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SENSITIVITY AND SELECTIVITY OF COMPOUNDS INTERACTING WITH NEUROPATHY TARGET ESTERASE

FURTHER STRUCTURE–ACTIVITY STUDIES

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Abstract—Assay of neuropathy target esterase (NTE) which accounts for about 70% of paraoxon-resistant phenyl valerate (PV) esterase activity of hen brain depends on the fact that it is selectively inhibited by mipafox. A previous study of structure/activity relationships (*Biochem. Pharmac.* **24**, 797, 1975) has been extended. Among 14 potential substrates NTE hydrolysed phenyl phenoxyacetate and phenyl thiophenoxyacetate faster (1.5–1.7×) than PV, but selectivity of these substrates for NTE among the paraoxon-resistant esterases was only 35–52%.

Seventy-seven other potential inhibitors (organophosphates, phosphonates, phosphoramidates, phosphinates and carbamates) were examined to determine I_{50}^{NTE} and effects on both NTE and "non-NTE" at 3–4 × I_{50}^{NTE} (I_{85-95}) and, where possible, at 6–20 × I_{50}^{NTE}. Hydrophophic inhibitors with small/flexible leaving groups were generally very inhibitory: several 2,2-dichlorovinyl phosphates and fluorides were active at low nanomolar concentrations. In the dichlorovinyl phosphate series increasing dialkyl chain length beyond *n*-pentyl decreased inhibitory power, presumably due to steric hindrance since the methyl/*n*-decyl ester was 15× more active than di-*n*-decyl. Chloro-substitution of both ortho-positions of a phenyl leaving group for benzylcarbamates reduced inhibitory power more than 20× but had little effect in a phenyl leaving group of methyl phenylphosphonates where the acyl-leaving group bond is longer and less subject to steric hindrance. *N*-phenylbenzohydroxamyl benzylcarbamate is 10× more potent than any previously described carbamate against NTE. Among stereo-isomers differences of activity ranged from <2- to 15-fold.

Only diphenylphosphinyl fluoride appeared to be virtually specific for NTE: at 0.5–1 μM it inhibited *ca.* 92% of NTE and 10–13% of "non-NTE" which is similar to the specificity found for 2,6-dichlorophenyl methyl phenylphosphonate which has been claimed to be specific. Diphenylphosphinyl fluoride has an advantage in that it is easily synthesized and should be protective rather than neuropathic, but it is not stable in store.

We cannot repeat experiments purporting to show a substantial proportion of a second isozyme of NTE. However, according to first-order kinetics, concentrations of inhibitor >6 × I_{50} should inhibit NTE > 98% and for 19 out of 26 compounds a residue greater than 3% (limit of precision) was found under these conditions: in nearly every case the quantity was 3–5%. This quantity may not be "true NTE" but it cannot be the target for organophosphate-induced delayed neuropathy since it is resistant to various neuropathic and protective compounds. The error of including this "non-NTE" in assays using the standard protocol is negligible

Nervous tissue of the hen contains a number of esterases which will hydrolyse phenyl phenylacetate, phenyl valerate and related esters. The two most active enzymes were shown to be inhibited by low concentrations of paraoxon [1, 2]. The paraoxon-resistant esterases include one which is now known as NTE: it is inhibited *in vivo* by those OP esters which cause delayed neuropathic effects seen as degeneration of long axons (OPIDP)* [3–6]. The intoxication process is unusual in that it 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* [4, 5].

Assay *in vitro* of NTE of neural tissue taken from hens dosed with organophosphates has provided a measure of neuropathic potential [3–5, 7, 8]. The activity of a large number of progressive and non-progressive inhibitors when they are added to NTE *in vitro* has been reported [9, 5], and some generalizations about structure–activity relationships and comparison of NTE with other esterases were made. Such studies may help in understanding the topography of the active site and are of immediate practical relevance in design both of OP pesticides with little neuropathic hazard and of protective compounds.

The preferred assay of NTE [10] is a differential procedure. Paired samples of tissue are preincubated

* Abbreviations used: OP, organophosphorus; OPIDP, OP ester-induced delayed polyneuropathy; DFP, di*iso*propyl phosphorofluoridate; PV, phenyl valerate; NTE, neuropathy target esterase, formerly called neurotoxic esterase; Cmpd, compound; I_{50}, the concentration of a substance which inhibits 50% of a given enzyme activity under defined conditions (of preincubation in the case of OP inhibitors of esterases).

with paraoxon to inhibit two irrelevant enzymes [1]; one tube also contains the neuropathic OP ester mipafox which, at the concentration used, selectively inhibits NTE. Phenyl valerate is then added to both tubes and the rate of hydrolysis is determined. The difference between the rates of hydrolysis in the two samples is a measure of NTE.

