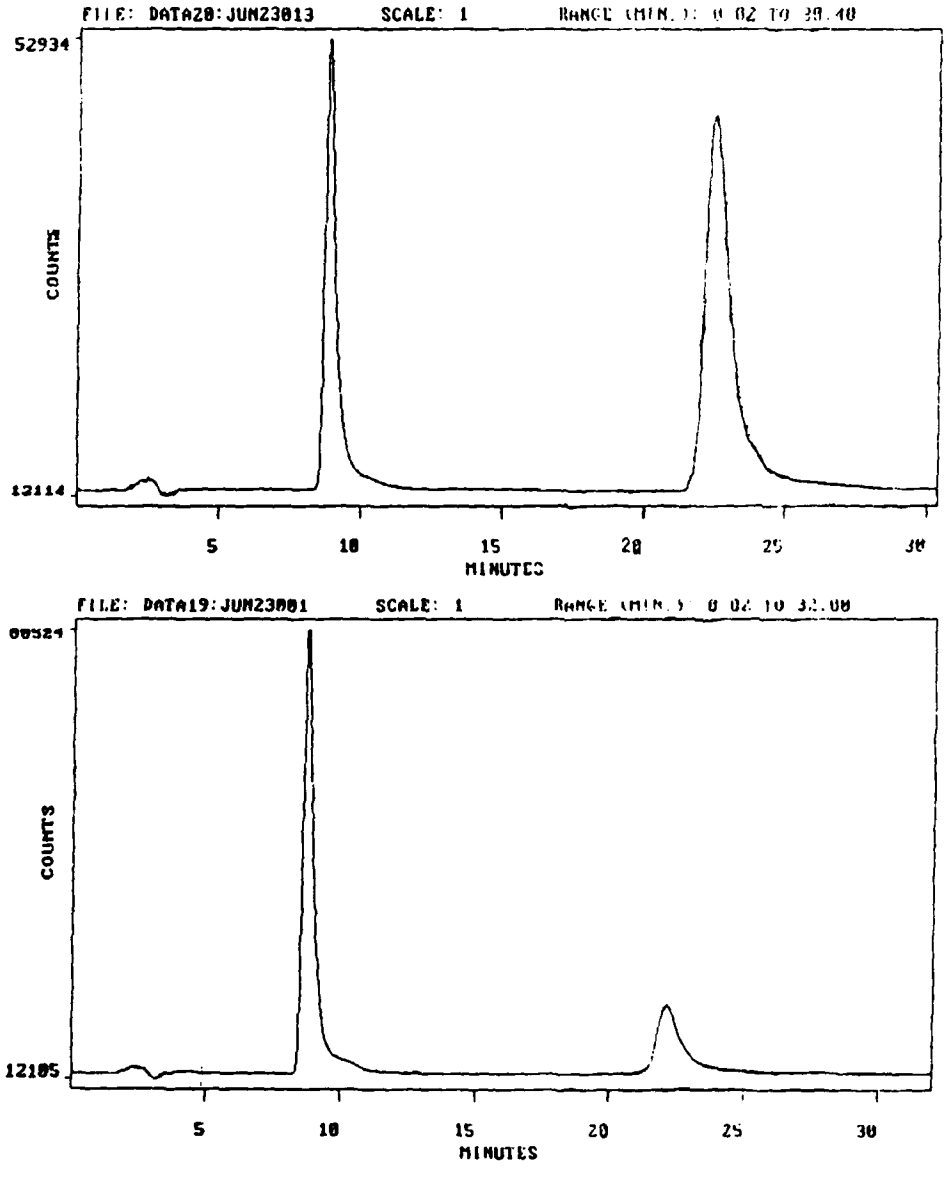


Figure 3. Decomposition of TCMPP in methanol at 24°C. Top panel is 0.020 ml of 1.45×10^{-3} M TCMPP after 78 min in solution. Bottom panel is the same solution after 170 min. The peak at 8.8 min is the decomposition product and the peak at 22 min is TCMPP.

Table 4. Chiral-phase high performance liquid chromatography of 4-nitrophenyl organophosphinates on (R)-N-(3,5-dinitrobenzoyl)phenylglycine ionically bonded column in 2-propanol/hexane at a flow of 1 ml/min at 18°C.

Series	P-C Bonded substituents		Retention time, min±s.e.		n	% 2-propanol
	R_1	R_2	1st enantiomer	2nd enantiomer		in mobile phase
Methyl	CH_3	2-naphthyl	47	a	2	10
Phenyl	$(CH_3)_2CH$	phenyl	16.67±0.048	17.18±0.062	4	10
			26.40±0.210	27.54±0.226	4	5
	CH_3CH_2	phenyl	20.06±0.205	20.54±0.219	4	10
			33.53±0.208	34.57±0.229	5	5
Halogen	$CHCl_2$	phenyl	19.15±0.108	19.55±0.099	7	10
			29.92±0.245	30.77±0.244	4	5
	CH_2Cl	phenyl	50	a	2	10
bis-substituted	phenyl	phenyl	28.12±0.129	b	4	10
			48.48±0.258	b	4	5
	2-thienyl	2-thienyl	33.53±0.176	b	7	10
			55.71±0.292	b	2	5

a. Not observed because of degeneration of compound during chromatography.
b. Not applicable.

Achiral, bis-substituted organophosphinates chromatographed as single peaks (Table 4). Chiral separations were enhanced by decreasing the polarity of the mobile phase and also by decreasing the column temperature to 18°C.

Enantiomers of 4-nitrophenyl isopropyl(phenyl)phosphinate (IPP) were isolated by collecting the eluate from baseline to the point of greatest absorbance of the first peak, discarding the middle portion, and then collecting from the point of greatest absorbance of the second peak to baseline; accumulated collections were rechromatographed on the next day to observe the partially purified enantiomers (Fig. 4). When enantiomers of the ethyl(phenyl) compound were collected, accumulation from 22 injections of 0.05 mg each yielded 0.19 mg of the lesser retained enantiomer and 0.40 mg of the greater retained enantiomer. Concentration and rechromatography of these fractions indicated that the first peak was approximately 95% pure while the second peak was only 80% pure. The second peak was purified further by repeating the process.

Recovery of 4-nitrophenyl diphenylphosphinate following intramuscular injection of mice was 83.1% (±14.3 s.e., n=5) when doses ranged from 0.625 mg to 2.5 mg per mouse. Clean-up of samples was not necessary since the phosphinate was retained for 10 min and no other peaks were observed between 5 min and 20 min. No phosphinate was observed in a control mouse injected with vehicle only. The recovery of phosphinate plus 4-nitrophenol from rabbit serum spiked with 4-nitrophenyl diphenylphosphinate was 80.0% (±3.6 s.e., n=4). There were no interfering peaks in the serum extracts. The actual limit of detection was not approached in this study and it appeared that phosphinate metabolism could be examined by this method.

Several HPLC methods were applied to organophosphinates; the chiral-phase separation of organophosphinate enantiomers should be particularly useful for preparation of small quantities to be tested as enzyme substrates or inhibitors. Organophosphinates were readily chromatographed from biological samples.

3. Inhibition and Reactivation of Carboxylester Hydrolase

3a. Introduction

Nitrophenyl dialkylphosphinates were inhibitors of horse liver carboxylesterase, although they were less inhibitory than their phosphonate and phosphate homologs (10). Organophosphinates also inhibited acetylcholinesterase (12,13); however, spontaneous recovery of phosphinylated acetylcholinesterase (18) was more rapid than that of phosphorylated acetylcholinesterase (21).

Organophosphinates did not produce delayed neurotoxic effects in hens and they also protected hens from effects of subsequent administration of several organophosphates which were neurotoxic in the absence of organophosphinates (22). It appeared that the lack of dealkylation (aging) of phosphinylated enzymes enhanced the rate of recovery and precluded delayed neurotoxicity (23).

We report very rapid inhibition of porcine liver carboxylesterase by 4-nitrophenyl organophosphinates containing aryl or heteroaryl substituents. While recovery from organophosphate inhibition has been studied for acetylcholinesterase from many sources (24), there are few reports of recovery of other hydrolases. We investigated the spontaneous and chemically induced reactivation of rabbit liver monomeric carboxylesterase following its inhibition by a wide variety of 4-nitrophenyl organophosphinates.

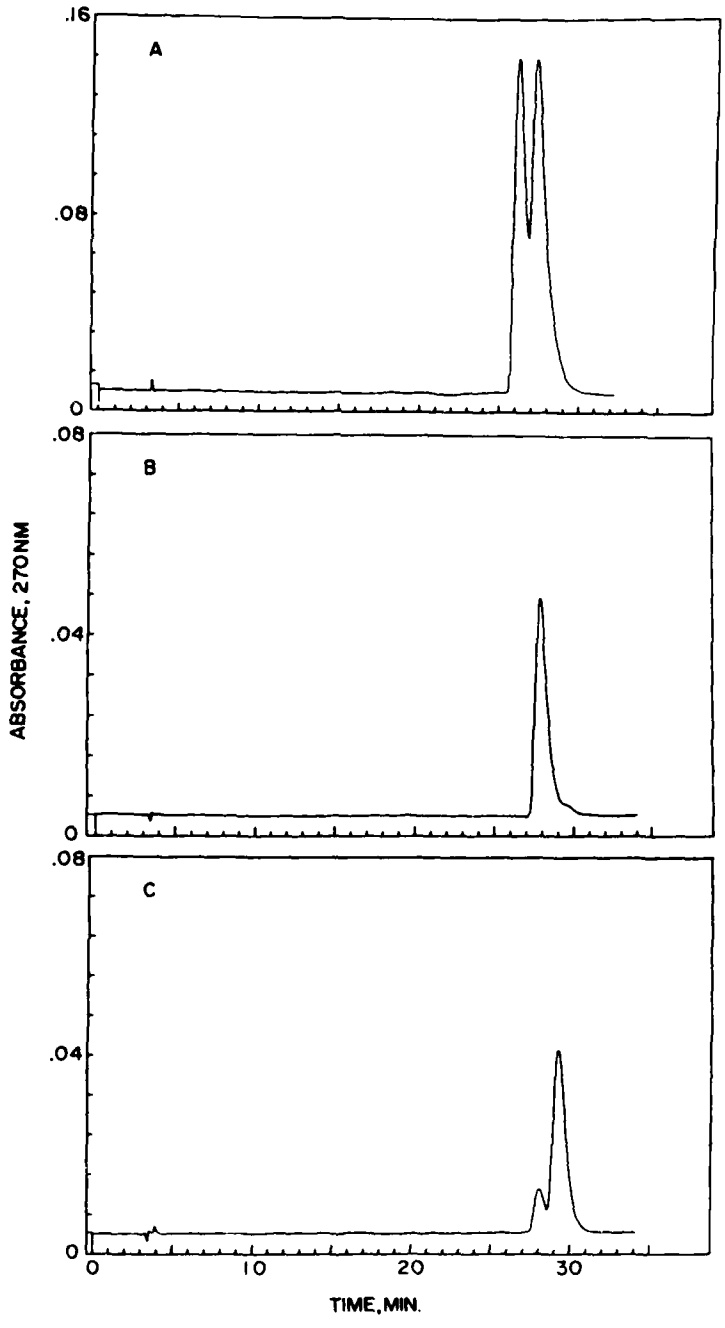


Figure 4. Chiral-phase separation of enantiomers of 4-nitrophenyl isopropyl-(phenyl)phosphinate (III). Panel A: chromatogram of racemic mixture; panel B: chromatogram of lesser-retained enantiomer; panel C: chromatogram of greater-retained enantiomer. Peaks shown in B and C were collected by preparative chromatography.

3b. Materials and Methods

The organophosphinates used in these experiments were considered as four series of analogous compounds (Table 1). They were synthesized by Ash-Stevens, Incorporated, Detroit, Michigan and provided to us by Mr. C. N. Lieske of the U.S. Army Medical Research Institute for Chemical Defense, Aberdeen Proving Ground, Maryland. Reagents were obtained from Sigma Chemical Company, St. Louis, MO.

Inhibition experiments employed 1 μg/ml Type II porcine liver carboxylesterase (Sigma Chemical Co., St. Louis, MO) which was incubated with inhibitor in the absence of substrate in 0.1 M sodium phosphate buffer, pH 7.5 at 37°C. At intervals from 1 to 5 min after adding inhibitor, aliquots were taken for 260-fold dilution into substrate solution to measure the hydrolase activity remaining. When 1-naphthyl acetate was used as the hydrolase substrate (25), an apparent K_m of 0.0356 mM was computed from an Eadie-Hofstee plot. Enzymatic hydrolysis of 0.35 mM 1-naphthyl acetate in 2.6 ml sodium phosphate buffer was terminated after 10 min by adding 0.5 ml 8.4 mM tetrazotized o-dianisidine in 0.425 M aqueous sodium dodecyl sulfate. The blue color, which developed in 10 min, was measured at 600 nm against a reagent blank. Typical hydrolase activity in the control with acetone carrier added was 0.29 mmoles min^{-1} mg $protein^{-1}$. In a confirmatory experiment, phenylthiobutyrate was substituted as substrate and the hydrolysis product was measured after a chromogenic reaction (26).

Spontaneous reactivation experiments were performed with rabbit liver monomeric carboxylesterase (27), generously provided as powder by Dr. A. R. Main, North Carolina State University. This carboxylesterase was not available to us during the earlier phase of the study in which commercial porcine liver carboxylesterase was employed for inhibition studies. We preferred to use the monomeric carboxylesterase in reactivation experiments because it was described in the literature (27) and also because of the greater simplicity of its monomeric form. Procedures were similar to those used with phosphinylated acetylcholinesterase (18). A molecular size exclusion column 10.8 cm by 0.8 cm was prepared from 1 g Sephadex G-25-150, swollen 3 h in 0.1 M sodium phosphate buffer, pH 7.6, and calibrated with Blue Dextran, N-2, 4-dinitrophenyl-L-phenylalanine and phosphinate II. Monomeric carboxylesterase at 1.53×10^{-6} M was inhibited at room temperature for 3 min with 4.87×10^{-4} M phosphinate added in acetonitrile (1% final concentration). It was necessary to use 9.74×10^{-4} M concentration of the 2-furyl derivatives, 4-nitrophenyl methyl(2-Furyl)phosphinate (MFuP) and 4-nitrophenyl di-2-Furylphosphinate (DFuP), for 30 min to inhibit > 90% of the enzyme.

The phosphinylated enzyme was separated from inhibitor by adding 0.5 ml of the mixture to the column and collecting the initial 4 ml eluate flowing at 0.5 ml/min. The eluate was diluted to 50 ml in 0.1 M sodium phosphate, pH 7.6, and held at 37°C in a shaking water bath. Recovery of enzyme activity from the column was 97.1% in this initial eluate and spectrophotometry indicated that unbound EPP was completely separated from the enzyme by this technique.

Spontaneous reactivation was assessed by adding 20 l of the purified, phosphinylated enzyme to 2.6 ml 0.2 mM 1-naphthyl butyrate for 10 min at 37°C and then terminating the reaction with tetrazotized o-dianisidine and measuring the absorbance as above. To examine reactivation at 25°C, the diluted eluate from the column was divided and a subsample was held in a second water bath. This sample was assayed for activity at 25°C and compared to a companion control. Inhibition and reactivation were replicated for each phosphinate on four separate days.

Oxime-induced reactivation was assessed following organophosphinate inhibition of rabbit liver monomeric carboxylesterase and column chromatography as previously described; the eluate was divided and aqueous TMB-4 was added to one half to give 0.1 mM final concentration while water was added to the remainder as a control. Uninhibited enzyme was passed through a second column and treated identically to assess the effect of the oximes. Reactivation at 37°C was determined as described above except that 3-[N-morpholino]propanesulfonic acid (MOPS) was substituted for sodium phosphate buffer; the experiments were performed in quadruplicate.

Comparative spontaneous reactivation of three different carboxylesterases employed the same methods described above for spontaneous reactivation of rabbit liver monomeric carboxylesterase with the exception that MOPS buffer was substituted again for sodium phosphate buffer throughout the method. The enzymes compared were rabbit liver monomeric carboxylesterase, rabbit liver oligomeric carboxylesterase (also obtained from A. R. Main), and porcine liver oligomeric carboxylesterase described above.

A preliminary study of malathion hydrolysis and the effect of EPP was performed with rabbit liver oligomeric carboxylesterase (from A. R. Main). After 10 min incubation of 7.6×10^{-5} mM carboxylesterase with 0.067 mM EPP, malathion was added to a final concentration of 0.82 mM. The buffer was 0.10 M MOPS, pH 7.5, and the temperature was 23°C. Samples of 0.01 ml were injected directly onto an octadecylsilyl-bonded HPLC column with a mobile phase of 70% methanol in water pumped at 1.0 ml/min. Malathion and EPP were detected at 205 nm by the Micromeritics 788 variable wavelength ultraviolet detector and the chromatograms were stored on an IBM Instruments 9000 computer. This experiment was performed only once.

3c. Results

Three heterocyclic organophosphinates and five phenylphosphinates were inhibitors of carboxylesterase (Table 6). According to the data in Table 6, DTP was the most inhibitory of eight compounds tested; IPP was least inhibitory. Six other compounds within these series were similar in potency, having median inhibitory values within a three-fold range of concentrations. Replacing the phenyl moiety with the thienyl group resulted in a three-fold increase in inhibition (Table 6). This was observed in both the methyl(2-thienyl) compound and the di-2-thienyl compound when compared to their phenyl analogues.

Table 6. Inhibition of porcine liver carboxylester hydrolase by organophosphinates as estimated by median inhibitory concentration for 2 min incubation at 37°.

Inhibitor	I_{50} (2 min), M
DTP	7.4×10^{-9}
MTP	1.7×10^{-8}
DPP	2.1×10^{-8}
MFuP	2.5×10^{-8}
DCMPP	2.8×10^{-8}
MPP	5.1×10^{-8}
EPP	5.3×10^{-8}
IPP	9.6×10^{-7}
Paraoxon	8.0×10^{-8}

Bimolecular reaction constants of inhibition could not be determined since many plots of log percentage of control activity versus time were non-linear or did not pass through 100% activity at zero time (28). The curvature in the lines obtained from results for most of these phosphinates was expected since the calculated enzyme concentration (29) was 1.4×10^{-8} M and most inhibitors were used at approximately an equivalent concentration rather than the 10-fold excess necessary to obtain linear plots. This problem cannot be overcome in our method since a 10-fold excess resulted in nearly total inhibition in 1 min.

In these experiments, enzyme and inhibitor were incubated first in the absence of substrate; then the inhibition mixture was diluted into substrate solution to measure the enzyme activity remaining. The substrate used to assess the residual activity had no effect on the results as observed by substituting either phenylthioacetate or phenylthiobutyrate for 1-naphthyl acetate.

Spontaneous recovery of phosphinylated rabbit liver monomeric carboxylesterase varied with the chemistry of the phosphinyl group so that in general the enzyme recovered more rapidly from inhibition by the more polar phosphinyl groups (Figs. 5-8).

Rabbit liver monomeric carboxylesterase recovered more than 80% of control activity after complete inhibition by compounds MPP, EPP and DPP of the phenyl series (Fig. 5). Initially, spontaneous reactivation was extremely rapid from the methyl(phenyl)phosphinylated carboxylesterase, which recovered one-half its activity in only 4 h. Following inhibition by the phenyl series, carboxylesterase reactivation ranked as methyl > ethyl > phenyl >>isopropyl. Reactivation was generally faster following

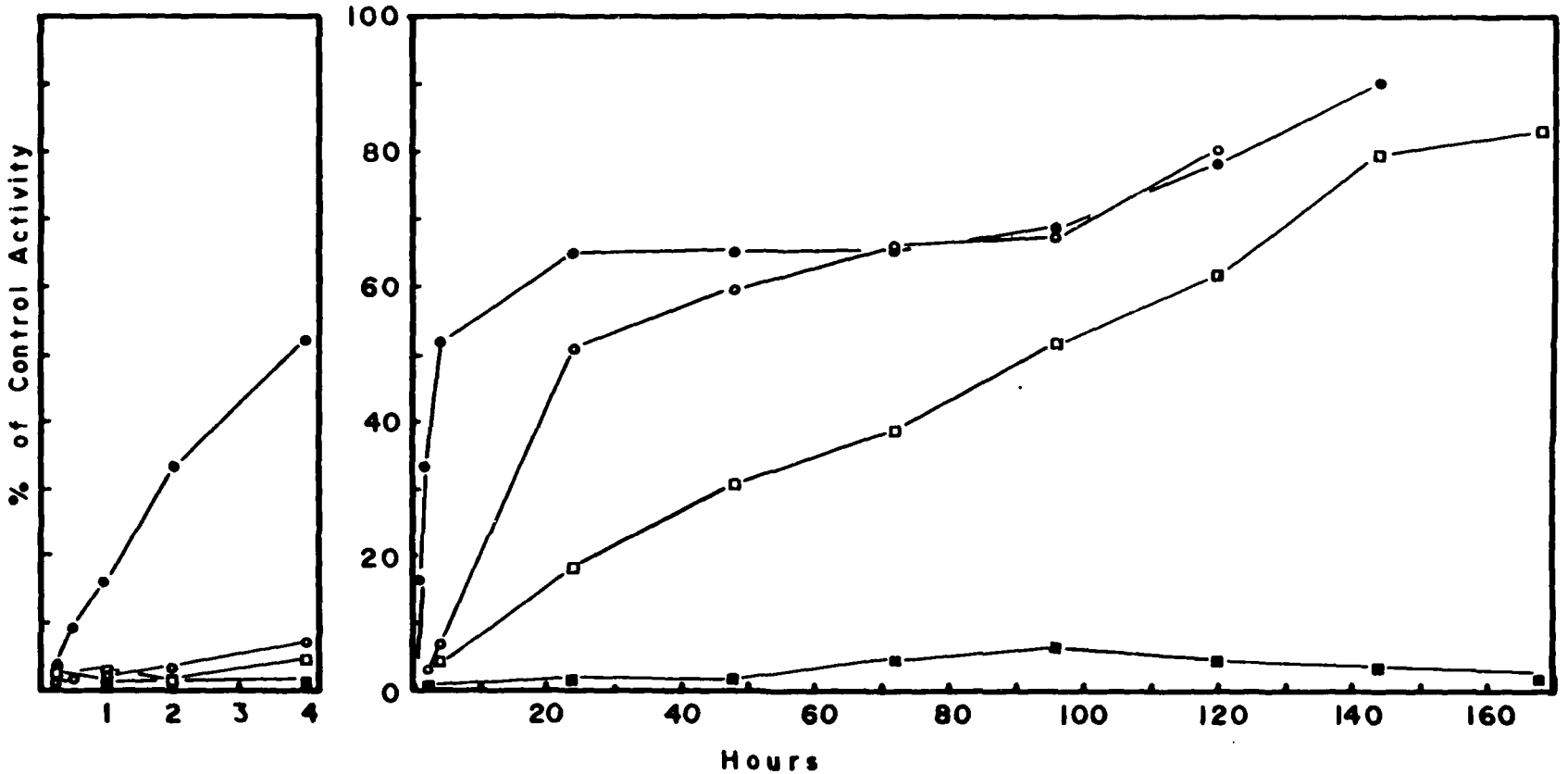


Figure 5. Spontaneous reactivation at 37°C of rabbit liver monomeric carboxylester hydrolase following inhibition by 4-nitrophenyl substituted organophosphinates of the phenyl series. Closed circle is methyl (phenyl), open circle is ethyl(phenyl), closed square is isopropyl(phenyl), and open square is diphenyl.

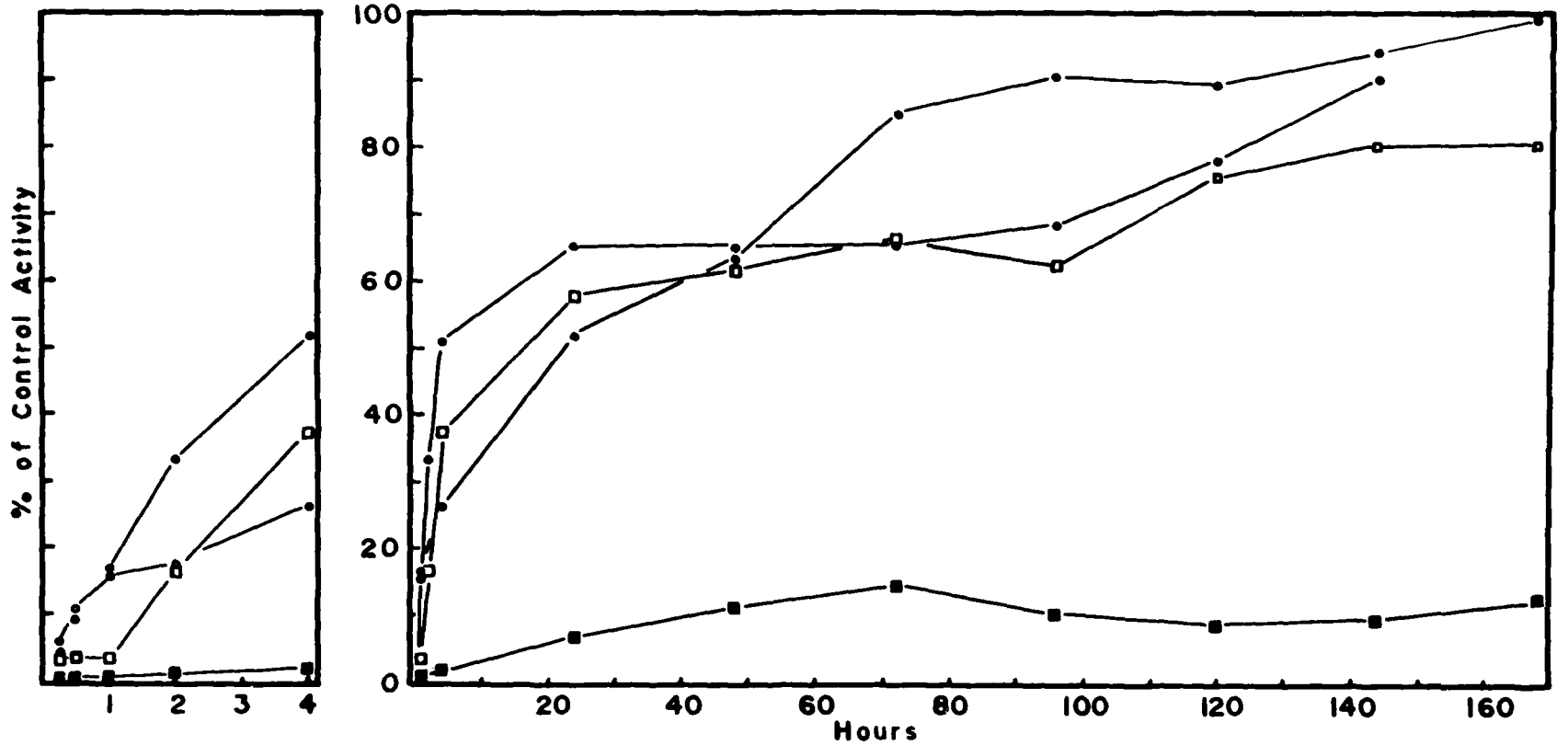


Figure 6. Spontaneous reactivation at 37°C of rabbit liver monomeric carboxylester hydrolase following inhibition of 4-nitrophenyl-substituted organophosphinates of the methyl series. Closed circle is methyl (phenyl), open circle is methyl (2-furyl), closed square is methyl(naphthyl), and open square is methyl (2-thienyl).

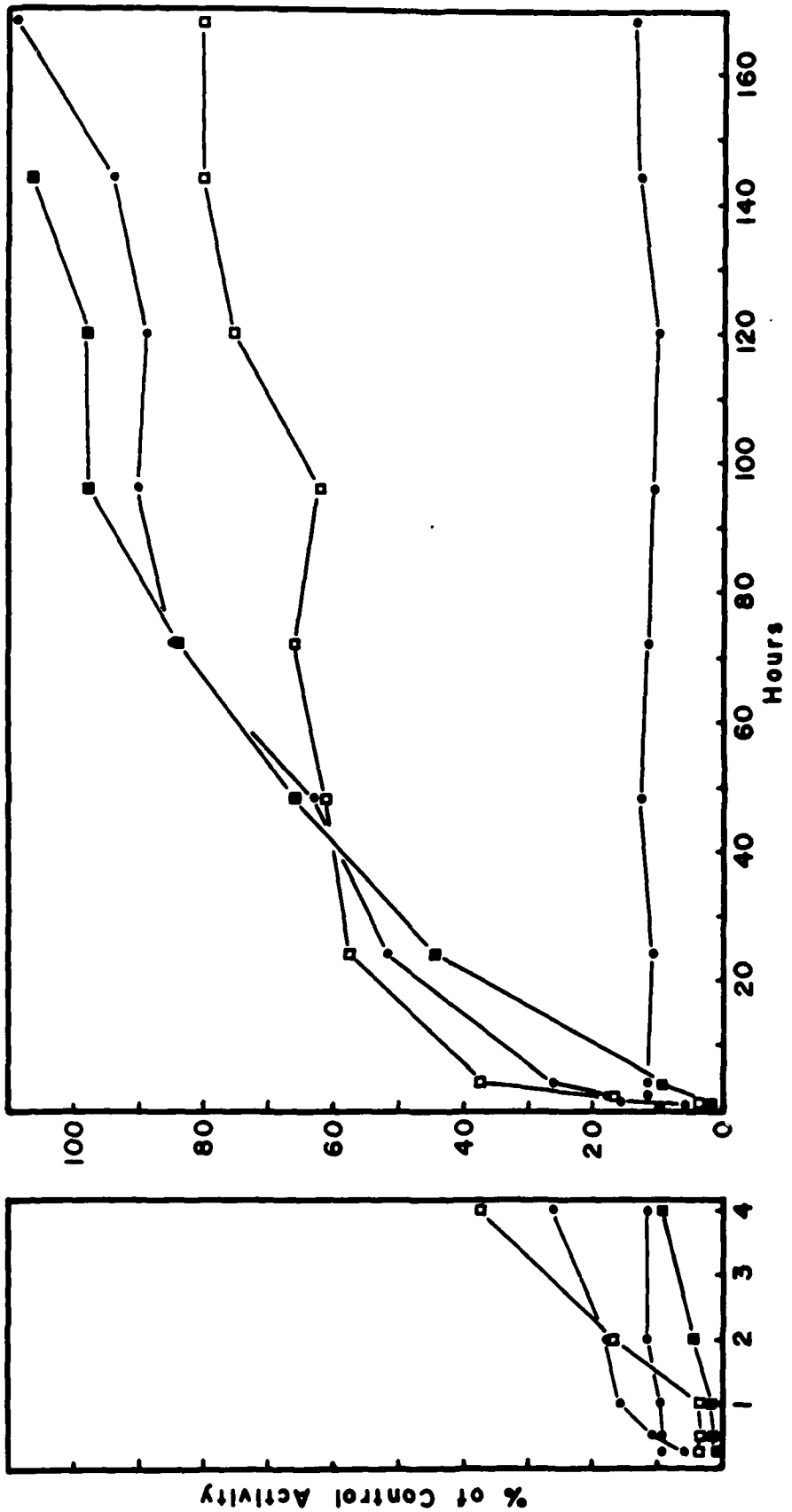


Figure 7. Spontaneous reactivation at 37°C of rabbit liver monomeric carboxylester hydrolase following inhibition by 4-nitrophenyl substituted organophosphinates of the heterocycle series. Closed circle is methyl(2-furyl), open circle is di-2-furyl, closed square is di-2-thienyl, and open square is methyl (2-thienyl).

