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REACTIVATION OF CARBOXYLESTER HYDROLASE FOLLOWING INHIBITION BY 4-NITROPHENYL ORGANOPHOSPHINATES

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Abstract—Porcine liver carboxylester hydrolase (EC 3.1.1.1; 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 7×10^{-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 it recovered rapidly while lacking an alkyl substituent. Oxime reactivation by TMB-4 doubled rates of recovery.

Nitrophenyl dialkylphosphinates are inhibitors of horse liver carboxylesterase although they are less inhibitory than their phosphonate and phosphate homologs [1]. Organophosphinates also inhibit acetylcholinesterase [2, 3]; however, spontaneous recovery of phosphinylated acetylcholinesterase [4] is more rapid than that of phosphorylated acetylcholinesterase [5].

Organophosphinates do not produce delayed neurotoxic effects in hens, and they also protect hens from the effects of subsequent administration of several organophosphates which are neurotoxic in the absence of organophosphinates [6]. It appears that the lack of dealkylation (aging) of phosphinylated enzymes enhances the rate of recovery and precludes delayed neurotoxicity [7].

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 [8], 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.

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, Inc., Detroit, MI, 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 the Sigma Chemical Co., St. Louis, MO.

Inhibition experiments employed 1 μg/ml Type II porcine liver carboxylesterase (Sigma Chemical Co.) which was incubated with inhibitor in the absence of substrate in 0.1 M sodium phosphate buffer, pH 7.5, at 37°. 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 [9], an apparent K_m of 0.0356 mM was computed from an Eadie–Hofstee plot. Enzymic 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 of 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 mmole · min^{-1} · mg protein^{-1}. In a confirmatory experiment, phenylthiobutyrate was substituted as substrate and the hydrolysis product was measured after a chromogenic reaction [10].

Spontaneous reactivation experiments were performed with rabbit liver monomeric carboxylesterase [11] 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 [11] and also due to the greater simplicity of its monomeric form. Procedures were similar to those used with phosphinylated acetylcholinesterase [4]. A molecular size exclusion column 10.8 cm by 0.8 cm was prepared from 1 g Sephadex G-10-120, swollen for 3 hr in 0.1 M sodium phosphate buffer, pH 7.6, and calibrated with Blue Dextran, *N*-2,4-dinitrophenyl-L-phenylalanine and

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

Number	Compound	Molecular weight
	Phenyl series:	
I	4-nitrophenyl methyl(phenyl)phosphinate	277
II	4-nitrophenyl ethyl(phenyl)phosphinate	291
III	4-nitrophenyl isopropyl(phenyl)phosphinate	305
IV	4-nitrophenyl diphenylphosphinate	339
	Methyl series:	
V	4-nitrophenyl methyl(2-furyl)phosphinate	267
VI	4-nitrophenyl methyl(2-thienyl)phosphinate	283
(I)	4-nitrophenyl methyl(phenyl)phosphinate	277
VII	4-nitrophenyl methyl(1-naphthyl)phosphinate	327
	Heterocycle series:	
(V)	4-nitrophenyl methyl(2-furyl)phosphinate	267
VIII	4-nitrophenyl di-2-furylphosphinate	335
(VI)	4-nitrophenyl methyl(2-thienyl)phosphinate	283
IX	4-nitrophenyl di-2-thienylphosphinate	351
	Halogen series:	
X	4-nitrophenyl *bis*-chloromethylphosphinate	284
XI	4-nitrophenyl monochloromethyl(phenyl)phosphinate	312
XII	4-nitrophenyl dichloromethyl(phenyl)phosphinate	347
XIII	4-nitrophenyl trichloromethyl(phenyl)phosphinate	382

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 a 9.74×10^{-4} M concentration of the 2-furyl derivatives, V and VIII, 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° in a shaking water bath. Recovery of enzyme activity from the column was 97.1% in this initial eluate, and spectrophotometry indicated that unbound II was completely separated from the enzyme by this technique. Spontaneous reactivation was assessed by adding 20 μl of the purified, phosphinylated enzyme to 2.6 ml of 0.2 mM 1-naphthyl butyrate for 10 min at 37°, and then terminating the reaction with tetrazotized *o*-dianisidine and measuring the absorbance as above. To examine reactivation at 25°, 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° and compared to a companion control. Inhibition and reactivation were replicated for each phosphinate on 4 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 1,1′-trimethylene-bis(4-formylpyridinium bromide) dioxime (TMB-4) was added to one half to give a 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° 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.