The above procedure has limitations. With an ideal substrate, (i) all the paraoxon-resistant activity would be due to NTE: no activity would be insensitive to mipafox so that the differential procedure could be abandoned, (ii) the catalytic rate would be high, and (iii) the K_m would be so far below the physical solubility limit that the enzyme active centre could be fully saturated by added substrate so as to displace completely any pre-added inhibitors which had not yet reacted covalently. During a previous major screening [9], and using criteria (i) and (ii) above, I found that PV was a much better substrate than phenyl phenylacetate which had been used until that time. However, the differential assay [10] is still required since only about 65–70% of the paraoxon-resistant PV esterase was sensitive to mipafox and represents NTE. The whole assay procedure is also less than ideal in that it requires use of mipafox which, although it is non-volatile and not easily absorbed through the skin, has caused neuropathy in man. Also PV has a high K_m for NTE [5]: this is of little import for routine assays but hinders kinetic studies.

I present here results of structure/activity studies since 1975 and of attempts to produce safer or more sensitive or selective reagents for assay of NTE.

MATERIALS AND METHODS

Assays

Full descriptions of materials and procedures for assay of NTE and of the paraoxon-sensitive related hydrolases in homogenates of hen brain prepared in Tris buffer (50 mM, adjusted with HCl to pH 8.0) containing EDTA (0.2 mM) have been published previously [3, 10]. The standard substrate is PV and alternative substrates were dispersed in Triton X-100 (0.03% in water) in the same way as PV. Test inhibitory compounds were prepared as stock solutions in dry acetone and added to the preincubation medium immediately after tissue and in such quantity as gave a final concentration of acetone of 1% (v/v): control samples received only solvent: preincubation was for 20 min at pH 8.0 and 37° before addition of substrate. Screening was carried out with inhibitor concentrations adjusted by factors varying from 10-fold initially to 2- to 4-fold for more definitive measures until approximate I_{50} values for NTE had been determined. If possible, inhibition of NTE by test compounds was also increased to the 85–95% level to determine what effect the required concentration of inhibitor had on the "Non-NTE" activity (that PV hydrolase activity which was resistant to both paraoxon and mipafox under the conditions of the standard NTE assay). All measurements were made in duplicate and few inhibitors were tested at concentrations above 100 μM, which, in many cases, was near the limit of physical solubility of the compound in buffer.

Chemicals

Test compounds are identified by a number as listed with their molecular formulae in Tables 1 and 2. Compound 63 and various precursors were available from Lancaster Synthesis Co., Aldrich Chem. Co. and other suppliers. Precursor hydroxamic acids for Cmpds 11-12, 68-69 and 79-80 were gifts from FMC Co. (Princeton, NJ) (Dr T. Cascieri).

Acid chlorides were usually redistilled before use but all other precursors were used as received. Solvents and general reagents were of "Analar" or equivalent grade where possible. Many compounds were synthesized in this laboratory and, with few exceptions, the nature of the product was presumed from the fact that a good yield of "clean" product was obtained in a reaction for which analogies were well-known. Progress of reactions was monitored by TLC (Silica) or GC. When progress of reaction appeared unsatisfactory, further investigations were sometimes performed with IR or GC/MS analysis to identify components of the reaction product mixture. Most products of synthesis were purified simply by crystallization or vacuum distillation and were presumed to be of >90% purity where only single spots were detected on TLC analysis in two solvent systems or >97% in cases where the compounds approved by TLC analysis were sharp-melting solids. In only two cases (listed below) were significantly inhibitory impurities detected and separated. Brief details of synthetic procedures are as follows.

Carboxylic acid ester substrates. As described previously for analogous compounds [7], compounds 1–7 and 14–15 were made by direct interaction of the available acid chloride added dropwise to the molten phenol. A similar reaction was used for compounds 9–13 after the acid chloride had been formed in two steps involving treatment with thionyl chloride of the appropriate acid obtained by condensation of sodium iodoacetate and the thiol, oxime or hydroxamate in aqueous tetrahydrofuran. Compound 8 was obtained by heating phenol with phenoxyacetic acid in trifluoracetic acid anhydride as solvent and catalyst.

Benzylcarbamate esters. Compounds 71–82 were made by condensation of benzyl isocyanate with the appropriate phenol, oxime or hydroxamate in dichloromethane in the presence of triethylamine as catalyst as described previously for analogous compounds [7].

Benzenephosphinic acid esters. Attempts to condense benzene–phosphinic acid with various phenols in hot trifluoracetic anhydride did not yield the desired products. Reaction of benzene–phosphinyl dichloride in dichloromethane with stoichiometric amounts of phenols and of triethylamine with added 5–10% molar proportion of 4-dimethylaminopyridine as catalyst gave modest yields of the esters 59–62.