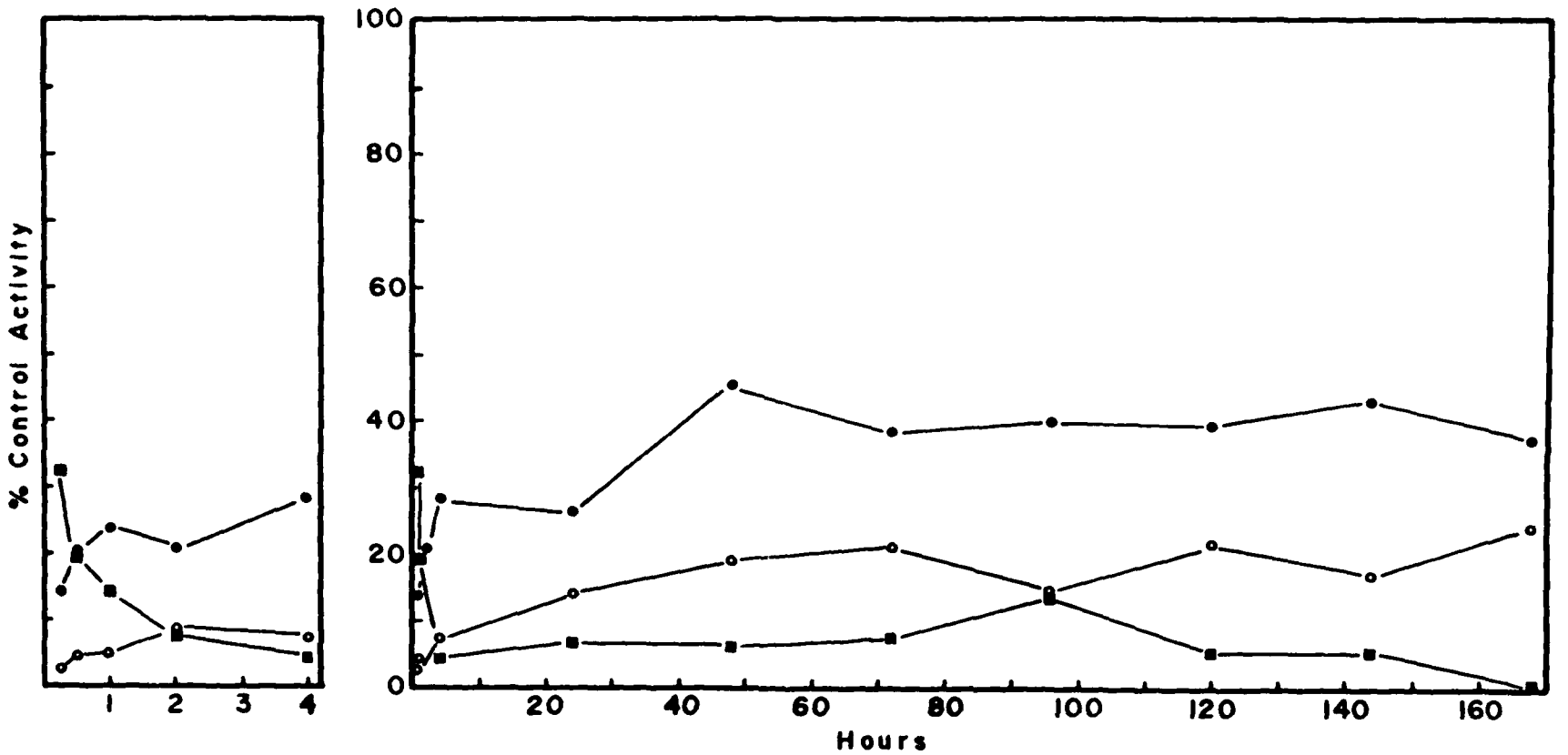


Figure 8. Spontaneous reactivation at 37°C of rabbit liver monomeric carboxylester hydrolase following inhibition by 4-nitrophenyl-substituted organophosphinates of the halogen series. Closed circle is monochloromethyl(phenyl), open circle is dichloromethyl(phenyl), and closed square is trichloromethyl(phenyl).

inhibition by compounds of the methyl series (Fig. 6) than it was following the phenyl series. Recovery from MPP, MFuP and 4-nitrophenyl methyl (2-thienyl)phosphinate (MTP) exceeded 25% in 4 h and it exceeded 80% in six days. Reactivation from 4-nitrophenyl methyl(2-naphthyl)phosphinate (MNP) was very slow. Based on initial reactivation rates, recovery from the phenyl series was in the order methyl > 2-thienyl > 2-furyl >> 2-naphthyl. Reactivation plots of carboxylesterase inhibited by compounds MFuP, MTP and DTP of the heterocycle series produced similar results, approximately 60% recovery at 40 h (Fig. 7). There was no progressive reactivation following inhibition by DFuP. Based on recovery during 24 h, reactivation from compounds of the heterocycle series was ranked as methyl(2-thienyl) > methyl(2-furyl) > di-2-thienyl >> di-2-furyl. Following inhibition by compounds CMPP, DCMPP and TCMPP of the halogen series, reactivation was inversely related to the number of chlorine atoms in the inhibitor (Fig. 8). While TCMPP did not completely inhibit carboxylesterase in the 3 min reaction, enzyme activity declined following elution from the molecular-size exclusion column; thereafter, it did not reactivate. Recovery from 4-nitrophenyl bis-chloromethylphosphinate (bCMP) was slow and data were highly variable. Recovery rates for the chloro-methyl(phenyl)phosphinylated carboxylesterase were ranked as monochloro > dichloro > trichloro.

Spontaneous reactivation did not proceed to completion as a first order reaction; however, some phosphinylated forms of carboxylesterase did recover initially as a first order reaction and the rate constants for those partial reactions ranged from 9.9×10^{-6} min^{-1} for IPP to 6.7×10^{-4} min^{-1} for MPP (Table 7). Those from which recovery was greatest contained both one alkyl substituent smaller than isopropyl and one aryl or heteroaryl substituent smaller than naphthyl. The exception was DTP, which contained two thienyl groups yet produced rapid recovery. Compounds can be grouped, according to the rates at which recovery proceeded, as follows: fast recovery, MPP, EPP, MFuP, MTP, and DTP; moderate recovery, DPP and CMPP; slow progressive recovery, DCMPP; and no progressive recovery, IPP, MNP, DFuP, bCMP, TCMPP and paraoxon.

At 25°C, reactivation rates from MPP and EPP were reduced from the rates at 37°C (Table 8).

Oxime-induced reactivation of ethyl(phenyl)phosphinylated carboxylesterase was twice as rapid as spontaneous recovery so that 91% reactivation was induced in 24 h by TMB-4 (Table 9). TMB-4 itself did not affect activity of carboxylesterase, as seen in the comparison of the control activities with and without the oxime treatment. MOPS buffer, which was substituted for sodium phosphate in this experiment only, had no effect on spontaneous recovery, which was 45.7% in 24 h as compared to 50.8% with sodium phosphate (Table 7). Reactivation of isopropyl(phenyl)phosphinylated carboxylesterase was also enhanced by oxime treatments.

Three carboxylesterases were compared for spontaneous reactivation (Table 10). Following inhibition by EPP, no spontaneous recovery was observed in the rabbit liver oligomeric enzyme or in the porcine liver enzyme at either inhibitor concentration; however, the rabbit liver monomeric enzyme was found to have recovered 45.7% of its activity 24 h

after exposure to 4.87×10^{-4} M EPP (Table 10). Neither rabbit liver carboxylesterase recovered from paraoxon inhibition; however, the porcine liver enzyme exhibited spontaneous reactivation from paraoxon with 69% recovery following exposure to 4.87×10^{-6} M paraoxon.

Malathion hydrolysis by rabbit liver oligomeric carboxylesterase was inhibited by EPP at 0.067 mM. Both the loss of parent malathion and the formation of an unidentified product (possibly malathion monoacid) were negligible in the presence of EPP. Malathion is an aliphatic phosphorodithioate insecticide which is primarily detoxified in mammals by the hydrolysis of one or both of its ethyl esters in the leaving group.

Table 7. Spontaneous reactivation of carboxylester hydrolase following inhibition by substituted 4-nitrophenyl organophosphinates.

Compound	Substitutions on phosphinate	% Reactivation % of control activity ± s.e. 24 h	72 h	k, min^{-1} [a]
MPP	methyl(phenyl)	65.3 ± 5.6	65.5 ± 4.8	6.7×10^{-4}
MTP	methyl(2-thienyl)	57.6 ± 2.8	66.0 ± 2.5	5.8×10^{-4}
MFuP	methyl(2-furyl)	51.9 ± 4.9	85.1 ± 2.1	3.9×10^{-4}
EPP	ethyl(phenyl)	50.8 ± 1.9	65.8 ± 2.6	4.8×10^{-4}
DTP	di-2-thienyl	44.4 ± 8.2	84.0 ± 9.1	6.0×10^{-4}
CMPP	monochloromethyl(phenyl)	26.5 ± 2.2	40.2 ± 14.6	- [b]
DPP	diphenyl	18.5 ± 3.9	39.0 ± 3.0	1.2×10^{-4}
DCMPP	dichloromethyl(phenyl)	14.2 ± 3.5	21.4 ± 3.5	-
bCMP	bis-chloromethyl	12.4 ± 4.6	13.6 ± 9.4	-
DFuP	di-2-furyl	10.6 ± 1.3	11.4 ± 1.0	-
MNP	methyl(2-naphthyl)	6.8 ± 0.4	14.7 ± 4.7	-
TCMPP	trichloromethyl(phenyl)	6.5 ± 2.0	7.5 ± 5.2	-
-	paraoxon	5.9 ± 2.1	5.4 ± 3.6	-
IPP	isopropyl(phenyl)	1.5 ± 0.9	4.4 ± 1.0	9.9×10^{-6}

[a] Reactivation rate constant based on first-order portion of reactivation curve, which included data through 24 h for MPP, EPP and MTP and data through 96h for IPP, DPP, MFuP and DTP.
[b] Dashes indicate recovery was not first-order.

Table 8. Effect of temperature on spontaneous reactivation of carboxylester hydrolase following inhibition by organophosphinates.

Inhibitor	Time (h)	Reactivation (% of control activity)		
		25°C	37°C	25°C/37°C
MPP	4	21.7	51.8	0.42
MPP	24	46.5	65.3	0.71
EPP	4	1.9	6.9	0.27
EPP	24	26.1	50.8	0.51

Table 9. Oxime-induced reactivation of rabbit liver monomeric carboxylester hydrolase following inhibition by organophosphinates.

Time, h	Spontaneous reactivation			Reactivation by 0.1 mM TMB-4		
	Activity ($mmoles\ min^{-1}\ mg^{-1}$)			Activity ($mmoles\ min^{-1}\ mg^{-1}$)		
	Inhibited	Control	% Reactivated	Inhibited	Control	% Reactivated
	Inhibition by 4-nitrophenyl ethyl(phenyl)phosphinate					
0.5	14 ± 4	596 ± 19	2.3	20 ± 3	579 ± 15	3.4
1	19 ± 3	526 ± 45	3.7	50 ± 4	540 ± 29	9.4
2	37 ± 5	545 ± 38	6.8	108 ± 8	475 ± 20	20.0
4	102 ± 22	459 ± 7	22.3	193 ± 19	485 ± 26	39.8
24	196 ± 17	429 ± 12	45.7	424 ± 23	467 ± 45	90.8
	Inhibition by 4-nitrophenyl isopropyl(phenyl)phosphinate					
24	16 ± 2	509 ± 192	3.2	32 ± 10	598 ± 14	5.4

Table 10. Comparative spontaneous reactivation of liver carboxylester hydrolase at 37°C 24 h after inhibition.

Inhibitor	Concentration	Activity (% of control + s.e.) (n)		
		Rabbit liver monomeric	Rabbit liver oligomeric	Porcine liver oligomeric
EPP	4.87×10^{-6} M	nd[d]	0 (2)	0 (2)
	4.87×10^{-4} M	45.7 ± 1.3 (4)[a]	0 (2)	0 (2)
paraoxon	4.87×10^{-6} M	0 (2)	0 (1)	69.0 ± 17.9 (4)[b]
	4.87×10^{-4} M	0 (2)	0 (1)	13.6 ± 6.5 (4)[c]

[a]Recovery at 1.0 h was 3.7%.
[b]Recovery at 1.0 h was 6.6%.
[c]Recovery at 1.0 h was 3.1%.
[d]nd (not determined)

3d. Discussion

It was clear that 4-nitrophenyl organophosphinates were extremely rapid inhibitors of carboxylesterase. While bimolecular reaction constants could not be found under these experimental conditions, the values would be > 10^7 moles^{-1} min^{-1} for all compounds tested except IPP; therefore, these aryl or heteroaryl-containing phosphinates were more rapid inhibitors than seven 4-nitrophenyl dialkylphosphinates for which bimolecular reaction constants ranged from 6×10^3 to 2×10^6 mole^{-1} min^{-1} with horse liver carboxylesterase (10).

Monomeric carboxylesterase appeared to be unusually refractory to recovery from paraoxon inhibition. We observed negligible reactivation of rabbit liver monomeric carboxylesterase following inhibition by paraoxon. In a previous study of phosphorylated porcine liver oligomeric carboxylesterase recovery from paraoxon, spontaneous reactivation was first order through 55% recovery with a rate constant of 2.1×10^{-3} min^{-1} (30). In a purified insect carboxylesterase, reactivation from paraoxon was a first order reaction through 48% recovery with a rate constant of 5.5×10^{-3} min^{-1} (31). This would suggest that phosphorylated carboxylesterase from various sources should be compared in one reactivation experiment.

The only previous spontaneous reactivation study with organophosphinates demonstrated first-order reactivation of eel acetylcholinesterase from MPP, with a rate constant of 3.6×10^{-4} min^{-1} at 25°C and slower recovery from DPP and from the dimethyl analogue (18). Our results with these phosphinates in monomeric carboxylesterase recovery were very similar except that reactivation with MPP, EPP and MTP appeared to be biphasic, with monomeric carboxylesterase recovering much more slowly

after one-half the activity was restored. This result was probably due to two forms of monomeric carboxylesterase in the preparation as was noted during the purification of the enzyme (27); however, MPP, EPP and MTP were racemic mixtures of enantiomers and stereoselective recovery was also a possible contribution to the result.

Induced reactivation of ethyl(phenyl)phosphinylated carboxylesterase was similar to that of di-n-butylphosphinylated acetylcholinesterase (32), slower than methyl(phenyl)phosphinylated acetylcholinesterase, and faster than diphenylphosphinylated acetylcholinesterase (18). Those studies found that 2-pyridine aldoxime methylochloride (2-PAM) was less effective than TMB-4 and our preliminary results with 2-PAM indicated the same relationship in monomeric carboxylesterase. We observed carboxylesterase inhibited by IPP to be refractory to TMB-4 reactivation and it should be tested with acetylcholinesterase. Comparison of spontaneous and induced reactivation following inhibition by EPP indicated that the portion of activity with slow spontaneous recovery was not resistant to TMB-4-induced reactivation.

Comparison of the recoveries of three different carboxylesterases after inhibition revealed a distinct response for each enzyme. Rabbit liver oligomeric carboxylesterase failed to recover from either phosphinylation or phosphorylation. Rabbit liver monomeric carboxylesterase recovered from phosphinylation but it did not recover following phosphorylation. This result could be explained hypothetically by assuming that the phosphorylated form of the enzyme was susceptible to aging which produced irreversible inhibition. Porcine liver carboxylesterase recovered from phosphorylation, as has been reported previously (30), but it failed to recover from phosphinylation which is a very unexpected result.

It is suggested from these data that carboxylesterases may be highly variable in spontaneous recovery from inhibition. This implies that studies must be carried out to find an appropriate model for man. It also suggests that such studies could be very informative concerning the mechanisms of the process of spontaneous reactivation of serine-active sites of enzymes.

Another interesting observation in these results is that the concentration of the inhibitor appeared to influence the rate of reactivation. A possible explanation would be the failure to separate the inhibitor from the enzyme in the gel filtration chromatographic cleanup procedure; however, our calibration of the column refutes this explanation since unbound phosphinate or phosphate did not elute from the column. Binding of parent inhibitor to the enzyme and release subsequent to elution with the enzyme is another possibility. It is also possible that a change in conformation of the enzyme occurs when it is exposed to very high concentrations of inhibitor. Conformation changes affecting the active sites of enzymes and the binding sites of receptor molecules and ion channel are common phenomena. Thorough investigation of these phenomena is necessary to clarify this unexpected observation in which dephosphorylation was more facile than was dephosphinylation.

When rat plasma was exposed to soman, inhibited carboxylesterase was reactivated by sodium fluoride treatment but inhibited acetylcholinesterase was not reactivated (33). While liver carboxylesterase has been recognized as important in xenobiotic detoxication (29), blood carboxylesterase is gaining attention both as a possible storage site and as a detoxication site due to its relative abundance. Monomeric carboxylesterase did not recover from paraoxon inhibition; however, we observed progressive reactivation after inhibition by certain organophosphinates and effective oxime-induced reactivation in one case. Organophosphinates may prove to be useful in further understanding of toxicokinetics involving carboxylesterase.

4. Enzyme-catalyzed Hydrolysis of Organophosphinates

4a. Partial Purification of Arylester Hydrolase from Rabbit Serum

4a.i. Introduction

Arylester hydrolase (EC 3.1.1.2), commonly referred to as paraoxonase, has as substrates a range of toxic organophosphates and organophosphinates (34,3). Because of its target substrates, arylester hydrolase is an important enzyme in xenobiotic organophosphorus detoxication and in environmental detoxification. While nothing is known about the natural substrates of arylester hydrolase (35,36), the enzyme is found in high levels in mammals, especially in rabbit serum, (37,38) while it is found in low levels in birds (37). Recently the gene for arylester hydrolase has been localized on human chromosome 7 and has found to be of use as a genetic marker for cystic fibrosis (39).

Although several research groups have worked with arylester hydrolase from crude tissue or serum, only Main (40) has published a complete purification (from sheep serum). Mackness and Walker (41) have published a partial purification from the same source. Various estimates of the molecular weight have been made; 35-50000 daltons (sheep serum) (40), greater than 200,000 daltons (sheep serum) (41), and 500,000 daltons (rabbit serum) (38).

This paper introduces a relatively rapid method for purifying rabbit serum arylester hydrolase to approximately the same specific activity as Main's (40) preparation as well as conditions for maintaining enzymatic activity over an extended period of time.

4a.ii. Materials and Methods

Paraoxon was purchased from Sigma Chemical Co. (St. Louis, MO). A stock solution was made by dissolving 114.4 mg of paraoxon in acetonitrile and bringing the volume to 1.0 mL (416 mM). Fresh rabbit blood was purchased from Blue Chip Farms, Taylors, SC. Polyethyleneglycol 4000 (PEG-4000), DEAE-Sepharose, and biological materials were purchased through Sigma Chemical. Ultrogel® AcA34® resin was purchased from LKB Instruments. GelBond PAG® and Acrylaide® were purchased from FMC Corporation (New York, NY). Centricon™ 10 microconcentration tubes were purchased from Amicon Corporation.

Two buffer systems were used extensively and are listed below. Buffer A was composed of 10 mM MOPS [3-(N-morpholino) propanesulfonic acid], pH 7.0, containing 2.5 mM Ca^{2+} and 0.02% sodium azide. Buffer B is the same as Buffer A but with the addition of 100 mM NaCl.

Fractions containing arylester hydrolase activity in the effluent from chromatographic columns were detected using a spot assay developed by us. A spot-assay paraoxon solution was made by adding 25 µL of paraoxon stock (above) to 7.0 mL of 100 mM MOPS, pH 7.5, containing 2.5 mM Ca^{2+}. The vial was immediately placed in an ultrasonic bath for 2 min. Two hundred microliter aliquots of the spot-assay solution were added to each well of a white spotplate. Ten to fifty microliters (depending on the anticipated activity) of the effluent to be assayed was added to individual wells. After 5-30 min of observation, the color development of each well was graded. Active fractions showed a bright yellow coloration.

Quantitative enzymatic analyses were done by preparing a tube containing 100 µL of the sample (appropriately diluted with assay buffer when necessary) and adding 1.2 mL of a solution containing 100 mM MOPS, pH 7.5, and 2.5 mM Ca^{2+}. The reaction was initiated by the addition of 10 µL of stock paraoxon followed by mixing on a Vortex mixer. The solution was then transferred to a 1.0 cm path length semi-micro-cuvette and the change in A_{405} with time measured in a continuous manner using a Cary 219 recording spectrophotometer. The extinction coefficient for p-nitrophenolate ion, under the experimental conditions, was determined to be 13.56 mM^{-1} cm^{-1}.

Protein concentrations were measured by the biuret method when possible or, for dilute solutions, by a fluorescamine assay. Biuret measurements were made by adding 200 µL of sample to 1 mL of biuret reagent (42), mixing, and measuring the absorbance at 540 nm. Bovine serum albumin was used as a standard.

For fluorescamine assays a stock solution of 11.2 mg of fluorescamine was dissolved in, and brought to 50 mL with, spectroscopic grade, anhydrous acetone. The assay consisted of adding 100 µL of sample to 1.4 mL of 0.05 M boric acid buffer, pH 8.5, in an acid-washed tube. To this was added 0.5 mL of fluorescamine stock and the mixture placed on a Vortex mixer. The contents of the tube were transferred to an acid-washed cuvette and the fluorescence measured on a Perkin-Elmer Model 65-40 fluorescence spectrophotometer using 390 nm as the excitation wavelength and 475 nm as the emission wavelength. Both excitation and emission slit widths were 10 nm. Bovine serum albumin was used as a standard.

Discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was done according to the method of Laemmli (43). Gels were 7.5 or 10% acrylamide, cast on GelBond PAG at a thickness of 0.75 mm and cross-linked with Acrylaide®. Samples were prepared by mixing (in a 1.5 mL polyethylene microcentrifuge tube) one volume (usually 50 µL) of protein sample with one volume of a solution of 0.2 M Tris,

pH 8.0, 8 M urea, 2% SDS, and containing 6 mg/mL dithiothreitol. This mixture was placed over a steam bath. At the end of 4 min, an aliquot of 0.2 M Tris, pH 8.0, 8 M urea, 2% SDS and 92 mg/mL iodoacetamide, equal to one-half the volume of the protein aliquot, was added and the mixture left in the steam bath for another 2 min. After the mixture cooled, 5 µL of bromphenol blue in 10 mM HCl was added as a marker. Gels, 12 x 14 cm, were run at 10°C at a constant amperage of 17½ ma/gel. When the bromphenol blue tracking dye reached the bottom of the gel, the gels were silver stained according to the method of Neilsen and Brown (44), increasing only the time of the silver nitrate wash from 20 min to 30 min to compensate for the presence of GelBond PAG.

Purification. Fresh rabbit blood was obtained by decapitation at a commercial rabbit slaughtering house. The blood was allowed to clot at room temperature for approximately 2 h. The clotted material and the serum were funnelled through cheesecloth and the serum made 2.5 mM Ca^{2+} and 0.02% in NaN_3. All subsequent steps were performed at 4°C. The filtrate from the previous step was centrifuged for 10 minutes at 700 x g to remove any non-clotted red blood cells. The light reddish supernatant was carefully and gently aspirated into a vacuum flask and saved.

Dry PEG-4000 was added to the supernatant (14 g/100 mL supernatant) and the tannish solution stirred overnight. The solution was then centrifuged at 16300 x g for 30 minutes. The resulting clear ruby-red supernatant was easily decanted. Eleven grams of solid PEG-4000 per 100 mL of supernatant was added with stirring. The mixture was stirred 3 h and then centrifuged at 16,300 x g for 30 min. The reddish supernatant was easily poured off and discarded. The gummy, tannish precipitate was slowly resuspended in Buffer A.

The resuspended material was added to a 4.5 x 25 cm column of DEAE-Sepharose previously equilibrated in Buffer A. When all of the dark red solution had entered the gel, the column was washed with Buffer A until the A_{280} again reached baseline values. The breakthrough material was a deep red while the column showed definite red, orange, and blue bands. The column was eluted with a linear gradient consisting of Buffer A in the mixing chamber and Buffer A plus 400 mM NaCl in the feed chamber. Several colored peaks were observed. Arylester hydrolase activity eluted late in the gradient appearing just after (and overlapping) a peak of dark red material and just before (and almost completely overlapping) a deep blue peak (Fig. 9A). Very small differences from run to run varied the overlap of the three peaks.

The pooled material was dialyzed overnight versus several changes of Buffer A and then applied to a column of DEAE-Sepharose previously equilibrated with Buffer B. After the material was loaded, the column was washed with Buffer B until the A_{280} began to return to baseline values. The effluent of this isocratic elution showed two peaks, the first being light orange and then, clearly separated from the first peak, was a bright red peak that tailed considerably. A very small amount of arylester hydrolase activity was found in this peak. After the isocratic wash the column still showed a greyish-blue band at the top. Arylester

hydrolase activity was eluted with a linear gradient running from Buffer B with no added NaCl to Buffer B with 0.4 M NaCl added (Fig. 9B). The peak containing essentially all of the arylester hydrolase activity also contained other protein components and a green color (See Table 11 for specific activity). Grothusen et al. (3) have used material from this stage of purification to measure substrate specificity and kinetic parameters. A single band of esterase activity was observed on isoelectric focusing gels using a 2-naphthyl acetate stain; no esterase bands were observed with a 1-naphthyl acetate stain.

To concentrate the enzyme, the pooled fractions with arylester hydrolase activity were brought to 80% ammonium sulfate saturation by the addition of four volumes of saturated ammonium sulfate previously buffered at pH 7.0 and containing 2.5 mM Ca^{2+}. This mixture was stirred overnight and then centrifuged at 48,000 x g for 60 min. The clear white precipitate was resuspended in a minimal volume of Buffer B. Material in this concentrated form loses activity only slowly over a period of months.

For later studies a final step was included immediately before use. One milliliter of the 80% saturated ammonium sulfate resuspension was applied to a 2.5 x 76 cm column of Ultrogel AcA34 previously equilibrated in Buffer B. After the sample entered the gel, the column was eluted with Buffer B. Arylester hydrolase activity eluted as a broad peak between two other peaks (Fig. 9C). The relative size of the two contaminating peaks varied from preparation to preparation, but the contaminant peaks were always sharper than the arylester hydrolase peak.

Physical methods. Fluorescence scans were made using a Perkin-Elmer Model 65-40 fluorescence spectrophotometer. Excitation and emission maximal wavelengths of 282 nm (excitation) and 334 nm (emission) were determined in the Pre-Scan mode as described in the instructional manual. All emission scans were done in the constant ratio mode using the excitation wavelength of 282 nm and emission and excitation slit widths of 5 nm.

