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STRUCTURE–ACTIVITY RELATIONSHIPS FOR SUBSTRATES AND INHIBITORS OF HEN BRAIN NEUROTOXIC ESTERASE

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Abstract—(1) Neurotoxic esterase is one of several paraoxon-resistant esterases of hen brain. It has previously been assayed with phenyl phenylacetate as substrate by a differential assay using Mipafox as selective inhibitor. The Mipafox-sensitive activity is a greater proportion (55 per cent) of the total when phenyl valerate is used as substrate and this activity behaves as a single enzyme according to several tests. Phenyl esters of other acids and esters of other phenols are less specific substrates and most are hydrolysed slower than phenyl valerate. (2) Neurotoxic esterase does not significantly hydrolyse peptides or amides but some hydrophobic peptides and amides are non-progressive inhibitors. (3) Inhibition by a range of organophosphorus, carbamic and sulphur-acid esters has been investigated. (4) Neurotoxic esterase is similar to chymotrypsin and trypsin but unlike acetylcholinesterase in the pattern of inhibition by organophosphorus esters. The structure–activity relationships presented give some guidance for design of non-neurotoxic pesticides. (5) More stable and less toxic alternatives to Mipafox as a selective inhibitor of neurotoxic esterase have been found. (6) Benzenesulphonyl fluoride is a selective inhibitor of some of the Mipafox-resistant esterases. (7) All the esterases were inhibited by PCMB (0·1 mM) and by Zn^{2+} (0·4 mM).

Nervous tissue of the hen contains a number of esterases which will hydrolyse phenyl phenylacetate. The two most active were shown to be inhibited by low concentrations of paraoxon [1, 2]. The paraoxon-resistant esterases include one which is now called neurotoxic esterase: it is inhibited *in vivo* by those organophosphorus esters which cause delayed neurotoxic effects seen as degeneration of long axons [3, 4]. The intoxication process is unusual in that the metabolic disturbance is dependent on the nature of the chemical group which becomes bound to the esterase active site: it does not depend on the loss of catalytic activity *per se* [5, 6].

Assay *in vitro* of neurotoxic esterase activity of brain taken from hens dosed with organophosphates has provided a measure of neurotoxic potential [3, 5]. However, activity of compounds added directly to an *in vitro* system can be more easily related to chemical structure provided that they do not require metabolic activation. The activity *in vitro* of a large number of progressive and non-progressive inhibitors of neurotoxic esterase is reported in this paper. Some generalizations about structure-activity relationships and comparison of neurotoxic esterase with other esterases are made. Such a study may ultimately help in understanding the topography of the active site, and is of immediate practical relevance in design both of pesticides with little neurotoxic hazard and of protective compounds.

The published assay of neurotoxic esterase [5] is a differential procedure. Paired samples of tissue are preincubated with paraoxon to inhibit two irrelevant enzymes [1]. One tube also contains the neurotoxic organophosphate Mipafox which selectively inhibits neurotoxic esterase. Phenyl phenylacetate is then added to both tubes and the rate of hydrolysis determined. The difference between the rates of hydrolysis in the two samples is a measure of neurotoxic esterase. The above procedure has limitations. It is desirable that the Mipafox-sensitive activity should be a large fraction of the total, but with phenyl phenylacetate as substrate only about 30 per cent of the total activity is Mipafox-sensitive. Moreover phenyl esters are unsuited to histochemical assays since the liberated phenol cannot be converted to an insoluble coloured product. A number of analogues of phenyl phenylacetate have therefore been examined as alternative substrates and inhibitors have been sought which would eliminate the Mipafox-insensitive activity. Also although Mipafox is a selective inhibitor it is unstable and neurotoxic in man. Among the compounds screened, a stable non-toxic carbamate has been found which can be used as an alternative to Mipafox.

MATERIALS AND METHODS

Tissue. Homogenates (10% w/v) of whole brain were prepared in buffer by using the rotating (1800 rev/min) smooth Perspex® pestle with 0·005 in. difference in diameter between tube and pestle [7]. The buffer was usually Tris (50 mM) containing EDTA (0·2 mM) adjusted with HCl to pH 8·0 at 20°. A less turbid preparation was obtained by centrifuging a homogenate prepared in 0·3 M sucrose at 9,000 *g* for 15 min; the supernatant contained about 85 per cent of the original activity of neurotoxic esterase. A paraoxon-treated semi-solubilised preparation in 0·04% sodium deoxycholate was obtained from a centrifuged particulate fraction as described previously [8]: this contained about 35 per cent of the activity of neurotoxic esterase compared with original homogenate and was almost clear; it was used in direct fluorimetric assays.

Chemicals. Sources of compounds are listed below. Unless specifically noted these were not less than 90–95 per cent pure and contained no known active impurities.