RESULTS

Three heterocyclic organophosphinates and five phenylphosphinates were inhibitors of carboxylesterase (Table 2). By this estimate, IX was the most inhibitory of the eight compounds tested; III was the least inhibitory. Six other compounds within these series were similar in potency having median inhibitory values within a 3-fold range of concentrations. Replacing the phenyl moiety with the thienyl group resulted in a 3-fold increase in inhibition (Table 2). This was observed in both the methyl(2-thienyl) compound and the di-2-thienyl compound when compared to their phenyl analogues.

Bimolecular reaction constants of inhibition could not be determined since many plots of log % of control activity versus time were non-linear or did

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

Inhibitor	I_{50} (2 min), M
IX	7.4×10^{-7}
VI	1.7×10^{-6}
IV	2.1×10^{-6}
V	2.5×10^{-6}
XII	2.8×10^{-6}
I	5.1×10^{-6}
II	5.3×10^{-6}
III	9.6×10^{-6}
Paraoxon	8.0×10^{-7}

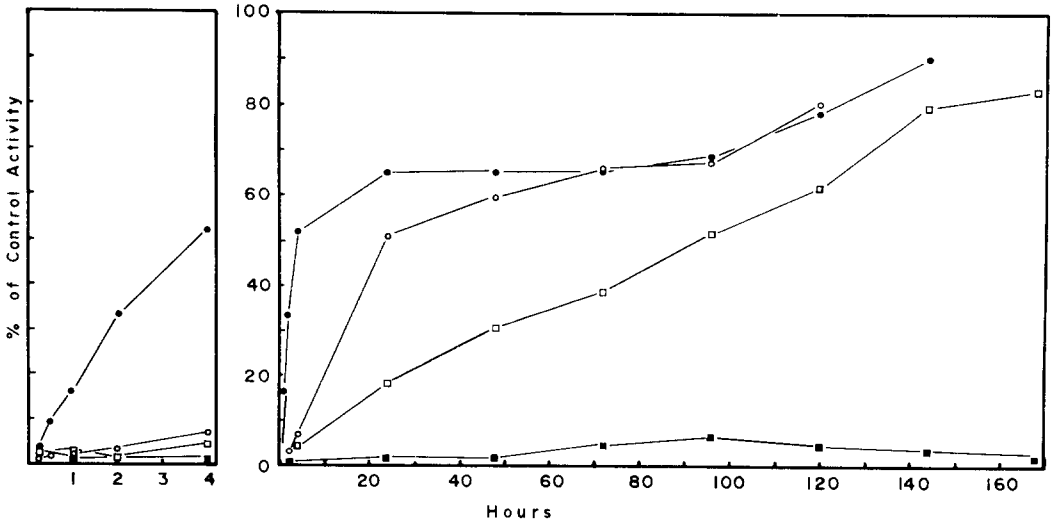

Fig. 1. Spontaneous reactivation at 37° of rabbit liver monomeric carboxylester hydrolase following inhibition by 4-nitrophenyl substituted organophosphinates of the phenyl series. Key: (●) methyl(phenyl), (○) ethyl(phenyl), (■) isopropyl(phenyl), and (□) diphenyl.

not pass through 100% activity at zero time [12]. The curvature in the lines obtained from results for most of these phosphinates was expected since the calculated enzyme concentration [13] 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. 1–4).