4a.iii. Results and Discussion

Purification. Arylester hydrolase has been purified 50-fold from rabbit serum, with yields varying from 14-30% (Table 11). The enzyme resulting from any purification of arylester hydrolase, from any source, must be compared with that of Main (40) since this work has provided by far the highest specific activity of any arylester hydrolase studied. The simple and relatively rapid procedure described here routinely provides yields with a specific activity near those obtained by Main (40) for arylester hydrolase from sheep serum and greater than that reported by Mackness and Walker (41) from sheep serum. The highest specific activity recovered in any Ultrogel AcA34 pool was 1.15 μmole/min/mg, slightly greater than Main's (40). To obtain this activity, a variation of the concentration step was performed on a very small DEAE-Sepharose column, but this method was unsuitable for overall recovery of activity. A direct comparison of the specific activities of the current preparation with that of Main (40) is not possible because Main's assay contained

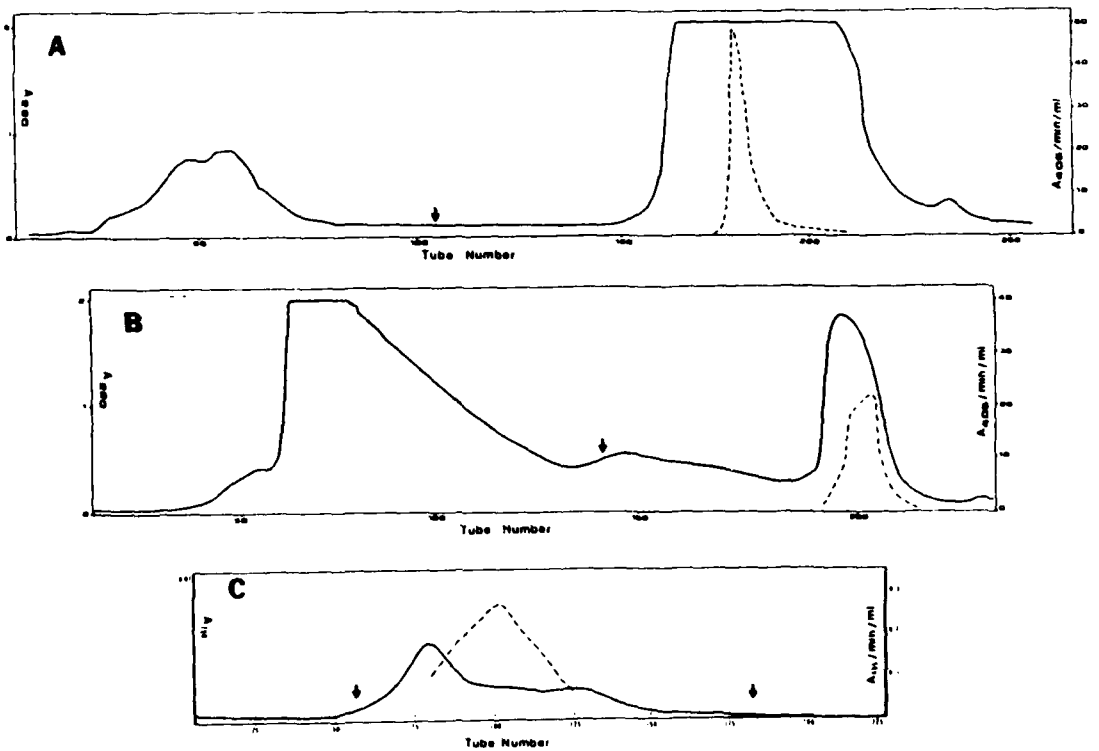


Figure 9. Chromatographic Separations

A. Recorder tracing of first DEAE-Sepharose chromatography. The column measured 4.5 x 25 cm. The monitor was set at 2 (AUFS) with 99 drops/tube, the path length of the flow cell of the monitor was 0.5 cm. The flat reading at 2 AU corresponds to the signal giving off scale. At the arrow a linear gradient of 400 mL of Buffer A (see Materials and Methods) to 400 mL of Buffer A + 0.4 M NaCl was initiated. The dashed line is the measured arylester hydrolase activity.

B. Recorder tracing of second DEAE-Sepharose chromatography. The column measured 4.5 x 25 cm. The monitor settings were the same as in A. The flat readings at 2 AU correspond to the signal giving off scale. At the arrow a linear gradient of 400 mL of Buffer B (see Materials and Methods) to 400 mL of Buffer B + 0.4 M NaCl was initiated. The dashed line indicates the measured arylester hydrolase activity.

C. Recorder tracing of Ultrogel AcA34 chromatography. The column size was 2.5 x 76 cm. The monitor settings were 40 drops/tube and 0.1 AUFS. The monitor flow cell had a 0.5 cm path length. The dashed line indicates the measured arylester hydrolase activity. The two arrows indicate the elution volumes of the void volume (blue dextran) and total included volume (ammonium sulfate).

Table 11. Purification of arylester hydrolase

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	58.42	4.9
80% Ammonium Sulfate	7.5	46	---	---	25.3	---
Ultrogel[b] AcA34	1140	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, resulting in a pool of 76 mL.

ethanol and phosphate, had not deliberately added Ca^{2+}, was done at 37°C and was from a different animal source. The current assay had no ethanol. MOPS replaced phosphate when phosphate was found to be an inhibitor, Ca^{2+} was found to be necessary for optimal activity, and the assay was done at 25°C. The specific activity values in Table 11 are underestimates, since the concentration of substrate used (3.2 mM final concentration) is only five times the K_m value for arylester hydrolase (0.609 mM) (3) under our experimental conditions but the paraoxon concentration could not be increased because of limited solubility. Table 11 shows the results from the current purification procedure. The apparent loss of activity in the 14-25% PEG-4000 fraction is because PEG inhibits the reaction strongly. This is confirmed by the "gain" in total units seen after the first DEAE-Sepharose column. The major purification occurs in the second DEAE-Sepharose column and the Ultrogel AcA34 fractionation. The major loss occurs during the resuspension of the 80% ammonium sulfate pellet to minimize the volume for passage over the Ultrogel AcA34 column (it is of note that the pooled arylester hydrolase from the AcA34 effluent often had a higher activity than could be expected from the average of

the velocities of individual tubes). In this respect, Walker and Mackness (35) reported four very closely spaced peaks in their preparative gel electrophoresis of sheep serum arylester hydrolase and Kojima and O'Brien (45) reported at least four enzyme forms in rat liver. We have seen occasional evidence for two peaks of arylester hydrolase activity in the AcA34 effluent.

Even though the colorless arylester hydrolase pool at this stage matches, or exceeds, the highest reported specific activity for arylester hydrolase, densitometer scans at 525 nm of AcA34 effluent material show the preparations to be only 20-50% pure. Accurate quantitative analysis of these silver stains is not possible because, in the system of Neilsen and Brown (44), different proteins show different colors.

The final step of the current purification (the Ultrogel AcA34 column) is not yet satisfactory. While adequate separation was achieved using the AcA34 column, the fact that it is a size fractionation method limits the volume of material that can be applied to the column in any one run. Concentration of the material by ultrafiltration, using any of a number of filters, resulted in a severe loss of activity, as does concentration by ammonium sulfate precipitation (see above). Centricon 10™ tubes do concentrate the protein without major inactivation, but at a limit of 2 mL/tube this method is impractical on a preparative scale. Several alternative methods have been tried. A concanavalin A column failed to bind any of the residual proteins; the use of a thiol-agarose column to find the free SH group (45) bound some protein, but not arylester hydrolase; and a Matrex Red® column bound an impurity but the arylester hydrolase was in the breakthrough volume, not the property desired for a final step. When arylester hydrolase was applied to two separate Sepharose affinity columns, using compounds known to be competitive inhibitors of arylester hydrolase (46), arylester hydrolase bound very tightly, was highly active, but could not be eluted even with paraoxon. Other methods are being tested but, because of the relatively large volume of material, the desired column matrix should selectively bind (and therefore concentrate) arylester hydrolase while allowing the two remaining impurities to pass through.

During the development of the assay system it was found that the commonly used phosphate ion (40,46,36,47) is a potent arylester hydrolase inhibitor and was replaced with MOPS. Very recently McIlvain et al. (1984) also found that phosphate inhibited the assay and they replaced phosphate with either borate or HEPES.

We also confirmed the work of McIlvain et al. (47) and Mackness and Walker (41) concerning the requirement for Ca^{2+}. In earlier stages of this work, an ammonium sulfate cut was included and without the presence of added Ca^{2+} the yields were drastically reduced. The yield at the PEG fractionation step is also enhanced if Ca^{2+} is added although it is not as critical at this point because the PEG cut occurs as the first purification step and Ca^{2+} is naturally present in serum. For high yields and to maintain activity, the presence of Ca^{2+} throughout the preparation is imperative. As further indication of the requirement for Ca^{2+}, the

addition of 5 mM EGTA completely abolished arylester hydrolase activity.

One of the frustrating problems in working with arylester hydrolase is its reported instability in the latter stages of purification (40,38, 48,36). Main (40) had some success in stabilizing the activity by freezing the enzyme in a weak bicarbonate solution. We have succeeded in stabilizing the activity by rapid dropwise freezing in liquid nitrogen, allowing the excess nitrogen to boil off, and storing the beads at -80°. As observed by Main, an immediate loss of activity occurs but the remaining activity is maintained; in our case for over a year. This method of preservation can be used at any stage in the preparation but is most effective when the total protein concentrations are high. Highly concentrated ammonium sulfate suspensions at 4° also retain activity for long periods of time (months). Inclusion of 0.02% sodium azide in all solutions greatly reduced the rapid loss of arylester hydrolase activity reported by others.

Main (40) and Mackness and Walker (41) have indicated that they believe that during purification a cofactor is lost. We have tried to add back fractions separated from those fractions containing arylester hydrolase activity and have had no success at restoring activity. The addition of 10 mg/mL BSA (to increase total protein concentration and to coat all glass surfaces) did not affect the activity but did slow the rate of inactivation. Because Walker and Mackness (41) indicated that arylester hydrolase activity is located in the HDL_2 fraction of sheep serum and may be an activity of one of the apo A proteins, the effect of phosphatidyl choline was examined. There was no effect on enzyme activity over the range of 20-100 μM phosphatidyl choline.

In a search for a natural physiological activity of arylester hydrolase, the enzyme was also tested for tyrosine phosphatase activity. Serine phosphatase and threonine phosphatase activities were used as controls. In none of the cases was any phosphatase activity found.

Physical Studies. Rabbit serum arylester hydrolase seems to differ in its physical properties from those reported by Main (40) for sheep serum arylester hydrolase. The rabbit protein does not show strong blue fluorescence reported by Main (40).

Reduced and alkylated SDS-PAGE of the enzyme after silver staining showed two closely spaced bands with molecular weights of 40,000-45,000 and 47,000-54,000 which directly correlated with arylester hydrolase activity. The results were the same under non-reducing conditions. On SDS-PAGE of the effluent of the AcA34 column, fractions containing these two bands are found to elute between peaks with estimated molecular weights of 150,000-165,000 and 170,000-190,000. The position of elution of arylester hydrolase activity from the AcA34 column gave an estimated molecular weight of 180,000-200,000. This information is consistent with native arylester hydrolase having an $\alpha_2\beta_2$ structure. Main (40) estimated the molecular weight of sheep serum arylester hydrolase to be between 35,000 and 50,000 daltons, based on a single, uncorrected sedimentation coefficient measurement of 3.69 Svedbergs. Walker and Mackness

(41) state that the molecular weight of the enzyme from sheep serum is greater than 200,000, based on its behavior on Sephadex G-200, but no K_d or standard curve was given. Zech and Zurcher (38) claim a molecular weight of 500,000 for rabbit serum arylester hydrolase, also based on its elution behavior on Sephadex G-200, but the K_d value of 0.063 reported is far too small to obtain a reliable molecular weight estimate.

Further Purification of Rabbit Serum Arylester Hydrolase

As indicated in the purification procedure above, despite the fact that a specific activity was obtained equalling or exceeding all others in the literature several protein bands are still evident on silver stained SDS-PAGE gels. This also results in ambiguity as to the molecular weight of the protein. Hydrophobic chromatography and chromatofocusing have been employed recently in attempts to improve the purification.

Hydrophobic Interaction Chromatography. Initial attempts were made using a phenyl-Sepharose column. The rationale for using this resin was the apparent requirement of the enzyme for an aromatic group. Material from a 50-75% ammonium sulfate fraction stored at -80°C was applied at room temperature to the column equilibrated previously with 50% saturated ammonium sulfate, buffered at pH 7.0 and containing 2.5 mM Ca^{2+}. The column was eluted as a linearly decreasing gradient to 10 mM MOPS, pH 7.0, containing 2.5 mM Ca^{2+}. This procedure did not release paraoxonase activity, apparently because of the enzymes affinity for the aromatic group.

Subsequently, material was applied to the column following equilibration in 100 mM NaCl, 10 mM MOPS, pH 7.0, containing 2.5 mM Ca^{2+}. This was sufficient to bind the paraoxonase to the column while allowing most of the protein of the sample to elute through unbound. This breakthrough material contained a protein with a deep red color, allowing one to monitor this progress visually. Elution from the column was attempted using a variety of steps of linear gradients (up to 80%) involving ethylene glycol, propylene glycol, or polyethyleneglycol-600 (all containing 2.5-5 mM Ca^{2+}). All produced a wash-off peak (or peaks) but all had tremendous adverse effects on the flow rate and on the optical properties of the gel. The only case in which reasonable amounts of activity were recovered was when 50% PEG-600 was used, then when the gel became transparent and the flow rate almost stopped, the system was washed with 10 mM MOPS, pH 7.0, containing 2.5 mM Ca^{2+}. The activity was found at the interface of the PEG-600 and the buffer. In a separate experiment paraoxonase activity was maintained far better in PEG-600 than in any of the other glycol solvents used. This technique possibly shows some promise but other alternatives needed to be explored.

Because the phenyl resin bound so tightly, hexane-agarose was tried. The enzyme did require the presence of high salt to absorb to the resin but activity could not be detected when eluted with a decreasing gradient of either ammonium sulfate or sodium sulfate. Fractions were added to other fractions in all possible binary combinations but this did not restore activity.

Hydrophobic interaction chromatography on hexane agarose is not worth pursuing. Chromatography on phenyl-Sepharose shows some promise but should be explored only if the combination of size exclusion and chromatofocusing still proves unsatisfactory.

Chromatofocusing. Initial attempts at chromatofocusing used a column of PBE 94 resin equilibrated with 25 mM imidazole, pH 6.2, containing 2.5 mM Ca^{2+}. The sample was from a DEAE-2 fraction that had been frozen at -80°C, thawed, and dialyzed against the above buffer. After sample application and an extensive wash with buffer, the column was eluted with polybuffer 74 diluted 1 to 10, containing 2.5 mM Ca^{2+} and adjusted to pH 4.0. Paraoxonase activity eluted between two not completely separated major peaks, with the activity overlapping the second peak heavily. A third peak was obtained when the column was washed with 1 M NaCl.

4-Nitrophenyl ethylphenylphosphinate was substituted for the paraoxonase assay. This had earlier been shown by Grothusen et al. (3) to have an even lower Km than paraoxon and gave almost no background.

Based on the initial pattern I used a narrower gradient, pH 5-4. While waiting for the proper cationic acid (piperazine) buffer to arrive, I used 25 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 5.5, with 5 mM Ca^{2+}. I failed to dialyze the paraoxonase sample against this buffer so the column pH did get re-adjusted after the sample was applied. After the sample was applied the column was washed with the above MES buffer and eluted with 100 ml of polybuffer 74 diluted 1 to 10, made 10 mM in Ca^{2+}, and adjusted to pH 4.0. The column was then washed with 1 M NaCl. Two well-separated activities were seen. The first peak was pooled and reapplied to a reequilibrated column and eluted in the same way.

The narrow pH range was again attempted when piperazine became available. The buffer for dialysis and column equilibration was 25 mM piperazine-HCl made 5 mM in Ca^{2+} and adjusted to pH 5.2, and the column was eluted as before. Active fractions again appeared between two prominent protein peaks. Because the activity did not clearly separate into two peaks, all the activity was pooled and put back on a reequilibrated column. The reequilibrated column was eluted with a 1 to 8 dilution of PBE 74 containing 5 mM Ca^{2+} and adjusted to pH 3.9. Two peaks were separated completely to baseline.

Chromatofocusing over the pH range 5-4 will aid tremendously in the last few steps of purification. This procedure should now be attempted with the active fraction from an AcA34 column, as described by Zimmerman and Brown (49). The fact that chromatofocusing can concentrate very dilute samples is a definite advantage to this combination.

Conclusion

This paper presents a relatively rapid purification of rabbit serum arylester hydrolase with good recovery of enzymatic activity. Crucial factors include the presence of Ca^{2+} at all steps and the inclusion of low levels of azide ion as a bacteriostat in all solutions. Activity at all stages can be maintained for long periods of time by rapid, dropwise freezing and storage at -80°C.

The enzyme characterized in this report appears to be substantially different than the arylester hydrolase purified by Main (40) from sheep serum. There are differences in estimated molecular weights as well as in fluorescence characteristics. It may be that the differences in separation procedures have resulted in the characterization of different isozymes. So little is known about the enzyme(s) that give arylester hydrolase activity that a true comparison must wait.

4b. Hydrolysis of Organophosphinates by Rabbit Serum Arylester Hydrolase

4b.i. Introduction

Arylester hydrolase (arylesterase, EC 3.1.1.2) is an enzyme which can detoxify the oxon metabolites of various organophosphorothioate insecticides, such as paraoxon. The enzyme was first studied by Mazur (50) and Aldridge (34). Mazur found that rabbit sera were capable of hydrolyzing paraoxon and diisopropylflurorphosphage (DFP). Aldridge demonstrated that the paraoxon hydrolyzing activity was enzymatic; that it was heat labile, pH dependent, and substrate concentration dependent. This enzyme has been referred to as paraoxonase, since paraoxon hydrolysis has been used to measure its activity, and also as phosphotriesterase because it does not hydrolyze monoesters of orthophosphoric acid (24). Paraoxonase has been purified from sheep serum by Main (40), but much of the work on paraoxonase specificity has been done with crude serum or tissue (46,38,36). Since there have been few purifications of arylesterase, its substrate specificity is unclear. A few organophosphonates were not hydrolyzed by rabbit serum (46).

We report hydrolysis of certain 4-nitrophenyl organophosphinates by arylesterase partially purified from rabbit serum. The study of the metabolism of phosphinates is very important because they are a class of organophosphorus compounds that do not undergo the "aging" reaction, and as such should not be capable of producing organophosphate-induced delayed neuropathy. These compounds provide transient protection against organophosphorus compounds that do cause delayed neuropathy (23). Phosphinates could also provide prophylaxis against acute poisoning by phosphates and phosphonates.

4b.ii. Materials and Methods

Chemicals. Organophosphinates (Table 1) were provided by the U.S. Army Medical Research Institute of Chemical Defense (Aberdeen Proving Ground, MD) following synthesis by Ash-Stevens, Incorporated (Detroit,

MI) (12,11). Paraoxon, MOPS 1- and 2-naphthyl acetate, 4-nitrophenylacetate and 4-nitrophenylbutyrate were from Sigma Chemical Company (St. Louis, MO), methyl paraoxon was from the U.S. Environmental Protection Agency, and parathion and methyl parathion were from Monsanto Agricultural Products Company. Phenylthioacetate, phenylthiopropionate and phenylthiobutyrate were generously provided by Dr. A. R. Main, North Carolina State University. Ethanethiol and butyryl chloride were purchased from Aldrich Chemical Co. (Milwaukee, WI). Ethylthioacetate and ethylthiopropionate were from Fairfield Chemical Co. (Blythewood, SC) and were distilled before use. Bio-Rad protein assay reagent was purchased from Bio-Rad Laboratories (Richmond, CA).

Handling of Organophosphinates. The organophosphinates have mammalian toxicities comparable to paraoxon and were handled accordingly. The LD_{50} values for some of the phosphinates in rats are published (11).

Synthesis of Ethylthiobutyrate. Ethylthiobutyrate was synthesized by an adaptation of procedures described by Booth and Metcalf (51). Butyryl chloride and ethanethiol were added in a 1.5:1 molar ratio and refluxed for 2 hours. The reaction mixture was cleaned up by distillation. Material which distilled at 115°C and lower was discarded. The material which distilled between about 135°C and 143°C was collected and then run through a 1 x 4.5 cm activated silicic acid column with methylene chloride as the solvent. The methylene chloride was removed from the sample by nitrogen stream. Purity was checked by thin layer chromatography on silica gel using hexane ethyl acetate (60:40) as the developing solvent. The silica gel plates had a fluorescent dye (Silica Gel 60 F254, E. Merck, Darmstadt Germany) which enabled visualization of the spots under ultraviolet light. A sample of the ethylthiobutyrate was checked for reaction with DTNB, prior to and after being treated with 1 M NaOH, to confirm that the sample was in fact a thioester.

Preparation of Arylester Hydrolase. The arylester hydrolase used for this study was purified 25-fold from rabbit serum. The purification involves two PEG 4000 fractionations and two DEAE-Sepharose column procedures. The 14-25% PEG-4000 fraction of the serum is resuspended in 10 mM MOPS with 2.5 mM $CaCl_2$ (pH 7.0) and applied to a DEAE-Sepharose column. A linear gradient, 0-400 mM NaCl in MOPS buffer, was run and active fractions were pooled and dialyzed before being applied to a second DEAE column. The active fractions from the second DEAE column were pooled and frozen dropwise in liquid nitrogen and stored at -60°C. Details of the complete purification procedure are available (49).

Determination of Enzyme Activity. Organophosphinates were assessed as substrates for paraoxonase in 1.2 ml of 0.1 M MOPS buffer (pH 7.5 with 2.5 mM calcium chloride) containing 10 µl of the organophosphinate in acetonitrile. Absorbance at 405 nm was measured at 22-24°C on a Bausch and Lomb Spectronic 2000 uv/vis spectrophotometer against a blank lacking only the phosphinate and recorded on an X-Y recorder to obtain a rate of spontaneous hydrolysis. Then 25 µl enzyme solution was added and the hydrolysis was determined. Replicate determinations were performed on separate days, each with 5 substrate concentrations. Replicates were

done with at least 2 different enzyme preparations. Non-enzymatic hydrolysis was determined also by adding 25 µl of the same enzyme solution from an aliquot which had been denatured by holding in a sealed plastic microcentrifuge tube in a steam bath for 10 min.

Velocities of hydrolysis (v) at the various substrate concentrations ([s]) were corrected for non-enzymatic hydrolysis at each concentration in which the non-enzymatic rate exceeded 10% of total hydrolysis. Michaelis constants were obtained from computer analysis of Woolf-Augustinsson-Hofstee (52) plots of v versus v/[s]. The results are presented in Table 12.

Specific activities were measured with high substrate concentrations and diluted enzyme samples. To insure saturation kinetics, assays were done at half the substrate concentration as that used for the Vmax assay. If no difference in rate was observed, then it was assumed that the higher substrate concentration was saturating. The values were corrected for non-enzymatic hydrolysis as described above. Protein concentration was measured using the Bio-Rad Protein Assay with gamma globulin as the standard.

Enzyme assays for ethyl and methyl parathion and for 4-nitrophenylacetate and 4-nitrophenylbutyrate were done the same as the saturating substrate assays for the phosphinates. The liberation of 4-nitrophenol in each case was followed continuously at 405 nm at 22-24°C.

Assays for activity against *ethylthio and phenylthio* esters were done using Ellmans reagent (53). The assay mixture consisted of 1 ml of MOPS buffer (pH 7.5) (54), 1 ml of DTNB in MOPS buffer (15.9 mg/100 ml), 20 µl of substrate in acetonitrile or acetone and 20 µl of enzyme solution. The reactions were followed contiuously at 405 nm at 22-24°C.

Polyacrylamide Gel Electrophoresis. Native slab gel electrophoresis was run on 5% acrylamide gels, 1.5 mm thick, made with 0.37 M Tris-HCl (pH 8.8) with a 3% stacking gel made with 0.12 M Tris-HCl (pH 6.8). The gels were run at 10°C in a 5 mM Tris, 38 mM glycine buffer (pH 9.0) with a constant current of 35 mA per slab gel. The gels were stained with 1- or 2-naphthyl acetate using a modification of the procedure of Saul et al. (55), in which MOPS buffer, 0.1 M MOPS, 2.5 mM $CaCl_2$, pH 7.5 is used.

While 10 of 13 4-nitrophenyl organophosphinates tested were substrates of rabbit serum arylester hydrolase (3), IPP, DPP and TCMPP 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, IPP and DPP could be tested as inhibitors in mixtures with paraoxon since the product could originate only from the substrate.

Arylester hydrolase was purified 25-fold from rabbit serum following the procedure described in Section 4 through the second DEAE-Sepharose chromatography step. The assays were carried out in 2 ml of 0.1M 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 from substrate 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 EPP as substrate. Substrate concentrations were near Michaelis constants which were 0.609 mM for paraoxon and 0.285 mM for EPP (3).

4b.iii. Results and Discussion

The results of K_M determinations for the organophosphinates are presented in Table 12. The values span a wide range but all are lower than that for ethyl paraoxon. In general, the K_M increases with increasing size of the second substituent within a group and with the retention times of these phosphinates on octasilyl-bonded silica. The retention times listed are from Brown and Grothusen (16) and are in excellent agreement with octanol-water partition values for these phosphinates (C. N. Lieske, personal communication). The only phosphinates tested that did not show measurable enzymatic or spontaneous hydrolysis were IPP, DPP and TCMPP. Although the K_M is often used as a measure of substrate suitability or of substrate affinity, it does not necessarily give information on how good a substrate is. A substrate with a low K_M could also have a low Vmax and, as such, could be considered a competitive inhibitor. The results of saturating substrate assays for a number of the compounds are presented in Table 12. These values for specific activity (µmoles/min/mg protein) indicate that these organophosphinates are good substrates for this enzyme preparation, since the Michaelis constants are lower and the specific activities are higher than paraoxon.

Additional proof that the enzyme preparation is an A esterase is given in Table 13. The specific activities for a number of other compounds studied are presented. Aldridge and Reiner (24) state that for A esterases, acetate esters are hydrolyzed faster than butyrate esters. In all three groups of esters, the specific activity against the acetate compound is greater than that of the butyrate. It should also be noted that the phenyl compounds have higher specific activities than the ethyl compounds.

Incubation of the enzyme for 40 minutes at 25°C in the presence of 0.18 mM p-chloromercuribenzoic acid inhibited more than 90% of the paraoxonase activity and more than 92% of the 4-nitrophenyl(ethyl)phenyl phosphinate hydrolysis. This is further evidence that the enzyme preparation is arylester hydrolase since Aldridge and Reiner (24) state that A esterases unlike B esterases are -SH enzymes and as such are inhibited by mercurials or other reagents capable of forming mercaptides. The inhibition of phosphinate hydrolysis by the p-chloromercuribenzoic acid is further evidence that phosphinates are hydrolyzed by paraoxonase.

Table 12. Michaelis constants and specific activities of 4-nitrophenyl organophosphinates, paraoxon, and methyl paraoxon with arylester hydrolase from rabbit serum.

Abbrev-iation	Substituents	Rt[a]	K_M, mM±s.e. (n)	Specific activity µmoles/min/mg
		Phenyl series		
MPP	methyl(phenyl)	3.85	0.0750 ± 0.047 (4)	2.9
EPP	ethyl(phenyl)	5.05	0.285 ± 0.048 (4)	0.64
IPP	isopropyl(phenyl)	6.58	b (2)	0.0025
DPP	diphenyl	9.68	b (2)	0.0025
		Methyl series		
MFuP	methyl(2-furyl)	3.12	0.0210 ± 0.014[c] (2)	1.4
MTP	methyl(2-thienyl)	3.68	0.0309 ± 0.0054 (4)	2.5
MPP	methyl(phenyl)	3.85	0.0750 ± 0.047 (4)	2.9
MNP	methyl(2-naphthyl)	7.31	0.124 ± 0.0621 (4)	0.19
		Heterocycle series		
MFuP	methyl(2-furyl)	3.12	0.0210 ± 0.014[c] (2)	1.4
MTP	methyl(2-thienyl)	3.68	0.0309 ± 0.0054 (4)	2.5
DTP	di-2-thienyl	7.67	0.0744 ± 0.0134 (4)	0.039
		Halogen series		
bCMP	bis chloromethyl	3.83	0.434 ± 0.104 (4)	nd[d]
CMPP	monochloromethyl(phenyl)	5.74	0.0567 ± 0.0082 (4)	1.6
DCMPP	dichloromethyl(phenyl)	8.89	0.167 ± 0.011 (4)	0.33
TCMPP	trichloromethyl(phenyl)	15.87	b (2)	0.0025
TFPMP	methyl-(trifluoromethylphenyl)	9.13	0.123 ± 0.039 (3)	1.5
	paraoxon	5.1	0.609 ± 0.023 (17)	0.056
	methyl paraoxon	nd	nd	0.036

[a]Retention times on octasilyl bonded column from Brown and Grothusen (16) corrected for dead volume.

[b]Enzymatic hydrolysis was below detection.

[c]Corrected for spontaneous hydrolysis.

[d]nd; not determined

Table 13. Specific activities for ester compounds with arylester hydrolase from rabbit serum.

	specific activity μmoles/min/mg protein
ethylthioacetate	0.012
ethylthioproprionate	0.010
ethylthiobutyrate	0.0039
phenylthioacetate	6.1
phenylthioproprionate	0.71
phenylthiobutyrate	0.22
4-nitrophenylacetate	1.2
4-nitrophenylbutyrate	0.062
ethyl parathion	0.002
methyl parathion	0.002

Native polyacrylamide gel electrophoresis was run on the enzyme preparations. Gels stained with 2-naphthyl acetate showed one red staining esterase band only, and gels stained with 1 naphthyl acetate showed no esterase band.

This is the first known report of hydrolysis of phosphinates by arylester hydrolase. A goal of the work presented here is the development of structure function relationships for this important detoxicating enzyme. We are currently extending this study to examine stereoselectivity of hydrolysis by using Pirkle chiral stationary phase HPLC columns.