Compounds 1–5 were from Shell Research Ltd., and Cmpds 6–8, 61 and 63 were synthesised by reaction of the appropriate amine or phenol with 2,2-dichlorovinyl phosphoryl dichloride supplied by Shell Research Ltd. Cmpds 9, 11–12, 28–31, 36 and 65 were from Albright & Wilson Ltd., who also supplied the dialkyl phosphites used to synthesize Cmpds 13 and 14 via the dialkyl phosphoryl chlorides [9]. Compounds 15–17 and 45 were from Cooper Research Laboratories; Cmpds 19, 23, 24, 46, 47 from A. G. Bayer Farbenfabricken; Cmpds 20–22 from Plant Protection Ltd.; Cmpds 25–27 from World Health Organization Vector Control Section; Cmpds 32, 40–43 from Prof. D. Henschler; Cmpds 33–35, 38–39 from Coalite Chemical Co.; Cmpd 44 from Prof. M. Eto; Cmpd 48 from Velsicol Co.; Cmpds 49–53 from Prof. E. Becker; Cmpd 54 from Prof. R. Hollingworth; 4-nitrophenyl benzyl carbonate and Cmpd 64 from C.D.E., Porton; Cmpds 91–96 from Prof. R. Fukuto; phenylglyoxaldoxime and Cmpd 105 from Ralph Emanuel Ltd; amino-acid naphthylamides and phenyl nicotinate from Koch-Light & Co.; phenyl benzoate from B.D.H. Ltd.; 4-methylumbelliferyl phenylacetate from Dr. D. Robinson; peptides from Sigma Chemical Co.; and benzohydroxamic acid from Schuchardt Chemical Co. Compounds 10, 54–60, 62, 67, 93, 95, 96 were the same samples as used previously [3, 5, 6, 10]. Where no source is listed the compound was prepared in this laboratory by a standard method from commercially available intermediates. Solid products were recrystallised to constant m.p. and liquids were redistilled *in vacuo*. Identity was presumed from the nature of the reaction and confirmed where possible by i.r. and n.m.r. analysis. Elemental analyses were not performed. Carboxylic acid esters were synthesized from commercially supplied acid chlorides and phenols. Carbamates were prepared by reaction of the appropriate amine with a chloroformate or by reaction of an isocyanate with the appropriate phenol in the presence of an equimolar amount of trifluoracetic acid. Typical carbamate and ester preparations and characterizations have been described previously [5].

Esterase assays. When hen brain homogenate is incubated for 30 min at 25° with paraoxon (10 μM) about 85 per cent of the hydrolytic activity against phenyl phenylacetate is inhibited while most of the remaining activity is resistant to concentrations of 10–100 μM [3]. The paraoxon-sensitive activity is due to two enzymes [1, 2]. These were completely inhibited in the following experiments by including paraoxon (40 μM) in all preincubation media. All measurements were made in duplicate.

For phenyl ester substrates the conditions were adapted to 37° from the method of Johnson [3, 4]. Paired samples of homogenate were preincubated for 20 min with paraoxon (40 μM) plus either (a) Buffer or (b) Mipafox (50 μM) in a final volume of 3 ml Tris–HCl buffer (50 mM, pH 8·0) containing EDTA (0·2 mM). After preincubation, 3 ml of substrate dispersion was added and incubation was continued for a further 15 min. Dispersions of substrate were prepared by adding a solution of Triton X-100 (0·03% in water) (30 vol) to a solution of substrate in redistilled dimethylformamide (1 vol) and mixing thoroughly. Except where noted in Table 1, undissolved substrate was present throughout the incubation. Reaction was stopped by addition of 3 ml of $HClO_4$ (0·3 M). Tubes were cooled in ice and centrifuged at 1500 *g* for 15 min. Phenol in the supernatant was estimated with aminoantipyrine as described previously [3]. The rate of production of phenol was linear for all substrates and was not altered by addition of progressive inhibitors immediately before the substrate. For 2- and 3-chlorophenylacetate, product could be estimated with aminoantipyrine but ϵ could not be determined since pure samples of the phenols were not available. The rate reported assumes the same ϵ (13,900) as for phenol.

Hydrolysis of *a*-naphthyl and 3-dimethylaminophenyl was estimated exactly as for phenyl esters: ϵs were 16,400 and 12,000 respectively.

Hydrolysis of phenyl thiolovalerate was carried out as for phenyl esters. To estimate liberated thiophenol, 4 ml of acid-quenched medium was added to 2 ml of 0·5 M Tris–HCl (pH 9·0) followed by 1 ml of a solution of 5,5′-dithiobis-(2-nitrobenzoic acid) (0·04% in 0·3 M Na_2HPO_4 pH 7·7). The stable yellow colour was read at 412 nm (ϵ = 12,400).

Hydrolysis of 4-nitrophenyl benzyl carbonate was carried out as for phenyl esters. To estimate liberated nitrophenol, 4 ml of acid-quenched medium was added to 3 ml of 0·5 M Tris–HCl (pH 9·0). The pH of the mixture was about 8·3 and ϵ was 13,000 at 410 nm.

For 4-methylumbelliferyl phenylacetate a fluorescence method was used. Pairs of samples of paraoxon-treated semi-solubilised brain particles (see above: 1 ml equivalent to about 15 mg original brain) were preincubated in a total of 3 ml phosphate buffer (10 mM pH 7·0) alone or containing Mipafox (128 μM) for 20 min at 37°. A stock solution of substrate in acetone (0·1 ml) was added to give a final concentration of 15 μM (which gave maximum enzymic rate). Reaction was linear for 7·5 min and was stopped by addition of 3 ml of a mixture of glacial acetic acid–water–ethanol (4·5:25·5:70 v/v) giving a clear solution pH 4–4·2. Fluorescence of liberated 4-methylumbelliferone was read immediately in a 1-cm cell of an Aminco–Bowman spectrofluorimeter at 460 nm with excitation at 330 nm. Sensitivity was set $\times$0·1. Under these conditions an approximately linear calibration curve was obtained (40% $T \equiv 0{\cdot}5$ μM). The non-enzymic rate was about one-third of the total paraoxon-resistant rate and increased rapidly at higher pH.