Rabbit liver monomeric carboxylesterase recovered more than 80% of control activity after complete inhibition by compounds I, II and IV of the phenyl series (Fig. 1). Initially, spontaneous reactivation was extremely rapid from the methyl(phenyl)phosphinylated carboxylesterase, which recovered one half its activity in only 4 hr. Following inhibition by the phenyl series, carboxylesterase reactivation ranked as methyl > ethyl > phenyl ≫

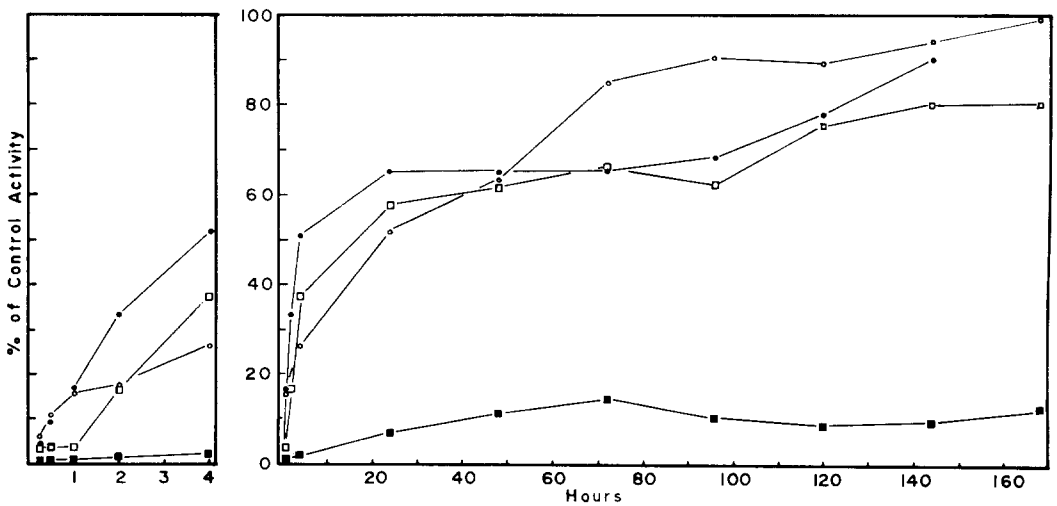

Fig. 2. Spontaneous reactivation at 37° of rabbit liver monomeric carboxylester hydrolase following inhibition of 4-nitrophenyl substituted organophosphinates of the methyl series. Key: (●) methyl(phenyl), (○) methyl (2-furyl), (■) methyl(naphthyl), and (□) methyl(2-thienyl).

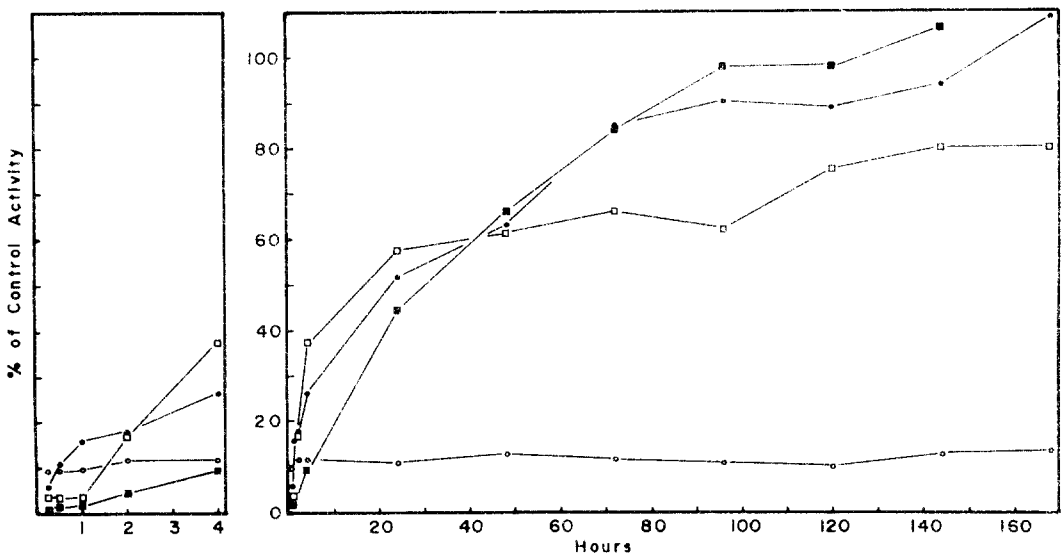

Fig. 3. Spontaneous reactivation at 37° of rabbit liver monomeric carboxylester hydrolase following inhibition by 4-nitrophenyl substituted organophosphinates of the heterocycle series. Key: (●) methyl(2-furyl), (○) di-2-furyl, (■) di-2-thienyl, and (□) methyl(2-thienyl).