Arylester hydrolase was partially inhibited by IPP at 8.69×10^{-5}M and by DPP at 4.35×10^{-5}M, respectively (Table 14). More concentrated solutions containing these inhibitors and substrate could not be made. The greatest inhibition of paraoxon hydrolysis observed was 42.5% when IPP was present at approximately one-fourth the concentration of paraoxon. Least inhibition observed was 6.7% when IPP was present at about one-tenth the concentration of paraxon. Inhibition of arylester hydrolase by DPP was observed when either paraoxon or EPP was the substrate (Table 14).

While arylester hydrolase was partially inhibited by IPP and DPP at 4.35×10^{-5} M (Table 14), this enzyme was much less sensitive to these compounds than carboxylester hydrolase, in which 70% inhibition was observed upon 1 min exposure to DPP at 4×10^{-8} M concentration (8). At the highest substrate concentrations tested, there was not more than 22.7% inhibition by either phosphinate. Data with EPP (Table 14) confirmed our previous report that certain organophosphinates were excellent

Table 14. Inhibition of rabbit serum arylester hydrolase by organophosphinates.

Inhibitor, mM	Substrate, mM	Activity, nmol/min/mg ±s.e. control	inhibited	% inhibited
IPP[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
DPP[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
DPP[c]	EPP[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

substrates for arylester hydrolase (3).

The degree of inhibition was inversely related to the concentration of substrate present. This would be expected in the case of a competitive mechanism of inhibition. It is possible that IPP and DPP were competitive inhibitors acting by occupation of the active site of the enzyme; their structural similarity to EPP and to other organophosphinates which were substrates (3) 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 usually 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.

5. Stereoselectivity of Acetylcholinesterase and other Hydrolyses toward Organophosphinates

5a. Introduction

Organophosphorus enzyme inhibitors possessing chirality at a phosphorus center often react stereoselectively with acetylcholinesterase (56,57) and with other hydrolases (58,23). Stereoselectivity has been examined most often in organophosphonate derivatives which include nerve agents such as soman (59) and organophosphonothioate insecticides such as ethyl 4-nitrophenyl phenylphosphonate, EPN (60,61). S-propyl phosphorothioate insecticides also exhibit stereoselective reactions with hydrolases (62,63).

This study was performed to determine the stereoselectivity of several hydrolases toward organophosphinates, since this class of compounds had not been investigated previously in this regard. Organophosphinates are monoesters containing 2 direct phosphorus-to-carbon bonds and many are rapid but transient inhibitors of acetylcholinesterase (EC 3.1.1.7) (11) and carboxylesterase (EC 3.1.1.1) (7).

We have applied chiral-phase HPLC techniques (16) to this investigation to isolate organophosphinate enantiomers directly from racemic mixtures for inhibition of acetylcholinesterases and -chymotrypsin (EC 3.4.21.1). Previous studies have relied upon relatively complex stereospecific synthesis to obtain the optically pure enantiomers or, in one case, upon the stereospecific, enzymatic degradation of one enantiomer followed by clean-up of the remaining enantiomer (59). We have employed chiral-phase HPLC also for analysis of reaction products after the racemic mixture was exposed to arylester hydrolase (EC 3.1.1.2) or proteinases; chiral-phase capillary gas-liquid chromatography has been used for this purpose by others (64).

5b. Materials and Methods

Preparation and analysis of phosphinate enantiomers. Racemic mixtures of EPP, IPP, DCMPP and TCMPP were provided by the U.S. Army Medical Research Institute of Chemical Defense. Their formulas and chemical syntheses have been described (12,11). Enantiomers were partially resolved by chiral-phase HPLC and the isolation of pure enantiomers was accomplished as described previously (16) with the following changes in the method: all solvents were HPLC grade (Burdick and Jackson Laboratories, Muskegon, MI) and columns contained either the D or L form of 3,5-dinitrobenzoylphenylglycine (DNBPG) covalently bonded to 5 μm aminosilica particles (Regis Chemical Company, Morton Grove, IL).

Mobile phase was 7% 2-propanol in n-hexane and, upon collection of peaks, it was removed by evaporation with a stream of nitrogen at 40°C and replaced with acetonitrile. For EPP, DCMPP and TCMPP, 0.1% glacial acetic acid was added to the mobile phase.

Each enantiomer was obtained from the respective column at 95% purity by collecting the lesser retained peak to its apex. Collection of the greater retained peak after its apex gave the opposite enantiomer at approximately 80% purity; these less pure samples were used only in confirmatory experiments. Quantitation of collected enantiomers was

accomplished by octylsilyl reversed-phased HPLC versus standard curves (16). Optical rotatory dispersion spectra of IPP enantiomers revealed that the lesser retained peak from the L-DNBPG column was P(-) and the lesser retained peak from the D-DNBPG column was P(+); spectral crossover occurred at 275 nm (Broomfield). The lesser retained peak of EPP from the D-DNBPG column was also P(+).

Inhibition of acetylcholinesterase. Electric eel acetylcholinesterase (Type V-S, Sigma Chemical Company, St. Louis, MO) or bovine erythrocyte acetylcholinesterase (Type XII-S, Sigma Chemical Company) solutions at 2 units/ml in 0.10 M MOPS buffer, pH 7.5, were exposed to EPP or IPP enantiomers added in acetonitrile (1% of final volume). Mixtures were held in a shaking water bath at 24°C for the eel enzyme or 37°C for the bovine enzyme and aliquots were removed at intervals up to 120 min for spectrophotometric measurement of remaining hydrolytic activity against acetylthiocholine using a Spectronic 2000 with X-Y recorder to monitor the continuous formation of product at 412 nm (26). The bimolecular reaction constant (k_i) was determined for each mixture (28). Two separate preparations of enantiomers were used, each on 1 or 2 days.

Inhibition of chymotrypsin. Bovine pancreatic α-chymotrypsin (Type II, Sigma Chemical Company) solution at 2 units/ml in 0.10 M MOPS, 2.5 mM calcium chloride, and 0.002% sodium azide, pH 7.5, was exposed to phosphinate enantiomers added in acetonitrile (0.3% final volume). The mixture was held at 37°C in a shaker bath and aliquots were removed at intervals up to 95 min for measuring remaining activity against N-benzoyl-L-tyrosine ethyl ester by the continuous spectrophotometric monitoring of product at 256 nm (65) with the instrument described above. The k_i for each mixture was computed (28) and each experiment was replicated on four days; two preparations of enantiomers were used, each in one or two replicates.

Reaction of racemic phosphinate with proteinases. Bovine α-chymotrypsin, bovine pancreatic trypsin (EC 3.4.21.4, Type III-S, Sigma Chemical Company) protease (EC 3.4.21.14, Type VII, subtilisin BPM' Sigma), or porcine intestinal mucosa peptidase (Sigma) at 0.34 mM was dissolved in 0.1 M MOPS, 2.5 mM calcium chloride, and 0.002% sodium azide, pH 7.5. Racemic phosphinate in acetonitrile was placed in a tube and the solvent was removed under nitrogen to near dryness. Proteinase solution was added and the final concentration of phosphinate was 0.34 mM for EPP or IPP, 0.45 mM for DCMPP and 0.55 mM for TCMPP. The mixture was held in a shaker bath at 37°C and aliquots were removed at intervals for analysis.

Aliquots were mixed with two volumes of 50% acetonitrile in acetone to precipitate the enzyme which was removed by centrifugation at 9220 x g for 20 min at 4°C. Solvents were removed from the supernatant by rotating evaporator and the remaining solution was extracted three times with dichloromethane. The extract was evaporated to near dryness and dissolved in acetonitrile, from which chiral-phase HPLC analysis was performed as described above. The experiments were replicated four times on separate days.

Inhibition of carboxylester hydrolase. Rabbit liver monomeric carboxylester hydrolase (prepared by A. R. Main) and porcine liver oligomeric carboxylester hydrolase (Sigma) in 0.10M MOPS buffer, pH 7.5, were exposed to purified enantiomers of EPP added in acetonitrile. At 1.0-min intervals to 5.0 min, aliquots were taken and the enzymatic activity remaining was determined against 1-naphthyl butyrate (7; vide Section 3b). There were three replicates with the rabbit enzyme and two replicates with the porcine enzyme.

Hydrolysis catalyzed by arylester hydrolase. Rabbit serum arylester hydrolase (EC 3.1.1.2) was purified 25-fold by fractional precipitation and Sepharose chromatography described elsewhere (49). At 24°C, 0.027 units (determined with paraoxon as substrate) of arylester hydrolase in 0.10 M MOPS, 2.5 mM calcium chloride, and 0.002% sodium azide, pH 7.5, were incubated with 7.5 mM racemic EPP added in acetonitrile (1% final volume). At intervals up to 2.5 h, 0.1 ml aliquots were transferred into 1 ml 0.1% glacial acetic acid in ethyl acetate, which then was partitioned against 1 ml water. Ethyl acetate layers of two extractions were collected, dried by a stream of nitrogen, and dissolved in 0.1 ml acetonitrile for chiral phase HPLC analysis as described above. The experiment was repeated on each of four days.

Activities of arylester hydrolase toward the resolved enantiomers of EPP were determined by monitoring the continuous production of 4-nitrophenol spectrophotometrically at 405 nm at 24°C (3). These assays were performed in the buffer described above with each enantiomer added in acetonitrile (2% final volume); two preparations of enantiomers were used, each in two replicates.

5c. Results

Acetylcholinesterase was inhibited stereoselectively by both EPP and IPP; P(+) EPP and P(+) IPP were more inhibitory than their opposite enantiomers in both eel and bovine forms of this enzyme (Table 15). Stereospecificity, as expressed by r_a, was four-fold greater for EPP than IPP, and P(+)EPP was 10^4-fold more active than P(+)IPP when bimolecular reaction constants were compared.

Stereoselectivity of inhibition of α-chymotrypsin was reversed from that of acetylcholinesterases (Table 15). This proteinase was inhibited more rapidly by P(-)EPP and P(-)IPP than by their opposite enantiomers. However, stereospecificity was not as pronounced in α-chymotrypsin as it was in acetylcholinesterase. Results with IPP in the three enzymes were consistent regardless of which column was used to prepare the enantiomers.

Selective loss of P(-)IPP was observed upon incubation of racemic IPP with α-chymotrypsin, thereby confirming that this was the more inhibitory enantiomer (Fig. 10). Less selectivity was found with trypsin, although P(-)IPP was again lost more rapidly than P(+)IPP (Fig. 11). These chromatograms were typical results of four independent replications analyzed on the D-DNBPG column. A fifth replication analyzed on the opposite (L-DNBPG) column indicated the same result, and the resolution

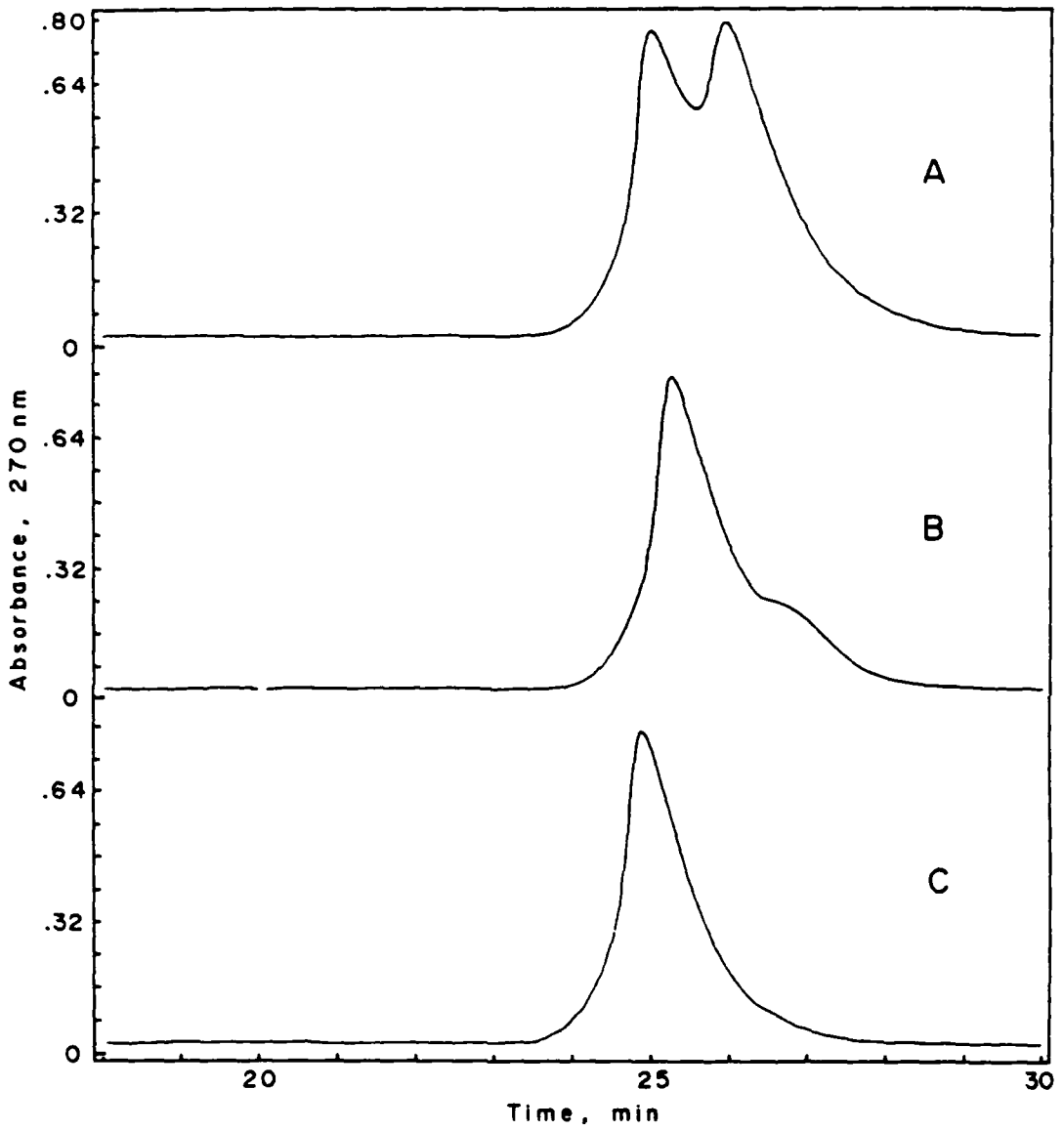


Figure 10. Stereoselective reaction of 0.34 mM racemic 4-nitrophenyl isopropyl-(phenyl)phosphinate with 0.34 mM α-chymotrypsin in 0.1 M MOPS buffer, pH 7.5, at 37°C as monitored by D-DNBPG chiral-phase HPLC of extracts. (A) control chromatogram of IPP in buffer without enzyme for 40 min. (B) chromatogram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

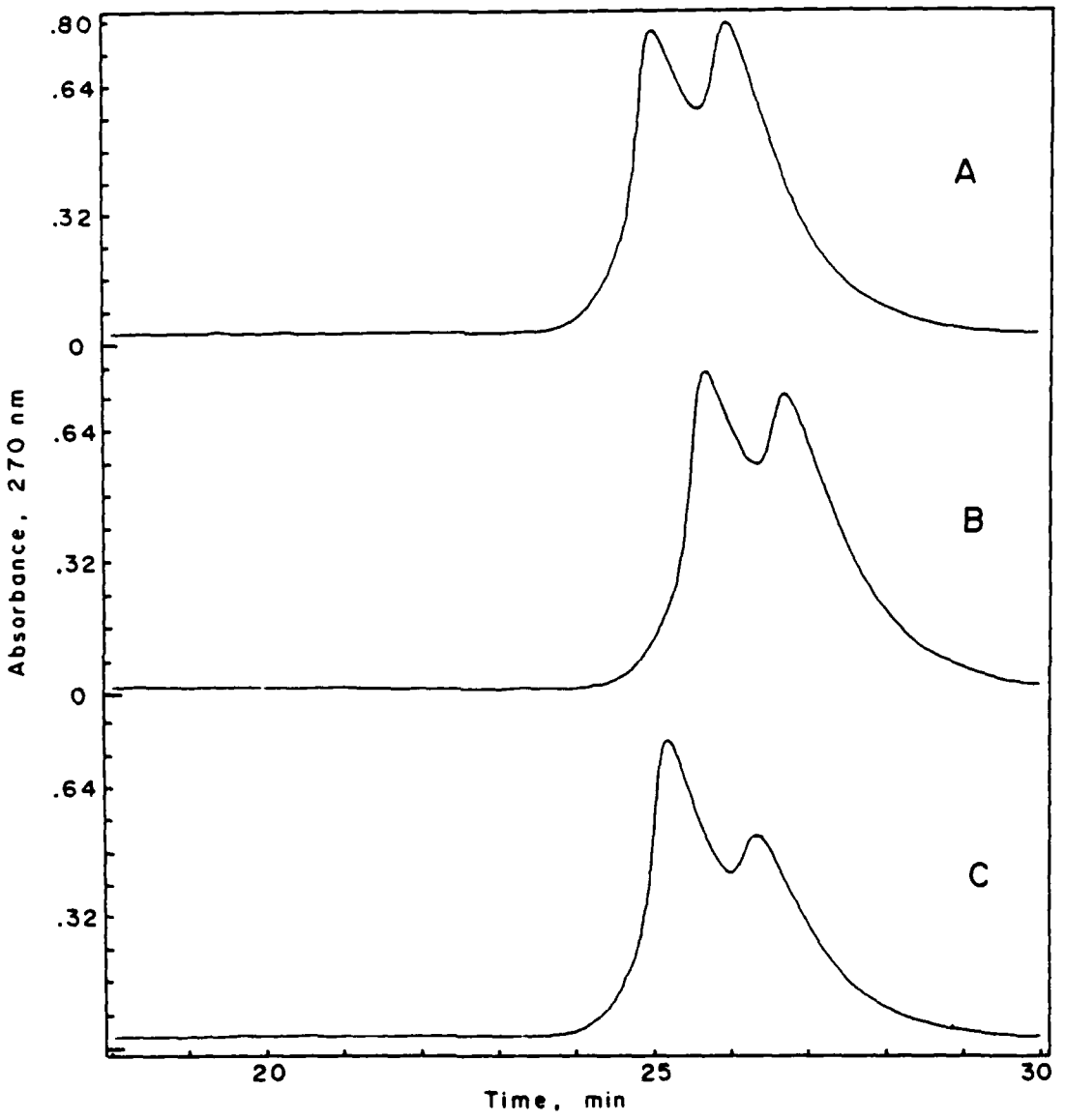


Figure 11. Stereoselective reaction of 0.34 mM racemic 4-nitrophenyl isopropyl(phenyl)phosphinate with 0.34 mM trypsin in 0.1 M MOPS buffer, pH 7.5, at 37°C as monitored by D-DNBPG chiral-phase HPLC of extracts. (A) control chromatogram of IPP in buffer without enzyme for 40 min. (B) chromatogram of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

of enantiomers (which appeared in the reverse order of elution) was improved slightly.

Protease also was stereoselective reacting with (-)IPP when exposed to the racemic IPP (Fig. 12). Inclusion of 0.1% glacial acetic acid in the HPLC mobile phase in this experiment with IPP resulted in sharper peaks and the detection of 4-nitrophenol produced in the reaction. This acidification was routinely used with EPP, DCMPP and TCMPP analyses, but it was only included in the IPP analysis in this single case. A preliminary experiment with peptidase versus alkaline phosphatase resulted in slight loss of (-)IPP from the racemic mixture with alkaline phosphatase, but only after 40 min; there was no stereoselective reaction detectable with peptidase (Fig. 13).

Two halogen series compounds were investigated also with chymotrypsin. While methyl series compounds could not be analyzed for stereoselective reaction, DCMPP and TCMPP were substituted as chlorinated methyl analogues. With racemic DCMPP, there was a stereoselective loss of (-)DCMPP from the mixture (Figs. 14 and 15). Unlike the phenyl series, this stereoselective loss did not go to completion but appeared to occur only in the initial reaction since there was no additional stereoselective loss during the 8 min to 40 min interval. With TCMPP, there was no detectable spontaneous hydrolysis and there was a detectable reaction with chymotrypsin producing 4-nitrophenol (Figs. 16 and 17); however, this reaction was not stereoselective.

Stereoselectivity of inhibition of carboxylester hydrolase was slight and it was more pronounced for the rabbit enzyme than it was for the porcine enzyme (Table 16). Once again, stereoselectivity was opposite that of acetylcholinesterase with (-)EPP exhibiting slightly faster inhibition than (+)EPP. Slower inhibition by the racemic EPP than by the less inhibitory enantiomer was observed for carboxylesterase only. This suggests a possible antagonistic interaction of the enantiomers in the mixture.

Arylester hydrolase from rabbit serum catalyzed hydrolysis of P(-) EPP with nine-fold greater activity than observed for P(+)EPP (Table 17). Again, this stereoselectivity was reversed from that observed in acetylcholinesterase inhibition (Table 15).

Confirmation of stereoselective hydrolysis of EPP was found when P(-)EPP disappeared more rapidly from the racemic mixture incubated with arylester hydrolase (Fig. 18). These experiments were performed with EPP only since IPP is not a substrate for arylester hydrolase (3).

Arylester hydrolase was also stereoselective toward the halogen series compound DCMPP; the lesser retained peak from the L-DNBPG column was hydrolyzed slightly faster than the opposite enantiomer at 37°C (Fig. 19).

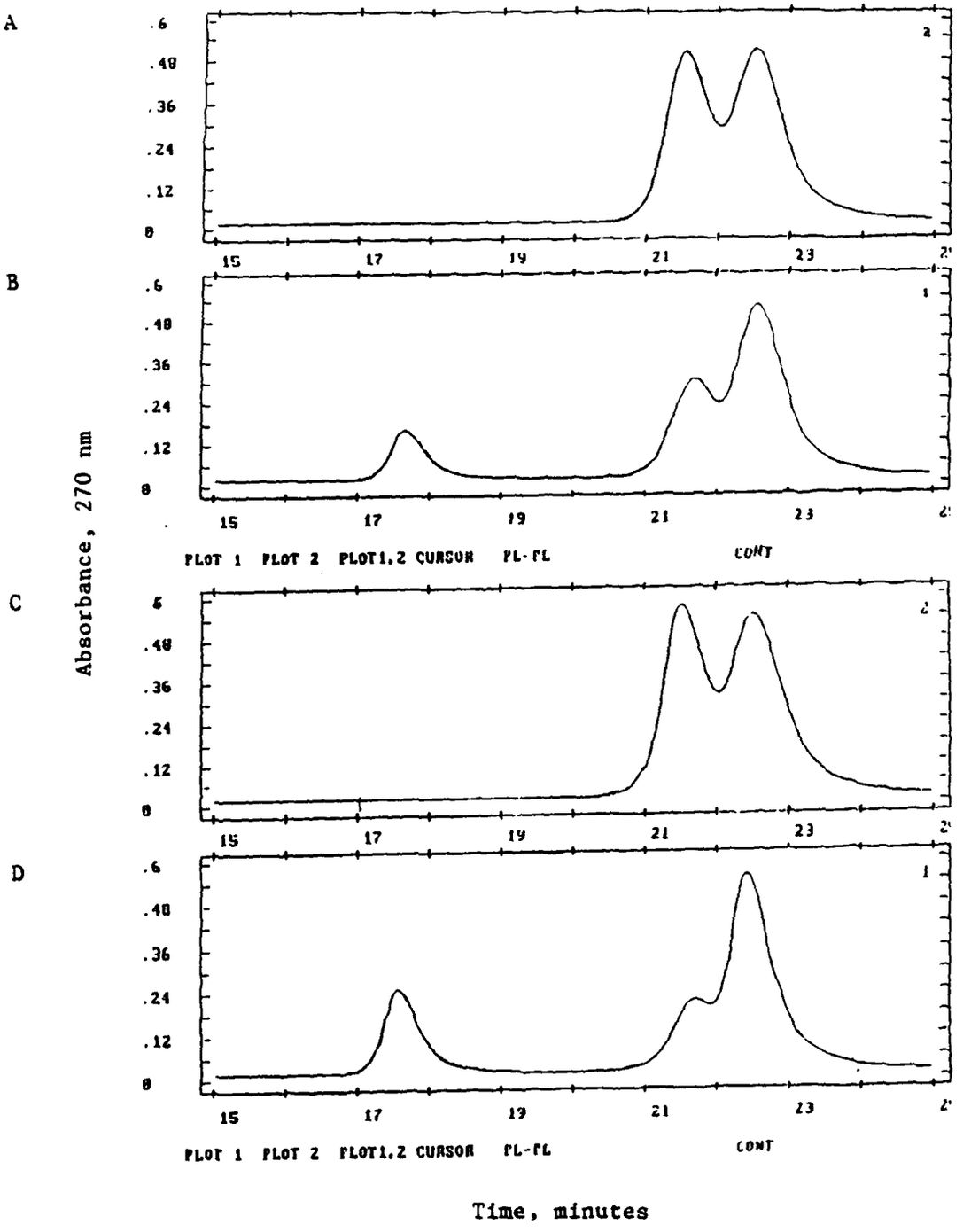


Figure 12. Stereoselective reaction of 0.34 mM racemic 4-nitrophenyl isopropyl (phenyl)phosphinate with 0.34 mM protease (subtilisin) in 0.1 M MOPS buffer, pH 7.5, at 37°C as monitored by L-DNBPG chiral-phase HPLC of extracts. (A) chromatogram of IPP in buffer for 8 min. (B) chromatogram of IPP with protease for 8 min. (C) chromatogram of IPP in buffer for 40 min. (D) chromatogram of IPP in protease for 40 min.

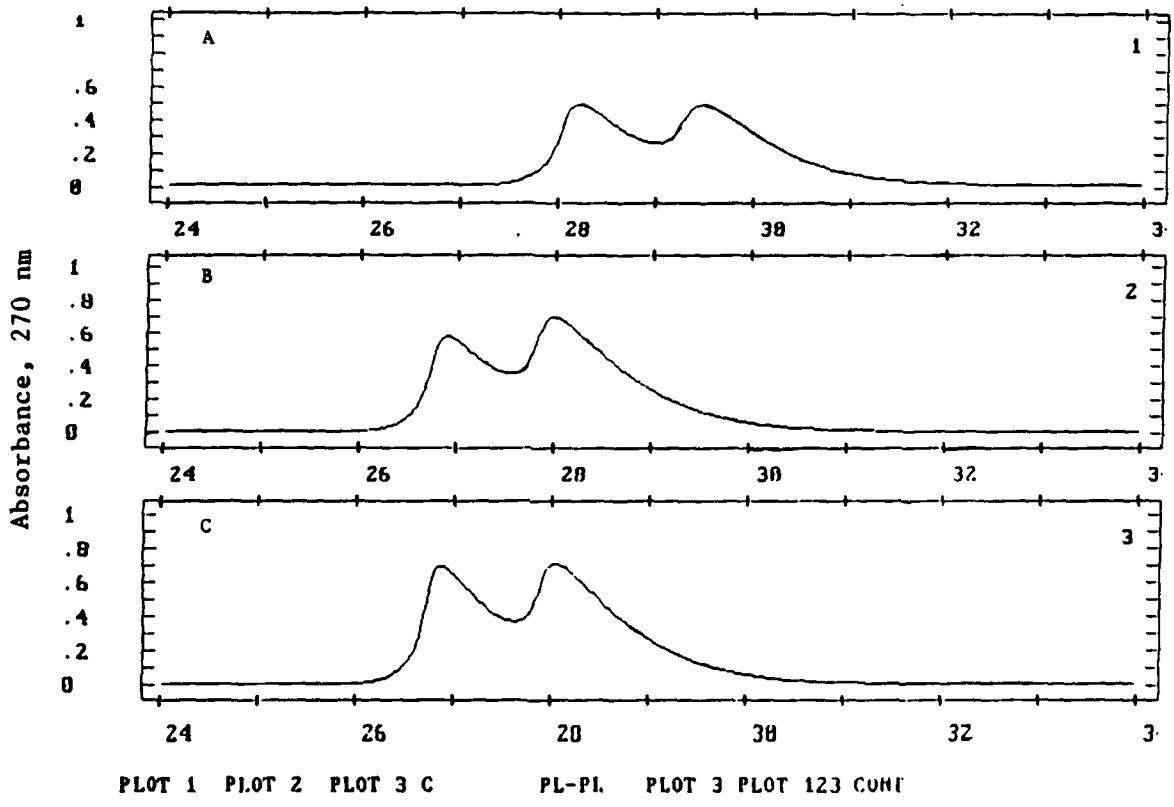


Figure 13. Reaction of racemic 4-nitrophenyl isopropyl(phenyl)phosphinate in 0.1 M MOPS buffer, pH 7.5, at 37°C. (A) in buffer alone, (B) with acid phosphatase for 40 min. and (C) with peptidase for 40 min. as monitored by L-phenylglycine chiral-phase HPLC of extracts.