Attempts to estimate 4-hydroxyazobenzene by diazo-coupling methods were unsuccessful. A direct but insensitive spectrophotometric assay of hydrolysis of the valeryl ester was possible in solutions where turbidity was reduced by using very dilute substrate and the low-speed supernatant fraction from brain homogenate as enzyme source (see above). To avoid colour interference by 4-nitrophenol, paraoxon was replaced by tetraethylpyrophosphate (40 μM) which inhibits both paraoxon-sensitive phenyl phenylacetate esterases without affecting the remainder [1, 3]. Paired tissue samples (equivalent to 25 mg original brain) were preincubated for 20 min at 37° in 1·5 ml Tris–HCl buffer (15 mM, pH 8·0) containing EDTA (0·06 mM) with tetraethylpyrophosphate (40 μM) $\pm$ Mipafox (100 μM). Two millilitres of a dispersion of valeryl ester of 4-hydroxyazobenzene (5 μg/ml in 0·03% sodium deoxycholate dissolved in 0·5 M Tris–HCl buffer, pH 9·0) was added and the increase of extinction at 420 nm was followed for 3 min in 1-cm cells with a Unicam SP800. The rate was approximately linear provided the change was $<$0·07 extinction units. At pH 9

ϵ = 1,860 (maximum value is 2130 at pH 11) and there was no interference from unhydrolysed ester.

Inhibition experiments. In screening experiments, concentrated solutions of potential progressive inhibitors in acetone (50 μl) were added to the medium at the start of the 20-min preincubation period: acetone alone caused a slight stimulation of esterase activity and was added to the controls. Several active inhibitors were shown to cause no inhibition when added at the end of the preincubation period and immediately before the substrate. It is clear, therefore, that addition of substrate effectively halted the progressive reaction of inhibitors with enzyme. For more detailed examination, the inhibitors were included in the preincubation by adding them in buffer and reducing the volume of paraoxon concentrate. Non-progressive inhibition by substrate analogues was examined by adding test compounds to the stock solution of substrate in dimethylformamide before it was dispersed in Triton X-100.

RESULTS

Comparison of substrates related to phenyl phenylacetate

The paraoxon-resistant activity of hen brain homogenate against a number of substrates is shown in Table 1. The proportion of activity which is sensitive to Mipafox increases to a maximum up the homologous series to phenyl valerate. The Mipafox-sensitive activity was found to persist when phenyl valerate concentration was reduced below the solubility limit, and there was no increase in the proportion of Mipafox-sensitive activity when phenyl propionate was used in emulsion instead of true solution. One carbonate and several carboxylic acid esters made from homologues of phenol are included in Table 1: the proportion of the esterase activity susceptible to Mipafox was low with these substrates. Several peptides and amides derived from phenylalanine were hydrolysed by hen brain but the activity was not affected by either paraoxon or Mipafox.

Effect of compounds administered in vivo *on hydrolysis of substrates.* Table 2 shows the effect of a neurotoxic dose of DiPF (di-isopropylphosphorofluoridate) (1·7 mg/kg s.c.) given to a hen 17 hr before removal of the brain for assay *in vitro*. For all the substrates the Mipafox-sensitive activity was much inhibited while the Mipafox-insensitive activity was less affected. It is likely therefore that in every case the Mipafox-sensitive activity was due to neurotoxic esterase. Because the analytical error of the differential assay is larger with high inhibition, it is doubtful whether there is a real difference between the amounts of inhibition measured by the different substrates.

Table 2 shows also the effect of administration of 4 other inhibitors of neurotoxic esterase with structures very different from that of DiPF. These compounds all profoundly inhibited the Mipafox-sensitive activity against phenyl valerate but had less effect on the remainder.

Further examination of phenyl valerate. The Mipafox-sensitive activity against phenyl phenylacetate is due only to neurotoxic esterase [3]. The results in

Table 1. Activity of paraoxon-resistant esterases of hen brain against various substrates and the effect of Mipafox

Substrate	Final concn (mg/ml)	Esterase activity: Total (nmoles/min per g brain)	Esterase activity: Mipafox-sensitive (% of total)
Phenyl acetate	0·16*	1890	5, 6
Phenyl propionate	0·16*	670	5
	1·5	1730	5
Phenyl butyrate	0·16	880, 980	30, 29
Phenyl valerate	0·16	2580, 2850	52, 58
	0·03*	1130	37
Phenyl caproate	0·16	2600, 2480	49, 46
Phenyl benzoate	0·16	90	5
Phenyl phenylacetate	0·16	540, 490, 480	32, 31, 26
Phenyl 3-phenylpropionate	0·16	3030, 2990	23, 25
Phenyl 4-phenylbutyrate	0·16	1380, 1220	32, 34
Phenyl nicotinate	0·16	155	11
2-Chlorophenyl phenylacetate	0·16	<500	29
3-Chlorophenyl phenylacetate	0·16	<500	19
4-Methylumbelliferyl phenylacetate	0·005*	50†	12
α-Naphthyl propionate	0·12	300	<5
α-Naphthyl valerate	0·10	445	5
Benzenethiolyl valerate	0·10	3300	3
4-Hydroxyazobenzene valeryl ester	0·003*	1000‡	20
4-Nitrophenyl benzyl carbonate	0·05	900	6
3-Dimethylaminophenyl valerate	0·13	1020‡	8

After preincubation of hen brain homogenate with inhibitor(s) as described in Methods, a dispersion of substrate was added and the rate of hydrolysis was determined. In all cases except where noted (*) the final concentration of substrate was above the solubility limit. Each value is the result of a single experiment with good duplicate measurements. †,‡: tissues were respectively the low-speed centrifugation supernatant and the deoxycholate-treated particles as described (Methods).