Esters of diphenylphosphinic acid. Compounds 68 and 69 were prepared by dropwise addition of the acid chloride in dry dichloromethane to stoichiometric amounts of the hydroxamic acid and triethylamine in solvent. Under these conditions, benzaldoxime did not give a desired product but underwent a dehydration reaction. Propargyl, 2-fluoroethyl and ethyl esters (not listed in the tables) were all formed in similar reactions but decomposed within a few days.

Table 1. *Esteratic activity of hen brain homogenate against potential substrates for NTE*

Compound No.	Substrate	Final concn (mg/ml)	Esterase activity nmole/min/g After preincubation with: A Buffer	B Paraoxon	C Paraoxon + Mipafox	"NTE" (B − C)	Selectivity $= \frac{B - C}{B}$ (%)
I. Esters of valeric acid							
(1)	3—CH_3.CO.—Ph.O—	0.57	3000	834	801	34	4
(2)	2,6—Cl_2.—Ph.O—	0.083	180	20	12	8	40
(3)	2,4,6—Cl_3—Ph.O—	0.066	<30	ND	ND	—	—
(4)	3—Cl—5—(CH_3O)—Ph.O—	0.33	3210	713	594	119	17
II. Phenyl esters of:							
(5)	C_4H_9.CO.—	0.24	13,100	3780	1000	2780	74
(6)	Ph.CH_2.CH_2.CO.—	0.16	18,400	2540	1500	1040	41
		0.24	18,500	2620	1490	1130	43
(7)	Ph.CH=CH.CH_2.CO—	0.25	ND	462	305	157	34
(8)	Ph.O.CH_2.CO—*	0.1	>30,000	13,100	8500	4600	35
(9)	Ph.S.CH_2.CO—†	0.25	34,000	10,000	5150	4850	48
		0.16	55,500	10,600	5800	4800	45
		0.11	40,700	9900	5900	4000	41
		0.05‡	18,900	6230	2990	3240	52
(10)	*n*Oct.S.CH_2.CO—	0.16	19,800	3510	1730	1780	52
		0.11	21,800	3980	1710	2270	57
(11)	Ph.CO.N(Ph).O.CH_2.CO—	0.25	ND	1060	785	275	26
(12)	Ph.CH_2.CO.N(Ph).O.CH_2.CO—	0.25	ND	826	669	157	19
(13)	Ph.CH=N.O.CH_2.CO—	0.25	ND	3760	2180	1580	42
III. Other esters							
(14)	2,3—Cl_2—Ph.O.OC.CH_2.CH_2.Ph	0.085	5000	2030	1690	340	17
(15)	2,5—Cl_2—Ph.O.OC.CH_2.CH_2.Ph	0.085	5300	2300	1450	850	37

* Non-enz. hydrolysis >NTE rate under chosen conditions.

† Non-enz. hydrolysis = *ca.* 50% NTE rate.

After preincubation of hen brain homogenate with inhibitor(s) as described in Methods, a dispersion of substrate was added and the hydrolytic rate 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 duplicate measurements within 5% range. For most substrates (except those marked * or †) non-enzymic hydrolysis did not significantly hinder the assay procedure. N.D. = not determined.

Table 2. Relationship of chemical structure to progressive inhibitory power against NTE and to selectivity for NTE among "paraoxon-resistant" esterases hydrolysing phenyl valerate

Compound No.		I_{50}^{NTE} (μM)	"Non-NTE" activity removed (%) by pre-incubation with test compound at concentration which inhibits NTE to degree shown below	
			50%	85–95%
(A) PHOSPHATES				
Di-n-alkyl 2,2-dichlorovinyl phosphates				
(16)	Pro/Pro	<0.05		19
(17)	Bu/Bu	<0.003		17
(18)	Pent/Pent	<0.0025		20
(19)	Hex/Hex	0.013	12	>20
(20)	Hept/Hept	0.06		26
(21)	Oct/Oct	0.13	15	17
(22)	Dec/Dec	1.1	18	17
(23)	Me/Oct	0.05	17	17
(24)	Me/Dec	0.07	17	17
Other				
(25)	$(CH_3O)_2.P.(O).F$	2.7	7	15
(26)	$(Ph.O)_2.P.(O).F$	0.2	25	
(27)	$[Ph.(CH_2)_4.O]_2.P.(O).F$	0.001	15	42
(28)	$[Ph.(CH_2)_4.O].[(CH_3)_2N].P.(O).F$	0.08	17	44
(29)	$C_2H_5.O.[1\text{-Pyrene-}4\text{-}C_4H_9.O].P.(O).F$	0.001	16	30
(30)	$(4\text{-}NO_2\text{-}Ph.O)_2.P(O).O.Et$	2–3		>72
(31)	$(CH{\equiv}C.CH_2O)_2.P.(O).OEt$	⩾100		
(B) PHOSPHONATES				
(32)	$Ph.P(O).F_2$	10§	70	
(33)	$Ph.CH_2.P(O).F_2$	1	19	
(34)	2-Dansylaminoethyl $CH_3.P(O).F$	20	92	
(35)	$EtO.(Me).P(O).O.(4\text{-}NO_2.Ph)$	90	31	
(36)	$EtO.(Ph).P.(O).O.(2,6\text{-}Cl_2Ph)$	0.4	12	8
		0.4*	8*	18*
		0.35†	10†	16†
(37)	$PhO(n.C_5H_{11}).P(O).OPh$	2	8	22
(38)	$3\text{-}Cl.PhO.(Ph.CH_2).P(O).O.3\text{-}Cl.Ph$	2.5	20	36
	$EtO.(Ph).P(O).S.CH_2.4\text{-}Cl.Ph$			
(39)	R(+) Isomer	>100	0	
(40)	S(−) Isomer	>100	0	
	$EtO.(Ph).P(O).S.CH_2.2,4\text{-}Cl_2Ph$			
(41)	R(+) Isomer	100	0	
(42)	S(−) Isomer	>100	24	
	$EtO(Ph).P(O).S.CH_2.C{\equiv}CH.$			
(43)	R(+) Isomer	12	7	17
(44)	S(−) Isomer	10	7	17