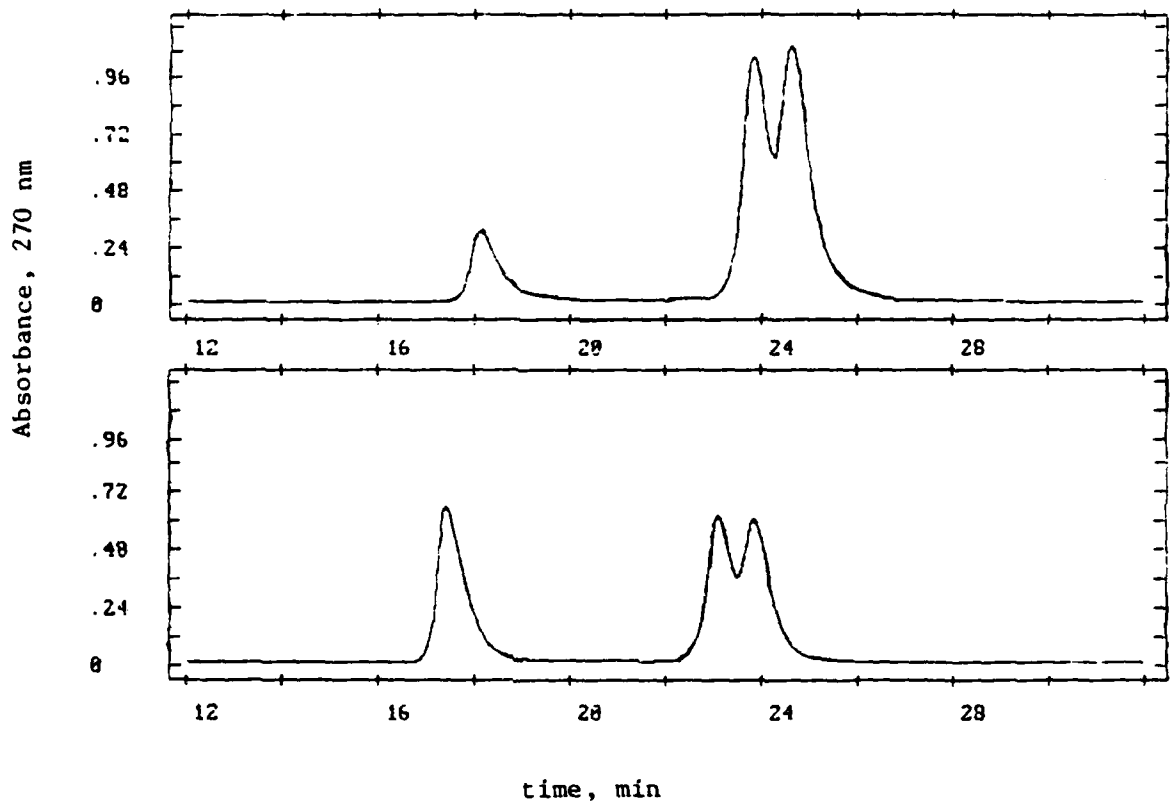


Figure 14. Spontaneous hydrolysis of 4-nitrophenyl dichloromethyl(phenyl) phosphinate in MOPS buffer. The top panel is the chromatogram on the L-phenylglycine column of the extracted sample after 8 min incubation at 37°C in MOPS buffer. The bottom panel is the extracted sample after 40 min incubation. The absorbance at 270 nm was monitored. The DCMPP peaks are at 23-25 min retention. The peak at 18 min is 4-nitrophenol.

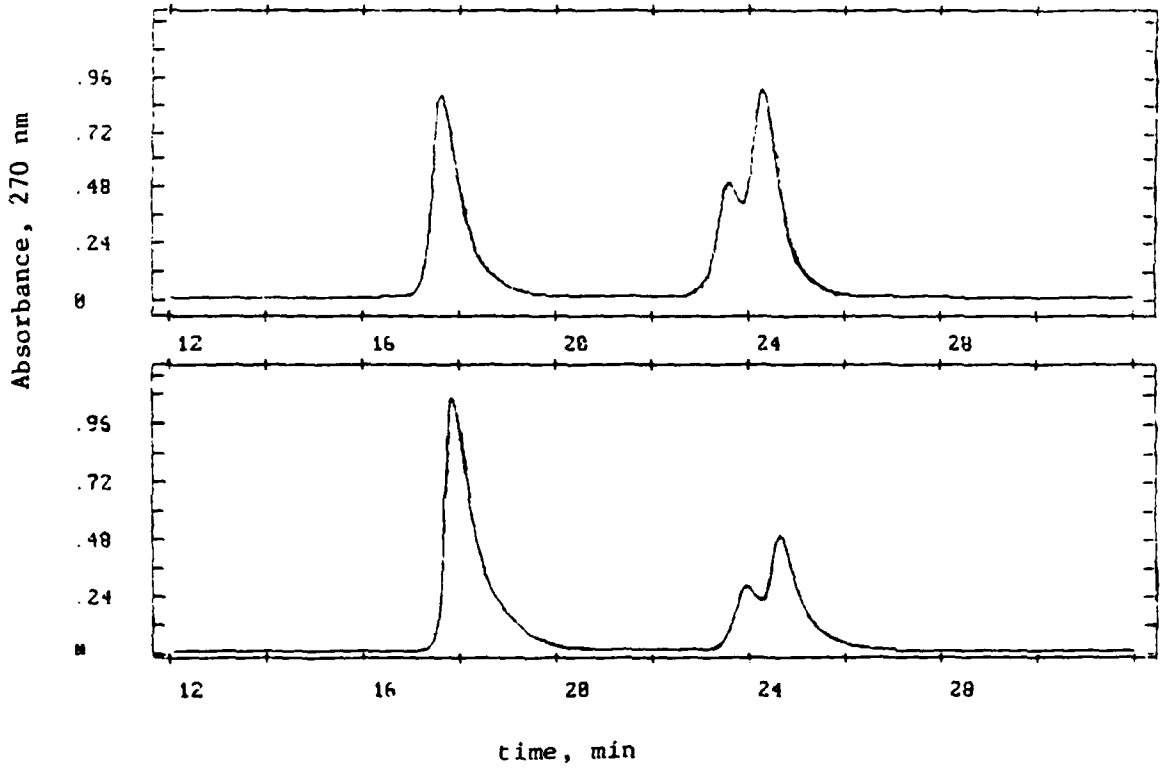
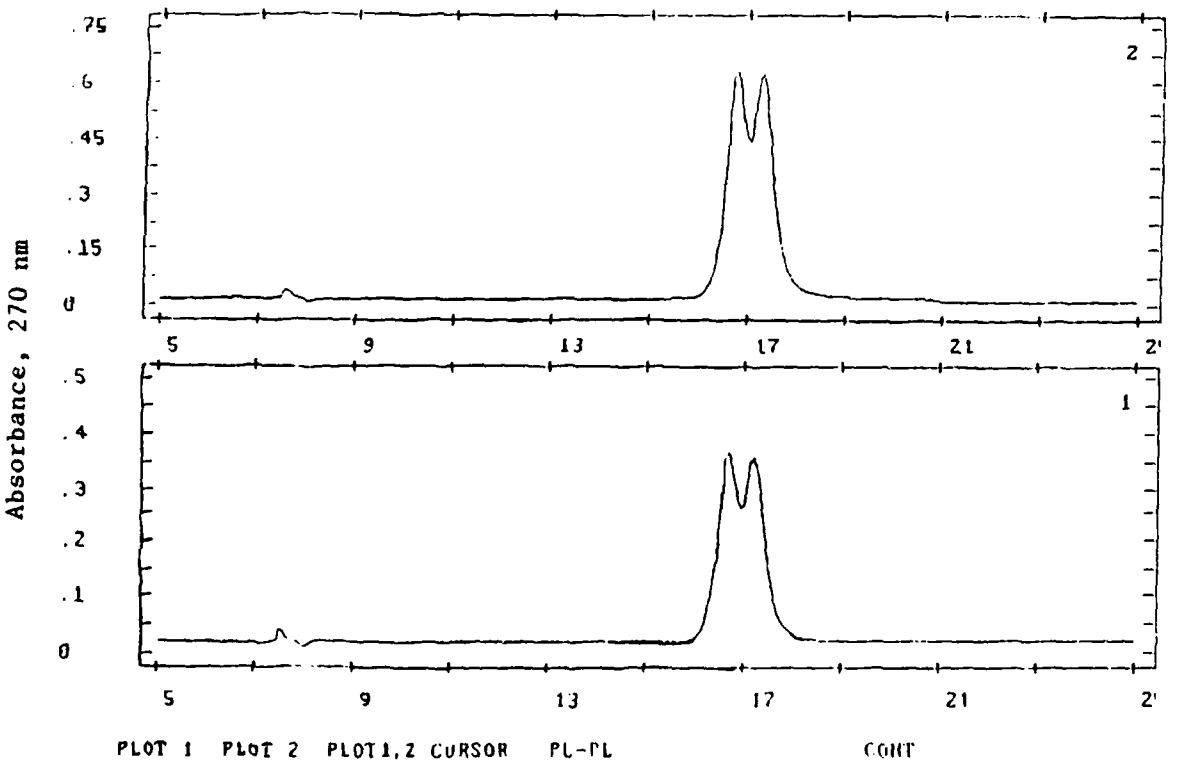


Figure 15. Hydrolysis of 4-nitrophenyl dichloromethylphenyl phosphinate in the presence of chymotrypsin. The top panel is the chromatogram on the L-phenylglycine column of the extracted DCMPP after 8 min incubation at 37°C. The bottom panel is the extracted sample after 40 min incubation at 37°C. The absorbance at 270 nm was monitored. The peak at 18 min is 4-nitrophenol.



Time, minutes

Figure 16. Spontaneous hydrolysis of 4-nitrophenyl trichloromethyl(phenyl)-phosphinate in MOPS buffer. Top panel is extract after 8 min and bottom panel is extract after 40 min; the L-DNBPG chiral-phase column was used.

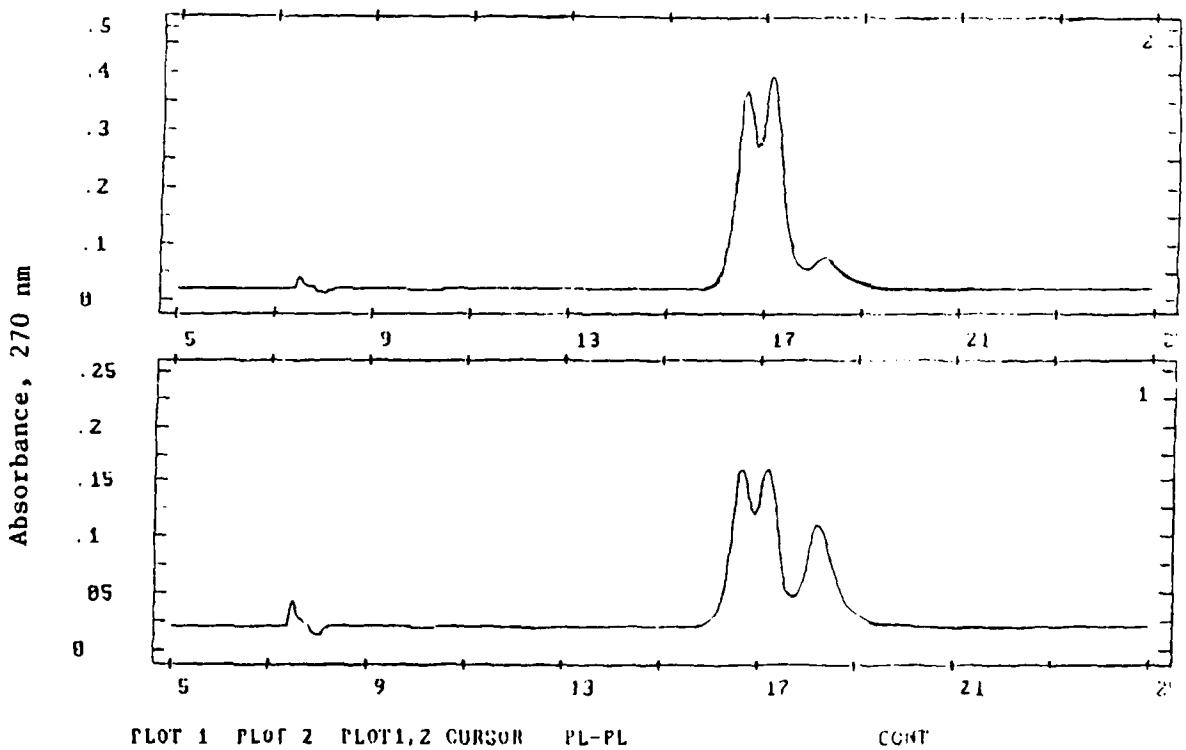


Figure 17. Hydrolysis of 4-nitrophenyl trichloromethyl(phenyl)phosphinate in the presence of chymotrypsin as analyzed on the L-DNBPG column. Top panel is extract after 8 min and bottom panel is extract after 40 min exposure; the peak at 18 min is 4-nitrophenol.

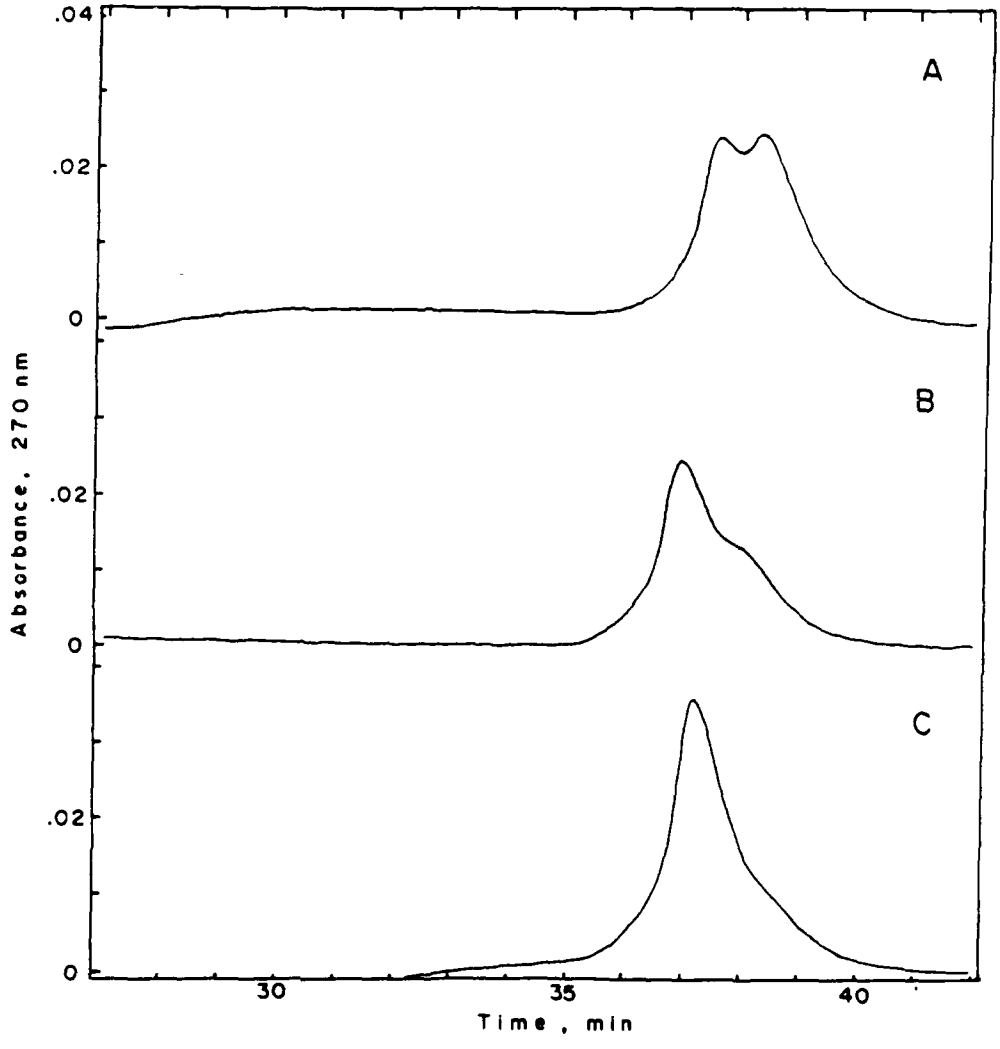


Figure 18. Stereoselective hydrolysis of 7.5 mM racemic 4-nitrophenyl ethyl-(phenyl)phosphinate by arylester hydrolase partially purified from rabbit serum at 24°C as monitored by D-DNBPG chiral-phase HPLC of extracts. (A) Control chromatogram of EPP in buffer without enzyme for 24 hr, (B) chromatogram of EPP after 1 hr with arylester hydrolase, and (C) chromatogram of EPP after 2.5 hr with arylester hydrolase.

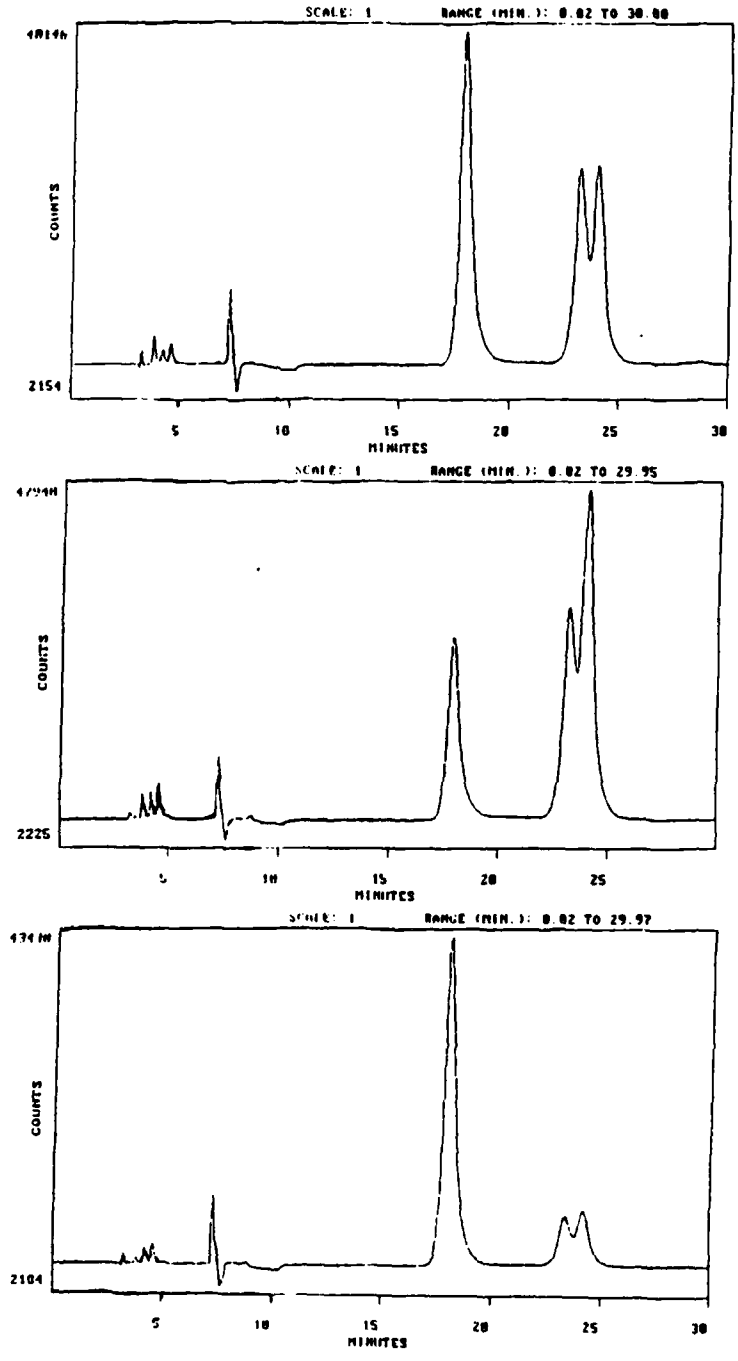


Figure 19. Stereoselective hydrolysis of 0.34 mM 4-nitrophenyl dichloromethyl(phenyl)phosphinate by rabbit serum arylester hydrolase. Top panel is DCMPP extracted after 40 min in MOPS buffer at 37°C; middle panel is DCMPP extracted after 8 min exposure to arylester hydrolase; and bottom panel is DCMPP extracted after 40 min in arylester hydrolase. Peak at 18 min is 4-nitrophenol and analysis is performed on L-DNBPG column.

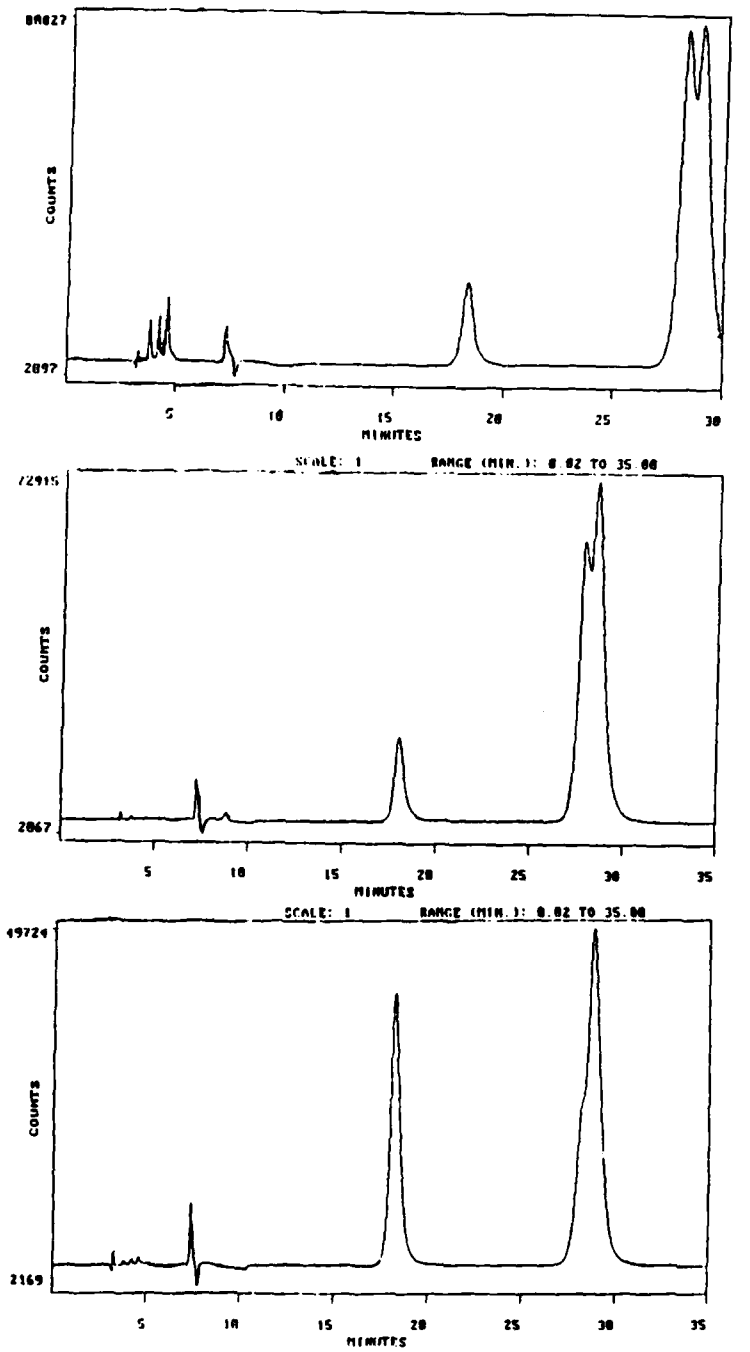


Figure 20. Stereoselective hydrolysis of 0.34 mM 4-nitrophenyl ethyl(phenyl) phosphinate by 0.0585 mM phosphorylase kinase at 37°C. Top panel is EPP extracted from buffer after 200 min; middle panel is EPP extracted after 40 min in phosphorylase kinase; and bottom panel is EPP extracted after 200 min in phosphorylase kinase. Analysis is by L-DNBPG column and 18 min peak is 4-nitrophenol.

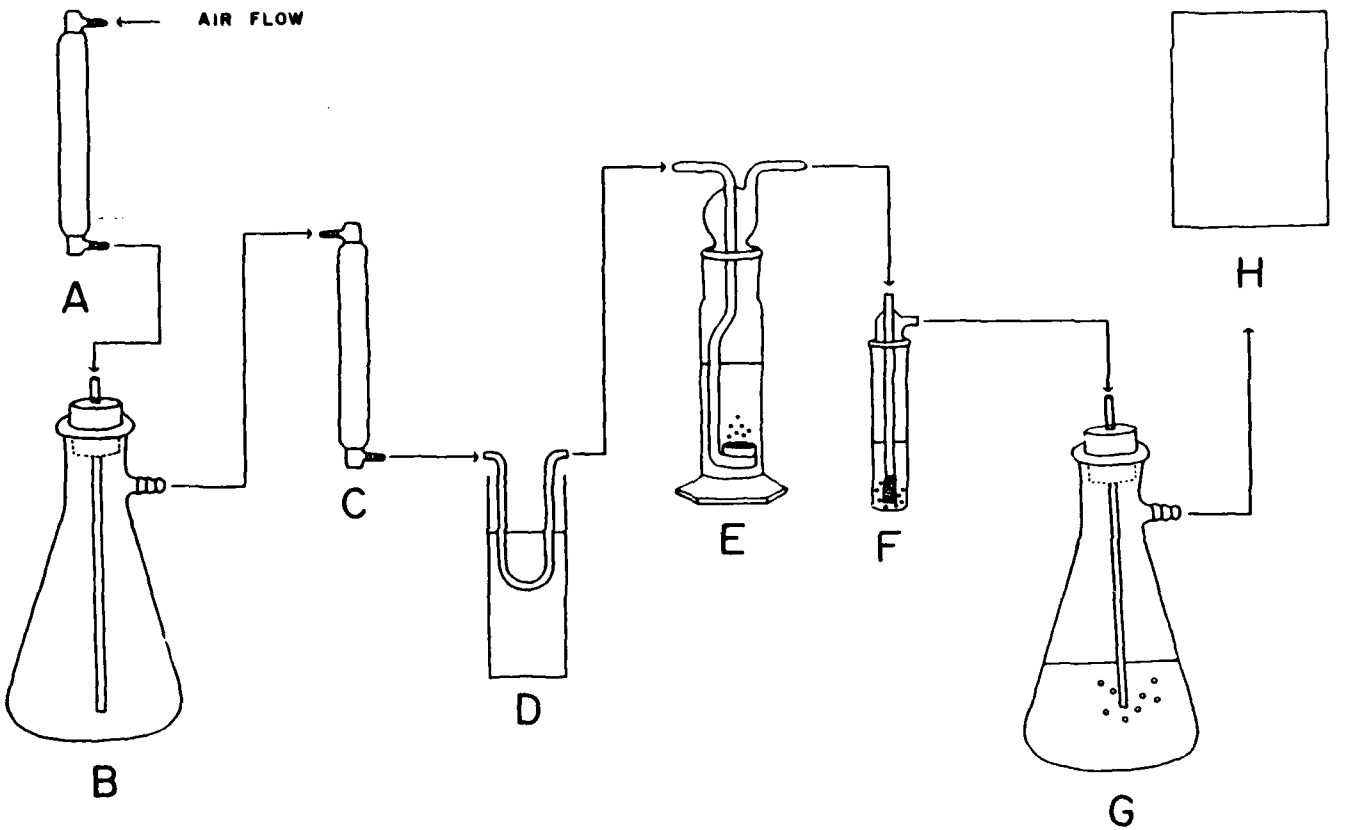


Figure 21. Apparatus for collection of [^{14}C]carbon dioxide expired from mice; (A) Ascarite^{R} column, (B) 250 ml flask for holding mouse, (C) Drierite^{R} column, (D) dry ice and acetone bath for glass U-trap for expired organic compounds other than carbon dioxide, (E) primary trap for carbon dioxide containing 100 ml 25% (V/V) Carbo-sorb^{R} in scintillation cocktail, (F) secondary trap for carbon dioxide containing 10 ml 25% (V/V) Carbo-sorb^{R} in scintillation cocktail, (G) safety trap containing 5% aqueous potassium hydroxide, (H) diaphragm vacuum pump for pulling air through the system.

Table 15. Stereoselectivity of organophosphinates in the inhibition of acetylcholinesterases and α-chymotrypsin.

Inhibitor	Preparation[a]	Eel Acetylcholinesterase k_i[b]	r_a[c]	Bovine Acetylcholinesterase k_i[b]	r_a[c]	α-Chymotrypsin k_i[b]	r_a[c]
racemic EPP	-	$2.69\times10^6 \pm 2.9\times10^5$(3)		$9.08\times10^5 \pm 1.85\times10^4$(2)		11698 ± 1133(3)	
(+) EPP	D-peak 1	$3.98\times10^6 \pm 3.2\times10^5$(3)	34.91	$1.88\times10^6 \pm 8.85\times10^4$(2)[d]	83.92	1860 ± 460(3)	0.0845
(-) EPP	L-peak 1	$1.14\times10^5 \pm 3.2\times10^4$(3)		$2.24\times10^4 \pm 2.24\times10^4$(2)[d]		22007 ± 867(3)	
racemic IPP	-	231.9 ± 35.5(3)		535.7 ± 28.5(4)		1390 ± 87(3)	
(+) IPP	D-peak 1	316.3 ± 56.3(3)	7.90	1023.6 ± 17.9(4)	21.19	560 ± 282(4)	0.1984
(-) IPP	L-peak 1	40.0 ± 20.3(3)		48.3 ± 28.3(4)		2824 ± 95(4)	
(+) IPP	L-peak 2	230.7 ± 30.9(2)		906.7 ± 15.3(4)		696 ± 7(2)	
(-) IPP	D-peak 2	46.5 ± 46.4(2)		211.0 ± 24.6(4)		2875 ± 115(2)	

a Enantiomer collected from D- or L-DNBPG HPLC column; peak 1 was 95% pure and peak 2 was 80% pure in each case.

b Bimolecular reaction constant (M^{-1} min^{-1}) ± s.e. (n); eel enzyme at 24°C and other enzymes at 37°C.

c Ratio of activity was the quotient of k_i of (+)enantiomer divided by k_i of (-)enantiomer; all other data were obtained using two separate preparations.

d These replicates were done with one preparation of EPP enantiomers.