Table 2. Effect of administration of compounds which inhibit neurotoxic esterase on paraoxon-resistant esterases of hen brain

Compound and dose (mg/kg and route)	Substrate (phenyl ester of named acid)	Esterase activity (% of normal) (A) Mipafox-sensitive	(B) Mipafox-insensitive
DiPF (1·7 s.c.)			
1st Expt.	Phenylacetic	20	87
	4-Phenylbutyric	27	71
	Butyric	20	80
2nd Expt.	3-Phenylpropionic	19	72
	Caproic	20	79
	Valeric	9	78
2-Methylphenyl diphenyl phosphate (120 oral)	Valeric	0	50
Di-2-chloroethyl 4-nitrophenyl phosphate (120 oral)	Valeric	13	72
Phenylmethanesulphonyl fluoride (40 s.c.)†	Valeric	28	60
4-Nitrophenyl di-*n*-pentylphosphinate (3 s.c.)	Valeric	10*	85*

Compounds were obtained and administered as described previously [3, 6]. After 24 hr hens were killed and assays at 37° were performed as described Methods. Whole homogenates were used except in the case marked (*) where the particulate fraction [8] was used. s.c. = Subcutaneous.

† Dose given 2 days before assay.

Tables 1 and 2 suggest that phenyl valerate has advantages as a substrate provided that the Mipafox-sensitive activity represents a single enzyme. Figure 1 shows that for this activity there is a linear relationship of log-(percentage activity remaining) to inhibitor concentration for Mipafox, DiPF and for 4-nitrophenyl dipentylphosphinate and that the lines extrapolate through log value 1·99–2·00 (each ± 0·01 S.D.) at zero inhibitor concentration. I_{50}s are 7 μM, 0·7 μM and 18 nM respectively. Concentrations of these inhibitors 7–8 × I_{50} inhibited > 93 per cent of the Mipafox-sensitive activity. Also, the ratio of activity against phenyl valerate and phenyl phenylacetate remained unaltered within experimental error in the range of Mipafox concentrations used in Fig. 1 so that the same I_{50} was obtained with either substrate. All this data indicates that the activity with phenyl valerate as substrate is due only to neurotoxic esterase.

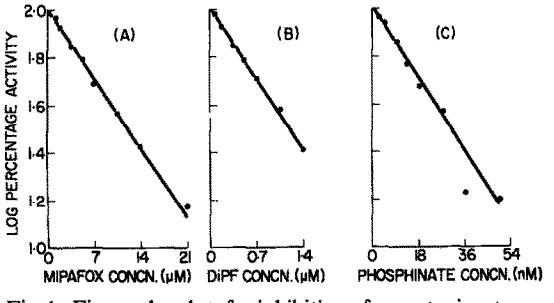


Fig. 1. First-order plots for inhibition of neurotoxic esterase by (A) Mipafox, (B) DiPF, (C) Di-*n*-pentylphosphinate. Substrate was phenyl valerate and conditions are described in Methods.

Effects in vitro *of inhibitors on neurotoxic esterase and on the Mipafox-insensitive esterases*

Neurotoxic esterase is distinguished from other paraoxon-resistant esterases by the fact that it is selectively inhibited by Mipafox. The relationship of chemical structure to activity of other inhibitors of the

Table 3. Relationship of chemical structure of phosphorus compounds to ability to inhibit neurotoxic esterase and the Mipafox-resistant esterases of hen brain

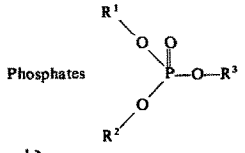
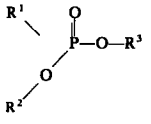
No.	R^1	R^2	R^3	Neurotoxic esterase I_{50} (μM)	Inhibition (%) of Mipafox-resistant activity at concentration: (A)	(B)	(C)
Phosphates							
1	As R^2	CH_3-	$Cl_2C{=}CH$-	70	28		
2		C_2H_5-		2	0	<10	
3		i-.C_3H_7-		0·05	0	<10	
4		n-.C_5H_{11}-		0·003	0	<10	
5		C_6H_5-		0·007	13	47	

Table 3. (*contd.*)