	Pinacolyl.O(Me)P.(O).F (soman)			
(45)	C(−)P(+) isomer	0.11	*ca.* 20	
(46)	C(−)P(−) isomer	0.75	*ca.* 20	
(47)	C(+)P(+) isomer	0.78	*ca.* 20	
(48)	C(+)P(−) isomer	1.7	*ca.* 20	
	MeO.(Ph).P(O).O.2,5-Cl_2Ph.			
(49)	R(+) isomer	1.2	8	19
(50)	S(−) isomer	0.5	22	31
(C) PHOSPHORAMIDATES				
	[RO.(NH_2).P(O).S.Me]			
(51)	Me: Racemic	2,150	15	20
(52)	R(+) Isomer	1,020	15	20
(53)	S(−) Isomer	10,900	>20	
(54)	Et.	550	22, 10	33, 28
(55)	*Iso*-Pr.	630	0	17
(56)	*n*-Pr.	55	11, 12	27, 50
(57)	*n*-Bu.	5	10, 25	40, 57
(58)	*n*-Hex.	0.44	15	35
(D) PHOSPHINATES				
I. Esters of Benzenephosphinic acid‡ (Ph.PH.(O).—)				
(59)	Ph.O	20	20	
(60)	2-Cl-6-Me.Ph.O	>50	0	
(61)	2,6-Cl_2.Ph.O	>50		
(62)	3-Cl-5-MeO.PhO	>50		
II. Esters and Halides of diphenylphosphinic acid (Ph_2.P(O).—)				
(63)	Cl§	>50		
(64)	F§	*ca.* 0.07	7, 0	13, 10
(65)	Cl_2.C═CH.C(═C.Cl_2).O	6	60	75
(66)	Cl_2·C═CH.C(═C.H.Cl).O	6	10	30
(67)	Ph.O	⩾30		
(68)	Ph.C(O).N(Ph).O	*ca.* 60	0	16‖
(69)	Ph.CH_2.C(O).N(Ph).O	⩾50		
III. Other				
(70)	(*n*-C_5H_{11})$_2$.P(O).OPh	0.27	14	18
(E) CARBAMATES				
Esters of benzylcarbamic acid ($PhCH_2$.NH.CO.—)				
(71)	PhO	5	12	33
(72)	2,6-Me_2.Ph.O	⩾50		
(73)	3,5-(MeO)$_2$.PhO	4	23	45
(74)	3-Cl-5-(MeO).PhO	4	8	18
(75)	2,5-Cl_2.PhO	20	16	25
(76)	3,5-Cl_2.PhO	*ca.* 0.5		31
(77)	2,6-Cl_2.PhO	>100		
(78)	Ph.CH═N—O	25	35	

continued

Table 2 (*continued*)

Compound No.		I_{50}^{NTE} (μM)	"Non-NTE" activity removed (%) by pre-incubation with test compound at concentration which inhibits NTE to degree shown below	
			50%	85–95%
(79)	$Ph.CH_2.C(O).N(Ph).O$	⩾100		
(80)	$Ph.C(O).N(Ph).O$	*ca.* 0.5		38
(81)	$CH_3CH{=}N.O$	10		60
(82)	$Ph{-}\overset{\overset{O}{\|}}{C}{-}CH{=}N{-}O$	⩾100		
(F) OTHER ESTERS				
(83)	$(CH_3.O)_2.P.(O).S.CH_3$	⩾100		
(84)	$CH_3.O.P.(O).(S.CH_3)_2$	⩾100		
(85)	$(C_2H_5.O)_2.P.(O).S.C_2H_5$	⩾100		
(86)	$C_2H_5.O.P.(O).(S.C_2H_5)_2$	⩾100		
(87)	$Ph.CH_2.SO_2.O(2,6\text{-}Cl_2.Ph)$	⩾50		
(88)	$Ph.CH_2.SO_2.NH(2,6\text{-}F_2.Ph)$	⩾50		
(89)	$Ph.CH_2.NH.SO_2.O(2,6\text{-}Cl_2.Ph)$	⩾50		
(90)	$Ph.CH_2.NH.SO_2.O(2\text{-}Cl\text{-}6\text{-}Me.Ph)$	⩾50		
(91)	$Ph_2N.C(O).F$	0.05	40	70
(92)	$(Ph.NH)_2.P.(O).Cl$§	120	18	24