Table 16. Stereoselectivity of carboxylester hydrolases toward EPP in 0.1M MOPS buffer, pH 7.5, at 37°C.

Inhibitor	Concentration, M	Rabbit liver k_i, $M^{-1}min^{-1}$	Rabbit liver r_a	Porcine liver k_i, $M^{-1}min^{-1}$	Porcine liver r_a
racemic EPP	6.8×10^{-8}	1.47×10^6		1.19×10^6	
(+)EPP	3.75×10^{-8}	2.24×10^6	0.28	2.29×10^6	0.72
(-)EPP	2.78×10^{-8}	7.99×10^6		3.20×10^6	

Phosphorylase kinase was tested for stereoselectivity by similar methods but using 0.0585 mM enzyme exposed to 0.34 mM EPP at 37°C. It was clear that (-)EPP was lost preferentially so that after 200 min there was only a shoulder of the (-) enantiomer on the (+) enantiomer peak (Fig. 20). This hydrolysis appeared to be due to catalytic turnover, as contrasted with a large initial burst which occurred with chymotrypsin (vide Section 7). This experiment confirmed that phosphorylase kinase catalyzed EPP hydrolysis (vide Table 22).

Table 17. Stereoselectivity of rabbit serum arylester hydrolase for hydrolysis of 4-nitrophenyl ethyl(phenyl)phosphinate.

Substrate	Concentration (mM)	Activity[a]	r_a[b]
racemic EPP	0.049	28.02 ± 1.52(2)	
(+) EPP	0.049	6.29 ± 0.42(4)	0.115
(-) EPP	0.049	54.62 ± 4.76(4)	

[a]Expressed as nanomoles of 4-nitrophenol released/min/mg protein ± s.e. (n) at 24°C.

[b]Ratio activity was the quotient of activity with (+) enantiomer divided by activity with (-) enantiomer.

5d. Discussion

Acetylcholinesterase, arylester hydrolase and α-chymotrypsin are stereoselective toward organophosphinates. This is consistent with their reported stereoselectivity toward organophosphonates. Carboxylester hydrolases, protease, α-chymotrypsin and trypsin exhibited similar

selectivity toward (-)EPP, which is consistent with their classification as B-esterases. It is apparent also that chiral-phase HPLC techniques are very useful in studies of stereoselectivity of enzymes.

The reversed stereospecificity between acetylcholinesterase and α-chymotrypsin observed for inhibition by EPP and IPP has been observed for EPN-oxon (60), which resembles EPP, and for 4-nitrophenyl S-methyl- and S-ethyl methylphosphonothiolate (66,67). On the other hand, when the chain length of the 4-nitrophenyl S-alkyl methylphosphonothiolates was increased to propyl and longer, then both acetylcholinesterase and α-chymotrypsin were more sensitive to the identical enantiomer (66). This relationship of like stereospecificity was observed again when both acetylcholinesterase and α-chymotrypsin were more sensitive to inhibition by P(-) soman than to inhibition by P(+) soman (59).

Reversed stereoselectivity between acetylcholinesterase inhibition and arylester hydrolase hydrolysis as observed for EPP was found previously with five 4-nitrophenyl S-alkyl methylphosphonothiolates (66). The previous study employed bovine erythrocyte acetylcholinesterase and sheep serum arylester hydrolase partially purified and having a specific activity toward paraoxon of 0.048 mol/min/mg protein (66); this value for our rabbit serum preparation was 0.056 mol/min/mg protein (3). Other studies (59,60) have noted this reversed stereoselectivity; however, they have employed rabbit serum in the study of soman (59) and rat liver microsomes in the study of EPN-oxon (60), rather than partially purified arylester hydrolase. There have been no published reports to our knowledge of chiral organophosphorus compounds with like stereospecificity toward both acetylcholinesterase inhibition and catalytic arylester hydrolase degradation. This suggests similarity of their active sites in the regions which confer stereoselectivity.

The bimolecular reaction constant for inhibition of eel acetylcholinesterase by racemic EPP (Table 15) was 1.6-fold greater than that determined previously using a stop-flow apparatus at 25°C and at pH 6.9 (12). The value for inhibition of bovine pancreatic α-chymotrypsin by P(-)EPP (Table 15) was 1.04-fold less than that reported for 4-nitrophenyl dipentylphosphinate, which was the most potent and largest of a series of dialkylphosphinates (68); 10-fold lower activity toward P(-) IPP indicates that an optimum size for inhibition has been exceeded in this case.

It appears that exposure of EPP or IPP to pancreatic proteolytic hydrolases, to serum arylester hydrolase and to liver carboxylester hydrolase would result in more rapid loss of that enantiomer which is the less potent acetylcholinesterase inhibitor. These organophosphinates, which are not subject to aging reactions on phosphinylated enzymes, may be useful probes in future investigations of active sites, especially when coupled with molecular techniques currently employed with acetylcholinesterase (69) and trypsin (70).

Direct analysis of enzyme stereospecificity by chiral-phase HPLC of the product from a racemic mixture was demonstrated here for α-chymotrypsin, trypsin and arylester hydrolase. This technique provided

results which agreed with those determined with the resolved enantiomers. Therefore, this method can provide a rapid preliminary analysis without the laborious prior synthesis or separation of enantiomers.

6. Drug Interactions with Organophosphinates in Mice

6a. Introduction

Organophosphinates are an uncommon chemical group in relation to organophosphonates and organophosphates which are used extensively as pesticides and drugs. Organophosphinates contain only one phosphorus-ester bond and, while they are rapid inhibitors of acetylcholinesterase, their effects are relatively more reversible since there is no possibility of O-dealkylation (aging) of the phosphinylated enzyme as occurs following poisoning by many phosphonates and phosphates (18). Organophosphinates were found to protect against delayed neurotoxicity in hens (22) and have been considered as pretreatment agents in protection against organophosphate poisoning.

Certain organophosphorus esters are known to potentiate pesticides and drugs which contain carboxylester groups due to the inhibition of carboxylesterase (carboxylester hydrolase, EC 3.1.1.1) which normally catalyzes their hydrolytic detoxication. The remarkable interaction of malathion and EPN in rats and in dogs (71) was likely due to this phenomenon (72,73). A similar interaction occurred in the potentiation of procaine by tri-o-tolylphosphate in rats (74,75). The possible interaction of organophosphinates with drug metabolism has not been investigated previously.

Carboxylesterase was inhibited very rapidly by 4-nitrophenyl alkyl (aryl)- and diarylphosphinates and the phosphinylated forms of the enzyme recovered activity at various rates (7). The experiments described here were performed to determine whether or not organophosphinates, represented by two compounds of different biochemical properties, would interfere with hydrolytic metabolism of carboxylester-containing drugs, aspirin and procaine.

6b. Methods

Crystalline O-4-nitrophenyl diphenylphosphinate (DPP) and O-4-nitrophenyl methyl(phenyl)phosphinate (MPP) were provided by the U.S. Army Medical Research Institute of Chemical Defense (18,12). They were administered to mice im in the left rear leg in polyethylene glycol-200 (Sigma Chemical Company, St. Louis, MO) containing 0.1% glacial acetic acid and 10% dichloromethane (v/v) which was necessary to dissolve DPP.

Toxicity of organophosphinates was determined in CF1 mice (Charles River Breeding Laboratories, Wilmington, MA) held with ear-tags at least 7 days prior to treatment at 23°C and 12 h photoperiod in 27 x 21 x 14 cm polycarbonate cages with hardwood bedding and provided mouse chow and water ad libitum; five mice of like sex were held per cage. Mice 66 to 88 days of age and 23-35 g were selected randomly for doses of MPP of 0.0, 1.19, 1.41, 1.68 and 2.00 mg/kg (based on pilot study) injected im

in 0.015 to 0.023 ml; males and females were treated on different days and observed for 7 days; probit analysis of mortality was performed (76). Since DPP was less toxic, higher doses were administered in up to 0.076 ml.

The effect on drug metabolism of pretreatment with LD01 MPP (1.11 mg/kg) and the highest practical dose of DPP (100 mg/kg) was assessed in three experiments in which the phosphinates were administered to male mice im 2 h prior to drug injection ip. The first two experiments assessed aspirin metabolism by tracing each of the primary hydrolysis products (Appendix D). Male CF1 mice were held 3 per 28 x 18 x 12 cm cage with dried corncob bedding at 21°C and 12 h photoperiod, fed rat chow and water ad libitum, and used at 19-26 g.

In the first experiment, phosphinate-pretreated mice were injected ip with 10 µCi [carboxyl-^{14}C]-aspirin (7.25 mCi/mmol, Pathfinder Laboratories, St. Louis, MO) in 0.076 ml 10 mM sodium bicarbonate and liquid scintillation counting (LSC) of trapped peaks from HPLC was used to determine [carboxyl-^{14}C]aspirin and -salicylic acid in blood. Blood from the cut tail was measured in a heparinized capillary tube (0.028-0.036 ml), expelled into a 0.4 ml plastic centrifuge tube, diluted with 0.1 ml acetonitrile containing unlabeled standards, mixed, sonicated 15 s and centrifuged 19,400 x g for 10 min at 4°C. For separation, 0.01 ml of supernatant was injected onto an octadecylsilane-bonded HPLC column (25 cm x 4.6 mm) (ODS-3 Partisil-10, Whatman Chemical Separation, Clifton, NJ) preceded by a guard column (6 cm x 4.6 mm) (Pellicular ODS, Whatman) and eluted with a mixture of 0.2 M monobasic potassium phosphate, pH 2.5, water and acetonitrile (40:35:25) at 1 ml/min (77); the instrument has been described (16). Peaks coincident with the standards were collected, scintillation mixture added (78), and LSC performed with external standard quench correction (TM Analytic Model 6895, Elk Grove Village, IL). A randomized block, split plot experimental design was employed. The 3 treatments were repeated on 4 different days (blocks) so that 12 mice were employed; subtreatments of the split plot were the times at which each mouse was repeatedly sampled. Data were converted to dpm in the total blood assuming 0.0778 ml/g body wt (79) and analysis of variance was performed (80) using a general linear model computer routine (SAS Institute, Raleigh, NC). Least significant difference (LSD) was also computed for the test of significance of differences among means (81).

In the second experiment, phosphinate-pretreated mice were injected ip with 1.0 µCi [acetyl-1-^{14}C]aspirin (5 mCi/mmol, Research Products International, Mount Prospect, IL), held in a closed respiration apparatus (Fig. 21) and expired [^{14}C]carbon dioxide was trapped and measured by LSC. Each injected mouse was placed immediately in a 250-ml filtering flask. Air filtered by Ascarite® was pulled at 0.75 standard cu ft/h through the flask, then through Drierite®, through a glass U-trap at -76°C, and through 100 ml 25% Carbo-sorb® (United Technologies, Downers Grove, IL) in scintillation mixture (v/v) held in a model 7164 gas washing bottle with 175 µm porosity (Ace Glass, Vineland, NJ). Safety traps of Carbo-sorb® and 5% potassium hydroxide followed in series. At intervals, 2 ml was removed from the primary Carbo-sorb® trap for LSC.

Each organophosphinate-pretreated mouse was compared simultaneously to a control mouse using paired holding flasks in a parallel apparatus on 4 different days. Since acetic acid, and not carbon dioxide, was the direct product of hydrolysis, this experiment was performed again with 1.0 μCi [1-^{14}C]acetic acid (57 mCi/mmol, Amersham, Arlington Heights, IL) substituted for aspirin. Data were analyzed as above.

In the third experiment, phosphinate-pretreated mice were injected ip with 3.5 μCi [carboxyl-^{14}C]procaine in 0.125 ml 20% ethanol solution and blood was analyzed for parent and [carboxyl-^{14}C]4-aminobenzoic acid by HPLC and LSC. Methods were identical to the first experiment with aspirin except the mobile phase for HPLC contained 5 mM 1-pentane sulfonate (Regis Chemical, Morton Grove, IL) in a mixture of 0.1 M sodium phosphate, pH 4.5, and acetonitrile (85:15) and five replicates were done. Synthesis of [carboxyl-^{14}C] procaine was performed from [carboxyl-^{14}C]4-aminobenzoic acid (10.32 mCi/mmol, Pathfinder Laboratories) using a previously described method (82), with the reaction volume reduced to 2.3 ml. After purification by thin layer or column chromatography, average radiochemical purity of 2 preparations was 90% by thin layer chromatography.

To assess the degree of carboxylesterase inhibition by pretreatment, each mouse was sacrificed 2 h after phosphinate injection and its liver excised and homogenized with glass-against-Teflon in 0.1 M sodium phosphate, pH 7.5. The homogenate was mixed well by Vortex and centrifuged at 19,400 x g for 20 min at 4°C. The supernatant was diluted 100-fold and activity toward 1-naphthyl butyrate was determined (7); there were four replicates on different days.

The effect of DPP pretreatment on procaine loss in liver homogenates was determined in an additional experiment. Male CF1 mice were pretreated with 50 mg/kg of DPP injected in the left rear leg im or treated with vehicle as previously described. After 2 h, mice were sacrificed and livers excised, rinsed and homogenized for 25 s, using a Kinematica homogenizer (Brinkmann Instruments Co.) in four volumes 0.1M MOPS buffer, pH 7.5, per g fresh weight. Homogenates were centrifuged at 10,500 x g for 20 min at 4°C and supernatants were collected. Aliquots were held at 37°C and aqueous procaine HCl was added to give 0.107 mM final concentration. The reaction was terminated by addition to an octadecylsilyl-bonded Bond Elut^{R} column (Analytichem International) with vacuum and three rinses with 10% methanol in water separate procaine (bound to column) from the homogenate (washed through to waste). Procaine was eluted with two aliquots which totaled 0.4 ml 0.1% acetic acid in methanol and the amount eluted was determined by high performance liquid chromatography as described above except that quantitation of unlabeled procaine was by ultraviolet absorbance at 289 nm. Limit of detection was 72 ng procaine; detector response was linear to > 2500 ng, and recovery from spiked samples (10 mM NaF added) was 80.1%. No procaine peak was detected in control homogenates.

6c. Results

In CF1 mice, MPP was highly toxic and produced a very homogeneous response (Table 18). Tremors were observed in males within 5 min after administration of 1.19 mg/kg, while females exhibited no symptoms at that dose. Most mortality occurred within 4 h. There was no mortality from DPP, although paralysis of the injected leg was rapid and persisted for 7 days at 100 mg/kg. Paralysis of the leg was observed also with MPP, but survivors recovered noticeably in 3 days.

In the three experiments with drug metabolism, MPP at 1.11 mg/kg killed none of 17 mice treated, DPP at 100 mg/kg killed none of 17 mice, and the vehicle killed none of 25 mice.

Complete resolution of aspirin from salicylic acid, and of 4-aminobenzoic acid from procaine, was achieved by reversed-phase HPLC (Fig. 22). Radiolabeled standards and actual blood samples were analyzed and it was found that 98% of recovered radioactivity (75% of injected radioactivity) was associated with aspirin or salicylic acid peaks. Data were not corrected for recovery from the column.

At 8 min following ip administration of approximately 9.9 mg/kg (10 μCi/mouse) [carboxyl-^{14}C]aspirin, blood concentration reached 3.68 μg/ml in DPP-pretreated mice as compared to 1.41 μg/ml in control mice (Fig. 23); at 16 min there was no difference in those concentrations. There was no effect of MPP pretreatment and the overall main effect due to treatment was not statistically significant.

Table 18. Toxicity (mg/kg) of O-4-nitrophenyl methyl(phenyl)phosphinate and O-4-nitrophenyl diphenylphosphinate following intramuscular injection in mice.

Organophosphinate	Sex	n	LD_{01}	LD_{50}	LD_{95}	Slope
MPP	combined	50	1.21(0.10-1.32)[a]	1.61 (1.53-1.70)	1.97	18.8
MPP	male	25	1.11(0.75-1.26)[a]	1.51 (1.38-1.63)	1.87	17.6
MPP	female	25	1.61	1.72	1.81	74.3
DPP	female	11	> 100	-	-	-

[a]95% confidence limits.

There was not a corresponding decline in blood [carboxyl-^{14}C] salicylic acid, as would be expected had DPP pretreatment blocked aspirin hydrolysis (Fig. 23); in fact [carboxyl-^{14}C]salicylic acid concentration

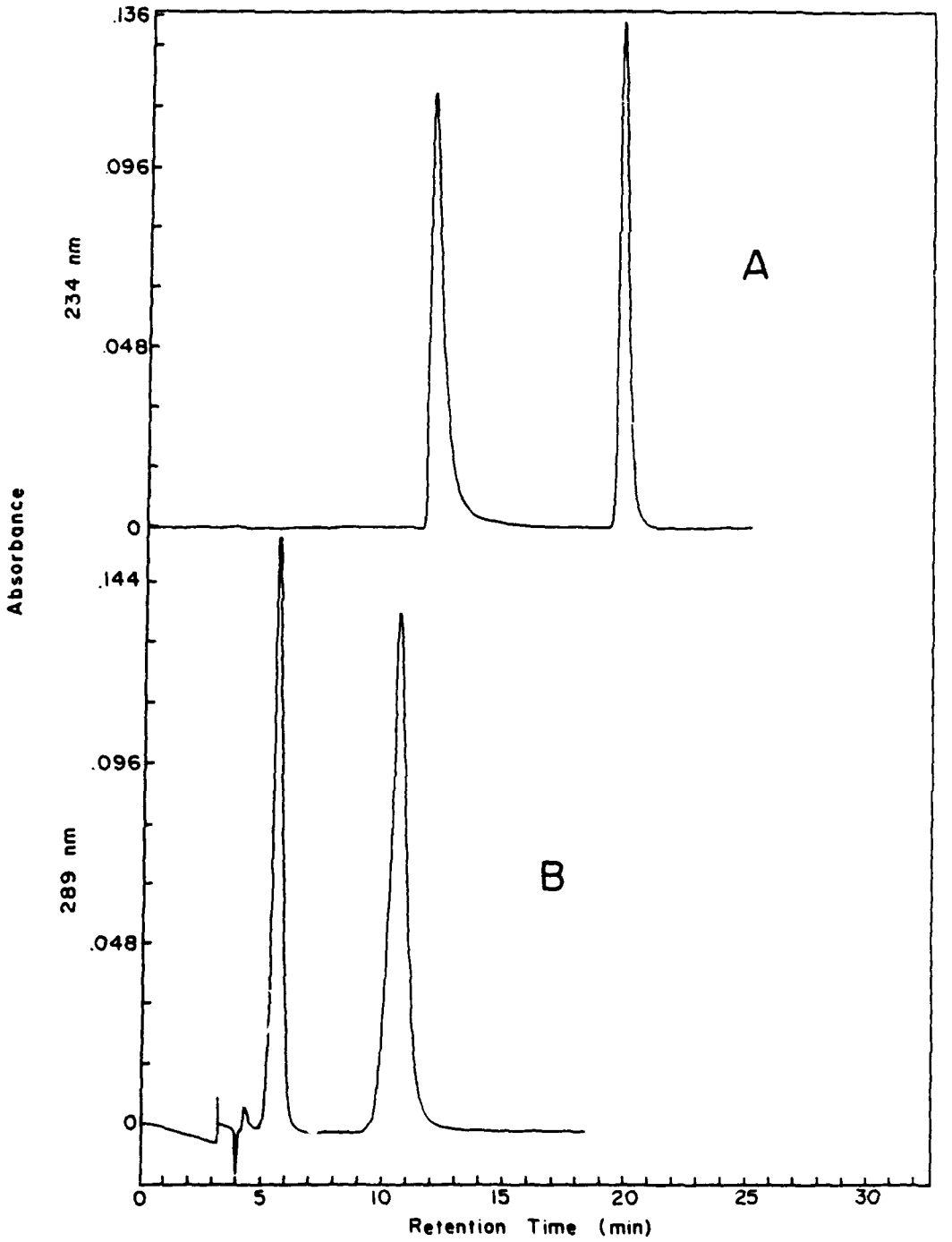


Figure 22. High performance liquid chromatography on octadecylsilyl reversed-phase column with mobile phases pumped at 1 ml/min; (A) peak at 11.9 min was 0.0025 mg aspirin and peak at 20 min was 0.0025 mg salicylic acid; mobile phase was 0.2 M monopotassium phosphate pH 2.5, water, and acetonitrile (40/35/25); (B) peak at 6.0 min was 0.0025 mg 4-aminobenzoic acid and peak at 10.5 min was 0.025 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.

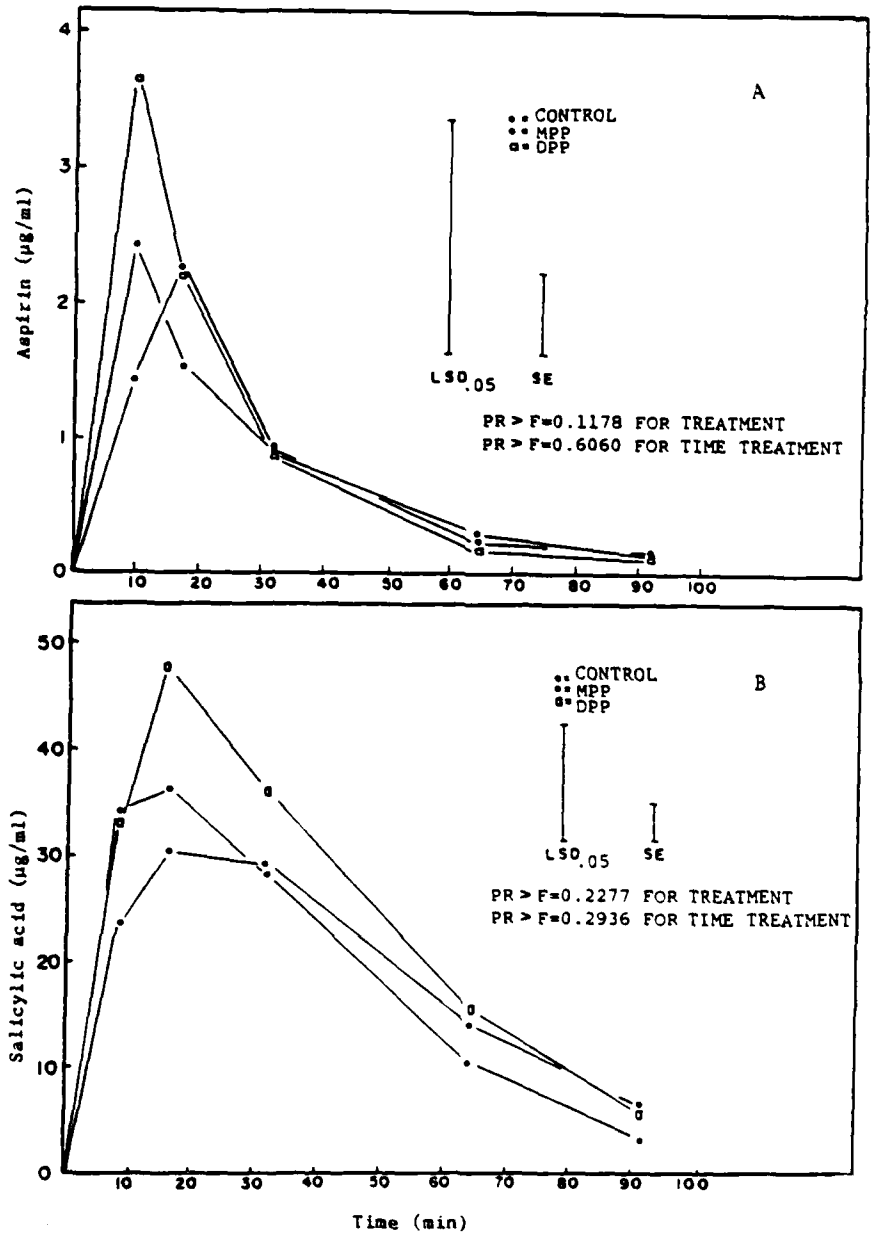


Figure 23. Radioactivity as aspirin (A) or its metabolite salicylic acid (B) in blood following ip injection of 0.01 mCi[carboxyl-^{14}C]aspirin into organophosphinate-pretreated mice. Mice were injected im with LD01 organophosphinate 2 h prior to aspirin administration.

reached 47.6 μg/ml in DPP pretreated compared to 36.8 μg/ml in control mice at 16 min. Again, there was no effect of MPP pretreatment and the overall main effect was not significant. At the highest levels reached in control mice, total blood [carboxyl-^{14}C]salicylic acid represented 37% of the injected dose, while parent [carboxyl-^{14}C]aspirin in total blood was 1.7% of the injected dose.

There was no effect of MPP pretreatment or DPP pretreatment (Fig. 24) on expiration of [1-^{14}C]carbon dioxide through 172 min following administration of [1-^{14}C]acetic acid. This result demonstrated that the effect of phosphinate pretreatment on aspirin hydrolysis could be assessed by the alternative method of determining [^{14}C]carbon dioxide expired following administration of [acetyl-1-^{14}C]aspirin which is hydrolyzed to [1-^{14}C]acetic acid and nonradioactive salicylic acid.

There was no effect of either MPP or DPP pretreatment on expiration of [^{14}C]carbon dioxide from 1.44 mg/kg [acetyl-1-^{14}C]aspirin in CF1 mice (Fig. 25). Approximately 70% of the injected dose was collected as [^{14}C]carbon dioxide in 243 min in the respiratory apparatus. This result was obtained with very little variation in the replicates and clearly demonstrated a lack of effect of MPP or DPP pretreatment on primary hydrolysis of aspirin.

In 9 min after administration of approximately 3.2μmg/kg (3.5 μCi/mouse) [carboxyl-^{14}C]procaine, the concentration reached 0.836 μg/ml in blood of DPP pretreated mice, while it was only 0.348 μg/ml in control mice (Fig. 26). There was a significant main effect due to treatment and the concentration in DPP pretreated mice remained 3.3-fold greater than control mice at 27 min. There was no effect of MPP-pretreatment on [carboxyl-^{14}C]procaine levels.

There was no significant difference among measurements of [carboxyl-^{14}C]4-aminobenzoic acid concentrations in blood which at 9 min were 0.16 μg/ml in DPP-pretreated mice and 0.14 μg/ml in control mice. Only 1.2% of the injected dose in control mice and 2.4% of the injected dose in DPP pretreated mice was accounted for as procaine plus 4-aminobenzoic acid in total blood volume at 9 min, indicating a very small mobilization of applied procaine as compared to aspirin and its primary hydrolysis product. A preliminary experiment revealed a 40-fold increase in [carboxyl-^{14}C]procaine in the liver of a DPP pretreated mouse as compared to the control at 30 min after administration.

When areas under the curves were computed for these results (Table 19), the greatest change observed was the increase in procaine areas under the curves following DPP treatment. Again it is apparent from areas under the curves values that neither organophosphinate altered aspirin metabolism by more than 28%; this was confirmed by the second experiment with aspirin in which organophosphinate pretreatment did not alter expiration of radiolabeled carbon dioxide for aspirin (Figs. 24 and 25).

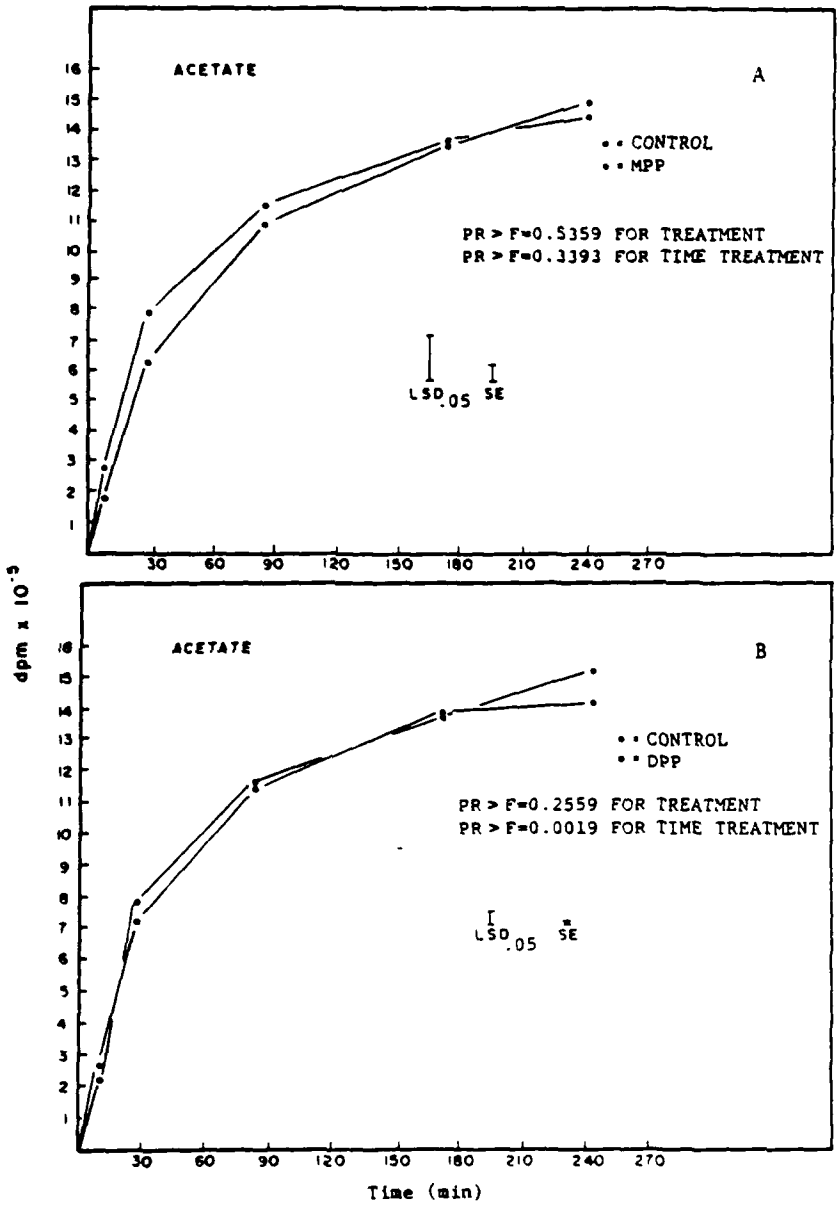


Figure 24. Radioactivity in cumulative carbon dioxide trapped following 0.001 mCi[1-^{14}C]acetate ip injection of mice which had been injected im 2 h earlier either with 1.11 mg/kg O-4-nitrophenyl methyl(phenyl)phosphinate (A) or with 100 mg/kg O-4-nitrophenyl diphenylphosphinate (B).