No.	R^1	R^2	R^3	Neurotoxic esterase I_{50} (μM)	Inhibition (%) of Mipafox-resistant activity at concentration: (A)	(B)	(C)
6		4-tert.C_4H_9.C_6H_4-	Cl_2C=CH-	0·8		3	40
7		2,4,6-$(CH_3)_3$.C_6H_2-	Cl_2C=CH-	>100	0		
8		2-[$(CH_3)_2$CH]-4-CH_3.C_6H_3-	Cl_2C=CH-	>100	0		
9		CH_3-	4-NO_2.C_6H_4-	NS	0		
10		C_2H_5-	4-NO_2.C_6H_4-	>100	0		
11		n.C_3H_7-	4-NO_2.C_6H_4-	100	35		
12		$(CH_3)_2$CH-	4-NO_2.C_6H_4-	NS	15		
13		n.C_4H_9-	4-NO_2.C_6H_4-	6		50	
14		n.C_5H_{11}-	4-NO_2.C_6H_4-	2		60	
15		Cl.C_2H_4-	4-NO_2.C_6H_4-	10		30	
16	As R^2	CH_3-	Cl.C_2H_4-	>100	0		
17	As R^2	C_2H_5-	Cl.C_2H_4-	>100	10		
18	As R^2	n.C_4H_9-	Cl.C_2H_4-	3		40	
19	As R^2	CH_3-	Cl.CH=CH-	60		10	
20	As R^2	CH_3-	2-(CH_3CO).C_6H_4-	NS	0		
21	As R^2	CH_3-	3-(CH_3.CO).C_6H_4-	NS	0		
22	As R^2	CH_3-	4-(CH_3.CO).C_6H_4-	NS	0		
23	As R^2	CH_3-	CH≡C.CH_2-	NS	0		
24	As R^2	CH_3-	C_2H_5.S.C_2H_4-	NS	0		
25	As R^2	CH_3-	1-(4-chlorophenylthio)-vinyl-	60		50	
26	As R^2	CH_3-	1-(4-chlorophenyl)-2-chlorovinyl-	60		25	
27	As R^2	CH_3-	2,5-Cl_2-4-Iodo-C_6H_2-	20	20	70	
28	As R^3	As R^3	CH_3.CHCl.CH_2-	NS	0		
29	As R^3	As R^3	CH_2Cl.CH_2.CH_2-	NS	0		
30	As R^3	As R^3	CH_2Cl.CHCl.CH_2-	NS	30		
31	As R^3	As R^3	CH_2Br.CHBr.CH_2-	NS	20		
32	As R^3	As R^3	C_6H_5-	NS	0		
33	As R^3	As R^3	2-CH_3.C_6H_4-	NS	0		
34	As R^3	As R^3	3-CH_3.C_6H_4-	NS	0		
35	As R^3	As R^3	4-CH_3.C_6H_4-	NS	0		
36	As R^3	As R^3	2-C_2H_5.C_6H_4-	NS	0		
37	As R^3	As R^3	3-C_2H_5.C_6H_4-	NS	0		
38	As R^3	As R^3	4-C_2H_5.C_6H_4-	NS	0		
39	2-CH_3.C_6H_4-	C_6H_5-	C_6H_5-	NS	0		
40	4-C_2H_5.C_6H_4-	As R^1	4-(CH_3.CHOH).C_6H_4-	NS	0		
41	4-C_2H_5.C_6H_4-	As R^3	4-(CH_3.CHOH).C_6H_4-	NS	0		
42	4-C_2H_5.C_6H_4-	As R^1	4-(CH_3CO)C_6H_4-	100		38	
43	4-C_2H_5.C_6H_4-	As R^3	4-(CH_3CO)C_6H_4-	20		50	
44	As R^3	As R^3	4-(CH_3CO)C_6H_4-	30		50	
45	Cl.C_2H_4-	As R^1	7-(3-chloro-4-methyl-coumarinyl)-	3		50	
Phosphonates							
46	CH_3-	CH_3-	2,4-Cl_2.C_6H_3-	15		20	
47	C_2H_5-	C_2H_5-	2,4,5-Cl_3.C_6H_2-	2		20	
48	C_6H_5-	CH_3-	2,5-Cl_2-4-Br.C_6H_2-	0·4		30	
49	C_6H_5.CH_2-	C_2H_5-	4-NO_2.C_6H_4-	2		50	
50	$C_6H_5(CH_2)_2$-	C_2H_5-	4-NO_2.C_6H_4-	0·3		30	
51	C_6H_5.$(CH_2)_3$-	C_2H_5-	4-NO_2.C_6H_4-	0·5		40	
52	C_6H_5.$(CH_2)_4$-	C_2H_5-	4-NO_2.C_6H_4-	80		35	
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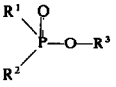
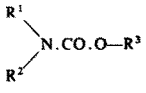
Phosphinates							
54	As R^2	C_2H_5-	4-NO_2.C_6H_4-	100		15	
55	As R^2	n.C_4H_9-	4-NO_2.C_6H_4-	0·3		20	
56	As R^2	n.C_5H_{11}-	4-NO_2.C_6H_4-	0·02		10	15
57	As R^2	n.C_5H_{11}-	Cl_2C=CH-	0·06		10	20
Other phosphorus esters							
58	[$(CH_3)_2$CH.O]$_2$P.O.F			0·7		0	0
59	[$(CH_3)_2$.CH.NH]$_2$P.O.F.			7		0	0
60	$(C_2H_5O)_2$.PO.O.PO.$(OC_2H_5)_2$			>100	10		
61	$(C_6H_5$.NH$)_2$.PO.OCH=CCl_2			5		10	20
62	(n.$C_4H_9S)_3$.PO			>100	10		
63	N,N'-Dipyrollidinyl.PO.OCH=CCl_2			3		15	40
64	Phenyl O,O-Saligenin phosphate			0·005		15	25
65	Tris-(2,3-dichloropropyl)phosphite			>100	0		

Neurotoxic esterase was assayed with either phenyl phenylacetate or phenyl valerate as substrate and the I_{50} for each inhibitor was determined as described in Methods. Using phenyl valerate as substrate the Mipafox-insensitive activity was assayed with concentrations of inhibitor causing (A) slight inhibition of neurotoxic esterase (concn $\leqslant I_{15}$), (B) about half inhibition (concn = I_{40}–I_{60}) or (C) great inhibition (concn $\geqslant I_{85}$). NS = No significant inhibition at 100 μM or at saturating concentration.