"Paraoxon-resistant" and "Non-NTE" esterases are defined respectively as activity remaining after preincubation with paraoxon alone (40 μM for 20 min at 37° and pH 8.0) or by paraoxon plus mipafox (50 μM). NTE and other phenyl valerate esterases were assayed and I_{50} values were determined as described in Materials and Methods. The inhibition of "Non-NTE" activity (approx. %) was determined at concentrations near I_{50}^{NTE} and at I_{85-95}^{NTE}.

* Acetone solvent concentration = 3.25%.

† Solvent as in * and tissue was supernatant from centrifuged homogenate.

‡ High zero-time blanks suggest these compounds were impure and/or unstable.

§ Inhibitor decomposed within 2 min in pH 8 buffer.

‖ Preincubation 100 μM/60 min.

Esters of dialkylphosphinic acids. Phenyl and substituted phenyl esters of di-*n*-octylphosphinic acid made by more than one route decomposed in the preparation solution to yield dioctylphosphine oxide; 4-nitrophenyl di-*n*-pentylphosphinate was rather unstable but the phenyl ester (compound 70) prepared as described recently [11] was stable.

Fluoridates. All were made by metathesis of the appropriate chloride: compound 64 used saturated aqueous KF plus a phase-transfer catalyst, compound 32 used NaF in tetramethylene sulphone at 100–135° for 2 hr and compound 91 used KSO_2F in refluxing xylene for 2–3 days: attempts to make No. 91 with aqueous KF yielded only decomposition products.

Other compounds. Compounds 38 and 87–88 were prepared from the precursor acid chlorides by direct reaction of the acid chloride with the appropriate phenol or amine. Compounds 89 and 90 were prepared by interaction of equimolar amounts of the precursor amine plus triethylamine with excess sulphuryl chloride in cold dichloromethane followed by removal of sulphuryl chloride and reaction with the appropriate phenol plus triethylamine. Compound 30 was separated chromatographically as an impurity in some syntheses of paraoxon [12] and compound 37 was separated as an impurity in the synthesis of compound 70 [11].

All other compounds were gifts kindly provided from the sources listed below with the compound numbers in parentheses: Shell Research Limited, Sittingbourne (16–24, 65, 66); Chemical Defence Establishment, Porton Down (25–28, 67) (Dr L. Leadbeater and Mr G. Sainsbury); World Health Org., Division of Vector Biology and Control (31); Bayer Chem. Co., Leverkusen (Dr W. Hofer) (33, 51, 54–58); Dr G. Amitai, Weizman Inst., Rehovat (29, 34); Dr A. Aaviksaar, Talinn, Estonia (35); Dr L. G. Hansen, Urbana, U.S.A. (36); Dr H. Yoskikawa, Fukuoka, Japan (39–44); Dr H. P. Benschop, T.N.O., Rijswijk, The Netherlands (45–48); Dr T. R. Fukuto, Riverside, U.S.A. (49, 50, 52, 53); Dr W. N. Aldridge of this Unit (83–86); Dr P. Farmer of this unit (92).

RESULTS AND DISCUSSION

Substrates

Table 1 lists the hydrolytic activity of hen brain homogenate against a range of substrates compared with PV (compound 5), the currently preferred substrate for NTE. Activities are those expressed after preincubation of the tissue with (A) buffer alone, (B) paraoxon or (C) paraoxon + mipafox and the (presumed) NTE activity is calculated as B–C. The possibility that B–C represented more than a single enzyme was not investigated for the alternative substrates. The homogeneity of the B–C activity against PV is considered below (see "Selectivity of mipafox"). The selectivity of compounds as substrates for NTE was calculated as the ratio (B–C)/(B)%.