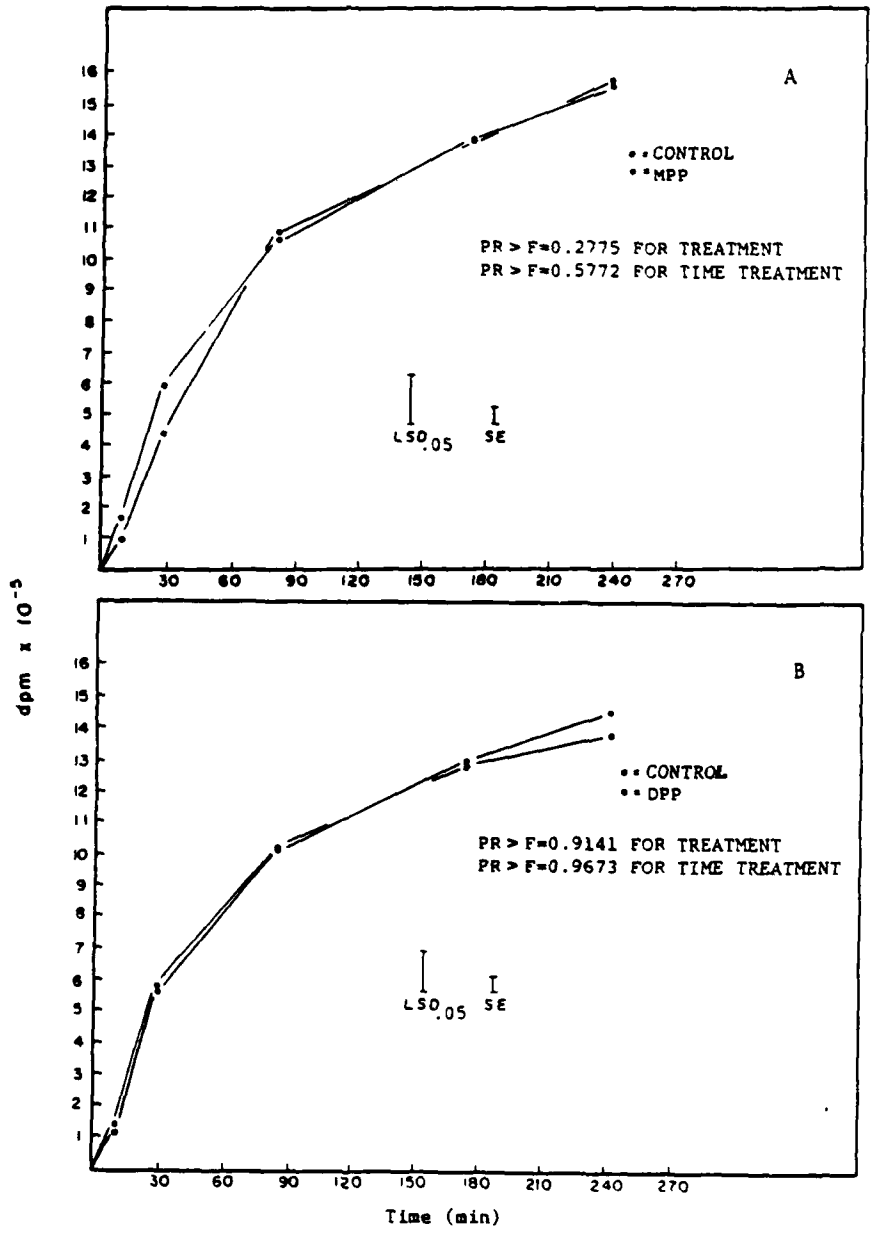


Figure 25. Radioactivity in cumulative carbon dioxide trapped following 0.001 mCi[acetyl-1-^{14}C]aspirin ip injection of mice which had been injected im 2 h earlier either with 1.11 mg/kg O-4-nitrophenyl methyl(phenyl)phosphinate (A) or with 100 mg/kg O-4-nitrophenyl diphenylphosphinate (B).

Carboxylesterase activity in excised livers 2 h following DPP pretreatment was only 11% of control activity (Table 20). Reduction of activity by MPP pretreatment was not significant.

In homogenates of liver from DPP pretreated mice, procaine was lost at less than one-half the rate of homogenates from control mice (Table 21). These homogenates from DPP mice also had similarly lower activities against 1-naphthyl butyrate, indicating inhibition of carboxylester hydrolase, although at this dose (50 mg/kg), inhibition was not as great as in the previous experiment in which 100 mg/kg was administered.

6d. Discussion

The four-fold greater toxicity of MPP to mice found here than to rats (11) may be due to the acidification of the vehicle in our study which was not done in the study with rats. We noticed the rapid decomposition of this compound by the yellow color formed in the vehicle; this was eliminated by acidification; acidification of vehicle also stabilized DPP as confirmed by octylsilyl reversed-phase HPLC determination of DPP and 4-nitrophenol (16).

The rapid hydrolysis of aspirin to salicylic acid in mice was similar to previous results in other mammals. Introduction of aspirin at 12 mg/kg into the hepatic portal vein of dogs resulted in peak plasma concentrations of 9.7 μg/ml aspirin at 23 min and 34 μg/ml salicylic acid at 35 min (83), while similar administration of 10 mg/kg aspirin to rats produced 3.5 g/ml aspirin at 4 min (84); our dose of 9.9 mg/kg [carboxyl-^{14}C]aspirin gave peak blood concentrations of 1.41 μg/ml aspirin and 37 μg/ml salicylic acid in mice. Oral doses of approximately 9 mg/kg in two of many studies in man have resulted in peak plasma concentrations, 5.9 and 23 μg/ml aspirin and 40 and 43 μg/ml salicylic acid, respectively (77,85).

In the rat, aspirin hydrolysis and removal from the plasma were computed to be 33% of the applied dose in the liver and 47% in the gastrointestinal tract (84) as compared to 36% and 28%, respectively, in the dog (83). While it is clear that aspirin is rapidly converted to salicylic acid, and that 4 isozymes of liver carboxylesterases in rat liver (86) and one carboxylesterase in guinea pig liver (87) can catalyze this hydrolysis, the relative importance of non-enzymatic acetylation of protein has not been addressed, although it is known to occur extremely rapidly with cyclooxygenase (88). Since hydrolysis of aspirin in mice was not reduced despite 89% inhibition of liver carboxylesterase activity (Table 20), it appears that the contribution of enzyme catalyzed hydrolysis to total aspirin hydrolysis is not significant. Further studies will address the phosphinate sensitivity of specific forms of carboxylesterase in mice.

While organophosphinates did not reduce aspirin hydrolysis, there was an increase in blood concentration of [carboxyl-^{14}C]procaine with DPP pretreatment, which could have resulted from decreased hydrolysis or other metabolic or elimination processes. Liver homogenates of DPP pre-

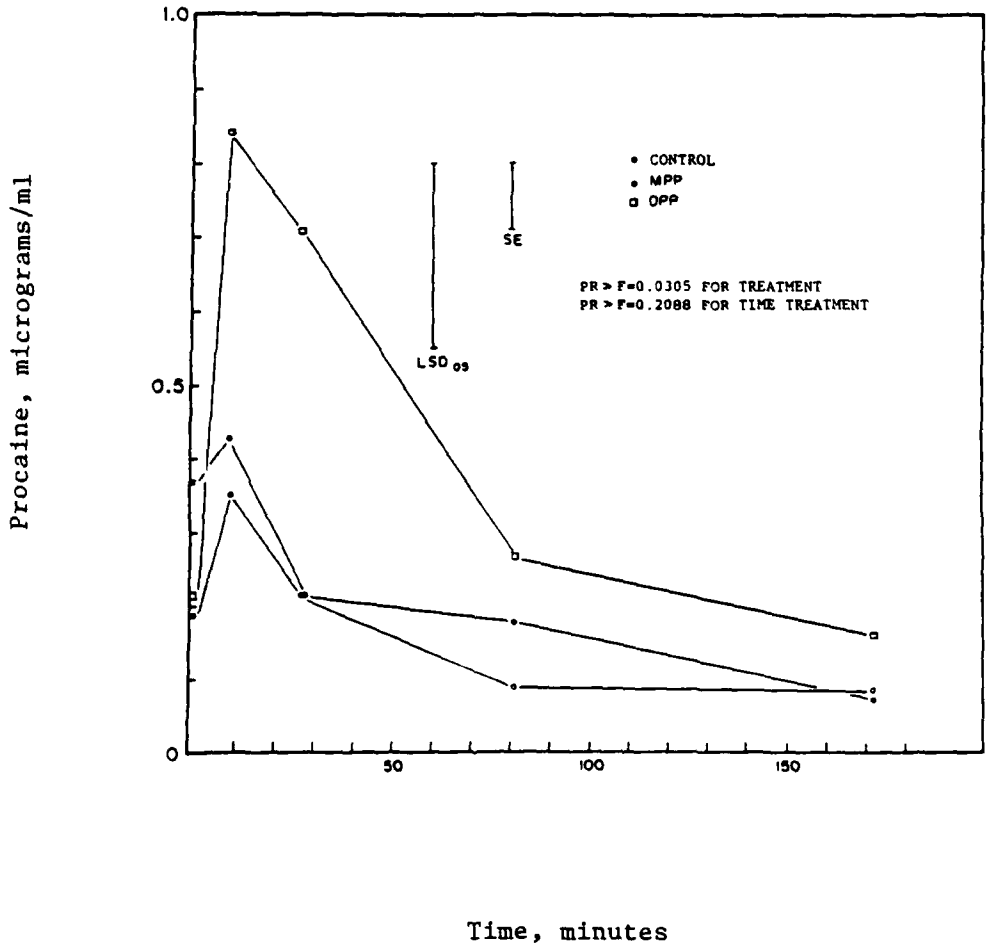


Figure 26. Radioactivity as procaine in total body blood following ip injection of 0.0035 mCi [carboxyl-^{14}C]procaine into organophosphinate-pretreated mice. Mice were injected im with LD01 organophosphinate 2 h prior to procaine administration.

Table 19. Pharmacokinetics of aspirin and procaine in mice 2 h after administration of O-4-nitrophenyl methyl(phenyl)phosphinate or O-4-nitrophenyl diphenylphosphinate.

Treatment	Aspirin[a]		Salicylic Acid[a]		Procaine[b]	
	AUC[c]	% of Control	AUC	% of Control	AUC	% of Control
Control	6.69	100	1735	100	20.4	100
MPP	5.87	88	1752	100	27.6	135
DPP	6.76	101	2221	128	58.7	287

[a]Data for aspirin and its metabolite, salicylic acid are from curves in Fig. 2.

[b]Data for procaine are from curves in Fig. 5.

[c]AUC is area under the curve and all units are $\mu g\ ml^{-1}$ min.

Table 20. Carboxylesterase activity remaining in excised mouse livers 2 h following *in vivo* inhibition by O-4-nitrophenyl methyl(phenyl) phosphinate (MPP) and O-4-nitrophenyl diphenylphosphinate.

Treatment	Specific Activity[a]	% of Control
Control	2.725 ± 0.357	-
MPP	2.170 ± 0.276	79.6
DPP	0.300 ± 0.162[b]	11.0

[a]Expressed as micromoles of 1-naphthyl butyrate hydrolyzed/min/mg protein ± s.e., n = 4.

[b]Significantly different from control and MPP at the a = 0.001 level.

treated mice lost procaine at only one-half the control rate confirming the interaction observed *in vivo* and suggesting that metabolism (probably hydrolysis) of procaine was inhibited by DPP.

Table 21. Hydrolysis of procaine in homogenates of mice following pretreatment with 4-nitrophenyl diphenylphosphinate.

Time, min	n	Procaine Concentration, μg/ml ± s.e. Control	DPP pretreated
0	3	17.8 ± 2.3	21.2 ± 0.6
10	3	9.3 ± 2.9	18.0 ± 1.2
20	2	5.2 ± 3.2	14.8 ± 3.2
40	3	1.2 ± 0.8	9.4 ± 1.4

Procaine was hydrolyzed very slowly by rabbit plasma (89), while one of five rat liver carboxylesterases hydrolyzed procaine at 3% of the maximum velocity at which aspirin was hydrolyzed by a different liver carboxylesterase (86). Procaine hydrolysis in rats was apparently inhibited by tri-o-tolylphosphate, although those studies did not separate procaine from 4-aminobenzoic acid, but relied upon spectrophotometric analysis of the mixture for quantitation (74,75). It appears that carboxylesterase, which prefers larger acyl groups over analogous acetic acid esters, was a significant factor in the total hydrolysis of procaine *in vivo* in rats and mice.

The interaction of DPP, and not MPP, with procaine kinetics would be expected, not only due to the higher dose employed, but also due to the two-fold greater inhibitory potency of DPP toward rabbit liver carboxylesterase which was followed by five-fold slower recovery of enzyme activity (7). On the other hand, MPP is the more potent acetylcholinesterase inhibitor with a bimolecular rate constant of $2.9 \times 10^4 M^{-1} s^{-1}$, while DPP is approximately 100 (11). In addition, MPP is rapidly hydrolyzed enzymatically by serum arylester hydrolase, while DPP is not a substrate for this enzyme (3).

7. Interactions of Organophosphinates with Other Hydrolases and Kinases

7a. Screen for Reaction of 4-Nitrophenyl Ethyl(phenyl)phosphinate with Hydrolases and Kinases

We have screened three additional hydrolases and three transferases for EPP hydrolysis (Table 22). Of hydrolases, alkaline phosphatase and protease were active while phospholipase was inactive. Of transferases, phosphorylase kinase was active, while phosphorylase b and pyruvate kinase were inactive. Phosphorylase kinase susceptibility may be important since this is a controlling enzyme in the cell.

Table 22. Hydrolases and transferases screened for reactivity with 4-nitrophenyl ethyl(phenyl)phosphinate.

Enzyme Commission Number	Name	Source
2.4.1.1	phosphorylase b	rabbit muscle (Sigma-P-6635)
2.7.1.38	phosphorylase kinase	rabbit muscle (Sigma P-2014)
2.7.1.40	pyruvate kinase	rabbit muscle (Boehringer-Mannheim)
3.1.1.1	carboxylester hydrolase	porcine liver (Sigma type I;E-3128)
3.1.1.2	arylester hydrolase	rabbit serum (Zimmerman)
3.1.3.1	alkaline phosphatase	human placenta (Sigma Type XVII;P-1391)
3.1.4.4	phospholipase D	peanut (Sigma Type III, P-0640)
3.4.21.1	α-chymotrypsin	bovine pancreas (Sigma Type II, 4129)
3.4.21.14	protease	Bacillus amylolique faciens (Sigma Type VII, P-5255)
3.4.21.4	trypsin	bovine pancreas (Sigma Type III-3,T-2395)

Hydrolysis of EPP was catalyzed rapidly by arylester hydrolase, confirming our previous results (Table 23). With the serine hydrolases, chymotrypsin, carboxylester hydrolase and protease, there was an apparent burst of product probably due to the phosphorylation of the active site. All data were corrected for spontaneous hydrolysis. The subsequent very slow hydrolysis probably reflects the spontaneous reactivation and re-phosphorylation of these enzymes. The initial burst was greatest with carboxylester hydrolase which we have found to be rapidly inhibited by EPP (7). Two other hydrolases, alkaline phosphatase and phospholipase D, did not react with EPP initially, but appear to produce a very slow hydrolysis.

When human placenta alkaline phosphatase was compared directly to bovine intestinal mucosa alkaline phosphatase of 130-fold greater purity, EPP hydrolysis in the higher specific activity enzyme was only one-third the rate found in the lower specific activity enzyme. This inverse relationship of EPP hydrolysis to alkaline phosphatase purity suggests that EPP hydrolysis is not a result of alkaline phosphatase, but is probably due to some impurity.

Table 23. Hydrolysis of racemic 4-nitrophenyl ethyl(phenyl)phosphinate (0.34 mM) by hydrolases and transferases at 22°C in pH 7.5 MOPS buffer with 2.5 mM calcium.

enzyme	μmole x 10^3	4-nitrophenol produced, μmol x 10^3 at 0 min	at 10 min	rate after 2 min., μmol x 10^3/min
arylester hydrolase	1.3	3.9	327.9	30.70
chymotrypsin	12.4	12.2	18.7	0
phosphorylase kinase	12.4	1.0	10.5	1.13
alkaline phosphatase	50	0	4.9	0.45
chymotrypsin	100	24.0	50.5	0.13
carboxylester hydrolase	100	45.2	65.3	0.51
protease	100	41.7	55.2	0.19
phospholipase D	100	0.97	5.8	0.42
phosphorylase b	100	3.5	8.8	0.61
pyruvate kinase	100	3.2	8.4	0.59

Of three transferases examined, phosphorylase kinase appeared to catalyze EPP hydrolysis, while phosphorylase b and pyruvate kinase did not. Phosphorylase kinase activity toward EPP was about 300-fold less that that of arylester hydrolase; however, this interaction could be significant because phosphorylase kinase is an important factor in cellular response to extracellular signals.

7b. Attempted Purification of Pig Kidney Fluorohydrolase (DFPase)

Attempts were made to reproduce fluorohydrolase purification (90). Porcine kidney was homogenized in unbuffered solvent. After centrifugation to remove cellular debris, the supernatant was adjusted to pH 5.5 and another centrifugation was performed. The supernatant material was precipitated with cold ethanol (60 ml ethanol/100 ml supernatant), centrifuged, the precipitate resuspended, again centrifuged, and the supernatant subjected to an ethanol fractionation (5 ml ethanol/100 ml supernatant discarding the pellet and then an additional 10 ml ethanol/100 ml supernatant retaining the pellet). The resuspended pellet fraction was passed successively over Sepharose 4B and Sephadex G-200, heat treated at 65°C, centrifuged and finally run over Sephadex G-100. The assay method involved the use of a pH stat to follow the initial release of hydrogen ion. The procedure reported a 3% yield with a purification of 483-fold.

As proposed in this contract, a fluoride electrode was employed to assay for activity by the release of fluoride ion from DFP. This was unsuccessful because of extreme baseline drift. Assays using phenylthioacetate and Ellman's reagent (26) give activities approximately corresponding to positions indicated by Storkebaum et al. (90) until the 15% ethanol fractionation. Since phenylthioacetate is a general esterase substrate, most of the activity was left in the 15% supernatant when fluorohydrolase separated from other esterases. The amount of activity remaining in the 5-15% ethanol fraction was small and, even if it were indicative of fluorohydrolase activity, was diluted so rapidly in later column procedures it became of no value.

Elution over Sepharose 4B showed only a single peak eluting quite near the included volume (approximately 80% of the total volume of the column). The position of the peak vs. activity vs. total column volume was essentially identical with Storkebaum et al. (90), but additional low molecular weight material reported by Storkebaum et al. (90) was not observed.

Material from the Sepharose 4B effluent was concentrated on a PM-30 membrane and run over Sephadex G-200. Under Storkebaum's conditions this step required 80 h and resulted in two peaks: one near the void volume, and a second near a partition coefficient of approximately 0.5. The fluorohydrolase activity is in the non-resolved valley in between. Our Sephadex G-200 column showed proteins distributed over a wide size distribution with two peaks roughly corresponding to Storkebaum's. No activity assays were available to us that were specific enough or sensitive enough so fractions were pooled that corresponded to the relation-

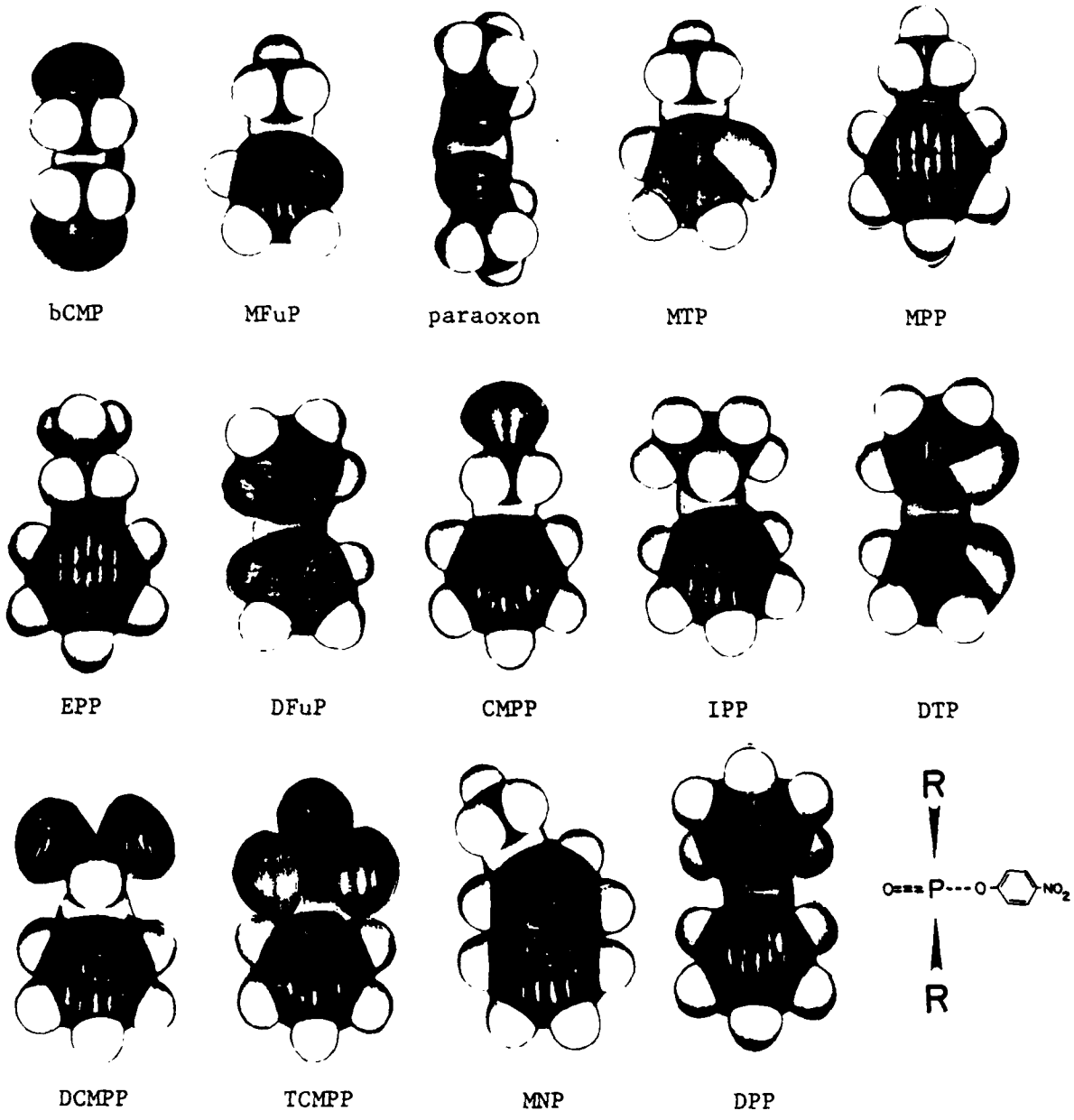


Figure 27. Silhouettes of the P-C bonded substituents of the organophosphinates. CPK models were xeroxed with the P-C bonded substituents flat against the glass with minimum overlap between the substituents. See Table 1 for chemical names.

ship (partition coefficients) shown in Storkebaum's paper. These were concentrated using a PM-30 ultrafiltration unit.

The concentrated material was heated to 65°C for 40 min, as indicated in the Storkebaum et al. (90) procedure, the precipitate discarded, and the supernatant material placed on a Sephadex G-100 column. Neither protein nor phenylthioacetate-hydrolyzing activity was observed in the eluate. The loading protein concentration was much too small at this point.

The use of three successive size exclusion columns, followed by necessary reconcentration steps, seemed to be wasteful and poor separation technology. In an alternative procedure suggested by Dr. Charles White at the 1983 ACS Meeting in Washington, DC material both just after the pH 5.5 centrifugation and after the 60% ethanol precipitation step was applied to DEAE-Trisacryl column (1 mM $MnCl_2$, 0.1 mM dithiothreitol (DTT), 10 mM MOPS, pH 7.5, 0.02% sodium azide).[2] Separations were multiple and excellent, with five peaks of activity eluting and resolved from most of the broad peaks of protein. Rechromatography of selected peaks was also very useful. Progress in this very promising area will require addition of a reliable, reproducible assay procedure for fluorohydrolase. It should be noted that the autotitration procedure used by Storkebaum et al. (90) and now available to us would preclude the use of these ion exchange techniques which require buffer.

8. Conclusions

Organophosphinates used in this study were all 4-nitrophenyl esters and four series of analogous compounds were investigated; they were phenyl, methyl, heterocycle and halogen (as listed in Table 1). Diversity among silhouettes of their space-filling models is illustrated by facing the common 4-nitrophenyl group away to observe only the P to C substituents (Fig. 27).

Arylester hydrolase was not inhibited by most phosphinates; conversely this enzyme catalyzed rapid hydrolysis of 10 of 13 phosphinates tested (Fig. 28). Arylester hydrolase was purified 25-fold from fresh rabbit serum by DEAE-Sepharose chromatography collecting those fractions hydrolyzing paraoxon. Methyl series phosphinates were excellent substrates with, Michaelis constants five-fold to 30-fold less than that for paraoxon. In the phenyl series, increasing the alkyl substituent from methyl (MPP) to ethyl (EPP) reduced specific activity from 2.9 to 0.64 μmoles/min/mg protein and increased the Michaelis constant. The IPP- and DPP-substituted derivatives were not hydrolyzed.

Six phosphinates observed by Lieske (11) as acetylcholinesterase inhibitors were also substrates of arylester hydrolase (Fig. 28). Nonsubstrates IPP and DPP were relatively impotent inhibitors of paraoxon hydrolysis when compared to their anti-carboxylesterase activities. It appears that phosphinate pretreatment poses minimal risk to xenobiotic detoxication by arylester hydrolase.

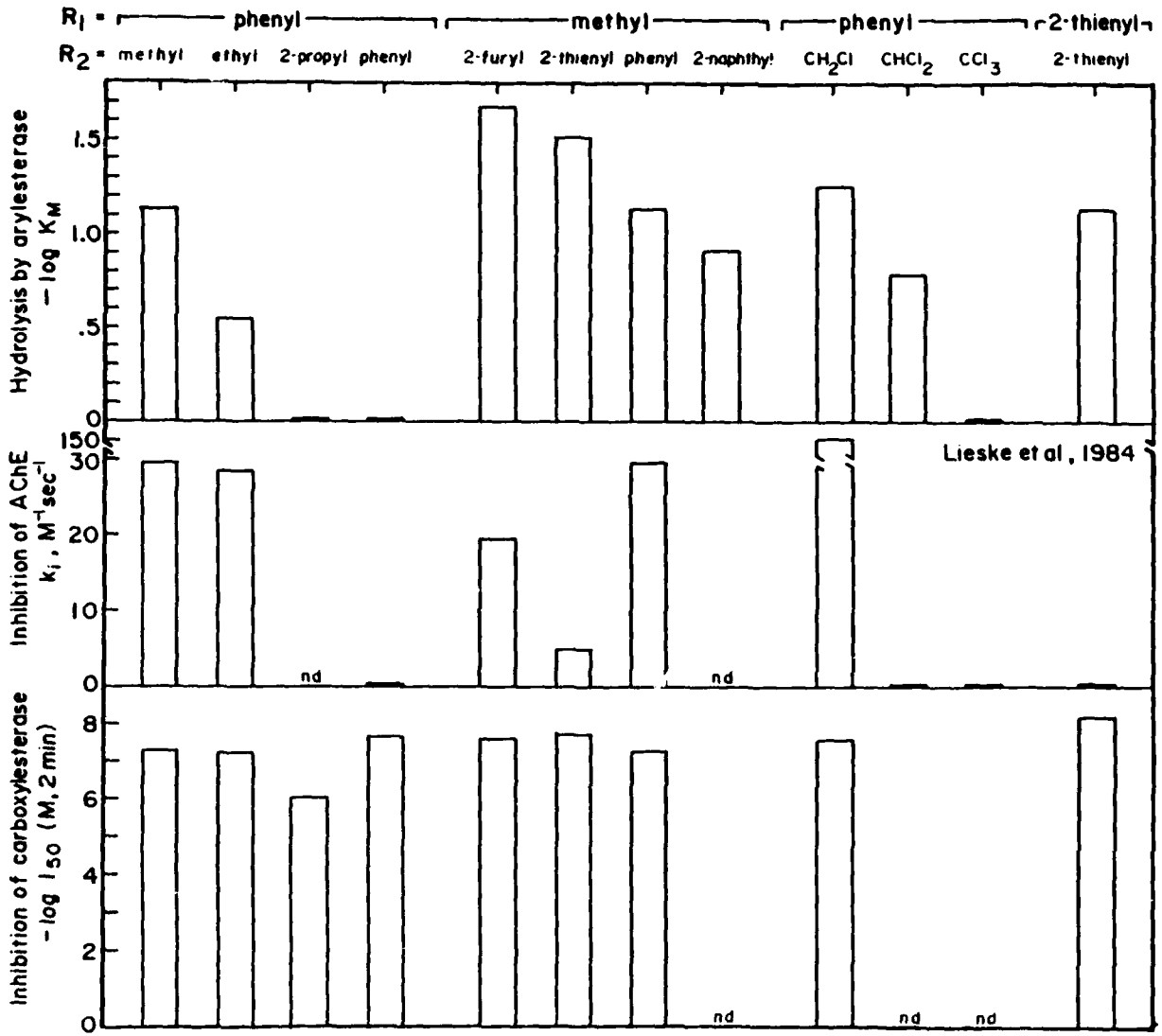


Figure 28. Comparison of enzymatic hydrolysis of 4-nitrophenyl organophosphinates with inhibitory activities toward acetylcholinesterase (AChE) and carboxylesterase; nd is not determined.