enzymes is indicated in Tables 3 and 4. The first column of data in Table 3 shows the I_{50} for organophosphorus compounds tested on neurotoxic esterase. Column B shows that at concentrations near the I_{50} many compounds also inhibited the Mipafox-insensitive activity but a few inhibited this activity markedly less or more than the neurotoxic esterase. No single compound was found to inhibit all the Mipafox-insensitive activity but mixtures were more effective, showing that more than one enzyme is involved. Column A shows the effect on Mipafox-resistant activity when the inhibitor concentration was such that inhibition of neurotoxic esterase was <15 per cent. *n*-Propyl paraoxon (Cmpd 11), and Tris–2, 3-dichloropropyl phosphate (Cmpd 30) selectively inhibited some of the Mipafox-insensitive activity. Column C shows the effect when the concentration was such that inhibition of neurotoxic esterase was >85 per cent. The higher alkyl dichlorovinyl phosphates (Cmpds 2–4) and DiPF (Cmpd 58) selectively inhibited neurotoxic esterase.

Table 4. Relationship of chemical structure of carbamates and sulphur compounds to their ability to inhibit *in vitro* the neurotoxic esterase and the Mipafox-resistant esterases of hen brain

No.	R^1	R^2	R^3	Neurotoxic esterase I_{50} (μM)	Inhibition (%) of Mipafox-resistant activity at concentrations: (A)	(B)	(C)
Carbamates							
66	C_6H_5-			40		20	
67	$C_6H_5.CH_2$-			10		10	15
68	$C_6H_5.CH_2CH_2$-			>100	10		
69	$CH_3.CH(C_6H_5)$-			NS	20		
70	4-Br.C_6H_4-			>100	25		
71	4-$CH_3.C_6H_4$-			50		50	
72	4-$NO_2.C_6H_4$-			>100	0		
73	2-Pyridyl-			>100	0		
74	4-Pyridyl-			>100	0		
75	4-COOH.C_6H_4-	H-	C_6H_5-	>100	0		
76	2-Cl.$C_6H_4.CH_2$-			55		10	
77	3-Cl.$C_6H_4.CH_2$-			2		15	20
78	4-Cl.$C_6H_4.CH_2$-			5		15	25
79	3,4-$Cl_2.C_6H_3.CH_2$-			25		20	
80	4-Br.$C_6H_4.CH_2$-			5		15	25
81	n.C_4H_9-			65		25	
82	1-Naphthyl-			50	0	5	
83	cyclo-Hexyl-			>100	0	20	
84	$C_6H_5.CH_2.CH(COOH)$-			>100	0		
85	C_6H_5		$C_6H_5.CH_2$-	>100	10		
86			4-Br.C_6H_4-	>100	30		
87			4-$NO_2.C_6H_4$-	>100	20		
88			1-Naphthyl-	>100	15		
89			2-Naphthyl-	>100	15		
90			2-Cl.C_6H_4-	80			
91	4-$CH_3O.C_6H_4$-	H-	3-$(CH_3)_2N.C_6H_4$-	NS	0		
92	$C_6H_5.CH_2$-		C_2H_5-	NS	0		
93	4-COOH.C_6H_4-		C_2H_5-	>100	0		
94	4-Cl.C_6H_4-		n.C_8H_{17}-	NS	0		
95	4-Cl.C_6H_4-		CH_2=CH.CH_2-	NS	0		
96	4-$(CH_3)_2N.C_6H_4$-		Cl.$CH_2.CH_2$-	NS	0		
97	$C_6H_5.CH_2$-		4-$NO_2.C_6H_4.CH_2$-	>100	0		
98	C_6H_5-	CH_3-	C_6H_5-	>100	20		
99	$C_6H_5.CH_2$-	CH_3-		35		40	
100	$C_6H_5.CH_2$-	C_2H_5-		NS	35		
101	n.C_4H_9-	CH_3-		>100	15		
Thiocarbamates							
102	$C_6H_5.CH_2.NH.CS.SC_6H_5$			NS	0		
103	$C_6H_5.CH_2.NH.CS.OC_6H_4(4\text{-}NO_2)$			NS	35		
Sulphonyl fluorides R.SO_2.F							
	R						
104	C_6H_5-			NS	35		
105	2-$NO_2.C_6H_4$-			NS	0		
106	$C_6H_5.CH_2$-			30		50	
107	n.C_4H_9-			50		50	
Sulphamyl compounds							
108	$C_6H_5.CH_2.NH.SO_2.OC_6H_5$			NS	0		
109	$C_6H_5.CH_2.NH.SO_2.Cl$			100		35	
110	$C_6H_5.CH_2.N(CH_3).SO_2.Cl$			100		50	
Thiamyl compounds							
111	$C_6H_5.CH_2.NH.SO.OC_6H_5$			>100	0		
112	$C_6H_5.CH_2.N(CH_3).SO.OC_6H_5$			NS	0		
113	$C_6H_5.CH_2.NH.SO.Cl$			NS	0		
114	$C_6H_5.CH_2.N(CH_3).SO.Cl$			NS	0		

For details see Legend to Table 3.