The substituted phenyl esters of valeric acid (compounds 1–4) were very poor substrates for NTE although the activities in column A for compounds 1 and 4 were about one quarter that for PV. Four other compounds had faster "A" activity rates than PV (No. 6, 8, 9 and 10) and compound 8 and 9 had NTE rates markedly faster than PV. However, the selectivity of these latter were about 35 and 40–50% respectively compared with 74% for PV assayed at the same time and, moreover, the non-enzymic rate of hydrolysis of compounds 8 was unacceptably high and was substantial for compound 9. Thus of the 15 potential substrates investigated only phenyl phenylthioacetate (compound 9) could be considered as a more sensitive substrate if the non-enzymic rate could be manipulated by lowering pH. Phenyl *n*.Octylthioacetate (compound 10) was the most selective after PV and had NTE rate about 70–80% that of PV. Attempts to synthesize substrates with —NH— or —NR— groups replacing the sulphur atom in the above compounds were unsuccessful although several routes were tried. The esters derived from hydroxamylacetic or oximylacetic acids (compounds 11–13) were unremarkable in hydrolysis rates. The halophenylesters were uniformly worse substrates than the unsubstituted parents (compare compounds 2 or 3 with compound 5, and No. 14 or 15 with No. 6).

Inhibitors

Potency. Table 2 lists inhibitory power (I_{50} for 20 min preincubation at 37° and pH 8) against NTE in the standard assay [10] using tissue preincubated with paraoxon with or without mipafox and with PV as substrate.

It has been shown previously [9, 13] that longer-chain dialkyl phosphates were more active than dimethyl and diethyl analogues and this is clearly shown with the 2,2-dichlorovinyl phosphates (compounds 16–24) (I_{50} for dichlorvos is about 25 μM [9]), and with the fluoridates (compounds 25–27) and the phosphoramidates (compounds 51–58). However, there is a limit to this chain-length effect with I_{50} increasing again with the di-*n*-hexyl to decyl compounds (compounds 19–22). It seems likely that this reflects steric hindrance since the mixed methyl/octyl and methyl/decyl esters (compounds 23 and 24), are more active than their di-long-chain analogues.

This study adds more evidence to that presented previously [9] that small or flexible-chain leaving groups such as fluoride or dichlorovinyl tend to good anti-NTE activity. Likewise, although sulphur-linked leaving groups do not usually make for good NTE inhibition the *S*-propargyl group in compounds 43 and 44 increased the activity at least 10-fold compared with the poorly inhibitory analogous *S*-halobenzyl phosphonates (compounds 39–42).

Previous study showed that benzylcarbamates were more active NTE inhibitors than analogous esters [9]. Among the benzylcarbamates studied here, two interesting features were seen. (i) Substitution of halogen in the 2- or the 2,6-positions of the phenyl leaving group markedly reduced the inhibitory power although electronic considerations suggest they should be more labile to hydrolysis (compare compounds 75 and 77 with Nos. 71, 74 and 76). Presumably, the effect of the ortho-substituents is that of steric hindrance but it is interesting that no such effect is seen with ortho-halophenyl leaving groups in phosphonates such as compounds 36, for

which a large series of analogues has been studied [14]; the overall P–O–phenyl bond-length would be somewhat greater than the C–O–phenyl and so reduce spatial crowding. (ii) The N-phenyl benzohydroxamyl ester (compounds 80) was very potent whereas the analogue (No. 79) with one —CH_2— group inserted was inactive. It is not clear whether the conjugated unsaturation of the former can account for all the difference especially since the conjugated oxime ester (compound 78) was only weakly inhibitory.

The largest differences of activity between stereoisomers was only 15× (compounds 45 and 48 with two centres of asymmetry) or 11× (compounds 52 and 53). By contrast compounds 45 and 48 differ about 1000× in their anticholinesterase activity [15].

Two compounds listed are of interest because they were formed as unexpected impurities in syntheses of desired compounds. Bis-(4-nitrophenyl) ethyl phosphate (compound 30) contaminated some preparations of paraoxon (4-nitrophenyl diethyl phosphate) made in this laboratory by direct interaction of commerical ("97%") diethyl phosphoryl chloride with sodium nitrophenate [12]. With an I_{50} for NTE of 2–3 μM it caused these preparations of paraoxon to interfere significantly in standard NTE assays: I_{50}^{NTE} of pure paraoxon is 400–500 μM [5]. Diphenyl *n*-pentylphosphonate (compound 37) was formed in almost equal amounts with the desired phenyl di-*n*-pentylphosphinate (compound 70) during the reaction of *n*-pentylmagnesium bromide with carefully purified phenyl phosphoryl dichloride [11]. Fortunately, the phosphonate was easily separated and, also, it was at least 10 × less inhibitory than the phosphinate which is a conveniently stable member of the class of phosphinates which protect against OPIDP and has been useful in studies of NTE *in vivo* [16].

Selectivity. As a measure of selectivity the % of "non-NTE" (i.e. mipafox-resistant) activity which was sensitive to each compound is given, usually at two different assay conditions: these were assay being performed with (i) about the I_{50}^{NTE} concentration, or (ii) the concentration which would inhibit nearly all the NTE (85–95% inhibition). It is noticeable that for a number of compounds the effect on "non-NTE" was not obviously more at the higher concentration (see, for instance, the dichlorovinyl phosphates (compounds 21–24), diphenylphosphinyl fluoride (No. 64) and others) but this "plateau" effect was not universal (see, for instance, the phosphoramidates (compounds 51–58), the carbamates (compounds 71–75) and others).