isopropyl. Reactivation was generally faster following inhibition by compounds of the methyl series (Fig. 2) than it was following the phenyl series. Recovery from I, V and VI exceeded 25% in 4 hr and 80% in 6 days. Reactivation from VII was very slow. Based on initial reactivation rates, recovery from the methyl series was in the order phenyl > 2-thienyl > 2-furyl ≫ 1-naphthyl. Reactivation plots of carboxylesterase inhibited by compounds V, VI and IX of the heterocycle series produced similar results: approximately 60% recovery at 40 hr (Fig. 3). There was no progressive reactivation following inhibition by VIII. Based on recovery during 24 hr, 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 XI, XII and XIII of the halogen series, reactivation was inversely related to the number of chlorine atoms in the inhibitor (Fig. 4). While XIII 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 X was slow and data were highly variable. Recovery rates for the chloro-methyl(phenyl) phosphinylated carboxylesterase were ranked as monochloro > dichloro > tricholoro.

Spontaneous reactivation did not proceed to com-

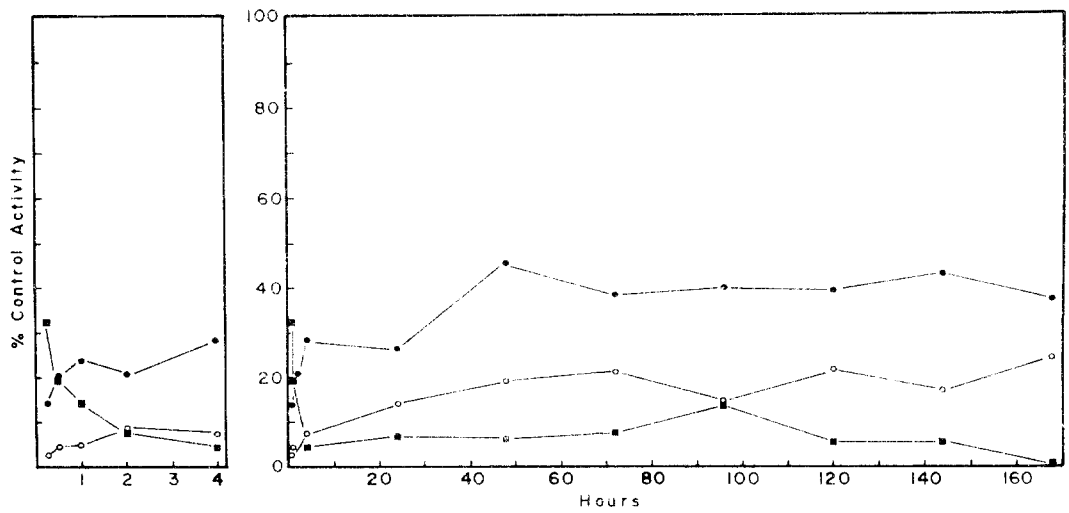

Fig. 4. Spontaneous reactivation at 37° of rabbit liver monomeric carboxylester hydrolase following inhibition by 4-nitrophenyl substituted organophosphinates of the halogen series. Key: (●) monochloromethyl(phenyl), (○) dichloromethyl(phenyl), and (■) trichloromethyl(phenyl).