On the other hand, carboxylesterase was very susceptible to phosphinate inhibition as compared to acetylcholinesterase (Fig. 28). With the exception of IPP, these phosphinates were more potent inhibitors than 4-nitrophenyl diarylphosphinates tested by Ooms and Breebaart-Hansen (10). It was noted that very slow acetylcholinesterase inhibitors DPP and DTP were the most potent carboxylesterase inhibitors and it would be expected that this was due to lipophilic association and not to reactivity.

Spontaneous recovery of rabbit liver monomeric carboxylesterase was most rapid from phosphinyl groups, with one alkyl substituent smaller than isopropyl and one aryl or heteroaryl substituent smaller than naphthyl; an exception was the di-2-thienylphosphinylated enzyme which recovered rapidly while lacking an alkyl substituent (Fig. 29). This general pattern of recovery appeared to be similar to the pattern observed for two cholinesterases (94). Oxime TMB-4 doubled the reactivation rate of ethyl(phenyl)phosphinylated carboxylesterase (Fig. 30).

Carboxylesterase inhibition was observed in mice upon 2 h pretreatment with LD_{01} DPP injected im. While blood aspirin was transiently elevated by DPP, the hydrolysis product salicylic acid was not increased by phosphinates and was rapidly produced in all mice. Expired [^{14}C]carbon dioxide from aspirin hydrolysis was not reduced by phosphinate pretreatment. When [carboxyl-^{14}C]procaine was synthesized and administered to phosphinate pretreated mice, its metabolism was inhibited by DPP. Procaine in blood was increased nearly three-fold; confirmation was found when liver homogenates of DPP mice were only one-third as active as controls in hydrolyzing procaine. Pretreatment with MPP did not reduce drug metabolism in mice.

It is clear that carboxylesterase is very susceptible to organophosphinates inhibition and that drug interaction must be considered with pretreatment. It is also clear that each specific combination of pretreatment agent and drug should be considered until enough data are accumulated to generalize concerning the effect. It is also possible that transitory inhibition of xenobiotic-degrading hydrolases could be beneficial in protection against an exposure to a nerve agent such as soman in the same manner in which acetylcholinesterase would be protected.

Enantiomers of four phosphinates were separated by HPLC, using the chiral-phase, amino-bonded (R)-N-(3,5-dinitrobenzoyl)phenylglycine. Arylester hydrolase was selective in hydrolysis of (-)EPP nine times faster than (+)EPP. This was confirmed when (-)EPP was lost more rapidly from the racemic mixture exposed to arylester hydrolase. Likewise, (-)IPP from racemic IPP reacted more rapidly with equimolar chymotrypsin than did (+)IPP. On the other hand, acetylcholinesterase was selectively inhibited by (+)EPP and by (+)IPP, thereby reacting with opposite stereochemistry from that of both arylester hydrolase and chymotrypsin. Stereo selectivity of EPP and IPP resembles that of EPN-oxon and is unlike stereoselectivity of soman regarding the acetylcholinesterase vs. chymotrypsin relationship (Table 24). It appears that acetylcholinesterase and arylester hydrolase may be similar in stereochemical mechanism since

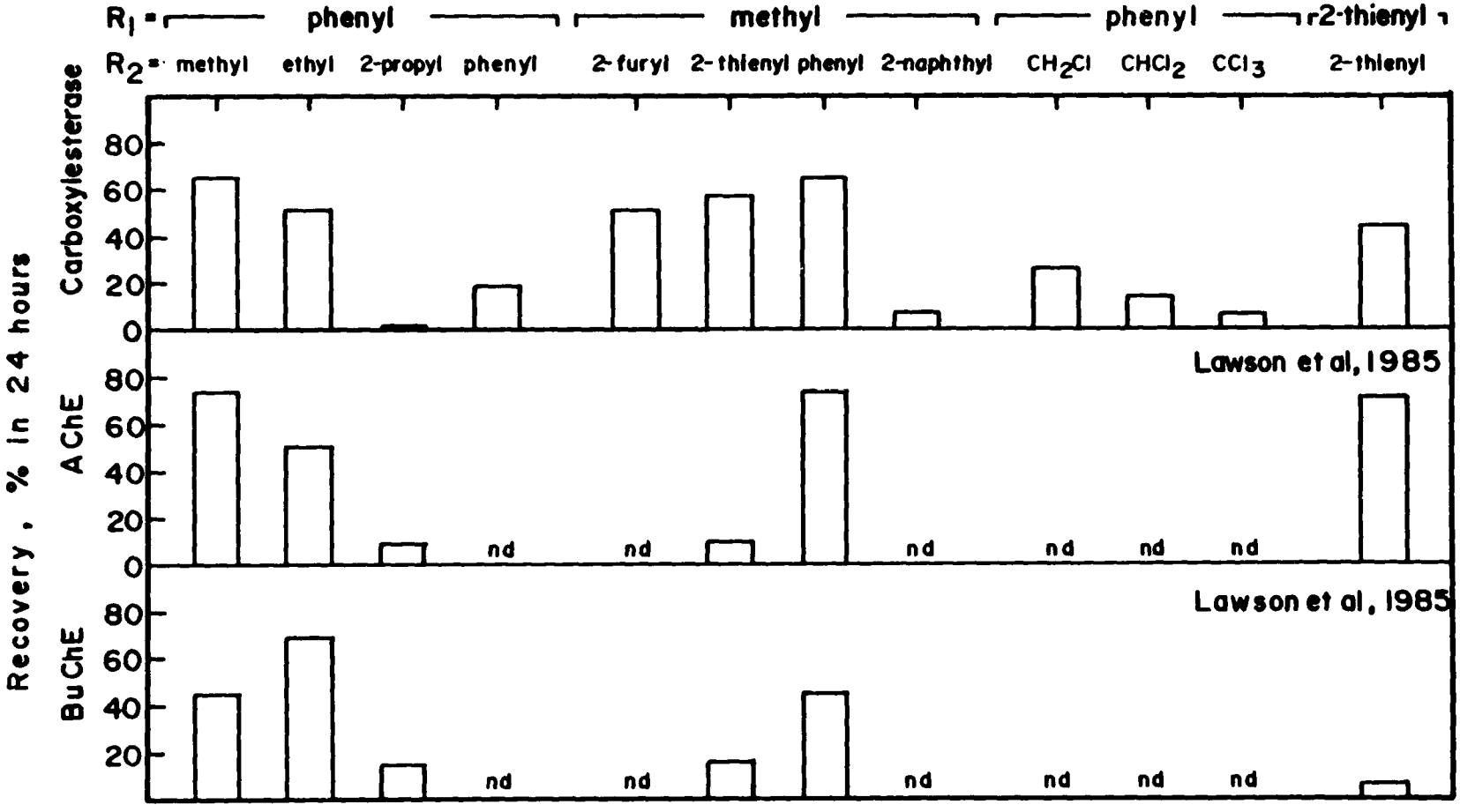


Figure 29. Comparative spontaneous reactivation of three enzymes following inhibition by 4-nitrophenyl organophosphinates. AChE, acetylcholinesterase; BuChE, butyrylcholinesterase; and nd, not determined.

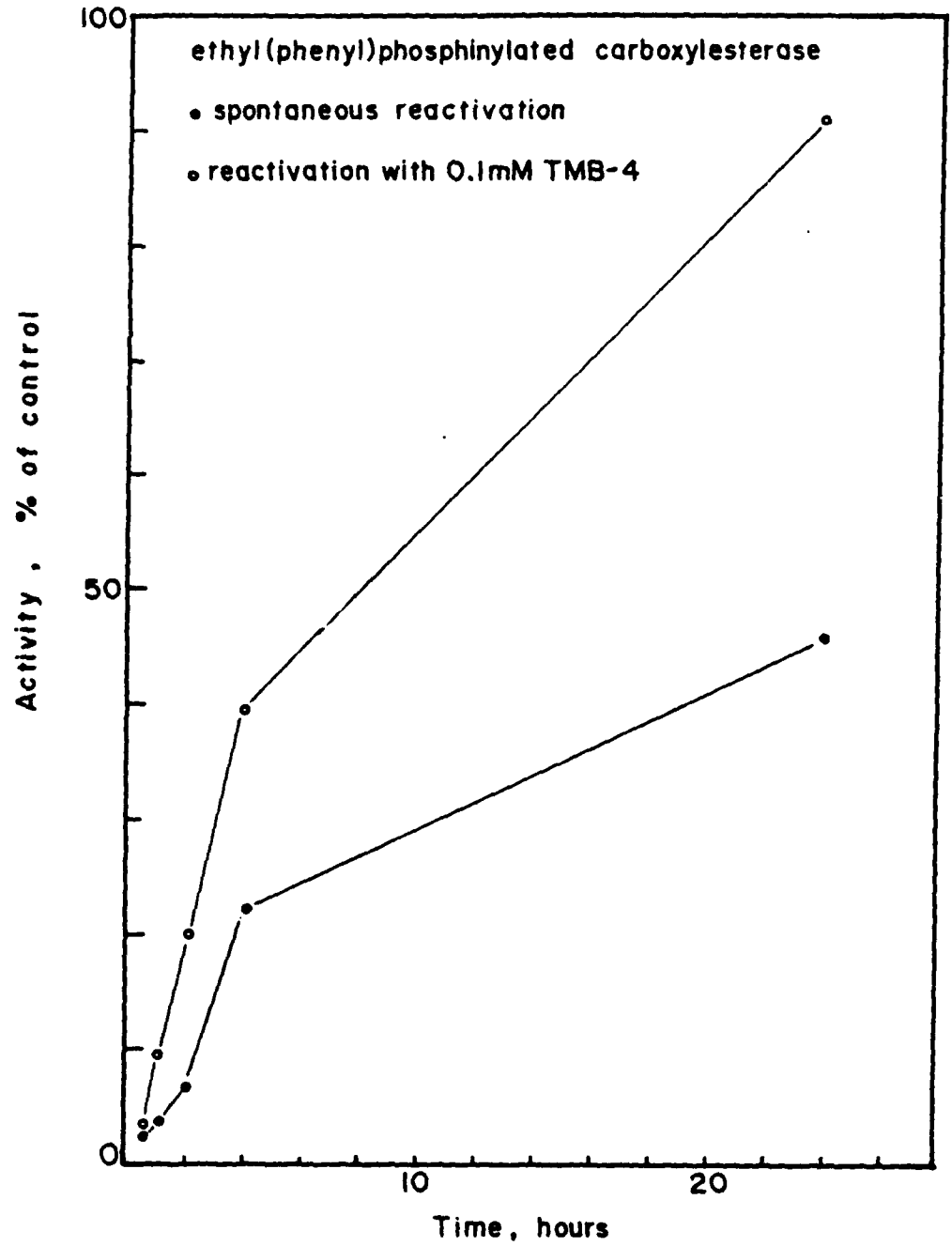


Figure 30. Oxime-induced reactivation of carboxylester hydrolase following inhibition by 4-nitrophenyl ethyl(phenyl)phosphinate.

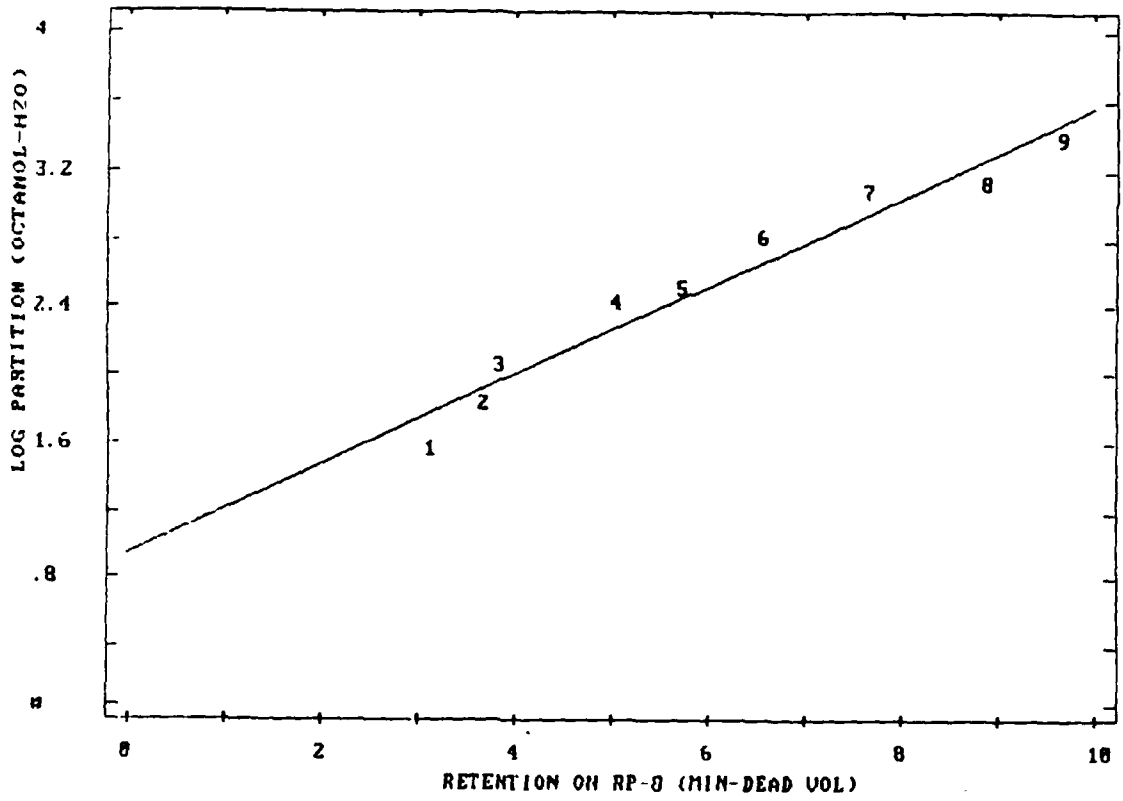


Figure 31. Correlation of retention time on reversed phase HPLC with the octanol-water partition values of organophosphinates. The retention times on octasilyl are from Brown and Grothusen (16), with the solvent front time subtracted. The octanol-water values were provided by C. N. Lieske (personal communication). The slope of the line is 0.261 with a Y intercept value of 0.971 and a correlation coefficient of linear least squares of 0.975.

Table 24. Stereoselectivity of enzymes toward organophosphinates and other chiral organophosphorus compounds.

Compound	Acetylcholinesterase	Arylester Hydrolase	Chymotrypsin
EPP	+	-	-
IPP	+	nd[a]	-
EPN-oxon (63)	+	-	-
soman (61)	-	+	-
4-nitrophenyl S-alkyl methylphosphonothiolates (68,69)			
methyl	+	+ (r_a[b]=1.2)	-
ethyl	-	+	+
propyl	-	+	-
butyl	-	+	-
pentyl	-	+	-

[a] nd: Not determined.
[b] r_a: Ratio of activity of enantiomers

they are opposite in stereoselectivity. On the other hand, the relationship between acetylcholinesterase and chymotrypsin varies with the size of the alkyl ether (or thioether).

There are many factors that need to be considered in any attempt to develop quantitative structure-activity relationships for the phosphinates with enzymes. Two factors that we attempted to use in correlations were hydrophobicity and size of the phosphinates.

Hydrophobicity is commonly measured using octanol-water partitioning. Reversed phase high performance liquid chromatography has been used as an alternate method to octanol-water values (92,93). We obtained octanol-water partition values for a number of phosphinates from C. N. Lieske (94) and compared them to the reversed phase HPLC retention times we have previously reported (16). We get an excellent linear correlation between the log of the octanol-water partition values and the retention times on the C_8 reversed phase column (Fig. 31). The use of HPLC to estimate hydrophobicity of phosphinates is fast, easy, and unlike octanol-water partitioning, is not complicated by spontaneous breakdown of the phosphinates.

Table 25. Linear correlation coefficients for enzyme data.

	R	N
Specific activity (arylester hydrolase) vs. size	-0.79	10
- Log K_i (acetylcholinesterase) vs. size	-0.70	10
Specific activity (arylester hydrolase) vs. retention time	-0.66	10
- Log K_i (acetylcholinesterase) vs. retention time	-0.59	10
Recovery (carboxylesterase) vs. retention time	-0.55	13
Recovery (carboxylesterase) vs. size	-0.53	13
- Log K_m (arylester hydrolase) vs. retention time	-0.29	9
- Log K_m (arylester hydrolase) vs. size	-0.18	9
I_{50} (carboxylesterase) vs. size	-0.097	8
I_{50} (carboxylesterase) vs. retention time	-0.039	8

We built models of each of the phosphinates in this project using a CPK molecular model kit. Each model was placed on a copy machine with the two P-C bonded substituents flat against the glass. The conformation used was that which gave the minimum overlap between the two substituents. The silhouettes were cut out and the areas determined by weighing. This silhouette procedure is similar to that described by Amoore (95). To convert from model size to molecular size the value of 1 Å/1.25 cm of model length was used. Fig. 27 shows the silhouettes of the P-C bonded substituents of the phosphinates arranged from smallest (4-nitrophenyl bis-chloromethylphosphinate) to largest surface area (4-nitrophenyl diphenylphosphinate).

We attempted linear correlations of our data from carboxylesterase, paraoxonase and data from acetylcholinesterase (11) with the two parameters hydrophobicity (retention on C_8 HPLC) and size (silhouettes of P-C substituents) and used the correlation coefficient of linear least squares as a first approximation of fit (Table 25). It is apparent from these results that neither size nor hydrophobicity alone accurately define structure-activity relationships with these phosphinates and enzymes. The relationships are more complex than a simple linear fit. We need to look at non-linear as well as multiparameter relationships.

Where R is the correlation coefficient of linear least squares and N the number of phosphinates. The specific activity and K_m data are from the Second Annual Progress Report. The recovery and I_{50} data are from the First Annual Progress Report. The k_i data are from Lieske et al. (11). While it is apparent that these attempts at quantitative structure-activity relationships revealed little useful information, the best correlations reinforce the observation that acetylcholinesterase and arylester hydrolase are similar in interactions with organophosphinates; i.e., rapid acetylcholinesterase inhibitors are usually good substrates for arylester hydrolase and are easily hydrolyzed. It appears that for most relationships a greater diversity of chemical structure must be employed to produce meaningful correlations.

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10. Publications and Presentations:

10a. Publications in Scientific Journals:

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10b. Papers Presented at Scientific Meetings:

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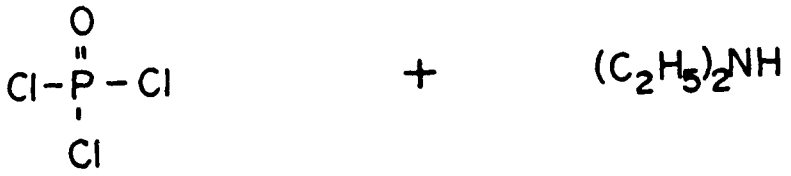
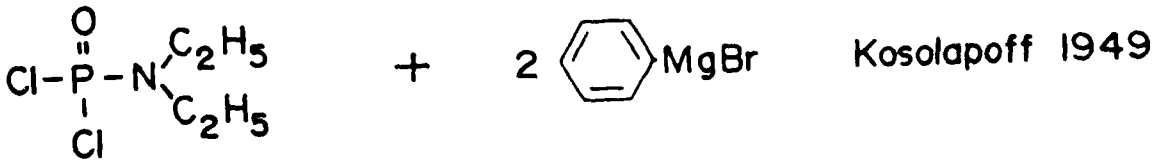
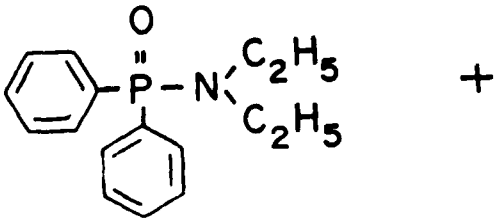
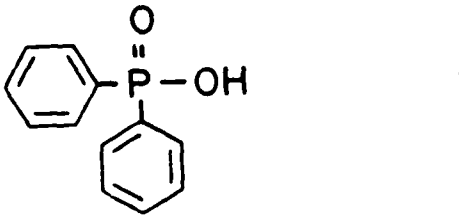
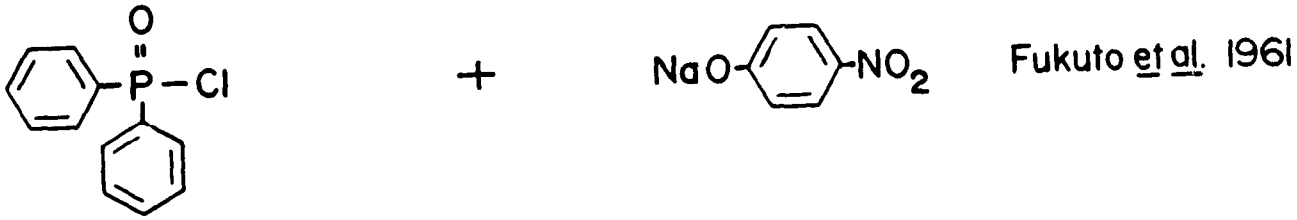
Appendix A. Conventional route of synthesis of 4-nitrophenyl diphenylphosphinate (13, 14).

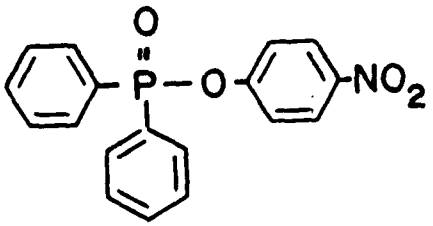


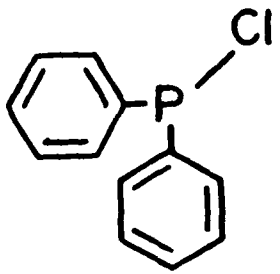




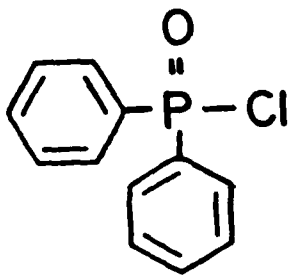


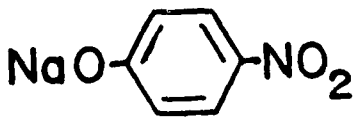




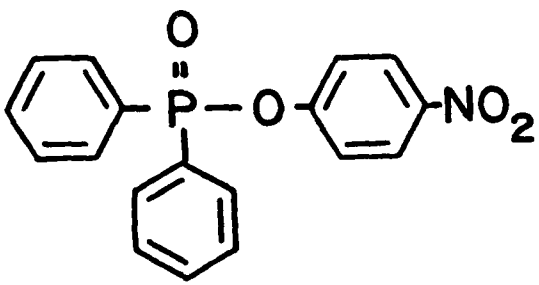


+ $1/2\ O_2$ Tyssee et al. 1973



+ 

Fukuto et al. 1961

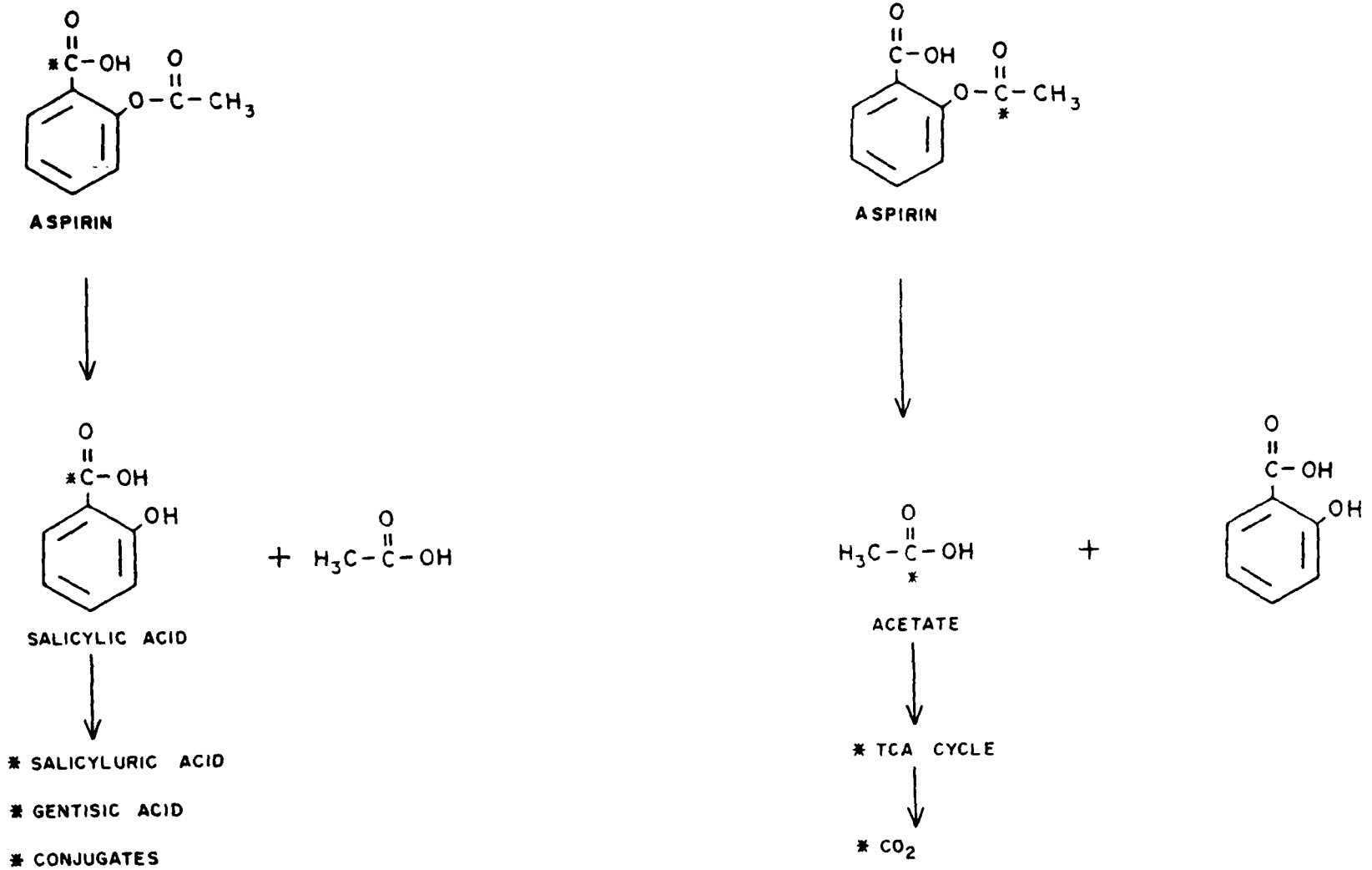


Appendix B. Abridged route of synthesis of 4-nitrophenyl diphenylphosphinate (13, 15).

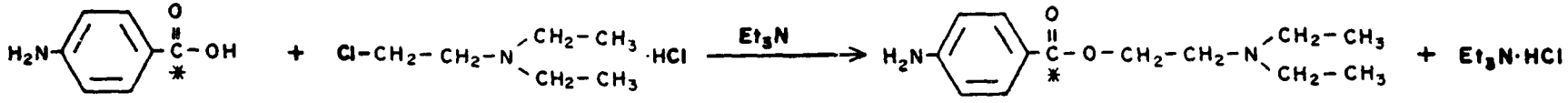
Appendix C. Chromatography of methyl-series phosphinates on various HPLC chiral stationary phases with mobile phases pumped at 1.0 ml/min at 18°C.

Compound	Chiral Stationary Phases			
	L-DNBPG[a]	leucine[a]	leucine[b]	urea[a]
	Retention times of peaks (min)			
4-nitrophenol	14.9	12.6	31.2	10.1
(MPP) methylphenyl	15.3, 33.5	12.7, 20.0	31.4, 43.2	9.1
(MNP) methyl-2 naphthyl	15, 44.6	12.9, 23.6		
(MTP) methyl-2-thienyl	15.3, 33	12.7, 22.2		
(MFuP) methyl-2-furyl	14.9, 25.5, 26	12.8, 14.4		
(TFPMP) trifluoromethylphenyl	15, 22.4	12.6, 26		
(EPP) ethyl phenyl		12.8, 14.5	27.5, 30.5	7.0

[a] Mobile phase was 7% 2-propanol in hexane with 0.1% HOAc added
[b] Mobile phase was 3.5% 2-propanol in hexane with 0.1% HOAc added



Appendix D. Use of radiolabeled (*) aspirin for measuring production of hydrolysis products in two experiments; TCA is tricarboxylic acid.



Appendix E. Synthesis of [carboxyl-^{14}C]procaine.

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