None of the compounds which were active enough to give high inhibition of NTE at workable concentrations was totally selective at the I_{85-95}^{NTE}. The best was diphenylphosphinyl fluoride (compound 64) which, because inhibited NTE obtained from this compound could not age, has the advantage of being a potentially protective rather than neuropathic compound [4]. It would, therefore, be less hazardous than mipafox and, also, it is very easily synthesized but, unfortunately it is extremely rapidly hydrolysed ($t_{\frac{1}{2}}$ = 20 sec) in the assay buffer and tends to decompose even in storage. Experience in our laboratory suggests it is not likely to become a satisfactory substitute for mipafox. Phenyl benzylcarbamate (compound 71) was suggested earlier [9] as the most convenient almost specific substitute but the more recent data shown in the table and other unpublished experiments do not confirm its specificity. Re-examination of the earlier raw data show that the control NTE rates were somewhat lower than those we have found in recent years and the selectivity ratio (B – C/B in Table 1) was only 64–67%; both these facts may be related to use in the older assays of paraoxon containing traces of compound 30 as mentioned above. It may be that, besides reducing the NTE activity of tissue, the contaminant also inhibited some "non-NTE" which is now being picked out by phenyl benzylcarbamate.

Ethyl 2,6-dichlorophenyl phenylphosphonate (compound 36) was recommended as a totally specific alternative to mipafox [14, 17]. The table shows that I cannot reproduce this selectivity either under conditions of assay as practised in this laboratory or with the higher concentration of added acetone or with centrifuged homogenate as used by Reinders *et al.* [17] (8–18% of "non-NTE" is sensitive). More recent work in that laboratory also failed to demonstrate complete specificity of compound 36 (Dr E. Cozzi, private communication). The claim [18] that leptophos oxon is a specific alternative to mipafox was not substantiated according to two other studies [9, 14].

Selectivity of mipafox

In earlier studies from this laboratory the hydrolytic activity characterized as NTE in whole hen brain homogenate appeared to be homogeneous up to about 90% inhibition by mipafox as determined by linearity of first-order plots and constancy of ratio of residual activities against two substrates (phenyl phenylacetate and PV) [3, 9]. However, it has been claimed [19] that closer kinetic analysis using up to 28 closely-spaced concentrations of mipafox or DFP in fixed-time incubations and a sequential (curve-stripping) analysis of semi-log plots revealed the presence of two distinct mipafox-sensitive activities dubbed NTE_A and NTE_B in the proportion of 18:82; NTE_A was considered to be 5× more sensitive to mipafox and 13× less sensitive to DFP than NTE_B. Numerous attempts to reproduce these findings in our laboratory have failed (Johnson and Read unpublished): this holds whether we used paraoxon and the mipafox or DFP range mixed together in preincubation or adopted the Chemnitius *et al.* [19] procedure of preincubation with paraoxon followed by a dilution step before a further preincubation with mipafox or DFP. We have recently also used a computer curve-fitting procedure on our untransformed data. This procedure does not introduce error-bias at the higher inhibitions as occurs when processing semi-log data as was done in Ref. 19. In four experiments we found that the data was fitted best as only two activities, i.e. NTE and "Non-NTE" (the latter is virtually insensitive to mipafox although, by other criteria, it is heterogeneous). In one out of 5 experiments, assumption of a third activity representing about 4–7% of the total mipafox-sensitive activity improved the fit slightly.

Further kinetic analysis by Carrington and Abou-Donia [20] also discounts the presence of two NTE-like activities: they also used computer-fitting of untransformed data, and, unlike either my or the Chemnitius and Zech studies, measured the time-dependence at various concentrations of inhibitor and also allowed for the fact that the K_m for PV is so high that addition of substrate at physically saturated solution concentrations does not necessarily completely discharge the Michaelis complex between NTE and high concentrations of inhibitor. However, I do have evidence of an entirely different sort that NTE, as usually assayed in our laboratory, is not entirely homogeneous. When an OP-sensitive esterase is titrated with an inhibitory compound, first-order kinetics indicate that concentrations about 6–7× the approximate I_{50} should inhibit more than 98% of the activity. Table 3 shows the residual mipafox-sensitive activity found with such concentrations in my screening of 26 compounds. It should be borne in mind that the limit of precision in such routine assays was a difference in extinction of 0.010–0.015 between paired samples (preincubated with/without mipafox at 50 μM) which would represent about 3% of the control activity. For many compounds no significant residue of mipafox-sensitive activity remained at $>6 \times I_{50}$. However, the table shows that for several compounds a residual 3–6% did remain uninhibited. The three independent experiments with compound 18 are supported by numerous further experiments (unpublished) with this compound in which the conditions of the assay were varied in small ways or in which sub-cellular particle fractions were assayed rather than fresh whole homogenate. In the case of 4-nitrophenyl di-*n*-pentylphosphinate (two experiments) the resistant residue resisted $21 \times I_{50}$ and a similar situation was found when dissecting mipafox-sensitive sites during the electrophoretic characterization of NTE [21]. Thus, although the published kinetic basis for suggesting there is a substantial second component to NTE seems flawed there does seem evidence for a much smaller quantity not detectable by such procedures. Could this small, rather than the major, component be the "true" NTE target for initiation of OPIDP? Many of the compounds which do not