Table 3. Spontaneous reactivation of carboxylester hydrolase following inhibition by substituted 4-nitrophenyl organophosphinates

Compound	Substitutions on phosphinate	Reactivation (% of control activity ± S.E.) 24 hr	72 hr	k, min^{-1}*
I	methyl(phenyl)	65.3 ± 5.6	65.5 ± 4.8	6.7×10^{-4}
VI	methyl(2-thienyl)	57.6 ± 2.8	66.0 ± 2.5	5.8×10^{-4}
V	methyl(2-furyl)	51.9 ± 4.9	85.1 ± 2.1	3.9×10^{-4}
II	ethyl(phenyl)	50.8 ± 1.9	65.8 ± 2.6	4.8×10^{-4}
IX	di-2-thienyl	44.4 ± 8.2	84.0 ± 9.1	6.0×10^{-4}
XI	monochloromethyl(phenyl)	26.5 ± 2.2	40.2 ± 14.6	
IV	diphenyl	18.5 ± 3.9	39.0 ± 3.0	1.2×10^{-4}
XII	dichloromethyl(phenyl)	14.2 ± 3.5	21.4 ± 3.5	
X	*bis*-chloromethyl	12.4 ± 4.6	13.6 ± 9.4	
VIII	di-2-furyl	10.6 ± 1.3	11.4 ± 1.0	
VII	methyl(1-naphthyl)	6.8 ± 0.4	14.7 ± 4.7	
XIII	trichloromethyl(phenyl)	6.5 ± 2.0	7.5 ± 5.2	
	paraoxon	5.9 ± 2.1	5.4 ± 3.6	
III	isopropyl(phenyl)	1.5 ± 0.9	4.4 ± 1.0	9.9×10^{-6}

* Reactivation rate constant based on first-order portion of reactivation curve which included data through 24 hr for I, II and VI and data through 96 hr for III, IV, V and IX.

pletion 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 III to 6.7×10^{-4} min^{-1} for I (Table 3). 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 IX which contained two thienyl groups, yet recovered rapidly. Compounds can be grouped according to the rates at which recovery proceeded as follows: fast recovery—I, II, V, VI, and IX; moderate recovery—IV and XI; slow progressive recovery—XII; and no progressive recovery—III, VII, VIII, X, XIII and paraoxon.

At 25°, reactivation rates from I and II were reduced from the rates at 37° (Table 4).

Oxime-induced reactivation of ethyl(phenyl) phosphinylated carboxylesterase was twice as rapid as spontaneous recovery so that 91% reactivation was induced in 24 hr by TMB-4 (Table 5). 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 hr as compared to 50.8% with sodium phosphate (Table 3). Reactivation of isopropyl(phenyl)phosphinylated carboxylesterase was also enhanced by oxime treatments.

Table 4. Effect of temperature on spontaneous reactivation of carboxylester hydrolase following inhibition by organophosphinates

Inhibitor	Time (hr)	Reactivation (% of control activity) 25°	37°	25°/37°
I	4	21.7	51.8	0.42
I	24	46.5	65.3	0.71
II	4	1.9	6.9	0.27
II	24	26.1	50.8	0.51

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 III; 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 $moles^{-1}$ min^{-1} with horse liver carboxylesterase [1].

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} [14]. 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} [15]. 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 I with a rate constant of 3.6×10^{-4} min^{-1} at 25°; there was lesser recovery from IV and from the dimethyl analogue [4]. Our results with these phosphinates in monomeric carboxylesterase recovery were very

Table 5. Oxime-induced reactivation of rabbit liver monomeric carboxylester hydrolase following inhibition by organophosphinates

Time (hr)	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

similar except that reactivation with I, II and VI 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 [11]; however, I, II and VI 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 [16], slower than methyl(phenyl)phosphinylated acetylcholinesterase, and faster than diphenylphosphinylated acetylcholinesterase [4]. Those studies found that pyridine-2-aldoxime methochloride (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 III to be refractory to TMB-4 reactivation, and it should be tested with acetylcholinesterase. Comparison of spontaneous and induced reactivation following inhibition by II indicated that the portion of activity with slow spontaneous recovery was not resistant to TMB-4-induced reactivation.

When rat plasma was exposed to soman, inhibited carboxylesterase reactivated from sodium fluoride treatment while inhibited acetylcholinesterase did not reactivate [17]. While liver carboxylesterase has been recognized as important in xenobiotic detoxication [13], 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.

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