Table 4 contains data for carbamate, sulphonate and related inhibitors set out as in Table 3. The nearest to a selective inhibitor for neurotoxic esterase among these was phenyl benzylcarbamate (Cmpd 67). The most selective inhibitors of some of the Mipafox-resistant activity were phenyl *N*-benzyl-*N*-ethyl-carbamate (Cmpd 100). 4-nitrophenyl benzylthionocarbamate (Cmpd 103) and benzenesulphonyl fluoride (Cmpd 104). I_{50}s for neurotoxic esterase of several carbamates added in acetone were found to be 1·5–3 times higher compared with those found when the inhibitors were added in buffer. This effect was less evident for the Mipafox-resistant esterases.

Effect of other inhibitors

The thiol-attacking inhibitors *N*-ethyl maleimide and *p*-chloromercuri-benzoate inhibited both Mipafox-sensitive and insensitive esterases (preincubation for 20 min with 0·1 mM reagent) but iodoacetate or iodoacetamide were ineffective at 1 mM. Zinc acetate (0·4 mM) totally inhibited the Mipafox-resistant activity and caused 80 per cent inhibition of neurotoxic esterase. Mg^{2+}, Mn^{2+} and Co^{2+} ions had negligible effect at this concentration. These ions are known to inhibit some and to activate other brain amidases and peptidases [11–13].

A number of compounds having some structural relationships to phenyl phenylacetate were investigated as possible competitive inhibitors. No specific inhibitors were found but three observations were of interest:

(1) Amino-acid naphthylamides (0·5–1 mM) were inactive except those of the hydrophobic amino-acids tryptophan and phenylalanine which were moderately inhibitory. Likewise, the only dipeptides which inhibited at 3 mM were Trp–Trp, Trp–Phe and Phe–Trp: dipeptides containing only one of these amino-acids did not inhibit. It is interesting that none of these compounds was hydrolysed by neurotoxic esterase.

(2) High concentrations (7 mM) of phenyl nicotinate which is a slowly-hydrolysed substrate (Table 1) inhibited phenyl valerate hydrolysis by 45 per cent. Presumably this compound could compete with inhibitors *in vivo*, thereby causing the slight protective effect observed when hens poisoned with triorthocresyl phosphate were repeatedly dosed with large amounts of this ester [14].

(3) Phenylglyoxaldoxime and benzohydroxamic acid (2–3 mM) inhibited activity by 40–50 per cent. These compounds also reduced the severity of ataxia produced *in vivo* by DiPF but only when administered before the DiPF (Johnson, unpublished): it is likely that they competed with inhibitor for the enzyme active site rather than that they reactivated phosphorylated enzyme.

DISCUSSION

Structure–activity relationships

Substrates. The substrates most rapidly hydrolysed by neurotoxic esterase were the hydrophobic phenyl esters although these were less soluble than the lower esters and V_{max} was probably not achieved. The data of Tables 1 and 2 and Fig. 1 show that phenyl valerate is a good alternative substrate for neurotoxic esterase which has previously been assayed with phenyl phenylacetate. The proportion of the paraoxon-resistant activity which is inhibited by Mipafox is about 55 per cent (30 per cent for phenyl phenylacetate) and this activity is homogenous by several criteria. Also, the rate of hydrolysis is about 9 times greater. Naphthyl, 4-methylumbelliferyl or thio-esters which are commonly used in histochemistry are clearly not suitable for localisation of neurotoxic esterase. The failure of neurotoxic esterase to hydrolyse amides or peptides is noteworthy, since the response to organophosphate inhibitors is not unlike that of trypsin and chymotrypsin (see below).

Organophosphorus inhibitors. Most of the dimethyl phosphates in Table 3 are known to be active anticholinesterases but only one (Cmpd 27) had much activity against neurotoxic esterase. Increase in hydrophobic nature of R^1 and R^2 increased the inhibitory power against neurotoxic esterase in three homologous series [the dichlorovinyl phosphates (Cmpds 1–4), 4-nitrophenyl phosphates (Cmpds 9–15), and the phosphinates (Cmpds 54–56)]. This pattern is similar to that seen for inhibition of chymotrypsin and trypsin but different from that for inhibition of cholinesterase and acetylcholinesterase where increase in chain-length has far less effect [15–18]. The lesser activity of 4-nitrophenyl ethyl 4-phenylbutylphosphonate (Cmpd 52) compared with the lower homologues (Cmpds 49–51) is also true for chymotrypsin but not for acetylcholinesterase [18]. This suggests there may be an upper limit to the known tendency for increase of neurotoxic activity *in vivo* as chain-length is increased [19–21]. Longer-chain phenylalkyl esters might be found to be anticholinesterases with low neurotoxic activity and prove useful candidate pesticides.

The 4-nitrophenyl phosphinates (Cmpds 55–56) are much more active than the analogous phosphates (Cmpds 13, 14). This may be due partly to their known greater reactivity. However, it is possible to inhibit neurotoxic esterase with these phosphinates *in vivo* without causing severe anticholinesterase effects whereas the phosphates are lethal at doses which would be expected to inhibit neurotoxic esterase [6, 22]. The ratio of affinity of phosphinate for enzyme: affinity of phosphate for enzyme must be higher for neurotoxic esterase than for acetylcholinesterase although absolute values are not known.