Table 3. Residual percentages of NTE which remain uninhibited by test compounds at various concentrations above 6× apparent I_{50} under standard assay conditions

Test compound*	Approx. I_{50} (μM)	Multiple of I_{50} tested	NTE activity remaining (%)
I. 2,2-Dichlorovinyl phosphates			
No. 18	0.003	6–8	5, 5, 4
No. 19	0.013	12	4
No. 24	0.07	7	Nil
Di-2-chloroethyl	0.011	8	5
Diphenyl	0.008	12	9
Di-4-*tert*butylphenyl	0.6	8	1–2
Di-*N*,*N*-pyrollidinyl	3	7	Nil
II. Phosphonates			
4-nitrophenyl *n*-pentyl *n*-pentylphosphonate	0.06	12	1
4-nitrophenyl *n*-decyl ethylphosphonate	0.2	12	0
2,2-dichlorovinyl 2-chloroethyl ethylphosphonate	0.06	8	4
No. 36	0.4	10	3, 3, 5
No. 43	12	8	5
No. 44	10	10	3
No. 50	0.45	20	2.4
III. Benzylcarbamates			
No. 71	5	10	3
No. 73	4	10	3
No. 74	4	6	3
		12	<1
No. 76	0.5	8	5
		16	4
		20	2.3
No. 80	0.5	10	3
No. 81	10	10	3
IV. Other			
DFP	0.7	7	0, 4
4-nitrophenyl di-*n*-pentylphosphinate	0.018	7	5, 6
		21	4
Phenyl di-*n*-pentylphosphinate	0.275†	21†	5†
No. 58	0.7	6	3
No. 64	0.07	14	8
No. 92	120	8	6

* For compounds identified by full name the source of chemical is given along with earlier reports of I_{50} determination [8, 9].

† Data from Ref. 22.

inhibit the residual 4–6% do inhibit NTE *in vivo* and do cause the expected response of initiation of OPIDP (phosphates and phosphonates) or of prophylaxis against the effect (phosphinates and phenyl benzylcarbamate) [5, 8]. Therefore the proposal can be discounted and the usual assay of paraoxon-resistant mipafox-sensitive PV hydrolase continues to be an adequate assay of the target for initiation of OPIDP.

GENERAL DISCUSSION

The screening of potential substrates and inhibitors of NTE reported here extends previous reports and confirms some structure/activity generalizations made previously [9, 8, 5]. It has revealed a few substrates which are hydrolysed at rates comparable to or higher than those for PV, the usual assay substrate, but their selectivity is not greater than 50%. It has also revealed that diphenylphosphinyl fluoride is a potent and almost specific non-neuropathic alternative to mipafox for NTE assay procedures but the compound is inconveniently unstable. Kinetic analysis suggest that about 4–7% of NTE activity, as usually assayed, is due to a different enzyme but the inhibitor-specificity of this activity is incompatible with that for a primary target for initiation of OPIDP.

In the event of further detailed kinetic studies on NTE being required, a substrate with K_m well below saturated solution concentration would be desirable (see Results and Discussion Section) but precision of assays also requires highly selective substrates. If any of the best alternative substrates have a low K_m, then attempts to improve selectivity could be made. Although it is impossible to predict how successful the strategy might be with other substrates, the addition of nickel sulphate (2 mM final) along with the substrate might improve selectivity markedly since, with PV as substrate, this additive was shown to inhibit nearly half the "non-NTE" without affecting the NTE [9]. If selectivity of alternative substrates could be improved by this or other means then a re-screening of some of the compounds which Table 2 shows to be nearly specific alternatives for mipafox might become justified. However, it cannot be predicted whether the mipafox-resistant ("non-NTE") enzyme(s) which are somewhat sensitive to "mipafox-substitutes" may be expressed proportionately less than NTE in a new assay using a substrate other than phenyl valerate.

In conclusion, we now have a large stock of structure/activity data for this intriguing enzyme but the study shows that the standard NTE assay is unlikely to be superseded for most purposes although a series of small incremental changes might lead to better conditions for rigorous kinetic studies.

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