Several of the alkyl-substituted tri-aryl phosphates (Cmpds 32–39) have been shown to inhibit some liver esterases *in vitro* including those which hydrolyse phenyl phenylacetate (Ref. [1], and Aldridge, personal communication). In contrast, these compounds did not inhibit the brain activity studied here nor the paraoxon-sensitive esterases normally removed by preincubation. However, a number of these phosphates are neurotoxic and known to be metabolised *in vivo* to active esterase inhibitors which are themselves neurotoxic. Thus the very active phenyl saligenin phosphate (Cmpd 64) is derived from the neurotoxic diphenyl 2-methylphenyl phosphate (Cmpd 39) [23–25]. Compounds 40–43 are produced *in vivo* from the neurotoxic tri-(4-ethylphenyl) phosphate (Cmpd 38) and are themselved neurotoxic at lower doses than the parent compd [26–29]. However, *in vitro* only the two with acetyl substituents (Cmpds 42, 43) are active against neurotoxic esterase. It is well known that unsaturated groups inserted in the 4-position of a phenyl ester labilise the ester bond while an α-hydroxyethyl group (as

in Cmpds 40, 41) does not: the former compounds would therefore be more active phosphorylating agents.

The low activity *in vitro* of tri-(*n*-butylthiolo-) phosphate (Cmpd 62) which is neurotoxic at high doses [30] raises the question whether there is a more active metabolite. The fully oxygenated analogue would undoubtedly be too stable to inhibit but an intermediate with two butyloxy groups would probably have high affinity for the enzyme while the remaining butylthio group would be a suitably labile leaving group.

A comparison of Cmpds 30 and 65 show that while the phosphate has a little activity the phosphite is inactive. This comparison has not been extended to more active phosphates.

Carbamate and sulphur-acid inhibitors. Among the phenyl mono-*N*-substituted carbamates there is a sharp optimum for inhibition of neurotoxic esterase at benzyl (Cmpd 67) compared with 2-phenylethyl (Cmpd 68) or phenyl (Cmpd 66). *N*-pyridyl and *N*-cyclohexylcarbamates (Cmpds 73, 74, 83) were inactive. The other esters of phenylcarbamic acid which were tested (Cmpds 85–90) were all poorer inhibitors than the phenyl ester. This is analogous to the pattern seen with substrates (Table 1). Among the phenyl *N*,*N*-disubstituted carbamates (Cmpds 98–101) it seems likely that steric hindrance reduced activity against neurotoxic esterase more than against the remaining esterases (see also the branched monosubstituted Cmpd 69). The thiocarbamates (Cmpds 102, 103) were also inactive against neurotoxic esterase but Cmpd 103 affected the remainder. This correlates with the fact that benzenethiolyl valerate was hydrolysed almost exclusively by the Mipafox-resistant esterases (Table 1).

Benzenesulphonyl fluoride (Cmpd 104) does not inhibit neurotoxic esterase but this cannot be due to lack of reactivity since it is an unusually good inhibitor of the remaining esterases. Phenylmethanesulphonyl fluoride (Cmpd 106) is much more inhibitory.

It is interesting that the sulphamyl chlorides (Cmpds 109, 110) were inhibitory while the analogous thiamyl compounds derived from sulphurous acid (Cmpds 113, 114) were not.

Activity and specificity

Interaction of substrates, progressive and non-progressive inhibitors with neurotoxic esterase is favoured by the presence of large hydrophobic side-chains in the molecule although the longest-chain ω-phenylalkylphosphonate tested was much less active. This pattern is similar to that for chymotrypsin although neurotoxic esterase does not hydrolyse peptides or amides. However, the active sites of the two enzymes must differ in accessibility. Comparison of Cmpds 49 and 53 shows that insertion of an acetoxy group in the α-position of the benzyl group decreased activity against neurotoxic esterase while it causes an 11-fold increase in activity against chymotrypsin [31]. Also, the very bulky diphenylcarbamyl chloride is a potent inhibitor of chymotrypsin but far less active against neurotoxic esterase. The I_{50}s are <1 μM (calculated from Ref. [32]), and 25 μM respectively.

Several compounds were noted to be virtually specific for neurotoxic esterase. Both DiPF (Cmpd 58) and di-*n*-pentyl 2,2-dichlorovinyl phosphate (Cmpd 4) are more stable than Mipafox but are very toxic. Phenyl *N*-benzylcarbamate (Cmpd 67) is not entirely specific, but for screening purposes provides a useful stable and non-toxic alternative to Mipafox.

Experience with the various compounds which preferentially inhibit the Mipafox-resistant esterases has shown that benzensulphonyl fluoride (Cmpd 104) is the most effective single inhibitor and little is gained by using mixtures of these compounds to improve the specificity of the assay.

CONCLUSION

The esterase activity of neurotoxic esterase seems to serve no physiological purpose but the whole protein may have an essential function which is disrupted by phosphorylation of the esterase site and subsequent 'ageing' of the inhibited esterase [6]. To understand this process further it is important to locate this enzyme within the nervous system. The present study has shown that substrates suitable for histochemistry are unlikely to be found but the increased sensitivity and selectivity of the assay with phenyl valerate makes sub-cellular fractionations *in vitro* feasible. The enzyme was originally identified by a differential labelling reaction with [^{32}P]DiPF [10]. Table 3 includes several inhibitors more active than DiPF and investigations of the selectivity of triated Cmpd 4 are proceeding with a view to developing an autoradiographic localization method.

The data of Table 3 undergird the suggestion [21] that among anticholinesterase organophosphates prepared as potential pesticides less neurotoxic hazard will be associated with dimethyl esters than with higher homologues.

A simplified and unhazardous assay suitable for routine toxicological screening using phenyl benzylcarbamate instead of Mipafox is to be published.

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